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

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ABSTRACT

Coevolutionary relationships between parasites and their hosts can lead to bizarre phenotypes over time. As such, certain *Ophiocordyceps* fungi have evolved to manipulate the behaviour of insect and arachnid hosts. The most conspicuous examples are found in ants of the Camponotini tribe, colloquially known as "zombie ants", which climb to elevated positions to aid fungal spore transmission. While the unusual behaviours induced during infection are well described, the molecular driving force behind these changes is still unknown. Recent genomics and transcriptomics analyses have identified several candidate secreted proteins and secondary metabolites, highly upregulated during infection. Amongst these compounds is an ergot alkaloid related to the mycotoxin aflatrem, known to cause neurological issues in cows collectively called "staggers syndrome". Staggering is also observed in *Camponotus floridanus* ants during the late

stages of *Ophiocordyceps* infection and manipulation. To test if aflatrem-like compounds could be responsible for this behavioural phenotype, we injected healthy ants with purified aflatrem, and recorded their behaviour for 30 minutes. Using both the machine learning tool MARGO and manual behavioural quantification, we found that aflatrem caused reduced ant activity and speed, and increased the number and duration of staggering behaviours. To examine changes at the transcriptome level that might underlie these phenotypes, we performed RNA-Seq on the heads of aflatrem-injected ants. We identified 261 genes that were significantly up- or down-regulated in the aflatrem-injected ants compared to sham-injected controls. When compared with RNA-seq data available for ants manipulated by *Ophiocordyceps camponoti-floridani*, we found that 108 of these genes were also differentially regulated in the heads of late-stage *Ophiocordyceps*-infected ants. These shared dysregulated genes included *sensory neuron membrane protein* genes, several *odorant-binding protein* genes, and musculoskeletal genes such as *titin* and *obscurin*. Together, these results indicate that aflatrem-like compounds, produced by *O. camponoti-floridani* during behavioural manipulation, significantly affect neuromuscular and sensory functioning, leading to a staggers phenotype in *C. floridanus*.

1. INTRODUCTION

As parasites adapt to their hosts, the two often develop an intense relationship driven by selective pressures presented in the host environment. As the parasite becomes more successful, the selective burdens shift to favour emergent resistance phenotypes in the host. Over time, these co-evolutionary pressures can lead to host-specificity and the subsequent emergence of unique manipulation phenotypes. Host manipulations provide an adaptive advantage to the parasite, for instance through the induction of specific behaviours that increase its reproduction success

(Sánches and Biron, 2019). These adaptive phenotypes are different from sickness behaviours, defined as actions exhibited by infected individuals that are not conducive to the fitness of the host nor the parasite (e.g., lethargy). One example of behavioural manipulation 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 behaviour known as "summit disease" (Wesołowska and Wesołowski, 2013). Summiting behaviour makes snails more susceptible to predatory birds, the primary host of *L. paradoxum*, ultimately increasing the parasite's likelihood of completing its lifecycle (Wesołowska and Wesołowski, 2013).

Such behaviour-manipulating parasites are often colloquially referred to as "zombie parasites" because the phenotypes exhibited by their hosts are reminiscent of zombies from pop culture and sci-fi media (e.g., the movies *28 Days Later* and *The Body Snatchers*). While the manifestations of behavioural manipulation vary across species, many host-manipulating parasites have convergently evolved to induce summiting behaviour to increase their transmission (Araújo & Hughes, 2019; de Bekker et al., 2021; Latchininsky et al., 2016; Steinkraus et al., 2017; Wesołowski & Wesołowski, 2013). One of the largest known groups of parasites that cause summit disease are the "zombie fungi" of the genus *Ophiocordyceps*, which infect various species of insects and arachnids globally (Arruda et al., 2021; Cooley et al., 2018; de Bekker et al., 2021). 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)). However, a large portion of the currently described species belong within the species complex *Ophiocordyceps unilateralis*; one of the more well-

70 studied groups of zombie fungi that collectively infect and manipulate carpenter ants with extreme
71 host-specificity (Araújo et al., 2018, Araújo et al., 2020).

72 *Ophiocordyceps unilateralis* species begin their lifecycle as a spore, ejected from their
73 parental fruiting body. These spores sail on the wind to infect new ants, either through immediate
74 contact or through the production and attachment of secondary capillispores from the forest floor
75 (Evans et al., 2018). Once inside the ant, the fungus begins to secrete an array of biomolecules in
76 a time-specific manner; some of which alter the behaviour of their host (de Bekker et al., 2015;
77 Will et al., 2020). The first notable change occurs as these ordinarily social hosts begin to show a
78 lessened ability to communicate and participate in foraging tasks (Trinh et al., 2021).
79 Subsequently, infected ants abandon the nest and climb nearby vegetation. Once at an elevated
80 position, they latch on with their mandibles, such that the ant is perched on the underside of its
81 biting substrate (Andersen et al., 2009). The fungus then completes its lifecycle through the
82 formation of an endosclerotium that siphons the remaining nutrients from the host for the
83 formation of a new fruiting body (Fredericksen et al., 2017). Closer observation and quantification
84 of behaviours in both the lab and the field have led to the identification of other, more subtle
85 behavioural changes. Altered behaviours include changes in daily rhythms (Hughes et al., 2011;
86 Trinh et al., 2021) and the loss of motor coordination and balance, resulting in ants that fumble
87 their way around as in a "drunkard's walk" (Supplemental Video 1) (Hughes et al., 2011; Trinh et
88 al., 2021; Will et al., 2020). These conspicuous behavioural changes caused by the fungus during
89 infection make the *O. unilateralis* species complex and their hosts particularly well-suited for the
90 study of behaviour-manipulating parasites in greater detail (de Bekker et al., 2015).

91 Recent work establishing fungal culturing and ant infection techniques, as well as the
92 genomic sequencing of several *O. unilateralis s.l.* species, has laid the groundwork for research

93 aimed at understanding the molecular framework driving behavioural manipulation (de Bekker et
94 al., 2015; de Bekker et al., 2017; Will et al., 2020). Transcriptomics analyses of the North
95 American species *Ophiocordyceps kimflemingiae* and *Ophiocordyceps camponoti-floridani* have
96 identified secreted proteins and secondary metabolite clusters highly upregulated during
97 summiting behaviour (de Bekker et al., 2015; Will et al., 2020). One of these secondary metabolite
98 clusters demonstrated a ~5,900 (in *O. kimflemingiae*) and ~12,000-fold (in *O. camponoti-floridani*)
99 increase in expression during manipulation compared to fungi grown in culture (Will et al., 2020).
100 This cluster was first identified as an ergot alkaloid-producing pathway based on the functional
101 annotation of its tryptophan dimethyltransferase (Pfam: TRP_DMAT (PF11991)) backbone gene
102 (de Bekker et al., 2015). More detailed annotation of the genes within and surrounding the cluster
103 demonstrated that it was homologous to the aflatrem-producing cluster in the plant pathogen
104 *Aspergillus flavus* (Will et al., 2020). Genomic comparisons with other *Ophiocordyceps* genomes
105 revealed that this secondary metabolite cluster appears to be conserved across species within *O.*
106 *unilateralis* but not in other *Ophiocordyceps* species outside the species complex (e.g.,
107 *Ophiocordycpes australis s.l.*) (de Bekker et al., 2017). This begs the question; what evolutionary
108 purpose may aflatrem-like products serve for the fitness of *Ophiocordyceps* species that
109 manipulate the behaviour of *Camponotus* hosts?

