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Emergency measures: Adaptive response to pathogen intrusion in the ant nest

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ABSTRACT

Ants have developed prophylactic and hygienic behaviours in order to limit risks of pathogenic outbreaks inside their nest, which are often called social immunity. Here, we test whether ants can adapt the "social immune response" to the level of pathogenic risk in the colony. We challenged *Myrmica rubra* colonies with dead nestmates that had either died from being frozen or from infection by the fungus *Metarhizium anisopliae*. Ant survival was compromised by the presence of the fungus-bearing corpses: workers died faster with a significantly lower survival from the 4th day compared to workers challenged with freeze-killed corpses. When faced with fungus-bearing corpses, workers responded quickly by increasing hygienic behaviours: they spent more time cleaning the nest, moving the corpses, and self-grooming. Ants in fungus-threatened colonies also decreased contact rates with other workers, and moved corpses further in the corners of the nest than in colonies in contact with non-infected corpses. These results show that ant colonies are able to assess the risk level associated with the presence of corpses in the nest, and adjust their investment in terms of hygienic behaviour.

1. Introduction

Pathogens represent a particular challenge to insect societies due to the increased risk of transmission between nestmates (Schmid-Hempel, 1998). Indeed, social insects live in nests with confined and humid conditions that are favourable to the development of most pathogens. Furthermore, nestmates are often genetically highly related, live at high densities and frequently interact with each other to perform cooperative tasks or to share food. All of these factors facilitate the propagation and outbreak of diseases.

In response to these increased sanitary risks, social insects have developed a wide range of behavioural defences that limit the intake and transmission of pathogens in the colony. This set of behaviours that protects individual and colony health is known as "social immunity" (Cotter and Kilner, 2010; Cremer et al., 2007). Several analogies appear when comparing social immunity and the internal immune system of the individual (Cotter and Kilner, 2010; Cremer and Sixt, 2009). Indeed, the colony, seen as a "superorganism", is capable of detecting and reacting to the presence of parasites, just as individuals do. The set of behaviours performed to protect the colony against pathogens can either be prophylactic and happen even in the absence of disease, or can be triggered as a response to the intrusion of pathogens.

Prophylactic behaviours such as avoiding contacts with possible sources of infection act as a barrier limiting the intake and transmission of parasites in the colony. Ants avoid settling their nest in a contaminated soil (Drees et al., 1992; Franks et al., 2005; Oi and Pereira, 1993). All social insects are known to isolate corpses from the colony either by burying them, as in termites and a few ant species (Ballari et al., 2007; Ulyshen and Shelton, 2012), or by transporting them outside the nest as for most ant species and bees (Ataya and Lenoir, 1984; Diez et al., 2012; Wilson et al., 1958). In ants, the maintenance of nest hygiene by removing dead nestmates has shown its prophylactic value with a beneficial impact on ant survival (Diez et al., 2014). If pathogens nevertheless manage to enter the nest, termites and ants can respond by modifying their behaviour (Jaccoud et al., 1999; Oi and Pereira, 1993; Rosengaus et al., 1999, 1998a; Tranter et al., 2014). The termite *Zootermopsis angusticollis* displays an alarm signal (Rosengaus et al., 1999), and many social species increase self- and allogrooming in the presence of pathogens on the cuticle (Konrad et al., 2012; Reber et al.,

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2011; Rosengaus et al., 1998b). The ability to detect and reject larvae infected with the fungus *Ascosphaera apis* is well correlated to colony resistance in honeybee hives (Gilliam et al., 1988). Concerning allogrooming, its impact on colony fitness is less clear: it has a positive effect on survival in the termite *Z. angusticollis* (Rosengaus et al., 1998b), but none on the ant *Formica selysi* (Reber et al., 2011). Moreover, the hygienic efficiency of allogrooming varies according to the level and stage of infection. Indeed, removing the fugal spores is not relevant once the fungus have had time to penetrate the cuticle (Hajek and Leger, 1994; Reber et al., 2011). Allogrooming may even have a vaccination effect on healthy nestmates who had removed spores from weakly infected ants (Konrad et al., 2012; Traniello et al., 2002 Ugelvig and Cremer, 2007) but this «immune priming» phenomenon currently remains controversial (Reber and Chapuisat, 2012).

In this study, we ask whether ant colonies are capable of assessing the risk associated with the presence of dead nestmates within the colony, and respond adaptively to this risk level. Therefore we introduced in *Myrmica rubra* nests two types of corpses – uninfected or infected with *Metarhizium anisopliae* – and compared the survival of ants and their behavioural responses to these different sanitary risks.

2. Material and methods

M. rubra colonies were excavated from earth banks in a semi-open area on the campus of Gembloux Agro-Bio Tech, Belgium. In the laboratory, a total of 18 colonies were kept in plaster nests (Janet type, 85 × 85 × 2 mm) connected to a foraging arena (13,5 × 18,5 × 5 cm) with borders coated with polytetrafluoroethylene (Whitford, UK) to prevent ants from escaping. The nest entrance consisted of a 15 mm hole perforated in the middle of the glass roof. Nests contained no queens, 170–230 workers, and 58–60 larvae. Queens were not included in the nest to avoid new ants to be born during the experiment. Laboratory conditions were kept at 25 ± 1 °C and 45 ± 5 % HR, with a constant photoperiod of 12 h per day. Nest humidity was maintained by adding 75 mL of water 3 times a week in the two ditches surrounding the nest. Each colony was provided ad libitum with water and an artificial diet with a 2:1 sugar/protein ratio (Dussutour and Simpson, 2008).

