Title:

Does fungal infection produce cheaper workers?

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Abstract

Cuticle quality is of vital importance in insects. Amongst others, it prevents desiccation, and protects against pathogens. Therefore the development of thicker cuticle would be advantageous. The within-species variation of cuticle structure is affected by many factors, both physiological and ecological. Parasites can also affect the exoskeleton structure either to facilitate intrusion or to aid transmission to the next host. In the frame of our study we investigated the effect of an ectoparasitic fungus, *Rickia wasmannii* on the cuticle of its host. We measured the thickness of the exoskeleton in infected and uninfected ants originating from three European populations. Infected ants had thinner cuticle than uninfected ones, while there were also differences among populations. It is unclear though, whether this is the host's response investing less into cuticle formation, or the fungus infects colonies with thin cuticle. Either way, within-species variation is linked to response to parasitic pressure.

Introduction

The cuticle of insects is a complex structure with considerable variations with regards to both composition and function (Hopkins and Kramer 1992; Vincent and Wegst 2004; Nation 2016). It is characterized by high mechanical strength, resistance to chemical or enzymatic degradation, prevents desiccation, and also serves as the primary defense line against parasites (Hopkins and Kramer 1992; Vincent and Wegst 2004; Nation 2016; Peeters et al. 2017). There are many constraints that could act on the structure of the cuticle, from physiological to ecological (Nation 2016). According to the hypothesis formulated by Peeters et al. (2017) in social insects, as ants, within and between species variation in cuticle thickness could have important implications for cost-optimization related to colony size. Thus, the production of more cost-efficient or cheaper workers with thinner cuticle would make less costly the development of large colonies.

It is widely documented that parasites can cause alterations in the behaviour, physiology, and morphology of their insect hosts, as ants for example (Schmid-Hempel 1998; Shik et al. 2011; Verble et al. 2012; Hughes et al. 2016; Csata et al. 2017a). Constant parasitic pressure could impose considerable costs on the development of cuticle as well. Ants possess a wide array of fungal pathogens, and some developed a series of adaptations to penetrate the cuticle of host ants, as the well-known generalist entomopathogenic fungus *Metarhizium anisopliae* (Leger et al. 1987), or the lethal ant specialized *Ophiocordyceps* and *Pandora* species (Roy et al. 2006; Hughes et al. 2016; Małagocka et al. 2017). A specific threat to ants could represent those ectoparasitic fungal species that do not penetrate the cuticle, but reside on it. Such epicuticular parasites are the fungi of the order Laboulbeniales, which obligatorily parasitize insects (Weir and Blackwell 2005). Specifically, *Rickia wasmannii* exploits ants of the genus *Myrmica*

Latreille (Espadaler and Santamaria 2012). The fungal thalli attach to the outer layer of the cuticle, and covers the whole body of the ant host in high abundance (Markó et al. 2016; Tragust et al. 2016). It was demonstrated that it affects the water-consumption (Báthori et al. 2015), the grooming behaviour, and also the lifespan of infected ants (Csata et al. 2014), besides causing changes in the composition of CHC-profile (Csata et al. 2017a). These point to the existence of certain effects on the host's cuticle that could involve thinning of the cuticle, or even its thickening as a counter-adaptation to the constant presence of parasites on the exoskeleton.

In the frame of our study we analyzed the cuticle thickness of ants from infected and uninfected colonies from three different regions of Europe to elucidate whether the presence of this fungal parasite correlates with changes in the exoskeleton.

Materials and methods

Rickia wasmannii Cavara (1899) is one of the most widely distributed myrmecoparasitic Laboulbenian fungal species in Europe (see Santamaria and Espadaler 2015). It obligatorily exploits *Myrmica* ants (Espadaler and Santamaria 2012; Witek et al. 2014; Santamaria and Espadaler 2015). Altogether 6 infected and 6 uninfected *Myrmica scabrinodis* colonies (2+2 colonies/populations) were collected for the purpose of our study from Borşa Cătun, Romania (N 46.88909, E 23.70067), from Kraków, Poland (N 50.01592, E 19.51753), and from Gessertshausen, Germany (N 48.32339, E 10.71942) in 2014, and 2016. Ants were identified to species level based on Czekes et al. (2012). Since old individuals are the most infected and thus the most affected (Csata et al. 2017b) only old individuals were used for simplicity, selected on

the base of darker cuticular pigmentation (see Moroń et al. 2008). All selected individuals were screened for fungal thalli using an Olympus SZ51 stereomiscroscope (×80).

