**File S3 – Additional results & discussion**

*Pathogen-Host Interaction database annotations*

We also compared DEGs upregulated and downregulated during manipulation using the Pathogen-Host Interaction (PHI) database (Urban *et al.* 2017). We counted PHI descriptions that contained any annotation other than “unaffected\_pathogenicity” as genes putatively involved in virulence and manipulation. Upregulated genes comprised 57 hits with pathogenicity annotations, while only 22 were present in the downregulated gene set. In both cases, numerous gene products without PHI annotation results were present (201 upregulated, 32 downregulated). Of these genes absent in the PHI database, 54 genes in the upregulated set putatively encoded secreted proteins (i.e., SignalP annotation), whereas only 4 such genes were present in the downregulated group. These genes, that lack PHI database annotation but are part of the secretome, could contain novel undescribed fungal effectors that are relevant to *Ophiocordyceps*-*Camponotus* interactions*.*

*Host gene expression patterns related to tissue destruction and nutrition.*

The expression levels of three ant myogenesis-regulating glycosidase genes, decreased from healthy control ants, to live manipulated, and further to dead manipulated ants, as well as a gene annotated as “actin, muscle” (10-fold decrease from control to live manipulation). However, its homolog in *C. castaneus*, followed the opposite expression pattern.

In starved *Drosophila* flies, lipase 3, an enzyme involved in fat metabolism, was found to be up-regulated (Zinke *et al.* 1999). Similarly, we identified an upregulated lipase 3 gene (2-fold increase from control to live manipulation) in ants sampled during manipulation. However, an additional four lipase 3 genes and an overrepresentation of the GO term “lipid metabolic process” among the downregulated subset of genes suggested that overall lipid metabolism might be diminishing in manipulated ants.

As we note changes in gene expression not in line with reports in other insects (i.e., lipase 3, apolipophorin III, and cytochrome P450 4C1), this could mean that the ants’ energy reserves have been fully depleted at our nearly terminal time points of sampling. Alternatively, genetic starvation mechanisms are different in ants compared to flies and cockroaches, or the fungal parasite has disrupted the ants’ typical starvation responses.

We found putatively secreted metalloprotease encoding genes upregulated during live manipulation that could be involved in infection and affect host IIS pathways. These genes carried PFAM PF05572|Peptidase\_M43 annotations and additional MEROPS protease annotations. Genes with MEROPS M43.002 putatively function similarly to *mep1*, which assists fungi to counteract mammalian immune systems (Hung *et al.* 2005; Shende *et al.* 2018). Putative *mep1* genes in *O. camponoti-floridani* were upregulated during live manipulation relative to both culture and dead manipulated samples (Fig 6). One homologous *mep1* metalloprotease was also significantly upregulated during manipulation in *O. kimflemingiae* (i.e., 71-fold increase from culture to live manipulation in *O. camponoti-floridani*, 3-fold in *O. kimflemingiae*) (de Bekker *et al.* 2015).

Other M43 annotations present in the *O. camponoti-floridani* genome predicted the presence of ulilysins (MEROPS M43.007) and pappalysins (MEROPS M43.004 or M43.005). Ulilysins and pappalysins are known to interact with IGF binding proteins that regulate levels of free IGF (Tallant *et al.* 2007). Two M43 metalloproteases carrying both ulilysin and pappalysin MEROPS annotations were upregulated from culture to manipulation in *O. camponoti-floridani* but not in *O. kimflemingiae*.

