Pathogen-mediated sexual selection and immunization in ant colonies

by

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Abstract

Social insects are a common model to study disease dynamics in social animals. Even though pathogens should thrive in social insect colonies as the hosts engage in frequent social interactions, are closely related and live in a pathogen-rich environment, disease outbreaks are rare. This is because social insects have evolved mechanisms to keep pathogens at bay – and fight disease as a collective. Social insect colonies are often viewed as "superorganisms" with division of labor between reproductive "germ-like" queens and males and "somatic" workers, which together form an interdependent reproductive unit that parallels a multicellular body. Superorganisms possess a "social immune system" that comprises of collective disease defenses performed by the workers - summarized as "social immunity". In social groups immunization (reduced susceptibility to a parasite upon secondary exposure to the same parasite) can e.g. be triggered by social interactions ("social immunization"). Social immunization can be caused by (i) asymptomatic low-level infections that are acquired during caregiving to a contagious individual that can give an immune boost, which can induce protection upon later encounter with the same pathogen (active immunization) or (ii) by transfer of immune effectors between individuals (passive immunization).

In the second chapter, I built up on a study that I co-authored that found that low-level infections can not only be protective, but also be costly and make the host more susceptible to detrimental superinfections after contact to a very dissimilar pathogen. I here now tested different degrees of phylogenetically-distant fungal strains of *M. brunneum* and *M. robertsii* in *L. neglectus* and can describe the occurrence of cross-protection of social immunization if the first and second pathogen are from the same level. Interestingly, low-level infections only provided protection when the first strain was less virulent than the second strain and elicited higher immune gene expression.

In the third and fourth chapters, I expanded on the role of social immunity in sexual selection, a so far unstudied field. I used the fungus *Metarhizium robertsii* and the ant *Cardiocondyla obscurior* as a model, as in this species mating occurs in the presence of workers and can be studied under laboratory conditions. Before males mate with virgin queens in the nest they engage in fierce combat over the access to their mating partners.

First, I focused on male-male competition in the third chapter and found that fighting with a contagious male is costly as it can lead to contamination of the rival, but that workers can decrease the risk of disease contraction by performing sanitary care.

In the fourth chapter, I studied the effect of fungal infection on survival and mating success of sexuals (freshly emerged queens and males) and found that worker-performed sanitary care can buffer the negative effect that a pathogenic contagion would have on sexuals by spore removal from the exposed individuals. When social immunity was prevented and queens could contract spores from their mating partner, very low dosages led to negative consequences: their lifespan was reduced and they produced fewer offspring with poor immunocompetence compared to healthy queens. Interestingly, cohabitation with a late-stage infected male where no spore transfer was possible had a positive effect on offspring immunity – male offspring of mothers that apparently perceived an infected partner in their vicinity reacted more sensitively to fungal challenge than male offspring without paternal pathogen history.

Acknowledgments

First and foremost, I would like to thank my supervisor, Sylvia Cremer, who has taken me under her wing over the past few years. You always took your time when I was stuck and gave me the freedom to be creative, yet the guidance to be productive. I especially enjoyed our brainstorming sessions - whether we came up with new ideas or tried to find the common thread in the data, you were always there when I needed your advice. Thank you for giving me the opportunity to attend so many conferences and workshops, the adventurous ant collection trips at the most exciting locations (Tenerife was definitely my favorite!) and also for supporting me in my enthusiasm for scientific outreach. You sharpened my way of thinking and made me a scientist – thank you so much for everything!

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All this work would not have been possible without such a wonderful group – I would like to express my gratitude to the entire Social Immunity Team for creating such a wonderful working atmosphere. Thanks to Anna, Jenni and Lisi for keeping the lab running and for being extremely supportive. Thank you Anna for your patience in showing me all the molecular techniques – I really learnt a lot from you!

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I have met so many wonderful people on my scientific journey - this time was truly enriching and unforgettable!

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About the Author

Sina Metzler completed her BSc and MSc studies in Biology with a main focus on Evolutionary Biology, Neurobiology and Medical Microbiology at the University of Regensburg. After working one year at the Wolf Science Center in Ernstbrunn, studying cooperative behavior in dogs and wolves, she joined IST Austria in February 2015. Her main research interests are animal behavior, evolutionary ecology and immunology. Specifically, Sina's research has focused on social immunity in ants, in particular how social interactions can lead to long-term immunological effects in the individuals. She presented her work on different international conferences, such as the international IUSSI conference in Brazil 2018 and attended several workshops e.g. the Evolutionary Biology workshop in Guarda 2017. During the past years Sina has discovered her interest in communicating science to the public and presented her work in "P.M. Wissen" and gave an interview for an article in "Der Standard" about her research.

List of Collaborators and Publications

All collaborators that contributed to experimental work are outlined in each chapter.

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List of Abbreviations

β-1,3-GBP	Beta-1,3-glucan binding protein
COXME	Mixed effects cox models
ddPCR	droplet digital PCR
Def	Defensin
Dor	Dorsal
GLMM	Generalized linear mixed model
HR	Hazard ratio
IMD	Immune deficiency
LD	Lethal dose
LR	Likelihood ratio
Rel	Relish
SE	Standard error
SD	Standard deviation
Spn27A	Serpin 27A
PCR	Polymerase chain reaction
PO	Phenoloxidase
PPAF	Prophenoloxidase activating factor
PPO	Prophenoloxidase
Psh	Persephone
TGIP	Transgenerational immune priming
Tx	Triton-X
qPCR	quantitative real-time PCR

1. Introduction

Memory-like effects in invertebrate immunity

The discovery of immunization dates back to the 9th century when low doses of an otherwise deadly pathogen, e.g. the smallpox virus, were administered to trigger (lifelong) immunity against common diseases – termed "variolation" or "inoculation" (Greenough, 1980). In 1796 Edward Jenner set a milestone in the science of immunology by showing that an infection with less lethal cowpox protects against a subsequent infection with highly virulent smallpox (Jenner, 1798). Based on Jenner's findings, modern vaccinations work via killed or attenuated pathogens (e.g., surface components or toxoids) (Quan *et al.*, 2007).Vaccinations demonstrate how we can benefit from the ability of our immune system to show an improved reaction after a previous contact with the same or a very similar infectious agent (termed "immunization") that is based on the formation of specific immunological memory (Janeway *et al.*, 2007).

In vertebrates immune memory is based on lymphocytes and antibodies and leads to a specific targeted immune response (adaptive immunity) and complements the initial rather unspecific defense (innate immunity) (Janeway *et al.*, 2007). Vaccines do not only protect the vaccinated individual, but also non-immunized individuals by herd-immunity (Anderson and May, 1985). Here, the risk of disease spread is reduced if "the herd" becomes immune to a disease and thereby protects the fraction of susceptible individuals.

Until very recently, it was thought that invertebrates rely only on robust innate defenses, but numerous studies have shown that also the innate immune system of invertebrates can be "adaptive" to some degree (Kurtz, 2005; Sadd and Schmid-Hempel, 2006; Pham *et al.*, 2007; Roth and Kurtz, 2009; Rosengaus *et al.*, 2013; Milutinović and Kurtz, 2016). Immune protection in invertebrates was found to exist from being very unspecific and broad (Moret and Siva-Jothy, 2003) to be memory-like and very specific. These memory-like effects in invertebrates are often referred to as "priming" (Kurtz and Franz, 2003; Pham *et al.*, 2007; Roth and Kurtz, 2009).

The mechanisms so far are very different from those of vertebrates and are still mainly elusive (but see Tassetto *et al.*, 2017). However – even though insects lack the acquired branch of the adaptive immune system – some of them are long lived, such as ant queens, and have therefore developed some solutions for when they encounter pathogens.

Besides individual immunity there are further mechanisms to prevent disease e.g. by the collective achievement of "social immunity" (Cremer *et al.*, 2007).

Social immunity

Social immunity of a group is achieved by cooperative disease defense mechanisms performed collectively by its members and occurs in a wide range of social species, but is primarily studied in eusocial insects (social bees, wasps, ants and termites) where social immunity is especially pronounced (Cremer *et al.*, 2018).

Even though social insects have a lifestyle that should favor pathogen thrive and spread - such as frequent social interactions among highly related individuals and homeostatic nests in pathogen-rich environments (Cremer *et al.*, 2018) – epizootics are very rare (Evans, 1974). The collective anti-pathogen defenses of social immunity can be categorized in behavioral, physiological and organizational components that jointly protect the colony from pathogen uptake, establishment, replication and transmission (Cremer *et al.*, 2007). To prevent or at least reduce the risk of disease, ants perform undertaking behaviors like waste management (Farji-Brener *et al.*, 2016), burial and removal of the corpses (Pull and Cremer, 2017) or nest disinfection with antimicrobial compounds such as collected resin (Castella *et al.*, 2009) or self-produced antimicrobials like formic acid (Pull *et al.*, 2018b).

Another important component is sanitary care, including grooming, that is especially effective against externally-infecting pathogens such as spores of entomopathogenic fungi (Reber *et al.*, 2011) that are plugged off contagious individuals. Sanitary care is also performed towards brood (larvae and pupae) and comprises of (i) grooming (Tragust *et al.*, 2013b) and (ii) removal of infected brood from the colony (Ugelvig *et al.*, 2010; Tragust *et al.*, 2013a) termed "hygienic behavior". Hygienic behavior was initially described in honeybees and by definition is restricted to the immobile brood of social Hymenoptera (Rothenbuhler and Thompson, 1956; Wilson-Rich *et al.*, 2009). However, if these hygienic prophylactic behaviors have failed - e.g. when a pathogen successfully invaded an individual in the ant colony such as in the case of infected pupae - the colony moves to pathogen-triggered intensified grooming and finally to "destructive disinfection". The latter is a multicomponent behavior where ants target infected pupae utilizing chemical "sickness cues" emitted by the pupae. The pupa is unpacked from its cocoon, perforated and eventually sprayed with poison which is killing both the pupa and the fungus inside (Pull *et al.*, 2018a).

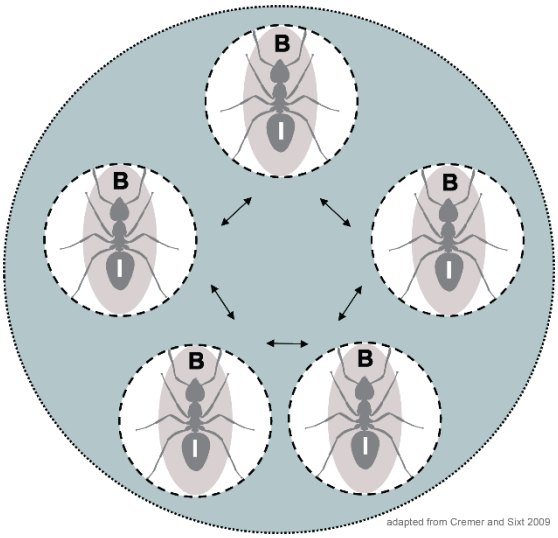

Figure 1.1. **Collective disease defenses.**

Collective defenses (light blue; dotted line) of a group (here described for an ant colony) comprises of all individual defenses (white; dashed line) of the colony members and their interactions (arrows). Each individual has its own individual immune defense comprising of their immune system (I) and their anti-parasitic behaviors (B, grey ellipse) e.g. pathogen avoidance behaviors or sanitary care (more examples in the text). Cooperative disease defense mechanisms that are performed collectively by the colony members lead to social immunity of the colony (Cremer *et al.*, 2007). The figure is adapted from Cremer and Sixt 2009.

Social immunity can be viewed as the immune system of the colony and is conceptually analogous to the immune system of multicellular organisms (Cremer and Sixt, 2009). Similar to a multicellular body, social insect colonies function as a single reproductive unit – consisting of queens and males analogous to the germ line and sterile workers that perform tasks for colony maintenance that represent the soma (Cremer and Sixt, 2009). Both components are highly interdependent and cannot reproduce without each other. Therefore, social insect colonies are often viewed as "superorganisms" (Wheeler, 1911; Boomsma and Gawne, 2018)

Social immunization

One particular component of social immunity is social immunization where protection acts at the group-level e.g. when group members are less susceptible to a pathogen after initial pathogen contact of other group members (Cremer and Sixt, 2009; Konrad *et al.*, 2012; Masri and Cremer, 2014). Social immunization has so far been reported in termites (Traniello *et al.*, 2002) and ants (Ugelvig and Cremer, 2007; Hamilton *et al.*, 2011; Konrad *et al.*, 2012). Social immunization can either occur (i) by transfer of immune effectors between individuals (passive immunization) or (ii) through transfer of low amounts of the pathogen between contaminated individuals and their nestmate ants that often causes low-level infections in the caring individual that do not cause disease, but instead stimulate the immune system and can lead to a protective effect against future infection with the same pathogen (active immunization) (Konrad *et al.*, 2012). This is analogous to the above described early variolation in humans and provides the ant with a survival benefit upon secondary encounter with the same pathogen (Konrad *et al.*, 2012). However, if the ant comes in contact with a second, very different

(heterologous) pathogen, instead of being protected at secondary contact - it can even be more susceptible to this pathogen as it might develop a highly detrimental superinfection (Konrad *et al.*, 2018). In the latter study, we also found that the disease susceptibility of ants was found to affect their behavior towards other group members as well. Low-level infected ants adjusted their sanitary care to their infection history and the presented challenge. Interestingly, we showed that ants allo-groomed their nestmates more when the threat was the same strain (homologous) as their low-level infection, yet sprayed more poison when presented to a heterologous threat, to which they had become more susceptible due to the low-level infection (Konrad *et al.,* 2018). This study clearly demonstrated a survival disadvantage after a superinfection with a fungal strain from a different genus (*Beauveria*) than the low-level infecting strain (*Metarhizium*) – and vice versa – but also that ants can modify their behavior according to their own infection history and thereby reduce the risk of a superinfection. In the second chapter, I tested how low-level infections acquired during sanitary care affect ant mortality upon a heterologous challenge with strains that are phylogenetic more distant than the same clone which can lead to protection (Ugelvig and Cremer, 2007; Konrad *et al.*, 2012) but less distant than a different genus that can increase disease susceptibility (Konrad *et al.*, 2018), by testing different *Metarhizium* strains or species of increasing genetic distance to the initially encountered strain in *Lasius neglectus*.

Transgenerational immunization

While the above stated social immunization occurred within a colony, among cohabiting workers, social immunization can also occur across generations. As ant colonies are rather sessile (Hakala *et al.*, 2019) and consist of overlapping generations, individuals from a colony are expected to experience a stable pathogen community over time and therefore transgenerational immunization is particularly beneficial. Due to the overlapping generations with a strict division of labor between the reproducing queen and her sterile workers (that take care of the next generations, i.e. their own younger siblings), the boundaries between individual and social transgenerational immunization blur (Masri and Cremer, 2014). Transgenerational immunization was found in several lineages of social Hymenoptera e.g. in bumble bees (Sadd *et al.*, 2005; Sadd and Schmid-Hempel, 2007; Barribeau *et al.*, 2016), honey bees (Salmela *et al.*, 2015; Hernández López *et al.*, 2017) and ants (larvae: Fuchs *et al.*, 2018; workers: Bordoni *et al.*, 2018; Casillas-Pérez *et al.*, 2022). When the current worker force is protected (either by individual immunization e.g. triggered by socially acquired low-level infections or by transgenerational immunization provisioned by the queen) the protection feeds back into protection of the current maternal colony and the queen herself (Casillas-Pérez *et al.*, 2022). However, according to the same principle, even the next generation of colonies – the daughter colonies - can be protected by immunizing the (dispersing) sexuals, leading to transgenerational protection of the new superorganism. Dispersing sexuals could (i) found novel daughter colonies outside the risky parasitic zone and (ii) prime (additionally) their offspring (Masri and Cremer 2014).

In species where queens solitarily found new colonies, transgenerational protection of daughter colonies requires immunization of the sexual offspring, i.e. the daughter queens and males. In contrast to this, in species where colony foundation involves help from the workers, as in the case of colony fission or budding (Cronin *et al.*, 2013), immunized workers might directly influence the immune status of the daughter colony after it has budded off. Transgenerational protection might especially play an important role in the success of many invasive species that often have multiple queens and new colonies are founded by budding, which facilitates the rapid growth of these colonies (Tsutsui and Suarez, 2003). In the fourth chapter of my thesis, I studied – amongst other questions – the transgenerational effects of parental pathogen history

(disease of the mother or the father) on the immunocompetence of the offspring in the tramp ant *Cardiocondyla obscurior*.

