



Temporal and spatial gradients of humidity shape the occurrence and the behavioral manipulation of ants infected by entomopathogenic fungi in Central Amazon

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ABSTRACT

Some species of the fungal genus *Ophiocordyceps* that parasitize ants have evolved some degree of behavioral manipulation to increase their own transmission. Carpenter ants (*Camponotus* spp.) infected by *Ophiocordyceps unilateralis* species climb vegetation and bite leaves or other plant parts before death, while other ants infected by other *Ophiocordyceps* species die buried within or on the leaf-litter, attached to stems and tree trunks. The microenvironment of the host's death location is important for fungal development and dispersal to new hosts, being an interesting example of an extended phenotype. This study investigated the effect of humidity variation in space (15 km²) and time (14 months) on the occurrence and expression of the extended *Ophiocordyceps* phenotype parasitizing ants in Central Amazonia. We found *O. unilateralis* s.l., *O. kniphofoides* s.l. and *O. australis* s.l. parasitizing 30 ant species, but *O. unilateralis* s.l. was by far the most abundant fungus. Parasitized *Camponotus atriceps* and *C. bispinosus* were more abundant and died in higher places in periods with greater air humidity. Otherwise, the abundance and height of *C. senex* cadavers were higher in drier plots. These results provide insights on how the spatial and temporal variation in air humidity may shape the occurrence and behavioral manipulation of ants infected by entomopathogenic fungi at larger scales.

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1. Introduction

Antagonistic interactions in which an individual consumes or parasitizes another organism represent the most common interaction in natural communities (Moore, 2002; Poulin, 2010). Specially between parasites and hosts, this interaction can promote multiple reciprocal adaptations related to increasing the infection or prevalence of the parasite (Pierce, 1995; Frank and Schmid-Hempel, 2008) and to counter attack the parasite infections by the hosts (Poulin, 1998; Eberhard, 2000; Thomas et al., 2002; Biron et al., 2006; Yanoviak et al., 2008; Maure et al., 2011). This arms race can result in specific interactions, such as the ones in which parasites manipulate the behavior of their host (Zuk and McKean, 1996; Omacini et al., 2001; Altizer et al., 2006; Hatcher et al., 2006). In such interactions, the behavioral manipulation can be viewed as an

“extended phenotype” in which the parasite genotype is expressed through manipulation of behavioral, physiological or morphological traits of the host, leading to increased parasite fitness (Dawkins, 1982).

Spatial and temporal variation in climatic conditions can constrain the ability of a parasite developing and infecting new hosts, reducing the spatial and temporal overlap of host and parasite distribution (Hassell et al., 1991; Bouchier and Smith, 1996). Even when parasites and the hosts are able to coexist, parasites can adjust the manipulation of the host behavior to avoid the occupation of microhabitats climatically unfavorable for its development and reproduction (Dawkins, 1982; Meiners and Obermaier, 2004). For instance, ants parasitized by fungi of the *Ophiocordyceps unilateralis sensu lato* (s.l.) (Hypocreales, Ophiocordycipitaceae) display a stereotypical biting behavior just before host death that does not occur in healthy ants (Hughes et al., 2011a; Semenova et al., 2011). However, the location where the ant bites (leaf, twig, green twig and trunk) varies between forest types (Loreto et al., 2018). Therefore, much of the strategies of the parasites that

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exhibit behavioral manipulation on their hosts might have evolved in response to local environment in which the interaction has been embedded (Loreto et al., 2018). Both spatial and temporal variation in climatic conditions could not only affect the patterns of host-parasite coexistence, but also promote variation in patterns of manipulative behavior exhibited by different parasites species.

The effect of humidity on parasite performance and host manipulation has been observed in ants infected by fungi belonging to *O. unilateralis* s.l. complex. Ant hosts infected by these parasites die outside their nests, on locations where microenvironment conditions are ideal for fungal sporulation and dispersion (Andersen et al., 2012; Loreto et al., 2014). However, the height at which dead parasitized ants are more commonly found seems to vary between ecosystems. For example, based on the experimental relocation of ants in Thailand, Andersen et al. (2009) showed that locations at 25 cm from the soil (the average natural height of cadavers), favored the development and sporulation of *O. camponoti-leonardi* infecting *Camponotus leonardi*. Spots at this height exhibited lower variation in humidity and temperature favoring the fungus to complete its life cycle (Andersen et al., 2009). Conversely, in the Atlantic Rainforests of Minas Gerais, Ceará and Central Amazon, Brazil, the fungi *O. camponoti-rufipedis* and *O. camponoti-atricipis* that infect ants *Camponotus rufipes* and *C. atriceps* respectively, died at higher height compared to Thailand, with a greater variance around the mean 60–180 cm (Andersen and Hughes, 2012; Loreto et al., 2014; Sobczak et al., 2017; Andriolli et al., 2019). The reason for this phenotypic difference is unknown (Roy et al., 2006; Andersen and Hughes, 2012; Evans et al., 2018), but it is possible that such variation in height of the hosts death between ecosystems is a reflex of extended phenotype adjustment accordingly with local environmental characteristics.

Biting plant parts is not the only behavioral manipulation described for the *Ophiocordyceps* species complex (Fig. 1). Currently, species belonging to *O. kniphofioides* s.l. are known to

infect four ant genera (i.e. *Cephalotes*, *Dolichoderus*, *Daceton* and *Paraponera*) inducing the infected hosts to die among the moss carpets at the base of large trees (Evans and Samson, 1982). In contrast, *O. australis* s.l. infect the ant genera *Neoponera*, *Pachycondyla*, *Paltothyreus*, *Crematogaster*, *Paraponera* and *Odontomachus*, leading most species to die grasped to the stems and branches of herbs and small shrubs (Mains, 1949; Evans, 1982; Sanjuán et al., 2001). Although air humidity is a factor directly linked with sporulation, different entomopathogenic groups may have developed strategies to optimize the horizontal transmission of spores, through morphological, physiological or ecological adaptations at local scales (Mongkolsamrit et al., 2012; Araújo and Hughes, 2017; Evans et al., 2018).

