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28 minutes later: investigating the role of aflatrem-like compounds in *Ophiocordyceps* parasite manipulation of zombie ants

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Coevolutionary relationships between parasites and their hosts can lead to the emergence of diverse phenotypes over time, as seen in *Ophiocordyceps* fungi that manipulate insect and arachnid behaviour to aid fungal spore transmission. The most conspicuous examples are found in ants of the Camponotini tribe, colloquially known as 'zombie ants'. While the behaviours induced during infection are well described, their molecular underpinnings remain unknown. Recent genomics and transcriptomics analyses of *Ophiocordyceps camponoti-floridani* have identified several highly upregulated biomolecules produced by the fungus during infection of *Camponotus floridanus*. Among them is an ergot alkaloid related to the mycotoxin aflatrem, known to cause 'staggers syndrome' in cows. Staggering, defined as unsteady movements side to side, is also observed in *C. floridanus* ants during late-stage infection. To test whether aflatrem-like compounds could be responsible, we injected healthy ants with aflatrem and recorded their behaviour for 30 min. Using both the automated object-tracking software MARGO and manual behavioural quantification, we found that aflatrem reduced ant activity and speed and increased staggering behaviours. To examine underlying transcriptomic changes, we performed RNA-seq on the heads of aflatrem-injected ants, keeping in step with previous transcriptomic work on *Ophiocordyceps*-manipulated ants. We identified 261 genes that were significantly dysregulated in the aflatrem-injected ants compared to sham-injected controls. When compared with RNA-seq data from *Ophiocordyceps*-manipulated ants, we found that both groups shared 113 differentially regulated genes. These 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 significantly affect neuromuscular and sensory function in *C. floridanus*. The conservation of staggers phenotype between *C. floridanus* and *Bos taurus* suggests that behaviour-manipulating strategies exhibited across the Tree of Life may be more similar in approach, if not widely different in application, than we realize.

When a parasite infects a host, an intense coevolutionary relationship can develop over time, driven by selective pressures exhibited by the host acting as the new environment for the parasite. As the parasite becomes more successful, the selective burdens shift to favour emergent resistance phenotypes in the host. Over time, these reciprocal selective pressures can lead to host specificity and the subsequent emergence of unique manipulation phenotypes (Fain, 1994; Flor, 1956; McLaughlin & Malik, 2017). Host manipulations provide an adaptive advantage to the parasite (Dawkins, 1982), for instance through the induction of specific behaviours that increase its reproduction success (Sánchez & Biron, 2019). These adaptive phenotypes are different from sickness behaviours, defined as actions exhibited by infected individuals that aid in the fitness of the host (e.g. lethargy) (Breed & Moore, 2010) and nonadaptive behaviours that benefit neither the host nor the parasite. One clear example of behavioural manipulation can be observed in the parasitic flatworm *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 & Wesołowski, 2013). Summiting behaviour makes

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snails more susceptible to predatory birds, the primary host of *L. paradoxum*, ultimately increasing the parasite's likelihood of completing its life cycle (Wesołowska & Wesołowski, 2013).

Such behaviour-manipulating parasites are often colloquially referred to as 'zombie parasites'. Not because they reanimate the dead, but because the drastically altered behavioural phenotypes exhibited by infected hosts favour parasite transmission, which is reminiscent of the behaviours of zombies in pop culture and science fiction media (e.g. the movies 28 Days Later and *The Last of Us*). 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; Wesołowska & 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. *Ophiocodyceps humbertii*: Sobczak et al., 2020), beetles (e.g. *Ophiocodyceps curculionum*: Shrestha et al., 2016), scale bugs (e.g. *Ophiocordyceps clavulata*: Petch, 1933), caterpillars (e.g. *Ophiocodyceps sinensis*: Guo et al., 2021), flies (e.g. *Ophiocodyceps dipterigena*: Chhetri et al., 2020) and spiders (e.g. *Ophiocodyceps 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-studied groups of zombie fungi that collectively infect and manipulate carpenter ants with extreme host specificity (Araújo et al., 2018, 2020).

Ophiocordyceps unilateralis species begin their life cycle 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 from 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 behaviour of their host (de Bekker et al., 2015; Will et al., 2020). The first notable change 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 abandon the nest and climb nearby vegetation. Once at an elevated position, they latch on with their mandibles, such that the ant is perched on the underside of its biting substrate (Andersen et al., 2009). The fungus then completes its life cycle through the formation of an endosclerotium that siphons the remaining nutrients from the host for the formation of a new fruiting body (Fredericksen et al., 2017). Closer observation and quantification of behaviours in both the laboratory and the field have led to the identification of other, more subtle behavioural changes. Altered behaviours include changes in daily rhythms (Hughes et al., 2011; Trinh et al., 2021) and the loss of motor coordination and balance, resulting in ants that fumble their way around as in a 'drunkard's walk' (Video S1) (Hughes et al., 2011; Trinh et al., 2021; Will et al., 2020). These conspicuous behavioural changes caused by the fungus during infection make the *O. unilateralis* species complex and their hosts particularly well suited for the study of behaviour-manipulating parasites in greater detail (de Bekker et al., 2015).

Recent work establishing fungal culturing and ant infection techniques, as well as the genomic sequencing of several *O. unilateralis s.l.* species, has laid the groundwork for research aimed at understanding the molecular framework driving behavioural manipulation (de Bekker et al., 2015, 2017; Will et al., 2020). 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 behaviour (de Bekker et al., 2015; Will et al., 2020). One of these secondary metabolite clusters demonstrated a ~5900-fold (in *O. kimflemingiae*) and ~12 000-fold (in *O. camponoti-floridani*) increase in expression during manipulation compared to fungi grown 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 annotation of the genes within and surrounding the cluster demonstrated that it was homologous to the aflatrem-producing cluster in the plant pathogen *Aspergillus flavus* (Will et al., 2020). 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 (Duran et al., 2007; Nicholson et al., 2009). Homologues to the *atmQ, atmD, atmP, atmM, atmC, atmB, atmG and idtS* genes involved in the synthesis of aflatrem in *A. flavus* were all found in both the *O. camponoti-floridani* and *O. kimflemingiae* genomes (Will et al., 2020). Further genomic comparisons with other *Ophiocordyceps* genomes revealed that this secondary metabolite cluster appears to be conserved across species within *O. unilateralis* but not in other *Ophiocordyceps* species outside the species complex (e.g. *Ophiocordycpes australis s.l.*) (de Bekker et al., 2017). Taken together, this suggests that *Ophiocordyceps* species that infect ants of the tribe Camponotini can produce a secondary metabolite that is relatively similar to aflatrem, which they upregulate at the time the ant behaviour is being manipulated. This raises the question: what role might aflatrem-like compounds play in the manipulation of *Camponotus* behaviour?

The effects of aflatrem on animal behaviour have previously been studied in *Bos taurus* where the symptoms of aflatrem exposure were first observed in livestock that unintentionally ingested *A. flavus*-contaminated grain feed (Selala et al., 2008; Valdes et al., 1985). In these cases, cows exhibit neurological problems exhibited by muscle tremors and hyperexcitability, followed shortly by incoordination, ataxia and seizures (Selala et al., 2008; Valdes et al., 1985). These neurological symptoms, linked directly to aflatrem's positive allosteric modulation of gamma-aminobutyric acid (GABA) receptors, are referred to as 'staggers syndrome' (Eldefrawi et al., 1990; Gant et al., 1987; Yao et al., 1989). Should aflatrem-like compounds exhibit similar effects in *Camponotus* species, it could have important ramifications for host manipulation given the suspected involvement of GABA receptors in caste and behavioural differentiation in *Camponotus* ants (Gospocic et al., 2017; Graham, 2018). The likelihood that an aflatrem-like compound may be used during the manipulation of carpenter ants is further supported by the 'drunkard's walk' observed during late-stage infection; a behaviour reminiscent of the staggers syndrome detailed in vertebrates (Video S1). We, therefore, hypothesized that aflatrem-like compounds are responsible for the stagger phenotype observed during the behavioural manipulation of carpenter ants by *O. unilateralis s.l.* To test this hypothesis, we formulated two research questions: (1) does aflatrem have any behavioral effect on carpenter ants and (2) how are genes dysregulated during induced behaviours? If aflatrem-like compounds are responsible for some of the motor impairment previously described as the 'drunkard's walk' in *Ophiocordyceps*-infected ants, then increasing doses of aflatrem should increase the duration and severity of staggering symptoms as previously studied in *B. taurus*. Furthermore, we expected neuromuscular genes required for coordination to be dysregulated.

In this study, we administered aflatrem to the Florida carpenter ant, *Camponotus floridanus*, the host of *O. camponoti-floridani* (from here simply referred to as *Ophiocordyceps*). We used microinjections to mimic the in vivo secretion of aflatrem-like compounds by

fungal cells in the haemolymph during infection. 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., 1991; Trienens & Rohlfs, 2011). Following injections, we observed and quantified ant behaviour to address whether aflatrem can induce behaviours consistent with those exhibited during infection by *Ophiocordyceps*. 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 differentially expressed genes, including genes that encode neuromuscular and chemosensory proteins.

