Corpse Removal as Simple Defense Mechanism that Keep Pandora’s Box Closed in an Ant Nest System Parasitized by a Highly Pathogenic Fungus

Social systems are attractive targets for parasites due to the high abundance of potential hosts and the spatial stability of colonies. Among social insects, ants in particular have developed various defense mechanisms in order to fight parasites, for instance nest cleaning, allogrooming, and corpse handling. The ultimate organizational form of the colonies of specific ant species is so-called supercolony: a system made up of a high number of related nests, which are connected through permanent worker and information exchange. These interconnections can facilitate the dispersal of parasites. However, in the Formica exsecta polydomial system that we studied the lethal endoparasitic fungus Pandora myrmecophaga had very low prevalence. The fungus manipulates infected workers that climb and die on grass blades near the nest, which then ensures the efficient distribution of the parasite by covering the mound surface with the conidia that is then produced. This strategy predicts a high prevalence of the fungi. We tested a hypothesis regarding the existence of simple defense mechanisms in ants: workers would dispose of every corpse appearing on grass blades as potential source of infection, thus lowering the chances of spore dispersal. We imitated the appearance of infected ants by fixing fresh corpses on grass blades near the nests at two different distance classes, while using dummies as control objects. The results confirmed our hypothesis: ants discovered and disposed of corpses efficiently, primarily those that were close to the nest, whereas dummies were dealt with less frequently. While the discovery rate was independent of the mound size and the activity of ants, the disposal rate was positively influence by the activity of ants around their mounds. This simple defense mechanism does not require any specific adaptation on the part of the ant and it ensures the low occurrence of a lethal pathogen in a supercolony.

Katalin Erős
Bálint Markó, PhD
László Rákosy

During manuscript preparation KE was provided by a “Young successful researchers’ professional development in an international and interdisciplinary environment” scholarship from the Sectoral Operational Program for Human Resources Development 2007–2013, co-financed by the European Social Fund under project no. POSDRU/159/1.5/S/132400. During manuscript preparation BM was provided by the Bolyai János scholarship of the Hungarian Academy of Sciences.
Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding.

**Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If they had no role in any of the above, include this sentence at the end of your statement: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript."

If the study was **unfunded**, provide a statement that clearly indicates this, for example: "The author(s) received no specific funding for this work."

**Competing Interests**

You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests.

Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)?

If **yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:*

If **no** authors have any competing interests to declare, please enter this statement in the box: "The authors have declared that no competing interests exist."

* typeset
<table>
<thead>
<tr>
<th>Ethics Statement</th>
</tr>
</thead>
<tbody>
<tr>
<td>You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should also be included in the Methods section of your manuscript. Please write &quot;N/A&quot; if your study does not require an ethics statement.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Subject Research (involved human participants and/or tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript.</td>
</tr>
</tbody>
</table>

Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research.

<table>
<thead>
<tr>
<th>Animal Research (involved vertebrate animals, embryos or tissues)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, &quot;The use of non-human primates in research.&quot; The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement.</td>
</tr>
</tbody>
</table>

If anesthesia, euthanasia or any kind of
animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied.

Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained.

Field Permit
Please indicate the name of the institution or the relevant body that granted permission.

Data Availability
PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found.

Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. Please note that simply stating 'data available on request from the author' is not acceptable. If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?

| No - some restrictions will apply |

Please describe where your data may be found, writing in full sentences. Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted. If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details.

Data are available from the first author KataIin Eros, who can be contacted by katika_eros@yahoo.com e-mail address. Currently there is no appropriate web surface at the institution where the data could be uploaded.
If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.”

If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX).” If this information will only be available after acceptance, please indicate this by ticking the box below.

If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example:

“Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.”

“Data are from the XXX study whose authors may be contacted at XXX.”

