28 Minutes Later: A behavioral and transcriptomic investigation into the potential role of aflatrem-like compounds in establishing *Ophiocordyceps* extended phenotypes in zombie ants

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ABSTRACT

Parasites and their hosts often share intimate coevolutionary relationship that can lead to bizarre phenotypes over time. *Ophiocordyceps* is one such genus that has evolved to manipulate the behavior of its various insect and arachnid hosts, the clearest examples of which are found in ants of the Camponotus tribe, colloquially known as “zombie ants”. While the unusual behaviors induced during infection are well described, the molecular driving force behind these changes are still unknown. Recent genomic and transcriptomics analyses have identified several candidate secreted proteins and secondary metabolites highly upregulated during infection. Of particular interest amongst these compounds is an aflatrem-like ergot alkaloid related to the mycotoxin aflatrem known to cause neurological issues in other animals, issues collectively called “staggers syndrome”. To test if aflatrem-like compounds play a role in *Camponotus floridanus* manipulation,

healthy ants were injected with purified aflatrem and their behavior was monitored for 30 minutes. RNA-Seq was also performed on the heads of aflatrem-injected ants to compare any changes at the transcriptomic level to data available for ants infected by *Ophiocordyceps camponoti-floridani*. Behavioral assay footage was analyzed using the machine learning tool MARGO, which showed a negative correlation between activity and speed, and dose of aflatrem. Behaviors were also scored manually, identifying a positive coloration between aflatrem dose and staggering behavioral phenotypes. RNA-seq discovered 262 genes significantly up or down regulated compared to sham injected ants. Of these genes, 108 were also differentially regulated in the heads of late-stage *Ophiocordyceps*-infected ants. Together, these results indicate that aflatrem-like compounds may play an important role during late-stages of infection by reducing activity in ants, keeping them in elevated microclimates optimal for the production and dispersal of spores.

1. INTRODUCTION

Parasites and their hosts often share an intimate coevolutionary history; associations that over time can lead to unique phenotypes and subsequent host-specificities. Perhaps one of the more remarkable symbiotic phenomena known to have convergently evolved in zootic parasites is the ability to manipulate host behavior. Host manipulations provide an adaptive advantage to the parasite through induction of specific behaviors that increase its reproduction success (Sánches and Biron, 2019); behaviors that can be viewed as an example of extended parasitic phenotypes (Dawkins, 1982). These adaptive phenotypes are different from sickness behaviors, which are defined as actions exhibited by the infected individuals which are not conducive to the fitness of the host nor the parasite (e.g., lethargy, in most cases). The difference between behavioral manipulations and sickness behaviors can be observed in the parasitic flat worm *Leucochloridium*

paradoxum, which manipulates amber snails from the genus *Succinea*. Once inside a snail, the worm drives the host to ascend nearby structures, a common manipulation behavior known as "summit disease" (Wesołowska and Wesołowski, 2013). This summiting behavior makes snails much more susceptible to predatory birds, the primary host of *L. paradoxum*, ultimately increasing the parasite's likelihood of completing its lifecycle at the expense of its secondary host (Wesołowska and Wesołowski, 2013). Furthermore, the return of the worm to its bird host allows it to spread much more effectively through bird droppings, increasing its fitness and providing a selectable pressure to maintain behavioral manipulation in the snails.

A variety of taxa are implicated in behavioral manipulations across the Tree of Life, including crustaceans (e.g., *Loxothylacus panopaei* infecting crabs (Blakeslee et al., 2021)), nematomorphs (e.g., *Spinochordodes tellinii* infecting katydids (Biron et al., 2005)), protozoans (e.g., *Toxoplasma gondii* infecting mice (Tong et al., 2021)), insects (e.g., *Cotesia congregate* parasitizing caterpillars (Adamo, 2019)), and fungi (e.g., *Entomophthora muscae* infecting flies (Elya and Licht, 2021)), and are even found to occur in non-living viruses (e.g., *Rabies lyssavirus* infecting mammals (Hueffer et al., 2017)). While behavioral manipulation includes a wide array of tactics, many of these parasites induce summiting behavior to increase their transmission chances (de Bekker et al., 2021; Araújo & Hughes, 2019; Steinkraus et al., 2017; Latchininsky et al., 2016; Wesołowski & Wesołowski, 2013). Collectively, behavior-manipulating parasites are referred to colloquially as "zombie parasites" due to the peculiar actions of infected hosts, many of which are reminiscent of zombies from pop culture sci-fi media (e.g., movies such as *28 Days Later* and *The Body Snatchers*).

Amongst these mind-altering parasites, the "zombie fungi" are one of the largest known groups, infecting various species of insects and arachnids globally (Arruda et al., 2021; de Bekker

et al., 2021; Cooley et al., 2018). This includes wasps (e.g., *O. humberti* (Sobczak et al., 2020)), beetles (e.g., *O. curculionum* (Shrestha et al., 2016)), scale bugs (e.g., *Ophiocordyceps clavulata* (Petch, 1933)), caterpillars (e.g., *O. sinensis* (Guo et al., 2021)), flies (e.g., *O. dipterigena* (Chhetri et al., 2020)), and spiders (e.g., *O. engleriana* (Sung et al., 2007)). While the genus is capable of infecting a diverse group of hosts, a large portion of the currently described species infect and manipulate carpenter ants of the Camponotini tribe with extreme host-specificity (Araújo et al., 2018, Araújo et al., 2020). These *Camponotus*-infecting fungi belong to the species complex *Ophiocordyceps unilateralis sensu lato*; one of the more well-studied groups of zombie fungi known for their clear and tractable behavioral manipulations compared to other *Ophiocordyceps* species.

Ophiocordyceps unilateralis species begin their lifecycle as a spore, ejected from their parental fruiting body. These spores sail on the wind to infect new ants, either through immediate contact or through the production and attachment of secondary capillispores after falling onto the forest floor (Evans et al., 2018). Once inside the ant, the fungus begins to secrete an array of biomolecules in a time-specific manner; some of which alter the behavior of their host (Will et al., 2020; de Bekker et al., 2015) One of the first notable changes occurs as these ordinarily social hosts begin to show a lessened ability to communicate and participate in foraging tasks (Trinh et al., 2021). Subsequently, infected ants climb nearby vegetation and latch on with their mandibles (Andersen et al., 2009). The fungus then completes its lifecycle through the formation of anastomosis tubes and an endosclerotium that siphons the remaining nutrients from the host and uses these materials to create a new fruiting body (Fredericksen et al., 2017). Closer observations and quantification of behaviors in both the lab and the field have led to the classification of other, more subtle behavioral changes including changes in daily rhythms (Trinh et al., 2021; Hughes et

al., 2011) and the loss of motor coordination and balance in ants that fumble their way around in a manner described as by Hughes et al. as a “drunkard’s walk” (Supplemental Video 1) (Trinh et al., 2021; Will eat al., 2020; Hughes et al., 2011). These conspicuous behavioral changes caused by the fungus during infection makes the *O. unilateralis* species complex and their hosts particularly well-suited for the study of behavior-manipulating parasites in greater detail (de Bekker et al., 2015).

Recent work establishing techniques for culturing the fungus in a laboratory setting and manually infecting ants, as well as the genomic sequencing of several *O. unilateralis* species, has laid the groundwork for research aimed at understanding the molecular framework driving these behavioral changes (Will et al., 2020; de Bekker et al., 2017; de Bekker et al., 2015). Transcriptomics analyses of the North American species *Ophiocordyceps kimflemingiae* and *Ophiocordyceps camponoti-floridani* have identified secreted proteins and secondary metabolite clusters highly upregulated during summiting behavior (de Bekker et al., 2015; Will et al., 2020). Of particular interest is a secondary metabolite cluster with a TRP_DMAT backbone that had a ~5,900 (in *O. kimflemingiae*) and ~12,000-fold (in *O. camponoti-floridani*) increase in expression during manipulation compared to fungal growth in culture (Will et al., 2020). This cluster was first identified as an ergot alkaloid producing pathway based on the functional annotation of its tryptophan dimethyltransferase (Pfam: TRP_DMAT (PF11991)) backbone gene (de Bekker et al., 2015). More detailed gene-by-gene annotation also found homologs of genes within and surrounding the cluster to gene that code for aflatrem production proteins in the plant pathogen *Aspergillus flavus* (Will et al., 2020). Genomic comparisons with other *Ophiocordyceps* genomes revealed that this complete secondary metabolite cluster appears to be conserved across species within *O. unilateralis* but not in other *Ophiocordyceps* species outside the species (e.g.,

Ophiocordycpes australis s.l.)(de Bekker et al., 2017). This begs the question, what evolutionary purpose may aflatrem-like products, particularly those produced by this cluster, serve for the fitness of *Ophiocordyceps* species that manipulate the behavior of *Camponotus* hosts.

Aflatrem, a secondary metabolite of the indole-diterpene group called aflatoxins, is a potent tremorgenic and carcinogenic mycotoxin known to cause neurological disorders in animals (Nicholson et al., 2009; Duran et al., 2006). Cows exposed to aflatrem through ingestion of *A. flavus*-contaminated grain feed exhibit neurological problems include muscle tremors and hyperexcitability, followed shortly by incoordination, ataxia, and seizures (Selala et al., 2008; Valdes et al., 1985). Collectively, these neurological symptoms linked directly to aflatrem's positive allosteric modulation of Gamma-aminobutyric acid (GABA) receptors are now referred to as "staggers syndrome" (Eldefrawi et al, 1990; Yao et al., 1989; Grant et al., 1987). The potential involvement of GABA receptors in host manipulation of *Camponotus* species could have important ramifications given their suspected role in caste and behavioral differentiation (Graham, 2018; Gospocic et al., 2017). The likelihood that an aflatrem-like compound may be employed during the manipulation of carpenter ants is further supported by observations of the "drunkard's walk" behaviors exhibited during infection, which are reminiscent of the staggers syndrome detailed in vertebrates (Supplemental Video 1). We, therefore, hypothesize that aflatrem-like compounds play a role in the behavioral manipulation of carpenter ants during *O. unilateralis* infection.

