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 produce proteins that modulate virulence, alter host physiology, and trigger host responses. These proteins, classified as a type of "effector," often act via protein-protein interactions (PPIs). The fungal parasite *Ophiocordyceps camponoti-floridani* (zombie ant fungus) manipulates *Camponotus floridanus* (carpenter ant) behavior to promote transmission. The most striking aspect of this behavioral change is a summit disease phenotype where the infected host ascends and attaches to an elevated position. Plausibly, cross-species PPIs drive aspects of *Ophiocordyceps* infection and host manipulation. Machine learning PPI predictions 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 2,083 host proteins and 129 parasite proteins, which are encoded by genes upregulated during manipulated behavior. We identified multiple overrepresentations of functional annotations among these proteins. The strongest signals in the host highlighted neuromodulatory G-protein coupled receptors and oxidation-reduction processes. We also detected *Camponotus* structural and gene-regulatory proteins. In the parasite, we found enrichment of *Ophiocordyceps* proteases and frequent involvement of novel small secreted proteins with unknown functions. From these results, we provide new hypotheses on potential parasite effectors and host targets underlying zombie ant behavioral manipulation.

Introduction

Fungal parasites employ a diverse array of secreted molecules to defend themselves, promote infection, and modify their hosts. In certain cases, infection can even lead to parasitic manipulation of host behavior. Often termed "effectors," secreted fungal molecules play a

42 critical role in host-parasite dynamics that include both widely shared and highly specific
43 mechanisms [1–4]. Parasite effectors have been suggested to play key roles during the infection
44 of insects by entomopathogenic fungi through their interactions with host nucleic acids,
45 carbohydrates, lipids, small metabolites, and proteins [3,5–7]. Bioinformatic exploration of
46 effector protein biology offers a high-throughput method to develop mechanistic hypotheses of
47 how some parasites can modify the behavior of their hosts. Here, we use such approaches to
48 investigate the behavior-manipulating fungus *Ophiocordyceps camponoti-floridani* (Florida
49 zombie ant fungus) and its insect host *Camponotus floridanus* (Florida carpenter ant).

50 Myrmecophilous *Ophiocordyceps* fungi are typically species-specific parasites that have
51 co-evolved with their ant hosts over millions of years [8]. This close relationship has resulted in
52 fungus-ant interactions that alter host behaviors in ways that are adaptive for the parasite.
53 Manipulated host ants succumb to a summit disease, affixing themselves to elevated positions
54 and dying at locations that promote fungal growth and transmission [9–13]. Behavioral changes
55 preceding this final summit could include host-adaptive responses, parasite-adaptive
56 manipulations, or general symptoms of disease that span hyperactivity, uncoordinated foraging,
57 decreased nestmate communication, and convulsions [9,14–17]. While these modified ant
58 behaviors have been observed in nature for some time [18], explorations of the fungal effectors
59 involved are relatively more recent endeavors [14,19–25]. These multi "-omics" studies have
60 provided hypotheses about the parasite and host molecules that play a role in establishing the
61 behavioral phenotypes observed, but leave their potential interactions open to interpretation.

62 A large number of fungal molecules and ant pathways may be involved in
63 *Ophiocordyceps* behavioral manipulation, including neuro-modulators and -protectants, insect
64 hormones, feeding, locomotion, circadian rhythms and light-sensing, and muscular hyperactivity

65 [14,17,19,21,22,25–29]. Fungal proteins such as bacterial-like enterotoxins, protein tyrosine
66 phosphatases, peptidases (e.g., S8 subtilisin-like serine proteases), and undescribed small
67 secreted proteins (uSSPs) have been proposed as mechanisms of manipulation that presumably
68 function by PPIs. These hypotheses are supported by strong gene upregulation of these
69 candidates during active manipulation of the ant and genomic comparisons among the
70 *Ophiocordyceps* [14,19,24]. Numerous other such proteins are supported as plausible effectors as
71 well. Testing them *in vivo* will be a costly and laborious process in an emerging, non-traditional
72 model organism such as *O. camponoti-floridani*. New evidence linking previously hypothesized
73 candidate proteins and pathways to host-parasite protein-protein interactions (PPIs) would give
74 strong support to select top predictions for functional validation.

75 Proteome-scale bioinformatic prediction of PPIs has largely been employed to describe
76 protein interaction networks within a single organism, as was done for the host species, *C.*
77 *floridanus* [30]. However, PPI predictions in some cross-species host-parasite relationships have
78 also been made, including animal or plant hosts and bacterial, viral, or fungal parasites [31–34].
79 Predicting PPIs is typically done using either an interolog or machine learning method.
80 Interologs are orthologous interactions conserved across species: each protein in one PPI has
81 orthologs in the second PPI [35]. While a useful prediction tool, detecting an interolog in a new
82 system requires established reference PPIs from other systems. This constraint by available data
83 would result in a very limited number of predictions when analyzing interactions between non-
84 model organisms. Machine-learning methods offer a more flexible alternative. Here, predicted
85 PPIs are still informed by a known set of PPIs used for training data. However, once trained, the
86 program is able to predict novel interactions outside the original dataset [33,36]. The machine
87 learning tool D-SCRIPT does this by training models on putative protein structures rather than

88 primary sequence data [33,34]. The underlying predicted structural interactions were shown to
89 largely agree with a benchmarking dataset of experimentally measured docked proteins [33,37].
90 This physically-based approach improves cross-species generalizability of D-SCRIPT's
91 predictive power, an important step for research on non-model or multi-species systems.
92 Notably, *Ophiocordyceps* and other fungi secrete a range of taxonomically distinct uSSPs that
93 are often thought to be effectors in host-parasite interactions [14,19,24,38–40]. Therefore,
94 bioinformatic techniques that hinge upon well described protein annotations or are subject to
95 strong species-specific biases can only have limited success in assigning uSSPs to PPIs.
96 We used D-SCRIPT to predict possible *O. camponoti-floridani* and *C. floridanus* PPIs
97 involved in behavioral manipulation. We filtered these predictions into subsets we considered
98 most likely to include relevant interactions. As such, our study focuses on PPIs involving
99 putatively secreted *O. camponoti-floridani* proteins encoded by genes that were previously found
100 to be upregulated during manipulated summiting [19]. These fungal proteins are plausibly
101 exported to the host environment during manipulation, providing support for their involvement
102 in hypotheses of manipulation of host behavior. We subsequently performed group-level
103 enrichment analyses for both host and parasite proteins involved in these PPIs. We chose this
104 approach in consideration of D-SCRIPT error rates having shown ca. 35% recall (i.e., 65% false
105 negatives) and ca. 80% precision (i.e., 20% false positives) [33], which indicate this tool is
106 strongest in broad characterizations rather than validating specific, previously hypothesized PPIs.
107 Our results should well represent group-level trends (enriched annotations) and offer a subset of
108 the mostly true PPIs to begin focusing attention on their functional characterization.
109 We further highlight aspects of functional enrichments after filtering out *Camponotus*
110 protein interactions with fungi that do not naturally infect or manipulate this host ("aspecific"

111 fungi). These aspecific fungi cover different lifestyles and phylogenetic relationships to
112 *Ophiocordyceps*: (*i*) *Cordyceps bassiana* (i.e., *Beauveria bassiana*), a generalist entomopathogen
113 in the order Hypocreales (which includes *Ophiocordyceps*), (*ii*) *Trichoderma reesei*, a plant-
114 degrading saprophyte in the order Hypocreales (although the genus also includes
115 entomopathogens), and (*iii*) *Saccharomyces cerevisiae*, a phylogenetically distant saprophytic
116 yeast in the order Saccharomycetales with a smaller secretome. This approach brought the
117 unique *Ophiocordyceps* PPIs to the forefront but does not discount the aspecific predictions.
118 Rather it brings additional attention to a narrower set of specific PPIs that could include co-
119 evolved adaptations underlying *Ophiocordyceps*-manipulated *Camponotus* behavior.
120

121 **Materials and Methods**

122 **Protein sequences and initial selection:** To predict PPIs between *O. camponoti-*
123 *floridani* secreted proteins and *C. floridanus* proteins, we retrieved sequence information for both
124 organisms from high-quality genome assemblies integrating long-read technology (GenBank
125 accessions GCA_012980515.1 and GCA_003227725.1, respectively) [19,41]. For *Camponotus*
126 proteins, we used the sequence represented by the single longest isoform (n = 12,512). We
127 predicted *Ophiocordyceps* proteins to have a localization outside of the cell by combining eight
128 annotation tools (SignalP v6.0, TMHMM, Phobius, Prosite, PredGPI, NucPred, and TargetP
129 v2.0) [42–48] following the relatively conservative approaches outlined in Beckerson et al., 2019
130 [1]. We focused on secreted proteins (n = 586) and excluded transmembrane proteins (n = 1,301)
131 in our downstream analyses, as we considered these to most plausibly constitute effectors.
132 Moreover, our preliminary findings showed that transmembrane proteins were generally less
133 specific in their binding (File S1). All remaining non-secreted, non-transmembrane proteins were

134 considered intracellular (n = 5,568). We annotated secreted proteins as uSSPs if they were under
135 300 amino acids in length and lacked any BLAST description, GO term, or PFAM domain (n =
136 154) [19].