2.1. Comparison of the effect of infected and uninfected corpses on ant survival

We investigated to what extent the colony survival is altered when corpses infected by spores of *M. anisopliae* fungus were introduced.

Therefore, we used colonies for which corpse removal was hampered and we compared the survival of "uninfected" colonies in contact with 10 frozen-killed corpses ($N = 8$) to that of "infected" colonies in contact with 10 infected sporulating corpses ($N = 8$). For each condition, one colony (among the nine colonies that were originally tested) was excluded from data analysis due to the fortuitous escape of ants before the end of survival experiments. To hamper the ability of ants to get rid of corpses, we covered the entrance with a 20 × 20 × 20 mm Plexiglas cube, which was perforated with 12 holes of 2 mm diameter each. The small section of holes permitted only one ant to pass at a time and made it hard to remove corpses from the nest. Preliminary experiments showed that the setup with transport-limiting holes at the nest entrance had no direct influence on ant's survival (Diez et al., 2014).

For the "uninfected" condition, 10 corpses were put in the nest through its entrance at the beginning of the experiment. Corpses were nestmates killed by freezing for 35 min at −24 °C and then left at room temperature for 3 h before introduction into the nest. Preliminary experiments showed that freshly killed corpses were discriminated from live workers by nestmates after 2.3 ± 0.47 h ($N = 15$). Following the introduction of corpses inside the nest, we counted the number of live and dead ants and larvae twice a week during 7 weeks.

For the "infected" condition, corpses were nestmates infected by *Metarhizium anisopliae* strain F52 (Novozymes Biochemicals) that showed sporulation with conidiophores having grown out of the ant corpse. To get infectious corpses, we gathered 10 healthy individuals in a closed 1 ml Eppendorf tube together with one sporulating corpse. This tube was vortexed at a speed of 2500 rpm, 4 times during 10 s each in order to untie spores from the corpse, and blend it with live ants. After having undergone this contamination procedure, we separated the ants in individual Petri-dishes (55 × 14.2 mm) for 2 h in order to prevent allogrooming and to limit the removal of spores from the cuticle. After these two hours, we gathered the ants together in a Petri dish and provided them with food and water until death of the individuals, which took 3–7 days. In order to favour fungus sporulation after death of the infected ant, the corpse's cuticle was cleaned of other microorganisms. First, we put the corpses into ethanol and then in a bath of distilled water for 10 s each. Then we placed it during one minute in 0.01% sodium hypochlorite, which prevents opportunistic microorganisms to develop (Vega and Blackwell, 2005). Then the corpses were successively rinsed in 3 different baths of distilled water and dried on a filter paper for a few minutes. Finally we transferred the corpses in hermetically closed Petri dishes, on humidified filter paper and kept them in a 23 °C incubator during 14 days. The sporulation of the *M. anisopliae* fungus could be easily identified with the naked eye because of its cuboid shape and greenish colour. After sporulation of the fungus, corpses were transferred to the experimental colonies. In order to estimate the quantity of spores introduced to the colonies, we counted the number of spores that were present on one corpse at day 14 post-mortem. This estimate was done with a microscope by using a Thoma's cell grid and led to an average quantity of $2.7 \times 10^6 \pm 1.6 \times 10^6$ spores ($N = 20$) covering one *M. rubra* corpse.

We counted the number of live and dead ants as well as the number of dead larvae each day during the first two weeks, then twice a week until week 7. We also checked whether ants dying during the experiment were actually contaminated by the fungus *M. anisopliae*. Therefore, we removed all corpses that were found outside the nest. When the found corpse was whole, we put it in favourable conditions for fungus sporulation by using the same protocol as explained above. Because of fungus growth in the body, infected corpses tended to fall apart more easily, therefore the proportion of corpses tested for contamination was lower in infected colonies.

2.2. Impact of fungal pathogen on hygienic behaviours

We investigated how the presence of *M. anisopliae* spores influences ant's behaviour towards corpses. All behavioural experiments were repeated on the same eight colonies used for the survival experiment, plus one extra colony ($N = 9$ for each treatment). After the ten corpses, either infected or uninfected, were introduced into the nest, we temporarily closed the entrance to prevent corpse removal during the 6 h observation period. We quantified whether the interest of workers towards infected and uninfected corpses changed over time. Therefore the number of contacts of workers with each of the 10 corpses was monitored for 1 min, every hour for 6 h following their introduction to the nest.

We also investigated the effects of the contaminating status of a corpse on the behavioural profile of ants having contacted it, when they are more likely to transmit spores onto nestmates. Therefore, for each experiment, we observed and monitored during 5 min the behaviour of five workers after they had contacted a corpse. An