Additional data availability information:
Corpse Removal as Simple Defense Mechanism that Keep Pandora’s Box Closed in an Ant Nest System Parasitized by a Highly Pathogenic Fungus

Katalin Erős¹, Bálint Markó¹¶, László Rákosy⁴

Social systems are attractive targets for parasites due to the high abundance of potential hosts and the spatial stability of colonies. Among social insects, ants in particular have developed various defense mechanisms in order to fight parasites, for instance nest cleaning, allogrooming, and corpse handling. The ultimate organizational form of the colonies of specific ant species is so-called supercolony: a system made up of a high number of related nests, which are connected through permanent worker and information exchange. These interconnections can facilitate the dispersal of parasites. However, in the Formica exsecta polydomial system that we studied the lethal endoparasitic fungus Pandora myrmecophaga had very low prevalence. The fungus manipulates infected workers that climb and die on grass blades near the nest – almost as in myrmecoparasitic Ophiocordyceps fungal species –, which then ensures the efficient distribution of the parasite by covering the mound surface with the conidia that is then produced. This strategy predicts a high prevalence of the fungi.

Despite its elaborate manipulation technique, we know very little about this fungus. Mostly data on its distribution and host species have been published with a recent publication on its molecular biology, but nothing is available on how ants could fight it, and behavioural interactions. Thus, we tested a hypothesis, which has been never tested before, regarding the existence of simple defense mechanisms in ants: workers would dispose of every corpse appearing on grass blades as potential source of infection, thus lowering the chances of spore dispersal. We imitated the appearance of infected ants by fixing fresh corpses on grass blades near the nests at two different distance classes, while using dummies as control objects. The results presented in the frame of this research article confirmed our hypothesis: ants discovered and disposed of corpses efficiently, primarily those that were close to the nest, whereas dummies were dealt with less frequently. While the discovery rate was independent of the mound size and the activity of ants, the disposal rate was positively influence by the activity of ants around their mounds. This simple defense mechanism does not require any specific adaptation on the part of the ant and it ensures the low occurrence of a lethal pathogen in a supercolony.

There were no prior interactions with PLoS One regarding the submitted manuscript. We suggest Darren Ward as Academic Editor to handle our manuscript. We do not nominate anyone as opposed reviewer.
Corpse Removal as Simple Defense Mechanism that Keep Pandora’s Box
Closed in an Ant Nest System Parasitized by a Highly Pathogenic Fungus

Katalin Erős1,2#a, Bálint Markó1,3#b*, László Rákosy4

1 Hungarian Department of Biology and Ecology, Babeş-Bolyai University, Cluj-Napoca, Romania
2 Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen, Hungary
3 Department of Ecology, University of Szeged, Szeged, Hungary
4 Department of Taxonomy and Ecology, Babeş-Bolyai University, Cluj-Napoca, Romania

#a Current address: Hungarian Department of Biology and Ecology, Babeş-Bolyai University, Cluj-Napoca, Romania
#b Current address: Hungarian Department of Biology and Ecology, Babeş-Bolyai University, Cluj-Napoca, Romania

*Corresponding author
E-mail: balintm@gmail.com (BM)

¶These authors contributed equally to this work
Abstract

Social systems are attractive targets for parasites due to the high abundance of potential hosts and the spatial stability of colonies. Among social insects, ants in particular have developed various defense mechanisms in order to fight parasites, for instance nest cleaning, allogrooming, and corpse handling. The ultimate organizational form of the colonies of specific ant species is so-called supercolony: a system made up of a high number of related nests, which are connected through permanent worker and information exchange. These interconnections can facilitate the dispersal of parasites. However, in the *Formica exsecta* polydomial system that we studied the lethal endoparasitic fungus *Pandora myrmecophaga* had very low prevalence. The fungus manipulates infected workers that climb and die on grass blades near the nest, which then ensures the efficient distribution of the parasite by covering the mound surface with the conidia that is then produced. This strategy predicts a high prevalence of the fungi. We tested a hypothesis regarding the existence of simple defense mechanisms in ants: workers would dispose of every corpse appearing on grass blades as potential source of infection, thus lowering the chances of spore dispersal. We imitated the appearance of infected ants by fixing fresh corpses on grass blades near the nests at two different distance classes, while using dummies as control objects. The results confirmed our hypothesis: ants discovered and disposed of corpses efficiently, primarily those that were close to the nest, whereas dummies were dealt with less frequently. While the discovery rate was independent of the mound size and the activity of ants, the disposal rate was positively influence by the activity of ants around their mounds. This simple defense mechanism does not require any specific adaptation on the part of the ant and it ensures the low occurrence of a lethal pathogen in a supercolony.
Introduction

