# Social immune defence in ants

# Different aspects of hygienic behaviour and the infestation with Laboulbeniales in *Lasius neglectus* ants.

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# General Introduction

The innate immune system in insects

Parasites and pathogens represent a constant threat to all organisms. In response to this threat all multicellular organisms have evolved mechanisms for the recognition and elimination of invading microorganisms, the so called innate immune system (Medzhitov and Janeway 1997). Despite similarities in some defence components (e. g. gremline-encoded receptors for the recognition of microbial-associated molecular patterns) and defence outputs (e. g. the production of reactive oxygen species) among the innate immune systems of plants (Jones and Dangl 2006), vertebrates (Janeway et al. 2007) and insects (Hultmark 2003), these innate immune systems are not the result of divergent evolution from an ancient unicellular eukaryote but are likely the consequence of convergent evolution (Ausubel 2005).

In recent years, with the aid of molecular and genetic studies in *Drosophila* great advances have been made in our understanding of the molecular pathways and regulation mechanisms leading to signalling (Toll, Imd, Jak/Stat and JNK pathways) and the recognition (PGRPs or β-GRPs) of attacks by bacteria, fungi, parasites and viruses in the innate immune system of insects (Lemaitre and Hoffmann 2007). Understanding of these processes is basic for an understanding of the immune responses observed in insects upon insult.

Immune responses of insects can be manifold and are often divided by convention in either constitutive and induced defences or humoral and cellular bound defences (Gillespie and Kanost 1997, Vilmos and Kurucz 1998). They involve for example coagulation and melanization at the site of wounding upon injury or cuticle breakage through parasites (Theopold et al. 2004), phagocytosis, encapsulation or nodule formation around microorganisms with subsequent melanization of invading parasites (Strand 2008), the induction of antimicrobial peptides (Zasloff 2002, Bulet and Stöcklin 2005) or the rapidly activated phenoloxidase enzyme cascade (Söderhäll and Cerenius 1998, Cerenius and Söderhäll 2004, Cerenius et al. 2008).

Traditionally insects are seen to lack the highly specific immune response of the adaptive immune system of vertebrates that can confer lifelong protection upon reinfection with the same pathogen (Janeway et al. 2007). This adaptive immune system is generated through somatic recombination of immune receptor genes and clonal expansion of activated lymphocytes (Litman et al. 2010) and has evolved twice in agnathans and gnathostomes using different recombinatorial systems for lymphocyte antigen receptor diversification (Pancer and Cooper 2006). However in insects and other invertebrate immune systems phenomena that show memory and specificity and are thus functionally equivalent to characteristics of the adaptive immune system in vertebrates have also been proofed to partly exist

(Little et al. 2005, Kurz 2005, Schmid-Hempel 2005a, Browden et al. 2007). As these phenomenological observations mostly on whole-organisms studies lack behind the knowledge of existing molecular mechanisms leading to them their existent has stirred significant controversy (Hauton and Smith 2007, Rowley and Powell 2007). But novel discoveries such as the Dscam molecule (immunoglobulin superfamily receptor Down syndrome cell adhesion molecule) involved in the innate immune system of *Drosophila* and possibly being expressed in more than 18000 isoforms (Watson et al. 2005) potentially provide a basis for the specificity and memory phenomena reported in insects.

The limits of the innate immune system of insects

All in all the existence of such and other phenomena seen in insects indicates either our still incomplete understanding of the innate immune system of insects or it calls for a broader understanding of how immunity is achieved apart from molecular pathways and the hemocoelic immune responses. The innate immune system is only the last line of defence, with boundary defences such as the cuticle (Armitage and Siva-Jothy 2005) and behavioural adaptations such as avoidance behaviour (Cremer and Sixt 2009) preceding them. Parasites and pathogens in the environment represent important selective forces on their hosts with reciprocal effects leading to the coevolution between hosts and parasites (Woolhouse et al. 2002). Therefore a broad understanding of insect immunity also necessitates taking into account evolutionary and ecological forces that shape insect immune defences (Schmid-Hempel and Ebert 2003, Schmid-Hempel 2003, Siva-Jothy et al. 2005, Schulenburg et al. 2009). The activation and use of the immune system is associated with costs (Sheldon and Verhulst 1996, Moret and Schmid-Hempel 2000) and trade-offs between immune defences and other functions or activities that share common resources and contribute to an animal's fitness are to be expected. Insect life history traits such as the nesting ecology, e. g. in pathogen and parasite rich soil, or food consumption, foraging behaviour, colony organization or type of reproduction need to be taken into account as they contribute in shaping immune defences (Zuk and Stoehr 2002, Boomsma et al. 2005, Schmid-Hempel 2006).

Group living

Under evolutionary, ecological and life history traits aspects group living insects are especially interesting for the study of how immunity is achieved. Living in groups has many benefits, such as cooperative brood care, foraging or anti-predator defences. But the close interaction and high density of often closely related individuals is also thought to be associated with the increased transmission of diseases and increased parasitism (Alexander 1974, Anderson and May 1979, Côté and Paulin 1995, Schmid-Hempel 1998). This is likely to have driven the evolution of increased or special immune defences under group living conditions. Solitary insects for example with temporal crowding such as migratory locusts or caterpillar larvae show a phenomenon called density dependent prophylaxis

(DDP) which refers to changes in pathogen resistance in response to conspecific crowding (Wilson and Reeson 1998, Barnes and Siva-Jothy 2000, Wilson et al. 2002, reviewed in Wilson and Cotter 2008). In social termites DDP could not be found (Pie et al. 2005), however a recent study in bumble bees has proposed that annual and perennial social insect societies might also modulate their base immune function in a adaptive way when they go through population fluctuations (Ruiz-Gonzàlez et al. 2009).

Social insects

The social insects, i. e. bees, wasps, ants and termites, constitute perhaps the most extreme example of group living with the homogeneity of most of these groups in terms of physical and genetic environment together with close contact among individuals representing a rich arena with diverse host-parasite strategies to be studied under evolutionary, ecological and life history traits aspects. Thus, not surprisingly, immune defences employed in insect societies can range from a genetic level to behavioural, physiological or organisational mechanisms.

As the first social insect genome, that of the honeybee *Apis mellifera* (Honey Bee Genome Sequencing Consortium 2006) was sequenced it revealed that compared to *Drosophila melanogaster* and *Anopheles gambiae* only a low number of immune genes were found. Although the main components of immune pathways were conserved, the genome contained smaller numbers of gene family members at all points along these pathways (Evans et al. 2006). As more genome sequences were available it became clear that dipterans have unusually large immune gene repertoires (Fischmann et al. 2011). In addition molecular evolutionary approaches provided evidence that individual immune genes in social Hymenoptera are subjected to positive selection and that sociality has driven immune gene sequence evolution (Viljakainen and Pamilo 2008, 2009).

The evolution of eusociality in general and particularly in Hymenoptera has been explained using kin selection theory with helping behaviour towards kin and intracolonial reletadness as driving forces in the evolution and maintenance of social behaviour (Hamilton 1964). However, as already mentioned previously, high relatedness among individuals in insect societies can also enhance parasite transmission (Shykoff and Schmid-Hempel 1991) making them susceptible to the same parasites. In recent years it has become clear that intracolonial genetic diversity through polyandry (females mating with several males) and polygyny (the presence of several functional queens in the same colony), sometimes found in social Hymenoptera (Strassmann 2001), is one line of defence in insect societies which leads to a decrease in parasite loads and improves disease resistance (Baer and Schmid-Hempel 1999, Tarpy 2003, Hughes and Boomsma 2004, Seeley and Tarpy 2007, Reber et al. 2008).

Another line of defence against parasites and pathogens in insect societies is given through the so

called “social immune system” (Cremer et al. 2007).

This social immune system can be given through organisational or spatial mechanisms. The sophisticated waste management strategies found in ants (Howard and Tschinkel 1976, Hart and Ratnieks 2002) are a good example of organisational immunity (Naug and Camazine 2002, Naug and Smith 2007, Fefferman et al. 2007) that can combine task partitioning, division of labour and nest compartmentalisation to collectively isolate hazardous waste (Hart and Ratnieks 2001). Social immunity is also given through behavioural and physiological mechanisms which are not only found at the individual level but also at the level of the society. For example grooming behaviour, i. e. the removal of fungal spores (Oi and Pereira 1993) or mites (Büchler et al. 1992) from the surface of the cuticle, can be performed at an individual level, e. g. self-grooming, or at a group level, e. g. allo-grooming, (Schmid-Hempel 1998). Other behavioural defences are only possible at a group level such as for example exhibited social fever in honeybees, whereby many bees simultaneously raise their body temperature to heat-kill bacteria in their hive (Starks et al. 2000). Physiological responses at an individual level such as the production of antimicrobial substances in the metapleural gland of ants (Maschwitz 1974) can be used to limit autoinfection (Fernándes-Marín 2006). On the other hand antimicrobial substances can be externalized to improve nest hygiene in termites (Chen et al. 1998, Rosengaus et al. 1998a, Hamilton et al. 2011) and antimicrobially active substances from the environment are used to enrich the nest material in ants (Christe et al. 2003, Chapuisat et al. 2007), thus providing a benefit for the whole society.

The social immune system found in insect societies is thus characterized by evolved cooperative social defences that complement the immune response of individual group members. Furthermore defence mechanisms employed in social immunity are based on interactions between two or more individuals of the society. Interestingly, these social interactions with for example diseased nest-mates can directly affect the susceptibility of individual group members increasing their survival upon a later infection with the same pathogen (Traniello et al. 2002; Ugelvig and Cremer 2007).

Social immune defences employed by ant societies will constitute the core part of the investigations performed in this thesis.

The study ant species

As main study ant species (except for chapter I) we used throughout the thesis workers and brood of *Lasius neglectus*. This ant species has only recently been formally described from a location in Budapest, Hungary, where it has been discovered in the early seventies (Van Loon et al. 1990). Unusual for *Lasius*, *Lasius neglectus* is highly polygynous with intranidal mating and can form huge supercolonies without clearly defined colony boundaries (Boomsma et al. 1990). Between nests of one population aggression is practically absent (Cremer et al. 2008). Upon introduction *Lasius neglectus* has a negative impact on the local ant fauna and other non arthropods (Nagy et al. 2009). Dispersal over short distances is thought to occur by budding and by human-mediated intervention over long

distances (Espadaler et al. 2007). All these features qualify them as invasive ant species (Tsutsui and Suarez 2003, Holway et al. 2002). Since its discovery, Europe has seen a rapid expansion of *Lasius neglectus* with currently (July 2011) 151 localities in 19 countries (Espadaler and Bernal 2011, for a comprehensive review of its introduction history also refer to Ugelvig et al. 2008). The probable origin of this ant species lies in West Asia from which it invaded Europe (Seifert 2000). In Europe this ant has become a major pest problem in some areas where it has been introduced, invading houses and causing damage to electrical equipment and circuitry, to which it is attracted (Espadaler 1999, Rey and Espadaler 2004).

This ant species has also been proofed to exhibit some remarkable features in gaining immunity, showing behavioural adaptations upon parasite pressure together with a survival benefit after contact with the same parasite upon secondary exposure (Ugelvig and Cremer 2007). Furthermore the unicoloniality of populations, multiple queens, large colonies and low aggression (Cremer et al. 2008) make them ideal laboratory animals, easy to manipulate for a diverse array of experimental investigations.

*Lasius neglectus collection and mantainance*

For the experiment performed in the first chapter colonies from Italy, Spain, Germany, and Turkey collected before 2008 were used (for details on collection and maintenance refer to Ugelvig et al. 2008 and Cremer et al. 2008). All other used *Lasius neglectus* colonies were collected throughout the years 2008 to 2010 from the populations of Jena, Germany, Volterra, Italy, L'Escala, Spain, Gif-sur-Yvette, France, and Douarnenez, France (for exact location of the populations refer to Espadaler and Bernal 2011). Queens, workers and brood from these collections were housed in large plastic boxes (approx. 30 x 20 x 15 cm, Length x Width x Depth) with plaster ground and fed at a diet of cockroaches and honey. Throughout the year we mimicked an annual temperature and light cycle keeping the colonies in an incubator (Rumed) with eight months summer condition (27/21°C with 14h/10h day/night cycle), one month autumn and spring conditions (15/10°C with 10h/14h day/night cycle) and 2 months winter condition (8/4°C with 6h/18h day/night cycle). For experiments only colonies in summer condition were used.

The pathogen

To elicit antiparasite defence and to insult the immune system of our study ant species we used the entomopatogenic fungus *Metarhizium anisopliae* var. *anisopliae*. This fungus is one of the most commonly isolated insect pathogenic fungi with over 200 insect host-species and a cosmopolitan distribution (Roberts and St. Leger 2004). Thus, not surprisingly, *Metarhizium* has also been found to occur commonly in the soil near leaf-cutting ant nests (Hughes et al. 2004) and has been isolated from *Formica selysi* ants (Reber et al. 2008). The evaluation of *Metarhizium* as biocontrol agent has a long standing history (Zimmermann 1993, Shah and Pell 2003) and several commercial endeavours have

registered strains of *Metarhizium* for insect pest management (Bidochka and Small 2005). Apart from their function as insect parasites it has recently been discovered that entomopathogenic fungi play additional ecological roles in the environment as endophytes, antagonists of plant pathogens, associates with the rhizosphere and possibly even plant growth promoting agents (for an overview refer to Vega et al. 2009).

The infection process of suitable hosts through entomopathogenic fungi (reviewed in Clarkson and Charnley 1996, Castrillo et al. 2005) includes recognition of a suitable host, attachment to the host, penetration of the integument through physical and enzymatic mechanisms, evasion of host immune defences, proliferation and re-emergence from the host for the next round of spore production (Bidochka and Small 2005). Attachment to the host cuticle is mediated via non-specific hydrophobic interactions between conidial spores and the insect cuticle (Boucias and Pendland 1991). Thereafter the fungus germinates and penetrates the cuticle with a combination of physical and enzymatic mechanisms (Hajek and St. Leger 1994). Once the fungus has successfully invaded the hemocoel, the host is killed by a combination of mechanical damage produced by fungal growth, nutrient exhaustion and toxic products from the fungus (Gillespie and Clayton 1989). Finally, the next round of dispersal spores is produced.

*Metarhizium anisopliae* is also very well suited for the experiments performed in this thesis as the fungus can be easily identified and death of individuals attributed to the fungus. Furthermore the fungus infects its hosts through the cuticle making it accessible for behavioural actions such as grooming during this stage.

*Metarhizium origin and maintenance*

Two different *Metarhizium anisopliae* var *anisopliae* strains where used throughout the thesis: strain KVL 03-143 obtained from Jorgen Eilenberg, Faculty of Life Sciences, University of Copenhagen, Denmark and strain ARSEF 2575, obtained from Mike J. Bidochka, Department of Biological Sciences, Brock University, Canada. Conidiospores of fungal strains were grown on either Sabaroud destrose agar (SDA, Sigma) or Malt extract agar (Merck) by plating out spore suspensions in 86% glycerol and 10% skimmed milk stored at -80°C (long term storage) or by plating out previously harvested spore suspensions. The fungal strains were let grown on the agar for 10 to 21 days at 24°C until conidiospores were visible. Then conidiospores were harvested by gently scraping off the spores with a glass scraper in Triton X-100 solution. The gained spore suspension was thereafter stored at 4°C and used for fungal exposure within two weeks after harvest.

Aim of this thesis

Aim of this thesis is to elaborate on how immunity in social insect societies is achieved focusing on behavioural aspects. As model ant species I will mainly use *Lasius neglectus* and the entomopathogenic fungus *Metarhizium anisopliae* will be used to elicit antiparasite defence. In the first three chapters I will especially explore behaviours directed against contaminated brood as brood represents a high future value for the colony and is likely to bear special protection provided by the society (Ayasse and Paxton 2002).

In Chapter I I will explore the disease susceptibility of larvae and pupae in ant colonies under the hypothesis that the trait of spinning a silk-cocoon enclosure around larvae when pupating affects susceptibility. At the same time I will investigate hygienic behaviour of adult workers directed against brood, e. g. allogrooming behaviour which reduces the spore load on exposed brood and hygienic brood removal which is likely to prevent disease transmission through behavioural observations.

In Chapter II I will specifically investigate allo-grooming behaviour in the ant *Lasius neglectus*. Through the count of spores from the surface of fungus exposed workers and brood I will first establish if spores are removed through allo-grooming behaviour in this ant species and how allo-grooming is influenced by fungal pathogenesis. Thereafter I will assess if allo-grooming might be responsable for an increased survival of fungus exposed workers living in a group reported in the literature. Furthermore I will assess the impact of hygienic care including worker-brood allo-grooming on fungus exposed brood.

In Chapter III I will present data on the use of antimycotic substances during hygienic care of the brood. Therefore I will seal diverse worker body openings in the presence of fungus exposed pupae and through combining the count of spores from exposed pupae together with assessing their viability try to elucidate the origin of antimycotic substances. Furthermore I will employ behavioural observations to see how antimycotic substances are applied to brood.

Although *Lasius neglectus* ants possess various lines of defences against parasites and pathogens partly shown in the first three chapters, the specialist ectoparasitic fungi *Laboulbenia formicarum* are able to breach these barriers and cause permanent infections of workers. In Chapter IV I will elaborate on the infestation history of *Lasius neglectus* ants with this fungus from one *Lasius neglectus* population and try to transmit the infestation to a previously uninfested population in a cross fostering experiment. Furthermore I will investigate the impact of such an infestation on host survival and a possible parasite-parasite interaction between *Laboulbenia formicarum* and *Metarhizium ansiopliae*.

# Chapter I

# Pupal cocoons limit fungal infections in ants


## Abstract

The trait of spinning a silk cocoon-enclosure when pupating is a highly variable trait in ants. We explored if a cocoon-enclosure around pupae acts as a protective shell against entomopathogenic fungi. We therefore exposed brood of four unrelated ant species, two having cocoon-enclosed pupae and two having free pupae, to *Metarhizium ansiopliae* in a between species approach. In addition we followed a within species approach exposing brood of one species, having both types of pupae within a single nest, to the same pathogen. We found that live spore fungus exposed larvae and free pupae – but not cocooned pupae – were removed more quickly and in higher proportions from the brood chamber than control treated brood in all species. The expression of hygienic brood removal was adaptive as removed brood also suffered higher fungal growth. Cocoon-enclosed pupae suffered least fungal growth of all brood thus indicating that a cocoon-enclosure might be beneficial under upon fungal infection.


Prospective manuscript. This work was done in collaboration with Line V. Ugelvig (University of Regensburg; 10%) and Michel Chapuisat (University of Lausanne, Switzerland; 5%).

# Introduction

Brood of social insects enjoys a high degree of care for successful development. In addition to food and favourable temperature and humidity control, the worker force provides a protected shelter (Ayasse and Paxton 2002). However, brood is also a formidable target for parasites and pathogens (Schmid-Hempel 1998). Thereby it can only rely on its innate immune system (Vilmos and Kurucz 1998) and is more susceptible to entomopathogenic fungi (Petterson and Briano 1993), while the cuticle is still not fully sclerotized and melanized (Thompson and Hepburn 1978, Hopkins and Kramer 1992). Behavioural defences provided by workers are likely to be of great importance to the usually immobile brood. Workers of social insects possess a large array of collective behavioural defences ranging from the intake of tree resin to prevent fungal and bacterial growth in the nest (Christe et al. 2003, Chapuisat et al. 2007) to removal of infectious particles via grooming (Oi and Pereira 1993) and elaborate waste management forms (Bot et al. 2001). This provides insects societies with a social immune system (Cremer et al. 2007) complementing the individual immune system of individuals. Yet, diseased brood often gets removed from the nest upon detection. This form of hygienic behaviour has originally been described in bees (Rothenbuhler and Thompson 1956, Wilson-Rich et al. 2009) and only recently found to occur in the ant species *Cardiocondyla obscurior* (Ugelvig et al. 2010). In contrast to bees where each larva is placed in a single comb, ants pile their brood thus increasing the probability of transmission. Up until know it is not known how wide spread this behaviour is in ants, but it is likely to play an important role in disease control in ants.

Interestingly, the brood of ants falls into two categories: free pupae and pupae in a silk cocoon enclosure (larvae are always "free" as they need constant feeding). The trait of spinning a silk cocoon enclosure is remarkably variable in ants (Wheeler 1915, Baroni-Urbani et al. 1992) – sometimes present, sometimes absent and in some subfamilies present and absent.
An ultimate explanation of cocoon presence / absence and also of its function is lacking. It is assumed that the cocoon in insects generally has a protection related function (Danks, H. V. 2004), either against 1) environmental fluctuation in temperature and dryness, or 2) parasites and pathogens. The former is not very likely in social insects given the effort put in homeostatic nest condition, while the latter hypothesis has been brought forward also for the cover of naked brood with symbiotic fungus found in the Attini ants (Armitage et al. 2011, submitted). To our knowledge this hypothesis has so far not been formally tested in ants. We therefore designed an experimental study to investigate the effect of cocoon presence / absence on ant pupae for their susceptibility to fungal disease and occurrence of hygienic behaviour. As fungal pathogen we chose the entomopathogenic fungus *Metarhizium anisopliae*. This fungus infects insects by penetration of the insect cuticle to reach the host hemocoel (Clarkson and Charnley 1996) and can be found in the nesting area of ants (Hughes et al. 2004, Reber

et al. 2011). We first performed a comparative *between species approach* using four ant species from different ant subfamilies, two of which have cocoon-enclosed pupae whereas the other two have free pupae. All four ant species have a similar nesting ecology and either nest in the soil directly or in rotten logs near the ground (Schilder et al. 1999, Van Loon et al. 1990, Oettler et al. 2008, Heller 2004). Presence / absence of pupal cocoons was our best predictor for differences in ant hygienic behaviour and brood susceptibility to the fungal disease, yet a between species comparison is always flawed by potential phylogenetic constraints or other underlying species differences. We therefore also performed a *within species approach* analysing a single ant species (*Formica selysi*) that simultaneously in the same nests can have either free or cocooned pupae.

# Materials and Methods

Host ants

Four ant species from different subfamilies were chosen for the between species comparison: *Platythyrea punctata* (Ponerinae) and *Lasius neglectus* (Formicinae) with cocoon-enclosed pupae, as well as *Linepithema humile* (Dolichoderinae) and *Crematogaster smithi* (Myrmicinae) with free pupae. All ants were collected in the years 2005 to 2008 from different populations (*P. punctata*: Puerto Rico, Dominican Republic, Barbados, see Kellner and Heinze 2011; *La. neglectus*: France, Turkey, Spain, Germany, Italy; for details see Ugelvig et al 2008 and Cremer et al 2008; *C. smithi*: Southeast Arizona, see Oettler et al. 2008; *Li. humile* (Catalan supercolony, Spain, Vogel et al. 2010), and reared in the laboratory under species specific conditions (*P. punctata* and *C. smithi*: 27°C, respectively 26°C; 12/12h day/night light cycle, Kellner and Heinze 2011, and Oettler et al. 2008 respectively; *La. neglectus* and *Li. humile*: 23/18°C; 14/10h day/night cycle; Ugelvig and Cremer 2007). For the within species analysis, *Formica selysi* (Formicinae) was collected in 2008 from a population in Switzerland in which cocoon-enclosed and free pupae coexist in the same nest. All ant colonies were fed with honey and cockroaches, and regularly watered.

Fungal pathogen and exposure

The entomopathogenic fungus *Metarhizium anisopliae var. anisopliae* (strain KVL 03-143, obtained from the Faculty of Life Sciences, University of Copenhagen, Denmark) was reared on agar plates and suspensions of either live or UV-killed conidiospores (1x10^9 spores/ml in 0.05% Triton X-100; Sigma) were produced as detailed in Ugelvig and Cremer 2007. The germination rate of the live-spore suspension was 98%, whereas none of the spores in the UV-spore suspension germinated. In addition to the live-spore and the UV-killed spore suspension, we used the solvent Triton X-100 as a sham control. Individual ant brood items were treated with the three suspensions by placing them in quantities of 0.3µl of the corresponding suspension applied on parafilm.

Experimental setup

For each ant species, we set up 12 replicates of five individually colour marked (Edding 780) adult workers in experimental nests (diameter 9 cm, height 2 cm; as detailed in Ugelvig and Cremer 2007), which contained a protected brood chamber (2x1 cm) and a foraging arena in which food (10% sucrose solution on a cotton ball) was offered ad libitum. One day after set up, we started the experiment by simultaneously adding three groups of brood items into each experimental nest, i.e. brood treated with the sham control, UV-killed spores and live spores (each group of brood on a 1x1 cm filter paper, laid out in equal distance to the brood chamber). In the four species of the between species comparison each group of brood consisted of two larvae and two pupae (with the exception of

four *P. punctata* nests where only one pupa per group could be added and all *Li. humile* nests for which only a single larva per group was available; to take those differences into account, statistical analyses were based on the single brood item level). For the within species analysis of *F. selysi,* each brood group consisted of two free and two cocoon-enclosed pupae. The brood items of a group were colour coded with two dots of an individual colour representing one of the three treatments. The affiliation of a colour to a certain treatment was shifted randomly between the experimental nests to prevent an observer bias.

Behavioural observations

Immediately upon placing the treated brood item groups in the experimental nests and for the next four days the location of the brood (removal from the filter paper, intake into the nest or removal from the nest chamber), the hygienic actions of the ant workers towards the brood (worker-brood allogrooming), and the status of the brood (fungal growth) were observed for each brood item at least 5 times per day (scan sampling as described in Ugelvig et al. 2010). After this time, the infection status of both brood and workers and the location of the brood items were observed for another seven days once a day.

Workers that died during the time of the experiment were removed from their experimental nests, surface sterilized (Lacey and Brooks 1997) and their cleaned bodies then transferred to Petri dishes containing damp filter paper (21°C +/- 3°). To confirm if dead workers died from *Metarhizium* infection, corpses were scanned for hyphal growth and spore production for three weeks after the end of the experiment.

Statistical analysis

We analysed *location of the brood* (1. brood taken into the nest and 2. brood removed from the nest if previously brought into the nest) and *fungal growth*, using the Kaplan-Meier procedure with Tarone-Ware statistics, as they contain censored data. For the between species comparison of brood location, we used species as stratum to control for species specific differences in brood handling through the workers. Location data from *F. selysi* were analysed using brood type (free vs. cocoon-enclosed pupae) as stratum.

As fungal growth occurred infrequently on brood inside the nest chamber, statistical analysis of fungal growth was only performed on brood that had been removed from the nest. We performed a similar analysis as with brood location and used brood type (larvae, free pupae and cocoon-enclosed pupae) as stratum for both the within and the between species comparison. Following overall significant results of applied treatment at a stratum level for as well location of the brood and fungal growth we performed posthoc pairwise comparisons between treatments adjusting alpha to 0.017 due to multiple testing.

To test for a correlation between the behavioural observation of brood removal by the ants and the infection state of the brood, we performed a Kendall's tau rank correlation between the day of fungal growth appearance on removed brood and the day of brood removal. The same analysis was also performed between the proportion of fungal growth on removed brood and the proportion of brood removal.

These statistical analyses were performed with SPSS 17.0 (IBM Statistics). The following analyses were performed in R version 2.13.0 (R Development Core Team 2011).

A differential intake and removal of brood depending on broodype (larva or pupa for the between species comparison and free pupa or cocoon-enclosed pupa for the within species comparison) was analysed over all treatments at the species level using a Pearsons's $\chi^2$-test with Yates' continuity correction.

Prior to statistical analysis observed worker-brood allo-grooming behaviour was expressed as grooming frequency, i. e. number of daily grooming events per number of daily performed scan samplings and standardized to the number of workers performing grooming behaviour and the number of brood items present in the nest. For the between species analysis grooming frequency averaged over the first five days of the experiment was squareroot-transformed and analyzed in a Generalized Linear Model (GLM) with broodtype (larva or pupa), treatment (sham control, UV spores, live spores) and their interaction as predictor variables and species identity as random variable. Statistical significance of predictor variables and interactions were tested with Likelihood–ratio-tests, by comparing the full model with reduced models until ending up with the minimal adequate model (Crawley 2007). For the within species analysis we proceeded similarly but used a GLM with quasipoisson errors on untransformed data and tested statistical significance with an F-test.

Upon statistical significance of a predictor variable all pairwise comparisons between levels of the predictor variable were carried out using the package "multcomp" (Hothorn 2008). The family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010).

# Results

Intake of brood into the nest

*Between species comparison.* In total 72% of all presented brood was brought into the nest chamber by all four species within the first two days of the experiment. While the two species with free pupae brought in a slightly higher percentage of presented larvae than pupae (*C. smithi*: 69% larvae vs. 44% pupae, Chisquare test: $\chi^2 = 8.186$, d. f. = 1, p = 0.004; *Li. humile*: 67% larvae vs. 51% pupae $\chi^2=1.699$, d.f.=1, p=0.192) the species with cocoon-enclosed pupae brought in larvae and pupae at equal numbers (*La. neglectus*: 75% larvae and pupae, $\chi^2 = 0.037$, d.f. = 1, p = 0.847; *P. punctata*: 100% larvae and pupae, n. s.). Free pupae were thus taken in at a slightly lower percentage than larvae, whereas cocoon-enclosed pupae seemed equally attractive to bring into the nest as the larvae of the respective species.

Larval intake was not affected by whether the larvae had received a live-spore, UV-killed spore or sham control treatment (Kaplan Meier: $\chi^2 = 0.805$, d. f. = 1, p = 0.370). Pupal intake gave a similar picture, with the only exception that *C. smithi* brought in fewer live-spore treated pupae than the two controls (Kaplan Meier: $\chi^2 = 4.898$, d. f. = 1, p = 0.027; *C. smithi*: $\chi^2 = 8.527$, d. f. = 2, p = 0.014; pairwise comparisons: live spores vs. sham control: p = 0.003; live spores vs. UV-killed spores: p = 0.098, UV-killed spores vs. sham control: p = 0.156; all other species n.s.).

*Within species analysis. F. selysi* workers brought a total of 69% of all presented pupae into the nest chamber, with cocooned-enclosed pupae being taken in at higher numbers than free pupae (78% and 61% of presented cocoon-enclosed and free pupae respectively, $\chi^2 = 3.96$, d. f. = 1, p = 0.047). Again, brood treatment (live or dead spores, sham control) did not influence the intake of pupae into the nest chamber (Kaplan Meier: $\chi^2 = 2.891$, d. f. = 1, p = 0.089).

All brood that was taken into the nest was piled irrespective of brood type and received treatment in all species.

Worker-brood allo-grooming

After intake, workers of all species performed allo-grooming of exposed brood in the nest chamber.

*Between species comparison.* The minimal adequate model for the grooming frequency of brood only contained a significant treatment effect (GLM: Likelihood-ratio = 10.306, p = 0.006). Post hoc pairwise comparisons showed that live spore treated brood was groomed at a higher frequency than sham control and UV-killed spore treated brood, with the latter being marginally non significant

(live spore vs. sham control: p = 0.003; live spore vs. UV killed spore: p = 0.052). No difference could be found between sham control and UV-killed spore treatment (p = 0.229).

*Within species comparison.* The minimal adequate model for the grooming frequency of brood in *F. selysi* also contained only a significant treatment effect (GLM: $F_{1,54} = 3.344$, p = 0.074). However, post hoc pairwise comparisons did not yield statistical significant differences between treatments (all comparisons, p = n. s.).