In this study, we administered aflatrem to the Florida carpenter ant *Camponotus floridanus*, the host of *O. camponoti-floridani*, using microinjections to mimic the *in vivo* secretion of aflatrem-like compounds by fungal cells in the hemolymph during infection (Figure 1).

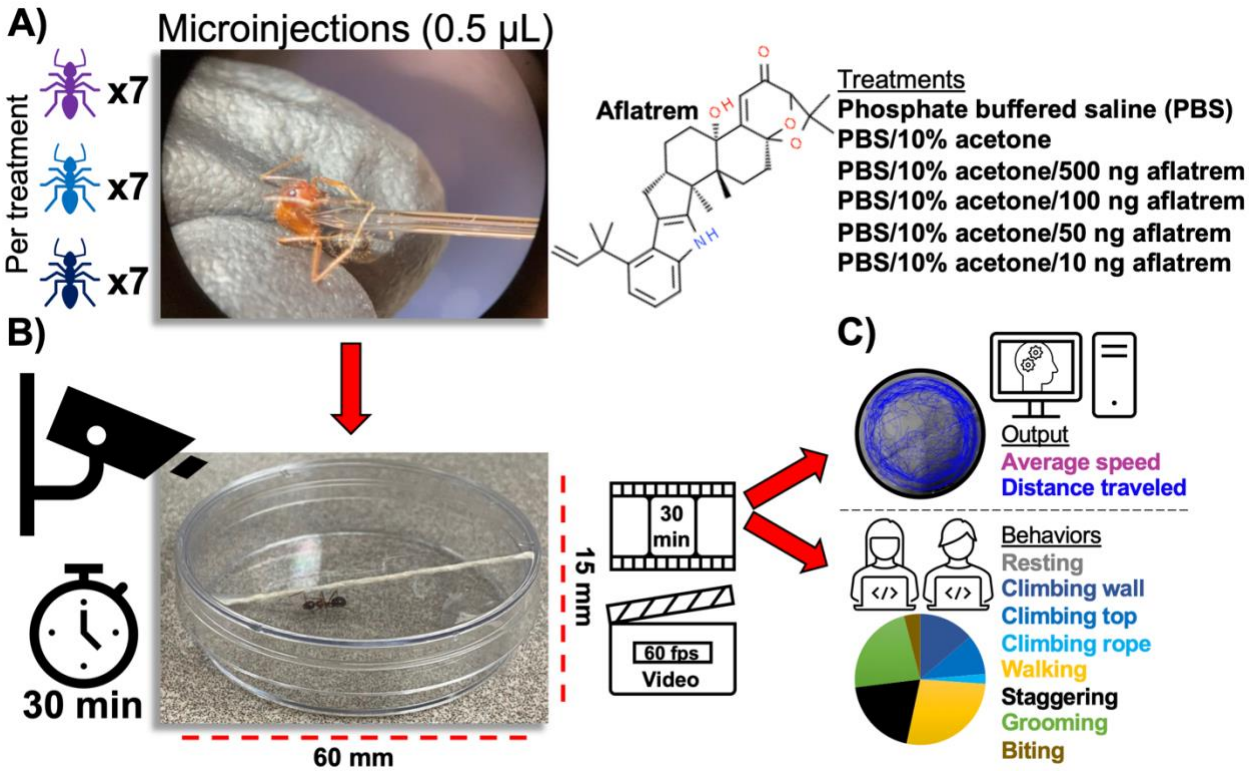

Figure 1. A project overview of (A) the treatment groups, each consisting of 7 individuals from 3 different colonies of *Camponotus floridanus*, and an example of microinjection, (B) the arena setup for behavioral assays and parameters for the observational periods, and (C) the behavioral analyses using machine learning and behavior scoring software.

Given the ease of its procurement and its previous use in other animal models, high purity pre-synthesized aflatrem (>98%) was used in our experiments (TePaske et al., 1992; Trienens & Rohlfs, 2011). Through the observation and quantification of various behaviors following injection, we demonstrate that aflatrem on its own can induce behaviors consistent with both those exhibited during infection by *O. camponoti-floridani* and those reported for vertebrates with staggers syndrome. To investigate which host pathways are affected by aflatrem, and potentially give rise to the exhibited behavioral phenotypes, we also conducted transcriptomics analyses of aflatrem-injected individuals. Comparison of these results to transcriptomics data

from *Ophiocordyceps*-infected *C. floridanus* ants (Will et al., 2020) yielded several consistent differentially expressed genes, including genes that encode neuromuscular and chemosensory proteins.

2. MATERIALS AND METHODS

2.1 Ant Collection

Three separate colonies of *Camponotus floridanus* were collected using a DeWALT Cordless 2 Gallon Wet/Dry ShopVac at the University of Central Florida's arboretum, coordinates: Colony 1 (28.602748, -81.194269), Colony 2 (28.603918, -81.191682), and Colony 3 (28.601430, -81.191251). Colonies were housed in 9.4 L plastic containers (Rubbermaid) lined with talcum (Acros Organics), applied using a viscous 20% w/v solution of powder suspended in 100% ethanol and let dry, to prevent ants from climbing the walls. Throughout the study, ants were provided with fresh supplies of both double distilled water (ddH2O) and 15% sucrose solutions, *ad libitum*. The housing was kept in an incubation chamber (Percival) set to the following program: 20°C – 0 lux – 80% relative humidity (RH) with a linear increase in temperature, light, and decrease in humidity over 4 hours to reach 28°C – 2,100 lux – 65% RH, at which conditions were held for 4 hours followed by a linear decrease in the temperature, light, and increase in humidity, returning to 20°C – 0 lux – 80% RH over 4 hours; and held for 12 hours.

2.2 Microinjections

To test our hypothesis and identify the effect of aflatrem-like compounds on *C. floridanus*, we established a behavioral assay using microinjections to introduce aflatrem in a manner

biologically relevant to the secretion of aflatrem-like compounds by *Ophiocordyceps* species in the hemolymph of their ant hosts (Figure 1). For these assays, we utilized high purity aflatrem, >98% $C_{32}H_{39}NO_4$, manufactured from *A. flavus* by BioCrick Science Solution Specialists in non-water-soluble crystal form, which we suspended in 100% acetone (Fisher Chemical) to a concentration of 10 mg/mL. All injections were performed using borosilicate glass micropipettes (Fisher) pulled with a glass puller (model: PC-100, Narishige) under the following parameters: single step, all weights, heated to 62°C. Each ant was held by the dorsal side of the thorax before piercing the carapace on the ventral side between the first and second pair of legs, followed by slowly injecting 0.5 μL of solution into the ant while simultaneously releasing grip to alleviate intracavitary pressure.

Because the aflatrem used in this study is non-water soluble, a preliminary trial was performed to identify acetone tolerance in ants by injecting subjects with 100%, 50%, 25%, 10%, and 5% acetone solutions diluted with phosphate buffered saline (PBS) (137 mmol NaCl, 2.7 mmol KCl, 10 mmol Na_2HPO_4, 1.8 mmol KH_2PO_4, pH adjusted to 7.4), along with a 100% PBS solution as a control. Prior to injection, containers housing ant colonies were removed from the incubator and set on the lab bench top to acclimate to lab conditions for 10 minutes. All injections were performed in triplicate, one ant per colony per treatment. Injected ants were placed into individual 60 mm x 15 mm petri dishes (USA Scientific) and returned to the incubator. Ants were kept under observation for 48 hours post injection using an infrared GoPro Hero6 set to record time-lapse footage with 60 second intervals and a CMVision IR30 Illuminator lamp for observation during the night cycle. Dead and resting *C. floridanus* ants were easily distinguishable from one another as dying ants coiled their legs and fell to one side upon death while resting ants remained prone with legs supporting their bodies. Time of death for each ant was estimated by noting the frame in

which each ant exhibited death behaviors, allowing us to estimate time of death within an interval of 1 minute. Any surviving ants after the 48-hour test period were euthanized by freezing at -20°C for 24 hours. Results from these preliminary analyses indicated that a PBS/10% acetone solution was the highest concentration for which there were no statistical differences in average survivability compared to PBS-injected and non-injected controls.

Using the tolerable PBS/10% acetone standard, a second preliminary trial was performed to test the survivability of ants post injections and to determine if any observed behavioral phenotypes may be attributed to "dying behaviors" resulting from aflatrem exposure. To do so, 7 ants were injected with 0.5 μL of a PBS/10% acetone solution containing 500 ng aflatrem. This was the highest dose possible using the 10 mg/mL aflatrem stock diluted with PBS. Post injection, ants were recorded for 48 h using the same GoPro setup as the previous preliminary test. Resulting behaviors were compared to 7 ants injected with a PBS/10% acetone solution without aflatrem and 7 ants injected with a 100% PBS solution. No differences in mortality were observed, with all individuals surviving for at least 24 h and an average time of death arising at 34 h, indicative of desiccation from lack of water.

