


# Evidence for convergent evolution of host parasitic manipulation in response to environmental conditions

Raquel G. Loreto,<sup>1,2</sup>  João P. M. Araújo,<sup>2,3</sup> Ryan M. Kepler,<sup>4</sup> Kimberly R. Fleming,<sup>1</sup>  Corrie S. Moreau,<sup>5</sup>   
and David P. Hughes<sup>1,2,6</sup>

<sup>1</sup>Department of Entomology, Pennsylvania State University, University Park, Pennsylvania

<sup>2</sup>Center for Infectious Diseases Dynamics, Pennsylvania State University, University Park, Pennsylvania

<sup>3</sup>Department of Biology, Pennsylvania State University, University Park, Pennsylvania

<sup>4</sup>Sustainable Agricultural Systems Laboratory, United States Department of Agriculture, Beltsville, Maryland

<sup>5</sup>Department of Science and Education, Field Museum of Natural History, Chicago, Illinois

<sup>6</sup>E-mail: dph14@psu.edu

Received October 31, 2017

Accepted March 27, 2018

Environmental conditions exert strong selection on animal behavior. We tested the hypothesis that the altered behavior of hosts due to parasitic manipulation is also subject to selection imposed by changes in environmental conditions over time. Our model system is ants manipulated by parasitic fungi to bite onto vegetation. We analyzed the correlation between forest type (tropical vs. temperate) and the substrate where the host bites (biting substrate: leaf vs. twigs), the time required for the fungi to reach reproductive maturity, and the phylogenetic relationship among specimens from tropical and temperate forests from different parts of the globe. We show that fungal development in temperate forests is longer than the period of time leaves are present and the ants are manipulated to bite twigs. When biting twigs, 90% of the dead ants we examined had their legs wrapped around twigs, which appears to provide better attachment to the plant. Ancestral state character reconstruction suggests that leaf biting is the ancestral trait and that twig biting is a convergent trait in temperate regions of the globe. These three lines of evidence suggest that changes in environmental conditions have shaped the manipulative behavior of the host by its parasite.

**KEY WORDS:** behavioural manipulation, *Camponotus ants*, convergent adaptation, host-parasite interaction, *Ophiocordyceps unilateralis*.

Convergent adaptations are important evidence of evolution by natural selection and have been reported for different levels of biological organization. For instance, transitions from free-living to parasitic life histories have occurred independently hundreds of times across unrelated lineages converging to a few modes of parasitism, transcending to genomes, and reflecting in convergent strategies of host exploitation and transmission (Poulin and Randhawa 2015). Another well-studied convergent trait is the behavioral response to similar environment conditions. For instance, changes in temperature and photoperiod trigger long-distance mi-

gration of birds, insects, whales, and turtles, as a response to environmental changes (Milner-Gulland et al. 2011).

In some cases, the behaviors observed in nature are adaptations on the part of parasites that have evolved to infect animals and manipulate their behavior as a transmission strategy (Moore 2002; Mehlhorn 2015). In these circumstances, the behavior of the host (i.e., its phenotype) is an extension of the genotype of the parasite; a phenomenon known as the extended phenotype (Dawkins 1982). The number of examples of parasites that adaptively manipulate the behavior of their hosts has recently escalated

a reflection of the expansion of the field (Hughes et al. 2012). Many aspects have been considered in the study of parasitic manipulation, such as the molecular mechanisms of behavioral manipulation (Biron et al. 2005; Biron et al. 2006; Hoover et al. 2011; de Bekker et al. 2014; de Bekker et al. 2015), the epidemiological significance of behavioral manipulation (Loreto et al. 2014), and the ecological importance of manipulated hosts in the environment (Thomas et al. 1998; Sato et al. 2011; Sato et al. 2012). What has not been examined is whether parasite manipulation of animal behavior responds to changes in the environmental conditions which the host experiences. Because environmental changes are known to result in adaptive shifts of phenotypes, such as animal behavior (Losos et al. 2004; Hoekstra et al. 2005; Langkilde 2009; Kingsley et al. 2017), it is reasonable to suppose that the same environment may act as a selective force on the extended phenotypes of parasites inside those animals.

One system where one might expect the environment to play a significant role in behavioral manipulation is the “zombie ant.” In this system, many species of parasitic fungi in the complex *Ophiocordyceps unilateralis sensu lato* (*s.l.*) manipulate ants from the tribe Camponotini to climb and bite onto aerial vegetation, attaching themselves to the plant tissue (Andersen et al. 2009). Uninfected ants never display this stereotypical biting behavior. The biting behavior displayed by the infected ants is the extended phenotype of the fungus and has been experimentally demonstrated to be adaptive for this parasite, which has zero fitness if the host falls on the ground or is moved to the forest canopy or inside the ants’ nest (Andersen et al. 2009; Loreto et al. 2014). The death of the ant, shortly after the manipulated biting behavior, is the end point of the manipulation and marks the transition for the fungus, from feeding parasitically on living tissue to feeding saprophytically on the dead tissue of its recently killed host (de Bekker et al. 2015). Besides providing nutrients, the carcass of the ant will serve as a platform for the fungus to grow a long stalk, externally from its dead host, to release the spores (termed ascospores in this group of fungi), ultimately infecting new hosts (Evans et al. 2011; Hughes et al. 2016). Once it starts growing externally from the dead ant, the fungus is exposed to environmental conditions outside the body of its host. Fungal development is known to be strongly affected by environmental conditions, notably changes in humidity and temperature (Baxter and Illston 1980; Kerry 1990). Species in the *O. unilateralis* complex have been recorded at latitudes ranging from 34° north (de Bekker et al. 2014) to 20° south (Loreto et al. 2014), which implies a wide range of environmental conditions exist in which behavioral manipulation of the ant and the subsequent postmortem development of the fungus occur.

Previous observations have suggested two kinds of behavioral manipulation occurring in distinct forest types. In tropical forests, ants infected by species of fungi in the *O. unilateralis* complex are predominantly manipulated to bite leaves (Andersen

et al. 2009; Loreto et al. 2014; Araujo et al. 2015). By contrast, in northern temperate systems (USA, Japan), manipulated ants have been described as biting onto twigs (Kepler et al. 2011; de Bekker et al. 2014). The seasonal leaf shed observed in temperate forests represents a major difference in comparison to tropical forests, where the majority of the trees are evergreen with leaves present throughout the year. For a parasite that manipulates the host to bite leaves before using the host cadaver as a platform for transmission, the permanence of a leaf as a platform may impact its fitness. Although there are other parasites that manipulate ant behavior, including other fungi (Boer 2008), cestodes (Beros et al. 2015), nematodes (Poinar and Yanoviak 2008), trematodes (Krull and Mapes 1952; Carney 1969), and flies (Henne and Johnson 2007), none of them have been as extensively studied as the zombie ants. This deeper understanding of the biology implies the zombie ant system may be a more suitable model for studying how environmental variation affects the behavioral manipulation of hosts by parasites.

We hypothesized that biting different substrates (leaf *versus* twig) is an adaptation of the parasite extended phenotype to the distinct seasonality and environmental conditions present in the two forest types (i.e., tropical vs. temperate). To test this hypothesis, we focused on three lines of evidence. First, it was necessary to confirm if the substrate to which the hosts bite (henceforth called biting substrate) (leaf vs. twig) varies consistently across the South–North cline from tropical forests to temperate woods (in the northern hemisphere). To this end, we analyzed the global distribution of species belonging to the *O. unilateralis* complex to determine how geography relates to the biting substrate. Second, we hypothesized that twig biting may confer an adaptive advantage in temperate forests where the leaves are shed annually, especially if the fungus requires an extended period of time to fully develop. Thus, we evaluated, across 20 months, the development of a species belonging to the *O. unilateralis* complex in a temperate forest located in South Carolina after the fungus manipulated its host. Finally, because temperate forests occur in different locations, we tested the hypothesis that behavioral manipulation of ants by fungi to bite twigs is an adaptation that has convergently evolved in geographically distinct temperate forests. To achieve this, we inferred the phylogenetic relationships and conducted ancestral state reconstruction (ASR) between different species of fungi within the *O. unilateralis* complex that manipulate the host to bite leaves and those that manipulate their hosts to bite twigs, in both Old and New World temperate and tropical forests. Taken together, we present multiple lines of evidence, suggesting the parasite extended phenotype has responded to long-term changes in environmental conditions by shifting biting behavior from leaves to twigs. Furthermore, the shift in the behavioral manipulation of the host is a convergently evolved extended phenotype in different areas of the globe.