METHODS

Ant Collection

For use in behavioural and transcriptomics experiments, we collected three separate queenless *C. floridanus* colonies of unknown age using a DeWALT Cordless 2 Gallon Wet/Dry ShopVac (https://www.dewalt.com) at the University of Central Florida's arboretum, coordinates: colony 1 (28°36′10″N, 81°11′39″W), colony 2 (28°36′14″N, 81°11′30″W) and colony 3 (28°36′5″N, 81°11′29″W) (see Supplementary keyhole markup language (KML) map). All colonies were collected from fallen logs using a tarp to ensure that nearly all ants that resided within were collected. In total, each collected colony contained ~100–200 major and minor worker ants without larvae, indicating that all were likely satellite nests of bigger colonies in the area. We housed these colonies in 9.4-litre plastic containers (Rubbermaid, https://www.rubbermaid.com) lined with talcum (Acros Organics, ThermoFisher Scientific, Waltham, MA, U.S.A.), applied using a viscous 20% w/v solution of powder suspended in 100% ethanol to prevent ants from climbing the walls. Throughout the study, we provided the ants with fresh supplies of both double-distilled water (ddH_2O) and 15% sucrose solutions, ad libitum, as well as aluminium foil-wrapped falcon tubes that functioned as darkened nest chambers. We also kept the housing in an incubation chamber (Percival Scientific, Perry, IA, U.S.A.) set to the following program: 20 °C, 0 lx and 80% relative humidity (RH), with a linear increase in temperature and light and a decrease in humidity over 4 h to reach 28 °C, 2100 lx and 65% RH, at which conditions were held for 4 h, followed by a linear decrease in the temperature and light and an increase in humidity, returning to 20 °C, 0 lx and 80% RH over 4 h and held for 12 h. Only minor worker ants were used in this study, without formally determining their caste differentiation into foragers and nurses. However, the ants chosen for injection were outside and away from the darkened nest tubes at the time of collection, exploring the edges of the containers.

Microinjections

To test our hypothesis and identify the effect of aflatrem-like compounds on *C. floridanus*, we established a behavioural assay using microinjections to introduce aflatrem in a manner biologically relevant to the secretion of aflatrem-like compounds by *Ophiocordyceps* species in the haemolymph of their ant hosts (Fig. 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 (Chengdu, Sichuan, People's Republic of China) in non-water-soluble crystal form. Without an approximation for the level of aflatrem-like compounds secreted in the host in vivo, we suspended the aflatrem to a concentration of 10 mg/ml in 100% acetone (ThermoFisher Scientific). This allowed us to make further dilutions to reach concentrations informed by other studies on the effects of toxic compounds in insects (Buczkowski & Wossler, 2019; Klotz & Moss, 1996; Sakamoto & Goka, 2021). We performed

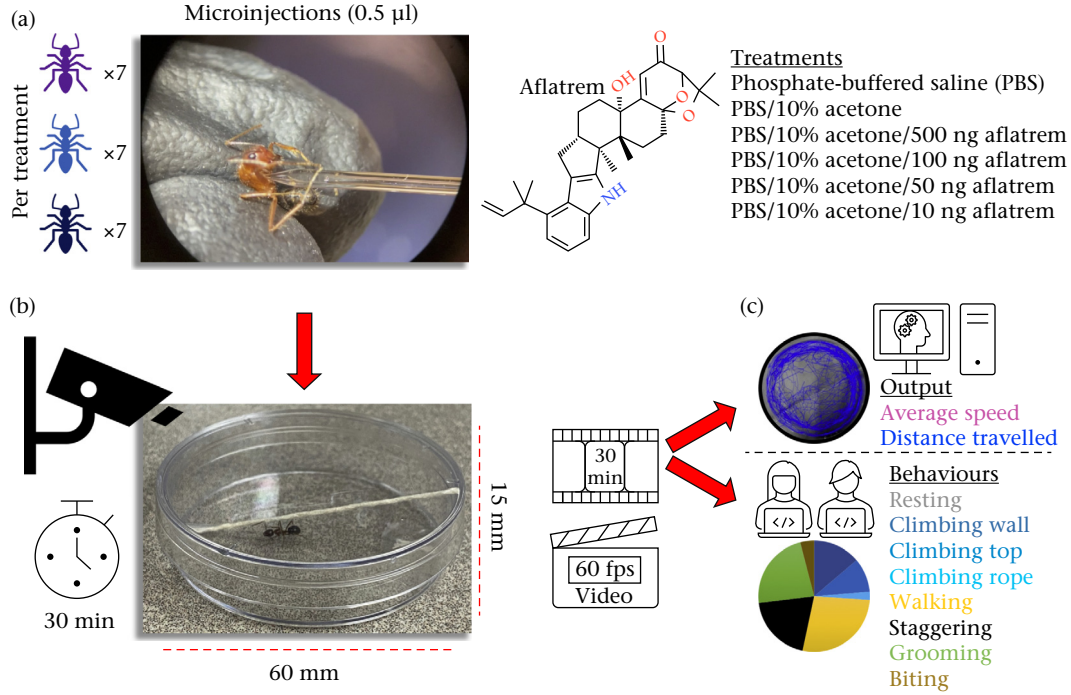

Figure 1. Experimental overview of (a) the treatment groups, each consisting of seven individuals from three different colonies of *Camponotus floridanus*, and an example of microinjection, (b) the arena set-up for behavioural assays and parameters for the observational periods and (c) the behavioural analyses using automated and manual behaviour scoring software. fps = frames/s.

injections using borosilicate glass micropipettes (ThermoFisher Scientific) pulled with a glass puller (model: PC-100, Narishige International, London, U.K.) under the following parameters: single step, all weights, heated to 62 °C. To inject the ants, we held each one 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. This procedure is similar to those routinely used to infect carpenter ants with fungal cells (de Bekker et al., 2014, 2015; Trinh et al., 2021; Will et al., 2020). Performing injections in the soft tissue between the joints allowed us to avoid puncturing the hard exoskeleton. This combined with constant pressure from the legs during walking holds the wound closed and prevents the ant from bleeding and allows them to recover.

Because the aflatrem used in this study was non-water-soluble, we performed a preliminary trial to identify acetone tolerance in ants. As such, we injected 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. Ten minutes prior to injection, we removed containers housing ant colonies from the incubator and set them on the laboratory bench top to acclimate to laboratory conditions. We also performed all injections in triplicate, one ant per colony per treatment. After injection, we placed the ants into individual 60 × 15 mm petri dishes (USA Scientific, Ocala, FL, U.S.A.) and returned them to the incubator. We kept these ants under observation for 48 h postinjection using an infrared GoPro Hero6 set to record time-lapse footage with 60 s intervals and a CMVision IR30 Illuminator lamp (CMVision, Houston, TX, U.S.A.) for observation during the night cycle. From these videos, we were able to easily differentiate dead and resting *C. floridanus* ants from one another; dying ants coiled their legs and fell to one side upon death while resting ants remained prone with legs supporting their bodies. As such, we were able to estimate the time of death of each ant within an interval of 1 min by noting the frame in which they exhibited 'death behaviours' (Video S2). We euthanized any ants that survived the 48 h test period by freezing at −20 °C for 24 h. Results from these preliminary analyses indicated that a 10% acetone solution was the highest concentration for which there were no statistical differences in average survivability compared to PBS-injected and noninjected controls.

To test the survivability of ants postinjection and determine whether any observed behavioural phenotypes could be attributed to 'dying behaviours' resulting from aflatrem exposure, we performed a second preliminary trial. We injected seven ants with 0.5 µl 10% acetone solution containing 500 ng aflatrem. This was the highest dose possible using the 10 mg/ml aflatrem stock. After injection, we recorded the ants for 48 h using the same GoPro setup as the previous preliminary test. We then compared the two groups and found no significant differences in mortality, with all individuals surviving for at least 24 h and an average time of death arising at 34 h. The results indicated that all ants that died in the trial did so as a result of desiccation from lack of water.

