

INVITED REVIEW

Hijacking time: How *Ophiocordyceps* fungi could be using ant host clocks to manipulate behavior

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Abstracts

Ophiocordyceps fungi manipulate ant behaviour as a transmission strategy. Conspicuous changes in the daily timing of disease phenotypes suggest that *Ophiocordyceps* and other manipulators could be hijacking the host clock. We discuss the available data that support the notion that *Ophiocordyceps* fungi could be hijacking ant host clocks and consider how altering daily behavioural rhythms could benefit the fungal infection cycle. By reviewing time-course transcriptomics data for the parasite and the host, we argue that *Ophiocordyceps* has a light-entrainable clock that might drive daily expression of candidate manipulation genes. Moreover, ant rhythms are seemingly highly plastic and involved in behavioural division of labour, which could make them susceptible to parasite hijacking. To provisionally test whether the expression of ant behavioural plasticity and rhythmicity genes could be affected by fungal manipulation, we performed a gene co-expression network analysis on ant time-course data and linked it to available behavioural manipulation data. We found that behavioural plasticity genes reside in the same modules as those affected during fungal manipulation. These modules showed significant connectivity with rhythmic gene modules, suggesting that *Ophiocordyceps* could be indirectly affecting the expression of those genes as well.

KEYWORDS

behavioural plasticity, circadian plasticity, entomopathogens, infectious disease, Zombie ants

1 | INTRODUCTION

Parasitism is one of the most successful organismic interactions on the planet.^{1,2} Virtually, all organisms are plagued by other living entities in the form of viruses, prokaryotes or eukaryotes. Through co-evolution, parasites have evolved a myriad of strategies to invade their hosts, exploit their resources and physiology and transmit effectively. In certain cases, this has given rise to complex parasite traits that involve the hijacking of host behaviour to facilitate parasite survival and transmission. The carpenter ants that are infected by *Ophiocordyceps* fungi represent one of the most famous examples. Myrmecophile *Ophiocordyceps* species break into the behavioural machinery of the ants that they infect, take the reins

and induce precise, stereotypical behaviours that result in their distancing from the ant colony and attachment at vantage points that promote fungal development and transmission.^{3–5} These bizarre *Ophiocordyceps*–ant interactions are certainly not unique. Many other bodysnatching fungi have been discovered so far,^{6–8} as well as viruses, protozoans, worms and insects that target both invertebrate and vertebrate hosts.^{9–15} Despite the relative ubiquity of bodysnatching parasites, our knowledge of the mechanisms that underlie parasitic hijacking of host behaviour is limited. Efforts are underway to expose these mechanisms in various emerging parasite–host model systems. The focus of these efforts largely centres around the discovery of the candidate parasite neuromodulating compounds involved and the potential host tissues that are

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mechanically (i.e., tissue invasion and degradation) and chemically (i.e., bioamine imbalances) affected.^{16–24} While these are excellent avenues towards unveiling some of the complex mechanisms that underlie the parasitic manipulation of host behaviour, they neglect the exquisite daily timing with which manipulated behavioural phenotypes have been reported to present themselves.^{21,23,25–31} As such, they do not consider the role of biological clocks in parasite–host interactions and their potential involvement in establishing behavioural manipulations.

Biological rhythms have extensive fitness impacts as they coordinate the physiology and behaviour of living organisms with predictable environmental rhythms. The same is true for parasites and their hosts. Host responses to infection exhibit clock-driven daily rhythms^{32,33}; Innate immune defences, such as inflammation, peak during an organism's active phase while repair mechanisms peak during the resting phase.^{34–38} In turn, the within-host survival, development and transmission strategies of parasites, as well as their metabolism, appear to be under circadian control.^{39–42} As such, rhythms exhibited as part of pathogen–host interactions after infection could benefit either, both or neither of the antagonistic parties involved.⁴³ Parasite-adaptive manipulations of host behaviour also appear to exhibit conspicuous rhythms, which gave rise to the hypothesis that manipulation of host behaviour is, at least in part, achieved through parasitic hijacking of behavioural outputs that are driven by biological clocks.^{8,44–47} This review discusses the current evidence for the involvement of biological clocks in the hijacking of carpenter ant behaviours by *Ophiocordyceps* fungi. We additionally address the potential fitness effects of specifically timing altered behaviours and the premises for parasitic hijacking of the host clock. We follow this assessment with a meta-analysis that links transcriptomics data on the behavioural manipulation of *Ophiocordyceps*-infected carpenter ants with data that explore carpenter ant behavioural plasticity through the lens of rhythmic gene expression. By conducting a weighted gene co-expression network analysis (WGCNA),⁴⁸ we identify modules of highly co-expressed genes that support the notion that the expression of carpenter ant genes that allow for behavioural plasticity and rhythmicity is corruptible by *Ophiocordyceps*. As such, we aim to inspire future studies on parasitic manipulation of host behaviour to record potential parasite-adaptive rhythms in model systems beyond the ones mentioned here, as well as a deeper exploration of the underlying rhythms in gene expression and protein levels to provide new, more detailed hypotheses about the clock-related molecular mechanisms that could underlie them.