## Material and Methods

### THE GLOBAL DISTRIBUTION OF THE ZOMBIE ANT FUNGI *O. unilateralis* s.l. AND VARIATION IN BITING SUBSTRATE

To survey the global distribution of fungi species of the *O. unilateralis* complex, we collected species records from around the world. We examined material in museums and herbarium collections, as well as pictures available on the internet (under the terms “*Ophiocordyceps*,” “*Cordyceps*,” and “zombie ants”). Additionally, we used the senior author’s laboratory collection, which includes samples collected by the authors of this study and other collaborators. We categorized the sources as: internet (flicker, blogs, etc.), internet by academics (flicker, blogs, etc., maintained by academics, such as museum curators), laboratory collection, museum collection, and publication. All samples could be easily ascribed to the *O. unilateralis* species complex that has a very distinctive macromorphology, where the ascospore-producing structure (ascoma) distinctly occupies one side of the stalk (hence the epithet *unilateralis*) or the immature stage emerges as a long stalk from between the head and thorax on the dorsal side of the ant (Evans and Samson 1984). For each record, we collected the following information (when available): country, most precise location available (e.g., national park, nearest city), geographic coordinates, ant host, biting substrate, collector, year, and source. We classified the substrate as “bark” when the host was biting the base or main trunk of the tree, as well as when it was encountered inside fallen logs (which only occurred in Missouri, USA). The substrate “twig” was designated when host ants bite the wooden material of the vegetation other than the main trunk (i.e., twigs). We classified the substrate as “leaf” when the host was biting leaves and its variations, such as spines. The “green twig” classification, which only occurred twice (in Costa Rica and Thailand), was designated when host ants were biting early stages of stems, which were photosynthetically active (green indicating the presence of chlorophyll *a*) and lacking cambium. For the specimens we genotyped during the study, we visually inspected the substrate in the field before collecting the samples. For the specimens we did not genotype but rather used the genetic data available on GenBank, we relied on the accuracy of the description of the samples in the original publication, as well as the figures that accompanied those publications. We performed ANOVA to test if there is a correlation between latitudinal gradient and type of biting substrate. For the analysis, we included only the samples with precise location (park, reserve, closest city) ( $n = 53$ ) and excluded the samples were assigned only to country level. Because of the reduced records for some biting substrate categories (bark and green twig), we summarized the response variable to “green” ( $n = 45$ ) and “brown” ( $n = 8$ ) biting substrate. The analysis was performed in R.

### POST-MORTEM PARASITE DEVELOPMENT IN A TEMPERATE FOREST

We hypothesized that the plant substrate the ants are manipulated to bite (leaf vs. twig) was related to leaf shed. The rationale for this hypothesis is that leaf shed in temperate biomes would limit the available time for the fungus to reach maturity if the ants were manipulated to bite leaves in this environment. To provide support for this hypothesis, it was necessary to document the time required for *O. kimflemingiae* to develop reproductive maturity postmortem of the ant in a temperate wood setting. This study was conducted in a private temperate forest patch located in Abbeville County, South Carolina (georeference: 34.375215, –82.346937). This woodland is dominated by deciduous trees that shed their leaves in the fall (Fig. S1A and B). Data collection was done during the entire year (between December 31, 2009, and August 23, 2011) to capture leaf biting if and when it occurred. During this period, we spent 3 h each day searching twigs and leaves for the presence of manipulated ants. All the cadavers of infected manipulated ants (attached to the vegetation) were tagged, photographed, and the biting substrate was recorded ( $n = 287$ ). For the newly killed ants attached to the vegetation ( $n = 29$ ), ants were labeled with a number and the date they were first found to ensure future identification. The 29 newly killed ants were photographed on a daily basis for the first 60 days, and then every two weeks to a month, until August 2011, to record the phenology of the fungus. One can determine if the fungus is sexually mature, and thus capable of releasing ascospores, because the mature ascoma are recognizable by the erumpent ostioles, which are holes through which ascospores are released.

### PHYLOGENETIC ANALYSES

To infer the evolution of substrate use and if this preference is a monophyletic or a convergent trait, we selected fungal species from as many different geographic locations as we could, to reconstruct the phylogenetic relationships between taxa. Note that our sampling was limited by previous collections that were not designed to sample all regions. Indeed, some regions such as the eastern Palearctic were not sampled because the *O. unilateralis* complex is likely extinct in that region, despite clearly having been present based on fossil evidence from the mid-Eocene (Hughes et al. 2011). DNA extractions were done following the protocol as previously described (Kepler et al. 2011). Briefly, the genomic DNA was isolated using chloroform and purified with GeneClean III Kit (MP Biomedicals). Many of the specimens in the senior author’s laboratory collection, collected in 1970–1980s, were dry and degraded, resulting in low-quality DNA templates. These samples were excluded from the phylogenetic analyses.

From the genomic templates, four genes were amplified by PCR. We used two ribosomal genes, nu-LSU (954 bp) and nu-SSU (1144 bp), and two protein-coding genes, RPB1 (813 bp) and TEF

(1,012 bp). The cleaned PCR products were sequenced by Sanger DNA sequencing (Applied Biosystems 3730XL) at the Genomics Core Facility of The Pennsylvania State University. The raw sequence reads were manually edited using Geneious version 8.1.8 (Biomatters, Ltd.) (Kearse et al. 2012). Individual gene alignments were generated by MUSCLE (Edgar 2004). For this study, we generated 123 new sequences (31 for SSU, 31 for LSU, 32 for RPB1, and 29 for TEF). More details for the molecular work performed for this study can be found in the Supporting Information S1. The alignment of each gene was inspected manually and concatenated into a single dataset using Geneious version 8.1.1 (Kearse et al. 2012). Ambiguously aligned regions were excluded from phylogenetic analysis and gaps were treated as missing data. The GenBank accession number and Herbarium voucher for all the specimens and genes used in this work are listed in Dataset S2. The aligned length of the concatenated four gene dataset was 3923 bp. Maximum likelihood (ML) analysis was performed with Randomized Axelerated Maximum Likelihood (RAxML) version 8.2.4 (Stamatakis 2006) through the online platform CIPRES (phylo.org) (Miller et al. 2010). The dataset was divided into eight partitions (one each for SSU and LSU, plus separate partitions for the three codon positions of protein-coding RPB1 and TEF) and the GTR+G model of molecular evolution was applied independently to each partition. Branch support was estimated from 1000 bootstrap replicates. Bayesian phylogenetic reconstruction was performed with MrBayes version 3.2.6 (Ronquist et al. 2012), applying the GTR model with gamma distributed rates and invariant sites using the same partition scheme as the ML analysis. The analysis was run with four independent chains for five million generations, sampling trees, and writing them to file every 500 generations. Runs were examined for convergence with Tracer version 1.6.0 (Rambaut et al. 2014). The first 25% of trees were discarded as burn-in and posterior probabilities mapped onto a 50% consensus tree. In addition, we performed an ASR. This analysis was implemented in Mesquite version 3.10 (Maddison and Maddison 2015). Ancestral character states were estimated across our single most likely topology with each taxon coded according to biting location preference (twig, leaf, or trunk). We implemented the Mk1 likelihood reconstruction method (with default settings), which maximizes the probability the observed states would evolve under a stochastic model of evolution (Schluter et al. 1997; Pagel 1999).

To test for correlation between character states for biting substrate and geographic location (i.e., tropical vs. temperate), we implemented a test of dependence of character evolution as implemented in Mesquite 3.2 (Pagel 1994). This analysis tested the relationship between two discrete characters across a phylogeny, which takes into account branch lengths, develops estimates of rates of changes for the characters and tests for correlated evolution without relying on ASR (Robson et al. 2015).

To discriminate whether a four-parameter or eight-parameter model is a better fit to the data, a likelihood ratio test statistic was used. In this analysis, the null hypothesis is that the substrate where the infected ants bite is random, rather than the correlated evolution of such trait in response to environmental conditions.

