UNIVERSITATEA BABEŞ-BOLYAI DIN CLUJ-NAPOCA

Facultatea de Biologie şi Geologie

Şcoala Doctorală Biologie Integrativă

DOCTORAL THESIS

Summary

Immune defense responses elicited by a non-lethal fungal parasite in *Myrmica scabrinodis* (Hymenoptera: Formicidae)

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Contents

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I. Introduction

Multicellular organisms are continuously exposed to different pathogens, e.g. viruses, bacteria, and fungi, which can harm the host, causing behavioral, morphological, and functional changes, or even lead to death (Schmid-Hempel, 1998; Verble et al., 2012; Csata et al., 2014; Hughes et al., 2016; Csata et al., 2017a; Csata et al., 2017b; Csata et al., 2018; Csősz et al. 2021). The host-parasite relationship is based on a strong connection. The parasite to become successful must adapt to the host body, and thus evolve co-evolution between the host and parasite species (Van Valen, 1973).

Insects evolved a complex and effective immune system (Hoffmann, 1995), which differs from the immune system of vertebrates. In insects, physical barriers, as well as cellular and humoral immune responses make up part of the innate immune system (Rosales, 2017). Their immune defense mechanisms involve the hemocytes, the midgut, their salivary glands, their fat body, and other tissues (Hillyer, 2016). The cuticle as a physical barrier plays a really important role in insects' defense mechanisms. Other physical barriers can be the midgut pleiotropic membrane or the tracheal chitin-cuticles. However, if pathogens pass these barriers, innate immune reactions, such as cellular and humoral immune responses are activated (Hauling, 2012).

Encapsulation, nodulation, melanization, phagocytosis, and coagulation make up part of cellular immune responses (Rolff and Raynolds, 2009). During the humoral immune response pattern recognition proteins play an important role in the recognition of pathogens (for ex. a peptidoglycan recognition protein PGRP, β-glucan recognition protein). Moreover, the use of some signaling pathways (Toll, Imd, JNK, JAK/STAT) stimulates the synthesis of antifungal and/or antibacterial peptides in the fat body, and these peptides will be emitted into the hemolymph. The Toll pathway plays a role in the recognition of Gram-positive bacteria, virulence factors, and fungi. The Imd pathway is involved in the recognition of Gram-negative bacteria, and DAMPs (Damage-Associated Molecular Patterns). The Jak/Stat pathway is activated by stress/injury (Broderick et al., 2009; Dubovskiy et al., 2016) and the JNK pathway mostly activates against Gram-negative bacteria (Libert et al., 2008). Humoral immune responses contain some enzymatic cascades, which regulate the melanization and coagulation of hemolymph, and the production of ROS (reactive oxygen species) and RNS (reactive nitrogen species) (Marmas and Lampropouou, 2009). In conclusion, fighting against pathogens involves a chain reaction of complex processes, and the cellular and humoral immune responses are highly correlated.

Social immunity means the collective defense of the group, emerging through the interaction among constituent individuals, from diseases and parasites. It is essentially different from the sum of individual immune reactions.

Social insects have evolved collective immune responses that enhance the overall resistance of the colony against diseases, since living in a group can ease the spread of the infection among the group members (Cremer et al., 2007; Stroeymeyt et al., 2018). When an individual is exposed to a pathogen, it triggers an immune response that can spread to other colony members through physical contact or shared resources. This collective immune facilitation helps to activate the immune system of other individuals, making them more resistant to similar pathogens (Konrad et al., 2012). In some cases, social insects can also modify their behavior to reduce the risk of disease transmission (Cremer et al., 2007; Stroeymeyt et al., 2018).

Social insects, like ants, are the preferred host organisms of several parasitic fungi. Both generalist and specialist, behavioral manipulators and neutrals (do not manipulate host behavior), ecto- and endoparasites. Most entomopathogenic fungi are thought to be lethal to ants.

The predominant focus of current scientific research lies on the lethal pathogen mentioned earlier. What sets the ectoparasitic fungus *Rickia wasmannii* apart is its intriguing characteristic of not causing the death of its host, unlike other parasites that quickly destroy the host organism. Our knowledge on the prolonged coexistence of a non-lethal pathogen with its host, its impact on the host organism, and the social implications of its coexistence over an extended period is limited. Hence, the *R. wasmannii* ectoparasitic fungus is exceptional in that it is recognized for inducing both individual and social changes over the long term. The primary focus of the thesis is on *Rickia* and its effects at the individual level.

1.1.Aims of study

- The switch to social lifestyle leads to evolutionary changes in the immune system. Ants, being eusocial (Ward, 2006), are ideal for this study. We analyzed proteins in the Toll and Jak/Stat signaling pathways in ants and other insects to see if the evolution of immune response proteins can be linked to social lifestyle.

