A fungal pathogen that robustly manipulates the behavior of *Drosophila melanogaster* in the laboratory

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Abstract

Many microbes induce striking behavioral changes in their animal hosts, but how they achieve these effects is poorly understood, especially at the molecular level. This is due in large part to the lack of a robust system amenable to modern molecular manipulation. We recently discovered a strain of the behavior-manipulating fungal fly pathogen *Entomophthora muscae* infecting wild adult *Drosophila* in Northern California, and developed methods to reliably propagate the infection in lab.-reared *Drosophila melanogaster*. Our lab.-infected flies manifest the moribund behaviors characteristic of *E. muscae* infections: on their final day of life they climb to a high location, extend their proboscides and become affixed to the substrate, then finally raise their wings to strike a characteristic death pose that clears a path for spores that are forcibly ejected from their abdomen to land on and infect other flies. Using a combination of descriptive, histological, molecular and genomic techniques, we have carefully characterized the progress of infection in lab.-reared flies in both the fungus and host. Enticingly, we reveal that *E. muscae* invades the fly nervous system early in infection, suggesting a direct means by which the fungus could induce behavioral changes. Given the vast toolkit of molecular and neurobiological tools available for *D. melanogaster*, we believe this newly established *E. muscae* system will permit rapid progress in understanding how microbes manipulate animal behavior.

Introduction

Among the most extraordinary products of evolution are microorganisms that are able to manipulate animal behavior to their advantage. Some have achieved fame in the popular press, like the fungus *Ophiocordyceps*

unilateralis that makes ants wander away from their nests and climb to an optimal height before sprouting through their heads to rain down infectious spores [1], or the protozoan *Toxoplasma gondii* that suppresses rodents' innate fear of cat odors to aid the return of the parasite to a cat's stomach where it can sexually reproduce [2]. However, the mechanisms by which these and other microbes hijack the animal nervous system have remained elusive.

Entomophthora, from the Greek meaning "insect destroyer", is an aptly-named genus within the basal fungal lineage Zoopagomycota consisting of species that infect, alter the behavior of, then kill their insect hosts [3]. *Entomophthora muscae*, first described in 1855 [4] in house flies (*Musca domestica*), is a fungal species complex that exclusively targets dipterans [5,6]. A fly infected with *E. muscae* exhibits a striking set of behaviors: shortly before sunset on its final day of life, the fly climbs to a high location (a behavior known as "summiting"), extends its proboscis and becomes affixed to the substrate on which it stands via fungal holdfasts [7–9]. The fly's wings then lift up and away from its dorsal abdomen, striking a final death pose that is thought to be ideal for fungal dispersal [9].

Over the course of the next few hours, the fungus within the dead fly differentiates into structures called conidiophores that emerge through the weakest points in the fly's cuticle, usually the intersegmental membranes of the dorsal abdomen, giving the cadavers a distinct banding pattern [10]. A primary conidium (also referred to as a "spore") forms at the tip of each conidiophore; once mature, these conidia are forcibly ejected into the surrounding environment in order to land on the cuticle of a susceptible fly host [11,12].

Launched primary conidia are polynucleated, campanulate (bell-shaped) and are surrounded by a sticky "halo" that serves to adhere the conidium where it lands. If successful in landing on the cuticle of a new host, the conidium germinates, using both mechanical and enzymatic force to bore through the cuticle and into the fly's hemolymph [10,13]. If the primary conidium misses its target or fails to germinate upon landing on the host [14], it can sporulate anew to generate a smaller secondary conidium [11]. Off-target conidia can continue to re-sporulate and give rise to smaller, higher order conidia until a host is reached or resources are exhausted [11]. Once inside the fly, the fungus is initially sustained by nutrients in the hemolymph then later consumes the fat body as an energy source [10]. When available resources are depleted, the fungus elicits the end of life behaviors and the fungal life cycle begins again.

A range of fly species and even non-dipterans can be infected and killed by *E. muscae* in the laboratory, though not all infected hosts manifest the stereotypical end-of-life behaviors, and susceptibility has not been found to track with host phylogeny [15,16]. *E. muscae* has almost exclusively been observed and studied in muscoid flies (especially the house fly, *Musca domestica*), organisms for which we have very few experimental tools [17]. Thus, despite inspiring curiosity and intrigue for over a century, how *E. muscae* achieves control of its host remains poorly understood, with essentially no information as to what is occurring at the molecular level in either fungus or host.

In 2015, we observed several wild *Drosophila* in separate sites in Northern California with the characteristic death pose and fungal banding of *E. muscae* infections, and saw an unprecedented opportunity to study a behavior-changing fungus in the laboratory species, *Drosophila melanogaster*. Here, we describe the isolation and subsequent characterization of this *E. muscae* strain and its impact on *D. melanogaster* behavior in the laboratory, and present the *E. muscae-D. melanogaster* as a model for developing a mechanistic understanding of parasitic manipulation of host behavior.

Results

Discovery and isolation of *E. muscae* from wild *Drosophila*

In June 2015, we established a stable food source (organic fruits in a clean dish pan, referred to henceforth as the "fendel") at a field site in Berkeley, CA to collect wild *Drosophila* for a separate study (see [18]). In late July 2015, we noticed that several flies had died with raised wings at the bottom of the fendel and, upon closer inspection, observed remnants of fungal growth and sporulation on these dead flies (Fig S1A). We suspected that these animals had been killed by the fungal pathogen *Entomophthora muscae*, though there have been only a few reports of *E. muscae* infection in wild *Drosophila [19–21]*.

We first confirmed that these flies had been killed by *E. muscae* by genotyping a dozen representative cadavers at the ITS and 28S (LSU) rDNA loci and searching for similar sequences with BLAST (Fig S1B,C). PCR genotyping of the host at the cytochrome oxidase II (COII) locus [22] demonstrated that susceptible host species included *D. melanogaster*, *D. immigrans, D. simulans* and *D. hydei*, which are all commonly observed in Berkeley, CA. The fungal sequences for all of the typed cadavers were identical at these two loci, consistent with one *E. muscae* strain being responsible for this epizootic event. Species identification within the *E. muscae* species complex (which will hereafter be referred to as *E. muscae*) has historically relied on conidial morphology (and, to a lesser extent, host species), but is expanding to include molecular data [23]. Still, the taxonomic boundaries between strains and species within this group are still unclear. To distinguish our strain (or possibly species) from others reported, we will henceforth refer to our isolate as *E. muscae Berkeley*.

We were intrigued by the possibility that the presence of *E. muscae* in *Drosophila* would allow us to establish an infection in lab.-reared flies. However, our initial observations were all of dead flies that had already ejected their spores (Fig S1A). Studies in *M. domestica* have shown that, at room temperature, the majority of *E. muscae'*s infectious spores are ejected within the first approximately twelve hours of an infected host's death, and lose infectivity within 48 hours of landing on a non-host substrate [24]. Thus, to culture *E. muscae Berkeley* we needed to procure freshly-killed flies to ensure access to viable conidia.

101 The repeated observation of *E. muscae Berkeley*-killed *Drosophila* demonstrated that the infection was
102 circulating in the population of flies at our field site. We therefore reasoned that some of the flies that were
103 visiting our fendel should be infected. Previous *E. muscae* research had demonstrated that the fungus only
104 kills hosts once a day, around sunset [9]. Thus, we collected flies once every morning (1-2 hours after
105 sunrise) from the fendel and monitored them nightly (1-3 hours after sunset), looking for animals that had
106 recently died in the stereotyped death pose.

107 Using a single, wild cadaver, we first established a culture of *E. muscae Berkeley in vitro,* by
108 inoculating liquid media previously reported to support *E. muscae* growth [23]. Genotyping the resultant
109 culture at both the ITS and 28S loci verified that we had isolated the same strain as the one that had killed
110 the previously observed cadavers (Fig S2).

111 To establish an *in vivo* infection, wild cadavers were co-housed overnight in a confined space with
112 healthy, lab.-reared CantonS *D. melanogaster*, and exposed flies were monitored nightly for two weeks to
113 identify *E. muscae Berkeley* cadavers. We repeated this process daily for several weeks before we were
114 able to passage the infection. We were aware that our standard fly diet contained a small amount of the
115 preservative tegosept (0.09%), but did not anticipate that this would be problematic since infected wild flies
116 still died of infection after being housed on this diet for up to eight days (Fig S3). However, it was only
117 when we began housing flies on food devoid of the preservative tegosept that we were able to successfully
118 passage the infection.

119 Once we had transferred *E. muscae Berkeley* to lab.-reared flies, we assessed the impacts of several
120 variables on infection efficacy, ultimately arriving at an optimized propagation protocol (Fig S4). Briefly,
121 we expose flies to *E. muscae* by embedding six freshly-killed, infected cadavers headfirst in sucrose agar
122 and confining 50 young (eclosed within the past 24 hours) CantonS adults of mixed sex with these cadavers
123 for 24 hours in a cool, humid environment on an inverted 12:12 light:dark cycle. After 24 hours,
124 confinement is relieved and flies are transferred to a medium free of tegosept. Exposed flies are housed at
125 room temperature with moderate humidity and monitored daily for death by fungus.
126

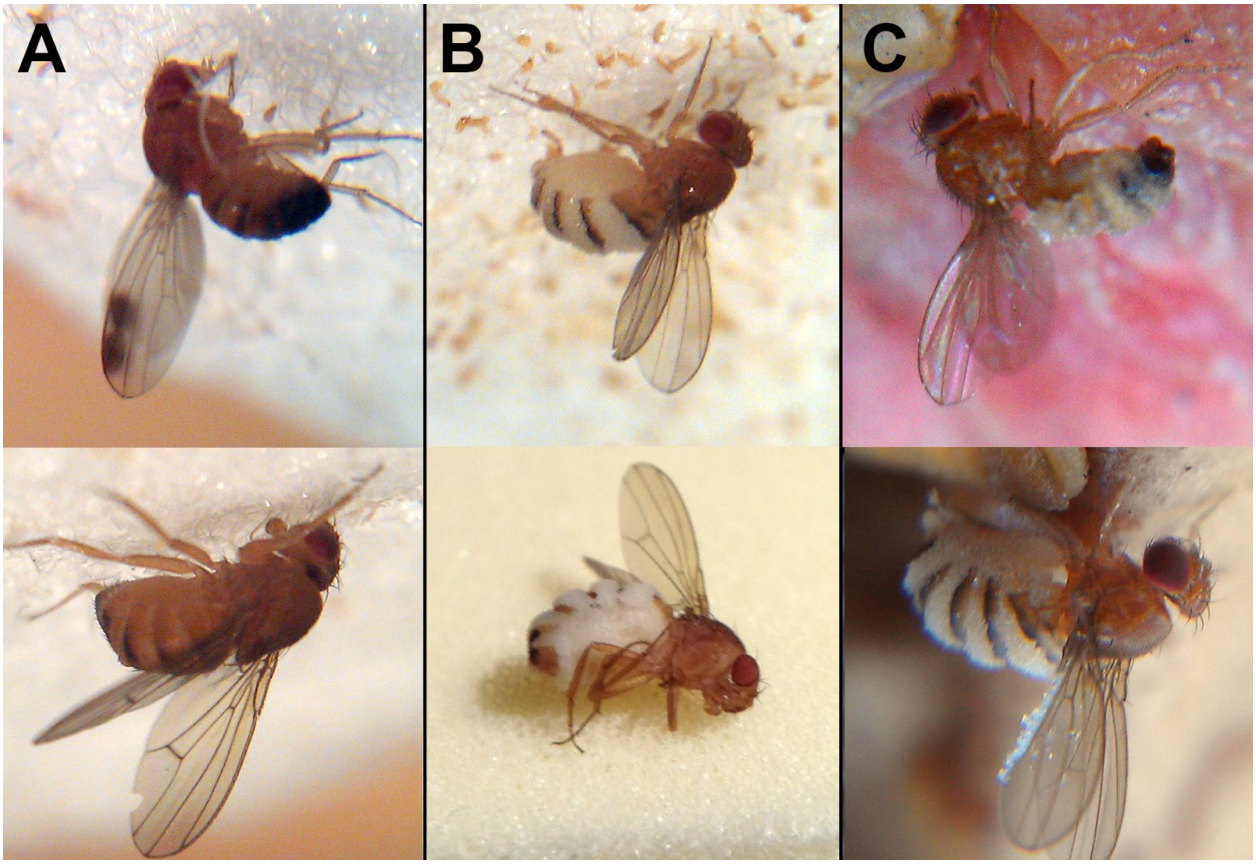
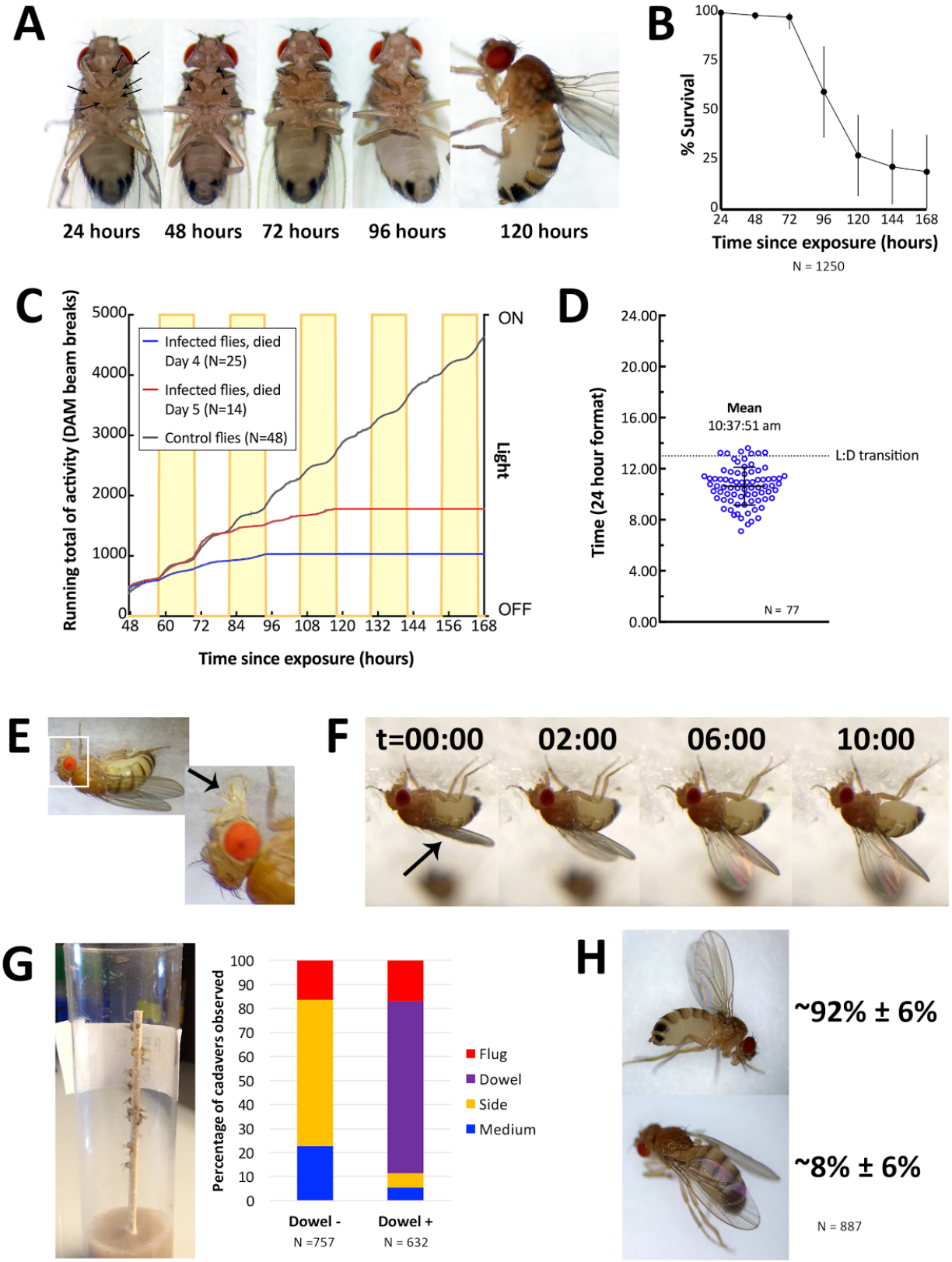


Figure 1. Wild drosophilids killed by *Entomophthora muscae* Berkeley. A) Cadavers found among sampled flies 65 minutes (above) and 40 minutes (below) after sunset. *E. muscae Berkeley* has not grown through the host cuticle. B) Cadavers found among sampled flies 120 minutes (above) and 160 minutes (below) after sunset. *E. muscae Berkeley* has grown through the host cuticle and will soon start to eject conidia. C) Cadavers as discovered *in situ* in fendel at least 12 hours after sunset. *E. muscae Berkeley* has grown through the host cuticle and ejected conidia, some of which have landed on the cadavers' wings.

