

1 **Complex behavioral manipulation drives mismatch between host and parasite**  
2 **diversity**

3

4 **Short title:** Mismatch between host and parasite diversity

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24

25 **Abstract**

26 Parasites and hosts are intimately associated such that changes in the diversity of one  
27 partner are thought to lead to changes in the other. We investigated this linked  
28 diversity hypothesis in a specialized ant-*Ophiocordyceps* system in three forests  
29 across 750 km in Central Amazonia. All species belonging to the fungal genus  
30 *Ophiocordyceps* associated with ants have evolved some degree of behavioral control  
31 to increase their own transmission, but the leaf-biting behavior is the most complex  
32 form of host manipulation. Such a system requires control of the mandibular muscles  
33 and a distinct shift in behavior, from climbing vegetation to walking on leaves to  
34 rasping leaf veins in the seconds before death. The need to induce complex behavior  
35 may limit host availability and represent a constraint on parasite diversity. The  
36 consequence for community structure is that complex behavioral manipulation leads  
37 to a mismatch between ant hosts and the diversity of their fungal parasites.

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39 **Keywords:** ants, behavior manipulation, interaction, *Ophiocordyceps*, tropical forests.

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## 42 **Introduction**

43           Species diversity varies considerably between habitats and regions [1], and the  
44 factors driving such heterogeneity typically depend on the scale of the analysis [2]. At  
45 the local level, interactions among species are known to play an important role in  
46 structuring communities [3]. This is especially the case when the interacting species  
47 occupy different trophic levels, which leads to a stronger link between the diversity of  
48 consumers and the diversity of resources [4–6]. Parasite-host interactions are  
49 examples of such trophic effects. Parasites tend to be host specific with hosts serving  
50 as both the habitat and the dispersal agents for parasites [7]. This implies that changes  
51 in host abundance often lead to changes in parasite abundance. Such specificity is  
52 considered to lead to arms races that promote overall diversity at the community level  
53 [8].

54           Within the framework of linked diversity in host-parasite systems, the sub-set  
55 of parasites that manipulate behavior has not been considered. The effect of parasites  
56 on their hosts is not only to reduce host fitness but in some cases also involves a  
57 manipulation of host behavior that directly increases parasite fitness [9]. In these  
58 cases, other constraints acting on the parasite related to its need to control behavior as  
59 a life-history strategy may affect the coupling of diversity across scales. The  
60 interaction between ants and the ascomycete fungus *Ophiocordyceps* provides a  
61 convenient model for understanding the roles of behavioral manipulation on patterns  
62 of host and parasite diversity. Ants infected by *Ophiocordyceps* species die in specific  
63 locations outside the nest where the microenvironment is ideal for fungal sporulation  
64 and subsequent dispersal to new hosts [10-12]. Dying outside the nest is considered  
65 adaptive for the fungus, because it avoids the cleaning behavior of ant workers that  
66 may prevent the completion of the fungal lifecycle inside the colony [10]. Depending

67 on which fungal species is involved, infected ants may die attached to stems (Fig.  
68 1C), buried in the leaf-litter, attached to tree bark (Fig. 1D) or biting leaves (Fig. 1E).  
69 Biting leaf veins or leaf tissue is the most complex form of manipulation and  
70 maintains the ant in situ after death giving the fungus the necessary 24-48 hours to  
71 grow adhesive mycelia that bind the ant to the plant [11]. This behavioral  
72 manipulation is ancient with vein biting occurring at least since the Eocene [12].  
73 Recent evidence has shown that this host-parasite relationship is highly specific with  
74 each host species examined having its own specific parasite species [13].

75 To explore the linked diversity hypothesis between parasite and host we  
76 worked with a large dataset of more than 70,000 samples representing 340 ant species  
77 with knowledge on parasite diversity built up from 2,700 samples collected from three  
78 Amazonian sites across a 750km transect (Fig. 1A). We specifically compare the  
79 composition of infected and non-infected ant species among sites and how the  
80 complex behavioral manipulation by the fungus *Ophiocordyceps* can affect the  
81 parasite assemblage structure.