The topography in Central Amazon favors the maintenance of high microclimatic heterogeneity: bottomlands (valleys) are more humid through the year than higher areas (plateaus) (Oliveira et al., 2008). Valleys tend to remain more humid because of their drainage patterns and proximity to water bodies, the mycelial activity and the production of fruit bodies of non-pathogenic fungus is higher in such areas than in the plateau (Sylvia et al., 1999; Braganeto et al., 2008). Most of *C. atriceps* ants infected by *O. camponoti-atricipis* are found during the rainy season in Amazon forests, suggesting that the lack of humidity may also limit fungal development (Sylvia et al., 1999; Sanjuán et al., 2001; Sánchez et al., 2002; Araújo et al., 2015). Here, for over 14 months, we monitored ants infected by the most abundant entomopathogenic fungi complexes (*O. unilateralis* s.l., *O. kniphofioides* s.l. and *O. australis* s.l.) in both local (250 m²) and meso (15 km²) scales in Central Amazon to address the following hypothesis: (i) patterns of dead ant occurrence are positively related to temporal (seasons) and spatial (valleys-plateaus) humidity gradient, and (ii) entomopathogenic fungi are able to perform fine adjustments in response to the temporal and spatial humidity gradients.



Fig. 1. Some of the fungal species found in this study. (A) *Camponotus atriceps* parasitized by *Ophiocordyceps unilateralis* s.l. biting a leaf edge. (B) *Cephalotes atratus* killed by *O. kniphofioides* s.l. buried in the mosses of a tree trunk. (C) *Daceton armigerum* infected by *O. daceti* attached to a stem. (D) *Odontomachus hastatus* infected by *O. australis* s.l. found dead on moss carpet at the base of trees. Photo credit: João Araújo.

2. Material and methods

2.1. Study area

We carried out this study at Reserva Ducke, 26 km North of Manaus-AM, Brazil ($3^{\circ} 05' S$, $60^{\circ} 00' W$) (Fig. 2). The average annual precipitation in the reserve is approximately 2,400 mm with rainy season during March and April and dry season during August and September (Marques-Filho et al., 1981). The terrain is moderately rugged, with elevation varying between 40 and 120 m (Hopkins, 2005). The elevation associated with other edaphic factors creates a mosaic of forests with different structures (Ribeiro et al., 1999). Plateau areas are characterized by high, flat areas with clayed and well-drained soils (Ribeiro et al., 1999). In these areas, the height of the forest canopy reaches up to 60 m and the understory is relatively open compared with bottomlands (Hopkins, 2005). The bottomlands or valleys are close to water bodies with sandy soils. Some areas in the valley may be flooded for short periods (few hours), especially during the rainy season (Ribeiro et al., 1999).

Reserva Ducke has a system of perpendicular trails, which gives access to a set of permanent plots of 250 m distributed along the landscape. This system is maintained by the Research Program on Biodiversity (PPBio) and the Long-term Ecological Studies (PELD) (Pezzini et al., 2012; Costa et al., 2015). In this study, we sampled 15 plots from three trails (Fig. 1). We sampled each plot seven times, every two months from November 2014 to December 2015.

2.2. Dead ant monitoring

We followed the number of infected ant cadavers bimonthly

over 14 months to estimate the history and infection rate of each fungus complexes in our study plots. In each plot, we walked along the 250 m central line of the plots observing closely 1 m wide by 2 m high for the right and left side, carefully inspecting all the plants and litter within this area. We measured the height that each dead parasitized ant was found and individually tagged those ants. Throughout the study, a reference collection was created with ants parasitized by the three fungi complex.

We identified the parasitized ants to species level using taxonomic keys (MacKay, 1993; Andrade and Urbani, 1999; Fernández, 2002; Wild, 2005; Jiménez et al., 2007; Mackay and Mackay, 2010; Ortiz and Fernández, 2011; Baccaro et al., 2015) and by comparison with the reference Entomological Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA). We identified the fungi complexes based on their external morphology (Evans, 1982; Evans and Samson, 1984; Hughes et al., 2011b; Araújo et al., 2015) and were confirmed by a specialist (Araújo, J. P. M.). Given that the interaction between ants and their parasite is species-specific (Evans et al., 2011a; Araújo et al., 2015, 2018), we used the ant species identity as a template to fungal species identity. We deposited the species examined in this study in the Prof. Paulo Bührnheim Zoological Collection - CZPB, at Federal University of Amazonas – UFAM.

2.3. Environmental factors

The meteorological station at Reserva Ducke monitored the relative air humidity of our study site on a daily basis. We used the mean relative air humidity from the 60 d before each sampling campaign as a measure of temporal humidity. The values

