Entomopathogenic *Rickia wasmannii* fungal infection generated colony-level stress effects cause size decline in *Myrmica* ants

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Abstract

Parasitism-generated negative effects on ant societies are multifaceted, implying individual and colony-level responses. Though laboratory based evidence shows that the sublethal fungus *Rickia wasmannii* is responsible for physiological and behavioral responses that may negatively affect individual workers’ resilience and life expectancy in *Myrmica* ant workers, colony-level stress response to this parasite is largely unknown. Here, we focus on understanding of a long-term, colony-level effect of *Rickia* infection on *Myrmica scabrinodis* ant populations by tracking trait size-based changes. We collected worker specimens from infected and uninfected colonies from the same population in order to: (i) compare body size in response to parasitism, (ii) assess the extent to which possible changes in size are associated with the severity of
infection, and (iii) investigate shifts in body size in response to infection over time by testing
correlation of workers’ ages and sizes. We found that workers from infected colonies were
significantly smaller than their healthy congeners, but neither infection level nor the age of the
workers showed significant correlation with the size in infected colonies. Decreasing body sizes
in infected colonies can be ascribed to workers’ mediated stress toward developing larvae,
which are unable to attain the average body size before they pupate.

Introduction

Ants (Hymenoptera: Formicidae), the most widespread social organisms on Earth, attract an
amazing diversity of parasitic organisms, such as viruses [1], bacteria [2], fungi [3,4], and an array
of uni- and multicellular animal organisms [5,6]. Many of these parasites cause lethal diseases
[7], but most are sublethal, i.e. they do not necessarily pose an imminent danger, though they are
assumed to have detrimental effects on the quality of the hosts’ lives [4,8].

Entomopathogenic fungus, Rickia wasmannii Cavara, 1899 (Ascomycota: Laboulbeniales) is a
typical sublethal parasite of several Myrmica (Hymenoptera: Formicidae) species which has
long been believed to have no detrimental effect on its host individuals [9,10,11]. For a century,
very little was known about this parasitic organism, and only scant information was available
on their distribution and host specificity [4,12,13]. The effect of the fungus on the physiology of
its host species was largely unknown. In recent years, modern research has shed light on the
real nature of this widespread organism and has shown that it has negative effects on individual
host ants. Based on previous studies on the physiological response of infected workers of the
main host Myrmica scabrinodis Nylander, 1846, infected workers show higher mortality under
laboratory conditions [14,15]. Infected M. scabrinodis workers were also shown to exhibit
improved sanitary behavior [14] and a decreased level of intraspecific aggressivity [16,17] which
may be disadvantageous for the infected colonies in competitive interactions.

Although R. wasmannii is known to elicit different detrimental physiological responses in
individual workers, colonies seem to resist and compensate for the negative effects of the
infection; they contain queens, rear larvae and pupae, and all age-classes of workers are present
(see Csata et al. [17]). This may be due to the fact that it is often challenging to study colony-
level effects of an infection under in situ conditions, particularly when complex environmental
parameters and multifactorial relationships with a number of other organisms [8,15,18] must be
taken into consideration. This may be one of the reasons why a colony-level stress effect of 
*Rickia* fungal parasites has never been the subject of focused research.

We propose that the colony-level negative effect of the infection is detectable through the 
decline in size of colony workers. Here, we hypothesize that the infection has detrimental 
effects on the fitness of brood caring workers, making them unable to engage in foraging 
activity and feeding and grooming behavior toward their larvae to the necessary extent, and the 
declining larval growth rate ultimately leads to a decrease in the size of the next generation of 
workers. The size of the first workers infected is not affected, but *R. wasmannii* infection is 
transferred over generations, causing a long-term chronic, life-long infection in a colony, which 
is known to expand year by year in the nest with some level of seasonal fluctuation [19]. The 
detrimental colony-level effect of the infection accumulated over time can be measured in 
workers’ sizes. We hypothesized that if *R. wasmannii* infection is widespread in an ant colony, 
it will detrimentally affect the size of workers groomed by infected sisters owing to the parental 
colony’s reduced fitness. To test this hypothesis, we compared the sizes of randomly sampled 
workers from infected and uninfected colonies.

How can we rule out the possibility that the size decline is caused not by direct larval infection 
but by mediated colony-level stress among workers? *Rickia wasmannii* fungus is found to grow 
on imago [14,20] and is not known to infect ant larvae [11,21] but in order to make sure that a 
possible size decline is ascribed to stress mediated by nursing workers and is not caused by 
direct larval infections, trait scaling patterns were also observed. The background of this 
approach is that infections in the larval stage often cause scrambled trait combinations in adults 
[22] via altered static trait allometries. If significant shifts in trait scaling were detected in 
infectected colonies, parasitism generated stress might not only be mediated by infected workers.