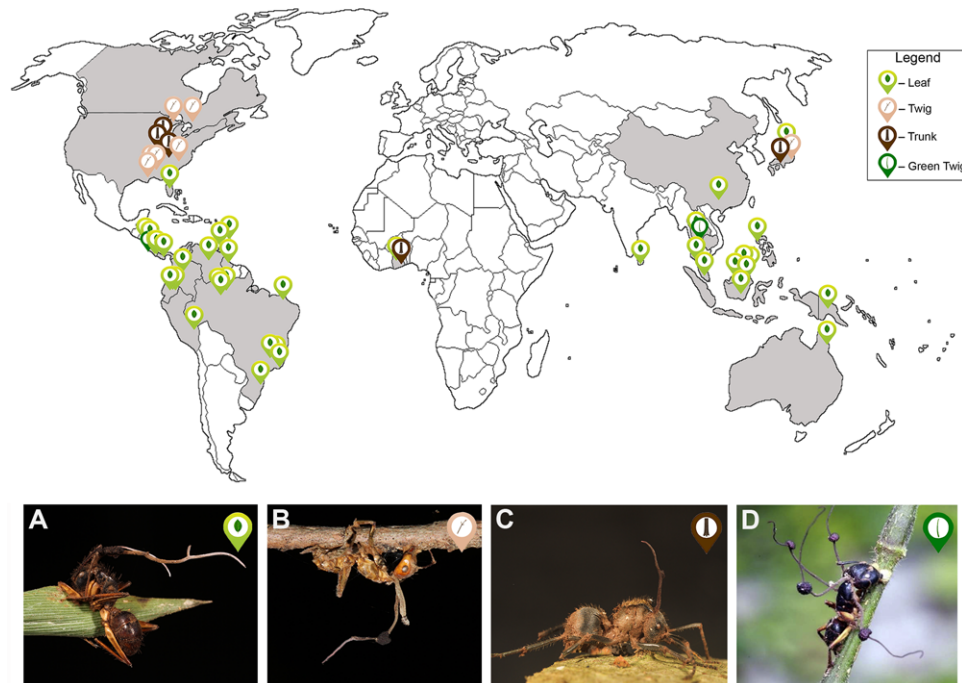
## Results

### THE GLOBAL DISTRIBUTION OF THE ZOMBIE ANT FUNGI *O. unilateralis s.l.* AND VARIATION IN BITING SUBSTRATE

We determined that species in the *O. unilateralis* complex have been recorded in 26 countries (Fig. 1; Dataset S1). Laboratory collections ( $n = 24$ ), publications ( $n = 16$ ), and museum collections ( $n = 14$ ) accounted for the majority of the records found. The other seven records originated from images on webpages maintained by academics, such as museum curators. Only 11 of 72 were collected from images posted online with no scientific affiliation. These 11 records are listed in Dataset S1, but were not included in further analyses performed on this study. We found reports of zombie ant fungi in North, Central and South America, Africa, Asia, and Oceania (Fig. 1; Dataset S1). The latitudinal distribution of *O. unilateralis s.l.* is 74°, ranging from 47° North (Ontario, Canada) to 27° South (Santa Catarina, Brazil). Our dataset was constructed based on different sources and methods of collection (see methods) and for this reason, we are not able to infer the relative abundance in different locations of the globe. However, in some cases, records came from more detailed studies (e.g., Mongkolsamrit et al. 2012; Loreto et al. 2014), so we were able to estimate the abundance for those specific areas. This permitted us to confirm previous observations that most of the occurrence records for *O. unilateralis s.l.* are from tropical forests. In the tropics, the majority of records were of ants manipulated to bite onto leaves. Exact numbers of *O. unilateralis s.l.* killed ants encountered was not recorded, but it is in excess of 10,000 samples based on 12 years of field work in the Atlantic rainforests of Brazil (Loreto et al. 2014), Amazonian forest of Brazil (Araujo et al. 2015), and Colombia (Sanjuan et al. 2001) and lowland forests of Peninsular Thailand (Andersen et al. 2009; Pontoppidan et al. 2009; Mongkolsamrit et al. 2012). Although leaf biting predominates in the tropics, we know of two exceptions; one in Costa Rica (online record, Dataset S1) and another in Thailand (Kobmoo et al. 2015). In both cases, the ants are found biting chlorenchymous stems (green stems/twigs that are photosynthetically active and lack cambium) and detailed information about behavior and ecology of these can be found in the Appendix.

In temperate regions, species in the complex *O. unilateralis s.l.* have been, so far, reported for three countries with predominantly temperate forests: United States, Japan, and Canada. In





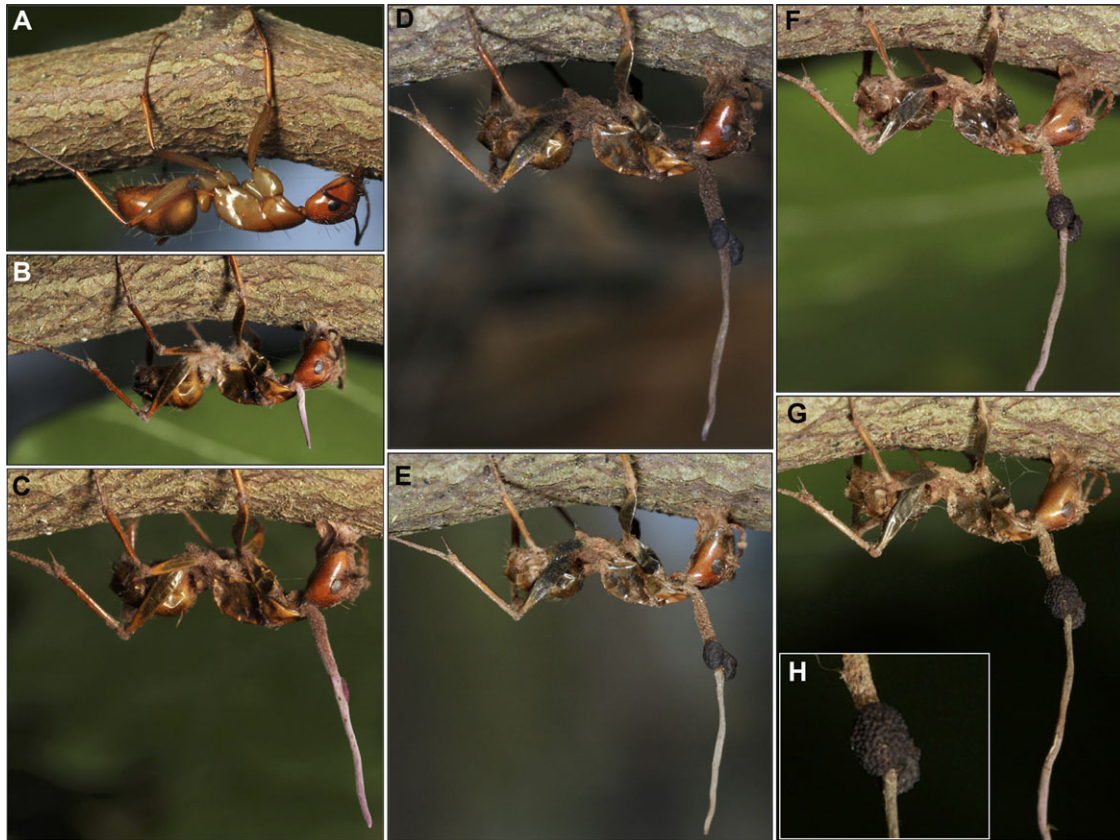
**Figure 1.** Global distribution and behavioral manipulation by *Ophiocordyceps unilateralis sensu lato* infecting ants. The light green markers represent records of ants manipulated to bite leaves. The light brown represents the records of ants manipulated to bite twigs. The dark brown represents records of ants manipulated to bite tree trunks. The dark green markers represent the record of the one species of ant manipulated to bite green twigs. (A) *Camponotus atriceps* manipulated to bite onto a leaf (Brazilian Amazon). (B) *Camponotus castaneus* manipulated to bite onto twig (South Carolina, USA). (C) *Polyrhachis militaris* manipulated to bite onto bark (Atewa, Ghana). (D) *Camponotus* sp. manipulated to bite green twigs (Nakhon Nayok, Thailand), image modified from Kobmoo et al. (2015).