82

### 83 **Materials and Methods**

84 We sampled ants and their fungal parasites in three Amazonian forests. Two  
85 of them (Maracá Ecological Station, 3° 22'N, 6° 127'W and Viruá National Park, 1°  
86 27'N, 61° 01'W) are situated in forest reserves in Roraima State (extreme North of  
87 Brazil). The third (Ducke Reserve, 2° 57'S, 59° 56' W) is situated 25 km North of  
88 Manaus, Central Amazonia (Fig 1A). The sites cover a latitudinal gradient (~ 750 km)  
89 in Amazonian forests and encompass wide environmental heterogeneity, including  
90 areas of open and dense forests, and areas subject to different degrees of seasonal  
91 flooding [14]. We sampled both, parasitized and non-parasitized ants in 9 plots per

92 site covering approximately an area of 9 km<sup>2</sup>. In each plot, we carefully searched for  
93 infected ants buried in soil/litter, and attached to vegetation and tree trunks: habitats  
94 where the infected ants are most commonly found. The three-dimensional volume  
95 sampled per plot was ~ 500 m<sup>3</sup>: 250 m length, 1 m wide and 2 m in height, resulting  
96 in 13,500 m<sup>3</sup> in total. Two persons sampled each plot for at least 1.5 hours (~ 40  
97 hours/person over the three sites); one focusing on all infected ants and the other on  
98 non-infected ants belonging to the genera infected by *Ophiocordyceps*. In the Upper  
99 Amazon, this and previous research [15] has shown that the following seven ant  
100 genera are infected: *Camponotus*, *Cephalotes*, *Daceton*, *Dolichoderus*, *Ectatomma*,  
101 *Pachycondyla* and *Paraponera* genera. To contrast the assemblage of infected ants  
102 with the whole ant community, we used a comprehensive ant survey of 30 plots per  
103 site, with over 70,000 samples collected. This survey included 900 1 m<sup>2</sup> litter samples  
104 (Winkler sacks), 900 pitfall-traps and 900 sardine baits regularly distributed among  
105 the three sites to describe the ant assemblage composition (see [14] for additional  
106 details).

107         The data were organized in three matrices: 1) all ant species collected at the  
108 three sites, 2) all species from the genera known to be suitable hosts (i.e. *Camponotus*,  
109 *Cephalotes*, *Daceton*, *Dolichoderus*, *Ectatomma*, *Pachycondyla* and *Paraponera*  
110 genera) and 3) only the species we discovered to be infected. This last category was  
111 created because not all species in a genus are infected. To provide further  
112 understanding of the role of complex behavioral manipulation on ant community-  
113 level patterns, we also constructed matrices of ant species according to the type of  
114 manipulation (Fig 1B). We reduced the dimensionality of all matrices using Principal  
115 Coordinate Analysis (PCoA) based on the Sørensen dissimilarity index.  
116 Presence/absence data were used to avoid overestimation of species with larger nests.

117 We compared assemblage composition between the three areas using non-parametric  
118 multivariate analysis of variance [16]. The statistical significance of each analysis was  
119 based on 9,999 Monte Carlo permutations.