137 **PPI predictions:** We used D-SCRIPT (v 0.1.5) with the default human-protein pre-
138 trained model and settings [33] to predict PPIs. D-SCRIPT assigns each tested protein interaction
139 an edge value (i.e., a confidence score to rank possible PPIs) with a default threshold for positive
140 prediction of ≥ 0.5, on a scale of zero to one. These parameters have been previously used to
141 predict PPIs within insect and fungal model organisms [33]. With these settings, we tested every
142 PPI combination between secreted *O. camponoti-floridani* proteins and the entire *C. floridanus*
143 proteome (Fig. 1 step 1). We used proteins shorter than 2,000 amino acids due to computational
144 constraints. Limiting the length of input proteins removed 63 (1.1%) intracellular
145 *Ophiocordyceps* proteins and 299 *Camponotus* proteins (2.4%).

146 To identify self-interacting *Ophiocordyceps* PPIs, we tested secreted *Ophiocordyceps*
147 proteins against intracellular *Ophiocordyceps* proteins (Fig. 1 step 2). We then identified
148 reciprocal homologs (putative orthologs) between *Ophiocordyceps* and *Camponotus* proteins
149 using Proteinortho (v 5.0) with default settings [49]. We filtered out PPIs that contained a fungal
150 secreted protein predicted to bind both one of *Ophiocordyceps*' own intracellular proteins and a
151 *Camponotus* protein that was orthologous to that fungal intracellular protein (Fig. 1 step 3). We
152 reasoned that a cross-species PPI would more likely indicate an interaction related to infection or
153 manipulation when the *Ophiocordyceps* protein was predicted to only bind a *Camponotus*
154 protein, without also binding a fungal ortholog to that ant protein. We considered this to be a
155 favorable tradeoff with possibly overlooking parasite effectors that may target well-conserved
156 biological processes.

157 We narrowed the remaining PPIs to those involving secreted fungal proteins encoded by
158 differentially expressed genes (DEGs) (Fig. 1 step 4). These genes were upregulated during
159 manipulated summiting behavior in *C. floridanus* ants experimentally infected with *O.*
160 *camponoti-floridani*, as compared to their expression in culture [19]. By incorporating empirical
161 gene expression data, we anchored our computational analyses in predictions that are most
162 biologically relevant during manipulation. Although the chance exists that some parasite
163 effectors act during manipulation without increased gene transcription, we presumed that in most
164 cases, gene expression would be informative.

165 **Hypergeometric enrichment analyses:** We performed hypergeometric enrichment
166 analyses on proteins from PPIs with secreted, upregulated *Ophiocordyceps* proteins that only had
167 predicted interactions with the host. For enrichment analyses of the fungal proteins in these PPIs,
168 we used the *O. camponoti-floridani* secretome as the background. For enrichment analyses of the
169 ant proteins in these PPIs, we used the *C. floridanus* proteome as the background (Fig. 1, step 5).

170 We examined annotated GO terms (biological processes and molecular functions
171 combined) [50], PFAM domains [51], and weighted gene co-expression network analysis
172 (WGCNA) [52] module memberships reported in the transcriptomics study from which we also
173 obtained the above-mentioned DEGs [19]. These WGCNA modules correlated expression of
174 gene-networks to sample types in that study, i.e., *i*) healthy ant controls, *ii*) *in vitro* fungus
175 controls, *iii*) active fungus-manipulated ants, and *iv*) dying fungus-manipulated ants. These
176 modules were additionally correlated directly between *Ophiocordyceps* gene modules and
177 *Camponotus* gene modules [19]. We referenced their previously assigned names following the
178 convention of ant-module-1 (A1) and fungus-module-1 (F1) as used in that study [19]. For
179 *Camponotus* proteins, we also tested for enrichments of DEG class (i.e., upregulated or

180 downregulated gene expression from healthy controls to active manipulated ants) [19]. For
181 *Ophiocordyceps* proteins, we additionally tested for enrichment of uSSP annotations.
182 We performed the hypergeometric enrichment analyses with the R package
183 timecourseRNAseq (v 0.0.9000) using default settings (significant enrichment at FDR ≤ 0.05) in
184 R studio (v 2021.09.2) with R (v 4.1) [53–55]. We plotted GO terms in semantic space to cluster
185 related terms based on R code generated from REVIGO but did not remove any GO terms (R
186 package ggplot2, v 3.3.5) [56,57].
187 "**Aspecific" fungus-*Camponotus* PPI combinations:** For more selective analyses of
188 species-specific *Ophiocordyceps*-*Camponotus* PPIs, we produced "aspecific" PPIs from the
189 secretomes of other fungi tested against the *C. floridanus* proteome. Removal of more common
190 PPIs, leaving only those potentially unique to *Ophiocordyceps*, provided a view that was easier
191 to interpret in the context of specialized parasite-host interactions, such as behavioral
192 manipulation. However, a PPI need not be unique to a certain cross-species interaction to be
193 relevant [1]. Additionally, computationally predicted protein binding does not indicate that all
194 such interactions occur in dynamic physiological environments. In and around the cell, protein
195 localization, transcriptional control, post-translational modification, competing PPIs, and
196 biochemical conditions (e.g., pH) could affect the likelihood of a predicted PPI to occur. The
197 cellular environments within fungal cells and insect cells could plausibly differ in many ways,
198 shaping protein activity. Therefore, we used the removal of aspecific interactions to help us focus
199 on some of the, likely, more important PPIs involved in establishing *Ophiocordyceps*
200 manipulation of ant behavior, but still anchored our analyses and discussion on the larger dataset.
201 We chose three fungi based on lifestyle, phylogeny, and availability of well-annotated
202 genomes to generate aspecific PPIs: *C. bassiana* (GenBank accession GCA_000280675.1), *T.*

203 *reesei* (GenBank accession GCA_000167675.2), and *S. cerevisiae* (GenBank accession
204 GCF_000146045.2) [7,58,59]. As for *Ophiocordyceps* (see above), we predicted each fungal
205 secretome and removed secreted proteins over 2,000 amino acids long, which only removed one
206 *T. reesei* protein (0.002%). The secretomes of *C. bassiana*, *T. reesei*, and *S. cerevisiae* fungi
207 included 833, 505, and 153 proteins, respectively. These proteins were then paired with the
208 *Camponotus* proteome for D-SCRIPT predictions (see above).

209 To find orthologous proteins between *Ophiocordyceps* and each of the aspecific fungi we
210 used Proteinortho (v 6.0) with default settings while enforcing blastp+ and single-best reciprocal
211 hits (i.e., -sim=1) [49]. Using this information, we identified shared PPIs between
212 *Ophiocordyceps* and *Camponotus*, and aspecific fungi and *Camponotus*; i.e., interactions
213 between orthologous fungal proteins and the same ant protein (Fig. S1). More stringently, we
214 also applied a filter that eliminated shared host proteins with aspecific fungal interactions,
215 regardless of orthology. This resulted in a set of *Ophiocordyceps* PPIs where the *Camponotus*
216 protein was never predicted in any aspecific PPI (Fig. S1). After removing either shared PPIs or
217 shared host proteins, we again performed enrichment analyses (see above) to investigate
218 overrepresented annotations among the *Ophiocordyceps*-specific PPIs (Fig. 1 step 6). To
219 visualize changes in PPI connectivity and abundance that resulted from these aspecific filters, we
220 used Cytoscape (v 3.8.2) to produce networks of host proteins and their respective
221 *Ophiocordyceps* binding partners [60].