- Long-term exposure to infections can exert serious pressure on the host's immune system and affect its response to new parasites. There is limited information on how the fungus *R. wasmannii* affects the immune systems of ants. We aimed to determine the strength of defense of *Myrmica scabrinodis* ant species by standard immunocompetence assays: encapsulation efficiency in young and old, infected and uninfected workers completes by other measurements as fat body quantification, morphometry, infection intensity assessment.
- Immune investment depends on an individual's age and pathogen pressure and can be revealed by identifying the level of certain proteins involved in immune pathways. We studied the immunocompetence of *M. scabrinodis* ant workers of different health statute and age by measuring levels of active phenoloxidase (PO) and total PO (PPO) (reflecting the amount of both active and inactive forms of the enzyme).
- To examine how fungal infection can influence the immune system on molecular level, we investigated how different categories within a colony (queens, workers, brood) respond to fungal threat. Immune gene activity was measured, focusing on *defensin 1* and *prophenoloxidase* genes.

II. Materials and methods

2.1. Study species

2.1.1. The ectoparasitic fungus *Rickia wasmannii* Cavara, 1899

Laboulbeniales (Ascomycota: Laboulbeniomycetes) are fungal biotrophic parasites, which attach to the surface of their host (Haelewaters, 2012). The parasite negatively affects infected individuals' lifespans (Csata et al., 2014). Parasitized individuals have higher water consumption (Báthori et al., 2015), reduced cuticle thickness (Csata et al., 2018), and smaller body size (Csősz et al., 2021). The fungus also caused behavioral changes in infected ants, increasing allogrooming (Csata et al., 2014; Báthori et al., 2015; Báthori et al., 2017). Infected individuals had altered cuticular hydrocarbon profiles (Csata et al., 2017a), and were more inclined to accept foreign workers and queens (Csata et al. 2017a, Csata et al. 2023).

2.1.2. The host ant, *Myrmica scabrinodis*

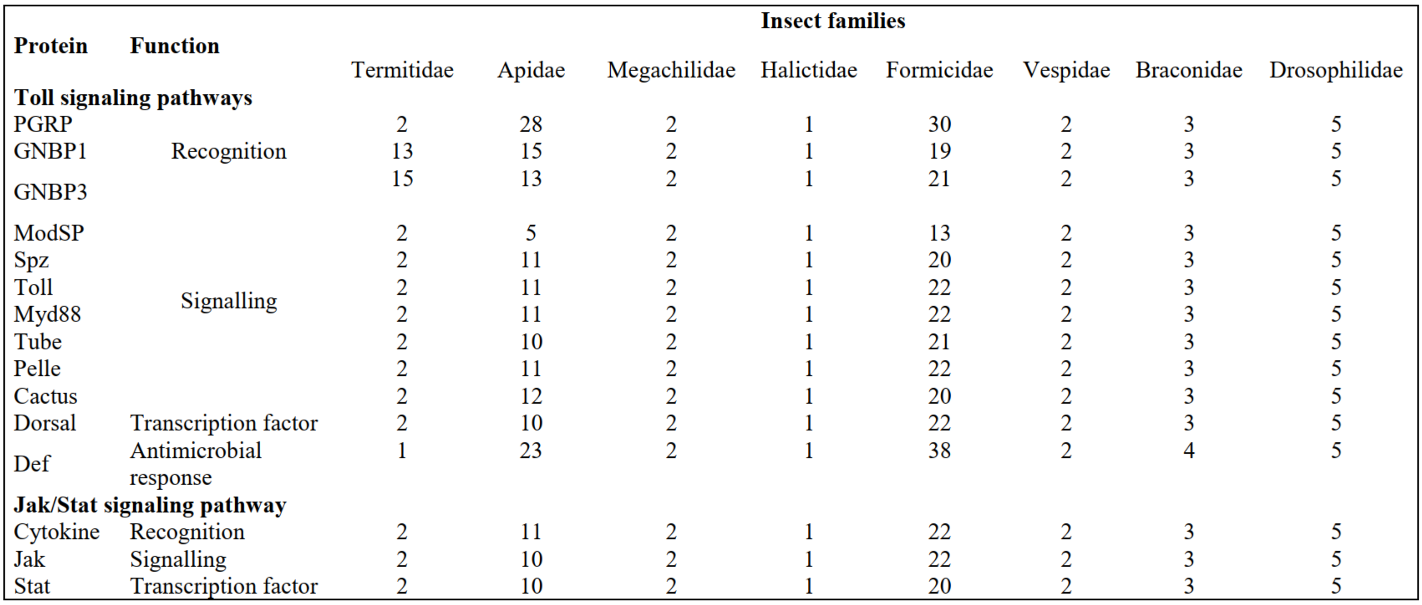
M. scabrinodis is a Europe-Siberian species, which can be found in diverse habitats, mostly in wet patches of grasslands, but in woodlands, and open forests as well. They are tolerant species and only avoid dry places. In our studied sites *M. scabrinodis* is the most common host for the ectoparasitic fungus *R. wasmannii* (Csata et al., 2013; Witek et al., 2014).

