Using machine learning to predict protein-protein interactions between a zombie ant fungus and its carpenter ant host

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Abstract

Parasitic fungi are known to produce proteins that modulate virulence, alter host physiology, and trigger host responses. Due to their various effects on the host, these proteins are often considered a type of "effector" molecule, many of which act via protein-protein interactions (PPIs) between host and parasite. Cross-species PPIs between *Ophiocordyceps camponoti-floridani* and *Camponotus floridanus* likely underlie aspects of infection and parasitic behavioral manipulation in this host-parasite relationship. The fungal parasite *Ophiocordyceps* (zombie ant fungus) manipulates *Camponotus* (carpenter ant) behavior to induce a summit disease phenotype: the infected host ascends and attaches to an elevated position that promotes fungal growth and transmission. Machine learning approaches offer high-throughput methods to produce mechanistic hypotheses on how this behavioral manipulation occurs. Using D-SCRIPT to predict host-parasite PPIs, we found ca. 6,000 interactions involving 129 parasite genes that were previously found to be upregulated during manipulated summiting behavior. We analyzed the predicted PPIs to identify overrepresentation of functional annotations among these participating proteins hypothesized to mediate *Ophiocordyceps*-*Camponotus* interactions. Additionally, we compared these PPIs to predictions made by testing *Camponotus* proteins with secretomes from three fungi that differ from *Ophiocordyceps* by degrees of lifestyle and phylogeny, to find which PPIs were unique for *Ophiocordyceps*-ant interactions. Intriguingly, *Ophiocordyceps*-*Camponotus* specific PPIs carried strong signals for neuromodulatory G-protein coupled receptors in the host and frequent involvement of

unannotated small secreted *Ophiocordyceps* proteins in a variety of PPIs. We also detected less specific overrepresentation of *Camponotus* oxidation-reduction, structural, and gene-regulatory proteins, in addition to *Ophiocordyceps* proteases. These hypothesized interactions include potential fungal drivers of ant infection and manipulation, whether by increasing, decreasing, or modulating host protein activity. As such, this work provides a springboard for targeted molecular investigations of the mechanisms underlying fungal manipulation of ant behavior.

Introduction

Fungal parasites use a diverse array of molecules to defend themselves, promote infection, and modify their hosts. Often termed "effectors," these molecules play a critical role in host-parasite dynamics that include both widely shared and highly specific mechanisms (Beckerson et al., 2019; Hogenhout et al., 2009; Haiyang Wang et al., 2021; Win et al., 2012). Effector biology research has frequently centered on parasite effector proteins that interact with host nucleic acids, carbohydrates, lipids, small metabolites, or other proteins. Such proteins have been suggested to play key roles in fungal infections of insects (Cen et al., 2017; C. Wang & Wang, 2017; Haiyang Wang et al., 2021; Xiao et al., 2012). Bioinformatics and machine learning tools provide a good starting point for the investigation of fungal effectors and their host targets in lesser studied, non-model organisms. In this study, we used such tools to explore possible protein effectors and protein targets between the behavior-manipulating entomopathogenic fungus *Ophiocordyceps camponoti-floridani* (Florida zombie ant fungus) and its insect host *Camponotus floridanus* (Florida carpenter ant).

Myrmecophilous *Ophiocordyceps* fungi are typically species-specific parasites that co-evolved with their ant hosts for millions of years (Araújo & Hughes, 2019). This close relationship has resulted in presumably complex fungus-ant interactions that give rise to altered host behaviors adaptive for the parasite. Manipulated host ants succumb to a summit disease, affixing themselves to elevated positions and dying at these locations that promote fungal growth and transmission (Andersen et al., 2009; de

Bekker et al., 2021; Lovett et al., 2020). Behavioral changes preceding this final summit can include host-adaptive responses, parasite-adaptive manipulations, or general symptoms of disease that span hyperactivity, uncoordinated foraging, decreased nestmate communication, and convulsions (Andersen et al., 2009; de Bekker et al., 2015; Hughes et al., 2011; Pontoppidan et al., 2009; Trinh et al., 2021). Identifying host-parasite protein-protein interactions (PPIs) can offer fundamental insights into the molecular basis of how these organisms interact and how the resulting infection and manipulated behavioral phenotypes are produced.

Recent research into the mechanisms of parasitic manipulation by *Ophiocordyceps* have reported a number of fungal molecules and ant pathways that may be involved, including neuro-modulators and -protectants, insect hormones, feeding, locomotion, circadian rhythms and light-sensing, and muscular hyperactivity (de Bekker, Merrow, et al., 2014; de Bekker et al., 2015; de Bekker, 2019; de Bekker & Das, 2022; Kobmoo et al., 2018; Loreto & Hughes, 2019; Mangold et al., 2019; Trinh et al., 2021; Will et al., 2020; Zheng et al., 2019). Among these candidates, fungal proteins such as bacterial-like enterotoxins, protein tyrosine phosphatases, peptidases (e.g., S8 subtilisin-like serine proteases), and various undescribed small secreted proteins (uSSPs) may mediate important host-parasite PPIs. These hypotheses are supported by strong gene upregulation of these candidates during active manipulation of the ant and genomic comparisons in the *Ophiocordyceps* (de Bekker et al., 2015; de Bekker, Ohm, et al., 2017; Will et al., 2020). Moreover, in the case of the protein tyrosine phosphatase, links to baculovirus-induced behavioral changes in caterpillars appear to exist (Kamita et al., 2005; Katsuma et al., 2012). However, if the protein products of these and other highly upregulated fungal genes are true effectors remains to be tested. This is a costly and time-consuming process, especially in non-model organisms, that would benefit from more evidence indicating effector-like capabilities such as engaging in host-parasite PPIs.

Proteome-scale bioinformatic prediction of PPIs has largely been employed to describe protein interaction networks within a single organism, as was done for the host species, *C. floridanus* (Gupta et al., 2020). However, PPI predictions in cross-species host-parasite relationships have also been made (Loaiza et al., 2021; Ma et al., 2019; Sledzieski et al., 2021). Bioinformatic approaches like these can

offer rapid, large-scale predictions of PPIs prior to labor and resource intensive functional gene assays or laboratory screens (e.g., yeast-two-hybrid). Predicting PPIs is typically done using either an interolog or machine learning method. Interologs are orthologous interactions conserved across species. That is, each protein in one PPI has orthologs in the second interolog PPI (Walhout et al., 2000). While a useful tool for predicting PPIs, detecting an interolog in a new system requires that a reference PPI has already been established in previous systems. This direct one-to-one dependency on previous work offers some confidence in the clear matching to known interactions but is also directly constrained by available data. Even though interologs can be supplemented by homologous domain-domain interactions in the absence of full protein homology (Loaiza et al., 2021), the number of predictions will likely still be very limited with novel proteins not described in model organisms. Machine-learning methods offer a more flexible alternative. These methods are still informed by known PPI training data, but can predict novel interactions that are not exact orthologs to established PPIs (M. Chen et al., 2019; Sledzieski et al., 2021). The machine learning tool D-SCRIPT does this by training models on putative protein structures rather than primary sequence data (Sledzieski et al., 2021). This cross-species generalizability of D-SCRIPT's predictive power is an important step for researchers working on non-model or multi-species systems that lack experimentally supported PPIs. Notably, fungi secrete a range of taxonomically distinct uSSPs that are often thought to be effectors in host-parasite interactions (Qiang Cheng et al., 2014; Feldman et al., 2020; Fischer & Requena, 2022), not unlike the many candidate manipulator uSSPs found for *Ophiocordyceps* species (de Bekker et al., 2015; de Bekker, Ohm, et al., 2017; Will et al., 2020). Bioinformatic techniques that hinge upon well described protein annotations or are subject to strong species-specific biases can only have limited success in assigning uSSPs to PPIs.

To investigate possible *Ophiocordyceps*-*Camponotus* PPIs involved in behavioral manipulation, we used D-SCRIPT to predict PPIs, and filtered these predictions into subsets we consider most likely to include the ones relevant to infection and manipulation. As such, our study focuses on PPIs involving putatively secreted *Ophiocordyceps* proteins that were previously found to be upregulated during manipulated summiting (Will et al., 2020). These fungal proteins were plausibly produced and exported

to the host environment during manipulation, making these PPIs seemingly the most relevant to understanding manipulation of host behavior. We subsequently performed enrichment analyses for both host and parasite proteins involved in these PPIs. We further highlight aspects of those enrichments after filtering out shared orthologous PPIs or PPIs involving host proteins shared with fungi that have not co-evolved with and do not naturally manipulate *Camponotus* ("aspecific" fungi). Co-evolved adaptive manipulation of ant behavior, like summiting, may involve key PPIs specialized to such species interactions. To remove less specific PPIs, we used D-SCRIPT to predict fungus-*Camponotus* interactions with three "aspecific" fungal species. These fungi cover different lifestyles and phylogenetic relationships to *Ophiocordyceps*: (*i*) *Cordyceps bassiana* (i.e., *Beauveria bassiana*), a generalist entomopathogen in the order Hypocreales (which includes *Ophiocordyceps*), (*ii*) *Trichoderma reesei*, a plant-degrading saprophyte in the order Hypocreales (although the genus also encompasses entomopathogens), and (*iii*) *Saccharomyces cerevisiae*, a phylogenetically distant saprophytic yeast in the order Saccharomycetales with a smaller secretome. Removing aspecific interactions brings unique *Ophiocordyceps* PPIs to the forefront as an additional line of evidence for our proposed hypotheses of fungus-ant interactions that could be involved in the establishment of behavioral manipulation phenotypes.