We carried out five histological cross-sections on the level of the first dorsal abdominal segment (Fig. 1) in 21 infected and 21 uninfected individuals coming from different colonies (3-4 individuals/colony). The abdomens of the ants were removed to allow penetration of the fixative gluteraldehyde, and they were fixed for 12 h in cold 2% glutaraldehyde buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. One hour after post-fixation in 2% osmium tetroxide in the same buffer, tissues were dehydrated in a graded acetone series, embedded in Araldite and sectioned with an Autocut Reichert Jung 2040 microtome (serial semithin 0.5 μm sections). For each individual five cross-sections were prepared, which were then and measurements were taken at five different points per cross-sections resulting in 25 individual measurements for each individual. In some cases we excluded a few cross sections as the different layers of the cuticle were not measurable due to technical errors. The following cuticular layers were considered for measurements: exocuticle, endocuticle, and altogether the procuticle (= endocuticle + exocuticle). Measurements were taken in μm in blind manner by the same person with a Soliger Camera attached to a Zeiss Axioplan microscope at ×200 magnification and with the use of the analySIS Digivision Pro software.

We tested all data for assumptions of normality (Kolmogorov-Smirnov test). Linear Mixed-effect Model approach (LMM) was applied to test the potential effect of infection on the cuticle thickness of individuals. The thickness of different cuticle layers was introduced as response variable, while the infection status of the individuals, the ID of different populations and their interaction were included as explanatory factors. The IDs of cross-sections, of individuals, and of colonies were introduced as nested random factors in order to handle

dependencies. All statistical analysis were carried out using the R 3.2.4 statistical environment (R Development Core Team 2016). LMMs were performed using the *lme* function of the *lme4* R package (Pinheiro et al. 2016). The exact significance levels of input variables were retrieved with the use of *Anova* function in *car* R package (Fox and Weisberg 2015). *Tukey* function of *multcomp* package was used in order to carry out sequential comparisons among factor levels (Hothorn et al. 2008). The graphs were illustrated using the *ggplot2* R package (Wickham 2009).

Results and Discussion

A few studies hypothesize that Laboulbenian fungi produce penetration structures to extract resources from hosts (Benjamin 1971), but Tragust et al (2016) found no hard evidence that ant-parasitizing Laboulbeniales fungi, as e.g. *R. wasmannii* and the related *Laboulbenia formicarum* would indeed penetrate the cuticle. On the other hand, *R. wasmannii* infected ants are known to consume water longer (Báthori et al. 2015), have shorter lifespan (Csata et al. 2014), and their CHC profile also shows significant changes (Csata et al. 2017a), which all suggest that infection could have a direct or indirect effect on the cuticle. Our LMM results came to support this hypothesis, as the cuticle of infected individuals was significantly thinner, than those of uninfected in each cases: procuticle $\chi^2 = 8.5$, $p < 0.003$ (Fig. 2), exocuticle $\chi^2 = 7.42$, $p < 0.006$ and endocuticle $\chi^2 = 4.38$, $p = 0.036$. The interaction between the infection and population was not significant in either of the cases (LMM $\chi^2 \leq 1.30$, $p = NS$).