Confident that neither 10% acetone nor 500 ng aflatrem killed the ants or induced death-related behaviours, we conducted an assay to determine the effects of aflatrem on ant behaviour using 10% acetone solutions containing the following doses of aflatrem; 10 ng, 50 ng, 100 ng and 500 ng. For each dose of aflatrem, we injected seven ants from each of the three colonies ($N = 21$, per dose) for a total sample size of 126. We individually lifted each ant from its housing by hand wearing neoprene gloves (NeoTouch, Ansell Ltd, Richmond, Australia) and allowed it to acclimate to the laboratory conditions by placing it in a 60 × 15 mm petri dish (USA Scientific) on the laboratory benchtop for 10 min. We fitted each petri dish with a thread of twine spanning the dish from the bottom of one side to the top of the other. This provided ants with a substrate for biting and more dexterous climbing behaviours (Fig. 1b). To allow ants to walk around the perimeter of the dish unimpeded, we made sure to tape (Scotch, 3M, St Paul, MN, U.S.A.) the twine pressed flat against the bottom corner of the petri dishes. We created a new petri dish arena for each test subject ($N = 126$) to avoid any behavioural changes that could be induced via chemical cues left by prior occupants. After the 10 min acclimation period, we injected ants 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 we measured body length to the nearest 0.5 mm for each individual, weight was measured in groups of seven ants corresponding to their treatment and colony identity (ID). We recorded these measurements immediately after the ants were euthanized via freezing, preserving the approximate water weight for each ant. To account for the approximate weight of solution injected into each ant, we also subtracted 3.5 mg from the total weight of each group. Based on an average of 8.28 mg weight and 6.68 mm length calculated from measurements of all 126 subjects used in this study (Appendix, Table A1), the four doses of aflatrem that we used were 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 (Buczkowski & Wossler, 2019; Klotz & Moss, 1996; Sakamoto & Goka, 2021).

Using an independent two sample *t* test to compare the behaviour of ants injected with PBS to those injected with 10% acetone, we were able to rule out any significant differences 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. To determine whether a small amount of staggering behaviour is normal in ants, which would need to be accounted for in our results, we reviewed the footage at time stamps logged for these events with an increased level of scrutiny. Upon closer review, we discerned that nearly all staggering observed in both groups fell into the 'gaster wagging' subtype (Video S3) and nearly always occurred in the first 30 s of the recording. This trend was also observed in the aflatrem treatment groups as well, indicating that this particular type of staggering behaviour is likely a transient physical response to the trauma resulting from the injection process. We confirmed this hypothesis through a quick follow-up experiment pricking ants with empty glass capillaries, which also induced gaster wagging in some.

Video Scoring of Behavioural Assays Postinjection

To record behaviours postinjections, we used an iPhone 13 pro. We recorded three or four arenas at a time in a rolling format to ensure high-quality resolution at 60 frames/s. This generated video files approximately 45 min in length. In cases where ants exhibited signs of physical injury from the injection, e.g. immobility in the hindlegs or body curling, we euthanized the subject and replaced it with a new ant and a new arena. If any ants were exhibiting resting behaviour at the end of their recording period, we tapped their arenas several times to induce movement. All instances of tapping elicited activity, confirming that all test subjects were still alive.

To crop the rolling videos into clips showing only individual arenas, we first converted the iPhone .MOV files to .mp4 files using iMovie. We then exported these .mp4 files to Movavi Video Editor Plus 2022 (Movavi, St Louis, MO, U.S.A.) and trimmed each clip to

exactly 30 min, beginning as soon as each arena was positioned in the field of view. The names for each of these clips were blinded before two observers manually scored the videos using the behaviour tracking software CowLog3 (Pastell, 2016). Before formal scoring, we trained each observer using eight test run videos; four videos of ants injected with 500 ng aflatrem and four videos 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. We used the results from these practice runs to form a consensus on scoring methodology. From these training videos, the observers identified eight scorable behaviours: climbing the wall, climbing on the top of the petri dish, climbing the rope, walking, staggering, grooming, resting and biting (Video S3). While most of these behaviours are self-explanatory, we defined 'staggering' as a slow, sluggish walking behaviour with repeated lateral movements (Video S4). We also categorized behaviours as 'staggering' when ants had trouble balancing or they listed to one side, fell over, walked in tight circles, flipped over or showed dramatic wagging of the gaster (Video S4). Additionally, each observer kept notes on unique behaviours observed during the scoring period, including walking backwards, lunging/bucking and two instances of convulsions (Video S5).

During the formal scoring, we assigned the blinded videos to the observers in a randomized block format (Appendix, Fig. A1a) with each observer scoring three videos at random per treatment group per colony. Both observers scored the seventh video for each treatment group, from which the results were compared and used as a formative metric to ensure consistent scoring throughout the evaluation period. While most behaviours 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 (Appendix, Fig. A1b).

In addition to manual observations, we also analysed each video using the MATLAB-based automated object-tracking software Massively Automated Real-time GUI for Object-tracking (MARGO) (Werkhoven et al., 2019). Using this tool, we were able to measure the speed and distance travelled by each subject. While we calculated distance travelled by measuring the total distance between centroid coordinates over the entire 30 min duration, we only measured speed, calculated as the distance travelled (mm)/s, during activity. To optimize the tracking within the field of view for each video, we used the following metrics: 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 at 70–80, distance of the arena was set to 60 mm using the custom measuring tool, frame rate was set to 30 and minimum/maximum area was set to 5 and 80, respectively.

Before testing the effects of aflatrem on ant behaviour, we compared the results from the 10% acetone controls to the 100% PBS controls to identify any potential behavioural phenotypes induced by the addition of acetone to the injections. To test for any statistical significance between the two groups, we used a two-sample *t* test in RStudio version 1.3.959. Following the comparison of these two controls, we tested the effect of aflatrem exposure on ant behaviour with a linear regression model using the 'lme4' package in RStudio, by comparing the 10% acetone control (0 ng aflatrem) with 10% acetone containing 10 ng, 50 ng, 100 ng and 500 ng aflatrem treatments.

RNA Sequencing

To understand the effects of aflatrem exposure in *C. floridanus* at the genomic level, we injected a total of 10 worker ants from colony 1 with either 10% acetone (control group, $N = 5$) or 500 ng aflatrem (treatment group, $N = 5$). We then placed each ant into their own petri dish arena for 4 min. We chose this time point based on the scaling levels of activity observed in our behavioural assays (Appendix, Fig. A2). After this period, we quickly placed the ants into 2 ml microcentrifuge tubes and snap-froze them in liquid nitrogen. Subsequently, we removed the head from each ant with forceps and placed it in a prefrozen 2.0 ml Self-Standing Impact Resistance Microtube (USA Scientific) containing two Grade 25 5/32″ (3.97 mm) metal ball bearings (Wheels Manufacturing, Louisville, CO, U.S.A.) for mechanical tissue disruption in a SPEX® SamplePrep 1600 MiniG (Spex CertiPrep, Metuchen, NJ, U.S.A.) at 1500 rpm for 30 s. We dissolved the frozen samples in 500 µl TRIzol (Ambion, ThermoFisher Scientific) to extract the RNA and isolated it using the Qiagen RNeasy® MiniElute® Cleanup Kit (Will et al., 2020). The resulting RNA was stored at −80 °C and shipped to Azenta US for Next Generation Sequencing (Burlington, MA, U.S.A.). Libraries were prepared by Azenta US using PolyA selection and sequenced as 150 bp paired end reads on an Illumina HiSeq (Illumina Inc., San Diego, CA, U.S.A.), resulting in ~350 million reads. Read quality 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. We then aligned the transcripts to the *C. 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). After alignment, we checked the resulting .bam files for quality using the standard metric of uniquely mapped reads >70%, sorted and indexed using Samtools (Danecek et al., 2021). We next calculated the number of gene hits for each sample 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. After removing hits with low level of expression (counts less than 100 per gene, i.e. an average less than 10), we normalized the remaining counts using DESeq2 (Love et al., 2014) eliminating 3227 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). To visualize the overlap between the differentially expressed genes found in this study and those found in a previous transcriptomics data set obtained from *Ophiocordyceps*-infected ants (Will et al., 2020), we developed a kaleidoscope diagram using a custom R package made by Andrew Swafford (Supplementary material S1, R code). Finally, we performed a GO enrichment analysis using the R package from Das and de Bekker (2022) made publicly available at https://github.com/biplabendu/timecourseRnaseq (Das, 2022).

Ethical Note

We performed all injections and behavioural analyses in this study on the invertebrate ant species *C. floridanus*, which is not endangered and is a prevalent species in central Florida, U.S.A. We collected colonies of this species at the University of Central Florida Arboretum, which did not require prior approval from an animal welfare or ethics committee. Only three colonies were collected to meet the minimal scientific standard of triplicate replication. Collection utilized a fast and handling-minimizing method. We applied the following standards in effort to reduce the amount of stress incurred by the ants throughout our study as much as possible. Housing was conducted in an environmental chamber set to mirror the subjects' natural conditions. We maintained colonies by providing ad libitum food and water and dark enclosures for

nesting. We furthermore conducted experiments on the minimum required number of subjects to reach the sample size needed for statistical analysis. Ants were quickly euthanized at −20 °C in instances where they were injured during treatment and immediately following all experiments.