Brood removal from the nest chamber

*Between species comparison.* Overall 49% of all brood that was previously brought into the nest chamber was removed from the nest chamber over the course of the experiment. The two species with free pupae removed fewer larvae than pupae (*C. smithi*: 30% larvae vs. 63% pupae, Chisquare test: $\chi^2 = 7.148$, d. f. = 1, p = 0.008; *Li. humile*: 29% larvae vs. 70% pupae, $\chi^2 = 8.318$, d. f. = 1, p = 0.004). On the opposite, the two species with cocoon-enclosed pupae removed more larvae than pupae (*La. neglectus*: 69% larvae vs. 41% pupae, $\chi^2 = 7.322$, d. f .= 1, p = 0.007; *P. punctata*: 72% larvae vs. 6% pupae, $\chi^2 = 47.867$, d. f .= 1, p < 0.001).

In contrast to brood intake, larval removal was highly influenced by the applied treatment (Fig. 1 A; Kaplan Meier: $\chi^2 = 50.281$, d. f. = 1, p < 0.001) with only *Li. humile* showing a non-significant result at the species level (*C. smithi*: $\chi^2 = 33.535$, d. f. = 2, p < 0.001; *Li. humile*: $\chi^2 = 1.939$, d. f. = 2, p = 0.379; *La. neglectus*: $\chi^2 = 33.784$, d. f. = 2, p < 0.001; *P. punctata*: $\chi^2 = 12.277$, d. f. = 2, p < 0.001). For the other three species, larvae treated with live spores were consistently removed earlier and at higher rates than both sham treated larvae (all species p < 0.017) and larvae treated with UV-killed spores (all species p < 0.003), whereas there was no difference in larval removal after treatment with the sham control or UV-killed spores (all species p = n. s.). This indicates that the removal of larvae is performed by the species according to treatment risk they were exposed.

The removal of pupae was also highly influenced by their treatment (Kaplan Meier: $\chi^2 = 15.234$, d. f. = 1, p < 0.001). However, at the species level, only the species with free pupae (*C. smithi* and *Li. humile*) but not the species with cocooned pupae (*La. neglectus* and *P. punctata*) showed this significant effect (Fig. 1 A; *C. smithi*: $\chi^2 = 12.061$, d. f. = 2, p = 0.002; *Li. humile*: $\chi^2 = 8.721$, d. f. = 2, p = 0.013; *La. neglectus*: $\chi^2 = 2.531$, d. f. = 2, p = 0.282; *P. punctata*: $\chi^2 = 2.122$, d. f. = 2, p = 0.346). Similar to larval removal patterns *C. smithi* and *Li. humile* removed live-spore treated pupae at a significantly higher rate and sooner than sham treated pupae (both species $p \leq 0.001$). For the comparison live-spore treated pupae to UV-killed spores treated pupae this result was only significant for *C. smithi* (*C. smithi*: p = 0.014; *Li. humile*: p = 0.318), but again in both species there was no difference in pupal removal after treatment with the sham control or UV-killed spores (both p = n. s.).

The general emerging pattern was thus that removal of larvae and free pupae was increased after the live-spore treatment, whereas pathogen-treated cocoon-enclosed pupae were not removed from the nest in higher frequencies than the two controls (Fig. 1 A).

*Within species analysis. F. selysi* workers removed 54% of all pupae previously taken into the nest, with cocoon-enclosed pupae being removed at a lower percentage than free pupae (43% cocoon-enclosed pupae vs. 61% free pupae, $\chi^2 = 5.383$, d. f. = 1, $p = 0.02$).
Also in *F. selysi*, there was an overall significant effect of treatment on the removal of pupae (Kaplan Meier: $\chi^2 = 7.032$, d. f. = 1, $p = 0.008$), yet this was only significant for free pupae but not for cocoon-enclosed pupae (Fig. 1 B; free pupae: $\chi^2 = 7.564$, d. f. = 2, $p = 0.023$; cocoon-enclosed pupae: $\chi^2 = 3.647$, d. f. = 2, $p = 0.161$). For free pupae, we found a significantly higher and sooner removal of live-spore treated pupae compared to sham control treated pupae ($p = 0.008$) and a similar tendency for pupae treated with UV-killed spores compared to the sham control ($p = 0.030$), yet no significant difference to the treatment with UV-killed spores ($p = 0.726$).

All species created a common dump pile outside of the nest, where the removed rood was placed.

Fungal growth on brood

Fungal growth occurred very rarely on brood inside the nest chamber (10 out of all 188 brood items brought into the nest, i.e. 8%). Interestingly, only 4/10 brood items showing this fungal growth had been treated with infectious, live spores of *M. anisopliae*, and the remaining 6 with either UV-killed spore (n=2) or sham control (n=4) brood items, indicating spore transfer between brood items piled together on the same brood pile in the nest. This low occurrence of fungal growth on brood remaining in the nest was contrasted by 74% fungal growth being detectable on removed brood in *C. smithi*, *Li. humile*, *La. neglectus* and *P. punctata* and 55% in *F. selysi.* Noteworthy, brood removal always preceded the appearance of fungal growth indicating that ants detected infection before outgrowth of fungal material.

*Between species comparison.* Fungal growth on removed brood was clearly influenced by brood treatment (Fig. 2; Kaplan Meier: $\chi^2 = 32.879$, d. f. = 1, $p < 0.001$). However, at the brood level, only larvae and free pupae but not cocoon-enclosed pupae showed a significant treatment effect (larvae: $\chi^2 = 20.912$, d. f. =2, $p < 0.001$; free pupae: $\chi^2 = 53.969$, d. f. = 2, $p < 0.001$; cocoon-enclosed pupae: $\chi^2 = 1.266$, d. f. = 2, $p = 0.531$). For the larvae and the free pupae, treatment with live fungal spores, as expected, lead to higher appearance of fungal growth than sham control and UV-killed spore exposed brood (Fig. 2; all pairwise comparisons: $p \leq 0.001$), whereas we could not detect a difference between the treatment with sham control or UV-killed spores (both larvae and free pupae p

= n. s.). In contrast to this, cocoon-enclosed pupae did not develop more fungal growth when treated with live spores than with either sham control or UV-killed spores (both comparisons p = n. s.).

We also found a significant positive correlation between the day of removal and the day of first appearance of fungal growth (Kendall's tau rank correlation: $t_{182} = 0.374$, $p < 0.001$), with only sham control and UV-killed spore treated free pupae showing a slightly sooner removal than fungal growth compared to the other treated brood (Fig. 3 A). Between the proportion of fungal growth on removed brood and proportion brood removal we could not find a significant correlation (Fig. 4 A; Kendall's tau rank correlation: $t_9 = 0.254$, $p < 0.345$). This was most likely due to the fact that sham control and UV-killed spore treated free pupae showed a much lower fungal growth than suggested by their removal rate compared to the other treated brood.

*Within species analysis.* The appearance of fungal growth on removed brood was also highly influenced by treatment for *F. selysi* (Kaplan Meier: $\chi^2 = 12.194$, d. f. = 1, $p < 0.001$), but as for the between species comparison significant differences were limited to free pupae ($\chi^2 = 10.809$, d. f. = 2, p = 0.004) and did not occur in the cocoon-enclosed pupae ($\chi^2 = 4.988$, d. f. = 2, p = 0.083). In free pupae, treatment with live spores resulted in a higher and earlier fungal growth compared to sham control (p = 0.015) and UV-killed spores treatment (p = 0.004; Fig. 2), whereas both these comparisons were non-significant for the cocoon-enclosed pupae (p = n. s.). Again, we could not detect any differences in the occurrence of fungal growth on pupae treated with either a sham control or UV-killed spores (both free pupae and cocoon-enclosed pupae p = n. s.).

For *F. selysi*, we could not detect a significant correlation between day of brood removal and day of first appearance of fungal growth (Fig. 3 B; Kendall's tau rank correlation: $t_{54} = 0.114$, p = 0.302) and also not between the proportion of fungal growth and the proportion of brood removal (Fig. 4 B; $t_6 = 0.467$, p = 0.188).

Fungal infection of workers

Only two workers out of all 300 (one in *C. smithi* and one in *La. neglectus*) died from an infection with *M. anisopliae.*

# Figures and Tables

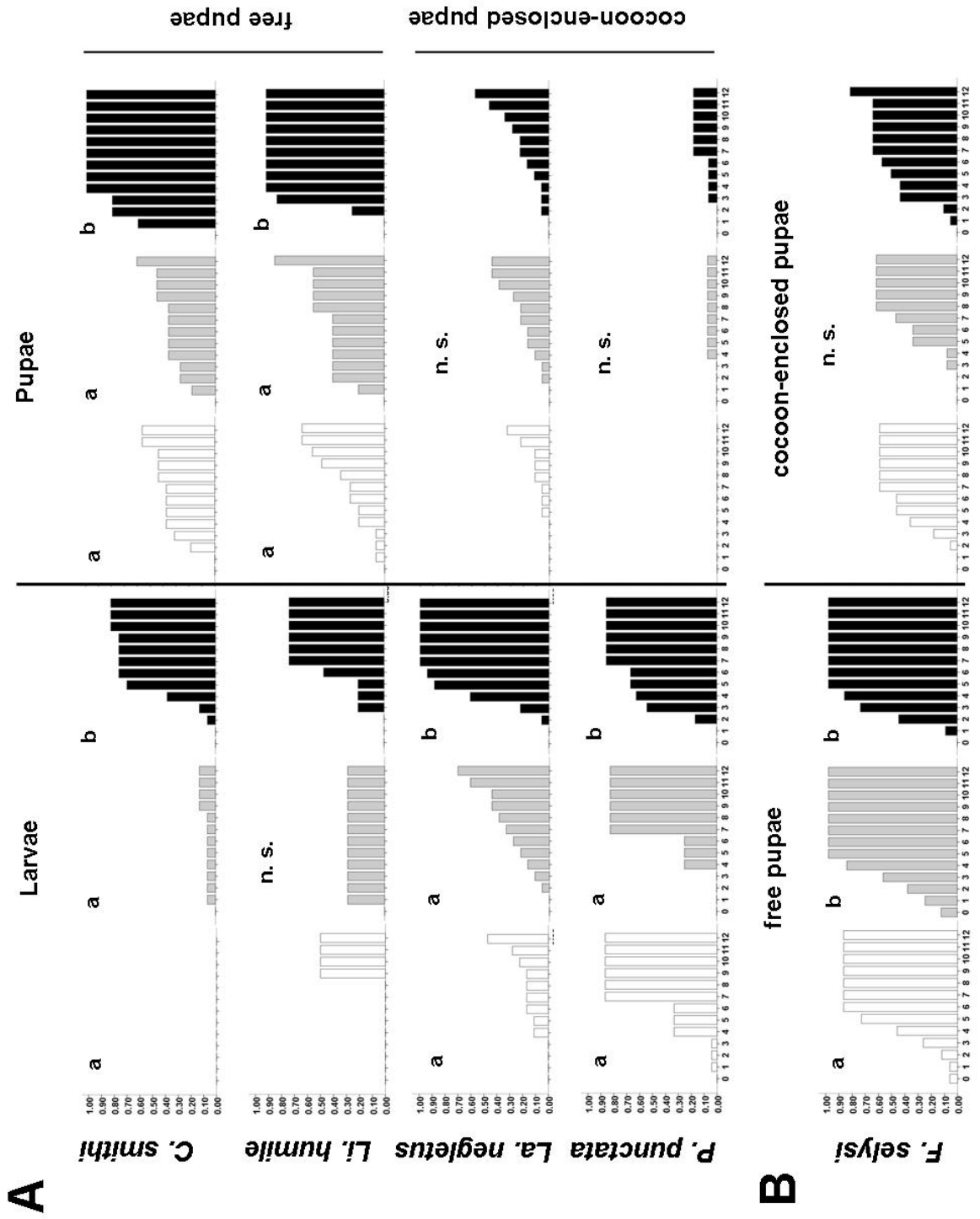

**Fig. 1**: Cumulative proportion of larvae and pupae removed from the nest chamber (y-axis) plotted against the experimental day after exposure (x-axis). For the between species comparison (A) with *C. smithi*, *Li. humile*, *La. neglectus* and *P. punctata* we found a significantly higher and/or sooner removal of larvae (exception *Li. humile*) and free pupae treated with live spore suspension (black bars) compared to sham control exposed (white bars) and UV-killed spore exposed (grey bars) larvae and free pupae, the latter two treatments not being different. In contrast there was no difference in the removal of neither control nor live or dead pathogen exposed cocoon-enclosed pupae (treatment = n. s.). For the within species comparison (B) we also found a higher and sooner removal of live-spore treated free pupae compared to sham control treated free pupae and a similar tendency for free pupae treated with UV-killed spores compared to the sham control, yet no significant difference between live and UV-killed spores treatment. Again there was no treatment effect on the removal of cocoon-enclosed pupae (p = n. s.).

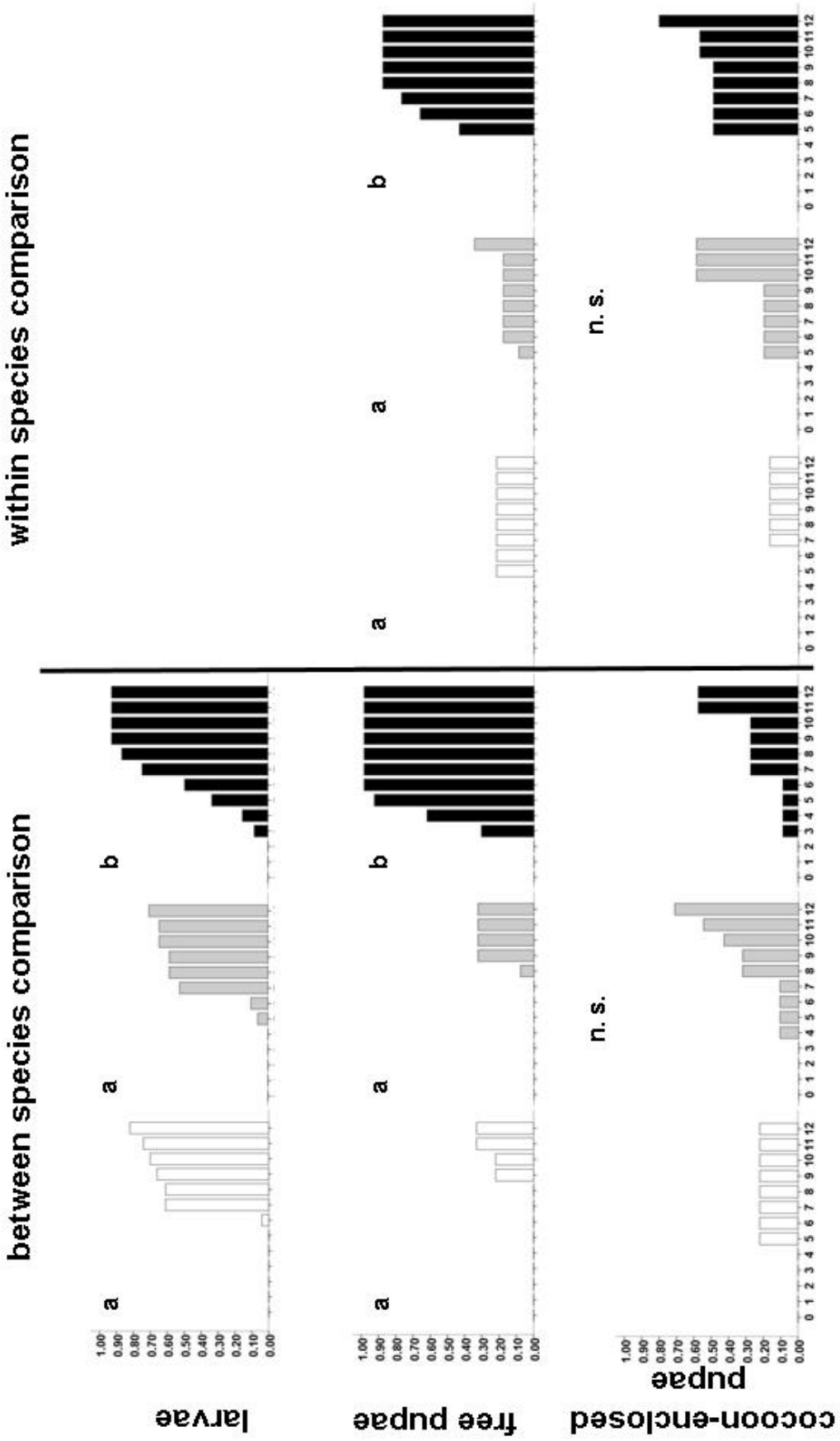
between species comparison
within species comparison
larvae
free pupae
cocoon-enclosed pupae
a
b
n. s.

**Fig. 2:** Cumulative proportion of fungal growth (y-axis) on larvae, free pupae and coccon-enclosed pupae removed from the nest chamber plotted against the experimental day after exposure (x-axis). For the between species comparison with *C. smithi*, *Li. humile*, *La. neglectus* and *P. punctata* we found a significantly higher and sooner fungal growth appearance on larvae and free pupae treated with live spore suspension (black bars) compared to sham control (white bars) and UV-killed spore (grey bars) treatment, the latter two treatments not being different. In contrast there was no difference in the appearance of fungal growth on cocoon-enclosed pupae (treatment = n. s.). For the within species comparison we also found a higher and sooner appearance of fungal growth on live-spore treated free pupae compared to sham control treated and UV-killed spores treated free pupae, but no difference between these two. Again there was no treatment effect for fungal growth appearance on cocoon-enclosed pupae (p = n. s.).

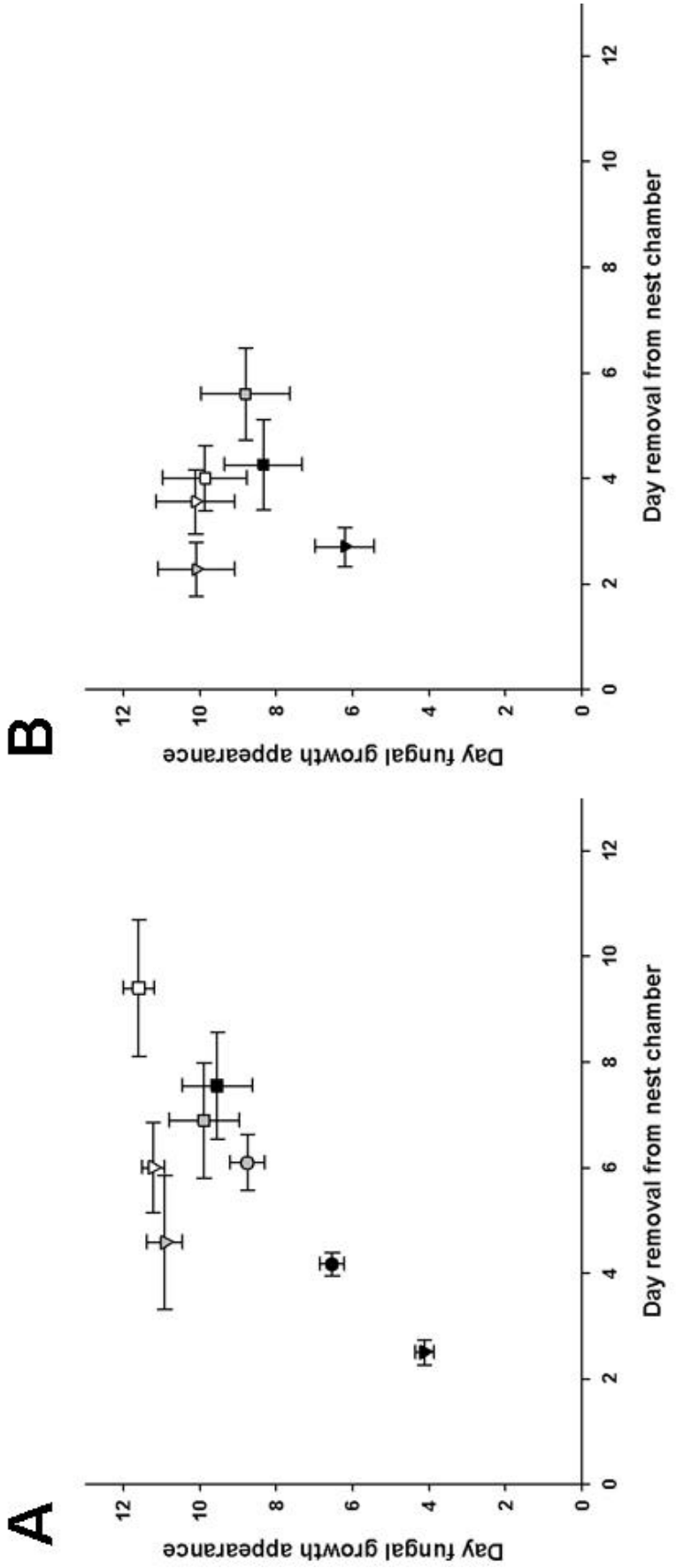


**Fig. 3:** Mean day +- s. e. of fungal growth appearance on removed brood (circles: larvae, triangles: free pupae, squares: cocoon-enclosed pupae) against day brood was removed from nest chamber. The between species comparison (A) with *C. smithi*, *Li. humile*, *La. neglectus*, *P. punctata* resulted in a significant correlation between day of brood removal and day of fungal growth appearance whereas the correlation was not significant for the within species comparison (B) with *F. selysi*. Color coding corresponds to treatment (white: sham control, grey: UV-killed spores, black: live spores).

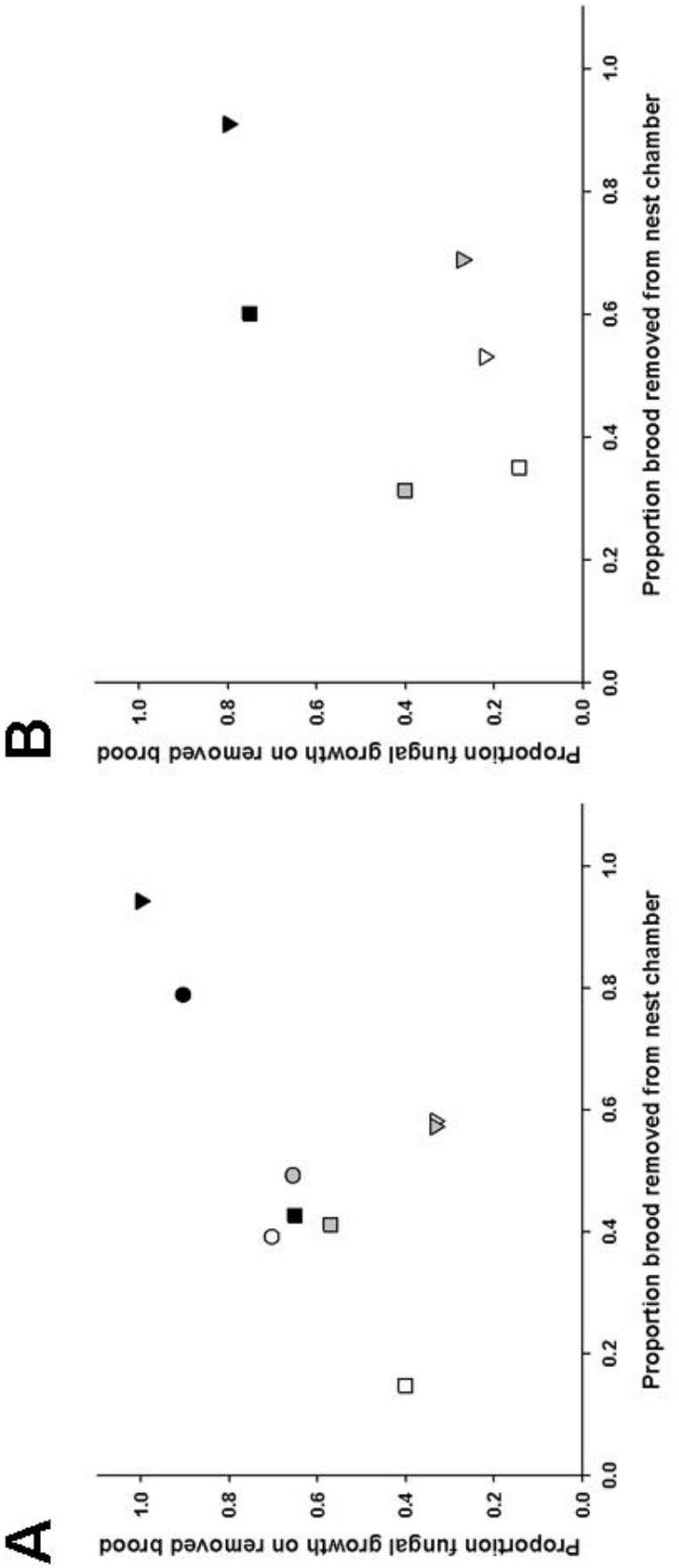


**Fig. 4:** Proportion of fungal growth on removed brood (circles: larvae, triangles: free pupae, squares: cocoon-enclosed pupae) against proportion of brood removed from nest chamber. Neither in the between species comparison (A) with *C. smithi*, *Li. humile*, *La. neglectus*, *P. punctata* nor in the within species comparison (B) *F. selysi* a significant correlation could be found. Color coding corresponds to treatment (white: sham control, grey: UV-killed spores, black: live spores).

# Discussion

In the present study we explored the impact of a cocoon-enclosure around pupae in ants by confronting a group of adult workers with either live spore exposed brood or brood exposed to control treatments. The experiment was performed once with four unrelated ant species (between-species comparison), two with and two without pupal cocoon-enclosure, and their respective brood and once with one species (within species comparison) having cocoon-enclosed as well as free pupae.

We could show that the addition of live-spore and control exposed brood was detected quickly by the workers and the brood brought into the nest chamber. Thereby the ant species did not distinguish between treatments applied (exception pupae in *C. smithi*). The same result has been obtained in another ant species, *Cardiocondyla obsucrior*, when confronted with exposed larvae (Ugelvig et al. 2010). Furthermore, it has been shown in *La. neglectus* (Ugelvig and Cremer 2007) and termites (Mburu et al. 2009) that the presence of a pathogen can be detected quickly and the behaviour adjusted accordingly. Apparently the presence of brood seems to be a stronger trigger than the presence of a pathogen during the initial infection course. Brood as a strong behavioural elicitor is also exploited by parasitic *Maculinea* butterflies that mimic larvae of the ant *Myrmica* and are therefore picked up and brought into the nest (Akino et al. 1999). We also found an elevated rate of larval intake compared to the intake of free pupae in the between-species comparison (n. s. in *Li. humile*) and a higher intake rate of cocoon-enclosed pupae compared to free pupae in the within species comparison. This result might indicate some sort of brood preference and cannot be explained otherwise.

Upon intake, workers started to perform hygienic brood behaviour, grooming the brood. Thereby they did not treat larvae and pupae differently but distinguished between treatments showing an elevated grooming frequency towards live spore treated brood compared to sham control treated and UV-killed spore treated brood in the between species comparison. The adaptive nature of grooming behaviour according to treatment was also shown in the above mentioned study with exposed larvae of the ant *Cardiocondyla obscurior* (Ugelvig et al 2010). Furthermore grooming behaviour can be modulated by dosage of fungal exposure (Okuno et al. 2011) and previous experience with the fungus (Walker and Hughes 2009, Reber et al. 2011). The benefit from performed allo-grooming is immediately evident as infectious particles from the surface of exposed adult individuals have been shown to be removed (Hughes et al. 2002, Reber et al. 2011). In the within species comparison we could not detect an elevated grooming frequency of live spore treated brood. This cannot be explained as *F. selysi* ants have been shown to be able to modulate grooming behaviour according to experience (Reber et al. 2011).

After a few days however, all ant species started to remove brood from the nest chamber. Thereby larvae and free pupae were removed according to treatment risk, with live spore exposed brood being removed sooner and at a significantly higher rate than UV-killed spore treated and sham control

treated brood (exception UV-killed spore exposed free pupae in the within species analysis; Fig. 1). This is expected if ants can detect the live spore exposure or later the infection and do not simply react to any treatment (sham control) or the presence of fungal particles (UV-killed spore treatment). The same result has been obtained with the ant species *Cardiocondyla obsucrior*, when confronted with exposed larvae (Ugelvig et al. 2010). Contrary to the removal of larvae and free pupae however, removal of cocoon-enclosed pupae was not influenced by treatment, with live spore exposed pupae not being removed significantly different than sham control or UV-killed spore exposed pupae. Furthermore in the between and the within species comparison we found that this was also associated with a indifferent fungal growth on removed cocoon-enclosed pupae, whereas fungal growth on removed larvae and free pupae was higher after live spore exposure than sham control or UV-killed spore exposure (Fig. 2). This might imply that the silk cocoon enclosure interfered with normal fungal pathogenesis. Entomopathogenic fungi such as *Metarhizium anisopliae* need to attach to the host surface then germinate and penetrate the surface to enter the host hemocoel. How these processes are mediated is fairly well understood on the insect cuticle (Clarkson and Charnley 1996) and differences between developmental stages in cuticle composition (Chilhara et al. 1982) and thus susceptibility (Castrillo et al. 2008) are to be expected. However almost nothing is known of how a silk cocoon-enclosure might affect fungal pathogenesis. It has been shown that Lepidopteran silk proteins have an immune function (Korayem et al. 2007) and peptides in the cocoon act as bacterial and fungal proteinase inhibitors (Nirmala et al. 2001). But in weaver ants *Polyrhachis dives* survival of *Metarhizium* exposed ants was not found to be influenced by the presence of silk (Fountain and Hughes 2011). On the other hand in the lesser spruce sawfly *Pristiphora abietina* it was not possible to infect cocoon-enclosed pupae with *Metarhizium anisopliae* (Führer et al. 2001). It thus could well be that the silk cocoon-enclosure around pupae in ants may form a protective shell, limiting fungal infections. The hypothesis of a protective shell is further supported by a significant correlation between the day of removal and the day of fungal outgrowth (Fig. 3, n. s. in the within species comparison). In as well the between species comparison as the within species comparison live spore treated cocoon-enclosed pupae are removed later and show lower fungal growth than live spore treated free pupae (within species comparison) or larvae (between species comparison), clustering more together with sham control or UV-killed spore treated brood. The same pattern emerges between the proportion of brood removal and the proportion of fungal outgrowth (Fig. 4) although there we could not find a significant correlation neither in the between species comparison nor in the within species comparison. Taken together this might indicate that cocoon-enclosed pupae were the least susceptible brood type for fungal infection.