III. Results

3.1. Phylogenetic analyses of the proteins involved in encapsulation signaling pathways in ants[1]

We studied the phylogeny of proteins involved in immune responses in ants to reveal whether there are ant-specific and sociality specific patterns, respectively. We identified several homologue sequences from a high number of species in the studied insect groups (Table 1). The alignments generated by the T-coffee program yielded the best scores, therefore we used these to generate phylogenetic trees.

Table 1. Number of protein homologue sequences identified in five social (Termitidae, Apidae, Halictidae, Formicidae, Vespidae) and three solitary insect families (Megachilidae, Braconidae, Drosophilidae).



Protein	**Function**	**Insect families**							
		Termitidae	Apidae	Megachilidae	Halictidae	Formicidae	Vespidae	Braconidae	Drosophilidae
Toll signaling pathways									
PGRP	Recognition	2	28	2	1	30	2	3	5
GNBP1		13	15	2	1	19	2	3	5
GNBP3		15	13	2	1	21	2	3	5
ModSP	Signalling	2	5	2	1	13	2	3	5
Spz		2	11	2	1	20	2	3	5
Toll		2	11	2	1	22	2	3	5
Myd88		2	11	2	1	22	2	3	5
Tube		2	10	2	1	21	2	3	5
Pelle		2	11	2	1	22	2	3	5
Cactus		2	12	2	1	20	2	3	5
Dorsal	Transcription factor	2	10	2	1	22	2	3	5
Def	Antimicrobial response	1	23	2	1	38	2	4	5
Jak/Stat signaling pathway									
Cytokine	Recognition	2	11	2	1	22	2	3	5
Jak	Signalling	2	10	2	1	22	2	3	5
Stat	Transcription factor	2	10	2	1	20	2	3	5

<ins>Toll signaling pathway</ins>

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[1] Published: **Orbán-Bakk K**, Csata E, Markó B, Kósa F. 2023, Phylogenetic analyses of the proteins involved in encapsulation signaling pathways in ants. *Studia Universitatis Babeş-Bolyai Biologia* **68**(1):67–101.

We reconstructed the phylogenetic trees of the 11 proteins involved in the Toll signaling pathway. The different insect families grouped separately in well-defined clades in most phylogenetic trees (bootstrap proportion 70-99%).

In the case of *Pelle* and *Dorsal* proteins, the phylogenetic tree structure followed the general insect phylogeny with Hymenoptera grouping separately. The different members of the order belonged to a well-supported clade, while species of the other two orders (Blattodea, Diptera) formed outgroups on the phylogenetic trees.

The other proteins of the Toll signaling pathway (*PGRP*, *GNBP1*, *GNBP3*, *ModSP*, *Spz*, *Toll*, *Myd88*, *Tube*, *Cactus*) did not follow the classical insect phylogeny, and neither did they reflect differences in social lifestyle.

Ants formed a single well-supported clade in the case of *ModSP*, *Toll*, *Myd88*, *Tube*, and *Dorsal*, whereas in the case of *PGRP*, *GNBP1*, *GNBP3*, *Spz*, *Pelle*, and *Cactus* they did not group together. Phylogenetic trees of recognition proteins such as *PGRP*, *GNBP1*, and *GNBP3* were especially diverse.

Jak/Stat signaling pathway

The different insect families grouped in well-defined separate clades in the majority of phylogenetic trees.

In the case of the recognition protein *Cytokine* receptor, we found that the phylogenetic tree structure followed the general insect phylogeny, thus there was a well-supported clade of the different social and solitary Hymenoptera species, with two outgroups as Blattodea and Diptera. The phylogenetic trees of the signaling molecule *Jak* and the transcription factor *Stat* did not show the same structure and did not reflect differences with regards to lifestyle either.

All things considered ants did not group in a single well-supported clade in any of the studied proteins.

3.2. Under pressure: the effect of long-term fungal infection on the encapsulation response in ants[2]

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[2] Published: **Orbán-Bakk K**, Marczin MJ, Gál L, Heinze J, Csata E, Markó B. 2022, Under pressure: the effect of long-term fungal infection on the encapsulation response in ants. *Insectes Sociaux* 69(4):361–367. https://doi.org/10.1007/s00040-022-00879-z

We examined the immune competence of *M. scabrinodis* workers of different age categories either coming from infected colonies or from healthy control colonies using encapsulation assays.