222 **Data availability –** The genome assemblies used for this study are available on NCBI:
223 *O. camponoti-floridani* (GCA_012980515.1), *C. floridanus* (GCA_003227725.1), *C. bassiana*
224 (GCA_000280675.1), *T. reesei* (GCA_000167675.2), and *S. cerevisiae* (GCF_000146045.2)
225 [7,19,41,58,59]. Transcriptomic sequence data for *O. camponoti-floridani* and *C. floridanus* are

226 also hosted on NCBI (BioProject PRJNA600972) [19]. Analyzed transcriptomic data used here
227 are available on figshare, as supplemental information associated with their original publication
228 (https://doi.org/10.25387/g3.12121659) [19].
229
230 **Results**
231 ***Ophiocordyceps-Camponotus* PPIs:** We tested a total of 7,156,818 potential cross-
232 species PPIs between the parasite secretome and host proteome (Fig. 1 step 1). Of these possible
233 protein-protein combinations, 0.33% were positively predicted as PPIs (n = 23,629 PPIs) (Table
234 1, Fig. S2). This result is in line with a previous 0.95% positive D-SCRIPT prediction rate from a
235 single-species dataset testing 50 million potential PPIs [33].
236 From these positively predicted cross-species interactions, we removed 2,850 PPIs that
237 were homologous to within-*Ophiocordyceps* interactions (Fig. 1 step 2 and 3, Table 1). We then
238 selected those involving *Ophiocordyceps* proteins encoded by upregulated DEGs during
239 manipulation. This resulted in 6,011 predicted PPIs. We used this set of putative effector-host
240 PPIs, containing 129 unique *Ophiocordyceps* proteins and 2,083 unique *Camponotus* proteins,
241 for further analyses (Fig. 1 step 4, Table 1, File S2).
242 **PPIs between aspecific fungi and *Camponotus* in comparison to *Ophiocordyceps*:** The
243 proportions of positive PPI predictions between aspecific fungi and *C. floridanus* (range 0.26-
244 0.28%) and secreted proteins predicted to be in at least one PPI (range 68-76%) were similar to
245 the values observed for *Ophiocordyceps* (0.33% and 69%, respectively) (Table 1, Table S1).
246 Eliminating orthologous PPIs between *Ophiocordyceps* and any of the aspecific fungi (i.e.,
247 shared PPIs) removed 1,776 of the 6,011 (30%) *Ophiocordyceps-Camponotus* PPIs (Table 2,
248 Fig. S1). Eliminating PPIs involving host proteins that were also predicted to be bound by

249 aspecific fungi, regardless of orthology of the fungal binding partner (i.e., shared host proteins),
250 removed 1,936 of 2,083 (93%) host proteins, leaving only 157 *Ophiocordyceps-Camponotus*
251 PPIs (3%) (Table 2, Fig. S1). In line with secretome size, phylogeny, and entomopathogenic
252 lifestyle, *C. bassiana* shared the most overlap with *Ophiocordyceps* predictions (Fig. S1). As
253 expected, removing PPIs involving shared host proteins led to major changes in enrichment
254 results while removing shared PPIs led to more modest changes (Table 2, File S3). We primarily
255 report the results based on all three fungi that we included in this study. However, those based on
256 *C. bassiana* alone were largely similar (Fig. S1, File S3). While these filters assisted us in
257 ordering and emphasizing results (i.e., signals maintained through the strictest filters are being
258 prioritized), we did not use this approach to wholly select PPIs of interest. Results presented
259 below only use aspecific filters where stated.

260 ***Ophiocordyceps* enrichment signals:** We found that *Ophiocordyceps* proteins in PPIs
261 with *Camponotus* proteins were enriched for one PFAM domain and three WGCNA modules
262 (Fig. 2, Table 2).

263 **S8 serine peptidases –** The enriched PFAM domain was a "peptidase S8 domain,"
264 indicating an overrepresentation of fungal serine proteases that could cleave host proteins. The
265 five fungal peptidase S8 proteins in this enrichment were predicted to interact with 34 host
266 proteins across 39 PPIs (File S2). Among these host proteins, there were seven kinesin-like
267 proteins (motor proteins), five nuclear pore proteins, and a (pro-)resilin that is an insect cuticle
268 and connective tissue protein [61]. Even after removing PPIs that were shared with aspecific
269 fungi, the enrichment signal for S8 peptidases was retained. However, the much more stringent
270 removal of shared host targets eliminated the S8 peptidase enrichment result (Fig. 1 step 6, Table
271 2).

272 **Fungal manipulation WGCNA modules –** We also found fungal WGCNA modules F1,
273 F2, and F3 to be enriched (Fig. 2). Modules F1 and F2 were previously described to have
274 significant positive correlations to manipulation. This means that most of the fungal genes in
275 those modules were upregulated, in a similar co-expressed manner, during manipulated
276 summiting behavior. As such, these modules contained multiple genes hypothesized to mediate
277 infection or manipulation processes (uSSPs and hypothesized effectors modules) (Fig. 2). With
278 fungal gene expression clearly negatively correlated with control cultures, module F3 was
279 modestly positively correlated to fungi in both manipulated hosts and dying hosts. This module
280 largely contained genes related to core cell processes in the fungus (e.g., DNA packaging) (Fig.
281 2). All three fungal modules also had significant direct correlations to ant WGCNA modules,
282 with F1 being negatively correlated to ant modules A14 and A15 – both associated with host
283 neuronal processes (Fig. 2). Core cell processes module F3 was positively correlated with
284 neuronal process module A15 [19]. The overrepresentations of all three fungal WGCNA
285 modules was retained after eliminating shared PPIs, but uSSP and effectors module F1 was lost
286 after removing shared host proteins (Table 2).

287 ***Camponotus* enrichment signals:** We found that *Camponotus* PPI proteins were
288 enriched for 50 GO terms, 74 PFAM domains and nine WGCNA modules (Table 2). As GO
289 term and PFAM domain enrichments often indicated similar functions, we present results largely
290 from the GO term perspective. We highlight PFAM domains for emphasis or to discuss
291 biologically interesting results not well captured by GO terms alone.

292 **G protein-coupled receptor signaling –** We detected GO term enrichments for "G
293 protein-coupled receptor (GPCR) activity" and "GPCR signaling pathway" (Fig. 3). These
294 enrichments resulted from 71 GPCR-related PPIs with 16 unique *Ophiocordyceps* proteins (File

295 S2). All but two receptor proteins in these PPIs carried the PFAM domain "7 transmembrane
296 receptor (rhodopsin family)." We found 33 unique GPCR-related protein genes (of 128 possible
297 with these GO terms), with a range of putative ligands: 11 neuropeptides (n = 12 receptors), four
298 biogenic monoamines (dopamine, serotonin, octopamine, tyramine) (n = 11 receptors),
299 acetylcholine (n = 2 receptors), a spider venom toxin protein (alpha-latrotoxin) (n = 1 receptor),
300 and opsin blue- and ultraviolet-sensitive receptors (n = 2 receptors). Fourteen of these 33
301 *Camponotus* receptors (or receptor subunits) belonged in ant-neuronal processes WGCNA
302 modules A14 and A15, which showed overall reduced expression during manipulation compared
303 to healthy ants (i.e., the modules were negatively correlated to manipulation) [19]. Host GPCR-
304 related PPI proteins were predicted to bind 16 fungal proteins that included five uSSPs, a
305 carboxylesterase, a glycosyl hydrolase, a CAP cysteine-rich secretory protein, and a protein
306 tyrosine phosphatase. Most of these fungal proteins were previously assigned to fungal uSSPs
307 and effectors module F2 (n = 9) but included some in modules F1-4 (File S2), which all were
308 also correlated to infection and manipulation of hosts.

309 The enrichment signals for GPCRs were retained after we removed both PPIs and host
310 proteins shared with aspecific fungi (Fig. 4). Twenty-eight of the 33 GPCR host proteins
311 remained after removing shared PPIs. However, only seven remained after filtering out shared
312 host proteins. These host proteins, predicted to be uniquely bound by *Ophiocordyceps* during
313 manipulation, were a putative dopamine receptor 2, Oamb octopamine receptor, methuselah-like
314 10 receptor, orexin receptor, trissin receptor, cAMP-dependent protein kinase subunit, and
315 receptor without an informative BLAST description ("uncharacterized protein"). The four
316 *Ophiocordyceps* proteins binding these host receptors were predicted in many PPIs (range = 101
317 to 458). The dopamine receptor 2, Oamb octopamine receptor, and uncharacterized receptor

318 shared the same fungal uSSP partner. The orexin and trissin receptors also shared an unannotated
319 secreted fungal protein (larger than an uSSP).