2 | CURRENT EVIDENCE FOR THE HIJACKING OF ANT BEHAVIOURAL RHYTHMS BY *OPHIOCORDYCEPS*

Parasite hijacking of host behaviour, in which the modified host's behaviour increases parasite fitness and serves as an extended phenotype,⁴⁹ has been discovered for a wide variety of taxa. Nevertheless,

detailed studies investigating the interactions between both parties only exist for a handful of manipulation systems. This is likely because most of the organisms involved in manipulative interactions do not represent classic model systems with readily available molecular tools, genomes and culturing/rearing protocols. Nevertheless, detailed field studies and natural history observations have provided intriguing hypotheses about the manipulation strategies of parasitic hijackers and how they would corrupt their hosts. One example is the conspicuous synchronized timing of host behavioural phenotypes that are crucial to the parasite's life cycle, as well as the loss of the host's regular daily activity patterns. To name a few, *Ophiocordyceps* and *Entomophthora* fungi infecting ants and flies, respectively, *Dicrocoelium* trematodes infecting ants, and baculoviruses infecting caterpillars all give rise to phenotypes in which daily rhythms are seemingly disrupted or altered.^{21,23,25–31} These observations have resulted in the hypothesis that these behaviour-hijacking parasites affect the clock of their hosts in some capacity.

Samples of dead, *Ophiocordyceps*-infected ants have been collected from tropical and temperate forests all over the globe.^{50–55} These biodiversity studies so far suggest that every ant species that is parasitized by a unique *Ophiocordyceps* species has specialized to infect that host. When a given species interaction results in an elevated death position (i.e., the summing position), or when its fungus-sprouting cadavers are found in areas outside of the ants' general ecological niche,^{53,55} this suggests that the fungal infection has caused the ant to end up there. While manipulation of host behaviour does not occur for some *Ophiocordyceps*-ant interactions, most species pairs seem to end up in elevated positions, hanging onto plant tissues with the ant's mandibles and legs. This final position is determined to be the result of fungal manipulation of ant behaviour, and thus an extended phenotype of the fungus, because (1) the biting behaviour that attaches the ant upon summing is not part of the ant's regular behavioural repertoire and (2) the elevated position of the cadaver benefits fungal spore transmission.⁵⁶

Field and lab studies that noted the timing of the summing and biting behaviour of infected individuals found that these manipulation phenotypes are synchronized to a certain time of day. A carefully conducted field study in Thailand noted that ants of the canopy-dwelling species *Camponotus Leonardi* ascend and bite understory vegetation around solar noon when infected with *Ophiocordyceps camponoti-leonardi*.²⁵ This is in line with opportunistic field observations made in Florida, USA, for *Ophiocordyceps camponoti-floridanus* infections in the local *Camponotus floridanus* ant.²⁰ Nevertheless, when this species pair was moved into the laboratory for more detailed behavioural studies, the summing behaviour remained synchronized but shifted to take place in the early morning before the onset of light.²⁰ A similar synchronized shift was observed in lab studies with another species pair (i.e., *Camponotus castaneus* and *Ophiocordyceps kimflemingiae* from South Carolina, USA).^{22,23} Environmental conditions experienced by organisms in nature that serve as daily entrainment cues are difficult to exactly replicate in the laboratory. Despite our best efforts, the provided daily fluctuations in light, temperature and humidity levels are bound to be

different, which could be causing the phase shifts observed in the laboratory. However, these synchronized phase shifts suggest that the timing of manipulated summing in *Ophiocordyceps*-infected carpenter ants occurs with a daily rhythm that is synchronized by those environmental cues.