The average model included 15 individual models, taking into account all factors and variables. The strength of encapsulation response (area covered by the hemocytes) depended on morphological characteristics of individuals: smaller individuals (with smaller heads) had a stronger encapsulation response than larger ones (z = 2.42, p = 0.015). Neither fat content (z = 0.39, p = 0.69) nor age (z = 0.39, p = 0.69), infection status (z = 0.78, p = 0.43), nor infection intensity (z = 0.43, p = 0.66) had a significant effect on the encapsulation response, but the interaction of infection status of colonies and age of workers did display a significant effect (z = 2.09, p = 0.036) (Fig. 1). Young individuals from infected colonies had a significantly stronger encapsulation response than uninfected young workers (Fig. 1).

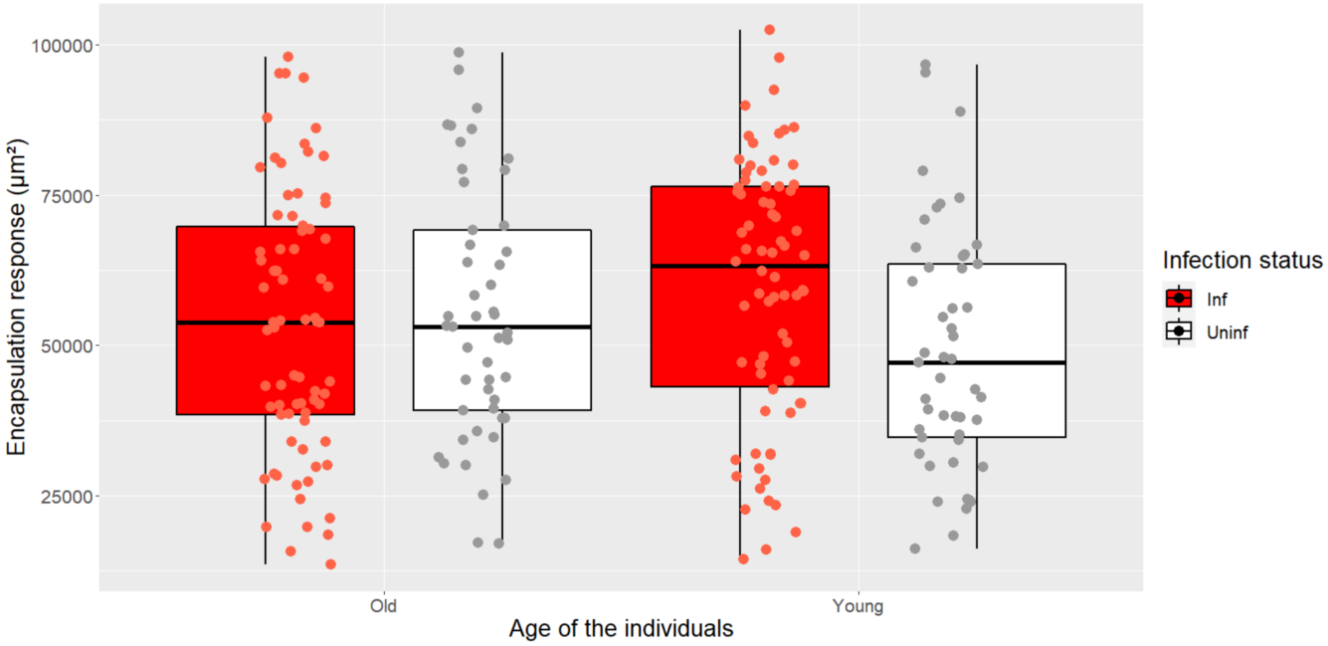

Figure 1. Encapsulation response of ant workers belonging to different age categories from infected and uninfected *M. scabrinodis* colonies.

3.3. Infection with a non-lethal fungal parasite is associated with increased immune investment in the ant *Myrmica scabrinodis*[3]

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[3] Published: **Orbán-Bakk K**, Witek M, Dubiec A, Heinze J, Markó B, Csata E. 2024, Infection with a non-lethal fungal parasite is associated with increased immune investment in the ant *Myrmica scabrinodis*. *Journal of Invertebrate Pathology* 202: 108027. https://doi.org/10.1016/j.jip.2023.108027

We investigated the general immunocompetence of *M. scabrinodis* ant workers associated with non-lethal fungal infection by measuring the levels of active phenoloxidase (PO) and total PO (PPO) (reflecting the amount of both active and inactive forms of the enzyme) in two age classes.

