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2022-01

Rissanen , J , Helanterä , H , Will , T & Freitak , D 2022 , ' Lack of self-medication by fungus infected Lasius platythorax (Formicidae, Formicinae) ants in a multitrophic experiment ' , Annales Zoologici Fennici , vol. 59 , no. 1 , pp. 205-213 . https://doi.org/10.5735/086.059.0117

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https://doi.org/10.5735/086.059.0117

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Source: Annales Zoologici Fennici, 59(1) : 205-213

Published By: Finnish Zoological and Botanical Publishing Board

URL: https://doi.org/10.5735/086.059.0117

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Ann. Zool. Fennici 59: 205–213 ISSN 1797-2450 (online)
Helsinki 23 August 2022 © Finnish Zoological and Botanical Publishing Board

Lack of self-medication by fungus infected *Lasius platythorax* (Formicidae, Formicinae) ants in a multitrophic experiment

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Received 29 Mar. 2022, final version received 27 July 2022, accepted 26 July 2022

Rissanen, J., Helanterä, H., Will, T. & Freitak, D. 2022: Lack of self-medication by fungus infected *Lasius platythorax* (Formicidae, Formicinae) ants in a multitrophic experiment. — *Ann. Zool. Fennici* 59: 205–213.

In laboratory conditions, ants can combat a pathogen infection by means of the medicinal use of reactive oxygen species (ROS). However, it is still unknown where they obtain medicinal compounds in the wild and how they use them. Due to an upregulation of ROS in response to herbivory, aphid-infested plants have been suggested to be a potential source of ROS for ants in the wild. We investigated whether infection would cause *Lasius platythorax* ants to change their foraging on extrafloral nectar on aphid-infested plants. We found no clear evidence for the ants significantly changing their foraging behaviour in response to the pathogen, nor for the extrafloral nectar to contain ROS. The aphids in our experiment had a relatively high concentration of ROS and future research should determine whether predation on aphids could be a potential source of both protein and ROS needed to combat a disease.

Introduction

Ants, like other social insects, live in dense colonies with a low genetic diversity among frequently interacting nestmates, making them susceptible to contracting and transmitting disease within their nests (Schmid-Hempel 1998). Although there is evidence that pathogens are abundantly present both within and outside their nests (Hughes *et al.* 2004, Valles *et al.* 2010, Reber & Chapuisat 2012), colony collapse caused by an infectious disease seems to be quite rare in nature (Evans 1974). Apart from having physiological and behavioural strategies to help combat pathogens on an individual level, the interplay of individual defence strategies of ants can mitigate the threat and effects of pathogens in their society as a form of social immunity (Cremer *et al.* 2007, 2018). However, social immunity does not make ant societies impregnable to disease. It is suggested, that social immunity is better equipped to limit the

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effects of generalist pathogens than specialist pathogens (Małagocka *et al.* 2019). Specialist pathogenic fungi such as *Pandora* and *Ophiocordyceps* manipulate ant behaviour to benefit their survival and transmission in ant colonies (de Bekker *et al.* 2014, Małagocka *et al.* 2017, Araújo *et al.* 2018), but their occurrence in the natural environment of ants is considered to be rare as compared with that of generalist pathogens, which are common in the surroundings of ant nests (Reber & Chapuisat 2012), and thus predicted to have far greater effect on the colony fitness.

With the emergence of the social-immunity theory, interest in pathogen-caused changes in host behaviour as beneficial for the host instead of for the survival and transmission of the pathogen has increased. Although the use of generalist entomopathogenic fungi in laboratory conditions has been questioned in terms of how relevant they are compared with natural conditions (Loreto & Hughes 2016), there is a substantial amount of reported evidence that these fungi present a constant, even if not critical, threat to ants in nature (Hughes *et al.* 2004, Valles *et al.* 2010, Reber & Chapuisat 2012). Due to the constant nature of the pathogen threat, it is likely that behaviour patterns of ants have adapted to deal with it through social immunity and individual behaviours. Behavioural changes in response to a pathogen infection which benefit ants has largely been studied with generalist entomopathogenic fungi such as *Metarhizium* and *Beauveria* (Cremer *et al.* 2018). In response to these pathogens, ants have been shown to regulate their rate of grooming (Tranter & Hughes 2015) and allogrooming (Konrad *et al.* 2018), cut social ties and leave the nest if infected (Heinze & Walter 2010, Bos *et al.* 2012), and alter their food intake to incorporate biologically active compounds as a form of self-medication (Bos *et al.* 2015, Rissanen *et al.* 2022).