Not only manipulated summing behaviour but also host death and eventual spore release exhibit diurnal patterns. Manipulated individuals succumb to their infection within a predictable amount of time after manipulation^{20,23,25} as *Ophiocordyceps* switches from parasitic to saprophytic growth, quickly consuming the host's innards to begin stalk, and eventually, fruiting body formation. Studies that recorded the time of death for insect hosts infected by *Entomophthorales* fungi also reported such diurnal patterns.^{21,30,31,57,58} Moreover, field-collected ant cadavers with mature *Ophiocordyceps* fruiting bodies appeared to only release their spores sometime between dusk and dawn (personal observations). This is again consistent with the timing of spore release found for various other insect infecting fungi,^{59–63} including those that are known to also induce a timed summing behaviour in their hosts.^{21,64} What mechanisms drive these temporal aspects of infection and transmission are currently unclear but are probably either circadian outputs, products of entrainment to environmental conditions or simply light-regulated.⁶⁵ Regardless of the mechanisms, the fact that these temporal aspects are seemingly conserved and have convergently evolved in insect infecting fungi across phyla indicates that they would have measurable fitness effects. It has been proposed that spore release during the night-time would ensure more favourable conditions for spore survival and germination due to lower temperatures and higher humidity levels.⁶³ Moreover, in the case of *Ophiocordyceps* spores, which are thin-walled and lacking in melanin, release during the night-time would protect them from damaging UV rays and desiccation.⁶⁶ Furthermore, the spores of insect infecting fungi are said to be short-lived.^{62,66,67} This would make it pertinent that spores are released at a time when hosts are present or soon to be present. Indeed, the carpenter ant species that have been found to be infected with *Ophiocordyceps* so far generally forage during the night-time (e.g. *C. castaneus*), while abundant day-active species that reside in the same habitat (e.g., *Camponotus pennsylvanicus*) remain seemingly unaffected by *Ophiocordyceps*.²²

In addition to the conspicuous timing of *Ophiocordyceps*-induced summing behaviour being an indicator that the fungal parasite could co-opt and manipulate ant daily rhythms, maintaining 24-h dark-light cycles under laboratory conditions appeared to be a crucial factor in successful infection and manipulation studies. Our infection studies that failed to incorporate these cycles or that had them accidentally interrupted failed to result in any behavioural manipulations.⁴⁴ Moreover, infection studies with *O. camponoti-floridani* and its host *C. floridanus* demonstrated that infected individuals lose their daily rhythms during the incubation stages prior to manipulated summing. Instead of showing a heightened foraging activity during the night-time, like their healthy sisters, they demonstrated a constant, high extranidal activity throughout the day.⁶⁸ While perturbation of daily activity rhythms could also be a

hallmark of infectious disease in general, ants infected with the non-manipulating generalist fungus *Beauveria bassiana* did not demonstrate significant changes in daily oscillations.⁶⁸

Further evidence for the proposal that biological clocks play a role in synchronized biting and the disruption of foraging behaviour has been provided by two independent transcriptomics analyses.^{20,23} Both these studies found that the core clock gene, *circadian locomotor output cycles kaput (clk)*, was significantly down-regulated in manipulated carpenter ants of the species *C. castaneus* and *C. floridanus* as compared to their time-matched healthy conspecifics. Homologs of the clock-controlled gene *takeout (to)*, which encodes for a protein that interacts with juvenile hormone (JH) and is thought to be involved in insect foraging and feeding behaviours,^{69,70} was similarly down-regulated. Moreover, TO has been proposed as a potential target for baculovirus disruption of caterpillar behaviour and locomotor activity.⁴⁶ In this parasite manipulation of host behaviour model, viral protein tyrosine phosphatase (PTP) of *Bombyx mori* nucleopolyhedrovirus and *Autographa californica* nucleopolyhedrovirus induced and enhanced locomotion activity in its larval hosts *B. mori* and *Spodoptera exigua*.^{16,71} This protein has been suggested to potentially affect clock-mediated signalling pathways through the dephosphorylation of PTP targets in the host, which in turn affect levels of TO and JH, cascading into altered neuropeptides and neurotransmitters that lead to changes in host behaviours.⁴⁶ Myrmecophile *Ophiocordyceps* species appear to have several genes encoding for *ptp* of which some were found to be up-regulated during manipulated summing and biting behaviour in *O. camponoti-floridani* and *O. kimflemingiae*.^{20,23} Moreover, locomotion activity is heightened in *Ophiocordyceps*-infected individuals prior to summing.^{20,23,68} As such, a clock-mediated signalling pathway as proposed for certain behaviour-hijacking baculoviruses, involving PTP and TO, could potentially be underlying some of the observed changes in host behaviour caused by *Ophiocordyceps* as well.