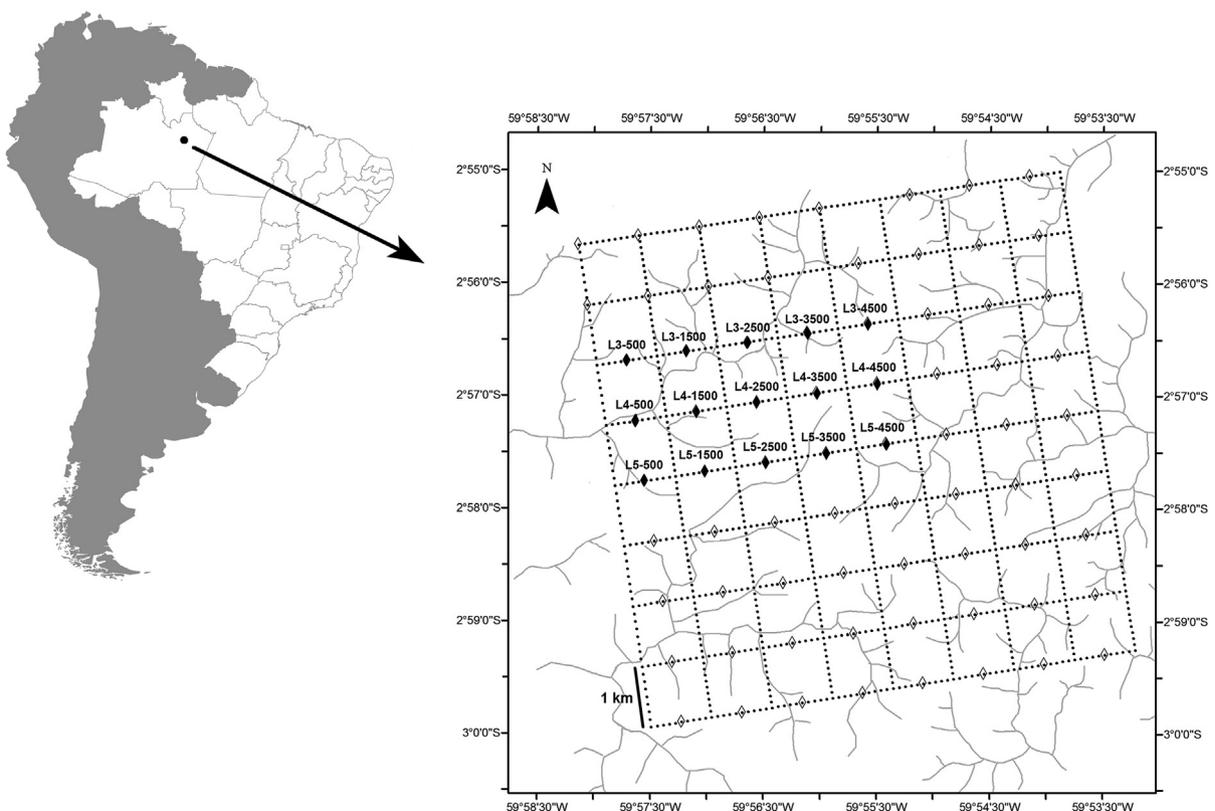


Fig. 2. Location of the Reserva Ducke near Manaus – AM. Black diamonds represent the 250 m long sampled transects regularly distributed at every 1 km. The dotted lines represent the grid of trails.

corresponded to local air humidity for the period in the whole Reserve area.

We estimated the spatial variation in local humidity according to the topography of our plots using the height above the nearest drainage system (HAND). The values of HAND for each plot were obtained with pictures of Digital Elevation Models (DEM) from the Shuttle Radar Topography Mission (SRTM), using the algorithm of Rennó et al. (2008). This algorithm normalizes the altitude according to the vertical distance of the points to the closest drainage system. Thereby, it estimates the correlation between the water table and topographic humidity, providing an individual measure of water/moisture availability for each plot. The HAND data is available on the Research Program on Biodiversity (PPBio) website (<http://ppbio.inpa.gov.br/knb/metacat>). Plots with higher HAND values are more elevated, exhibited conditions typical of drier plateau areas. Likewise, plots with lowest HAND values exhibited environmental conditions similar to the moister valleys habitat.

2.4. Data analysis

We used Generalized Linear Mixed Models (GLMM) with Poisson error distribution to access the relationship between abundance of major groups of parasites (*O. unilateralis*, *O. kniphofioides* and *O. australis*) and our gradients of temporal and spatial air humidity. We used the mean relative air humidity from the 60 d before the sampling (one temporal humidity measure for all the plots) and HAND (local humidity measure for each plot) as fixed effects in the models. We also included the plot identity as random factors in the models to control temporal and spatial autocorrelation of the dead ants (Zuur et al., 2009). For this analysis, we only considered new ant individuals that appeared between monitoring campaigns and the most abundant ant species for each fungal complex. We also used GLMMs with Poisson error distribution to investigate the relationships between the number of dead *Camponotus atriceps*, *C. bispinosus*, and *C. senex* infected by *O. unilateralis s.l.* and the species *Cephalotes atratus* infected by *O. kniphofioides sensu stricto* against the same temporal and local humidity gradients. The general setup for this analysis was similar to the previous one, in which we considered only new ant species and used plot identity as a random factor in the models (Zuur et al., 2009).

We followed the same analytical framework described above to evaluate how height from the soil of parasitized dead ants varies in response to the temporal and spatial humidity gradients. We used GLMMs with Gaussian error distribution to investigate the relationship between the average height where we found the infected ants per plot (response variable), against the average of the mean relative air humidity from the 60 d before the sampling and the HAND (fixed variables) with the plot identity as a random factor.

We compared the Akaike's Information Criterion (AIC) of each GLMM with the respective AIC of the null model (intercept and random effect only). Full GLMM models were selected when the delta AIC (difference between full and null models) were >2 (Akaike, 1982). We also calculated the marginal R² for each GLMM. Marginal R² provide the variation explained only by the fixed effects in the model (Nakagawa and Schielzeth, 2013). We also performed residual analysis to verify the adequacy of the model predictions. We performed all the analysis and graphs in R (R Team Core, 2015), using "lme4" (Bates et al., 2014) and performed the residual analysis using the "DHARMA" package (Hartig, 2016). We also used partial graphs to represent the modeling results. Each partial graph shows expected values of the dependent variable and expected values of the target independent variable if all other independent variables in the analysis were maintained at their median values (Breheny and Burchett, 2016).

