



# Getting lost: the fungal hijacking of ant foraging behaviour in space and time

Thienthanh Trinh<sup>1</sup>, Renee Ouellette<sup>1</sup>, Charissa de Bekker<sup>1,\*</sup>

<sup>1</sup>Department of Biology, College of Sciences, University of Central Florida, Orlando, FL, U.S.A.

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Many parasites have evolved strategies to exploit host behaviour for successful transmission. *Ophiocordyceps* manipulations of carpenter ant behaviour represent an evident example. Manipulated ants are coerced to ascend vegetation and clamp down their mandibles in a stereotypical 'death-grip' bite. The fungus then kills the ant, sprouts a stalk and releases infective spores. Research has focused on this final manipulation, leaving the subtler behavioural changes prebiting largely unexplored. Field and transcriptome studies found that the host circadian clock, olfaction and communication may be disrupted, which suggests that the fungus is affecting ant foraging activity and effectiveness. To test this hypothesis, we investigated if the foraging behaviour of *Camponotus floridanus* ants is notably affected during early stage *Ophiocordyceps* infection. Specifically, we used a maze to quantify foraging patterns and trail optimization. Moreover, by comparing infected individuals to healthy ants and those infected with non-manipulating *Beauveria bassiana*, we aimed to distinguish between nonmanipulator-specific and manipulator-specific changes. We found that *Ophiocordyceps*-infected ants became arrhythmic in their activity patterns, were less likely to participate in effective foraging efforts and seemed less able to communicate with their nestmates compared to healthy ants. We hypothesize that these changes in behaviours are adaptive to *Ophiocordyceps* transmission since they reduce the chance of aggressive interference by nestmates. Indeed, *Beauveria*-infected individuals remained rhythmic; however, they also seemed to lose their ability to forage optimally, suggesting that, in part, these changes in behaviour might be mere general behavioural side-effects of infection. Overall, this study informs future work on parasitic strategies underlying host manipulation, other parasite–host interactions and the behavioural ecology of infectious diseases in general.

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In a constant arms race with their hosts, parasites have evolved myriad strategies to bypass their defences and take advantage of host pathways and resources. One successful strategy to increase parasite fitness is the manipulation of host behaviour such that it promotes transmission. The manipulations induced can range from slight changes in pre-existing phenotypes to novel behaviours that lie outside of the host's typical behavioural repertoire (de Bekker et al., 2018; Poulin & Thomas, 1999; Thomas et al., 2002). These manipulated host behaviours are considered the extended phenotypes of the parasites that infect them (Dawkins, 1999). By studying extended phenotypes, we can further our understanding of the mechanisms underlying host manipulation, parasite–host interactions and the behavioural ecology of infectious diseases.

One evident example of behavioural extended phenotypes is provided by parasitic fungi of the genus *Ophiocordyceps*. Popularly known as 'zombie ant fungi', *Ophiocordyceps* species manipulate the behaviour of ants (Araújo et al., 2015; Evans et al., 2011; Sung et al., 2007). Ants live in colonies that have a decentralized division of labour in which some sterile workers stay in the nest to care for the brood (i.e. nurses) while others leave to forage for food (i.e. foragers) (Tripet & Nonacs, 2004). The foraging caste is thought to be the most prone to infection since foragers venture the most and the furthest outside the nest, giving them a higher chance to encounter fungal spores. After initial infection, the fungus colonizes the ant and manipulates it to abandon the colony and ascend forest vegetation about 16–25 days later (Andersen et al., 2009; de Bekker et al., 2015; Will et al., 2020). Once elevated, the ant is coerced into a final 'death grip' by clamping down onto the vegetation with its mandibles. The fungus then kills the ant prior to sprouting a stalk through the dorsal surface of the ant's thorax and producing a

\* Corresponding author.

E-mail address: [debekker@ucf.edu](mailto:debekker@ucf.edu) (C. de Bekker).

fruiting body to release infective spores (Andersen et al., 2009). The induced biting lies outside of the ant's regular behavioural repertoire and is adaptive to the fungal parasite as it can only grow a fruiting body postmanipulation and when the ant has died outside of the nest at an elevated position (Loreto et al., 2014). The studies currently available on *Ophiocordyceps*-infected ant behaviour focus largely on this stereotypical 'death grip' at the end of the infection since it is most conspicuous (Andersen et al., 2009; de Bekker et al., 2015; Fredericksen et al., 2017; Hughes et al., 2011; Will et al., 2020). As such, the details regarding disease progression and the accompanying behaviours that lead up to the final summing and biting behaviour have remained largely anecdotal.

Previous studies suggest that the timing of manipulated biting behaviour is synchronized. Manipulated foragers of the crepuscular species *Camponotus leonardi* have been observed to bite the vegetation at solar noon (Hughes et al., 2011). Similarly, laboratory studies with the nocturnal species *Camponotus castaneus* and *Camponotus floridanus* consistently recorded manipulated biting during the subjective morning (de Bekker et al., 2015) and just before subjective dawn, respectively (Will et al., 2020). In all studied cases, active biting was, thus, synchronized to a certain time of day with the difference in timing across species interactions likely being due to differences in available light cues (Will et al., 2020). This suggests that light-entrainable molecular clocks could play an important role in the adaptive biting behaviour of *Ophiocordyceps*-infected ants (de Bekker et al., 2014). Moreover, the synchronization of biting indicates that *Ophiocordyceps* infections may be affecting the host's biological clock, either by shifting the phase of daily ant behavioural rhythms or causing ants to become arrhythmic. Circadian clocks are endogenous timekeepers that are important in regulating physiological processes, including immune responses (Dickmeis & Foulkes, 2011; Masri et al., 2013). As such, disrupting the host circadian clock may aid the parasite in bypassing host immune defences and increase its chances of successful manipulation (de Bekker et al., 2014; Hevia et al., 2015). However, it is currently unclear whether clock disruption would only occur at the ultimate stages of infection or if the host clock is already disrupted earlier in the disease progression.

The current literature on *Ophiocordyceps*–ant interactions assumes that, prior to displaying biting behaviour, infected foragers lose their ability to follow foraging trails. This is partly based on field observations of manipulated *C. leonardi*, which, instead of foraging with their canopy-dwelling nestmates, descend to the forest floor (Hughes et al., 2011). *Camponotus* ants rely heavily on olfactory cues to locate food sources and follow chemical foraging trails (Hölldobler & Wilson, 1990). Therefore, *Ophiocordyceps* fungi may be disrupting ant olfactory pathways, leading them to become disoriented, wander from their colony's established foraging trails and bite vegetation in locations away from the nest that promote fungal growth and spore dispersal. This way, *Ophiocordyceps* may be facilitating its own transmission by circumventing social immunity within the ant nest (Loreto et al., 2014). This hypothesis is supported by transcriptomics studies in which odour receptors and other sensory perception and chemical communication proteins were dysregulated in carpenter ants that displayed manipulated biting behaviour (de Bekker et al., 2015; Will et al., 2020). In addition to using chemical-based foraging trails, ants also rely on tactile stimulation (i.e. antennal and foreleg contact) and trophallaxis (i.e. sharing food and chemical cues through oral liquids) to directly communicate with their nestmates (Leboeuf et al., 2019). Indeed, manipulated *C. castaneus* and *C. floridanus* appear to be largely unresponsive to environmental stimuli and agitation by other ants (de Bekker et al., 2015; T. Trinh, R. Ouellette, & C. de Bekker, personal observations). As such, the ability of

*Ophiocordyceps*-infected ants to effectively interact with nestmates and forage seems to be disrupted at several sensory levels.

Taken together, studies that focused on the final manipulated biting stages of *Ophiocordyceps*-infected ants suggest that earlier in the infection, normal foraging behaviour might be disrupted. However, truly revealing whether there are any measurable early changes in foraging behaviour (i.e. slight changes in a pre-existing phenotype instead of a conspicuous novel behaviour such as biting) requires detailed high-interval observations and quantification of infected individuals within the colony's foraging caste. As such, we investigated the early behavioural effects of the fungal parasite *Ophiocordyceps camponoti-floridani* on its ant host *C. floridanus* to answer (1) how the ability of *C. floridanus* to participate in foraging efforts is affected and (2) how the daily foraging rhythms of *C. floridanus* might change. To distinguish between manipulator-specific changes and those related to general infections, we also assessed the behavioural effects of the generalist pathogen *Beauveria bassiana*. To answer the first question, we quantified the foraging activities of *Ophiocordyceps*-infected (OI), *Beauveria*-infected (BI) and healthy control foragers (i.e. *Ophiocordyceps*-control: OC, *Beauveria*-control: BC) throughout disease progression. We hypothesized that effective foraging is disrupted in *Ophiocordyceps*-infected ants as they would show less directional behaviour, wander more and not be able to optimize. If the disruption of foraging activity and effectivity is solely a manipulator-specific change that may be adaptive to *Ophiocordyceps*, we would expect *Beauveria*-infected ants to behave similarly to healthy controls and to continue to contribute to effective group-foraging efforts. To answer the second question, we analysed the daily foraging rhythms of healthy foragers and those infected with *O. camponoti-floridani* and *B. bassiana*. We hypothesized that *Ophiocordyceps*-manipulated ants would become arrhythmic or experience a phase shift (e.g. switch from nocturnal to diurnal foraging) in contrast to healthy controls and *Beauveria*-infected ants. Confirming these hypotheses would support that the disruption of the host circadian clock is a manipulator-specific strategy and may be adaptive to furthering *Ophiocordyceps* manipulation and transmission.

## METHODS

### Ethical Note

We performed infection and behavioural studies on colonies of the invertebrate ant species *C. floridanus*, which is not rare or endangered. Therefore, no additional permits or prior approval by an animal welfare or ethics committee were required beyond the local field site permits that we obtained for ant collection. Fieldwork was conducted with minimal damage and disruption to the subjects and surroundings of the wider ecosystem. We kept the number of colonies and experimental replicates to the minimum required to produce meaningful data and treated colonies as well as possible given the constraints of our experiments. We kept live colonies in a controlled laboratory setting in which food and water were always provided and readily accessible. To this end, colonies underwent minimal stress and suffering.

### Ant Colony Collection, Husbandry and Entrainment

We collected three *C. floridanus* ant colonies from the University of Central Florida Arboretum in Orlando, Florida, U.S.A. (28°35'28"N, 81°11'17"W), between October and December 2019. Each colony was large and estimated to have >2000 workers. Colony 1 was queenless and had lots of pupae while Colony 2 was

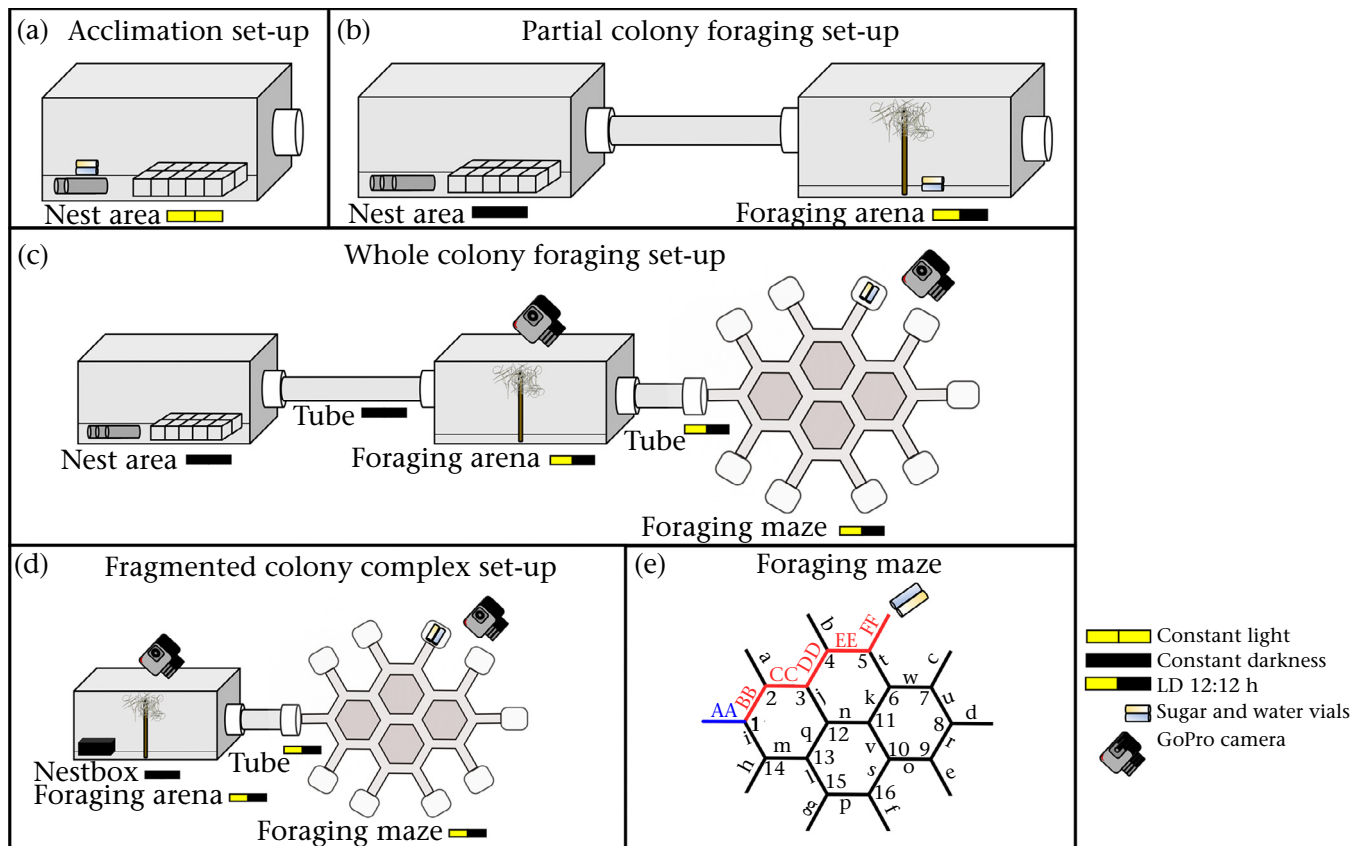
queenright and had minimal brood. Both colonies were used for behavioural experiments as soon as possible after collection (<2 months). Colony 3 was also queenless and consisted mostly of pupae at the time of collection. Before using this colony, we made sure that enough pupae had eclosed and had the time to age to provide enough foragers for experimentation (~2 months). While queen presence/absence effects on collective behaviour and disease susceptibility have been reported for other ant species with smaller colony sizes (Keiser et al., 2018), we did not note any substantial differences between our colony experiments since all led to comparable results with regards to foraging activity and survival rate (see below). This can potentially be attributed to the much larger size of the colonies that we collected, the fact that *C. floridanus* is polydomous or the minimal time between collection and experimentation.