Confident that neither 10% acetone nor 500 ng aflatrem killed the ants or induced death-related behaviors, an assay to determine the effects of aflatrem on ant behavior was conducted using PBS/10% acetone solutions containing the following doses of aflatrem; 10 ng, 50 ng, 100 ng, 500 ng. For each dose of aflatrem, 7 ants from each of the 3 colonies were injected (N=21, per dose) for a total sample size of N=126. Ants were individually lifted from their housing by hand wearing premium disposable neoprene gloves (NeoTouch) and placed into individual 60 mm x 15 mm petri dish (USA Scientific) arenas on the lab benchtop to acclimate for 10 minutes. These arenas were fitted with a thread of twine spanning the dish from the bottom of one side to the top

of the other, providing a substrate for biting and more dexterous climbing behaviors (Figure 1-B). To allow ants to walk around the perimeter of the dish unimpeded, the twine was pressed flat and taped to the bottom corner of the petri dishes using a small piece of tape (Scotch). To avoid any behavioral changes that may be induced via chemical ques left by prior occupants, a new arena was created for each test subject (N=126). After the 10-minute acclimation period, ants were injected with aflatrem following the microinjection protocols established in the preliminary trials. The doses of aflatrem used were based on approximate ant equivalent weights, verified by measuring *C. floridanus* body volume through length and weight following each experiment. While body lengths were measured to the nearest 0.5 mm for each individual, weight was measured in groups of seven ants corresponding to their treatment and colony ID. Measurements were recorded immediately following euthanization at -20°C, preserving the approximate water weight for each ant. To account for the approximate weight of solution injected into each ant, 3.5 mg was subtracted from the total weight of each group. Based on an average 8.28 mg weight and 6.68 mm length calculated from measurements of all 126 subjects used in this study (Supplemental Table 1), the four doses of aflatrem that were used are approximately 6.04e-5, 1.21e-5, 0.60e-5, and 0.12e-5 ant equivalents. These ant equivalent doses are consistent with pervious works testing insecticidal compounds in ants (Sakamoto & Goka, 2021; Buczkowski & Woosler, 2019; Klotz & Moss, 1996).

Ant behaviors were recorded for 30 minutes post injections using an iPhone 13 pro. Any ants exhibiting signs of physical injury from the injection, e.g., immobility in the hind legs or body curling, were euthanized immediately and a new subject and arena were used in its place. Ants were recorded three or four arenas at a time in a rolling format to ensure high quality resolution at 60 fps, generating video files of approximately 45 minutes in length. At the end of each recording

period, arenas containing resting ants were tapped several times to induce movement, confirming the subjects were still alive. All instances of tapping elicited activity.

2.3 Video scoring of behavioral assays post injection

Videos were converted from .MOV to .mp4 files using iMovie for editing in Movavi Video Editor Plus 2022, which, unlike iMovie, allows for cropping files to show only single arenas. The cropped files were additionally trimmed to exactly 30 minutes of footage beginning as soon as each arena was positioned in the field of view. The names for each file were then blinded before manual scoring by two observers using the behavior tracking software CowLog3 (Pastell, 2016). The observers were trained using eight test run videos; four of ants injected with PBS/10% acetone/500 ng aflatrem, and four of ants injected with PBS. These videos were made during proof-of-principle testing and are not included in the data presented in this study. Results generated for these practice runs were then reviewed together by both observers to form a consensus on scoring methodology. From these training videos, the observers identified eight scorable behaviors: Climbing Wall, Climbing Top, Climbing Rope, Walking, Staggering, Grooming, Resting, and Biting (Supplemental Video 2). While most of these behaviors are self-explanatory, “staggering” was generally defined as a slow, sluggish walking behavior with repeated lateral movements (Supplemental Video 3). Behaviors were also marked as “staggering” when trouble balancing, listing to one side, falling over, walking in tight circles, flipping over, or dramatic wagging of the gaster were observed (Supplemental Video 3). Each observer also kept notes on unique behaviors observed during the scoring period, including walking backwards, lunging/bucking, and two instances of convulsions (Supplemental Video 4).

Blinded videos were assigned to the observers in a randomized block format (Supplemental Figure 1-A) with each observer scoring three videos at random per treatment group per colony. The seventh video for each treatment group was scored by both observers and used as a formative metric to ensure consistent scoring standards throughout the evaluation period. While most behaviors were readily distinguishable, resulting in very similar scores between observers, walking and staggering provided the most discrepancy. We therefore used staggering as the primary measurement to evaluate scoring consistency across observers (Supplemental Figure 1-B).

In addition to the manual observations, videos were also analyzed using the MATLAB-based machine learning tool Massively Automated Real-time GUI for Object-tracking (MARGO) (Werkhoven et al., 2019). This tool was used to measure the speed and distance traveled by each subject. Speed was only measured during activity by calculating the distance between centroid coordinates in mms traveled per second during movement. Distance traveled was measured as a total value for distance between centroid coordinates over the entire 30 min duration. To optimize tracking for the field of view in each video, the following metrics were used: region of interest (ROI) was set to circular shape and manually overlayed around the arena, background referencing was set to detect dark objects on light backgrounds, tracking threshold was set between 70-80, distance of the arena was set to 60 mm using the custom measuring tool, frame rate was set to 30, and min/max area was set to 5 and 80, respectively.

The results from the PBS/10% acetone controls were first compared to those from the 100% PBS controls to identify any potential behavioral phenotypes induced by exposure to acetone. Significant differences between the two groups were tested using a two-sample t-test in RStudio v1.3.959. To test for significant behavioral changes in the aflatrem treatment groups, a linear regression model using the lme4 package in RStudio was performed to compare the

PBS/10% acetone control (0 ng aflatrem) with PBS/10% acetone containing 10 ng, 50 ng, 100 ng, and 500 ng aflatrem treatments.

2.4 RNA sequencing

To understand the effects of aflatrem exposure in *C. floridanus* at the genomic level, a total of 10 ants were injected with either PBS/10% acetone (control group, N=5) or PBS/10% acetone/500 ng aflatrem (treatment group, N=5). Ants were each placed into their own petri dish arena for 4 minutes post injection. This timepoint was selected based on the scaling levels of activity observed in our behavioral assays (Supplemental Figure 2). Ants were then quickly placed into 2 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Subsequently, for each ant, the head was removed using a forceps and placed in a pre-frozen 2.0 mL Self-Standing Impact Resistance Microtube containing 2 Wheels Manufacturing Grade 25 5/32" metal ball bearings for mechanical tissue disruption in a SPEX® SamplePrep 1600 MiniG at 1500 rpm for 30 sec. Disrupted samples were defrosted while dissolving in 500 uL TRIzol (Ambion) to extract RNA followed by sample clean up using the Qiagen RNeasy® MiniElute® Cleanup Kit (Will et al., 2020). The resulting RNA was stored at -80C and shipped to Azenta US for Next Generation Sequencing. Libraries were prepared by Azenta US using PolyA selection and sequenced in duplicate on Illumina HiSeq with 150 bp paired end reads, resulting in ~350M reads. The quality of resulting paired RNA-seq reads was verified using FastQC (Andrews 2010). We used Trimmomatic (Bolger et al., 2014) to trim reads for adapters and quality using the following parameters: Leading: 3, Trailing: 3, MinLen: 36. Transcripts were then aligned to the *Camponotus floridanus* reference genome (Gene bank ascension number GCA_003227725.1) using Spliced Transcripts Alignment to a Reference (STAR) 2.7.10a (Dobin et al., 2013). The resulting .bam files were checked for quality using the

standard metric of uniquely mapped reads > 70%, sorted, and indexed using Samtools (Danecek et al., 2021). The number of gene hits for each sample were calculated using FeatureCounts following the suggested counting of strictly uniquely mapped reads within exonic regions (Liao et al., 2019). The quality of the resulting files was validated using a benchmark of 15-20 million reads per sample. The hit counts were normalized using DESeq2 (Love et al., 2014) after removing low level expression (counts less than 100 per gene, i.e., an average less than 10), eliminating 3,227 genes from further analysis. We performed differential expression analysis on the remaining 10,443 genes using DESeq2 with an alpha value of 0.05 and functionally annotated the differentially expressed genes using Blast2GO (Conesa et al., 2005).

3. RESULTS

3.1 The behavioral effects of aflatrem in *C. floridanus* ants

Before we could analyze the behavioral effects of aflatrem in ants, we screened for any potential behavioral changes caused by the presence of 10% acetone in the injections. Using an independent two sample t-test to compare the behavior of ants injected with PBS to those injected with PBS/10% acetone, we were able to rule out any significant differences in behavior between the two groups: rest ($p = 0.787$), speed ($p = 0.997$), distance ($p = 0.947$), stagger ($p = 0.205$). Even though neither group was exposed to aflatrem, we unexpectedly observed a few instances of staggering in both. To determine if a small amount of staggering behavior is normal in ants, which would need to be accounted for in our results, we reviewed the footage at timestamps logged for these staggering events with an increased level of scrutiny. Upon closer review, we discerned that nearly all events of staggering observed in both groups fell into the “gaster wagging” sub-type of staggering (Supplemental Video 2), and nearly always occurred in the first 30 seconds of the

recording. This trend was also observed in the aflatrem treatment groups, indicating that this particular type of staggering behavior is likely a transient physical response to the trauma resulting from the injection process. This was confirmed by a quick follow-up experiment pricking ants with empty glass capillaries, which also induced gaster wagging.