3. Results

3.1. Temporal and local humidity patterns

March and April 2015 were the most humid months with mean air humidity reaching 90% (min = 82%, max = 94%) and 91% (min = 85%, max = 95%), respectively. August and September 2015 were the least humid months with a mean humidity of 62% (min = 50%, max = 72%) and 73% (min = 55%, max = 83%), respectively (Fig. 3). On average, the 15 studied plots were at 23.18 m from the water table level (HAND), ranging from ~1 m to 45 m.

3.2. Ant and fungus occurrence

We found 4,277 parasitized ants distributed in 26 species and four morphotypes. The four species with the largest number of parasitized ants were: *Camponotus atriceps* and *C. bispinosus* both found in all 15 plots, infected by *Ophiocordyceps camponoti-atricipis* and *O. camponoti-bispinosi*, respectively. *Cephalotes atratus* infected by *O. kniphofioides s.s.* was present in 14 plots, and *Camponotus senex* (infected by *Ophiocordyceps sp.* belonging to *O. unilateralis* complex) in 13 plots. The least abundant species, with only one ant infected were: *Neoponera cavinodis*, *Dolichoderus bispinosus*, *Cephalotes pusillus*, *Camponotus sp.1*, *Camponotus sp.2* and *Camponotus sp.3*, all infected by undescribed species of *Ophiocordyceps* (Table 1).

3.3. Occurrence of *O. unilateralis s.l.*

Ophiocordyceps unilateralis s.l. was the most abundant fungus complex, accounting for 89.1% of the total number of individuals. The most abundant infected ants were *Camponotus atriceps* (N = 2,599, 60.7%), *C. bispinosus* (N = 766, 17.9%) and *C. senex* (N = 158, 3.6%). Average air humidity and HAND explained ~13% of the total variation in the abundance of parasitized ants and the full model had a lower AIC than null model (Delta AIC > 2). More dead ants infected by *O. unilateralis s.l.* were found during periods with higher air humidity (b = 0.213, p < 0.001). Variation in spatial humidity (HAND) did not influence the number of parasitized ants by *O. unilateralis s.l.* (b = 0.062, p = 0.630). However, the relationship between the abundance of parasitized ants, air humidity and HAND varied between ant species.

We found infected individuals of *C. atriceps* mainly in palm trees, biting leaves and thorns. For this host species, there was an increase

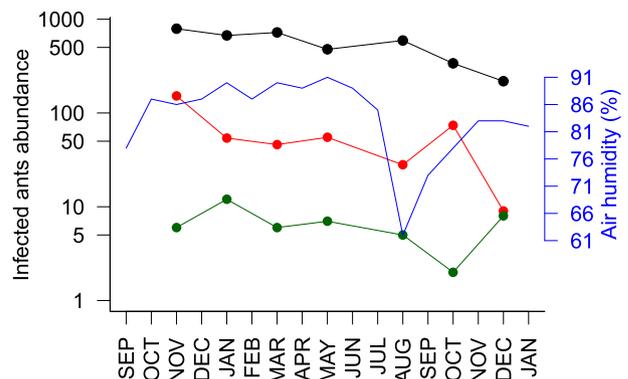


Fig. 3. Abundance of fungal complexes (*O. unilateralis s.l.* in black, *O. kniphofioides s.l.* in red and *O. australis s.l.* in green) in relation to sampling event and air humidity (%) monthly average from September 2014 to January 2016. The y-axis is a log scale to account for the differences in abundance among fungal complexes.

Table 1

Species of parasitized ants and their respective entomopathogenic fungi in 15 km² at Reserva Ducke, Central Amazon, Brazil. For each listed ant species, the number of plots where each species was found and the total number of collected individuals were reported.

Fungal complex	Ant species	Number of plots	Number of individuals
<i>Ophiocordyceps unilateralis</i> (3,811 individuals)	<i>Camponotus ager</i>	3	8
	<i>Camponotus atriceps</i>	15	2599
	<i>Camponotus balzani</i>	3	5
	<i>Camponotus bispinosus</i>	15	766
	<i>Camponotus cacticus</i>	2	2
	<i>Camponotus chartifex</i>	10	54
	<i>Camponotus fastigatus</i>	2	2
	<i>Camponotus femoratus</i>	5	39
	<i>Camponotus hippocrepis</i>	7	95
	<i>Camponotus latangulus</i>	3	7
	<i>Camponotus nidulans</i>	5	65
	<i>Camponotus senex</i>	13	158
	<i>Camponotus sexgutattus</i>	4	8
	<i>Camponotus</i> sp. 1	1	1
	<i>Camponotus</i> sp. 2	1	1
	<i>Camponotus</i> sp. 3	1	1
<i>Ophiocordyceps kniphofioides</i> (418 individuals)	<i>Cephalotes atratus</i>	14	398
	<i>Cephalotes pusillus</i>	1	1
	<i>Daceton armigerum</i>	1	3
	<i>Dolichoderus bispinosus</i>	1	1
	<i>Dolichoderus diversus</i>	2	6
	<i>Paraponera clavata</i>	4	9
	<i>Neoponera cavinodis</i>	1	1
<i>Ophiocordyceps australis</i> (48 individuals)	<i>Neoponera crenata</i>	3	4
	<i>Neoponera foetida</i>	2	4
	<i>Neoponera unidentata</i>	2	2
	<i>Neoponera villosa</i>	5	20
	<i>Odontomachus hastatus</i>	4	4
	<i>Pachycondyla impressa</i>	4	11
	<i>Pachycondyla</i> sp.	2	2

of 25% in the number of infected ants in the period of the highest air humidity compared to periods with the lowest air humidity (Table 2; Fig. 4A). *Camponotus bispinosus* also presented similar results, but we found parasitized individuals from this species mainly biting thorns of palm trees, not leaves. The abundance of this ant species was 10 times higher during months with the highest air moisture compared to the least humid periods (Table 2; Fig. 4C). The abundance of these two ant species (*C. atriceps* and *C. bispinosus*) was not affected by HAND values (Table 2; Fig. 4B and D). We also found parasitized individuals of *C. senex* mainly in palm trees, biting thorns. However, the abundance of this species was three times higher in areas with the highest HAND values compared to areas with lowest HAND, with no clear pattern related to the temporal gradient of air humidity (Table 2; Fig. 4E and F). The full model of the three parasite *Camponotus* species had a better fit than its respective null models (Delta AIC > 2 in all cases).