1.1 Study organisms

In the current work two different host-pathogen systems were used:
In the second chapter, *Lasius neglectus* was used as host, and different strains of *Metarhizium robertsii* (KVL 13-12, KVL 12-38, and KVL 12-35), *Metarhizium brunneum* (KVL 13-13, KVL 12-30, KVL 12-37) and *Metarhizium majus* (KVL 12-29) as fungal pathogens (Steinwender *et al.*, 2014). The fungal strains were kindly provided by B. Steinwender, N. Meyling and J. Eilenberg (University of Copenhagen).
In the third and fourth chapters, *Cardiocondyla obscurior* and *Metarhizium robertsii* (ARSEF2575) with a stable insertion of the *mRFP1* gene was used. This strain was produced and kindly provided by M. Bidochka according to the methods published in Fang *et al.* (2006).
Both ant species are invasive, but differ greatly in their life-histories. Each of these organisms is described in more detail below.

1.1.1 The invasive garden ant *Lasius neglectus*

Figure 1.2.
Photograph of *L. neglectus.*
Photo credit: S. Metzler and R. Ferrigato

L. neglectus is a Formicine ant with presumed origin in Asia Minor (Seifert, 2000) and has by now spread all over Europe – likely by unintentional human activities (Espadaler *et al.*, 2007) and has reached pest status (Seifert, 2000). It invades urban premises such as parks and gardens (Espadaler *et al.*, 2004) and outcompetes native ant species (Nagy *et al.*, 2009). Workers collect huge amounts of honeydew from aphids (Paris and Espadaler, 2009) whereby they cause great harm of native ecosystems e.g. by vast tree damage (Nagy et al., 2009). In contrast to other invasive species, *L. neglectus* can cope with colder temperature and even survive extended periods of frost (Seifert, 2000). As a common characteristic shared with other invasive ant species, it exhibits the "supercolonial syndrome" (Cremer *et al.*, 2008b) and its reproductive biology enables fast growth and spread: interconnected and cooperating nests where individuals are in constant exchange, containing multiple non-dispersing queens that mate in the nest (Van Loon *et al.*, 1990; Cremer *et al.*, 2008b), leading to unicoloniality.
Over the past decade *L. neglectus* became a well-studied model organism for the study of social immunity (Konrad *et al.*, 2012, 2015, 2018; Tragust *et al.*, 2013a, 2013b; Pull *et al.*, 2018a, 2018b). *L. neglectus* can be collected in large numbers in the field and relatively easily maintained in the laboratory. As queens readily mate in the nest and produce new sexuals, their colonies grow fast and replenish worker-losses caused by experimental setups.

Study population and rearing conditions
In our experiments we used *L. neglectus* collected in 2015 from Jena, Germany (N 50° 55.910 E 11° 35.140). All experiments were performed at a constant temperature of 23°C with 65% humidity and a day/night cycle of 14h light/10h dark. Experiments were performed in petri dishes with plastered ground and 10% sucrose solution as food supply for ad libitum consumption. Collection of this unprotected species, rearing and all experimental work complied with European law and institutional guidelines.

1.1.2 The tramp ant *Cardiocondyla obscurior*

Figure 1.3.
Photograph of *C. obscurior.*
Photo credit: S. Metzler and R. Ferrigato

Cardiocondyla obscurior (Wheeler 1929), is a small invasive Myrmicine ant (worker and male size ~1.5mm; queens ~2mm) with its presumed origin in the tropics and subtropics of Southeast Asia. Colonies of *C. obscurior* nest arboreally, living among others in leaves of trees (Heinze *et al.*, 2006) or rotten branches. Colonies in the field are rather small and consist of less than a hundred nest members and new colonies are founded by budding (Heinze *et al.*, 2006), where groups of workers and queens leave the nest with brood and settle in a nest nearby (Heinze and Delabie, 2005). Colonies can grow massively in size under laboratory conditions.
C. obscurior is a tramp species with typical characteristics: multiple queens (polygyny), sterile workers and mating in the maternal nest (Heinze *et al.*, 2006). This has led to an environmentally determined male diphenism (Cremer and Heinze, 2003) with winged disperser males that are peaceful and locally competing wingless males. Winged males are competing with their wingless opponents for access to virgin female sexuals (queens). Shortly after emergence, the young winged males have been shown to mimic female sexuals chemically, which protects them from being attacked by wingless males (Cremer *et al.*, 2002, 2008a). After roughly ten days, winged males disperse, presumably to mate again in another nest (Cremer *et al.*, 2002) with a limited amount of sperm, as their spermatogenesis ceases shortly after eclosion and their testes degenerate (Heinze and Hölldobler, 1993).
Wingless males however, are well adapted to exclude rival males from the nest by enduring fights (more details in chapter 3). The winner of the combat gains the benefit of monopolized mating with many virgin queens present in the nest (Heinze and Hölldobler, 1993; Cremer and Heinze, 2002). A remarkable adaptation to this is that they have a lifelong spermatogenesis (Heinze and Hölldobler, 1993) such that their lifetime number of matings is no longer constrained (Metzler *et al.*, 2016) - a unique characteristic amongst all Hymenoptera.
Ergatoid males are readily produced all year round, whereas under stressful conditions (e.g. temperature or colony size reduction) winged males are produced in addition (Cremer and Heinze, 2003). All experiments in the present study were exclusively performed with wingless males.
The unique biology of *C. obscurior* allowed us to directly observe and manipulate mating behavior in the laboratory. Furthermore, their short generation time (of approximately 6-8 weeks) made transgenerational studies possible. As queens are rather short-lived (average lifespan six month) the total lifespan of a queen can be monitored and her lifetime reproductive success can be determined (Schrempf *et al.*, 2005; Heinze and Schrempf, 2012)

Study population and rearing conditions
All ants used in the experiments (of chapters 3 and 4) were taken from stock colonies and respective splits that were collected in Tenerife in 2013 and 2017 (N 28° 23.403 E 16° 35.78). All experiments were performed at constant 27 °C with a day/night cycle with 14h of light and 10h of dark and 65% humidity. If not stated otherwise, all experiments were performed in experimental containers (Ø25mm) with a plastered ground and 10% sucrose was provided for ad libitum consumption.
Ant collection followed the rules for Access and Benefit-Sharing following the Nagoya protocol and was granted by the Spanish Ministry of Agriculture, Fisheries and Environment

(ABSCH-IRCC-ES-237603-2 number ESNC2), as well as the Council of Tenerife (Expte: AFF 119/17). All experimental work is in line with European law and institutional guidelines. *C. obscurior* is an invasive species and is not protected.

1.1.3 The entomopathogenic fungus *Metarhizium*

As a model pathogen, we used various strains of the obligate killing pathogen *Metarhizium* that is a genus of Ascomycete (Hajek and St Leger, 1994). *Metarhizium* has a worldwide distribution (Roberts and St Leger, 2004) and kills its host by causing the green muscardine disease. Entomopathogenic *Metarhizium* are generalist fungi that occur at high densities in the soil where up to 5000 conidiospores per gram soil (Keller *et al.*, 2003; Cremer *et al.*, 2018) can be found. Sporulating cadavers can have up to 12 million conidiospores (Hughes *et al.*, 2002; Hughes *et al.*, 2004b), thereby bearing a great contamination risk and are responsible for natural infections of ants in field populations (Hughes *et al.*, 2004b; Reber and Chapuisat, 2012; Casillas-Pérez *et al.*, 2022). Some species are known to infect a large array of hundreds of different species (from at least seven orders), which has led to its use as a biological insecticide (Gillespie and Claydon, 1989; Brunner-Mendoza *et al.*, 2019) as an alternative to chemical pesticides.

All *Metarhizium* species follow approximately the same lifecycle: infectious conidiospores (in the following referred to as "spores") are acquired from the environment (Hajek and St Leger, 1994; Hughes *et al.*, 2004b; Reber and Chapuisat, 2012) and initially loosely attach to the insect's cuticle via unspecific hydrophobic interactions. Under humid conditions the spores swell and give rise to a specialized infection organ – called "appressorium" that penetrates into the hemocoel by enzymatic breakage of the cuticle (Sun and Zhou, 2013). Once in the hemocoel, budding occurs, which leads to the production of single cells called "blastospores". These replicate in the host body, spread via the hemolymph and weaken the host by sugar consumption and the production of destruxins. The latter are toxins that e.g. suppress the immune system and kill cells (Pedras *et al.*, 2002) which finally leads to the host's death. Eventually, mycelia grow out of the corpse through the intersegmental regions and sporulate from their tips to produce new (asexual) conidiospores (Sun and Zhou, 2013).

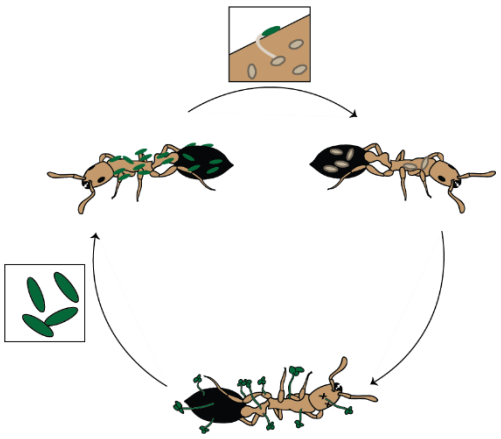

Figure 1.4. **Host-pathogen lifecycle.**

Conidiospores (green ellipse) get picked up from the environment and first loosely attach to the cuticle. Under humid conditions they penetrate the cuticle (upper picture). Once in the hemocoel, blastospores (beige ellipse) replicate in the host body and toxins are released that finally leads to the host's death. Eventually, mycelia grow out of the corpse and sporulate to produce new conidiospores (more details in text).

Metarhizium acts in a dose-dependent manner

Metarhizium acts in a dose-dependent manner, i.e. the infection success increases with the number of spores encountered within a certain time period. This means that a higher exposure dose leads to higher and often earlier mortality. However, depending on the context even very low dosages can cause lethal infections (Hughes *et al.*, 2004a; Boomsma *et al.*, 2014), but with a lower probability.

Time after topical spore exposure determines the disease stage

As *Metarhizium* infects its host via the cuticle, the time after pathogen contact influences the probability of transmitting spores from one individual to the other as well as the stage of infection.

Individuals that just freshly came in contact with spores we refer to as "contaminated". At this early stage of infection, intense allogrooming of the spore-exposed individuals can typically be observed as an initial social immune response, namely sanitary care (Rosengaus *et al.*, 1998; Konrad *et al.*, 2012). Before the spores have attached to the ant's cuticle – which is described to be completed latest after 48h (Walker and Hughes, 2009) - the spores can easily be removed by grooming and spread between individuals via social interactions or even by sharing the same environment (Rojas *et al.*, 2018; Kurze *et al.*, 2020) as spores that have not attached might fall to the ground from where they can be picked up again.

Individuals that were freshly exposed before the experiment and that are therefore initially contagious to their nestmates we refer to as "early-stage infected" individuals hereafter.

After 48h, when spores have firmly attached to the host's cuticle and an internal infection has established - allogrooming is no longer effective and spores cannot spread between individuals anymore (Walker and Hughes, 2009; Pull *et al.*, 2018a).

Individuals that already carried established infections at the start of our experimental setups are referred to as "late-stage infected" individuals. Late-stage infected individuals were exposed to fungal spores and individually kept for 48h before the experimental start.

Fungal culturing

Aliquots of conidiospore suspensions were kept in long-term storage at – 80°C. Prior to each experiment, the conidiospores were grown on Sabouraud dextrose agar (SDA) at 23°C for approximately three weeks until they have sporulated. Conidiospores were harvested by scraping them off the plates and suspending them in 0.05% sterile Triton X-100 (0.05% Tx, Sigma Aldrich, Austria). The concentrations of the spore suspensions were counted and adjusted with a Cellometer® Auto M10 counter (Nexcelom). The germination rate of each spore suspension was determined before the start of each experiment and was >95% in all cases (unless stated differently).

Fungal exposure of ants

All individuals were exposed by submerging them shortly in an aliquot of either 0.05% Tx as sham-treatment or a conidiospore-suspension for treatment and placed on filter paper that absorbed excess liquid until the ants recovered enough to walk. The aliquots were regularly vortexed to prevent spore clumping and to ensure a homogeneous spore suspension. As *L. neglectus* releases formic acid during the handling process which can interfere with the germination of the spores, a maximum of 15 ants was dipped in an aliquot of 150 µl.

1.2 Thesis aims and outline

Sanitary care is one of the major components of social immunity (Cremer *et al.*, 2007). Ants that are contaminated with infectious disease particles receive intensive sanitary care by their nestmates, which often contract the pathogen themselves – but usually only in a very small quantity. These small pathogen doses can lead to low-level infections that do not cause disease, but instead can reduce the disease susceptibility of the nestmates by a protective immune stimulation upon secondary contact with the same (homologous) pathogen or have neutral effects (Konrad *et al.*, 2012). However, low-level infections can also be costly and make the host more susceptible to superinfections after contact to a dissimilar (heterologous) pathogen, such as an unrelated genus (Konrad *et al.*, 2018).

In the **second chapter**, I dissected in detail the benefit-cost aspect of fungal strain combinations on ant survival. I tested whether strains of increasing genetic distance to the low-level infecting strain can provide cross-protection and characterized the combinations of strains where cross-protection occurred by looking at the first and second strain's relative virulence (virulence difference) and their immunogenic ability to induce immune gene expression in *Lasius neglectus*.

In the **third** and **fourth chapters**, I studied the role of social immunity in sexual selection in ants, a so far unstudied aspect of social immunity. I was interested to see how a pathogen affects mating behavior and how disease alters success of queens and males, colony development and the immunity of the offspring generation. To study pathogen-mediated sexual selection, I had to use a model organism where I was able to look at mating behavior under laboratory conditions. Unlike most ant species – where mating occurs in mating flights far away from the maternal nest - sexuals of the ant *Cardiocondyla obscurior* stay and mate in their maternal nest and males fight with competitor males over access to emerging virgin queens.

In the **third chapter**, I aimed to determine how different stages of disease influence male-male competition and fighting ability. As fighting occurs in the presence of workers, I was able to study the interplay of social immunity by workers and a possible interference of the workers on male-male competition. At early-stage of infection, workers could on one hand care for contaminated individuals that just recently came in contact with fungal spores and thus hamper disease development for the individual, but on the other hand also prevent transmission through the colony by preventing close contact of healthy males to contagious rivals.

In the **fourth chapter**, I looked at how different stages of disease may affect mating behavior and success of queens and males and whether workers interfere in risky mating attempts from contagious or infected mating partners. As I found that males at early- and late-stage of infection are still able to mate successfully and are able to retain high sperm quality, I evaluated the long-term effect on queen survival and fertility, colony development and caste composition and finally the immunocompetence of the offspring when the potential mating partner of a queen was (i) either at an early-stage of infection where spore transmission was still possible or (ii) at the late-stage of infection (when the male was sick, but no longer contagious to the queen).

2. Social contact to an infectious colony member can provide cross-protection to related pathogens in ants

Photograph shows *Lasius neglectus* workers grooming each other on a leaf.
Photo credit: S. Metzler and R. Ferrigato.

2. Social contact to an infectious colony member can provide cross-protection to related pathogens in ants

Project conceptualization with Matthias Konrad and Sylvia Cremer: own contribution 30%

Survival experiment with MK: own 50%

Social setups for immune gene expression analysis: own 100%

qPCR immune gene expression: Elisabeth Naderlinger 100%

Data analysis with MK: own 80%

Genetic distance analysis: Anna Grasse 100%

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2.1 Abstract

Social insects fight diseases by sophisticated collective defense behaviors. Ants that are contaminated with infectious disease particles receive intensive sanitary care by their nestmates, which often contract the pathogen themselves during their social interactions with the contaminated individuals. The transfer of these typically small pathogen doses can lead to low-level infections that do not cause disease, but instead reduce the disease susceptibility of the nestmates by a protective immune stimulation upon secondary contact with the same pathogen. Due to the diversity and high abundance of pathogens in their natural habitat, infections with several pathogens are very likely. We found that socially acquired low-level infections can also provide a survival benefit following challenge with related fungal strains of the same pathogen species, but that protection only occurred when the initially encountered stain was less virulent than the subsequent, superinfecting one. When testing for the immunogenicity of these strains, we found that protection only occurred in the combinations in which the strain that induced higher immune gene expression in two effector genes (Defensin and *PPO*), was the first strain. Given the high pathogen diversity in the field, the cross-protective nature of social immunization we have discovered, thus is expected to increase overall protection of the colony.