We also tested whether the extent of infection (i.e., number of thalli on the cuticular surface of 
the ants) and the workers’ ages affect the detected changes in imaginal size. Therefore, we 
registered both infection level and estimated age of the workers in the colony.

Understanding the colony-level effect of *R. wasmannii* infection on *Myrmica* colonies in a 
natural environment would foster a better understanding of the dynamics of this 
entomopathogenic fungus and the host-parasite system. This issue has interesting 
implications, because *Myrmica* ants are a known host species of a guild of ant guests and 
socially parasitic organisms which live together with their colonies [8,23]. This network is very
sensitive, and every single component of this very complex system might have a regulatory role of its own.

**Results**

A total of 300 workers from 30 colonies were measured (15 colonies of each class). The calculated Intra Class Correlation between 16 pairs of repeated measures was very high (R = 0.980 to 1.0), i.e. measurement error was negligible. Body size was significantly decreased in infected colonies in comparison to uninfected ones ($\beta = -0.038$, SE = 0.014, $t = -2.66$, $P = 0.013$, Fig. 1). We found no significant effect of age ($\beta = -0.003$, SE = 0.005, $t = -0.65$, $P = 0.514$) or thalli number ($\beta = 0.0004$, SE = 0.006, $t = 0.06$, $P = 0.950$, Fig. 2) on body size among infected colonies. Variance inflation factor was 1.025 for both independent variables, suggesting no substantial multicollinearity. Nevertheless, based on the Poisson GLMM, age had a significant positive effect on thalli number ($\beta = 0.405$, SE = 0.011, $t = 35.71$, $P < 0.001$, Fig. 3). Furthermore, we found no significant difference between trait correlation matrices of uninfected and infected colonies, indicating that they exhibit the same allometries ($X^2 = 1.313$, $P = 0.999$, Fig. 4).
Fig. 1. Distribution of PCA axis values representing body size in uninfected and infected colonies. Asterisk marks statistically significant difference.

Fig. 2. Visualization of how log-transformed thalli number (left panel) and age (right panel) are associated with body size; based on our LMM neither of them had significant effect on the workers' size.
Fig. 3. Association of age and thalli number of workers among infected colonies. The solid line represents the association predicted on the Poisson GLMM.

| PPW | SL  | FL  | FR  | CW  |
|-----|-----|-----|-----|-----|
| 0.69| 0.59| 0.79| 0.64| 0.84|
| 0.79| 0.67| 0.83| 0.71|

| PPW | SL  | FL  | FR  | CW  |
|-----|-----|-----|-----|-----|
| 0.68| 0.57| 0.68| 0.73| 0.87|
| 0.73| 0.64| 0.75| 0.83|

Fig. 4. Correlograms visualizing the trait associations of measured body size variables in uninfected (left panel) and infected (right panel) colonies.
Discussion

Our results show that *R. wasmannii* infected *M. scabrinodis* workers are significantly smaller in a native environment than uninfected ones. This decline in body size in the infected population is consistent across all observed characters. It is important to note that we did not detect shifts in allometries in the characters measured. The significant decrease in size took place synchronously. The lack of shifts in static trait allometries indicates that the size decline in the next generation of workers is not ascribed to physiological stress due to direct larval infections but to a colony-level stress effect caused by widespread infection of *R. wasmannii* fungus across the colonies’ adults. Discrete factors, such as parasitism, are known to modify larval development through altered physiological processes, and these developmental perturbations leave their mark on the allometry of some traits of subsequent adults [22], but no traces of such parasitism are detected in natural *Myrmica* populations. We suppose that a colony-level stress in response to *R. wasmannii* infection is mediated toward larvae by nursing workers, resulting in significantly smaller static trait size among the next generation of their nest mates.

Earlier, laboratory based findings and field studies have shown that this fungal infection does not attack *Myrmica* larvae [11,21,24,25], but is known to cause an array of detrimental changes in physiology in individual ant workers, such as a decrease in life expectancy, particularly in cases of water and food deprivation [15], increased sanitary behavior [14], and a reduced ability to fight against competitors [16,17]. Yet, despite the number of negative effects detected under laboratory conditions, infected colonies in the field have been thought to function relatively well, as they contain queens, larvae and pupae, and all age-classes of workers (see Csata et al. [17]).

Our research is the first undertaking that provides quantitative evidence concerning a decline in worker size in infected nests, dispelling the previous concept concerning the seeming health of infected colonies and revealing that chronic *R. wasmannii* infection has a long, intergenerational, detrimental colony-level impact in the natural environment.