Description of *E. muscae Berkeley* infection in CantonS flies

With *E. muscae Berkeley* stably propagating *in vivo*, we next focused on carefully observing the process of infection in CantonS flies. By eye, infected flies are hard to distinguish from their healthy counterparts both morphologically and behaviorally until they begin to exhibit end-of-life behaviors (Fig 2A). Exposed flies bear melanized scars that form following spore entry through the cuticle, which are most apparent when the point of entry is the pale ventral abdomen. However, not all flies that are penetrated by the fungus are successfully infected and killed, as we have observed animals with scarring that survive beyond seven days after exposure, and have found that housing exposed flies on diet with anti-fungal significantly improves survival (Fig S4). At 72 hours after exposure and beyond, infected flies generally have more opaque abdomens than uninfected flies due to abundant fungal growth. Under our conditions, ~80% of

145 CantonS flies are killed four to seven days after exposure to *E. muscae Berkeley*, with the majority of deaths
146 occurring at 96 and 120 hours (Fig 2B). While by eye infected animals behave normally until the onset of
147 end-of-life behaviors, analysis of infected fly activity revealed that infected flies exhibit a marked decrease
148 in total activity compared to healthy counterparts beginning about 36 hours before time of death, which
149 presently is the best indication of imminent mortality for a given fly (Fig 2C).
150 On the last day of life, *E. muscae Berkeley* infected flies stop moving 0-5 hours before sunset (Fig 2D).
151 Taking time of last movement as a proxy for time of death, this observation agrees with reports of *E. muscae*
152 in house flies [9]. Also consistent with previous reports, flies exposed to *E. muscae Berkeley* and housed
153 under complete darkness die sporadically throughout the day rather than in a gated fashion (Fig S5, [9]).
154 As healthy flies housed for 168 hours in complete darkness maintain circadian rhythm, this suggests that
155 environmental cues and/or a fungal clock are required to coordinate the timing of death, as has been
156 previously suggested [9]. Of note, flies housed in complete darkness are still observed to die in elevated
157 positions. This suggests that summiting behavior relies predominantly on gravitaxis rather than phototaxis.
158


A
24 hours
48 hours
72 hours
96 hours
120 hours
B
% Survival
100
75
50
25
0
24 48 72 96 120 144 168
Time since exposure (hours)
N = 1250
C
Running total of activity (DAM beam breaks)
5000
4000
3000
2000
1000
0
Infected flies, died Day 4 (N=25)
Infected flies, died Day 5 (N=14)
Control flies (N=48)
ON
Light
OFF
48 60 72 84 96 108 120 132 144 156 168
Time since exposure (hours)
D
Time (24 hour format)
24.00
20.00
16.00
12.00
8.00
4.00
0.00
Mean
10:37:51 am
L:D transition
N = 77
E
F
t=00:00
02:00
06:00
10:00
G
Percentage of cadavers observed
100
90
80
70
60
50
40
30
20
10
0
Flug
Dowel
Side
Medium
Dowel -
N =757
Dowel +
N = 632
H
~92% ± 6%
~8% ± 6%
N = 887

159

Figure 2. Characteristics of *E. muscae Berkeley* infected CantonS. A) Typical female fly over the course of infection. Arrows denote conidia that have landed on the cuticle but not yet bored into the hemolymph. Arrowheads indicate melanization of the fly cuticle that has occurred in response to conidia boring into hemolymph. B) Time of death for flies infected as per standardized protocol (Fig S4). C) Activity profile of control flies or *E. muscae Berkeley*-infected flies measured using the *Drosophila* activity monitor (DAM). D) Time of last movement as measured using the DAM. Each blue circle represents the time of last movement observed for one cadaver. Flies were exposed to *E. muscae Berkeley* as per standardized protocol. Dotted line indicates the light-dark transition (L:D transition). E) *E. muscae Berkeley*-infected fly exhibiting proboscis extension tens of minutes before death. Arrow indicates extended proboscis adhered to the surface. Real time footage of an *E. muscae Berkeley*-infected fly undergoing proboscis extension is available as Movie S1. F) *E. muscae Berkeley*-infected fly exhibiting wing raising immediately prior to death. Arrow indicates original positioning of wings. Time elapsed is given in minutes:seconds. Real time footage of an *E. muscae Berkeley*-infected fly undergoing wing raising is available as Movies S2, S3. G) *E. muscae Berkeley*-killed CantonS summited and adhered to a wooden dowel. Graph to the right indicates position of death for flies housed in vials without (Dowel -) or with (Dowel +) a wooden dowel. H) Most commonly observed wing positions of *E. muscae Berkeley*-killed CantonS. Complete wing raising is observed in most cadavers; wing lowering is consistently observed in a small fraction of cadavers.

On the last day of life, flies infected with *E. muscae Berkeley* show a precipitous decline. The first portent of imminent death is that flies cease to fly. Though they can still walk and are responsive to perturbations (i.e. poking with a paintbrush or jostling their container), they will not take flight. After they have lost the ability (or desire) to fly, moribund flies will begin to exhibit a shaky and slowed gait which is usually coincident with an upward climbing or movement towards a vertical surface. Many flies reach elevated positions before they lose the ability (or desire) to continue moving (even when perturbed by the experimenter), but some succumb to immobility before they leave the ground. When provided a thin, wooden dowel as a summiting substrate, more flies are observed to die in elevated positions, mostly on the dowel itself (Fig 2G). Interestingly, we have noticed that when drips of medium are present on the side of a vial, flies that die on the side of the vial are preferentially found on these drips. It is unclear if this indicates a preference for the medium as a climbing substrate (versus the smooth plastic of a fly vial) or if the flies are attempting to eat until their very last.

Once the fly stops walking, it extends its proboscis until it makes contact with the surface on which it is standing (Fig 2E). The extension of the proboscis is shaky and can occur slowly relative to extension in response to a nutritive stimulus, and we have observed in multiple instances that the labella of infected flies do not spread as is typically observed when uninfected flies eat (see Movie S1). Typically, once the

194 proboscis has made contact with the surface, the fly may move its legs in what appears to be an apparent
195 attempt to escape, but the material that emanates from the proboscis is sufficient to keep it anchored in
196 place. After the proboscis has adhered, the fly then begins to raise its wings up and away from the dorsal
197 abdomen (Fig 2F). This process has been observed to take on the order of ~10 minutes, with wing raising
198 occurring in small bursts, reminiscent of the inflation of a balloon (see Movies S2 and S3). Curiously, a
199 persistent minority of infected flies die with their wings lowered down onto their abdomen rather than with
200 wings elevated (Fig 2H). By applying pressure to the thorax of these flies, the wings are observed to
201 "toggle" into the upright position, suggesting that the same muscles are involved in raising and lowering.
202 The fly may continue to twitch its legs and antenna for several minutes after the wings have reached their
203 final position but will shortly cease moving.
204 After death, the fungus inside of the fly continues to differentiate into conidiophores, conidia-
205 launching structures, that grow out into the environment through weak points in the fly's cuticle. Over the
206 course of several minutes, each conidiophore forms a single primary conidium (Movie S7) which, upon
207 maturation, is forcibly ejected into the environment. Using time lapse imaging, we observe that conidia
208 begin to launch approximately five hours after sunset and continue doing so for several hours at ambient
209 temperature and humidity (Fig 3A). We observed that conidia form and launch asynchronously within a
210 given cadaver, and not all conidiophores are guaranteed to launch what appear to be mature conidia. Using
211 high speed videography, we were able to capture the motion of conidial ejection (Fig 3B), and determine
212 that conidia leave the conidiophore at an initial velocity of ~21 miles per hour (~9.4 meters/second). These
213 speeds are comparable to those observed in coprophilous fungi, which are among the fastest observed
214 velocities of organisms relative to their size known in the natural world [25]. In addition, we obtained high
215 speed footage of primary conidia landing (Fig 3C), which shows conclusively that conidia and halo land
216 concurrently, an observation that supports the fungal canon mechanism of spore discharge [26].
217

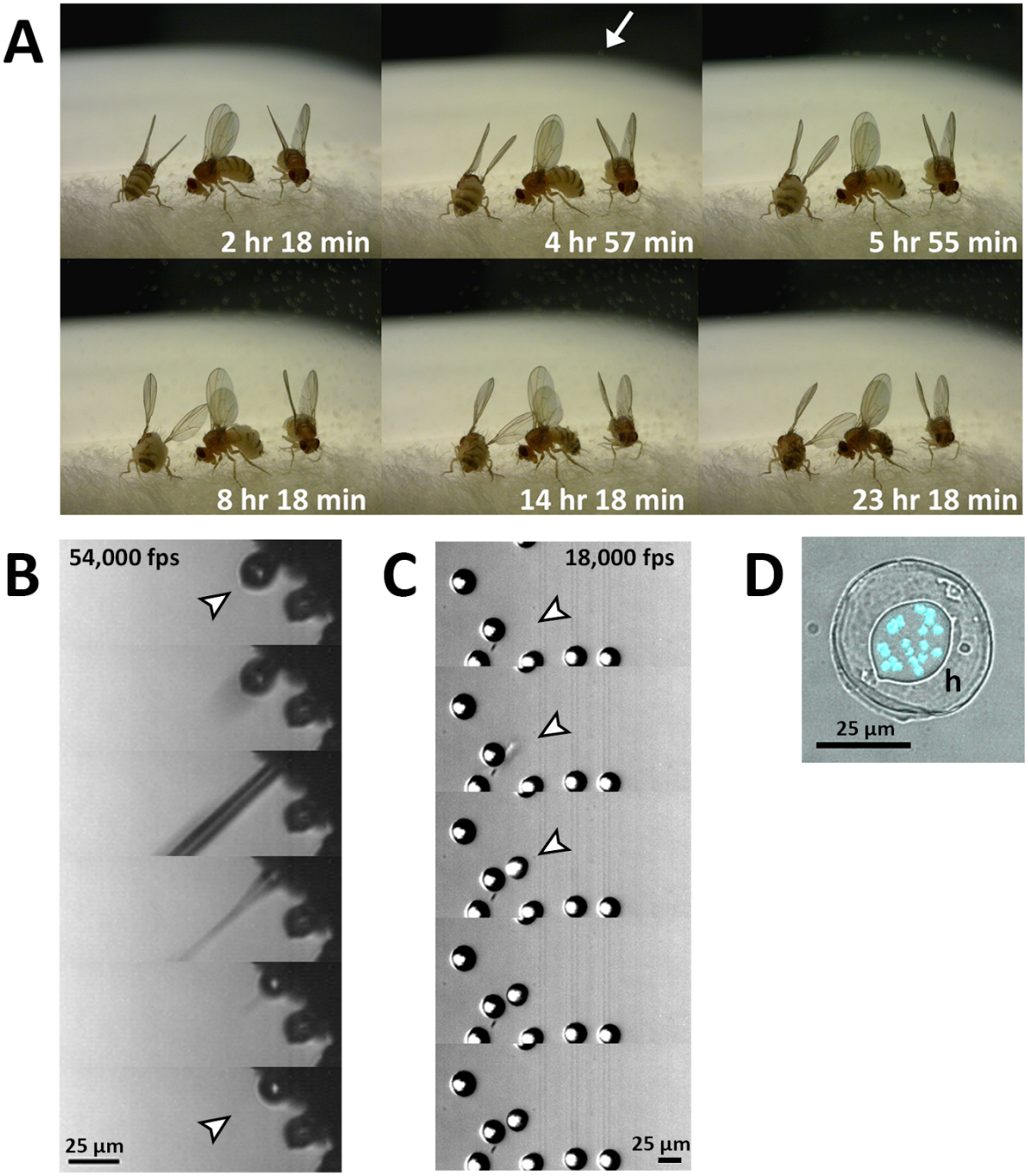


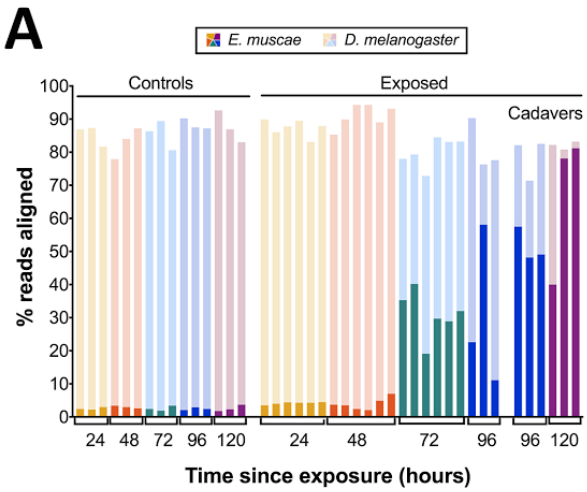
Figure 3. Fungal transmission from *E. muscae Berkeley* killed cadavers. A) Sporulation time lapse in *E. muscae Berkeley* killed cadavers. Time listed in each frame is the time that has elapsed since the light-dark transition. One image was taken every minute for ~24 hours with three cadavers situated on a cotton flug at ambient temperature and humidity. The arrow in the second frame indicates the first primary conidium observed to land on the camera's lens, indicating the start of conidial ejection (i.e. sporulation). Animated time lapse available as Movie S4. B) Time lapse of the ejection of a primary conidium from a

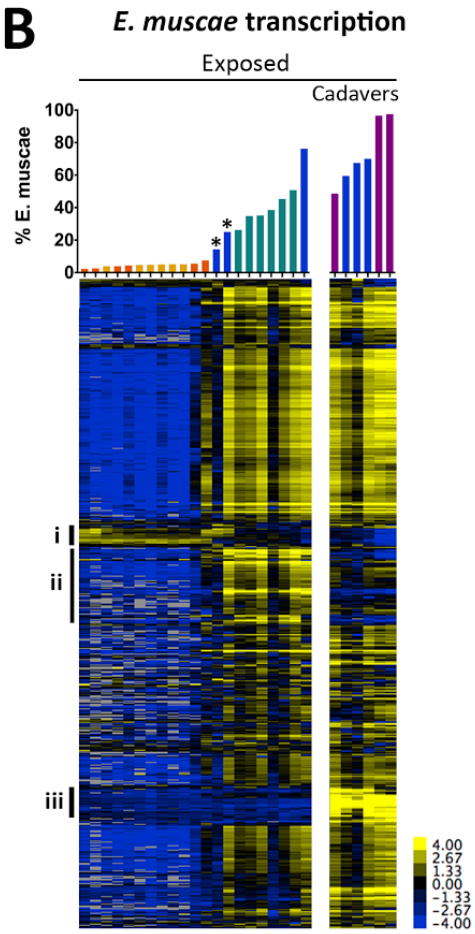
225 sporulating cadaver as captured at 54,000 frames per second (fps). Arrowheads indicates conidium that
226 launches and the vacant conidiophore that remains after launch. Animated time lapse available as Movie
227 S5. C) Time lapse of a primary conidium landing on the lid of a coverslip as captured at 18,000 fps. The
228 conidium lands as one complete unit, supporting the fungal cannon mechanism of primary conidium
229 ejection in *E. muscae*. Arrowheads indicates the position where the primary conidium lands. Animated time
230 lapse available as Movie S6. D) Primary conidium adhered to glass coverslip and stained with fluorescent
231 nuclear dye (Hoechst 33342). The conidium is surrounded by a halo of co-ejected material (h).
232
233 To compare *E. muscae Berkeley* with other reported isolates, we collected primary conidia and
234 measured their key morphological traits (e.g. Fig 3D). Our measurements are most similar to primary
235 conidia from *E. muscae sensu strictu* rather than other members of the *E. muscae* species complex (Table
236 1). As expected, secondary conidia were observed to form from primary conidia that had landed on non-
237 productive surfaces (host wing or agar substrate) (Movie S8).
238
239 **Table 1. Morphology of primary conidia of *E. muscae Berkeley* compared to other reported *E. muscae***
240 **strains.**

