Effect of Recent Pathogen Experience on Induced Sanitary Brood Care in ant *L. neglectus*

Project report (Summer 2022)

A report on the research project carried out under the guidance of Linda Sartoris and Prof Sylvia Cremer at the Institute of Science and Technology Austria (ISTA)

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Introduction

Lasius Neglectus or the invasive garden ant is a unicolonial ant characterised by the presence of multiple egg-laying queens in its vast super-colony. The number of queens in a 14 ha super-colony in Seva, Spain, was estimated to be around 35,500 by counting the number of queens under stones. By analysing soil cores, the number of workers in this colony (excluding those foraging on trees) was estimated at around 112 million (Espadaler et al., 2014).

Similar to the invasive garden ants, other social insects – ants, bees, wasps and termites – also live in colonies with high densities and have frequent social contact with close relatives who have high genetic relatedness. This makes social insects susceptible to the spread of parasitic infections. To counter this, they have evolved a suite of colony-level anti-parasite defences to avoid infection and prevent its spread in the colony (Cremer et al., 2007).

This colony-level anti-parasitic defence, called social immunity, is achieved by the cooperation of all individuals in the colony across social insects. It is characterised by hygiene behaviour (e.g., allogrooming), physiological defence (e.g., encapsulating the parasite in a layer of propolis) and spatial organisation (e.g., centrifugal polyethism and contact frequency regulation). This defence can be prophylactic – such as building the nest with anti-microbial tree resin (Chapuisat et al., 2007); or it can be activated – for instance, when fungus-contaminated ants are socially distanced (Stroeymeyt et al., 2018).

Social immunity in insect colonies has the same underlying organisational principles as that of individual-level immune system. Three layers of defence can be described in both – i) *border defence* to avoid the intake of parasites, ii) *soma defence* to prevent the establishment and multiplication of parasites, and iii) *germline defence* to prevent the infection of reproductive individuals and protect the daughter generation (Cremer and Sixt 2009). Protecting the queen and the brood ensures that the colony survives and prevents vertical transmission of infection. As a part of germline defence in social insect colonies, queens and the brood are found in the centre of the colony and tended by young individuals that are unlikely to have been outside the nest and infected by parasites. Also, the offspring are protected against infection by sanitary care such as increased grooming and use of anti-microbial poison-spraying (Tragust et al., 2013, Seibeneicher et al., 1992).

Previous studies have shown that pathogen-experience modulates sanitary care behaviour in ants. It has been shown that contaminated L. neglectus ants decreased the time spent in brood chamber and the naïve nestmates increased brood care (Ugelvig and Cremer 2007). In F. selysi ants, it was shown that the amount of allogrooming towards noncontaminated individuals was higher when the group had been previously exposed to the pathogen (Reber et al., 2011). It has also been shown that ants modulate sanitary care by performing less grooming and more antimicrobial disinfection when caring for nestmates contaminated with heterologous pathogens compared with homologous ones (Konrad et al., 2018). Moreover, clonal ants that were trained with contaminated larvae over four days groomed larvae for a longer duration, and were more effective at removing conidiospores from them as compared to naïve ants (Westhus et al., 2014).

Recent studies in the Cremer lab by Linda Sartoris had shown that recent pathogen experience modulates constitutive sanitary care. Fungus-experienced ants (ants that were exposed to a fungus-contaminated larvae for 30 minutes) reduced sanitary care towards the larvae, as compared to a naïve nestmate. qPCR analysis of these experienced ants showed that they had picked up a low-level contamination from their exposure (Sartoris unpublished).

In the context of these studies, we wanted to test the effect of recent pathogen experience on *induced* sanitary brood care by presenting experienced L. neglectus ants with fungus-treated or TritonX-treated (control) larvae. So, this set of experiments is also called the Induced Grooming experiment. I expected that fungus-experienced ants would increase grooming of fungus-contaminated larvae and decrease grooming towards triton-treated larvae. We also wanted to test whether reduced sanitary care towards the larvae by experienced ants in the previous experiment was because the naïve larvae were more susceptible to a low-level contamination as compared to naïve nestmates. To test this, we did a survival experiment with workers and larvae of *L. neglectus* being exposed to a low-level fungal solution.