Group-living is frequently associated with increased level of parasitism, since the transmission of parasites is often considered host density-dependent [1-3]. Nevertheless, several recent studies dispute the generality of this theory, since group-living results in new, social strategies of reducing the frequency and virulence of pathogens and parasites [4-7]. Social insects like ants are extreme examples of group-living, and several life traits make them sensitive to the transmission of pathogens [8]: they live in highly aggregated groups, which are composed of genetically related individuals and overlapping generations, and contacts among colony members are very frequent, thus facilitating the transmission of pathogens both vertically and horizontally. In addition, these societies usually persist in more or less the same location (nest) under stable climatic conditions for several years. As they are exposed to parasites and pathogens, ants have developed a wide variety of defense strategies, both physiological and behavioral: they produce fungicidal secretions and practice auto- and allogrooming, pathogen avoidance, nest hygiene, and exclusion or emigration of infected individuals from colonies, and, in extreme cases, they even move the nest [8-16]. The relocation of dead or diseased individuals to external or internal refuse piles is also a widespread practice in these social insects [17-19], since corpses can be potential carriers of infection [20-23].

Under certain environmental conditions [24-27] colonies of specific ant species can evolve to form a highly complex polydomous system consisting of a network of related nests formed through nest budding, which in its largest form is often referred to as a supercolony. Polydomous systems involve food-source sharing and exchange of individuals and brood among nests [24, 28-32]. Thus, they are even more vulnerable to attacks from pathogens, as infections
may easily spread to different well-connected nests within the colony [33, 34]. On the other hand, a polydomous system may also be more resilient, as pathogens or parasites can be isolated by cutting off contact with an infected nest [6, 7, 33, 35]. According to Tragust et al. [34] in order for a parasite to spread rapidly in the supercolonial host population (a) the parasite must not be very virulent and must not cause disruption of the network of interconnected nests since this would lead to its quick isolation and (b) the host species must not possess special adaptations to the parasite which could limit the parasite’s spread independent of the supercolonial organization.

*Pandora myrmecophaga* is a myrmecopathogenic fungus that parasitizes ants of the genus *Formica*, among them those species that are known to form large supercolonies [36, 37]. In the last stage, infected individuals climb on grass blades next to the nest, from where spores can be efficiently poured onto the mound and its surroundings [36, 38]. Even a single or a few conidia are assumed to be sufficient to achieve the spread of infection [39]. On the basis of this efficient transmission mechanism, considerable prevalence of the fungus would be expected in a parasitized social system. Moreover, the fungus has been found in the largest known polydomial system in European *Formica* ants, belonging to *F. exsecta*, and consisting of more than 3,000 nests [40]. Despite expectations based on the considerations outlined above, the prevalence of the fungi is extremely low, hardly reaching 1% of the nests, and with a few exceptions only a single infected individual is found in a nest [37]. Since the fungus clearly has an efficient dispersal strategy, the explanation for its low prevalence must be that the host has developed a specific defense strategy that hinders its transmission. This could be either a specific physiological adaptation in order to reduce the virulence of the fungus on the level of the host or
a particular behavioral act that would prevent or limit infected individuals from dispersing the pathogen.