After collection, we introduced each colony to their own individual acclimation set-ups (Fig. 1a), which consisted of a large 9.5-litre plastic container (41.86 × 29.16 × 14.27 cm, Rubbermaid) with a bottom layer of damp plaster (Plaster of Paris). Within this container, we provided ad libitum food (i.e. crickets and 15% sugar water) and water. We also provided three large 50 ml glass tubes (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and a plastic jewellery box with connected compartments (13.49 × 13.97 × 2.23 cm, Bead Landing, Michaels, Irving, TX, U.S.A.) to serve as nest chambers. To prevent ants from escaping, we applied polytetrafluoroethylene fluon (BioQuip, Rancho Dominguez, CA, U.S.A.) to the lower half of the container walls and talcum powder (Fisher) to the upper half. We kept the acclimation set-up in a climate-controlled incubator (136VL, Percival Scientific, Perry, IA, U.S.A.)

under constant light (100% of the incubators lights on at 1991.32 lx) at 25 °C and 80% relative humidity until the majority of ants housed inside the provided nest chambers (1–3 days). Since ants can use light cues to keep time, continuous light was chosen during the acclimation phase to reset and synchronize their biological clocks (Aschoff, 1960; Chen et al., 2008; Daan & Pittendrigh, 1976).

Following continuous light and temperature exposure, we entrained the colony to a 12:12 h light:dark cycle and temperature cycle in the partial colony foraging set-up (Fig. 1b) for 2–9 days prior to starting our behavioural experiments. We converted the acclimation set-up into a nest area by moving it out of the incubator into a temperature-controlled dark room at 25 °C, with an extension into a foraging arena inside the incubator. We covered the nest area with a sheet of Plexiglas and a blackout curtain to block any potential incoming light and connected the nest area to the foraging arena with 1.2 m plastic tubing. Another 9.5-litre container with a layer of damp plaster and fluon/talcum walls served as the foraging arena. The arena also contained 13.5 cm tall trees made of wooden sticks and *Tillandsia usneoides* to serve as climbing structures and potential biting substrates. After connection to the foraging arena, we removed the food and water from the nest area and began to provide them ad libitum in the foraging arena instead (Fig. 1b). The arena aided in the sampling of foragers for infection since it was open and easily accessible.

We also used the foraging arena for entrainment purposes by keeping it under an LD 12:12 h cycle and temperature cycle (Fig. 1b). To simulate dawn, lights progressively turned on and temperature progressively increased from 22 °C to 28 °C between Zeitgeber time, ZT (standardized 24 h notation), ZT0 and ZT3.



**Figure 1.** Experimental set-ups used in this study to (a) acclimate ants to the laboratory after field collection and reset and synchronize their biological clocks, (b) entrain ants to an LD 12:12 h cycle and temperature cycle and sample foragers, and (c, d) observe and quantify ant foraging patterns and optimization to a constant food source. (e) Section labelling of the foraging maze with unique section IDs to facilitate location data collection. The maze entrance is indicated in blue. Edges in red refer to the shortest path to the food source.

Subsequently, from ZT3 until ZT9, light levels were kept at the incubator's maximum light levels (1991.32 lx) and temperature was maintained at 28 °C. To simulate dusk, we programmed the lights to progressively turn off and temperature to progressively decrease from 28 °C to 22 °C between ZT9 and ZT12. Following this simulated daytime, we kept the incubator lights off and maintained temperature at 22 °C between ZT12 and ZT0 to simulate night-time. We kept humidity constant at 70% relative humidity. We validated these environmental conditions with a HOBO data logger (model U12, Onset) that logged light levels, temperature and humidity at 5 min intervals (Appendix 1, Fig. A1).

### Behavioural Experiments

For each ant colony, we ran three sets of behavioural experiments and accompanying observations to record and analyse (1) large colony foraging activity, (2) changes in behaviour upon *Ophiocordyceps* infection and (3) changes in behaviour upon *Beauveria* infection. Since *Ophiocordyceps* and *Beauveria* infections have vastly different incubation times (10+ days and up to 5 days, respectively), and we aimed to maximize ant numbers per infection, we conducted each experiment back to back, allowing colonies to replenish their foragers in between. We randomized the order of the three experiments as follows: Colony 1: *Ophiocordyceps* infection – large colony foraging – *Beauveria* infection; Colony 2: large colony foraging – *Ophiocordyceps* infection – *Beauveria* infection; Colony 3: large colony foraging – *Beauveria* infection – *Ophiocordyceps* infection.

After colony acclimation and entrainment, colony set-ups were expanded to whole colony complex foraging set-ups (Fig. 1c) or fragmented colony complex foraging set-ups (Fig. 1d), depending on the experiment order. We recorded colony behaviour in the whole colony complex foraging set-up for 2 days. *Ophiocordyceps* and *Beauveria* infection experiments were recorded for 12–15 days and 5 days, respectively. We recorded OI in parallel with OC ants and BI in parallel with BC ants.

For the large colony foraging experiments, we converted the partial colony set-up to a whole colony complex foraging set-up by connecting a monochrome-coloured, ABS plastic 3D-printed foraging maze to the foraging arena with a 0.3 m long tube on the opposite end of where ants could enter the arena from the nest (Fig. 1c). We kept the maze inside the incubator, subjecting it to the same LD 12:12 h cycle and temperature cycle as the arena. The maze (40 × 28.5 × 2.5 cm) consisted of four identical, equally spaced hexagons with 16 bifurcations, nine different outlets and Plexiglas placed on top to prevent ants from escaping. The pathways along the maze in which ants moved were 1.5 cm in width and 2 cm in height. At each maze outlet, we connected a small container (7.62 × 7.62 × 5.08 cm, Rubbermaid) with talcum-powdered rims. When we converted the partial colony set-up to the large colony complex foraging set-up, we removed sugar and water from the foraging arena and provided them ad libitum in one of the outlets of the foraging maze instead. The maze was washed in between trials with 10% bleach, followed by 70% ethanol, then 70% hexane to remove any pheromones or residual odours that may have affected behaviour in subsequent experiments.

The experiments that we performed to test our behavioural set-up (Appendix 2, Experimental Verification) demonstrated that healthy *C. floridanus* ants are aggressive towards infected nest-mates. Therefore, infected (i.e. OI and BI) and control groups (i.e. OC and BC) were taken from the whole colony set-up and placed side by side in their own respective fragmented colony complex foraging set-ups (Fig. 1d). These set-ups were similar to the large colony complex foraging set-up and kept under the same LD

12:12 h cycle and temperature cycle. However, the nest area and foraging arena were condensed into one container to be able to fit the behavioural set-ups for treatment groups inside the same climate-controlled incubator. To create the darkened nest, we formed a circular indentation (60 × 15 mm) in the plaster and covered it using a pipette tip box lid (17 × 8.25 cm) with blackout tape. The presence of larvae has been shown to increase worker foraging behaviour (Ulrich et al., 2016). Moreover, *C. floridanus* workers seem to accept brood from other colonies (Carlin & Schwartz, 1989). As such, to induce foraging after placing treatment groups in their respective complex foraging set-ups, we provided each with 7–16 larvae from a separate *C. floridanus* queenright colony (i.e. not belonging to Colony 1, 2 or 3). As soon as these larvae were introduced, ants in all treatment groups immediately carried them to the nest.

To aid in the quantification of optimization to a food source, we labelled the sections of the foraging maze with unique section IDs so that we could collect location data in addition to activity data based on counts (Fig. 1e). We labelled maze edges with letters and maze nodes with numbers. Although it was possible to provide food at any of the maze outlets, we chose to provide 15% sugar and water in 2 ml tubes ad libitum at the third outlet container to the left of the maze entrance. There is only one optimized trail to reach this outlet, which we defined as the shortest trail taken from the maze entrance to the food source (Fig. 1e, indicated in red). The experiments that we performed to verify our behavioural set-up demonstrated that this approach allowed us to measure trail optimization by healthy ants (Appendix 2, Experimental Verification). Although there may be a potential left–right bias due to food placement, we kept its placement constant across all trials, so infection status was the sole changing variant. This allowed us to measure potential differences in trail optimization between healthy and infected ants more easily.

### Forager Selection for Infection

After colony acclimation to the LD 12:12 h cycle and temperature cycle in the partial colony foraging set-up, we sampled ants of the foraging caste for infection experiments. The experiments that we conducted to test our behavioural set-up showed that most foragers were recurrent in the foraging arena (Appendix 2, Experimental Verification). Therefore, we classified all worker ants in this location, belonging to the smaller 'minor' morphological caste, as foragers. To maximize the number of foragers in our infection experiments, we checked the foraging arena every hour starting at ZT11 on the day of infection (i.e. 1 h before the foraging peak) up until we were able to treat them all (on average 14 h later). This resulted in 118 foragers for Colony 1, 106 foragers for Colony 2 and 145 foragers for Colony 3 for *Ophiocordyceps* infection experiments. For *Beauveria* infection experiments, we sampled 89 foragers for Colony 1, 54 foragers for Colony 2 and 91 foragers for Colony 3. We divided foragers equally over the infected and control groups for each behavioural trial.

### Fungal Infections

We performed *Ophiocordyceps* infections (OI) with the *O. camponoti-floridani* Arb2 strain, previously isolated from a manipulated *C. floridanus* ant cadaver (Will et al., 2020) and *Beauveria* infections (BI) with the *B. bassiana* strain Bb0062 (Huang et al., 2019). Fresh blastospores (i.e. yeast-like single cells) of both strains were produced using previously described protocols (de Bekker et al., 2017; Will et al., 2020; Ying & Feng, 2006). We performed infection assays by injecting (*O. camponoti-floridani*) or



pricking (*B. bassiana*) ants with these blastospores using 10 µl borosilicate capillary tubes (Fisher) pulled with a PC-100 Narishige instrument (settings: all weights at two-step pulling, 60% of the maximum output heat level). We infected OI ants by injecting 0.5 µl of a freshly prepared blastospore solution ( $2.58 \pm 0.88 \times 10^7$  cells/ml) in Grace's Insect Medium (Gibco, Thermo Fisher Scientific) with 2.5% FBS (Gibco) between their legs on the ventral side of the thorax, breaking through the epidermis layer, using an aspirator tube (Drummond Scientific, Broomall, PA, U.S.A.). We sham-infected OC ants by injecting 0.5 µl Grace's Insect Medium with 2.5% FBS without cells. Our preliminary infection experiments to establish a protocol for *B. bassiana* infections (data not shown) indicated that pricking ants with *Beauveria* instead of injecting fungal solution extended the initial survival time of ant hosts, while still reliably causing infection. As such, we infected BI ants by pricking ants between their legs on the ventral side of the thorax with a pulled borosilicate capillary tube, covered in blastospores ( $1.99 \pm 0.739 \times 10^7$  cells/ml Grace's Insect Medium with 2.5% FBS). We sham-treated BC ants by pricking them with a capillary tube covered in Grace's Insect Medium with 2.5% FBS. We then introduced infected and control groups to their own respective fragmented colony complex forager set-ups (Fig. 1d).

#### In-person Observations, Video Recordings and Data Collection

To record the foraging activities of large entire colonies and fragmented treatment groups, we mounted infrared lights (CMVision IR30, wavelength = 850 nm) and infrared-enabled GoPro Hero 6 cameras above the foraging arena and foraging maze (one of each per foraging location). This allowed us to record ant activity and location during the day and night-time. We recorded time-lapse videos in which the camera took a picture every minute. Using the Media Player Classic Home Cinema program (MPC-HC), we paused time-lapse videos at every 30th frame (i.e. every 30 min) to count all live ants present in a given frame (Supplementary material 1, Data set 1; also see Supplementary material 4 for further details). An ant was deemed dead in a video frame when no movement for that individual was detected in the thirty frames (i.e. 30 min) before and after the frame being scored. This ant count data was collected by two observers who followed this exact protocol to keep data collection consistent. Additionally, the first observer trained the second one and checked their initial data collection for consistency with their own, to assure recordings were comparable. We removed time points in which there was camera malfunction, or the camera view was obstructed by the researcher. Overall, we scored and analysed a total of 3711 observations in the arena and 3631 observations in the maze in *Ophiocordyceps* experiments (i.e. OI = 1896 observations; OC = 1815 observations in the arena; OI = 1851 observations; OC = 1780 observations in the maze). For *Beauveria* experiments, we scored and analysed a total of 1306 observations in the arena and 1304 observations in the maze (i.e. BI = 658 observations; BC = 648 observations in the arena; BI = 660 observations; BC = 644 observations in the maze).

One observer also performed daily in-person observations to note any changed behaviours that could not be recorded through time-lapse videos. These were performed in conjunction with daily collection of survival data and maintenance activities, such as spraying the plaster in the foraging arena with water to retain high humidity and replacing the water and food source before it got depleted. Notable qualitative behaviours were recorded with an infrared-enabled GoPro Hero 6 or an iPhone 7 to provide video evidence.