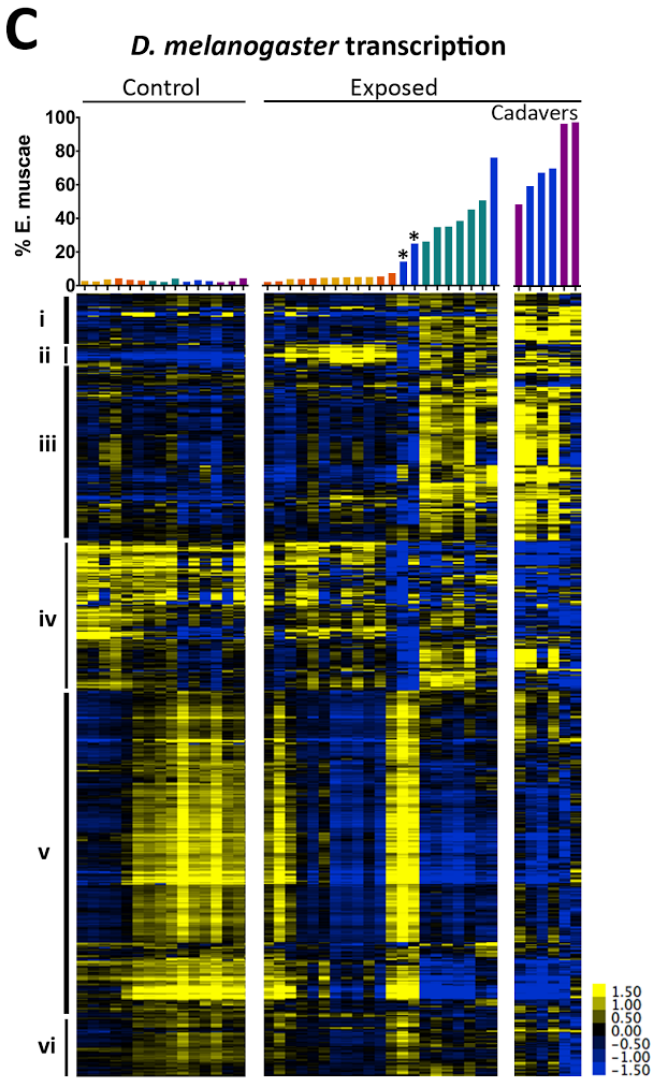
Report	Isolated from	Infected host	# of nuclei	Diameter of nuclei (µm)	Conidium length (µm)	Conidium width (µm)
Present study[1]	*Drosophila spp.*	*D. melanogaster*	13.9-14.9 (8-22)	3.7-4.1 (2.8-5.8)	23.5-26.1 (19.2-31.8)	18.4-20.5 (14.6-26.7)
[21][2]	*Drosophila spp.*	*Drosophila spp.*	15.3-15.9	4.4	26.0-27.6 (23-30)	21.7-22.9 (17-27)
[27][3]	*M. domestica*	*D. suzukii*	12.8-13.8 (11–16)	NA	27.9-29.1 (25.2-36.8)	22.4-23.2 (19.7-27.6)
[28][4]	*M. domestica*	*M. domestica*	15.2-20.2 (10-27)	3.9-4.4 (3.5-5.5)	26.9-31.1 (21-35)	20.4-24.2 (16-29)

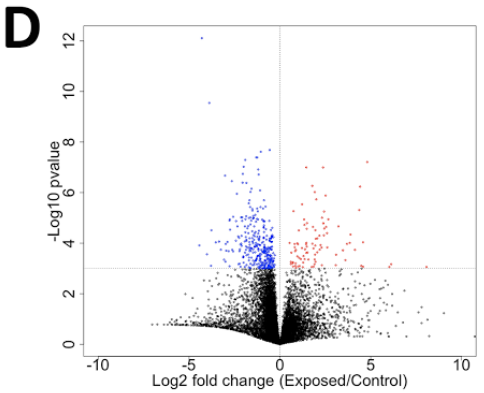
241 [1] Measurements are given as range in means of three series of 50 objects per host.
242 [2] Measurements given as reported, unknown number of conidia measured
243 [3] Measurements are given as reported, nuclei of 12 conidia from one animal were counted, 20 conidia were
244 measured for length and width

245 [4] Redescribed type species of *E. muscae* in type host, *M. domestica*, for reference. Measurements were taken
246 of 8-27 series of 50 objects per host.
247
248 Transcriptional profiles of *E. muscae Berkeley* and *D. melanogaster* over the course of infection
249 To gain a first comprehensive look into how *E. muscae Berkeley* infection progresses in *D.*
250 *melanogaster* at the molecular level, we next measured how transcription changes in both host and fungus
251 at 24 hour time points. We knew that in any given exposure vial there are a mix of infected and uninfected
252 animals and faced the complication that, early on, infected flies are phenotypically indistinguishable from
253 uninfected animals. However, we felt confident that we would be able to distinguish infected from
254 uninfected exposed animals after sequencing mRNA since a) only a few *E. muscae Berkeley* reads would
255 be needed to confirm that *E. muscae Berkeley* was present and b) it would be unlikely that infected and
256 uninfected exposed animals wouldd demonstrate identical transcriptional profiles. To favor our chances of
257 collecting three infected flies for each time point, we collected six *E. muscae Berkeley* exposed CantonS
258 females at each 24, 48 and 72 hours, three exposed flies at 96 hours, and six fresh cadavers, three at each
259 96 hours and 120 hours. In parallel, we collected three CantonS females at each time point that were
260 subjected to a "mock" exposure (housed under identical conditions but in the absence of cadavers). We
261 prepared and sequenced mRNA libraries from each individual sampled, then aligned the reads to the *D.*
262 *melanogaster* transcriptome reference and a bespoke *E. muscae Berkeley* transcriptome reference, which
263 was assembled from reads that failed to align to the *D. melanogaster* reference. This initial *E. muscae*
264 *Berkeley* transcriptome was contaminated by some fly RNAs. After removal of the bulk of contaminating
265 transcripts based on BLAST alignment and expression levels, our final *E. muscae Berkeley* reference
266 contained 86,509 transcripts, including a small number of contaminating *Drosophila* transcripts to which
267 an average of 2.6% of reads from uninfected, control flies aligned (see Methods for details).
268 We first examined the percentage of reads that aligned to host or fungus in each of our time course
269 samples (Figure 4A). We observe that *E. muscae Berkeley* reads are low abundance until 72 hours after
270 exposure at which point a significant portion of the total reads align to the *E. muscae Berkeley*
271 transcriptome. This likely reflects that the fungus does not begin to actively divide until between 48 and 72
272 hours and is consistent with our previous observation that *E. muscae Berkeley* rRNA is not reliably
273 detectable by endpoint reverse transcription PCR until at least 72 hours after exposure (Fig S6). Notably,
274 the majority of reads from cadavers align to *E. muscae Berkeley* rather than fly. Strikingly, two of our
275 cadavers show only trace amounts of *D. melanogaster* RNA at the point of sampling.
276









GO term	Fold enrichment	P-value
arginine metabolic process (GO:0006525)	33.45	2.08E-02
glutamine family amino acid metabolic process (GO:0009064)	10.84	1.37E-02
cellular amino acid metabolic process (GO:0006520)	4.51	5.22E-03
hexose metabolic process (GO:0019318)	8.87	4.95E-02
oxidation-reduction process (GO:0055114)	3.75	2.04E-12
transmembrane transport (GO:0055085)	2.71	4.73E-03
regulation of cellular process (GO:0050794)	0.53	2.74E-03
regulation of macromolecule metabolic process (GO:0060255)	0.34	7.72E-03

GO term	Fold enrichment	P-value
regulation of melanization defense response (GO:0035007)	68.46	3.59E-02
peptidoglycan catabolic process (GO:0009253)	42.6	7.69E-03
regulation of Toll signaling pathway (GO:0008592)	39.94	5.66E-04
antibacterial humoral response (GO:0019731)	39.94	3.26E-05
Toll signaling pathway (GO:0008063)	33.88	5.79E-06
positive regulation of antibacterial peptide biosynthetic process (GO:0006963)	33.63	1.94E-02
negative regulation of proteolysis (GO:0045861)	24.96	5.60E-03
defense response to fungus (GO:0050832)	19.17	2.34E-03
innate immune response (GO:0045087)	17.55	1.01E-06
proteolysis (GO:0006508)	3.67	8.50E-03
cellular macromolecule metabolic process (GO:0044260)	< 0.2	1.28E-02

277

278 **Figure 4. Gene expression time course of *E. muscae Berkeley*-infected CantonS flies.** A) Percentage of
279 reads aligned to *D. melanogaster* reference versus *E. muscae Berkeley* reference using Kallisto. Samples
280 are separated into controls (healthy animals who were mock exposed), exposed (animals who exposed to
281 *E. muscae Berkeley* and were alive at the time of sampling) and cadavers (animals who were killed by *E.*
282 *muscae Berkeley,* dead at the time of sampling) and are color-coded according to the time point at which
283 they were collected (i.e. 24, 48, 72, 96 or 120 hours). B) *E. muscae Berkeley* expression data from *E. muscae*
284 *Berkeley*-exposed and cadaver samples. Complete linkage hierarchical gene clustering by gene was
285 performed in Gene Cluster 3.0 after filtering out across all genes that are expressed at least at ten TPM in
286 at least three out of 27 samples (10,809 transcripts total), then log transforming and centering on the mean
287 value for each transcript. Samples are ordered by percentage of *E. muscae Berkeley* reads as a fraction of
288 the total reads aligned (above). The scale bar for the heatmap is given to the right of the plot. Two 96 hour
289 exposed samples that show an aberrant immune response compared to all other exposed samples are
290 indicated by asterisks. C) *D. melanogaster* expression data from control, *E. muscae Berkeley*-exposed and
291 *E. muscae Berkeley*-killed cadavers. Complete linkage hierarchical gene clustering by gene was performed
292 in Gene Cluster 3.0 after filtering out across all genes that are expressed at least at two TPM in at least three
293 out of 42 samples (10,875 transcripts total), then log transforming and centering on the mean value for each
294 transcript. Samples are ordered by percentage of *E. muscae Berkeley* reads as a fraction of the total reads
295 aligned (above). The scale bar for the heatmap is given to the right of the plot. Two 96 hour exposed samples
296 that show an aberrant immune response compared to all other exposed samples are indicated by asterisks.
297 D) Genes that are consistently over or under-expressed compared to controls over the first 72 hours after
298 exposure to *E. muscae Berkeley*. Top: Volcano plot for all genes over the first 72 hours after exposure. P-
299 value is determined by ANOVA grouping 24-72 hour control vs. 24-72 hour exposed samples. Genes with
300 p-value below 0.001 are shown in color. Bottom: Panther GO-term analysis (complete biological process)
301 of genes overexpressed (red) or under-expressed (blue) in exposed animals compared to controls.
302

303 We next surveyed gene expression in *E. muscae Berkeley* across our exposed samples. As different
304 exposed individuals vary in their rate of infection by *E. muscae Berkeley*, we reasoned that it would be most
305 informative to order our samples based on *E. muscae Berkeley* titer, which we approximated using the
306 proportion of reads that aligned to *E. muscae Berkeley* of total reads aligned to either the *E. muscae Berkeley*
307 or *D. melanogaster* references (Figure 4B). The bulk of transcripts are not expressed until three days after
308 exposure, which could simply be a consequence of the fungus being low abundance until this time point.
309 Interestingly, there are three groupings of genes (Groups i-iii) that demonstrate patterns that cannot be
310 explained merely by fungal abundance in the samples. Group i consists of genes that are expressed early
311 and depressed later on in infection, Group ii contains genes that turn on during the later phases of growth

312 in the living host but are turned off after the fly is killed and Group iii consists of genes that do not turn on
313 until after the fungus has killed the host. At present, there is almost nothing known what gene products
314 these transcripts encode, as there is little homology between entries in protein databases and the translated
315 open reading frames in these transcripts.

316 Next, we examined host gene expression patterns across all of our samples, again ordering samples
317 based on the proportion of *E. muscae Berkeley* aligned reads among all total and clustering genes by
318 expression pattern (Figure 4C). Host gene expression segregates into six major groupings (Table S1). Group
319 i and Group iii contain genes with low expression in controls and early infection, but increased expression
320 as infection continues, and are enriched for genes involved in epithelial integrity and sensory processes,
321 respectively. Group ii shows induction of expression all exposed samples except for two taken at 96 hours
322 (asterisks) and is highly enriched for genes involved in the innate immune response to fungi. Group iv
323 contains genes that, broadly speaking, are expressed in controls and early infection but not during later
324 infection and is enriched for genes involved in the metabolism of carbohydrates, steroids and lipids, as well
325 as cellular respiration. Group v and Group vi largely trend in the same direction, with genes in both groups
326 tending to be expressed in control samples 72 hours and later and a handful of exposed samples, including
327 three samples (one 48 hour sample and two 96 hour samples) that behave aberrantly compared to other
328 biological replicates. These groups are enriched for genes with functions in broad and basic cell functions
329 including DNA synthesis and repair, transcription, translation and cell cycle control (Group v) as well as
330 protein localization, mitochondrial translation, autophagy and homeostasis (Group vi).

331 Following our initial overview of host transcription, we next looked at genes that were consistently
332 different between control and exposed samples from 24-72 hours (Figure 4D). We excluded all cadaver
333 samples (both 96 and 120 hours) from this pooled analysis because the animals are dead, and variations in
334 gene expression would be confounded by mRNA degradation. We also opted to exclude animals at 96 hours
335 because two of these three samples do not show immune induction (Figure 4C, Group ii). One-way
336 ANOVA analysis between exposed and control animals from 24-72 hours demonstrated that genes that are
337 under-expressed in exposed animals are enriched for a handful of metabolic processes, including arginine
338 and gluatamine synthesis. Interestingly, both arginine and glutamine are amino acids synthesized from the
339 Kreb's cycle intermediate alpha-ketoglutarate. In times of starvation, the cell would be expected to
340 prioritize generating ATP via the Kreb's cycle over synthesizing these amino acids. The idea that the fly is
341 starving is consistent with these enrichments and also with the observation that basic cell metabolism
342 (macromolecule synthesis) is substantially decreased at 72 hours (Fig S7).

343 The same analysis shows that genes that are over-expressed in exposed animals are enriched for
344 immune function, including the melanization defense response and Toll-dependent pathways. *E. muscae*
345 *Berkeley* infection relies on boring through the host cuticle which should elicit an initial melanization

346 response, consistent with our observation. However, it is generally thought that *E. muscae Berkeley* evades
347 the host immune response once inside the fly because it grows without a cell wall (i.e. protoplastically) and
348 therefore does not present antigens that can alert the fly immune system to infection [29,30]. Examining
349 expression patterns of all genes annotated as having immune function, we see a large induction of immune
350 gene expression at 24-48 hours which includes genes both involved in the melanization response and genes
351 that specifically respond to fungal infection. In addition, we see overexpression of several groups of
352 immune genes compared to uninfected controls that persists into late infection (72 and 96 hours) and even
353 into death (96 and 120 hours) (Fig S8) These data suggest that the initial immune response may not be
354 strictly limited to wound repair and show that the host immune system remains engaged throughout
355 infection.
356
357 *E. muscae Berkeley* is present in the fly nervous system 48 hours after exposure
358 To better understand the process of *E. muscae Berkeley* infection in *D. melanogaster*, we next used
359 a histological approach to examine the interior anatomy of exposed flies. Analogous to the transcriptomic
360 time course, we collected adult flies (a mix of 50 males and females) every 24 hours for the first 168 hours
361 after *E. muscae Berkeley* or mock exposure. Flies were fixed before embedding and sectioning in paraffin
362 then stained with Safranin O/Fast Green (SFG), a contrast staining method that facilitates the differentiation
363 of fungal versus host cells (Richard Humber, personal communication), though is more commonly used for
364 plant histology. We identified *E. muscae Berkeley* morphology by examining *E. muscae Berkeley*-killed
365 hosts. While there is slide-to-slide variability in the exact hue for a given tissue stained with SFG, generally,
366 we observed that SFG-stained *E. muscae Berkeley* hyphal bodies have nuclei that stain red (or dark purple)
367 and cytoplasm that stains purple (Figure 5). *E. muscae Berkeley* nuclei are consistently sized throughout
368 the host which helps in distinguishing them from host *D. melanogaster* cells.
369