320 **Oxidation-reduction –** We also found the enrichment of oxidation-reduction functions
321 among *Camponotus* proteins in PPIs (six supporting GO terms) (Fig. 3, File S2). Altogether, 159
322 host proteins had oxidation-reduction annotations, forming 321 PPIs with 48 parasite proteins. A
323 plurality of the host proteins were encoded by cytochrome P450 genes (70 unique genes, 15
324 putative subtypes). This abundance of cytochrome P450, a hemoprotein, also contributed to the
325 enrichment of "heme-binding" and "iron ion binding" GO terms (Fig. 3) and the PFAM domain
326 "cytochrome P450" (File S2). These *Camponotus* cytochrome P450 proteins paired with 10
327 unique *Ophiocordyceps* genes, consisting of uSSPs and fungal cytochrome P450s. Other notable
328 contributors to the host enrichment of oxidation reduction related functions were peroxiredoxins,
329 laccases, dehydrogenases, and a vat-1 synaptic vesicle membrane-like protein (File S2). The 48
330 fungal proteins included cytochrome P450s, oxidases and oxidoreductases, amidases, peptidases,
331 and an enterotoxin.

332 Enrichment signals for four of the six oxidation-reduction GO terms were retained after
333 we removed PPIs that were shared with the aspecific fungi. Only two remained after we applied
334 the more stringent shared host protein filter (File S3). Additionally, indicators of cytochromes,
335 heme and iron binding GO terms and the "cytochrome P450" PFAM domain, continued to be
336 enriched after either filter (File S3). Most host proteins passed the shared PPI filter (140 of 159),
337 but only 19 remained after removing shared host proteins (Fig. 4). Cytochrome P450 proteins
338 shifted from a plurality to a dominant majority after shared host protein filtering (17 of 19
339 proteins), with eight cytochrome P450 subtypes: 3A19, 315a1, 4C1, 49a1, 6a13, 6a14, 6k1, and
340 9e2.

341 **Transcription and DNA-binding –** Multiple enrichments that can be associated to gene
342 transcription and regulation were enriched. We found 204 host proteins annotated for any of
343 "DNA binding," "DNA-binding transcription factor activity," "sequence-specific DNA binding,"
344 "DNA helicase activity," "regulation of transcription, DNA-templated," and "mRNA binding"
345 GO terms (Fig. 3). These proteins similarly contributed to the high enrichment of PFAM
346 domains such as "helix-loop-helix DNA-binding domain," "zinc Finger C4 type," "'paired box'
347 domain," and "bZIP transcription factor" (File S2). These 204 host proteins interacted with 43
348 fungal proteins to generate 922 PPIs. Among the 204 host proteins, we found transcription
349 factors and regulators with many functions, which included examples associated with insect
350 behavior and neuronal function such as Mushroom body large type Kenyon specific protein 1
351 (e.g., elevated in worker bees, foraging behavior), Gooseberry (e.g., neuromuscular junction
352 function), Hairy (e.g., juvenile hormone mediated gene repression), photoreceptor specific
353 nuclear receptor (light sensing), CLOCK (circadian clocks), and steroid and ecdysone nuclear
354 receptors and induced proteins [62–66]. Other proteins, most clearly understood from the
355 neuronal development perspective thus far, produce atypical behavioral or locomotor phenotypes
356 when dysregulated and include Jim Lovell, Dead ringer, Hairy/enhancer-of-split related,
357 Achaete-scute complex proteins, and Forkhead [67–72]. These 34 proteins taken as biologically
358 relevant examples account for 186 of the 922 gene regulation PPIs, interacting with 20
359 *Ophiocordyceps* proteins covering a similar breadth of protein types as the 43 parasite proteins
360 overall (File S2). The 43 fungal proteins included five uSSPs that were also found in the PPIs
361 reported above (Fig. 4A), three that were not, and various hydrolases, oxidoreductases,
362 peptidases, and carboxylesterases.

363 All of the aforementioned GO terms and PFAM domains continued to be enriched after
364 removing shared PPIs (191 of 204 host proteins retained), but none of these annotations
365 continued to be enriched after removing shared aspecific host proteins (five host proteins
366 retained) (Fig. 4, File S3).

367 **Cuticular proteins –** We found 28 ant proteins that shared the enriched GO term
368 "structural constituent of cuticle" (Fig. 3) and PFAM domain "insect cuticle protein" (File S2).
369 We predicted these ant proteins interacted with 22 fungal proteins to form 138 PPIs (Fig. 4A).
370 These host proteins included (pro-)resilin, endocuticle structural proteins, and a range of "cuticle
371 proteins" (e.g., "cuticle protein 7"). The fungal proteins included a CAP protein and a
372 carboxylesterase also predicted to bind GPCRs (see above). Four fungal uSSPs were shared with
373 the transcription and DNA-binding host proteins. We additionally identified a glycosyl
374 hydrolase, a disintegrin/reprolysin-like peptidase, a S8 subtilisin-like serine peptidase, M43
375 peptidases, an uSSP, and an unannotated secreted protein (larger than an uSSP). These
376 enrichments were only kept under the aspecific shared PPI filter (with all 28 original cuticular
377 proteins) and lost with the shared host protein filter (none of the 28 proteins were uniquely bound
378 by *Ophiocordyceps*) (Fig. 4, File S3).

379 **Ant DEGs and WGCNA modules –***Camponotus* genes that were previously determined
380 to be differentially expressed during manipulation, as well as host gene modules, which were
381 found to be correlated to manipulation, were enriched among PPI proteins (File S2). Both up-
382 and downregulated host DEGs were enriched. The upregulated host DEGs were enriched for
383 gene/DNA regulatory functions and the downregulated DEGs for oxidation-reduction (File S2).
384 Three ant WGCNA modules were enriched that had significant correlations to manipulation,
385 with an additional six that did not (of 22 possible modules total). Neuronal processes module

386 A14 was negatively correlated to manipulation and fungal uSSP and effectors module F1 (Fig.
387 2). This host module included many genes putatively associated with neuronal function,
388 including GPCRs reported above [19]. The other host neuronal processes module, A15, was not
389 enriched for PPIs. Module A10 was positively correlated to manipulation and contained
390 enrichment signals for GO terms indicating signal transduction and transcription [19] (Fig. 2).
391 Module A4 had a positive correlation to manipulation and contained enrichment signals for
392 proteasomes and odor detection [19] (Fig. 2). All three of these host modules passed the shared
393 PPI filter but did not persist after removing shared host targets with aspecific fungi.
394

395 **Discussion**

396 The inter-species molecular interactions during manipulation of *C. floridanus* by *O.*
397 *camponoti-floridani* are likely mediated by parasite effector proteins that target host proteins. To
398 predict such protein-protein interactions (PPIs), we used the machine learning tool D-SCRIPT,
399 which infers structural relationships between proteins without relying on protein-wide sequence
400 homology to known interactions. This flexibility allows D-SCRIPT models to extend predictions
401 across organisms and beyond previously documented PPIs. As such, D-SCRIPT was able to
402 predict several thousand PPIs between *Camponotus* host proteins and upregulated, secreted
403 *Ophiocordyceps* proteins.

404 Due to the unfamiliar terrain of leveraging this approach between non-model species, we
405 sought to propose hypotheses balancing biological plausibility and the possibility of novel
406 interactions. On the one hand, we considered PPI predictions in the light of previous knowledge
407 of protein and cellular function. However, since the molecular aspects of parasitic manipulation
408 are still largely to be uncovered, we also considered that undescribed or unusual mechanisms

409 may underlie these interactions. Often, we found that single proteins, especially from
410 *Ophiocordyceps*, were in multiple host-parasite PPIs. Although in some cases these could be
411 errant predictions (D-SCRIPT's precision rate is 80%) [33], *Ophiocordyceps* effectors may
412 indeed bind many host proteins across different pathways. This is a commonly observed
413 phenomenon in microbe-plant interactions, and is possibly even the norm [2,4]. Moreover, we
414 found that only the S8 peptidase annotation term was enriched among *Ophiocordyceps* secreted,
415 upregulated proteins that were in PPIs with *Camponotus* proteins. This finding indicates that the
416 PPI proteins are not a sharply distinct subset from the fungal secretome (i.e., background
417 proteins of the enrichment analysis), which is functionally enriched for pathogenesis and
418 proteolytic activity overall [19]. In contrast, we detected many enriched annotations for the host
419 side of the interactions.