Confident that behaviors were not significantly affected by the addition of 10% acetone in the injections, we compared behaviors across increasing doses of aflatrem. A linear regression model showed that time spent resting in the arena was positively correlated with increasing doses of aflatrem ($F_{4,105}$ = 5.54, p = 2.05e-2) (Figure 2-A), while the average speed of aflatrem-treated ants was negatively correlated ($F_{4,105}$ = 4.91, p =2.89e-2) (Figure 2-B). However, the average distance traveled did not correlate with aflatrem treatment ($F_{4,105}$ = 3.036, p = 8.44e-2, ns) (Figure 2-C). Finally, bouts of staggering did positively correlate with increasing doses of aflatrem ($F_{4,105}$ = 36.65, p = 2.32e-8) (Figure 2-D).

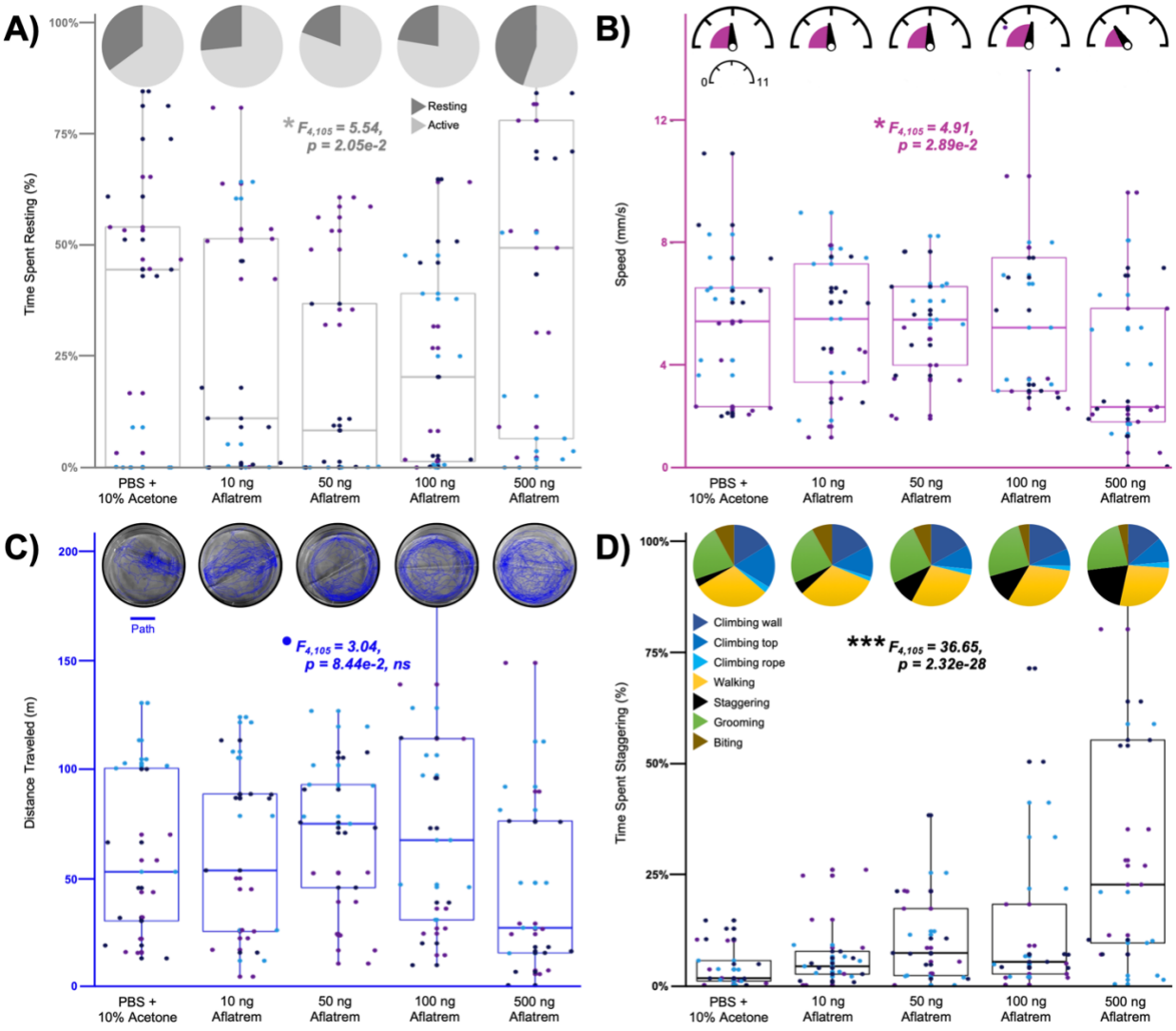

Figure 2. Behavioral results collected from *C. floridanus* ants injected with increasing doses of aflatrem. Each boxplot depicts the quartiles of the distribution for each treatment with ants from colonies 1, 2, and 3 shown as purple, light blue, and dark blue dots, respectively. A) shows the proportion of time spent resting for each ant, B) shows their average speed during activity in millimeters per second, C) shows their total distance traveled in meters, and D) shows the proportion of activity spent staggering for each ant. The linear regression model results are shown in the center of the graph for each behavior. A & D) both contain pie charts illustrating the relative

duration of each behavior, while B) contains speedometers for each treatment and C) presents the median tracer plot from MARGO for each group.

3.2 RNA-seq results

With injection of *C. floridanus* with aflatrem resulting in a quantifiable phenotype, we next asked which genes could be underlying the emergence of staggering phenotypes. As such, we conducted transcriptomics to compare gene expression in the heads of ants treated with PBS/10% acetone/500 ng aflatrem (treatment group) to that of ants treated with PBS/10% acetone solutions (control group). We found 262 genes that were significantly differentially expressed (Supplementary File 1). Of these genes, 148 were upregulated and 114 were downregulated in the treatment group (Figure 3). While a comparable number of genes were up- and down-regulated in our treatment group, the largest changes in expression were found among the downregulated genes (Figure 3). We found the largest increase of expression (~22-fold increase) in a gene encoding an aminopeptidase N-like protein, a common cell surface hydrolase involved in a variety of cellular processes (Nocek et al., 2008), while to the largest decrease in expression (~512-fold decrease) was exhibited by a gene coding for a cytochrome P450 6k1, a protein involved in detoxification of insect cells (Xing et al., 2021) (Figure 3).

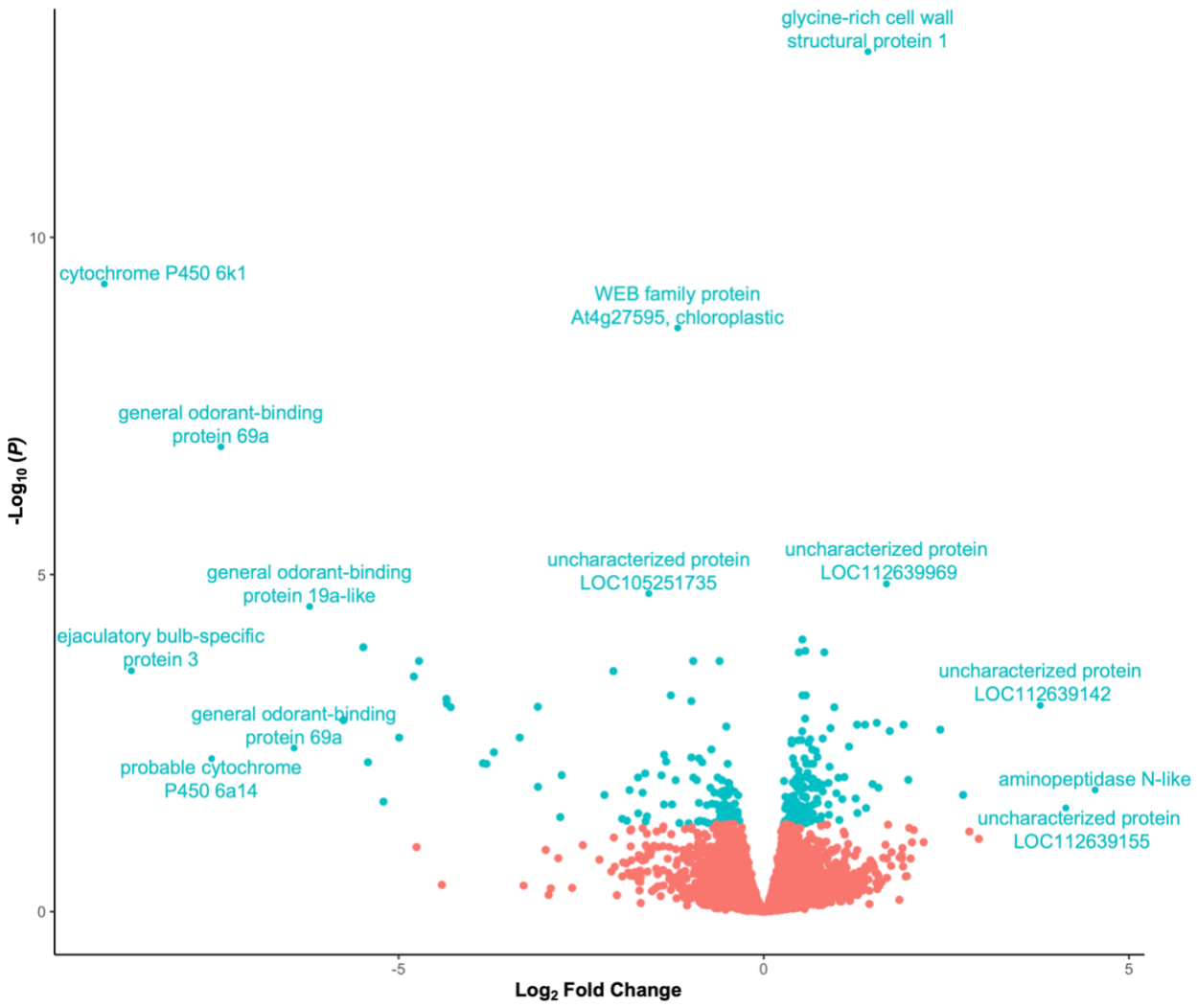

Figure 3. Volcano plot for the differential expression of 10,443 *Camponotus floridanus* genes in ants treated with PBS/10% acetone/500 ng aflatrem compared to the PBS/10% acetone control group. Dots in red represent non-significant changes in gene expression, while dots in blue represent significant change using an alpha value of 0.05. Functional annotations are shown for genes with adjusted *P* values less than 5e-5 and an absolute $\log_2$ fold changes lower than -6 or greater than 3.5.