Methods

General methods learned at the Cremer lab

The Cremer Group studies social immunity features in different ant species using a range of behavioural and molecular techniques. In the initial weeks of my internship, I learnt to handle ants and maintain ant colonies of *Lasius niger* and *Lasius neglectus* species.

Figure 1. Images of various experimental methods I learned – a) Sporulating cadaver of a *Linepithema humile* ant b) colour-marked L. neglectus individuals for the Induced grooming experiment c) Dead, surface-sterilized larvae were plated on wet filter paper to check for fungal outgrowth

An inexhaustive list of other methods I learned over the course of my internship are listed below and shown in Fig. 1.

a. Colour-marking ants for identification
b. Infecting ants and larvae with a fungal suspension
c. Behavioural annotation
d. Plating fungal spores on petri dishes
e. Extracting spores and constituting fungal suspensions of a particular concentration
f. Extracting spore packets from a cadaver
g. Checking the survival of larvae
h. Surface sterilisation and plating cadavers for sporulation
i. Quantitative PCR analysis

Induced grooming experiment methods

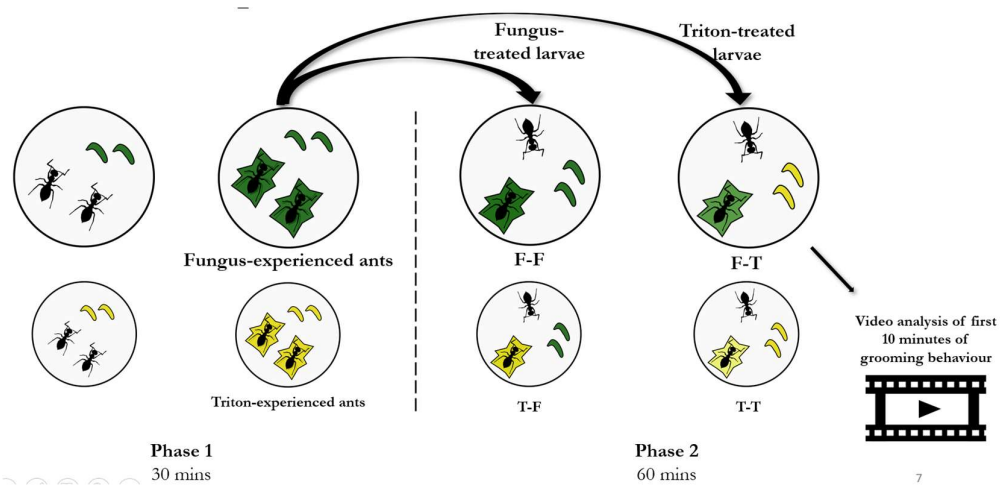

Figure 2. Schematic representation of the methods employed in Induced grooming experiment

The experiment was carried out in two phases – in Phase 1, two ants were exposed to fungus-treated larvae (*Metarhizium anisopliae* C17a strain, conc: 10^9 spores/ml) or TritonX-treated larvae. The larvae were exposed by pipetting 0.5 µL of the treatment liquid on them and allowing them to dry. Two ants and two larvae were kept in a container (because it was observed that when it was a single ant with a single larva, the ant didn't interact much with the larvae). The ants that were exposed to fungal or control-treated larvae for 30 minutes were considered as experienced ants.

In Phase 2, the experienced ants from Phase 1 were transferred to a container with a naïve nestmate and two exposed larvae, that were again either fungus-treated (conc: 10^8 spores/ml) or triton-treated. Thus, four combinations were possible – a fungus- or triton-experienced ant could be put in with fungus- or triton-treated larvae in Phase 2. Their behaviour in Phase 2 was recorded for 60 minutes, and the samples were frozen for further analysis.

The L. neglectus adult workers and larvae were chosen from the same super-colony (Jena_19). Larvae were chosen by size, around 2 ± 0.2 mm, by measuring a few larvae on millimeter paper and then using them as reference. The workers were collected from the inner chambers. The workers and larvae were collected from the colony a day before the experiment, and the workers were color-marked for identification.

Two containers with two conditions were set up in Phase 1, and four containers with four different treatment-combinations were set up in Phase 2. Sixteen replicates per treatment were filmed and analyzed in total. The recording room temperature was maintained at 23°C, and the containers were kept within a dome light and recorded with Blackfly S BFS-U3-120S4C camera. The videos were recorded using OBS Studio software and camera settings were adjusted using SpinView. The speed was set at 30 frames per second. First 10 minutes of Phase 2 were analyzed and grooming behaviour (by the experienced ant and the nest mate towards each other or the larvae) was annotated manually using custom behavioural annotation software developed by Jinook Oh at the Cremer Group.