There was no relationship between the levels of PO and PPO (t = 1.18, p = 0.23). The PPO level was influenced by age: old individuals had significantly higher levels than young ones ($\chi^2 = 25.81$, $p < 0.001$, Cohen's *d* = 1.21) (Fig. 2). Neither infection status ($\chi^2 = 0.36$, p = 0.54, Cohen's *d* = 0.1) nor the size of individuals (characterized through head size) ($\chi^2 = 0.24$, p = 0.61) had a significant effect on PPO level. The interaction of the infection status and age of workers had only a marginal effect ($\chi^2 = 2.94$, p = 0.08): young individuals from infected colonies had higher PPO levels than those from uninfected colonies (Fig. 2).

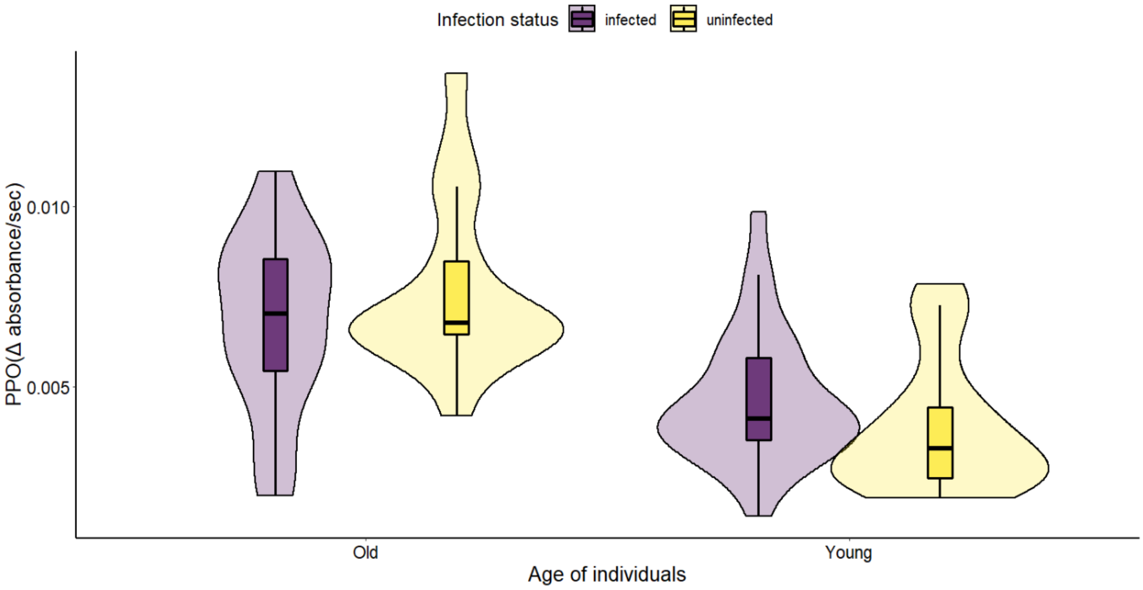

Figure 2. The amount of total phenoloxidase (PPO) (Δabsorbance/sec) in *Myrmica scabrinodis* workers belonging to different age categories from infected and uninfected colonies.

The level of PO was affected by infection: workers from infected colonies had significantly higher levels of PO ($\chi^2 = 6.7$, d.f. = 1, p = 0.009, Cohen's *d* = 0.53; Fig. 3) and high PO levels were connected to large body size (characterized by larger head size) as well ($\chi^2 = 18.63$, d.f. = 1, p = 0.001). Neither age ($\chi^2 = 1.23$, d.f. = 1, p = 0.26, Cohen's *d* = 0.64), nor the interaction of the infection status of colonies and the worker age ($\chi^2 = 0.1$, d.f. = 1, p = 0.74) displayed any significant effect on the level of PO though (Fig. 3).