We also found a significant amount of spore transmission to sham exposed and UV-killed spore exposed brood (Fig. 2, 3). This was probably caused by brood piling in the nest together with a common dump pile outside the nest. In contrast to brood, cross infection of adult workers through contact with live spore exposed brood was practically absent. A low susceptibility of workers when in

contact with live spore treated individuals has also been found in termites and other ant species (Rosengaus et al. 1998b, Hughes et al. 2002, Ugelvig et al. 2010). In the leaf-cutting ant *Acromyrmex echinator* death of workers was highest when in contact with sporulating cadavers (Hughes et al. 2002). This might emphasize the importance of hygienic brood removal before fungal growth.
Upon removal of brood, free pupae were removed at a significantly higher proportion compared to larvae and larvae at a significantly higher proportion compared to cocooned pupae in the between species comparison. In the within species comparison free pupae were removed at a significantly higher proportion compared to cocooned pupae. In contrast to the observed differential brood intake, the differential removal could be caused by the different susceptibility of brood to live spore exposure. A high susceptibility of larvae and free pupae would imply also a high proportion of spore transmission from live spore exposed brood to control exposed larvae and free pupae. However plotting the day of removal against the day of fungal growth revealed that although control exposed free pupae were removed soon they showed a late fungal growth relative to other control treated brood (Fig. 3). Similarly, plotting the proportion of brood removed against the proportion of brood showing fungal growth revealed that although the proportion of removed control exposed free pupae was high this was only associated with a low proportion of fungal growth relative to other control treated brood (Fig. 4). This is either indicative of some sort of brood preference, an argumentation already used for the differential intake of brood, the result of differences between developmental stages in cuticle composition (Chilhara et al. 1982) and thus susceptibility (Castrillo et al. 2008), or the result of some unknown factors.

Nontheless our results first demonstrate a potential protective function of the cocoon-enclosure around pupae in ants upon fungal pathogen exposure. Second, our data suggest that hygienic brood removal is widespread in ants, not only depending on exposure risk due to treatment of the brood but also taking into account the apparently low susceptibility of cocoon-enclosed pupae.

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# Chapter II

# The impact of hygienic care and grooming behaviour on *Lasius neglectus* workers and brood


## Abstract

The display of grooming behaviour is a fundamental part of hygienic care upon pathogen exposure in social insects. In the first part of this chapter we test the hypothesis that the removal of fungal spores through displayed allo-grooming behaviour in a group significantly contributes to an increased survival of individuals exposed to the entomopathogenic fungus *Metarhizium anisopliae*. We found that spore removal through allo-grooming behaviour is unlikely to be responsible for the increased survival of individuals maintained in a group after exposure and that other factors possibly associated with group living must play an important role.

In the second part of this chapter we investigate grooming behaviour and other hygienic care towards exposed brood. We found that allo-grooming is likely to result in a lower spore load on exposed brood, thus delaying and/or lowering fungal outgrowth. However displayed hygienic care is unable to prevent infection of pupae with the pathogen. We also found that upon pathogen exposure a large proportion of larvae is dead in the presence of workers 24h past exposure. Furthermore exposed pupae are unpacked from their silk cocoon-enclosure during hygienic care. Whereas the nature of larval death remains to be elucidated the behaviour of unpacking pupae is likely part of the hygienic repertoire of the ants which has not been reported so far.

# Introduction

Grooming, i. e. body surface care, is a common hygienic behaviour displayed almost ubiquitously among terrestrial vertebrates (Hart 1990, Mooring et al. 2004, Clutton-Brock et al. 2009). In social insects grooming behaviour is considered amongst other hygienic behaviours as part of the social immune system (Cremer et al. 2007). The expression of grooming behaviour in social insects can be constitutively as a preventive prophylactic response when returning to the nest after increased contamination risk outside the nest (Morelos-Juárez et al.2010, Reber et al. 2011) or conditionally dependent upon the presence of dangerous parasites (Hughes and Boomsma 2004, Walker and Hughes 2009, Rosengaus et al. 1998b, Ugelvig and Cremer 2010). Moreover grooming behaviour can be modulated by previous experience with parasites (Walker and Hughes 2009, Reber et al. 2011) and by the amount of present parasites (Okuno et al. 2011).

The benefit of grooming behaviour display is immediately evident as the load of ectoparasites and fungal particles on the surface of exposed animals is reduced through grooming (bees: Büchler et al. 1992; ants: Oi and Perreira 1993; termites: Shimizu and Yamaji 2003). Individuals in insect societies have the advantage that they can not only groom themselves (self-grooming) but can also be groomed by others (allo-grooming). Allo-grooming is potentially beneficial in addition to self-grooming as areas of the body surface can be targeted which are not accessible to self-grooming and more parasites can be removed. A higher removal of fungal particles through the combination of self- and allo-grooming in comparison to self-grooming alone could be shown upon exposure with entomopathogenic fungi (ants: Hughes et al. 2002, Reber et al. 2011; termites: Yanagawa and Shimizu 2007). This is likely also to be beneficial for the survival of exposed individuals living in a group compared to living in isolation. An increased survival of exposed individuals maintained in a group compared to isolated individuals has been found several times (termites: Rosengaus et al. 1998b, Yanagawa and Shimizu 2007; ants: Hughes et al. 2002, Okuno et al. 2011), but in none of these studies the contribution of increased spore removal through the combination of self- and allo-grooming on the increased survival has been assessed.

During fungal pathogenesis fungal spores not only attach to the host cuticle but also enter the host hemocoel within 24-48h (Boucias and Pendland 1991) making the fungus inaccessible for self- and allo-grooming. Thus fungal pathogenesis sets limits to the time self- and allo-grooming can be effective at spore removal after exposure to the fungus.

In the first part of this chapter we explore if self- and allo-grooming after fungal exposure leads to a reduction of fungal particles also on the surface of *Lasius neglectus* ant workers. At the same time we explore if the combination of self- and allo-grooming in a group is more effective at spore removal than self-grooming alone. Thereafter we try to assess if spore removal through the combination of self- and allo-grooming significantly contributes to the survival of an exposed individual living in a group

making use of the limits imposed by fungal pathogenesis and keeping exposed individuals isolated form the group for different time periods past exposure.

Most work on grooming behaviour in social insects in combination with fungal exposure has dealt with adult individuals. Apart from two studies where the authors could show that grooming behaviour towards larvae is performed according to exposure risk of larvae (Ugelvig et al. 2010) and exposure risk of workers (Ugelvig and Cremer 2007) we are not aware of any work performed with brood of social insects. This is surprising as brood of social insects can not groom itself and is likely to be highly dependent upon hygienic care provided by workers. In the second part of this chapter we therefore first establish the presence and possible differences of worker-brood allo-grooming towards fungus exposed larvae and pupae through behavioural observations. In a next step we quantify a possible reduction of fungal spores on the surface of exposed larvae and pupae in the presence of worker allo-grooming. At the same time we also explore the limits of allo-grooming on spore removal set through fungal pathogenesis. Thereafter we try to assess the impact of hygienic worker care including allo-grooming on exposed larvae and pupae by measuring fungal outgrowth on exposed brood after 24h of worker care compared to no worker care. In a last step we measure the impact of hygienic care on the hatching rate of fungus exposed pupae in comparison to control treated pupae.

# Materials and Methods

General methodology

Brood, queens and adult workers of the invasive garden ant *Lasius neglectus* were collected in the years 2008 to 2010 from the population at the botanical garden in Jena (Espadaler and Bernal 2011). Stock colonies from these collections, with several thousand workers, were housed in large plastic boxes with plaster ground and maintained at a diet of honey and cockroaches. At the time of performing the experiments all used stock colonies were kept in summer condition with temperatures of 27/21°C and a 14/10h day/night cycle. For experiments last instar larvae, pupae and adult workers were transferred to small petri dishes (diameter 5cm) with plaster ground and kept at 24°C at a 12/12h day/night cycle. Brood used in one experiment always belonged to the same stock colony. In the experimental conditions involving adult workers and brood, brood and workers never belonged to the same stock colony.

The GFP labelled and Benomyl resistant (Fang et al. 2006) entomopathogenic fungus *Metarhizium anisopliae var. anisopliae* (ARSEF 2575, obtained from Bidochka M. J., Department of Biological Sciences, Brock University, Canada) was grown on Malt extract agar (Merck; full medium according to manufacturer recipe) plates at 24°C. Another strain of *Metarhizium anisopliae var. anisopliae* (KVL 03-143, obtained from Eilenberg J., Faculty of Life Sciences, University of Copenhagen, Denmark) was cultured identically as the strain ARSEF 2575 but only used for the survival curves of adult workers (see experiment 2 of this chapter). For the experiments conidiospores were harvested from fully sporulating plates using 0.01% Triton X-100 (Sigma) as solvent. Concentrations were quantified using a Neubauer-improved counting chamber and adjusted to a working concentration of 1x10^9 spores/ml if not specified otherwise. For UV spore exposure the spore suspension with live spores was subjected to UV irradiation (312nm, 6X15W) for 1h to kill the spores. Each spore suspension (live spores and UV-spores) was plated out again and checked for germination capacity 14h later. UV-treated spores did not show any germination and live spore suspensions with germination <95% were not used. For the different experiments, different spore suspensions were used.

For fungal spore exposure brood and workers were taken out of the stock colonies not more than 1h before treatment and placed in a Petri dish if not noted otherwise. They were then treated by placing one brood item or one adult worker in quantities of 0.5µl of the treatment suspension (live or UV spore) applied on Parafilm. Thereafter they were put on a piece of filter paper to let the spore suspension dry on the surface. The use of fungal spore exposed brood and workers in the experiments always followed directly after exposure.

Remaining fungal spores on the surface of exposed brood and workers were washed off by vortexing brood or workers per experimental Petri dish setup for 1 min in an Eppendorf vial containing 0.01% Triton X solution supplied with 5ug/ml Benomyl, 50ug/ml Streptomycin and 50ug/ml Kanamycin (Sigma-Aldrich), thus removing all spores that did not adhere (Ment et al. 2010) or were not groomed off (Hughes et al. 2002, Reber et al. 2011). From each of the gained spore suspensions through washing 5 times 1µl spots were pipetted onto a malt extract agar plate. After 14-15h in an incubator at 24°C, 1-2 fields of vision per each pipetted µl spot were inspected at 200X or 500X magnification under a stereomicroscope for the number of visible spores. Washes containing UV-killed spores were not incubated but inspected immediately after pipetting onto the agar plate. The spore load on the surface of exposed brood was calculated as the sum of spores per one field of vision, averaged over all five inspected 1µl quantities from one washed off spore suspension.

Statistical analysis was performed in R version 2.13.0 (R Development Core Team 2011).

Experiment 1: Fungal attachment and self- and allo-grooming effectiveness on spore removal on exposed adult workers

To explore the strength of spore attachment and at the same time the effectiveness of self-grooming or the combination of self- and allo-grooming on the removal of fungal spores on the surface of exposed adult workers we created three different adult worker groups: workers that were held alone in experimental Petri dishes and thus could only self-groom (20 replicates), workers that were placed in groups of three in experimental Petri dishes and thus could self- and allo-groom (19 replicates) and a no grooming control at two time points (12 replicates for each time point). Adult workers were taken out of the stock colony the day before start of the experiment and placed alone or in groups of three in experimental Petri dishes. In the Petri dishes containing three workers two were colour coded receiving a small paint droplet (Edding 751) on their gaster. Workers for the no grooming control were placed together in a big Petri dish. All Petri dishes were supplemented with a piece of filter paper soaked with 10% sucrose solution on a metal plate (stainless steel). Before fungal spore exposure the next day, the 24 workers for the no grooming control were freeze killed by placing the Petri dish in the freezer at -20°C for 1h and the other workers (alone or in group) were briefly cold anesthetized before fungal spore exposure by placing the Petri dishes on ice. In the Petri dishes containing three adult workers only the non colour coded worker was exposed to the fungal spore suspension. After fungal spore exposure, exposed workers were put back in their respective Petri dishes, thereby separating the freeze killed workers of the no grooming control in single experimental Petri dishes.

10 min. after fungal exposure, the spores on the surface of 12 freeze killed workers of the no grooming control were washed off. 5h after fungal exposure the spores on the surface of all other exposed workers (alone, in group and the remaining 12 freeze killed workers) were washed off. Living workers

(alone or in group) were again briefly chilled by placing the Petri dish on ice before washing off spores.

Data were analyzed by constructing a Generalized Linear Model (GLM) with a negative binomial error structure. Spore load was treated as response variable and treatment group (alone for 5h, in group for 5h, freeze killed 0h = 10min. or freeze killed 5h) as predictor variable. A global F-test on the model and all pairwise comparisons between means in this model were carried out using the package "multcomp" (Hothorn 2008). The family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010). This allowed us to assess the strength of spore attachment to the cuticle of workers comparing the total spore load in the no grooming control 0h = 10 min. after fungal exposure to the spore load 5h after exposure on freeze killed workers. Furthermore we could determine the effectiveness of self-grooming or the combination of self- and allo-grooming at spore removal comparing total spore load on exposed workers kept alone to exposed workers kept in group for 5h after exposure.

Experiment 2: Impact of hygienic care and allo-grooming on the survival of exposed adult workers

To explore the impact of experienced hygienic care including allo-grooming on the survival of exposed workers at different periods past exposure we created three isolation treatments. Adult workers were colour coded the day before start of the experiments and transferred in groups of three in experimental Petri dishes. The Petri dishes were supplemented with a piece of filter paper soaked with 10% sucrose solution on a metal plate (stainless steel). The next day one worker in each Petri dish was exposed to a live fungal spore suspension and either isolated for the rest of the experiment (60 replicates), put back to the other two workers for 24h after exposure and then isolated for the rest of the experiment (60 replicates), or isolated for 24h after exposure until putting it back to the other two workers for the rest of the experiment (60 replicates). Furthermore we created another treatment group where the exposed worker was put back to the other two workers without isolation for the rest of the experiment (60 replicates). After fungal exposure the status of the exposed worker and the two group members was monitored for 12 days in all setups. Workers that died during the time of the experiment were taken out of the experimental nests, their surface sterilized (Lacey and Brooks 1997) and then their cleaned bodies transferred to Petri dishes containing damped filter paper at 24°C +- 2°C. To confirm if dead workers died from *Metarhizium* infection, corpses were scanned for hyphal growth and spore production for another 3 weeks after the end of the experiment. During this time the filter paper was continuously moistened with deionized water.

Survival data of exposed workers was analyzed using Cox proportional regression. This analysis was conducted twice, once with all workers that died during the experiment and once only with dead workers showing fungal growth. A global $\chi^2$-test on these models and all pairwise comparisons between isolation treatments were carried out using the package "multcomp" (Hothorn 2008). The

family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010).

Survival data of group members was analysed with a mixed-effects Cox proportional regression using the package "coxme" (Therneau 2011) with the two group members nested within replicated Petri dish as random factor and isolation treatment group as fixed effect. Again a global $\chi^2$-test on the model and all pairwise comparisons between groups were carried out as described before.

Experiment 3: Worker-brood allo-grooming behaviour displayed toward exposed larvae and pupae

To explore if adult workers react differently toward live spore exposed larvae or pupae, we observed hygienic behaviour toward exposed brood during the first hour after exposure. Last instar larvae and pupae were exposed to a live fungal spore suspension and then placed in groups of 4 to two workers in experimental Petri dishes for 15 min. Then behavioural observation began for an observation period of 15 min., followed by a break of 30 min. and another 15 min observation period. Only the behavioural category of worker-brood allo-grooming was assessed quantitatively. During the observation periods the number and duration of worker-brood allo-grooming bouts were recorded with the program BioLogic v2. In total 12 replicated setups per exposed larvae as well as pupae were observed.

For statistical analysis the number and the total duration of all recorded grooming bouts were analysed over all 30 min. of observation. The duration per worker-brood allo-grooming bout during these 30 min. of observation was calculated as the total duration of all allo-grooming bouts divided by the number of observed bouts. All three variables, i. e. the number, the total duration and the duration per bout were analysed separately using Welch two sample t-tests with brood identity (last instar larvae or pupae) as predictor.

We also calculated the percentage of time workers spent allo-grooming brood assuming 60min. of possible grooming behaviour display for both workers during the behavioural observation (30min. + 30min.).

Experiment 4: Effect of worker allo-grooming on the removal of spores from live spore and UV-killed spore exposed brood

In this experiment we assessed the effectiveness of worker-brood allo-grooming on the removal of spores from the surface of exposed brood by keeping exposed brood in the presence or in the absence of adult workers for 5h or 24h after exposure. The effectiveness was evaluated in the presence and absence of fungal spore attachment by performing the experiment once with live fungal spore exposed brood and once with UV-killed spore exposed brood.

Workers from a stock colony were transferred in groups of two to experimental Petri dishes. Directly after fungal exposure of the brood, one part of the experimental Petri dishes containing two adult workers was provisioned with either live fungal spore exposed pupae or last instar larvae whereas the other part was provisioned with either UV-killed spore exposed pupae or larvae. Furthermore

experimental Petri dishes not containing workers were also provisioned with live spore or UV-killed spore exposed pupae or live spore or UV- killed spore exposed larvae. Exposed brood was always placed in groups of four on a small piece of filter paper in the experimental Petri dishes.
In half of the Petri dishes the experiment was stopped at 5h after fungal spore exposure of the brood and spores were washed off the surface of all four brood items that had been placed together in one Petri dish. In the Petri dishes containing workers, the workers were already removed at 3h. In the remaining Petri dishes the experiment was stopped at 24h and spores on brood washed off.
Washes that showed too much bacterial or saprophytic growth upon spore counting were excluded from the analyses. Furthermore in the presence of workers a lot of larvae were dead and could not be washed off. For the obtained sample sizes of the different treatment groups refer to Table 1.
Effectiveness of worker-brood allo-grooming was inferred in the presence of spore attachment (live spore exposure) and in the absence of spore attachment (UV-killed spore exposure) by constructing Generalized Linear Models (GLMs) with negative binomial error structure separately for brood type (pupae or larvae) and for type of exposure. Thereby spore load was treated as response variable and time (5h or 24h), worker presence (absent or present) and their interaction as predictor variables. The significance of the predictor variables was assessed with the Anova command of the package "car" (Fox and Weisberg 2011) using type III sums of squares with orthogonal contrasts.

Experiment 5: Impact of worker absence compared to the presence of workers for 24h after exposure on fungal growth appearance on exposed brood

To determine the impact of hygienic care including allo-grooming on fungal growth appearance we compared live spore exposed brood in the absence of workers with live spore exposed brood in the presence of workers during the first 24h after exposure. Live spore exposed larvae were placed in groups of 3 in 25 experimental Petri dishes without workers and in 60 experimental Petri dishes together with one worker. Live spore exposed pupae were also placed in groups of 3 in 25 experimental Petri dishes without a worker and in groups of 2 in 60 experimental Petri dishes containing one worker. After 24h, workers were removed and the brood was placed in all Petri dishes on small metal plates (stainless steel) to facilitate visual inspection. Thereafter the status of the brood and appearance of fungal growth (first visible hyphae) was monitored for the next 11 days.
Statistical analysis of fungal growth appearance was performed separately for exposed larvae and pupae with worker presence/absence as predictor variable. We used the package "coxme" (Therneau 2011) to perform a mixed-effect Cox proportional regression with brood groups nested within experimental Petri dish as random factor and worker presence (absent/present) as fixed effect. Fungal growth appearance on larvae was thereafter analysed in more detail comparing larvae that were dead after worker presence for 24h and larvae that were still alive after worker presence for 24h separately to larvae without worker presence. Furthermore we compared fungal growth appearance on dead and alive larvae in the presence of workers for 24h to each other. Integrated partial likelihood (IPL)

estimating 3 parameters (fixed factor, random factor and nested factor in random factor) was used to assess the significance of the models.

Experiment 6: Impact of hygienic worker care on the hatching rate of live spore or UV-killed spore exposed pupae

In the last experiment we explored the impact of hygienic care through workers on the ultimate survival of exposed pupae, i. e. callow worker hatching, comparing the hatching rate of live spore exposed pupae to the hatching rate of UV-killed spore exposed pupae. Pupae exposed to a high concentration of live fungal spore suspension (1 x $10^9$ Spores/ml), pupae exposed to a low concentration of live fungal spore suspension (2 x $10^7$ Spores/ml) and pupae exposed to a UV-killed spore suspension (1 x $10^9$ Spores/ml) were placed in groups of four to two colour coded adult workers in experimental Petri dishes (30 replicates each). Furthermore pupae exposed to a high concentration of live fungal spore suspension were placed singly to one colour coded worker in experimental Petri dishes (60 replicates) as a control for hatched callow death due to contact with other exposed pupae. This treatment was not included in the statistical analysis as exposure was performed with a different spore suspension. All Petri dishes were supplemented with a piece of filter paper soaked with 10% sucrose solution on a metal plate (stainless steel). The filter paper on which exposed pupae were placed into the Petri dishes was removed as soon as the workers removed the exposed pupae from the filter paper. The hatching rate of exposed pupae was monitored for 12 days. Hatched callow workers that died during the course of the experiment and pupae that were unpacked from their cocoon enclosure, were taken out of the Petri dish, surface sterilized (Lacey and Brooks 1997) and then transferred to Petri dishes containing damp filter paper at 24°C +- 2°C. To confirm if death was caused by a *Metarhizium* infection, they were scanned for hyphal growth and spore production for three weeks after the end of the experiment.

A mixed-effect Cox proportional regression with the package "coxme" (Therneau 2011) was again used to analyse hatching rate, with pupae nested within experimental Petri dish as a random factor and exposure type (high dose live spore exposure, low dose live spore exposure and UV-killed spore exposure) as fixed effect. A global $\chi^2$-test on the model and all pairwise comparisons between groups in the model were carried out using the package "multcomp" (Hothorn 2008). The family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010).

# Results

Experiment 1: Fungal attachment and self- and allo-grooming effectiveness on spore removal on exposed adult workers

Treatment groups (alone for 5h, in group for 5h, no grooming control 0h and 5h) differed significantly in the amount of spores that could be washed off (Fig. 1; Global F-test on neg. binomial GLM: $F_{3,59} = 124.7$, $p < 0.001$). Post hoc pairwise comparisons showed a significantly lower spore load in the no grooming control at 5h after exposure compared to 0h ($p < 0.001$), indicating a significant reduction of spores due to attachment of the fungus to the cuticle of exposed dead workers. Nonetheless exposed workers maintained alone or in a group with two other workers after exposure showed both a significantly lower spore load compared to the no grooming control after 5h (no grooming control 5h vs. workers alone: $p = 0.002$; no grooming control 5h vs. workers in group: $p < 0.001$), with exposed workers in a group showing also a significantly lower spore load than workers maintained alone ($p < 0.001$).

Experiment 2: Impact of hygienic care and allo-grooming on the survival of exposed adult workers

General survival of fungus exposed workers did not depend upon the time period they spent with two other workers after exposure (Fig. 2 A; Global $\chi^2$ on Cox proportional regression = 4.514, d. f. = 3, $p = 0.211$). However death due to *Metarhizium* infection was significantly dependent upon time period spent in a group after exposure (Fig. 2 B; Global $\chi^2$ on Cox proportional regression = 22.61, d. f. = 3, $p < 0.001$). Exposed workers that were never isolated from the other two workers after exposure died significantly less due to *Metarhizium* infection than workers that were kept in isolation for different time periods after exposure (Post hoc: all pairwise comparisons: $p < 0.001$). Compared to isolation after exposure during the whole experiment, the isolation treatment for the first 24h past exposure and the isolation treatment at 24h past exposure and thereafter resulted both in a slightly lower number of workers dying of a *Metarhizium* infection which was however not at all statistically significantly (both pairwise comparisons: $p = 0.7$).

General survival of group members living together with exposed workers did not depend upon time period they spent with the fungus exposed worker (Fig. 3; Global $\chi^2$ on mixed-effects Cox proportional regression = 3.696, d. f. = 3, $p = 0.296$). However the hazard of dying was almost four times higher for group members living together with an exposed worker all the time compared to workers that never lived together with an exposed worker. From the few group members that died during the course of the experiment almost none died due to a *Metarhizium* infection indicating a low transmission of infective spores through contact and hygienic care (group members never living together with an exposed worker: 0 out of 7 dead group members; group members always living together with an exposed worker: 4 out of 20 deatd group members; group members living together

with an exposed worker for 24h after exposure: 3 out of 5 dead group members; group members living together with an exposed worker 24h after exposure: 1 out of 4 dead group members).

Experiment 3: Worker-brood allo-grooming behaviour displayed toward exposed larvae and pupae

Behavioural observations for 30min. during the initial 1.5h after exposure revealed that workers groomed exposed pupae and exposed larvae for 13% respectively 12% of the possible time for behavioural display. Hygienic behaviour towards fungus exposed larvae and pupae did neither differ in the number of worker-brood allo-grooming bouts, the duration of grooming bouts, nor the duration per allo-grooming bout (number of grooming bouts: $t = -0.2573$, d. f. = 18.303, $p = 0.8$; duration grooming bouts: $t = 0.1683$, d. f. = 19.68, $p = 0.868$; duration per grooming bout: $t = 1.541$, d. f. = 21.791, $p = 0.138$).

Experiment 4: Effect of worker allo-grooming on the removal of spores from live spore and UV-killed spore exposed brood

On *UV-killed spore exposed* pupae and larvae spore load was lower in the presence of workers compared to worker absence at 5h after exposure (29% and 61% on pupae and larvae respectively, Table 1, Fig. 4 B) and at 24h after exposure (74% and 99% on pupae and larvae respectively, Table 1, Fig. 4 B) (GLM worker effect: larvae: LR $\chi_1^2 = 58.583$, $p < 0.001$; pupae: LR $\chi_1^2 = 30.724$, $p < 0.001$), indicating spore removal due to worker-brood allo-grooming. We also found a significant time effect with spore load decreasing between 5h and 24h past exposure also in the absence of workers on the surface of the brood (21% and 26% on larvae and pupae respectively, Table 1, Fig. 4 B) (GLM time effect: larvae: LR $\chi_1^2 = 40.939$, $p < 0.001$; pupae: LR $\chi_1^2 = 28.151$, $p < 0.001$), although UV-killed spores should not be able to attach to the surface of the brood. The difference in spore load on the surface of brood between worker presence and worker absence increased significantly from 5h to 24h after spore exposure (GLM worker x time interaction: larvae: LR $\chi_1^2 = 35.204$, $p < 0.001$; pupae: LR $\chi_1^2 = 30.704$, $p < 0.001$), indicating that spore removal through allo-grooming increased over time. However, as we could only assess the spore load on UV-killed spore exposed larvae in the presence of workers 24h past exposure from 2 Petri dishes (see also Table 1) caution must be taken in the interpretation and statistical significance of the results for spore load on larvae.

On *live spore exposed* pupae and larvae spore load was also lower in the presence of workers compared to worker absence at 5h after exposure (50% and 82% on pupae and larvae respectively, Table 1, Fig. 4 A) and at 24h after exposure (51% and 11% on pupae and larvae respectively, Table 1, Fig. 4A) (GLM worker effect: larvae: LR $\chi_1^2 = 31.35$, $p < 0.001$; pupae: LR $\chi_1^2 = 22.938$, $p < 0.001$), indicating spore removal due to worker-brood allo-grooming. We also found a significant time effect with spore load decreasing between 5h and 24h past exposure also in the absence of workers on the surface of the brood (69% and 98% on pupae and larvae respectively, Table 1, Fig. 4 A) (GLM time effect: larvae: LR $\chi_1^2 = 342.55$, $p < 0.001$; pupae: LR $\chi_1^2 = 62.39$, $p < 0.001$), indicating a strong

attachment of the fungus on the surface of the brood over time. However in the presence of spore attachment the difference of spore load on the surface of the brood between worker presence and worker absence was unchanged between 5h and 24h after spore exposure on pupae, whereas it decreased significantly on larvae (GLM worker x time interaction: larvae: LR $\chi_1^2 = 19.02$, $p < 0.001$; pupae: LR $\chi_1^2 = 0.006$, $p < 0.937$), indicating that fungal attachment to the surface might counteract the effectiveness of allo-grooming on spore removal.

Experiment 5: Impact of worker absence compared to the presence of workers for 24h after exposure on fungal growth appearance on exposed brood

Worker presence for 24h after exposure resulted in both larvae and pupae in a delay of fungal growth compared to worker absence after exposure (Fig. 5 A, B) (mixed-effects Cox proportional regression, larvae: IPL $\chi^2 = 101.54$, d. f. = 3, N = 255, $p < 0.001$; pupae: IPL $\chi^2 = 62.96$, d. f. = 3, N = 195, $p < 0.001$). In the presence of workers 81% of exposed larvae (146 out of 180; most of them on a replicated Petri dish basis, e. g. all 3 brood items in one Petri dish) were dead after 24h. Both dead larvae as well as still living larvae after 24h of worker presence showed a delayed fungal growth appearance compared to larvae maintained in worker absence (death larvae: IPL $\chi^2 = 79.34$, d. f. = 3, N = 221, $p < 0.001$; living larvae: IPL $\chi^2 = 68.56$, d. f. = 3, N = 109, $p < 0.001$). However dead larvae showed a significantly sooner appearance of fungal growth compared to still living larvae with worker presence during 24h after exposure (Fig. 6; IPL $\chi^2 = 18.93$, d. f. = 3, N = 180, $p = 0.003$).

Experiment 6: Impact of hygienic worker care on the hatching rate of live spore or UV-killed spore exposed pupae

The hatching rate (Table 2) of exposed pupae was significantly affected by type of exposure (Global $\chi^2$ on mixed-effects Cox proportional regression = 23.14, d. f. = 2, N = 240, $p < 0.001$). High dose live spore exposure and low dose live spore exposure resulted in a significantly lower hatching rate compared to the UV-killed spore exposure (Post hoc comparisons: $p < 0.001$ for both), but hatching rate was not different between doses of live spore exposure ($p = 0.86$). Moreover from the 29 hatched pupae of the high live spore exposure 24 died with a median of 2 days after hatching (low dose exposure: 16 of 30, median death after hatching: 2 days), whereas only 2 of the 98 hatched pupae in the UV spore exposure died after hatching (Table 2). All hatched callow workers, except one, that died after hatching showed fungal growth upon high dose live spore exposure (low dose exposure: all hatched callow workers). This was also the case when exposed pupae were singly put together with a worker (10 out of 60 pupae hatched; 7 of the hatched callow workers died with a median of 2 days after hatching and 5 of these 7 workers showed fungal growth), indicating that callow death due to *Metarhizium* infection was not primarily caused through contact with other exposed pupae in the Petri dishes.

During the course of these experiments we could observe also another behaviour which has not been observed before (Table 2). 76% (91 out of 120) of the exposed pupae in the high dose live spore exposure did not hatch. Only half of these (43 out of 91) showed fungal growth during the course of the experiment whereas the others (48 out of 91) were prematurely unpacked from their silk cocoon envelope by the present adult workers. In the low dose live spore exposure 70% (79 out of 113) of the pupae did not hatch and here also only around half of them showed fungal growth (35 out of 79) whereas the other half was prematurely unpacked (44 out of 79). Upon being unpacked these pupae were dead but did still not show any visible signs of fungal growth. Surface sterilization and thereafter monitoring possible *Metarhizium* appearance for three weeks however revealed that all, except one, showed fungal growth in the high dose exposure and all except two in the low dose exposure. Interestingly, plotting the time course of this behaviour together with the time course of fungal growth appearance on pupae that were not cared for by workers for both high and low dose live spore exposure shows that the behaviour was predominantly performed in advance of fungal growth (Fig. 7).