Nutrition has recently been highlighted as of special interest in the interaction between ants and pathogens (Csata & Dussutour 2019). Immune responses to pathogen infections are costly in terms of energy and nutrients, and any deficiencies caused by disease can be offset through alteration in diet preferences as a form of compensatory diet choice (Ponton *et al.* 2011). Apart from merely replenishing diminished nutritional supplies, ants can use diet choices to directly combat pathogen infection through self-medication (Bos *et al.* 2015, Rissanen *et al.* 2022), i.e., the use of biologically active compounds in a way which would be harmful to uninfected individuals. Self-medication can be therapeutic or prophylactic depending on when the compounds are used in relation to encountering a pathogen (de Roode *et al.* 2013, Abbott 2014). Ants suffering from an infection by *Beauveria bassiana* successfully medicated themselves by incorporating food containing H_2O_2, a reactive oxygen species (ROS) under laboratory conditions (Bos *et al.* 2015, Rissanen *et al.* 2022), but the extent to which ants self-medicate and how they do it in nature in regard to obtaining and using biologically active compounds is still unclear.

Identifying self-medication behaviour in ants under natural condition can be challenging (Sapolsky 1994). If the pathogen threat to an ant nest is high and if the ants are capable of self-medicating, it is likely that self-medication will be incorporated in to their natural behaviour (de Roode & Lefèvre 2012). Therefore, changes in ant behaviour caused by an active pathogen infection may be hard to detect. Compounds that insects have been shown to use for self-medication were studied in their pure form (Bos *et al.* 2015, Poyet *et al*. 2017). Their effects, however, may be quite different in nature where they are inside e.g. specific plant organs. One of the criteria for self-medication suggested by de Roode *et al.* (2013) was that the self-medication behaviour must be relevant to the natural environment of the infected animals. Therefore, purely focusing on artificial foods in laboratory conditions is not sufficient to prove natural relevance, but instead there should be more focus on identifying natural pathways of medicinal compounds which ants could use. Changes in foraging choices caused by an infection in ants could also have ecological consequences as ants have a disproportionally high effect on the ecosystems they occupy, thus being often referred to as ecosystem engineers (Folgarait 1998).

Ants regularly enter symbiotic relationships with both plants (Rico-Gray & Oliveira 2008) and aphids (Offenberg 2001). Aphids offer ants

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honeydew, an excretion of excess sugars and liquids from the plant phloem on which they feed, in exchange for protection from predators (Offenberg 2001). Regarding plants, some have extrafloral nectaries (EFNs), usually on the stem or on the leaves, through which they reward ants with nectar for protection from herbivores and to keep them away from the flowers where they could disturb pollination (Rico-Gray & Oliveira 2008). It is possible that ROS, produced systematically in response to e.g. aphid infestation — weak in the case of a compatible reaction and strong when an incompatible reaction between plant and a non-specialist species of aphids occurs (Sun *et al.* 2020) — is also present in the nectar of infested plants, providing ants with a natural source of ROS. Furthermore, EFN nectar contains a variety of other compounds, including antifungal compounds (Escalante-Pérez *et al.* 2012, Nepi 2014), which ants could also potentially utilize. Aphids may also act as a protein source if additional proteins are required as this can be a form of self-medication as well (Lee *et al.* 2006).