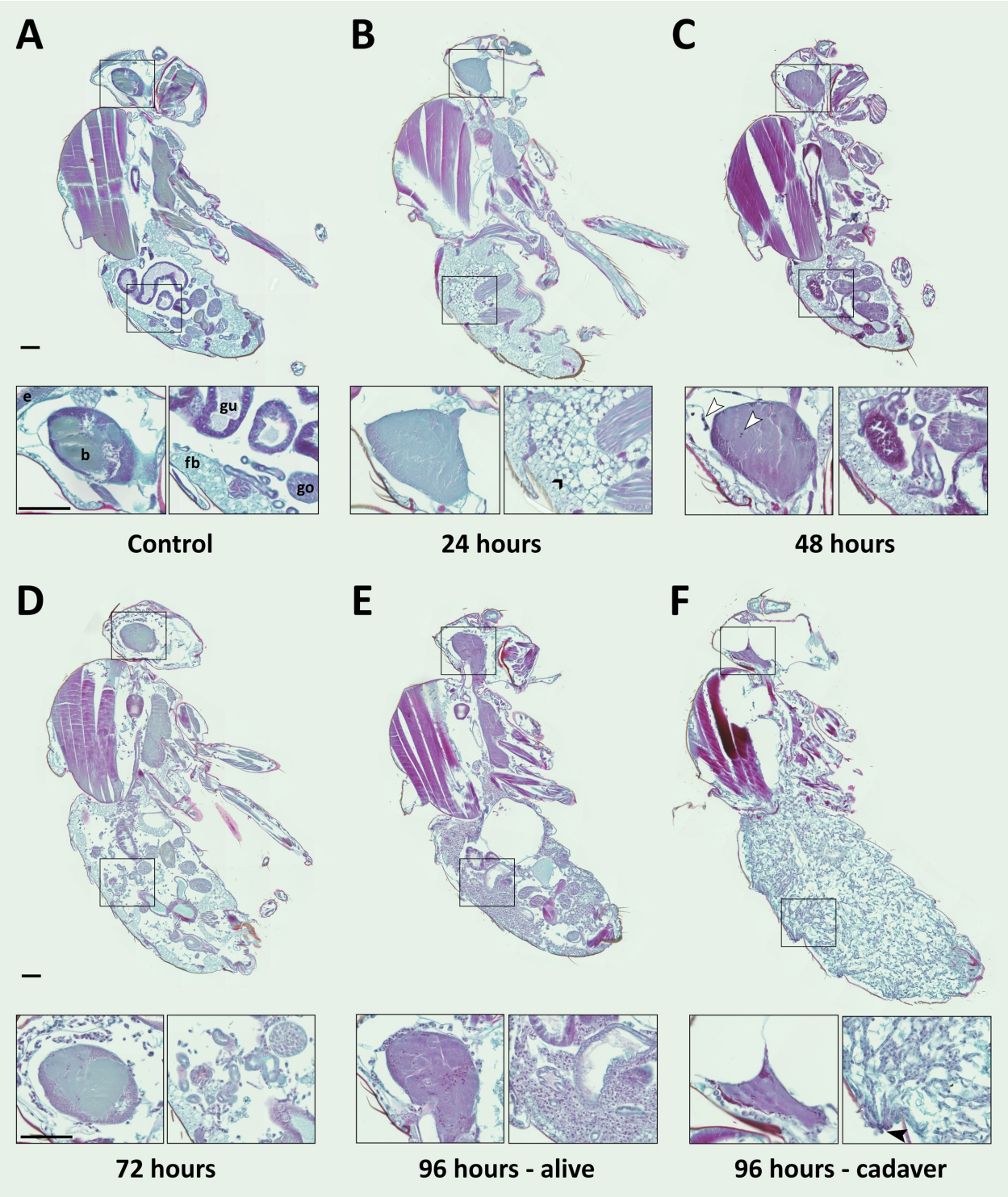
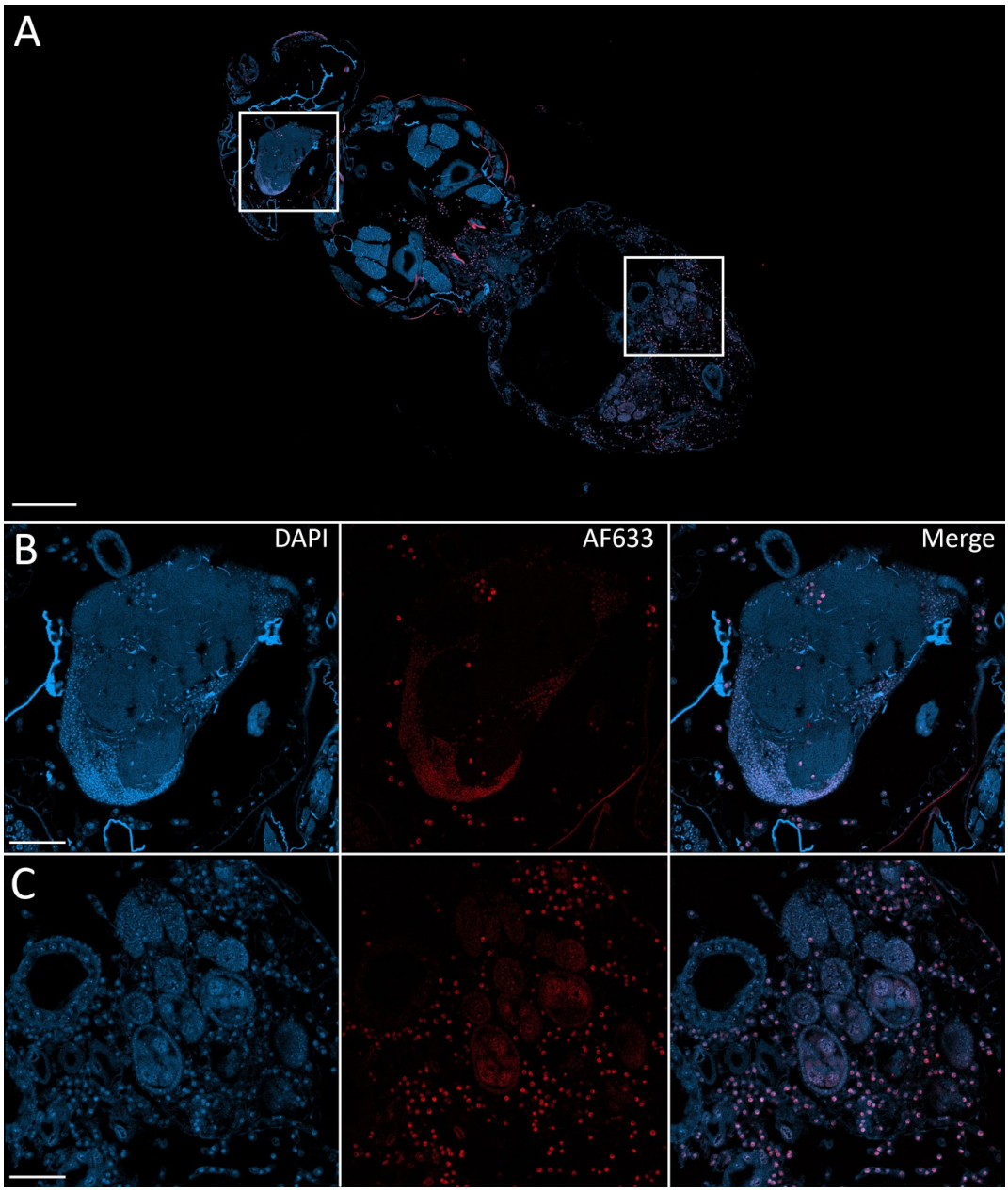


Figure 5. *E. muscae Berkeley* is consistently present in the nervous system starting 48 hours after exposure. CantonS flies were exposed or mock-exposed (control) to *E. muscae Berkeley* starting 3-5 hours after the light-dark transition and were subsequently sampled at 24, 48, 72 or 96 hours for histological analysis. For each time point, 4-6 individual, paraffin-embedded flies were sectioned at 8 µm, stained using

375 Safranin O/Fast Green to identify fungal morphology and location and imaged at 20x magnification (Zeiss
376 Axio Scan.Z1 slide scanner). Only male flies are shown here for ease of comparison. No differences in the
377 progression of the infection were observed between males and females. An inset of the brain and the
378 abdomen are shown for each sample. A) Uninfected fly with major anatomical features labeled as follows:
379 e - eye, b - brain, g - gut, t - testes, f - fat body. B) At 24 hours after exposure there is significant
380 immunological activity in the abdomen; the nervous system is devoid of fungal cells. C) At 48 hours after
381 exposure *E. muscae Berkeley* cells are present in the brain (white arrowheads) and/or ventral nerve cord
382 (VNC) of all but one sample where *E. muscae Berkeley* cells abut but have not yet entered brain. A handful
383 of *E. muscae Berkeley* cells are observed in the abdominal and/or thoracic hemolymph at 48 hours. The gut
384 and testes are not invaded by fungus. D) At 72 hours after exposure, *E. muscae Berkeley* can be found
385 throughout the body cavity and the amount of visible fat body has decreased. *E. muscae Berkeley* titers
386 have increased in the nervous system. E) In a living fly at 96 hours after exposure (the first point at which
387 a fly may be killed by *E. muscae Berkeley* infection), fungus occupies virtually all available volume in the
388 hemolymph. *E. muscae Berkeley* titers have increased in the nervous system, gut and gonads remain
389 uninvaded. F) In an *E. muscae Berkeley*-killed fly (cadaver) at 96 hours after exposure, only traces of host
390 organs remain in the abdomen and the nervous system has been considerably degraded. No fat body cells
391 are observed. *E. muscae Berkeley* cells differentiate into conidiophores, cell-walled structures that will
392 pierce through weak points of the cuticle to produce and launch infectious conidia. Black scale bars are 100
393 µm. All living animals shown are males; cadaver's sex is undetermined (the gonads have been consumed
394 by the fungus.)
395
396 We then carefully examined SFG-stained sections from exposed and control flies to determine
397 where the fungus resides and how fly tissue is impacted over the course of infection (Figure 5). As we
398 observed no difference in fungal localization between males in females, all samples from a given time point
399 are described regardless of sex. In control animals, sagittal sections consistently show abundant fat body
400 cells in the abdomen surrounding the gut and gonads. Fat body is also apparent, though less abundant, in
401 the head and thorax. The thorax is predominantly occupied by muscles (generally staining red), which are
402 also observed in the legs. At 24 hours after exposure, we observed hemocyte activity in the abdomen, with
403 all other tissues indistinguishable from controls. Though hemocyte activity indicates that the immune
404 system is responding to the fungus, we could not unambiguously identify the fungal cells anywhere in the
405 body cavity at this time point. We must therefore conclude that the fungus adopts a morphology that is
406 different from that of cadavers.
407 At 48 hours after exposure, fungal cells are consistently observed in the brain and/or ventral nerve
408 cord (VNC; 4 out of 5 samples). In the one case where fungus had not invaded the nervous system, hyphal

409 bodies were apparent immediately adjacent to the brain, abutting the blood brain barrier. A handful of
410 fungal cells are also observed in the abdomen or thorax, with some samples showing hemocyte activity as
411 in 24 hour samples. At 72 hours after exposure, fungal growth is apparent throughout the body cavity and
412 some hemocyte activity can still be observed. The fat body is depleted compared to earlier time points and
413 fungus is apparent between muscle fibers, but the gut and gonads all appear indistinguishable from controls.
414 In addition, fungal titers increase in the brain and VNC. In infected animals that survived 96 hours, fungal
415 growth is rampant throughout the entire body cavity (head, thorax and abdomen), with the fat body
416 substantially depleted and fungus residing between muscle fibers. There is no apparent damage to the gut
417 or gonads. Occasional hemocyte activity can still be observed; fungal titers continue to increase in the brain
418 and VNC. In *E. muscae Berkeley* killed cadavers, fungus is apparent throughout the body cavity, especially
419 in the abdomen. The gut and gonads have been completely degraded by the fungus, the brain has begun to
420 be degraded and the muscles are largely intact.
421 To confirm that the morphologies observed in the nervous system at 48 hours after exposure and
422 beyond were *E. muscae Berkeley*, we used fluorescence *in situ* hybridization (FISH) to specifically label
423 *E muscae* Berkeley cells within the context of an infected fly. By performing FISH with a fluorescently-
424 labeled DNA probe targeting the most abundant repeated 18mer in the *E. muscae Berkeley* genome
425 (~11,000 copies, Bronski, Elya and Eisen, unpublished), we verified that *E. muscae Berkeley* is present in
426 the brain and VNC in infected animals (Figure 6).
427



428

429 **Figure 6. Fluorescence in situ hybridization confirms that *E. muscae Berkeley* resides in the nervous**
430 **system during infection.** CantonS flies were exposed or mock-exposed (control) to *E. muscae Berkeley*
431 starting 3-5 hours after the light:dark transition and were subsequently sampled at 24, 48, 72 or 96 hours
432 for histological analysis. For time points 48 hours and later, at least 3 individual, paraffin-embedded flies
433 were sectioned at 8 µm and subjected to FISH with an *E. muscae Berkeley*-specific 18mer DNA probe
434 labeled at the 5' end with AlexaFluor633. Sections were imaged at 40x magnification on a confocal
435 fluorescent microscope (Zeiss 800 LSM). A) Psuedo-coronal section of a female sampled 96 hours after
436 infection stained with an *E. muscae Berkeley*-specific probe and DAPI. Regions shown at higher detail in
437 B and C located are denoted by white boxes. Scale bar is 200 µm. B) Enlargement of top region in A

showing each DAPI, AlexaFluor633 and merged images of this area. *E. muscae* Berkeley nuclei are strongly labeled and can be observed inside the host neuropil. Fungal nuclei are also observed in the head outside of the brain. Scale bar is 50 µm. C) Enlargement of bottom region in A showing each DAPI, AlexaFluor633 and merged images of this area. *E. muscae* nuclei are observed in abundance outside of gut and ovaries. Scale bar is 50 µm.

Discussion

A remarkably diverse array of microbes have independently evolved the ability to alter animal behavior. The prospect of understanding how they do this is intrinsically fascinating, and potentially of great practical value as a means to understand how animal behaviors are generated and how we might manipulate them in disease therapy, pest control and other contexts. While an increasing number of researchers have been drawn to these systems, sustained progress has been limited by the lack of anything approaching an ideal model system. We believe that the combination presented here of a strain of *E. muscae* that infects wild *Drosophila*, protocols for propagating this strain in lab.-reared flies, and the robust induction of behaviors in the laboratory has the potential as to serve the model system that finally allows us to successfully wield the tools of modern molecular genetics and neuroscience to describe the molecular mechanisms that underlie at least one example of microbial manipulation of animal behavior.

E. muscae in wild drosophilids

Though to our knowledge we are the first to study a naturally *Drosophila*-infecting strain of *E. muscae* in the laboratory, we are not the first to encounter *E. muscae* circulating in wild *Drosophila*. In 1927, Goldstein reported finding *Empusa muscae* (now *E. muscae*)-infected cadavers of *Drosophila repleta* as well as *Musca domestica* at Columbia University in New York state, stating that an epidemic of *E. muscae* had been observed for the previous four years in this location [20]. In 1969, Turian and Wuest reported observing *E. muscae*-infected cadavers of wild *Drosophila hydei* in a rotting fruit bait in Geneva, Switzerland [19]. In 2002, Keller et. al. reported morphological parameters for an *E. muscae* strain (putatively identified as *E. ferdinandii*, a member of the *E. muscae* species complex) infecting *Drosophila spp* in Switzerland [21].

Notably, the discovery of *E. muscae Berkeley* has not been our only observation of *E. muscae* infecting wild fruit flies. In fall of 2014, members of our group caught two individuals from the southern bay area that were some days later found killed by *E. muscae* (Quan and Schiabor, unpublished). During a return to the same site in fall 2015, we recovered several additional *E. muscae* cadavers. In the fall of 2016, a wild drosophilid collected from a site in the north bay was also killed by *E. muscae*. Interestingly, all of

472 these samples from Northern California, including those found in the fendel, are identical at the LSU and
473 ITS loci. Samples recovered from a colleague at two distinct locations in Southern California show distinct
474 sequences at these loci, suggesting that multiple strains (or species) are infecting wild *Drosophila*. Drawing
475 from these observations as well as other unpublished reports of *E. muscae* infections in fruit flies across the
476 continental United States (D. Tighe, S. Dara, B. de Bivort), we propose that *E. muscae* infections in wild
477 *Drosophila* populations are more common than heretofore recognized. Based on our experience, the
478 infections seem to be positively correlated with high, local densities of fruit flies in temperate habitats,
479 which is consistent with how *E. muscae* infection is propagated.

480 It remains unclear both 1) if the strain(s) or species infecting *Drosophila spp* are distinct from those
481 that infect other fly species and 2) the degree of specificity for *Drosophila spp* over other dipterans. We
482 have observed that *E. muscae Berkeley*-infected *D. melanogaster* cadavers are capable of infecting *M.*
483 *domestica* in the laboratory, but it is unclear if this infection occurs frequently in the wild. Whether infection
484 can occur naturally would depend on the ecology of the two different host species (i.e. if they interact
485 frequently enough to expect the exchange of *E. muscae* infection) Our understanding of strain diversity and
486 host specificity would greatly benefit from the collection of more molecular and ecological data.
487

488 <u>The progression of *E. muscae Berkeley* infection in lab.-reared *D. melanogaster*</u>

489 Taken together, our RNAseq and histology time course data describe the typical progression of *E. muscae*
490 infection in lab.-reared *D. melanogaster*. At 24 hours after exposure, flies show a robust antifungal immune
491 response, though the fungus is nearly undetectable within the fly by histology, indicating that it is at low
492 titer. At 48 hours, the fungus has begun to adopt the morphology which it will assume until killing the host.
493 Fungus is observable within the host brain and VNC, though overall fungal titer is still quite low. As the
494 abdomen is the most likely point of initial entry for the fungus (it is the biggest target for the fungus to hit),
495 we suspect that the fungus has travelled from the point of entry to the CNS, indicating tropism for neural
496 tissue early in the infection. Elements of the host's immune response are still activated. At 72 hours, fungus
497 was apparent throughout the body cavity, in the thorax (between muscle fibers), abdomen (surrounding but
498 not invading the gut and gonads) and also in the limbs and halteres. The fat body is significantly depleted
499 by this point; the host's dampened metabolism suggests an internal starvation state. At 96 hours, if the fly
500 has not succumbed to infection (i.e. there are still energy reserves available to the fungus), fungal titer will
501 continue to increase and the fat body will continue to be depleted. Two-three hours after death, flies that
502 have been killed by the fungus show no intact abdominal organs and nervous systems that are being broken
503 down.