# Figures and Tables

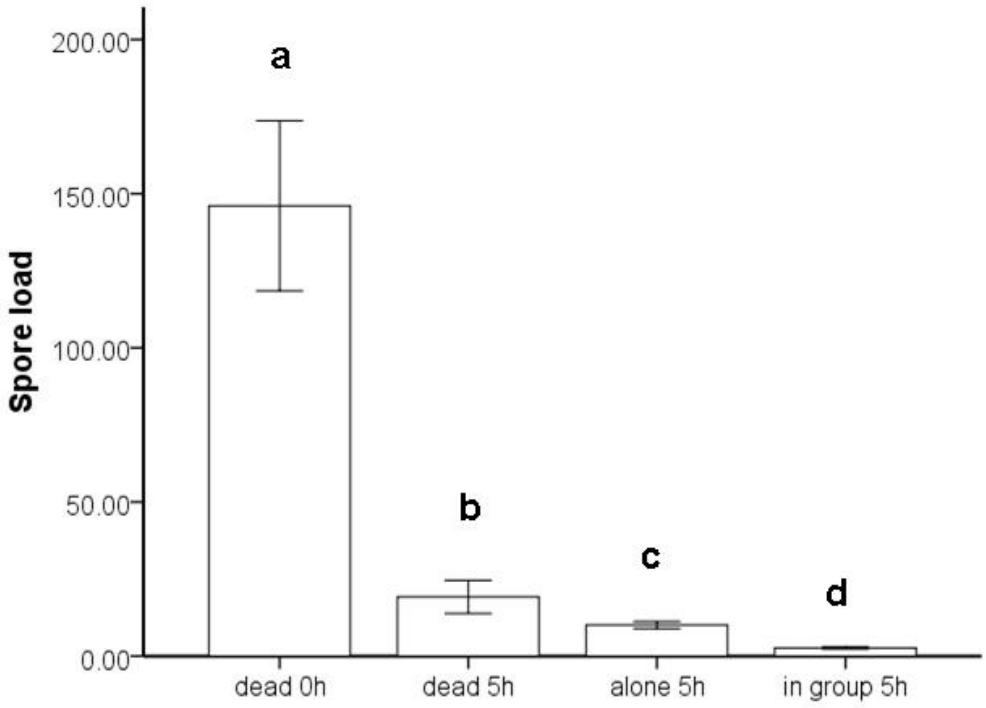


**Fig. 1:** Mean +- s. e. spore load on dead workers at 0h = 10min. (n = 12) and 5h (n = 12) after live spore exposure and on workers maintained either alone (n = 20) or in a group with two other workers (n = 19) 5h after live spore exposure. Small letters denote statistical significant differences at $\alpha < 0.05$. Although fungal spore exposure resulted in a significant decrease of the total spore load on dead workers between 0h and 5h presumably due to spore attachment, spore load on workers that were maintained alone and could self-groom and on workers that were maintained in a group and could self-groom and experience allo-grooming was significantly lower than on dead workers which were dead and thus not able to groom 5h after exposure. Spore load on exposed workers in a group was also significantly lower than on workers maintained alone.

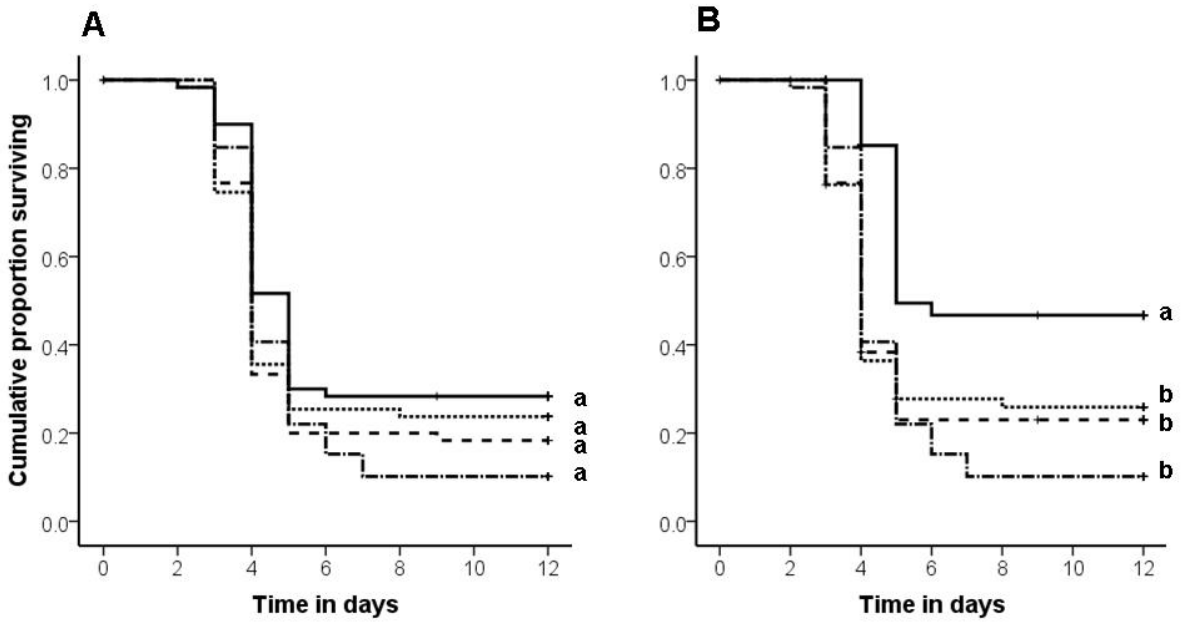


**Fig. 2**: Cumulative proportion survival of live spore exposed workers that were either kept isolated after fungal exposure (dash-dotted line), kept isolated after exposure for 24h and then put to two other workers for the rest of the experiment (dashed line), kept with two other workers for the first 24h after exposure and then isolated for the rest of the experiment (dotted line) or always kept with two other workers after exposure (solid line). A: General survival of workers. B: Survival of workers that died of a *Metarhizium* infection. Whereas the general survival of exposed workers was not affected by the isolation treatments (A), significantly fewer exposed workers died from a *Metarhizium* infection when introduced in a group directly after exposure (B). Small letters denote statistical significant differences at $\alpha < 0.05$.

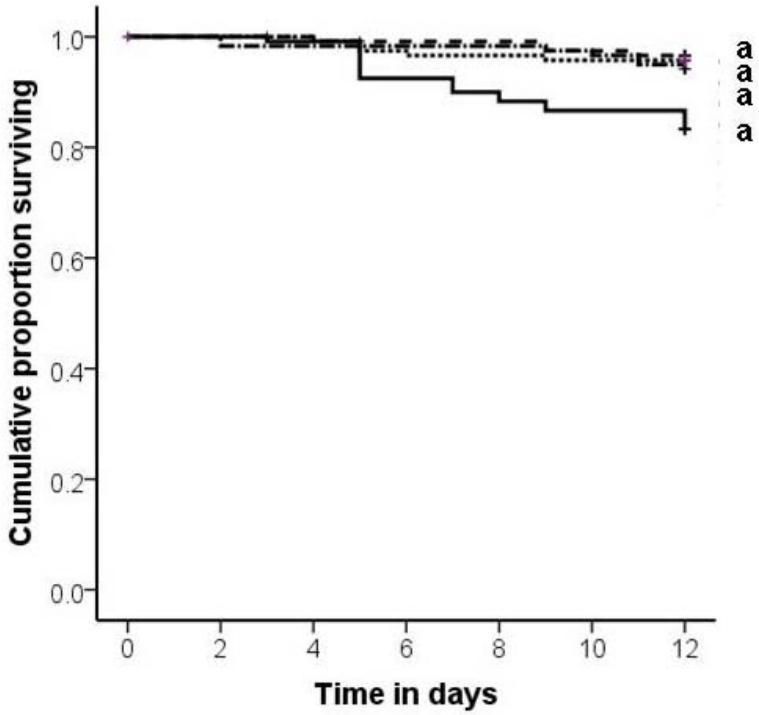


**Fig. 3**: Cumulative proportion survival of workers that never lived with a live spore exposed worker (dash-dotted line), that lived with a live spore exposed worker only after the exposed worker was isolated from them for 24h after exposure (dashed line), that lived with a live spore exposed worker only for the first 24h after exposure (dotted line) or that lived with a live spore exposed worker over the whole course of the experiment (solid line). There was no difference in survival between the four groups (i.e. all belonged to the same significance group assumuming statistical differences upon $\alpha < 0.05$).

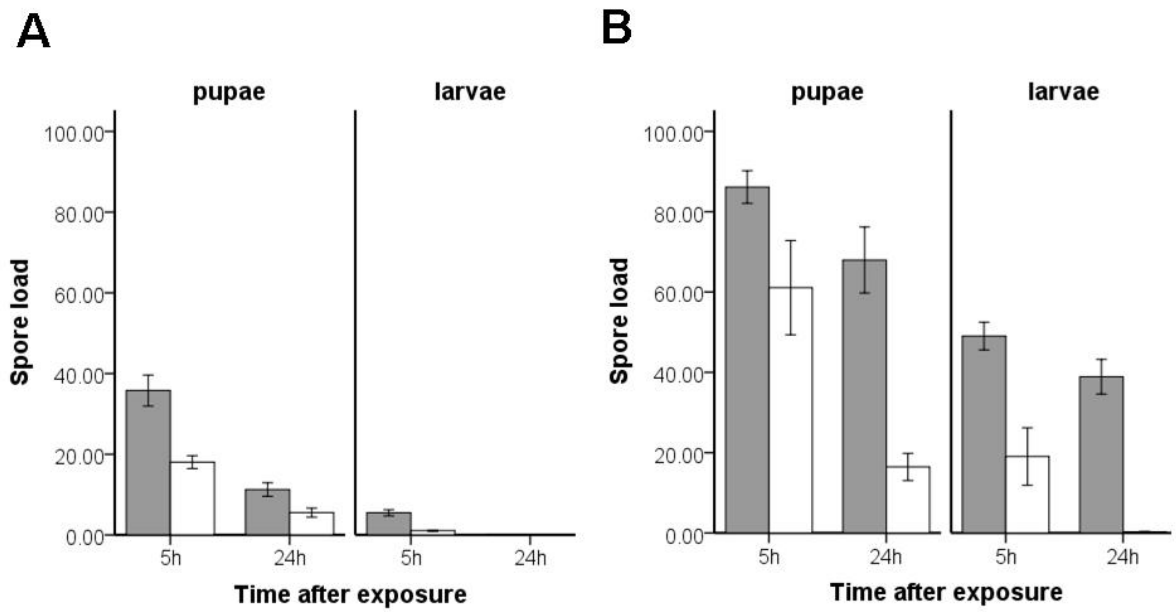


**Fig. 4:** Mean +- s. e. spore load on live spore exposed (A) and UV-killed spore exposed (B) pupae and larvae in the absence (grey bars) and presence (white bars) of workers for 5h and 24h after exposure. For the sample size of the different treatment groups refer to Table 2. The spore load on exposed brood in the presence of workers was lower than in the absence of workers at 5h and 24h past exposure indicating spore removal due to allo-grooming. Whereas this difference in total spore load between worker presence and absence increases on UV-killed spore exposed brood at 24h past exposure compared to 5h past exposure, the difference remains the same or decreases on live spore exposed brood, indicating that fungal pathogenesis limits spore removal through allo-grooming.

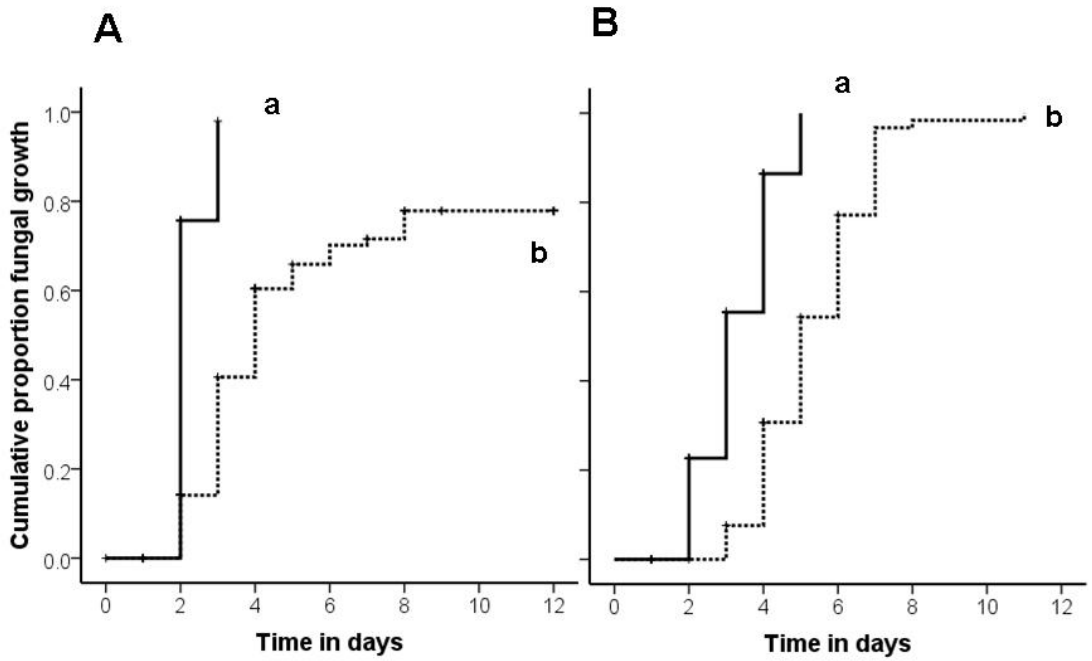


**Fig. 5**: Cumulative proportion of fungal growth on live spore exposed larvae (A) and pupae (B) int the absence of workers (black line) and in the presence of workers during the first 24h after exposure (dotted line). Worker presence results in both larvae and pupae in a delay of fungal outgrowth. Small letters denote statistical significant differences at $\alpha < 0.05$.

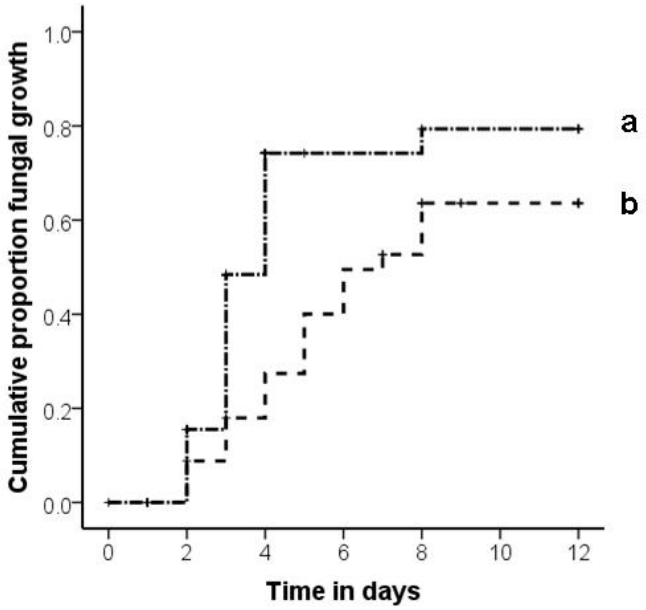


**Fig. 6:** Cumulative proportion of fungal growth on larvae that were dead (dash-pointed line) or still living (dashed line) after worker presence for 24h past exposure. Fungal outgrowth on dead larvae appeared significantly sooner and at a higher proportion than outgrowth on living larvae. Small letters denote statistical significant differences at $\alpha < 0.05$.

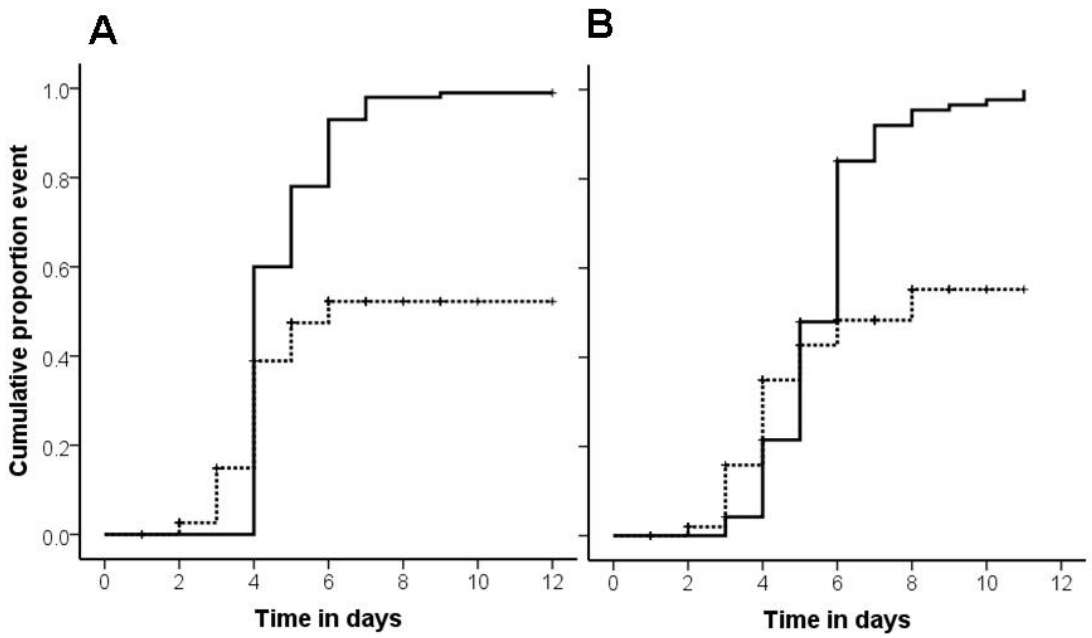


**Fig. 7**: Cumulative proportion of pupae that were prematurely unpacked from their silk cocoon enclosure (dotted line) in the presence of workers plotted against the cumulative proportion of fungal growth appearance (black line) in the absence of workers upon exposure of pupae to a high dose (1 X 10^9 spores/ml) (A) or a low dose (2 X 10^7) (B) spore suspension. The worker behaviour of unpacking the pupae from their cocoon was at both exposure treatments predominantly performed in advance before visible fungal growth would appear on pupae in the absence of workers.

**Table 1**: Overview of the spore load on the surface of larvae and pupae either exposed to a live spore suspension or a UV-killed spore suspension at 5h and 24h past exposure in the presence and absence of two workers obtained in experiment 4 of this chapter. On the right hand side of the table the mean spore load is presented, with the sample size mean values are based on in brackets. On the left hand side of the table the percentage spore difference between setups containing two adult workers compared to setups containing no workers at 5h and 24h after spore exposure is given. Furthermore the percentage spore difference from 5h to 24h in the worker absent and present condition is depicted. Percentages are based on the mean values given on the left hand side of the table.



**Mean spore load for the time points**

| live spores | | 5h | | 24h | |
|---|---|---|---|---|---|
| | worker | *absent* | *present* | *absent* | *present* |
| | pupae | 35.8 (16) | 18 (16) | 11.25 (16) | 5.5 (16) |
| | larvae | 5.5 (11) | 1 (12) | 0.09 (13) | 0.08 (10) |
| UV-killed spores | | | | | |
| | worker | *absent* | *present* | *absent* | *present* |
| | pupae | 86.1 (15) | 61.1 (13) | 64.1 (16) | 16.4 (16) |
| | larvae | 49 (14) | 19 (9) | 38.9 (13) | 0.3 (2) |

**Percentage spore difference**

| worker absent to present | | 5h to 24h | |
|---|---|---|---|
| *5h* | *24h* | *worker absent* | *worker present* |
| 50 | 51 | 69 | 69 |
| 82 | 11 | 98 | 92 |
| 29 | 74 | 26 | 73 |
| 61 | 99 | 21 | 98 |

**Table 2:** Summary of the hatching rate, the *Metarhizium* outgrowth and the behaviour of unpacking pupae from their silk cocoon from live spore exposed or UV-killed spore exposed pupae obtained in experiment 6. The percentages are calculated based upon the raw data in brackets. Live spore exposed pupae show a much lower hatching rate (Callows) than UV-killed spore exposed pupae. Furthermore a high number of callows in the live spore exposure died shortly after hatching, most of them of a confirmed *Metarhizium* infection. Pupae that did not hatch showed either fungal growth or were prematurely unpacked from their silk cocoon enclosure.

| | Live spores | | UV killed spores |
|---|---|---|---|
| exposure dose | high | low | high |
| **Callows** | | | |
| Hatched | 24% (29/120) | 25% (30/120) | 82% (98/120) |
| Death after hatching | 83% (24/29) | 53% (16/30) | 2% (2/98) |
| *Metarhizium* death after hatching | 96% (23/24) | 88% (14/16) | ---- |
| **Pupae** | | | |
| Death | 76% (91/120) | 70% (79/113) | 4% (5/120) |
| *Metarhizium* death | 47% (43/91) | 44% (35/79) | ---- |
| Premature unpacked | 53% (48/91) | 56% (44/79) | 4% (5/120) |
| *Metarhizium* on premature unpacked | 98% (47/48) | 95% (42/44) | ---- |

# Discussion

In the first part of this chapter we investigated the effect of allo-grooming on spore removal and survival of fungus exposed workers in the ant *Lasius neglectus.*

In experiment 1 we found that spore load on no grooming control workers was lowered by 87% between 0h and 5h after exposure indicating that a high proportion of fungal spores attached to the cuticle of exposed workers during this time period (Fig. 1, Boucias and Pendland 1991). Nonetheless spore load was lower in the presence of grooming behaviour 5h past exposure, with the combination of self- and allo-grooming in a group resulting in an increased removal of spores compared to self-grooming alone (Fig. 1). These results are in line with other studies on the removal of spores through grooming behaviour (ants: Oi and Perreira 1993; Hughes et al. 2002, Reber et al. 2011; termites: Yanagawa and Shimizu 2007) and suggest a potentially added benefit through allo-grooming for exposed individuals maintained in a group.

Several studies could already show that the survival of fungus exposed individuals is higher when maintained in a group than when maintained in isolation (termites: Rosengaus et al. 1998b, Yanagawa and Shimizu 2007; ants: Hughes et al. 2002, Okuno et al. 2011). In experiment 2 we also found that living in a group directly after exposure significantly decreases death due to *Metarhizium* infection compared to workers that were isolated after exposure (Fig. 2 B). As the fungus attaches to the host cuticle and starts to penetrate it within 24-48h to reach the host hemocoel (Boucias and Pendland 1991) the time period grooming can be effective at spore removal is limited through fungal pathogenesis. Concordantly, introducing exposed workers for 2.5h in a group of workers after an isolation period of 24h past exposure did not result in a different survival of exposed workers compared to exposed workers always kept in isolation in *Formica selysi* (Reber et al. 2011). Also in *Acromyrmex ecinatior* exposed workers introduced in a group directly after exposure survived better than workers introduced in a group 48h after isolation although allo-grooming frequency was not different after introduction (Walker and Hughes 2009). Similarly to the study in *Formica selysi* we found that the introduction of an exposed worker into a group after isolation of 24h past exposure did not result in less workers dying of a *Metarhizium* infection compared to always isolated workers and similarly to the study in *Acromyrmex ecinatior* death due to *Metarhizium* infection was significantly lower in workers introduced into the group directly after exposure than workers introduced only after 24h of isolation past exposure. However we also found that living in a group during the first 24h after exposure and isolation thereafter did not result in less workers dying due to *Metarhizium* compared to complete isolation or an introduction 24h past exposure. In our experimental setup only exposed workers living in a group all the time after exposure died less from a *Metarhizium* infection. Our limited experimental setup suggests that spore removal due to allo-grooming in a group impacts only marginally on the survival of exposed individuals and other factors associated with grooming (see also

chapter III) or group living must play an important role. It has for example been shown that *Camponotus pennsylvanicus* ants transmit antibiotic substances during trophallaxis (Hamilton et al. 2010) which could help the diseased individual also after penetration of the cuticle during fungal pathogenesis. On the other hand group size could influence the amount of spore removal through experienced allo-grooming and thus survival, however survival has not been found to be dependent upon group size in *Acromyrmex ecinatior* ants kept in groups of three or six after exposure (Hughes et al. 2002) and also not in *Formica selysi* ants with exposed workers introduced in small (three workers) or large groups (12 workers) after an isolation period of 24h past exposure (Reber et al. 2011).
Group members living together with an exposed worker did not differ significantly in their survival depending upon time period spent with the exposed worker. Only group members living together with an exposed worker all the time had a four times higher hazard of dying than group members never living with the exposed worker, possible indicating a contraction of infectious particles during hygienic care (Konrad et al. 2011, in preparation). However this can not explain why the group members living together with exposed workers during the critical 24h after exposure only had a 0.7 increased hazard of dying. Further experiments will need to address the nature of this phenomenon and if it might be connected to the increased resistance of group members after contact with a fungus exposed worker found in this species (Ugelvig and Cremer 2007). Almost none of the workers that had contact to the fungus exposed individual in our experiment died itself by contraction of the disease. A low transmission rate through hygienic care of fungus exposed workers and brood has also been observed in several other studies (Hughes et al. 2002; Rosengaus et al.1998b, Ugelvig et al 2010), indicating that workers are able to inactive removed fungal particles (Jaccoud et al. 1999, Fernández-Marín 2006).

In the second part of this chapter we extended our investigations on the allo-grooming behaviour display in the ant *Lasius neglectus* to grooming behaviour directed towards brood.
We first found in experiment 3 that upon exposure of brood workers spent a considerable amount of time allo-grooming the brood without showing differences in their grooming behaviour toward larvae and pupae at the initial stage after infection. This is in agreement with the results obtained in chapter I of this thesis where we also were not able to find differences in the display of grooming behaviour between larvae and pupae over five days of observation upon exposure.
The presence of grooming workers resulted in a lower spore load on the surface of exposed brood compared to exposed brood that was held in the absence of workers in experiment 4. This was true at 5h and 24h past exposure in the absence of spore attachment upon UV-killed spores exposure and in the presence of spore attachment upon live spore exposure (Fig. 4, Table 1) and indicates that worker-brood allo-grooming is effective at spore removal from the surface of exposed brood. Surprisingly we found upon both UV-killed spore exposure and live spore exposure a lower spore load on brood between 5h and 24h in the absence of workers (between 21% and 26% upon UV-spore exposure and

68% and 98% upon live spore exposure, Table 1). As the fungus attaches to the surface during fungal pathogenesis (Clarkson and Charnley 1996) and thus less spores are likely to be washed off the surface of exposed brood over time this was expected for live spore exposed brood but unexpected upon UV-killed spore exposure. Either dead UV-killed spores fall off the surface over time as they cannot attach or dead spores are able to attach partly to the surface as the first stages in conidial attachment to the surface of the cuticle are mediated via non-specific hydrophobic interactions between conidial spores and the insect cuticle (Boucias and Pendland 1991). Nonetheless the reduction of spore load over time in the absence of workers was much higher on live spore exposed brood than it was on UV-killed spore exposed brood (Table 1) suggesting a strong attachment to the surface of the brood upon live spore exposure. Interestingly the observed difference in spore load between worker presence and absence further increased from 5h to 24h on UV-killed spore exposed brood, but it either remained the same or even decreased on live spore exposed brood from 5h to 24h past exposure (Table 1). This suggests that the effectiveness of worker-brood allo-grooming on spore removal increases over time on UV-killed spore exposed brood but that it is likely counteracted due to fungal pathogenesis upon live spore exposure. As in experiment 1 and 2 with exposed workers fungal pathogenesis seems to set limits to the time grooming can be effective at spore removal making it possibly even less effective over time. Differences regarding spore load between larvae and pupae were not analysed statistically as the amount of spores applied to the brood might be different due to size differences between larvae and pupae. However the higher attachment of spores to exposed larvae compared to pupae over time in the absence of workers and the higher effectiveness of spore removal over time on pupae compared to larvae in the presence of workers at 24h past exposure might in part explain the results in chapter I of this thesis where we found that the silk cocoon around pupae interferes with fungal pathogenesis and most likely also serves a protective function.

Despite the apparent limits of worker-brood allo-grooming on spore removal set through fungal pathogenesis, we found that worker presence for the first 24h after exposure significantly delayed fungal growth appearance on as well larvae as pupae in experiment 5 (Fig. 6). This underlines the importance of behavioural defences provided by workers for the brood. Although we found that larvae were groomed at the initial stages after spore application in experiment 3 and that this resulted in a lower spore load in experiment 4 a large proportion of larvae was dead in the presence of workers 24h past exposure in this experiment 5. Larval death has already been observed in experiment 4 but there spores from dead larvae could not be washed off. In this experiment we found a significant delay of fungal growth also when comparing only dead larvae after worker presence for 24h to larvae maintained in the absence of workers. This suggests that death larvae are also likely cared for but die at some point after exposure in the presence of workers. Dead larvae also showed a higher and sooner fungal growth compared to still living larvae in the presence of workers for 24h past exposure (Fig. 7). Either worker care is stopped after larval death or the still functioning innate immune system of living larvae provides an added benefit in the fight against fungi over dead larvae. The death of larvae can

not be attributed to the treatment with spore suspension as no larvae were dead in the absence of workers 24h past exposure. At this point we cannot determine whether the death of larvae is caused by an active behaviour (killing) or whether it is a consequence of adult worker care. Further experiments including behavioural observations at different time periods after exposure will have to determine the nature of larval death in the presence of workers. Intensive grooming on the limit of biting has apparently also been observed in *Cardiocondyla* ants upon larval exposure to *Metarhizium* (Ugelvig and Cremer, unpublished results). Larval death might therefore not just simply represent an artefact due to experimental conditions in this experiment.

Although hygienic care including allo-grooming provided by workers resulted in a significantly lower and delayed fungal growth appearance on pupae in experiment 5 we found in experiment 6 that less callow workers hatched from live spore exposed pupae than from UV-killed spore exposed pupae (Table 2). The lowered hatching rate was not due to the possibility that workers are not able to cope with the amount of spores as even a much lower dose yielded the same result. Moreover almost all callow workers hatching from live spore exposed pupae died with a median of 2 days after hatching from a *Metarhizium* infection. A contraction of the disease from other exposed pupae in the experimental Petri dishes would be a possible explanation, however hatched callow workers also died from a *Metarhizium* infection when they were singly put together with one worker in an experimental Petri dish (Table 2). This indicates that spores are able to penetrate the silk cocoon-enclosure and infect the pupae inside. In a study on the lesser spruce sawfly it was not possible to infect pupae through their silk cocoon-enclosure with *Metarhizium* though (Führer et al. 2001). Our data thus possibly suggest basic differences between the silk cocoon-enclosure across hymenoptera. Taken together the death of callow workers from a *Metarhizium* infection in our experiment indicates that although worker care can lower the amount of spores on pupae through grooming, even a low amount of spores is sufficient to penetrate the silk cocoon-enclosure and infect callow workers. This contrasts the findings of a potentially protective function of the cocoon-enclosure around pupae obtained in chapter I of this thesis. Further experiments will need to determine a clear beneficial effect of the silk cocoon-enclosure upon fungal pathogen exposure.