Keywords: immunological cross-protection, superinfection, virulence, immunogenicity, social immunity, host-pathogen interactions

2.2 Introduction

The observation that a low-level infection with an otherwise deadly pathogen, e.g., the smallpox virus, can trigger (lifelong) immunity against common diseases – termed "variolation" or "inoculation" – dates back to the 9th century (Greenough, 1980) and ultimately led to the development of vaccines. In 1796 Edward Jenner set a milestone in the science of immunology as he developed the concept further and proved that an infection with cowpox protects against a subsequent infection with smallpox (Jenner, 1798). This was not only the start of vaccine development, but also the first reported case of cross protection – which means protection against a related organism (Vojtek *et al.*, 2019). Nowadays, instead of intentionally transferring low level infections, modern vaccines make use of dead or attenuated pathogens such as surface components or toxoids (Quan *et al.*, 2007), that are designed in a way that they elicit a broad immune response and confer a wide-ranging protection, also termed cross-protection (Vojtek *et al.*, 2019).
Vaccinations as well as natural infections can protect hosts against secondary infections of the same, homologous pathogen, a phenomenon known as "immune memory" in vertebrates and termed "immune priming" in invertebrates (Kurtz and Franz, 2003; Pham *et al.*, 2007; Roth and Kurtz, 2009; Masri and Cremer, 2014). In addition to immunological changes due to priming, infection outcome can also be severely affected by pathogen interactions within the host. Prior infection can also increase a host's susceptibility to a subsequent other, heterologous pathogen. These superimposed secondary infections - known as 'superinfections'- often have greater detrimental impacts on host health and survival than either pathogen alone. Pathogens might interact within the host (i.e. competition or cooperation) (Ben-Ami *et al.*, 2011; Jamieson *et al.*, 2013) and the response against one pathogen can be mal-directed against another pathogen. However, it can also be that the host response raised against the first pathogen is protective against another secondary pathogen, as previously described for cowpox that protected against smallpox (Greenough, 1980) or *Mycobacterium bovis* in the Bacillus Calmette–Guérin vaccine that protects against tuberculosis (Merle *et al.*, 2010). The way two pathogens interact with each other, depends on how similar the pathogens are. Compared to co-infections with different pathogen species, it is to be expected that strains of the same species compete less with each other and possibly even cooperate (Buckling and Brockhurst, 2008). Virulence is also an important factor in multiple infections as virulent pathogens often have higher competitive abilities within the host as they grow faster (Ben-Ami *et al.*, 2008) and less virulent pathogens may be completely suppressed in coinfections.

Cross-protection is especially relevant in species that live in environments where they are exposed to a broad pathogen spectrum which are very diverse in the host population, such as ants (Cremer *et al.*, 2018). Ants live in microbe-rich environments and face a high load (Keller *et al.,* 2003) and diversity (Steinwender *et al.*, 2014) of pathogens, i.e. fungal spores, in their natural habitats. As they often nest in the soil where several *Metarhizium* species can occur simultaneously (Steinwender *et al.*, 2014) co- and superinfections are very likely in the field. *Metarhizium* produces a large number of infectious spores which are found both in the soil as well as on sporulating cadavers that individuals encounter e.g. during foraging trips and make multiple sequential infections of individual colony members a serious risk. Therefore, individuals (especially foragers that regularly leave the nest) – even belonging to the same colony - are very likely to encounter different pathogen species and have repeated pathogen contact with different pathogens.

Disease prevention is particularly important in such social groups, as pathogens can easily spread once one of the group members gets infected. Ants live in high density, engage in a high

number of within-group interactions and share the same resources (Schmid-Hempel, 2011, 2017; Kappeler *et al.*, 2015; Hawley *et al.*, 2021), making them particularly susceptible to pathogen transmission. To counteract this risk, ants (and social animals in general) have evolved anti-pathogenic defenses at the individual as well as at the group-level. The underlying strategy and adaptations can be of physiological, behavioral or organizational nature (Cremer *et al.*, 2018; Stockmaier *et al.*, 2021). Ants display a particularly rich repertoire of group-level protective behaviors that prevent disease outbreaks known as 'social immunity' (Cremer *et al.*, 2007; Cremer and Sixt, 2009; Cremer *et al.*, 2018) that operates in conjunction with individual immunity.

One particularly important component of social immunity is mutual grooming (allogrooming) where external pathogens, i.e. fungal spores, are removed mechanically, which can be combined with chemical disinfection by application of antimicrobials, such as their poison (Graystock and Hughes, 2011; Reber *et al.*, 2011; Tragust *et al.*, 2013a; Tranter and Hughes, 2015). While this behavior effectively reduces the risk of disease for pathogen-contaminated nestmates, individuals in social contact with contagious individuals often contract the pathogen themselves and might become sick themselves (Rosengaus *et al.*, 1998; Hughes *et al.*, 2002). However, if only a low dose of the pathogen is picked up, the nestmates only develop low-level infections that typically do not lead to disease symptoms, but instead can even confer a significant survival benefit by protecting against the same, homologous pathogen, upon secondary pathogen contact. This "social immunization" (Konrad *et al.*, 2012) is functionally similar to acquired immunity by immune priming and has so far been described to occur in diverse social insect species (Rosengaus *et al.*, 1999; Ugelvig and Cremer, 2007; Hamilton *et al.*, 2011; Konrad *et al.*, 2012; Liu *et al.*, 2015).
Unlike the protective effect of low-level infections when ants were exposed to the homologous pathogen, we previously showed that when ants come in contact with a second, different pathogen they can even be more susceptible to this pathogen and can develop a detrimental superinfection (Konrad *et al.*, 2018). Although low-level infected ants in this study were more susceptible to superinfections, the ants continued providing care to their nestmates, but altered their sanitary care performance in a risk-averse manner depending on their individual infection state (disease susceptibility). In this case, low-level infected ants reduced grooming and applied more antimicrobial poison onto nestmates contaminated with a heterologous second pathogen (Konrad *et al.*, 2018) and therefore minimized their risk of contracting a superinfection. The superinfections in this study were induced by fungal strains belonging to different genera (*Metarhizium* and *Beauveria*), meaning that they are only very distantly related. In the present study, we investigated a range of genetic distance between two super-infecting strains to see when we find a switch from protection to cost to see until when the immune system can rely on cross-protection and tested different strains of the two most common *Metarhizium* species, *M. robertsii* and *M. brunneum* - and a different species (of the same genus) - *M. majus.*

We here test how low-level infections acquired during social contact to a contagious group member affect the future disease susceptibility, following heterologous pathogen challenge. For this, we reared *Lasius neglectus* workers with a pathogen-exposed individual that was treated with conidiospores of different strains of either *Metarhizium robertsii* or *Metarhizium brunneum* (or a sham-treatment with 0.05% Triton X) for five days as described in Konrad *et al.* (2012). We then tested how these socially acquired low-level infections (referred to as "first strain" hereafter) affect ant mortality upon a challenge (referred to as "second strain" hereafter) with strains of increasing phylogenetic distance and different virulence to the initially encountered strain. It is important to state that the ants carry an ongoing low-level infection

that is not cleared yet (Konrad *et al.*, 2012) at the time when they receive a challenge with the second strain.
We tested (i) whether the protective effect of socially acquired low-level infections (first strains) provides cross-protection against similar pathogens or has a high degree of specificity and (ii) analyzed the relationship of protection and the relative virulence (virulence difference) between the first and second strain and the immunogenicity of the first strain at the time point of challenge with the second strain.

2.3 Material and Methods

Ant host
We used the invasive, unicolonial ant species *Lasius neglectus* collected in 2015 from Jena, Germany (N 50° 55.910 E 11° 35.140) as host. All experiments were performed at a constant temperature of 23°C with 65% humidity and a day/night cycle of 14h light/10h dark. Experiments were performed in petri dishes with plastered ground and 10% sucrose solution as food supply for ad libitum consumption. Ants were randomly assigned to the respective treatment groups (details see below). Collection of this unprotected species, rearing and all experimental work complied with European law and institutional guidelines.

Fungal pathogens
We used entomopathogenic fungi belonging to the genus *Metarhizium*. For each, *M. robertsii* (abbreviated as *R*) and *M. brunneum* (abbreviated as *B*) we carried out an experiment, in which we exposed single ants to a first and a second fungal exposure of increasing genetic difference between the two fungi: (i) the same strain, yet a different isolate from the field, (ii) a different strain of the same species, and (iii) a different species (in both cases *M. majus*, abbreviated as *Maj*). All strains were isolated from the same location by B. Steinwender (Steinwender *et al.*, 2014), and obtained from the University of Copenhagen (research group of N.V. Meyling and J. Eilenberg). For *R* and *B,* we followed the genetic characterization as performed in Steinwender *et al.* (2014). In detail, the following strains were used: *M. robertsii:* two field isolates of the same strain: KVL 13-12 (*R1*a) and KVL 12-38 (*R1b*) and KVL 12-35 (*R2*) as different strain; *M. brunneum*: KVL 13-13 (*B1a*) and KVL 12-30 (*B1b*) as two different isolates from the same strain and KVL 12-37 (*B2*) as a different strain. *M. majus* (KVL 12-29, referred to as *Maj*) was used as the different species for both *M. robertsii* and *M. brunneum*.

Additionally, we determined the genetic distances of the different fungal strains based on a 729bp long part of the EF1-alpha gene. The sequences of the following strains were already published in Steinwender *et al.* (2014): *R1a* (GenBank Accession Number KM391926), *R2* (KM391927), *B1b* (KM391920), *B2* (KM391921) and *Maj* (KM391931). For *R1b* and *B1a* the sequence was amplified in the Cremer lab using the primers published in Bischoff *et al.* (2006) and sent for sequencing to LGC genomics (Berlin). All sequences were aligned with CLC Main Workbench (version 7.0.3) and the genetic distances were calculated with the MEGA software (version 11.0.10) using the p-distance method (Tamura *et al.*, 2021). The genetic distances of all pairwise comparisons are summarized in the table below (*Table 2.1*).

Table 2.1. Genetic distances based on a partial sequence of the EF1-alpha gene of all fungal strains used in our experiments. Larger values indicate a greater genetic distance.

	R1a	*R1b*	*R2*	*B1a*	*B1b*	*B2*	*Maj*
R1a							
R1b	0.0000						
R2	0.0028	0.0028					
B1a	0.0220	0.0220	0.0193				
B1b	0.0220	0.0220	0.0193	0.0000			
B2	0.0220	0.0220	0.0193	0.0000	0.0000		
Maj	0.0344	0.0344	0.0317	0.0316	0.0316	0.0316	

Looking at the sequenced part of the EF1-alpha gene, it was not possible to differentiate the three *M. brunneum* strains from each other. The genetic difference of strains *B1a*, *B1b* and strain *B2* can be seen when looking at the SSR loci that Steinwender *et al.* (2014) analyzed (more details see *Table 2.2* below). In more detail, when only considering loci for which the amplification could successfully be established in the Cremer lab for all strains that we used in our experiment, *R1a*/*R1b* and *R2* differ in 5, *R1a*/*R1b* and *Maj* in 10, *R2* and *Maj* in 9 loci, whereas *B1a*/*B1b* and *B2* differ in 2 and *B1a*/*B1b*, *B2* and *Maj* in 11. Together, the EF1-alpha gene sequence and the microsatellite information hence revealed a clear difference between the different strains in both *Metarhizium* species, albeit suggesting that the different *M. brunneum* strains are somewhat less different to one another than the two strains of *M. robertsii*.

Table 2.2. SSR loci for the individual strains and the corresponding allele sizes. This table is based on data from Steinwender *et al.* (2014) (supplemental table 2). Here only SSR loci are shown for which the amplification was successful for all strains used in our experiments.

Locus (Ma)	**2054**	**2055**	**307**	**2287**	**2049**	**2063**	**2296**	**2292**	**2283**	**145**	**2089**
Strain											
R1a=R1b	217	141	145	303	128	134	139	195	264	109	196
R2	230	141	161	302	130	134	139	195	264	110	196
B1a=B1b	238	149	186	297	128	140	139	196	278	113	200
B2	238	151	199	297	128	140	139	196	278	113	200
Maj	215	137	161	287	116	147	133	195	268	108	194

Aliquots of conidiospores of each fungal strain were kept in a long-term storage at -80 °C. Prior to the experiment the respective fungi were grown on Sabouraud dextrose agar plates at 23°C for approximately three weeks and harvested by suspending them in sterile 0.05% Triton X-100 (Sigma; in milliQ Water). All conidiospore suspensions had a germination rate of >94% as determined directly before the experiments, except for *Maj* that only had a germination rate of 90%.

Experimental design

To induce socially acquired low-level infections, we grouped a single exposed worker ("focal ant"; distinguishable by a color mark on its gaster (Edding 780)) for five days with five naïve nestmates in a petri dish (Ø=9cm) as in Konrad *et al.* (2012) that had been exposed to a fungal spore suspension (3 x 10^9 spores/ml) or sham-treatment of 0.05% sterile Triton-X (Tx, Sigma).

After five days of social contact the nestmates were challenged with a second fungal exposure, or a sham-treatment, as detailed in *Table 2.3* (see below).
Individuals were removed from the social setups, experimentally exposed with the respective conidiospore suspensions (3 x 10^9 spores/ml for all fungal pathogens) or 0.05 % Tx as sham-treatment and subsequently kept individually in petri dishes (Ø=3.5cm) with 10% sucrose ad libitum as food source. Different nestmates originating from the same initial petri dish (=replicate) were each exposed to a different fungal strain or sham-treated with 0.05% Tx. Survival of the challenged nestmates was monitored daily for a period of 12 days.

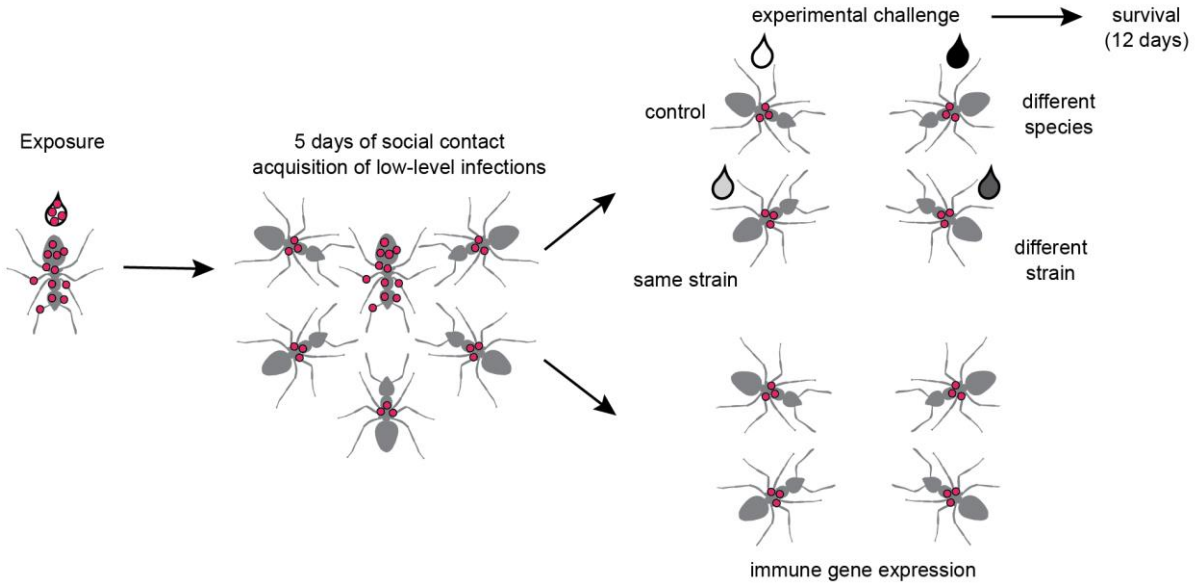

Figure 2.1. **Experimental design to induce socially acquired low-level infections.** To induce socially acquired low-level infections, we grouped a single exposed worker (=focal ant) for five days with five naïve nestmates as in Konrad *et al*. (2012). The focal ant had either been exposed to a fungal spore suspension (as shown in the figure, spores are indicated by pink dots, exposed to 3 x 10^9 spores/ml) or a sham-treatment of 0.05% Tx. The experiment was performed with different strains of *M. robertsii* and *M. brunneum* (and *M. majus* as a different species). After five days of social contact the nestmates were either challenged with a second fungal stain (3 x 10^9 spores/ml for all fungal strains) or sham-treated with 0.05% Tx or we measured the immune gene expression of low-level infected nestmates. Nestmate challenge was performed with either (i) a different field isolate of the same strain (same strain, here indicated in grey), (ii) a different strain of the same species (different strain, here indicated in dark grey), (iii) a different species (here indicated in black) or (iv) a sham-treatment with 0.05% Tx (control, here indicated in white). Challenged nestmates were kept individually in petri dishes (Ø=3.5cm) with 10% sucrose ad libitum as food source. Different nestmates originating from the same initial petri dish were each exposed to a different fungal strain or 0.05% Tx. Survival of the challenged nestmates was monitored daily for a period of 12 days.