#### Survival Analysis and Controlling for Differences in Treatment Group Density

To collect survival data, we counted and removed dead ants from the set-up. To confirm death by *Ophiocordyceps* infection, abdomens of expired individuals were screened for blastospores by squashing them in between a microscope slide and a cover glass. To confirm *Beauveria*-infected deaths, expired individuals were incubated in a petri dish with wet, sterile filter paper to promote fungal growth from the cadaver. Since *C. floridanus* nestmates tend to rip apart the cadavers of moribund or already dead individuals (T. Trinh, personal observations), we frequently recovered unattached bodies and abdomens in the set-ups. To avoid counting the same cadaver twice, we only included counts of whole cadavers or single abdomens to determine survival curves for all treatment groups.

As disease progressed, infected individuals died at a higher rate than their healthy controls. Differences in treatment group density could lead to differences in communication and greatly influence overall group foraging patterns. To account for diminishing numbers of ants in the infection treatments, we randomly selected and removed OC and BC ants and housed them in a separate box outside of the experimental set-up to match the number of OI and BI ants that had died. With this removal of healthy ants, we were able to quantify survival counts while accounting for differences in the number of ants that were present as infections progressed.

We analysed survival data with a log-rank test using the R packages 'survival' (Therneau, 2020) and 'survminer' (Kassambara et al., 2018). By determining the LD50 of each colony's OI and BI survival (i.e. the day on which 50% of the infected individuals had died), we were able to separate the data into early-stage and late-stage infections to analyse the overall changes in foraging activity in more detail. To confirm that the number of ants present in infected and control groups throughout the experiments (OI versus OC, BI versus BC) was not statistically different due to death or subsequent removal of healthy controls, we compared the treatment groups using Mann–Whitney *U* tests.

#### Zero-One Beta Models to Investigate the Effect of Infection on *C. floridanus* Foraging Efforts

To assess the effects of *Ophiocordyceps* and *Beauveria* infections on the ability of *C. floridanus* to participate in foraging efforts, we converted our count data into proportion data to account for the number of ants that perished due to infection. As such, we divided the number of ants in the arena and in the maze by the total number of ants that were present in the treatment group when the frame was taken. Additionally, *C. floridanus* foragers display a strong preference for foraging during the night-time, which resulted in an inflation of zero proportions in our data set. Therefore, we used zero-one inflated beta models (ZOIB) in the R package 'gamlss' (Rigby et al., 2005) to analyse activity data since ZOIBs account for (1) proportions between zero and one ( $\mu$  parameter), (2) proportions equalling to zero ( $\nu$  parameter) and (3) proportions equalling to one ( $\tau$  parameter) in separate model parameters that converge into a final ensemble model.

Moreover, infected and treatment ants behaved differently in the arena and maze (Appendix 1, Table A1), which led us to analyse the foraging activity in those areas separately to quantify potential location-specific differences between treatment groups. As such, we modelled (1) arena proportions in OI and OC, (2) arena proportions in BI and BC, (3) maze proportions in OI and OC and (4) maze proportions in BI and BC. For analyses on OI and OC, we

included 'treatment' (i.e. infected or control), 'ZT' (i.e. time of day), 'day(s) postinfection' (i.e. 'day') and their interactions as fixed effects in the  $\mu$  and  $\nu$  parameters in the ensemble ZOIB model. For analyses on BI and BC, we tested the same ZOIBs but with 'day' replaced by 'progression' (i.e. early versus late based on LD50) since BI individuals died three times faster. We held the  $\tau$  parameter constant since only a limited number of proportions were equal to one. Since we scored proportions of the same group of foragers across time in each trial, we accounted for dependencies and pseudoreplication by including treatment groups (i.e. OI and OC or BI and BC) nested within each colony (i.e. Colony 1, 2, 3) as random effects. We used Akaike's information criteria (AIC) (Akaike, 1973) to identify the most plausible model.

For ants that made it into the maze, we compared their ability to establish and follow an optimized trail towards the food source. To convert counts to 'on trail' versus 'off trail' proportions, we removed 'AA', the maze entrance, and 'node' data and divided the total number of ants on the optimized trail by the total number of ants scored in the maze. To compare on trail and off trail proportions, we used two independent ZOIBs for *Ophiocordyceps* infections and *Beauveria* infections with 'on versus off proportions' as the response variable. We included treatment, progression and treatment\*progression interaction as fixed effects in the  $\mu$  parameter. We held the  $\nu$  and  $\tau$  parameters constant. Again, treatment groups were nested within each colony as random effects to account for dependencies and AIC was used to identify the most plausible model.

In addition, we compared ants off the optimized trail based on how far away from the optimized trail they travelled by creating 'edge groups'. We grouped edges on the optimized trail (i.e. BB, CC, DD, EE, FF) in 'group 0', immediately adjacent to the optimized trail (i.e. a, b, i, j, t) in 'group 1', two edges away from the optimized trail (i.e. h, m, q, n, k, w) in 'group 2', three edges away (i.e. l, v, u, c) in 'group 3', four edges away (i.e. g, p, s, o, r, d) in 'group 4', and five edges away (i.e. e, f) in 'group 5'. The entrance to the maze, AA, and nodes were analysed as separate groups. To convert counts to 'location group proportions', we divided the daily total number of ants scored in each edge group by the daily number of ants scored in the entire maze. To compare foraging in edges close and far from the optimized trail, we used ZOIBs for each edge group with 'location group proportions' as the response variable and treatment as the fixed effect. Colony ID and subgroup were included as

random effects to account for the dependencies in the repeated measures design.

### Rhythmicity Analyses to Investigate the Effect of Infection on *C. floridanus* Foraging Rhythms

In addition to comparing the foraging activities of OI, OC, BI and BC spatially in the arena and in the maze, we also analysed the daily foraging rhythms of foragers to identify whether infection could be affecting ant biological clocks that drive these rhythms (de Bekker, 2019; de Bekker et al., 2014; Kay et al., 2018; Will et al., 2020). We calculated the mean 'arena proportions' and mean 'maze proportions' at each ZT for each treatment group (i.e. OI, OC, BI, BC) during the early stage, late stage and entire incubation period of the experiment. We then performed rhythmicity analyses using the R package 'rain' (Thaben & Westermark, 2014) to identify whether the daily activity patterns of OI, OC, BI and BC were significantly rhythmic. The following parameters were used: deltatt (sampling interval) = 0.5; period (period length) = 24; nr. series = 1; peak.b-order (partitions of the rising slope with respect to the whole period) = 0.3–0.7.

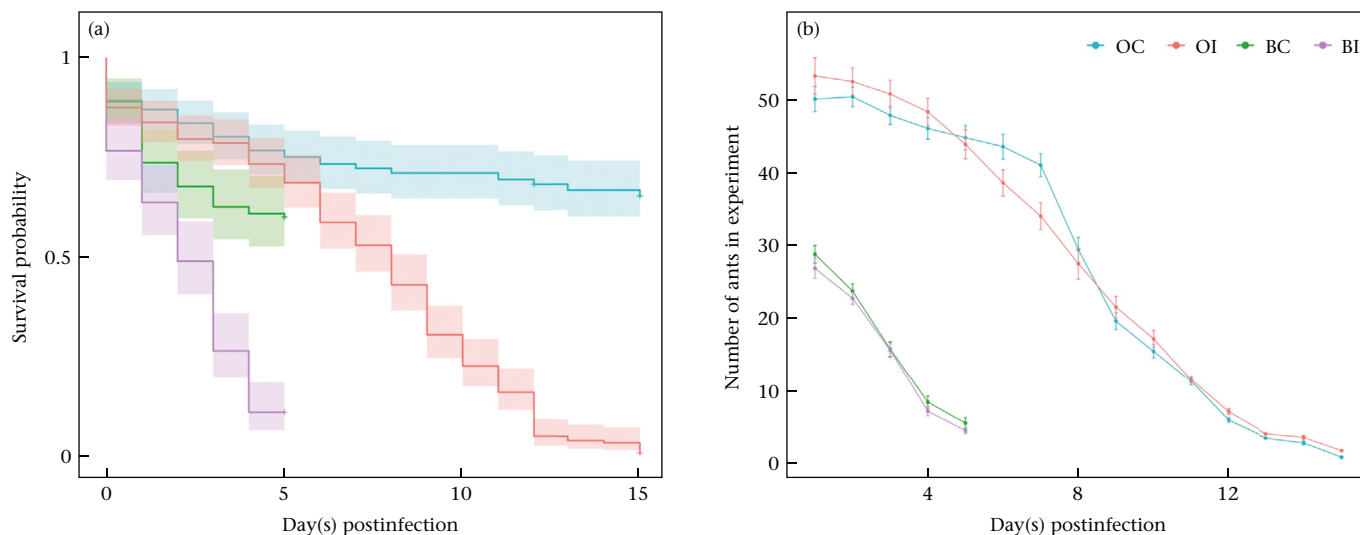
## RESULTS

### Experimental Set-up Verification

Prior to conducting our infection experiments, we evaluated whether our experimental set-up would allow us to (1) identify recurrent foragers to use in our infection trials, (2) quantify rhythmic foraging activities, (3) quantify optimization to a constant food source and (4) track survival and behaviour of infected individuals within a large colony context. The results compelled us to perform our experiments with the behavioural set-up that we designed but using separate colony fragments for the control and infected treatment groups instead of large colonies (Appendix 2, Experimental Verification).

### Camponotus floridanus Survival

Infection with *Ophiocordyceps* and *Beauveria* had a significant effect on forager survival (log-rank test:  $P < 0.001$ ). Generally, OI ants succumbed to the infection 12–15 days postinfection, with an



**Figure 2.** Survival curves and ants present in *Ophiocordyceps* and *Beauveria* experiments across days postinfection. (a) Survival of *Ophiocordyceps*-control (OC), *Ophiocordyceps*-infected (OI), *Beauveria*-control (BC) and *Beauveria*-infected (BI) ants. Shaded areas represent 95% confidence intervals. (b) The number of ants present in the experiments decreased over the day(s) postinfection due to death in OI and BI and subsequent removal of OC and BC.

LD50 at 5–7 days postinfection. In comparison, disease progressed faster in BI ants, which generally succumbed to the infection 5 days postinfection, with an LD50 around 1–3 days postinfection (Fig. 2a). Since disease progresses differently in OI and BI, we analysed *Ophiocordyceps* and *Beauveria* experiments separately. We used the LD50 of each infected colony replicate to divide our observations into early (i.e. before LD50) and late (i.e. after LD50) infection stages. Furthermore, to assure we were making fair behavioural comparisons between infected and control groups, we confirmed that our random selection and removal of ants in the control groups matched the number of ants dying in the infected groups. We found that the number of ants present in OI indeed did not statistically differ from OC at any given time during the infection experiments (Mann–Whitney  $U$  test:  $W = 105$ ,  $N_1 = N_2 = 15$ ,  $P = 0.77$ ). Likewise, the number of ants present in BI and BC did not statistically differ throughout the experiment (Mann–Whitney  $U$  test:  $W = 14$ ,  $N_1 = N_2 = 5$ ,  $P = 0.69$ ; Fig. 2b).

It should be noted that OI foragers never reached full manipulation in which they ascended and bit down on the provided substrate. All infected ants expired prior to making it to the late incubation stage in which manipulation normally takes place. However, as early as 4 days postinfection, we were able to detect blastospores in the abdomens of expired OI individuals (Appendix 1, Fig. A2a). This confirmed that they were infected successfully and likely perished due to the infection. Confirmation of successful infection in expired BI ants was done through cadaver incubation, which resulted in the emergence of fungal mycelium from all incubated ants (Appendix 1, Fig. A2b).

#### Healthy Ant Foraging Behaviour after Colony Fragmentation

To assess whether colony fragmentation would result in significant behavioural changes that we would have to account for in our

data analyses, we compared the activity patterns of healthy, large colonies with the healthy OC and BC fragmented groups taken from those colonies. Similar to large colonies, OC fragmented groups also displayed a distinct foraging peak at ZT12, with a more obvious peak in the arena than in the maze (Appendix 1, Fig. A3). However, BC fragmented groups displayed a foraging peak 5.5 h later in the day at ZT 17.5 (Appendix 1, Fig. A3). BC ants also rarely foraged in the maze. This could be an effect of the relatively brief period that BC ants were monitored upon rehousing, which was only 5 days (i.e. the maximum length of *B. bassiana* infections). In contrast, OC ants were monitored for 12–15 days (i.e. the maximum length of *O. camponoti-floridani* infections). It is, therefore, possible that the BC treatment ants did not have enough time to properly acclimate to the experimental set-up. Additionally, BC ants could have been less inclined to forage for food and water as the number of ants in the fragmented BC colonies quickly decreased to match the drop-off in the infection groups. Nevertheless, results showed that colony fragmentation did not necessarily affect overall foraging patterns since daily foraging rhythms persisted (Appendix 1, Fig. A3).