RESULTS

The Behavioural Effects of Aflatrem in C. floridanus

Confident that behaviours were not significantly affected by the addition of 10% acetone in the injections, we made comparisons 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 ($F_{4,105} = 5.54$, $P = 2.05e\text{-}2$; Fig. 2a), while the average speed of aflatrem-treated ants was negatively correlated ($F_{4,105} = 4.91$, $P = 2.89e\text{-}2$; Fig. 2b). However, the average distance travelled did not correlate with aflatrem treatment ($F_{4,105} = 3.036$, $P = 8.44e\text{-}2$, NS; Fig. 2c). Finally, bouts of staggering did positively correlate with increasing doses of aflatrem ($F_{4,105} = 36.65$, $P = 2.32e\text{-}8$; Fig. 2d).

The Effects of Aflatrem on Gene Expression in C. floridanus

While injection of *C. floridanus* with aflatrem resulted in quantifiable phenotypes, we next asked which genes could be underlying the emergence of staggering and diminished activity phenotypes. As such, we conducted transcriptomics to compare gene expression in the heads of ants treated with 500 ng aflatrem (treatment group) to the heads of ants treated with 10% acetone

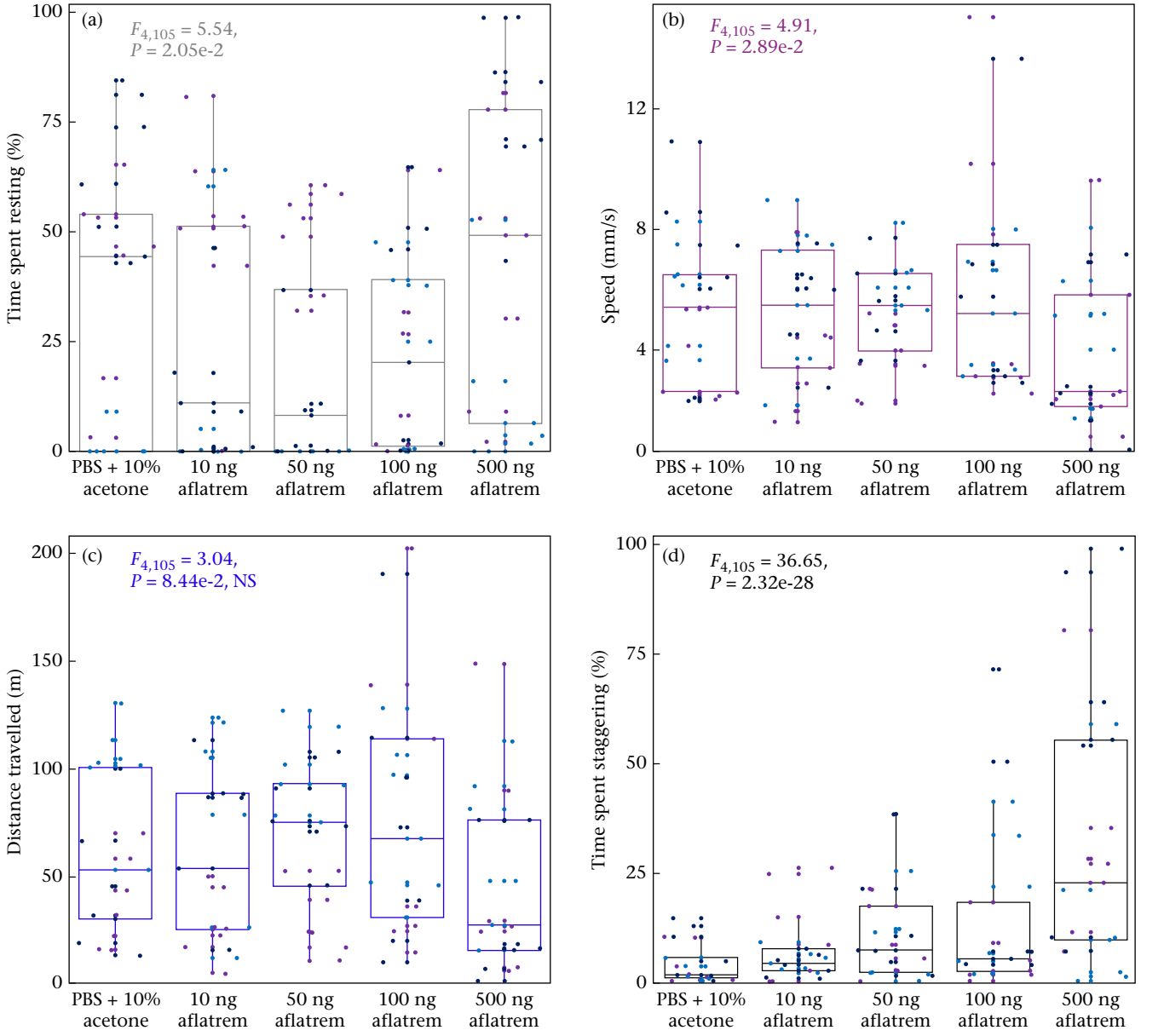

Figure 2. Behavioural results collected from *C. floridanus* ants injected with increasing doses of aflatrem showing (a) the percentage of time each ant spent resting, (b) their average speed during activity (in mm/s), (c) total distance travelled (m) and (d) the percentage of activity each ant spent staggering. Each box plot 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. The linear regression model results are shown in the top left of the graph for each behaviour.

solutions (control group). We found 261 genes that were significantly differentially expressed (Supplementary material S2, Data). Of these genes, 148 were upregulated and 113 were downregulated in the treatment group (Fig. 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). 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) (Fig. 3). A GO enrichment analysis of these 261 genes identified three significantly overexpressed GO terms, all three of which are broad categories: 'sequence-specific DNA binding' (GO:0043565, $P_{adj} = 0.006$), 'DNA-binding transcription factor activity' (GO:0003700, $P_{adj} = 0.006$) and 'regulation of transcription, DNA-templated' (GO:00635, $P_{adj} = 0.048$).

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 aflatrem-treated ants. We also detected a *membrane metallo-endopeptidase* (*Neprilysin*), a transmembrane protein found in neurological tissue that plays a role in tissue perception and preservation (Obulesu, 2019, pp. 39–44), which was slightly upregulated in aflatrem-treated ants. More specific to muscular function, we found two 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). Also upregulated was a *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). On the other hand, *caveolin-3*, which codes for a scaffold protein that organizes signalling molecules in the membranes surrounding muscle cells (Dewulf et al., 2019; Galbiati & Lisanti, 2013) was found to be downregulated, as well as two *myosin ID heavy chain-like protein* genes that act as the motor proteins of muscles (Wells et al., 1996) (Supplementary material S2, Data).

In addition to neuromuscular genes, we also identified many downregulated olfactory-related genes, including four general odorant-binding protein genes (OBPs) (i.e. *OBP19a*, *OBP56a* and two *OBP69a*). These 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 three 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 detected a differentially expressed gene coding for a circadian clock-controlled protein. Such genes could be of interest, given previous explorations of the time-dependent nature of behavioural manipulation and the disruption of clock-dependent foraging behaviours in *Ophiocordyceps*-infected ants (Das & de