It is not yet clear whether actually the fungus causes alterations in cuticle structure, or it prefers colonies containing individuals with thinner cuticle. The fact that infected ants adjust their water consumption (Báthori et al. 2015) and show changes in hydrocarbon profile (Csata et al. 2017a) could suggest that the presence of *R. wasmannii* drives these changes. Since the

prevalence of infection is higher in humid spots (Markó et al. 2016), it is fair to assume that ants could also adjust their microhabitat preference to morphological constraints: thinner cuticle selects for more humid environment to prevent desiccation. On the other hand, the reverse could also be valid, microhabitats with higher humidity could also allow ants to invest less in cuticle formation, and thus develop the less costly thinner exoskeleton. That would imply a certain degree of phenotypic plasticity from the part of the ants, as suggested also by Peeters et al. (2017). The existence of such within species plasticity is suggested by the clear differences among populations in the case of almost all cuticle layers as in procuticle and endocuticle (LMM $\chi^2 \geq 33.35$, $p < 0.0001$; see Fig. 2), with the exception of the exocuticle (LMM $\chi^2 = 1.38$, $p =$ NS). The pairwise comparison yielded detailed results: significant differences were registered between Polish and Romanian, and German and Romanian samples with regards to pro- and endocuticle (LMM $z \leq -3.65$, $p < 0.001$), whereas no differences were found between German and Polish populations (LMM$_{pro}$ $z \leq 0.32$, $p =$ NS).

Rickia wasmannii is known to influence in many ways its ant host (Csata et al. 2014, Báthori et al. 2015, Csata et al. 2017a), including the reduction of the host's lifespan (Csata et al. 2014), while infected colonies are still functioning well. In these conditions, maintaining a colony despite the high prevalence of the parasite and heavy parasitic load that manifests itself in old workers (Markó et al. 2016, Csata et al. 2017b) could be costly. The early loss of forager workforce could be compensated by higher degree of polygyny (see Csata et al. 2017a), that could compensate the negative effects by the production of more workers. Costs could present themselves though in the form of smaller workers or thinner cuticle (Peeters et al. 2017). There is no data available on the size of infected workers, but the results of the present study would point to the direction of the existence of anatomical costs on the level of exoskeleton. Whether cuticle

thinning appears because of mingling of the parasite with biochemical pathways related to cuticle formation, or more simply because of the reduction of protein input on colony level required for normal cuticle formation, needs to be cleared. We cannot dismiss either the more parsimonius explanation that *R. wasmannii* thrives mostly in host colonies with thinner exoskeleton adapted to more humid habitats. *Myrmica scabrinodis* is known to have a wide habitat preference (Radchenko and Elmes 2010), which actually might be reflected in the between population variation in cuticle thickness uncovered here. This phenotypic and ecological plasticity could serve as a basis to anatomical response to infection with this ectoparasite.