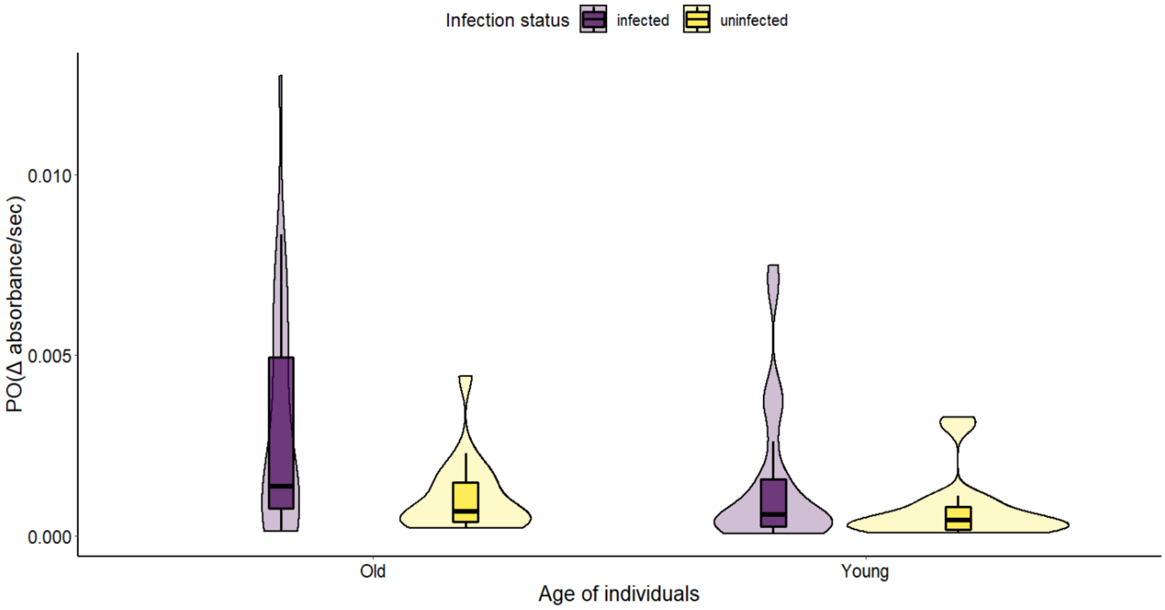

Figure 3. The amount of active phenoloxidase (PO) (Δabsorbance/sec) in *Myrmica scabrinodis* workers of different ages from infected and uninfected colonies.

3.4. Immune response varies among members of a fungus-infected ant society[4]

We investigated how different groups within the colonies respond to being exposed to the fungus by measuring the expression of the genes *defensin 1* and *prophenoloxidase*, both vital components of ant immunity.

Overall, *prophenoloxidase* expression varied relatively little between castes (LMM, larvae vs queens: p = 0.99; larvae vs workers: p = 0.2; queens vs workers: p = 0.31, Figure 4). In larvae, colony infection status also did not affect *prophenoloxidase* expression (LMM, χ^2 = 0.05, p = 0.94). In workers, colony infection status did not affect *prophenoloxidase* levels (LMM, colony infection status, χ^2 = 1.6, p = 0.2) and neither did fat content (χ^2 = 2.4, p = 0.12), nor body size (based on head size; χ^2 = 2.5, p = 0.11). However, workers carrying more thalli showed higher *prophenoloxidase* expression (χ^2 = 4.18, p = 0.04). In queens, almost all the measured variables had a significant effect on *prophenoloxidase* expression, with the exception of body size (based on head size; LMM, χ^2 = 0.13, p = 0.71). The number of thalli (χ^2 = 7.8, p = 0.005), fat content (χ^2 = 5.75, p = 0.01) and colony infection status (χ^2 = 6.67, p = 0.009) affected positively (increased) *prophenoloxidase* expression (Fig. 4).

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[4] published: **Orbán-Bakk K**, Schultner E, Heinze J, Markó B, Csata E. 2024, Immune response varies among members of a fungus-infected ant society. bioRxiv: xxx (in prep for publication). https://www.biorxiv.org/content/10.1101/2024.02.08.579503v1.full