3 | THE FITNESS EFFECTS OF TIMED SUMMING BEHAVIOURS

Why would infected ants summit around solar noon? What are the fitness effects, and which environmental cues mainly drive this behavioural synchronization? There is some evidence that light is involved in the answers to both these questions. Two independent field studies, again across different *Ophiocordyceps*-ant species pairs, suggest that infected individuals orient themselves and move towards certain incipient light levels in their final manipulated hours prior to death. These studies have been conducted in areas with high-density aggregations of *Ophiocordyceps*-infected ants, so-called graveyards.⁷² *Dolichoderus thoracicus* ants that were infected with *Ophiocordyceps pseudolloydii* in such graveyards in Taiwan were found in death positions in which the orientation of the ants' heads was correlated with the direction of sunlight falling through the present canopy openings.⁷³ To study the effect of light on ant cadaver positioning of *Camponotus atriceps* infected with *Ophiocordyceps*



camponoti-atricipi within graveyards in the Amazonian rainforest in Brazil, researchers altered light levels in identified graveyards with shading cloth. After reducing light levels in one half of the studied graveyards, significantly more *O. camponoti-atricipis*-infected ants latched onto vegetation in the half that was left unshaded to serve as control areas, demonstrating a certain preference for those light levels. Moreover, ants that did latch onto vegetation underneath the shading cloths often did so at the edges, where more light would have been present during sunup and sundown. Furthermore, the ant cadavers underneath the shading cloth had climbed significantly higher than in the areas in which incipient light was unaltered, suggesting that phototaxis played a role and they were 'seeking' higher light levels.⁷⁴ Both these studies indicate that light plays a role in establishing the manipulation phenotypes that determine the final position of *Ophiocordyceps*-infected ants before the fungus kills them.

It appears that the final positioning of the soon-to-be cadaver is important for fruiting body formation while infected *C. atriceps* that positioned themselves underneath the shading cloth produced a fruiting body significantly less often than those that had summited in the control areas where incipient light was left unaltered.⁷⁴ This is in line with previous work that suggested that *Ophiocordyceps*-manipulated ant behaviour would cause the host to die in a location that provides a microclimate for the fungus to thrive and develop.⁴ As such, timing the manipulated summing behaviour around solar noon and pairing it with phototaxis could be a fungal strategy to increase the chance that the ant host dies at a position that experiences abiotic factors, which benefit fungal growth and fruiting body development. The development of the stalk and fruiting body is necessary for fungal spore production and transmission, which does not occur when the manipulation is unsuccessful, and the ant dies elsewhere.⁷⁵ Moreover, ants display aggression towards sick individuals, eventually killing and removing them as part of their social immunity,⁷⁶ which has also been observed for *Ophiocordyceps* infections in *C. floridanus*.^{20,68} Many carpenter ant species, including *C. floridanus*, are nocturnal; their foragers leave the nest to search for food during the night-time.^{68,77} Assuring that infected individuals wander away from the nest and summit during hours that are outside of the activity peak of healthy nest mates could, therefore, be another way for the fungus to increase its transmission chances.

4 | PREMISES FOR *OPHIOCORDYCEPS* HIJACKING OF THE ANT CLOCK

The consistent daily timing of summing and biting behaviours, as well as the dysregulation of certain clock genes in ants infected with *Ophiocordyceps*, suggest that the host circadian clock is a likely targeted pathway. Moreover, light, which is generally considered to be the most important Zeitgeber, appears to play an important role in the establishment of manipulated behaviours and is a prospective entrainment cue of the underlying parasite–host interactions. For the hypothesis of behavioural hijacking via the host circadian clock to hold, both the parasite and the host would, thus, need to have a

functional, light-entrainable clock. Moreover, the host clock would need to be plastic enough for the fungus to find a way to break in and turn the dials to its favour.