In this study, we took the first steps into studying the availability and use of medicinal compounds by infected ants in more natural conditions by using a multi-trophic approach, reflecting the natural environment of ants to investigate how an infection by the generalist entomopathogenic fungus *Beauveria bassiana* affects the foraging behaviour of *Lasius platythorax* ants feeding on EFN-bearing broad bean plants (*Vicia faba*) infested with *Megoura viciae* aphids. *Lasius platythorax* is a common palearctic species of ants which generally builds its nest in soil or through excavating rotting wood (Seifert 1991). *Megoura viciae* is a generalist pest on legume plants and does not engage in mutualistic behaviours with ants (Novgorodova 2002) and is considered unpalatable or even potentially toxic to some insect predators (Tsaganou *et al.* 2004). *Megoura viciae* was chosen due to its non-myrmecophilic nature and its overlapping distribution and habitats shared with *L. platythorax*, as aphid tending would have presented the ants with a secondary source of carbohydrates that would have competed with the EFNs. As *M. viciae* is a generalist on legumes, it could potentially cause a more intense immunological reaction in broad beans than a more specialized aphid (Sun *et al.* 2020). If the ants started to prey upon the aphids when infected, despite their unpalatableness and potential toxicity, then this would be a clear qualitative change in diet behaviour caused by infection meeting the criteria of self-medication.

The aim of this study was to investigate whether ants suffering from a pathogen infection change their foraging choices to medicate themselves against the disease. The ants in our experiment had a quantitative feeding choice to forage on the EFNs and a qualitative choice to consume aphids. As apterous pea aphids (*Acyrthosiphon pisum*) were found to contain a relevant concentration of hydrogen peroxide (Łukasik *et al.* 2012), we analysed aphids for the presence of ROS to investigate whether aphids served as a natural source of ROS that ants could use to self-medicate. We analysed ants for the presence of ROS to see whether a change in foraging activity would correlate with changes in ROS concentrations in ants, possibly indicating a presence of ROS in the EFN nectar.

Material and methods

Experimental design

For the experiment, we first assessed the effect of *B. bassiana* on the survival of *L. platythorax* ants through an infection test to gain information on the lethality of the pathogen. We then set up 40 experimental colonies of *L. platythorax*, 20 infected and 20 control colonies, each in a separate nest-box with one aphid-infested plant in every box as a food source.

Infection test

Beauveria bassiana was cultivated in petri dishes on potato glucose agar (Sigma-Aldrich) in the dark at room temperature. Spores were collected from plates with visibly sporulating fungus by pipetting 10 ml of 1 × phosphate-buffered saline (PBS) directly on the plate and carefully rubbing it with a sterile glass rod. The spores were then centrifuged in 3000 rpm for 3 min and then sus-

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pended in 10 ml of Milli-Q H_2O. The spore concentration was evaluated with a haemacytometer.

To assess the ability of *B. bassiana* to infect and kill the ants and when it takes place, we collected four wild nests of *L. platythorax* from tree stumps in a wood clearing in Lappohja, Hanko, Finland (59°54′46.9′′N, 23°15′53.8′′E). Each nest was split into four separate sub-colonies of 20 ants each, two of which were infected and the other two served as controls. There were thus 16 colonies of which 8 were infected and 8 were used as control. The colonies used in the infection experiment were infected by submerging the ants in a solution containing 1×10^7 spores ml^{-1} of *B. bassiana* for 5 seconds. The control colonies were submerged in Milli-Q H_2O for 5 seconds. Dead ants were counted and removed from the jars daily at 10:00 for a total of seven days. The colonies were all fed *ad libitum* with the Bhatkar & Whitcomb diet (Bhatkar & Whitcomb 1970) and water. The nests used in the infection test were not used in the other experiment.

Experimental setup

We collected 20 wild nests of *L. platythorax* from tree stumps in a wood clearing in Lappohja, Hanko, Finland (same location as above). Each nest was split into two sub-colonies of 500 workers one to be infected and one to serve as control. There were thus 40 colonies of which 20 were infected and 20 were used as control. The colonies used in the infection experiment were submerged in a solution containing 10^8 spores ml^{-1} of *B. bassiana* for 5 seconds. The control colonies were submerged in Milli-Q H_2O for 5 seconds. Each colony was then placed in an individual plastic box (35 × 20 × 20 cm, length × width × height) lined with a mixture of talcum powder and ethanol to keep the ants from escaping (Ning *et al.* 2019). Each box contained a 2-cm layer of garden peat and a ceramic tile (10 × 10 cm) for nest material.