504 While the trajectory of infection is consistent, it is important to recognize that just because two
505 animals have been exposed for the same duration of time that these two animals will not progress through

506 infection identically. In our RNAseq data, we noticed that the host gene expression in exposed animals at
507 24 and 48 hours tended to be more variable than those for 72 hours. We imagine that this was due to chance,
508 that we picked animals that were at similar points in infection at 72 hours, whereas we picked animals that
509 were more variable at other time points. This may at least partly explain why we observed so much
510 differential expression at the 72 hour time point in exposed versus controls whereas less was observed at
511 earlier time points, especially at 48 hours. It is likely that several factors play into whether or not an infection
512 succeeds and how quickly it progresses (e.g. initial exposure titer, size of host, nutritional status of host
513 etc.). Thus, future work should consider how to determine a metric to gauge progress of infection so that
514 similarly-progressed samples can be compared.
515
516 *E. muscae Berkeley* infection and host immune response
517 The entomopathogen community has believed that *E. muscae* evades the host response by growing
518 protoplastically (i.e. without a cell wall, components of which would be recognized and targeted by the host
519 immune system). In both the gypsy moth and the greater wax moth, it has been shown that the host immune
520 cells recognize walled *Entomophaga* fungal cells, but there is little cellular response to protoplasts [31,32].
521 Based on these findings, it has been posited that the host does not detect the ever-increasing fungal mass
522 within until the end of infection when the fungus puts on a cell wall that contains epitopes that the host can
523 recognize [29,30]. As a result of ostensibly evading the immune system, it has also been hypothesized that
524 *E. musca*e does not generate toxins, as it would have no incentive to do so in the absence of attack by the
525 host [29].
526 Our data show that there is a robust initial response to *E. muscae Berkeley* exposure. Many of the
527 immune genes that are induced with *E. muscae Berkeley* have also been observed to be induced by exposure
528 to other, more generalist fungal pathogens (e.g. *Beauveria bassiana*, *Metarhizium anisopliae* [33,34]).
529 These data clearly indicate that the host detects an invader early on in infection. Furthermore, there is a
530 detectable immune response through the length of infection (Fig S8), though at this point we cannot say if
531 this response is a slow disengagement of the initial response or stimulated *de novo* by the growing fungus.
532 Interestingly, the living animals sampled at 96 hours for RNAseq are inconsistent in their host
533 transcriptional immune response: two of the three animals more closely resemble control animals than
534 infected animals in host transcription (Figure 4C). At least two scenarios could explain this observation. It
535 is possible that both of these animals were in the process of recovering from infection (i.e. the immune
536 system was effectively combatting the fungus) or there was a delay in the course of infection compared to
537 contemporaneous samples. The proportion of fungal reads present in these samples is lower than what
538 would be expected for late time points, which is consistent with either scenario. At this point we simply do
539 not know if every instance of a spore hitting a fly leads to a productive fungal infection. There is some

evidence to the contrary: we have consistently observed that some highly-exposed flies die prematurely. These animals are generally smaller than others in the vial and are often covered in spores. This could indicate that getting hit by too many spores (an unlikely outcome in the natural world) leads to an overwhelmed fly (e.g. overactive immune system or accelerated fungal growth) that dies before being manipulated. These flies do not sporulate, though it is possible that they do produce resting spores. On the other hand, we have observed that survival of exposed flies is substantially increased when flies are exposed to small quantities of anti-fungal. This indicates that there are ways of either halting or slowing an infection, though whether the fly's immune system is generally capable of doing this is unknown.

Though five of our six cadavers sampled for transcriptomics have similar levels of immune gene transcripts compared to *E. muscae Berkeley*-exposed animals sampled at 72 hours, the sixth cadaver exhibits higher expression of anti-fungal peptides Drosomycin and Metchkinowin, the beta-glucan receptor GNBP3-like and several IM family genes. It is possible that this fly is demonstrating the proposed immune-system overload and resultant spike in immune system gene expression [29]. It might be the case that this immune spike occurs in all animals; we could have sampled too late to observe it in the other five samples but were able to see it in one sample that was late to respond. However, one can imagine that in the presence of copious fungal epitopes the fly immune system would continue to go be highly engaged until death, not drop back down to levels comparable to earlier time points (i.e. 72 hours), as seen in the majority of sampled cadavers. At present, we are inclined to interpret this odd-sample-out as a fluke rather than an indication of a moribund immune spike.

Why is *E. muscae Berkeley* in the brain?

Our work demonstrated that *E. muscae Berkeley* is present in the nervous system system relatively early in infection, just 48 hours following exposure. *E. muscae Berkeley*'s invasion of the nervous system grants the fungus direct access to host neurons and may be mechanistically important for achieving behavioral manipulation of the host fly. However, we should be careful to consider any and all possible ways *E. muscae Berkeley* could alter host behavior before jumping to this conclusion.

We can imagine four general mechanisms by which *E. muscae* is able to achieve behavioral manipulation. The fungus could invade the nervous system in order to localize adjacent to and impinge on the activity of particular neurons through chemical or physical means. However, we are skeptical that this is the case as our observations do not support specific localization of the fungus in the CNS.

A second possibility is that the fungus invades the nervous system in order to gain access to either a particular group or groups of neurons or all neurons generally, but does not localize within the CNS in a stereotyped manner. Rather it is sufficient that it has crossed the blood brain barrier, which insulates the nervous system from the activities in the hemolymph and allows for the selective transport of compounds

574 to and from the hemolymph, allowing the fungus to modulate the activity of neurons by secreting
575 compounds that diffuse throughout the CNS. The secreted compounds could be specific, only altering the
576 activity of a subset of susceptible neurons, or could be more general, changing activity over many or all
577 neurons.

578 A third possibility is that the fungus does not need to invade the nervous system in order to change
579 the host's behavior. The fungus could be secreting a compound into a hemolymph that is capable of crossing
580 the blood brain barrier and altering neuronal activity. Alternatively, the fungus could be secreting a
581 compound into the hemolymph that changes the host's internal state (either directly or by leading the host
582 to respond in a way that causes the internal state to change) which leads the animal to respond by executing
583 one or more of the end-of-life behaviors.

584 Lastly, it's possible that the fungus does not secrete compounds to induce these behaviors, but by
585 destroying fly tissues elicits the series of observed behaviors. While we believe this last scenario to be
586 highly unlikely, it cannot yet be ruled out.

587 For these last two proposed mechanisms, the fungus would not need to invade the CNS in order to
588 affect behavior. In these cases, the fungus could be invading the CNS as a means of escaping immune
589 surveillance. By establishing a reservoir in the CNS, the fungus could replenish dying cells in the
590 hemolymph in order to ensure that the infection took hold. Alternatively, the fungus could invade the CNS
591 because it provides a rich, nutritive substrate to sustain the fungus. This scenario is inconsistent with our
592 histological data both from flies that are not executing end-of-life behaviors (Figure 5) and flies that are
593 executing end-of-life behaviors show that the brain is largely intact, indicating that the fungus abstains from
594 consuming these tissues until host death.

595 Our observation that *E. muscae Berkeley* invades the host nervous system contrasts with the recent
596 report that *Ophiocordyceps unilateralis,* a fungal pathogen that induces very similar end-of-life phenotypes
597 in the ant host *Camponotus castaneus* is absent from the brain at the point of behavioral manipulation [35].
598 Interestingly, another Entomophthoralean fungus, *Strongwellsea magna,* is also known to invade the
599 nervous system of its lesser house fly host (*Fannia canicularis*) during infection [36]. In this case, the
600 author proposed that this did not have consequences for behavior.

601 Surprisingly, transcriptomic analysis of dissected brains from exposed females at 24, 48 and 72
602 hours with confirmed *E. muscae Berkeley* infections failed to show differential gene expression compared
603 to uninfected controls but did show an increase in *E. muscae Berkeley* titer (taking *E. muscae Berkeley*
604 reads as a proxy) (Fig S9). Though these samples were not collected at the point of behavioral manipulation
605 by the fungus, it is surprising that there are no major transcriptional changes within the brain at these time
606 points, and suggests that behavioral modification may be largely independent of transcriptional changes in
607 the brain.

608

609 Behavior and beyond: the utility of the *E. muscae Berkeley-D. melanogaster* system

610 The past decade has seen an explosion of tools for characterizing and manipulating the nervous system of
611 *D. melanogaster*, including a catalog of the types and corresponding expression patterns of its
612 approximately 100,000 neurons, a complete map of connections in the brain, reagents for conditionally
613 activating or disabling specific sets of neurons as well as purifying these cells, and methods for
614 automatically tracking and classifying behaviors in populations. Our development of a robust system of
615 microbially induced behavior manipulation in *D. melanogaster* will allow us, and we hope many others, to
616 leverage the powerful molecular and neurobiological toolkit of *D. melanogaster* to explore the molecular
617 basis of this fascinating but still mysterious biological phenomenon.

618

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620

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633 entomopathogens. CE also acknowledges the support of Nora, Bruce and Kevin.

634

635 **Author Contributions**

636

637 CE discovered *Drosophila* infected with *Entomophthora* in her backyard, figured out how to reliably
638 passage the infection to lab-reared flies, carried out all of the experiments described in this paper, made all
639 the figures and wrote the manuscript. TCL and QES helped maintain populations of infected flies for
640 experiments and provided experimental assistance, and HM assisted with the early characterization of the

641 *Entomophthora* infection. MBE encouraged the pursuit of this project by CE, obtained funding for the
642 research, provided feedback and ideas throughout, and edited and advised on figures and the manuscript.
643
644 **Funding**
645
646 All of the research described in this paper was funded by an HHMI Investigator award to MBE. CE was
647 supported by a National Science Foundation Graduate Research Fellowship.
648
649 **Materials and Methods**
650
651 Fly husbandry
652 Healthy wild-type, CantonS Wolbachia-free (WF) D. melanogaster were reared on Koshland diet (0.68%
653 agar, 6.68% cornmeal, 2.7% yeast, 1.6% sucrose, 0.75% sodium tartrate tetrahydrate, 5.6 mM CaCl2, 8.2%
654 molasses, 0.09% tegosept, 0.77% ethanol, 0.46% propionic acid) supplemented with activated dry yeast
655 pellets (Red Star) at 21C on a 12:12 light:dark photoperiod. Any time the photoperiod needed to be adjusted,
656 flies were reared from third instar or earlier on the desired photoperiod to ensure that emerging adults were
657 properly entrained.
658
659 Fendel tending
660 Wild fruit flies were caught by directly aspirating from an uncovered plastic dishwashing pan (aka the
661 "fendel") that was baited with quartered organic watermelon and an assortment of other organic fruits.
662 Aspirated flies were transferred onto Koshland diet and housed at ambient temperature and ambient
663 humidity. Baiting and capture was performed in the spring through early fall of 2015 at a personal residence
664 in Berkeley, CA.
665
666 PCR genotyping
667 DNA was extracted from individual cadavers or 1.5 mL of *in vitro* culture using the QIAamp Micro Kit
668 (QIAGEN) following the tissue protocol. These DNA preparations were used to amplify the desired
669 sequences. Entomophthora-specific ITS primers (emITS: emITS-1 5'-
670 TGGTAGAGAATGATGGCTGTTG-3', emITS-4 5'- GCCTCTATGCCTAATTGCCTTT-3') or fungal-
671 specific large subunit primers (LSU: LR3-1 5'- GGTCCGTGTTTCAAGAC-3', LR0R-4 5'-
672 GTACCCGCTGAACTTAAGC-3') were used to genotype Entomophthora (James et al 2006); cytochrome
673 oxidase II primers (tLEU: 5' ATGGCAGATTAGTGCAATGG 3' and tLYS: 5'

674 GTTTAAGAGACCAGTACTTG 3') were used to genotype infected Drosophila hosts (Liu and
675 Beckenbach (1992). Each reaction was performed using GoTaq 2x colorless mastermix (Promega) with
676 800 nM of each forward and reverse primer with the following thermocycling conditions: 95C for 5 min
677 followed by 35 iterations of 95C for 30 seconds, 51C for 30 seconds then 72C for 1 min/kb then 72C for
678 an additional 10 minutes. Reactions were checked by gel electrophoresis in 1% agarose. Successful
679 reactions were prepared for sequencing using ExoSap-IT (Affymetrix) per manufacturer's instructions and
680 submitted with each amplification primer for Sanger sequencing. Assembly of forward and reverse reads
681 was attempted to generate a consensus sequence using Seqman Pro (DNA Lasergene v.10). Sequences were
682 searched against the BLAST NT database using blastn.
683
684 Isolating *E. muscae Berkeley in vitro*
685 To grow E. muscae Berkeley in vitro, first spores were collected using the ascending conidia collection
686 method (i.e. by placing a fresh cadaver in the bottom of a sterile petri dish and allowing the cadaver to
687 sporulate overnight) (144). The following morning, the lid of the dish was rinsed with 10 mL of Grace's
688 insect medium (1x) supplemented with L-glutamine, 3.33g/L lactalbumin hydrolysate, 3.33g/L yeastolate
689 (ThermoFisher Scientitfic # 11605- 094) containing 5% fetal bovine serum (Invitrogen) and transferred to
690 a vented, treated T25 tissue culture flask (Corning # 10-126-10) using sterile technique. The culture was
691 then incubated at room temperature in the dark until growth was apparent (non- homogenous white spheres
692 floating in the medium). The culture was genotyped with emITS and LSU primers to confirm that it was E.
693 muscae and was an identical match to the cadaver that produced the spores which started the culture
694 ("Fendel mama") at these loci. The culture was periodically examined at 100-400x on a compound
695 microscope to confirm proper morphology and absence of contamination.
696
697 Isolating and optimizing *in vivo E. muscae* Berkeley infection
698 Wild fruit flies sampled from the fendel and housed on Koshland food were monitored nightly for two
699 weeks after capture for deaths due to *E. muscae* Berkeley. Freshly killed cadavers were separated from their
700 living conspecifics by briefly anesthetizing live flies via cold anesthesia (incubating 2-3 minutes in a
701 residential freezer). Fresh cadavers (anywhere from 1-10, depending on availability) were placed on chunks
702 of organic banana in a wide Drosovial with kimwipes to sop up excess moisture. Approximately 50 healthy
703 flies were then transferred onto the prepared vial by flipping (no anesthesia was used). The plug of the vial
704 was pushed down to confine the flies within a few centimeters to improve the likelihood that they would
705 encounter flying spores. Leaving the exposed flies with the spent cadavers was initially problematic as we
706 were working without access to anesthesia or a microscope and had to identify new cadavers by naked eye.
707 Additionally, the raw banana began to ferment and break down, leading to excess moisture which was

prematurely killing some of our exposed flies. To avoid these issues, the exposed flies were transferred to a new banana/kimwipe vial after the first 48 hours. This was done by allowing the living flies to climb into an empty "holding" vial then flipping them onto the fresh vial. The flies were monitored daily for deaths; cadavers were removed after allowing healthy flies to climb into a "holding" vial and used to start new exposure vials.

In vivo propagation of *E. muscae Berkeley* infection

Cadavers are collected daily 2-5 hours after the end of the 12 hour light cycle from exposure vials that are between 96 and 168 hours (4 and 7 days) old. All flies that will die on this day because of *E. muscae* Berkeley infection will be dead by this time and will be extremely swollen with fungal growth, making them obvious among the living flies. CO2 is used to anesthetize the living flies in exposure vials and collect cadavers, which are placed in a petri dish with a piece of Whatman paper wetted with DI water to mitigate static. Molten cadaver embedding medium is preparing by microwaving solidified AS solution (1.5% agar, 10% sucrose) and poured into a clean 100 x 15 mm petri dish just enough to cover the bottom of the dish. As soon as the agar has set, six cadavers are embedded head first in a circle of diameter <2 cm with their wings on the outside of the circle. The wings are pressed into the agar to ensure they do not intercept any launched conidia. The agar is allowed to completely set before continuing. The cadaver circle is cut out from the set agar by using an empty, wide-mouth Drosophila vial (FlyStuff). The agar disc containing the fly circle is then transferred, cadaver-side up, into an empty, wide-mouth Drosophila vial. A ruler is used to mark 2 cm above the surface of the agar. CantonS flies reared on the same 12 hour light cycle on Koshland medium are collected from eclosing vials using CO2 anesthesia. Fifty healthy flies are added to the vial container the agar disc with cadavers and tapped down until all flies are under the 2 cm mark. A Droso-plug (FlyStuff) is pushed into the vial such that its bottom is flush with the 2 cm mark. The vials is incubated for the first 24 hours at 18C in a humid chamber (~95% humidity, 2L plastic beaker lined at the bottom with wetted paper towels or kimwipes and covered with foil), to encourage sporulation. After 24 hours, the Droso-flug is lifted to relieve the confinement of the flies and the vial is moved to a 21C incubator (~60% humidity). After 48 hours, the exposed flies are transferred onto GB+ medium (40% organic banana [w/v], 2% agar, 0.3% propionic acid) without anesthesia; incubation continues at 21C. Cadavers are collected daily 2-45 hours after the end of the 12 hour light cycle from exposure vials that are between 96 and 168 hours (4 and 7 days) old. The process is repeated daily to supply cadavers for experiments and ensure the infection is maintained.