420 ***Ophiocordyceps* proteolysis of core intracellular host proteins –** Fungal PPI proteins
421 were enriched for S8 peptidase domains, indicating that a significant number of these proteins
422 had proteolytic functions with possibly diverse targets [73]. Cuticular host proteins are a
423 canonical target for such effectors as we discuss below. Additionally, multiple host kinesin
424 motor proteins were in PPIs with these *Ophiocordyceps* proteases. Kinesins play a role in mitotic
425 and intracellular transport processes – with key functions in vesicle transport towards the
426 periphery of neurons [74]. Defects in kinesin activity can lead to dysregulated neurotransmitter
427 release at synapses, thereby contributing to impaired neurological functions and development
428 [74,75]. From these predicted S8 peptidase-kinesin interactions, we infer that the fungus may be
429 dysregulating core host cell processes. The effects of which possibly impair neuron signaling and
430 neurotransmitter release to contribute to modified behavioral phenotypes. We also predicted
431 these peptidases to interact with multiple *Camponotus* nuclear pore proteins. Although some

432 components of the nuclear pore undergo proteolytic post-translational modification to remove
433 SUMOylation [76,77], those peptidases are in a different family than S8 peptidases. If the
434 predicted S8 peptidase-nucleopore proteins interactions are genuine, this could mean the parasite
435 alters transport of molecules in and out of the host nucleus. Speculatively, this could facilitate
436 entry of the fungal proteins that bind host transcription factors.

437 ***Camponotus* G protein-coupled receptors as targets to modify behavior –** We
438 detected over 30 ant GPCRs or GPCR subunits in PPIs with fungal proteins. Most GPCRs were
439 from the rhodopsin/A family and included receptors related to biogenic monoamines,
440 acetylcholine, and neuropeptides. These receptors offer a wide range of hypothetical connections
441 to manipulated ant behavior. Commonly, these receptors and their ligands have been linked to
442 locomotion, feeding, and circadian rhythms – processes that have been previously highlighted in
443 hypotheses of *Ophiocordyceps* and other fungus-insect summit-disease behavioral manipulations
444 [10,14,19,78,79].

445 The monoamine neurotransmitters dopamine, serotonin, octopamine (analogous to
446 vertebrate norepinephrine), and tyramine have been implicated in modulating locomotor,
447 foraging, learning, social, reproductive, and aggressive behaviors in many insects, with
448 experimental evidence in social insects, including ants [80–85]. Compared to the aspecific fungi
449 tested, both a *C. floridanus* dopamine and octopamine receptor were uniquely bound by
450 *Ophiocordyceps* upregulated secreted proteins. This interaction hypothetically implicates these
451 host receptors in specific *Ophiocordyceps* interactions such as behavioral manipulation. We
452 additionally detected two muscarinic acetylcholine receptors associated with sensory and motor
453 neurons in non-insect animals [86–88]. In bees, excessive activation of acetylcholine receptors
454 can cause changes in locomotor, navigational, foraging, and social behaviors [89]. Changes in

455 such behaviors have also been observed in *C. floridanus* ants infected with *O. camponoti-*
456 *floridani*. Locomotor behavior is enhanced in these ants, while social behaviors are diminished,
457 daily timed foraging activities become arrhythmic and navigation capabilities appear less
458 effective [17].

459 The neuropeptide GPCRs in PPIs had a range of putative ligands linking parasite
460 interference with behavior-regulating processes such as circadian rhythm, nutritional signaling,
461 insect hormones, and odor reception. These GPCRs included a host trissin receptor and an
462 allatotropin receptor (i.e., annotated as an "orexin" receptor, but likely an allatotropin receptor in
463 insects), which, compared to the aspecific fungi, only *Ophiocordyceps* was predicted to bind.
464 Between these two receptors, dysregulation of feeding and circadian control of locomotor
465 activity, juvenile hormone production, immune activation, and muscle stimulation are possible
466 effects [90–96]. Circadian rhythm is related to ant foraging behavior (and, perhaps indirectly,
467 feeding) and is affected by *Ophiocordyceps* infection [17]. The synchronized time-of-day
468 summiting and biting behavior further suggests a role for circadian control of activity [15,19,23].
469 Interlaced with feeding and foraging processes in ants, juvenile hormone mediates
470 insulin/feeding related signaling and social caste identity (e.g., foraging workers) [28,97–99].
471 Among the host neuropeptide GPCR targets shared with the aspecific fungi, we predicted
472 binding of receptors also associated with locomotion and activity levels, feeding, aggression,
473 muscle control, juvenile hormone, reproductive behavior, or pheromones that included those
474 activated by: allatostatin-A [100–103], adipokinetic hormone or corazonin (i.e., a "gondaotropin-
475 releasing hormone" receptor) [104], cholecystokinin-like peptides [105], CCHamide-2 [106], and
476 pyrokinin, pheromone biosynthesis activating neuropeptide (PBAN) and/or capa type
477 neuropeptides [107–109]. Odor and pheromone detection are key elements of ant

478 communication, behavioral regulation, and social interactions [110–112]. Possibly, GPCRs
479 typically involved in odorant response could become dysregulated by fungal effectors to dampen
480 nestmate interaction and/or induce aberrant behavior without normal cues. Taken together, we
481 found multiple PPIs involving ant GPCRs putatively involved in signaling systems that could
482 control which and when certain behaviors are performed by manipulated ants.

483 We also predicted PPIs involving a putative host cAMP-dependent protein kinase
484 catalytic subunit, found only between *Ophiocordyceps* and *Camponotus*, and a host G-protein
485 subunit alpha, both of which serve important roles as components of GPCRs. This alpha subunit
486 was also found previously to be downregulated in manipulated *Camponotus* [19].
487 *Ophiocordyceps* interference with host GPCR signaling has been previously hypothesized to be
488 mediated, at least in part, by enterotoxins and other fungal protein toxins that interfere with
489 cAMP levels and signal transduction [14,19,24,25].

490 Given the signal for GPCR interference, with both our broadest and narrowest analyses,
491 and that many of these receptors/ligands overlap in their behavioral associations, we suggest that
492 this provides evidence for host-parasite GPCR PPIs as one mechanism of behavioral
493 manipulation used by *Ophiocordyceps*. The fungal counterparts in these host GPCR-parasite
494 effector PPIs were often predicted in many interactions. These fungal proteins included five
495 uSSPs upregulated during manipulation and correlated to manipulation in WGCNA modules
496 [19]. Given that these fungal proteins lack annotations, we cannot yet determine if they might be
497 agonists or antagonists and if they bind the ligand-site or elsewhere on the receptor. As these
498 uSSPs were not predicted to be specific to only binding these host proteins, their interaction with
499 the receptors may be less likely mediated by specific receptor-ligand binding sites than more
500 generic contact sites elsewhere on the host protein.

501 **Host-pathogen interactions involving oxidation-reduction –** We predicted over 300
502 PPIs that contributed to an enrichment of oxidation-reduction processes among the host proteins.
503 Oxidation-reduction processes can be involved in a range of metabolic, development, and host-
504 parasite interaction pathways that have been previously hypothesized to play a role in
505 *Ophiocordyceps* infections [14,19,113,114]. Approximately half of the host proteins were
506 putative cytochrome P450s, with enrichment signals for cytochrome P450s maintained after
507 aspecific fungi filtering. In insects, cytochrome P450 proteins are often involved in hormone
508 metabolism (e.g. ecdysone and juvenile hormone), stress response, xenobiotic detoxification
509 processes, and cuticular development [115–121]. For example, we found multiple cytochrome
510 p450 proteins bound uniquely by *Ophiocordyceps* of the 4C1 subtype that has been implicated in
511 stress response (e.g., social isolation, temperature, UV, or starvation) and insecticide/xenobiotic
512 detoxification, and is regulated by hormones including juvenile hormone [117,122–125]. In turn,
513 D-SCRIPT predicted that many of those host cytochrome P450s interacted with fungal
514 cytochrome P450s. Although cytochrome P450 proteins often act independently, homo- and
515 hetero-dimer formation of cytochrome P450 proteins can modulate protein function in positive,
516 negative, and substrate-specific manners [126]. Oxidation-reduction related PPIs might most
517 strongly represent the physiological stress and antagonistic cellular processes between host and
518 parasite. However, fungal interference with these pathways may also be critical elements of
519 creating a host susceptible to manipulation.