Methods

Predicting PPIs: To prepare PPI test combinations between *O. camponoti-floridani* and *C. floridanus*, we retrieved protein sequence information from high-quality genome assemblies integrating long-read technology for both organisms (GenBank accessions GCA_012980515.1 and GCA_003227725.1) (Shields et al., 2018; Will et al., 2020). We annotated *Ophiocordyceps* proteins as putatively secreted (hereafter, simply "secreted") if they passed the methods used in Beckerson et al., 2019, which combined eight protein sequence-based tools. Proteins that were not annotated as secreted, but did have at least one putative transmembrane domain, were considered transmembrane proteins. To predict PPIs, we used D-SCRIPT (v 0.1.5) with the default human-protein pre-trained model and settings

(Sledzieski et al., 2021). At the time of analysis, D-SCRIPT (v 0.1.5) did not support new model training. Therefore, we report results only from the default model, which has predicted PPIs in insect and fungus model organisms (Sledzieski et al., 2021). D-SCRIPT assigns each tested protein interaction an edge value (i.e., a confidence score to rank possible PPIs) with default threshold for positive prediction ≥ 0.5, on a scale of zero to one. We tested every PPI combination between extracellular (i.e., secreted or transmembrane) *O. camponoti-floridani* proteins and all proteins of *C. floridanus* (i.e., the proteome) (Fig. 1 step 1). We used proteins shorter than 2,000 amino acids due to computational constraints. Limiting the length of input proteins removed six extracellular (0.3%) and 63 (1.1%) intracellular *Ophiocordyceps* proteins and 299 *Camponotus* proteins (2.4%). To statistically test the similarity of PPI connectivity of uSSPs compared to other secreted fungal proteins, we plotted the data with R package ggplot2 (v 3.3.5) and performed a Wilcoxon test through R studio (v 2021.09.2) with R (v 4.1) (R Core Team, 2021; RStudio Team, 2015; Wickham, 2016).

We reasoned that predicted cross-species PPIs would be most likely to indicate specific host-parasite interactions when *Ophiocordyceps* proteins were predicted to only bind *Camponotus* proteins and not any of the fungus's own proteins. To identify self-interacting *Ophiocordyceps* PPIs, we tested extracellular *Ophiocordyceps* proteins with intracellular *Ophiocordyceps* proteins (Fig. 1 step 2). We then searched for reciprocal homologs (putative orthologs) between *Ophiocordyceps* and *Camponotus* proteins using Proteinortho (v 5.0) with default settings (Lechner et al., 2011). If a parasite extracellular protein was predicted to bind one of *Ophiocordyceps*' own intracellular proteins, as well as a *Camponotus* protein homologous to that intracellular protein, we deemed it likely to be a conserved PPI not directly related to infection and manipulation of the host. Any predicted cross-species PPI that had a homologous predicted fungal self-interaction PPI was, therefore, not included in our further analyses (Fig. 1 step 3).

We focused our analyses on PPIs involving secreted fungal proteins encoded by differentially expressed genes (DEGs) upregulated during manipulated summiting behavior as compared to their expression in culture (Fig. 1 step 4). This information was obtained from a previous transcriptomics study

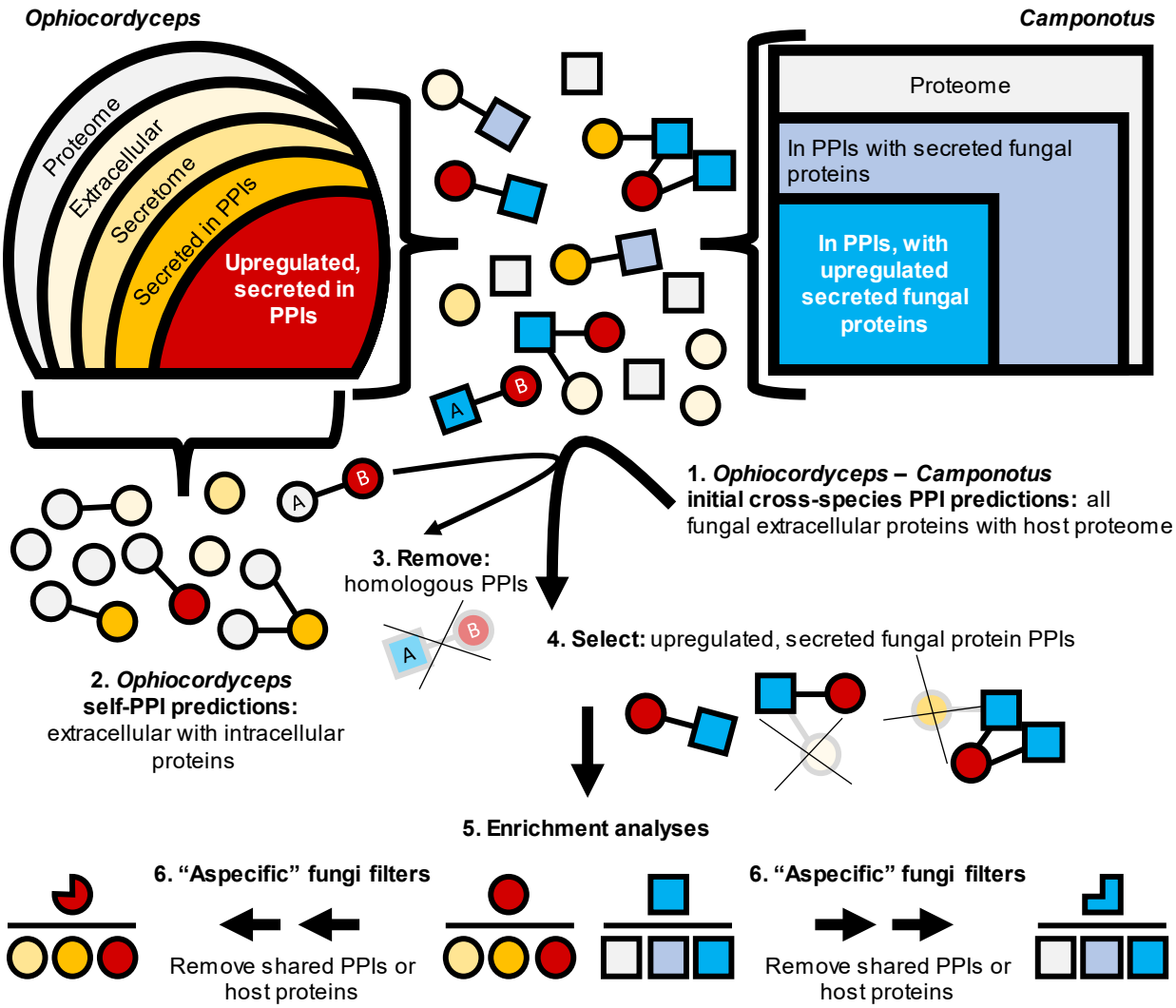

Figure 1. Conceptual framework for PPI testing, selection, and analysis.

First, we tested every extracellular *O. camponoti-floridani* protein with every *C. floridanus* protein in the host proteome (1). If a cross species PPI and an *Ophiocordyceps* self-interaction PPI shared a common fungal protein paired with a protein homologous between both species, we removed that PPI from our cross-species predictions (2, 3). Subsequently, we filtered predicted PPIs to focus on those that involved fungal proteins that were secreted (rather than transmembrane) and upregulated during manipulation (4). Finally, we analyzed host and parasite proteins from these PPIs with hypergeometric enrichment analyses (5). For enrichment analyses, the background protein set was either the parasite secretome (*Ophiocordyceps*) or host proteome (*Camponotus*). We also performed enrichment analyses after further filtering PPIs or host proteins that were predicted in interactions between "aspecific" fungi with *C. floridanus* (6). Fungal proteins are shown as circles and ant proteins as squares. Proteins are color-coded by functional subset, with *Ophiocordyceps* upregulated, secreted proteins in cross-species PPIs as red and their interacting *Camponotus* proteins in bright blue. Other extracellular fungal protein categories are shades of orange. Other host proteins in PPIs are blue-gray. All other proteins of either organism are gray. Proteins "A" are *Ophiocordyceps* and *Camponotus* homologs to each other, and protein "B" is a single fungal protein.

that investigated the expression of *O. camponoti-floridani* and *C. floridanus* genes in experimentally infected ants that displayed summiting behavior under laboratory conditions (Will et al., 2020).

Hypergeometric enrichment analyses: We performed hypergeometric enrichment analyses on proteins from PPIs with secreted, upregulated *Ophiocordyceps* proteins that only had predicted interactions with the host. For enrichment analyses of the fungal proteins in these PPIs, the background protein set was the *Ophiocordyceps* secretome. For enrichment analyses of the ant proteins involved in these PPIs, the background protein set was the *Camponotus* proteome (Fig. 1, step 5). We also performed enrichment analyses on our PPIs of interest after removing those shared as "aspecific" interactions, identified by using three non-*Ophiocordyceps* fungi (see section below). *Ophiocordyceps* and *Camponotus* PPIs that had a corresponding PPI with any of the three "aspecific" fungi when tested against *Camponotus*, such that the ant protein was the same and the fungal proteins were orthologous between *Ophiocordyceps* and an "aspecific" fungus, were removed (Supp. Fig. S1). We also removed every *Ophiocordyceps* and *Camponotus* PPI with an ant protein predicted to be bound by any fungal secreted protein in the "aspecific" fungi tests (Fig. 1 step 6).