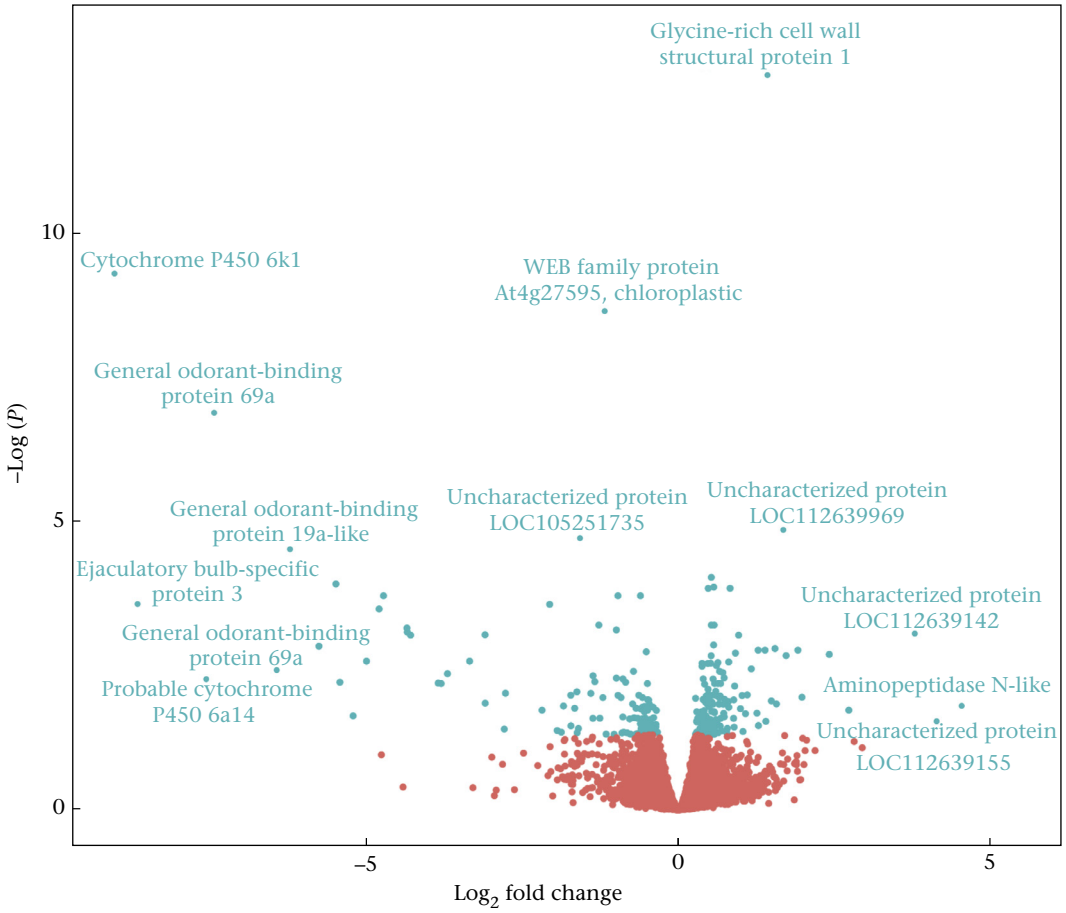

Figure 3. Volcano plot for the differential expression of 10 443 *Camponotus floridanus* genes in ants treated with 500 ng aflatrem compared to the 10% acetone control group. Dots in red represent nonsignificant changes in gene expression, while dots in blue represent significant changes using an alpha 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.

Bekker, 2022; de Bekker et al., 2015; Will et al., 2020). This gene, which showed an 18-fold decrease in expression in aflatrem-injected ants compared to 10% acetone controls, also contains a haemolymph juvenile hormone-binding protein Pfam domain. Such binding proteins, driven by circadian rhythms, are known to act as carrier proteins, chaperoning juvenile hormones, which play a wide array of roles in insect physiology and development, to their target tissues (Zalewska et al., 2009).

Comparison of Aflatrem-induced DEGs with Ophiocordyceps *Infection DEGs*

To investigate whether any of the differentially expressed genes were affected during *Ophiocordyceps*-induced behavioural manipulation, we compared our RNA-seq results from the heads of aflatrem-injected ants to transcriptomics data obtained from the heads of *C. floridanus* manipulated by *Ophiocordyceps* (Will et al., 2020). We found that 113 of the 261 differentially expressed genes observed in aflatrem-treated ants were also differentially regulated in late-stage *Ophiocordyceps* infections (Supplementary material S2, Data). Of these genes, 30 were differentially regulated in the same direction, while 83 shared an inverse relationship (Fig. 4).

The previously mentioned *cytochrome P450*, *sensory neuron membrane protein* encoding gene, *OBP56a* and *caveolin-3* genes were among the similarly expressed genes, all of which were downregulated. We also found an upregulated *nuclear hormone receptor E75*, a member of a family of transcription factors that are known to be activated by juvenile hormone signalling pathways in Diptera and Lepidoptera (Dubrovskaya et al., 2004). In addition, we detected an upregulated circadian clock steroid-producing gene linked to the maintenance of circadian rhythms during stress in *Drosophila* (Kumar et al., 2014). Moreover, a *neurogenic locus protein delta*, capable of coding for multiple translational products, including neural differentiation factors and growth hormones (Kopczynski et al., 1988), was also upregulated in both data sets.

Among the differently expressed genes, the aforementioned *OBP19a-like* gene, 5 *cytochrome P450s* and *ejaculatory bulb-specific protein 3* were all significantly downregulated in aflatrem-treated ants but upregulated in *Ophiocordyceps*-infected ants. More genes

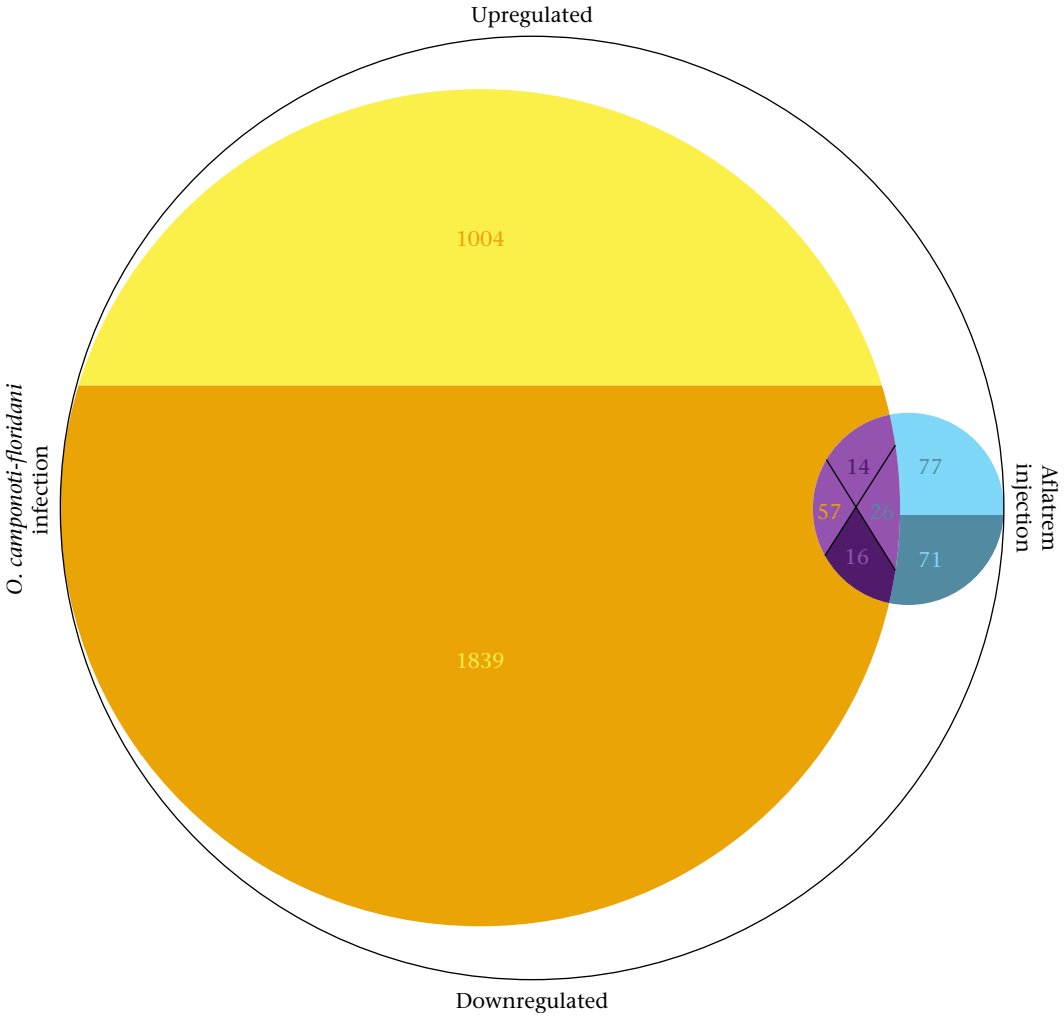

Figure 4. A modified Venn diagram (i.e. a 'kaleidoscope diagram') representing the overlap and differences between the differentially expressed genes (DEGs) in the heads of *Ophiocordyceps*-infected ants (left) (Will et al., 2020) and the DEGs in the heads of aflatrem-treated ants (right) (this study), as compared to gene expression in their respective control groups. The lighter top colours represent upregulated genes while the darker bottom colours represent downregulated genes. In the centre overlapping region, the lighter north quadrant and darker south quadrant represent DEGs that are dysregulated in the same direction in both groups, either up or down, respectively. Conversely, the west quadrant with orange text represents DEGs with opposite expression from the perspective of upregulated *Ophiocordyceps*-infection genes and downregulated aflatrem-injection genes. The east quadrant with blue text represents DEGs with opposite expression from the perspective of upregulated aflatrem-injection genes and downregulated *Ophiocordyceps*-infection genes.

shared this same inverse relationship, including an *odorant receptor coreceptor* gene, coding for proteins expressed in chemosensory organs that form heteromultimeric complexes within odorant receptors (Mukunda et al., 2014). We also detected a *short neuropeptide F*, which is implicated in the regulation of animal behaviour including circadian rhythms 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). *Sialin*, a sialic acid transporter-coding gene that prevents neurodegenerative disorders caused by accumulation of sialic acid (Tarailo-Graovac et al., 2017) was additionally among these differentially expressed genes.