Infected colonies can somehow manage to compensate for the negative effects of *R. wasmannii* infection (colonies operate with queens, larvae and pupae and they have the capacity to produce sexual forms and maintain their populations), but certain functions are clearly impaired, which is reflected in the decrease in body size among the next generation of workers.

The background of the workers’ mediated colony-level stress as a consequence of parasitism is not entirely clear. Similarly to other members of the order Laboulbeniales, *R. wasmannii* does...
not penetrate the cuticle of the host, so the most likely hypothesis concerning the feeding of the parasitic fungus is that it absorbs the necessary nutrients from the workers’ cuticle surface or directly from the environment\cite{25}. Ants have numerous exocrine glands, the secretions of which are spread on the cuticle surface by self-grooming and allogrooming. This may be confirmed by the fact that infected *M. scabrinodis* workers show increased sanitary behavior \cite{14}. This behavior has been observed in the invasive garden ant (*Lasius neglectus* van Loon boomsma et Andrásfalvy, 1990) infected by *Laboulbenia formicarum* Thaxt. \cite{26}, where fungus also was not found to penetrate the cuticle of its hosts \cite{25}. This increased sanitary behavior could mean that ant workers have less time and energy to care for and feed the brood, which could be another explanation for the decline in body size in subsequence generations in infected colonies.

This might be explained by the discrete, i.e., presence-absence type of stress caused by *Rickia* infections on colonies, though we do not find this explanation the most persuasive one. The most plausible reason might be lurking in the dynamics and phenology of the infection, namely the level of infection might not correlate with the number of visible thalli on the worker individuals’ cuticular surface. We also know that the intensity of infection varies throughout the year, and the peak number of thalli takes place in late autumn \cite{19}. This fluctuation, combined with the emergence of smaller workers from the overwintering larvae of *Myrmica* ants \cite{27}, makes it difficult to map the exact processes.

Although only negative effects of the fungus are currently known, we speculate that the increased sanitary behavior may be advantageous against other parasites, and *R. wasmannii* may also have unknown positive effects on its hosts, similar to the *L. formicarum* \cite{14,26}.

We also speculate that the decline in size among workers in infected *Myrmica* ant colonies is part of the colony-level strategy to minimize the adverse effects of the infection in order to mitigate increased mortality and maintain the ability to produce sexuals. From an evolutionary biological perspective, whether a *R. wasmannii* infection impacts the fitness of *M. scabrinodis* colonies is an important question. We do not know whether infected colonies produce smaller or perhaps fewer sexuals or this ultimate function is not impaired. This topic merits further investigation.

**Materials and Methods**
Study site

Material for the present study was collected from a relatively small, one-acre fragment of a natural marshland surrounded by deciduous, oak-dominated forest near Gyöngyös, Sár-hegy: Gyilkos-rét (23.06.2016; 47.811206, 19.988027; 320 m a.s.l) in Hungary (Fig. 5). This area is part of the Bükk National Park and has not been disturbed or extensively cultivated over the course of the past two decades.

Fig. 5. Field site where *M. scabrinodis* workers for our study were collected. Field site is located in Europe (a), Hungary (b) near Pálosvörösmarty (c). Wet meadow (Gyilkos-rét) surrounded by deciduous forest (d).

Sampling

Infected (Fig. 6) and uninfected *M. scabrinodis* colonies were found via hand searching in the grassland by FB and AT. Sampling activities were concentrated in a short, one-day period of time (23.06.2016) because *R. wasmannii* infection is known to show seasonal fluctuation \[^{[19]}\]. Altogether 30 *M. scabrinodis* colonies were used as samples, and 15 uninfected and 15 infected specimens were taken from each colony. The nests were carefully opened and the presence of
\textit{R. wasmannii} on the workers’ body surfaces was checked in every colony with the use of a 40x magnifying glass in the field. After inspection, a small fragment of colony workers was collected from each nest. The sampled workers were stored in 1.5 mL Eppendorf tubes with 67.5\% EtOH until observation in the laboratory.

\textit{Thallus number on the workers’ cuticular surfaces}

Altogether, 225 \textit{M. scabrinodis} workers (15 individuals from each infected colony) were screened (by FB) for fungal thalli (Fig. 6). All fungal thalli on the whole ant body were counted using a Leica MZ125 stereomicroscope at \times 10-160 magnification as described by Báthori et al. [20]. After the fungal thalli had been counted, colony infection level was calculated based on arithmetic mean thalli number of 15 randomly sampled workers from each colony.

Fig. 6. Habitus image of an infected \textit{Myrmica scabrinodis} worker. \textit{Rickia wasmannii} thalli cover the head, mesosoma, and, to a lesser extent, the appendages. Photo: Zsolt Újvári.
Estimating worker age

As was done by Báthori et al. [20], all infected *M. scabrinodis* workers screened for fungal thalli were separated into different age groups. Based on the degree of cuticular pigmentation, five different age categories were described by Cammaerts-Tricot [28]. The infected individuals were classified into categories according to cuticle coloration from younger to older (1-5). The highly pigmented oldest workers were given the highest numbers.

