Fungal Ecology 25 (2017) 22–28

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Zombie ant heads are oriented relative to solar cues

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ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form
23 September 2016
Accepted 10 October 2016
Available online 24 November 2016

Corresponding Editor: Nicolai Vitt Meyling

Keywords:
Death grip
Dolichoderus thoracicus
Extended phenotype
Ophiocordyceps
Zombie ant

ABSTRACT

Parasite manipulation of host behavior is a compelling example of an extended phenotype. Some parasitic *Ophiocordyceps* species manipulate their ant victims to behave like zombies, causing them to walk abnormally, have convulsions that make them fall down, and climb vegetation to bite the veins or margins on the underside of plant leaves. Manipulation of the ants' behavior supports the fungus's growth and reproductive success. This study reports that *Ophiocordyceps pseudolloydii* can naturally infect *Dolichoderus thoracicus* ants in a broad-leaved forest in central Taiwan. The infected ants behaved as zombies but did not display 'death grip' behavior. Instead, the entire ant was covered in a dense matrix of fungal hyphae, securing the abdominal segments and the mandibles, but not the legs, to the leaf. Furthermore, the direction of the canopy opening and the orientation of the ants' heads were correlated, suggesting that a direct solar cue orients the dying ant. By detailed survey records from three squares with different canopy geometries within the graveyard areas, our data indicate that sunlight plays an important role in manipulation of ant hosts. These findings provide further indications of how the parasites have evolved to manipulate the host's behavior to increase fitness of the fungus.
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1. Introduction

The concept of the extended phenotype was first proposed by Dawkins (1982). The observable properties of an organism constitute its phenotype, which is defined as the product of genes and the environment. The extended phenotype refers to the influence of gene expression on the behavior of an organism rather than its physical composition. Some parasites (or parasitoids) can change their hosts' behavior or physiology. This behavior is considered the extended phenotype of these parasites; the expression of their genes modifies the host's behavior. Such manipulation is genetically selected to increase the reproductive success of the parasite. This concept has been the stimulus for a substantial amount of research activity recently (Hughes, 2013, 2014), the most extreme case being the behavioral control of ants by some parasitic *Ophiocordyceps* species to position their fruitbodies in places that provide spores with good opportunity to infect foraging ants. Inside the host, fungal cells damage the ant's muscles and central nervous system (Hughes et al., 2011a; de Bekker et al., 2014), causing a drunkard's walk, vegetation climbing, clamping to the underside of a leaf or twig, and finally death. The fungus then produces spores from a fruiting body on the back of the victim's head, discharged spores landing on ants on the ground below.

Infected ants in the forest understorey are driven by the fungus to select leaves of saplings that are a particular height off the ground, where the humidity and temperature are optimal for fungal growth (Andersen et al., 2009; Andersen and Hughes, 2012). They reported that these 'zombie ants' (ants in the tribe Camponotini, Formicinae: Formicidae) regularly fell from the vegetation as they walked because of repeated convulsions, precluding them from returning to the canopy (Hughes et al., 2011a). Furthermore, the mandibles of the ants penetrated deeply into the vein tissue, and this was accompanied by extensive atrophy of the mandibular muscles (Andersen et al., 2009). They also demonstrated that the 'death grip' occurs when the sun reaches the highest point in the sky. This lockjaw is induced to permanently secure the dead ant to the leaf against the force of gravity. Subsequently, the cells from the fungus proliferate around the ant's brain and between the surrounding muscle fibers, causing the infected ant to eventually stop on the underside of a leaf which is crucial for the success of the

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http://dx.doi.org/10.1016/j.funeco.2016.10.003
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fungus. Without the death grip, the ant would fall to the ground and the launching point for the fungus's spores would be lost.

The manner in which the *Ophiocordyceps* species change their hosts' behavior or physiology has been researched for a long time. However, two crucial questions remain. (1) How different is the zombie behavior of different ant species infected by different *Ophiocordyceps* fungi? (2) How do solar cues influence the fungus's steering of the ant's position? This study reports on *Ophiocordyceps pseudolloydii*, which infected the ant *Dolichoderus thoracicus* (= *Dolichoderus bituberculatus*) (Dolichoderinae: Formicidae) in a broad-leaved forest in central Taiwan. Infected ants behaved as zombies but did not display death grip behavior. Instead, the entire ant was covered in a dense matrix of fungal hyphae, securing the abdominal segments and the mandibles, but not the legs, to the leaf. Furthermore, evidence of the potential use of solar cues for the orientation of these zombie ants is presented.

2. Materials and methods

2.1. Fieldwork

Fieldwork was conducted in an evergreen, broad-leaved forest at Lianhuachi Experimental Forest, Nantou County, in central Taiwan (23°55′7″N, 120°52′58″E), and it is part of the Taiwan Forest Dynamics Monitoring Network. The substrate consists of alternating argillaceous sandstone and shale, and the dominant soils can be classified into typical Dystrochrept and typical Hapludult red soils. The average annual temperature is 20.8 °C and the annual precipitation is 2285.0 mm with seasonal variation throughout the year (Chang et al., 2010). In September 2015, the high-density areas (>30 dead ants/m^2) of dead *O. pseudolloydii*–infected ants were located. These ant graveyards were still active in December 2015 and thus were the primary focus of the sampling efforts in this work.