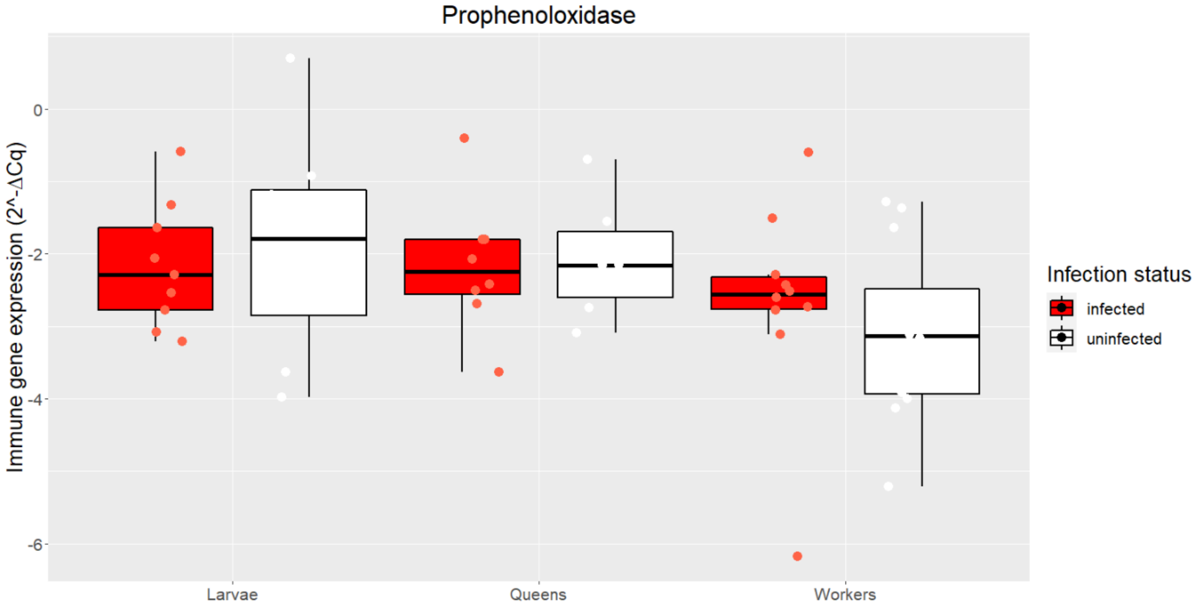

Figure 4. Immune gene expression ($2^{-\Delta Cq}$ values) in larvae, queens, and workers from *Rickia*-infected and uninfected colonies of the ant *Myrmica scabrinodis*.

In contrast to *prophenoloxidase*, *defensin 1* expression was highly variable across groups: larvae having higher *defensin 1* expression compared to queens and workers (LMM, larvae vs queens: $p < 0.001$; larvae vs workers: p = < 0.001; queens vs workers: $p = 0.10$; Figure 5). *Defensin 1* expression in larvae was not affected by the colony's infection status (LMM, colony infection status, $\chi^2 = 0.09$, $p = 0.75$).

Workers carrying more thalli showed higher *defensin 1* expression (LMM, number of thalli, $\chi^2 = 7.67$, $p = 0.005$). Worker body size (reflected in head size; LMM, $\chi^2 = 0.07$, $p = 0.78$), fat content (LMM, fat content, $\chi^2 = 1.53$, $p = 0.21$) and colony infection status had no significant effect (LMM, colony infection status, $\chi^2 = 2.01$, $p = 0.15$). Queens from infected colonies exhibited higher *defensin 1* expression (LMM, colony infection status, $\chi^2 = 3.91$, $p = 0.04$, Figure 5), whereas large queens expressed more *defensin 1* (reflected in head size; LMM, $\chi^2 = 27.61$, $p < 0.001$). Neither the number of thalli (LMM, number of thalli, $\chi^2 = 0.37$, $p = 0.54$), nor fat content (LMM, fat content, $\chi^2 = 3.08$, $p = 0.07$) affected *defensin 1* expression in queens, though fat content showed a slight trend towards increasing expression.