110 Aflatrem, a secondary metabolite of the indole-diterpene group called aflatoxins, is a potent
111 tremorgenic and carcinogenic mycotoxin known to cause neurological disorders in animals (Duran
112 et al., 2006; Nicholson et al., 2009). Cows exposed to aflatrem through ingestion of *A. flavus*-
113 contaminated grain feed exhibit neurological problems exhibited by muscle tremors and
114 hyperexcitability, followed shortly by incoordination, ataxia, and seizures (Selala et al., 2008;
115 Valdes et al., 1985). These neurological symptoms, linked directly to aflatrem's positive allosteric

116 modulation of Gamma-aminobutyric acid (GABA) receptors, are referred to as "staggers
117 syndrome" (Eldefrawi et al, 1990; Grant et al., 1987; Yao et al., 1989). Should aflatrem-like
118 compounds exhibit similar effects in *Camponotus* species, it could have important ramifications
119 for host manipulation given the suspected involvement of GABA receptors in caste and
120 behavioural differentiation in *Camponotus* ants (Gospocic et al., 2017; Graham, 2018). The
121 likelihood that an aflatrem-like compound may be employed during the manipulation of carpenter
122 ants is further supported by the "drunkard's walk" observed during late-stage infection; a
123 behaviour reminiscent of the staggers syndrome detailed in vertebrates (Supplemental Video 1).
124 We, therefore, hypothesize that aflatrem-like compounds are responsible for the stagger phenotype
125 observed during the behavioural manipulation of carpenter ants by *O. unilateralis* s.l.

126 In this study, we administered aflatrem to the Florida carpenter ant *Camponotus*
127 *floridanus*, the host of *O. camponoti-floridani*. We used microinjections to mimic the *in vivo*
128 secretion of aflatrem-like compounds by fungal cells in the haemolymph during infection (Figure
129 1).

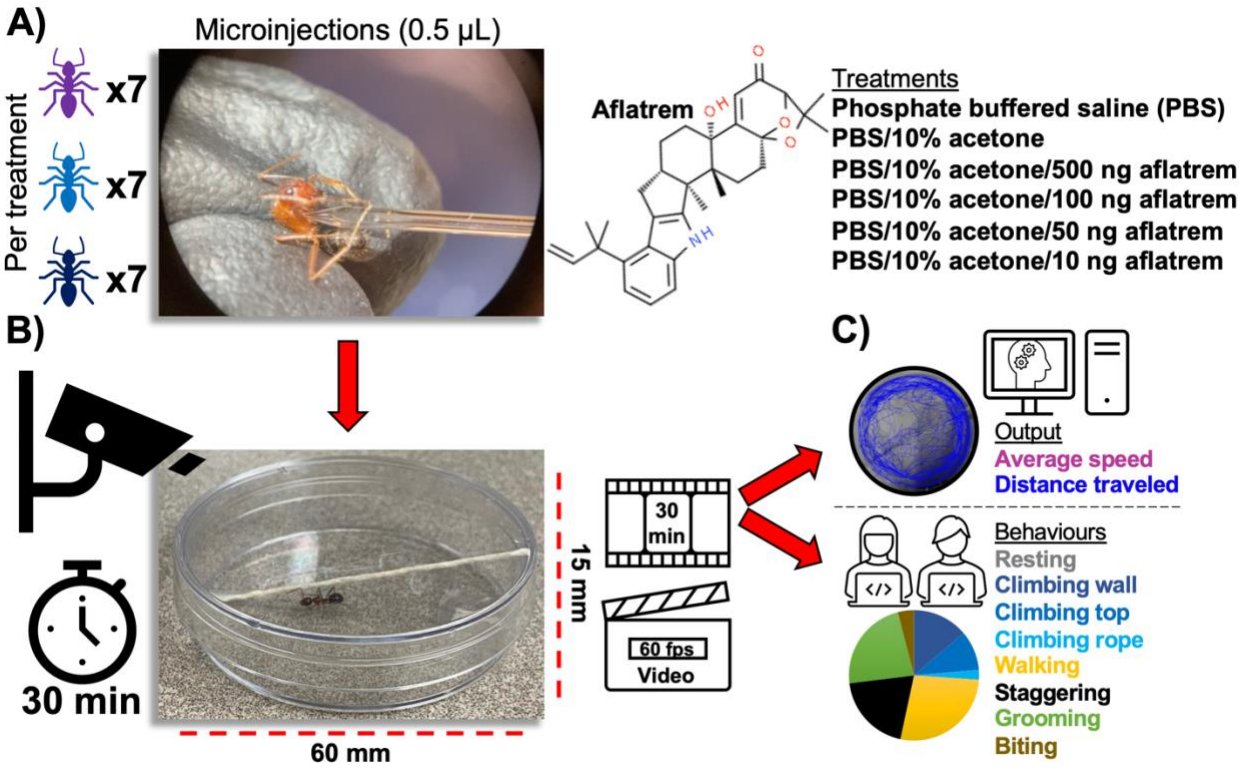

Figure 1. Experimental overview of (A) the treatment groups, each consisting of 7 individuals from 3 different colonies of *Camponotus floridanus*, and an example of microinjection, (B) the arena setup for behavioural assays and parameters for the observational periods, and (C) the behavioural analyses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

Given the ease of its procurement, and its previous use in other animal models, we used high purity synthesized aflatrem (>98%) in our experiments (TePaske et al., 1992; Trienens & Rohlfs, 2011). Following injections, we observed and quantified ant behaviour to address if aflatrem can induce behaviours consistent with those exhibited during infection by *O. camponoti-floridani*. Furthermore, to investigate which host pathways are affected by aflatrem and potentially give rise to the exhibited behavioural 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

144 differentially expressed genes, including genes that encode neuromuscular and chemosensory
145 proteins.
146
147 **2. MATERIALS AND METHODS**
148
149 **2.1 Ant Collection**
150 For use in behavioural and transcriptomics experiments, we collected three separate colonies of
151 *Camponotus floridanus* using a DeWALT Cordless 2 Gallon Wet/Dry ShopVac at the University
152 of Central Florida's arboretum, coordinates: Colony 1 (28.602748, -81.194269), Colony 2
153 (28.603918, -81.191682), and Colony 3 (28.601430, -81.191251). We housed these colonies in 9.4
154 L plastic containers (Rubbermaid) lined with talcum (Acros Organics), applied using a viscous
155 20% w/v solution of powder suspended in 100% ethanol to prevent ants from climbing the walls.
156 Throughout the study, we provided the ants with fresh supplies of both double distilled water
157 (ddH2O) and 15% sucrose solutions, *ad libitum*. We also kept the housing in an incubation
158 chamber (Percival) set to the following program: 20°C – 0 lux – 80% relative humidity (RH) with
159 a linear increase in temperature, light, and decrease in humidity over 4 hours to reach 28°C – 2,100
160 lux – 65% RH, at which conditions were held for 4 hours followed by a linear decrease in the
161 temperature, light, and increase in humidity, returning to 20°C – 0 lux – 80% RH over 4 hours;
162 and held for 12 hours.
163
164 **2.2 Microinjections**
165 To test our hypothesis and identify the effect of aflatrem-like compounds on *C. floridanus*,
166 we established a behavioural assay using microinjections to introduce aflatrem in a manner

167 biologically relevant to the secretion of aflatrem-like compounds by *Ophiocordyceps* species in
168 the haemolymph of their ant hosts (Figure 1). For these assays, we utilized high purity aflatrem,
169 >98% $C_{32}H_{39}NO_4$, manufactured from *A. flavus* by BioCrick Science Solution Specialists in non-
170 water-soluble crystal form, which we suspended in 100% acetone (Fisher Chemical) to a
171 concentration of 10 mg/mL. We performed injections using borosilicate glass micropipettes
172 (Fisher) pulled with a glass puller (model: PC-100, Narishige) under the following parameters:
173 single step, all weights, heated to 62°C. To inject the ants, we held each one by the dorsal side of
174 the thorax before piercing the carapace on the ventral side between the first and second pair of
175 legs, followed by slowly injecting 0.5 μL of solution into the ant while simultaneously releasing
176 grip to alleviate intracavitary pressure.