Acknowledgements

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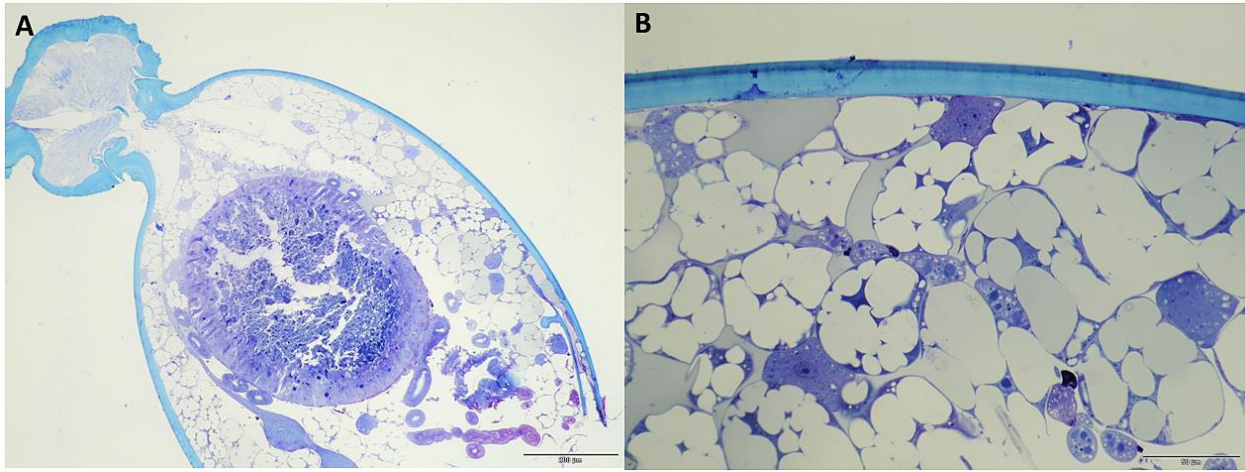
Fig. 1. Longitudinal section through prothorax of uninfected *M. scabrinodis* individual (**a**), and cross-section of the abdomen of the individual (mf: muscle fibres) (**b**).
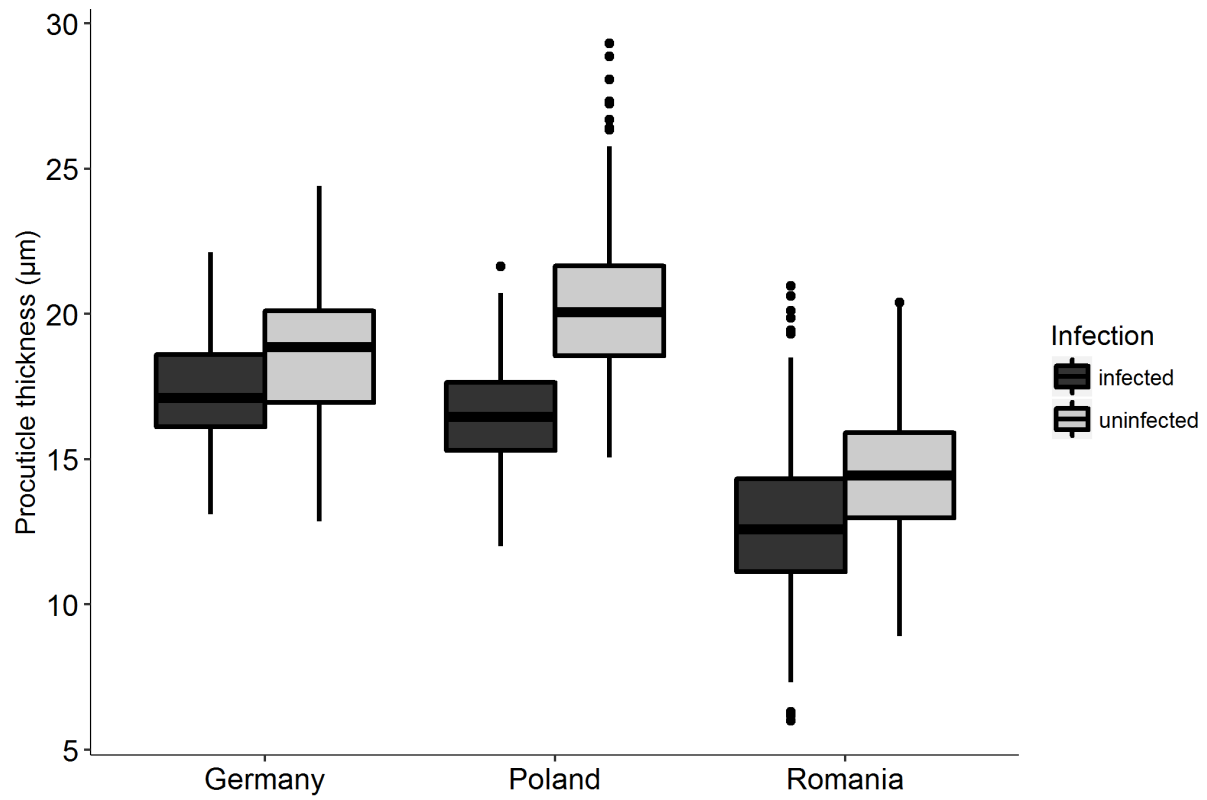
Fig. 2. The thickness (μm) of the procuticle in infected and uninfected *M. scabrinodis* individuals' from different populations (median, quartiles, min-max values, outliers).