Photography and videography

741 Pictures and videos of dead and dying flies (extending probosces and raising wings) were taken with a
742 Nexus 5x (Google) or iPod Touch (Apple) aided by attaching a macrolens (Luxsure) to the device camera
743 lens or by aligning the device camera lens with the eyepiece of a dissecting scope. Images were taken under
744 ambient light, humidity and temperature.

745 Time lapse microscopy was taken via a USB microscope (DinoLite Digital Microscope Pro) using
746 DinoLite software v1.12 (Figure 3A) or on a Nikon 80i compound microscope equipped with a Hamamatsu
747 black and white camera (C11440) using MetaMorph software (v. 7.8.00, Molecular Devices) (Figure 3B,C).
748 Each time lapse consists of images collected once a minute for the indicated duration. Images were taken
749 under ambient temperature and humidity.

750 High-speed videos (18,000-54,000 fps) were filmed with a 5x objective on a Axiovert 200
751 microscope (Zeiss) equipped with an Photron Fastcam 1024PCI. Cadavers were mounted in 1.5% agar on
752 a glass slide and arranged such that forming conidiophores and conidia were visible (for spore ejection) or
753 such that cadavers sporulated onto a #1 glass coverslip in the plane of the camera (for spore landing). Video
754 was captured via Photron Fastcam Viewer software, running at the indicated frames per second in end
755 trigger mode (new frames were continually captured and old frames dumped until the user triggered the
756 filming to stop). Spores or landing sites were manually watched until a spore disappeared or appeared, then
757 video was stopped and last ~10 seconds of footage were manually searched for the spore launching or
758 landing event.

759

760 Circadian timing of death

761 CantonS WF flies were reared on a 12:12 light cycle (photophase 1 am – 1 pm or 7 pm - 7 am PDT, as
762 indicated). For experiments run in complete darkness, 25 flies were exposed to E. muscae Berkeley via the
763 standard anesthesia-dependent protocol. All exposure vials were set up before the utilized cadavers
764 sporulated (within 2 hours of the light-dark transition). Exposure vials were housed in a humid chamber in
765 a dark 21C incubator wrapped in blackout cloth for approximately 24 hours before loading into Drosophila
766 activity monitors (DAMs, Trikinetics). Before loading flies, DAM tubes (5 mm x 65 mm, polycarbonate)
767 were prepared containing such that one end of the tube held approximately one cm of 1.5% agar, 10%
768 sucrose and was wrapped in parafilm to prevent drying out.

769 To load the flies, all accessible light sources were disabled before removing the humid chamber
770 from 21C and placing on the bench at RT. Vials of flies were kept under a foil-lined box as they waited to
771 be processed. One vial at a time was retrieved from the box, knocked out with CO2 under a dissecting scope
772 whose LED light ring was covered with a red gel filter (Neewer), sorted by sex and loaded into individual
773 DAM tubes with the aid of a red LED flashlight (KMD Aero) before capping each tube with an autoclaved
774 cotton plug. For each vial, eight females and eight males were chosen for loading into DAM vials.

775 Unexposed flies (i.e. controls) were always processed before proceeding to exposed flies. CO2 pad was
776 wiped down with 70% ethanol between vial types to present cross-contamination. DAMs were loaded from
777 bottom to top row, filling a row and securing each tube with rubber bands before proceeding to the next.
778 Loaded tubes were kept under a foil-lined box to prevent light exposure. When all loading was finished,
779 DAMs were covered with blackout cloth and transported back to the 21C incubator. There they were
780 attached to the DAM interface unit and recording began, binning counts in 30 second intervals. Recording
781 continued for ~170 hours until the experiment was stopped.

782 Like with loading, experiments were stopped by first disabling all light sources, then carefully
783 disconnecting and removing DAMs from the 21C incubator to not disturb adjacent experiments, and sealing
784 incubator before turning on the overhead lights for manual inspection. Each DAM tube was inspected to
785 see if the fly was dead or alive. If dead, the tube was inspected for evidence of sporulation to determine if
786 the cause of death was patent *E. muscae* infection. For experiment run on a 12:12 light cycle, flies were
787 exposed as above but without concern for light contamination; resultant DAMs were housed on a 12:12
788 light cycle for the duration of the experiment. Each channel was checked daily following sundown to see
789 which flies had died within the previous 24 hours.

790 DAM data were processed using Python to determine time of last movement (accurate to 30
791 seconds) and to plot movements over time. For each channel, the reported time of last movement was
792 manually cross-checked using the plot of activity data. In cases where there was an erroneous movement
793 (i.e. a signal occurring more than 24 hours after the fly's last movement), the time of last death was manually
794 re-assigned. For data visualization, data were binned into 15-30 minute intervals and the average
795 movements of unexposed animals (controls), exposed or entrained light cycle of exposed flies and cadavers
796 and the time of last movement for each observed cadaver were plotted in Prism (GraphPad). There were
797 generally no obvious differences between male and female activity for the unexposed animals so sexes were
798 combined for data analysis.
799

800 <u>Collection and staining of primary conidia</u>

801 Three to six fresh cadavers (i.e. those who had not yet sporulated) were collected from exposure vials using
802 the anesthesia dependent methods detailed above. Sporulation chambers were prepared as follows: a small
803 piece of Whatman paper was placed in the base of a small petri dish (60 mm x 15 mm) and wetted with DI
804 water. A bloated cadaver was chosen for each chamber and its wings were removed. The cadaver was
805 placed in the middle of the Whatman paper and the chamber was topped with a custom, 3D-printed top that
806 included a square opening slightly smaller than a standard 22x22 mm coverslip. The top and bottom were
807 sealed using parafilm and a new coverslip was placed over the opening. Cadavers were left in the chambers
808 at room temperature to sporulate. Coverslips were changed every 30 minutes to 1 hour, as needed, and

809 promptly stained for microscopy by applying Hoechst (1 ug/mL). Spores were imaged on a compound
810 microscope at 40x for measuring conidia attributes; exact distances were determined by calibration with a
811 0.01 mm micrometer (OMAX). For each attribute (number of conidia, length and width of conidia, diameter
812 of nuclei), at least 50 different primary conidia were counted from three different cadavers.
813
814 RNA experiments
815 RNA was prepared from each thawed sample by homogenizing with an RNase-free pestle (Kimble Chase),
816 washing the pestle with 750 uL Trizol, then proceeding using the manufacturer's protocol. For reverse-
817 transcription PCR (RT-PCR) and transcriptomic experiments, three mock vials and three Three mock vials
818 and three exposure vials were started with 25 CantonS WF flies 0-1 days old (RT-PCR, whole flies) or 1-2
819 days old (dissected brains) with either 0 (mock) or 6 (exposure) cadavers embedded in AS. Flies were
820 incubated for the first 24 hours at 18C confined to 2 cm with cadavers, then moved to 21C where the
821 confinement was relieved. Flies were transferred to GB+ at 48 hours where they continued to be housed at
822 21C. (Vials were sampled every 24 hours for 72 (dissected brains), 96 (RT-PCR) or 120 (whole flies) hours
823 by anesthetizing the entire vial with CO2. Exposed animals were preferentially selected based on evidence
824 of contact with fungus (e.g. spores or melanization responses thereto visible on cuticle). Sampling for each
825 time point consistently occurring between 2-3 hours following the light-dark transition. Before sampling,
826 all equipment used to manipulate flies (e.g. CO2 pad, forceps etc.) were treated with 10% bleach, wiped
827 with DI water then sprayed with 70% ethanol. All materials that handled flies (CO2 gun, pad, forceps) were
828 treated with 10% bleach and rinsed with DI water between sampling exposure vials. Control vials were
829 always sampled first. Sampled material (either whole fly or dissect brain) was immersed in 250 uL Trizol
830 then immediately flash frozen with liquid nitrogen. Samples were stored at -80C until extraction.
831
832 RT-PCR
833 One control female (24-96 hours) and two infected females (24-72 hours) or one fresh cadaver (96 hours)
834 from each vial were collected as described above. RNA was prepared from each thawed sample by
835 homogenizing with an RNase-free pestle (Kimble Chase), washing the pestle with 750 uL Trizol, then
836 proceeding using the manufacturer's protocol. RNA was then treated with Turbo DNase (ThermoScientific)
837 per the manufacturer's protocol and quantified using a Qubit Fluorometer (Qubit RNA HS assay kit,
838 ThermoFisher Scientific). For each sample, 1 µL or 160 ng of DNase-treated RNA, whichever was more,
839 was added to a new, nonstick tube and mixed with two pmol primer emITS1, 770 nM dNTPs in a final
840 volume of 13 µL. The reaction was incubated at 65C for 5 minutes then incubated on ice for at least 1
841 minute before proceeding. To the mixture was added 5x First Strand Buffer (1x final, ThermoFisher
842 Scientific), 100 mM DTT (5 mM final, ThermoFisher Scientific), 1 uL RNaseOUT (ThermoFisher

843 Scientific) then 200 units of SuperScript III RT (ThermoFisher Scientific). After thorough mixing, each
844 tube was incubated at 55C for 60 minutes to reverse transcribe then 70C for 15 minutes to heat kill the
845 transcriptase. To amplify E.muscae-specific cDNA, 2 uL of the reverse transcription reaction was mixed
846 with GoTaq 2x colorless mastermix (1x final, Promega) and 500 nM each primers emITS1 and emITS4
847 (5'- GCCTCTATGCCTAATTGCCTTT-3') then run on a thermocycler with the following settings: 95C
848 for 5 min followed by 35 iterations of 95C for 30 seconds, 61C for 30 seconds then 72C for 30 seconds
849 then 72C for an additional 10 minutes. Four µL of each reaction was analyzed by gel electrophoresis in 1%
850 agarose.
851
852 Whole fly *in vivo* RNAseq time course
853 One control female (24-120 hours) and two infected females (24-72 hours), one infected female and one
854 cadaver (96 hours) or one cadaver (120 hours) from each vial were collected as described above. RNA was
855 prepared from each thawed sample by homogenizing with an RNase-free pestle (Kimble Chase), washing
856 the pestle with 750 uL Trizol, then proceeding using the manufacturer's protocol. RNA was quantified
857 using a Qubit Fluorometer (Qubit RNA HS assay kit, ThermoFisher Scientific) and quality was checked by
858 running on a RNA 6000 Pico chip on a Bioanalyzer 2100 (Agilent Technologies). High quality RNA was
859 then treated with Turbo DNase (ThermoScientific) per the manufacturer's protocol. RNAseq libraries were
860 prepared with the TruSeq RNA v2 kit (Illumina) using 500 ng of input RNA per sample. Samples were
861 multiplexed 21 samples to a lane and sequenced using 100 bp paired-end reads on a HiSeq 4000 at the QB3
862 Vincent J. Coates Genomic Sequencing Facility at UC Berkeley.
863
864 Dissected brain RNAseq
865 Brains were individually dissected and sampled from first three control and then three exposed females.
866 Each animal was dissected in sterile 1x PBS in its own disposable dissection chamber (35mm petri dish
867 lined with 2-3% agar) and dissecting forceps were treated with 3.5% hydrogen peroxide then rinsed with
868 sterile water between samples to prevent nucleic acid carryover. The body of each animal was saved and
869 subjected to a DNA extraction using the manufacturer's provided protocol for the isolation of genomic
870 DNA from tissues (QIAamp DNA Micro kit, QIAGEN) eluting in 20 µL of buffer AE. For each fly body,
871 1 µL was used to template a PCR reaction consisting of 12.5 µL GoTaq, 2 µL of each primer emITS1 and
872 emITS4 (10 µM stocks), and 7.5 µL water for a final volume of 25 µL. Reactions were cycled with the
873 following conditions: 95C for 5 minutes followed by 35 cycles of 95C for 30 seconds, 51C for 30 seconds
874 and 72C for 1 minute, then a final 10 minute extension at 72C. Reactions were analyzed via gel
875 electrophoresis to confirm that all exposed animals had come into contact with *E. muscae* Berkeley and that
876 control animals were uninfected.

877 RNA was prepared from each thawed sample by homogenizing with an RNase-free pestle (Kimble
878 Chase), washing the pestle with 750 uL Trizol, then proceeding using the manufacturer's protocol. RNA
879 was quantified using a Qubit Fluorometer (Qubit RNA HS assay kit, ThermoFisher Scientific) and quality
880 was checked by running on a RNA 6000 Pico chip on a Bioanalyzer 2100 (Agilent Technologies). One
881 replicate control RNA sample for the 48 hour time point was lost prior to library preparation so was omitted.
882 High quality RNA was then treated with Turbo DNase (ThermoScientific) per the manufacturer's protocol.
883 RNAseq libraries were prepared with the TruSeq RNA v2 kit (Illumina) using all of the extracted RNA for
884 each brain, 17-75 ng of input RNA per sample. Samples were multiplexed 17 samples to a lane in equimolar
885 amounts and sequenced using 100 bp paired-end reads on a HiSeq 4000 at the QB3 Vincent J. Coates
886 Genomic Sequencing Facility at UC Berkeley.
887
888 *E. muscae* reference transcriptome assembly
889 An initial reference (Emus-Ref1) was assembled from reads from exposed *in vivo* time course samples that
890 had first failed to align as pairs to the D. melanogaster transcriptome (r6.11, HiSat2) then failed to align as
891 singletons to the D. melanogaster genome (r.611, bowtie2) using TRINITY with the developer's
892 recommended settings [37]. After assembly, all *in vivo* time course reads were aligned to Emus-Ref1 to
893 assess contamination of non-*E. muscae* sequences. All Emus-Ref1 transcripts were searched using blastn
894 for homology (evalue 1e-50 or smaller) to organisms not annotated as fungi or virus. These transcripts were
895 removed to generate Emus-Ref2. All *in vivo* time course reads were aligned to Emus-Ref2 to assess
896 contamination of non-E. muscae sequences. Transcripts that were not expressed by any sample (TPM = 0)
897 or where TPM of uninfected samples accounted for more than 10% of TPM summed across all samples
898 were removed to generate Emus-Ref3. Transcriptome completeness was estimated by BUSCO v1.1
899 analysis using the fungal reference set (1438 BUSCOs).
900
901 RNAseq data analysis
902 To calculate gene expression, reads were pseudo-aligned to the appropriate reference (dmelDBGP6.rel85
903 for D. melanogaster or Emus-Ref3 for E. muscae) and transcript abundance was estimated using Kallisto
904 [38]. Data were analyzed using hierarchical clustering by gene (Cluster 3.0), ANOVA between grouped
905 treatments (scipy.stats) and GO term analysis (Panther [39]). Hierarchical clustering heatmaps were
906 generated in Java TreeView; other data were plotted in matplotlib (Python), Prism (GraphPad) or Excel
907 2013 (Microsoft).
908
909 Paraffin embedding and microtomy of whole flies

910 Two mock and two exposure vials were started daily for seven days each with 50 CantonS WF flies 0-1
911 days old with either 0 (mock) or 6 (exposure) cadavers embedded in AS. Flies were incubated for the first
912 24 hours at 18C confined to 2 cm with cadavers, then moved to 21C where the confinement was relieved.
913 Flies were transferred to GB+ at 48 hours where they continued to be housed at 21C. Vials were sampled
914 every 24 hours via CO2 anesthesia then infiltrated and embedded in paraffin. For detailed protocol, see
915 https://www.protocols.io/u/carolyn-elya. Briefly, flies were fixed 24-36 in ice-cold Carnoy's (6:3:1
916 ethanol:chloroform:glacial acetic acid) at 4C. Samples were next dehydrated by stepping through a series
917 of increasing ethanol concentrations Samples were then transitioned into Histoclear (National Diagnostic)
918 before slowly introducing Paraplast (Sigma). Samples were infiltrated with Paraplast for at least 84 hours
919 at 60C with gentle shaking before embedding in base molds with embedding rings (Thermo Scientific) and
920 drying overnight. Samples were stored at room temperature until they were sectioned at 8 µm with an
921 RM2255 microtome (Leica), applied to Polysine slides (ThermoFisher Scientific) and dried overnight at
922 42C. Sections were stored at room temperature for up to three weeks before Safranin O/Fast Green FCF
923 staining or up to one week before fluorescence in situ hybridization (FISH).
924
925 Safranin O/Fast Green FCF staining of paraffin sections
926 Slide-mounted sections were dewaxed with two, 10 minute changes of Histoclear then rehydrated to 70%
927 ethanol with a decreasing ethanol series. Sections were then stained one-at-a-time following Johansen's
928 Safranin and Fast Green protocol [40] then checked under a dissecting scope before mounting in DEPEX
929 mounting medium (Electron Microscope Sciences) and drying overnight. Slides were imaged using a 20x
930 objective with the Axio Scan.Z1 (Zeiss).
931
932 Fluorescent *in situ* hybridization (FISH) of paraffin sections
933 Slide-mounted sections were dewaxed with two, 10 minute changes of Histoclear then rehydrated to 70%
934 ethanol with a decreasing ethanol series. Slides were incubated in 0.2 M HCl at 37C for 45-60 minutes and
935 rinsed in DI water before applying 80 µL of hybridization solution (20 mM Tris-HCl pH 8.0, 0.9 M NaCl,
936 0.01% sodium dodecyl sulphate, 30% formamide) containing 100 pmol/µL of an *E. muscae* Berkeley-
937 specific DNA probe (AlexaFluor633-5'-TGCTAAAACAGCACAGTT-3', ThermoFisher Scientific).
938 Slides were incubated overnight in a humid chamber at room temperature. The following day, slides were
939 briefly washed in 1x PBS with 0.3% Triton-X100, rinsed in 1x PBS and mounted in ProLong Gold with
940 DAPI (ThermoFisher Scientific). Slides were cured for 24 hours before imaging on a LSM 800 confocal
941 microscope (Zeiss) with 5x-40x air objectives.