Upregulated in our aflatrem-treated ants, but downregulated in the *Ophiocordyceps*-infected ants, was a *receptor-type tyrosine-protein phosphatase delta* (*PTPRD*). This gene is part of the PTP family involved in many known signalling pathways for which delta is shown to promote neurite growth and regulate axon guidance (Tomita et al., 2020). In addition, we found an *obscurin*, which, like *titin*, codes for a large protein expressed in skeletal muscles that anchors myofibrils to the sarcoplasmic reticulum for muscle contraction (Feher, 2017, pp. 305–217), and a *juvenile hormone acid O-methyltransferase-like* gene, which codes for an enzyme that converts precursor molecules into juvenile hormones (Shinoda & Itoyama, 2003). Finally, the two data sets also shared 26 genes with statistically significant changes in expression coding for proteins of unknown function (Supplementary material S2, Data).

DISCUSSION

Many parasites from across the Tree of Life utilize behavioural manipulation to increase their reproduction, survivability and transmission at a cost to the host. Examples include *Loxothylacus panopaei* infecting crabs (Blakeslee et al., 2021), *Spinochordodes tellinii* nematomorphs infecting katydids (Biron et al., 2005), the protozoan *Toxoplasma gondii* infecting mice (Tong et al., 2021), *Cotesia congregata* wasps parasitizing caterpillars (Adamo, 2019), the virus *Rabies lyssavirus* infecting vertebrate animals (Hueffer et al., 2017) and *Entomophthora muscae* fungi infecting flies (Elya & De Fine Licht, 2021). Despite the growing awareness of this phenomena, the fundamental processes in which such behavioural manipulations are established, both in terms of the parasite's role and the pathways affected within their animal hosts, remain poorly understood. In recent years, multi-omics work on 'zombie ants' has provided myriad candidate compounds that *Ophiocordyceps* fungi might use to manipulate ant behaviour. However, their causal relationships with manipulated host behaviours still need to be functionally tested. This study aimed to test aflatrem-like compounds previously implicated in the establishment of *Ophiocordyceps*-extended phenotypes in zombie ants and known to cause neuromuscular impairment in vertebrates (Selala et al., 2008; Valdes et al., 1985; Will et al., 2020). This 'drunkard's walk' behaviour has not been described in ants infected by other disease-causing agents (e.g. *Beauveria bassiana*) (Trinh et al., 2021), making it less likely that staggering should be considered a general sickness behaviour. Understanding the transferability of behaviour-affecting compounds and their effects at the genetic level is a step forward in elucidating the mechanisms underlying behavioural manipulation phenotypes and for determining whether neurological pathways affected during infection are conserved in nature. Moreover, learning how parasites can dysregulate host behaviour will provide insights into the regulation of animal behaviour in general.

We tested both the behavioural and genetic effects of aflatrem on carpenter ants by exposing healthy *C. floridanus* ants to high purity aflatrem and quantifying its behavioural effects. Following injections, we found the dose of aflatrem to be positively correlated to the amount of time that treated individuals spent staggering. To rule out that this observed behaviour was a symptom related to death, we performed a longevity assay to demonstrate that aflatrem does not kill *C. floridanus*. These preliminary tests demonstrated that aflatrem-like compounds are unlikely to be used by *Ophiocordyceps* species as a means to kill their hosts. Instead, the observed staggering behaviours indicate that the aflatrem-like compounds produced by *Ophiocordyceps* during infection are, at least in part, responsible for the drunkard's walk phenotype exhibited by infected carpenter ants (Hughes et al., 2011; Trinh et al., 2021).

In vertebrates, staggers syndrome results from the potentiation of GABA chloride currents (Yao et al., 1989). Given that these receptors are conserved in ant species (Wnuk et al., 2014), it is plausible that similar mechanisms are at play here. Additionally, our RNA-seq results suggest dysregulation of several genes that could explain dysfunction in neuromuscular activity. Among them, we found *anoctamin-4*, which was downregulated in aflatrem-treated ants and encodes a Ca^{2+}-dependent nonselective monovalent cation channel (Reichhart et al., 2019). These channels play a vital role in regulating cation currents that drive excitation of neuronal and muscular activity (Hartzell et al., 2005). Likewise, *ryanodine receptor* also plays a role in cation regulation by releasing Ca^{2+} ions during contraction in skeletal muscles (Lanner et al., 2010). Dysregulation in both genes could impact proper muscle contraction in aflatrem-treated ants, resulting in difficulty walking. Furthermore, *caveolin-3*, a gene coding for structural components of the plasma membrane that regulate signal transduction events in skeletal muscle cells (Galbiati & Lisanti, 2013), was also dysregulated. Mutations in caveolin-3 proteins are known to cause a variety of different muscle diseases in humans (Galbiati & Lisanti, 2013), including caveolinopathy and distal myopathy. Both diseases are characterized by the onset of muscle weakness (Aboumousa et al., 2008). Moreover, caveolin-3-related rippling muscle disease is a disorder that can lead to repeated rapid contraction in muscles, particularly proximal muscles (Torbergsen, 2002). As such, *caveolin-3* dysregulation in aflatrem-treated ants could have given rise to the staggers that we observed. Our findings provide additional insight into the downstream effects on gene expression resulting from GABA potentiation as our RNA-seq data reveals genes that may be associated with neuromuscular dysfunction as a result of aflatrem-induced staggering. Given the similarity of effects observed between our finding with invertebrates and previous work in cows, our data could be informative for future research in vertebrate models.

Our experiments further demonstrated that aflatrem has a significant effect on the mobility of ants, decreasing overall activity as dose increased. Ants injected with higher doses of aflatrem moved less often, did so much slower when they were active, and as a result travelled shorter distances. We also observed that they had more difficulties climbing, particularly when it involved the twine in the arena, which required greater dexterity than climbing on the wall or top of the petri dish. Previous field studies suggest that *Ophiocordyceps* development and fruiting body formation, needed for spore transmission, are dependent on precise levels of light, humidity and temperature (Andersen & Hughes, 2012; Andriolli et al., 2019; Cardoso Neto et al., 2019; Hughes et al., 2011; Lavery et al., 2021; Will et al., 2023). The reduced speed and disruption of normal gate induced by the upregulation of aflatrem-like compounds during late infection could aid to keep the ant within these optimal microclimates prior to biting, by preventing it from climbing too high into the canopy. However, further studies would be needed to test this hypothesis as lethargy is a universally common sickness behaviour as well.

While we could reason a parasite-adaptive function for aflatrem-reduced activity, it seemingly contradicts previous studies where *Ophiocordyceps*-infected individuals showed hyperactive locomotion prior to summiting (de Bekker et al., 2015; Trinh et al., 2021; Will et al., 2020). Nevertheless, transcriptomics studies on *Ophiocordyceps*-manipulated ants suggest that the fungus expresses manipulation compounds in a time-specific manner (de Bekker et al., 2015; Will et al., 2020). It is reasonable to suggest that, prior to the upregulation of aflatrem-like compounds, *Ophiocordyceps* secretes effectors that induce hyperactivity and climbing behaviours to drive the ant away from the nest and up nearby structures. An example of one such effector could be protein tyrosine phosphatase (PTP). Genes encoding this protein are upregulated during *Ophiocordyceps* manipulation (de Bekker et al., 2015; Will et al., 2020) and have been found to induce hyperactivity and enhanced locomotion in caterpillars that are infected with a behaviour-manipulating baculovirus (Han et al., 2015). Enhanced locomotion could be parasite-adaptive by leading hosts away from aggressive conspecifics, which can recognize infected individuals and attack them (Trinh et al., 2021; Will et al., 2020) as part of their social immunity (Cremer et al., 2007). After the ant is safely away from the adaptive behaviours of the colony, the production of 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 & Hughes, 2012; Andriolli et al., 2019; Loreto et al., 2018; Will et al., 2023). 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. This indicates that if aflatrem-like compounds do play a role in maintaining optimal positioning for adherence, it requires the aid of other compounds to induce the death grip phenotype.

As part of the death grip phenotype, *Ophiocordyceps*-infected *C. floridanus* are often found wrapping their limbs around thin pieces of Spanish moss, *Tillandsia usneoides*, which they most frequently use as a biting substrate (Fig. 5) (Will et al., 2023). This 'hugging' behaviour has also been observed in other *Ophiocordyceps*-infected ants where smooth substrates, such as twigs, are primarily bitten (Loreto et al., 2018). The transcription-level dysregulation of sensory perception and neuromuscular functioning that we found, as well as the observed staggers, suggest that unsteadiness during climbing might cause ants to stumble and hug the vegetation. As such, the hugging behaviour itself could act as a secondary means by which hosts are kept in elevated positions should biting itself fail. Indeed, the adherence to vegetation through biting is sometimes missing in *Ophiocordyceps*-manipulated ants, both in the field and in the laboratory (Will et al., 2020) while hugging is always observed. In fact, our group has witnessed multiple examples of such instances in the field where hosts with fully grown fruiting bodies are held to strands of Spanish moss by just their limbs, either due to failed biting or the complete loss of the ant's head from the sample. Hugging may be unique to ant hosts of *Ophiocordyceps* species as the phenotype is absent from other host-manipulating entomopathogens, such as *Eryniopsis lampyridarum* infecting the goldenrod soldier beetle, *Chauliognathus pensylvanicus*, which similarly induces summiting and death grip behaviours on thin, huggable foliage (Steinkraus et al., 2017).