both the United States and Japan, some species of fungi manipulate the ants to bite twigs, while others manipulate their host ants to bite leaves (Dataset S1). In the United States, an undescribed member of this fungal species complex that manipulates the ants to bite onto leaves was reported from an evergreen wetland forest, near the eastern coast of Florida (28° North, Dataset S1). In Japan, we encountered another undescribed species within the *O. unilateralis* complex in temperate forests (30° North), manipulating the ant *Polyrhachis moesta* to bite onto leaves. Interestingly, all the specimens collected for this species were found on evergreen plants (in which there is no leaf fall) in a forest in Kyoto (Dataset S1). In both the United States and Japan, we also encountered ants being manipulated by *O. unilateralis s.l.* to bite onto the bark of trees, although it was not frequent. This bark biting behavior was previously observed for two ant species collected from Ghana, in cocoa plantations (Samson et al. 1982). In Missouri, we discovered trunk biting where ants were manipulated to bite wood on the inside of logs. Confirming this observed pattern, we found that the use of biting substrate is correlated to the latitude. The latitudinal mean of infected ants biting brown substrates ( $38.94 \pm 13.76$ ) is significantly higher than the latitudinal mean of infected ants biting green substrates ( $14.59 \pm 2.17$ ,  $P < 0.0001$ ).

#### POSTMORTEM PARASITE DEVELOPMENT IN A TEMPERATE FOREST

For the vast majority of the temperate samples (286/287, 99.7%), we only found cadavers attached to the underside of twigs (Fig. S1C) and across our 20-month survey, we never found infected ants biting leaves. We also found that only two species of ants, *Camponotus castaneus* and *Camponotus americanus* were infected. We conducted extensive searching over the entire year and only discovered newly killed ants between June 20 and October 24 ( $n = 29$ ). Although the newly killed ants were found during the summer and at the beginning of autumn, when both leaves and twigs are available for the manipulated ants to bite onto, all of them were found biting onto twigs. For some ants (7/29), we determined that the cadaver was discovered within the first 24 h after manipulation and death of the ant, because of the stereotypical appearance of the gaster (terminal portion of the ant's abdomen) which was noticeably swollen due to abundant fungal tissue inside the body (Fig. 2A; Video S1). The remaining 22 ants were within two to three days of death as they were all discovered before the fungus grew from inside to the outside of the ant's body.

We discovered that the development of the fungal stalk for all specimens was delayed until the year following the behavioral



**Figure 2.** The development of the fungus, *Ophiocordyceps kimflemingiae*, postmanipulation and postmortem of the ant host, *Camponotus castaneus*, in South Carolina. These photographs were taken of the same ant, under natural conditions, across a year (A) Freshly killed individual (between 0 and 24 h after the ant was killed) on July 25, 2010, (B) August 10, 2010, (C) August 26, 2010, (D) November 14, 2010, (E) March 13, 2011, (F) June 4, 2011, (G) July 16, 2011, and (H) close up of the mature fungal sexual structure.

manipulation and death of the ants (Fig. 2). Of the 29 cadavers identified in 2010 (June 20–October 24) as recently manipulated and killed, 14 fell from the tree soon after biting. The average duration was 25.2 days, ranging from one to 138 days, with the majority (9/14) lasting less than 20 days. In addition, we discovered for ants manipulated by *O. kimflemingiae*, the behavioral manipulation also involves wrapping the legs of the ant around the twig (Fig. S2 and Movie S2). This is not a behavior observed in healthy ants when they walk or rest on twigs because ants do not walk with their tibia or femurs touching the substrate. Instead, they use the most distal segment of the tarsus: the third to fifth tarsomeres (Endlein and Federle 2015), which means they essentially walking on their “toes.” Of the 287 samples we observed during this study, 48 were missing legs, perhaps as a result of a long period in the field. From the 239 of which, we could clearly observe details from their legs, 90% (216) had their legs wrapped around the biting substrate. Only 23 (out of 239) ants were attached to the twigs by their mandibles alone. In some cases, as shown in the Movie S2, it is clear that the leg grasping behavior prevented the dead ant from falling from the twig,

consequently enabling the parasitic fungus to complete its life cycle. The first pair of legs typically crossed the second and in some cases the third pair of legs, which may provide increased purchase (Fig. S2). Both the legs touching other legs and the legs touching the wood developed dense mats of hyphae at their contact points that stitched the ant to the substrate (Fig. S2). This leg wrapping behavior has never been seen in ants manipulated by *O. unilateralis s.l.* in tropical forests.

Based on the morphology of ascoma, the remaining 15 specimens (out of 29 freshly manipulated and killed in 2010) did not reach maturity until the summer of the following year (2011), which can be determined either based on the presence/absence of the ascoma or the erumpent nature of the ostioles on the ascoma (Fig. S3). The minimum time required to reach sexual maturity was 310 days (October 16, 2010–August 22, 2011). The average duration of these cadavers in the environment was 572 days, ranging from 510 to 615 days. Note that not all samples reached sexual maturity over the course of this study. In some cases, the stalk broke off (Fig. S4A and B) or hyperparasitic fungi infected *O. kimflemingiae* (Fig. S4C), preventing the parasite from

reaching sexual maturity. It is notable that during winter, the fungus experiences severe weather with snow and ice rain (Fig. S1D and E).

## PHYLOGENY

Based on our phylogenetic reconstruction, we find that the species complex *O. unilateralis s.l.* forms a monophyletic group (maximum likelihood analysis boot strap value = 100). The RAxML inference topology is presented in Figure 3. In addition, we performed a Bayesian analysis that produced a topology consistent with the ML approach, as well as similar support values for the majority of the nodes (Fig. S5). Within the *O. unilateralis s.l.* clade, we recovered two major subclades: one formed by Asia–Oceania species and another with mostly American species, with a single exception, *Ophiocordyceps pulvinata* (from Japan). Within the two subclades (continental scale), the fungal species did not cluster according to geographic origin of the samples (country scale). Within the Asia–Oceania cluster, where the fungus is found infecting both carpenter and spiny ants, the genera *Camponotus* and *Polyrhachis*, respectively, there was also no clustering of fungal species by the ant host although there is host specificity at the species level. Thus, below the continent level, there is no clear phylogenetic pattern either related to geographic location or the host species. This implies that neither host associations nor geographic locations are reliable indicators of phylogenetic relationships.

The result of the ancestral character–state reconstruction analysis on our maximum likelihood topology analysis is presented in Figure 3 (colored branches). We find that leaf biting is strongly inferred as the ancestral character state for ants manipulated by fungi within the *O. unilateralis* complex. The shift in biting different substrates occurred at least four times in the evolutionary history of this group of manipulative parasites. In node 1 (Fig. 3), the shift from leaf to twig biting was reported for samples from North America. The ancestral biting substrate for node 2 was ambiguous. This node consists of fungi manipulating ants to bite onto twigs (from Japan), and tree trunks (from Missouri, USA). Node 3 (Fig. 3), representing the change from leaf biting to green twig, a behavioral manipulation found in a fungal species from Thailand. Nodes 1–3 were well supported in the phylogenetic analyses (bootstrap > 70). The fourth change on the biting substrate is reported for the fungus species *O. ootakii* (node 4 Fig. 3), which manipulates ants to bite twigs. However, this node is not well supported in the phylogenetic analyses (bootstrap < 70). Tests of correlated characters performed in Mesquite using Pagel's correlation analysis method (Pagel 1994) strongly supported the correlation between biting substrate (leaf vs. nonleaf substrate) and geographic range of species (tropical vs. temperate) (likelihood difference = 5.45429;  $P = 0.01$ ). Thus, the substrate where