Experimental procedure
Individual workers were exposed by dipping them shortly in a conidiospore suspension of 3 × 10^9 conidiospores/ml or in 0.05% Tx. Exposed ants were subsequently placed on filter paper to absorb excess liquid.

We only used nestmates (i) if their focal ant had survived the first 24h of the experiment to assure enough social contact for successful establishment of low-level infections in the nestmates, (ii) who themselves survived the five days of social interaction and (iii) could be surveilled until the end of the experiment in their petri dishes. For each *M. robertsii* and *M. brunneum*, we tested 21 different strain combinations, leading to a total sample size of 5448 challenged ants (2738 in the *M. robertsii* experiment and 2710 in the *M. brunneum* experiment)

that we set up in eight independent experimental blocks. The exact strain combinations and the sample sizes can be found in *Table 2.3*.

Table 2.3. All tested strain combinations and respective sample sizes. Left side indicates the first strain (low-level infecting strain) and second strain (strain used for challenge) is presented above. c=sham-treatment with 0.05% Tx.

		M. robertsii						***M. brunneum***				
Second strain		*C*	*1a*	*1b*	*2*	*Maj*		*C*	*1a*	*1b*	*2*	*Maj*
First strain	*C*	131	131	132	135	130	*C*	134	138	136	133	137
	1a	126		132	123	126	*1a*	121		126	125	123
	1b	127	132		128	129	*1b*	131	133		131	129
	2	127	132	129		129	*2*	123	121	125		123
	Maj	133	134	139	136		*Maj*	131	129	129	132	

Virulence determination of the different strains

To characterize the strains for which we found a protective effect of low-level infections, we assessed their virulence. To do so, we compared the survival of nestmates that were kept with a sham-treated focal ant and only received a sham-challenge with 0.05% Tx to the survival of nestmates that were kept with a sham-treated focal ant and received a pathogen challenge with one of the strains.

Immune gene expression of ants with socially acquired low-level infections

To determine the immune status of the low-level infected nestmates at the time point of secondary pathogen challenge five days after social contact to contaminated nestmates, we measured immune gene expression using quantitative real-time PCR (qPCR). We chose three immune genes known to be involved in the antifungal immune response of ants, namely β-1,3-glucan binding protein (*β-1,3-GBP*), a receptor gene encoding for a protein that recognizes and binds to β-glucans on the surface of fungal cell walls (Ma and Kanost, 2000), and two genes that encode for insect immune effectors: the enzyme prophenoloxidase (*PPO)* (Cerenius and Söderhäll, 2004, 2021) that is activated to phenoloxidase after infection by a variety of pathogens, including entomopathogenic fungi, or by tissue damage (Gillespie *et al.*, 1997; Cerenius *et al.*, 2008; Cerenius and Söderhäll, 2021) as well as the antimicrobial peptide defensin (Viljakainen and Pamilo, 2005, 2008) that destroys fungal cell walls and is known to be upregulated by fungal infection in insects (Gillespie *et al.*, 1997; Lemaitre and Hoffmann, 2007). As a housekeeping gene we used 28S ribosomal protein S18a *(28S RP S18a).*

We induced socially acquired low-level infections as described previously and instead of receiving a pathogen challenge – individuals were individually snap-frozen in 1.5 ml safe-lock Eppendorf-tubes and stored at -80 °C until further processing.

We initially set up eight social setups for each fungal strain and excluded setups where the focal individual died within the first 24h. Whenever possible, we analyzed the immune gene expression of three nestmates per setup for all *M. robertsii* and *M. brunneum* strains, resulting in the following sample sizes per fungal strain and gene (*Table 2.4*).

Table 2.4. Sample sizes for immune gene expression analysis per strain and gene.

Fungal strain	*Defensin & β-1,3-GBP*	*PPO*
R1a	16	16
R1b	21	20
R2	22	21
B1a	20	19
B1b	20	19
B2	19	18

RNA extraction, DNaseI treatment and cDNA preparation
Each sample was homogenized using a TissueLyser II (Qiagen) with a mixture of one 2.8 mm ceramic bead (Qiagen) and ~100 mg 425-600 µm glass beads, acid washed (Sigma-Aldrich). Homogenization was carried out in two steps 2 x 2 min at 30 Hz. After the first 2 min the tube racks were rotated to ensure uniform disruption and proper homogenization of all samples.

The total RNA was extracted using the Maxwell® RSC instrument together with the Maxwell® simplyRNA Tissue Kit (Promega) according to the manufacturer's instructions with a final elution volume of 60 µl. Afterwards, to ensure complete removal of residual DNA contaminations an additional DNase-I treatment (Sigma-Aldrich) step was performed prior to reverse transcription. The subsequent cDNA synthesis was done using the iScript cDNA synthesis kit (Bio-Rad) as per manufacturer's recommendations. The cDNAs of all genes except for *PPO* (which was used undiluted due to low gene expression values) were diluted 1:5 with H_2O and stored at -20 °C for later use.

Analysis of immune gene expression by qPCR
Gene expression analysis was performed for the housekeeping gene *28S RP S18a* (Pull *et al.*, 2018a) and the immune genes prophenoloxidase (*PPO*), β-1,3-glucan binding protein (*β–1,3-GBP*) (Pull *et al.*, 2018a) and defensin (*def*) (Konrad *et al.*, 2012), using the following primer sequences and the annealing temperatures (Ta) (*Table 2.5*).

Table 2.5. Primer sequences and annealing temperature for housekeeping and immune genes used for qPCR.

Gene	Primer Sequence 5'-3'	Ta (°C)
28S RP S18a	Forward: CGGCTGTATGCTACCACGTA Reverse: AAGCCTGCTTTCTGAGCCAT	60
Defensin	Forward: AAGAACACCATCGCGCACGTAG Reverse CTGAGAATGCAGTGAGCAGCGC	55
β-1,3-GBP	Forward: CTGCGCATATCAATTCCCGAC Reverse: TTCGCTATCTGTCCCGCTTC	55
PPO	Forward: TCTTTCTCGCGGTCTTGACT Reverse: TTGTTGGCGACGATTCTGTA	63

qPCR reactions with a total volume of 20 µl were prepared as follows: 1 x Luna Universal qPCR Master Mix (New England Biolabs), 250 nM of the respective primers (Sigma Aldrich), except for *PPO* , where 200 nM were used, 2 µl cDNA template and 7 µl of nuclease-free water (Sigma).

The amplification protocol was as follows: 95 °C for 1 min (initial denaturation), followed by 40 cycles of 95 °C for 15 sec (denaturation) and Ta °C for 30 sec (annealing and extension). Samples were analyzed in triplicates (or duplicates in case one sample was very different) on a Bio-Rad CFX96 real-time PCR detection system. Each plate included a H_2O negative control, a -RT control, a calibrator sample (pool of naïve ants) and a standard curve (1:5 dilution series of a cDNA sample derived from a pool of naïve ants) to monitor the PCR efficiency. The same calibrator sample was used on all qPCR plates. Primer efficiency was > 90% for all primer sets. Primer specificity was monitored by including a melting curve analysis at the end of each run. To calculate the differential immune gene expression the "Pfaffl method" was used (Pfaffl, 2001).

Statistical analysis

All statistical analyses were carried out in the program 'R' version 4.0.3 (R Core Team 2020). Unless otherwise stated, we used a generalized mixed modelling approach ('lme 4' (Bates *et al.*, 2015)) in which the significance of the model predictors were estimated by comparing each model to a null model only containing the intercept using Likelihood Ratio (LR) tests (Bolker *et al.*, 2009). We checked whether residuals were normally distributed and if the model variances were homogeneous ('DHARMa' (Hartig, 2020)) and assessed models for instability and checked whether there were any influential data points (dfbetas). When multiple inferences were made, all p-values were corrected using the Benjamini-Hochberg procedure to protect against a false discovery rate of 5% (Benjamini and Hochberg, 1995). Likewise, when posthoc tests were performed p-values were corrected with the 'multcomp' package (Hothorn *et al.*, 2008) using the Benjamini-Hochberg correction and we report adjusted p-values. All figures were made with the 'ggplot2' package (Wickham *et al.*, 2018).

The protective effect of low-level infections. To determine whether there was an overall effect of low-level infections on nestmate survival upon pathogen challenge depending on the nature of the challenge (very similar to less similar pathogenic challenge), we ran a Cox mixed effect model (package 'coxme'(Therneau, 2015)). We included the nature of challenge as 'combination' (containing four levels: pathogen 1 and pathogen 2 of (i) the same strain, (ii) a different strain, (iii) a different species and (iv) a control that had no first pathogen (contact to sham-treated focal ant, followed by a pathogenic challenge) together with 'experiment' (containing two levels: *M. robertsii* or *M. brunneum*) as main effects. Additionally, 'pathogen strain used for challenge' and 'social setup replicate nested in experimental block' were included as random effects. We checked for an interaction of our main effects by comparing the model with the interaction term to a model without an interaction using a LR test and found the interaction not to be significant and therefore refitted the model without the interaction. To obtain the significance for each main effect of our model, we removed the effect of interest from the model and compared it to the full model again using a LR test. To determine how broadly a low-level infection affected the nestmate survival after pathogen challenge, we performed Dunnet post hoc comparisons to the control.
This general overall analysis was followed by a detailed analysis where we looked at each challenge separately and determined in which exact strain combinations we found low-level infections to affect the nestmates survival after pathogen challenge and determined the "intensity" of this effect (details hereafter). To do so, we analyzed each challenge separately and compared the survival of nestmates that have had contact to a pathogen-exposed focal ant and received a pathogen challenge to the survival of nestmates that have had contact to a sham-treated focal ant and received a pathogen challenge (=control). To that end, we ran Cox mixed-effect models with 'first strain' as fixed factor and 'experimental block' as random effect and

performed Dunnet post hoc comparisons to the control. For each pathogen combination, we excerpted the inverse hazard ratios (1/hazard ratio=protective effect) for further analysis. The inverse hazard ratios are a measure of the survival benefit (indicated by values >1) nestmates have due to the first pathogen (low-level infection) upon challenge with the second pathogen (*Table 2.6*).

Virulence of each strain. To determine the virulence of each strain, we compared the survival of nestmates without low-level infections (contact to sham-treated focal ant) that were pathogen-challenged to the survival of nestmates without low-level infections that only received a sham-challenge with 0.05% Tx and determined the hazard ratio HR. Here, we used Cox mixed-effect models for both experiments (*R* and *B*) separately with 'pathogen challenge' as main effect and 'experimental block' as random effect and performed Tukey post hoc comparisons to test for differences between the fungal strains of each experiment (*Figure S 2.3*). As we did not find any protective effect of low-level infections with *M. majus*, we excluded this strain from further analysis.

Effect of low-level infection on nestmate survival. We also assessed the baseline mortality of low-level infected nestmates (=costs of a low-level infection) by comparing the survival of low-level infected nestmates that received a sham-challenge to the survival of nestmates that were reared with a sham-treated focal ant and only received a sham-challenge. To do so, we used Cox mixed-effect models for both experiments (*R* and *B*) separately with 'low-level infection' as main effect and 'experimental block' as random effect and performed Tukey post hoc comparisons to test for differences between the fungal strains (*Figure S 2.4*)

Relative strain virulence and the protective effect of low-level infections. Based on the virulence (as HR across strains) and the protective effect of each strain (as inverse HR compared to the same challenge in the absence of low-level infection), we evaluated whether the order of infection matters and whether we find a bidirectional protective effect upon pathogen challenge. Therefore, we ran a generalized linear mixed model with the relative virulence (virulence difference) of the first and the second strain as predictor and the inverse hazard ratio of the first strain in the respective strain combination as response variable and included 'first strain' and 'second strain ' as random effects (*Figure 2.1*).

Relative immunogenicity and the protective effect of low-level infections. To determine whether the degree of immune stimulation following low-level infection of each strain can predict its protective effect, we analyzed the immune gene expression of three different immune genes: *β-1,3-GBP* on receptor level and defensin and *PPO* on effector-level.
For statistical analysis we log transformed the gene expression values to reach near normal distribution and calculated the geometric mean per strain and gene. We formulated a generalized linear mixed model per gene with the difference in mean gene expression of the first and second strain as predictor and the protective effect of the first strain in its respective pairing with the second strain as response and added 'first strain' and 'second strain' as random effects (*Figure 2.2*).

2.4 Results

Low-level infections can provide cross-protection against pathogens of the same strain or species

Overall, we found an effect of the nature of pathogen combination on nestmate survival for both *M. robertsii* and *M. brunneum* (Cox mixed effects model [COXME]: overall LR: χ^2=35.441, d.f.=4, p<0.001; combination: χ^2=33.239, d.f.=3, p<0.001; experiment: χ^2=1.699, d.f.=1, p=0.192). The survival of nestmates that carried low-level infections differed to the survival of nestmates without any previous low-level infections (=control) after pathogen challenge on strain and species level (both p<0.001), but not on genus level with *M. majus* (p=0.225).

In more detail, we found a protective effect (1/HR; see *Table 2.6* for all pathogen combinations, *Table S 2.7* for overall survival models and *Table S 2.8* for p-values of post hoc comparisons) of low-level infections when nestmates were challenged with a different isolate of the same strain once each for *M. robertsii (R1a-R1b,* but not *R1b-R1a)* and *M. brunneum (B1b-B1a*, but not *B1a-B1b).* Furthermore, initial contact with a different strain (of the same species) provided protection twice in *M. robertsii (R2-R1a, R2-R1b,* but not *R1a-R2* and *R1b-R2)* and in *M. brunneum (B1a-B2, B1b-B2,* but not *B2-B1a* and *B2-B1b)*. In all reverse combinations of the above-mentioned and on genus level, there were only neutral effects of existing low-level infections after pathogen challenge, except for one case, where low-level infected individuals with the most virulent strain -*B2*- were challenged with *Maj*. In this case, we found a survival cost after secondary pathogen challenge (*Table 2.6*).

Table 2.6. Effect of previous low-level infections by means of inverse hazard ratios. The inverse hazard ratio corresponds to 1/hazard ratio. Significant values are indicated in bold (p<0.05). Values >1 indicate a survival benefit compared to control nestmates without low-level infections. Statistics of the overall models can be found in *Table S 2.7* whilst detailed p-values of Dunnet post hoc comparisons to the control can be found in *Table S 2.8*.

genetic distance ↓

	combination		*M. robertsii*		*M. brunneum*	
same strain	1a→1b	1b→1a	**1.602**	1.216	1.172	**1.464**
different strain same species	1a→2	2→1a	0.903	**1.594**	**1.384**	1.266
	1b→2	2→1b	1.172	**1.606**	**1.481**	1.192
different species same genus	1a→Maj	Maj→1a	1.337	1.195	1.208	0.972
	1b→Maj	Maj→1b	1.194	1.256	1.200	1.056
	2→Maj	Maj→ 2	1.443	0.756	**0.617**	1.045

Protective effect of low-level infections depends on the order of infection with the protection only occurring when the first pathogen strain is less virulent than the second strain

Next, we wanted to see whether i) we find a bidirectional protection of each strain combination and ii) whether the virulence difference of the first and second strain affects the ants survival after pathogen challenge.