Broad-bean plants had been grown indoors in small plastic pots (6 × 6 × 6 cm, length × width × height) in gardening soil. Of those, 40 in the best condition with developed two pairs of EFNs were chosen for the experiment. Between 24 and 30 *M. viciae* aphids were transferred from stock plants to the plants chosen for the experiment the day before the pots containing the plants and aphids were put into the nest boxes with the ants. Variable numbers of aphids were used to take the size differences and developmental stages of individual aphids into account. The aphids were allowed to reproduce freely during the experiment.

Foraging observation

The number of ants visiting each EFN in each colony was recorded six times per day at 09:00, 11:00, 13:00, 15:00, 17:00 and 19:00, for a total of six days. Each observation lasted for 2 seconds to give us a snapshot of the foraging activity. The plants had two nectaries, parallel to each other, and typically between 0 and 2 ants were present on the nectaries during each observation, thus 2 seconds was enough to count the ants foraging on nectar. An ant was considered to be a forager if it was within a body length of an EFN and facing it. We also observed the ants for any signs, e.g. biting and carrying aphids into the nest, indicating that they would use the aphids as food.

ROS analysis

Ants were sampled from each colony on day 0 (beginning of the experiment), day 3 (middle point), and in the morning on day 7 (end of the experiment). Each sample consisted of three replicates of randomly chosen ants (10 ants per replicate). A sample of three replicates of aphids of mixed age were collected from each colony on day 7 (15 aphids per replicate). The ant and aphid samples were placed in eppendorf tubes with 250 µl of 1 × PBS 3-amino-1,2,4-triazole solution (2 mg ml^{-1}) to prevent ROS from reacting, and stored in –80 °C until further processing. The samples were homogenized with the TissueLyser II (Qiagen) for 5 min at 1800 rpm. The samples were then centrifuged at 150 000 rpm for 10 minutes in 4 °C and the supernatant was collected for further analysis.

The protein concentration in the samples was analysed with the Bicinchonic Acid Protein Assay Kit (Sigma-Aldrich) according to

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the manufacturer's protocol with a following minor modification: instead of using 25 µl of the sample and 200 µl of working reagent suggested by the manufacturer, we used 12.5 µl of the sample in 100 µl of working reagent thus preserving the suggested dilution ratio. The change was made to make sure that we had enough sample for further analyses. Due to the high concentration of protein in the original samples, following the manufacturer's protocol, for the analysis we diluted 2.5 µl of the ant sample in 10 µl of 1 × PBS and 5 µl of the aphid sample in 7.5 µl of 1 × PBS. Each assay was made in duplicate and their average was used in calculations. The absorbance of each sample was measured at 562 nm wavelength in a microplate reader (SpectraMax iD3, Molecular Device).

The ROS content was analysed with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) which detects hydrogen peroxide (H_2O_2) in samples. The analysis was done according to the manufacturer's protocol with a following minor modification: we used 25 µl of the sample in 25 µl of reagent instead of 50 µl of the sample in 50 µl of reagent suggested by the manufacturer. Due to the high concentration of H_2O_2 in the original samples, according to the manufacturer's protocol, for the analysis we diluted 12.5 µl of the ant sample in 12.5 µl of reagent buffer provided in the kit, and 5 µl of the aphid sample in 20 µl of reagent buffer provided in the kit. Each assay was made in duplicate and their average was used in calculations. The fluorescence of the samples was measured at 573–608 nm after excitation at 530–560 nm in a microplate reader (SpectraMax iD3, Molecular Device). The ratio of ROS to protein content was calculated to avoid differences caused by variation in ant and aphid sizes.