For the enrichment analyses we examined GO terms (biological processes and molecular functions combined) (Ashburner et al., 2000), PFAM domains (Finn et al., 2014), and weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008) module membership annotated and reported in Will et al., 2020. These WGCNA modules were correlated to sample types, either healthy ant and *in vitro* fungus controls or live fungus-infected manipulated ants. These modules were additionally correlated between parasite and host, directly between *Ophiocordyceps* gene modules and *Camponotus* gene modules (Will et al., 2020). We reference their previously assigned names that follow the basic patterns of ant-module-1 (A1) and fungus-module-1 (F1), where the number has no special meaning other than to uniquely identify the module (Will et al., 2020). For *Camponotus* proteins, we also tested for enrichments of DEG class (i.e., upregulated or downregulated gene expression from healthy controls to live manipulated ants) (Will et al., 2020). For *Ophiocordyceps* proteins, we additionally tested for enrichment of uSSP annotations. We annotated proteins as uSSPs when they were found to be

putatively secreted using the methods of Beckerson et al., 2019 and were previously annotated as small secreted proteins without any BLAST description, GO term, or PFAM domain (Will et al., 2020).

We performed hypergeometric enrichment analyses with the R package timecourseRNAseq (v 0.0.9000) using default settings (significant enrichment at FDR ≤ 0.05) in R studio (v 2021.09.2) with R (v 4.1) (Das, 2022; R Core Team, 2021; RStudio Team, 2015). We plotted GO terms in semantic space to cluster related terms based on R code generated from REVIGO but did not remove any GO terms (R package ggplot2, v 3.3.5) (Supek et al., 2011; Wickham, 2016).

Aspecific fungus-*Camponotus* PPI combinations: To assist us in assessing and filtering D-SCRIPT predictions for species-specific *Ophiocordyceps*-*Camponotus* PPIs, we tested secretomes from "aspecific" fungi, which have not co-evolved with *C. floridanus* to establish infection and manipulation, against the *C. floridanus* proteome. We chose three fungi based on lifestyle, phylogeny, and availability of well-annotated genomes: *C. bassiana* (GenBank accession GCA_000280675.1), *T. reesei* (GenBank accession GCA_000167675.2), and *S. cerevisiae* (GenBank accession GCF_000146045.2) (Engel et al., 2022; Martinez et al., 2008; Xiao et al., 2012). We functionally annotated each fungal proteome for secreted proteins as for *Ophiocordyceps* (see above) and paired those secreted proteins with the *Camponotus* proteome for D-SCRIPT predictions (as above). To establish orthology between *Ophiocordyceps* and each of the "aspecific" fungi we used Proteinortho (v 6.0) with default settings while enforcing blastp+ and single-best reciprocal hits (i.e., -sim=1) (Lechner et al., 2011). For PPIs we identified as biologically interesting through enrichment analyses and "aspecific" fungi filtering, we created an interaction network to visualize host proteins and their respective *Ophiocordyceps* binding partners with Cytoscape (v 3.8.2) (Shannon et al., 2003).

Results

PPI predictions: We generated 22,972,653 possible PPIs to test by combining 1,881 *Ophiocordyceps* extracellular proteins with 12,213 *Camponotus* proteins (Fig. 1 step 1). Of the

approximately 23 million PPIs tested, 0.41% were positive predictions (n = 94,215 PPIs) (Table 1 row 1, Supp. Fig. S2). This result is in line with a previously predicted 0.95% true PPIs from a 50 million PPI test-set with single-species data (Sledzieski et al., 2021). To be able to remove possibly spurious or uninformative cross-species predictions, we also tested for fungal self-interacting PPIs composed of *Ophiocordyceps* extracellular and intracellular proteins, which resulted in 65,809 PPIs from a possible 10,354,905 combinations (0.64%) (Fig. 1 step 2) (Table 1 row 2).

We found *Ophiocordyceps* transmembrane proteins to often be more promiscuous binding partners than secreted proteins and involved in possibly less informative predicted PPIs (Fig. 2). For example, the extracellular proteins with the two highest number of predicted interactions with the host were a *Ophiocordyceps* transmembrane Sec61 subunit (n = 1392 PPIs) and maltose permease (n = 983 PPIs). Both of these functions appear to be more indicative of core cell functions and possible spurious PPI predictions; the Sec61 protein transporter plays a role in translocating proteins to the endoplasmic reticulum and the maltose permease is involved in sugar transport (Q. Cheng & Michels, 1989; Osborne et al., 2005). Secreted *Ophiocordyceps* proteins had fewer predicted interactions (range = 1 to 644, median = 15) than transmembrane *Ophiocordyceps* proteins (range = 1 to 1392, median = 21). Similarly, the resulting binding partners in *Camponotus* had fewer interactions with secreted fungal proteins (range = 1 to 94, median = 3) compared to fungal transmembrane proteins (range = 1 to 252, median = 4) (Fig. 2). Also, as secreted proteins would be wholly contained in the host-environment, we inferred that these proteins would more often constitute effectors. In total, D-SCRIPT predicted 23,629 host-parasite PPIs to involve secreted *Ophiocordyceps* proteins (Table 1 row 3).

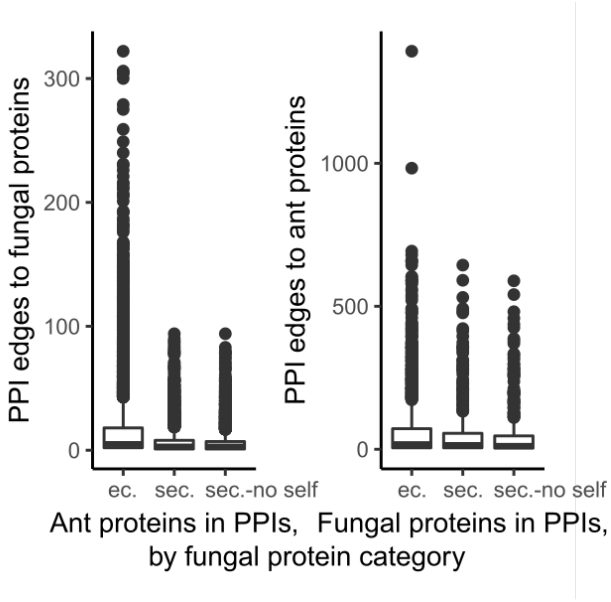

Figure 2. PPI connectivity after filtering predictions based on *Ophiocordyceps* protein category. Box plots demonstrating the predicted number of PPIs per protein, organized by fungal protein category. PPI networks between extracellular fungal proteins ("ec.") and host proteins had the most connected proteins (i.e., protein nodes with the most PPI edges). Removing PPIs involving fungal transmembrane proteins left only secreted proteins ("sec.") and further filtering out homologous PPIs across species ("sec.-no self") reduced connectivity. Note the difference in scale between ant protein connectivity (maximum > 300) and fungal protein connectivity (> 1,000).

Table 1. PPI sets overview.

We tested every combination of an extracellular *O. camponoti-floridani* (*O.*) protein (n = 1,881) to every *C. floridanus* (*C.*) protein (represented by the single longest isoform, n = 12,213) (except any proteins > 2,000 amino acids long). Predicted PPIs were all combinations returned with an edge value of ≥ 0.5. We then created PPI subsets based on fungal gene regulation, secretion and/or transmembrane signals, and homologous interaction between extracellular (i.e., either secreted or transmembrane) and intracellular fungal proteins. For the *Ophiocordyceps*-self interaction set, the extracellular proteins were tested against all remaining intracellular proteins (n = 5,505).

PPI set	Predicted PPIs (% of protein combinations tested)	*Ophiocordyceps* proteins in PPIs	*Camponotus* proteins in PPIs
Ophiocordyceps (*O.*)-extracellular & *Camponotus* (*C.*)	94,215 (0.41%)	1358	5740
O.-extracellular & self-intracellular	65,809 (0.64%)	1275-extracellular 3326-intracellular	NA
O.-secreted & *C.*	23,629 (0.33%)	402	3289
O.-secreted, upregulated & *C.*	6,792 (0.30%)	130	2230
O.-secreted, upregulated & *C.*, no homologous self PPIs	6,011 (0.26%)	129	2083

From the *Ophiocordyceps* proteins that are secreted, the ones that are most likely to be effectors involved in host manipulation are those that were found to be upregulated during summiting behavior (Will et al., 2020). Upregulated, secreted *Ophiocordyceps* proteins in combination with the *Camponotus* proteome (n = 2,271,618 possible PPIs) resulted in 6,792 predicted PPIs (Table 1 row 4). Removing 781 PPIs that were homologous to within-*Ophiocordyceps* interactions left 6,011 PPIs. We used this core set of PPIs, which contained 129 unique *Ophiocordyceps* proteins and 2,083 unique *Camponotus* proteins, for further analyses (Fig. 1 step 4, Table 1 row 5, Supp. File S1).

"Aspecfic" fungi in comparison to *Ophiocordyceps-Camponotus* PPIs: The tested secretomes of *C. bassiana*, *T. reesei*, and *S. cerevisiae* fungi included 833, 505, and 153 proteins, respectively. The proportions of positive PPI predictions with *C. floridanus* (mean 0.27%) and secreted proteins predicted to be in at least one PPI (mean 72%) were similar among these three species as well as to *Ophiocordyceps* values (0.33% positive PPI predictions and 69% of secreted proteins, prior to filtering based on DEGs or self-interactions, Table 1 row 3) (Supp. Table S1). Applying a filter based on shared PPIs between *Ophiocordyceps* and any of the "aspecific" fungi removed 1,776 of the 6,011 (30%) core set *Ophiocordyceps-Camponotus* PPIs (Table 2, Supp. Fig. S1). Similarly, we produced a filtered data set by removing 1,936 of 2,083 (93%) of the host proteins predicted to be bound by *Ophiocordyceps* that were shared binding partners with "aspecific" fungi, leaving 157 PPIs (3%) (Table 2, Supp. Fig. S1). In line with secretome size, phylogeny, and entomopathogenic lifestyle, *C. bassiana* shared the most overlap with *Ophiocordyceps* predictions with both filtering methods (Supp. Fig. S1). Filtering by shared host proteins led to major changes in enrichment results and filtering by shared PPIs led to modest changes (Table 2, Supp. File S2). Filtering based on *C. bassiana* alone was largely similar to combining it with *T. reesei* and *S. cerevisiae* (Supp. Fig. S1, Supp. File S2). Results presented below reflect *Ophiocordyceps-Camponotus* results without these filters unless otherwise stated. These filters assisted us in ordering and emphasizing results (i.e., signals maintained through the strictest filters as being prioritized), but we did not use this approach to wholly select PPIs of interest (see Discussion).