We found for all within strain and species combinations protection only in one direction, and only when the first strain was less virulent than the second strain (χ^2=5.534, d.f.=1, p<0.001, see *Figure 2.1*), indicating that the order of superinfection matters.

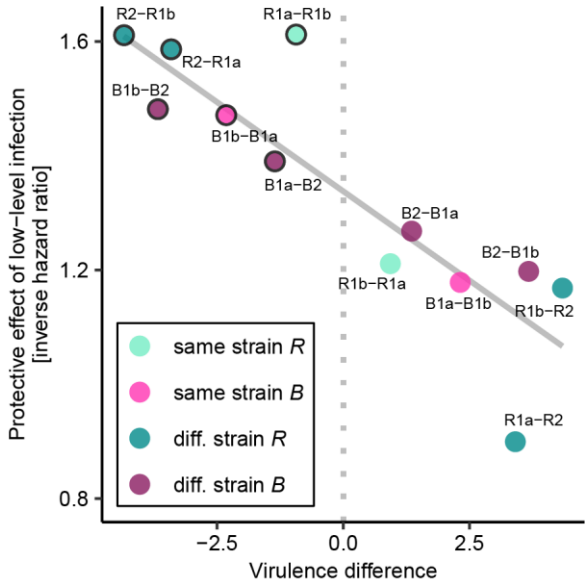

Figure 2.1. **Relative strain virulence can predict the protective effect of low-level infections.** We analyzed the protective effect of low-level infections by means of their inverse hazard ratio (1/HR; values >1 indicate survival benefit compared to control ants that were not low-level infected) and the relative virulence difference of the first and second strain (negative values indicate that the first strain is less virulent than the second strain). The dashed line represents the scenario when both strains are equally virulent. Data points indicate different combinations of first (low-level infecting) and second (challenging) strain. Strain combinations with the same strain are indicated in light and combinations with different species are indicated in dark - shades of pink for *M. brunneum* combinations and shades of turquoise for *M. robertsii* combinations. A protective effect of an existing low-level infection was only found when the first strain was less virulent than the second strain (significant values are indicated by black outline), indicating that the order of superinfection matters. The grey line represents a linear regression (R^2= 0.746).

Higher cross-protection when first strain elicits higher immune gene expression

As successful immunization in *L. neglectus* is known to rely on the ants increasing the expression of immune genes during the first infection in homologous situations (Konrad *et al.*, 2012), we checked immune gene expression of low-level infected ants at the time point at which the second strain would infect the ants in our social immunization setups. To characterize the strains for which we found a protective effect, we determined the degree of immune stimulation (immunogenicity) following low-level infection. We found no relationship between protection upon challenge and gene expression elicited by the low-level infection on receptor level (*β-1,3-GBP:* χ^2=0.743, d.f.=1, p=0.389), but on effector level we found cross-protection when the first strain was more immunogenic than the second strain for both genes tested (Defensin: χ^2=4.614, d.f.=1, p=0.049, *PPO*: χ^2= 4.360, d.f.=1, p=0.049, see *Figure 2.2*).

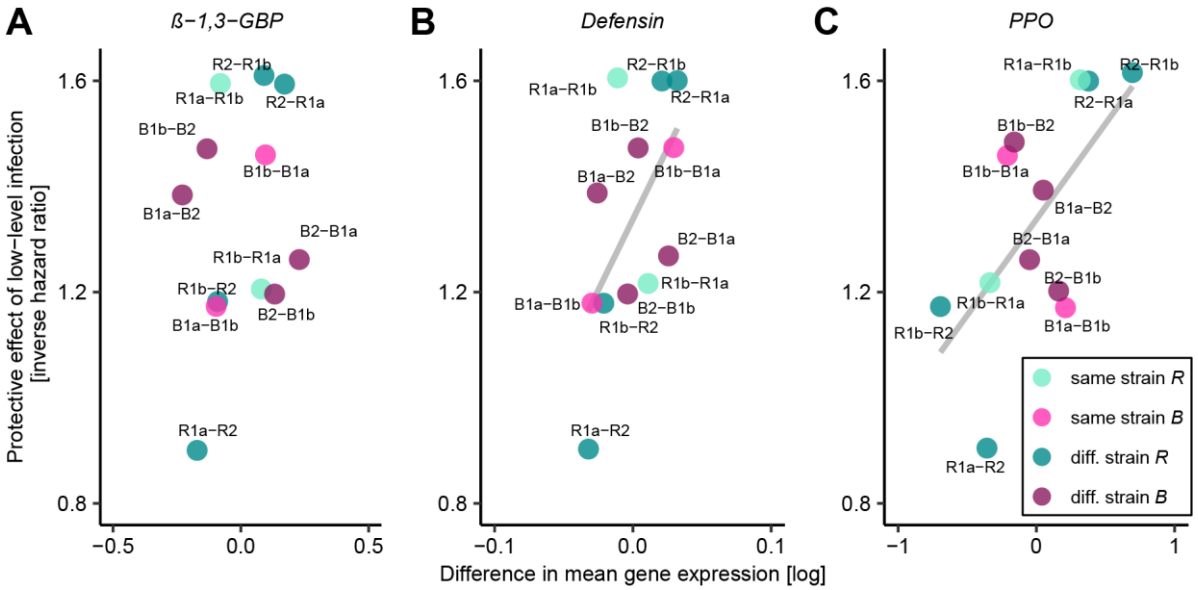

Figure 2.2. ***Relative immunogenicity can predict the protective effect of low-level infections.*** We analyzed the protective effect of low-level infections by means of their inverse hazard ratio (1/HR; values >1 indicate survival benefit compared to control ants that were not low-level infected) and the difference in immunogenicity of the first and second strain. We log transformed the gene expression values and calculated the geometric mean per fungal strain. Afterwards, we calculated the difference in immune gene expression of the first and second strain (positive values indicate that the first strain has higher immune gene expression than the second strain). Data points indicate different combinations of first (low-level infecting) and second (challenging) strain. Strain combinations with the same strain are indicated in light and combinations with different species are indicated in dark - shades of pink for *M. brunneum* combinations and shades of turquoise for *M. robertsii* combinations. We found that low-level infections provide a higher protection when the immunogenicity of the first strain is higher than that of the second on effector level (Defensin and *PPO*), but not on receptor level (*ß-1,3-GBP*). The grey lines represent linear regressions for each gene (Defensin: R^2=0.293, *PPO*: R^2= 0.341*).*

2.5 Discussion

In this study, we tested whether socially acquired low-level infections can provide broad protection against heterologous fungal strains and found cross-protection up to species level. Overall, it became clear that the effect of low-level infections seemed to strongly depend on the pathogen combination used for socially acquired low-level infections and subsequent pathogen challenge. In some combinations, we found cross-protection between different strains of the same species, illustrating that protection is not restricted to homologous pathogen combinations only.

These findings match previous studies in various invertebrate species, showing specificity at the strain level (Kurtz and Franz, 2003; Roth and Kurtz, 2009; Roth *et al.*, 2009) and species level (Sadd and Schmid-Hempel, 2006; Pham *et al.*, 2007) even up to protective effects within the same genus (Pham *et al.*, 2007) – the latter we did not find in our study.

In most cases, we found either neutral or protective effects of low-level infections on ant survival, except for one case where we found survival costs – this was the case when the ants were low-level infected with the most virulent strain and challenged with a different species (*B2-Maj*). This matches findings in another study of low-level infected *L. neglectus* ants whose susceptibility was consistently increased to superinfections with a pathogen of a different genus (Konrad *et al.*, 2018). Similar deleterious effects have been documented for heterologous challenges after individual and transgenerational immune priming (Sadd and Schmid-Hempel, 2009; Ulrich and Schmid-Hempel, 2012) and superinfections (Ben-Ami *et al.*, 2011; Jamieson *et al.*, 2013; Konrad *et al.*, 2018) when pathogens interact within the host (i.e. cooperation and

competition) and thereby negatively affect the host's immune system (Griffin *et al.*, 2004). As we only found a protective effect up to species level suggests that the strains have to be fairly similar to elicit cross-protective immunization.
Furthermore, our study revealed that the order of infection matters, as we found - in each case where protection occurred - it only occurred in one direction, namely when the first strain was less virulent and more immunogenic than the second strain. The effects of a superinfection on the host are an interplay of the host's immune system (i.e. immune response mounted against the first pathogen) and pathogen-pathogen interactions within the host. Therefore, the virulence that is experienced in a superinfection is a result of the interaction between the co-infecting pathogens (Seppälä *et al.*, 2012). Therefore, potential protective effects of existing low-level infections may get cancelled out, e.g., when the immune response against one strain is mal-directed against another one. If the first strain is more virulent, it could be that the host's immune system is overwhelmed and therefore immune memory cannot be formed (Duneau *et al.*, 2016). The more dissimilar two pathogens are, the more likely it is that they compete for resources within the host that eventually leads to its death.
Moreover, we found that strains that elicited a higher immune gene expression, and were therefore more immunogenic, provided better protection, but only on an effector level (defensin and *PPO*), and not on a receptor level (*β-1,3-GBP*) – likely because we measured immune gene expression five days post initial contact to the fungal spores and therefore the receptor gene that gets typically differentially expressed first, may not be up-regulated anymore.
Many pathogens have evolved to manipulate the host immune system, e.g., some fungal strains are known to produce catalase (=virulence factor) to overcome the host immune system by reducing the ability of the host's immune system to produce reactive oxygen species that are used to break down pathogens (Hernandez *et al.*, 2010; Medina-Gómez *et al.*, 2018). Medina-Gómez *et al.* (2018) hypothesized that more virulent strains might produce more catalase and thereby conceal possible immunization, which could indicate a direct link between host immunity and strain virulence. Based on this, one could speculate that less virulent pathogens potentially stimulate the immune system stronger (are more immunogenic). It could also be that highly virulent strains overwhelm the immune system and the host activates survival mechanisms instead of investing into priming for the secondary challenge. In this sense, analyzing the correlation of strain virulence and immune gene expression would be a valuable extension to our current experiment to be tested in the future as our current sample size of six strains is too low to test this hypothesis.
As expected, we did not find survival costs of low-level infected ants (Konrad *et al.*, 2012) - except for *R1b* where low-level infected nestmates survived significantly worse than nestmates that were kept with a sham-treated focal ant and received a sham-challenge. *R1b* is one of the most virulent strains that we have tested and for which we did not find a protective effect when it was used as a first strain. It could be that this strain does not provide protection as the immune system is overwhelmed with fighting off this strain.
So far we only found a protective effect of low-level infections when the first strain was less virulent than the second strain, but in this context, it would be very interesting in future experiments to see whether the protective effect would disappear when the dose of the less virulent strains would be increased or the dose of highly virulent strains would be reduced. As we worked with socially acquired low-level infections where we naturally expect a variation in pathogen load between different nestmates tested - and pathogen load within the host was shown to be the key determinant of host immune gene expression (Tate and Graham, 2017) - experiments including pathogen load can provide important insight in the dynamics of strain virulence, pathogen load and the host's immune system.

Cross-protection is a widespread phenomenon in nature that is also used in human medicine, e.g., in the case of cross-protective vaccines that are used against multiple virus infections. Here, an immune response is triggered against pathogen types that are not specifically targeted by the antigens in the vaccine itself, providing protection against related species within the same genus, e.g., cowpox against smallpox or *mycobacterium bovis* against tuberculosis (Vojtek *et al.*, 2019).
It is also utilized in plant virus disease control whereby tolerance or resistance of a plant to one virus strain is induced by infecting it with another strain. This phenomenon was discovered more than 80 years ago in tobacco plants (McKinney, 1929). Plants that were systematically infected with a less virulent virus strain were protected against subsequent infection with a more virulent strain of the virus (Pechinger *et al.*, 2019). We found the same phenomenon – that a less virulent strain protects against a more virulent strain – in a social system which can lead to protection of the colony.
The cross-protective nature of socially acquired low-level infections that we have discovered benefits the protection of the whole colony as ants are naturally exposed to a broad pathogen spectrum of related pathogens (e.g. Steinwender *et al.,* 2014), making super-and co-infections very likely.

2.6 Supplement

Strain virulence

Most of the different strains differed significantly in their virulence in the *M. robertsii* experiment (overall χ^2=125.820, d.f.=3, p<0.001, post hoc all pairwise comparisons p<0.006, except *R1a-R1b* p=0.520, *Figure S 2.3 A*) as well as in the *M. brunneum* experiment (overall χ^2=171.530, d.f.=3, p<0.001, post hoc pairwise comparisons all p<0.05, except *B1a-B2* p=0.310, *Figure S 2.3 B*).

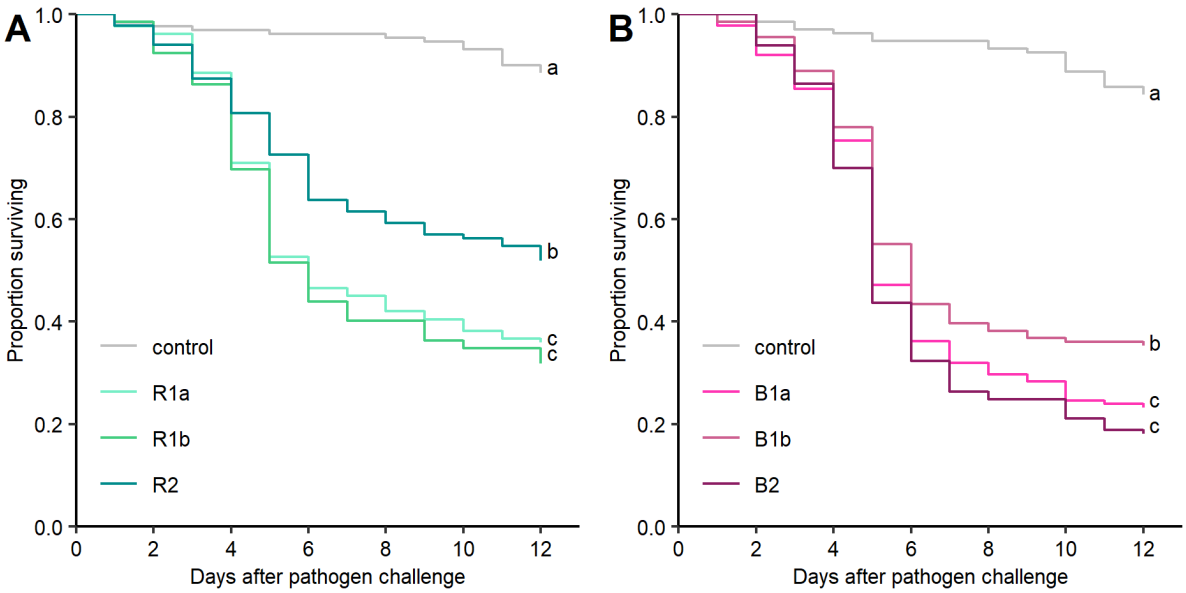

Figure S 2.3. **Virulence of the different strains for the *M. robertsii* and *M. brunneum* experiment.** We compared the survival of nestmates that were reared with a sham-treated focal individual that received a pathogen challenge (sham low-level infected - pathogen challenged) to nestmates that were reared with a sham-treated focal individual that were sham-challenged (sham low-level infection – sham-challenge; =control). Control nestmates are indicated in grey, shades of turquoise represent different strains of *M. robertsii* and different shades of pink different strains of *M. brunneum*. Different letters indicate significance groups of all pairwise post hoc comparisons after Benjamini–Hochberg correction at α=0.05.