177 Because the aflatrem used in this study is non-water soluble, we performed a preliminary
178 trial to identify acetone tolerance in ants. As such, we injected subjects with 100%, 50%, 25%,
179 10%, and 5% acetone solutions diluted with phosphate buffered saline (PBS) (137 mmol NaCl,
180 2.7 mmol KCl, 10 mmol Na_2HPO_4, 1.8 mmol KH_2PO_4, pH adjusted to 7.4), along with a 100%
181 PBS solution as a control. Ten minutes prior to injection, we removed containers housing ant
182 colonies from the incubator and set them on the lab bench top to acclimate to lab conditions. We
183 also performed all injections in triplicate, one ant per colony per treatment. After injection, we
184 placed the ants into individual 60 mm x 15 mm petri dishes (USA Scientific) and returned them to
185 the incubator. We kept these ants under observation for 48 hours post injection using an infrared
186 GoPro Hero6 set to record time-lapse footage with 60 second intervals and a CMVision IR30
187 Illuminator lamp for observation during the night cycle. From these videos, we were able to easily
188 differentiate dead and resting *C. floridanus* ants from one another; dying ants coiled their legs and
189 fell to one side upon death while resting ants remained prone with legs supporting their bodies. As

190 such, we were able to estimate the time of death of each ant within an interval of 1 minute by
191 noting the frame in which they exhibited "death behaviours". We euthanized any ants that survived
192 the 48-hour test period by freezing at -20°C for 24 hours. Results from these preliminary analyses
193 indicated that a 10% acetone solution was the highest concentration for which there were no
194 statistical differences in average survivability compared to PBS-injected and non-injected controls.

195 To test the survivability of ants post injection, and determine if any observed behavioural
196 phenotypes may be attributed to "dying behaviours" resulting from aflatrem exposure, we
197 performed a second preliminary trial. We injected 7 ants with 0.5 μL 10% acetone solution
198 containing 500 ng aflatrem. This was the highest dose possible using the 10 mg/mL aflatrem stock.
199 After injection, we recorded the ants for 48 h using the same GoPro setup as the previous
200 preliminary test. We then compared the two groups and found no significant differences in
201 mortality, with all individuals surviving for at least 24 h and an average time of death arising at 34
202 h. The results indicate that all ants that died in the trial did so as a result of desiccation from lack
203 of water.

204 Confident that neither 10% acetone nor 500 ng aflatrem killed the ants or induced death-
205 related behaviours, we conducted an assay to determine the effects of aflatrem on ant behaviour
206 using 10% acetone solutions containing the following doses of aflatrem; 10 ng, 50 ng, 100 ng, 500
207 ng. For each dose of aflatrem, we injected 7 ants from each of the 3 colonies (N=21, per dose) for
208 a total sample size of N=126. We individually lifted each ant from their housing by hand wearing
209 neoprene gloves (NeoTouch) and allowed them to acclimate to the lab conditions by placing them
210 in individual 60 mm x 15 mm petri dishes (USA Scientific) on the lab benchtop for 10 minutes.
211 We fitted each petri dish with a thread of twine spanning the dish from the bottom of one side to
212 the top of the other. This provided ants with a substrate for biting and more dexterous climbing

213 behaviours (Figure 1-B). To allow ants to walk around the perimeter of the dish unimpeded, we
214 made sure to tape (Scotch) the twine pressed flat against the bottom corner of the petri dishes. We
215 created a new petri dish arena for each test subject (N=126) to avoid any behavioural changes that
216 may be induced via chemical ques left by prior occupants. After the 10-minute acclimation period,
217 we injected ants with aflatrem following the microinjection protocols established in the
218 preliminary trials. The doses of aflatrem used were based on approximate ant equivalent weights,
219 verified by measuring *C. floridanus* body volume through length and weight following each
220 experiment. While we measured body lengths to the nearest 0.5 mm for each individual, weight
221 was measured in groups of seven ants corresponding to their treatment and colony ID. We recorded
222 these measurements immediately following euthanization at -20°C, preserving the approximate
223 water weight for each ant. To account for the approximate weight of solution injected into each
224 ant, we also subtracted 3.5 mg from the total weight of each group. Based on an average of 8.28
225 mg weight and 6.68 mm length calculated from measurements of all 126 subjects used in this study
226 (Supplemental Table 1), the four doses of aflatrem that were used are approximately 6.04e-5,
227 1.21e-5, 0.60e-5, and 0.12e-5 ant equivalents. These ant equivalent doses are consistent with
228 pervious works testing insecticidal compounds in ants (Buczkowski & Woosler, 2019; Klotz &
229 Moss, 1996; Sakamoto & Goka, 2021).
230

231 **2.3 Video scoring of behavioural assays post injection**

232 To record behaviours post injections, we used an iPhone 13 pro. We recorded three or four arenas
233 at a time in a rolling format to ensure high quality resolution at 60 fps. This generated video files
234 approximately 45 minutes in length. In cases where ants exhibited signs of physical injury from
235 the injection, e.g., immobility in the hind legs or body curling, we euthanized the subject and

236 replaced it with a new ant and new arena. If any ants were exhibiting resting behaviour at the end
237 of their recording period, we tapped their arenas several times to induce movement. All instances
238 of tapping elicited activity, confirming that all test subjects were still alive.

239 To crop the rolling videos into clips showing only individual arenas, we first converted the
240 iPhone .MOV files to .mp4 files using iMovie. We then exported these .mp4 files to Movavi Video
241 Editor Plus 2022 and trimmed each clip to exactly 30 minutes, beginning as soon as each arena
242 was positioned in the field of view. The names for each of these clips were blinded before two
243 observers manually scored the videos using the behaviour tracking software CowLog3 (Pastell,
244 2016). Before formal scoring, we trained each observer using eight test run videos; four of ants
245 injected with 500 ng aflatrem, and four of ants injected with PBS. These videos were made during
246 proof-of-principle testing and are not included in the data presented in this study. We used the
247 results from these practice runs to form a consensus on scoring methodology. From these training
248 videos, the observers identified eight scorable behaviours: Climbing Wall, Climbing Top,
249 Climbing Rope, Walking, Staggering, Grooming, Resting, and Biting (Supplemental Video 2).
250 While most of these behaviours are self-explanatory, we defined "staggering" as a slow, sluggish
251 walking behaviour with repeated lateral movements (Supplemental Video 3). We also categorized
252 behaviours as "staggering" when trouble balancing, listing to one side, falling over, walking in
253 tight circles, flipping over, or dramatic wagging of the gaster were observed (Supplemental Video
254 3). Additionally, each observer kept notes on unique behaviours observed during the scoring
255 period, including walking backwards, lunging/bucking, and two instances of convulsions
256 (Supplemental Video 4).

257 During the formal scoring, we assigned the blinded videos to the observers in a randomized
258 block format (Supplemental Figure 1-A) with each observer scoring three videos at random per

259 treatment group per colony. Both observers scored the seventh video for each treatment group,
260 from which the results were compared and used as a formative metric to ensure consistent scoring
261 throughout the evaluation period. While most behaviours were readily distinguishable, resulting
262 in very similar scores between observers, walking and staggering provided the most discrepancy.
263 We, therefore, used staggering as the primary measurement to evaluate scoring consistency across
264 observers (Supplemental Figure 1-B).

265 In addition to manual observations, we also analysed each video using the MATLAB-based
266 machine learning tool Massively Automated Real-time GUI for Object-tracking (MARGO)
267 (Werkhoven et al., 2019). Using this tool, we were able to measure the speed and distance travelled
268 by each subject. While we calculated distance travelled by measuring the total distance between
269 centroid coordinates over the entire 30 min duration, we only measured speed, calculated in mms
270 travelled per second, during activity. To optimize the tracking within the field of view for each
271 video, we used the following metrics: region of interest (ROI) was set to circular shape and
272 manually overlayed around the arena, background referencing was set to detect dark objects on
273 light backgrounds, tracking threshold was set between 70-80, distance of the arena was set to 60
274 mm using the custom measuring tool, frame rate was set to 30, and min/max area was set to 5 and
275 80, respectively.