Morphometric character recording

The measured morphometric characters are defined as in [22]. The measurements of altogether 300 *M. scabrinodis* workers (10 from each colony) were made with an ocular micrometer using a Leica MZ125 stereomicroscope at a magnification of ×50 for CL, CW and FR, ×100 for FL, SL and PPW (all measurements were recorded in μm). All measurements were made by FB. Measured characters are defined in Table 1. Raw data are available in Supplementary table 1.

Measurement error assessment

All measurements are subject to error, therefore repeatability, i.e., the degree of agreement between pairs of observations made on the same measurand under the same conditions, i.e. made by the same observer, using the same microscope, following the same measurement protocol as defined in Csősz et al. [29], was tested before the statistic framework was created. The repeatability of the recorded size parameters was assessed via Intraclass Correlation Coefficients (ICC) on repeated measurements of 16 ant specimens using Package ICC (Wolak et al. 2012). ICC scores are given for each characteristic in Table 1.

| Abbr. | Description of traits | ICC       |
|-------|-----------------------|-----------|
| CL    | Cephalic length measured from the anterior-most point of clypeal margin to the mid-point of the occipital margin, in full-face view. | 0.990 [0.980, 1.000] |
| CW    | Cephalic width measured in full-face view, including compound eyes. | 0.997 [0.994, 1.000] |
| Abbr. | Definition                                                                 | ICC     |
|-------|---------------------------------------------------------------------------|---------|
| FR    | Frons width measured according to the minimum distance between the frontal carinae. | 0.995 [0.990, 1.000] |
| FL    | Frontal lobe width measured according to the maximum distance between external borders of the frontal lobes. | 0.995 [0.991, 1.000] |
| SL    | Scape length measured from the neck to the distal end of the scape. | 0.993 [0.986, 1.000] |
| PPW   | Postpetiole width measured according to the maximum width of the postpetiole in dorsal view. | 0.996 [0.991, 1.000] |

Table 1. Verbatim trait definitions for morphometric character recording. Abbreviation (Abbr.), verbatim character definition and intraclass correlation coefficients (ICC) of certain morphometric traits are provided. Upper and lower bounds of ICC scores, separated by a coma, are also given in parentheses.

Statistical analysis

All data analyses were performed with R version 4.0.2. To reduce the number of variables on body size we used principal component analysis (PCA) by non-linear iterative partial least squares (NIPALS) with the R-package “nipals”[^31]. We preferred this method over classical PCA because in a small number of cases (7 in total) size measurements of some body parts for a given ant were not feasible (hence the missing measurements); NIPALS can use data with missing observations, whereas in classical PCA we should have excluded all those specimens for which any measurement was missing. Variables were centered at zero (by subtracting variable mean from each value) and rescaled (by dividing all values by the variable standard deviation) in order to bring them to the same scale. We retained only the first PCA axis (being the only axis with an eigen-value higher than 1), which corresponded to 77% of the total variation in the six body measurement variables and was positively correlated with all variables.
To test whether there is a significant difference in body size between infected and uninfected colonies, we used a mixed-effects linear regression model (LMM) with Gaussian error distribution using the R-packages “lme4” and “lmerTest” \cite{32,33}, specifying the abovementioned PCA axis representing body size as the dependent variable and infection as independent factor.

To test how the severity of fungal infection and age affected body size, we fitted another LMM (naturally, only using data from infected colonies) with the body size PCA axis as a dependent variable and the log-transformed number of thalli and age as independent variables. Because previously it had been shown that age and thalli number can be correlated, we estimated variance inflation factors to see whether there is substantial multicollinearity between the independent variables used and the “car” package \cite{34}. In addition, we tested the association between age and thalli number using a Poisson generalized LMM (GLMM), specifying thalli number as a dependent variable. In both LMMs and in the GLMM, colony number was used as a random effect to control for the non-independence of observations from the same colonies.

Furthermore, to see whether the infection causes changes in static trait allometries across the measured body size indices, we estimated trait correlations (Pearson’s $\rho$) separately for uninfected and infected colonies and compared the resulting two correlation matrices \cite{35}. 


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Author contributions

SC and FB contributed to the study conception and design. Data preparation, data collection was performed by FB, LEB. Data analyses were performed by SC and ZR. The first draft of the manuscript was written by SC, FB and AT and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

Raw data are available from the Supplementary table 1.

Additional Information

The authors declare that there is no conflict of financial and non-financial interest.