Of all differentially expressed genes, 31 had a greater than 4-fold change in expression in the heads of aflatrem-treated ants. Among these genes were several that have been predicted to

play a role in neuromuscular systems, e.g., a downregulated *anoctamin-4*, which codes for an ion channel protein involved in Ca^{2+}-dependent conductance (Reichhart et al., 2019), and *membrane metallo-endopeptidase* (*Neprilysin*), a transmembrane protein found in neurological tissue that plays a role in tissue perception and preservation (Obulesu, 2019), slightly upregulated in our aflatrem-treated ants but significantly downregulated in *Ophiocordyceps*-infected ants. This group also contained many olfactory-related genes, including 4 general odorant-binding protein genes (OBPs) (i.e., *OBP19a*, *OBP56a*, and 2 *OBP69a*), all of which were downregulated. Such proteins are located in the antennal sensillary lymph of insects and bind to pheromones that play a role in insect communication and interactions (Gaubert et al., 2020). Moreover, we found 3 significantly downregulated *ejaculatory bulb-specific* genes, involved in the release of pheromones in insects (Pelosi et al., 2018), and a downregulated *sensory neuron membrane protein* gene, which has been found to play a role in pheromone sensitivity in the moth *Heliothis virescens* (Pregitzer et al., 2014).

We also found expression signatures of neuromuscular gene dysregulation in the larger group of 231 differentially expressed genes with a less than 4-fold degrees of change. Amongst them were 2 upregulated *titins* genes, which code for very large proteins that play an essential role in sarcomere function (Freiburg et al., 2000; Zhang et al., 2000). We also found an upregulated *ryanodine receptor* gene, responsible for releasing Ca^{2+} in the smooth endoplasmic reticulum of muscle cells during excitation-contraction coupling (Lanner et al., 2010; Shakiryanova et al., 2007). Furthermore, we detected a downregulated *caveolin-3*, which codes for a scaffold protein to organize signaling molecules in the membranes surrounding muscle cells (Dewulf et al., 2019; Galbiati et al., 2000-2013), and 2 downregulated *myosin ID heavy chain-like protein* genes, the motor proteins of muscles (Wells et al., 1996) (Supplementary File 1). Also of interest, given our

previous exploration of the time-dependent nature of behavioral manipulation and the disruption of clock-dependent foraging behaviors in *Ophiocordyceps*-infected ants (de Bekker & Das, 2022; Will et al., 2020; de Bekker et al., 2015), was a gene coding for a circadian clock-controlled protein with a Haemolymph Juvenile Hormone Binding Protein Pfam domain showing an 18-fold decrease in expression. These proteins, affected by circadian rhythms in the ant, act as a carrier for juvenile hormone to its target tissues (Zalewska et. Al., 2009).

Because the behaviors of aflatrem-infected ants are reminiscent of the extended behavioral phenotype of *Ophiocordyceps*-infected ants, and due to expression data that demonstrates an aflatrem-like secondary metabolite pathway is highly upregulated during late-stage infection of carpenter ants by *Ophiocordyceps unilateralis* species (de Bekker et al., 2015; Will et al., 2020), we also compared our RNA-seq results to transcriptomics data obtained from *C. floridanus* manipulated by *O. camponoti-floridani* (Will et al., 2020). From this comparison, we found that 108 of the 262 differentially expressed genes observed in aflatrem-treated ants were also found to be differentially regulated in late-stage *O. camponoti-floridani*-infections (i.e., Alive/Control and Dead/Control, Supplementary File 1). Of these genes, 34 were differentially regulated in the same direction, while 74 shared an inverse relationship. The similarly expressed genes contained the previously mentioned *cytochrome P450*, a *sensory neuron membrane protein* encoding gene, *OBP56a*, and *caveolin-3* genes, as well as an upregulated *nuclear hormone receptor E75*, a circadian clock steroid-producing gene discovered in *Drosophila* linked to the maintenance of circadian rhythms during stress (Kumar S et al., 2014). Additionally, *neurogenic locus protein delta*, capable of coding for multiple translational products including neural differentiation factors and growth hormones (Kopczynski et al., 1988), was upregulated during *Ophiocordyceps* infection and isolated aflatrem exposure.

Among the genes that were differentially expressed in inverse directions versus the fungal manipulation dataset, we found the aforementioned *OBP19a-like* gene, *cytochrome P450*, and *ejaculatory bulb-specific protein 3*, as well as an *odorant receptor coreceptor*, which is also expressed in chemosensory organs and forms heteromultimeric complexes with odorant receptors (Mukunda et al., 2014). The gene set also contained a *short neuropeptide F*, which is implicated in the regulation of animal behaviors including circadian rhythm and hunger (Lee et al., 2004) and a *netrin receptor UNC5C*, which aids in the formation of axon connections through axon extension and cell migration (Kim & Ackerman, 2011). We also found a *receptor-type tyrosine-protein phosphatase delta* (*PTPRD*), part of the PTP family involved in many known signaling pathways for which delta is shown to promote neurite growth and regulate axon guidance (Tomita et al., 2020). Moreover, in both infection and sole aflatrem exposure, *sialin*, a sialic acid transporter-coding gene that prevents neurodegenerative disorders caused by accumulation of sialic acid (Tarailo-Graovac et al., 2017), was dysregulated, as well as *obscurin*, which, like *titin*, codes for another large protein expressed in skeletal muscles that anchors myofibrils to the sarcoplasmic reticulum for muscle contraction (Feher, 2017). Both datasets also indicate the dysregulation of a *juvenile hormone acid O-methyltransferase-like* gene, which codes for an enzyme that converts precursor molecules into juvenile hormones, regulating many aspects of insect physiology (Shinoda & Itoyama, 2003). Finally, the two data sets also shared 26 genes with statistically significant changes in expression coding for proteins of unknown function.

4. DISCUSSION

While sickness behaviors are common in animals infected by parasites, only behaviors that confer an adaptive advantage and increase the reproduction and/or transmission of parasites are true

behavioral manipulations. It is, therefore, imperative that behaviors exhibited during parasitism be thoroughly vetted before causal relationships are drawn between parasite genotypes and resulting host phenotypes. This study aims to test the previously implicated involvement of aflatrem-like compounds in the establishment of *Ophiocordyceps* extended phenotypes in zombie ants (Will et al., 2020) by exposing healthy *C. floridanus* ants to high purity aflatrem and quantifying the behavioral effects, a hypothesis formed from the tremor-like staggers that have been observed in both *Ophiocordyceps*-infected ants and mammals following ingestion of aflatrem through the consumption of *A. flavus*-infected feed, (de Bekker et al., 2017; Selala et al., 2008; Valdes et al., 1985). As such, we observed and quantified staggering, which we found to be positively correlated to aflatrem dose. This suggests that this behavior is caused by the presence of aflatrem. To rule out other possible explanations for the staggering behavior, such as death behaviors that may contradict its status as a true behavioral manipulation, we performed a longevity assay to demonstrate that aflatrem does not kill *C. floridanus*. This makes it unlikely that the aflatrem-like compound produced by *Ophiocordyceps* is used as a killing compound, but is rather employed to induce staggering behaviors prior to death. We were further able to demonstrate that aflatrem has a temporary yet significant effect on the mobility and coordination of ants and decreased overall activity. Ants injected with higher doses of aflatrem moved much slower and had more difficulties participating in climbing behaviors, particularly the more dexterous activities involving the twine in the arena.

While we do not yet have a direct answer as to how the behaviors observed in this study confer an adaptive advantage to the fungal parasite during late-stage infection, time-specific hypotheses regarding the situational use of such a compound provide a presumptive explanation. Indeed, a reduction in overall activity may seem contradictive to the goal of positioning the host

away from the nest at a higher elevation; however, if we consider the temporal variation and time-specific expression of fungal genes that have been hypothesized to play a vital role throughout the infection process, the induction of immobility during late-stage manipulation, during which natural "drunkard walking" is observed, could have some biological relevance. It is reasonable to suggest that, prior to the upregulation of aflatrem-like compounds, genes responsible for producing effectors that induce hyperactivity and climbing behaviors are first produced. This would lead the host up nearby vegetation and away from aggressive conspecifics of the nest enacted as part of social immunity before late-stage production of compounds would be required to maintain the ant in a desirable location (Cremer et al., 2007). In support of this hypothesis, transcriptomics data from *Ophiocordyceps*-infected ants show that a *protein tyrosine phosphatase* (*ptp*) is upregulated during manipulation (de Bekker et al., 2015; Will et al., 2020), a gene found to induce hyperactivity and enhanced locomotion in caterpillars that are infected with a behavior-manipulating baculovirus (Han et al., 2015). Moreover, enhanced locomotion has been observed in *Ophiocordyceps*-infected ants during the stages leading up to the final summiting behavior (Trinh et al., 2021). Once away from the nest, following successful completion of summiting behaviors, the upregulation of compounds that reduce activity would become vital to maintain the host at optimal microclimates where conditions suitable for fruiting body production.