276 Before testing the effects of aflatrem on ant behaviour, we compared the results from the
277 10% acetone controls to the 100% PBS controls to identify any potential behavioural phenotypes
278 induced by the addition of acetone to the injections. To test for any statistical significance between
279 the two groups, we used a two-sample t-test in RStudio v1.3.959. Following the comparison of
280 these two controls, we tested the effect of aflatrem exposure on ant behaviour with a linear

281 regression model using the lme4 package in RStudio, by comparing the 10% acetone control (0 ng
282 aflatrem) with 10% acetone containing 10 ng, 50 ng, 100 ng, and 500 ng aflatrem treatments.
283

284 **2.4 RNA sequencing**

285 To understand the effects of aflatrem exposure in *C. floridanus* at the genomic level, we injected
286 a total of 10 ants with either 10% acetone (control group, N=5) or 500 ng aflatrem (treatment
287 group, N=5). We then placed each ant into their own petri dish arena for 4 minutes. We chose this
288 timepoint based on the scaling levels of activity observed in our behavioural assays (Supplemental
289 Figure 2). After this period, we quickly placed the ants into 2 mL microcentrifuge tubes and snap
290 froze them in liquid nitrogen. Subsequently, we removed the head from each ant with forceps and
291 placed it in a pre-frozen 2.0 mL Self-Standing Impact Resistance Microtube (USA Scientific)
292 containing 2 Grade 25 5/32" metal ball bearings (Wheels Manufacturing) for mechanical tissue
293 disruption in a SPEX® SamplePrep 1600 MiniG at 1500 rpm for 30 sec. We dissolved the frozen
294 samples in 500 uL TRIzol (Ambion) to extract the RNA and isolated it using the Qiagen RNeasy®
295 MiniElute® Cleanup Kit (Will et al., 2020). The resulting RNA was stored at -80C and shipped to
296 Azenta US for Next Generation Sequencing. Libraries were prepared by Azenta US using PolyA
297 selection and sequenced as 150 bp paired end reads on an Illumina HiSeq, resulting in ~350M
298 reads. Read quality was verified using FastQC (Andrews 2010). We used Trimmomatic (Bolger
299 et al., 2014) to trim reads for adapters and quality using the following parameters: Leading: 3,
300 Trailing: 3, MinLen: 36. We then aligned the transcripts to the *Camponotus floridanus* reference
301 genome (Gene bank ascension number GCA_003227725.1) using Spliced Transcripts Alignment
302 to a Reference (STAR) 2.7.10a (Dobin et al., 2013). After alignment, we checked the resulting
303 .bam files for quality using the standard metric of uniquely mapped reads > 70%, sorted, and

304 indexed using Samtools (Danecek et al., 2021). We next calculated the number of gene hits for
305 each sample using FeatureCounts, following the suggested counting of strictly uniquely mapped
306 reads within exonic regions (Liao et al., 2019). The quality of the resulting files was validated
307 using a benchmark of 15-20 million reads per sample. After removing hits with low level of
308 expression (counts less than 100 per gene, i.e., an average less than 10), we normalized the
309 remaining counts using DESeq2 (Love et al., 2014) eliminating 3,227 genes from further analysis.
310 We performed differential expression analysis on the remaining 10,443 genes using DESeq2 with
311 an alpha value of 0.05 and functionally annotated the differentially expressed genes using
312 Blast2GO (Conesa et al., 2005).
313

314 **2.5 Ethical Note**

315 We performed all injections and behavioural analyses in this study on the invertebrate ant species
316 *Camponotus floridanus*, which is not endangered and is a prevalent species in Central Florida. We
317 collected colonies of this species at the University of Central Florida Arboretum, which did not
318 require prior approval from an animal welfare or ethics committee. Only three colonies were
319 collected to meet the minimal scientific standard of triplicate replication. Collection utilized a fast
320 and handling-minimizing method. We applied the following standards in effort to reduce the
321 amount of stress incurred by the ants throughout our study as much as possible. Housing was
322 conducted in an environmental chamber set to mirror the subjects' natural conditions. We
323 maintained colonies by providing *ad libitum* food and water, and dark enclosures for nesting. We
324 furthermore conducted experiments on the minimum required number of subjects to reach the
325 sample size needed for statistical analysis. Ants were quickly euthanized at -20°C in instances
326 where they were injured during treatment and immediately following all experiments.

327

328 **3. RESULTS**

329 **3.1 The behavioural effects of aflatrem in *C. floridanus***

330 Before we could analyse the behavioural effects of aflatrem in ants, we screened for any potential
331 behavioural changes caused by the presence of 10% acetone in the injections. Using an
332 independent two sample t-test to compare the behaviour of ants injected with PBS to those injected
333 with 10% acetone, we were able to rule out any significant differences between the two groups:
334 rest ($p = 0.787$), speed ($p = 0.997$), distance ($p = 0.947$), stagger ($p = 0.205$). Even though neither
335 group was exposed to aflatrem, we unexpectedly observed a few instances of staggering. To
336 determine if a small amount of staggering behaviour is normal in ants, which would need to be
337 accounted for in our results, we reviewed the footage at timestamps logged for these events with
338 an increased level of scrutiny. Upon closer review, we discerned that nearly all staggering observed
339 in both groups fell into the "gaster wagging" sub-type (Supplemental Video 2), and nearly always
340 occurred in the first 30 seconds of the recording. This trend was also observed in the aflatrem
341 treatment groups as well, indicating that this particular type of staggering behaviour is likely a
342 transient physical response to the trauma resulting from the injection process. We confirmed this
343 hypothesis through a quick follow-up experiment pricking ants with empty glass capillaries, which
344 also induced gaster wagging in some.

345 Confident that behaviours were not significantly affected by the addition of 10% acetone
346 in the injections, we made comparisons across increasing doses of aflatrem. A linear regression
347 model showed that time spent resting in the arena was positively correlated with increasing doses
348 of aflatrem ($F_{4,105} = 5.54$, $p = 2.05e\text{-}2$) (Figure 2-A), while the average speed of aflatrem-treated
349 ants was negatively correlated ($F_{4,105} = 4.91$, $p = 2.89e\text{-}2$) (Figure 2-B). However, the average

distance travelled did not correlate with aflatrem treatment ($F_{4,105} = 3.036$, $p = 8.44e\text{-}2$, ns) (Figure 2-C). Finally, bouts of staggering did positively correlate with increasing doses of aflatrem ($F_{4,105} = 36.65$, $p = 2.32e\text{-}8$) (Figure 2-D).