120

121

## 122 **Results**

123 We found that the ant assemblage composition was markedly different  
124 between areas (npMANOVA,  $p < 0.001$ ; Fig 1F). We recorded 343 species from 58  
125 different genera in 11 sub-families. We found very little overlap of species between  
126 areas; only 72 out of 343 ant species (~20%) were sampled in each of the three sites.  
127 Although we recorded 58 genera of ants only 7 genera contain species that are  
128 infected by *Ophiocordyceps* (these are *Camponotus*, *Cephalotes*, *Daceton*,  
129 *Dolichoderus*, *Ectatomma*, *Pachycondyla* and *Paraponera*). The assemblage  
130 composition of 68 species of ants belonging to these 7 genera also were different  
131 between areas (npMANOVA,  $p < 0.001$ , Fig S1). However, the assemblage of  
132 infected ants did not mirror the community structure; either of all ants from all genera  
133 or all ants from the genera that we identified as containing infected species. The  
134 assemblage composition of species of ants that are infected by *Ophiocordyceps* was  
135 not different between sites (npMANOVA,  $p = 0.109$ ; Fig 1G). Put another way,  
136 despite the fact that ~20% of the ant species were shared among the three locations  
137 the infected ant species were similar between these three very geographically and  
138 ecologically different sites. We had expected that different areas would have different  
139 infected species assemblages reflecting the general pattern of ant diversity across  
140 three sites, i.e. linked diversity between hosts and parasites. The linked diversity in  
141 the host-parasite system only matched when the infected ants that are manipulated to

142 bite into plant tissue are removed from the analysis (npMANOVA,  $p < 0.001$ , Fig.  
143 1H).

144

## 145 **Discussion**

146 To infect an ant worker, *Ophiocordyceps* fungus produces spores that are  
147 released onto the forest floor or onto vegetation, over both of which foraging ants  
148 move. The spores of this fungus are very large, with a relatively thin cell wall and  
149 devoid of pigmentation [i.e. hyaline 17], making them sensitive to dehydration and  
150 UV radiation. Therefore, for fungal life cycle completion, an ant must pass, at the  
151 correct time of the day, over the spores scattered on the forest floor to be infected. In  
152 addition, the parasite must overcome the host population structure to complete their  
153 life cycle. Ants are very interactive organisms, and the competition between colonies  
154 of the same species at local scale has been frequently demonstrated [18]. Colony-  
155 colony competition implies an additional barrier for the transmission between  
156 colonies of a specialized parasite. However, the complex behavioral manipulation by  
157 *O. unilateralis* seems to circumvent these barriers by creating a relative large  
158 minefield areas ( $\sim 30\text{m}^2$ , [11]) where the foraging ants can be infected by spores. This  
159 is achieved by very high densities of manipulated/killed hosts in a small area known  
160 as graveyards (Pontop et al). Virtually, in all the plots where we found species of ants  
161 known to be a host to *O. unilateralis* complex we also found ants infected by fungi  
162 within the *O. unilateralis* complex. And over the 750 km range the same group of  
163 *Camponotus* species were infected despite those areas having limited overlapp in  
164 *Camponotus* species assemblage (Fig 2). Other groups of *Ophiocordyceps* that infect  
165 ant species with large colonies, such as the ant genera *Cephalotes* and *Dolichoderus*  
166 (both included in this study) also create graveyards, but in smaller areas (personal

167 observations). In the latter case, the dead ants died attached in one tree trunk [17], and  
168 sites without any infected ants were more common (Fig 2).

169           Biting behavior requires a control of the mandibular muscles that involves a  
170 reduction in muscle organelle abundance [12]. It also requires a distinct shift in  
171 behavior in the seconds before biting as infected ants shift from a wandering behavior  
172 to rasping of either the major veins or leaf edges. Other complexes of *Ophiocordyceps*  
173 cause ants to die on leaves (*O. lloydii*, 17) but the do not cause ants to bite into the  
174 plant tissue. We suggest that the nature of complex manipulation and the necessary  
175 additional control of the host's phenotype that is entailed limit the potential host range  
176 of fungi investing in manipulation. Transmission requires this complex control of  
177 behavior, which in turn requires multiple effects at the physiological and neuronal  
178 level. The consequence for community structure is that even across large geographical  
179 areas, complex behavioral manipulation results in a mismatch between host and  
180 parasite diversity patterns. However, despite this apparent constraint the evolution of  
181 behavioral manipulation seems to be a successful strategy and ant hosts of *O.*  
182 *unilateralis* group were by far the most abundant hosts we discovered.

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184

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191 (<http://ppbio.inpa.gov.br/knb/style/skins/ppbio/>).