520 **Alteration of *Camponotus* gene regulation –** We found signs that suggest
521 *Ophiocordyceps* could interfere with *Camponotus* gene regulation via proteins that bind host
522 transcription factors and DNA- or mRNA-binding proteins. Using the host's own cells to
523 dysregulate behavioral pathways could be metabolically favorable for the parasite. Some of the

524 fungal proteins in these PPIs were uSSPs and we, therefore, cannot predict the functional result
525 of those PPIs. However, several parasite proteins were hydrolases. We suggest that these
526 hydrolases most plausibly cleave host proteins or their post-translational modifications.
527 Depending on the role of the protein and specific bond broken, parasite hydrolase activity could
528 increase or decrease transcription of host genes. Among the ca. 200 host proteins in the
529 nucleotide-binding category, we found many tied to behavioral activity via changes in
530 locomotion, feeding/foraging, gravitaxis, light perception, circadian clocks, development and
531 social caste related hormones, and neuronal maintenance. Covering a similar breadth of
532 processes as the host GPCRs in PPIs, transcriptional interference may be a complementary
533 parasite strategy to modify their hosts. However, very few of these putative targets passed the
534 strictest shared aspecific host protein filter. As such, specific hypotheses about individual
535 transcription factors are difficult to parse. However, as a broader functional category, many of
536 these host proteins intersect with processes that appear relevant to manipulated ant behavior.
537 **Destruction of *Camponotus* structural proteins –** During manipulation, the fungus also
538 secretes proteins predicted to bind host cuticle and connective tissue proteins. Being an
539 endoparasite, *Ophiocordyceps* has direct access to internal host connective tissues, the
540 endocuticle layer (once the epidermis is breached), and the cuticular lining of the gut and
541 trachea, which include tissues that contain chitin [61,127]. However, the fungal cell wall also
542 contains chitin. As such, we cannot fully discount that some of these secreted fungal proteins
543 may simply be cell wall modifying proteins that have falsely been predicted to bind chitin-
544 associated ant proteins. Nevertheless, the fungus could be degrading cuticular proteins for
545 nutrition, detaching muscles, or in preparation for emerging hyphae to later grow out of the host
546 cadaver. Corroborating evidence for the disruption of the musculature stems from detailed

547 microscopy work on *Ophiocordyceps*-infected ants at the time of manipulated summiting and
548 biting behavior. These reports describe visual signs of fungal invasion, tissue degradation, and
549 atrophy of manipulated ant mandible muscles [15,22,29,128].

550 The predicted fungal interactors with host structural proteins included multiple proteases
551 such as a M43-like metalloprotease and S8 subtilisin-like serine peptidase. S8 peptidases can
552 degrade insect cuticle and are found in many fungi [129,130]. We also detected unannotated
553 secreted proteins in cuticular protein PPIs that could have similar or different functions. The
554 aspecific fungi included a generalist entomopathogen capable of degrading a wide variety of
555 insect tissue (*C. bassiana*); consistent with this, we found no *Ophiocordyceps*-specific cuticular-
556 protein host targets.

557 **Gene networks and expression broadly implicate PPIs in manipulation –**
558 *Ophiocordyceps* PPI proteins were enriched for WGCNA modules transcriptionally associated
559 with manipulation and containing uSSPs and other previously hypothesized effectors (F1 and F2)
560 [19]. In *Camponotus*, gene modules associated with neuronal processes and GPCRs (A14) and
561 signaling and transcription (A10) further support the involvement of these processes with
562 manipulation [19]. Assuming "guilt-by-association," in some cases this could indicate these PPIs
563 are related to infection and manipulation processes even if we yet lack clear mechanistic
564 hypotheses of how (e.g., PPIs with uSSPs) [131–133]. Additionally, both upregulated and
565 downregulated host DEGs [19] were enriched in predicted PPIs, possibly indicating homeostatic
566 responses to fungal modulation of functional protein levels. As such, the proteome-level
567 predictions that we made in this study are well in line with previous transcriptomics work.
568 Therefore, this work serves as an additional line of evidence for certain host processes and
569 narrows the pool of well-supported effector candidates involved with behavioral manipulation.

570 **Conclusion –** Faced with the challenge of uncovering how a parasitism with two non-
571 model species may operate at the molecular level, we combined multiple datatypes and tools to
572 predict host-parasite PPIs. These approaches are feasible in many study systems, even for those
573 that may lack the tools for sophisticated functional testing. We employed a structurally aware
574 machine learning program, D-SCRIPT, to predict PPIs beyond well-documented interactions. By
575 combining genomic and transcriptomic data we selected PPIs of interest with multiple
576 bioinformatic tools to: (*i*) annotate secreted fungal proteins, (*ii*) filter interactions predicted
577 across different species, and (*iii*) select proteins linked to increased gene expression during
578 infection. Using the resulting PPIs, we performed enrichment analyses to identify functional
579 categories that should be robust to computational error rates at the individual PPI level. Taken
580 together, we produced multiple hypotheses connecting host-parasite cross-species PPIs to
581 processes related to infection and manipulation of *Camponotus* by *Ophiocordyceps*. We
582 highlighted evidence for PPIs involving fungal S8 proteases and PPIs involving host oxidation-
583 reduction processes, cuticular proteins, gene regulation, and GPCRs. Especially, with
584 analytically robust results and direct biological links to behavior, we emphasize parasitic
585 dysregulation of GPCR signaling as a mechanism of parasitic behavioral manipulation.
586

587 **Key Points**

588 - Producing high-throughput predictions of host-parasite protein interactions can offer initial
589 insights and hypotheses that lay the foundation for future functional validation. However, this
590 can be a challenge if the taxa and proteins studied are dissimilar from preexisting data.
591 - To reduce the constraints based on approaches only using protein sequence homology, we used
592 a sequence-to-structure based machine learning tool (D-SCRIPT).

593 - We also used multiple supporting bioinformatic steps to select PPIs based the plausibility of the
594 parasite protein being an effector (secretion and gene upregulation during infection) and
595 specificity of the interaction across multiple species tested.
596 - Group-level enrichment analyses indicated multiple host-parasite processes may be at play,
597 with notable signals for interactions with host GPCRs related to behavioral processes.
598

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609

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913

914 # Tables

915 **Table 1. PPI sets overview.**

916 We tested 7,156,818 combinations of secreted *O. camponoti-floridani* (*O.*) proteins (n = 586) with every *C.*
917 *floridanus* (*C.*) protein (n = 12,213). Predicted PPIs were combinations returned with an edge value of ≥ 0.5. We
918 then created PPI subsets based on homologous interaction between secreted and intracellular fungal proteins and
919 fungal gene upregulation during manipulation.

PPI set	**Predicted PPIs (% of protein combinations tested)**	***Ophiocordyceps* proteins in PPIs**	***Camponotus* proteins in PPIs**
All secreted *O.* & *C.*	23,629 (0.33%)	402	3289
no homologous fungal-self PPIs	20,779 (0.29%)	399	3143
no homologous fungal-self PPIs & *O.* upregulated	6,011 (0.08%)	129	2083

920

921

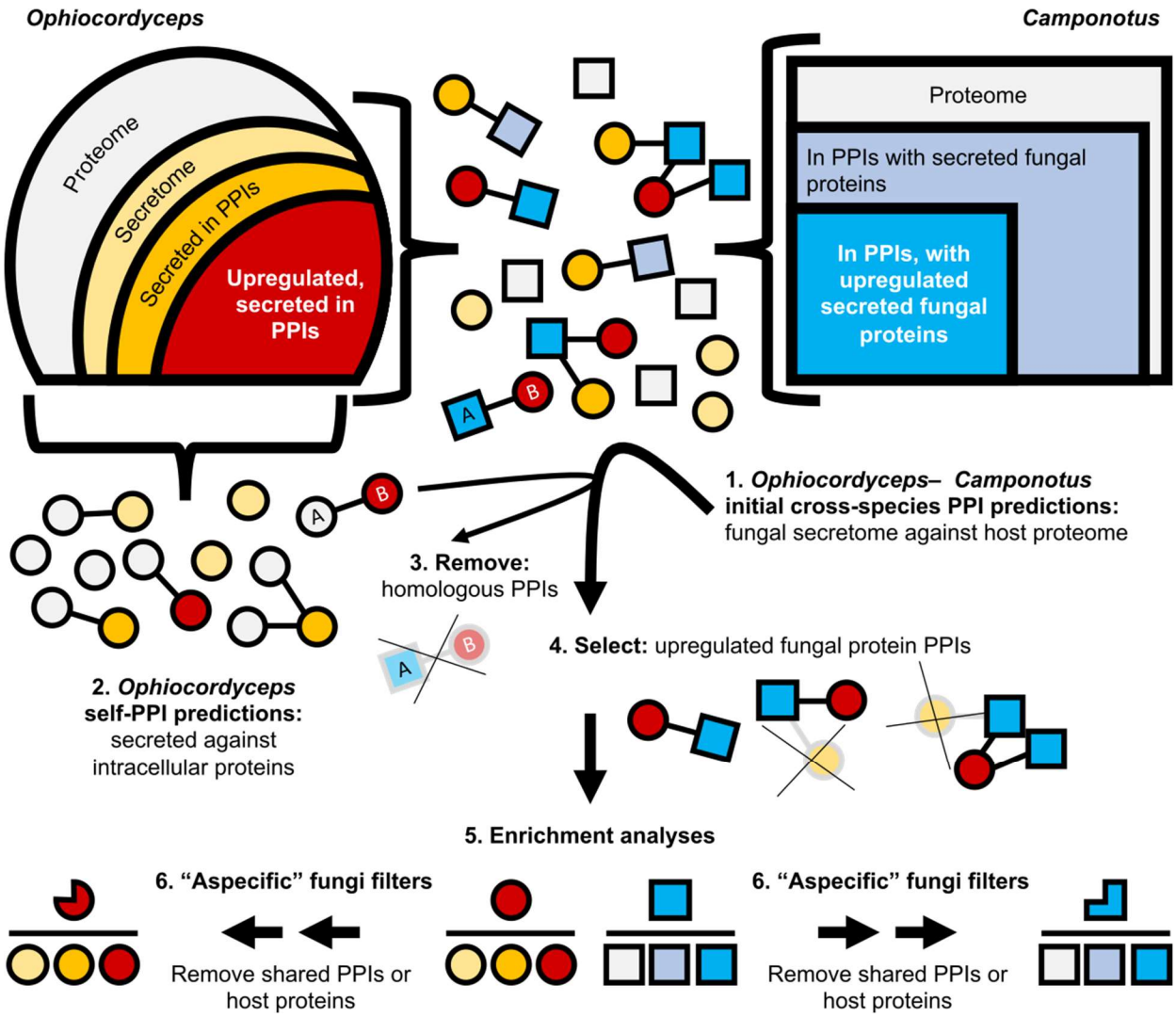
922 **Table 2. Annotation enrichments of PPI proteins.**