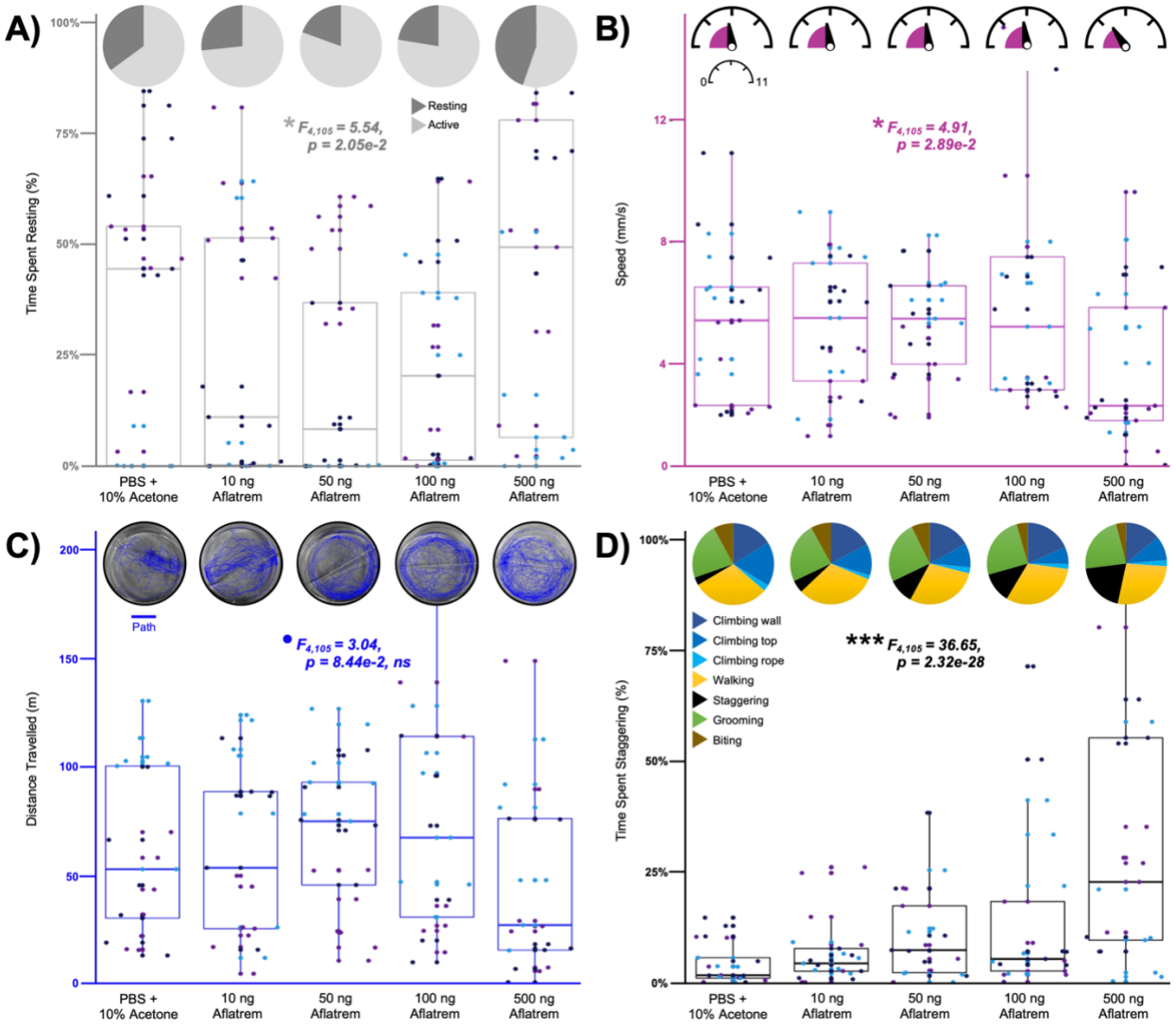

Figure 2. Behavioural results collected from *C. floridanus* ants injected with increasing doses of aflatrem. Each boxplot depicts the quartiles of the distribution for each treatment with ants from colonies 1, 2, and 3 shown as purple, light blue, and dark blue dots, respectively. A) shows the proportion of time spent resting for each ant, B) shows their average speed during activity in millimetres per second, C) shows their total distance travelled in meters, and D) shows the

359 proportion of activity spent staggering for each ant. The linear regression model results are shown
360 in the centre of the graph for each behaviour. A & D) both contain pie charts illustrating the relative
361 duration of each behaviour, while B) contains speedometers for each treatment and C) presents the
362 median tracer plot from MARGO for each group.
363

364 **3.2 The effects of aflatrem on gene expression in *C. floridanus***

365 While injection of *C. floridanus* with aflatrem resulted in quantifiable phenotypes, we next asked
366 which genes could be underlying the emergence of staggering and diminished activity phenotypes.
367 As such, we conducted transcriptomics to compare gene expression in the heads of ants treated
368 with 500 ng aflatrem (treatment group) to that of ants treated with 10% acetone solutions (control
369 group). We found 261 genes that were significantly differentially expressed (Supplementary File
370 1). Of these genes, 148 were upregulated and 114 were downregulated in the treatment group
371 (Figure 3). We found the largest increase of expression (~22-fold increase) in a gene encoding an
372 aminopeptidase N-like protein, a common cell surface hydrolase involved in a variety of cellular
373 processes (Nocek et al., 2008). The largest decrease in expression (~512-fold decrease) was
374 exhibited by a gene coding for a cytochrome P450 6k1, a protein involved in detoxification of
375 insect cells (Xing et al., 2021) (Figure 3).

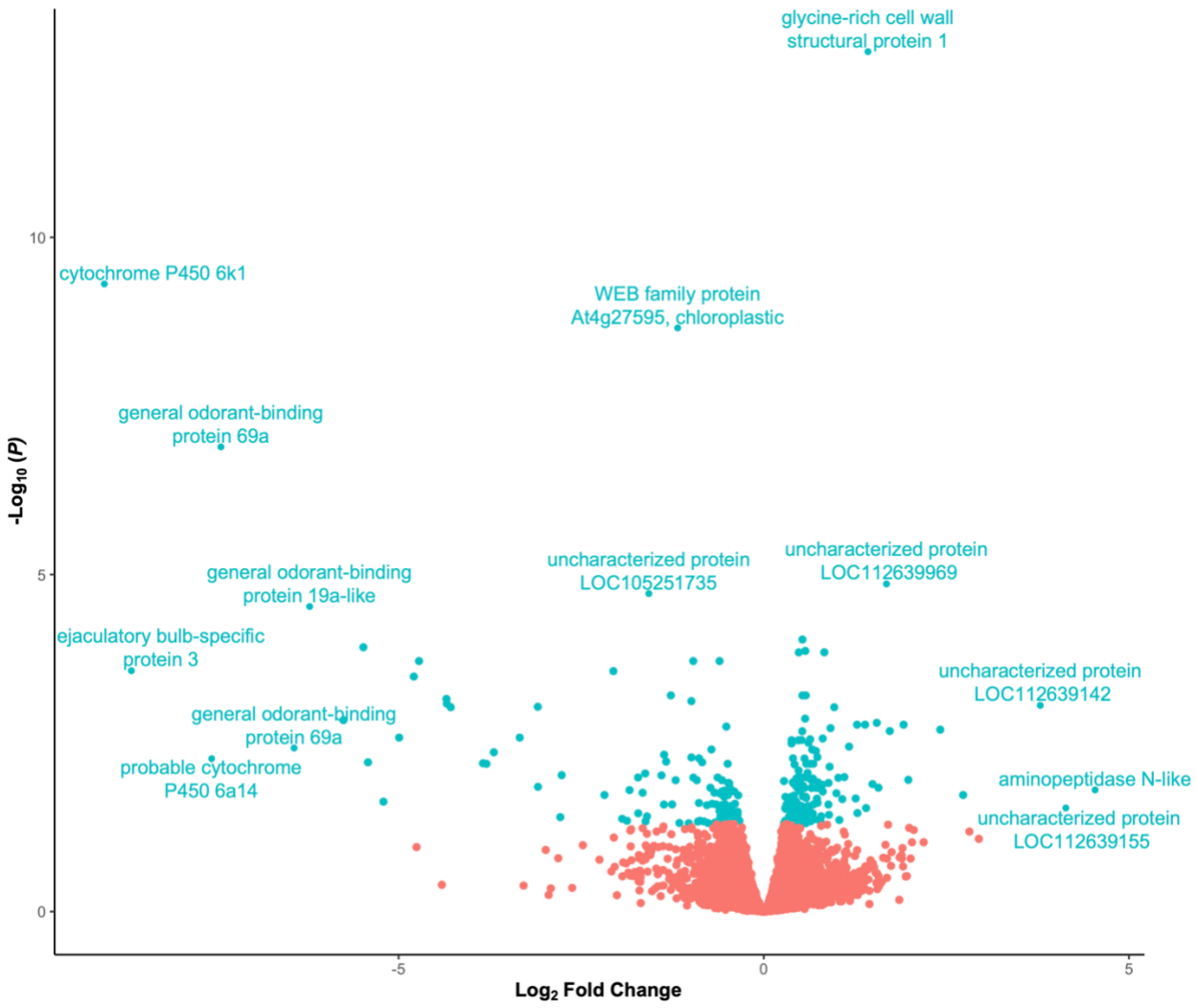

Figure 3. Volcano plot for the differential expression of 10,443 *Camponotus floridanus* genes in ants treated with 500 ng aflatrem compared to the 10% acetone control group. Dots in red represent non-significant changes in gene expression, while dots in blue represent significant changes using an alpha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