Baseline mortality of low-level infected ants

We found that low-level infections in most cases are not costly in terms of survival as low-level infected nestmates survived equally well as nestmates without low-level infections (*M. robertsii* overall χ^2=8.995, d.f.=3, p=0.029, all pairwise post hoc comparisons p>0.201, except control-*R1b* p=0.026, *M. brunneum* overall χ^2=5.874, d.f.=3, p=0.118, *Figure S 2.4)*

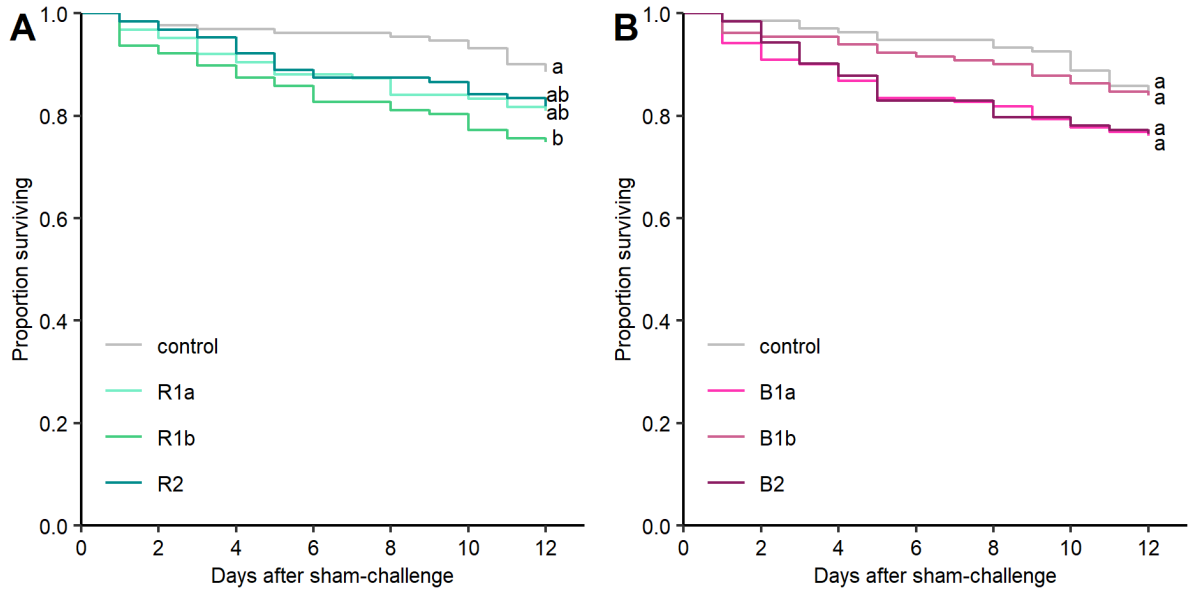

Figure S 2.4. **Baseline mortality of low-level infected nestmates for the *M. robertsii* and *M. brunneum* experiment.** We compared the survival of nestmates that were reared with a pathogen-exposed focal individual that only received a sham-challenge (low-level infected – sham-challenge) to nestmates that were reared with a sham-treated focal individual that were sham-challenged (sham low-level infection – sham-challenge; =control). Control nestmates are indicated in grey, shades of turquoise represent different strains of *M. robertsii* and different shades of pink represent different strains of *M. brunneum*. Different letters indicate significance groups of all pairwise post hoc comparisons after Benjamini–Hochberg correction at α=0.05.

Determination of the protective effect of low-level infections

To determine the protective effect of low-level infections for each strain combination, we analyzed the data for *M. robertsii* and *M. brunneum* separately for each challenge and performed post hoc comparisons to determine the inverse hazard ratios (1/HR=protective effect; details of the overall models are in *Table S 2.7* and p-values of Dunnett post hoc comparison for all strain combinations are in *Table S 2.8*).

Table S 2.7. Statistical values for the overall survival models for each pathogen challenge.

	Challenge	**[COXME] overall LR**
M. robertsii	*R1a*	χ^2=8.107, d.f=3, p=0.044
	R1b	χ^2=11.867, d.f.=3, p=0.008
	R2	χ^2=6.779, d.f.=3, p=0.079
	Maj	χ^2=4.162, d.f.=3, p=0.244
M. brunneum	*B1a*	χ^2=10.667, d.f.=3, p=0.014
	B1b	χ^2=1.721, d.f.=3, p=0.632
	B2	χ^2=11.286, d.f.=3, p=0.010
	Maj	χ^2=18.444, d.f.=3, p<0.001

Table S 2.8. P-values of Dunnett post hoc comparisons to control nestmates for all strain combinations. P-values are corrected for multiple testing according to the Benjamini-Hochberg procedure at α=0.05. Significant p-values are indicated in bold.

	combination		*M. robertsii*		*M. brunneum*	
same strain	1a→1b	1b→1a	**0.003**	0.260	0.310	**0.009**
different strain same species	1a→2	2→1a	0.570	**0.015**	**0.025**	0.110
	1b→2	2→1b	0.380	**0.003**	**0.006**	0.260
different species same genus	1a→Maj	Maj→1a	0.130	0.130	0.330	0.840
	1b→Maj	Maj→1b	0.140	0.340	0.340	0.720
	2→Maj	Maj→ 2	0.096	0.057	**0.005**	0.750

3. Pathogen effects on male-male competition

Photograph shows two *Cardiocondyla obscurior* males fighting on a leaf.
Photo credit: S. Metzler and R. Ferrigato.

3. Pathogen effects on male-male competition

3.1 Abstract

Fights are ubiquitous - and have the immediate purpose to monopolize or defend valuable resources, such as mating partners. The fighting over access to mating partners occurs mainly in male-male competition, where the winner of the combat usually gets the chance to mate. However, fighting is also energetically exhausting and increases the risk of injury and can even end lethal. We studied the effect of a fungal pathogen (*Metarhizium robertsii*) in male-male competition in the invasive tramp ant *Cardiocondyla obscurior.* In contrast to other ant species that have a scramble competition mating system, *C. obscurior* has developed fierce combats as mating occurs in the nest. We analyzed male fighting ability and the influence of workers at two different stages of disease when (i) males were at an early-stage of disease where pathogen transmission was possible and (ii) at a late-stage of disease when the males were sick but no longer contagious. We found that sanitary care (removal of the pathogen from the cuticle of contagious individuals) performed by workers reduced the risk of pathogen transmission to the healthy rival and thereby preventing disease spread through the colony. Early- and late-stage infected males were inferior to healthy males and were more likely to lose fights and die. In late-stage infected males this can be attributed to the progressed infection, as late-stage infected males also died in the absence of fighting, whereas this was not the case for early-stage infected males. Yet, we showed that the latter already invest strongly in an immune response, as we found their immune genes to be upregulated, which seemed to have impaired their fighting ability. The costs of mounting an immune response were hidden in non-fighting conditions and only became apparent with the additional stress of a fight. The high immune investment of males was unexpected in social Hymenoptera males, but is was likely selected in *Cardiocondyla* males as they are relatively long-lived and can be the dominant male in a colony for several weeks. Our work hence demonstrates the existence of an immunity-fighting trade-off in ant males.

3.2 Introduction

Fights are ubiquitous in the animal kingdom - and have the immediate purpose to either monopolize or defend valuable resources (Backwell and Jennions, 2004) e.g. shelter, prey or a mating partner (Clutton-Brock *et al.*, 1979; Andersson and Iwasa, 1996; Baxter *et al.*, 2015). The fighting over access to mating partners occurs mainly in male-male competition (intra-sexual selection) and are besides mate choice (typically performed by the female) the main drivers of sexual selection (Darwin, 1871). In such systems females tend to be the choosier sex and assess male quality by specific characteristics (mostly physiological or morphological traits or male behavior) that often as well correlate with the success in competition (Candolin, 1999; Hunt *et al.*, 2009). As mostly only healthy individuals are able to maintain costly secondary traits, females use these as indicators of health and resistance to parasites (Hamilton and Zuk, 1982; Milinski and Bakker, 1990) that reflect good condition and overall vigor.

Male-male competition serves to select a superior mate or to exclude another rival and is observed in a large number of species. Most examples are vertebrate species (Clutton-Brock *et al.*, 1979), but male combat also occurs in invertebrates such as cephalopods (Schnell *et al.*, 2015), weaponless butterflies (Kemp and Wiklund, 2001) and Hymenopterans (Abe *et al.*, 2005). In most cases fighting success correlates well with reproductive success - but at the same time fighting is costly. Fighting is not only energetically exhausting and takes time, it also increases the risk of predation and injury and in rare cases might even be lethal (Briffa and Elwood, 2004; Arnott and Elwood, 2009)

Overall vigor and physical strength determine fighting ability, but are negatively affected by disease. Sick individuals suffer from changes in their normal physiology and behavior – "sickness behaviors" - e.g. weakness, lethargy, reduced movement, loss of appetite and a disengagement from social activities (Hart, 1988; Kent *et al.*, 1992; Dantzer, 2001). To fight an infection, other behaviors e.g. territorial aggression (Owen-Ashley *et al.*, 2006), parental care (Bonneaud *et al.*, 2003), and sexual behavior (Avitsur and Yirmiya, 1999) are hampered to conserve energy. Contrary to a sickness-induced loss of aggression and lethargy, it is also possible that males that perceive a low probability of surviving the infection and are close to death, make a last-ditch attempt to rescue their reproductive success by fighting more violently against a rival as a terminal investment strategy (Clutton-Brock, 1984). Escalated lethal combat might be a worthwhile strategy for short-lived species with a low expected lifetime male mating success as each successful mating contributes to a considerably larger proportion of lifetime reproductive success (Liu *et al.*, 2017).

In many species – including humans – testosterone was found to facilitate aggression, such as territorial and dominance aggression (Mazur and Booth, 1998; Eisenegger *et al.*, 2011), but at the same time is known to have an immune suppressive effect in males (Alexander and Stimson, 1988; Zuk *et al.*, 1990; Hillgarth and Wingfield, 1997). This indicates that current reproductive success has a higher priority than the investment in long-term immunity and to survive until the next mating opportunity. As males gain fitness by increasing their mating success, they often invest less in immunity compared to females that profit from the number of offspring produced and raised (Schmid-Hempel, 2003) – and profit from a "live hard, die young strategy" (Zuk and Stoehr, 2002). In this sense, they should invest all their resources in reproduction, even at costs of their own survival and could become even more feisty when they "have nothing to lose" as a terminal investment strategy.

However, just mounting an immune response itself is costly and often involves the reallocation of resources away from growth and reproduction (Sheldon and Verhulst, 1996; Schwenke *et al.*, 2016; Barribeau and Otti, 2020). Therefore, both early- and late-stage infection can restrict the amount of resources a male has available for fight engagement. Therefore, males might already suffer from reduced physical strength (Adamo *et al.*, 2008; Westra *et al.*, 2015) and

therefore fighting ability (Ditchkoff *et al.*, 2001; Rantala and Kortet, 2004) during the incubation phase of an infection as well as from low levels of the pathogen before they have become fatally infected.

We studied the effect of a fungal pathogen in male-male competition in the invasive tramp ant *Cardiocondyla obscurior.* In contrast to other ant species that have a scramble competition mating system, *C. obscurior* has developed fierce combats due to local mate competition as e.g. known from several species of fig wasps and parasitoid wasps (West *et al.*, 2001; Abe *et al.*, 2005). So far, the effect of developing or established infections on male-male competition in social Hymenoptera are understudied and elusive. As fights occur in the maternal nest in the presence of workers, we were able to additionally study the interplay of social immunity and the interference of workers in male-male competition. Complementary to the individual immunity of each colony member, there is also social immunity at the colony level (Cremer *et al.*, 2007), which allows on one hand to care for contaminated individuals that just recently came in contact with external pathogens, such as fungal spores, and thus to stop disease development for the individual, but on the other hand also to prevent transmission through the colony by e.g. preventing close contact of healthy males to a contagious rival. Studying these aspects can provide important insights, as invasive species and are often controlled with biocontrol agents such as entomopathogenic fungi like *Metarhizium* (Brunner-Mendoza *et al.*, 2019) that commonly occur in the field, e.g. the soil.

C. obscurior has a particular male diphenism with non-aggressive winged disperser males and wingless ("ergatoid" – worker-like) males that mate in the natal nest and have developed fatal fighting over access to newly emerging female sexuals (virgin queens), for which they also have evolved special morphology (Kinomura and Yamauchi, 1987; Cremer and Heinze, 2003; Lenoir *et al.,* 2007; Cremer *et al.*, 2012).
Wingless males patrol through the nest, especially the brood piles, grab rival wingless males with their elongated, sickle-shaped mandibles and mark them chemically with hindgut secretions (Yamauchi and Kawase, 1992) which is known to elicit worker aggressive behavior towards the besmeared male. Mostly, the "winner" remains as the only single adult wingless male in the colony (Cremer and Heinze, 2002). In case males besmear each other reciprocally, it can be that the fights end lethal for both rivals. The fights can last for up to several days and the conflicts in male-male competition seem to be resolved in favor of the older males who consistently won against combatants younger than two days as their cuticle is still soft and not fully sclerotized yet which makes them very vulnerable to attacks (Cremer *et al.*, 2012). Thus, virgin female sexuals are limited in their mate selection - however they can still refuse mating attempts with the remaining survivor (winner). Survivors of male fights typically try to copulate with many virgin queens in the nest (up to 50 or more (Metzler *et al.*, 2016)) and thus male (health) status can shape the fitness of the entire colony.

First, we tested how fight outcome is affected when males just recently came in contact with fungal spores of *Metarhizium robertsii* and are at the early-stage of an infection. We hypothesized that fighting ability is not strongly amended yet as (i) we determined the fight outcome within the first 24h after pathogen contact and we predict that infections have not established yet and (ii) social immunity performed by the workers can still effectively operate as spores can be removed from the males (Cremer *et al.*, 2007; Konrad *et al.*, 2012).
As a next step, we tested how late-stage (established) infections affect male fighting ability and consequently the fight outcome. Here, we expected that males already suffer from the progressing infection and might be more likely to be inferior in fights against healthy combatants. However, it could also be that males close to death terminally invest all their

resources in fighting off a rival and are even more aggressive to monopolize the last mating opportunity. Besides being more likely to lose in male-male competition, infected males may not be able to show proper courtship behavior and might not even be able to mate anymore (a hypothesis we tested in chapter 4). As *Metarhizium* acts in a dose-dependent manner (Hughes *et al.*, 2004a), we performed all experiments with two different dosages, simulating scenarios when males have come in contact with a low respectively high dose of the pathogen.

3.3 Material and Methods

Experimental design

To study the effect of *Metarhizium robertsii* on male-male competition, we set up male fights consisting of a pair of males and five randomly chosen sham-treated workers originating from the same stock colonies as the males.

Opponents were roughly size matched even though body size per se seemed not to be an important predictor of fight outcome in *C. obscurior* (Cremer *et al.*, 2012), but size could affect the number of spores that remain on the males after pathogen exposure. All males were randomly assigned to one of three different treatment groups: (i) control (both males sham-exposed), (ii) one male fungus-exposed later referred to as "mixed fights" or (iii) both males fungus-exposed. After 24h the fight outcome was determined: fights either were decided (with a clear winner and loser) or undecided with either both males being dead or both being still alive.

We performed the experiments at an early- and a late-stage of infection (see details below) and with two different dosages each, simulating scenarios of contact to a low (e.g., when the nest environment is contaminated or contact is made with a contagious nestmate with a very low amount of spores) or high dose of fungal spores (e.g., when there is a disease outbreak in the colony or when they are fed by a forager that was in contact with a sporulating cadaver).

As the number of spores directly correlates with disease progression and the severity of infection (Hughes *et al.*, 2002; Konrad *et al.*, 2012; Boomsma *et al.*, 2014), we expected less pronounced (or later) effects with the lower dose (experimental setup see *Figure 3.1*).