Enrichment signals among *Ophiocordyceps* proteins involved in PPIs: We conducted enrichment analyses on the 129 *Ophiocordyceps* proteins found to be involved in the subset of PPIs that we deemed to be most likely to include ant host manipulation effectors (Fig. 1 step 5, Table 1 row 5). We tested these proteins against a background of the entire *Ophiocordyceps* secretome (n = 586) to ask if the upregulated set of host-binding proteins was functionally distinct from all proteins that *Ophiocordyceps* could secrete (Fig. 1 step 5, Table 4,). We do not consider D-SCRIPT predictions to necessarily be a comprehensive way to investigate single hypothesized PPIs, rather its outputs are best interpreted at the group level (e.g., enrichment analyses). However, previous work has hypothesized specific fungal effectors (Will et al., 2020) from which we selected top candidates to investigate and report in Supp. File S3.

We found enrichments for one PFAM domain and three WGCNA modules (Table 4). The enriched PFAM domain was a "peptidase S8 domain," indicating an overrepresentation of fungal serine proteases that could cleave host proteins. The five fungal peptidase S8 proteins in this enrichment were predicted to interact with 34 host proteins across 39 PPIs (Supp. File S1). Among these host proteins, there were seven kinesin-like proteins (motor proteins), five nuclear pore proteins, and a (pro-)resilin that is an insect cuticle and connective tissue protein (Burrows & Sutton, 2012). We also found fungal WGCNA modules F1, F2, and F3 to be enriched. Modules F1 and F2 were previously described to have significant positive correlations to manipulation and contained multiple genes hypothesized to mediate infection or manipulation processes. Module F3 largely contained genes related to core fungal cellular processes (e.g., DNA packaging), but was significantly negatively correlated to control samples, with modest positive correlation to manipulated ants. All three modules also had significant direct correlations to ant WGCNA modules, with F1 being correlated to ant modules A14 and A15 – both associated with host neuromodulation (Will et al., 2020). Module F3 was also positively correlated with A15 (Will et al., 2020). Even after removing PPIs that were shared with "aspecific" fungi the enrichment signals for S8 peptidases and all three WGCNA modules were retained. However, the much more stringent removal of shared host targets eliminated the S8 peptidase and WGCNA module F1 enrichment results.

Table 2. Annotation enrichments of PPI proteins.

The PPIs used for these enrichment analysis were all those without homology to *Ophiocordyceps* self-interactions and involving upregulated, secreted *Ophiocordyceps* proteins and any *Camponotus* proteins (Fig. 1 step 5) with or without additional filtering based on shared "aspecific" fungal interactions (Fig. 1 step 6). We found no enrichment signal for uSSPs in the fungus. Typically, more stringent filtering reduced the number of enrichments detected. Only for *Ophiocordyceps* GO terms were enrichments gained, which included oxidation-reduction terms and various general binding terms (e.g., "binding," "catalytic activity," "cofactor binding," or "ion binding"). *Ophiocordyceps* PPI proteins were tested against its secretome (586 background proteins) and *Camponotus* PPI proteins were tested against its proteome (12,213 background proteins). Upregulated DEGS are abbreviated as UP and downregulated DEGs as DN.

Test proteins (n)	**GO**	**PFAM**	**WGCNA**	***C.* DEG**
Ophiocordyceps (129)	0	1	3	NA
shared PPIs removed (125)	1	1	3	NA
shared host proteins removed (26)	11	0	2	NA
Camponotus (2,083)	50	106	9	UP, DN
shared PPIs removed (1,901)	44	64	9	none
shared host proteins removed (147)	12	3	2	none

Enrichment signals among *Camponotus* proteins involved in PPIs: We applied the same approach to the *Camponotus* proteins found to be in cross-species PPIs as we did for the fungus. Again, we compared the 2,083 host proteins to the full host proteome, which encompasses every protein that possibly could have bound an *Ophiocordyceps* protein (n = 12,213 host proteins) (Fig. 1 step 5) (Table 2). As GO term and PFAM domain enrichments often indicated similar functions, we present results largely from the GO term perspective, as there were fewer terms overall compared to PFAM domains. We do highlight PFAM domains for emphasis or to discuss biologically interesting results not well captured by GO terms alone.

G protein-coupled receptor signaling. We detected GO term enrichments for "G protein-coupled receptor (GPCR) activity" and "GPCR signaling pathway" (Fig. 3). We predicted 71 GPCR-related PPIs

with 16 unique *Ophiocordyceps* proteins (Supp. File S1). All but two receptor proteins in these PPIs carried the PFAM domain "7 transmembrane receptor (rhodopsin family)." We found 33 unique GPCR-related protein genes (of 128 possible with these GO terms), with a range of putative ligands: 11 neuropeptides (n = 12 receptors), four biogenic monoamines (dopamine, serotonin, octopamine, tyramine) (n = 11 receptors), acetylcholine (n = 2 receptors), a spider venom toxin protein (alpha-latrotoxin) (n = 1 receptor), and opsin blue- and ultraviolet-sensitive receptors (n = 2 receptors). Fourteen of these 33 *Camponotus* receptors (or receptor subunits) belonged in ant-neuronal WGCNA modules A14 and A15. Few of the encoding host genes were previously found to be DEGs during *Ophiocordyceps* manipulation. The only upregulated gene was a putative G-protein alpha subunit (Will et al., 2020) – a key component of GPCR signaling function. We also found downregulated receptors for neuropeptides (putative pyrokin-1 and gonadotropin-releasing hormone II), a biogenic monoamine (octopamine beta 2R), and acetylcholine (muscarininc gar-2) (Will et al., 2020). Host GPCR-related PPI proteins were predicted to bind 16 fungal proteins that included five uSSPs, a carboxylesterase, a glycosyl hydrolase, a CAP cysteine-rich secretory protein, and a protein tyrosine phosphatase. Most of these proteins were previously assigned to manipulation-associated fungal module F2 (n = 9), but included some in modules F1-4 (Supp. File S1).

Enrichment signals for GPCRs were among the few functional annotations that persisted after we filtered results based on both PPIs and host proteins shared with "aspecific" fungi results (Fig. 4). In addition to passing the shared PPI filter, both GPCR GO terms were still among the 12 enriched GO terms after filtering out shared host proteins (Supp. File S2). Similarly, a GPCR enrichment signal for "7 transmembrane receptor (rhodopsin family)" was preserved as one of three PFAM domains enriched after removing shared host proteins (Supp. File S2). Twenty-eight of the 33 GPCR host proteins remained after removing shared PPIs. However, only seven remained after filtering out shared host proteins. These host proteins, predicted to be uniquely bound by *Ophiocordyceps* during manipulation, were a putative dopamine receptor 2, Oamb octopamine receptor, methuselah-like 10 receptor, orexin receptor, trissin

receptor, cAMP-dependent protein kinase subunit, and receptor without an informative BLAST description ("uncharacterized protein"). The four *Ophiocordyceps* proteins binding these host receptors were predicted in many PPIs (range = 101 to 458). The dopamine receptor 2, Oamb octopamine receptor, and uncharacterized receptor shared the same fungal uSSP partner. The orexin and trissin receptors also shared an unannotated secreted fungal protein (larger than an uSSP). The cAMP-dependent protein kinase subunit PPI involved a fungal protein tyrosine phosphatase specifically hypothesized to be involved in behavioral manipulation by *O. camponoti-floridani* (Supp. File S3) (Will et al., 2020). Although *Ophiocordyceps* putatively bound seven specific host GPCRs uniquely among all tested fungi, the "aspecific" fungi were predicted to bind some GPCRs that *Ophiocordyceps* was not. Eight proteins