Several of the 261 differentially expressed genes were predicted to play a role in neuromuscular systems. Among them, we found an *anoctamin-4*, which codes for an ion channel protein involved in Ca^{2+}-dependent conductance (Reichhart et al., 2019), downregulated in

386 aflatrem-treated ants. We also detected a *membrane metallo-endopeptidase* (*Neprilysin*), a
387 transmembrane protein found in neurological tissue that plays a role in tissue perception and
388 preservation (Obulesu, 2019), which was slightly upregulated in aflatrem-treated ants. More
389 specific to muscular function, we found 2 upregulated *titins* genes, which code for very large
390 proteins that play an essential role in sarcomere function (Freiburg et al., 2000; Zhang et al., 2000).
391 Also upregulated was a *ryanodine receptor* gene, responsible for releasing Ca^{2+} in the smooth
392 endoplasmic reticulum of muscle cells during excitation-contraction coupling (Lanner et al., 2010;
393 Shakiryanova et al., 2007). On the other hand, *caveolin-3*, which codes for a scaffold protein that
394 organizes signalling molecules in the membranes surrounding muscle cells (Dewulf et al., 2019;
395 Galbiati et al., 2000-2013) was found to be downregulated, as well as 2 *myosin ID heavy chain-*
396 *like protein* genes that act as the motor proteins of muscles (Wells et al., 1996) (Supplementary
397 File 1).

398 In addition to neuromuscular genes, we also identified many downregulated olfactory-
399 related genes, including 4 general odorant-binding protein genes (OBPs) (i.e., *OBP19a*, *OBP56a*,
400 and 2 *OBP69a*). These proteins are located in the antennal sensillary lymph of insects and bind to
401 pheromones that play a role in insect communication and interactions (Gaubert et al., 2020).
402 Moreover, we found 3 significantly downregulated *ejaculatory bulb-specific* genes, involved in
403 the release of pheromones in insects (Pelosi et al., 2018), and a downregulated *sensory neuron*
404 *membrane protein* gene, which has been found to play a role in pheromone sensitivity in the moth
405 *Heliothis virescens* (Pregitzer et al., 2014).

406 We also detected a differentially expressed gene coding for a circadian clock-controlled
407 protein. Such genes could be of interest, given previous explorations of the time-dependent nature
408 of behavioural manipulation and the disruption of clock-dependent foraging behaviours in

409 *Ophiocordyceps*-infected ants (de Bekker et al., 2015; de Bekker & Das, 2022; Will et al., 2020),
410 This gene, which showed an 18-fold decrease in expression in aflatrem-injected ants compared to
411 10% acetone controls, also contains a Haemolymph Juvenile Hormone Binding Protein Pfam
412 domain. Such binding proteins, driven by circadian rhythms, are known to act as carrier proteins,
413 chaperoning juvenile hormones, which play a wide array of roles in insect physiology and
414 development, to their target tissues (Zalewska et. Al., 2009).

415 To investigate if any of the differentially expressed genes were affected during
416 *Ophiocordyceps*-induced behavioural manipulation, we compared our RNA-seq results to
417 transcriptomics data obtained from *C. floridanus* manipulated by *O. camponoti-floridani* (Will et
418 al., 2020). We found that 108 of the 261 differentially expressed genes observed in aflatrem-treated
419 ants were also differentially regulated in late-stage *O. camponoti-floridani* infections
420 (Supplementary File 1). Of these genes, 34 were differentially regulated in the same direction,
421 while 74 shared an inverse relationship. The previously mentioned *cytochrome P450*, *sensory*
422 *neuron membrane protein* encoding gene, *OBP56a*, and *caveolin-3* genes were amongst the
423 similarly expressed genes, all of which were downregulated. We also found an upregulated *nuclear*
424 *hormone receptor E75*, member of a family of transcription factors that are known to be activated
425 by juvenile hormone signalling pathways in Diptera and Lepidoptera (Dubrovskaya et al., 2004).
426 In addition, we detected an upregulated circadian clock steroid-producing gene linked to the
427 maintenance of circadian rhythms during stress in *Drosophila* (Kumar S et al., 2014). Moreover,
428 a *neurogenic locus protein delta*, capable of coding for multiple translational products including
429 neural differentiation factors and growth hormones (Kopczynski et al., 1988), was also upregulated
430 in both datasets.

431 Amongst the differently expressed genes, the aforementioned *OBP19a-like* gene, 5
432 *cytochrome P450s*, and an *ejaculatory bulb-specific protein 3*, were all significantly
433 downregulated in aflatrem-treated ants but upregulated in *Ophiocordyceps*-infected ants. More
434 genes shared this same inverse relationship, including an *odorant receptor coreceptor* gene, coding
435 for proteins expressed in chemosensory organs that form heteromultimeric complexes within
436 odorant receptors (Mukunda et al., 2014). We also detected a *short neuropeptide F*, which is
437 implicated in the regulation of animal behaviour including circadian rhythms and hunger (Lee et
438 al., 2004), and a *netrin receptor UNC5C*, which aids in the formation of axon connections through
439 axon extension and cell migration (Kim & Ackerman, 2011). S*ialin*, a sialic acid transporter-
440 coding gene that prevents neurodegenerative disorders caused by accumulation of sialic acid
441 (Tarailo-Graovac et al., 2017) was additionally among these differentially expressed genes.
442 Upregulated in our aflatrem-treated ants, but downregulated in the *Ophiocordyceps*-
443 infected ants, was a *receptor-type tyrosine-protein phosphatase delta* (*PTPRD*). This gene is part
444 of the PTP family involved in many known signalling pathways for which delta is shown to
445 promote neurite growth and regulate axon guidance (Tomita et al., 2020). In addition, we found
446 an *obscurin*, which, like *titin*, codes for a large protein expressed in skeletal muscles that anchors
447 myofibrils to the sarcoplasmic reticulum for muscle contraction (Feher, 2017), and a *juvenile*
448 *hormone acid O-methyltransferase-like* gene, which codes for an enzyme that converts precursor
449 molecules into juvenile hormones (Shinoda & Itoyama, 2003). Finally, the two data sets also
450 shared 26 genes with statistically significant changes in expression coding for proteins of unknown
451 function (Supplemental File 1).
452
453 **4. DISCUSSION**

454 Certain parasites can induce host behaviours that increase the reproduction, survivability, and
455 transmission of those parasites at a cost to the host. How such behavioural manipulations are
456 established is still poorly understood. In recent years, multi-omics work on "zombie ants" has
457 provided a myriad of candidate compounds that *Ophiocordyceps* fungi might use to manipulate
458 ant behaviour. However, their causal relationship to resulting host behaviours still needs to be
459 functionally tested. This study aimed to test aflatrem-like compounds previously implicated in the
460 establishment of *Ophiocordyceps* extended phenotypes in zombie ants (Will et al., 2020). We
461 accomplished this by exposing healthy *C. floridanus* ants to high purity aflatrem and quantifying
462 its behavioural effects. Following injections, we found dose of aflatrem to be positively correlated
463 to the amount of time that treated individuals spent staggering. To rule out that this observed
464 behaviour was a symptom related to death, we performed a longevity assay to demonstrate that
465 aflatrem does not kill *C. floridanus*. These preliminary tests demonstrated that aflatrem-like
466 compounds are unlikely to be employed by *Ophiocordyceps* as a means to kill hosts. Instead, the
467 observed staggering behaviours indicate that the aflatrem-like compounds produced by
468 *Ophiocordyceps* during infection are, at least in part, responsible for the drunkard's walk
469 phenotype exhibited by infected carpenter ants (Hughes et al., 2011; Trinh et al., 2021). How the
470 emergence of this behaviour would adaptively contribute to *Ophiocordyceps* fitness is, however,
471 unknown.