Light seems to be an important regulator of virulence in fungi that infect both animals and plants with the blue-light receptor and circadian regulator white collar-1 (WC-1) often playing a central role (reviewed in 65). This makes it plausible that the light-entrainable clock, which is highly conserved across the fungi,⁷⁸ is also involved in *Ophiocordyceps* interactions with its ant host. Indeed, when mining the *O. kimflemingiae* genome⁷⁹ for clock genes and photoreceptors that are under the control of the circadian clock in the model fungus *Neurospora crassa*, homologs of *frequency* (*frq*), *white collar-1* and *white collar-2* (*wc-1* and *wc-2*), *vivid* (*vvd*) and *phytochrome 1* (*phy-1*) were found.⁸⁰ The identification of these and other homologs suggests that *Ophiocordyceps* exhibits clock-controlled daily oscillations in gene expression. This was further strengthened by a time-course study in which fungal blastospores (i.e., the yeast-like state in which *Ophiocordyceps* and other fungal insect pathogens remain when growing inside a living host) were entrained to 12-h:12-h light–dark (LD) environmental conditions. Investigating the daily gene expression patterns using RNA-Seq resulted in the finding that genes such as *frq*, *wc-1*, *vvd* and blue-light photoreceptor *cryptochrome* (*cry-1*) cycled daily under LD conditions.⁸⁰ In addition, the expression of homologs of *N. crassa* involved in sexual and asexual spore formation was found to be rhythmic, as well as genes that encode secreted proteins that are potentially involved in manipulated summing and biting behaviour (i.e., *ptp* and several enterotoxins).^{23,80} Moreover, *Ophiocordyceps* appeared to exhibit a daily division of labour in gene expression under light cycling conditions: transcription factors were overrepresented among the genes that peaked in their expression during the light phase and secreted proteins, including the above-mentioned *ptp* and putative enterotoxins, were overrepresented among the night-peaking genes.⁸⁰ This suggests that daily rhythms in light conditions drive processes in *Ophiocordyceps* that are involved in parasite–host interactions, behavioural hijacking and transmission.

Like other insects, ants exhibit daily rhythms in gene expression and behaviour. In addition, individuals within the same colony can display vastly different daily rhythms depending on their age and worker ant caste.^{81–87} Ant colonies often have a complex social organization in which the division of labour is decentralized, and an individual's caste identity is tightly linked to their daily activities. Generally, the older ants that leave the nest to forage for food (i.e., forager caste) do so with a distinct daily timing, while their younger sisters remain inside to take care of the brood (i.e., nurse caste) with an around the clock activity. As ants age, they begin to transition from nurse-associated activities to forager-associated activities and daily rhythms. Therefore, worker castes could be viewed as highly distinct chronotypes since they display phenotypes that differ in their timing of activity relative to the predictable 24-h fluctuations in their environment. Although caste-related activity patterns are robust, ants, like other eusocial hymenoptera, can also move between caste identities at a moment's notice when their social

context changes.^{83,88–91} Taken together, this suggests that time-keeping and associated behavioural outputs are both distinct and highly plastic across worker ants of the same colony. Indeed, a 24-h time-course study on *C. floridanus* foragers and nurses of the same colony demonstrated that foragers, entrained to provide 12-h:12-h LD cues, showed robust nocturnal foraging peaks, while nurses remained within the nest.⁷⁷ Conducting RNA-Seq on the brains of ants collected from these behavioural castes throughout a 24-h day showed that evident chronobiological differences in gene expression are plausibly underlying these phenotypes. Forager brains exhibited 24-h rhythmicity in three times more genes than nurse brains. However, it would be too simplistic to conclude that nurses are, therefore, simply less rhythmic and that this is what is giving rise to their around the clock brood care activity. Half of the 24-h rhythmic genes in nurses overlapped with those found in foragers—both in function and in diurnal expression pattern—suggesting that nurses do have at least a somewhat functional clock. Moreover, a significant number of the 24-h rhythmic genes in foragers were cycling every 8h in nurses with largely synchronized expression patterns. Among these genes were homologs of animal core clock genes such as *Period* and *shaggy*.⁷⁷ Furthermore, several genes that are known to encode for proteins involved in the regulation of the division of labour in insects were found to be either differentially rhythmic (e.g., *vitellogenin*) or differentially expressed (e.g., trophallactic fluid proteins) in forager versus nurse brains. As such, this study found a link between chronobiological and behavioural plasticity in *C. floridanus*, which would allow a colony to have its worker ants swiftly transition between forager and nurse castes when conditions require it.⁷⁷ This high level of plasticity is essential for survival. However, plasticity can also serve as an Achilles heel^{92,93} as it could provide behaviour manipulating parasites such as *Ophiocordyceps* fungi with a mechanism that is flexible enough for them to corrupt.

5 | LINKS BETWEEN FUNGAL MANIPULATION AND ANT PLASTICITY AND RHYTHMICITY: A TRANSCRIPTOMICS-BASED META-ANALYSIS

The above-mentioned transcriptomics data on ant daily rhythms and behavioural plasticity in *C. floridanus* have been used to identify rhythmic genes and plasticity genes potentially involved in the behavioural division of labour into foragers and nurses.⁷⁷ Additionally, research on the behavioural manipulation of *C. floridanus* by *O. camponoti-floridani* has brought about transcriptomics data that, in addition to fungal genes, also identified ant genes that are potentially involved in manipulated summing and biting behaviour.²⁰ Even though the latter study was not designed with the detection of clock gene expression in mind, enrichment analyses indicated that ant genes that have been functionally annotated to be involved in circadian rhythms and light sensing are affected during the manipulation phase of the *Ophiocordyceps* infection. This suggests that a computational approach to link ant gene expression data from both

studies might be useful to infer new hypotheses that would begin to answer how *Ophiocordyceps* could induce changes to ant rhythmic behaviours.