923 We performed enrichment analyses on cross-species PPIs without homology to *Ophiocordyceps* self-interactions
924 and involving upregulated, secreted *Ophiocordyceps* proteins and any *Camponotus* proteins (Fig. 1 step 5) with or
925 without additional filtering based on shared aspecific fungal interactions (Fig. 1 step 6). We found no enrichment
926 signal for uSSPs in the fungus. Only in the unfiltered analysis were *Camponotus* DEGs overrepresented – for both
927 upregulated and downregulated genes. The number of gene co-expression modules enriched are given under column
928 WGCNA. While typically more stringent filtering reduced the number of enrichments detected, it increased the
929 number of enriched GO terms for *Ophiocordyceps*. These enrichments included oxidation-reduction terms and
930 various general binding terms (e.g., "binding," "catalytic activity," "cofactor binding," or "ion binding").

Test proteins (n)	**GO**	**PFAM**	**WGCNA**
Ophiocordyceps (129)	0	1	3
after shared PPIs removed (125)	1	1	3
after shared host proteins removed (26)	11	0	2
Camponotus (2,083)	50	74	9
after shared PPIs removed (1,901)	44	64	9
after shared host proteins removed (147)	12	3	2

931

932 **Figures**

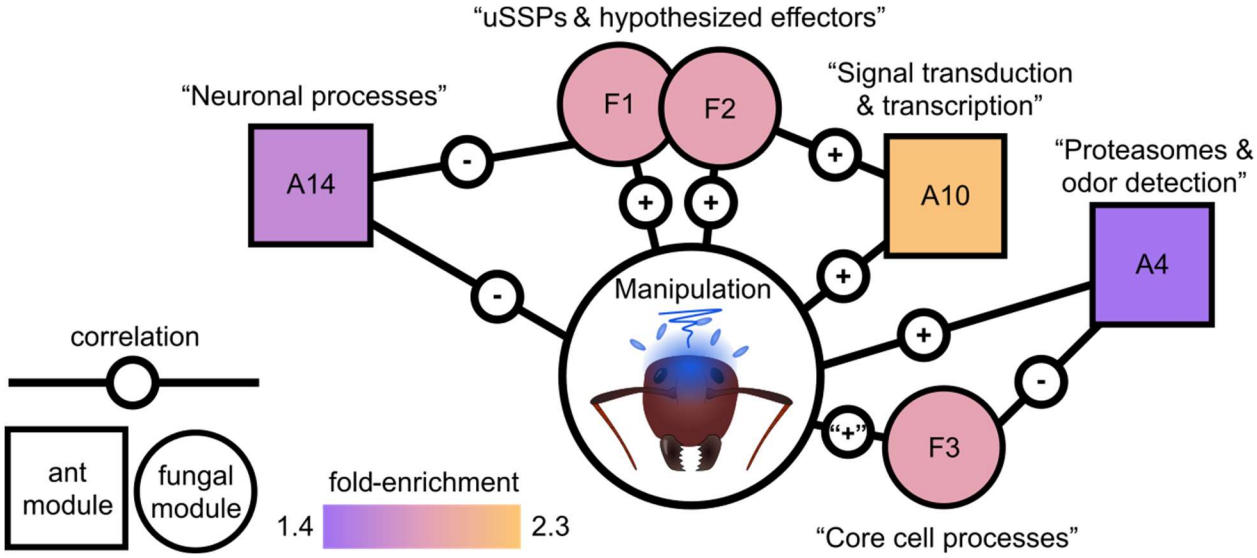


933

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

935 Step 1) We tested every secreted *O. camponoti-floridani* protein (secretome) with every *C. floridanus* host protein
936 (proteome). Steps 2, 3) If a cross-species PPI and an *Ophiocordyceps* self-interaction PPI shared a common fungal
937 protein paired with a protein homologous between both species, we removed that PPI from our predictions. Step 4)
938 Subsequently, we filtered predicted PPIs to focus on those that involved fungal proteins encoded by genes that were
939 upregulated during manipulation. Step 5) We analyzed host and parasite proteins from these PPIs with
940 hypergeometric enrichment analyses. For enrichment analyses, the background protein set was either the parasite
941 secretome (*Ophiocordyceps*) or host proteome (*Camponotus*). Step 6) We also performed enrichment analyses after
942 removing PPIs or host proteins that were predicted in interactions between aspecific fungi and *C. floridanus*. Fungal

943 proteins are shown as circles and ant proteins as squares. Proteins are color-coded, with *Ophiocordyceps*
944 upregulated, secreted proteins in cross-species PPIs indicated in red and their interacting *Camponotus* proteins in
945 bright blue. Shades of orange represent other secreted fungal protein categories while blue-gray indicates other host
946 proteins in PPIs. All other proteins are shown in gray. Proteins "A" are *Ophiocordyceps* and *Camponotus* homologs,
947 and protein "B" is a single fungal protein.
948



949

950 **Figure 2. Enriched WGCNA modules and their correlations to manipulation and each other.**
951 Module membership, correlations, and functional summary of modules were based on previous work [19]. Each
952 module is a mutually exclusive network of genes that are coexpressed across control, manipulated ant, and dying ant
953 conditions. Here, we only depict modules enriched among PPIs and only their correlations to the manipulated state
954 or between host-parasite modules directly. Core cell processes module F3 was clearly negatively correlated to
955 control conditions, with modest positive correlations divided between both live manipulation and dying ants [19], as
956 indicated by "+" here. While module F1 and F3 were also notably correlated to neuronal process module A15, this
957 ant module is not depicted here as it was not enriched among the PPIs detected in this study.
958
959