While previous genomic analyses have demonstrated that the secondary metabolite cluster responsible for the production of aflatrem-like compounds is conserved across *Ophiocordyceps* species infecting members of the Camponotini tribe, the exact structure and concentrations of these compounds produced in vivo are still unknown. Regardless, we were able to use reasonable doses of purified aflatrem (i.e. within body weight equivalents used in previous pesticide testing) to replicate the staggers/'drunkard's walk' phenotype induced in ants during *Ophiocordyceps* infection. This caused the differential expression of 108 ant host genes that were previously identified in *Ophiocordyceps*-infected ants exhibiting the summiting phenotype. As such, at least in part, similar host pathways appear to be targeted during isolated aflatrem-dosing and *Ophiocordyceps* infection, leading to comparable behaviours. This indicates that aflatrem-like compounds secreted by *Ophiocordyceps* species are likely responsible for some of the transcriptional and behavioural changes observed during manipulation. Future work characterizing the chemical composition and three-dimensional structure of aflatrem-like compounds produced by species of the *O. unilateralis* complex is needed to shed further light on the physiological effects of these alkaloids and their role in behaviour manipulation. Nevertheless, our work demonstrates that exposing hosts to hypothesized parasite manipulation compounds

Figure 5. Examples of 'hugging' behaviour portrayed by *Ophiocordyceps camponoti-floridani*-infected *Camponotus floridanus* ants during the death grip observed in a natural setting.

is a valuable avenue to test their proposed effects at the genome and phenome level and establishes a framework for future studies characterizing the effects of host-manipulating compounds. Furthermore, these sorts of in vitro studies provide a baseline for the comparison of host pathways affected by other manipulating parasites to determine whether other systems share a common approach towards manipulation. As such, integrative behavioural studies like this one bring us one step closer to characterizing the molecular mechanisms that drive not only zombie-making fungi, but other behaviour-manipulating parasites across the Tree of Life.

Author Contributions

W.C. Beckerson and C. de Bekker designed the experiment, analysed the data, performed the RNA extractions and wrote the paper. W.C. Beckerson, C. Krider and U.A. Mohammad collected the ants, performed the injections and recorded the video footage. C. Krider and U.A. Mohammad performed the behavioural analyses using CowLog. W.C. Beckerson ran the MARGO software and performed the statistical analyses.

Data Availability

The raw RNA-seq data used in these analyses can be found at NCBI's Sequence Read Archive under BioProject ID PRJNA914723 (https://www.ncbi.nlm.nih.gov/sra/PRJNA914723). The raw CowLog observation data can be accessed at: https://github.com/WCBeckerson/28-Minutes-Later-Data.

Declaration of Interest

None.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at https://doi.org/10.1016/j.anbehav.2023.06.011.

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Appendix

Table A1
The average length and weight of *Camponotus floridanus* in this study

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

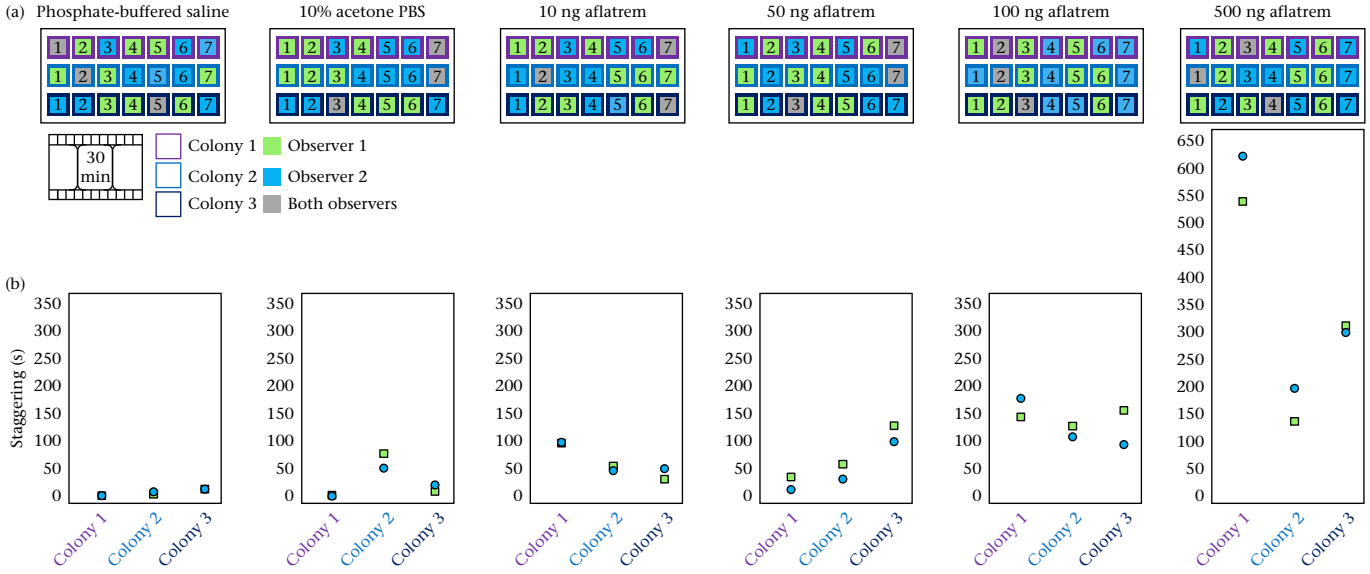

Figure A1. (a) An overview of the randomized block format used to assign videos to each observer. The videos assigned to observer 1 are shown in green, the videos assigned to observer 2 are shown in blue, and the videos assigned to both for formative analysis are shown in grey. (b) Duration of staggering 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.

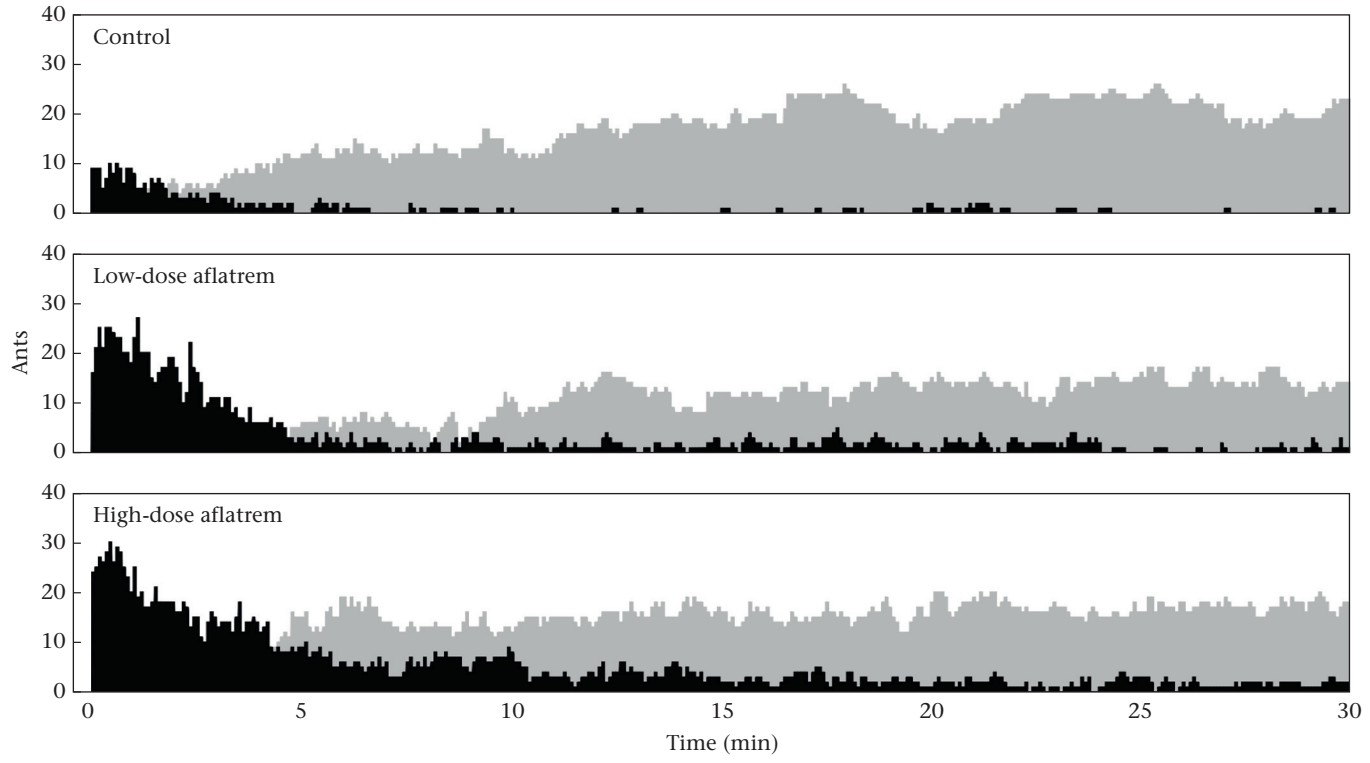

Figure A2. Bar graphs depicting the number of ants exhibiting staggering behaviour (black) and the number of ants resting (dark grey) across the 30 min observational period, broken down into 5 s intervals. Control refers to ants injected with 10% acetone solvent. Low-dose aflatrem shows the data for ants that received 10–50 ng aflatrem, while high-dose aflatrem visualizes staggering and resting for ants that received 100–500 ng.