#### The Effect of Fungal Infection on Location-dependent Foraging Activity

We compared the foraging activity between infected and control ants in the foraging arena and in the foraging maze in four ZOIB ensemble models (Table 1). To analyse the foraging activity of *Ophiocordyceps*-infected ants and their controls in locations farther from the constant food source (i.e. closer to the nest), we modelled the activity of OI and OC ants in the foraging arena (Table 1, Model 1). We found that OI ants occupied the arena at significantly higher proportions (0.17) compared to their healthy controls (0.11) (treatment:  $t = 5.382$ ,  $P < 0.001$ ; Fig. 3a, Table 1, Model 1, nonzero data). In addition, time of day affected the foraging intensity of OI

**Table 1**

Parameter estimates of the ZOIB for *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) ants in the arena (Model 1), OI and OC in the maze (Model 2), *Beauveria*-infected (BI) and *Beauveria*-control (BC) ants in the arena (Model 3) and BI and BC in the maze (Model 4)

Covariate	Model for nonzero data ( $\mu$ )				Model for zero data ( $v$ )			
	Estimate	SE	$t$	$P$	Estimate	SE	$t$	$P$
<b>Model 1: OI and OC in the foraging arena</b>								
Treatment (T)	0.653	0.121	5.382	<b>&lt;0.001</b>	−4.621	0.654	−7.071	<b>&lt;0.001</b>
Day	0.069	0.017	4.114	<b>&lt;0.001</b>	0.22	0.031	7.036	<b>&lt;0.001</b>
ZT	0.023	0.007	3.411	<b>0.001</b>	−0.158	0.022	−7.243	<b>&lt;0.001</b>
T*Day	0.015	0.018	0.815	0.415	0.105	0.063	1.679	0.093
T*ZT	−0.036	0.008	−4.33	<b>&lt;0.001</b>	0.147	0.049	2.981	<b>0.003</b>
<b>Model 2: OI and OC in the maze</b>								
Treatment (T)	−0.565	0.106	−5.306	<b>&lt;0.001</b>	0.617	0.618	0.999	0.318
Day	0.053	0.01	5.258	<b>&lt;0.001</b>	0.453	0.045	10.125	<b>&lt;0.001</b>
ZT	−0.024	0.005	−4.714	<b>&lt;0.001</b>	−0.111	0.038	−2.924	<b>0.003</b>
T*Day	−0.066	0.016	−4.044	<b>&lt;0.001</b>	0.134	0.066	2.029	<b>0.042</b>
T*ZT	0.006	0.008	0.766	0.444	0.166	0.048	3.488	<b>&lt;0.001</b>
<b>Model 3: BI and BC in the arena</b>								
Treatment (T)	0.717	0.132	5.418	<b>&lt;0.001</b>	−0.411	0.518	−0.794	0.427
Progression (P)	−0.423	0.187	−2.26	<b>0.024</b>	2.613	0.389	6.719	<b>&lt;0.001</b>
ZT	0.015	0.008	1.891	0.059	0.017	0.023	0.754	0.451
T*P	0.575	0.216	2.657	<b>0.008</b>	−1.53	0.599	−2.553	<b>0.011</b>
T*ZT	−0.015	0.01	−1.567	0.117	−0.046	0.04	−1.153	0.249
Day*ZT	0.04	0.013	3.041	<b>0.002</b>	−0.058	0.028	−2.097	<b>0.036</b>
T*Day*ZT	−0.036	0.015	−2.325	<b>0.02</b>	0.117	0.045	2.579	<b>0.01</b>
<b>Model 4: BI and BC in the maze</b>								
Treatment (T)	−0.276	0.166	−1.666	0.096	0.498	0.398	1.249	0.212
Progression (P)	0.474	0.152	3.113	<b>0.002</b>	1.043	0.342	3.049	<b>0.002</b>
ZT	0.006	0.008	0.767	0.443	−0.018	0.02	−0.874	0.382
T*P	0.308	0.223	1.383	0.167	−0.244	0.505	−0.482	0.63
T*ZT	−0.012	0.012	−1.024	0.306	0.03	0.03	0.994	0.32
P*ZT	0.012	0.011	1.071	0.284	−0.006	0.025	−0.245	0.806
T*P*ZT	0.017	0.016	1.016	0.31	0.01	0.038	0.256	0.798

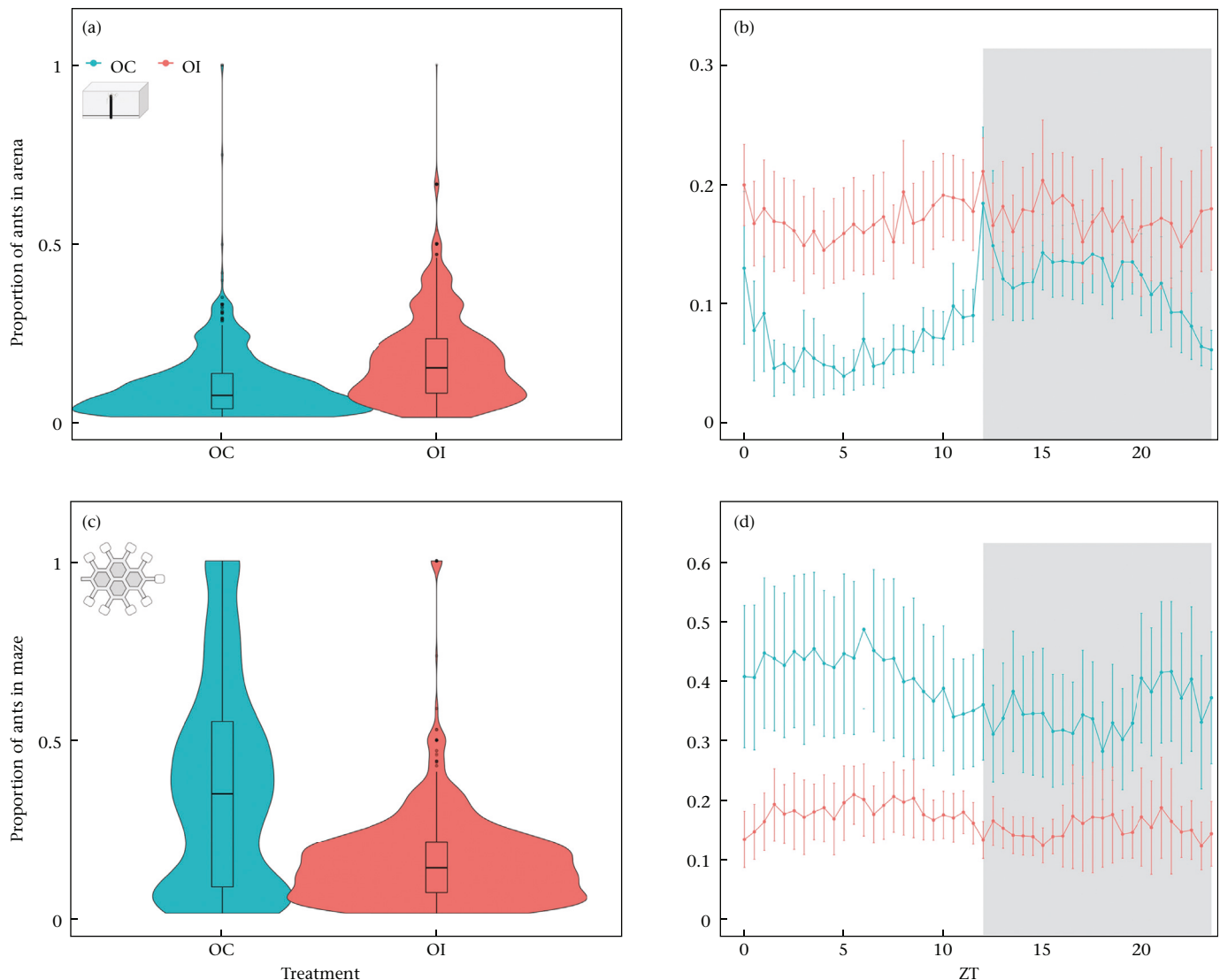
ZOIB: zero-one inflated beta models; ZT: Zeitgeber time.  $R^2$  values: model 1 = 0.535; model 2 = 0.772; model 3 = 0.300; model 4 = 0.372. Significant  $P$  values < 0.05 are indicated in bold.

and OC ants differently (treatment\*ZT:  $t = -4.33$ ,  $P < 0.001$ ; Fig. 3b, Table 1, Model 1, nonzero data). OC ants foraged at higher proportions during the night-time (ZT 12–23.5 mean proportion = 0.12) and occupied the foraging arena less during the daytime (ZT 0–11.5 mean proportion = 0.07) (Fig. 3b). In contrast, OI ants did not have a strong preference for the time of day in the arena as they did not have an obvious foraging peak and occupied the arena at the same proportions during the night-time and the daytime (ZT 12–23.5 mean proportion = 0.17; ZT 0–11.5 mean proportion = 0.17) (Fig. 3b).

These results aligned with our daily qualitative in-person observations. Towards the late stage of infection, we observed that OI ants were present all over the foraging arena while healthy OC ants were not. Healthy *C. floridanus* ants travelled a more direct route to the maze and did so predominantly during the night-time. We rarely observed healthy ants in the arena during the daytime. In contrast, OI ants occupied the foraging arena at all times of the day. They displayed constant locomotion behaviours by walking around

in the arena, seemingly without direction, and continuously attempting to climb up against its slippery walls. We did not observe these behaviours for healthy *C. floridanus* ants. Additionally, OI ants would oftentimes show a lack of motor coordination as they tripped, fumbled and flipped onto their dorsal side, followed by several seconds of scrambling to get back onto their legs. Their antennae were also irregularly displayed out and their gait was notably unsteady (Supplementary Video S2). It should be noted that we did not quantify these in-person observations. However, healthy ants never displayed the conspicuous motor coordination issues of late-stage infected individuals.

To analyse the foraging activity of OI and OC ants in locations closer to the constant food source, we modelled their maze foraging activity (Table 1, Model 2). We found that, overall, OI ants foraged significantly less in the maze compared to their healthy controls (treatment:  $t = -5.31$ ,  $P < 0.001$ ; Fig. 3c, Table 1, Model 2, nonzero data), as they occupied the maze at a lower mean proportion (0.16) compared to OC ants (0.38). Foraging intensity in the maze, as it



**Figure 3.** Effects of *Ophiocordyceps* infection on foraging activity. Pink represents *Ophiocordyceps*-infected (OI) ants and blue represents *Ophiocordyceps*-control (OC) ants. Proportional foraging intensity of ants present in the (a) foraging arena throughout the entirety of the experiment, (b) foraging arena throughout the day, (c) foraging maze throughout the entirety of the experiment and (d) foraging maze throughout the day. Time points during the night-time phase are shaded in grey. (a, c) Box plots represent the median, 25th, 50th and 75th percentiles and violin plots represent the kernel density of the foraging proportions. (b, d) Filled circles represent mean proportions and bars represent 95% confidence interval.

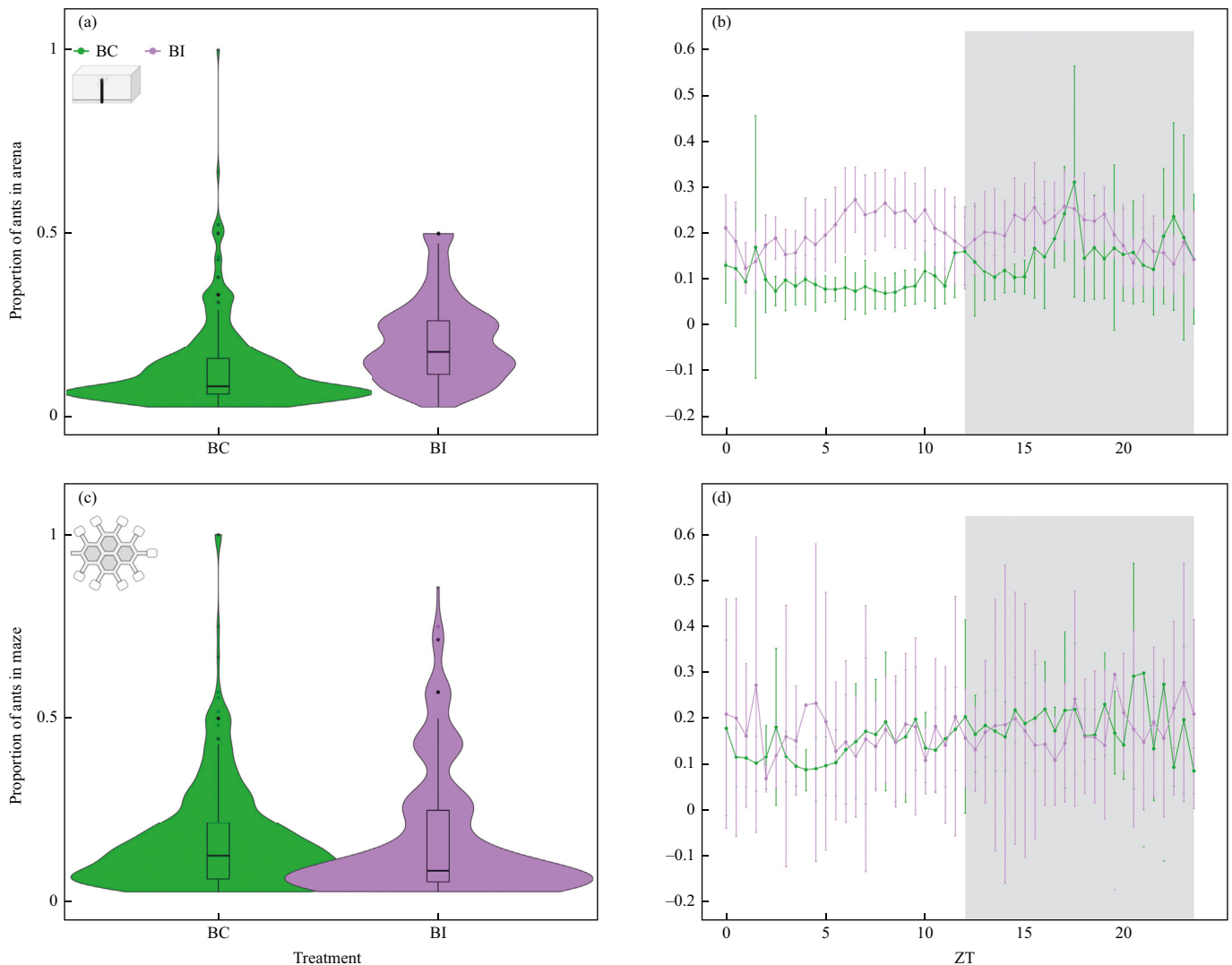


related to time of day, was not significantly affected in OI ants compared to OC ants (treatment\*ZT:  $t = 0.766$ ,  $P = 0.444$ ; Fig. 3d, Table 1, Model 2, nonzero data) as neither showed evident temporal bouts in maze activity.