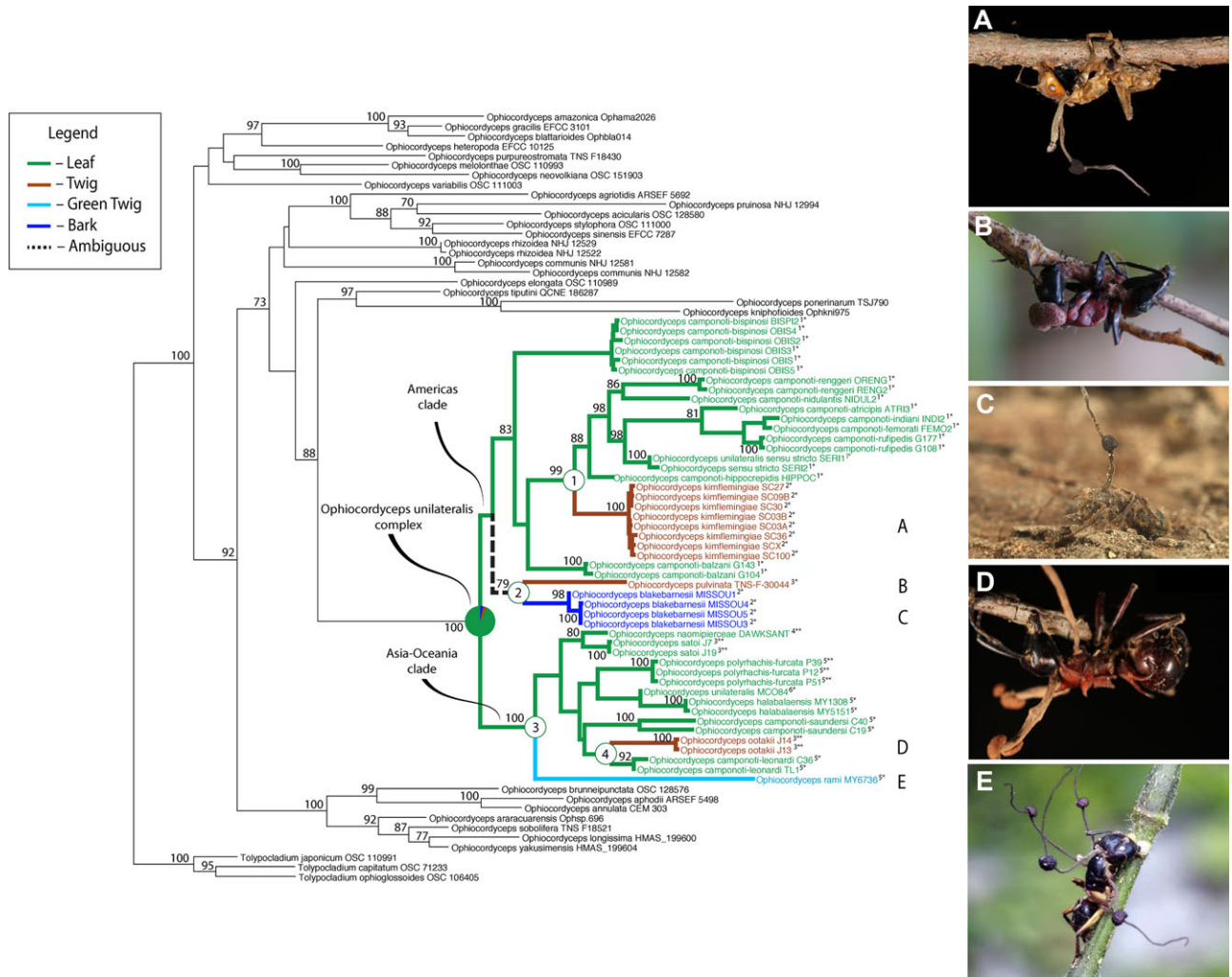
the ants are found attached is positively correlated with the geographical species range rather than the result of chance.

## Discussion

Convergence of traits in response to similar environment and convergence of parasitism strategies are central topics in evolutionary biology. Here, we combined these topics to investigate the interplay of animal behavior and parasite adaptation by testing the hypothesis that extended phenotypes (i.e., manipulation of host behavior by parasites) in phylogenetically distinct parasite species have responded to the environmental conditions experienced by both partners in similar manners. The overlap between the observed biting substrate (leaf/twig) and forest system (tropical/temperate), together with the time required for the fungus to complete its life cycle in each system, as well as the homoplastic nature of the biting substrate trait, suggests that environmental conditions have played an important role in shaping the mode of behavioral manipulation by this group of fungal parasites. Based on the ASR, we suggest that leaf biting, and not twig biting, is the ancestral condition and that twig biting evolved independently in multiple temperate forest biomes due to local environmental conditions and resource availability (i.e., ephemeral leaves as platforms versus stable twigs). Additionally, given the apparent difficulty in biting twigs, we suggest that in temperate systems twig grasping evolved in addition to the biting behavior, which arose first.

In this study, we focused on the species of fungi in the *O. unilateralis* complex, which manipulate their ant host behavior after infection giving them their common name of zombie ants. This complex occurs within the genus *Ophiocordyceps* that is one of the most speciose taxa of fungal parasites infecting insects (Quandt et al. 2014; Araujo and Hughes 2016). Tropical Asia is likely to be the center of origin of this entomopathogenic fungi group (Hywel-Jones 2002) and so they likely originated in moist, lowland tropical forests. The precise age of the *O. unilateralis* clade is unknown, but based on the chronogram of Sung et al. (2008), it is likely from the early Eocene, 47–56 million years ago. Our phylogenetic analysis and ASR analysis demonstrate that twig biting/grasping is not restricted to a single clade (Fig. 3). Rather, twig biting/grasping arose independently multiple times in the evolutionary history of this fungal group. Given the early Eocene origin of the *O. unilateralis* clade, this means that the group evolved in an ice-free world with high precipitation, average temperatures of 30°C, and minimal pole–pole temperature variations (Huber and Caballero 2011). It is likely then that this fungal group arose in evergreen biomes. Meanwhile, the deciduous forest spread in response to seasonal drought at the late Eocene cooling in the subtropics, and later became adapted to the seasonal cold in temperate regions (Willis and McElwain 2014).





**Figure 3.** The evolutionary relationships among closely related species of fungi from the *Ophiocordyceps unilateralis* complex that manipulates ants to bite different plant substrates. Phylogenetic relationship (based on Maximum Likelihood analysis) between fungi manipulating ants to bite leaves, twigs, and bark as inferred with molecular data. Each numbered node refers to a distinct event of biting substrate shift. The dashed black line is the only branch that the ASR could not define the biting substrate (e.g., ambiguous). The location of the fungal samples represented in this tree is indicated as (1) Brazil, (2) United States, (3) Japan, (4) Australia, (5) Thailand, and (6) Malaysia. The host of the fungal samples represented in this tree are indicated as (\*) for *Camponotus* and (\*\*) *Polyrhachis*. (A) *Camponotus castaneus* infected with *O. kimflemingiae*, manipulated to bite onto twigs. Samples originated from South Carolina. (B) *Camponotus obscuripes* infected with *O. (= unilateralis) pulvinata* manipulated to bite twigs. Sample originated from Japan; image from Kepler et al. 2011. (C) *Camponotus chromaiodes* infected with *Ophiocordyceps blakebarnesii*, manipulated to bite onto the interior surface of a tree trunk. Sample originated from Missouri. (D) *Polyrhachis lamellidens* infected by *Ophiocordyceps ootakii*, manipulated to bite twigs. Sample originated from Japan. (E) *Camponotus* sp. infected with *O. rami*, manipulated to bite nonwoody twigs. Sample originated from Thailand; image from Kobmoo et al. (2015).

It is known from fossil evidence that the highly characteristic pattern of leaf biting induced in ants by species in the *O. unilateralis* s.l. complex was present 47 million years ago in what is modern day Germany, which was then an evergreen biome and 10° further south than its current location (Hughes et al. 2010). Thus, based on past climate and forest type distribution, fossil evidence of leaf biting and our ancestral state character reconstruction, there are grounds to suggest that the species in the *O. unilateralis*

clade originally manipulated ants to bite leaves and subsequently experienced independent convergent evolution to twig biting by different fungal parasites in response to global climate change and the emergence of the deciduous forests in different areas of the globe. The emergence of the additional twig grasping presumably came later as it may increase the likelihood that the host cadaver, which the fungus requires for reproduction, stays in position over extended periods of time.