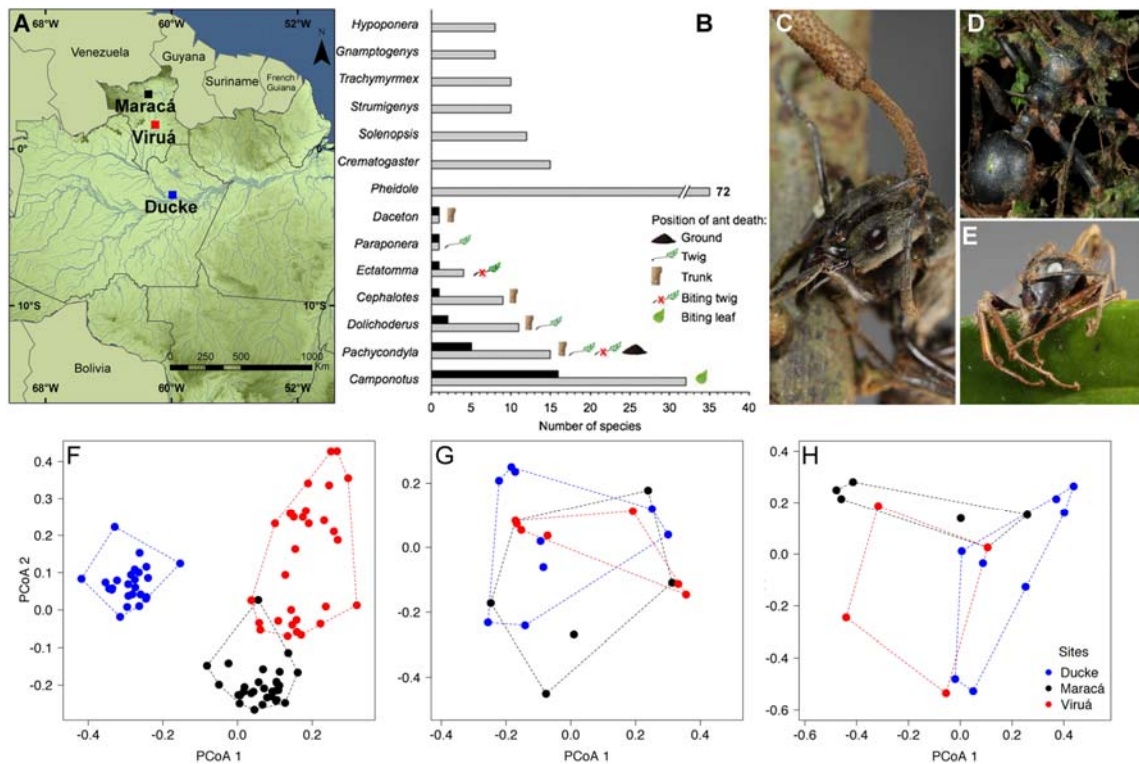
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246 **Figure 1.** (A) Map of study area. (B) Relative infection levels by ant genera showing

247 where the ants died and the seven more specious non-infected genera sampled in 27

248 plots (note that *Pheidole* bar is at different scale). Black bar shows the number of

249 infected species and gray bars the number of non-infected species. (C) *Pachycondyla*

250 *inversa* infected by *Ophiocordyceps kniphofioides* var. *ponerinarum* attached to a

251 stem. (D) *Cephalotes atratus* killed by *O. kniphofioides* var. *kniphofioides* buried in

252 the mosses of a tree trunk. (E) *Camponotus atriceps* parasitized by *O. unilateralis* s.l.