To determine the contamination level of experienced ants after Phase 1, I conducted some extra recordings and froze the samples after Phase 1. 12 fungal replicates and 6 triton replicates were filmed for the duration of Phase 1 and later frozen. The ants were separated into head and body parts and pooled (3 per vial) before they were analyzed in qPCR (based on lab protocols) to quantify the level of fungal contamination.

Low-level contamination survival experiment methods

50 workers each were exposed for control treatment and low-level-contamination treatment and observed for 12 days. 50 larvae each were exposed for control and low-level contamination treatment, observed for 7 days and their survival was plotted. TritonX was used for control treatment and a low concentration of fungal suspension – 10^6 spores/ml – was used to simulate a low-level contamination effect.

The two treatment solutions were blinded by a third person for the experimenters. A droplet of 0.5 µL of the treatment liquid was pipetted onto the larvae. In the case of workers, 0.5 µL of solution was pipetted on a glass slide, and the ant's gaster was rubbed on the droplet until it was dry (about 20-30 seconds). Each individual larva and worker were put in a small container and were not fed for the duration of the experiment.

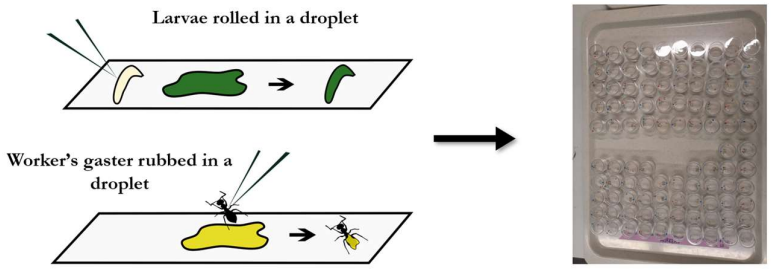

Figure 3. Schematic representation of methods used to set up the low-level contamination survival experiment.

For larvae survival, we checked for movement of the head parts by prodding the larvae with a blunt needle (Schultner et al., 2013). Discolored, immobile larvae were also considered dead. Ants' survival was checked every day by checking for movement of the antennae, activity and surface-adherence of the animal.

Dead larvae were surface sterilized (with ethanol and bleach – refer to the lab protocol) and plated on a wet filter paper petri dish to check for fungal outgrowth on Day 13 after exposure. 9 replicates of each treatment for workers and larvae were frozen to check the exact level of fungal contamination with this low-level exposure.

Results

Experiment 1: Recent Pathogen Experience Induced Grooming experiment

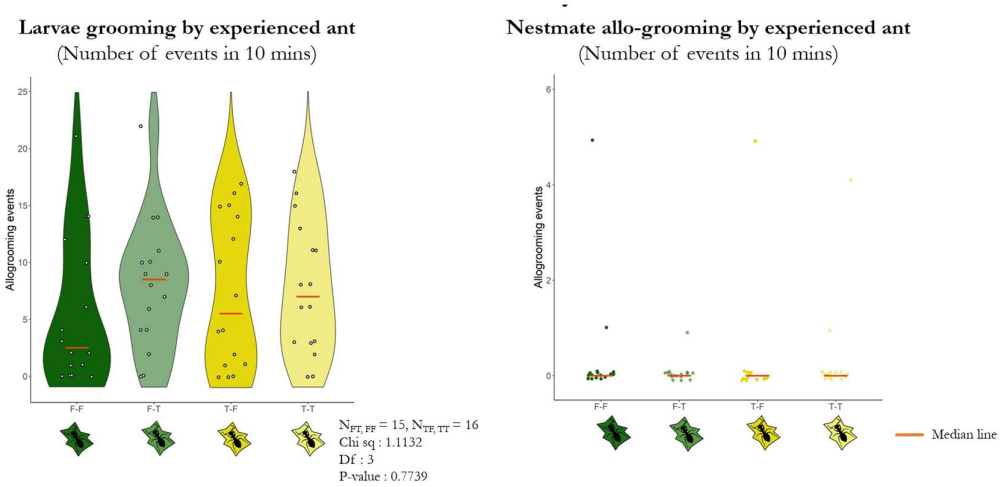

Figure 4. Larvae and nestmate grooming (as number of grooming events) by the experienced ant in first 10 minutes of Phase 2 of the experiment. 'F-F' stands for fungus-experienced ant grooming a fungus-treated larvae in Phase 2.