In addition to reducing the ant's overall activity, aflatrem-like compounds may also aid in the positioning of the host for the final and perhaps most iconic behavioral manipulation observed in zombie ants - the "death grip" - by inhibiting the host's motor capabilities. Under the time-specific scenario that we propose, the reduction of climbing behavior in concert with reduced dexterity of the host could aid the fungus in the completion of its lifecycle in a manner that confers a fitness advantage, as killing the host without proper adherence to the substrate would likely result

in the ant falling to the forest floor, away from the microclimate that promotes fruiting body formation (Andersen et al., 2012; Andriolli et al., 2019; Will et al., under revision) and negating the benefits of elevation position for spore dispersal. Field observations of body position for *Ophiocordyceps*-infected *Camponotus* species corroborate this hypothesis. In addition to the "death grip" adherence with their mandibles, infected *Camponotus castaneus* and *C. floridanus* ants infected with *Ophiocordyceps kimflemingiae*, and *O. camponoti-floridani*, respectively, are often found wrapping their limbs around the substrate (Figure 4) (Loreto et al., 2018; Will et al., 2020). Therefore, the discoordination of the ant host, as indicated by the dysregulation of neuromuscular and sensory genes in this and previous studies, could play a role in initiating these hugging behaviors as a prerequisite to biting or ensure adherance in case biting fails during the final stages of infection (Will et al., 2020).

Figure 4. Examples of "hugging" behavior portrayed by *Ophiocordyceps*-infected *Camponotus floridanus* ants during the death grip observed in a natural setting.

Even though the exact structure and concentrations of the aflatrem-like compounds produces by *Ophiocordyceps in vivo* are still unknown, this study was able to replicate the "drunkard's walk" phenotypes induced in the host during *Ophiocordyceps* infection, as well as

identify significant changes in the expression of 108 previously identified candidate manipulation genes in response to aflatrem exposure. While several of these genes were differentially regulated when compared with previously published transcriptomics data for *Ophiocordyceps*-infected ants (Will et al., 2020), the presence of changes in these genes nevertheless demonstrates their importance as targets for behavioral manipulations. Future work characterizing the 3-dimensional structure of the actual aflatrem-like compound produced by *O. unilaterial* species will shed further light on the role of these alkaloids in behavior manipulation and its precise physiological effects in the host. Even so, this work presents a hypothetical mechanism for time-specific expression of ergot alkaloids for the disruption of host activity in conjunction with summit disease that could play a vital role in facilitating the eventual attachment of the host at an elevated vantage point. By comparing the behaviors and gene expression profiles between ants treated with aflatrem and ants infected with *Ophiocordyceps*, we have glimpsed into the evolutionary purpose that secondary metabolites, upregulated during manipulation, may serve in *Ophiocordyceps* zombie fungi. As such, integrative behavioral studies like this one can bring us one step closer to characterizing the molecular mechanisms that drive zombie-making fungi. Furthermore, the protocols established as part of this research provide a framework for future studies that wish to examine the role of putative effectors produced and secreted by members of the *Ophiocordyceps* species complex, paving the way for further characterization of compounds produced by *Ophiocordyceps* as well as other behavior-manipulating species and their respective insect hosts.

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Author Contributions:

WCB and CdB designed the experiment, analyzed the data, performed the RNA extractions and wrote the paper. WCB, CK, and UAM collected the ants and performed the injections and video recordings. CK and UAM performed the behavioral analyses using CowLog. WCB ran the MARGO analyses and performed the statistical analyses.

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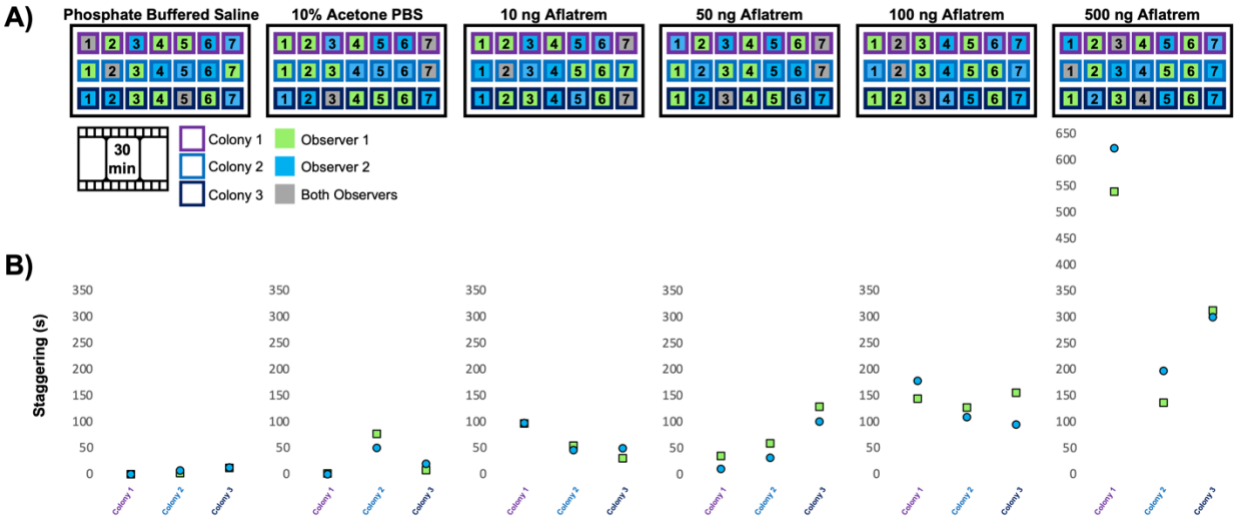
Supplemental Files

Supplemental Table 1: The average length and weight of *Camponotus floridanus* in this study

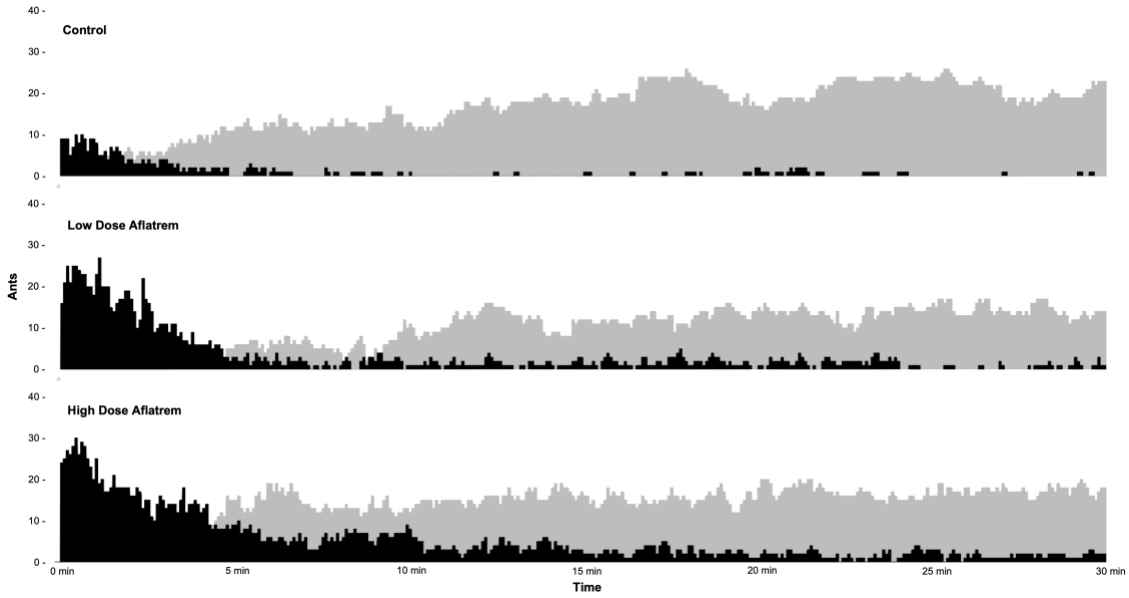
Colony #	Treatment Group	Average Length (mm)	Average Weight (mg)
Colony 1	C1-PBS	7.07	7.56
	C1-PBS/10% acetone	6.50	6.93
	C1-PBS/10% acetone/500ng aflatrem	6.78	7.94
	C1-PBS/10% acetone/100ng aflatrem	7.00	8.14
	C1-PBS/10% acetone/50ng aflatrem	6.43	8.44
	C1-PBS/10% acetone/10ng aflatrem	7.29	9.01
Colony 2	C2-PBS	7.07	9.49
	C2-PBS/10% acetone	7.07	10.27
	C2-PBS/10% acetone/500ng aflatrem	6.93	11.09
	C2-PBS/10% acetone/100ng aflatrem	6.93	10.48
	C2-PBS/10% acetone/50ng aflatrem	6.86	10.86
	C2-PBS/10% acetone/10ng aflatrem	6.86	10.13
Colony 3	C3-PBS	6.07	5.40
	C3-PBS/10% acetone	6.93	7.93
	C3-PBS/10% acetone/500ng aflatrem	6.79	6.44
	C3-PBS/10% acetone/100ng aflatrem	5.71	5.40
	C3-PBS/10% acetone/50ng aflatrem	5.64	6.23
	C3-PBS/10% acetone/10ng aflatrem	6.29	7.24
	Total	6.68 mm	8.28 mg