3.4. Occurrence of *O. kniphofioides* s.l. and *O. australis* s.l.

Ophiocordyceps kniphofioides s.l. represented 9.8% of the total number of individuals. The most abundant ant species infected was

Table 2

Summary of the generalized-linear mixed models of abundance of parasite ant species related to the average air humidity during the 60 d before the sampling and HAND (Height Above the Nearest Drainage) as a proxy to topographic humidity.

Ant species	R ² marginal	Fixed factors	b	p-value
<i>C. atriceps</i>	0.081	air humidity	0.158	<0.001
		HAND	0.309	0.294
<i>C. bispinosus</i>	0.206	air humidity	0.528	<0.001
		HAND	-0.047	0.849
<i>C. senex</i>	0.185	air humidity	0.038	0.631
		HAND	0.729	0.003

Cephalotes atratus (N = 398, 9.3%). All *C. atratus* were found concentrated in the moss at the base of the trees near to ant foraging trails. Following the general pattern observed for the whole *O. kniphofioides* s.l. group, the abundance of *C. atratus* parasitized with *O. kniphofioides* s.l. did not correlate with air humidity, neither to HAND values. The null model had better fit than the full model (Delta AIC = 1.94).

Ophiocordyceps australis s.l. was the least abundant fungus complex, representing only 1.1% of the total number of individuals. The most abundant ant species infected by these fungi was *Neoponera villosa* (N = 20, 0.4%). The abundance of parasitized ants by the fungi *O. australis* s.l. was not related to neither the average air humidity during the 60 d before the sampling nor with HAND. The *N. villosa* parasitized by *O. australis* s.l. was not abundant enough to be investigated separately.

3.5. Extended phenotype variation of *O. unilateralis* s.l.

The height of ant cadavers infected by *O. unilateralis* s.l. (mean = 109, min = 34, max = 181 cm) was not related to the air humidity neither HAND. However, the cadaver height varied according to the ant species parasitized. The height at which parasitized *C. atriceps* (Fig. 5A) and *C. bispinosus* (Fig. 5C) died doubled during months with higher air humidity (Table 3). In contrast, the height where *C. senex* cadavers was found was not related to the air humidity (Fig. 5E), but doubled in plots located at higher HAND values (Table 3, Fig. 5F). In this case, parasitized ants died at higher places in plots with higher HAND values.