We performed network analysis using WGCNA.⁴⁸ We have provided our step-by-step data processing, including the code that we used, in Supplementary File 1 such that it could be used as a guide for network analysis of other time-course datasets. We set out to answer¹ which modules, if any, of the ant host's gene expression network are seemingly affected by *Ophiocordyceps* during manipulation, and² if the fungus targets modules in the host network that are under clock control or drive behavioural plasticity. As such, we built a gene co-expression network (GCN) for *C. floridanus* using the time-course transcriptomics data generated for forager and nurse ant brains that captured gene expression every 2h over the course of a 24-h day under 12-h:12-h LD conditions for these behavioural castes.⁷⁷ We combined gene expression data for foragers and nurses to construct a generalized ant GCN and identified modules of highly co-expressed genes. This resulted in a total of twelve co-expressed gene modules for which we calculated the module-to-module similarity to determine how they were interconnected as part of the global gene expression network (Figure 1A). Next, we annotated the ant GCN using Fisher's exact tests to identify whether a significant overlap existed between the ant genes that make up the various modules and previously identified (1) rhythmic genes,² putative behavioural plasticity genes and³ candidate manipulation genes (Supplementary File 2).

We found that five of the twelve modules were overrepresented for 24-h rhythmic genes as previously identified using empirical JTK Cycle.⁷⁷ One of these modules (Module 4) was overrepresented for genes that displayed 24-h rhythmicity in foragers, two (Modules 1 and 11) for genes with 24-h rhythmicity in nurses, and two for both (Modules 7 and 12) (Figure 1A, B). We further annotated these modules by identifying whether the general expression pattern of all rhythmic genes in the module was peaking during the night or during the daytime (Figure 1C). This resulted in Modules 4, 11 and 12 being indicated as day-peaking clusters and the other two (Modules 1 and 7) as night-peaking clusters (Figure 1A). Additionally, we found that the 'plasticity genes' that were significantly higher expressed in forager brains as compared to nurses⁷⁷ (for-UP) were significantly overrepresented in Module 9. In contrast, the genes that were significantly higher expressed in nurse brains (or, lower expressed in foragers, for-DOWN) were significantly enriched in Module 6. No other modules in the ant GCN showed a significant overlap with the for-UP or for-DOWN gene sets (Figure 1B). This finding suggests that the co-expressed genes in Module 9 are at least to some extent involved in driving the physiological and behavioural state that characterize a forager ant. As such, we could refer to Module 9 as the forager module, whereas Module 6 could be referred to as the nurse module (Figure 1A). Similarly, we identified whether any of the modules contained a significant number of the previously identified 'manipulation genes'.²⁰ We found that the genes that were significantly up-regulated in the heads of *Ophiocordyceps*-manipulated individuals as compared to healthy controls (Ophio-UP) were also