253 biting a leaf edge. PCoA ordination plots indicating (F) the differences in species

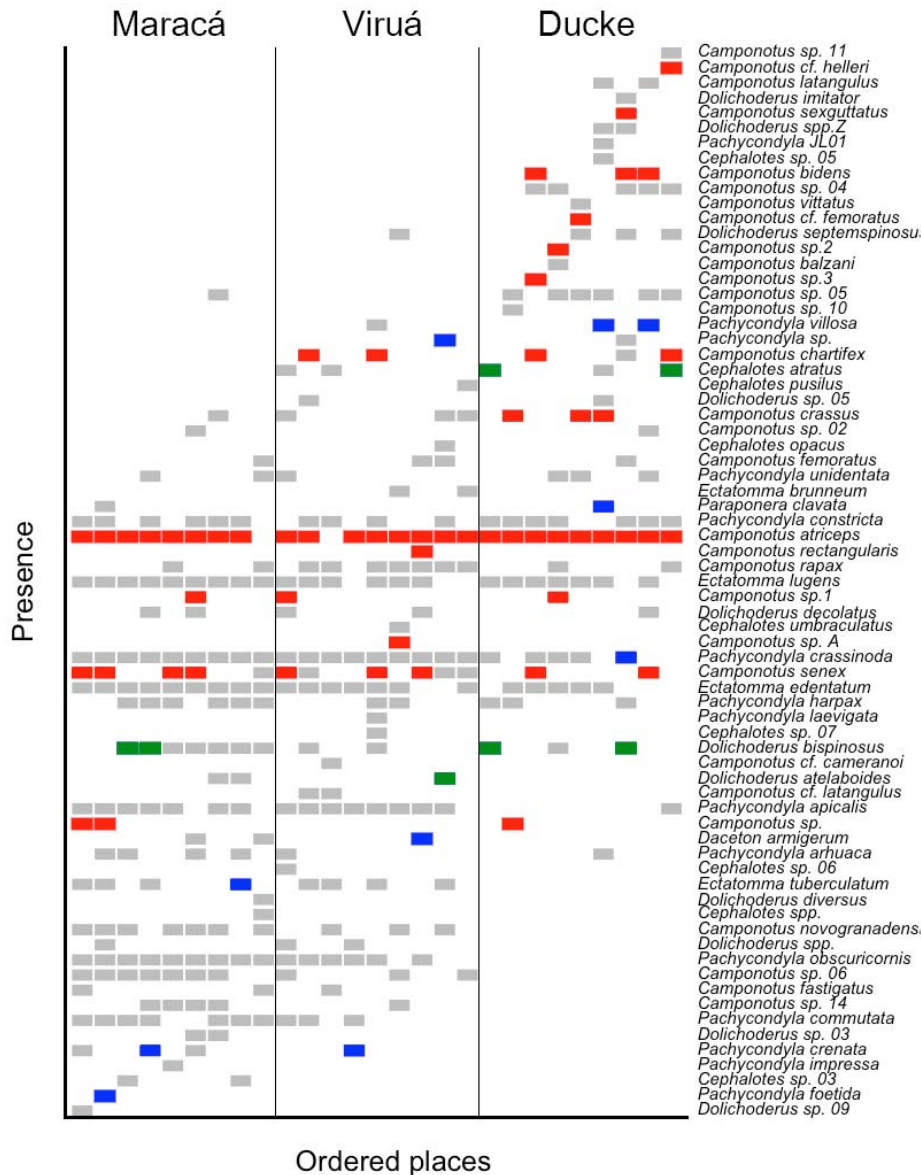
254 composition among the three sites using all data, (G) congruence in species

255 composition of all infected ant species found in 25 out of 27 plots and (H) different

256 assemblage composition of non-biting infected ants. Some plots are stacked in the last

257 two figures, because had the same infected ant species composition.

258



259

260 **Figure 2.** Distribution of ant species ordered by the first axis of PCoA analysis in  
 261 study plots at Maracá, Viruá and Ducke sites. Occurrence of uninfected ants are in  
 262 gray. Plots where ant species were infected by *unilateralis* complex (biting plant  
 263 tissue) are showed in red. Blue and green bars represent plots were ants infected by  
 264 *australis* and *kniphofioides* complex were found, respectively. In the later case, the  
 265 infected ants were found on litter and buried in tree trunks.