Marikovsky [38] anecdotally formulated a hypothesis concerning a simple social behavioral defense strategy on the basis of his field observations: “Whenever they (the ants) discovered an infested ant, they painstakingly removed it from the grass.” Simple as it was, the hypothesis was never tested experimentally. Could this defense mechanism indeed be specific, that is could it target only individuals who have been infected by a fungus, or is it a more general response on the part of the ants, thus valid for any conspecific ant corpse that is found? The first scenario would require identification of specific cues of infection, while the second strategy would be more general, but time consuming, since it would require the removal of all conspecific corpses. However, the second scenario would not require any specific ability to recognize the corpse’s status.

In the framework of our study we tested the hypothesis regarding the existence of a simple defense mechanism: whether workers would dispose of every conspecific ant corpse found on grass blades as a potential source of infection, thus decreasing considerably the chances of infection. Our findings confirmed the existence of such a defense mechanism and its efficiency.
Materials and Methods

Study Species and Site

Formica (Coptoformica) exsecta Nylander, 1846 is a relatively common mound-building, territorial Pan-Palaearctic ant species inhabiting areas of mixed and deciduous forests mostly at forest edges, forest clearings or mountain pastures [41]. Our study system, which is the largest known European polydomous system of F. exsecta [40], is located in a semi-wet meadow with Molinia caerulea (L.) Moench, Deschampsia caespitosa (L.) P. Beauv., Festuca pratensis Huds. in the southern part of the Giurgeului depression (46°36’N, 25°36’E, 780 m a.s.l.) in the Eastern Carpathians. The system contains 3,347 permanent nests over an area of ca. 22 ha with a density of ca. 153.25 nests/ha. The area is fairly intensely grazed by cows for most of the year [40]. The study site is not part of any protected area, it can be accessed freely as a communal grazing site, and the ant species is not protected under national law. No specific permits were required for our fieldwork for the previously stated reasons.

Pandora myrmecophaga (Turian et Wuest) S. Keller (2005) is an endoparasitic fungi with unclear taxonomic status. It is a member of the phylum Entomophthoromycota. The fungus produces infective spores that attach to, germinate on, and penetrate the cuticle of its ant hosts by enzymatic degradation, ultimately killing them. The fungus manipulates the behaviour of the hosts in a specific way: infected individuals look for an elevated position, usually parts of the vegetation (e.g. leaves, grass blades) next to their colonies and they attach themselves to distal parts of the plant (e.g. grass blades) using their mandibles and legs. This serves to ensure the effective spread of the spores (Fig. 1), and it is this behavior of the host after which the fungus, which is referred to as ‘summit disease’ [11, 36, 38], was named. Within hours, the rhizoids
grow out of the intersegmental parts of the basisternum and laterocervical plates and attach the ant to the leaf even more strongly [42]. Within one or two days, fur-like fungus appears on the intersegmental parts of the mesosoma and gaster, mainly the dorsal parts, and somewhat later also near the bases of the mandibles and the antennal insertions [36-38]. The switch from within-host growth to reproduction on the host surface results in the production of an arsenal of enzymes and other proteins which ensure the effective and quick digestion of host integument [43]. Pandora zombies do not produce fungal fruiting bodies, but sporulate directly from the mycelium on the surface of the dead ants [36]. Most probably Pandora combines rapid semelparous asexual reproduction with the production of persistent conidiospores as in Ophiocordyceps fungi [44]. A common transmission mode of spores is aerial by wind and rain in such cases [11]. The prevalence of the fungus within colonies is generally low: usually a few individuals can be found around a single nest [36, 45]. In the case of our study system, in general only a single individual infected with the fungus was found near a given nest [see 37 for details]. The fungus is quite widely distributed in Europe, but its presence is sporadic everywhere, and its known host range includes several species of the ant genus Formica [36, 37, 42, 43, 45, 46].

Fig. 1. Infected Formica exsecta worker attached to a grass-blade. The white fur-like conidia is visible at the intersegmental parts.