During the course of this experiment we could also observe a new behaviour, the unpacking of exposed pupae, which has not been described before. This behaviour has gone unnoticed in the first chapter of this thesis which might be attributable to the fact that behavioural observations were conducted blind with respect to pupal treatments. Directly after unpacking, pupae did not show visible fungal growth but developed fungal growth after having been surface sterilized and transferred to high humidity which is beneficial for fungal growth. Unpacked pupae therefore carried *Metarhizium* infections in their body at the time of unpacking. Interestingly, compared to the timeline of fungal growth appearance on pupae not cared for by workers this behaviour was predominantly performed before fungal growth would be visible on pupae in the absence of workers (Fig. 7). Together with the fact that this behaviour is only rarely expressed upon exposure with UV-killed spores (Table 2) this

indicates that the behaviour is part of the hygienic behaviour repertoire of *Lasius neglectus* ants. The function of this behaviour remains to be elucidated. Most likely it is part of the waste management. Unpacking of cocoons could lead to desiccation which is unfavourable for fungal growth. Some termites bite the legs off the corpses of their nestmates, thus killing infectious parasite stages by desiccating the cadaver (cited in Cremer et al. 2007). On the other hand unpacking of pupae labels them as dead and dead corpses get thrown on a rubbish pile usually located outside the nest (Howard and Tschinkel 1976, Hart and Ratnieks 2002). This behaviour might proof to be important to limit disease spread in the colony as brood gets usually piled in the nests of ants (Ugelvig et al. 2010, chapter I) and thus transmission is likely higher (chapter I) whereas it is known that exposed (Ugelvig and Cremer 2007) and diseased adult individuals close to death often leave the nest (Heinze and Walter 2010). This behaviour also resembles remarkably the description of hygienic behaviour in honeybees, which also uncap the brood cells from American foulbrood infested larvae (Rothenbuhler and Thompson 1956).

Taken together the results obtained in this chapter indicate that allo-grooming of workers and brood is effective at spore removal but severely limited through fungal pathogenesis. Spore removal through grooming is unlikely to contribute significantly to the increased survival of exposed individuals living in a group. Furthermore we could show that although spore removal through allo-grooming is likely to be responsible for a delay of fungal growth on brood it cannot prevent together with other hygienic care the infection of pupal cocoons. We also found that a large proportion of larvae were dead in the presence of workers after 24h. Furthermore we found the expression of a new behaviour directed towards live fungus exposed pupae: the premature unpacking of pupal cocoons. Whereas the premature unpacking of pupal cocoons is likely to be part of the hygienic behaviour repertoire of the ants the nature of larval death needs further investigations.

# Acknowledgements

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# Chapter III

# A novel function for an old behaviour: The use of antiseptic substances during hygienic brood care


## Abstract

The use of antimicrobial substances is commonly found in insect societies and an important addition to behavioural defences. Here we explore the possible use of antimycotic substances during hygienic brood care in the invasive garden ant *Lasius neglectus* when brood is exposed to the entomopathogenic fungus *Metarhizium anisopliae*. We will first show that during care of exposed brood fungal particles are not only mechanically removed due to worker-brood allo-grooming but in addition also fought with chemical weaponry using antimycotic substances. Sealing of diverse body openings will reveal that the site of production of these substances resides in the gaster involving the poison reservoir. Finally, behavioural observations of hygienic brood care will show how these substances from the gaster are applied on the brood, assigning the abdominal self-grooming behaviour of workers a new role during hygienic brood care.


This work will be published with additional behavioural and chemical data obtained by Vanessa Barone and Matthias Konrad, and Barbara Mitteregger respectively (all IST Austria).

# Introduction

Parasites are a common threat to all organisms including social insects. Social insects are thought to be especially vulnerable as they often live in high densities and have frequent interaction rates increasing the transmission risk (Alexander 1974, Côte and Poulin 1995).

As one line of defence insect societies employ antimicrobial substances against parasites and invading pathogens. In wasps and fungus-growing ants antimicrobial defences are linked to social complexity (Hoggard et al. 2011, Hughes et al. 2008) and in bees the evolution of sociality was suggested to be accompanied by the evolution of stronger antimicrobial compounds (Stow et al. 2007).

Antimicrobial substances used in insect societies can be derived from the environment. *Formica* ants are known for their intake of tree resin into the nest which inhibits the growth of bacteria and fungi in the nest material (Christe et al. 2003) and protects themselves (Chapuisat et al. 2007). Similarly, honey bees use propolis, a resinous substance collected from plants, for construction and maintenance of their hives with broad antimicrobial properties (Marcucci 1995). In fungus-growing ants antimicrobial substances are also provided through symbiosis with bacteria and used to protect their fungus-gardens (Currie et al. 1999). But social insects also produce themselves a plethora of chemical substances with antimicrobial activity in exocrine glands (ants: reviewed in Attygale and Morgan 1984, Schlüns and Crozier 2009; wasps: Park et al. 1995, Gambino 1993, Turillazzi et al. 2004, termites: Rosengaus et al. 2000, 2004, Hamilton et al. 2011), which are then used in a variety of contexts, such as for example nest hygiene (Chen et al. 1998, Rosengaus et al. 1998a), the protection of food in bees (Fujiwara et al. 1990) or the protection of the brood (Obin and Vander Meer 1985, Vander Meer 1995).

The use of antiseptic substances complements other cooperative social defences employed in insect societies (Cremer et al. 2007) such as allogrooming of exposed nest-mates and brood by which particles are removed (Oi and Pereira 1993, chapter II of this thesis). In fungus-growing ants the use of antiseptic compounds from the metapleural gland (Maschwitz et al. 1970) goes hand in hand with hygienic grooming behaviour inhibiting removed fungal particles in the infrabuccal pockets of the ants and thus limiting autoinfection (Fernández-Marín et al. 2006). The use of antiseptic substances and other behavioural defences are therefore not mutually exclusive but expected to interact in various ways.

Here we investigate a possible use of antimycotic substances during hygienic brood care in the invasive garden ant *Lasius neglectus* by exposing brood to the entomopathogenic fungus *Metarhizium anisopliae*. This fungus infects insects by penetration of the cuticle to reach the host hemocoel (Clarkson and Charnley 1996). In chapter II of this thesis we have already shown that worker-brood allogrooming can reduce the spore load on the surface of the brood. Here we will first show that hygienic brood care does not only involve the mechanical removal of spores but also the use of antimycotic substances. Thereafter we will explore the origin of these substances and their possible

route of application through sealing of diverse body openings and behavioural observations. We will reveal that the employed substances are most likely taken up from the gaster into the mouth during abdominal self-grooming of workers and are then applied on the surface of exposed brood during allo-grooming.

# Materials and Methods

General methodology

Brood, queens and adult workers of the invasive garden ant *Lasius neglectus* were collected in the years 2008 to 2010 from the population at the botanical garden in Jena (Espadaler and Bernal 2011). Stock colonies from these collections, with several thousand workers, were housed in large plastic boxes with plaster ground and maintained at a diet of honey and cockroaches. At the time of performing the experiments all used stock colonies were kept in summer condition with temperatures of 27/21°C and a 14/10h day/night cycle.

During experimental conditions adult workers and brood were kept in experimental nests (Petri dishes, diameter 5 cm and plaster ground) at 12/12 hr day/night cycle and a constant temperature of 24°C. Brood and workers in experimental setups always originated from a single stock colony; however they never belonged to the same stock colony.

Two strains of the entomopathogenic fungus *Metarhizium anisopliae var. anisopliae* (strain ARSEF 2575 with GFP labelling Benomyl resistance (Fang et al. 2006), obtained from Bidochka M. J., Department of Biological Sciences, Brock University, Canada; and strain KVL 03-143, obtained from Eilenberg J., Faculty of Life Sciences, University of Copenhagen, Denmark) were grown on Malt extract agar (Merck) plates at 24°C. Strain KVL 03-143 was used if not stated otherwise. Prior to experiments conidiospores where harvested from fully sporulating plates using 0.01% Triton X-100 (Sigma-Aldrich) as solvent. Concentrations were quantified using a Neubauer-improved counting chamber and adjusted to a working concentration of 1 x $10^9$ spores/ml. Each spore suspension was plated out again and checked for germination capacity 14h later. Spore suspensions with germination <95% were not used. For the different experiments, different spore suspensions were used.

For fungal spore exposure brood was taken out of the stock colonies not more than 1h before exposure and placed in a Petri dish. It was then exposed by placing a single brood item in quantities of 0.5µl of the working spore suspension applied on Parafilm. Exposed brood in groups of four was then placed on a small piece of filter paper to let the spore solution briefly dry on the surface. Use of fungal spore exposed brood for experiments always followed directly after exposure.

Remaining fungal spores on the surface of exposed brood were washed off by vortexing all four brood items for 1 min in an Eppendorf vial containing 0.01% Triton X solution supplemented with 5ug/ml Benomyl and 50ug/ml Streptomycin (Sigma-Aldrich), thus removing all spores that did not adhere (Ment et al. 2010) or were not groomed off (Hughes et al. 2002, Reber et al. 2011).

Thereafter from each of the gained spore suspensions through washing 5 times 1µl spots were pipetted onto a malt extract agar plate. After 14-15h in the incubator at 24°C, 1-2 fields of vision per each pipetted µl spot were inspected at 200X or 500X magnification under a stereomicroscope for the

number of viable and non viable spores. Viable spores were defined as spores which produced a visible germ tube within this 14-15h. The spore load on the surface of exposed brood was calculated as the sum of viable and non viable spores per one field of vision, averaged over all five inspected 1µl spots from one washed off spore suspension. The viability was calculated as the proportion of viable spores on the total spore load in one field of vision, averaged over all five inspected 1µl spots.

Statistical analyses were performed in R version 2.13.0 (R Development Core Team 2011).

Experiment 1: Use of antimycotic substances during brood care through workers

To assess if antiseptic substances are employed by workers during brood care we re-analysed the live spore data with strain ARSEF 2575 obtained in experiment 4 of chapter II in more detail.

In this experiment we placed live fungal spore exposed last instar larvae or pupae either to two workers in experimental Petri dishes or in experimental Petri dishes without workers. After 5h and 24h we washed the spores off the surface of the brood, plated the gained spore suspensions out and assessed the spore load on the surface of exposed brood. This resulted in 16 replicates for all pupal washes and 12 replicates for larval washes at 5h in the presence of workers, 11 replicates for larval washes at 5h in the absence of workers, 10 replicates of larval washes at 24h in the presence of workers and 13 replicates of larval washes at 24h in the absence of workers.

While in chapter II we only analyzed spore load on exposed brood, now we analyzed the *viability of spores* by dividing spore load in the number of viable and non viable spores. Generalized Linear Models (GLMs) were constructed for live spore exposed pupae and larvae separately with quasibinomial errors to account for overdispersion, spore viability as response variable and time (5h or 24h), worker presence (absent or present) and their interaction as predictor variables. The significance of the predictor variables was assessed with a Likelihood-ratio (LR) $\chi^2$-test using the Anova command of the package "car" (Fox and Weisberg 2011) with type III sums of squares and orthogonal contrasts. For statistical use the response variable viability was created by concatenating the count of viable and non viable spores (Crawley 2007).

Experiment 2: Origin of antimycotic substances part I

With this setup of adult worker treatments we wanted to infer if potentially used antimycotic substances during hygienic brood care originate from the mouth, the metapleural gland or the acidopore.

Adult workers were taken out of the stock colonies, cold anesthetized for 1-3 min. on ice, fixed and their mouth, their metapleural gland openings or their acidopore opening sealed with a small droplet of superglue (UHU brand). Control workers without a seal received a small droplet of superglue on their gaster, i. e. they were non-sealed but control-glued (sham control worker presence). After the treatment always two concordantly treated adult workers were placed in one experimental nest. To

guarantee full recovery of treated workers, experiments started 3-5h after adult worker treatment by placing fungal spore exposed pupae in groups of four to the workers in the Petri dish. We also set up a worker absence control by placing fungal spore exposed pupae in Petri dishes containing no workers. After 24h fungal spores on the surface of exposed pupae were washed off and spore load and viability of spores assessed. Treated adult workers were stored at -20°C to check later if seals of body openings were still intact. As it was sometimes difficult to assess if seals were still intact, we may have not detected all broken seals.

The complete experimental setup was conducted once with the fungal strain KVL 03-143 and once with the strain ARSEF 2575. Replicates, with dead adult workers after 24h of pupal care, hatched callow workers from exposed brood and treated workers with apparently broken seals were excluded from the analysis. For the strain KVL 03-143 this resulted in 18 replicates for the sham control worker treatment, 17 replicates for metapleural gland openings sealed worker treatment, 16 replicates for acidopore opening sealed worker treatment, 12 for the mouth sealed worker treatment and 28 replicates for the worker absent control. For the strain ARSEF 2575 this resulted in 19 replicates for the control sealed workers treatment, 15 replicates for metapleural gland openings sealed worker treatment, 19 replicates for acidopore opening sealed worker treatment, 11 for the mouth sealed worker treatment and 20 replicates for the worker absent control.

For statistical analysis one factorial Generalized Linear Models (GLMs) were constructed for the variable *spore load* and *viability of spores*. Data from the two strains were analysed separately. In the case of count data for the response variable spore load negative binomial errors were used and for proportion data in the response variable viability of spores quasibinomial errors were used. For statistical analysis of the response variable viability we concatenated the count of viable and non viable spores (Crawley 2007). A global F-test and all pairwise comparisons between means in these models were carried out using the package "multcomp" (Hothorn 2008). The family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010).

Experiment 3: Attachment of fungal strains to pupae

To determine the strength of spore attachment between pupal exposure and pupal wash after 24h, we exposed pupae to either the strain KVL 03-143 or the strain ARSEF 2575 and placed them in groups of four in experimental Petri dishes with no workers. After 10 min. (= 0h) spores on the surface of exposed pupae were washed off and the total spore load assessed. For both strains 10 replicates were set up for the time point 0h.

We then compared the spore load on exposed pupae at 0h to the spore load on exposed pupae after 24h in the absence of workers. For the time point 24h we used the data gained from experiment 2 in the control worker absent setup (28 replicates for strain KVL 03-143 and 20 replicates for strain ARSEF 2575).

For statistical analysis we constructed a GLM with negative binomial errors, spore load as response variable and strain identity (KVL 03-143 or ARSEF 2575), time (0h or 24h) and their interaction as predictor variables. The significance of the predictor variables was assessed with a Likelihood-ratio (LR) $\chi^2$-test using the Anova command of the package "car" (Fox and Weisberg 2011) with type III sums of squares and orthogonal contrasts.

Experiment 4: Origin of antimycotic substances part II

To elucidate antimycotic substances possibly originating from the acidopore in more depth we created two different acidopore sealing treatments. In one treatment, acidopore sealed workers were confronted with fungus exposed pupae within one hour after sealing (=0h) and in the other treatment acidopore sealed workers were sealed 24h before confrontation with fungus exposed pupae (=24h). We also set up again a mouth sealed worker treatment, a sham control worker treatment and a worker absent control in the same fashion as described in experiment 2. As the acidopore is the poison projecting opening we created additionally a poison sac depletion treatment by briefly cold anesthetizing adult workers, fixing them and upon recovery annoying them by poking at their gaster until release of most of their poison sac content as a defence reaction. The experiment started again 3-5h after worker treatment, except for the two acidopore opening sealing treatments, by placing fungal spore exposed pupae in groups of four to the workers in the Petri dish. After 24h fungal spores on the surface of exposed pupae were washed off and spore load together with spore viability assessed. Treated adult workers were again stored at -20°C to check if sealings were still intact.

This resulted in 13 replicates for the sham control worker treatment, 14 replicates for the worker absent control, 11 replicates for the mouth sealed worker treatment, 10 replicates for the acidopore opening sealed worker treatment for 0h, 10 replicates for the acidopore opening sealed worker treatment for 24h and 14 replicates for the poison sac depletion worker treatment.

Statistical analysis of data was performed as in experiment 2.

Experiment 5: Incubation of gaster droplet and pure formic acid with fungal spore suspension

To determine the antimycotic activity of substances originating from the acidopore, we proceeded similarly as in experiment 4 for the poison sac depletion treatment, by taking out adult workers from the stock colony, briefly cold anesthetizing them, fixing them and upon recovery annoying them by poking at their gaster until release of their poison sac content as a defence reaction. 0.05µl of the produced gaster droplet was collected in capillaries and immediately flushed into 0.05% Triton X-100 in Eppendorf cups. The used capillaries were pulled from pre-made capillaries (Servoprax 50µl) over an ethanol lamp to a fine point, washed with acetone, coated with Sigmacoat (Sigma-Aldrich) and then calibrated using a UMPIII micropump with a Micro 4 controller (WPI) by placing a small paint dot on the mark. In total gaster droplets from 24 workers were obtained this way. The droplet from 12 workers was each flushed into 2.5µl Triton X (50 fold dilution) and used to create 12 replicated 100

and 250 fold dilution steps. The droplet from the other 12 workers was each flushed into 5µl Triton X (100 fold dilution) and used to create 12 replicated 500, 750 and 1000 fold dilution steps. Dilution steps were achieved by mixing different quantities of the base 50 and 100 fold dilutions of the gaster droplets with a the desired dilution corresponding quantity of 1 X $10^6$ concentrated spore suspension. As the main constituent of the poison sac in Formicinae is formic acid (Attygale and Morgan 1984), we also incubated at the same time pure formic acid (98-100%, Merck), diluted either 200, 1000 or 10000 fold with Triton X, with the spore suspension. Each mixture of spore suspension and formic acid was replicated five times at all three dilution steps. After one hour of incubation the mixtures of gaster droplet with spore suspension and the mixtures of pure formic acid with spore suspension were plated out and the viability of spores assessed as outlined before.

Experiment 6: Application of antimycotic substances and impact of worker treatment on worker-brood allo-grooming

To observe the possible use of antimycotic substances and to determine a possible impact of worker treatment on hygienic behaviour, adult worker treatments were created as mentioned before in experiment 2 and 4, i. e. we created sham control treated workers, performed sealing of the mouth, the metapleural gland openings, the acidopore opening and we depleted the poison sac content, on separate groups of workers. Thereafter we placed fungus exposed pupae in groups of four on a filter paper to two identically treated workers in experimental nests for 15 min. Then behavioural observation began for an observation period of 15 min., followed by a break of 30 min. and another 15 min observation period. Only the behavioural category of worker-brood allo grooming was assessed quantitatively. During the observation periods the number and duration of worker-brood allo-grooming bouts were recorded with the program BioLogic v2. In total 12 replicated setups per adult worker treatment were observed.

For statistical analysis the number and the total duration of all recorded grooming bouts was analysed over all 30 min. of observation. The duration per worker-brood allo-grooming bout during these 30 min. of observation was calculated as the total duration of all allo-grooming bouts divided by the number of observed bouts. All three variables, i. e. the number, the total duration and the duration per bout of were analysed separately using a one factorial ANOVA with adult worker treatment as predictor. A global F-test and all pairwise comparisons between means in these models were carried out using the package “multcomp” (Hothorn 2008). The family wise error rate when performing multiple comparisons was adjusted using the method of Westfall implemented in the package (Bretz et al. 2010).

# Results

Experiment 1: Use of antimycotic substances during worker care

In Chapter II (experiment 4) of this thesis we found that worker presence resulted in a reduction of the spore load on live spore exposed larvae and pupae compared to the absence of workers. A re-examination of the data splitting the already used variable spore load in viable and non viable spores revealed that worker presence also decreased the viability of spores on exposed larvae and pupae significantly compared to worker absence (Fig. 1) (GLM worker effect: pupae: LR $\chi_1^2 = 184.202$, $p < 0.001$; larvae: LR $\chi_1^2 = 21.368$, $p < 0.001$), thus indicating the use of antimycotic substances during worker care. Over time, the viability loss of spores in the presence of workers compared to worker absence increased significantly on pupae (from 20% at 5h to 75% at 24h) but not on larvae (from 23% at 5h to 32% at 24h) (GLM: worker x time interaction; pupae: LR $\chi_1^2 = 17.445$, $p < 0.001$; larvae: LR $\chi_1^2 = 0.545$, $p = 0.46$). In the absence of workers the viability of spores also decreased slightly on pupae over time (2% from 5h to 24h) and much more on larvae (54% from 5h to 24h) (GLM time effect: larvae: LR $\chi_1^2 = 51.062$, $p < 0.001$; pupae: LR $\chi_1^2 = 20.671$, $p < 0.001$).

Experiment 2: Origin of antimycotic substances part I

Sealing of the metapleural gland openings, the acidopore opening or the mouth allowed us to investigate a variety of sources for the effect of reduced viability of spores on pupae in the presence of adult workers when compared to sham control treated workers and a worker absence control.

We found a significant difference of *spore viability* on exposed pupae across adult worker treatments for both fungal strains (strain KVL 03-143: Fig. 2 A; Global F-test: $F_{4,86} = 23.93$, $p < 0.001$; strain ARSEF 2575: Fig. 3 A; Global F-test: $F_{4,79} = 31.26$, $p < 0.001$).

*Fungal strain KVL 03-143:* As in experiment 1, viability of spores was significantly lower in the presence of sham control treated workers than in the worker absence control (pairwise comparison: $p < 0.001$). Sealing of the metapleural gland openings resulted in the same loss of spore viability as with sham control treated workers ($p > 0.8$). Sealing of the acidopore opening or the mouth on the other hand, resulted in a increased spore viability compared to sham control workers ($p < 0.001$), with both not being different from the worker absent control, although the acidopore sealing only weakly (acidopore sealed vs. worker absent $p = 0.06$; mouth sealed vs. worker absent $p = 0.91$).

*Fungal strain ARSEF 2575:* Exposure of pupae to this strain resulted in the same results obtained with the strain KVL 03-143 barring one exception: the acidopore sealing resulted now in a significantly lower viability than the worker absence control ($p = 0.009$).

We also found a significant difference of *spore load* on exposed pupae across adult worker treatments for both fungal strains (KVL 03-143: Fig. 2 B; Global F-test: $F_{4,86} = 35.71$, $p < 0.001$; ARSEF 2575: Fig. 3 B; Global F-test: $F_{4,79} = 3.298$, $p < 0.015$).

*Fungal strain KVL 03-143:* Presence of metapleural glands sealed workers, acidopore sealed workers or sham control workers resulted in a significantly lower spore load on exposed pupae compared to both mouth sealed workers and worker absent control (all pairwise comparisons $p < 0.001$). Acidopore sealed worker presence resulted in an even lower spore load compared to sham control or metapleural gland sealed workers ($p < 0.03$ for both comparisons). Spore load on exposed pupae in the presence of mouth sealed workers was on the other hand not different to worker absence control ($p = 0.732$).

*Fungal strain ARSEF 2575:* In contrast to the strain KVL 03-143 only the presence of acidopore sealed workers resulted in a lower spore load on exposed pupae compared to worker absent control and sham control workers ($p < 0.02$ for both post hoc pairwise comparisons), whereas spore load was not different in all other treatments (p = n.s for all pairwise comparisons).

Experiment 3: Attachment of fungal strains to pupae

Spore load in the absence of workers on exposed pupae was significantly lowered between exposure and wash 24h thereafter (Fig. 4) (GLM: time effect: LR $\chi_1^2 = 344.8$, $p < 0.001$). Thereby spore load reduction over time was significantly smaller for the strain KVL 03-143 (74% from 0h to 24h) than for the strain ARSEF 2575 (93% from 0h to 24h) (GLM: time x strain interaction: LR $\chi_1^2 = 34.2$, $p < 0.001$), indicating a higher attachment of spores from the strain ARSEF 2575.

Experiment 4: Origin of antimycotic substances part II

The use of two different acidopore sealed worker groups (one that was used within one hour after sealing (=0h) and one only after the workers had their acidopore opening sealed for 24h) and a poison sac content depleted worker group together with a mouth sealed worker group, a sham control worker group and a worker absent control also resulted in a significant difference of spore viability on exposed pupae across groups (Fig 5 A; Global F-test: $F_{5,66} = 16.74$, $p < 0.001$). As in experiment 1 and 2 viability of spores was significantly lower in the presence of sham control treated workers than in the worker absence control (post hoc pairwise comparison: $p < 0.001$). The viability of spores on the surface of exposed pupae was significantly lower when acidopore sealed workers were confronted with the pupae within one hour after sealing as when they were confronted with the pupae only 24h after sealing ($p = 0.03$), with the viability of the 0h acidopore sealed group not being different from the sham control worker group ($p = 0.8$). The viability of spores in the 24h acidopore sealed worker group was not different than the viability in the mouth sealed worker group, although only weakly ($p = 0.0631$), and significantly lower than the viability in the worker absent group ($p < 0.001$). Confrontation of poison sac content depleted workers with fungus exposed pupae resulted in a

significantly higher viability of spores compared to sham control treated workers ($p = 0.001$), not different from the viability with mouth sealed workers ($p = 0.428$), but significantly lower than the worker absent control ($p = 0.02$). Mouth sealed worker group and worker absent control did not differ in their viability ($p = 0.134$).

Also in this experimental setup we found a significant difference of spore load on exposed pupae across adult worker treatments (Fig. 5 B; Global F-test: $F_{5,66} = 10.37$, $p < 0.001$). The presence of 0h and 24h acidopore sealed workers, poison sac depleted workers and sham control workers resulted in a lower spore load on exposed pupae compared to the presence of mouth sealed workers and worker absence control (all post hoc pairwise comparisons $p < 0.02$), the first four treatments and the last two treatments not being different amongst each other (all pairwise comparisons $p > 0.6$).

Experiment 5: Incubation of gaster droplet and pure formic acid with fungal spore suspension

Incubation of a gaster droplet, gained through annoying adult workers, with a fungal spore suspension in a dilution series ranging from 1000, 750, 500, 250 and 100 fold dilution resulted in a increasing loss of spore viability (Fig. 6). Also the incubation of pure formic acid, the main constituent of the formicine poison, with fungal spore suspension in a dilution series ranging from 10000 over 1000 to 200 fold resulted in a increasing loss of spore viability. Thereby the viability of spores in a 1000 fold dilution of pure formic acid corresponded to the viability of spores in a 250 - 500 fold dilution of a gaster droplet.

Experiment 6: Application of antimycotic substances and impact of worker treatment on worker-brood allo-grooming

Worker treatment, i. e. sealing of the mouth, the metapleural gland openings, the acidopore opening, sham control sealing and depletion of the poison sac content, did not have an influence on the number of displayed worker-brood allo-grooming bouts (ANOVA, Global F-test: $F_{4,55}=1.779$, $p=0.146$), nor the total duration of bouts (ANOVA, Global F-test: $F_{4,55}=2.298$, $p=0.070$) or the duration per grooming bout (ANOVA, Global F-test: $F_{4,55}=1.657$, $p=0.173$) during behavioural observation. Even mouth sealed workers, which were not capable of a successful allo-grooming, displayed vigorous cleaning attempts. Apart from worker-brood allo-grooming, workers displayed self-grooming behaviour frequently, which was however not quantified. A peculiar worker self-grooming behaviour in all treatment groups was the forward bending of the gaster between the legs and at the same time the downward bending of the head to the gaster tip.

# Figures and Tables

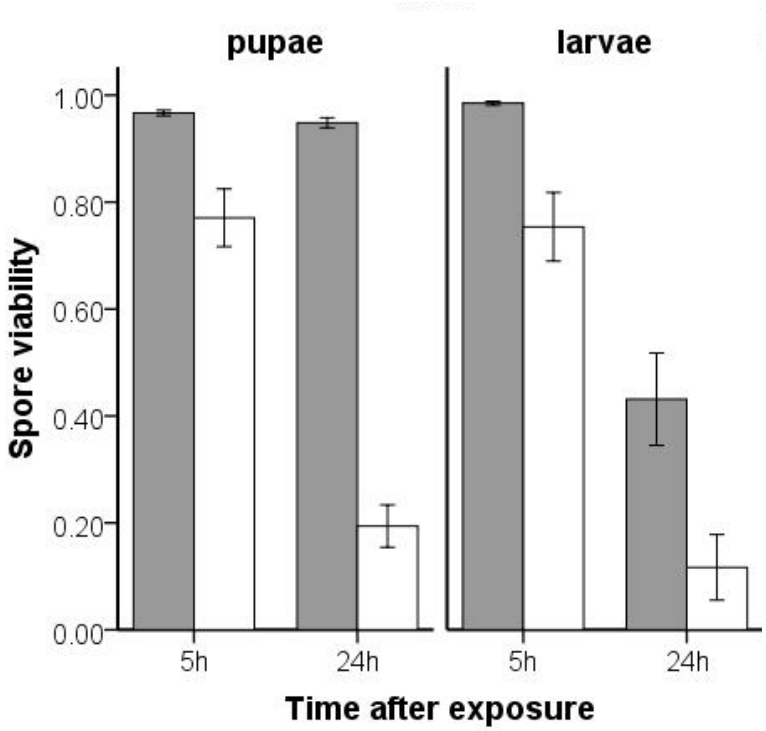


**Fig. 1**: Mean +- s. e. spore viability of spores washed off from live spore exposed pupae and larvae maintained in the absence (grey bars) or presence (white bars) of two workers for 5h and 24h after exposure. For the sample size of the different groups refer to material and methods experiment 1 in this chapter. On both pupae and larvae the viability of spores on the surface of the brood decreased in the presence of workers.

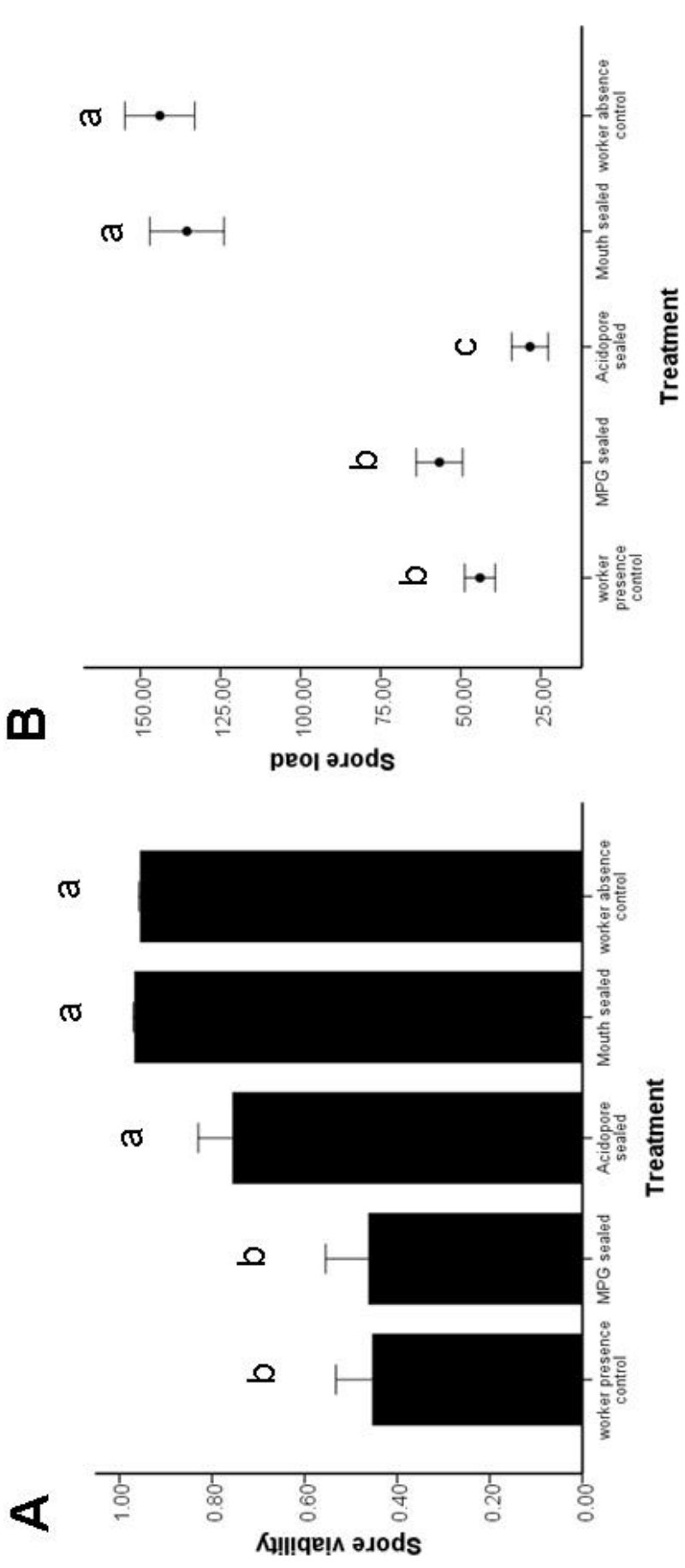


**Fig. 2**: Mean +- s. e. viability of spores (A) and mean +- s. e. spore load (B) on *Metarhizium anisopliae* strain KVL 03-143 exposed pupae. Adult worker presence during the first 24h after exposure results in a reduced viability (A) and a reduced spore load (B). Whereas sealing of metapleural gland openings (MPG) resulted in the same reduced viability as seen with non sealed adults (worker presence control), spore viability was not significantly reduced with acidopore sealed adult workers compared to mouth sealed workers, which were not able to groom. This was not due to a lowered grooming activity in acidopore sealed workers, as there was an even higher reduction in spore load with acidopore sealed workers compared to non sealed workers.