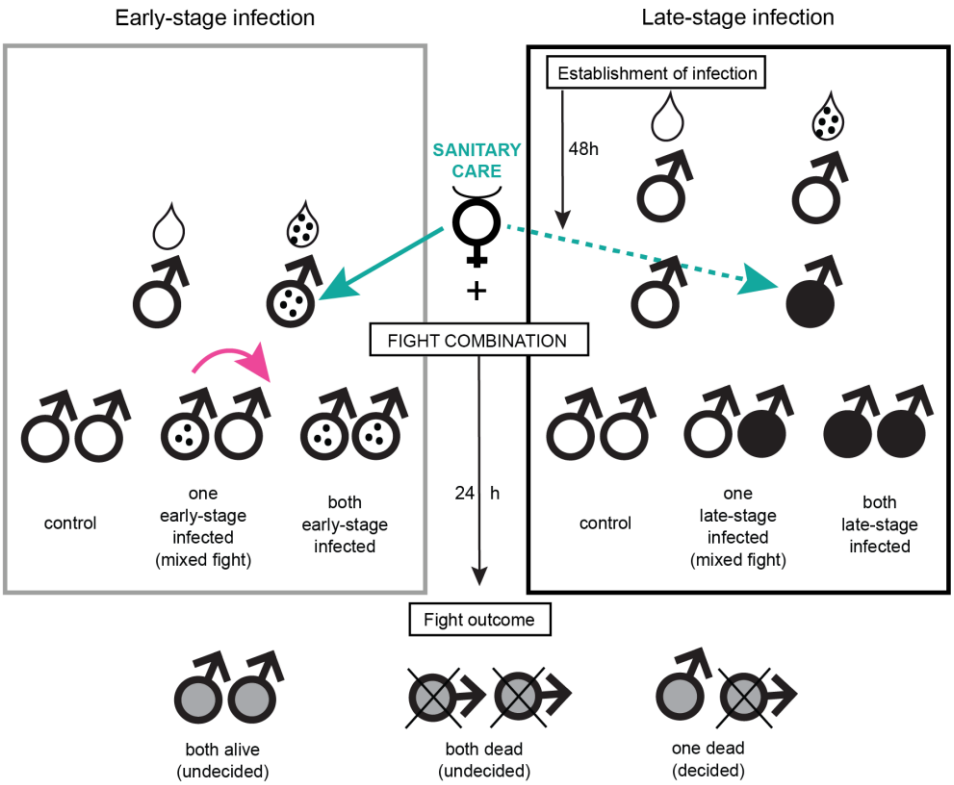

Figure 3.1. ***Experimental design of fights of early- and late-stage infected males.*** To determine how male infection affects fighting ability and fight outcome, we set up fights consisting of two males and five sham-treated workers each. We paired males in three different fight combinations: (i) control (both males sham-treated), (ii) one male infected (=mixed fights; either early- or late-stage infected) or (iii) both males infected (either early- or late-stage infected) and determined the fight outcome after 24h. Fights could either end undecided with either both males being dead or both still being alive or decided with only one male still being alive (=winner). Fights were performed at two different dosages each. Early-stage infected males were exposed either to (i) 1 x 10^9 spores/ml or sham-treated with 0.05% Tx (="high dose" fights) or (ii) 1 x 10^6 spores/ml or sham-treated with 0.05% Tx ("low dose" fights) and the fights were directly set up thereafter (grey box on the left; black dots indicate spores that can get transferred from the exposed male to its rival; indicated by the pink arrow). At this stage workers perform sanitary care and groom off the spores from contagious males (indicated by the turquoise arrow) and can thereby hamper disease development for the individual and spore transmission through the colony. At the late-stage of infection sanitary care is no longer effective anymore (indicated by the dashed turquoise arrow), as spores have already firmly attached to the cuticle. Late-stage infected males were exposed either to (i) 1 x 10^6 spores/ml or sham-treated with 0.05% Tx ("high dose" fights) or (ii) 1 x 10^4 spores/ml or sham-treated with 0.05% Tx ("low dose" fights) and individually kept for 48h so that the infections could establish (late-stage infected males are indicated in black). Male symbols indicating the fight outcome are colored in grey (as they represent the fight outcome of early- as well as late-stage infected males and the respective sham-treated males). In decided mixed fights the winner and the loser were determined either by color-marks or via spore load determination (droplet digital PCR).

Experimental procedure

Males were collected as pigmented "ready-to-hatch" pupae and kept with two nurse workers (that helped with eclosion) in a plastered experimental container with 10% sucrose. All males were approximately the same age and between three and six days old, which means their cuticle was fully sclerotized and their immune system should have been fully established.

"Early-stage infected" males were exposed to either 10^6 spores/ml ("low dose"), 10^9 spores/ml ("high dose") or 0.05% Tx as sham-treatment and the experiments were immediately set up thereafter. Between high and low dosage we chose a 1000-fold difference in application dose as we expected the workers to groom off spores effectively and to ensure that there was still a

pronounced difference between the two dosages tested. Nestmate workers were all sham-exposed with 0.05% Tx before the start of the experiments.

"Late-stage infected" males were exposed to a spore suspension with 10^4 spores/ml ("low dose"), 10^6 spores/ml ("high dose") or to 0.05% Tx as sham-treatment and individually put in experimental containers for 48h with 10% sucrose before the experimental start for the infections to establish and prevent contamination of the rival and the workers. We chose a 100-fold difference between the application dosages to ascertain a clear difference in physical condition – with mild or severe impact – at the time of the fight situation. Nestmate workers of the experimental setups were sham-treated with 0.05% Tx and kept in groups of 35 until the fights were set up. The table below summarizes the fight combinations and their respective sample sizes (*Table 3.1*).

Table 3.1. Fight combinations and sample sizes (early- and late-stage of infection). Control males received a sham-treatment with 0.05% Tx.

	Fight combination	**N**
Early-stage infected high	Control	43
	One male infected	65
	Both males infected	31
	total	*139*
Early-stage infected low	Control	30
	One male infected	30
	Both males infected	30
	total	*90*
Late-stage infected high	Control	53
	One male infected	75
	Both males infected	55
	total	*183*
Late-stage infected low	Control	20
	One male infected	31
	Both males infected	32
	total	*83*

Determination of the treatment of the loser

We determined whether the loser of decided mixed fights (one early- or late stage infected male rivalling a sham-treated male with a clear winner and loser) had been pathogen- or sham-treated by either color-marks of the males or by spore load determination via droplet digital PCR (details below). The data from both approaches were pooled for the analysis as the proportion of decided fights per treatment was not statistically different from each other, indicating that color-marks per se do not interfere with the fight outcome.

Color-marking of males

The males were marked with metallic emaille color (Revell) with a tiny dot on their gaster using a micro dissection needle holder with a fine tungsten needle with a 1 µm tip diameter the evening before the experiments were set up and kept in isolation to allow the color to dry properly.

DNA extraction & droplet digital PCR (ddPCR)

To determine which of the individuals was the initially exposed one (early-stage infected, respectively late-stage infected) in decided mixed fights, we determined the spore load of both combatants using a droplet digital PCR system (ddPCR, Bio-Rad). We inferred that initial exposure was reflected in a higher spore load in both cases.

For the DNA extraction, the samples were homogenized using a TissueLyser II (Qiagen) with a mixture of one 2.8 mm ceramic (Qiagen), five 1 mm zirconia (BioSpec Products) and ~100 mg of 425-600 µm glass beads, acid washed (Sigma-Aldrich) in 50 µl of nuclease-free water (Sigma). Homogenization was carried out in two steps (2 x 2 min at 30 Hz). After the first 2 min the tube racks were rotated to ensure uniform disruption and homogenization of all samples. In case the samples were not uniformly crushed yet, the homogenization was repeated. DNA extraction was performed using Qiagen DNeasy 96 Blood & Tissue Kit (Qiagen) following the manufacturer's instructions with a final elution volume of 50 µl.

As we used a fluorescently labelled strain of *Metarhizium robertsii* (ARSEF 2575) where each spore carries a single plasmid with a single copy of the *mRFP1* gene (Fang *et al.* 2006), we could quantify absolute spore numbers for each analyzed sample.

Primers and probe were designed to bind to the *mRFP1* gene (mRFP1_Forward: 5'-CTGTCCCCTCAGTTCCAGTA, mRFP1_Reverse: 5'- CCGTCCTCGAAGTTCATCAC, mRFP1_probe: 5'-[6FAM]AGCACCCCGCCGACATCCCCG[BHQ1]) using Primer3Plus software (Untergasser *et al.*, 2007).

The ddPCR reactions with a total volume of 22 µl were prepared as follows: 11 µl of 2x ddPCR Supermix for Probes (Bio-Rad), 900 nM of both primers (Sigma Aldrich) and 250 nM probe (Sigma Aldrich), 10 U of both enzymes EcoRI-HF and HindIII-HF (New England Biolabs), 5.27 µl nuclease-free water (Sigma) and 2.2 µl of template DNA.

20 µl of the ddPCR reaction and 70 µl Droplet Generator Oil for Probes (Bio-Rad) were transferred to DG8 Cartridges of the QX200 Droplet Generator (Bio-Rad) and sealed with the DG8 Gaskets. Subsequently, 40 µl of the generated droplets were transferred to a 96 well plate (twin.tec® PCR Plate 96, semi-skirted, Eppendorf) and sealed with pierceable PCR Plate Heat Seal (Bio-Rad) using the PX1 PCR Plate Sealer (Bio-Rad).

PCR amplification was carried out in a T100 Thermal Cycler (Bio-Rad). The amplification program was initiated with a first step at 95 °C for 10 min to activate the enzyme, followed by 40 cycles of 30 sec at 94 °C and 60 sec at 56 °C, and finished with 98 °C for 10 min to inactivate the enzyme. For the entire program the ramp rate was set to 2 °C/sec. After amplification droplets were analyzed on the QX200 Droplet Reader (Bio-Rad) for the readout of positive and negative droplets. Data analysis was done using the QuantaSoft Analysis Pro Software (Version 1.0.596; Bio-Rad). The threshold was manually set to 3000.

Overloaded samples (low amount of negative droplets) were diluted and re-run. Each run included a H_2O negative control. To calculate the absolute amount of spores per ant the value 'Copies/20µl Well' was multiplied with the dilution factor (if applicable), divided by 2 (amount of DNA used in the PCR reaction) and multiplied with 50 (final elution volume of the DNA extraction). Based on this calculation we can conclude that using this method at least 25 spores have to be on the ant to see a positive result (=detection threshold).

Determination of mortality risk of "non-fighters"
To determine the "baseline" mortality of the males due to the sham-treatment or different stages of infection, we compared the survival of early-stage and late-stage infected males that were kept with nestmate workers, but without a rival, to the survival of the respective sham-treated control males. We refer to these males as "non-fighters" hereafter.
Males were color-marked the evening before the experiment was set up. Thereafter, they were put in new plastered experimental vials together with five sham-treated workers (no rival present). After 24h we determined the survival of the males. We performed this experiment for early- and late-stage infected males each with the low and the high dose. The table below summarizes the sample sizes for all treatment groups (*Table 3.2*).

Table 3.2. Treatment groups and sample sizes of non-fighters (early- and late-stage of infection).

	Treatment	**N**
Early-stage infected	Sham-treated	60
	Low dose	29
	High dose	62
	total	*151*
Late-stage infected	Sham-treated	53
	Low dose	25
	High dose	39
	total	*117*

By comparing the mortality of non-fighters to the mortality of males that were in a fight setup, we could determine whether and to which extent rival presence and combat increased the mortality risk of the males - not only considering their own, but taking the rival's infection status into account as well (see details *Figure 3.6*).

Behavioral scan samples
For a subset of fights, we performed additional behavioral scan sampling (each scan taking one to several seconds). Scans were performed the first 8h after the experiments were set up every 30 min and a final scan after 24h where we determined the outcome as well. This resulted in 18 scans in total per experimental setup, where multiple behaviors could occur at the same time. In order to differentiate the males, they were color-marked. We looked at male-male and worker-male interactions, where we put the main focus on aggressive behaviors/interactions in our analysis that could be driven by either the presence of spores or the infection status of the males. Behaviors were classified into distinguishable, non-overlapping categories and were defined before the experiment.

Male-male aggressive interactions were *biting* (one male biting the rival), *holding* (grabbing the rival with the mandibles) and *besmearing* (male bending its gaster to apply hindgut secretion; often combined with holding the rival). When one of these behaviors was observed, we categorized the scan as a fight. *Antennation* was counted as a neutral, non-aggressive behavior. *Allogrooming* was not observed in males.

Similarly, *antennation* and *grooming* performed by workers towards the males were categorized as non-aggressive behaviors and *biting*, *carrying* (male is held with the mandibles and carried around), *dismembering* (body parts are intensively attacked or already bitten off) and *dragging* (male is held and pulled over the ground) were categorized as aggressive behaviors.

For analysis we added all neutral and all aggressive behaviors and analyzed them as total numbers. Early- and late-stage infected males exposed to the low dose did not show any behavioral differences to sham-treated control males, therefore they are not shown (all $p>0.05$). The table below summarizes the sample sizes for all fight combinations for which we analyzed behavior (*Table 3.3*).

Table 3.3. Sample sizes for behavioral scan sampling per fight combination (early- and late-stage of infection).

	Treatment	**N**
Early-stage infected high	Control	16
	One male infected	17
	Both males infected	17
	total	*49*
Early-stage infected low	Control	30
	One male infected	30
	Both males infected	30
	total	*90*
Late-stage infected high	Control	30
	One male infected	34
	Both male infected	30
	total	*94*
Late-stage infected low	Control	20
	One male infected	31
	Both males infected	32
	total	*83*

Activation of the immune system of early-stage infected males

As we found that early-stage infected males exposed to the high dose had a much higher risk of dying in a fight than one would expect by chance, we tested whether the males already mounted an immune response against the spores and therefore possibly faced an immunity-fighting ability trade-off, so that they performed poorly in a combat.

To this end, we exposed color-marked and age-controlled males (3-6 days old) by dipping them into 10^6 and 10^9 spores/ml or sham-treated them with 0.05% Tx, kept them individually and checked their immune gene expression to determine if they had altered gene expression within the fighting period when they were pathogen-treated.

Males were snap frozen after 12 and 24h post exposure in 1.5ml Safe-Lock Tubes (Eppendorf), which corresponds to the middle and the end time point of the fight setups. We analyzed 9-10 males for each treatment and both time points (see *Table 3.4* below) and measured the immune gene expression of three different genes that are involved in the cellular and humoral immune response against *Metarhizium* with ddPCR (details see below). We analyzed relish (*Rel*) – the transcription factor of the immune deficiency pathway whose upregulation would indicate a fungus-unspecific immune response (Myllymäki *et al.*, 2014; Sheehan *et al.*, 2020), prophenoloxidase-activating factor (*PPAF*) - a gene involved in the *PPO*-pathway and activates *PPO* in its active form (Gillespie *et al.*, 1997; Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008) and the antimicrobial peptide defensin (*Def*) involved in the Toll-pathway that is known to destroy fungal cell walls (Viljakainen and Pamilo, 2005, 2008) and the housekeeping gene elongation factor alpha 1 (*EF1*). Samples were stored at -80 °C until further sample procession. The table below summarizes the sample size of sham-treated and exposed males per gene and time point (*Table 3.4*).

Table 3.4. Sample sizes of sham-treated and pathogen-exposed males for immune gene expression analysis. Males were either sham-treated with 0.05% Tx or pathogen-exposed (1 x 10^6 and 1 x 10^9 spores/ml). Immune gene expression was measured 12 and 24h after exposure.

Treatment	12h	24h
Sham-treated	10	9
Low dose	10	9
High dose	9	10
Total	*29*	*28*

RNA extraction, cDNA synthesis & ddPCR

For RNA extraction individuals were homogenized in 200 µl homogenization buffer including 4 µl 1-Thioglycerol with a TissueLyser II (Qiagen) with a mixture of five 1 mm zirconia (BioSpec Products) and ~100 mg of 425-600 µm glass beads, acid washed (Sigma-Aldrich). Homogenization was carried out in two steps (2 x 2 min at 30 Hz). After the first 2 min the tube racks were rotated to ensure uniform disruption and homogenization of all samples. In case the samples were not uniformly crushed yet, the homogenization was repeated.

Total RNA was extracted using the Maxwell® RSC instrument together with the Maxwell® simplyRNA Tissue Kit (Promega) according to the manufacturer's instructions. To ensure the complete removal of residual DNA contamination an additional DNase-I treatment (Sigma-Aldrich) step was performed prior to the reverse transcription. The cDNA synthesis was done using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

To analyze the expression patterns of the immune genes, we used multiplex ddPCR assays, each targeting one immune gene and the housekeeping gene. The primer design and the method establishment were done by J. Kirchner within the framework of her Bachelor's thesis 2021. Primers were designed using Primer3Plus (Untergasser *et al.*, 2007) and "Multiple Primer Analyzer" (https://www.thermofisher.com) software according to the following sequence IDs of the *C. obscurior* genome Cobs.alpha.v.2.1 (Lukas Schrader, Institute for Evolution and Biodiversity, Münster, 2021, unpublished, *Table 3.5* and *Table 3.6*).

Table 3.5. Sequence IDs of genes used to design primers for the immune gene expression analysis.

Gene	ID in Cobs.alpha.v.2.1
EF1	COBS15396
PPAF	COBS07801
Rel	COBS06226
Def	COBS15672

Table 3.6. Sequences of primers and probes used for immune gene expression in ddPCR.