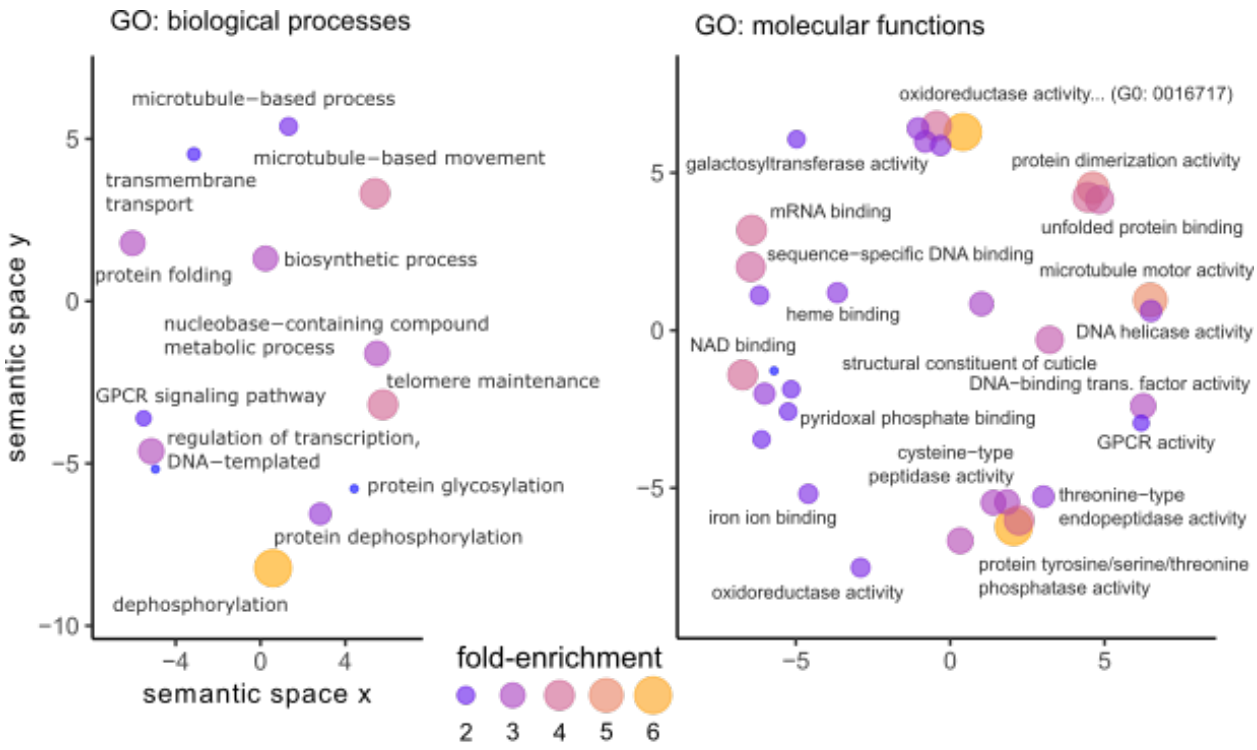

Figure 3. Enrichments found for *C. floridanus* proteins in PPIs with upregulated secreted fungal proteins against the *C. floridanus* proteome background.

GO terms are plotted in semantic space, which lacks any inherent unit or value other than to cluster terms by functional similarity. Some labels were omitted for readability and when not relevant to our discussion of the results.

(with GO terms “GPCR activity” or “GPCR signaling pathway”) were bound by at least one of the three “aspecific” fungi but not *Ophiocordyceps*.

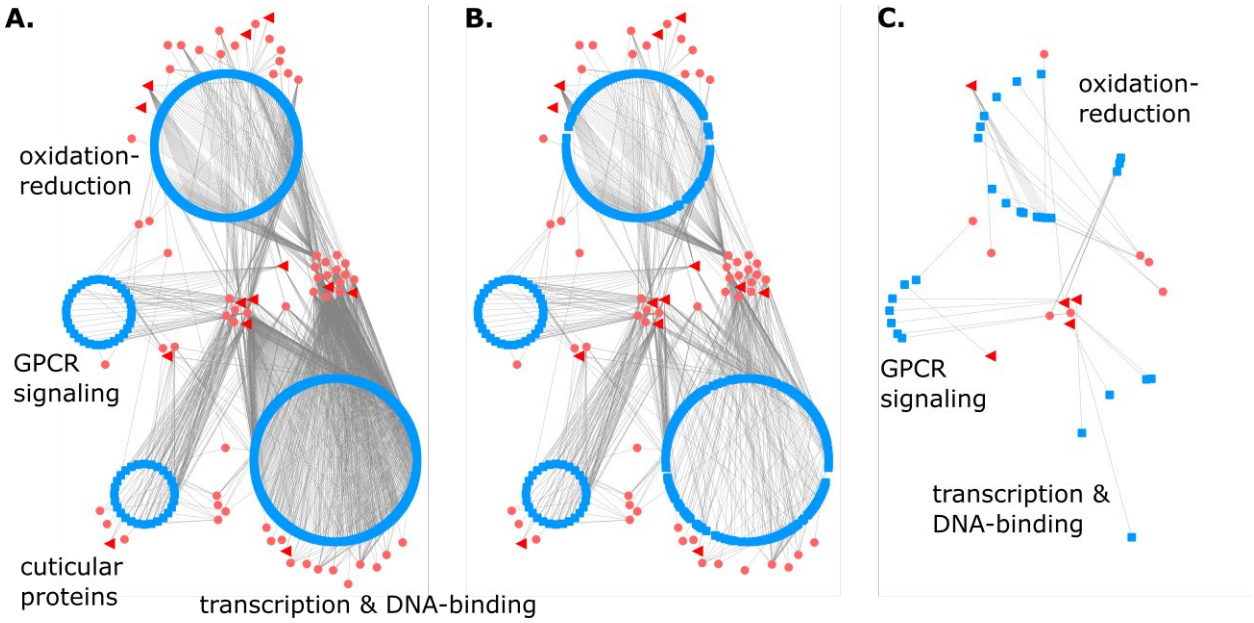

Figure 4. PPI network with host proteins contributing to selected GO term enrichments.
Host proteins (blue square nodes) are clustered and labeled by their general GO term functional category and connected (gray line edges) to their fungal PPI partners that are either secreted proteins (small pink circle nodes) or uSSPs (large red triangle nodes) that were found to be upregulated during *Ophiocordyceps* manipulation of *Camponotus* behavior. Proteins may have PPI connections outside of those depicted here. A) PPI network without “aspecific” PPI filtering. B) Network of remaining PPIs after removing shared “aspecific” PPIs. Although nearly 30% of PPIs (edges) were removed, most proteins (nodes) and enrichment results were kept. C) Network of remaining PPIs after removing PPIs with host proteins that also interacted with “aspecific” fungi. Some enrichments for GPCRs and oxidation-reduction persisted after this filter. Enrichment signals for transcription and DNA-binding were lost, but some individual proteins remained.

Oxidation-reduction. We detected the enrichment of oxidation-reduction functions among *Camponotus* proteins in PPIs (six supporting GO terms) (Fig. 3, File S1). Altogether, 159 host proteins had oxidation-reduction annotations, forming 321 PPIs with 48 parasite proteins. These fungal proteins included cytochrome P450s, oxidases and oxidoreductases, amidases, peptidases, and an enterotoxin. A plurality of the host proteins were encoded by cytochrome P450 genes (70 unique genes, 15 putative

subtypes). This abundance of cytochrome P450, a hemoprotein, also contributed to the enrichment of "heme-binding" and "iron ion binding" GO terms (Fig. 3) and the PFAM domain "cytochrome P450" (Supp. File S1). These *Camponotus* cytochrome P450 proteins paired with 10 unique *Ophiocordyceps* genes, consisting of uSSPs and fungal cytochrome P450s. Another ant protein with oxidation reduction function was a putative delta-1-pyrroline-5-carboxylate dehydrogenase. This enzyme catalyzes synthesis of glutamate, an amino acid with roles in metabolism and a key excitatory neurotransmitter of insect motoneurons (Johansen et al., 1989; Krnjević, 2010; Pflüger & Duch, 2011; Srivastava et al., 2012; Tiwari et al., 2013). We predicted this enzyme to bind a fungal uSSP that was in 31 other PPIs, 16 of which involved host cytochrome P450 proteins. Other notable contributors to the host enrichment of oxidation reduction related functions were peroxiredoxins, laccases, dehydrogenases, and a vat-1 synaptic vesicle membrane-like protein (File S1). Enrichment signals for four of the six oxidation-reduction GO terms were retained after we removed PPIs that were shared with the "aspecific" fungi and two after we applied the more stringent shared host protein filter (Supp. File S2). Additionally, indicators of cytochromes, heme and iron binding GO terms and the "cytochrome P450" PFAM domain continued to be enriched after either filter (Supp. File S2). The enrichment of "cytochrome P450"was one of only three PFAM domains detected after the shared host protein filter. Most host proteins passed the shared PPI filter (140 of 159), but only 19 remained after removing shared host proteins (Fig. 4). Cytochrome P450 proteins shifted from a plurality before shared host protein filtering to a dominant majority afterwards (17 of 19 proteins), with eight putative cytochrome P450 subtypes: 3A19, 315a1, 4C1, 49a1, 6a13, 6a14, 6k1, and 9e2.

Transcription and DNA-binding. Multiple enrichments that can be associated to gene transcription and regulation were enriched. We found 204 host proteins annotated for any of "DNA binding," "DNA-binding transcription factor activity," "sequence-specific DNA binding," "DNA helicase activity," "regulation of transcription, DNA-templated," and "mRNA binding" GO terms (Fig. 3). These proteins similarly contributed to the high enrichment of PFAM domains such as "helix-loop-helix DNA-binding domain," "zinc Finger C4 type," "'paired box' domain," and "bZIP transcription factor" (Supp.