Alternatively, the observed patterns of twig biting in temperate regions could be due to adaptive plasticity. The type of substrate to which the host bites would then be a plastic trait that responds to the environment inhabited by the host. There are many records of adaptive plasticity in response to environmental changes (Charmantier et al. 2008), including behavioral plasticity (Gross et al. 2010). If the biting substrate were a plastic trait that responds to the environment inhabited by the host, it would imply either one of the two following possibilities. The first possibility is that all or most of the fungi species in the *O. unilateralis* group would be able to manipulate its specific ant host to bite both onto twigs and leaves depending on the circumstances. We would then expect that in the tropical forests some proportion of the ant hosts would be manipulated to bite both twig and leaves, or that in temperate forests, some of the ants would be found biting leaves (although it would mean the death of the parasite). However, we only know of one possible record of plasticity in the biting substrate, which is the *Polyrhachis* sp. that was manipulated to bite both onto leaves and bark of cocoa trees in a Ghanaian cocoa farm (Samson et al. 1982). The second possibility is that the species of fungi in the temperate forest that have evolved the capability to induce twig biting, in addition to leaf biting. Unfortunately, we are not able to test this hypothesis because it would require either transplant of fungal/ant species or common garden experiments (cross-infections). These experiments are not possible for two reasons. The first is that these species of fungi are highly specific to the species of ant they infect and they cannot manipulate different species of ants (de Bekker et al. 2014). Comparative genomic analysis of the *O. unilateralis* shows a high degree of species-specific adaptation for the genes involved in host manipulation (de Bekker et al. 2017). The second reason is that ant hosts are restricted to either temperate or tropical forests, meaning transplant infections could not be carried out. Although we cannot exclude the possibility that plasticity explains the observed pattern of biting twigs in temperate regions, we suggest that convergently evolved extended phenotypes where different ant species are manipulated by different fungal species in different temperate forests (United States/Japan) is likely the most parsimonious interpretation of our data.

In tropical forests, twigs are abundant and are likely more stable than the leaves themselves, as the leaves in the tropical forest last between 1.5 months to four years depending on the plant species (Reich et al. 1992). However, the average duration of the cadavers attached to the leaves in tropical environment is approximately five months and where it has been tested, the leaves remain for longer than the development of the fungus (Mongkolsamrit et al. 2012). Thus, tropical leaf permanence is not a constraint for the development of the fungus. Perhaps then the preference exhibited by fungal species manipulating ants to bite leaves in tropical forests is related to benefits other than the long-term permanence

of the substrate in the environment. It is possible that the underside of leaves provide a favorable microclimate (Andersen et al. 2009; Pincebourde and Woods 2012), where the developing fungus is protected from UV damage and rain, and experiences more stable temperature and humidity. Compared to the dead tissue of stem bark, the living, vascularized tissue of leaves may also provide a nutritional supplement for the developing fungus. In line with this suggestion, several other entomopathogenic fungi are documented to grow as endophytes, including *Beauveria bassiana* (Bing and Lewis 1991; Vega 2008) in leaves, and *Metarhizium anisopliae* (Hu and Leger 2002) and *Ophiocordyceps sinensis* (Zhong et al. 2014) in plant roots. Leaf biting may create an opening where fungi gain ready access to plant nutrients. In fact, *O. unilateralis* s.l. fungal tissue has been identified inside the damage caused by biting in the leaf tissue in both modern and extinct leaves (Hughes et al. 2010); however, the direct interaction between these fungi and the plant substrate remains to be studied. Perhaps then the choice of leaves over twigs by manipulating fungi was adaptive and the switch to twig biting only emerged under the strong selective regime that deciduous plants present.

To complete its sexual reproduction, fungi within *O. unilateralis* complex grow and mature the ascoma, from where the ascospores will be produced and released to infect new hosts. The sexual reproduction is a key point in the life cycle of this parasite, which is dependent on the precise location where the ants are manipulated to die (Andersen et al. 2009). However, the pace of fungal development is generally regulated by temperature (Baxter and Illston 1980). In the tropics, the manipulation happens throughout the year (Mongkolsamrit et al. 2012; Loreto et al. 2014) and parasite development is completed in a few months after it kills the host (Mongkolsamrit et al. 2012; Araujo et al. 2015). In the temperate system, although the manipulation occurs during the summer when the temperature is elevated, our phenology study revealed that winter appears to interrupt development and so it takes at least one year for the fungus to complete the sexual cycle. Previous empirical work showed the placement of the ant cadaver on the forest floor resulted in zero fitness for the parasite (Andersen et al. 2009). If the infected ants were manipulated to bite leaves, the cadaver would fall onto the forest floor before the fungus can reproduce. Although the manipulation to bite twigs allows the fungus to avoid falling onto the ground due to leaf shed, almost 50% of the newly manipulated ants disappeared from the twigs, resulting in zero fitness for the parasite. This could be due to either weak attachment or predation. The same happens in the tropics, where ants suddenly disappear from the leaf substrate (Mongkolsamrit et al. 2012). Despite the possibility of falling or being predated, the fungus clearly increases its chance to survive and reproduce by avoiding the leaves. Thus, besides providing evidence that the environment has shaped the behavioral manipulation of the ants by the parasitic fungi, we are

able to suggest a possible mechanism by which this happens. We suggest that the slow growth rate, likely due to the lower average annual temperature, combined with the leaf fall that occurs between the manipulation and reproduction, selectively favored the fungi to manipulate their host to bite onto twigs. In contrast to the leaves of deciduous trees, twigs last for many seasons, providing a steady platform for the fungus to develop and release spores over extended periods of time. Additionally, the difficulty in biting twigs is likely a selective force for the manipulated ant to grasp the twigs with its legs, a novel behavior not previously observed in ants infected by this group of fungi.

The data presented here provide multiple lines of evidence suggesting a parasitic fungus inside ant hosts can respond to environmental change and alter the way it manipulates its host behavior over evolutionary time. We hypothesize that as the evergreen moist forests of the Eocene, which receded first to drier and then cooler deciduous woods (Huber and Caballero 2011), favored the selection of a switch in the manipulative behavior from biting leaves to biting twigs. Twig and leaf biting appears in both America and Asia-Oceania host ant clades, as well as in *Camponotus* and *Polyrhachis* ant hosts. In Ghana, some ant hosts were found biting both leaf and bark (the same ant species was found biting both substrates) (Samson et al. 1982). This indicates lability/plasticity across evolutionary time that may facilitate switching from biting one substrate to another. It is interesting that in tropical forests, where the abundance (Pontoppidan et al. 2009; Loreto et al. 2014) and diversity of species from the complex *O. unilateralis* is high (Evans et al. 2011; Kepler et al. 2011; Araujo et al. 2015; Kobmoo et al. 2015), the default manipulation is leaf biting. However, twigs are also in abundance on tropical forests. That is, when ants are being manipulated, the environment (a tropical forest) has both twigs and leaves available onto which manipulated ants could bite. Likewise, in temperate systems, the biting occurs during the summer, when the ants also have both leaves and twigs as possible biting substrates. We actively searched for infected *Camponotus* ants attached to leaves in our temperate system and did not encounter any. Therefore, the preference for twigs is not an artifact of leaf fall where only ants biting twigs remain to be sampled. A tantalizing question is which factor may have led the fungi to switch their biting substrate. There is some indication that the microclimate is important for the fungal development (Andersen et al. 2009; Hughes et al. 2011). Therefore, small changes in temperature, humidity, and/or CO<sub>2</sub> concentration could have been used as clues; but we can only speculate. Although it remains to be discovered how a microbe inside the body of its host can affect such precise choices in its manipulated host, our data suggest that the infected manipulated ants have a behavior, the extended phenotype, which is encoded by the fungus and results in the optimal selection of the plant tissue (leaf *versus* twig) to bite before being killed by the parasite.

## AUTHOR CONTRIBUTIONS

RGL and DPH conceptualized the study; RGL and DPH methodized the study; RGL, JPMA, KF, and DPH did all the investigation; RGL, JPMA, RMK, CSM, and DPH did formal analysis; RGL wrote original draft; RGL, JPMA, KF, RMK, CSM, and DPH wrote, reviewed, and edited the study; RGL, JPMA, and KF visualized the study; RMK, CSM, and DPH supervised the study; DPH acquired funding for the study.