Statistical analysis

The survival data of the infection test were analysed with a Cox proportional-hazard model implemented in the *coxme* function of the *coxme* package (https://CRAN.R-project.org/package=coxme). The day on which each ant died was used as a response variable with treatment (infection/control) used as a fixed factor, and colony as a random effect to account for pseudoreplication.

The foraging data were analysed with a Poisson generalized linear mixed model implemented in the *glmmTMB* function of the *glmmTMB* package (Brooks *et al.* 2017). The number of foragers (number of ants at nectaries) was used as a response variable and the treatment (infection/control) as an explanatory variable. The original nest of the colonies was used as a random factor to account for any differences between nests, and time was nested within colony as a random factor to account for pseudoreplication. Model diagnostics was carried out using the *DHARMa* package for diagnostics of *glmmTMB* models (Hartig 2018).

The ROS data were analysed using the *lmer* function in the *lme4* package (Bates *et al.* 2015). ROS/protein ratio was used as the response variable in the model with treatment (infection/control) as a fixed factor. The original nest was used as a random factor to limit any possible effects it might have on the result. The *lmer* function uses residual degrees of freedom, which we report in the results.

Pairwise comparisons of ROS concentrations between infected and control ants as well as between aphids and ants were done using the *emmeans* function in the *emmeans* package (https://CRAN.R-project.org/package=emmeans) with a Tukey's *p*-value adjustment when performing multiple comparisons.

Plants in two of the control colonies died during the experiment and therefore those colonies were not included in the analyses.

All statistical analyses were performed using the R software (ver. 4.1.2).

Results

Infection

Of all the ants in the control, 92.9% were still alive after seven days of the experiment, whereas only 72.1% of the infected ants survived to that point in time. The effect of infection on survival was significant ($\chi^2 = 16.809$, df = 1, $p < 0.001$) (Fig. 1). Most of the mortalities caused by the infection occurred during and after day 4.

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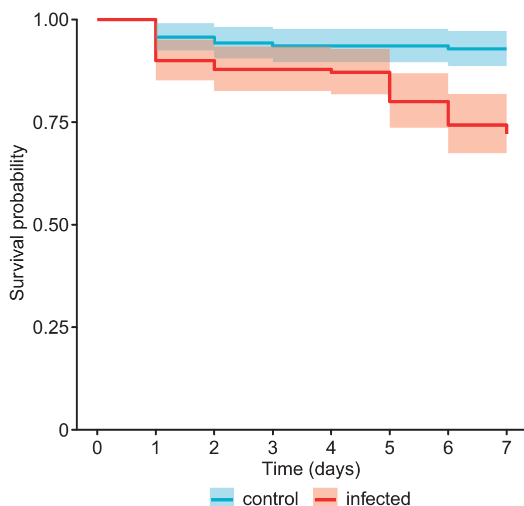

Fig. 1. *Lasius platythorax* survival during the infection test. The shaded areas along the lines indicate the 95% confidence intervals.

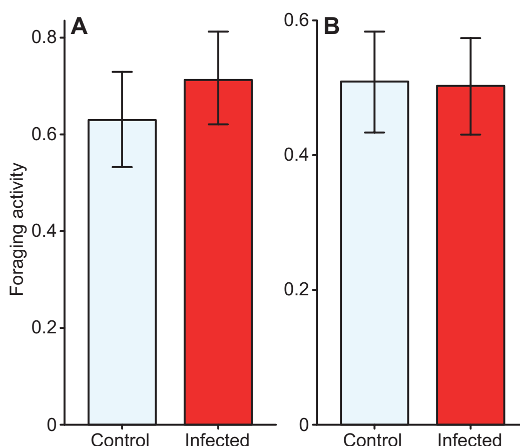

Fig. 2. Foraging activity (number of foragers found at the extrafloral nectaries, EFNs) during (**A**) the first four days and (**B**) the entire six-day *observation* period. The error bars represent the 95% confidence intervals.