File S1). These 204 host proteins interacted with 43 fungal proteins to generate 922 PPIs. These fungal proteins included five uSSPs that were also found in the PPIs reported above (Fig. 4A), three that were not, and various hydrolases, oxidoreductases, peptidases, and carboxylesterases. Among the 204 host proteins, we found transcription factors and regulators with many functions, which included examples associated with insect behavior and neuronal function such as Mushroom body large type Kenyon specific protein 1 (e.g., elevated in worker bees, foraging behavior), Gooseberry (e.g., neuromuscular junction function), Hairy (e.g., juvenile hormone mediated gene repression), photoreceptor specific nuclear receptor (light sensing), CLOCK (circadian clocks), and steroid and ecdysone nuclear receptors and induced proteins (Guo et al., 2018; F. Liu et al., 2022; Marie et al., 2010; Saha et al., 2016; Velarde et al., 2006). Other proteins, most clearly understood from the neuronal development perspective thus far, produce atypical behavioral or locomotor phenotypes when dysregulated and include Jim Lovell, Dead ringer, Hairy/enhancer-of-split related, Achaete-scute complex proteins, and Forkhead (Bjorum et al., 2013; Y. C. Cheng et al., 2015; García-Bellido & De Celis, 2009; Häcker et al., 1995; Shandala et al., 2003; Tang et al., 2012). These 34 proteins taken as biologically relevant examples account for 186 of the 922 gene regulation PPIs, interacting with 20 *Ophiocordyceps* proteins covering a similar breadth of protein types as the 43 parasite proteins overall (Supp. File S1). All of the aforementioned GO terms and PFAM domains continued to be enriched after removing shared PPIs (191 of 204 host proteins retained), but none of these annotations continued to be enriched after removing shared "aspecific" host proteins (Fig. 4, Supp. File S2). However, protein Jim Lovell remained as the sole host protein uniquely bound in an *Ophiocordyceps* PPI of the 34 discussed proteins above, and is one of five total DNA-regulatory proteins (originally 204) unique to predicted *Ophiocordyceps*-*Camponotus* PPIs (Fig. 4C). In *Drosophila* this protein relates to early neuronal development, larval locomotor and foraging defects, and adult atypical courtship and gravitaxis behaviors (Bjorum et al., 2013). We predicted this *Camponotus* Jim Lovell homolog in a single PPI, bound by an *Ophiocordyceps* uSSP. However, this uSSP was predicted in 541 PPIs, suggesting broad activity if many of these PPIs are true.

Cuticular proteins. We found 28 ant proteins that shared the enriched GO term "structural constituent of cuticle" (Fig. 3) and PFAM domain "insect cuticle protein" (Supp. File S1). We predicted these ant proteins interacted with 22 fungal proteins to form 138 PPIs (Fig. 4A). We found the same CAP protein, glycosyl hydrolase, and four of the uSSPs as above. We additionally identified a carboxylesterase (also predicted in the GPCR results), a disintegrin/reprolysin-like peptidase, a S8 subtilisin-like serine peptidase, M43 peptidases, an uSSP, and an unannotated secreted protein (larger than an uSSP). These enrichments were only kept under the "aspecific" shared PPI filter (with all 28 original cuticular proteins) and lost with the shared host protein filter (none of the 28 proteins were uniquely bound by *Ophiocordyceps*) (Fig. 4, Supp. File S2).

Ant DEGs and WGCNA modules. Camponotus genes differentially expressed during manipulation and host gene modules correlated to manipulation were enriched among PPI proteins (Supp. File S1). Both up- and downregulated host DEGS were enriched. Three ant WGCNA modules were enriched that had significant correlations to manipulation, with an additional six that did not (of 22 modules total). Module A14 was negatively correlated to manipulation and included many genes putatively associated with neuronal function, including GPCRs reported above (Will et al., 2020). Module A10 was positively correlated to manipulation and contained enrichment signals for gene regulatory processes (Will et al., 2020). Module A4 had a positive correlation to manipulation and contained enrichment signals for both proteasomes and odor detection (Will et al., 2020).

Connectivity of focal PPI proteins: The five general functional categories reported above (GPCR signaling, oxidation-reduction, transcription and DNA-binding, and cuticular proteins,) contained 1,451 PPIs of 424 host proteins and 74 parasite proteins (Fig. 4). Half of the fungal proteins interacted with host proteins in more than one of these enrichment categories (n = 37 fungal proteins), with the others possibly interacting with host proteins not highlighted here. Eight fungal proteins interacted with all five enrichment categories and were also highly promiscuous across all PPIs predicted in this study (much greater than the median secreted fungal protein connectivity of 15 PPIs). These fungal proteins were three uSSPs, a putative carboxylesterase, oxidase, glycosyl hydrolase, CAP cysteine-rich secretory

protein, and another unannotated secreted protein (n = 591, 482, 355, 395, 280, 263, 95, 110 PPIs, respectively) (Fig. 4). Taken together, this suggests that most fungal proteins of interest were predicted to bind multiple host proteins, with some predicted across a breadth of biological functions (Fig. 4). Although the three uSSPs were slightly less than half of the eight highly connected fungal proteins, such proteins, as a class, were not more specific in their binding pattern across the study (Wilcoxon rank-sum test, W = 16776, p = 0.093) (Fig. 5).

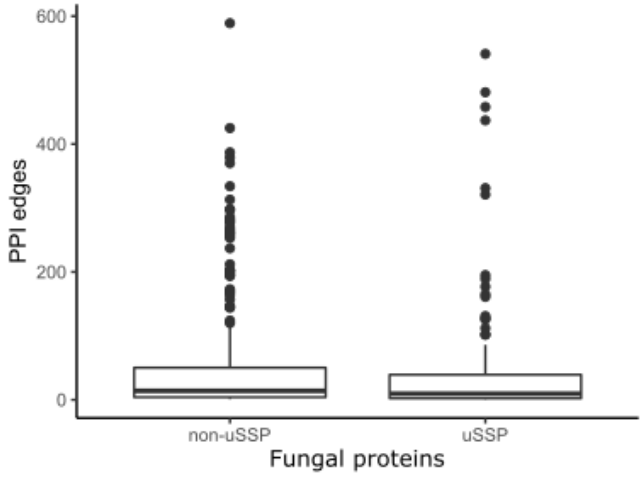

Figure 5. uSSPs have similar PPI connectivity to host proteins as other secreted fungal proteins. As our data was non-normally distributed, we applied a Wilcoxon rank sum test to ask if uSSPs had increased connectivity compared to all other secreted fungal proteins. We found that this was not the case (W = 16776, p = 0.093). Each point represents a single fungal protein, and the PPI edges count shows how many host proteins that fungal proteins was predicted to bind.

Discussion & conclusion

The inter-species molecular interactions during manipulation of *C. floridanus* by *O. camponoti-floridani* likely include parasite effector proteins with host protein targets. Bioinformatically predicting possible cross-species PPIs in non-model systems such as this one presents a special challenge with approaches still improving. As such, we used the machine learning tool D-SCRIPT, which infers structural relationships between proteins without relying on protein-wide sequence homology to known interactions. This flexibility allows D-SCRIPT models to extend predictions across organisms and beyond previously documented PPIs. In principle, D-SCRIPT better classifies novel interactions such as those involving unannotated fungal proteins or cross-species PPIs that, with currently available data, are less amenable to purely homology-based approaches (i.e., interologs). Indeed, D-SCRIPT was able to predict

several thousand PPIs between *Camponotus* host proteins and upregulated, secreted *Ophiocordyceps* proteins.

Due to the unfamiliar terrain of leveraging this approach between non-model species, we sought to propose specific hypotheses balancing biological plausibility and the possibility of novel interactions. On the one hand, we considered PPI predictions in the light of previous knowledge of protein and cellular function. However, since the molecular aspects of parasitic manipulation are still largely to be uncovered, we also considered that undescribed or unusual mechanisms may underlie these interactions. Weighing such possibilities also had to contend with the current limited availability of cross-species PPI experimental data compared to single-species data – let alone for insect-infecting parasitic manipulators. Often, we found that single proteins, especially from *Ophiocordyceps*, were in multiple host-parasite PPIs. Although in some cases these could be errant predictions, *Ophiocordyceps* effectors may indeed bind many host proteins across different pathways, as has been observed in other systems, such as microbe-plant interactions, and is possibly even the norm (Hogenhout et al., 2009; Win et al., 2012).

Fungal PPI proteins were enriched for S8 peptidase domains, indicating that a significant number of these proteins had proteolytic functions with possibly diverse targets (Muszewska et al., 2017). Multiple host kinesin motor proteins were in PPIs with these *Ophiocordyceps* proteins. Kinesins are involved in mitotic and intracellular transport processes – with key functions in vesicle transport towards the periphery of neurons (Klinman & Holzbaur, 2018). Defects in kinesin activity can lead to dysregulated receptor and neurotransmitter release at synapses, thereby contributing to impaired neurological functions (Klinman & Holzbaur, 2018). Zika viruses have been hypothesized to interfere with human kinesin, leading to blindness and microcephaly (L. Liu et al., 2021). From these predicted S8 peptidase-kinesin interactions, we infer that the fungus may be dysregulating core host cell processes, possibly resulting in impaired neuron signaling and neurotransmitter release underlying modified behavioral phenotypes. We also predicted these peptidases to interact with multiple *Camponotus* nuclear pore proteins. Although some components of the nuclear pore undergo proteolytic post-translational modification to remove sumoylation (Hang & Dasso, 2002; H. Zhang et al., 2002), these peptidases are in

a different family than S8 peptidases. If the predicted S8 peptidase-nucleopore proteins interactions are genuine, this could mean the parasite alters transport of molecules in and out of the host nucleus. Speculatively, this could facilitate entry of the fungal transcription factor-binding proteins that we noted. Furthermore, fungal PPIs were enriched for the WGCNA modules transcriptionally associated with manipulation from Will et al., 2020. Assuming "guilt-by-association," in some cases this could indicate these PPIs are related to infection and manipulation processes even if we yet lack clear mechanistic hypotheses of how (e.g., PPIs with uSSPs) (Carlson et al., 2006; Gillis & Pavlidis, 2012; Stuart et al., 2003). With only the S8 peptidase annotation and membership in three WGCNA modules, comparatively fewer terms were enriched among *Ophiocordyceps* than *Camponotus* proteins analyzed. In part, this could reflect the fungal secretome (i.e., background proteins of the enrichment analysis) being functionally specified toward host-pathogen processes overall, from which the PPI proteins might not be a highly distinct subset. In line with this possibility, we found most secreted proteins to be predicted in host-parasite PPIs.