Supplemental Figure 1



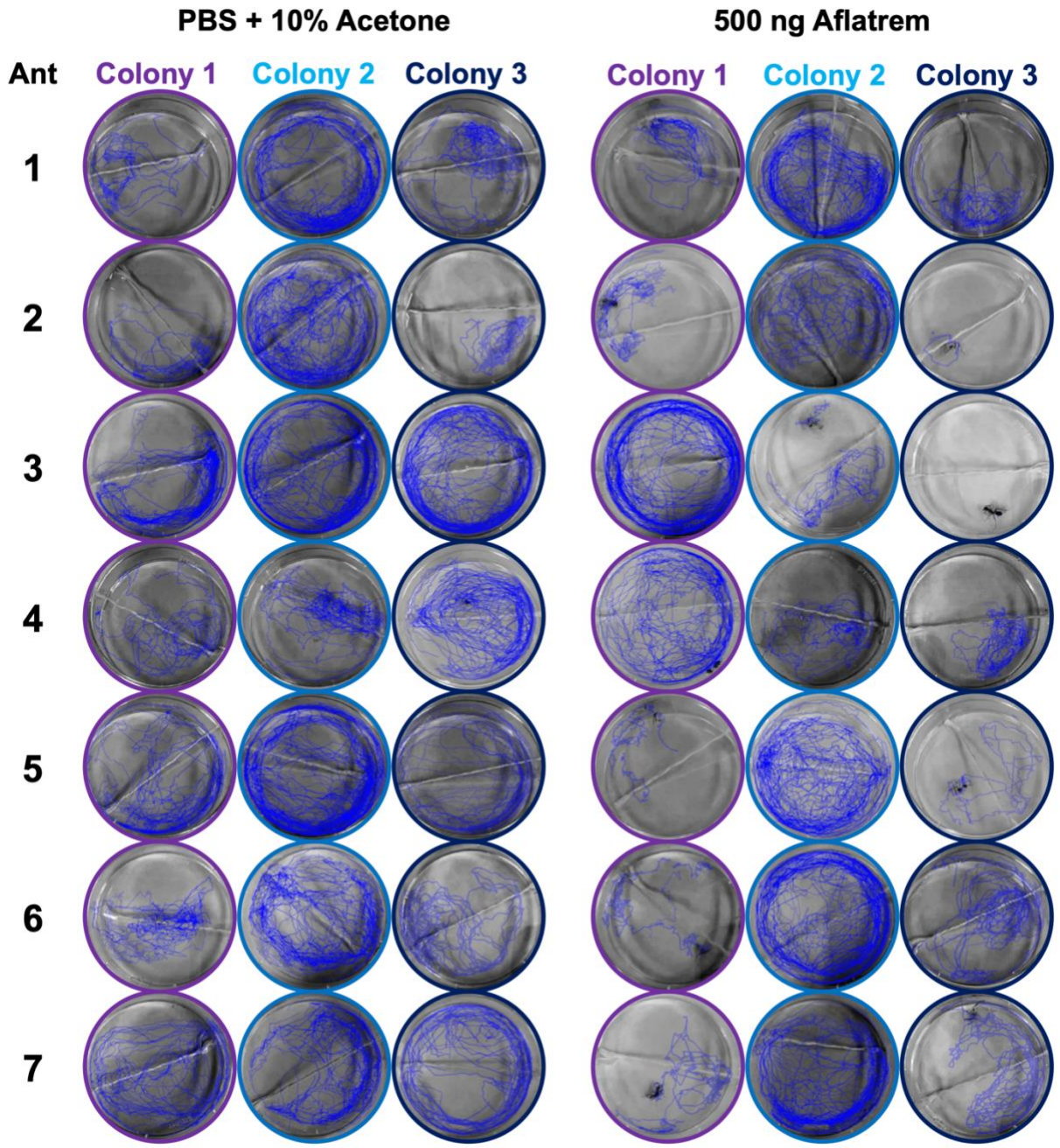
Supplemental Figure 1. An overview of the randomized block format used to assign videos to each observer (A). The videos assigned to observer 1 are shown in green, the videos assigned to observer 2 are shown in blue, and the videos assigned to both for formative analysis are shown in grey. B) depicts the duration of staggering scores for each grey assignment, with the scores for observer 1 shown as green squares and the scores for observer 2 shown as blue circles.

Supplemental Figure 2



Supplemental Figure 2. Bar graphs depict the number of ants exhibiting staggering behavior (black) and the number of ants resting (dark grey) across the 30-minute observational period, broken down into 5 second intervals.

Supplemental Figure 3



Supplemental Figure 3. The path traveled by each ant recorded by the MARGO tracking software during the 30-minute observational period is shown in dark blue. The three columns on the left shows the paths traveled by the control group ants injected with PBS/10% acetone, while the three

columns on the right shows the paths traveled by the ants in the PBS/10% acetone/500 ng aflatrem treatment group. Each group is further divided by colony ID using purple, blue, and dark blue outline to indicate individuals from colonies 1, 2, and 3, respectively.