## ACKNOWLEDGMENTS

We are thankful to Dr. T. Sato and S. Ootake for the hospitality, guidance, and help with the field work in Japan. We thank Dr. H. Evans for providing us access to the samples he collected. We are thankful to D. Shengjuler for revising our manuscript and to the anonymous reviewers for the comments that improved our work. RGL was partially supported by CAPES-Brazil (grant BEX6203-10-8). This work was supported in part by NSF grants IOS-1558062 (DPH) and NIH grant R01 GM116927-02 (DPH) and a grant from the National Academies Keck Futures Initiative-Collective Behavior: From Cells to Societies program (DPH and CSM).

## DATA ARCHIVING

The information collected for *Ophiocordyceps unilateralis s.l.* records from around the world is found on Dataset S1. Images used to evaluate the fungal development can be found in Figure S3. Taxon, specimen voucher and sequence information for specimens used in this study can be found in Dataset S2.

## LITERATURE CITED

- Andersen, S. B., S. Gerritsma, K. M. Yusah, D. Mayntz, N. L. Hywel-Jones, J. Billen, J. J. Boomsma, and D. P. Hughes. 2009. The life of a dead ant: the expression of an adaptive extended phenotype. *Am. Nat.* 174:424–433.
- Araujo, J., and D. Hughes. 2016. Diversity of entomopathogenic fungi: which groups conquered the insect body? *Adv. Genet.* 94:1–39.
- Araujo, J. P. M., H. C. Evans, D. M. Geiser, W. P. Mackay, and D. P. Hughes. 2015. Unravelling the diversity behind the *Ophiocordyceps unilateralis* (*Ophiocordycipitaceae*) complex: three new species of zombie-ant fungi from the Brazilian Amazon. *Phytotaxa* 220:224–238.
- Baxter, M., and G. M. Illston. 1980. Temperature relationships of fungi isolated at low-temperatures from soils and other substrates. *Mycopathologia* 72:21–25.
- Beros, S., E. Jongepier, F. Hagemeyer, and S. Foitzik. 2015. The parasite's long arm: a tapeworm parasite induces behavioural changes in uninfected group members of its social host. *Proc. R. Soc. B.* 282. <https://doi.org/10.1098/rspb.2015.1473>.
- Bing, L. A., and L. C. Lewis. 1991. Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environ. Entomol.* 20:1207–1211.
- Biron, D. G., C. Joly, L. Marche, N. Galeotti, V. Calcagno, A. Schmidt-Rhaesa, L. Renault, and F. Thomas. 2005. First analysis of the proteome in two nematomorph species, *Paragordius tricuspidatus* (Chordodidae) and *Spiniochordodes tellinii* (Spiniochordodidae). *Infect Geneti. Evol.* 5:167–175.
- Biron, D. G., F. Ponton, L. Marche, N. Galeotti, L. Renault, E. Demey-Thomas, J. Poncet, S. P. Brown, P. Jouin, and F. Thomas. 2006. "Suicide" of crickets harbouring hairworms: a proteomics investigation. *Insect Mol. Biol.* 15:731–742.
- Boer, P. 2008. Observations of summit disease in *Formica rufa* Linnaeus, 1761 (Hymenoptera: Formicidae). *Myrmecol. News* 11:63–66.
- Carney, W. P. 1969. Behavioral and morphological changes in carpenter ants harboring dicrocoeliid metacercariae. *Am. Midl. Nat.* 82:605–611.

- Charmantier, A., R. H. McCleery, L. R. Cole, C. Perrins, L. E. Kruuk, and B. C. Sheldon. 2008. Adaptive phenotypic plasticity in response to climate change in a wild bird population. *Science* 320:800–803.
- Dawkins, R. 1982. *The extended phenotype*. W.H. Freeman, Oxford, U.K.
- de Bekker, C., L. E. Quevillon, P. B. Smith, K. R. Fleming, D. Ghosh, A. D. Patterson, and D. P. Hughes. 2014. Species-specific ant brain manipulation by a specialized fungal parasite. *BMC Evol. Biol.* 14:166.
- de Bekker, C., R. A. Ohm, R. G. Loreto, A. Sebastian, I. Albert, M. Merrow, A. Brachmann, and D. P. Hughes. 2015. Gene expression during zombie ant biting behavior reflects the complexity underlying fungal parasitic behavioral manipulation. *BMC Genomics* 16:620.
- de Bekker, C., R. A. Ohm, H. C. Evans, A. Brachmann, and D. P. Hughes. 2017. Ant-infecting Ophiocordyceps genomes reveal a high diversity of potential behavioral manipulation genes and a possible major role for enterotoxins. *Sci. Rep.* 7. <https://doi.org/10.1038/s41598-017-12863-w>.
- Edgar, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform.* 5:1–19.
- Endlein, T., and W. Federle. 2015. On heels and toes: how ants climb with adhesive pads and tarsal friction hair arrays. *PLoS ONE* 10:e0141269.
- Evans, H. C., and R. A. Samson. 1984. Cordyceps species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems 2. The *Camponotus* (Formicinae) complex. *Trans. Br. Mycol. Soc.* 82:127–150.
- Evans, H. C., S. L. Elliot, and D. P. Hughes. 2011. Hidden diversity behind the zombie-ant fungus *Ophiocordyceps unilateralis*: four new species described from carpenter ants in Minas Gerais, Brazil. *PLoS ONE* 6:e17024.
- Gross, K., G. Pasinelli, and H. P. Kunc. 2010. Behavioral plasticity allows short-term adjustment to a novel environment. *Am. Nat.* 176:456–464.
- Henne, D. C., and S. J. Johnson. 2007. Zombie fire ant workers: behavior controlled by decapitating fly parasitoids. *Insectes Soc.* 54:150–153.
- Hoekstra, H. E., J. G. Krenz, and M. W. Nachman. 2005. Local adaptation in the rock pocket mouse (*Chaetodipus intermedius*): natural selection and phylogenetic history of populations. *Heredity* 94:217–228.
- Hoover, K., M. Grove, M. Gardner, D. P. Hughes, J. McNeil, and J. Slavicek. 2011. A gene for an extended phenotype. *Science* 333:1401.
- Hu, G., and R. J. S. Leger. 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol.* 68:6383–6387.
- Huber, M., and R. Caballero. 2011. The early Eocene equable climate problem revisited. *Clim. Past* 7:603.
- Hughes, D. P., T. Wappler, and C. C. Labandeira. 2010. Ancient death-grip leaf scars reveal ant–fungal parasitism. *Biol. Lett.* 7:67–70.
- Hughes, D. P., S. B. Andersen, N. L. Hywel-Jones, W. Himaman, J. Billen, and J. J. Boomsma. 2011. Behavioral mechanisms and morphological symptoms of zombie ants dying from fungal infection. *BMC Ecol.* 11:13.
- Hughes, D. P., J. Brodeur, and F. Thomas, eds. 2012. *Host manipulation by parasites*. Oxford Univ. Press, Oxford, U.K.
- Hughes, D., J. Araujo, R. Loreto, L. Quevillon, C. De Bekker, and H. Evans. 2016. From so simple a beginning: the evolution of behavioral manipulation by fungi. *Adv. Genet.* 94:437–469.
- Hywel-Jones, N. L. 2002. The importance of invertebrate-pathogenic fungi from the tropics. *Micromycetes* 2:133–144.
- Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, et al. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649.
- Kepler, R. M., Y. Kaitso, E. Tanaka, S. Shimano, and J. W. Spatafora. 2011. *Ophiocordyceps pulvinata* sp nov., a pathogen of ants with a reduced stroma. *Mycoscience* 52:39–47.
- Kerry, E. 1990. Effects of temperature on growth-rates of fungi from sub-antarctic macquarie island and casey, Antarctica. *Polar Biol.* 10:293–299.
- Kingsley, E. P., K. M. Kozak, S. P. Pfeifer, D. S. Yang, and H. E. Hoekstra. 2017. The ultimate and proximate mechanisms driving the evolution of long tails in forest deer mice. *Evolution* 71:261–273.
- Kobmoo, N., S. Mongkolsamrit, T. Wutikhun, K. Tasanathai, A. Khonsanit, D. Thanakitpipattana, and J. J. Luangsa-Ard. 2015. New species of *Ophiocordyceps unilateralis*, a ubiquitous pathogen of ants from Thailand. *Fungal Biol.* 119:44–52.
- Krull, W. H., and C. R. Mapes. 1952. Studies on the biology of *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899 (Trematoda: Dicrocoeliidae), including its relation to the intermediate host, *Cionella lubrica* (Müller). VII. The second intermediate host of *Dicrocoelium dendriticum*. *Cornell Vet.* 42:603–604.
- Langkilde, T. 2009. Invasive fire ants alter behavior and morphology of native lizards. *Ecology* 90:208–217.
- Loreto, R. G., S. L. Elliot, M. L. R. Freitas, T. M. Pereira, and D. P. Hughes. 2014. Long-term disease dynamics for a specialized parasite of ant societies: a field study. *PLoS ONE* 9:e103516.
- Losos, J. B., T. W. Schoener, and D. A. Spiller. 2004. Predator-induced behaviour shifts and natural selection in field-experimental lizard populations. *Nature* 432:505–508.
- Maddison, W. P., and D. R. Maddison. 2015. Mesquite: a modular system for evolutionary analysis. Version 3.10 (WWWdocument). Available at <http://mesquiteproject.org>.
- Mehlhorn, H. 2015. *Host manipulations by parasites and viruses*. Springer, Berlin.
- Miller, M. A., W. Pfeiffer, and T. Schwartz. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Pp. 1–8 in *Gateway computing environments workshop (GCE)*. Ieee, New Orleans, LA.
- Milner-Gulland, E., J. M. Fryxell, and A. R. Sinclair. 2011. *Animal migration: a synthesis*. Oxford Univ. Press, Oxford, U.K.
- Mongkolsamrit, S., N. Kobmoo, K. Tasanathai, A. Khonsanit, W. Noisriboom, P. Srikitkulchai, R. Somnuk, and J. J. Luangsa-ard. 2012. Life cycle, host range and temporal variation of *Ophiocordyceps unilateralis/Hirsutella formicidarum* on Formicine ants. *J. Invertebr. Pathol.* 111:217–224.
- Moore, J. 2002. *Parasites and the behavior of animals*. Oxford Univ. Press, Oxford, U.K.
- Pagel, M. 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proc. R. Soc. Lond. B Biol. Sci.* 255:37–45.
- . 1999. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. *Syst. Biol.* 48:612–622.
- Pincebourde, S., and H. A. Woods. 2012. Climate uncertainty on leaf surfaces: the biophysics of leaf microclimates and their consequences for leaf-dwelling organisms. *Funct. Ecol.* 26:844–853.
- Poinar, G., and S. P. Yanoviak. 2008. *Myrmeconema neotropicum* ng, n. sp., a new tetradonematid nematode parasitising South American populations of *Cephalotes atratus* (Hymenoptera: Formicidae), with the discovery of an apparent parasite-induced host morph. *Syst. Parasitol.* 69:145–153.
- Pontoppidan, M.-B., W. Himaman, N. L. Hywel-Jones, J. J. Boomsma, and D. P. Hughes. 2009. Graveyards on the move: the spatio-temporal distribution of dead *Ophiocordyceps*-infected ants. *PLoS ONE* 4:e4835.
- Poulin, R., and H. S. Randhawa. 2015. Evolution of parasitism along convergent lines: from ecology to genomics. *Parasitology* 142:S6-S15.
- Quandt, C. A., R. M. Kepler, W. Gams, J. P. Araújo, S. Ban, H. C. Evans, D. Hughes, R. Humber, N. Hywel-Jones, and Z. Li. 2014.

- Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in Tolypocladium. *IMA Fungus* 5:121–134.
- Rambaut, A., M. Suchard, D. Xie, and A. Drummond. 2014. Tracer v1.6. Available at <http://tree.bio.ed.ac.uk/software/tracer/>.
- Reich, P., M. Walters, and D. Ellsworth. 1992. Leaf life-span in relation to leaf, plant, and stand characteristics among diverse ecosystems. *Ecol. Monogr.* 62:365–392.
- Robson, S. K., R. J. Kohout, A. T. Beckenbach, and C. S. Moreau. 2015. Evolutionary transitions of complex labile traits: silk weaving and arboreal nesting in *Polyrhachis* ants. *Behav. Ecol. Sociobiol.* 69:449–458.
- Ronquist, F., M. Teslenko, P. Van Der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61:539–542.
- Samson, R., H. Evans, and E. Hoekstra. 1982. Notes on entomogenous fungi from Ghana. VI. The genus *Cordyceps*. *Proc. Nederl. Akad. Wetensch. C Biol. Med. Sci.* 85:589–605.
- Sanjuan, T., L. G. Henao, and G. Amat. 2001. Spatial distribution of *Cordyceps* spp. (Ascomycotina: Clavicipitaceae) and its impact on the ants in forests of the Amazonian Colombian foothill. *Rev. Biol. Trop.* 49:945–955.
- Sato, T., K. Watanabe, M. Kanaiwa, Y. Niizuma, Y. Harada, and K. D. Lafferty. 2011. Nematomorph parasites drive energy flow through a riparian ecosystem. *Ecology* 92:201–207.
- Sato, T., T. Egusa, K. Fukushima, T. Oda, N. Ohte, N. Tokuchi, K. Watanabe, M. Kanaiwa, I. Murakami, and K. D. Lafferty. 2012. Nematomorph parasites indirectly alter the food web and ecosystem function of streams through behavioural manipulation of their cricket hosts. *Ecol. Lett.* 15:786–793.
- Schluter, D., T. Price, A. Ø. Mooers, and D. Ludwig. 1997. Likelihood of ancestor states in adaptive radiation. *Evolution* 51:1699–1711.
- Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690.
- Sung, G.-H., G. O. Poinar, and J. W. Spatafora. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal–arthropod symbioses. *Mol. Phylogenet. Evol.* 49:495–502.
- Thomas, F., F. Renaud, T. de Meeus, and R. Poulin. 1998. Manipulation of host behaviour by parasites: ecosystem engineering in the intertidal zone? *Proc. R. Soc. B Biol. Sci.* 265:1091–1096.
- Vega, F. E. 2008. Insect pathology and fungal endophytes. *J. Invertebr. Pathol.* 98:277–279.
- Willis, K., and J. McElwain. 2014. The evolution of plants. Oxford Univ. Press, Oxford, U.K.
- Zhong, X., Q.-y. Peng, S.-S. Li, H. Chen, H.-X. Sun, G.-R. Zhang, and X. Liu. 2014. Detection of *Ophiocordyceps sinensis* in the roots of plants in alpine meadows by nested-touchdown polymerase chain reaction. *Fungal Biol.* 118:359–363.

Associate Editor: B. Koskella  
Handling Editor: P. Tiffin

## APPENDIX

Additional information on stem biting in tropical forests. The fungus *Ophiocordyceps rami*, which belongs to the *Ophiocordyceps unilateralis* complex (and was formerly described as *O. unilateralis*), was collected in the tropical forests of Thailand (14° north), and originally described to manipulate its host to bite twigs (Kobmoo et al. 2015). However, from the figure presented by the authors, the substrate onto which the ant was biting was green, and not woody, as we have observed in temperate areas (i.e., chlorenchymous stems lacking cambium). We could not find any further information on the specimen collected in Costa Rica.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Methods S1.** Detailed information on the molecular work methods.

**Dataset S1.** Summary of the information collected for *Ophiocordyceps unilateralis s.l.* records from around the world.

**Dataset S2.** Taxon, specimen voucher, and sequence information for specimens used in this study.

**Video S1.** Fungus development from July 24, 2010, to March 12, 2012.

**Video S2.** Importance of the grasping behavior on fixing the cadaver to the twig.

**Figure S1.** *Ophiocordyceps kimflemingiae* infected ants in temperate forest of South Carolina.

**Figure S2.** Cadaver of a *Camponotus castaneus* previously manipulated before having been killed by the parasitic fungus *Ophiocordyceps kimflemingiae*.

**Figure S3.** *Ophiocordyceps kimflemingiae* development.

**Figure S4.** *Ophiocordyceps kimflemingiae* might fail to reach sexual maturity even when the cadaver stays attached to the substrate.

**Figure S5.** Bayesian tree reconstruction with five million generations sampled in MrBayes (using the same model partitions as the ML analysis) produced a topology consistent with the ML approach.