We detected over 30 ant GPCRs or GPCR subunits in PPIs with fungal proteins. Most GPCRs were from the rhodopsin/A family and included receptors related to biogenic monoamines, acetylcholine, and neuropeptides. These receptors offer a wide range of hypothetical connections to manipulated ant behavior. Commonly, these receptors and their ligands have been linked to locomotion, feeding, and circadian rhythms – processes that have been previously highlighted in hypotheses of *Ophiocordyceps* and other fungus-insect summit-disease behavioral manipulations (de Bekker et al., 2015, 2021; de Bekker, Will, et al., 2017; Elya et al., 2018; Will et al., 2020).

The monoamine neurotransmitters dopamine, serotonin, octopamine (analogous to vertebrate norepinephrine), and tyramine have been implicated in modulating locomotor, foraging, learning, social, reproductive, and aggressive behaviors in many insects, with experimental evidence in social insects, including ants (Adamo, 2008; Adamo et al., 2013; Aonuma & Watanabe, 2012; Finetti et al., 2021; Kamhi et al., 2017; Verlinden, 2018). Compared to the other fungi we tested, both a *C. floridanus* dopamine and an octopamine receptor were uniquely bound by *Ophiocordyceps* upregulated secreted

proteins – hypothetically implicating these host receptors in specific *Ophiocordyceps* interactions such as behavioral manipulation. We additionally detected two muscarinic acetylcholine receptors associated with sensory and motor neurons in non-insect animals (Lee et al., 2000; Stankiewicz et al., 1999; Tsentsevitsky et al., 2017). In bees, excessive activation of acetylcholine receptors can cause changes in locomotor, navigational, foraging, and social behaviors (Grünewald & Siefert, 2019). Changes in such behaviors have also been observed in *C. floridanus* ants infected with *O. camponoti-floridani*. Locomotor behavior is enhanced in these ants, while social behaviors are diminished, daily timed foraging activities become arrhythmic and navigation capabilities appear less effective (Trinh et al., 2021).

The neuropeptide GPCRs in PPIs with secreted, upregulated *Ophiocordyceps* proteins had a range of putative ligands. This included a host trissin receptor and an allatotropin receptor (i.e., annotated as an "orexin" receptor, but likely an allatotropin receptor in insects), which, of the fungi we tested, only *Ophiocordyceps* was predicted to bind. Trissin has been shown to have specific neuronal expression, including in a subset of circadian core-clock neurons, and is implicated in insect feeding (Abruzzi et al., 2017; Nässel & Zandawala, 2019; Roller et al., 2016). Circadian rhythm is related to ant foraging behavior (and, perhaps indirectly, feeding) and is affected by *Ophiocordyceps* infection (Trinh et al., 2021). The intricately synchronized time-of-day summiting and biting behavior further suggests a role for circadian control of activity (de Bekker, Quevillon, et al., 2014; Hughes et al., 2011; Will et al., 2020). Allatotropin can also modulate feeding and circadian control of locomotor activity, as well as juvenile hormone production, immune activation, and muscle stimulation (Hernaândez-Martõânez et al., 2017; Lismont et al., 2015; Nagata et al., 2012; Nässel & Zandawala, 2019; Petri et al., 2002). Interlaced with feeding and foraging processes in ants, juvenile hormone mediates insulin/feeding related signaling and social caste identity (e.g., foraging workers) – GPCRs that are similarly connected to juvenile hormone may be one host target for parasite effector proteins (Ament et al., 2008; Chandra et al., 2018; de Bekker & Das, 2022; Schwinghammer et al., 2011; Will et al., 2020). Among the host GPCRs shared with other "aspecific" fungi, we also predicted binding to a host allatostatin-A receptor. Generally acting in opposition to allatoropin, allatostatin-A is also expressed in some circadian clock neurons and can act as a

feeding, locomotor, and wakefulness modulator, sometimes via juvenile hormone inhibition (Aguilar et al., 2003; J. Chen et al., 2016; Hergarden et al., 2012; Tsao et al., 2018). Other neuropeptide GPCRs in PPIs are also associated with locomotion and activity levels, feeding, aggression, muscle control, juvenile hormone, reproductive behavior, or pheromones included those activated by adipokinetic hormone or corazonin (i.e., a "gondaotropin-releasing hormone" receptor) (Sakai et al., 2017), cholecystokinin-like peptides (Nässel & Williams, 2014), CCHamide-2 (Ren et al., 2015), and pyrokinin, pheromone biosynthesis activating neuropeptide (PBAN) and/or capa type neuropeptides (Choi et al., 2012; Jurenka & Nusawardani, 2011; Ragionieri et al., 2017). Odor and pheromone detection are key elements of ant communication, behavioral regulation, and social interactions (Chang et al., 2015; Ferguson et al., 2020; Gotzek et al., 2007). Possibly, GPCRs typically involved in odorant response could become dysregulated by fungal effectors to dampen nestmate interaction and/or induce aberrant behavior without normal cues. Taken together, we found multiple PPIs involving ant GPCRs putatively involved in signaling systems that could control which and when certain behaviors are performed by manipulated ants.

Our PPI predictions also included a putative orphan receptor, GPCR 52, which is responsive to anti-psychotic drugs, implicated in models of depression and neurodegenerative disease (e.g., Huntington's). This receptor is coexpressed in neurons with dopamine receptors, where it can interact with dopamine signaling pathways that, again, affect locomotion (Komatsu et al., 2014; Mantas et al., 2021; Yao et al., 2015). Two of the three predicted fungal binding partners for this receptor were uSSPs – possibly offering insights to the currently unknown ligand of GPCR 52. In addition to these rhodopsin/A family GPCRs, we also found ant-*Ophiocordyceps* PPIs involving a latrophilin/CIRL-like receptor, which is a secretin/family B GPCR that gets its name from a sensitivity to alpha-latrotoxin in black widow venom. This receptor has been implicated in mechanosensation, interneuronal adhesion, neurological disorders, hyperactivity, and increased nighttime activity (Moreno-Salinas et al., 2019). These receptors, with their links to activity levels and locomotor function, provide further hypothetical targets for the fungal parasite to promote manipulated summiting in *Camponotus*.

We also predicted PPIs involving a putative host cAMP-dependent protein kinase catalytic subunit, found only between *Ophiocordyceps* and *Camponotus*, and a host G-protein subunit alpha, both of which serve important roles as components of GPCRs. *Ophiocordyceps* interference with host GPCR signaling has been previously hypothesized to be mediated, at least in part, by enterotoxins and other fungal protein toxins that interfere with cAMP levels and signal transduction (de Bekker et al., 2015; de Bekker, Ohm, et al., 2017; Kobmoo et al., 2018; Will et al., 2020).

Given the signal for GPCR interference, with both our broadest and narrowest analyses, and that many of these receptors/ligands overlap in their behavioral associations, we suggest that this provides evidence for host-parasite GPCR PPIs as one mechanism of behavioral manipulation used by *Ophiocordyceps*. The fungal counterparts in these host GPCR-parasite effector PPIs were often predicted in many interactions. These fungal proteins included five uSSPs upregulated during manipulation and correlated to manipulation in WGCNA modules (Will et al., 2020). Given that these fungal proteins lack annotations, we cannot yet determine if they might be agonists or antagonists and if they bind the ligand-site or elsewhere on the receptor. As these uSSPs were not predicted to be specific to only binding these host proteins, their interaction with the receptors may be less likely mediated by specific receptor-ligand binding sites than more generic contact sites elsewhere on the host protein. More specific functional testing would better reveal which of these GPCR PPIs, exactly, are genuine and how they act in detail.

We predicted over 300 PPIs that contributed to an enrichment of oxidation-reduction processes among the host proteins. Oxidation-reduction processes can be involved in a range of metabolic, development, and host-parasite interaction pathways that have been previously hypothesized to play a role in *Ophiocordyceps* infections (de Bekker et al., 2015; Dubovskii et al., 2010; Iwanicki et al., 2020; Will et al., 2020). Approximately half of the host proteins were putative cytochrome P450s, with enrichment signals for cytochrome P450s maintained after “aspecific” fungi filtering. In insects, cytochrome P450 proteins are often involved in hormone metabolism (e.g. ecdysone and juvenile hormone), stress response, xenobiotic detoxification processes, and cuticular development (Iga & Kataoka, 2012; Scott & Wen, 2001; Su et al., 2021; Sztal et al., 2012; Huidong Wang et al., 2018; Xing et al., 2021; B. zhong Zhang et

al., 2016). For example, we found multiple cytochrome p450 proteins bound uniquely by *Ophiocordyceps* of the 4C1 subtype that has been implicated in stress response (e.g., social isolation, temperature, UV, or starvation), insecticide/xenobiotic detoxification, and is regulated by hormones including juvenile hormone (Kim Lien et al., 2019; Lu et al., 1999; Scharf et al., 2021; Shen et al., 2021; Su et al., 2021). In turn, we predicted that many of those host cytochrome P450s interacted with fungal cytochrome P450s. Although cytochrome P450 proteins often act independently, homo- and hetero-dimer formation of cytochrome P450 proteins can modulate protein function in positive, negative, and substrate-specific manners (Kandel & Lampe, 2014). Oxidation-reduction related PPIs might most strongly represent the physiological stress and antagonistic cellular processes between host-parasite – however, fungal interference with these pathways may be critical elements of creating a host susceptible to manipulation.

These interpretations should bear in mind that cytochrome P450s, like some other fungal proteins in PPIs, are often associated with the endoplasmic reticulum. While we used multiple metrics to annotate the putative fungal secretome (Beckerson et al., 2019), these secretion signals are not foolproof and can be difficult to disentangle from organelle/membrane trafficking intracellular "secretion" functions. Whether or not all of these fungal proteins are being exported to the host environment and have functions there is, thus, unclear and will require functional validation.