Experimental Methods

We randomly chose 80 F. exsecta nests from the middle part of the supercolony, maintaining a distance of at least 3 m between neighboring nests. All nests were checked for Pandora zombies in their near vicinity prior to experiments. These checks were regularly
performed during the experiments. No zombie ants were found. The aboveground volume of
each experimental nest mound was calculated using the formula for the volume of an elliptic
paraboloid \([47]\): \( V = \frac{1}{2} \times \pi \times r_1 \times r_2 \times h \), where \( r_1 \) is the largest radius at the bottom, \( r_2 \) is the
radius perpendicular to \( r_1 \), and \( h \) is the height aboveground. Nest mound size is usually
considered a fair indicator of the number of ants residing within a nest. The first group of 40
nests was examined between 14–17.08, the second between 17–19.08, 2012. Climatic conditions
were the same during the two periods. Ant corpses were freshly obtained by collecting live,
uninjured individuals two days prior to the experiments, and they were placed in small plastic
vials, where they died within hours. In order to ensure that individuals were not initially infected
by the fungus the fresh corpses were placed on wet cotton and kept there for two days until the
day of the experiment. This method enhances the growth of the fungus, and it is generally used
to facilitate fungal growth \([36, \text{pers. obs.}]\). None of the corpses proved to be infected. All
experimental ant corpses originated from the nests that were included in the experiment, and they
were returned to their original nests in order to avoid the effect of any potential nest-specific
differences in chemical cues. In addition to the 80 experimental nests, 432 nests were also
checked for *Pandora* zombies in order to assess the prevalence of the fungus during the study
period. If dead ants were found fixed to the grass blades they were brought in and checked for
infection using the methods described above.

In field conditions we imitated the appearance of *Pandora* infected ant corpses by fixing
a single experimental carcass near the nest mound (further on nest corpse) on a grass blade
(*Festuca pratensis*) with a minutia pin by the thorax at ca. 8 cm height, as *Pandora* infected
individuals usually appear. In order to test for the effect of distance from the nest mound we
placed another corpse 0.5 m away from the nest mound (further on referred to as ‘distant
corpse”) in a similar manner on the same axis as the nest corpse. A white, plastic, ant-size
dummy made out of three small polystyrene balls was also placed near each nest mound, fixed in
the same manner, in order to test the ants’ reaction to foreign objects. The dummy was placed
opposite the nest corpse at the other side of the nest.

Corpses/dummies were placed in this positions 10 min prior to the first observation in the
afternoon, since *F. exsecta* is usually less active at noon. Altogether eight 1 min observations
were carried out at each experimental nest, separated by 15 min intervals between 4 and 6 PM. In
the course of the observations we recorded the number of ants on the grass around the nests, the
number of corpse/dummy discoverers, and their behaviour. We considered discovery to have
occurred when the first physical contact with the corpse/dummy was recorded. The time it took
to dispose of a corpse was also recorded. We considered disposal to have occurred when the
entire corpse was removed. If observed, the direction in which a corpse was taken was also
noted. After the observation session, each corpse/dummy that was not carried away was left at its
original place and the status of these corpses was checked the next day at 4 PM and also after
four days in the case of the first group of nests and three days in the case of the second.

**Data Analysis**

The relationship between worker activity on grass blades and the nest mound’s
dimensions was assessed using a Linear Mixed Model (LMM, *N* = 640 obs.) approach, in which
nest dimension was introduced as an input variable, while observation series and nest code were
random factors. Differences in corpse/dummy discovery rates were analyzed with the help of a
Cox regression model (proportional hazard approach, *N* = 240 corpses). The type of corpse was
introduced as an input factor, the mound size and the average number of ants active on grass
blades around ant nests were input variables, while nest code was included as a random factor in order to handle dependencies. A similar approach was applied for the analysis of corpse removal rates. Two slightly different data setups were applied in this latter case. First we analyzed data from the 2-hour observation session, whereas in the second setup we also included the two additional observations coming from the following and last observation days as 9th and 10th observations. In the case of this latter setup the abundance of ants on grass blades was not included as an input variable, since it was not considered relevant for the last two observations.