Supporting Information

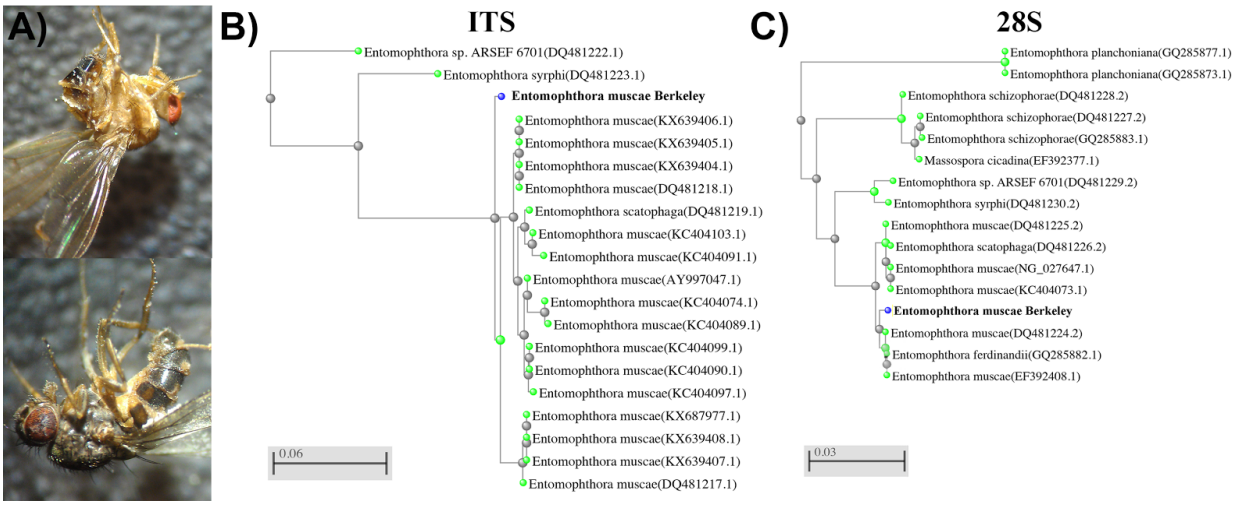


Figure S1. Discovery of *E. muscae Berkeley*. A) *E. muscae Berkeley*-killed cadavers discovered in bait on July 25, 2015. Note remnants of fungal growth through the intersegmental membrane of dorsal abdomen (above and below) and spores deposited on wings (above and below) and legs (below). B,C) BLAST (blastn) results as trees (fast minimum evolution) for B) consensus *E. muscae Berkeley* ITS (13 sequences) and C) *E. muscae Berkeley* 28S (11 sequences). Gray legend bars show difference in percent identity between sequences.

Figure S2. Confirmation that *E. muscae* Berkeley is growing *in vitro*. A) Alignment of ITS region from *in vitro* culture (iv_culture) and consensus ITS sequence from fendel cadavers (fendel). B) Same as A, but for LSU region.

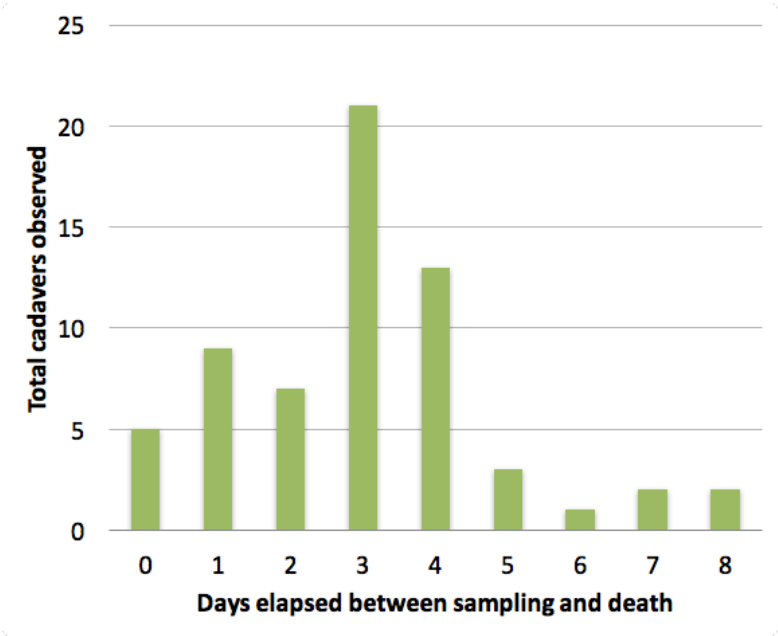
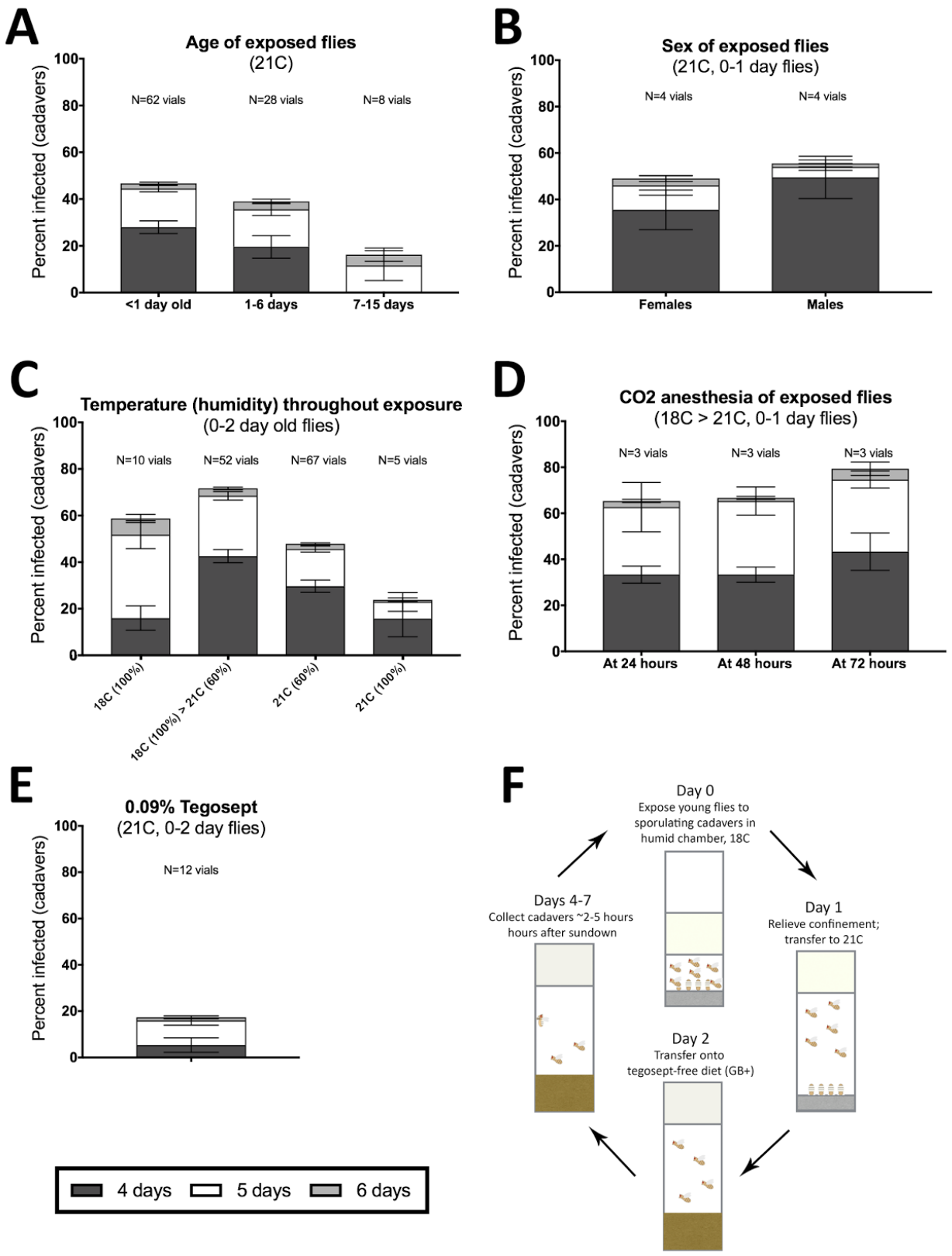


Figure S3. Time between capture of wild *Drosophila* and death by *E. muscae* infection when housed on Koshland diet. Flies were housed at ambient temperature and humidity in a second-story apartment during August and September 2015 in Berkeley, CA. A total of 63 cadavers of wild *Drosophila* were observed out of approximately 900 flies aspirated and monitored during this time.


A
Age of exposed flies
(21C)
Percent infected (cadavers)
N=62 vials
N=28 vials
N=8 vials
<1 day old
1-6 days
7-15 days
B
Sex of exposed flies
(21C, 0-1 day flies)
N=4 vials
N=4 vials
Females
Males
C
Temperature (humidity) throughout exposure
(0-2 day old flies)
N=10 vials
N=52 vials
N=67 vials
N=5 vials
18C (100%)
18C (100%) > 21C (60%)
21C (60%)
21C (100%)
D
CO2 anesthesia of exposed flies
(18C > 21C, 0-1 day flies)
N=3 vials
N=3 vials
N=3 vials
At 24 hours
At 48 hours
At 72 hours
E
0.09% Tegosept
(21C, 0-2 day flies)
N=12 vials
4 days
5 days
6 days
F
Day 0
Expose young flies to sporulating cadavers in humid chamber, 18C
Day 1
Relieve confinement; transfer to 21C
Day 2
Transfer onto tegosept-free diet (GB+)
Days 4-7
Collect cadavers ~2-5 hours hours after sundown

960

961 **Figure S4. Optimization of *in vivo E. muscae* infection of CantonS WF *D. melanogaster* under**
962 **laboratory conditions.** A-E) Percentage of infected cadavers at 96 hours (4 days), 120 hours (5 days) or
963 144 hours (6 days) after exposure to *E. muscae* Berkeley upon varying A) age; B) sex; C) temperature and
964 humidity; D) CO2 anesthesia for 20 minutes at the indicated post-exposure time or E) housing on 0.09%
965 tegosept for 120 hours beginning 48 hours after exposure. 18C > 21C indicates that vials were incubated
966 24 hours at 18C, 100% humidity then transferred to 21C, ~60% humidity. If no percent is indicated, then
967 humidity was ~60%. All vials used 4-6 cadavers to establish infection and were set up using the anesthesia-
968 independent protocol. Replicate vials for each condition are shown above the graph. For all panels each vial
969 contained 50 flies. F) Summary of method for *in vivo* propagation of *E. muscae* in CantonS WF *D.*
970 *melanogaster*. Briefly, 50 healthy, young (eclosed within the last 24 hours) CantonS flies of mixed sex are
971 confined within 2 cm of a circle of 6 cadavers embedded head-first in sucrose agar. Vials are incubated 24
972 hours at 18C and ~100% humidity. On Day 1 (24 hours since exposure) the vial plugs are raised to the top
973 of the vial and incubation continues for the next 24 hours at 21C with ~60% humidity. On Day 2 (48 hours
974 since exposure), flies are moved away from cadavers and onto GB+ diet. From Days 3-7 (96 to 168 hours
975 since exposure), vials are monitored 2-5 hours following "sundown" to collect fresh cadavers. These
976 cadavers are then used to begin new vials (Day 0).

977 https://youtu.be/111Wks5wYCs

978 **Movie S1. *E. muscae* Berkeley-infected CantonS fly undergoing end-of-life proboscis extension.** Video
979 recorded through the eyepiece of a dissecting microscope on a Nexus 5x phone (Google). Capture and
980 playback are in real time.

981

982 https://youtu.be/IiqJcpfBAGQ

983 **Movie S2. *E. muscae* Berkeley-infected CantonS fly undergoing end-of-life wing raising, viewed from**
984 **the side.** Video was captured with a Nexus 5x phone (Google) and macro lens (Luxsure). Capture and
985 playback are in real time.

986

987 https://youtu.be/DdR-iSdYG6A

988 **Movie S3. *E. muscae* Berkeley-infected CantonS fly undergoing end-of-life wing raising, viewed head-**
989 **on.** Video was captured with a Nexus 5x phone (Google) and macro lens (Luxsure). Capture and playback
990 are in real time.

991

992 https://youtu.be/qDCZJmhWkbU

993 **Movie S4. Animated time lapse of *E. muscae* Berkeley-infected CantonS cadavers undergoing spore**
994 **production and ejection.** Sporulation time lapse in *E. muscae Berkeley* killed cadavers. Time listed at the
995 top right corner of each frame is the time that has elapsed since the light-dark transition. One image was
996 taken every minute for ~24 hours with three cadavers situated on a cotton flug at ambient temperature and
997 humidity. Images are played back at 10 fps.

998

999 https://youtu.be/T5Pi0JoEm0I

1000 **Movie S5. A primary conidium is ejected from a conidiophore formed in an *E. muscae* Berkeley-**
1001 **killed cadaver.** Arrowheads indicates conidium that launches and the vacant conidiophore that remains
1002 after launch. Video was captured at 54,000 frames per second (fps) at 5x magnification; frames are played
1003 back at 5 fps. Scale bar is 25 µm.

1004

1005 https://youtu.be/B3BHDwvagzg

1006 **Movie S6. A primary conidium lands on the lid of a polystryene petri dish.** The conidium lands as one
1007 complete unit, supporting the fungal cannon mechanism of primary conidium ejection in *E. muscae*. Video
1008 was captured at 18,000 fps at 5x magnification; frames are played back at 5 fps. Scale bar is 25 µm.

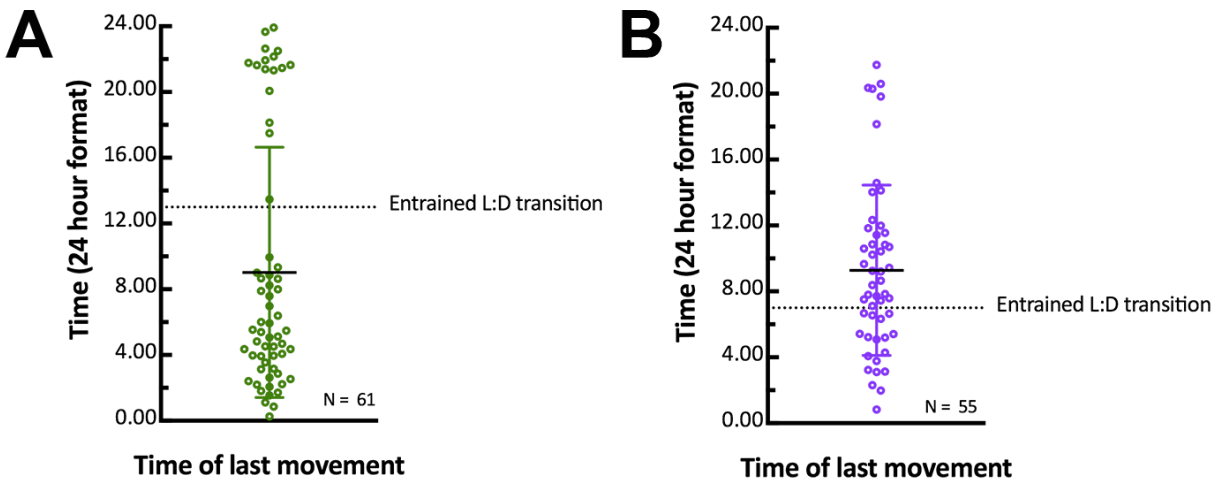


Figure S5. *E. muscae Berkeley*-infected CantonS flies housed in constant darkness do not consistently die in a gated fashion. Each circle represents the time of last movement observed for one cadaver as detected by the DAM. Flies were raised on the indicated 12:12 light:dark cycle then exposed to *E. muscae Berkeley* as per standardized protocol. Dotted line indicates the expected light-dark transition based on the entrained light cycle. A) Flies entrained on a 12:12 light:dark cycle with photophase beginning at 1 am and scotophase beginning at 1 pm. B) Flies entrained on a 12:12 light:dark cycle with photophase beginning at 7 pm and scotophase beginning at 7 am.