Beyond GPCR-mediated and oxidation-reduction processes, we also found signatures that suggest that *Ophiocordyceps* could interfere with *Camponotus* gene regulation via proteins that bind host transcription factors and DNA- or mRNA-binding proteins. Using the host's own cells to dysregulate behavioral pathways could be metabolically favorable for the parasite. Some of the fungal proteins in these PPIs were uSSPs and we, therefore, cannot predict the functional result of those PPIs. However, a number of the parasite proteins were hydrolases. We suggest that these hydrolases most plausibly cleave host proteins or their post-translational modifications. Depending on the role of the protein and specific bond broken, parasite hydrolase activity could increase or decrease transcription of host genes. Among the ca. 200 host proteins in the nucleotide-binding category, we found many tied to behavioral activity via changes in locomotion, feeding/foraging, gravitaxis, light perception, circadian clocks, development and

social caste related hormones, and neuronal maintenance. However, very few of these putative targets passed the strictest shared "aspecific" host protein filter nor did any stand out from the rest by merit of biological relevance. As such, specific hypotheses about individual transcription factors are difficult to parse; however, as a group, many of these host proteins intersect with processes that appear relevant to manipulated ant behavior.

During manipulation, the fungus also secretes proteins predicted to bind host cuticle and connective tissue proteins. Being an endoparasite, *Ophiocordyceps* has direct access to internal host connective tissues, the endocuticle layer (once the epidermis is breached), and the cuticular lining of the gut and trachea, which include tissues that contain chitin (Burrows & Sutton, 2012; Davies, 1988). However, the fungal cell wall also contains chitin. As such, we cannot fully discount that some of these secreted fungal proteins may simply be cell wall modifying proteins that have falsely been predicted to bind chitin-associated ant proteins. Nevertheless, the fungus could be degrading cuticular proteins for nutrition, detaching muscles, or in preparation for emerging hyphae to later grow out of the host cadaver. Evidence for the disruption of the musculature stems from detailed microscopy work on *Ophiocordyceps*-infected ant heads at the time of manipulated summiting and biting behavior. These reports describe visual signs of fungal invasion, tissue degradation, and atrophy of manipulated ant mandible muscles (Fredericksen et al., 2017; Hughes et al., 2011; Mangold et al., 2019; Zheng et al., 2019). Future investigations documenting fungal growth and host tissue destruction at different stages and in body compartments beyond the head could be very insightful. Such data could allow us to better understand the mechanical effects of the fungus and how much of the disease phenotype is potentially caused by structural disintegration (de Bekker et al., 2021).

The predicted fungal interactors with host structural proteins included multiple proteases: a M43-like metalloprotease, S8 subtilisin-like serine peptidase, and disintegrin/reprolysin-like metalloprotease. S8 peptidases can degrade insect cuticle and are found in many fungi (Arnesen et al., 2018; St Leger et al., 1996). A reprolysin-like (or disintegrin, in snake venom) and another serine protease have been found in parasitoid wasp venom and hypothesized as candidates involved in immune suppression and virulence,

but not necessarily by proteolytic activity (Andrews & Berndt, 2000; Parkinson, Conyers, et al., 2002; Parkinson, Richards, et al., 2002). We also detected unannotated secreted proteins in cuticular protein PPIs that could have similar or different functions.

In addition to enrichments in functional annotations, we detected an enrichment of host PPI proteins in WGCNA modules that have been previously correlated to *Ophiocordyceps*-manipulated *Camponotus* (Will et al., 2020). In particular, a module enriched for neuronal processes and GPCRs contained many of the GPCRs that we found to be in PPIs with secreted, upregulated fungal proteins. Another host module correlated to manipulation was enriched for gene regulatory processes – which we also propose as possible targets for fungal disruption based on the functional PPI enrichments. Additionally, both upregulated and downregulated host DEGs (Will et al., 2020) were enriched in predicted PPIs, possibly indicating homeostatic responses to fungal modulation of functional protein levels. As such, the proteome-level predictions that we made in this study are well in line with our previous transcriptomics work, serving as an additional line of evidence for certain host processes and narrowing the pool of well-supported effector candidates involved with behavioral manipulation. However, any single protein or PPI should be considered with attention to D-SCRIPT error rates, having shown ca. 36% recall and 80% precision (Sledzieski et al., 2021). That is, for a given true PPI, D-SCRIPT will detect it only 36% of the time. And, of the predicted PPIs D-SCRIPT reports, only 80% of those will indeed be true PPIs. Therefore, searching for a specific hypothesized PPI, will often lead to no PPI found and 20% chance of what is found being a false-positive. As such, our results should well represent group-level trends (enriched annotations) and offer a subset of the mostly true PPIs to begin focusing attention on their functional characterization.

To narrow our results towards focusing on *Ophiocordyceps* proteins most likely to be effectors involved in manipulation of the ant host, we applied *Ophiocordyceps* self-interaction (Fig. 1 step 3), DEG (Fig. 1 step 4), and "aspecific" interaction filters (Fig. 1 step 6). We assumed that self-interaction homologs would more likely contain spurious or irrelevant PPI predictions that related to core cell functions such as protein chaperoning and folding and intracellular transport. Even though this

assumption would mean that some true parasite effectors that may target well-conserved biological processes and pathways would be overlooked. Similarly, possible PPIs of biological importance may not always include fungal proteins encoded by DEGs upregulated during host manipulation, but we suspected that most manipulation related PPIs would be. Future experiments and data analysis incorporating RNAseq data at finer timescales of *Ophiocordyceps* infection would allow greater exploration of PPIs during the course of infection. Moreover, many *Ophiocordyceps*-*Camponotus* PPIs were "aspecific" in that homologous predictions were made between other fungi and *Camponotus*. Similarly, most host proteins predicted in *Ophiocordyceps*-*Camponotus* PPIs were also predicted in at least one other "aspecific" fungal interaction (although the associated fungal proteins could be vastly different). This aided in the focusing on uniquely *Ophiocordyceps* signals and provided a view that was easier to interpret in the context of specialized interactions with its host, such as behavioral manipulation. However, this requires a trade-off between clarity and comprehensiveness; and, a PPI need not be unique to a certain cross-species interaction to be relevant (Beckerson et al., 2019). Additionally, computationally predicted protein binding does not indicate that all such interactions occur in dynamic physiological environments. In and around the cell, protein localization, transcriptional control, post-translational modification, competing PPIs, and biochemical conditions (e.g., pH) could affect the likelihood of a predicted PPI to actually occur. The cellular environments within fungal cells and insect cells could plausibly differ in many ways, shaping protein activity. Therefore, we used the "aspecific" interaction filters to help us focus on some of the, likely, more important PPIs involved in establishing *Ophiocordyceps* manipulation of ant behavior, but still anchored our analyses and discussion on the unfiltered data.

In multiple PPIs that we investigated in more depth, we found recurring fungal interaction partners. Despite effectors having multiple binding partners in many cases (Hogenhout et al., 2009; Win et al., 2012), extremely broadly associated fungal proteins perhaps reflected limitations related to our methods and so may not yield the strongest first hypotheses of specific protein interactions. For example, in nearly 400 PPIs, we found a fungal carboxylesterase that could have produced PPIs that were erroneous, stemming from the particular qualities of this protein. Carboxylesterases can have a range of

substrates and hydrolyze ester or amide (peptide) bonds using a well-conserved mechanism and set of amino acid residues shared with serine hydrolases, peptidases, and lipases (Hosokawa, 2008; D. Wang et al., 2018). Furthermore, carboxylesterases are often localized in the endoplasmic reticulum and involved in detoxification functions (Hosokawa, 2008; D. Wang et al., 2018). Given this protein could have an endoplasmic reticulum trafficking secretion signal, a broad substrate repertoire, and key amino acid residues shared with other enzymes, this protein could be a prime example of a difficult situation for our methods to disentangle true and false host-pathogen PPIs. We suspect behavioral manipulation to be a multi-layered and complex process, with interacting players and redundant processes. However, taken superficially, if *Ophiocordyceps* releases this and many other protein effectors that each dysregulate hundreds of host proteins, this might lead to a degree of physiological chaos at odds with the apparently finer-tuned aspects of manipulation phenotypes, such as timed-summiting or species-specificity (Araújo et al., 2018; de Bekker et al., 2015; de Bekker, Quevillon, et al., 2014; Evans et al., 2011; Lin et al., 2020; Sakolrak et al., 2018; Will et al., 2020).

Taken together, we produced multiple hypotheses connecting host-parasite cross-species PPIs to processes related to infection and manipulation of *Camponotus* by *Ophiocordyceps* that can be followed up with functional testing and real-world validations. Within this rich dataset we highlighted evidence for PPIs involving fungal S8 proteases and PPIs involving host oxidation-reduction processes, cuticular proteins, gene regulation, and GPCRs. Especially, with analytically robust results and direct biological links to behavior, we propose parasitic dysregulation of GPCR signaling as one mechanism of parasitic behavioral manipulation in this interaction. For specific GPCR PPIs, additional computational analyses could be taken to estimate where, structurally, fungal proteins are binding host proteins using D-SCRIPT's contact map outputs. These contact maps show the predicted residues that mediate the PPI binding. This information allows for better estimations of the likelihood of interaction and the type of interaction (e.g., if the fungal protein binds allosterically, in an active/ligand-binding site, etc.). For orphan receptors, this may even provide hypotheses about the identity of unknown endogenous ligands.

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Data availability: Data and analysis results are contained in Supp. Files S1 and S2. Questions about these data during the preprint stage should be directed to the authors. All Supp. Figs and Supp. Tables are in a single file "Supplementals_Willetal2022_biorxiv.pdf".

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