All statistical analyses were carried out using the R 3.1.3 statistical package [48]. LMM was performed using lmer function in lme4 package [49], while a Cox regression analysis was performed with a coxme package [50]. Relevel function was used in order to carry out post-hoc sequential comparisons among factor levels when performing Cox regression analyses. We used table-wide sequential Bonferroni-Holm correction to reveal the exact significance levels in these cases.

Results

Only two potentially Pandora infected zombie ants were found at two separate nests out of the 512 verified mounds during the study period, and both individuals proved to have been infected by the fungus. Thus, the population-level prevalence of the fungus proved extremely low (0.4%) this year as well. Our experimental nests were of medium size (mean 90.11 dm\(^3\), SE ±80.41, min 8.13 dm\(^3\), max 467.82 dm\(^3\)) [see 29], and the activity of ants on grasses (mean 2.55, SE ±2.13) was clearly predicted by the size of the ant nest (LMM \(\chi^2 = 8.71, P = 0.003, N = 80\)): the bigger the ant nest, the higher the activity on the grass blades was.
Corpses were discovered by ants rapidly: after the first observations, 21 nest-corpses (26.25%) were discovered (three were removed), 10 distant corpses (12.5%) were found (two were removed), and six dummies (7.5%) were discovered (only one was removed). By the end of the 2 hrs observation session, the ants had discovered the majority of nest-corpses, while less than half of the distant corpses and dummies were found (Fig. 2). According to the results of the Cox regression analysis ($\chi^2 = 25.60, P < 0.01$) during this period nest-corpses were discovered at a significantly higher rate than both dummies ($z = -4.35, P < 0.001$) and distant corpses ($z = -3.26, P < 0.01$) (Fig. 3), while there was no difference between distant corpses and dummies in this respect ($z = 1.31, P = 0.19$). Discovery rate was independent both of the ant nest’s size or and the mean abundance of workers on grass blades ($z < 0.9, P = NS$).

Ant workers did not just discover, but also removed a larger proportion of nest-corpses than distant corpses and dummies within the 2 hrs observation session (see Fig. 2). A smaller proportion of the corpses (4.17%) was removed by taking them apart. In a few of the observed cases, when we actually spotted ants removing the cadavers, experimental corpses were taken...
right towards the ant nest. Based on the results of the Cox regression analysis ($\chi^2 = 17.32, P < 0.01$) nest-corpses were removed at a significantly higher rate than dummies ($z = -3.02, P < 0.01$), but they did not differ in this respect from distant corpses ($z = -1.82, P = 0.14$), while no difference was registered between distant corpses and dummies ($z = 1.6, P = NS$; Fig. 4). The removal rate was independent of the size of the ant nest ($z = 0.33, P = NS$), but it was significantly enhanced by the mean abundance of ant workers on grass blades ($z = -2.08, P = 0.04$).

After one day, the vast majority (77.5%) of the nest-corpses was gone, followed closely by distant corpses, while only 12.5% of the dummies had been removed (see Fig. 2). Within four days, almost every corpse had been removed: 92.5% of the nest-corpses and 85% of the distant corpses (see Fig. 2). In this case as well a smaller proportion of the dummies was missing.

Again, according to the results of the Cox regression analysis ($\chi^2 = 97.99, P < 0.001$) nest corpses were removed at a significantly higher rate than dummies ($z = -8.8, P < 0.0001$) and distant corpses ($z = -2.6, P < 0.01$). Distant corpses were not removed any more rapidly than dummies ($z = -6.99, P < 0.0001$). Ant nest size did not influence removal rate in this case ($z = -0.49, P = NS$).
Discussion