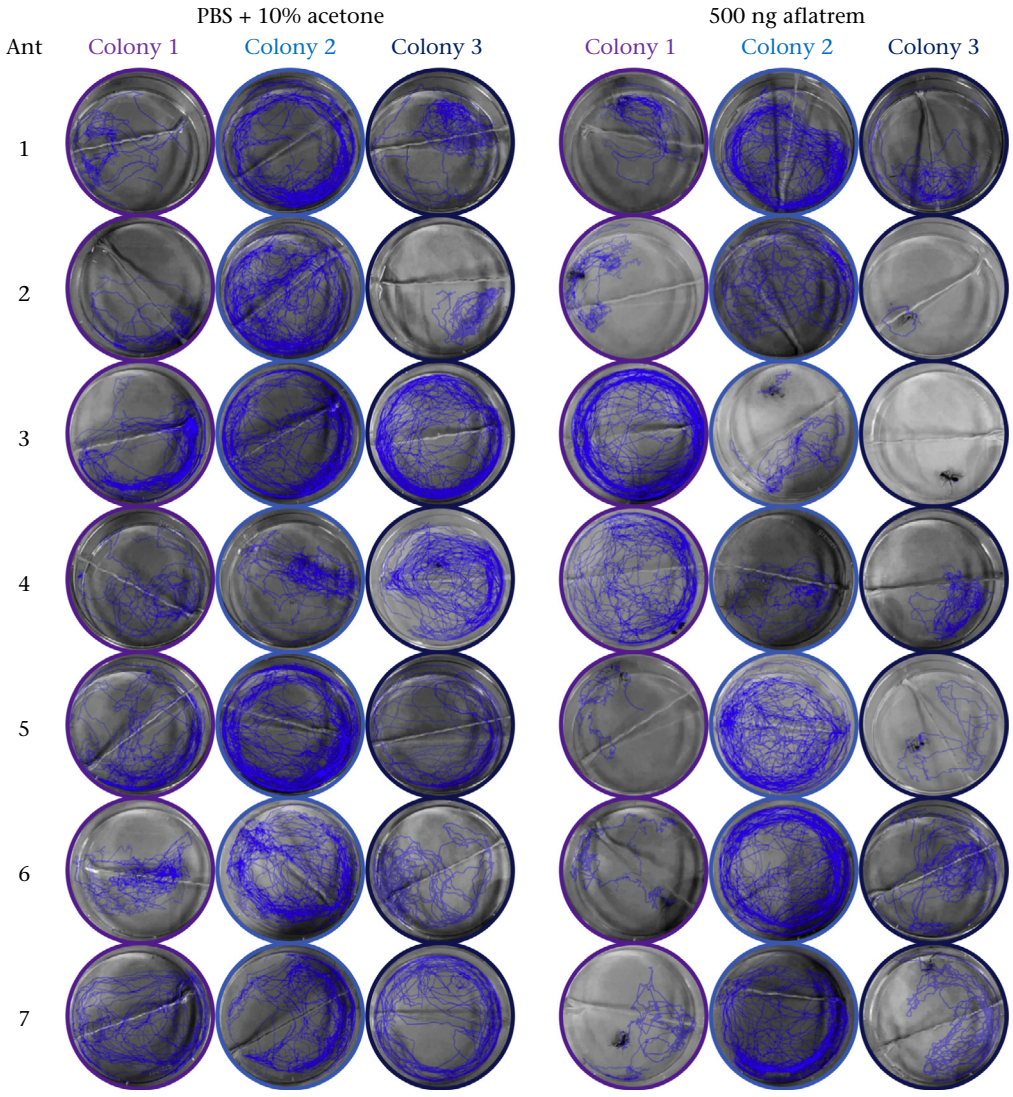

Figure A3. The path travelled by each ant recorded by MARGO tracking software during the 30 min observational period is shown in dark blue. The three columns on the left show the paths travelled by the control group injected with 10% acetone, while the three columns on the right show the paths travelled by ants in the 500 ng aflatrem treatment group. Each group is further divided by colony identity using purple, light blue and dark blue outline to indicate individuals from colonies 1, 2 and 3, respectively.

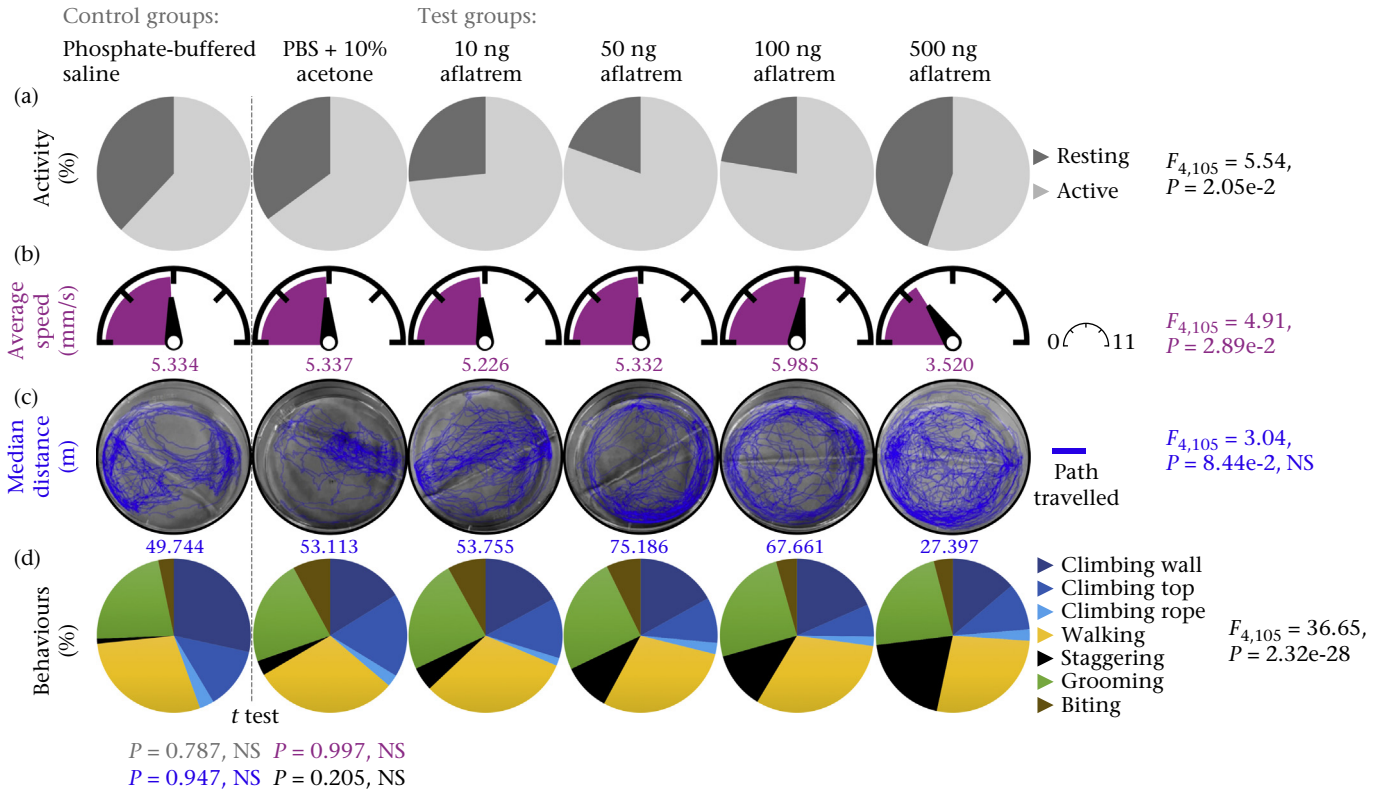

Figure A4. A graphical overview of (a) the relative time spent resting, (b) the average speed calculated by MARGO, (c) the path travelled for the median distance in each group and (d) the distribution of active time spent in the seven commonly observed behaviours. Column 1 represents a pure phosphate-buffered saline (PBS) control, column 2 represents the 0 dose control containing 10% acetone without aflatrem, and columns 3–6 represent the dose assay from 10 ng to 500 ng of aflatrem.