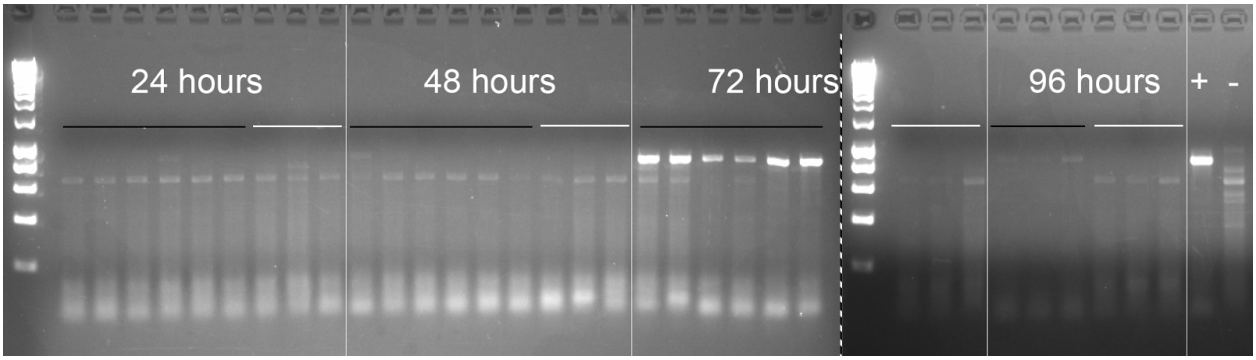


Figure S6. Reverse-transcription PCR of *E. muscae* Berkeley ITS sequence in exposed and control flies. All samples were run on the same gel with equal loading volumes; samples to the right of the dashed line were run on the lower half of the same gel containing samples to the left of dotted line (see methods for sample generation). DNA ladder (5 µL Hyperladder 1 kb, Bioline) was run in the first lane of each gel half. Samples are shown in chronological order, with the time point indicated above the left-most sample. Black lines indicated exposed flies; white lines indicate unexposed flies. Exposed flies collected at 96 hours were cadavers that had died of *E. muscae* Berkeley infection. Plus (+) indicates positive control (*E. muscae* Berkeley *in vitro* RNA template); minus (-) indicates additional negative control (*D. melanogaster* RNA from earlier experiment, before the discovery and introduction of *E. muscae* Berkeley to the laboratory).

https://youtu.be/GeTRUiBIW8s

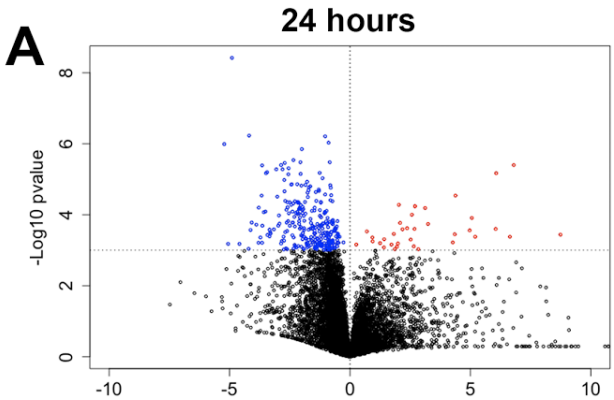
Movie S7. Formation of primary conidia atop conidiophores. The intersegmental membranes of a fresh *E. muscae Berkeley*-killed cadaver were imaged at 20x magnification every minute for 222 minutes beginning 4 hours and 10 minutes after sunset. The time lapse is played back at 10 fps. Scale bar is 50 µm.

https://youtu.be/1vrXbDWHQCw

Movie S8. Formation of secondary conidia from off-target primaries. The wing of an *E. muscae* Berkeley-killed cadaver was imaged at 20x magnification every minute for 361 minutes beginning 10 hours and 30 minutes after the light-dark transition. The time lapse is played back at 10 fps. Scale bar is 100 µm.

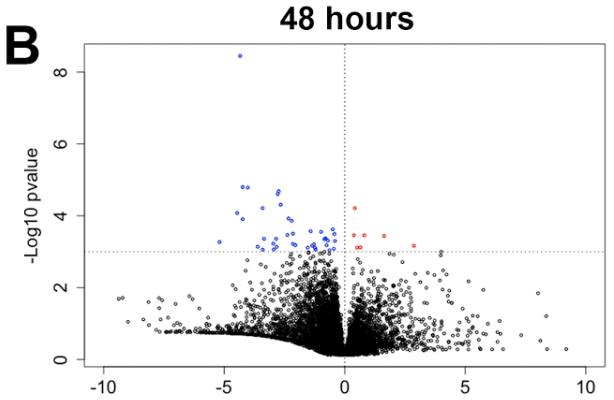
Table S1. GO term enrichments of host gene groups as given in Figure 4C. Panther GO-term analysis (complete biological process) for genes in Groups i-vi (Figure 4C).

- See file Table_S1.pdf

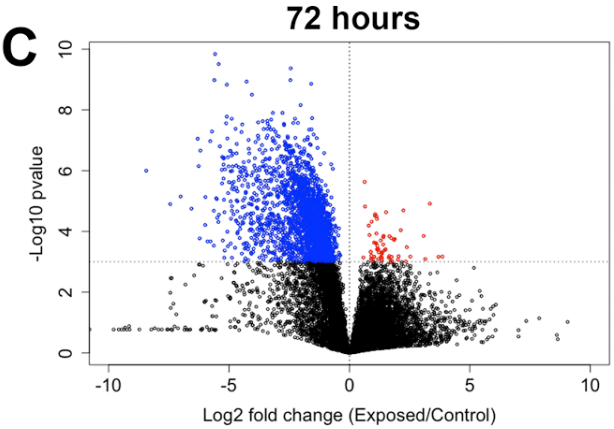


GO term	Fold enrichment	P-value
antifungal humoral response (GO:0019732)	150	6.17E-03
response to bacterium (GO:0009617)	13.23	2.31E-03
Toll signaling pathway (GO:0008063)	59.74	6.59E-05

GO term	Fold enrichment	P-value
glyceraldehyde-3-phosphate metabolic process (GO:0019682)	40.33	9.97E-03
glucose 6-phosphate metabolic process (GO:0051156)	40.33	9.97E-03
glutamine family amino acid metabolic process (GO:0009064)	13.07	4.03E-03
nicotinamide nucleotide metabolic process (GO:0046496)	11.63	3.35E-04
monosaccharide metabolic process (GO:0005996)	9.54	3.09E-02
single-organism catabolic process (GO:0044712)	5.6	3.82E-07
generation of precursor metabolites and energy (GO:0006091)	5.41	1.24E-05
monocarboxylic acid metabolic process (GO:0032787)	5.33	4.16E-03
oxidation-reduction process (GO:0055114)	4.08	2.06E-12
chitin-based cuticle development (GO:0040003)	3.98	2.23E-02
nucleic acid metabolic process (GO:0090304)	< 0.2	6.75E-03
gene expression (GO:0010467)	< 0.2	5.81E-04



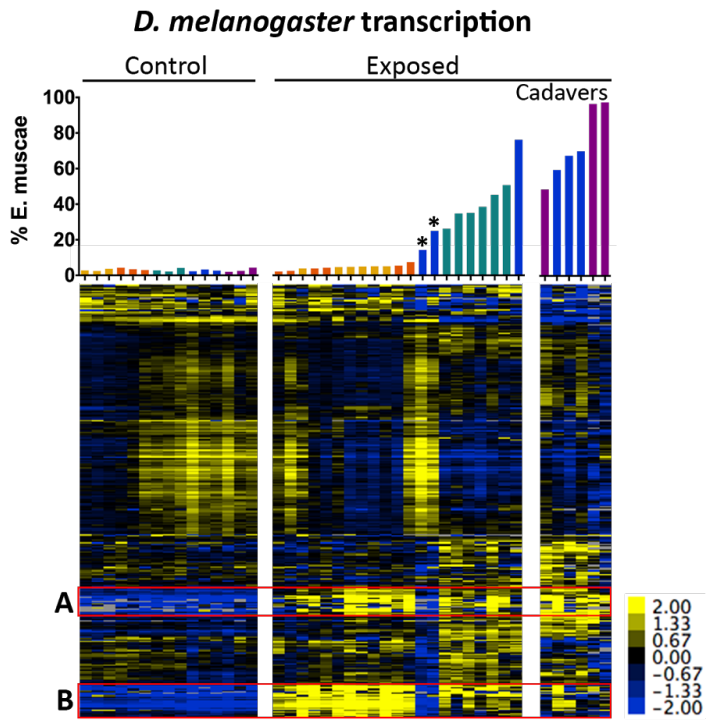
No significant GO term enrichments



GO term	Fold enrichment	P-value
chromatin assembly (GO:0031497)	6.22	4.35E-11
DNA replication (GO:0006260)	4.55	5.80E-19
regulation of cell cycle (GO:0051726)	3.93	3.45E-06
mRNA 3'-end processing (GO:0031124)	3.87	1.99E-02
RNA catabolic process (GO:0006401)	3.64	7.92E-06
rRNA metabolic process (GO:0016072)	3.48	3.23E-12
DNA repair (GO:0006281)	3.48	6.65E-14
tRNA aminoacylation for protein translation (GO:0006418)	3.22	6.04E-04
nuclear transport (GO:0051169)	3.05	3.34E-06
tRNA metabolic process (GO:0006399)	3	3.16E-07
nucleobase-containing compound transport (GO:0015931)	2.97	9.31E-05
chromosome segregation (GO:0007059)	2.75	4.10E-04
mRNA splicing, via spliceosome (GO:0000398)	2.62	2.37E-07
mitosis (GO:0007067)	2.57	1.55E-11
protein folding (GO:0006457)	2.57	9.01E-05
RNA splicing, via transesterification reactions (GO:0000375)	2.5	5.76E-05
protein targeting (GO:0006605)	2.05	1.40E-02
nitrogen compound metabolic process (GO:0006807)	2.02	1.31E-35
protein complex assembly (GO:0006461)	2.01	8.64E-03
chromatin organization (GO:0006325)	1.98	1.74E-05
response to stress (GO:0006950)	1.75	1.09E-04
phosphate-containing compound metabolic process (GO:0006796)	1.62	1.90E-07
catabolic process (GO:0009056)	1.61	9.31E-06
transcription from RNA polymerase II promoter (GO:0006366)	1.6	5.21E-05
cellular protein modification process (GO:0006464)	1.6	1.08E-05
biosynthetic process (GO:0009058)	1.56	1.55E-07
cell-cell signaling (GO:0007267)	< 0.2	6.60E-03
sensory perception (GO:0007600)	< 0.2	4.31E-07
mesoderm development (GO:0007498)	< 0.2	4.48E-04

Figure S7. Genes exhibiting differential expression between flies after mock exposure or exposure to *E. muscae* at 24, 48 or 72 hours. A) Left: Volcano plot for all genes at 24 hours. P-value is determined by ANOVA grouping control vs. exposed samples. Genes with p-value < 0.001 are shown in color. Right: Panther GO-term analysis (complete biological process) of genes overexpressed in exposed animals (red)

1046 or control animals (blue). B) Left: Volcano plot for all genes at 48 hours. P-value is determined by ANOVA
1047 grouping control vs. exposed samples. Genes with p-value under 0.001 are shown in color. Right: No
1048 significant GO term enrichments were found for differentially-expressed genes. C) Left: Volcano plot for
1049 all genes at 72 hours. P-value is determined by ANOVA grouping control vs. exposed samples. Genes with
1050 p-value under 0.001 are shown in color. Right: Panther GO-term analysis (complete biological process) of
1051 genes overexpressed in control animals (blue). There are no significant GO term enrichments for set of
1052 genes overexpressed in exposed samples.



Group A	Group B	
PGRP-LB	AttC	Tep2
CG10433	Mtk	MP1
Sp7	Dro	NimB3
PGRP-SA	DptB	Hayan
Spn28Dc	PGRP-SD	CG42259
grass	Drs13	
Spn27A	Drs12	
spirit	CG15065	
AttA	SPH93	
AttB	SPE	
TotM	GNBP-like3	
CecC	NimB1	
CecB	IM3	
Listericin	IM2	
IM18	IM14	
DptA	IM23	
edin	IM1	
CG30098	nec	
CecA2	IM4	
CecA1	Cpr49Ac	

Figure S8. Expression of immune genes over the course of infection of *D. melanogaster* by *E. muscae* Berkeley. Complete linkage hierarchical gene clustering by gene was performed in Gene Cluster 3.0 on all genes annotated with defense response (GO 0006952) after filtering out across all genes that are expressed at least at two TPM in at least three out of 42 samples (10,875 transcripts total), then log transforming and centering on the mean value for each transcript. Samples are ordered by percentage of *E. muscae* Berkeley reads as a fraction of the total reads aligned (above). The scale bar for the heatmap is given to the right of the plot. Two 96 hour exposed samples that show an aberrant immune response compared to all other exposed samples are indicated by asterisks.

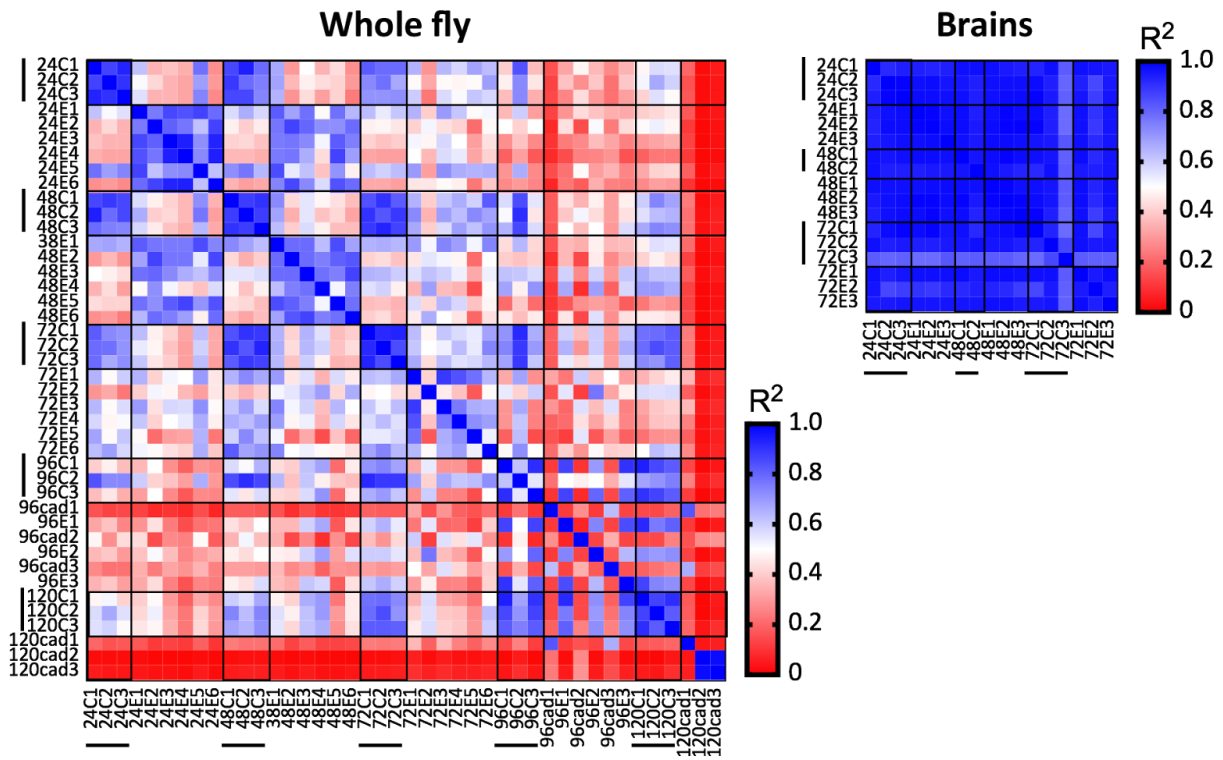


Figure S9. Host gene expression in the brain is stable over the first 72 hours of *E. muscae Berkeley* infection. Right) All pairwise linear correlations between samples from *E. muscae Berkeley*-infected whole fly RNAseq time course. Left) All pairwise linear correlations between samples from *E. muscae Berkeley*-infected dissected brain pilot RNAseq time course. Samples are named in the following format: HourTypeReplicate, with "C" indicating controls, "E" indicating exposed flies living at the time sampled and "cad" indicates that the fly had been killed by *E. muscae Berkeley* before sampling. For example, "24C1" indicates a the first replicate control sample (uninfected fly) taken at 24 hours after mock exposure. Black rectangles outline rows and columns containing correlation values for control samples. Control samples are denoted on each axis with a black bar.

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