Infection success of specialist insect pathogens is usually limited by the minimum infection doses required to overwhelm a healthy host [51, 52]. *Pandora myrmecophaga* has all the optimal conditions ensured to spread its conidia in necessary doses in the parasitized supercolonial system: high nest density [29, 40] paired with enhanced connectedness of the nests [29, 31], total acceptance of workers from different nests [53], and also considerable relatedness among nestmates [54]. It has been demonstrated that infection success by microsporidium *Thelohania solenopsae* is positively related to polygyny and multi-nest structure in the host ant *Solenopsis invicta*: epizootics only develop in populations dominated by colonies in which free exchange of individuals (queens, workers and brood) occurs between nests. In contrast, the parasite’s prevalence stays relatively low in populations dominated by colonies that show spatially dispersed patterns [55]. In a similar manner, the prevalence of the fungus *Laboulbenia formicarum* in the supercolonies of *Lasius neglectus* is much higher than the proportion of infected nests in ant species having discrete and spatially separated colonies [34]. In the framework of our study, however, we demonstrated that ants can keep the prevalence of *Pandora* at low levels with a simple, non-selective defense mechanism.

However, the true prevalence of *P. myrmecophaga* is difficult to estimate with current methods. We can only identify the manifestation of the infection, when the infected host is already dead on the grass and all the conditions have been met for the fungus to develop. We cannot know how many infected hosts actually reside in the nests. Based on the efficiency with which the ants remove any corpse from the grasses, most probably the true prevalence of the
fungus is much higher than occasional observation of the grass blades around nests would suggest.

Among ants, corpses can be regarded as important information carriers, since workers can discriminate even among corpses of different species, and their responses are consistent with the status of the ant species [56]. Usually, the appearance of dead colony members represents a threat of infection, which triggers general prophylactic behavior, such as aggression or corpse removal [18, 21, 23, 57]. Our findings clearly demonstrate that F. exsecta workers dispose of corpses appearing on grass blades mostly in the immediate vicinity of the nest mound, which represent a higher risk of infection, as suggested by Marikovsky [38]. This simple defense mechanism could significantly lower the chances of Pandora infection. The reaction of the ants to dummies also indicates the general nature of this behavior. The dummies did not represent sources of food or even signs of a threat of infection, yet they were also removed in quite high proportions.

After discovering the corpses, ants removed them quickly, and larger nests, which were more active, in general reacted more promptly. This efficient reaction prevents the development of the conidia, which needs at least 2-4 days in natural conditions [36, 38, pers. obs.], but in favorable conditions, with elevated temperatures and levels of moisture, the spores can develop after one day (pers. obs.). The generality of this prophylactic strategy is very important, since usually specific defense mechanisms are costly. Several studies have addressed the costs of individual immune defenses in social insects and the trade-offs between immunity and other life-history parameters within individuals [e.g. 9, 58, 59]. The fungus has to overwhelm the host’s immune system and ensure that the corpse can be exploited at maximum potential for the fungus’ own reproductive success, rather than serve to further the reproductive success of rival fungi and
bacteria. This would explain why in general Entomophthoralean fungi have very fast life cycles and accumulate single-cell structures without a cell wall that are not detected by the host’s immune system. Therefore, typically only a short time (hours or days) separates the collapse of the host’s immune system and the sporulation of the fungus [52].

It is unclear what happens with corpses that are carried away. There are different several scenarios, but all point in the same direction of hindering the outbreak of an epizootic. (1) Several specimens were seen taking bodies towards the nest, and among F. exsecta cadavers are collected in underground cemeteries [60], which, due to increased humidity, could facilitate the development of the fungus, but, on the other hand, may restrict its transmission. (2) In addition, carriers may apply formic acid to the corpses, and even contact with the mixture of gland secretions on the carriers’ body could slow down or stop the development of conidia [59]. (3) As also noticed by Marikovsky [38], the practice of taking apart the bodies may also hinder the development of the fungus if the fungus needs to whole body to develop. (4) In some cases ants are also known to eat ant corpses [see 61], which, in the case of corpses that have been infected, might enable the acquisition of appropriate immune-competence. Among Lasius neglectus low intensity infection by Metarhizium anisopliae reduces future susceptibility to the pathogen [14]. All things considered, however, once it has developed, the fungus might escape the ants, since as Marikovsky has observed [38], ants don’t handle corpses with conidia. Our pilot experiments (Erős unpubl.) also confirm that ants do not react to corpses with conidia. It is possible that once the conidia appear, due to sudden changes in the level of fungus [43], certain proteins are released by the fungus that repel ants or even make them neutral towards corpses by altering corpse specific clues on the surface of cadavers. On the other hand, contact with corpses covered
by conidia would be dangerous to ants, as it would expose them to infection. Specific tests would need to be conducted in order to test either of these hypothesis.