We found no difference in the number of grooming events by the experienced ants towards larvae (Negative binomial model: $N_{FT, FF} = 15$, $N_{TF, TT} = 16$, Chi sq: 1.1132, Df: 3, P-value: 0.7739) or the naïve nestmate (Negative binomial model: $N_{FT, FF} = 15$, $N_{TF, TT} = 16$, Chi sq: 0.8015, Df: 3, P-value: 0.8491) across different treatments. Grooming duration showed similar trends as grooming frequency. Nestmate allogrooming frequency and duration were both very low.

Results of qPCR analysis of the experiment are described below along with that of Survival experiment.

Experiment 2: Low-level Contamination Survival Experiment

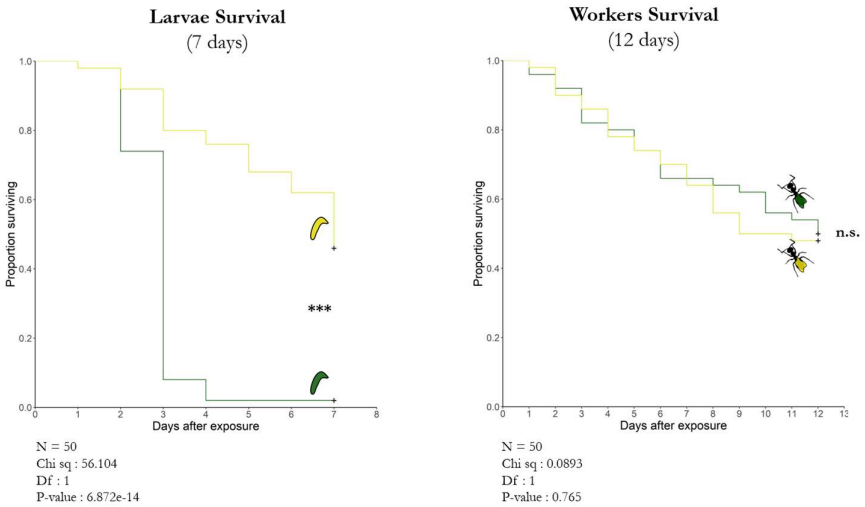

Figure 5. Survival graph of larvae and workers infected with a low-level fungal spore suspension.

The low-level contaminated, fungus-treated larvae have a significantly lower survivorship rate as compared to triton-treated larvae (Cox Mixed Effects model, ANOVA, N = 50, Chi sq: 56.104, Df: 1, P-value: 6.872e-14). Whereas, there is no difference in the survivorship rates of fungus-treated and control-treated adult workers (Cox Mixed Effects model, ANOVA, N = 50, Chi sq: 0.0893, Df: 1, P-value: 0.765).

After 14 days post exposure, among the surface-sterilised cadavers, 47 out of the 48 fungus-treated larval cadavers grew out *Metarhizium* fungus, whereas none of the triton-treated larvae had any *Metarhizium* outgrowth.