Foraging

Beauveria bassiana caused substantial mortality in *L. platythorax* workers starting from the 4th day after the initial exposure to the pathogen, and by day 7, only 72.1% of the infected ants survived as compared with 92.9% of the ants receiving the control treatment ($\chi^2 = 21.15$, df = 1, $p < 0.001$). We thus decided to check whether foraging during the first four days after infection and during the entire six-day observation period differed.

Infection did not affect the foraging (number of ants at nectaries) in a significant way during the first four days ($\chi^2 = 1.817$, df = 1, $p = 0.178$; Fig. 2A), nor during the entire six-day observation period ($\chi^2 = 1.061$, df = 1, $p = 0.303$; Fig. 2B).

ROS

The interaction of sampling day and infection did not affect the ROS content in the ants (sampling day × infection: $\chi^2 = 3.786$, df = 2, $p = 0.151$), neither did the fixed effect of infection ($\chi^2 = 0.927$, df = 2, $p = 0.336$) nor the sampling day ($\chi^2 = 5.190$, df = 1, $p = 0.075$). The ROS concentration in the infected ants was higher on day 7 than on day 0 ($t = -2.389$, $df_{residual} = 323$, $p = 0.046$), but it did not differ between day 0 and day 3 ($t = -1.283$, $df_{residual} = 323$, $p = 0.406$) or between day 3 and 7 ($t = -1.106$, $df_{residual} = 323$, $p = 0.511$). There were no differences in ROS concentration between the samples of the control colonies.

The ROS concentrations in the infected ants on days 0 and 7 were higher that in the control ants ($t = -2.389$, $df_{residual} = 323$ $p = 0.046$, and $t = -2.124$, $df_{residual} = 324$, $p = 0.035$, respectively).

On day 7, the ROS concentration in aphids was higher as compared with that in the ants ($t = -25.242$, $df_{residual} = 205$, $p < 0.001$) (Fig. 3).

Discussion

We found no clear evidence that an infection of *L. platythorax* by *B. bassiana* elicits a change in the frequency with which *L. platythorax* ants visit the EFNs of an aphid-infested plant. The ROS content in the infected ants increased with time, whereas in the control ants it remained unchanged. The increase ROS content in the infected ants did not mirror the foraging activity of the ants, which declined over time. The slight increase in ROS in the infected ants was most likely due to self-generation of ROS by the sick ants (Söderhäll & Cerenius 1998, Mikonranta *et al.* 2014) instead of ROS being present in the EFN nectar, which we were unable to measure

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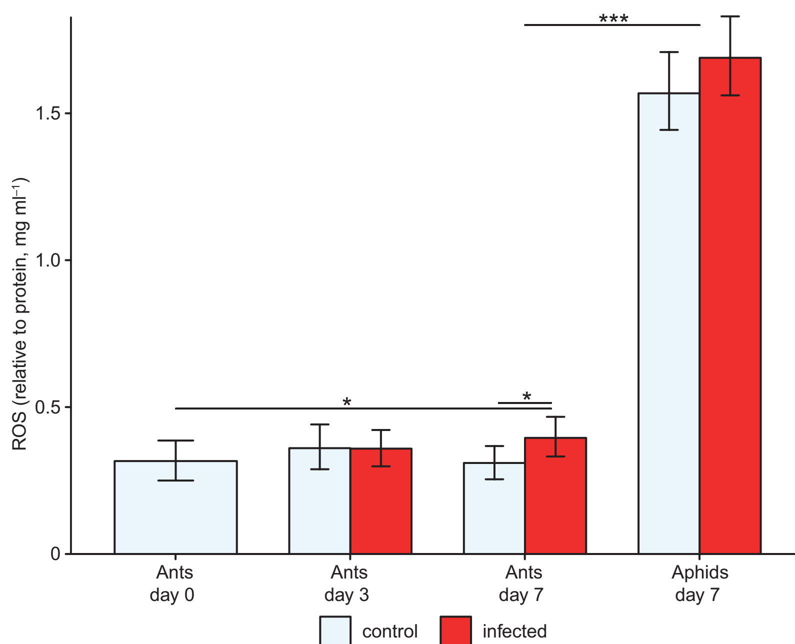