3.6. Extended phenotype variation of *O. kniphofioides* s.l. and *O. australis* s.l.

Ants infected by *O. kniphofioides* s.l. were found on average at

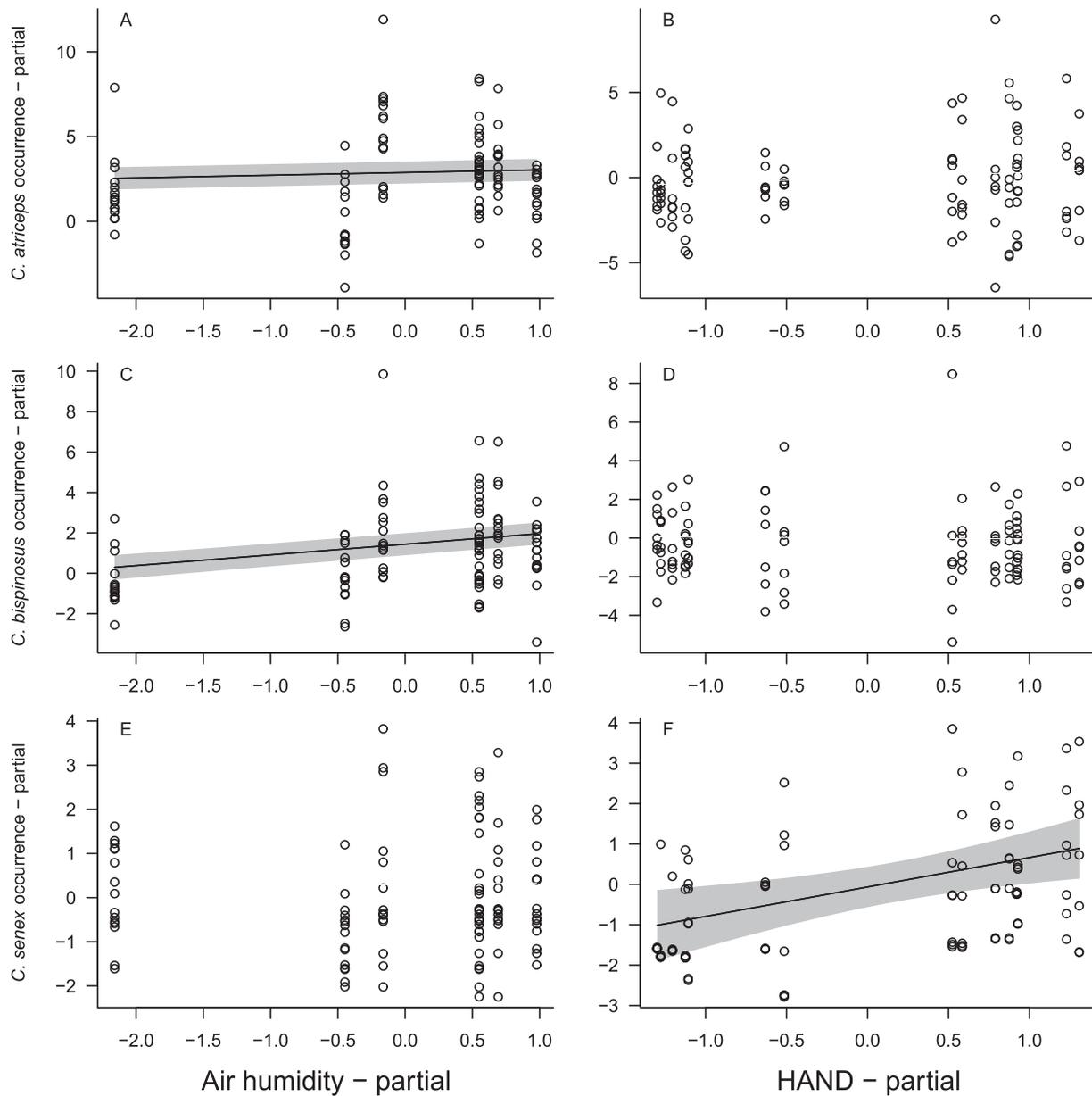


Fig. 4. Partial graphs of the number of host ants *Camponotus atriceps*, *C. bispinosus* and *C. senex* parasitized by the fungus *Ophiocordyceps unilateralis*, in relation to the average air humidity, during the 60 d before the sampling and HAND (topographic humidity). Circles represent the total number of parasitized ants in each plot by sampling event. The grey area represents the confidence interval of 95%.

17 cm above the ground (min = 0, max = 168 cm). The death height of ants infected by *O. kniphofioides s.l.* was not related to the air humidity, neither to the HAND values. A similar pattern was found for *C. atratus* infected by *O. kniphofioides s.l.* The null model had a better fit compared with full model (Delta AIC > 2 in both cases).

On average, the height of dead ants infected by *O. australis s.l.* was 15 cm above the ground (min = 0, max = 145 cm). The height at which infected ants died was not related to the air humidity nor to HAND. Because of the low number of individuals, the death height of ant species infected by this fungus complex was not abundant enough to be investigated separately.

4. Discussion

Temporal variation of the air humidity and spatial variation in HAND values only influenced the abundance of ants parasitized by

O. unilateralis s.l. The abundance of parasitized *Camponotus atriceps* and *C. bispinosus* were positively related to temporal air humidity. The extended phenotype of *C. atriceps* and *C. bispinosus* ants also seems to relate to the temporal gradient of air humidity, with ants found in higher places during periods of higher humidity. Conversely, abundance of parasitized *C. senex* was not related to air humidity but was higher in sites with deeper water table levels. Contrary to our predictions, we found more cadavers of parasitized *C. senex* in plots with higher HAND values, that is, with deeper water tables. Additionally, the height of dead *C. senex* was also higher in plots with higher HAND values. Altogether, our data suggests that, at least for ant species manipulated by the *O. unilateralis* complex, the effect of environmental humidity is species dependent, resulting in different patterns of host infection and extended phenotype.