qPCR Analysis

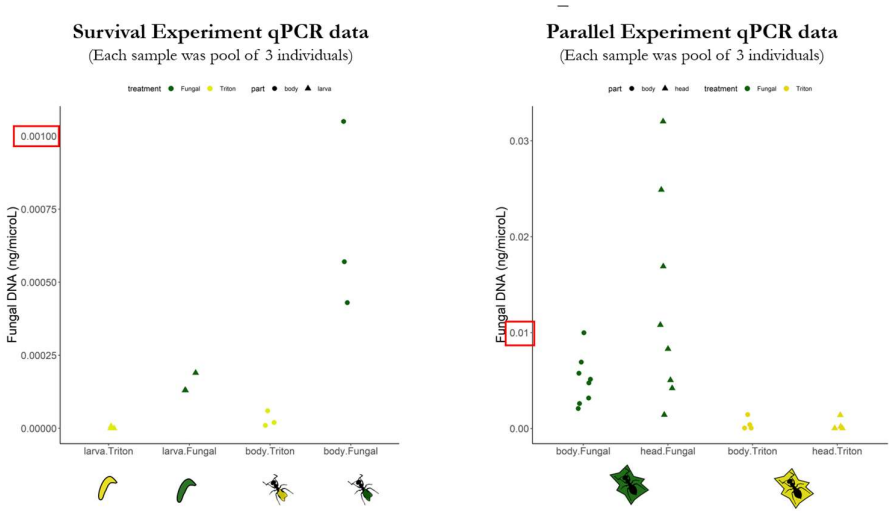

Figure 6. Data from Quantitative PCR of fungal DNA in different treatments of a) Low-level contamination survival experiment and b) Recent Pathogen Experience Induced grooming experiment

Samples from the Induced grooming Experiment – Fungal contamination is greater in fungus-experienced ants as compared to triton-experienced ants. Fungal DNA in the 'head' part is greater, as compared to the body of the ants, but it's also more variable.

Samples from the Survival Experiment – Ants rubbed in a droplet had higher fungal contamination as compared to larvae dosed with a droplet of fungal spores. Triton-treated larvae and workers had negligible level of fungal contamination.
The level of contamination from this low-level exposure (10^6 spores/ml) was much less than the level of contamination picked up by workers exposed to a fungus-treated worker in the Induced grooming experiment.

Discussion

Sanitary brood care is very important to ensure that the brood is protected from an infectious disease. In the presence of an infected individual in the nest, the workers increased the time spent in the brood chamber (Ugelvig and Cremer 2007). We wanted to test the effect of recent pathogen experience on induced sanitary care. We didn't observe any influence of recent pathogen experience or Phase 2 larval treatment on induced sanitary care (Fig. 4).

This could be because perhaps the fungus-experienced ants didn't want to get a higher contamination from fungus-treated larvae in Phase 2, and hence didn't increase grooming in an experimental set up quite removed from their natural social habitat. Another confounding factor could be the use triton-treated larvae as control. Triton-treated larvae are not the same as naïve larvae. Some studies have reported that treatment with a detergent (such as TritonX) elicits the same grooming intensity as fungus exposure (Graystock and Hughes 2011, Reber et al., 2011, Tragust et al., 2013b). We also saw that allogrooming of the naïve nestmate was very low in Phase 2, and this could be because of larval treatment in Phase 2, which would have made both ants focus on grooming the larvae.

Theis et al., 2015 proposed a theoretical model to predict the effects of allogrooming on disease transmission in the nest. According to the model, infected individuals should increase self-grooming and decrease allogrooming of susceptible individuals. Since we had seen that fungus-experienced ants decreased grooming of naïve larvae (Sartoris unpublished), we wanted to test whether larvae are susceptible to a low-level contamination.

We found that larvae are much more susceptible to a low-level contamination as compared to adult workers (Fig. 5). The larvae exposed with a low concentration of fungus died much faster as compared to workers treated with the same concentration. qPCR analysis of the samples show that the larvae died from a fungal contamination lower than what fungus-experienced ants would have picked up from the Induced grooming experiment (Fig. 6). Moreover, almost all of the fungus-exposed cadavers grew out *Metarhizium* fungi, validating that they had indeed died from the fungus infection and not from starvation or other factors. This finding explains the decreased allogrooming of fungus-experienced ants towards larvae in the Recent Pathogen Experience experiment (Sartoris unpublished) by showing that larvae are very susceptible to a low-level contamination.

Acknowledgements

I'm very grateful to Linda Sartoris for being a great mentor, showing me the ropes in the lab, finishing the qPCR of Induced grooming Experiment samples, and sharing her resources of illustrations and statistical analyses. I would like to thank Prof Sylvia Cremer for giving me the opportunity to work with her group at ISTA, and all the Cremer Group members for being helpful, supportive and a lot of fun to work with! I'm also grateful to my fellow summer interns and PhD students at ISTA, without whom this summer wouldn't have been the same.

I would also like to thank Prof Raghavendra Gadagkar, whose article on how insect societies deal with infectious diseases led me to explore social immunity literature and to this internship with Prof Cremer.

I acknowledge IISER Pune, Institute of Science and Technology Austria (ISTA) and the KVPY scholarship program which allowed me to pursue this internship.

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