During our daily qualitative in-person observations, we observed that a subset of OC ants grouped together at the maze entrance (AA) at all time periods of the day. These healthy individuals were stationary and performed frequent antennation and trophallaxis with nestmates. Individual foragers would travel back and forth between the group, food source and other sections of the maze, whereas this subset of foragers did not move from their position at the entrance (Supplementary Video S1). Since ants were not provided with individual IDs in this experiment, we were unable to determine whether this subset of foragers consisted of the same individuals or was consistently replaced by different foragers throughout the day. In contrast, OI ants did not typically group together at the maze entrance (AA). Indeed, as OI ants spent more time in the foraging arena, displaying directionless locomotion and crawling against the walls, they seemed less likely to reach the

maze than their healthy controls. Although we did not quantify interactions between nestmates during our in-person observations, OI ants were rarely observed to communicate through tactile stimulation or trophallaxis with one another.

To distinguish between potentially manipulator-specific changes of foraging activity and those being a result of general infection, we also modelled the foraging activity of BI individuals and their controls (Table 1, Model 3). Similar to our findings for OI ants in the arena, we found that BI ants occupied the arena at significantly higher proportions (0.21) compared to their healthy controls (BC) (0.13) (Fig. 4a, Table 1, Model 3: nonzero data: Treatment:  $t = 5.418$ ,  $P < 0.001$ ). However, in spite of foraging at different proportions, ZT did not affect the foraging intensity of BI and BC significantly differently (treatment\*ZT:  $t = -1.577$ ,  $P = 0.117$ ; Fig. 4b, Table 1, Model 3, nonzero data). Outside of the foraging peak at ZT 17.5 (ZT 17.5 mean proportion = 0.31), BC ants occupied the arena at much lower proportions than BI ants (day-time ZT 0–11.5 mean proportion = 0.10; night-time ZT 12–23.5 mean proportion = 0.16), while BI ants foraged with similarly



**Figure 4.** Effects of *Beauveria* infection on foraging activity. Purple represents *Beauveria*-infected (BI) ants and green represents *Beauveria*-control (BC) ants. Proportional foraging intensity of ants present in the (a) foraging arena throughout the entirety of the experiment, (b) foraging arena throughout the day, (c) foraging maze throughout the entirety of the experiment and (d) foraging maze throughout the day. Time points during the night-time phase are shaded in grey. (a, c) Box plots represent the median, 25th, 50th and 75th percentiles and violin plots represent the kernel density of the foraging proportions. (b, d) Filled circles represent mean proportions and bars represent 95% confidence interval.

higher proportions during the light phase (ZT 0–11.5 mean proportion = 0.21) and the dark phase (ZT 12–23.5 mean proportion = 0.21). Similar to BC, BI ants showed a foraging peak around ZT 17 (ZT 17 mean proportion = 0.26). However, BI ants appeared to have an additional peak at 6.5 (ZT 6.5 mean proportion = 0.27).

In our daily qualitative in-person observations, we observed that BI ants typically performed the same ‘wall-crawl’ behaviour as OI ants, in which they aberrantly climbed and stumbled up the sides of the container walls. Likewise, they frequently fell onto their dorsal sides and had difficulty scrambling to get back up. This may have led to the high proportions of BI ants that we observed in the arena. Like with our OC observations, we did not observe this same ‘wall-crawl’ behaviour in BC ants.

We also modelled the foraging activity of BI and BC ants in the maze (Table 1, Model 4). Overall, we did not find significant differences in maze foraging activity between BI and BC ants (treatment:  $t = -1.666$ ,  $P = 0.096$ ; Fig. 4c, Table 1, Model 4). In addition, the *Beauveria* treatment groups also did not differ in the timing of their maze foraging (treatment\*ZT:  $t = -1.024$ ,  $P = 0.306$ ; Fig. 4d, Table 1, Model 4). However, neither treatment group seemed to be present in the maze very much at any given time during the day, since on average, BI ants and BC ants occupied the maze at a low mean proportion of 0.17. These low numbers in the maze may have been due to the short experimental time during which BI and BC were monitored before all BI ants succumbed to infection (i.e. 5 days). Due to the short experimental time and the quick significant drop-off of ants present in the fragmented colony, there may have been a lower demand for food and water overall and, thus, a lessened urge for BC ants to use the same foraging strategy as OI ants and venture further from the nest. The BC baseline for maze foraging is, therefore, somewhat unreliable, making our results for this part of the data set inconclusive. This also complicated additional detailed analyses and interpretation of the maze data obtained from *Beauveria* treatment groups.

**Table 2**

Parameter estimates of the ZOIB for ants on the optimized trail to the food source in *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) treatments

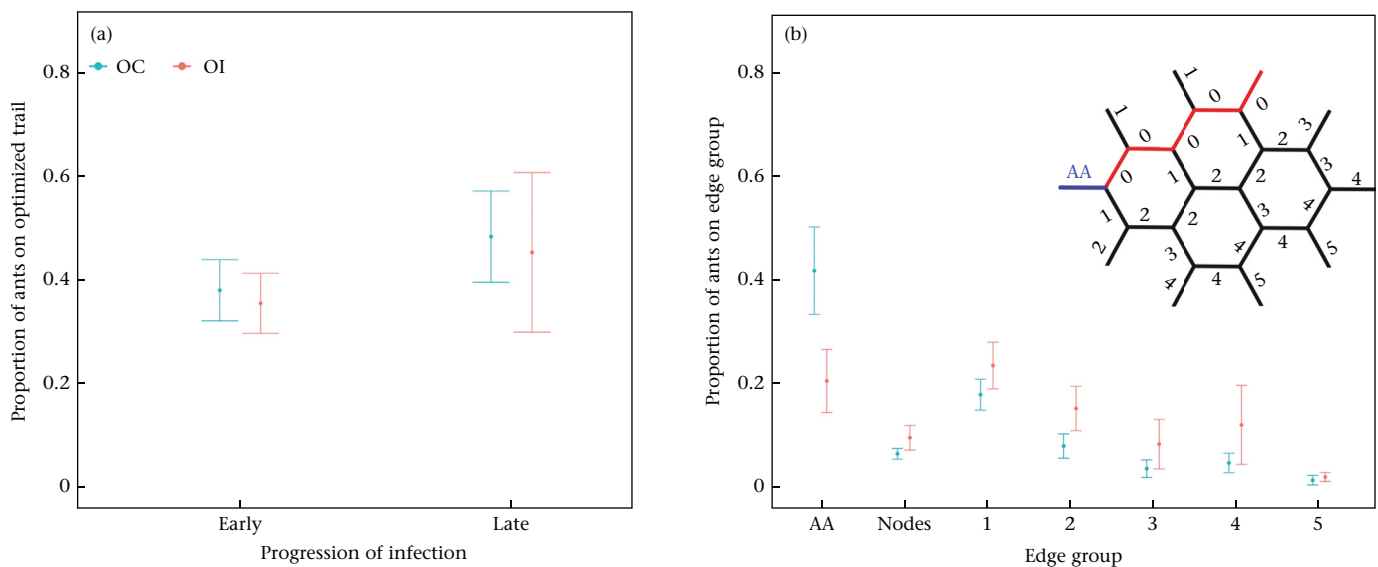
Covariate	Estimate	SE	<i>t</i>	<i>P</i>
Treatment (T)	−0.113	0.142	−0.8	0.426
Progression (P)	0.248	0.141	1.754	0.084
T*P	−0.204	0.216	−0.946	0.348

ZOIB: zero-one inflated beta models. Model's  $R^2 = 0.302$ .

### The Effect of Fungal Infection on Optimization to the Food Source

To identify the effect of infection on the ability of ants to optimize to a stable food source, we compared the proportions of healthy and infected ants in the maze that were present on and off the optimized trail towards that food source. We deemed this analysis to be relevant only for OI and OC ants since we found that both BI and BC ants rarely used the maze during their much shorter infection trials. This resulted in the scoring of a low number of BI and BC ants, rendering further detailed analyses inconclusive. To investigate whether disease progression played a role in the likelihood of finding OI more on or off trail than their healthy controls, we divided the data into early and late stages of infection.

Of the individuals that reached the maze, OI ants did not differ significantly from OC ants in their occupation of the optimized trail (Fig. 5a). This was true for early and late stages of the infection experiment (treatment\*progression:  $t = -0.946$ ,  $P = 0.348$ ; Fig. 5a, Table 2). However, OC ants occupied the maze entrance (AA) significantly more (Fig. 5b, Table 3). This is in line with our in-person observations that OC ants clustered in the maze entrance as part of their foraging strategy while OI ants did not. Additionally, there were some ants that were observed off trail. For those ants, we analysed their relative position away from the optimized path by scoring their presence on maze edges that we grouped based on



**Figure 5.** Proportions of *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) ants on the optimized trail and edge groups of the maze. Pink = OI ants; blue = OC ants. Filled circles represent mean proportions and bars represent 95% confidence interval. (a) Ants on the optimized trail during the early and late stages of infection. (b) Ants on each edge group of the maze based on their distance from the optimized trail to the food source; sections farther away from the optimized trail had a higher group number.

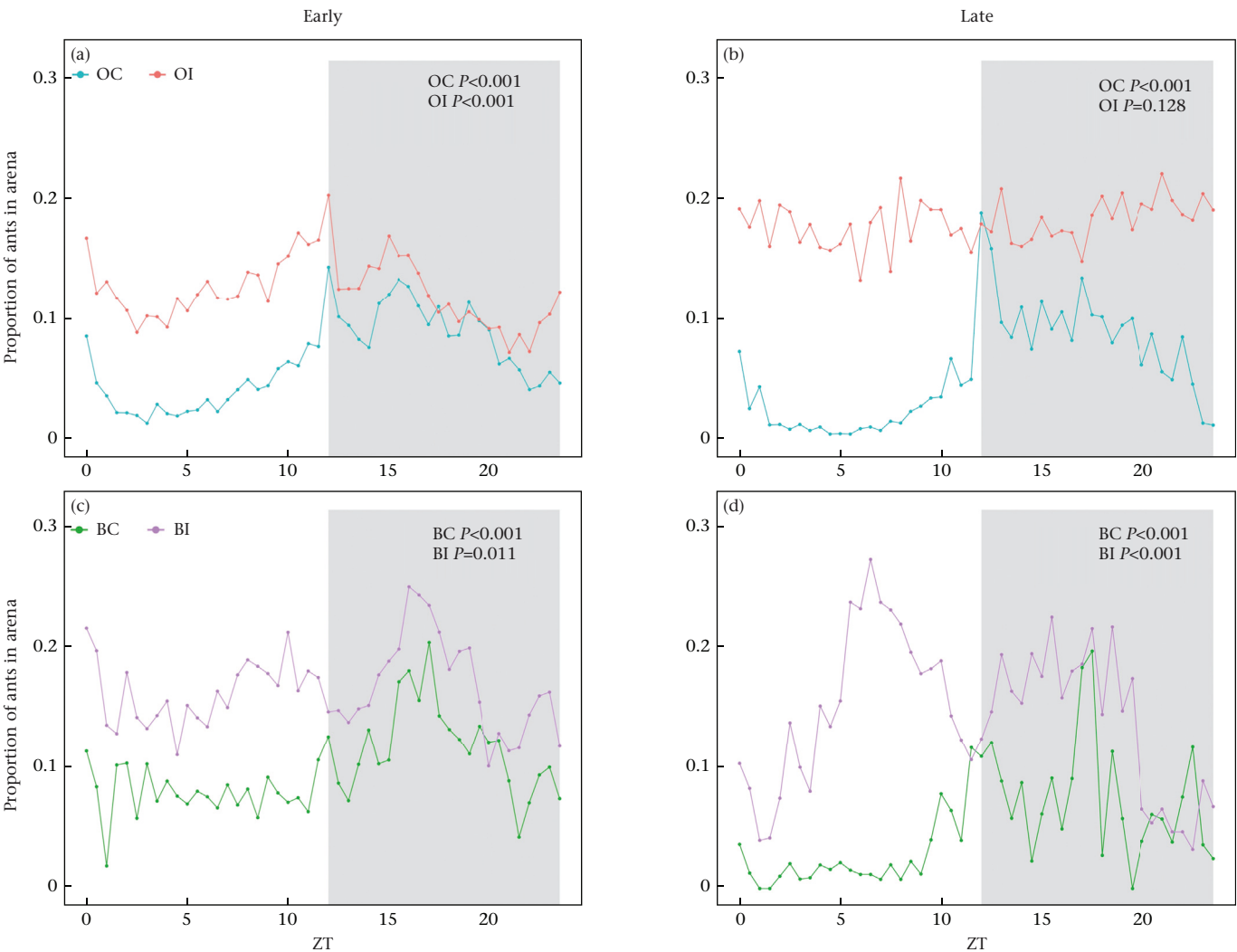
**Table 3**  
Differences between *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) ants in positions away from the optimized trail

OI			OC		
Group	Mean proportion	CI	Mean proportion	CI	P
AA	0.20	0.06	0.41	0.08	<b>&lt;0.001</b>
Nodes	0.09	0.02	0.06	0.01	<b>0.007</b>
1	0.23	0.04	0.18	0.03	<b>0.032</b>
2	0.15	0.04	0.08	0.02	<b>&lt;0.001</b>
3	0.08	0.05	0.03	0.02	<b>&lt;0.001</b>
4	0.12	0.08	0.05	0.02	<b>0.002</b>
5	0.018	0.01	0.012	0	<b>0.010</b>

Mean proportions, confidence intervals (CI) and *P* values from the zero-one beta regression models comparing the proportions of OI and OC at each edge group are shown. Significant *P* values < 0.05 are indicated in bold.

their number of edge distances away from the optimized path (i.e. 1 edge distance away, 2 edge distances away, etc.) (Fig. 1e). Of the ants that made it to the maze, OI ants occupied all five edge groups significantly more than the healthy control group (Fig. 5b, Table 3),

indicating that infected ants were found significantly more off trail than their healthy conspecifics. Although the differences in proportions of OI and OC ants in off trail edge groups were significant, these differences were small (Fig. 5b, Table 3).