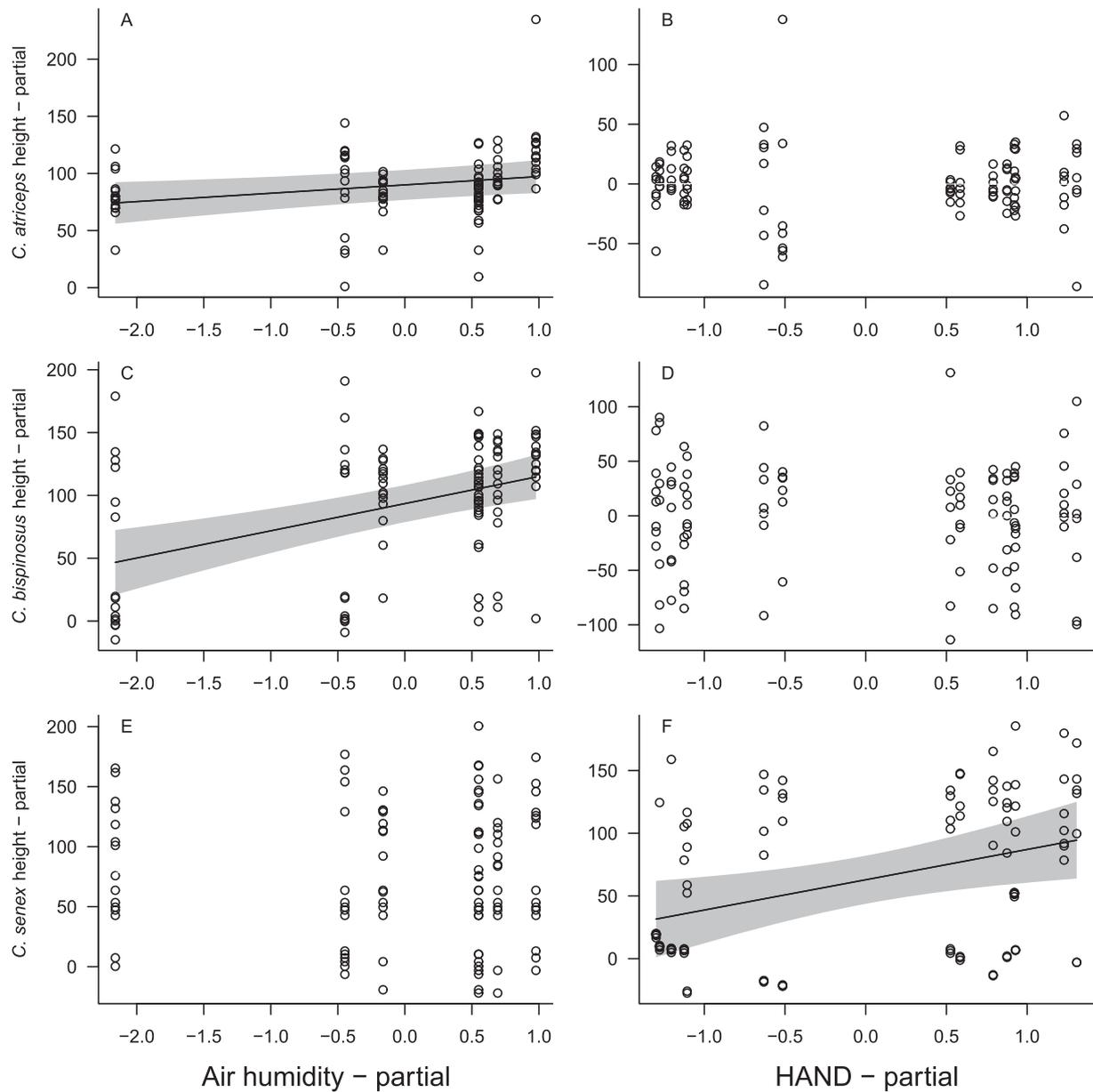


Fig. 5. Partial graphs of the height from the soil in centimeters of *Camponotus atriceps* (A, B), *C. bispinosus* (C, D) and *C. senex* (E, F) parasitized with the fungi *Ophiocordyceps unilateralis* related to the average air humidity during the 60 d before the sampling and to HAND values (topographic humidity). Circles represent the average height of ant cadavers in each plot and sampling event. The grey area represents the 95% confidence intervals.

Table 3

Summary of the generalized-linear mixed models of ant cadavers infected by *Ophiocordyceps* related to the average air humidity during the 60 d before the sampling and HAND (topographic humidity).

Height (cm)	R ² marginal	Fixed factor	b	p-value
<i>C. atriceps</i>	0.039	air humidity	7.308	0.015
		HAND	-0.179	0.976
<i>C. bispinosus</i>	0.14	air humidity	21.64	<0.001
		HAND	-1.861	0.785
<i>C. senex</i>	0.119	air humidity	-1.076	0.085
		HAND	24.145	0.022

4.1. Occurrence patterns of *Ophiocordyceps* complexes

Among the three fungal complexes sampled in our study site, only *O. unilateralis* has a pattern of infection associated with

temporal and spatial gradients of humidity. Fungal species belonging to this complex were also the most abundant in our study plots, representing 89% of all parasitized ants sampled. The other less abundant fungal complexes showed no clear pattern related to humidity: *Ophiocordyceps kniphofioides* represented 9.7% of the total number of collected ants and *O. australis*, only 1.1%. Therefore, it is possible that the lack of relationship between abundance of the less common fungus complexes and humidity may be biased by low sampling. Exploring some host functional traits may help us to establish fruitful avenues of investigation about how those relatively rare fungus complexes can respond to humidity variation.

Ant species commonly parasitized with *O. kniphofioides* s.l., including the species that we observed in this study, aggregate in small areas, usually in tree trunks (Evans and Samson, 1984, 1982). They live in relatively large colonies (>10,000 individuals) nesting

in the canopy or in the base of the trees (Hölldober and Wilson, 1990; Harada and Ketelhut, 2009), using the tree trunk as an access trail to the nest (Lanan, 2014). Non-infected *C. atratus* may remove cadavers of infected ants in the tree trunk, a behavior that may control the parasite prevalence (Evans and Samson, 1982). However, *O. kniphofioides* s.l. forms asexual spore-producing structures that remain in the environment, even after the host is removed (Araújo and Hughes, 2017; Araújo et al., 2018). These asexual structures consist of prostrate rhizoid-like outgrowths from the host that creep beneath the moss carpet and bark, giving rise to synnemata-like structures producing mucoid balls of conidia (Araújo et al., 2018). Thus, the spores have the chance to infect new hosts and continue the cycle of infection, resulting in larger numbers of infected ants close to foraging trails.

The relatively lower occurrence at assemblage level of *O. australis* s.l. was also observed in one forest in Colombian Amazon (Sanjuán et al., 2001) and may be related to some traits of their host species. In the current study, all ant species infected with *O. australis* s.l. (*Neoponera cavinodis*, *N. crenata*, *N. foetida*, *N. unidentata*, *N. villosa*, *Odontomachus hastatus* and *Pachycondyla impressa*) forage alone or in tandem (small groups of ant workers) (Lanan, 2014). Although information about the colony size of these ant species is scarce in the literature (Lanan, 2014; Delabie et al., 2015), they probably form relatively small colonies with up to 500 workers (Hölldober and Wilson, 1990; Harada and Ketelhut, 2009; Delabie et al., 2015). Therefore, it is reasonable to infer that the lower abundance of this parasite may be mirroring the abundance and colony size of their ant hosts and may not be a result of low sampling sufficiency. The behavioral manipulation caused by *O. australis* s.l. is still little studied and it is not known how the hymenostilboid spores infect their hosts. However, the infection is efficient, because even being less abundant, we found ants infected by this fungus complex in all plots. Therefore, considering the biology of *O. australis* and by *O. kniphofioides*, it is plausible to propose that these fungus complexes may be less dependent on humidity conditions, as the availability of hosts should be the main restrictive factor affecting their abundance in the environment.

Ophiocordyceps unilateralis infecting *Camponotus* ants was far the most abundant parasite in our site. Most Neotropical *Camponotus* species establish colonies in the canopy (Tobin, 1991) and create foraging trails that reach large areas (~10 m²) and last for years (Lanan, 2014). As a consequence, relatively large graveyards of *O. unilateralis* s.l. are commonly found near the foraging trails of *Camponotus* species (Pontoppidan et al., 2009; Loreto et al., 2014). The intense trail activity that these ants exhibit at night may also increase the chance of infection by the hirsutelloid spores, resulting in lower constraints in host availability compared to hosts of *O. kniphofioides* and *O. australis*.