472 Our experiments further demonstrated that aflatrem has a significant effect on the mobility
473 of ants, decreasing overall activity as dose increased. Ants injected with higher doses of aflatrem
474 moved less often, did so much slower when they were active, and as a result travelled shorter
475 distances. We also observed that they had more difficulties climbing. Particularly when it involved
476 the twine in the arena, which requires greater dexterity than climbing on the wall or top of the petri

477 dish. Previous field studies suggest that *Ophiocordyceps* development and fruiting body formation,
478 needed for spore transmission, are dependent on precise levels of light, humidity and temperature
479 (Andersen & Hughes, 2012; Andriolli et al., 2019; Cardoso Neto et al., 2019; Hughes et al., 2011;
480 Lavery et al., 2021; Will et al., under review). Aflatrem-mediated tremors and reduction of
481 dexterity and speed could aid to keep the ant within these optimal microclimates prior to biting,
482 by preventing it from climbing too high into the canopy.

483 While we could reason a parasite-adaptive function for aflatrem-reduced activity, it
484 contradicts previous studies where *Ophiocordyceps*-infected individuals showed hyperactive
485 locomotion prior to summiting (de Bekker et al., 2015; Trinh et al., 2021; Will et al., 2020).
486 Nevertheless, transcriptomics studies on *Ophiocordyceps*-manipulated ants suggest that the fungus
487 expresses manipulation compounds in a time-specific manner (de Bekker et al., 2015; Will et al.,
488 2020). It is reasonable to suggest that, prior to the upregulation of aflatrem-like compounds,
489 *Ophiocordyceps* secretes effectors that induce hyperactivity and climbing behaviours to drive the
490 ant away from the nest and up nearby structures. An example of one such effector could be protein
491 tyrosine phosphatase (PTP). Genes encoding this protein are upregulated during *Ophiocordyceps*
492 manipulation (de Bekker et al., 2015; Will et al., 2020) and PTPs have been found to induce
493 hyperactivity and enhanced locomotion in caterpillars that are infected with a behaviour-
494 manipulating baculovirus (Han et al., 2015). Enhanced locomotion could be parasite-adaptive by
495 leading hosts away from aggressive conspecifics, which can recognize infected individuals and
496 attack them (Trinh et al., 2021; Will et al., 2020) as part of their social immunity (Cremer et al.,
497 2007). After the ant is safely away from the adaptive behaviours of the colony, the production of
498 debilitating compounds could function to maintain the ant in a desirable, elevated location.

In addition, aflatrem-like compounds may also aid in the positioning of the host for the final, and perhaps most iconic, behavioural manipulation; the “death grip”. During the death grip, ants bite onto vegetation with their mandibles, firmly anchoring them in place. Shortly after, the fungus kills the host and quickly consumes the remaining nutrients to produce its reproductive structures. The attachment of the host is a vital step in host manipulation as it promotes infective spore formation and release. Without it, ants would fall back to the forest floor, which interferes with fruiting body formation and, eventually, transmission (Andersen et al., 2012; Andriolli et al., 2019; Loreto et al., 2014; Will et al., under revision). The large increase in resting behaviours seen in ants that received the highest dose of aflatrem could aid in positioning of the ant’s head for biting behaviours. Moreover, aflatrem injection resulted in the differential expression of muscle-related genes in the heads of treated ants as compared to the control group. Therefore, the muscle-related effects of aflatrem-like compounds secreted by *Ophiocordyceps* could potentially be directly involved in the locking of the mandible muscles as part of the biting phenotype (Hughes et al., 2011). However, we did not observe increased biting behaviour in aflatrem-treated ants in this study.

516 Figure 4. Examples of "hugging" behaviour portrayed by *Ophiocordyceps*-infected *Camponotus*
517 *floridanus* ants during the death grip observed in a natural setting.
518
519 As part of the death grip phenotype, *O. camponoti-floridani*-infected *C. floridanus* are
520 often found wrapping their limbs around thin pieces of Spanish moss, which they most frequently
521 use as a biting substrate (Figure 4) (Will et al., under review). This "hugging" behaviour has also
522 been observed in other *Ophiocordyceps*-ant interactions where smooth substrates, such as twigs,
523 are primarily bitten (Loreto et al., 2018). The transcription-level dysregulation of sensory
524 perception and neuromuscular functioning that we found, as well as the observed staggers, suggest
525 that unsteadiness during climbing might cause ants to stumble and hug the vegetation. As such,
526 the hugging behaviour itself could act as a secondary means by which hosts are kept in elevated
527 positions should biting itself fail. Indeed, the adherence to vegetation through biting is sometimes
528 missing in *O. camponoti-floridani*-manipulated ants, both in the field and in the lab (Will et al.,
529 2020) while hugging is always observed. In fact, our group has witnessed multiple examples of
530 such instances in the field where hosts with fully grown fruiting bodies are held to strands of
531 Spanish moss by just their limbs, either due to failed biting or the complete loss of the ant's head
532 from the sample. Hugging may be unique to ant hosts of *Ophiocordyceps* species as the phenotype
533 is absent from other host-manipulating entomopathogens, such as *Eryniopsis lampyridarum*
534 infecting the Goldenrod Soldier Beetle, which similarly induces summiting and death grip
535 behaviours on thin, huggable foliage (Steinkraus et al., 2017).
536 The exact structure and concentrations of the aflatrem-like compounds produced by
537 *Ophiocordyceps in vivo* are still unknown. Regardless, we were able to use reasonable doses of
538 purified aflatrem (i.e., within body weight equivalents used in previous pesticide testing) to

539 replicate the staggers/"drunkard's walk" phenotype induced in ants during *Ophiocordyceps*
540 infection. This caused the differential expression of 108 ant host genes that were previously
541 identified in *Ophiocordyceps*-infected ants exhibiting the summiting phenotype. As such, at least
542 in part, similar host pathways appear to be targeted during isolated aflatrem-dosing and
543 *Ophiocordyceps* infection, leading to comparable behaviours. This indicates that aflatrem-like
544 compound secreted by *Ophiocordyceps* is likely responsible for some of the transcriptional and
545 behavioural changes observed during manipulation. Future work characterizing the chemical
546 composition and three-dimensional structure of aflatrem-like compounds produced by species of
547 the *O. unilateralis* complex is needed to shed further light on the physiological effects of these
548 alkaloids and their role in behaviour manipulation. Nevertheless, our results demonstrate that
549 exposing hosts to hypothesized parasite manipulation compounds is a valuable avenue to test their
550 proposed effects at the genome and phenome level. As such, integrative behavioural studies like
551 this one can bring us one step closer to characterizing the molecular mechanisms that drive zombie-
552 making fungi. Furthermore, the protocols established as part of this research provide a framework
553 for future studies that wish to examine the roles of putative effectors produced and secreted by
554 *Ophiocordyceps* species, paving the way for further characterization of host-manipulating
555 compounds.
556

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Author Contributions:

WCB and CdB designed the experiment, analysed the data, performed the RNA extractions, and wrote the paper. WCB, CK, and UAM collected the ants, performed the injections, and recorded the video footage. CK and UAM performed the behavioural analyses using CowLog. WCB ran the MARGO software and performed the statistical analyses.