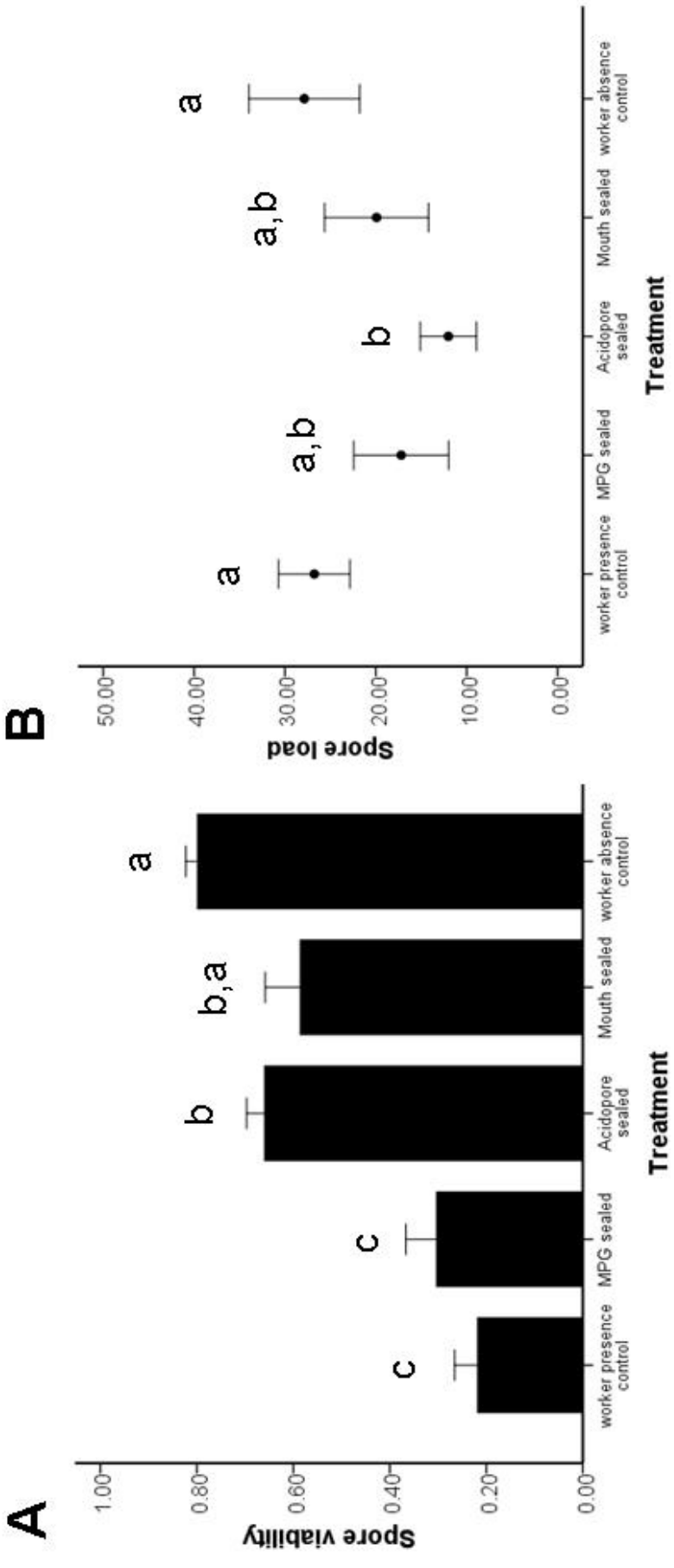


**Fig. 3**: Mean +- s. e. viability of spores (A) and mean +- s. e. spore load (B) on *Metarhizium anisopliae* strain ARSEF 2575 exposed pupae. Adult worker presence during the first 24h after exposure resulted in a reduced viability (A) and a reduced spore load (B). Whereas sealing of metapleural gland openings (MPG) resulted in the same reduced viability as seen with non sealed adults (worker presence control), spore viability was not significantly reduced with acidopore sealed adult workers compared to mouth sealed workers, which were not able to groom. This was not due to a lowered grooming activity in acidopore sealed workers, as acidopore sealing resulted in a significantly higher reduction of spore load compared to non sealed workers.

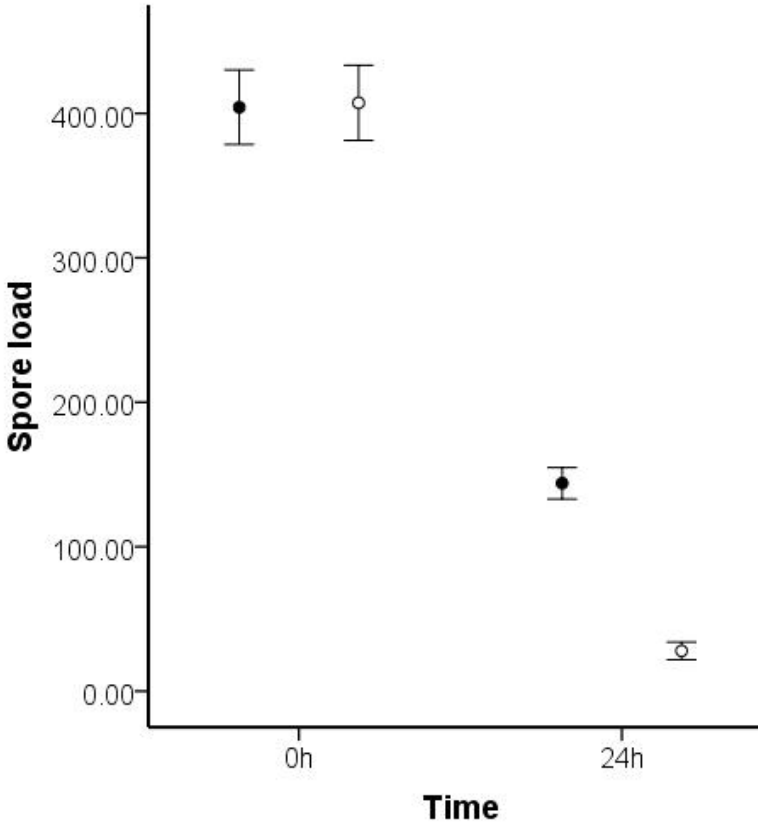


**Fig. 4**: Mean +- s. e. spore load in the absence of workers at 0h and 24h after exposure to the fungal strain KVL 03-143 (black) and ARSEF 2575 (white). The spore load on ARSEF 2575 exposed pupae after 24h shows a higher reduction than the spore load on KVL 03-143 exposed pupae.

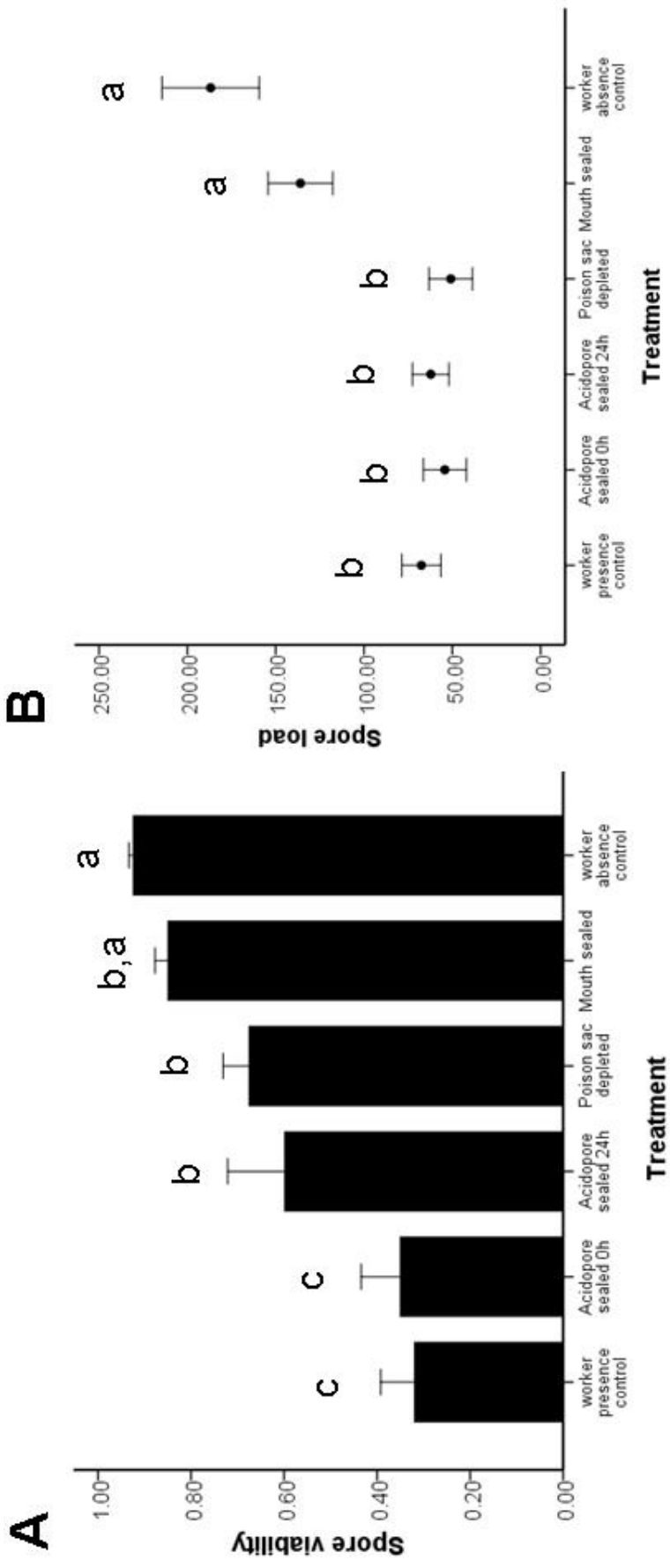


**Fig. 5**: Mean +- s. e. viability of spores (A) and mean +- s. e. spore load (B) on *Metarhizium anisopliae* strain KVL 03-143 exposed pupae. Adult worker presence during the first 24h after exposure resulted in a reduced viability (A) and a reduced spore load (B). Whereas the presence of adult workers 0h after sealing of the acidopore resulted in the same reduced viability as seen with non sealed adults (worker presence control), spore viability was not significantly reduced if the acidopore was sealed 24h before adult worker presence compared to mouth sealed workers, which were not able to groom. Furthermore spore viability was also not significantly reduced compared to mouth sealed workers if the poison sac content was depleted. The effects were not due to a different grooming activity as spore load was not significantly different in all treatments where workers were able to groom.

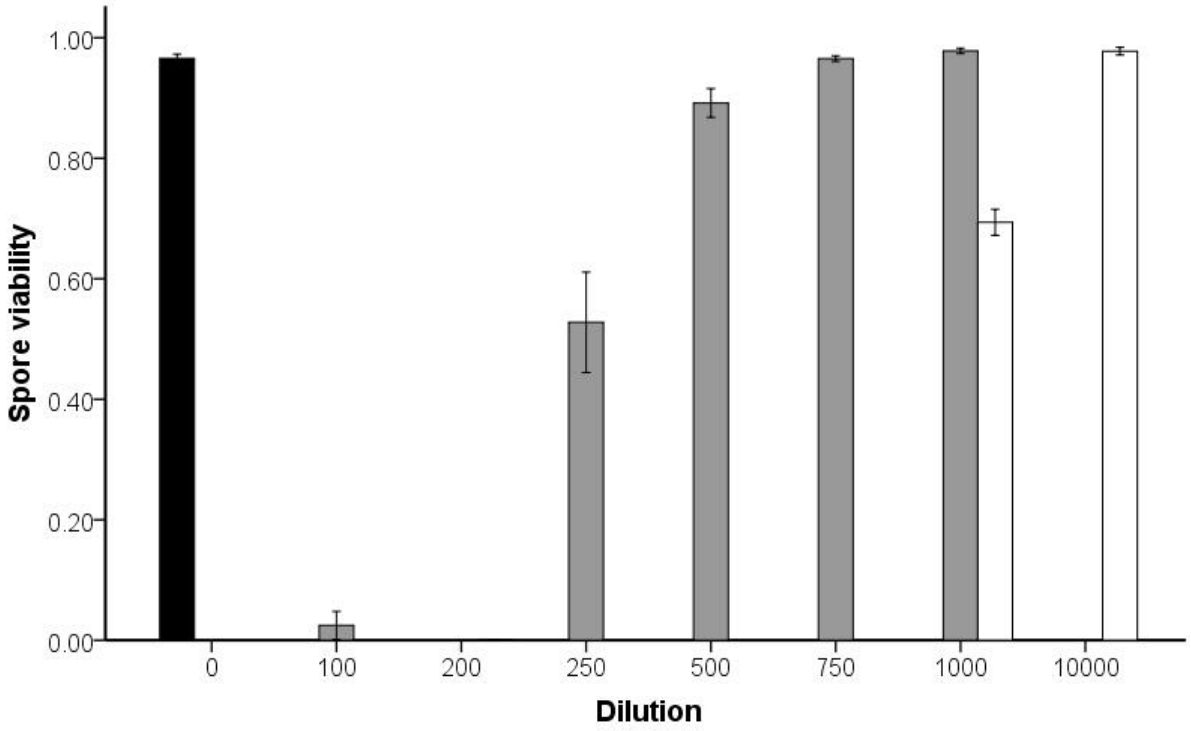


**Fig. 6:** The mean +- s. e. viability of spores incubated in a 200, 1000 and 10000 fold dilution of 100% pure formic acid (white bars) against the mean +- s. e. viability of spores incubated in a serial dilution of poison sac content (grey bars) from 12 adult workers. The viability of the used spore suspension is given as black bar. The viability of spores incubated in 1000 fold diluted pure formic acid lies between a spore incubation in 250-500 fold diluted poison sac content. This would correspond to a 25-50% concentration of formic acid in the poison sac.

# Discussion

In this chapter we first assessed in experiment 1 if antimycotic substances are used during worker care of fungus exposed brood, by re-analyzing the live spore dataset gained in chapter II (experiment 4) of this thesis with the fungal strain ARSEF 2575. In chapter II we could already show that worker presence resulted in a reduction of spore load on live exposed larvae and pupae compared to worker absence, indicating a removal of spores due to worker-brood allo-grooming. Now we could reveal that worker presence also decreased the viability of spores on exposed larvae and pupae significantly compared to worker absence (Fig. 1). This clearly indicates that antimycotic substances are being used during hygienic brood care. Several antiseptic substances with diverse origins in the body are known from ants (Attygale and Morgan 1984, Schlüns and Crozier 2009), the most prominent probably being the metapleural gland (reviewed in Huei Yek and Mueller 2010), the mandibular gland in the mouth and several glands in the gaster which can be discharged through the acidopore.
Sealing of the metapleural gland openings, the mouth and the acidopore opening in experiment 2 allowed us to narrow down potential candidates.

We found that sealing of metapleural gland openings did not result in a restored *viability of spores* compared to sham control glued workers (Fig. 2 A, 3 A). This was unexpected as a series of investigations have shown that metapleural gland secretions from different ant species have antiseptic properties against a range of microbes (reviewed in Huei Yek and Mueller 2010). Furthermore in the fungus-growing ant *Atta columbica* metapleural gland secretions have been shown to be used in the context of self-grooming to inactivate groomed off spores of *Metarhizium* in the infrabuccal pocket (Fernández-Marín et al. 2006). On the other hand sealing of the mouth or the acidopore opening both resulted in a restored viability of spores compared to sham control workers. Thereby spore viability on pupae in the presence of mouth sealed workers was not different to spore viability in the worker absent control, whereas in the presence of acidopore sealed workers spore viability was weakly non significantly different from the worker absent control when pupae where exposed with the strain KVL 03-143 ($p = 0.06$) and significantly different when pupae where exposed with strain ARSEF 2575 ($p = 0.009$). Already 1949 Pavan and Nascimbene could show that extracts from the head and gaster of *Lasius* ants have antibacterial properties. Our sealing of the mouth could have excluded a variety of sources. For example citral, contained in the mandibular gland of *Acantomyops claviger* (Reigner and Wilson 1968) and a variety of *Lasius* species (Bergström and Löfquist 1970) was shown to exhibit antifungal activity against a range of fungal pathogens in vitro (Cole et al. 1975). Also our sealing of the acidopore opening could have excluded a variety of sources. For example formic acid, the main constituent of the poison reservoir in formicine ants (Attygale and Morgan 1984), has been shown to exhibit antifungal activity (Pavan and Nascimbene 1949). Only recently it could furthermore be shown

that blocking of the acidopore opening in the formicine ant *Polyrhachis dives* resulted in a lower survival when exposed to *Metarhizium* compared to control treated ants (Graystock and Hughes 2011).

We also found that *spore load* was reduced on exposed pupae with the fungal strain KVL 03-143 in the presence of metapleural gland sealed workers, acidopore sealed workers and sham control workers compared to worker absence, but not in the presence of mouth sealed workers (Fig. 2 B). As mouth sealed workers should not be able to perform functional grooming this provides direct evidence that worker-brood allo-grooming behaviour is responsible for the reduction of spores. In contrast to strain KVL 03-143 spore load on exposed pupae with the fungal strain ARSEF 2575 was only reduced in the presence of acidopore sealed workers compared to sham control workers and worker absence, the latter two treatments and all other worker treatment comparisons not being different from each other (Fig. 3 B). This contrasts the findings obtained in experiment 1 where we also used strain ARSEF 2575 and found a significant decrease of spores on the surface of brood in the presence of workers compared to worker absence. A possible explanation might be a change in some traits of the fungus affecting fungal pathogenesis on exposed pupae (Clarkson and Charnley 1996). The spore suspension used in this experiment was gained from a plate incubated with spores isolated from sporulating *Lasius* cadavers infected previously with this fungal strain. A change in the infectivity of *Metarhizium* strains after re-isolation from sporulating cadavers has been found in ticks (Frazzon et al. 2000).

To explore possible differences during fungal pathogenesis between the two used strains we determined the strength of attachment for both strains in experiment 3 by comparing the spore load on pupae exposed to either strain directly after exposure to the spore load 24h after exposure in the absence of workers. The strength and speed of fungal spore attachment are likely to play an important role for the removal of spores during self- and allo-grooming as upon a certain strength and/or speed spore load reduction due to fungal pathogenesis may superimpose on the reduction in spore load due to worker allo-grooming. We found a 93% reduction of spore load on pupae exposed with the strain ARSEF 2575 between 0h and 24h after exposure, which was significantly higher than the reduction of 74% with the strain KVL-03-143, indicating differences in the attachment strength and/or speed between fungal strains (Fig. 4). This difference between the two strains highlights the possible importance of both mechanical removal of spores and use of antimycotic substances during hygienic care. If strength and speed of fungal pathogenesis are high, the use of antimycotic substances might be longer effective than their mechanical removal.

In experiment 4 we proceeded to elucidate antimycotic substances originating from the gaster in more detail. By creating two different acidopore opening sealed worker groups, we found that presence of acidopore sealed workers did not result in a restored viability of spores on exposed pupae compared to sham control workers when the pupae were presented within one hour after sealing but viability was restored when pupae were presented after 24h of sealing (Fig. 5 A). This result togegether with the indifferent viability of spores between mouth sealed workers and worker absent control in all

performed experiments suggests that antimycotic substances originating from the gaster are not applied directly through the acidopore opening and are still available some time after sealing of the acidopore.

We also found that presence of poison reservoir depleted workers resulted in a restored viability of spores on exposed pupae compared to sham control workers (Fig. 5 A). The reduction of spore load compared to worker absence however did not differ between them (Fig. 5 B). This suggests that contents of the poison reservoir are involved in the viability loss of spores on exposed pupae in the presence of workers. The main constituent of the poison reservoir in Formicinae ants is as mentioned formic acid (Attygale and Morgan 1984). However the presence of peptides and free amino acids has also been reported for *Camponotus pennsylvanicus* (Hermann and Blum 1968) as well as acetic acid (Lopez et al. 1993). Furthermore the contents of the adjacent Dufour gland are ejected simultaneously with the poison reservoir in *Lasius* and *Formica* ants (Maschwitz 1964), although morphological evidence exists that both glands can be operated independently (Billen 1982).

The involvement of formic acid is further corroborated in experiment 5 when the incubation of a gaster droplet, gained through depletion of the poison reservoir, and the incubation of pure formic acid with a spore suspension is compared. Under the assumption that only formic acid is involved in the viability loss of spores on exposed pupae in the presence of workers the formic acid content in the gaster droplet would correspond to a concentration of 25% to 50% formic acid (Fig. 6). This value matches the in the literature described values for formic acid concentration in the poison reservoir of other Formicinae ants (Stumper 1922, 1960: around 50% but with huge within species variation ranging from 30-70%). The antimicrobial activity of formic acid has long been known (Pavan and Nascimbene 1949) and its use against microorganisms suggested (Sauerländer 1961). Formic acid is also used by ants for defence against enemies and for predation (Osman and Kloft 1961), as pheromone (alarm: Löfquist 1976, recruitment and trail following: Kohl et al. 2001) and in one report as herbicide (Frederickson et al. 2005).

The behavioural observation of worker-brood allo-grooming in experiment 7 revealed that worker treatment, i. e. sealing of the mouth, the metapleural gland openings, the acidopore opening, sham control sealing and depletion of the poison sac content, did not have an influence on worker-brood allo-grooming behaviour. Instead, we observed the forward bending of the gaster between the legs and at the same time the downward bending of the head to the gaster tip during worker self-grooming in all treatment groups. This behaviour has been described as unique abdominal grooming behaviour in ants within hymenoptera (Basibuyuk and Quicke 1999), present in the ant subfamilies Formicinae, Dolichoderinae and Myrmicinae but absent in the subfamilies Myrmeciinae, Pseudomyrmacinae and Ponerinae (Farish 1972). In the light that antimycotic substances from the gaster are probably not applied directly through the acidopore opening it is possible that during this behaviour antimycotic substances from the gaster are taken up into the mouth and then used during hygienic care.

Taken together, based on all the results gained in this chapter, we propose that antimycotic substances from the gaster involving formic acid are likely responsible for part of the viability loss of spores seen in the presence of workers on fungus exposed pupae. Furthermore we propose that these substances are probably taken up into the mouth during worker abdominal self-grooming and likely applied during worker-brood allo-grooming. At this point, we cannot completely rule out that antimycotic substances from the mouth are additionally used, as sealing of the acidopore opening and depletion of the poison sac reservoir never restored spore viability to levels seen in the absence of workers.

Further data in addition to the performed experiments will be provided through collaborations with Vanessa Barone and Matthias Konrad (more detailed behavioural observations) and with Barabara Mitteregger (GC-MS analyses).

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# Chapter IV

# *Laboulbenia formicarum* infestation on *Lasius neglectus*: an invading parasite of an invasive ant

## Abstract

The invasive garden ant, *Lasius neglectus*, is a recently described pest ant currently spreading in Europe. It has now been found that four of its approximately 151 known populations suffer from infections with *Laboulbenia formicarum*, an ectoparasitic fungus hitherto unknown in continental Europe.

In the first part of this chapter we investigated the infestation history of one population of *Lasius neglectus* by *Laboulbenia formicarum*. Furthermore we tried to transmit an existing infestation to a not infested population during a cross fostering experiment. We found that the presence of *Laboulbenia formicarum* in Europe on *Lasius neglectus* is likely older than previously thought and show that it is possible to transmit the infestation with this ectoparasitic fungus to workers from a previously not infested population.

In the second part of this chapter we investigated this host-parasite system in more detail, by analysing how infestation with this rare specialist fungus affects the ants' survival under nutritional stress and exposure to the common generalist fungus *Metarhizium anisopliae* in a between and a within population comparison. We also tested a possible effect of *Laboulbenia formicarum* infestation on the immune status upon *Metarhizium anisopliae* exposure by performing measurements of the phenoloxidase activity. We found that *Laboulbenia formicarum* has a negative impact on the survival of *Lasius neglectus* ants under nutritional stress. Furthermore we found that *Laboulbenia formicarum* infested workers show an indifferent survival upon *Metarhizium* exposure and that their phenoloxidase activity is not changed upon *Metarhizium anisopliae* exposure in contrast to non infested workers. These results indicate a possible interaction between the ectoparasitic fungus *Laboulbenia formicarum* and the entomopathogenic fungus *Metarhizium anisopliae*, the nature of which remains to be elucidated.

# Introduction

Laboulbeniales are an order of the Ascomycota (Fungi) with more than 2000 known species. They entail an obligate biothropic relationship as ectoparasites on associated arthropods. The large majority of known Laboulbeniales parasitize insects, mostly Coleoptera and Diptera, but some are also known from millipedes and mites (after Weir and Blackwell 2005).

Among hymenoptera, only ants are known to harbour some species of Laboulbeniales:

*Rickia wasmannii* on *Myrmica* ants, *Dimorphomyces formicicola* on *Paratrechina* (collected only once), *Laboulbenia componoti* on *Camponotus* ants, *Laboulbenia ecitonis* on the army ants *Eciton* and *Laboulbenia formicarum* on several ant species of the subfamily Formicinae (after Espadaler et al. 2011).

*Laboulbenia formicarum* has only recently been described in Europe for the first time on *Lasius grandis* in Madeira (Espadaler and Santamaria 2003). In 2007 it was then discovered in continental Europe on *Lasius neglectus* in L'Escala, Spain (Herraiz and Espadaler 2007) and in Douarnenez, France. During a collection journey in June 2010 to the infested *Lasius neglectus* population in Douarnenez I also collected samples from a *Lasius neglectus* population in Gif-sur-Yvette near Paris, France, (Espadaler and Bernal 2011) and discovered that this population was also infested with *Laboulbenia formicarum*. Furthermore I could determine the infestation rate of both populations and differences between them in their infestation status. Both populations showed a high infestation rate (72% and 88% for Douarnenez and Gif-sur-Yvette respectively) but whereas infested workers from Gif-sur-Yvette also showed a high load of ectoparasitic fungi, bearing on average more than 3 thalli on their body, infested workers from Douarnenez showed a lower load, not always bearing thalli on their body but sometimes only showing dark spots indicative of spore attachment. This result was recently published together with a screen of 42 out of the 151 (July 2011) known populations of *Lasius neglectus* (Espadaler et al. 2011). Apart from the known infestation of the L'Escala population in Spain and the population in Douarnenez, France, only two other populations of *Lasius neglectus* from Gif-sur-Yvette and Saintes-Maries-de-la-Mare, France, were infested. It can be assumed that *Laboublenia formicarum* is exotic to Europe due to its limited distribution and host range (Espadaler et al. 2011). In North America the distribution of *Laboulbenia formicarum* is widespread and its host range comprises there 17 ant species (Espadaler et al. 2011). Other ant species collected together with infested *Lasius grandis* in Madeira and *Lasius negectus* in L'Escala and Saintes-Maries-de-la-Mare were not found to be infested (Espadaler and Santamaria 2003, Harreiz and Espadaler 2007, Espadaler et al. 2011). As outlined in Espadaler et al. 2011 the apparent affinity of *Laboubenia formicarum* to *Lasius negectus* in Europe deserves further attention as it might represent a new host-parasite system which has recently established in Europe between a exotic ectoparasitic fungus and an exotic ant species to Europe (Seifert 2000).

In the first part of this chapter the infestation history of *Lasius neglectus* ants by *Laboulbenia formicarum* in Gif-sur-Yvette near Paris, France, will be investigated by conducting a screen of samples of *L. neglectus* ants from this population on the presence of *Laboulbenia formicarum* infestation ranging from the years 2002 to 2010. Furthermore it will be established if it is possible to transmit a *Laboulbenia formicarum* infestation to a originally non infested population *Lasius neglectus* population in the laboratory by conducting a cross fostering experiment between the infested population of Gif-sur-Yvette and the non infested population of Jena, Germany.

Generally an infestation by Laboulbeniales is considered not to be deleterious to the host (Weir and Beakes 1995, Benjamin 1971). However Wheeler (1910) also noted that *Laboulbenia formicarum* infested colonies of *Lasius neoniger* Emery seemed to be less prosperous than non infested colonies nearby. Furthermore he also noted that infestations can be "so excessive that they resemble hedgehogs, fairly bristling with tufts of the fungus". Other authors report no apparent differences in activity and behaviour of infested *Lasius niger* var. *americana* ants by *Laboulbenia formicarum* (Smith 1917) and also by the Laboulbeniales *Rickia wasmannii* on Myrmicine ants (Rick 1903, Tartally et al. 2007). Due to the limited knowledge about Laboulbeniales in general and especially about the Laboulbeniales harboured by ants, where only notes on hosts and distributional data exist, in the main part of this chapter the impact of a *Laboulbenia formicarum* infestation on *Lasius neglectus* hosts will be investigated.

Laboulbeniales feed on their host (Scheloske 1969) and as well as entomopathogenic fungi such as *Metarhizium ansiopliae* use the insect cuticle as entrance point for infection. The use of entomopathogenic fungi for insect pest management is well established (Shah and Pell 2003) and a negative impact of an existing *Laboulbenia formicarum* infestation might prove to be a prospective way to control *Lasius neglectus* with entomopathogenic fungi. To this end first the impact of an existing *Laboulbenia formicarum* infestation on the survival of *Lasius neglectus* ants in a between population comparison will be investigated. Worker survival of the two infested *Lasius neglectus* populations of Gif-sur-Yvette and Douarnenez will be compared to worker survival of two non infested populations from Jena, Germany, and Volterra, Italy under nutritional stress, starving them, and upon exposure with *Metarhizium ansiopliae*. Thereafter the effects of a *Metarhizium* exposure and starvation will be analyzed in more detail in a single, heavily infested population of *Lasius neglectus* from L' Escala, Spain, (within population comparison) exploring worker survival of infested workers showing less than 10 thalli or more than 10 thalli of *Laboulbenia formicarum* on their body.