Fig. 3. Reactive oxygen species (ROS) content in each sample. The error bars indicate the 95% confidence intervals, the horizontal lines the groups compared, and the asterisks significant differences (* = $p < 0.05$, *** = $p < 0.001$).

in our experiment. The lack of ROS in the EFN nectar may be due to complex transport mechanisms in the plant because there is no close connection of glandular trichomes between the EFN and sieve tubes (Davis *et al.* 1988) potentially preventing ROS from entering the nectar even if the ROS response in the plant is systemic. Keeping the EFN nectar free of ROS during an aphid infestation could be beneficial for the plants in an evolutionary context, as the main purpose for a plant to invest in production of EFN nectar is to reward insect mutualists such as ants for protection against insect pests like aphids (Rico-Gray & Oliveira 2008). Had aphids triggered a ROS response in the EFN nectar, it would have reduced its quality for uninfected mutualists, thus possibly reducing the protection behaviour provided in exchange. The EFN nectar can also contain a variety of other biologically active compounds (Nepi 2014) as well as antifungal agents (Escalante-Pérez *et al.* 2012) which the ants could find attractive when infected.

Although we found no difference in foraging in response to the pathogen, when faced with a constant threat of a low-lethal disease, which *B. bassiana* is for ants in the wild (Reber & Chapuisat 2012), evolution will favour prophylactic medication becoming a fixed behaviour in ants (de Roode & Lefèvre 2012). It is possible, that other compounds present in the EFN nectar provide ants with medicinal benefits strong enough to combat low-level disease without a clear change in foraging behaviour. However, to prove that foraging on aphid-stressed plants is a form of fixed prophylaxis and not compensatory feeding, the costs and benefits of consuming nectar from aphid-stressed plants should be confirmed.

It is also important to consider the fact that we counted foraging ants six times per day, and feeding bouts of similar ants has been shown to last only up to 2 minutes (Portha *et al.* 2004), so it is possible, that even a small difference in the number of foragers when observed six times a day may accumulate to a bigger difference in nectar collection when considering the amount of nectar collected during the entire day. Apart from the number of foragers visiting the nectaries, it is also possible that there is a change in the amount of nectar collected per visit. Therefore, a continuous observation of foraging activity as well as measurement of the amount of nectar being foraged by each ant are necessary to detect any change in EFN nectar foraging. The ants in our experiment had access to only one plant, whereas in nature a colony of ants forages on several plants.

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In our study, the ROS content in the aphids was much higher than in the ants, presenting the ants with a potential source of both ROS and protein, both of which can be used for self-medication (Lee *et al.* 2006, Bos *et al.* 2015). However, we found no evidence for ants preying on aphids. *Megoura viciae* is not an ant-associated species and there is no evidence that ants would use them as food, which could be due to their potentially toxic effects on some insect predators (Tsaganou *et al.* 2004). Our assumption was that ants would prey on aphids due to their protein and ROS contents and regardless of potential toxic effects, but as stated above, this was not observed either because it did not take place or because we did not monitor the ants at all times, which makes it possible that they were in fact supplementing their diet with aphids, but we were not able to observe it. Its is also possible that the ants do not consume aphids if they find all the compounds they needed in the nectar.

Our results show, that an infection by a generalist entomopathogenic fungus may not induce a clear change in foraging behaviour in ants on a plant infested with *M. viciae* aphids. The extent of self-medication behaviour in ants and especially how they do it in the wild remain to be studied. Perhaps using palatable aphids could provide the ants with a more usable source of ROS, if they contain it in the same quantity as *M. viciae* in our experiment. It is also possible, that the ants do not acquire medicine through the ant–plant–aphid interaction, thus experiments using alternative sources of biologically active compounds available for ants, such as vertebrate carcasses (Paczkowski & Schütz 2011) and different species of plants, should be pursued.

Acknowledgements

We would like to thank Nick Bos for insights regarding the statistical analysis of the data and Onur Göktürk for help during the fieldwork.

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