*Dysregulation of odor detection.*

Two putative odorant receptor genes in *C. floridanus* were differentially expressed from control to live manipulation (i.e., upregulated *or1* and downregulated *or4*-like). Homologs of these genes have been proposed to encode for pheromone receptors in moths (Grosse-Wilde *et al.* 2011; Wicher *et al.* 2017). Other genes possibly associated with odor communication were also downregulated from control to live manipulation in *C. floridanus*. One of these genes was a putative *sensory neuron membrane protein 1* (17-fold decrease), which is involved in the detection of lipid-derived pheromones in pheromone-sensing antennal neurons (Pregitzer *et al.* 2014). We additionally detected two putatively encoding acyl-CoA Delta(11) desaturases (16- and 4-fold downregulated), which are key enzymes in the synthesis of pheromones in moths (Choi *et al.* 2002) and may speculatively play a role in chemical communication of ants as well. A homolog to the 16-fold downregulated acyl-CoA Delta(11) desaturase was also reduced in expression in *C. castaneus* (1.8-fold decrease) but does not meet our DEG threshold requirements (de Bekker *et al.* 2015).

Among the 11 differentially expressed PBP and general-OBP (GOBP) domain containing genes, four were annotated to putatively encode pheromone binding protein Gp9. Variation in *Gp9* influences colony dynamics and behavior by regulating queen number in colonies of fire ants (Ross 1997; Ross and Keller 1998; Krieger and Ross 2002; Gotzek and Ross 2007; Gotzek *et al.* 2007). Three of these *Gp9-*like genes were significantly upregulated from control to live manipulation in *C. floridanus*, while the fourth was down-regulated. Although the expression profiles of *C. castaneus* homologs did not match in this case, relatable patterns of odorant receptor and OBP dysregulation were found in manipulated *C. castaneus* (de Bekker *et al.* 2015).

*Fungal serine proteases are upregulated during manipulated biting behavior.*

The presence of an evolutionarily recent group of subtilases with unclear function that lack I9 domains may be associated with niche adaptation in the Entomophtoromycotina, fungal entomopathogens distantly related to *Ophiocordyceps*. Among the Entomophthoromycotina, the insect manipulating Entomophthora muscae and Pandora formicae have I9-lacking subtilases, albeit not exclusively (Arnesen et al. 2018). These subtilases have been suggested to represent hallmarks of niche specialization (Arnesen et al. 2018). A comparison of this protease group to subtilases of Ascomycetes demonstrated a notable dissimilarity. However, the representative of Ophiocordyceps in that analysis was a lepidopteran parasite outside the O. unilateralis species complex, Ophiocordyceps sinesis (Arnesen et al. 2018). Of the differentially expressed S8A subtilases in O. camponoti-floridani, two lacked inhibitor I9 domains (one with 38-fold increase, the other increasing from 0 RPKM to 4 RPKM).

*Fungal secondary metabolites involved in manipulation and infection.*

Cluster 12 is predicted to synthesize a compound similar to the polyketide citrinin. This mycotoxin is lethal to insects with nephrotoxic effects on Malpighian tubules, which perform kidney-like functions (Dowd 1989). Cluster 12 contains a backbone PKS that is a putative homolog to the citrinin PKS gene citS of *Monascus ruber* (BLASTp E-value = 4.49e-120) (He and Cox 2016). Additionally, genes in this cluster had high BLASTp matches to CitA and CitE that also participate in citrinin synthesis (E-value = 1.68e-60 and 1.56e-14 respectively). One cluster 12 gene was present in the manipulation correlated fungal WGCNA module F1 and two were present in F2. However, no suitable matches were found within this cluster for the remaining cluster synthesis genes *citC*, *citD*, or *citB*. Although, these genes did have hits elsewhere in the genome. Of these putative citrinin synthesis homologs, only *citA* appeared differentially expressed, being upregulated during manipulation and host death in both *O. camponoti-floridani* and *O. kimflemingiae*. Possibly, cluster 12 genes are active and play an earlier role in infection than our sampling regime was able to capture.