Aligned with our results, several studies have shown that environmental conditions such as humidity, light and temperature play an important role in the mode of behavioral manipulation of *O. unilateralis* fungi (Andersen et al., 2009; Evans et al., 2011b; Loreto et al., 2014, 2018; Andriolli et al., 2019). Here, *Camponotus atriceps* and *C. bispinosus* were the only infected ant species present in all study plots and exhibited a positive relationship with the temporal humidity gradient. Conversely, the number of *C. senex* parasitized with *O. unilateralis* s.l. was higher in areas distant from the water table, being the only species responding to the spatial gradient of humidity. Interestingly, *C. senex* is one of the smallest *Camponotus* host species (~0.3 cm length) (Mayr, 1877). As small body mass can increase desiccation risk (Hood and Tschinkel, 1990; Kaspari and Weiser, 2000) and may limit fungal growth, it was expected that fungi infecting this ant species were more dependent on water availability. As much of the speciation within *O. unilateralis* complex was driven by host species specificity (Evans

et al., 2011a; Kobmoo et al., 2012), it is possible that fungi associated with *C. senex* requires different environmental conditions compared to other fungal species parasitizing bigger *Camponotus* hosts. The relationship between *C. senex* and its fungal parasite seems to be a promising avenue for research due to the unexpected relationship with spatial humidity patterns.

4.2. Extended phenotype variation of *Ophiocordyceps* complexes

The height of ant cadavers infected with *O. australis* s.l. and *O. kniphofioides* s.l. was not related with temporal or spatial variation in humidity conditions. *O. kniphofioides* s.l. develops asexual structures located under the carpet of moss and the bark of the trees, which is a perfect trap to infect new hosts (Araújo et al., 2018). The carpet of moss on the tree trunks may act as a protective buffer maintaining moisture content independent of the height from the ground. Therefore, location of *Cephalotes atratus* cadavers may be more related to moss density and microhabitat conditions. The height of ants infected by *O. australis* s.l. was also not related to humidity conditions, but most cadavers were found close to the soil, where the humidity variation is lower. This pattern may be explained by two non-excluding mechanisms. First, it is possible that this fungal group is more limited by moisture conditions, leading ant hosts to die in locations in which spatial and temporal humidity variation is lower. Second, it is possible that we are missing possible patterns combining the extended phenotype expression of several fungal species. Even after 14-months monitoring over ~15 km², we do not have enough data to determine which mechanism may be acting. Given the lower abundance of this fungal group, accumulating species specific information and understanding their response in relating to environmental factors will be a challenge for this fungal group.

On the other hand, the cadaver height of the most abundant parasite group (*O. unilateralis* s.l.) was related to the humidity gradients. Positioning infected ants in higher locations may generate an ecological advantage for fungi that are not limited by host availability, as this causes fungal spores to spread through larger areas, increasing the probability of infection of new hosts (Roy et al., 2006; Evans et al., 2011a, 2018). However, at higher locations the fungi may face more moisture variation that may compromise fungus development (Andersen et al., 2009; Loreto et al., 2014). The tight relationship between ant cadaver height, fungal development and sporulation was previously demonstrated by the manual relocation of parasitized ants (Andersen et al., 2009). However, the mean height of infected cadavers vary according to fungus specificity, sunlight or vegetation structure, ranging from 25 cm in Thailand (Andersen et al., 2009), 62–110 cm in Taiwan (Chung et al., 2017) to up to 290 cm in Brazil (Sobczak et al., 2017; Andriolli et al., 2019). Our results add a new temporal perspective to this pattern, suggesting that the height of ant cadavers can be adjusted according to local environmental conditions. In dry periods, the most favorable location for fungal growth and development may be close to the soil, where the humidity is usually higher and more stable (Hedger, 1985; Lodge and Cantrell, 1995). During the rainy season, humidity is a less limiting factor and ant cadavers can be found at higher spots, allowing fungi to disperse their spores over larger areas.

Although air humidity plays an important role in the behavioral manipulation of fungal parasites, it does not affect equally the cadaver height of all ant species (Evans et al., 2011a). It is possible that each fungal species has an ideal temperature and moisture requirements for its growth and development, which results in differences of death height between host species (Andersen et al., 2009; Evans et al., 2018). Our results support this hypothesis: when we combine the data of all ant species infected with

O. unilateralis s.l., the relationship with the air humidity and HAND disappears.

Similarly to our observation about host abundance, fungus infecting *Camponotus senex* responded differently to variation in humidity conditions in comparison to fungus infecting *C. atriceps* and *C. bispinosus*. Infected *C. senex* were found in higher locations in plots more vertically distant from the water table. These results reinforce the idea that humidity is not a limiting factor for this specific fungus to develop or that other factors, not considered, here were driving its patterns of infection and behavioral manipulation.

The temporal and spatial variation in air humidity were related to both the occurrence and extended phenotype expression of ant parasite fungi. However, fungi from different complexes exhibited different responses. In one hand, *O. australis* s.l. and *O. kniphofioides* s.l. were not related with temporal or spatial variation in humidity. On the other hand, *O. unilateralis* s.l. that infects *C. atriceps* and *C. bispinosus* were positively related to air humidity, but its occurrence and extended phenotype in *C. senex* seems to follow the opposite direction. Such differences among fungal complex responses to humidity conditions seem to be a property that emerges from the interaction between traits of each fungus and host species. Humidity is likely to exert a stronger influence on the patterns of infection and host manipulation of fungal species in which constraints on host encounter is lower. Although much of our interpretation could not be uncoupled by some bias resulting from the natural variation in host occurrence, this is one of the few studies that simultaneously evaluated the occurrence and type of behavioral manipulation of different sympatric fungal parasite groups in different habitat conditions. Comparing the response of different fungal groups to environmental conditions shed light on some interesting patterns of parasite requirements, offering new avenues for investigation of ecological relationships between these parasite groups.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2019.100871>.

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