Pathogenic or parasitic pressures on host populations are presumed to be high in insect pathogenic fungi when the fungi are specialized on a specific host [52], thus virulence and defense traits are most probably shaped by coevolutionary arms races. This is particularly true for parasites that have developed ways of manipulating host behaviour, so that dying hosts express extended phenotypes that serve the parasites reproductive success [62, 63]. *Pandora myrmecophaga* manipulates its host by driving it to climb on vegetation, where it dies, attached to the grass by its mandibles (summit disease). The fungus then develops asexual conidia after the death of its host [46]. Intriguingly, a similar extended phenotype is known in *Cordyceps* fungal species that infect primarily tropical ants of the genus *Camponotus* [64-66], similar mechanisms can be found in the ant-parasitizing *Dicrocoelium* fluke worms, indicating that this behaviour is not necessarily linked phylogenetically. These analogous phenologies probably represent convergent evolutionary responses to evade social handling of infected hosts by ant workers, which would considerably decrease the pathogen’s reproductive success unless the pathogen evolved mechanisms to drive newly infected hosts out of the reach of their nestmates [52, 67].

In *F. exsecta*, simple behavioral acts, such as cutting grass for nest cover and general practices of nest protection involving patrolling on grasses, also serve as means of defense against pathogens. While the chances of escaping the ants’ defenses are higher for *P. myrmecophaga* in smaller nests and at bigger distances from the mound (as revealed by our study), the combination of these two factors might critically lower the transmission success of the fungus. We expect that other features of the fungus, e.g. its seasonality, or the timing of spore
development within a day when ants could be less active, might serve to ensure its stability within a supercolony.
Acknowledgments

We are greatly indebted to Norbert Fákó, Enikő Csata, Zsolt Czekes, and Réka Erős for their assistance during our fieldwork, and also to the Apáthy István Society for providing housing. We are grateful for the help of Joanna Małagočka, Gyöngyi Szigeti and János Varga with the identification of Pandora myrmecophaga.

References


42. Turian G, Wuest J. Description complémentaire de *Zoophthora* (Entomophthora) *myrmecophaga* Turian & Wuest, agent d'une mycose chez *Serviformica fusca* L.
471 Mitteilungen der Schweizerischen Entomologischen Gesellschaft / Bulletin de la Société
473
474 43. Małagocka J, Grell MN, Lange L, Eilenberg J, Jensen AB. Transcriptome of an
475 entomophthoralean fungus (Pandora formicae) shows molecular machinery adjusted for
477
478 44. Andersen SB, Ferrari M, Evans HC, Elliot SL, Boomsma JJ, Hughes DP. Disease
480
481 45. Sosnowska D, Bałazy S, Prishcheva L, Mikulskaya N. Biodiversity of arthropod
483
485
486 47. Bliss P, Katzerke A, Neumann P. The role of molehills and grasses for filial nest
487 founding in the wood ant Formica exsecta (Hymenoptera: Formicidae). Sociobiology
489
490 48. R Core Team. R: A language and environment for statistical computing. R Foundation
492
493 49. Bates D, Maechler M, Bolker B, Walker S. lme4: Linear mixed-effects models using
494 Eigen and S4. R package version 1.0-5, 2013; Available: http://CRAN.R-
495 project.org/package=lme4.
496
498 forge.r-project.org, published online on 15.05.2013.
499
501 of fungal entomopathogens in semi-natural populations of insects. In The Ecology of
503 23


Fig. 3
Click here to download Figure: Fig._3_cmyk_rgb.tif