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829

830 Supplemental Files

831 **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

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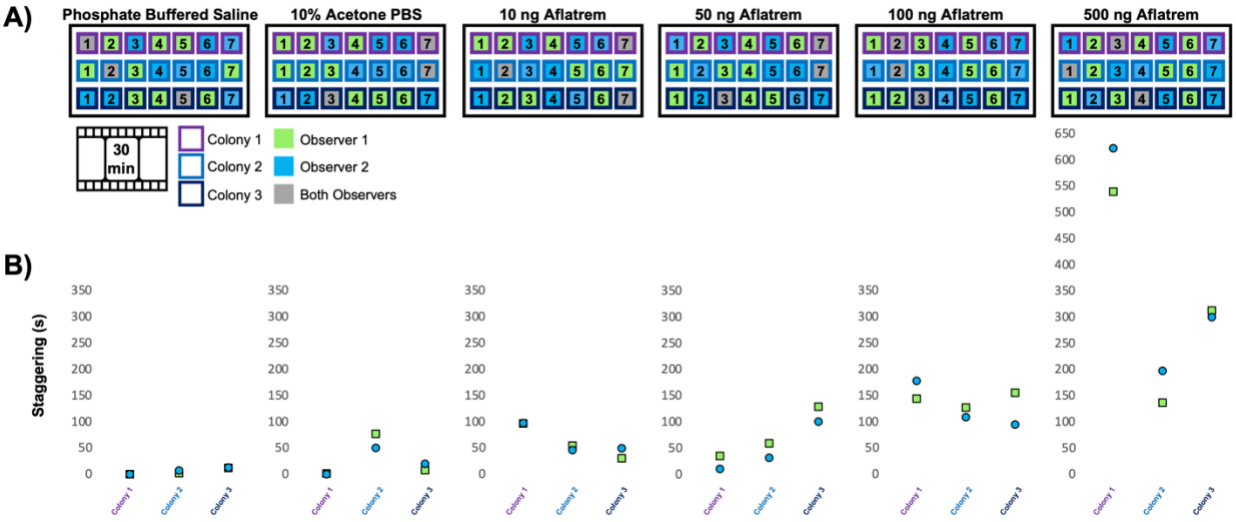
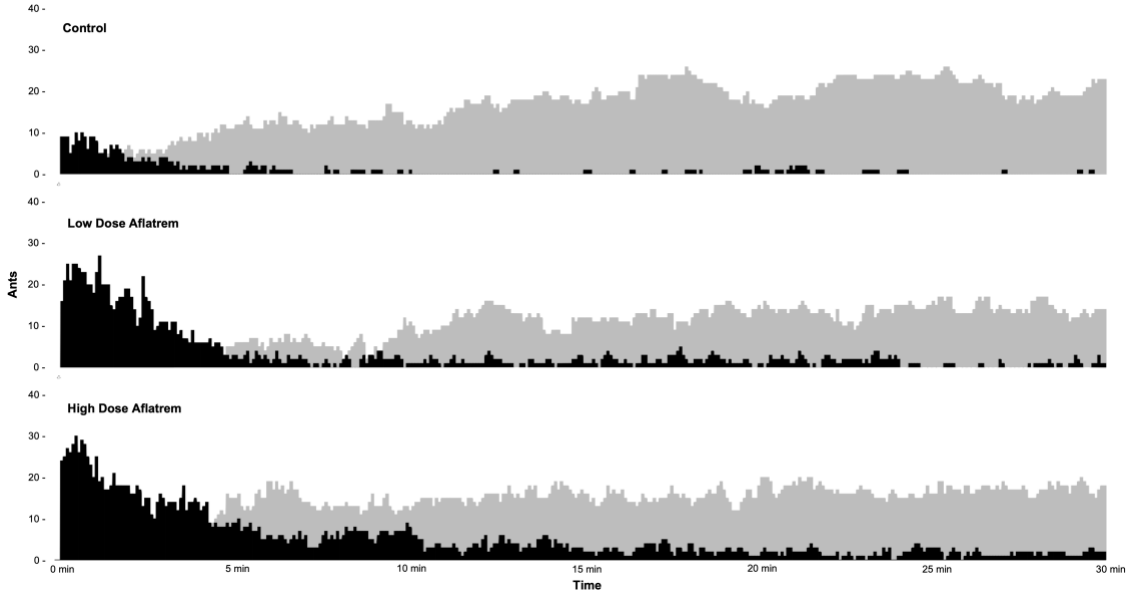
Supplemental Figure 1



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.

842 Supplemental Figure 2



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844 Supplemental Figure 2. Bar graphs depict the number of ants exhibiting staggering behaviour
845 (black) and the number of ants resting (dark grey) across the 30-minute observational period,
846 broken down into 5 second intervals. Control refers to ants injected with 10% acetone solvent.
847 Low dose aflatrem shows the data for ants that received 10-50 ng aflatrem, while high dose
848 aflatrem visualizes staggering and resting for ants that received 100-500 ng.

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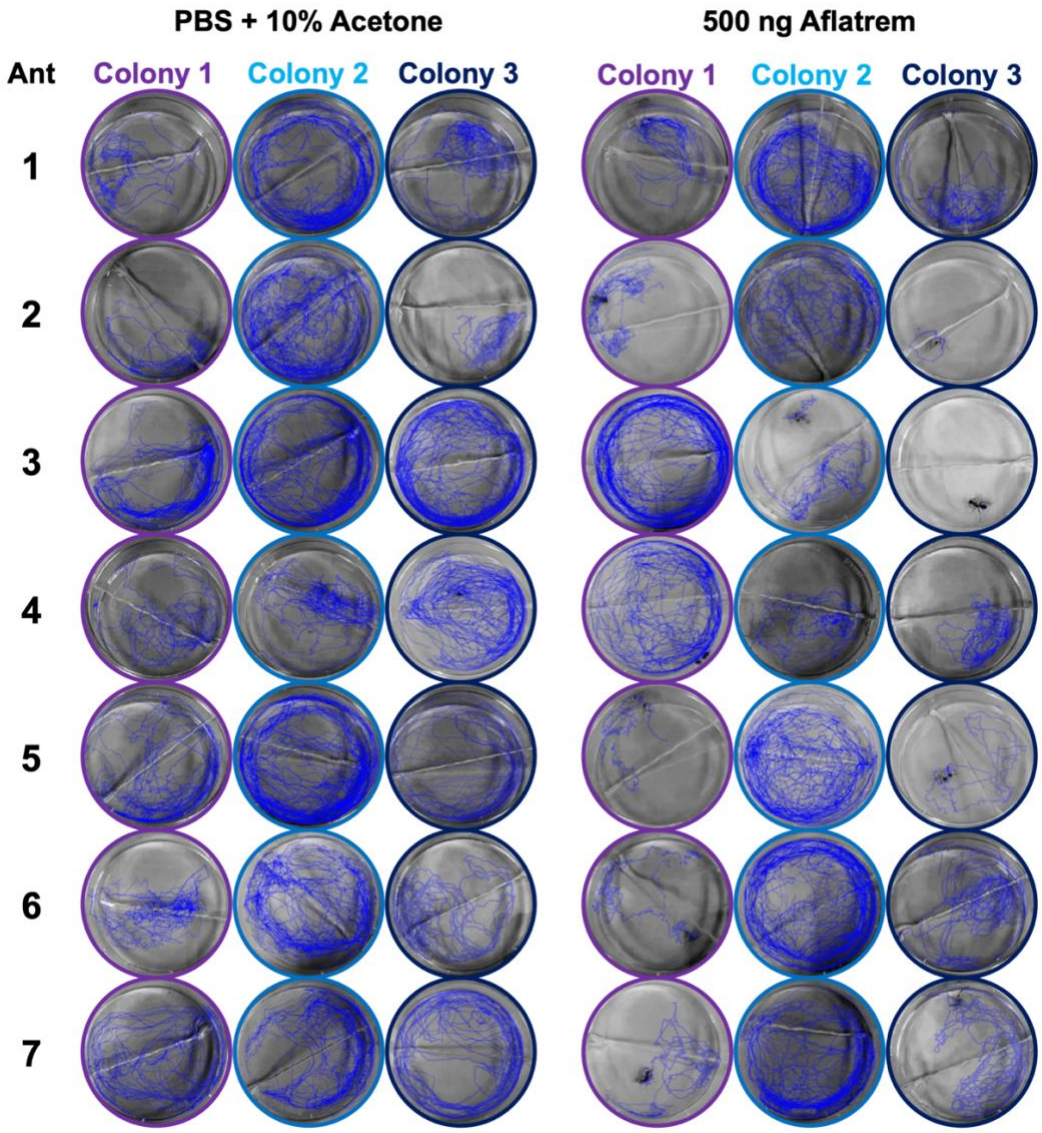
Supplemental Figure 3



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