Supplemental File 1

**RNAseq Data*

Supplemental Video 1

**O. camponoti-floridani* – infected *C. floridanus* staggers syndrome - mp4

Supplemental Video 2

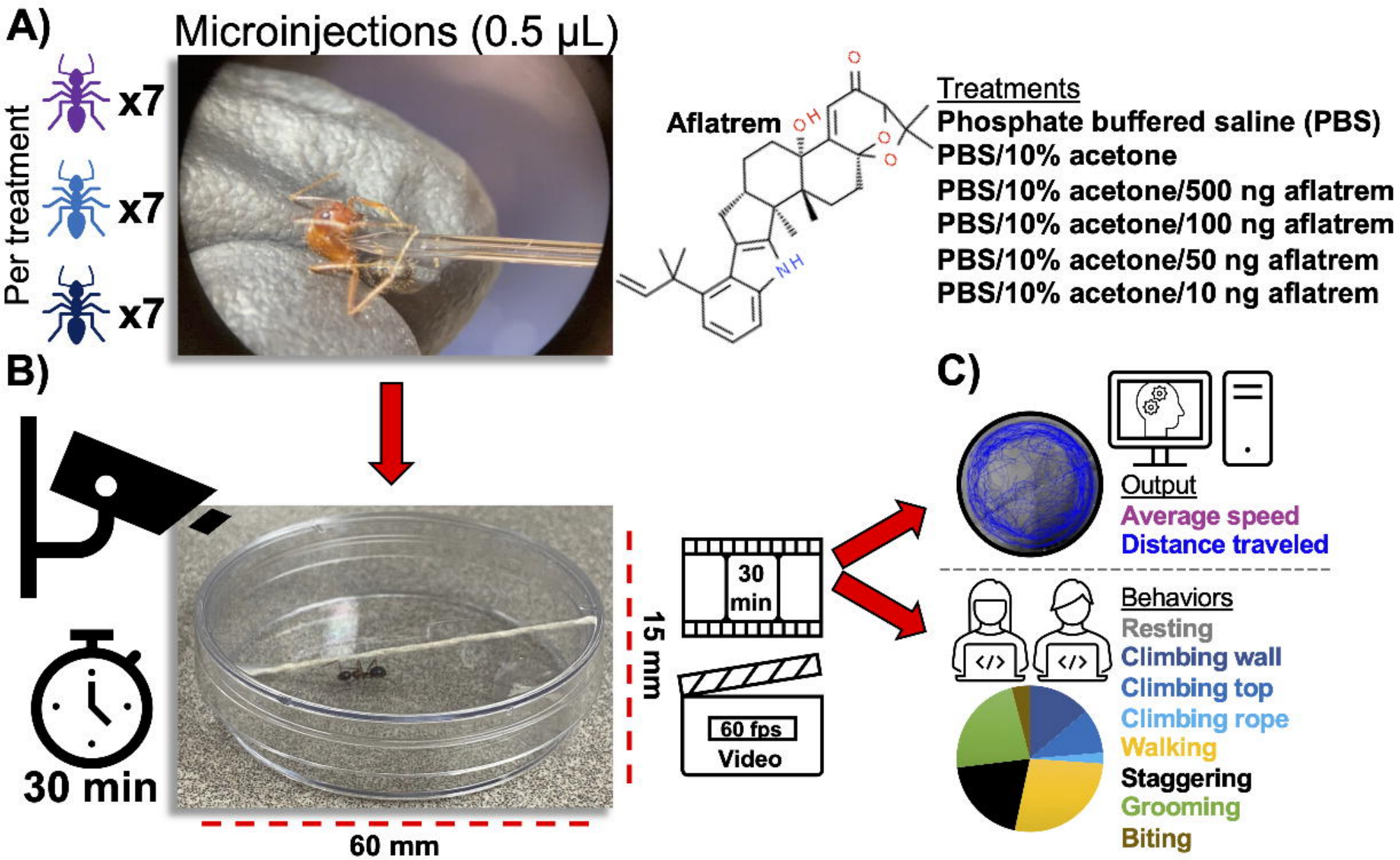
*Observed behaviors – mp4

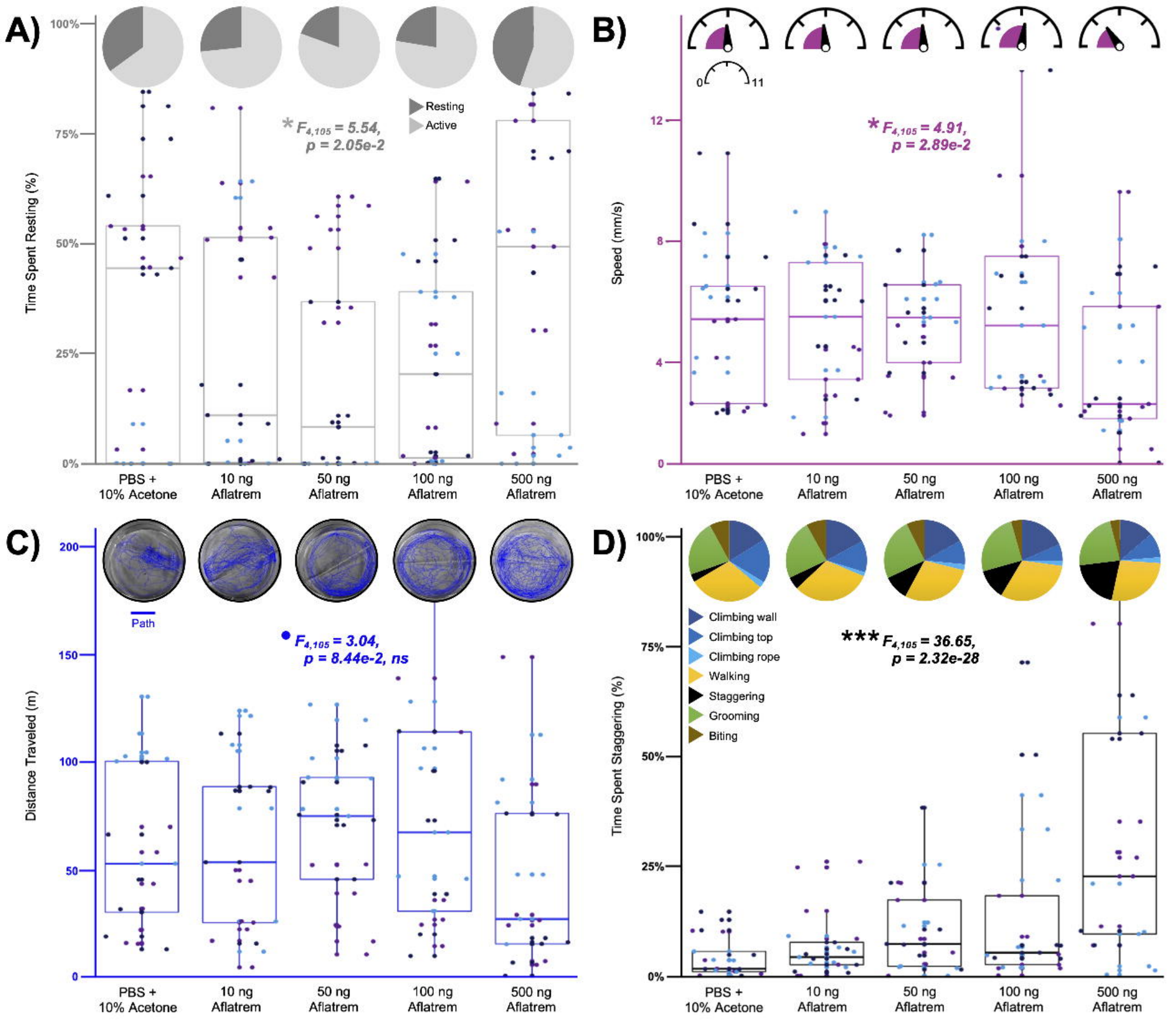
Supplemental Video 3

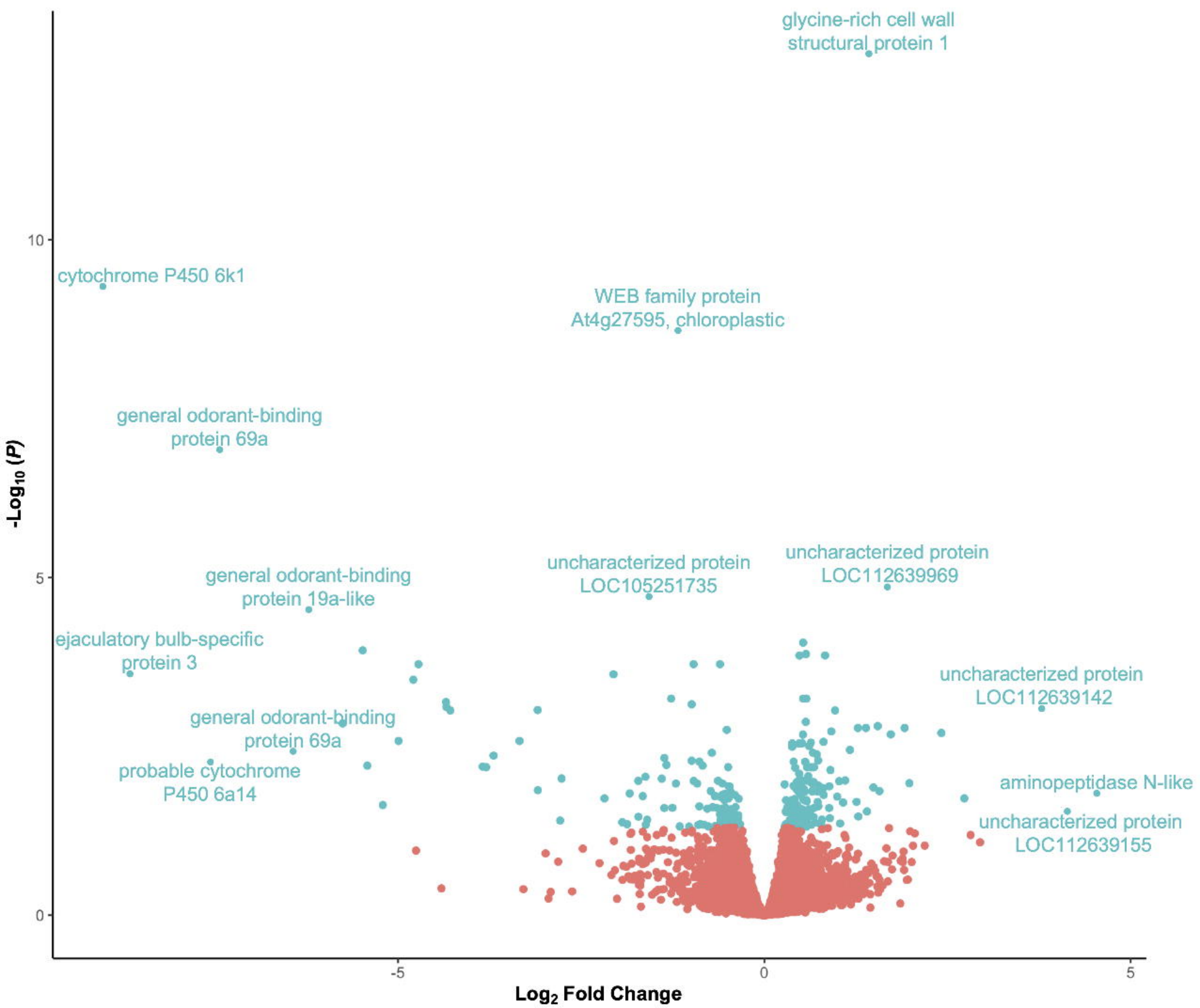
*Stagger behaviors – mp4

Supplemental Video 4

*Unique behaviors – mp4


A)
Microinjections (0.5 µL)
Per treatment
x7
x7
x7
Aflatrem
Treatments
Phosphate buffered saline (PBS)
PBS/10% acetone
PBS/10% acetone/500 ng aflatrem
PBS/10% acetone/100 ng aflatrem
PBS/10% acetone/50 ng aflatrem
PBS/10% acetone/10 ng aflatrem
B)
30 min
15 mm
60 mm
30 min
60 fps
Video
C)
Output
Average speed
Distance traveled
Behaviors
Resting
Climbing wall
Climbing top
Climbing rope
Walking
Staggering
Grooming
Biting


A)
Time Spent Resting (%)
100%
75%
50%
25%
0%
Resting
Active
* $F_{4,105}$ = 5.54,
p = 2.05e-2
PBS + 10% Acetone
10 ng Aflatrem
50 ng Aflatrem
100 ng Aflatrem
500 ng Aflatrem
B)
Speed (mm/s)
0
11
12
8
4
0
* $F_{4,105}$ = 4.91,
p = 2.89e-2
PBS + 10% Acetone
10 ng Aflatrem
50 ng Aflatrem
100 ng Aflatrem
500 ng Aflatrem
C)
Distance Traveled (m)
200
150
100
50
0
Path
• $F_{4,105}$ = 3.04,
p = 8.44e-2, ns
PBS + 10% Acetone
10 ng Aflatrem
50 ng Aflatrem
100 ng Aflatrem
500 ng Aflatrem
D)
Time Spent Staggering (%)
100%
75%
50%
25%
0%
Climbing wall
Climbing top
Climbing rope
Walking
Staggering
Grooming
Biting
*** $F_{4,105}$ = 36.65,
p = 2.32e-28
PBS + 10% Acetone
10 ng Aflatrem
50 ng Aflatrem
100 ng Aflatrem
500 ng Aflatrem


glycine-rich cell wall structural protein 1
cytochrome P450 6k1
WEB family protein At4g27595, chloroplastic
general odorant-binding protein 69a
uncharacterized protein LOC105251735
uncharacterized protein LOC112639969
general odorant-binding protein 19a-like
ejaculatory bulb-specific protein 3
uncharacterized protein LOC112639142
general odorant-binding protein 69a
probable cytochrome P450 6a14
aminopeptidase N-like
uncharacterized protein LOC112639155
10
5
0
-5
0
5
-Log10 (P)
Log2 Fold Change