**Figure 6.** Foraging rhythms in the arena. Daily activity patterns of *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) ants in the (a) early and (b) late stage of the infection. Daily activity patterns of *Beauveria*-infected (BI) and *Beauveria*-control (BC) ants in the (c) early and (d) late stage of the infection. Time points during the night-time phase are shaded in grey. Rhythmicity *P* values (i.e. significant rhythmicity *P* < 0.05) for each treatment group are indicated. Filled circles represent mean proportions.

### The Effect of Fungal Infection on Foraging Rhythmicity

Within the foraging arena, we found that the activity of the healthy control groups (i.e. OC and BC ants) was significantly rhythmic, both in the early and late stages of the experiment ( $P < 0.001$ ; Fig. 6a and b). In the early stage of the infection experiment, OI ants were also found to be rhythmic in their foraging arena occupation ( $P < 0.001$ ; Fig. 6a). However, as disease progressed to the late stage of the experiment, OI ants seemed to have lost rhythmicity ( $P = 0.128$ ; Fig. 6b). In contrast, BI ants appeared to maintain rhythmicity in the arena in both early and late stages of the infection ( $P = 0.011$  and  $P < 0.001$ , respectively; Fig. 6c and d). However, upon visually inspecting the activity patterns of BI ants, it seemed that BI ants began to display two activity peaks during the late stage of *Beauveria* infection (Fig. 6d). This could indicate a trend towards arrhythmicity in BI ants as well if the infection period had been longer.

### DISCUSSION

While the characteristic biting behaviour of carpenter ants infected with ‘zombie ant fungi’ of the genus *Ophiocordyceps* has been thoroughly studied, the potential fungal effects on other behaviours have not. Previous work on *Ophiocordyceps* suggests that ants become unresponsive to environmental stimuli, wander off the regular foraging trail and lose their daily rhythms in foraging activity. However, these trends are inferred from indirect evidence in which the ants investigated were always in the final manipulated biting and summing phase of the disease (de Bekker et al., 2015; Hughes et al., 2011; Will et al., 2020). To provide more direct evidence, our study aimed to quantify foraging behaviour preceding the characteristic summing and biting behaviour. To better distinguish between unique manipulation effects and general behavioural changes, we compared the foraging behaviours of *Ophiocordyceps*-infected individuals to healthy ants and contrasted this with the behaviour of ants infected with the generalist fungal parasite *B. bassiana*. To quantify foraging behaviours, we used a setup in which ants had to traverse a foraging arena to enter a foraging maze in which there was one optimized route towards a food source.

Our present study demonstrates that the foraging behaviour of *C. floridanus* ants is measurably disrupted when infected with *O. camponoti-floridani*. Most notably, *Ophiocordyceps*-infected ants became more day-active in the foraging arena and eventually lost their daily rhythmicity later in the infection. In contrast, healthy ants maintained rhythmicity in the foraging arena throughout the experiment as they did not typically leave the nest during the daytime, had a foraging peak in the arena when the lights turned off and foraged at a higher intensity during the night-time. The daily rhythmicity in *Beauveria*-infected ants was also not significantly disturbed. Our quantifications of ant foraging activity throughout the day, thus, suggest a disruption of host endogenous clocks in *Ophiocordyceps*-infected ants, but not in *Beauveria*-infected ants. Foraging activity is flexible to adapt to the constant changes of food availability and predators. Yet, performing foraging duties in a rhythmic pattern is important to facilitate the social organization in a eusocial context (Mildner & Roces, 2017; Sharma et al., 2004). Therefore, the disruption of rhythmicity in infected foragers may incur a fitness cost at the individual and colony level. These findings are consistent with the current hypothesis that *Ophiocordyceps* fungi interact with the biological clocks of their ant hosts as a strategy to manipulate behaviour.

Across *Ophiocordyceps* and *Camponotus* species, manipulated biting behaviour appears to be synchronized to a specific time of day outside of the hosts' normal foraging hours (de Bekker et al., 2015; Hughes et al., 2011; Will et al., 2020). Our findings suggest that this disruption of the biological clock may be occurring far ahead of the biting stage since *Ophiocordyceps*-infected ants at 6–8 days postinfection already showed loss of rhythmicity, while biting takes place much later. By affecting the host endogenous clock, *Ophiocordyceps* may be more efficient in infiltrating its host prior to the final manipulation time point. Given that *Ophiocordyceps* has its own endogenous clock (de Bekker et al., 2017), the interruption of the host endogenous clock may enable the fungal parasite to manipulate host behaviours at a time when it is most virulent. Additionally, light manipulation studies in the field with *Ophiocordyceps*-infected *Camponotus atriceps* suggest that incipient light levels are important to fungal fruiting body formation and, thus, spore release (Andrioli et al., 2019). As such, circadian disruption could be adaptive to fungal transmission by assuring that infected individuals are active and bite at a time and location where there are favourable light conditions that benefit fungal development and spore production.

It is, of course, also possible that the arrhythmicity we observed in *Ophiocordyceps*-infected ants is a mere by-product of infection. However, we found that *Beauveria*-infected ants largely maintained rhythmicity throughout disease progression. This suggests that the disruption of host rhythmicity potentially serves as an adaptive manipulation strategy for *Ophiocordyceps* rather than being a general symptom related to fungal infection. Nevertheless, during the late stages of our experiment, while *Beauveria*-infected ants maintained a foraging peak similar to their controls, they appeared to have gained an emerging additional activity peak during the daytime. As such, ants infected with *Beauveria* might have also eventually become arrhythmic, if only the fungus did not kill them so close to initial infection.

In addition, we found that *Ophiocordyceps*-infected ants lost the ability to efficiently forage for food, as indicated by the consistently higher numbers of infected ants in the foraging arena and lower numbers in the foraging maze as compared to healthy controls. Our daily qualitative observations confirmed that *Ophiocordyceps*-infected ants indeed were likely to spend more time in the foraging arena without reaching the maze as we observed that infected individuals climbed in a directionless manner up the slippery walls of the arena, stumbled and fell, then proceeded with this erratic climbing behaviour once again. In contrast, healthy control foragers went directly from their nest to the maze during foraging events. These observations are consistent with the disoriented climbing and falling behaviour described as a random ‘drunkard’s walk’ in *Ophiocordyceps*-infected *C. leonardi* ants (Hughes et al., 2011). Additionally, *Ophiocordyceps*-infected individuals that eventually ended up in the maze were not found on the optimized route to the food source and were further away from the food than their healthy counterparts. This could be due to an inability to optimize or an effect of increased exploratory behaviour. Nevertheless, healthy and infected individuals did not significantly differ in their ability to use the optimized trail to the food source. Although infection concentrations and methods were standardized, it is likely that infected ants progressed through the disease at slightly different speeds and behaved differently depending on their relative infection stage. As such, infected ants that optimized to the food source may simply have not been as far along in disease progression as those that did not optimize their route and never made it into the maze or had moved away from the trail to the food source.



The foraging maze in our experimental set-ups provided ants with minimal visual cues to guide their foraging efforts. The maze was monochrome in colour, with identical angles at each bifurcation. Therefore, ants had to rely mainly on olfaction and communication with nestmates to reach the constant food source. As such, the *Ophiocordyceps*-infected ants that were not able to make it into the maze as well as those that were further away from the food source, might not have been able to find their way due to their inability to detect or process olfactory cues. Indeed, manipulated *C. floridanus* and *C. castaneus* ants that displayed the final biting behaviour at the end of the infection period were dysregulated in genes related to odour perception and communication (de Bekker et al., 2015; Will et al., 2020). Our current study suggests that this dysregulation might already be occurring during the earlier stages of disease progression. Odour detection is central to effective foraging by *Camponotus* ants (Hölldobler & Wilson, 1990). The disruption of odour detection in the host could, therefore, be an effective strategy for *Ophiocordyceps* to lead hosts away from the nest in advance of the eventual summing and biting event. As we observed in the experiments that tested our behavioural set-up, healthy *C. floridanus* ants are aggressive towards their *Ophiocordyceps*-infected nestmates. By assuring that the host wanders away from the nest early on, the parasite may increase its chances of manipulation, which is crucial for transmission (Loreto et al., 2014). However, our data suggest that the potential disruption of olfaction may not be entirely unique to *Ophiocordyceps* infections. We found that *Beauveria*-infected ants were also present in the foraging arena in higher numbers than their healthy controls and showed similar directionless wall-climbing behaviour in the foraging arena. As such, they also seemed to have lost their ability to forage effectively. Indeed, *B. bassiana* has been shown to decrease feeding propensity and olfactory responses in *Anopheles gambiae* (George et al., 2011). However, the shorter experimental time and quickly dwindling number of ants in *Beauveria* experiments, make the data that we obtained difficult to interpret since this could have caused potential acclimation issues and a diminished overall foraging drive. Nevertheless, our findings suggest that the reduced ability to effectively forage, potentially through host olfactory disruption, could be a more general effect of fungal infection that happens to potentially benefit *Ophiocordyceps* transmission.

Direct communication and resulting cooperation with nestmates also seemed diminished in infected individuals. Among the healthy control ants, there was typically a subset of foragers that grouped together and constantly occupied the maze entrance. Ants displayed frequent antennation and trophallaxis, both within this more stationary group as well as with individual foragers entering and exiting the maze. Individual foragers oftentimes travelled to the food source or other sections of the maze after communicating with this group. The foraging behaviour of healthy control ants, thus, seemed like a collective process in which ants communicated and cooperated to retrieve food. This is reminiscent of the group recruitment strategies described for *Camponotus socius* and *Camponotus pennsylvanicus*, which rely on both chemical communication and group recruitment techniques (LeBoeuf et al., 2019). In contrast *Ophiocordyceps*-infected ants did not aggregate at the entrance of the foraging maze. As such, they did not seem to be using the same collective foraging strategy. To fully understand what is occurring, a follow-up study that aims to quantify and compare direct communication in individually-tracked infected and healthy *C. floridanus* is needed. Nevertheless, these findings suggest that diminished foraging efficiency could be partly due to a diminished direct communication with nest mates.

It is also plausible that the reduced foraging in the maze by both *Ophiocordyceps* and *Beauveria*-infected ants is in part caused by

starvation. One way in which insects are found to deal with pathogen infection is through induced starvation (Hsu et al., 2018). Indeed, genes involved in lipid metabolism and genes that are potentially involved in starvation were downregulated in *C. floridanus* during the manipulated biting stage (Will et al., 2020). Moreover, it has been found that starved *Drosophila melanogaster* show enhanced locomotion behaviour (Dietrich et al., 2015; Lee & Park, 2004; Yang et al., 2015; Yu et al., 2016), which is what may be exhibited in the constant, erratic wall climbing we observed in our infected ants. Furthermore, *Ophiocordyceps*-infected *C. floridanus* ants in our study generally died by day 15 postinfection across all experimental replicates. This is earlier compared to a previous laboratory study in which *Ophiocordyceps*-infected *C. floridanus* bit vegetation and died 19–23 days postinfection (Will et al., 2020), and might be due to starvation and desiccation. One stark difference with this previously published study is that we kept healthy and infected ants in separate foraging set-ups, whereas the previous study combined them (Will et al., 2020). It is possible that interactions with healthy conspecifics may elongate the life span of infected ants as *C. castaneus* was observed to share food with infected nestmates through trophallaxis (Solá Gracia et al., 2018). Our study found that infected ants, kept with only other infected individuals, were less capable of reaching the sugar and water source. This makes it entirely possible that desiccation and starvation, in combination with infection effects, shortened their life span and induced the enhanced locomotion activity that we observed.

## Conclusions

This study provides the first direct evidence for changes in foraging-related behaviours that lead up to the characteristic biting behaviour in ants, infected by the ‘zombie-making fungus’ *Ophiocordyceps*. We show that *Ophiocordyceps*-infected ants are less able to participate in foraging efforts to a constant food source, become arrhythmic throughout disease progression, display wandering behaviour, and are less able to communicate with their nestmates. In addition, we found that, in a large colony context, healthy *C. floridanus* nestmates show almost immediate aggression towards *Ophiocordyceps*-infected ants. Therefore, host disorientation may be adaptive to the parasite by reducing the chance of aggressive interventions by nestmates prior to transmission. Additionally, interference with the host’s biological clock could facilitate the seeking of a location with light levels that promote fruiting body growth and, thus, transmission. While perhaps adaptive to *Ophiocordyceps*, our infection studies with the nonmanipulating fungus *B. bassiana* suggest that these behavioural changes could extend to other fungal infections as well. Indeed, the billions of years of host-parasite coevolution across diverse species interactions have resulted in the selection for convergent strategies (Chetouhi et al., 2015). As such, investigating the extended phenotype of *Ophiocordyceps* in its ant host throughout disease progression could further our understanding of the behavioural effects of fungal disease in parallel systems. Therefore, this study is not only informing future work on parasitic strategies underlying host manipulation, but also fungal-insect, and other host-parasite interactions in general.

## Author Contributions

The study and experimental design were conceived by T.T. and C.d.B. T.T. performed all experiments, collected video recordings and performed personal observations. Data collections from video recordings were performed by T.T. and R.O. T.T. and C.d.B. analysed the data and wrote the manuscript.

## Declaration of Interests

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.2021.09.003>.

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## Appendix 1

**Table A1**

Parameter estimates of the ZOIB comparing proportions of ants in arena versus maze in *Ophiocordyceps*-infected (OI) and *Ophiocordyceps*-control (OC) ants and in *Beauveria*-infected (BI) and *Beauveria*-control (BC) ants

Covariate	Model for nonzero data ( $\mu$ )				Model for zero data ( $v$ )			
	Estimate	SE	<i>t</i>	<i>P</i>	Estimate	SE	<i>t</i>	<i>P</i>
Proportions of OI and OC ants in arena vs maze								
Location (L)	1.477	0.032	46.733	<b>&lt;0.001</b>	−1.022	0.078	−13.157	<b>&lt;0.001</b>
Treatment (T)	0.7	0.032	22.187	<b>&lt;0.001</b>	−2.015	0.09	−22.349	<b>&lt;0.001</b>
L*T	−1.617	0.042	−38.509	<b>&lt;0.001</b>	2.875	0.12	23.909	<b>&lt;0.001</b>
Proportions of BI and BC ants in arena vs maze								
Location (L)	0.217	0.057	3.798	<b>0.001</b>	−0.216	0.113	−1.914	0.056
Treatment (T)	0.542	0.053	10.24	<b>&lt;0.001</b>	−0.984	0.122	−8.098	<b>&lt;0.001</b>
L*T	−0.605	0.079	−7.679	<b>&lt;0.001</b>	1.607	0.168	9.561	<b>&lt;0.001</b>

ZOIB: zero-one inflated beta models.  $R^2$  values: model comparing OI and OC ants = 0.48; model comparing BI and BC ants = 0.16. Significant *P* values < 0.05 are indicated in bold.