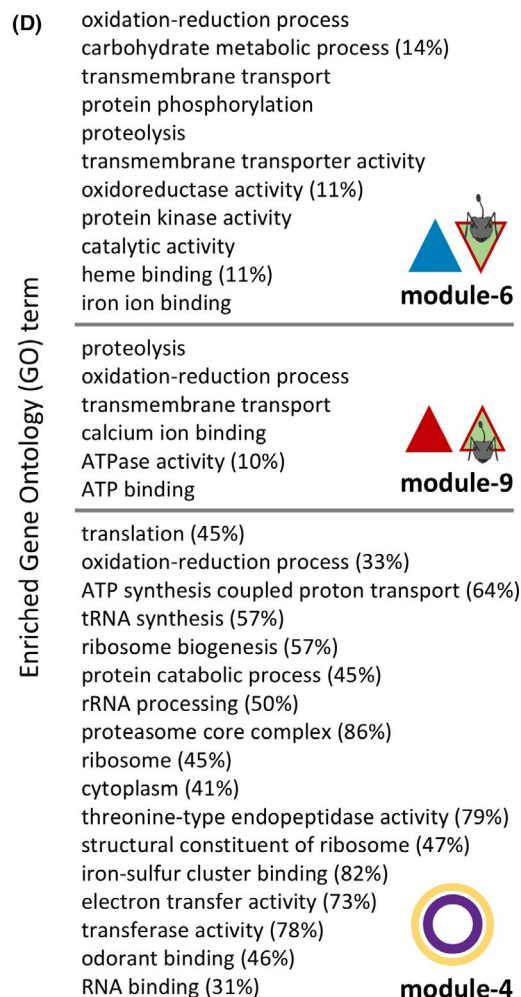
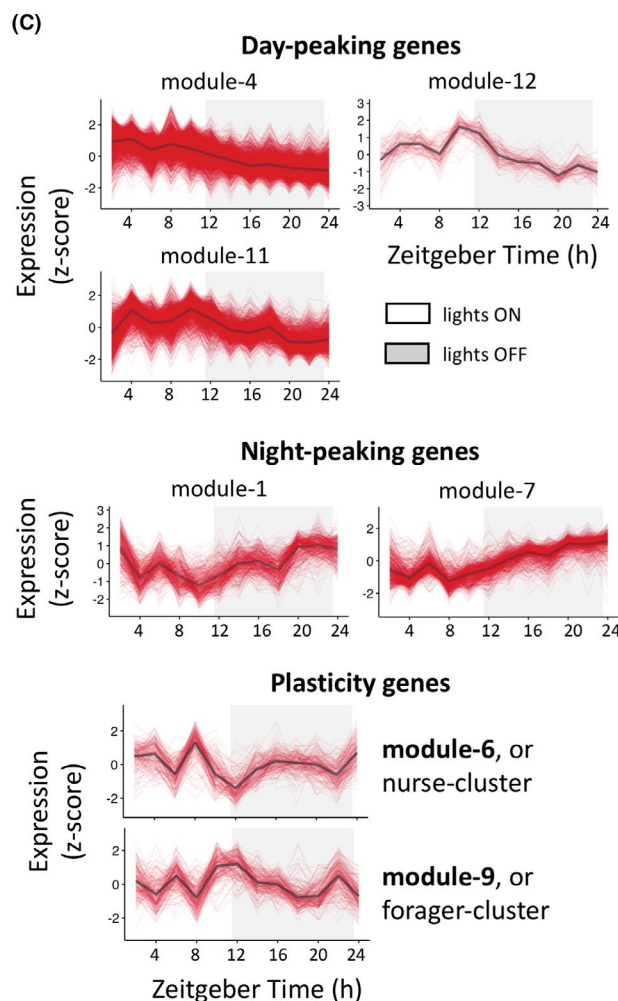
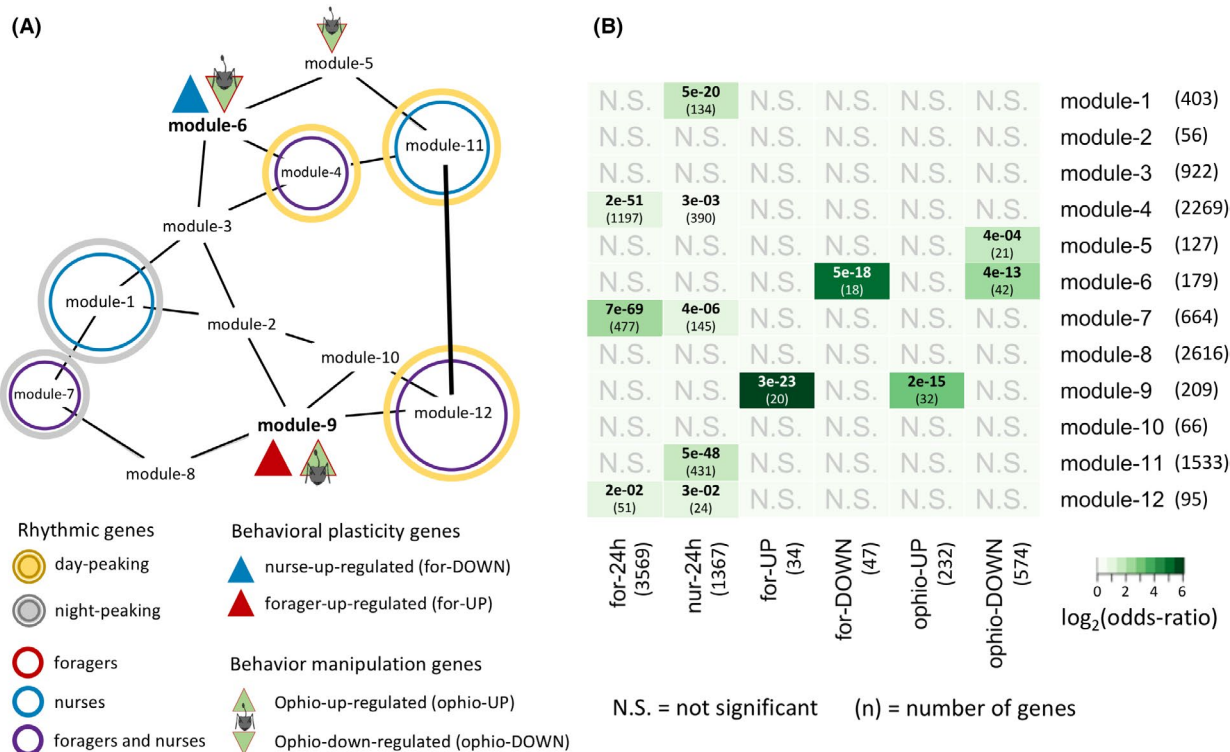