Three squares (3 m × 3 m) with different canopy geometries (open and closed canopies) were arbitrarily delineated within the graveyard areas (area A, B, and C). For all dead infected ants within these areas, species were identified and the height of the dead ant above ground, the direction of the ant head, the placement of the dead ant on the leaf, the leaf's compass orientation relative to the plant stem, and the development of fruit bodies were registered. This was completed for 350 *D. thoracicus* host ants.

2.2. Measurement of environmental factors

To further elucidate that the observed biased orientation was related to a solar cue, the solar irradiance of each of 10 directions was measured using a portable photo radiometer (HD 2102.1; Delta OHM, Padova, Italy) connected to an LP 471 PHOT probe (Delta OHM) from 7 a.m. to 5 p.m. Solar radiation was measured at 36° intervals (0° = north, 90° = east, 180° = south, and 270° = west) at an angle of 45° above the horizon. The data are presented as a percentage of the relative irradiance from each direction. Relative humidity and temperature were recorded with a data logger (EL-USB-2-LCD, Lascar Electronics, USA).

Hemispherical photography, also known as fisheye or canopy photography, was used for taking photographs oriented upward through an extreme wide-angle lens to estimate solar radiation and characterize the openness of the plant canopy. The camera was located in the center of each square. The vegetation coverage was determined using ImageJ analysis software version 1.49 (https://imagej.nih.gov/ij/).

2.3. Fungus species identification

Three fungus-infected ants from each square were transported to the laboratory and stored at 4 °C prior to analysis. DNA was extracted from small pieces of the ascoma/stroma through suspension in 200 μL of a lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride, 10 mM Tris, pH 8.0, and 1 mM ethylenediaminetetraacetic acid [EDTA]), to which 200 μL of phenol-chloroform-isoamyl alcohol (ratio of 25:24:1; isoamyl alcohol is optional) and 0.3 g of acid-washed glass beads (0.45–0.52 mm) were added and gently mixed. The samples were vortexed for 5 min to disrupt the cells and then centrifuged at 13,000–16,000*g* for 5 min. The aqueous layer of each sample was transferred to a clean tube and then 400 μL of 95% ethanol and 16 μL of 3 M sodium acetate, pH 5.2, were added. The samples were mixed through inversion and centrifuged at 13,000–16,000*g* for 5 min. The pellets were washed with 300 μL of 70% ethanol and then centrifuged at 13,000–16,000*g* for 2 min before the supernatant was discarded. The pellets were dried completely through aspiration of the ethanol solution for 30 min. Finally, genomic DNA from each sample was suspended in 100 μL of a Tris-EDTA buffer, pH 8.0.

Partial sequences of the large subunit (LSU) of ribosomal DNA (rDNA) were determined from the polymerase chain reaction (PCR) products generated by the genomic DNA that was extracted from the fungal cells. The sequences of partial LSU rDNA, 5.8S rDNA, and internal transcribed spacer (ITS) regions, were amplified using a PCR with the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) for species identification (White et al., 1990). Two other nuclear coding genes, elongation factor 1α and β-tubulin, were also amplified and sequenced to further confirm that the ants from the three sample areas were infected by the same species (strain). The elongation factor 1α was partially amplified using the primer 983F combined with EFgr, yielding a fragment of approximately 800 bp (Rehner and Buckley, 2005; Kobmoo et al., 2012). β-tubulin was amplified using the primers T1 and T22, spanning a fragment of approximately 1300 bp (Glass and Donaldson, 1995).

The polymerase chain reaction (PCR) mixture was prepared as follows. First, 1 μL of genomic DNA, used as the template; 5 μL of 2 mM dNTP; 1 μL of 10 μM forward primer; 1 μL of 10 μM reverse primer; 5 μL of Taq buffer; and 0.25 μL of Taq DNA polymerase were sequentially added to the reaction mixture. The total reaction volume was then adjusted to 50 μL by using sterile ddH_2O. PCR amplification was performed in a thermocycler (G02, ASTEC, Japan) as follows: (1) initial DNA denaturing at 95 °C for 5 min, (2) DNA denaturing at 95 °C for 1 min, (3) primer annealing at 50 °C for 30 s, and (4) DNA elongation at 72 °C for 1 min. Steps 2–4 were circulated 35 times, and the final step of extra elongation was conducted at 72 °C for 5 min. The DNA sequencing of these samples was performed at Tri-I Biotech, Inc. DNA sequences were aligned and examined using ClustalW in BioEdit software (Hall, 1999) with default alignment parameters. A BLAST search of the nucleotide sequences was conducted through the National Center for Biotechnology Information.

2.4. Histological cross sections

Five samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8, at 4 °C overnight. After fixation, the samples were washed in 0.1 M phosphate buffer, dehydrated using an ethanol series, and embedded in Technovit 7100 (Kulzer & Co., Germany), as described by Yeung and Chan (2015). Sections (3 μm in thickness) were cut with glass knives by using a Reichert-Jung 2040 rotary microtome (Heidelberg, Germany) and then stained with 0.1% (w/v) toluidine blue O in a benzoate buffer. The sections were observed and images were captured digitally using a CCD camera attached to a light microscope (Axio Imager A1, Carl Zeiss AG).

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