Some Laboulbeniales also produce haustoria entering the host hemocoel (Meola and Tavares 1982). It is not known if *Laboulbenia formicarum* also produces haustoria but dark spots indicative of the insertion point of living *Laboulbenia* can be easily seen on the cuticle of infested workers (Benjamin and Shannor 1950, Espadaler and Santamaria 2003). In arthropods the products of the phenoloxidase cascade are involved in wound healing, sclerotization of the cuticle and in the recognition and

melanization of foreign particles (Lemaitre and Hoffmann 2007). Upon cuticular injury or recognition of microbial components the inactive zymogen (proPO) of this cascade is converted into its active form (PO) (Cerenius and Söderhäll 2004). As both Laboulbeniales and *Metarhizium ansiopliae* use the cuticle as entrance point for infection a interaction between both fungi upon exposure of *Laboulbenia formicarum* infested workers with *Metarhizium* is possible. Additionally to worker survival, the phenoloxidase activity in the hemolymph one day after exposure with *Metarhizium anisopliae* in *Laboulbenia formicarum* infested workers from the population of L'Escala, Spain, and in non infested workers from the population of Jena, Germany will therefore be measured.

# Materials and Methods

Experiment 1: Infestation history of *Lasius neglectus* ants with *Laboulbenia formicarum* in Gif-sur-Yvette

We tried to assess the infestation history of *Lasius neglectus* ants with *Laboulbenia formicarum* in Gif-sur-Yvette near Paris, France, by conducting a survey on samples of *Lasius neglectus* hosts there. Samples stored in 96% ethanol were obtained from a big collection of *Lasius neglectus* at the Center of Social Evolution, University of Copenhagen (head: Prof. J.J: Boomsma). A total of 100 workers from 5 sampling sites in 2002, a total of 23 workers from 3 sampling sites in 2003 and a total of 96 workers from 5 sampling sites in 2007, of the invaded area by *L. neglectus* in Gif-sur-Yvette could be screened for infestation by *Laboulbenia formicarum*. Furthermore a total of 51 workers from 3 sampling sites in 2010 were accessible for screening due to own collection end of June 2010.

Workers were scanned for Laboulbeniales infestation under an Eschenbach stereomicroscope at 30-40X magnification. An infestation can be easily spotted by a trained eye, as thalli appear distinctly different in size and form from hairs. Furthermore, black spots on the cuticle, indicative of the insertion point of *Laboulbenia formicarum* (Benjamin and Shanor 1950) can sometimes be seen.

Experiment 2: Transmission of a *Laboulbenia formicarum* infestation during a cross fostering experiment

To explore if the transmission of a *Laboulbenia formicarum* infestation from an infested population to a non infested population is possible in the laboratory we set up a cross fostering experiment. Workers, brood and queens from the infested population of Gif-sur-Yvette near Paris, France and of the non infested population of Jena, Germany (Espadaler and Bernal 2011) were collected in June and April 2010 respectively. Until setup of the cross fostering experiment in August 2010 both populations were housed in large plastic boxes with plaster ground and maintained at a diet of honey and cockroaches. During this time and also during the cross fostering experiment stock colonies and cross fostering setups were kept in summer condition with temperatures of 27/21°C and a 14/10h day/night cycle. For the cross fostering experiment we set up six queens plus 60 to 80 copper wire marked workers from the population of Gif-sur-Yvette in one plastic box (15 x 10 x 5 cm L. x W. x H.) with plaster ground. We proceeded the same way with six queens plus 60 to 80 copper wire marked workers from the population of Jena. Thereafter we provisioned the cross fostering setup with queens and workers of Gif-sur-Yvette with around 400 pupae of the population of Jena and the setup with queens and workers of Jena with around 400 pupae of the Gif-sur-Yvette population. Thus pupae from the non infested population of Jena were raised in a *Laboulbenia formicarum* infested environment, whereas pupae of the infested population of Gif-sur-Yvette were raised in a Laboulbeniales free environment.

After seven months both cross fostering setups were frozen at -20°C and the hatched workers from pupae scanned for a *Laboulbenia formicarum* infestation.

Experiment 3: Impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure. Between population comparison.

To explore the impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure we set up a full factorial experiment with workers from two infested populations (Gif-sur-Yvette and Douarnenez) and two non infested populations (Jena and Volterra) (Espadaler and Bernal 2011). Workers, brood and queens from the *Lasius neglectus* populations in Jena, Germany, and Volterra, Italy, were collected in April and March 2010 respectively. Workers, brood and queens from the *Lasius neglectus* populations in Gif-sur-Yvette near Paris, France, and Douarnenez, France, were collected in June 2010. All populations collected were housed in large plastic boxes with plaster ground and maintained at a diet of honey and cockroaches at 27/21°C and a 14/10h day/night cycle until set up of the experiments. During the experiment animals were kept at constant 24°C and a 12/12h day/night cycle. The experiment was set up end of June, beginning of July 2010 within three weeks. For the experiment workers from all four populations were transferred in small experimental Petri dishes (diameter 5 cm) with plaster ground and either provisioned with 10% sucrose solution applied on a small piece of filter paper on a stainless steel metal plate (fed condition), not provisioned (starving condition) or provisioned with water applied on a small piece of filter paper on a stainless steel metal plate (water condition). The water condition was introduced to control that death in the starving condition was caused by starvation and not dehydration. 60 replicated experimental Petri dishes were set up for each of the three conditions with workers of all four populations (720 Petri dishes total). In addition to experimental Petri dishes with untreated workers in the described conditions we also established Petri dishes with workers from all four populations that were subjected to the following two treatments in all conditions. Workers were either exposed to a spore suspension of *Metarhizium ansiopliae* (720 Petri dishes total) or to the solvent Triton X-100 (Sigma) as sham control for the spore exposure (720 Petri dishes total). The used entomopathogenic fungus *Metarhizium anisopliae var. anisopliae* (strain KVL 03-143, obtained from the Faculty of Life Sciences, University of Copenhagen, Denmark) was reared on Malt extract (Merck) agar plates and suspensions of conidiospores (1x10^9 spores/ml in 0.05% Triton X-100; Sigma) were produced as detailed in Ugelvig and Cremer 2007. The germination rate of the used live-spore suspension was 99%. After setting up the experiment the survival of workers was monitored for 12 days.

Originally we intended to analyse the survival of workers in this full factorial experiment with a Cox-proportional regression including the population (Jena, Volterra, Gif-sur-Yvette or Douarnenez), the maintenance condition (fed, starving or water), the treatment (untreated, *Metarhizium* exposed or sham control exposed) and their interactions as predictor variables. However this full model as well as

several reduced models violated the basic assumption of proportional hazards. We therefore decided to analyse the survival data set at each condition and at each treatment separately using the Kaplan-Meier procedure with Tarone-Ware statistics implemented in SPSS 18. In each of the resulting six models (three for exposure treatments - unexposed, *Metarhizium* exposed and sham control exposed - and three for maintenance condition - fed, starving and water) we used population identity (Jena, Volterra, Paris and Douarnenez) as stratum to control for population specific differences upon exposed treatment or maintenance conditions. Posthoc pairwise comparisons between maintenance conditions or exposed treatments at a stratum level were performed following overall significant results, adjusting $\alpha$ to 0.017 due to multiple testing. Unfortunately with this type of analysis we cannot infer if worker survival is significantly increased or decreased in infested or non infested populations but only infer if the survival pattern is the same across populations upon treatment or maintenance condition.

Experiment 4: Impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure. Whitin population comparison.

To explore the impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure in more detail we set up a full factorial experiment with workers from a single infested population. Workers were collected in August 2009 from the heavily infested population of L' Escala, Spain (Espadaler and Bernal 2011), housed in a large plastic box with plaster ground and maintained at a diet of honey and cockroaches at 27/21°C and a 14/10h day/night cycle until set up of the experiment. During the experiment conducted in Oktober/November 2009 animals were kept at constant 24°C and a 12/12h day/night cycle. For the experiment workers were divided in two infestation status groups either carrying less than 10 thalli (low infestation status) or carrying more than 10 thalli of *Laboulbenia formicarum* on their body (high infestation status). Workers from each infestation status were transferred to experimental Petri dishes (diameter 5cm with plaster ground) and either provisioned with 10% sucrose solution applied on a small piece of filter paper on a stainless steel metal plate (fed condition) or not provisioned (starving condition). 30 replicated Petri dishes were set up for each condition with workers from both infestation levels (120 Petri dishes total). In addition to experimental Petri dishes with untreated workers in the described conditions we also established Petri dishes with workers from both infestation levels that were exposed to a spore suspension of *Metarhizium ansiopliae* and maintained in fed or starving conditions (120 Petri dishes total; for details on spore exposure refer to experiment 3 of this chapter). The survival of workers was thereafter monitored for 12 days.

As in experiment 3 it was not possible to analyse the data set with a Cox proportional regression. We therefore decided to analyse the survival of high and low infested workers at each condition and at each treatment separately. This resulted in four statistical models: two for treatment (unexposed and *Metarhizium* exposed) and two for condition (fed and starving). We used again the Kaplan-Meier procedure with Tarone-Ware statistics implemented in SPSS 18.

Experiment 5: Impact of a *Laboulbenia formicarum* infestation on the phenoloxidase activity in the hemolymph upon *Metarhizium* exposure of workers.

To examine a possible impact of an existing *Laboulbenia formicarum* infestation upon *Metarhizium* exposure we measured the phenoloxidase activity in the hemolymph of infested workers from the population of L' Escala, Spain, and the non infested population of Jena, Germany. Workers of both populations were collected in August 2009, housed in large plastic boxes with plaster ground and maintained at a diet of cockroaches and honey at 27/21°C and a 14/10h day/night cycle until performance of the experiment. During the experiment conducted in November 2009 animals were kept at constant 24°C and a 12/12h day/night cycle. Workers from both populations were either exposed to a spore suspension of *Metarhizium anisopliae* (for details of exposure refer to experiment 3 of this chapter) or were not treated and transferred to experimental Petri dishes (diameter 5 cm with plaster ground) provisioned with 10% sucrose solution applied on a small piece of filter paper on a stainless steel metal plate.

The next day hemolymph samples from *Metarhizium* exposed and untreated workers were collected. Therefore workers were cold anesthetized on ice for a maximum of 1h but at least for 20 min. Then the animals were fixed and one of their prolegs was cut off at the femure and 0.03µl of hemolymph collected in a self-pulled capillary. The capillaries were pulled from pre-made capillaries (Servoprax 50µl) over an ethanol lamp to a fine point, washed with actone, coated with Sigmacoat (Sigma-Aldrich) and then calibrated to 0.03µl using a micropump apparatus UMPIII with a Micro 4 controller (WPI) by placing a small paint dot on the mark. The collected hemolymph was flushed immediately in 3µl ice cold filtered Sodiumcacodylate buffer (0.01M Na-Ca, 0.005M CaCl2), put into liquid nitrogen and then stored at -20°C until further procedure.

Due to the low quantity of hemolymph gained from the workers we measured not only the active enzyme PO but also activated the inactive zymogen proPO with chymotrypsin, a exogenous serine proteases (Gillespie et al. 2000a). PO can be quantified by measuring the conversion of the substrate L-DOPA into dopachrome. The produced dopachrome results in a darkening of the test solution, which can be spectrophometrically quantified, thus obtaining the enzyme activity measured as the slope (Vmax), which is the velocity of the enzyme reaction when the substrate is at saturating concentration during the linear phase of the reaction (Armitage and Boomsma 2010). Enzyme measurements were modifications of the PO protocol of Armitage and Siva-Jothy (2005). Measurement of the total phenoloxidase activity (PO and activated proPO) in all obtained hemolymph samples was performed the same day as their collection. Hemolymph samples were first defrosted at 4°C and then centrifuged for 5 min at 2800rcf and 4°C. The whole sample (3µl) was then incubated together with 7.5µl of 2:1 diluted 0.1M PBS buffer in a half area well of a 96 well plate (Costar) on ice. Subsequently chymoptrypsin (5mg/ml) was added to each well and the plate then incubated at room temperature for 5 min. before final addition of 12.5ul L-Dopa (4mg/ml). L-Dopa was made the day prior measurement and shaked in the dark for 1h before filtering and storage at 4°C. Immediately

after addition of L-Dopa the plate was measured in a spectrophotometer (Versamax) for 1h with interval readings every 20 sec. at 490nm. Afterwards the activity was determined as the slope of the linear phase between 200 and 400 sec. in an interval of at least 100 sec. Every measurement was complemented with a measurement of a blank well containing every chemical but water instead of the hemolymph sample. These blank measurements were deduced from the sample readings before determining the slope of the hemolymph samples.

For statistical analyses only readings from clear hemolymph samples were taken into account, as turbid samples produced staggered activity curves where the slope in the linear phase could not be determined reliably. *Metarhizium* exposed animals were compared to non exposed animals with Mann-Whitney U-tests in SPSS 18.0 as data from workers of the L'Escala population did not follow a normal distribution.

# Results

Experiment 1: Infestation history of *Lasius neglectus* ants with *Laboulbenia formicarum* in Gif-sur-Yvette

The screening of *Lasius neglectus* samples from the population of Gif-sur-Yvette near Paris, France, revealed that the infestation with the ectoparasitic fungus *Laboulbenia formicarum* was already present in the population in the year 2002 (Fig. 1) with 2% of the scanned workers showing one to two thalli on their body. The infestation rate increased steadily over the years with 22% of the workers showing signs of infestation in 2003, 78% in 2007 and 88% in 2010. Over the years not only the percentage of workers showing signs of infestation increased but also the number of present thalli on the body of infested workers increased steadily with most of the infested workers in 2010 showing more than 10 thalli on their body. This however was assessed only qualitatively but not quantitatively.

Experiment 2: Transmission of a *Laboulbenia formicarum* infestation during a cross fostering experiment

Hatched workers from pupae of the Jena population raised in a *L. formicarum* infested environment showed 15% (14 out of 94) infestation rate after seven months with one to two thalli on the body of infested workers. Hatched workers from pupae of the Gif-sur-Yvette population raised in a non infested environment showed 0% (0 out of 88) infestation. In both cross fostering setups none of the copper wire marked workers survived. Queens of the infested population of Gif-sur-Yvette still showed thalli of *L. formicarum* on their body.

Experiment 3: Impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure. Between population comparison.

The survival of workers from all four populations did differ significantly depending upon maintenance condition when workers were not treated (Kaplan Meier: $\chi^2 = 281.473$, d. f. = 1, $p < 0.001$), exposed to *Metarhizium* (Kaplan Meier: $\chi^2 = 196.81$, d. f. = 1, $p < 0.001$) or sham control treated (Kaplan Meier: $\chi^2 = 277.839$, d. f. = 1, $p < 0.001$) with infested and non infested populations showing a significant result at the population level in all three treatments (*not treated*: Jena: $\chi^2 = 61.348$, d. f. = 2, $p < 0.001$; Volterra: $\chi^2 = 108.059$, d. f. = 2, $p < 0.001$; Gif-sur-Yvette: $\chi^2 = 100.157$, d. f. = 2, $p < 0.001$; Douarnenez: $\chi^2 = 90.390$, d. f. = 2, $p < 0.001$; *Metarhizium exposed:* Jena: $\chi^2 = 59.800$, d. f. = 2, $p < 0.001$; Volterra: $\chi^2 = 49.639$, d. f. = 2, $p < 0.001$; Gif-sur-Yvette: $\chi^2 = 54.860$, d. f. = 2, $p < 0.001$; Douarnenez: $\chi^2 = 96.016$, d. f. = 2, $p < 0.001$; *sham control exposed*: Jena: $\chi^2 = 67.774$, d. f. = 2, $p < 0.001$; Volterra: $\chi^2 = 96.724$, d. f. = 2, $p < 0.001$; Gif-sur-Yvette: $\chi^2 = 77.601$, d. f. = 2, $p < 0.001$; Douarnenez: $\chi^2 = 90.820$, d. f. = 2, $p < 0.001$). Post hoc pairwise comparisons revealed that for all populations in all three exposure treatments survival of workers was not different between water and

starving condition (all comparisons $p > 0.017$) but that survival was significantly higher in the fed condition compared to both starving and water condition (all comparisons $p < 0.001$ for both starving and water condition). This indicates that the death of workers in the starving condition was not due to dehydration and that *L. formicarum* infested populations do not differ from non infested populations in their reaction to the maintenance conditions they were subjected.

The survival of workers from all four populations did differ also significantly depending upon exposed treatment when workers were maintained in fed condition (Fig. 2; Kaplan Meier: $\chi^2 = 16.539$, d. f. = 1, $p < 0.001$), starving condition (Fig. 3; Kaplan Meier: $\chi^2 = 7.512$, d. f. = 1, $p = 0.006$) but not when maintained in water condition (Kaplan Meier: $\chi^2 = 1.587$, d. f. = 1, $p = 0.208$). At the population level only the non infested populations of Jena and Volterra and the infested population of Gif-sur-Yvette showed a significant different survival across treatments in the *fed condition* (Fig. 2; Jena: $\chi^2 = 14.695$, d. f. = 2, $p = 0.001$; Volterra: $\chi^2 = 20.752$, d. f. = 2, $p < 0.001$; Gif-sur-Yvette: $\chi^2 = 7.339$, d. f. = 2, $p = 0.025$; Douarnenez: $\chi^2 = 2.712$, d. f. = 2, $p = 0.258$) with *Metharizium* exposed workers from the population of Jena and Volterra surviving significantly worse than both sham control exposed workers and non treated workers ($p < 0.006$ for both populations in both treatment comparisons), the latter two not being significantly different ($p > 0.118$ for both populations). The survival of *Metarhizium* exposed workers from the population of Gif-sur-Yvette was only significantly lower than the survival of non treated workers ($p = 0.006$) but not of sham control treated workers ($p = 0.236$), with sham control treated workers also not surviving significantly worse than non treated workers ($p = 0.134$). This indicates that the survival of *L. formicarum* infested populations during normal maintenance, feeding them, might be generally not greatly influenced by exposure to an entomopathogenic fungus.

In the *starving condition* only the non infested population of Jena showed a significant result at the population level (Fig. 3;Jena: $\chi^2 = 18.712$, d. f. = 2, $p = 0.001$; Volterra: $\chi^2 = 3.729$, d. f. = 2, $p = 0.155$; Gif-sur-Yvette: $\chi^2 = 4.696$, d. f. = 2, $p = 0.095$; Douarnenez: $\chi^2 = 3.753$, d. f. = 2, $p = 0.153$) with *Metarhizium* exposed workers surviving significantly worse than sham control and non treated workers (both $p < 0.001$), the latter two not being significantly different ($p = 0.943$).

Experiment 4: Impact of a *Laboulbenia formicarum* infestation on the survival of workers under starving conditions and upon *Metarhizium* exposure. Whitin population comparison.

The survival of high and low infested non treated workers was not significantly different in the fed condition (Fig. 4 A; Kaplan Meier: $\chi^2 = 0.131$, d. f. = 1, $p < 0.781$) but significantly different in the starving condition (Fig. 4 B; Kaplan Meier: $\chi^2 = 4.826$, d. f. = 1, $p < 0.028$) with low infested workers showing a higher survival than high infested workers. Upon *Metarhizium* exposure high and low infested workers did not differ in their survival in the fed condition (Fig. 4 C; Kaplan Meier: $\chi^2 = 0.704$, d. f. = 1, $p < 0.401$) but differed significantly in the starving condition (Fig. 4 D; Kaplan Meier: $\chi^2 = 4.729$, d. f. = 1, $p < 0.030$) with again low infested workers showing a higher survival than high infested workers.

Experiment 5: Impact of a *Laboulbenia formicarum* infestation on the phenoloxidase activity in the hemolymph upon *Metarhizium* exposure of workers.

The total phenoloxidase activity, including PO and proPO, in the hemolymph of workers was significantly lower in workers that were exposed to *Metarhizium* than in workers that were not exposed to *Metarhizium* one day after exposure in the non *Laboulbenia formicarum* infested population of Jena (Fig. 5; Mann-Whitney U-test = 4, $p = 0.019$). In contrast no difference in the phenoloxidase activity between *Metarhizium* exposed and non exposed workers one day after exposure could be detected in the hemolymph of workers from the infested population of L'Escala (Fig. 5; Mann Whitney U-test = 102.5, $p = 0.913$).

# Figures and Tables

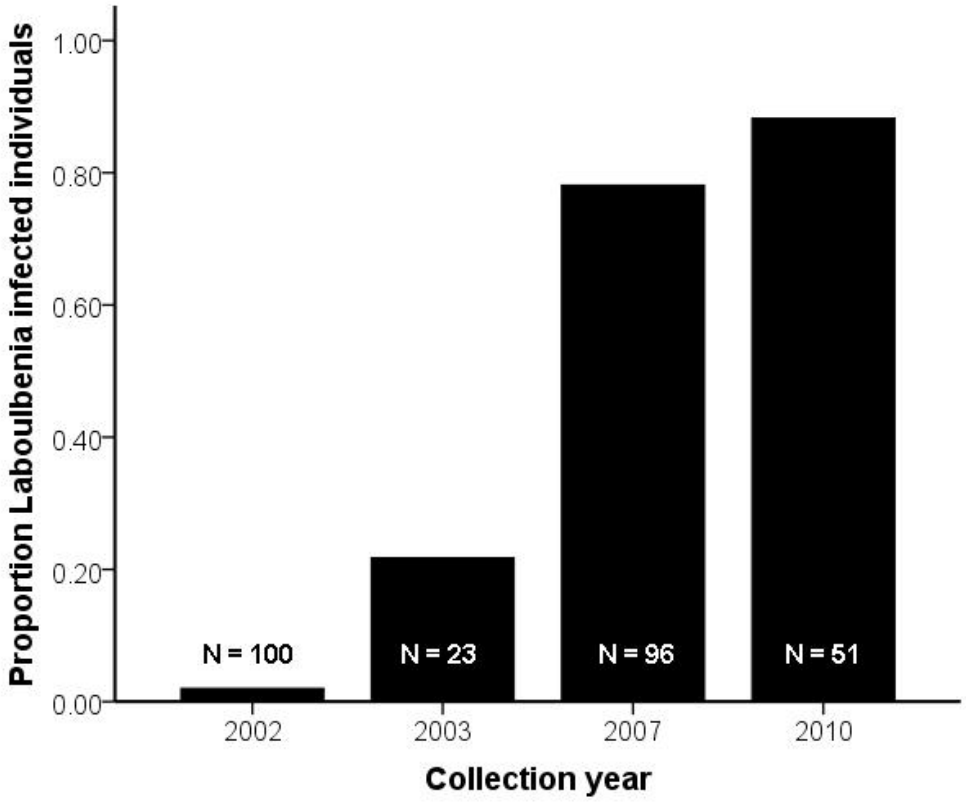


**Fig. 1:** Survey on the infestation status of *Lasius neglectus* with *Laboulbenia formicarum* in the population of Gif-sur-Yvette near Paris, France. The numbers of workers available for the survey in the different years are given in or above the bars. The infestation of *Lasius neglectus* with *Laboulbenia formicarum* was already present in the year 2002 and the infestation rate of the population shows a steady increase over the years.

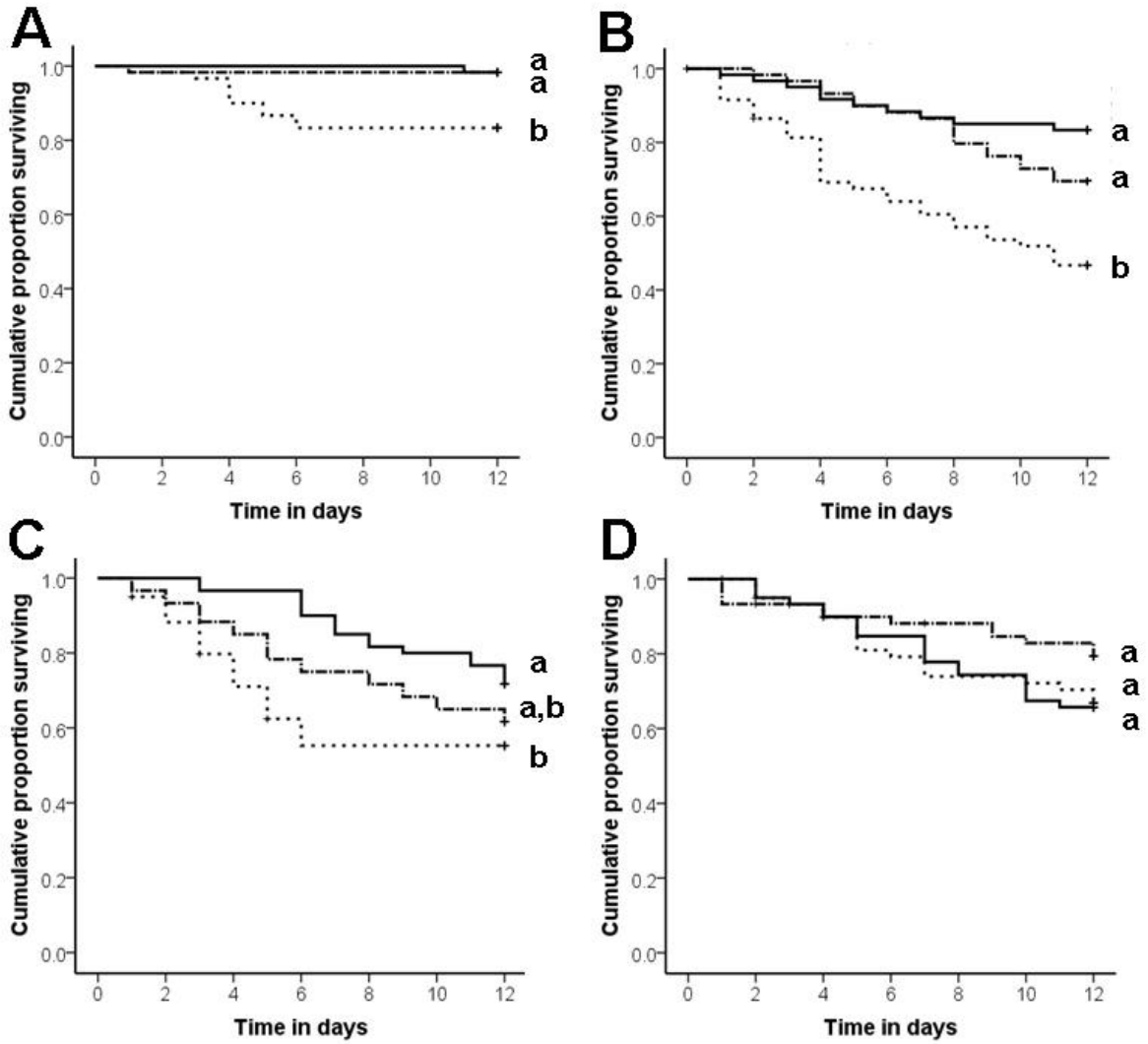


**Fig. 2:** Survival of single *Lasius neglectus* workers under fed condition from the non *Laboulbenia formicarum* infested populations of Jena (A) and Volterra (B) and the *Laboulbenia formicarum* infested populations of Gif-sur-Yvette (C) and Douarnenz (D). The workers of each population were either *Metarhizium* exposed (pointed lines), exposed to a sham control solution (dash-pointed lines) or not exposed (solid lines). Exposure of workers to *Metarhizium* resulted in a significantly lower survival compared to sham control or not exposed workers from the population of Jena and Volterra. In contrast survival of *Metarhizium* exposed workers from the population of Gif-sur-Yvette was only significantly lower compared to non exposed workers and survival of workers from the population of Douarnenez was indifferent with respect to exposure.

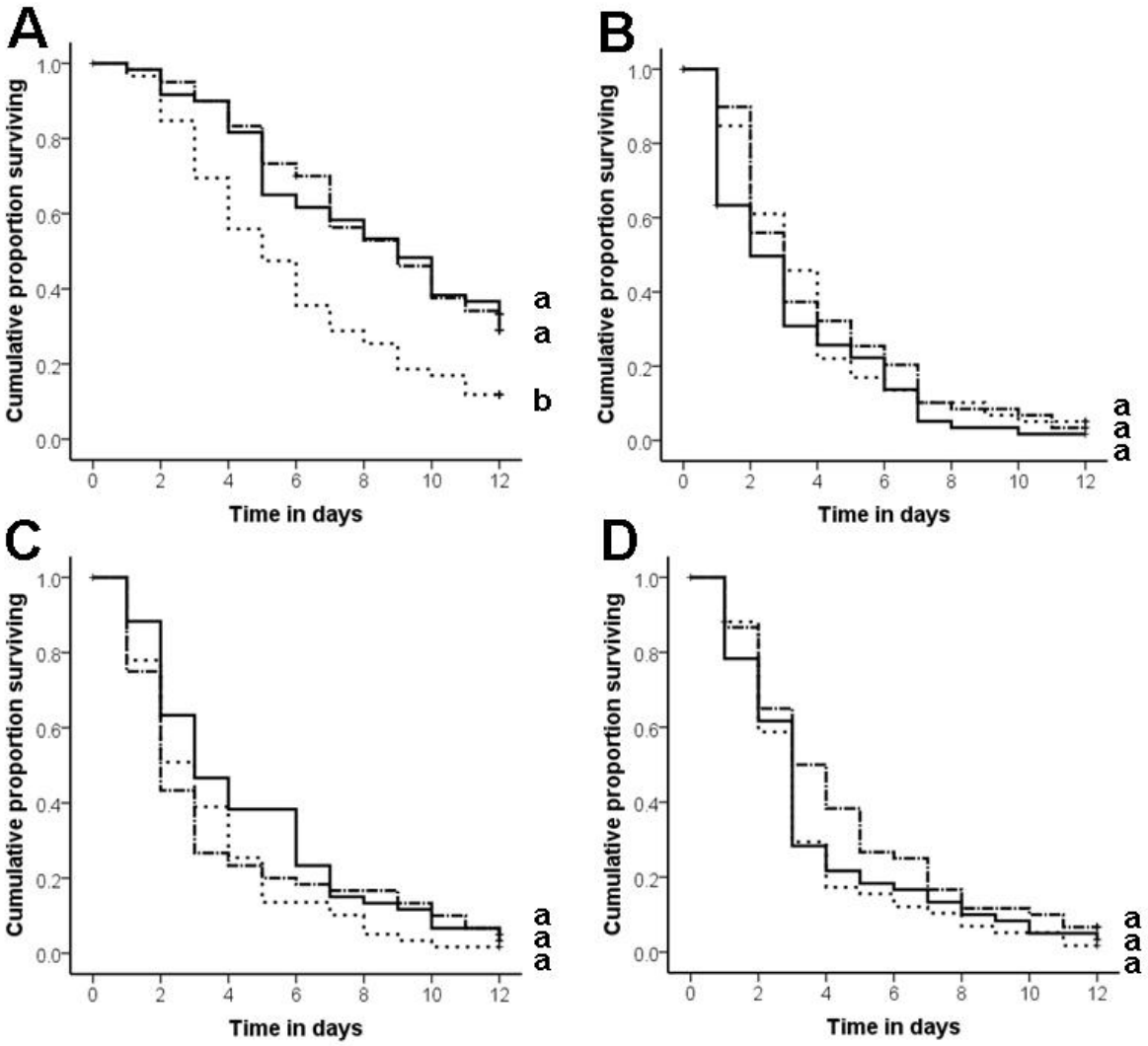


**Fig. 3**: Survival of single *Lasius neglectus* workers under starving condition from the non *Laboulbenia formicarum* infested populations of Jena (A) and Volterra (B) and the *Laboulbenia formicarum* infested populations of Gif-sur-Yvette (C) and Douarnenz (D). The workers of each population were either *Metarhizium* exposed (pointed lines), exposed to a sham control solution (dash-pointed lines) or not exposed (solid lines). Except for workers originating from the population of Jena, exposure to *Metarhizium* did not result in a decreased survival compared to non exposed workers or sham control exposed workers.