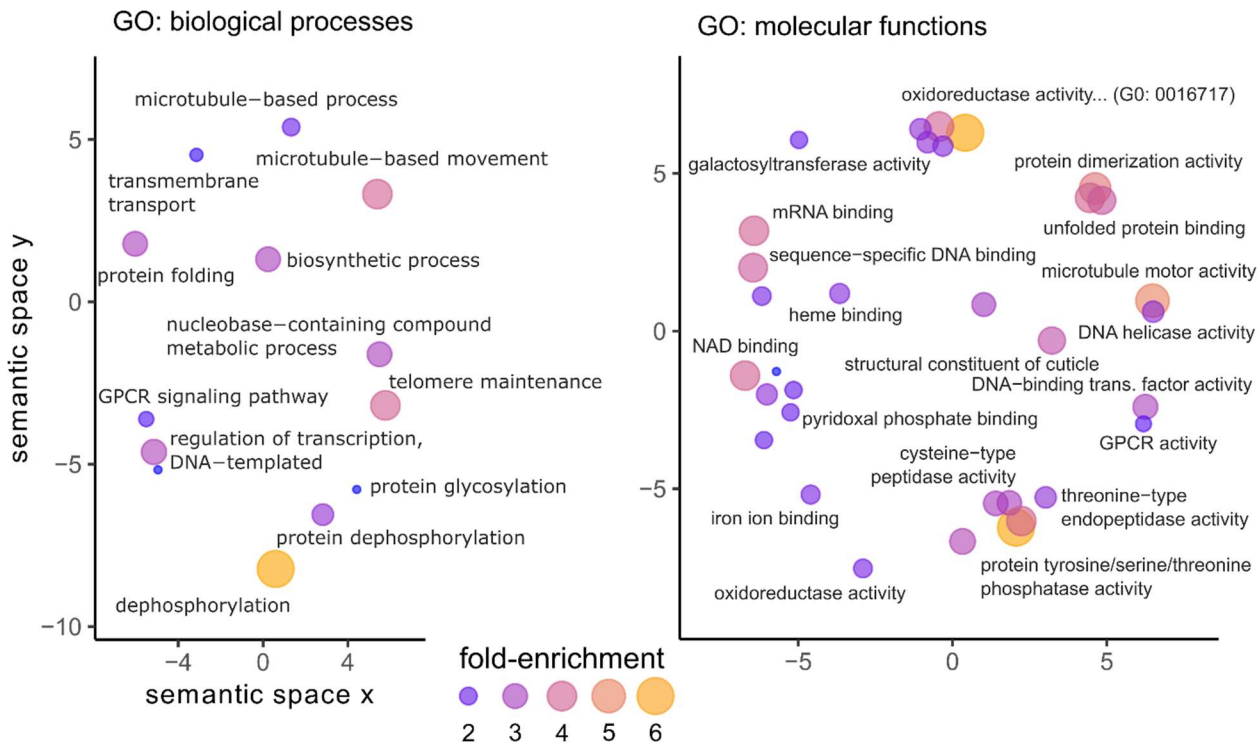
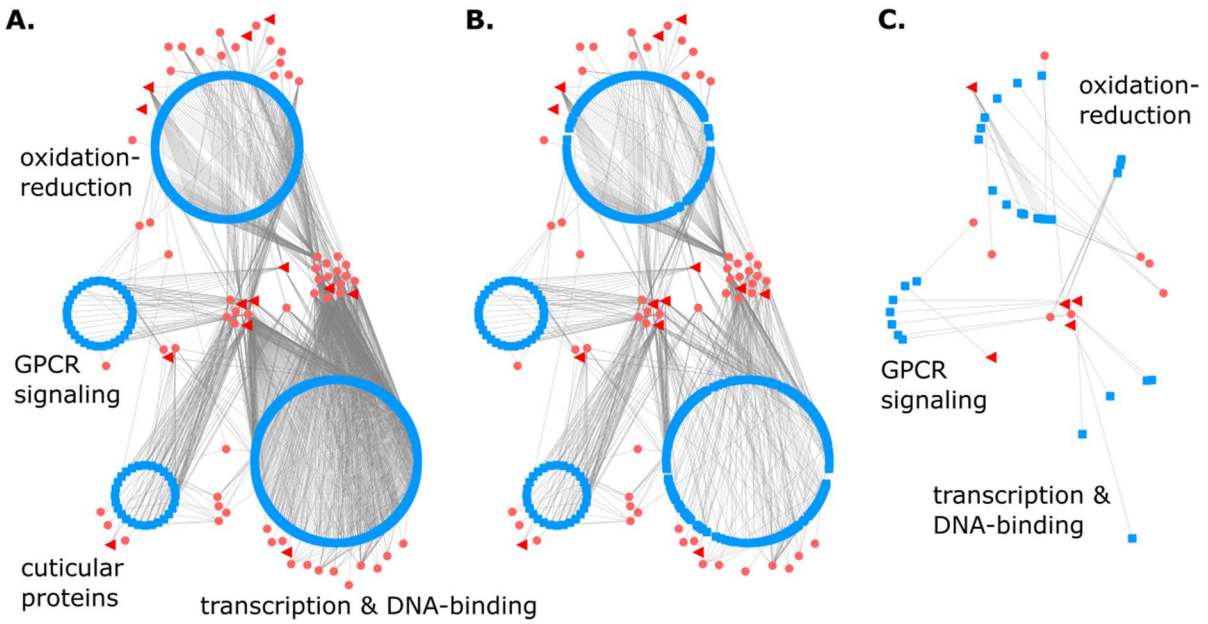


Figure 3. Enrichments for *C. floridanus* proteins in PPIs with upregulated secreted fungal proteins. GO terms are plotted in semantic space, which lacks any inherent unit or value other than to cluster terms by functional similarity. Some labels were omitted for readability and when not relevant to our discussion of the results.



969

970 **Figure 4. PPI network with host proteins contributing to selected GO term enrichments.**

971 Host proteins (blue square nodes) are clustered and labeled by their general GO term functional category and
972 connected (gray line edges) to their fungal PPI partners that are either secreted proteins (small pink circle nodes) or
973 uSSPs (large red triangle nodes) that were found to be upregulated during *Ophiocordyceps* manipulation of
974 *Camponotus* behavior. Proteins may have PPI connections outside of those depicted here. A) PPI network without
975 aspecific PPI filtering. B) Network of remaining PPIs after removing shared PPIs with aspecific fungi. Although
976 nearly 30% of PPIs (edges) were removed, most proteins (nodes) and enrichment results were kept. C) Network of
977 remaining PPIs after removing PPIs with all host proteins that also interacted with aspecific fungi, regardless of
978 orthology of the fungal binding partner. GO terms related to G-protein coupled receptor signaling and some
979 oxidation-reduction enrichments persisted after this filter. Enrichment signals for transcription and DNA-binding
980 were lost, but some individual proteins remained.

981

982

983 **Supplemental Material**

984 **File S1:** a Word document briefly describing preliminary analysis of transmembrane fungal
985 proteins.

986

987 **File S2:** *Ophiocordyceps-Camponotus* PPI protein and enrichment data in a single Excel file
988 with multiple tabs.

989

990 **File S3:** Aspecific fungi PPI data in a single Excel file with multiple tabs.

991

992 **Table S1: Aspecific fungi and *Ophiocordyceps* PPI overview.** Results do not reflect any DEG
993 or self-homology filtering steps, predictions are based on fungal secretome by host proteome.
994 "Predicted PPIs" reflect the percent of positive predictions from D-SCRIPT relative to the total
995 number of protein combinations tested, with the count of positive PPIs in parentheses. "Fungal
996 proteins" is the percent of fungal proteins that were in at least one positive PPI, relative to the
997 secretome size. "*Camponotus* proteins" is the precent of host proteins that were in at least one
998 positive PPI, relative to the proteome size. "Fungal connectivity" shows the range of how many
999 host proteins each fungal protein was predicted to bind and the median in parentheses.
1000 "*Camponotus* connectivity" shows the range of how many fungal proteins each host protein was
1001 predicted to bind and the median in parentheses.

1002

1003 **Figure S1: Filtering aspecific PPIs and host proteins shared between *Ophiocordyceps* and**
1004 **alternative fungi.** Aspecific filtering steps were performed after selecting PPIs based on
1005 *Ophicordyceps* secretion signals, upregulated DEGs, and no homology to self-PPIs. Both *C.*

1006 *bassiana* and *T. reesei* were similarly phylogenetically distant from *Ophiocordyceps*, with *S.*
1007 *cerevisiae* most distantly related. *C. bassiana* was most similar to *Ophiocordyceps* by lifestyle,
1008 being a generalist entomopathogen. A) All PPIs shared between *Ophiocordyceps* and at least one
1009 of *C. bassiana*, *T. reesei*, or *S. cervisiae* were removed. We defined aspecific homologous PPIs
1010 as those that involved a single host protein predicted to bind fungal proteins in *Ophiocordyceps*
1011 and an alternative fungus that were orthologous to each other (i.e., proteins "A" in the figure).
1012 Most PPIs were specific (4,235 of 6,011) to *Ophiocordyceps*. With most aspecific PPIs shared
1013 with *C. bassiana*. B) Removing aspecific host proteins (and therefore all PPIs and
1014 *Ophiocordyceps* proteins only predicted with these host proteins) was the strictest filter. Only
1015 147 of the initially predicted 2,083 host proteins passed this filter. Again, most were shared with
1016 *C. bassiana*.
1017
1018 **Figure S2: D-SCRIPT edge values for tested protein pairings between extracellular fungal**
1019 **proteins and the ant proteome.** D-SCRIPT edge values indicate the confidence of the model
1020 prediction for a given PPI to be true. We used the default edge value cutoff of ≥ 0.5 (green).
1021 These positive predictions comprised 0.41% of the ca. 23 million protein combinations tested. D-
1022 SCRIPT assigned most negative predictions with decisively low values far below the 0.5
1023 threshold.