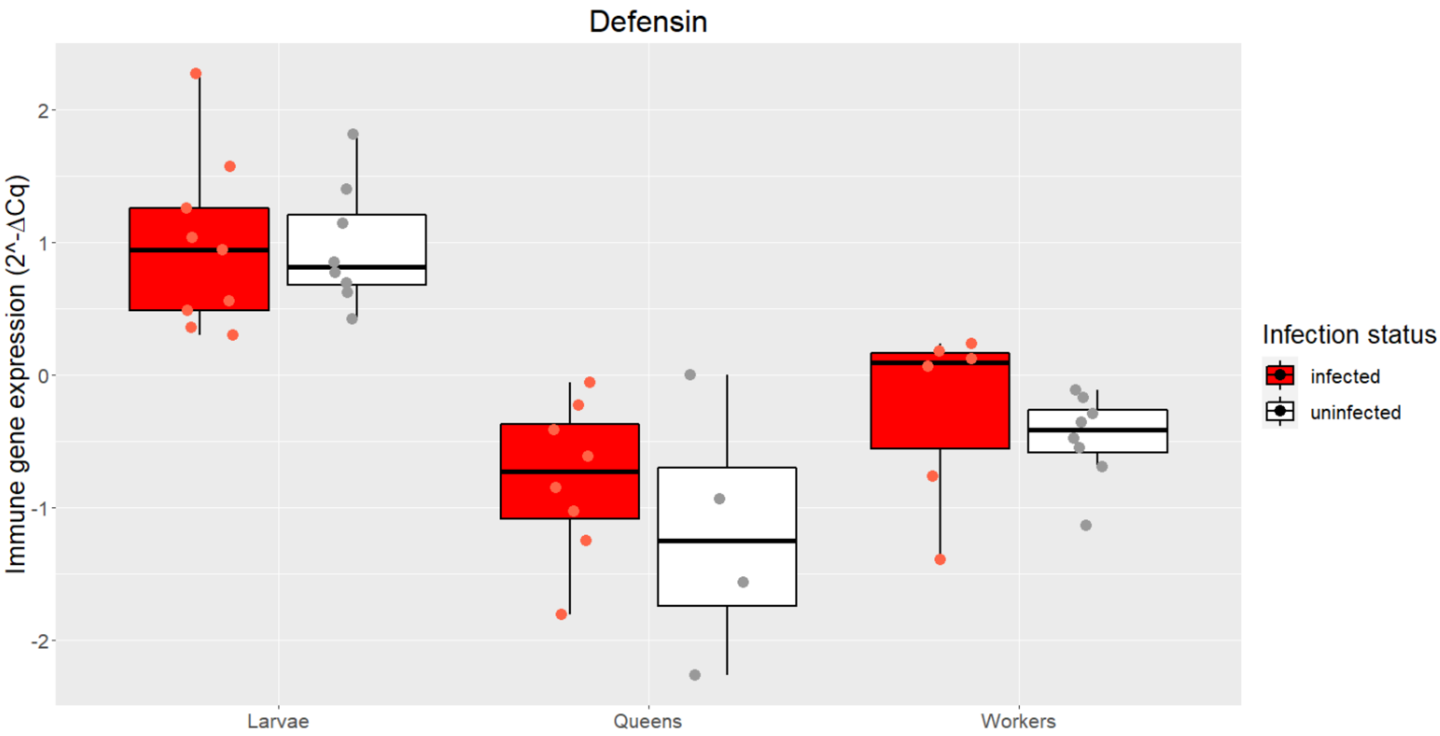

Figure 5. Immune gene expression ($2^{-\Delta Cq}$ values) in *Myrmica scabrinodis* larvae, queens and workers from infected and uninfected colonies (transformed data is presented).

IV. Discussion

Transition from solitary to social life may alter the immune system due to the development of social strategies to combat infections. In insects, there are two signaling pathways (Toll and Jak/Stat) that result in the expression of specific immune genes, which, on their turn, encode peptides, proteins and activate innate immune responses like encapsulation. We carried out phylogenetic analyses of 15 proteins involved in encapsulation signaling pathways in ants. Ants grouped separately from other insect groups in most cases, however, there were some notable exceptions mostly in the case of pattern recognition proteins, probably correlating with differences in potential pathogens. No major differences were revealed though between solitary and social insects with respect to proteins involved in encapsulation.

Despite the absence of significant variations in proteins associated with encapsulation between solitary and social insects, pathogens exhibit a diverse range of effects on host organisms, spanning from lethal consequences to minor behavioral changes (Hughes et al., 2012). Our data suggest that in the case of *M. scabrinodis* the constant pressure by *R. wasmannii* might facilitate the encapsulation response only in young individuals. In an infected colony, young individuals are from the beginning exposed to the fungi, which could result in maintaining their immune defenses at a higher level (Bull et al., 2012). Young individuals are the key to success of a colony. The increased reaction might therefore be the adequate strategy in an already challenging situation, keeping the fungus at bay for a given period and thus ensuring the survival of the colony.

Furthermore, several studies in ants, bees, and other insects show that the level of the active enzyme phenoloxidase (PO), is a good proxy for immune activity (Doums et al., 2002; Cerenius and Söderhäll, 2004; Bocher et al., 2007), and correlates positively with increased pathogen resistance (Adamo, 2004; Mucklow et al., 2004; but see Leclerc et al., 2006).Our study shows that long-term coexistence with a non-lethal fungal pathogen could be associated with increased immune activity of host ant workers regardless of their age. The general immunocompetence level of workers is age-dependent at the same time: old workers, which perform tasks outside the nest, maintain their total PO (PPO) at a higher level, which could be an adaptive response to increased risk exposure due to assumed tasks. High PO level could confer greater protection for the colony, which could even serve well to a parasite that managed to unlock the social fortress.

Based on literature data and our previous study results we predicted that infection with a non-lethal parasite may lead to the upregulation of two key immune genes, *prophenoloxidase* and *defensin 1* and that infection effects on immune gene expression may vary between workers, queens and larvae due to their different social roles. Workers displayed diverse profiles, with variable responses to infection. Both *prophenoloxidase* and *defensin 1* level increased with pathogen loads in *M. scabrinodis* same-age workers. Queens exhibited a more pronounced immune response, concerning both genes. Highly infected queens had a heightened immune response. Larvae did not show a discernible response. Morphological and physiological characteristics had limited effects, except in the case of queens, where larger individuals displayed higher *defensin 1* expression. Our study shows that these divergent responses likely stem from the differing physiological needs and priorities of various groups within the colony.

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