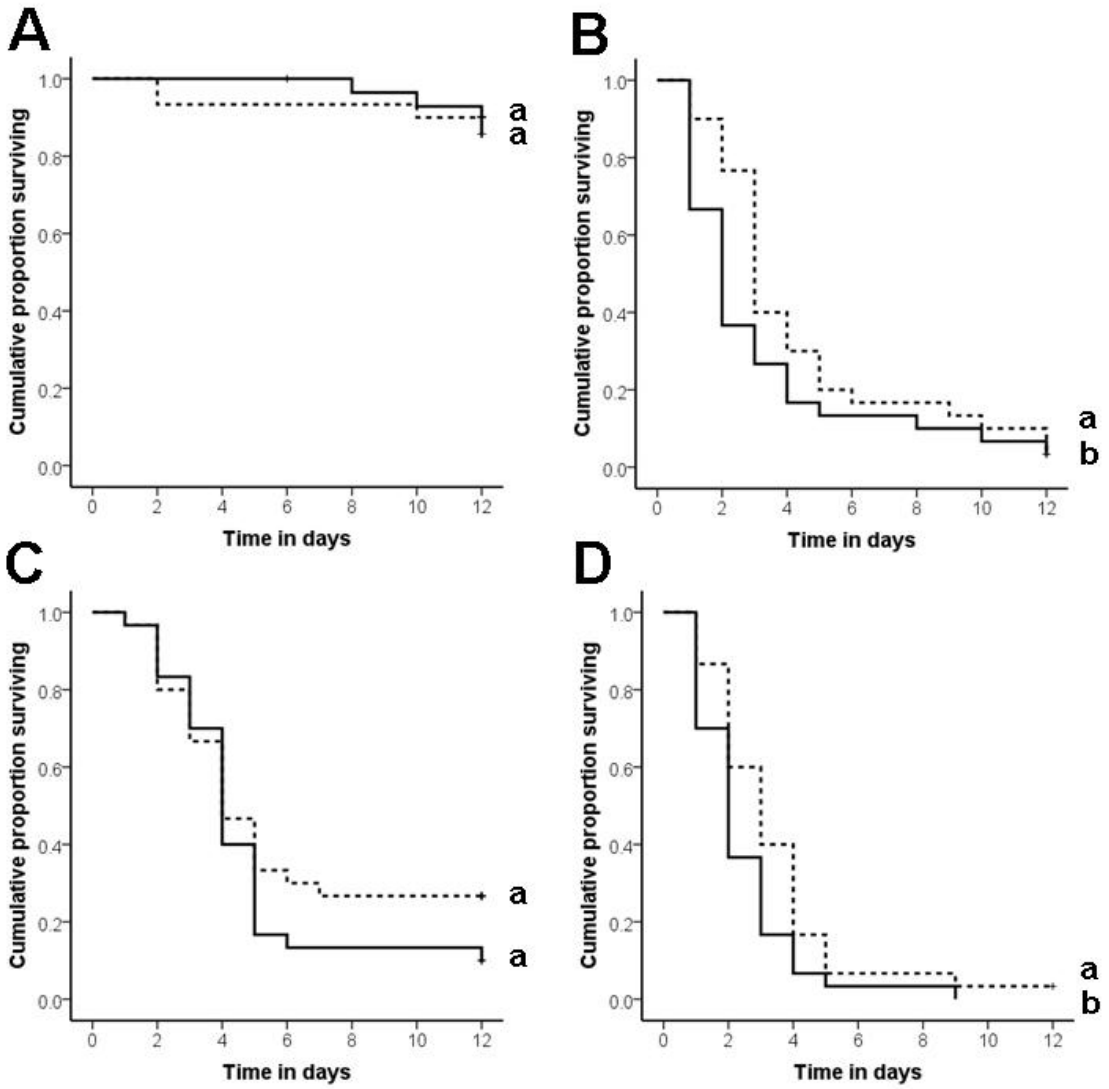


**Fig. 4**: Survival of single *Lasius neglectus* workers from the *Laboulbenia formicarum* infested population of L'Escala maintained under fed condition (A and C) or under starving condition (B and D) and that were either not treated (A and B) or exposed to *Metarhizium* (C and D). Infested workers of this population were divided in high infested workers showing more than 10 thalli of *Laboulbenia formicarum* on their body (solid lines) or in low infested workers showing less than 10 thalli on their body (dashed lines). Whereas survival of high and low infested workers was not different under fed condition (A), survival of low infested workers was significantly higher than of high infested workers under maintenance in starvation condition (B). *Metarhizium* exposure (C and D) did not result in a different survival between high and low infested workers compared to their survival when workers were not exposed.

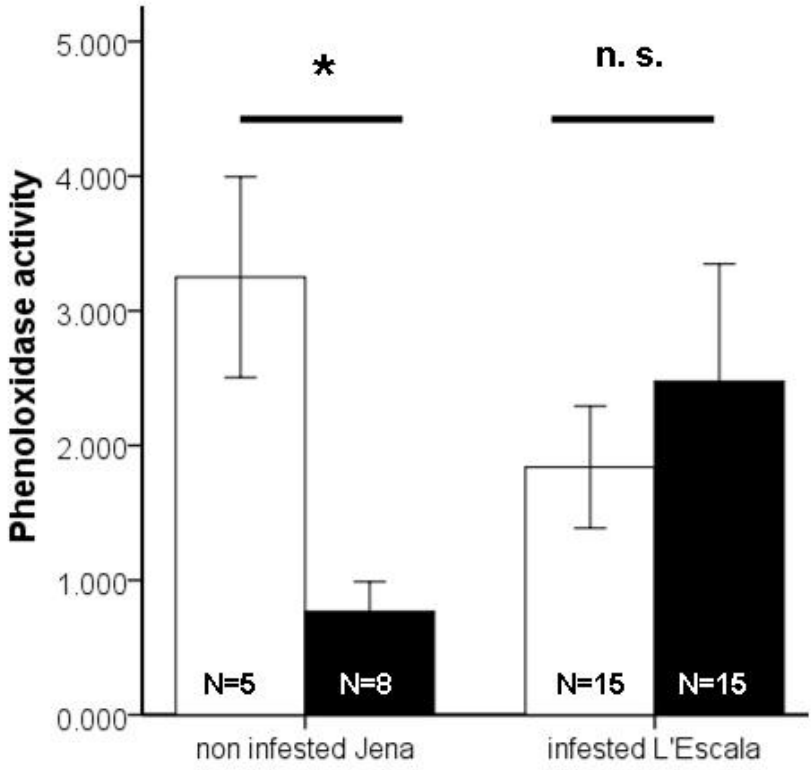


**Fig. 5:** The mean +- s. e. phenoloxidase activity (PO and proPO) one day after treatment of workers with the entomopathogenic fungus *Metarhizium anisopliae* (black bars) and one day after not treating workers (white bars). The phenoloxidase activity is given for *Laboulbenia formicarum* infested workers from the *Lasius neglectus* population of L'Escala and non infested workers from the population of Jena. Whereas there is a significant difference between *Metarhizium* exposed and non exposed workers in the phenoloxidase activity of workers from Jena, there is no difference between *Metarhizium* exposed and non exposed workers from the infested population of L'Escala.

# Discussion

In this chapter we investigated first the infestation history of *Lasius neglectus* ants by *Laboulbenia formicarum* in the population of Gif-sur-Yvette near Paris, France. In experiment 1 we found that an infestation by *Laboulbenia formicarum* in this population was already present in 2002. This is the same year samples for the first record of *Laboulbenia formicarum* in Europe on *Lasius grandis* (Espadaler and Santamaria 2003) were collected and much earlier than the first record of this ectoparasitic fungus on *Lasius neglectus* in Europe (Harreiz and Espadaler 2007). The presence of *Laboulbenia formicarum* on *Lasius neglectus* already in 2002 underlines the apparent affinity of *L. formicarum* to *Lasius neglectus* ants (Espadaler et al. 2011) and raises again the unresolved question of the origin of *L. formicarum* in Europe. Espadaler et al. 2011 found that from 42 out of 151 known populations of *Lasius neglectus* only four, located in Spain, France and Portugal (Madeira), showed an infestation by *Laboulbenia formicarum* suggesting an eastern expansion, maybe through the introduction of *L. formicarum* from North America. However it might also well be that *L. formicarum* was imported together with *Lasius neglectus* to Europe and got successively lost in most populations. More detailed screenings of the known *L. neglectus* populations will be needed including their native range in W Asia (Seifert 2000) as invasive species often show less parasites in their introduced ranges as in their native range (Torchin et al. 2003). The results of our survey in experiment 1 also showed a steady increase in the infestation rate over the years. We can not be completely sure if this increase in the infestation rate represents an increase of infestation in the whole *Lasius neglectus* population in Gif-sur-Yvette or whether it just reflects a sampling pattern, as we used animals from different sampling spots over the years for our survey. A throughout examination of further samples from the area should however answer this question and maybe also reveal the speed of transmission of *Laboulbenia formicarum* through the population.

In experiment 2 of this chapter we proceeded to investigate the possible transmission of a *Laboulbenia formicarum* infestation from an infested population of *Lasius negelctus* to a non infested population during a cross fostering experiment. We only found a low infestation rate of the previously non infested population in our setup after 7 months (15%) with infested workers also only showing a mild infestation status (one to two thalli on the body). This might be indicative of a long developmental time of *Laboulbenia formicarum* on *Lasius negelctus* ants. To our knowledge there are no data available on the developmental time of *Laboulbenia formicarum*. Developmental times for Laboulbeniales in general usually range between 2-3 weeks but can take up to 6 months (Benjamin 1971) starting with brownish-black areas on the cuticle of infested hosts indicative of spore attachment (Benjamin and Shanor 1950). In contrast to infested workers from the *L. neglectus* population of Douarnenenz (Espadaler et al. 2011) such black areas on the cuticle of infested workers were not prominent enough in our cross fostering experiment to be noted down. Males of *Lasius niger* have also

been reported to be infested by *Laboulbenia formicarum* (Benjamin and Shanor 1950). As males of ants are usually short lived (Hölldobler and Wilson 1990) this speaks against a long developmental time of *Laboulbenia formicarum*. On the other hand males of insects are known to show a lower immune response than their female counterparts (Schmid-Hempel 2005b), which has also been shown in social insects (Baer and Schmid Hempel 2006). Another explanation for the low infestation rate and status in our used setup might be explained by the mode of transmission of a *Laboulbenia formicarum* infestation, which is still unclear and remains to be elucidated. Direct contact, auto-infection and substrate infections are the most often discussed routes of transmission (De Kesel 1993). However there are also reports that parasitic mites on the ant *Eciton quadriglume* (Blum 1924, cited in Benjamin 1971) and beetles (Thaxter 1924, cited in Benjamin 1971; Seemann and Nahrung 2000) harbour the same species of Laboulbeniales as their hosts. As colonies of *Lasius neglectus* harbour a variety of mites, inquilines and commensals (Espadaler and Bernal 2011) an infestation through these could be a likely mode of transmission. As a last point unfavourable environmental conditions and host population density might also play a role in the transmission and maintenance of a Laboulbeniales infestation (De Kesel 1996). We do not know which environmental conditions are favourable to *Laboulbenia formicarum* growth on *Lasius neglectus* ants and how population density comes into play. Further experiments will have to shed light on these unresolved questions regarding the transmission of *Laboulbenia formicarum*.

In the second part of this chapter we explored the impact of an existing *Laboulbenia formicarum* infestation on the survival of workers that were maintained under starving conditions and/or exposed to *Metarhizium ansiopliae*. In the between population comparison of experiment 3 we found that the survival of workers from two non infested populations (Jena and Volterra) and two infested populations (Gif-sur-Yvette and Douarnenez) was severely reduced under starving conditions compared to normal maintenance, feeding the workers. This was the case upon *Metarhizium* exposure, sham control exposure and non exposure. Furthermore we found that under fed condition the survival of workers from the non infested population of Jena and Volterra was significantly lower upon *Metarhizium* exposure compared to sham control exposed or non treated workers (Fig. 2 A, B). In contrast, under fed condition the survival of workers from the infested populations of Gif-sur-Yvette was only significantly lower upon *Metarhizium* exposure compared to non treated workers but not to sham control treated workers, whereas the survival of workers from the infested population of Douarnenez was not affected at all by exposed treatment (Fig. 2 C, D). The difference between the two infested populations might be due to their different infestation status, with Gif-sur-Yvette workers showing a high infestation status, whereas workers from Douarnenez only showed a low infestation status (Espadaler et al. 2011). Generally however it appears that the survival of *Laboulbenia formicarum* infested workers is not as much affected by applied treatment as the survival of non infested workers. This might imply some sort of interaction between the ectoparasitic fungus *Laboulbenia formicarum* and the entomopathogenic fungus *Metarhizium anisopliae*. We also found

that starvation (Fig. 3) or only the access to water had such a strong negative impact on the survival of workers from all populations (exception workers from the population of Jena under starving condition) that exposure to *Metarhizium* was not able to cause further death.

Unfortunately our analysis did not allow us to determine if the survival of workers from *Laboulbenia formicarum* infested populations was lower than from non infested populations.

However the within population comparison of high and low infested *Laboulbenia formicarum* workers from the population of L'Escala, Spain, in experiment 4 showed that whereas the survival of workers was not different under normal maintenance, feeding the workers, the survival was significantly higher in low infested workers compared to high infested workers under starving condition (Fig. 4 A, B). This indicates a negative impact of *Laboulbenia formicarum* infestation under nutritional stress possibly due to the fact that these ectoparasitic fungi feed on their host (Scheloske 1969). Furthermore we found that the survival of high and low infested workers was not different upon *Metarhizium* exposure in the fed condition but only in the starving condition (Fig. 4 C, D). Therefore also in the within population comparison worker survival of *Laboulbenia formicarum* infested workers was apparently not different upon *Metarhizium* exposure compared to non treated workers. Only starvation resulted in a significantly different survival of low and high infested workers in this setup but not an exposure to *Metarhizium*.

Further evidence for a possible interaction between the ectoparasitic fungus *Laboulbenia formicarum* and the entomopathogenic fungus *Metarhizium anisopliae* seen with indifferent worker survival upon *Metarhizium* exposure compared to no exposure in experiment 3 and 4 was gained in experiment 5. In this experiment we observed a change of the total phenoloxidase activity (PO and proPO) in the hemolymph of workers from the non *Laboulbenia formicarum* infested population of Jena one day after *Metarhizium* exposure compared to not *Metarhizium* exposed workers (Fig. 5). Such a change in the PO activity after exposure to *Metarhizium anisopliae* has also been observed in the desert locust *Schistocerca gregaria* (Gillespie et al. 2000b). In our system phenoloxidase activity levels were lower upon *Metarhizium* exposure compared to no exposure. This could indicate that PO is not activated in response to a fungal challenge but that the activation of PO is suppressed upon exposure to *Metarhizium*. The suppression of PO activation has already been observed upon exposure to entomopathogenic fungi (Gillespie et al. 2000a). In contrast to workers from the population of Jena we were not able to detect a change in the phenoloxidase activity in workers from the heavily *Laboulbenia formicarum* infested population of L'Escala (Fig. 5) upon *Metarhizium* exposure. Why *Metarhizium* exposure of *Laboulbenia formicarum* infested workers does not lead to a change in the phenoloxidase activity remains to be elucidated. One possibility might be that PO is already suppressed by *Laboulbenia formicarum* or that the pattern of *Metarhizium* fungal pathogenesis (Clarkson and Charnley 1996) is somehow altered in the presence of *Laboulbenia formicarum*.

Taken together our results in the second part of this chapter suggest a negative impact of an existing *Laboulbenia formicarum* infestation under nutritional stress on the survival of *Lasius neglectus* ant workers. Furthermore we found that upon exposure to the entomopathogenic fungus *Metarhizium ansiopliae*, a *Laboulbenia formicarum* infestation leads to an indifferent survival of workers compared to non *Metarhizium* exposed workers and also to an altered pattern of phenoloxidase activation compared to non infested workers. At this point we are unable to determine how these two fungi interact and if this interaction has a negative or positive impact on the survival of infested workers.

# Acknowledgements

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# General conclusion

Aim of this thesis was to investigate the social immune defence of ant societies using mainly the invasive garden ant *Lasius neglectus* (Van Loon et al. 1990) and eliciting antiparasite defence by insulting the immune system with the entomopatogenic fungus *Metarhizium anisopliae*. Insect societies are especially interesting for the study of how immunity can be achieved as they have evolved cooperative defences that complement the immune response of individual group members and are based on interactions between two or more individuals of the society (Cremer et al. 2007).

In the first three chapters different behavioural aspects of hygienic care directed towards the brood were investigated. Brood of social insects enjoys a high degree of protection through the society (Ayasse and Paxton 2002). Furthermore brood represents a high future value for the colony and lacks important hygienic behaviours such as grooming behaviour (Oi and Perreira 1993). Therefore brood is likely to bear special protection.

In chapter I we found that fungus exposed brood gets intensively groomed by workers upon exposure to entomopathogenic fungi. Thereby worker-brood allo-grooming is performed adaptively according to exposure risk. The adaptive nature of grooming behaviour according to treatment toward brood has first been proofed with exposed larvae of the ant *Cardiocondyla obscurior* (Ugelvig et al 2010). As shown in chapter II the benefit from performed worker-brood allo-grooming is immediately evident as infectious particles from the surface of exposed brood are removed. Removal of fungal spores through grooming has so far only been shown in adult workers of insect societies (ants: Hughes et al. 2002, Reber et al. 2011; termites: Yanagawa and Shimizu 2007) where it is often associated with an increased survival of exposed individuals living in a group (termites: Rosengaus et al. 1998b, Yanagawa and Shimizu 2007; ants: Hughes et al. 2002, Okuno et al. 2011). Despite the hygienic care toward brood including worker-brood allo-grooming, we found in chapter I that a high proportion of exposed brood gets removed from the nest after some time. This hygienic brood removal has only been recently described from ants (Ugelvig et al. 2010). Hygienic brood removal has originally been described in the honey bee (Rothenbuhler and Thompson 1956, Wilson-Rich et al. 2009) and is probably targeted to decrease the transmission risk of an infection within the colony. The obtained results in chapter I show that hygienic brood removal can at least be found in another five ant species. Thus it probably constitutes a core element of hygienic behaviour in ants.

We could also show in chapter I that a silk-cocoon enclosure around pupal brood apparently interferes with normal fungal pathogenesis resulting in a indifferent removal of cocoon-enclosed pupae from the nest which is also associated with a indifferent fungal growth on removed cocoon-enclosed pupae in contrast to removed larvae or free pupae. The silk cocoon-enclosure around pupae may thus serve as protective shell, limiting fungal infection. In the lesser spruce sawfly *Pristiphora abietina* it was not possible to infect cocoon-enclosed pupae with *Metarhizium anisopliae* (Führer 2001), which suggests

that *Metarhizium* might not be able to penetrate through the silk cocoon. However in chapter II we found that less callow workers hatched from live fungus exposed pupae than from UV-killed spore exposed pupae. Moreover we found that those callow workers that managed to hatch from live fungus exposed pupae almost all died within a few days after hatching from a *Metarhizium* infection. This indicates that spores are able to penetrate the silk cocoon-enclosure and contrasts the findings of a potentially protective function of the cocoon-enclosure around pupae obtained in chapter I. Further experiments will be needed to determine a clear beneficial effect of the silk cocoon-enclosure upon pathogen exposure.

In chapter II we could also observe the premature unpacking of exposed cocoon-enclosed pupae, a behaviour which has not been reported in the literature so far. Premature unpacking of pupae is likely part of the hygienic repertoire of the ants, maybe as part of the waste management often reported in ants (Howard and Tschinkel 1976, Hart and Ratnieks 2001, Hart et al. 2002). Surprisingly in chapter II we also found that upon pathogen exposure a large proportion of larvae is dead in the presence of workers 24h past exposure. The nature of this behaviour cannot be explained and awaits further investigation. Both, larval death and unpacking of cocoon-enclosed pupae were not observed in chapter I maybe due to a plastic hygienic behaviour response according to experimental conditions expected for invasive species (Hughes and Cremer 2007).

In chapter II we also found that spore removal due to allo-grooming in a group impacts only marginally on the survival of exposed workers. Taken together with the observed high mortality of hatched callow workers from *Metarhizium* exposed pupae it therefore appears that although allo-grooming is able to remove spores from the surface of exposed brood and workers it is not able to prevent fungal infection. A similar conclusion has been recently obtained for worker survival in the ant *Formica selysi* (Reber et al. 2011). The restricted effect of self- and allo-grooming is likely imposed by fungal pathogenesis (Clarkson and Charnley 1993) as the fungus attaches to the host cuticle and starts to penetrate it within 24-48h to reach the host hemocoel (Boucias and Pendland 1991) thus limiting the time period grooming can be effective at spore removal. Other hygienic behaviours such as the hygienic brood removal in chapter I and the unpacking from the silk cocoon enclosure in chapter II are thereafter probably used to limit disease spread within the colony. The limits of grooming set throught fungal pathogenesis might also in part explain the differences in worker-brood-allogrooming effectiveness observed in chapter I and II between fungus exposed larvae and pupae. Although not analysed directly in chapter II, it appears that the fungus is able to attach stonger and/or faster on the cuticle of larvae compared to the silk cocoon envelope of pupae. On the other hand it appears that the workers are able to remove more spores on the surface of larvae shortly after exposure compared to pupae. These effects are likely to contribute to the differences in hygienic behaviour observed toward larvae and pupae (chapter I and II) although the display of worker-brood allo-grooming does not seem to be influence by this (chapter I and III).

Despite the limits of grooming at spore removal found in chapter II, we found in chapter III that during allo-grooming of brood antimycotic substances are employed by the workers. These antimycotic substances have their origin in the gaster involving formic acid. In fungus-growing ants the use of antiseptic compounds from the metapleural gland (Maschwitz 1970) goes also hand in hand with hygienic grooming behaviour inhibiting removed fungal particles in the infrabuccal pockets of the ants and thus limiting autoinfection (Fernández-Marín et al. 2006). The use of antimycotic substances during grooming behaviour in our system is likely to extend the period hygienic grooming behaviour can be effective, as such substances might still be effective when mechanical removal of spores is not possible anymore. This might also partly contribute to the higher survival of fungus exposed workers in a group seen in chapter II. However further experiments with extended periods of isolation of fungus exposed workers are needed to proof a contribution of chemical cleaning to the increased survival of workers living in a group. The use of formic acid during grooming might also provide an explanation for larval death seen in chapter II. As the cuticle of larvae is still soft in comparison with the cuticle of workers, formic acid could act there above a certain threshold as insecticide (Osman and Kloft 1961).

In chapter IV we investigated distinct aspects of the host-parasite system between *Lasius neglectus* ants and the ectoparastic fungus *Laboulbenia formciarum*, both exotic to Europe (Seifert 2000, Espadaler et al. 2011).

We first found that the association between *Lasius neglectus* and *Laboulbenia formciarum* in Europe is probably older than previously thought and that it is possible to transmit the infestation with this ectoparasitic fungus to previously uninfested populations of *Lasius neglectus* ants. The question about the origin of *Laboulbenia formciarum* found in Europe on *Lasius neglectus* remains unanswered but might be worthwhile to be studied in more detail as it either represents the early stages of a newly formed host-parasite system (Espadaler et al. 2011) or an example of an introduced ant species that shows less parasites in the introduced range compared to its native range (Torchin et al. 2003).

In the main part of chapter IV we explored a possible negative impact of an existing *Laboulbenia formciarum* infestation on *Lasius neglectus* ants. We found a negative impact of *Laboulbenia formicarum* infestation under nutritional stress possibly due to the fact that these ectoparasitic fungi feed on their host (Scheloske 1969). Furthermore we found that that upon exposure to the entomopathogenic fungus *Metarhizium ansiopliae*, a *Laboulbenia formicarum* infestation leads to an indifferent survival of workers compared to non *Metarhizium* exposed workers and also to an altered pattern of phenoloxidase activation compared to non infested workers. This indicates that the presence of these two fungi leads to an interaction with different outcome compared to non *Laboulbenia formicarum* infested workers, which could possible contribute to our understanding of the infection processes involved upon exposure of insects to entomopathogenic fungi. A better understanding of these infection processes might prove fruitful and contribute to the devolopment of *Metarhizium* as biocontrol agent against insect pests (Zimmermann 1993, Shah and Pell 2003).

# Summary

To study how immunity is achieved in insect societies I investigated the antiparasite defence of mainly the invasive garden ant *Lasius neglectus* when exposed to the entomopathogenic fungus *Metarhizium ansiopliae*. In the first three chapters I focused on behavioural aspects of the social immune system with special reference to hygienic actions toward brood.

In Chapter I I could first demonstrate a potential protective function of the cocoon-enclosure around pupae in ants when exposed to fungal pathogens. Second, I could prove that hygienic brood removal is widespread in ants probably targeted at lowering the transmission risk of a fungal infection within the colony.

In Chapter II I found that allo-grooming of workers and brood is effective at spore removal but severely limited through fungal pathogenesis. Spore removal through grooming is unlikely to contribute significantly to the increased survival of exposed individuals living in a group. Furthermore I could show that although spore removal through allo-grooming is likely to be responsible for a delay of fungal growth on brood it cannot prevent together with other hygienic care the infection of pupal cocoons. I also found that a large proportion of larvae were dead in the presence of workers after 24h. Furthermore I found the expression of a new behaviour directed towards exposed pupae: the premature unpacking of pupal cocoons. Whereas the premature unpacking of pupal cocoons is likely to be part of the hygienic behaviour repertoire of the ants the nature of larval death needs further investigations.

In Chapter III I could demonstrate that during care of fungus exposed pupae fungal particles are not only mechanically removed due to worker-brood allo-grooming but in addition also antimycotic substances are applied. Furthermore I could show that the production site of these substances resides in the gaster involving the poison reservoir. Finally, I could show that these substances are probably taken up from the gaster into the mouth before application, thus assigning the abdominal self-grooming behaviour of workers a new role during hygienic brood care.

In Capter IV I found that the association between *Lasius neglectus* and *Laboulbenia formciarum* in Europe is probably older than previously thought and that it is possible to transmit the infestation with this ectoparasitic fungus to previously uninfested populations of *Lasius neglectus* ants. Furthermore I found a negative impact of an existing *Laboulbenia formicarum* infestation under nutritional stress on the survival of *Lasius neglectus* ant workers and that upon exposure to the entomopathogenic fungus *Metarhizium ansiopliae*, a *Laboulbenia formicarum* infestation leads to an indifferent survival of workers and also to an altered pattern of phenoloxidase activation opposed to non infested workers.

# Zusammenfassung

In dieser Arbeit habe ich mich damit befasst wie Immunität in sozialen Insekten Gemeinschaften erlangt wird. Die antiparasitären Verteidigung wurde hauptsächlich an der invasiven Gartenameise *Lasius neglectus* mittels des entomopathogenen Pilzes *Metarhizium ansiopliae* untersucht. In den ersten drei Kapiteln standen dabei Verhaltensaspekte des sozialen Immunsystems mit spezieller Referenz zu hygienischem Verhalten gegenüber der Brut im Mittelpunkt.

In Kapitel I konnte zuerst gezeigt werden, dass der Seidenkokoon in dem sich die entwickelnden Puppen befinden wahrscheinlich eine schützende Funktion gegenüber der Infektion mit Pilzen besitzt. Desweitern konnte gezeigt werden, dass das hygienische Beseitigen von infizierter Brut vom Nest eine weit verbreitete Verhaltensweise in Ameisen ist, die wahrscheinlich dazu dient das Ausbreiten der Krankheit im Nest zu vermindern.

In Kapitel II konnte bewießen werden, dass durch gegenseitiges Putzen von Arbeiterinnen und durch das Putzen von Brut Pilzsporen von der Oberfläche Pilz exponierter Arbeiterinnen und Brut entfernt werden, die Effektivität dieses Verhaltens jedoch durch die Eigenheiten der Pilzpathogenese limitiert ist. Desweiteren ist es unwahrscheinlich, dass das Entfernen von Pilzsporen durch gegenseitiges Putzen signifikant zu einem höheren Überleben Pilz exponierter Arbeiterinnen in einer Gruppe führt. Auch konnte gezeigt werden, dass obwohl das Entfernen von Pilzsporen durch Putzen der Brut wahrscheinlich zu einem verzögertem Auswachsen des Pilzes auf exponierter Brut führt, eine Infektion von Puppen nicht verhindert werden kann. Desweiteren wurde gefunden, dass ein großer Anteil an Larven in Anwesenheit von Arbeiterinnen nach 24h Tod war, während gegenüber Pilz exponierten Puppen eine bisher unbekannte Verhaltensweisen entdeckt wurde: das übereilte Befreien von Puppen aus ihrem Kokoon. Während dieses übereilte Befreien von Puppen aus ihrem Kokoon wahrscheinlich Teil des hygienischen Verhaltensrepertoirs der Ameisen darstellt, bedarf das Phänomen der toten Larven noch weiterer Untersuchungen..

In Kapitel III konnte gezeigt werden, dass während des Putzens von Puppen nicht nur Pilzsporen von Puppen entfernt werden, sondern auch antimycotische Substanzen appliziert werden. Der Ursprung dieser Substanzen liegt dabei wahrscheinlich im Gaster der Ameisen mit Beteiligung des Giftreservoirs. Letztlich konnte gezeigt werden, dass diese Substanzen möglicherweise vor Applikation in den Mund aufgenommen werden. Damit könnte dem Putzen der Gasterspitze eine neue Rolle in der hygienischen Brutpflege zukommen.

In Kapitel IV konnte gezeigt werden, dass die Verbindung zwischen der Ameise *Lasius neglectus* und dem ectoparasitischem Pilz *Laboulbenia formciarum* in Europa möglicherweise älter ist als ursprünglich gedacht und dass es möglich ist den Befall mit diesem ectoparasitischem Pilz von einer befallenen zu einer vorher unbefallenen Population von *Lasius neglectus* Ameisen zu übertragen. Es wurde auch ein negativer Einfluss eines vorhandenen *Laboulbenia formicarum* Befalls unter

Ernährungsstress auf das Überleben von *Lasius neglectus* Arbeiterinnen festgestellt. Auch konnte gezeigt werden, dass Exposition von *Laboulbenia formicarum* befallenen Arbeiterinnen mit dem entomopathogenen Pilz *Metarhizium ansiopliae* zu einem indifferenten Überleben von befallenen Arbeiterinnen verglichen mit nicht befallenen Arbeiterinnen führt und dass eine Exposition mit *Metarhizium* von befallenen Arbeiterinnen auch zu einer veränderten Aktivierung der Phenoloxidase Kaskade im Vergleich zu nicht befallenen Arbeiterinnen führt.

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# Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für die Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form eine anderen Prüfungsbehörde vorgelegt.

Regenburg, Oktober 2011

Simon Tragust