Arnesen, J. A., J. Małagocka, A. Gryganskyi, I. V Grigoriev, K. Voigt *et al.*, 2018 Early Diverging Insect-Pathogenic Fungi of the Order Entomophthorales Possess Diverse and Unique Subtilisin-Like Serine Proteases. G3 8: 3311–3319.

de Bekker, C., R. A. Ohm, R. G. Loreto, A. Sebastian, I. Albert *et al.*, 2015 Gene expression during zombie ant biting behavior reflects the complexity underlying fungal parasitic behavioral manipulation. BMC Genomics 16: 620.

Choi, M.-Y., K. S. Han, K. S. Boo, and R. A. Jurenka, 2002 Pheromone biosynthetic pathways in the moths *Helicoverpa zea* and *Helicoverpa assulta*. Insect Biochem. Mol. Biol. 32: 1353–1359.

Dowd, P. F., 1989 Toxicity of Naturally Occurring Levels of the Penicillium Mycotoxins Citrinin., Ochratoxin A, and Penicillic Acid to the Corn Earworm., Heliothis zea, and the Fall Armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Environ. Entomol. 18: 24–29.

Gotzek, D., and K. G. Ross, 2007 Genetic regulation of colony social organization in fire ants: an integrative overview. Q. Rev. Biol. 82: 201–26.

Gotzek, D., D. D. Shoemaker, and K. G. Ross, 2007 Molecular variation at a candidate gene implicated in the regulation of fire ant social behavior. PLoS One 2: e1088.

Grosse-Wilde, E., L. S. Kuebler, S. Bucks, H. Vogel, D. Wicher *et al.*, 2011 Antennal transcriptome of Manduca sexta. Proc. Natl. Acad. Sci. U. S. A. 108: 7449–54.

He, Y., and R. J. Cox, 2016 The molecular steps of citrinin biosynthesis in fungi. Chem. Sci. 7: 2119–2127.

Hung, C.-Y., K. R. Seshan, J.-J. Yu, R. Schaller, J. Xue *et al.*, 2005 A metalloproteinase of Coccidioides posadasii contributes to evasion of host detection. Infect. Immun. 73: 6689–703.

Krieger, M. J. B., and K. G. Ross, 2002 Identification of a major gene regulating complex social behavior. Science (80-. ). 295: 328–32.

Pregitzer, P., M. Greschista, H. Breer, and J. Krieger, 2014 The sensory neurone membrane protein SNMP1 contributes to the sensitivity of a pheromone detection system. Insect Mol. Biol. 23: 733–742.

Ross, K. G., 1997 Multilocus evolution in fire ants: effects of selection, gene flow and recombination. Genetics 145: 961–74.

Ross, K. G., and L. Keller, 1998 Genetic control of social organization in an ant. Proc. Natl. Acad. Sci. 95: 14232–14237.

Shende, R., S. Sze, W. Wong, S. Rapole, R. Beau *et al.*, 2018 *Aspergillus fumigatus* conidial metalloprotease Mep1p cleaves host complement proteins. J. Biol. Chem. 239: 15538–15555.

Tallant, C., R. García-Castellanos, A. Marrero, M. Solà, U. Baumann *et al.*, 2007 Substrate specificity of a metalloprotease of the pappalysin family revealed by an inhibitor and a product complex. Arch. Biochem. Biophys. 457: 57–72.

Urban, M., A. Cuzick, K. Rutherford, A. Irvine, H. Pedro *et al.*, 2017 PHI-base: a new interface and further additions for the multi-species pathogen–host interactions database. Nucleic Acids Res. 45: D604–D610.

Wicher, D., S. Morinaga, L. Halty-deLeon, N. Funk, B. Hansson *et al.*, 2017 Identification and characterization of the bombykal receptor in the hawkmoth *Manduca sexta*. J. Exp. Biol. 220: 1781–1786.

Zinke, I., C. Kirchner, L. C. Chao, M. T. Tetzlaff, and M. J. Pankratz, 1999 Suppression of food intake and growth by amino acids in *Drosophila*: the role of pumpless, a fat body expressed gene with homology to vertebrate glycine cleavage system. Development 126: 5275–5284.