## Appendix 2. Experimental Verification

### Methods

To test our behavioural set-up for its ability to quantify foraging behaviours, we used one large, queenright colony of >2000 workers that we refer to as Colony A. This colony was collected from Chuluota Wilderness Area (28°22'23"N, 80°2'13"W) in August 2018. To evaluate the feasibility of quantifying changes in ant behaviour upon the infection of ants kept within a whole colony context, we used two separate large colonies of >2000 workers that we refer to as Colonies B and C. Colony B was queenright, whereas Colony C was not. These colonies were collected from the University of Central Florida Arboretum (28°35'28"N, 81°11'17"W) in September 2019.

Since the aim of this study was to investigate potential changes in foraging behaviour after infection, it was important to only include foragers in our infection experiments. To identify whether colony foragers consistently returned to the foraging arena while other colony members remained in the nest, we used a mark-and-recapture approach in the partial colony foraging set-up (Fig. 1b)

with Colony A. In this approach, we considered all ants in the foraging arena to be foragers and marked them with paint. We conducted painting trials for three consecutive days at ZT 12 after lights turned off in the incubator (i.e. the onset of night-time and presumed foraging peak). On the first day, we marked the abdomens of all ants that showed up in the foraging arena in the partial colony foraging set-up with a dot of purple acrylic paint (POSCA). These ants were classified as 'newly marked foragers' (Fig. A4). On the second marking day, we repeated the same procedure. We marked the abdomens of unpainted foragers with purple acrylic paint and classified them again as newly marked foragers. The ants that were previously marked with purple, now received a dot of green acrylic paint (POSCA) on their abdomen. These ants were classified as '2-day foragers', meaning that they had returned to the arena two nights in a row. On the third marking day, we repeated the same procedure from the second marking day. Additionally, the ants previously marked with green, were now marked with a dot of white acrylic paint. These ants were classified as '3-day foragers', meaning that they had returned to the arena three times (Fig. A4). This approach allowed us to identify whether ants consistently returned to the foraging locations in the experimental set-up over three consecutive days. As such, these experiments tested the functionality of our experimental set-ups in allowing us to identify recurrent foragers. We performed this mark-and-recapture method twice on Colony A.

To identify whether the complex set-ups that we designed allowed us to observe and quantify whole colony behaviour, we also tracked the behaviour of Colony A in the whole colony foraging set-up (Fig. 1c). To record colony activity patterns in the arena and maze of the whole colony complex foraging set-up, we mounted two infrared lights and two infrared-enabled GoPro Hero 6 cameras above the arena and maze to allow for day-and night-time recordings (Fig. 1c). We recorded 60 s time lapse videos (i.e. every hour in real time) and then scored the number of ants in both locations every 60 frames for three days. This resulted in 72 time points per foraging location (i.e. arena and maze). In addition to counts, we also collected location data within the maze at every 60th frame. To collect location data, we scored the number of ants on each maze section based on the maze section ID (Fig. 1e).

In addition, we evaluated the feasibility of quantifying changes in ant behavior upon the infection of ants kept within a whole colony context. Using Colonies B and C, we selected foragers and performed infection assays by ways of injecting with fungal blastospores ( $3.460 \times 10^7$  cells/ml). To differentiate between infected ants and other nestmates once we reintroduced them to the rest of the colony, we marked the remaining *Ophiocordyceps*-infected ants



(OI) with a dot of black paint on their thoraxes and a dot of white paint on their abdomens (Testors). We then returned OI to the colony by releasing them in the foraging arena of the experimental set-up.

## Results

After acclimation and entrainment, we found that nurses, brood, and the queen in Colony A housed in the designated nest area whereas foragers freely searched for food outside of the nest in the foraging arena in the partial colony set-up (Fig. 1b). To investigate if these foragers were largely reoccurring, which would indicate that the set-up allowed for the establishment of robust behavioural castes, we performed mark-recapture experiments in which we marked foragers for 3 consecutive days at ZT 12 (i.e. the onset of night-time and presumed foraging peak) (Supplementary material 2, Data set 2). We found that the number of foragers in the foraging arena was consistent across data collection days and that the group largely consisted of recurring individuals (first trial: 38% after the first day on day 2, 60% after the second day on day 3; Second trial: 83% after the first day on day 2, 91% after the second day on day 3) (Fig. A4). We also found that the percentage of minors (i.e. smaller physiological caste) and majors (i.e. larger physiological caste) in the foraging arena during the presumed foraging peak was consistent within each marking trial (first trial: 64–69% minors; second trial: 82–84% minors) (Fig. A4). As such, these results suggest that the partial colony set-up (Fig. 1b) indeed allowed for the identification of recurrent foragers, to later sample for infection treatments.

When assessing the overall foraging activity in both the arena and maze combined in the whole colony foraging set-up (Fig. 1c), we found that scoring the time-lapse videos at 60-minute intervals (i.e. 24 frames per day for 3 days) was enough resolution to capture foraging peak and rhythmic foraging patterns. As such, we found a visible activity peak at ZT 12 in Colony A (Fig. A5) (Supplementary material 3, Data set 3). This confirmed that the foraging caste showed nocturnal foraging activity in our experimental set-up as they would in nature. However, when we analysed activity in the arena and maze separately (Fig. A5), we found that foraging rhythmicity was most pronounced in the foraging arena with less obvious rhythmicity in the maze. This data indicates that foragers indeed establish measurable rhythmic foraging patterns in our whole colony complex foraging set-up, with a foraging peak at ZT12, when the lights turned off (Fig. A5). Yet, the data obtained from the foraging arena and the foraging maze might warrant separate analysis.

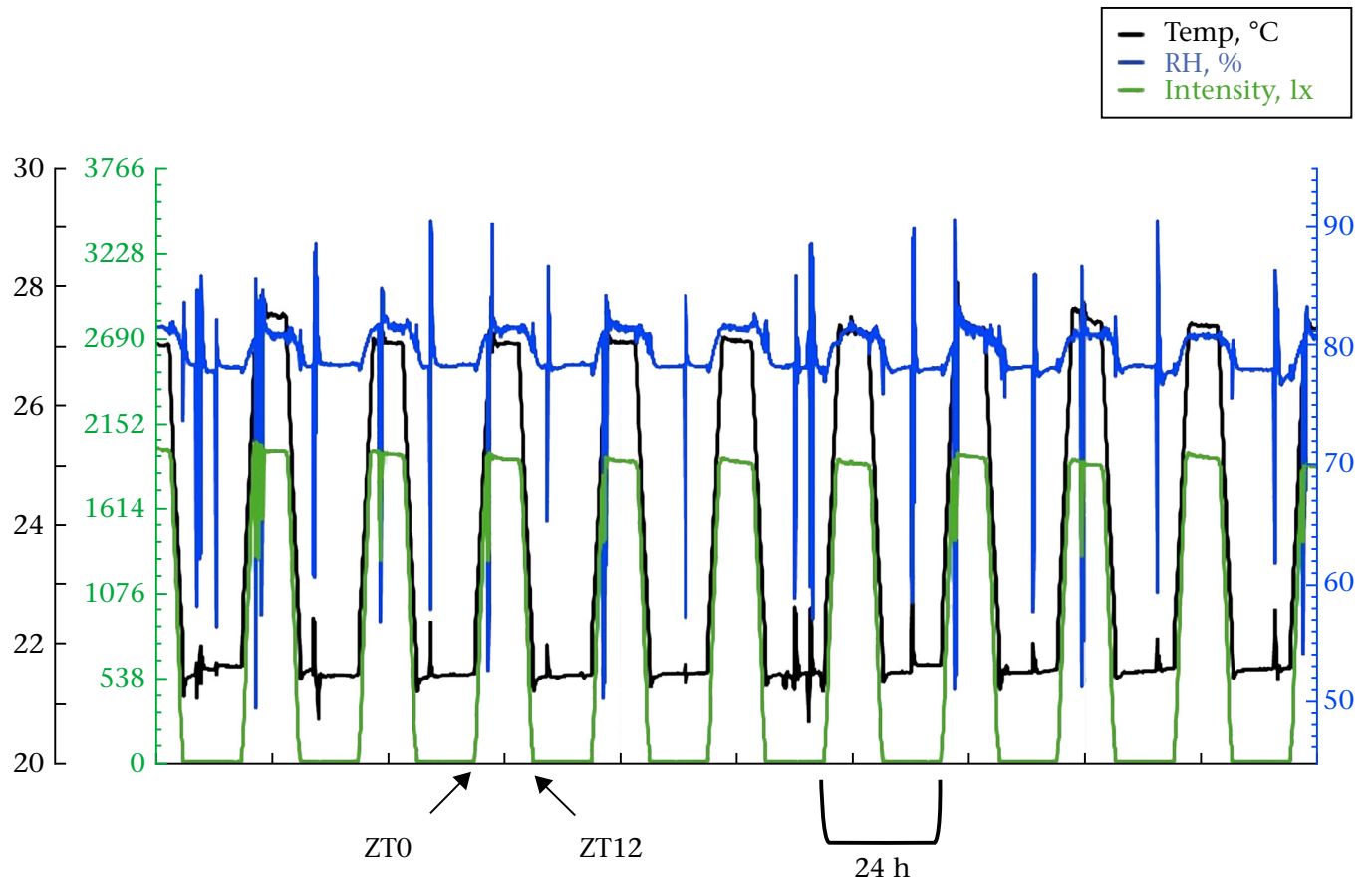
The location data we collected from the large *C. floridanus* colony (i.e. Colony A) that we housed in our complex foraging set-up (Fig. 1c) demonstrated that the 3D-printed foraging maze would indeed allow us to measure trail optimization to a stable food

source. The absolute number of ants on edges that represented the shortest route to the food (i.e. BB, CC, DD, EE, FF; Fig. 1e) was significantly greater than the absolute number of ants on the edges outside of the optimized trail (Mann–Whitney *U* test:  $W = 91.5$ ,  $N_1 = 15$ ,  $N_2 = 69$ ,  $P < 0.001$ ; Fig. A6). The maze entrance (i.e. AA) was the most occupied edge (Fig. A6). At this entrance edge, we observed a subset of foragers that constantly grouped together and occupied this edge throughout the three days of data collection (Video S1). We noticed frequent antennation and trophallaxis, both between ants within this group as well as with individual foragers entering and exiting the maze. This cooperation and constant communication seem to be a part of the foraging strategy of *C. floridanus*. This suggests that these ants were playing an active role in the foraging activities of the colony. Since ants were not provided with an individual ID, we were not able to determine the turnover rate of ants in this group and whether the same individuals were present at AA throughout the entirety of the experiment. As such, this persistent number of ants in the maze entrance likely affected the amplitude of measurable foraging activity, making rhythmicity less apparent.

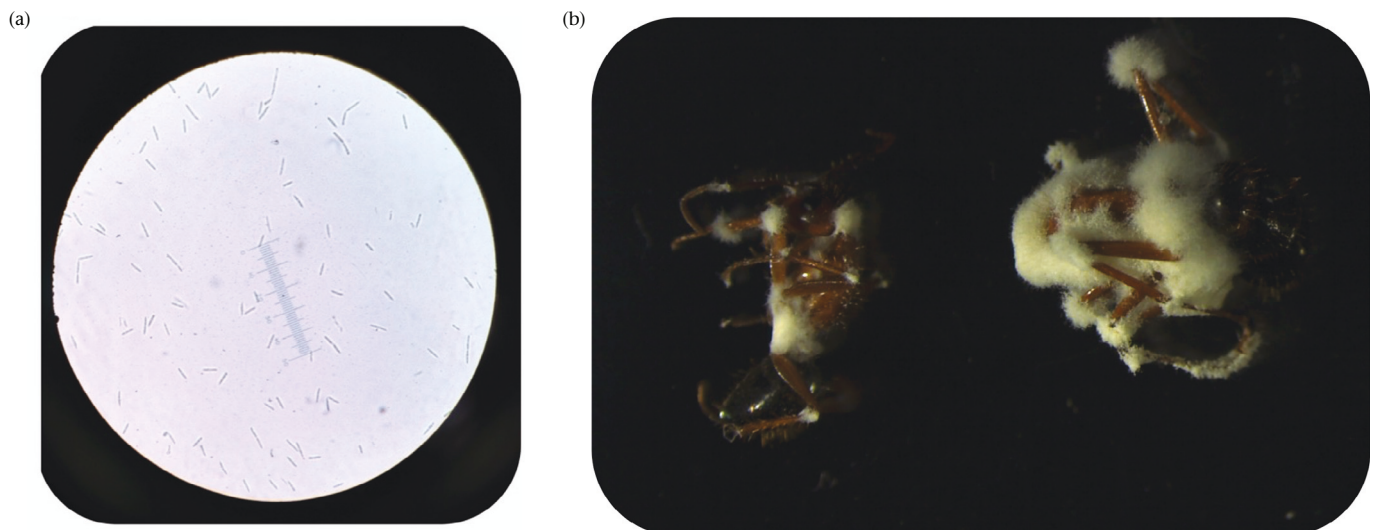
While our experimental set-up appeared to function well to identify foragers and quantify foraging activity and location, the large colony context caused issues when we began to infect individuals. When we infected individual ants with *Ophiocordyceps* and reintroduced them to the colony, we found that healthy nestmates displayed immediate aggression towards infected conspecifics. This interfered with our objective to quantify the potential changes in foraging behaviour of OI ants. In both the infection trials that we performed, and across the two large colonies that we tested (i.e. Colonies B and C), we observed that OI ants were immediately attacked by healthy nestmates upon their return to the foraging arena.