FIGURE 1 Gene co-expression network (GCN) in *Camponotus floridanus* ant brains. (A) The annotated gene co-expression network summarizes our overrepresentation analyses and identifies different modules of interest that are putatively important for the interplay of rhythmicity, behavioural plasticity and parasitic behavioural manipulation. The connectivity patterns between the gene modules are shown; thick edges indicate correlations ≥ 0.8 , thinner edges indicate correlations between 0.6 and 0.8, and no edges indicate correlations < 0.6 . (B) The heatmap summarizes the pairwise Fisher's exact tests used to annotate the ant brain GCN. Box colours represent odds ratio, and the Benjamini-Hochberg corrected p -values are shown together with the number of overlapping genes between module-geneset pairs in parenthesis. Non-significant overlaps are indicated as N.S., and the total number of genes in each module or geneset is shown in parenthesis as well. (C) Daily expression pattern of all genes in each of the rhythmic and plasticity modules are shown. Each red line represents the expression of a single gene, every 2 h over a 24-h day in forager brains. The black line represents the module's median gene expression. The x-axis shows the time of day or Zeitgeber Time (ZT) in hours, whereas the y-axis shows normalized gene expression as z-scores calculated from log2-transformed expression data. White background indicates the light phase (lights on at ZT24/ZT0), and grey background indicates the dark phase (lights turned off at ZT12). (D) The significantly overrepresented Gene Ontology (GO) terms in the behavioural plasticity/manipulation modules (Modules 6 and 9) and the correlated rhythmic modules (Modules 4 and 12). Module 12 is not depicted since it was only overrepresented in one GO term (i.e. membrane). The number in parenthesis indicates the percentage of all genes annotated with the GO term found in the module, if it was higher than 10%

significantly overrepresented in the forager module. Moreover, the genes down-regulated at manipulation (Ophio-DOWN), were significantly enriched in the nurse module (Figure 1B). Therefore, the same subsets of genes that are overrepresented in behavioural plasticity genes are seemingly overrepresented in genes that are significantly affected during manipulation. This suggests that *Ophiocordyceps* might manipulate behaviour by affecting behavioural plasticity genes that give rise to caste-specific behaviours. The manipulating parasite could be targeting some of the same genes and processes that allow for the behavioural plasticity in ants that gives rise to the nurse and forager castes, which display vastly different behaviours and daily rhythms. Furthermore, while these modules are not enriched for diurnal (24 h) genes, they are each directly connected ($0.6 \leq \text{correlation} < 0.8$) to one of the rhythmic modules in the GCN (Figure 1A). To alter or disrupt the processes that are under clock control, *Ophiocordyceps* could, therefore, indirectly be targeting these connected rhythmic modules by affecting the expression of genes in either of the behavioural plasticity modules. To provide some insight into the function of potential genes of interest, we performed a functional enrichment analysis (Figure 1D) and provided all genes, with their annotation and module identity in Supplementary File 2.

6 | CONCLUSION

There is mounting evidence from field and laboratory studies that *Ophiocordyceps* fungi and other behaviour manipulating parasites corrupt and potentially hijack host daily rhythms to further transmission. The plasticity in ant behaviour, given rise to by the circadian clock, could be a prerequisite for *Ophiocordyceps* manipulation. Future work that investigates rhythmic gene expression in infected individuals as well as functional gene studies that test the involvement of *Ophiocordyceps* clock genes in infection and manipulation would, therefore, be worthwhile avenues of research.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

The literature review and writing of the manuscript were done by CdB. The accompanying network analysis was conducted by BD.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets used in this study have been previously published and are publicly available through NCBI under the accession numbers BioProject PRJNA704762 and PRJNA600972. All code used and data output generated as part of this manuscript have been provided as supplementary files.

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