We observed nestmates working together to rip apart and carry OI ants into their nest area. This aggression behaviour against treated individuals interfered with our ability to (1) reliably record survival without having to disturb the workers inside the nest daily, which would cause stress and affect behaviour, and (2) quantify the behaviour of a large number of infected individuals for analyses. Without an accurate daily count of ant deaths, and a reasonable number of infected individuals to record, we would not be able to draw strong conclusions about the effects of infection on foraging behaviour. This immediate, strong aggression by healthy individuals against infected nestmates was not seen in previous infection experiments with colony fragments (de Bekker et al., 2015; Solá Gracia et al., 2018; Will et al., 2020). As such, this result compelled us to perform our experiments with the behavioural set-up that we designed but using separate colony fragments for the control and infected treatment groups instead of large colonies.

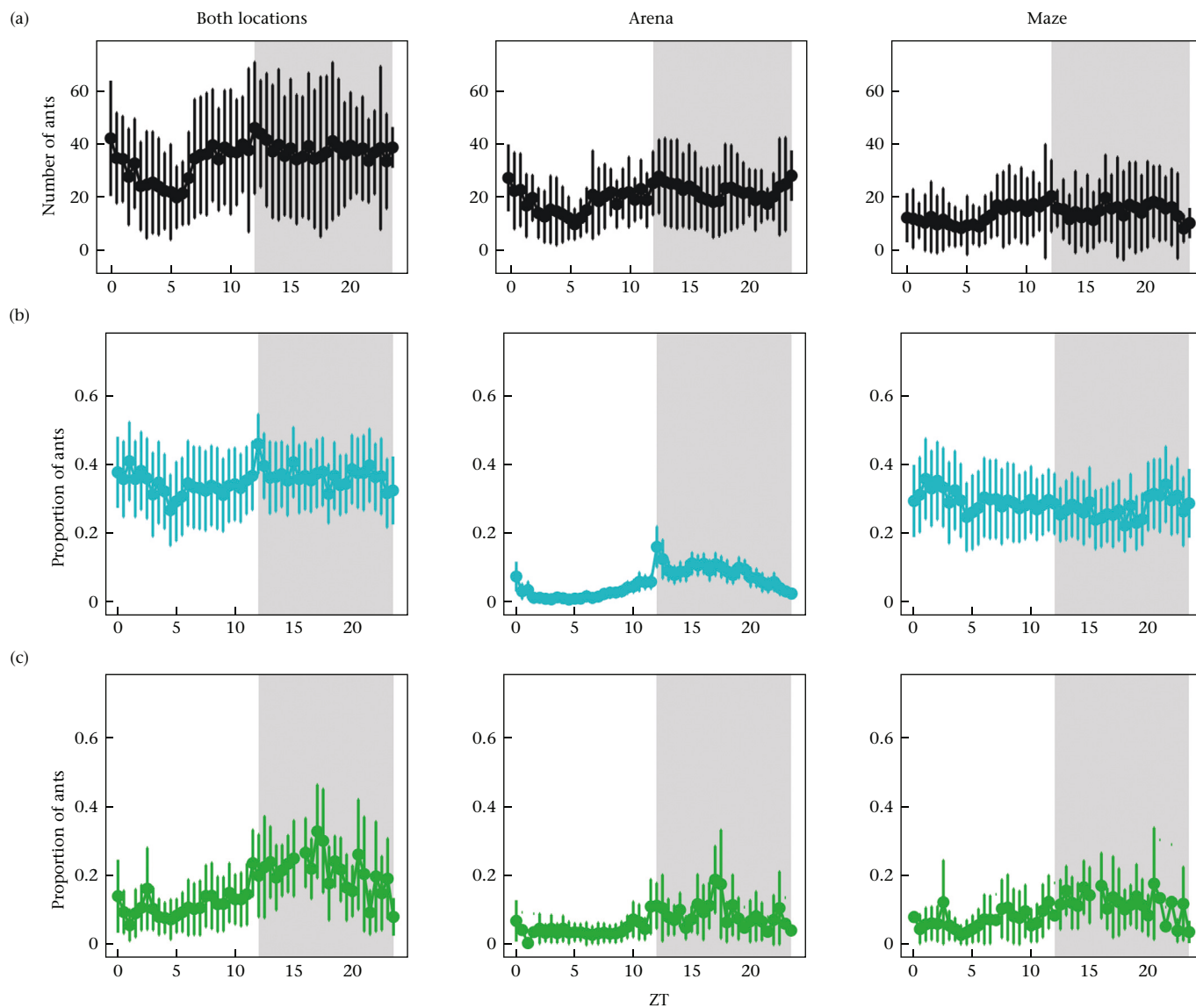




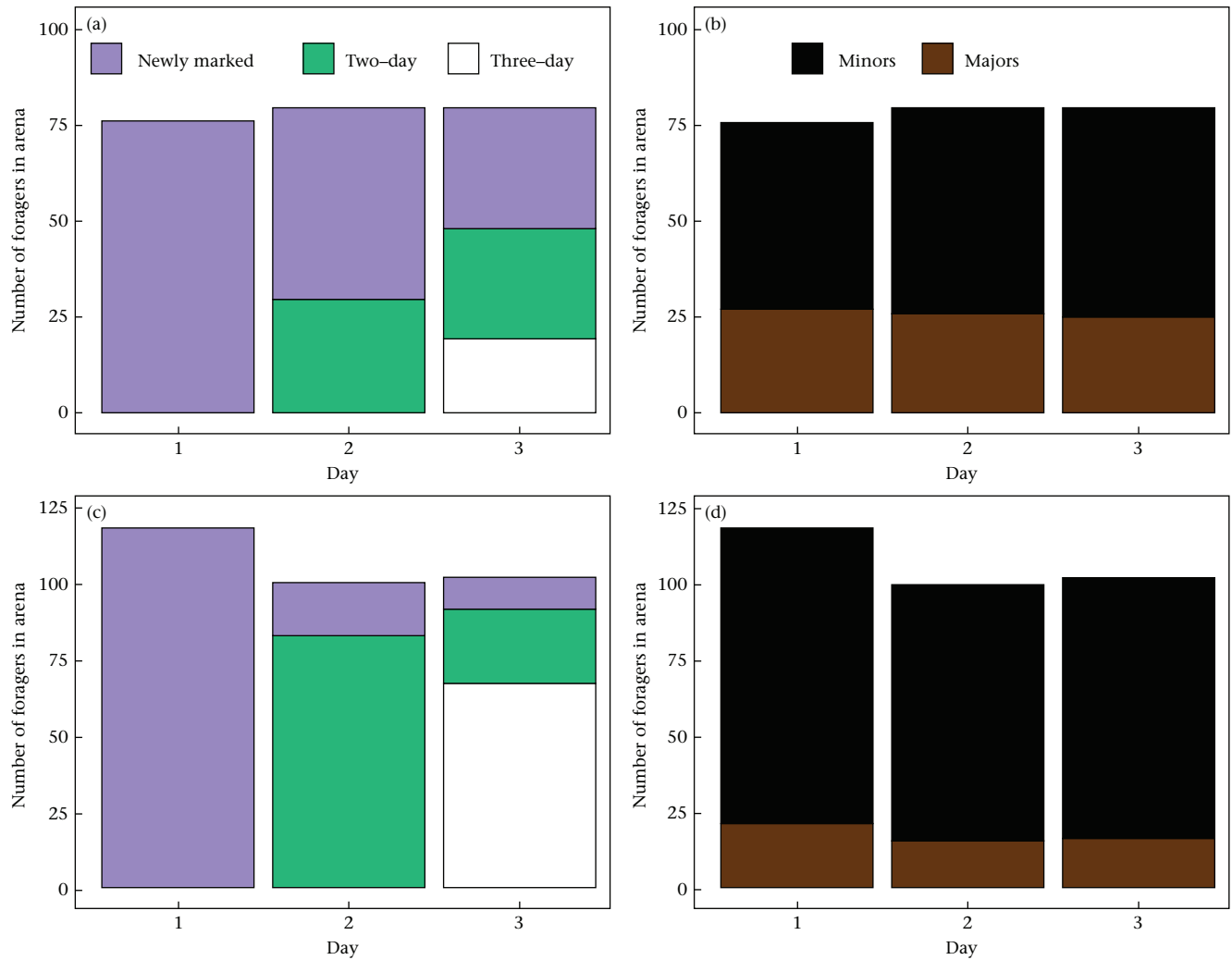
**Figure A1.** Zeitgeber time, ZT (LD 12:12 h cycle), and temperature cycle. Temperature, relative humidity and light intensity were validated with a HOBO data logger (model U12, Onset).



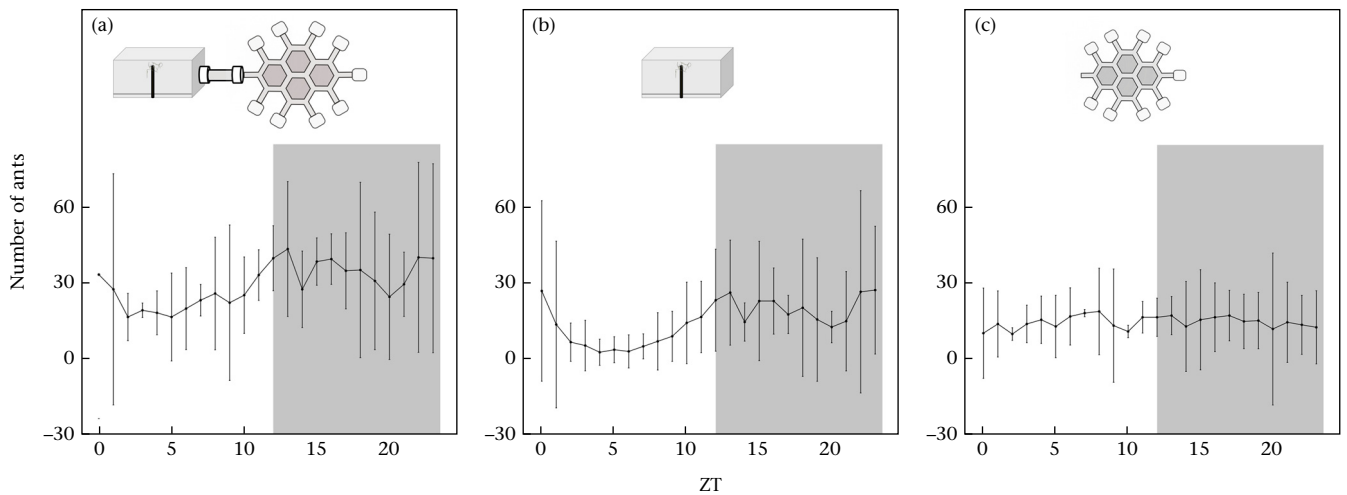
**Figure A2.** Confirmation of *Ophiocordyceps* infection and *Beauveria* infection. (a) *Ophiocordyceps camponoti-floridani* blastospores in the abdomens of expired *Ophiocordyceps*-infected (OI) individuals. (b) *Beauveria bassiana* growth from the body and leg segments of *Beauveria*-infected (BI) ant cadavers.



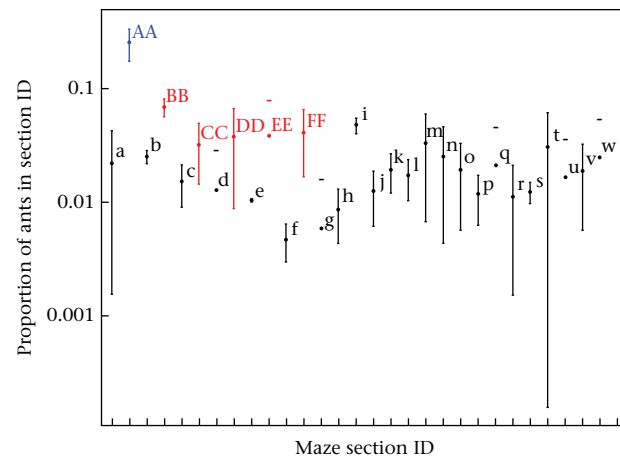
**Figure A3.** Fragmented, healthy control group versus whole colony foraging patterns. (a) Whole colonies were monitored for 2 days, (b) *Ophiocordyceps*-control (OC) fragments were monitored for 12–15 days and (c) *Beauveria*-control (BC) fragments were monitored for 5 days. Absolute counts are shown for whole colonies. Foraging proportions (ants scored/total ants in experiment) are shown for OC and BC.



**Figure A4.** Mark–recapture trials to identify recurrent foragers. Graphs show foragers based on (a) how recurrent they were during the 3 days (newly marked = purple, two-day = green, and three-day = white) and (b) whether they were from the minor or major physiological castes (black = minors; brown = majors). We repeated this experiment twice on Colony A. The top and bottom rows represent each iteration of the mark–recapture procedure.



**Figure A5.** Foraging patterns in (a) both the arena and maze, (b) the arena and (c) the maze separately. Foraging locations were scored every hour for three consecutive days. Time points during the night-time phase are shaded in grey. Filled-in circles represent the mean number of ants scored and bars represent 95% confidence intervals.



**Figure A6.** Whole colony optimization patterns. *Camponotus floridanus* ants were able to optimize in the foraging maze. The figure shows the number of ants at a certain section ID as a proportion of the total number of ants in the maze per day. Filled-in circles represent the mean number of ants scored and bars represent 95% confidence intervals. The absolute counts of ants on each of the edges belonging to the optimized path (i.e. BB, CC, DD, EE, FF, red data points) were greater than the absolute counts of ants located outside of the optimized path (black data points) (Mann–Whitney *U* test:  $P < 0.001$ ). The maze entrance (i.e. AA, blue data point) was the most occupied edge.