Gene	Primer sequence (5´- 3´)	
EF1	Forward	ATTGGAACAGTACCCGTTGG
	Reverse	CACCCTTCGGTGGGTTATTT
	Probe	[HEX]ACCTGGTATGGTCGTTACCTTTGCACCCGT[BHQ1]
PPAF	Forward	TGCTGCTCACTGTATCAAGG
	Reverse	TCTGTTTCAGTGTCGGTGTC
	Probe	[6FAM]ACTGGCGTCTGACCAGCGTCCGT[BHQ1]
Rel	Forward	ACGGATTTAGGATGGACACC
	Reverse	CTTGGTGGCTTCCTTCAACA
	Probe	[6FAM]TGCTCTCTTGTGCAGACTGGCGCAGA[BHQ1]
Def	Forward	ACGGGCCTACTTACGAATTG
	Reverse	CGCAAGCACTATGGTTGATG
	Probe	[6FAM]CGAAGAGGAGCCGTCACACCTGACGC[BHQ1]

The ddPCR reactions with a total volume of 22 µl were prepared as follows: 11 µl of 2x ddPCR Supermix for Probes (Bio-rad), 900 nM of each primer (Sigma-Aldrich) for the immune gene of interest as well as the housekeeping gene and 250 nM probe (Sigma Aldrich), 3.74 µl nuclease-free water (Sigma) and 2.2 µl of cDNA.
All further steps were performed as described above, except that the amplification program was slightly modified to improve the separation between positive and negative droplets.
The amplification program was initiated with a first step at 95 °C for 10 min, followed by 50 cycles of 30 sec at 94 °C and 50 cycles of 2 min at 56 °C, and finished with 98 °C for 10 min and 12 °C hold. All PCR steps were carried out with a ramp rate of 1°C/sec.
Relative immune gene expression was calculated by dividing the value 'Copies/20µl Well' for the immune gene by the value 'Copies/20µl Well' of the housekeeping gene in the same well. The thresholds were set manually as follows: *EF1* 4000, *PPAF* 1500, *Rel* 2000 and *Def* 4000.

To show whether immune genes were differentially expressed, we standardized the gene expression value of each male to the median of the respective sham-control per time point by subtracting the median from each individual sample. We present these "delta-values" as medians with 95% confidence intervals. An overlap of the confidence interval with the zero-baseline (median of the respective sham-treated males after 12 or 24h) therefore shows that the immune genes are not differentially expressed, whereas a non-overlap of the confidence interval and the baseline indicates differential gene expression. Values above the baseline indicate an upregulation, whereas values below the baseline indicate a downregulation of immune gene expression.

Spore transmission from early- and late-stage infected males to their rival in decided mixed fights and social immunity
To assess the risk posed by an early-stage infected male to contaminate its rival, we analyzed 20 decided mixed fights of a sham-treated and an early-stage infected male exposed to 10^9 spores/ml (five sham-treated workers) and determined the proportion of fights in which we were able to detect spore transfer with ddPCR. The experiment was not performed with the low dose, as the number of transferred spores would be below detection threshold (<25 spores). Additionally, we analyzed 16 decided mixed fights without workers and determined again the proportion of fights with detectable spore transfer. By comparing these proportions, we could quantify how effectively spore removal by sanitary care operated at the early-stage of infection when spores are still transmissible among individuals. We similarly also proceeded with 28

decided mixed fights of late-stage infected males with five sham-treated workers and 7 decided mixed fights without workers where no spore transmission was expected.

Statistical analysis

All statistical analyses were carried out in the program 'R' version 4.0.3 (R Core Team 2020) and all reported p-values are two-sided. Unless otherwise stated, we used a generalized mixed modelling approach (GLMM) ('lme 4' (Bates *et al.*, 2015)) in which the significance of the model predictors were estimated by comparing each model to a null model only containing the intercept and reduced models (containing all but the predictor of interest) using Likelihood Ratio (LR) tests (Bolker *et al.*, 2009). We tested for a significant interaction of the predictors by comparing models with the interaction included to models without the interaction. When the interaction of the predictors was significant, we did not assess the main effects, but directly performed post hoc testing. When the interaction turned out not significant, the models were refitted and run without the interaction to achieve better estimates for the main effects.
We assessed model assumptions (residual normality and heterogeneity, no multicollinearity and no overdispersion) with 'DHARMa' (Hartwig, 2021) and checked model stability and the presence of influential data points (Cook's distance and dfbetas).
Whenever multiple inferences were made from the same dataset, we corrected the overall p-values for multiple testing with the Benjamini-Hochberg procedure to protect against a false discovery rate of 0.05 (Benjamini and Hochberg, 1995). Only if the overall model revealed significance (after correction for multiple testing), Tukey post hoc tests were performed and corrected for multiple testing using the ghlt-function of the 'multcomp' package (Hothorn *et al.*, 2008) according to the Benjamini-Hochberg procedure. We report corrected p-values (unless otherwise stated). All graphs were made using the 'ggplot2' package (Wickham *et al.*, 2018). We ran separate models for early- and late-stage of infection and both dosages. All GLMMs included 'stock colony' as a random effect.

Proportion of fight outcome per fight combination. To determine whether the fight outcome (one male dead, both males dead, or both males alive) differed between the different fight combinations, we ran a GLMM with binomial error term and logit-link function for each outcome. We used the fight outcome as response variable and fight combination as predictor variables.

Proportion of early- and late-stage infected males losing in mixed fights. To test whether early- or late-stage infected males were losing more often in mixed fights against a sham-treated rival as expected by chance, we compared the proportion to 0.5 using χ^2-tests or Fisher's exact test (if the minimum expected frequency was less than 5).

Mortality risk of non-fighters. To determine the baseline mortality risk of the males due to early- and late-stage infection in the absence of a fight (non-fighters), we modeled survival (dead or alive) as a function of male infection status (sham-treated vs. infected). We ran a logistic regression implemented as GLMM with binomial error term and logit-link function.

Behavioral analysis
Fight intensity. When male aggressive behaviors (biting, besmearing or holding) towards the rival occurred, the scan was considered to be a fight. To determine whether fight intensity (the number of scans that were categorized as fights) differed between the fight combinations, we fitted a GLMM with negative binomial error term and log-link function as the data was

overdispersed when the analysis was performed with a GLMM with Poisson error term. The different fight combinations were used as predictor and the fight intensity as response.

Worker aggression and fight combination. To test whether worker aggression (number of aggressive interactions) towards the males was affected by the different fight combinations, we implemented GLMMs with Poisson error terms and log-link function. The number of aggressive behaviors performed towards each male was modeled as a function of fight combination. 'Stock colony' and 'experimental replicate' were included as random effects. This was important as worker aggression towards males in one fight setup (=replicate) was not independent from each other.

Worker aggression towards individual males in mixed fights. We checked whether the males in mixed fights were differently aggressed by workers depending on their infection status. We ran GLMMs with Poisson error terms and log-link function with male infection status as predictor and the number of worker aggressive behaviors as response. 'Stock colony' and 'experimental replicate' were included as random effects.

Male aggression performed in mixed fights. To test whether male aggression was affected by their own infection status, we compared the aggressive behavior performed by each male in mixed fights using GLMMs with negative binomial error term with log-link function and implemented 'stock colony' and 'experimental replicate' as random effects.

Losing a fight and worker aggression received. To evaluate whether the probability of losing in mixed decided fights was affected by own infection status and worker aggression received, we ran logistic regressions implemented as a GLMMs with binomial error structure and logit-link function with fight outcome (winning or losing) as response and infection status and worker aggression received as predictors. As there was no interaction of the main effects, we refitted the models without the interaction and reduced the models further to test for significance of the different main effects. We included 'experimental replicate' and 'stock colony' as random effects.

Losing a fight and male aggression performed. To determine whether the probability of losing a fight is affected by own infection status and own aggression performed, we ran a logistic regression implemented as a GLMM with binomial error structure and logit-link function with fight outcome (winning or losing) as response and infection status and male aggression performed as predictors and proceeded as described above.

As all the behavioral analyses for early- and late-stage infected males exposed to the low spore dose revealed no statistical significance, the results are not shown hereafter.

Contamination of the rival in mixed fights and sanitary care performed by workers. To test whether worker presence (sanitary care) reduced the proportion of cases with detectable spore transmission from the early-stage infected male to its sham-treated rival, we compared the proportion of cases with detectable spore transmission when workers were present to the proportion of cases with detectable spore transmission when workers were absent with a χ^2-test. As we found transmission in the late-stage setups only in one case, we did not perform any statistics.

3.4 Results

Mixed fights at early- and late-stage of infection end mostly decided
Mixed fights of early- or late-stage infected male exposed to the high dose ended more often decidedly, compared to other fight combinations. In detail, 62% (40/65) of the mixed fights of early-stage infected males and 72% (54/75) of the late-stage infected males ended decidedly. The proportion of cases where both males died was increased when two early-stage infected males were rivalling each other, whereas this was not the case for late-stage infected males (*Figure 3.2 A* and *B, Table 3.7*).

Table 3.7. Statistical values for the overall models of the fight outcome (early- and late-stage of infection, high dose). P-values are corrected for multiple testing according to the Benjamini-Hochberg procedure α=0.05.

Fight outcome	Early-stage infection	Late-stage infection
Both alive	χ^2=6.789, d.f.=2, p=0.034	χ^2=6.987, d.f.=2, p=0.030
Both dead	χ^2=24.584, d.f.=2, p<0.001	χ^2=22.263, d.f.=2, p<0.001
One dead	χ^2=16.059, d.f.=2, p<0.001	χ^2=21.962, d.f.=2, p<0.001

Early- and late-stage infected males lose fights over-proportionally
In mixed decided fights, we found that early- and late-stage infected males were significantly more often the losers than one would expect by chance. Early-stage infected males were in 85% (34/40) of the cases the loser (χ^2=11.168, d.f =1, p<0.001, *Figure 3.2 C*). Although early-stage infected males only recently have come into contact with the pathogen, they already seemed to have a disadvantage against their sham-treated combatants. Similarly, we found late-stage infected males to be the losers in 81% (44/54) of the cases (χ^2=11.881, d.f.=1, p<0.001, *Figure 3.2 C*).

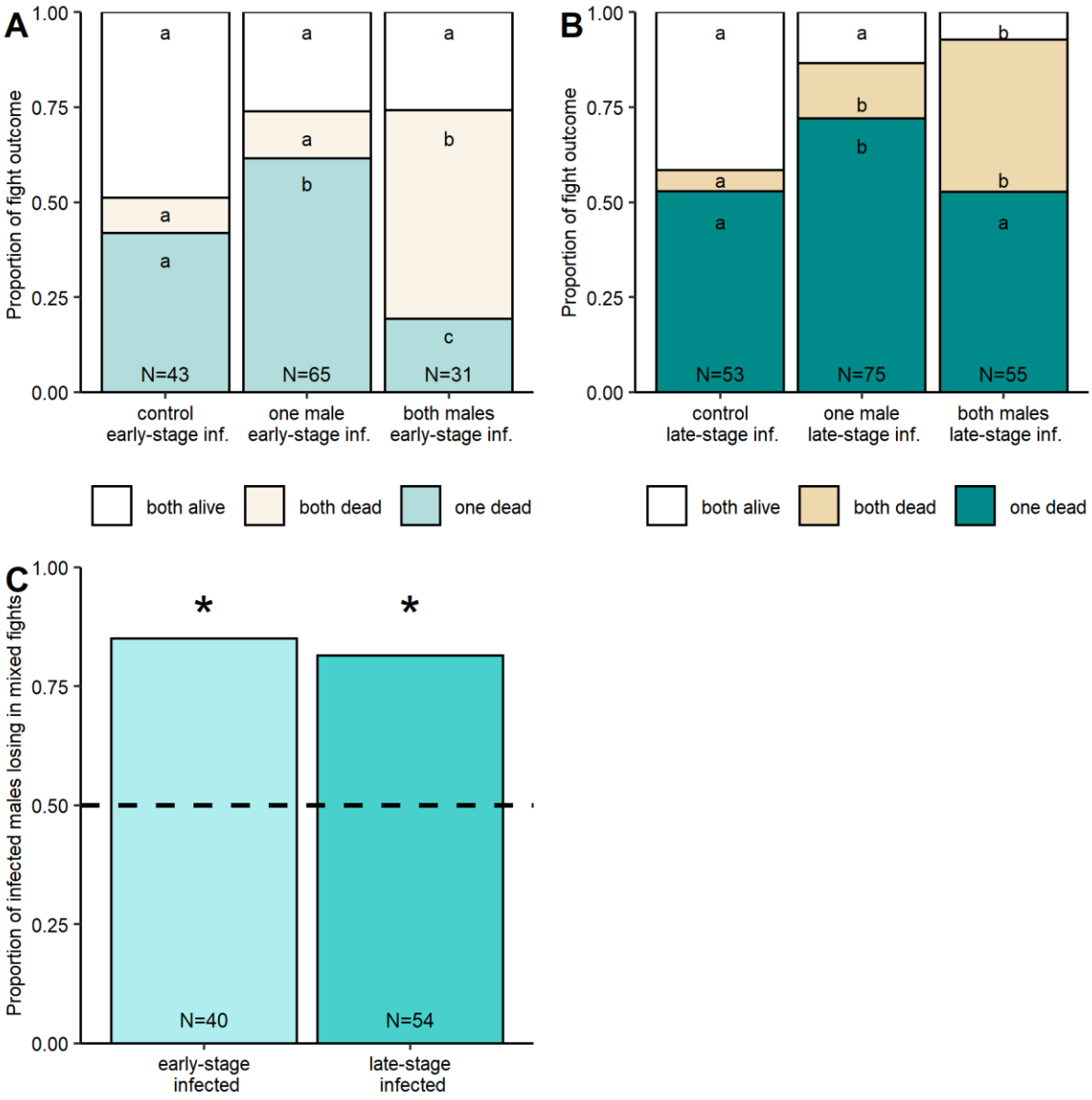

Figure 3.2. **Fight outcome depending on fight combination and the proportion of infected males losing in mixed fights (high dose).** We compared the fight outcome depending on the fight combination (A) at the early-stage and (B) at the late-stage of infection of males exposed to a high dose of fungal spores. The proportion of fights that ended undecidedly with both males being alive are shown in white, cases where both males died are shown in beige and decided fights where only one male died are shown in turquoise. We found that mixed fights usually end decidedly compared to other fight combinations. Different letters indicate significance groups of all pairwise post hoc comparisons after Benjamini–Hochberg correction at α=0.05. We further tested whether (C) early- or late-stage infected males are more likely to lose against their sham-treated rivals as one would expect by chance. Bars indicate the proportion of cases where the infected males (early-stage infected male represented in light blue and late-stage infected male represented in dark blue) were the losers in mixed fights. The dashed line indicates the proportion one would expect by change. We found that in mixed fights early- as well as late-stage infected males lost fights over-proportionally. N denotes the total sample sizes per bar. * represents statistical differences to the baseline of 0.5 ($p<0.05$).

Fights were not affected when males were only exposed to a low pathogen dose
Contrary to the findings in early- and late-stage infected males exposed to the high dose, where males in both cases performed notably poorly in mixed fights, this was not the case for males exposed to a lower dose (all p>0.324, *Table 3.8, Figure 3.3 A and B*). Early-stage infected males were only the loser in 40% (4/10) of the cases (Fisher's exact test p=1, *Figure 3.3 C*) and late-stage infected males in 38% (3/8) (Fisher's exact test: p=1, *Figure 3.3 C*). This illustrates that early- and late-stage infections with a low pathogen dosage did not affect fights, indicating that it needs a disease-inducing dose to find an impact on fighting ability in males.

Table 3.8. Statistical values for the overall models of the fight outcome (early- and late-stage of infection, low dose). P-values are corrected for multiple testing according to the Benjamini-Hochberg procedure α=0.05.

Fight outcome	**Early-stage infection**	**Late-stage infection**
Both alive	χ^2=2.551, d.f.=2, p=0.380	χ^2=0.145, d.f.=2, p=0.923
Both dead	χ^2=4.454, d.f.=2, p=0.324	χ^2=2.366, d.f.=2, p=0.460
One dead	χ^2=1.933, d.f.=2, p=0.380	χ^2=2.421, d.f.= 2, p=0.460

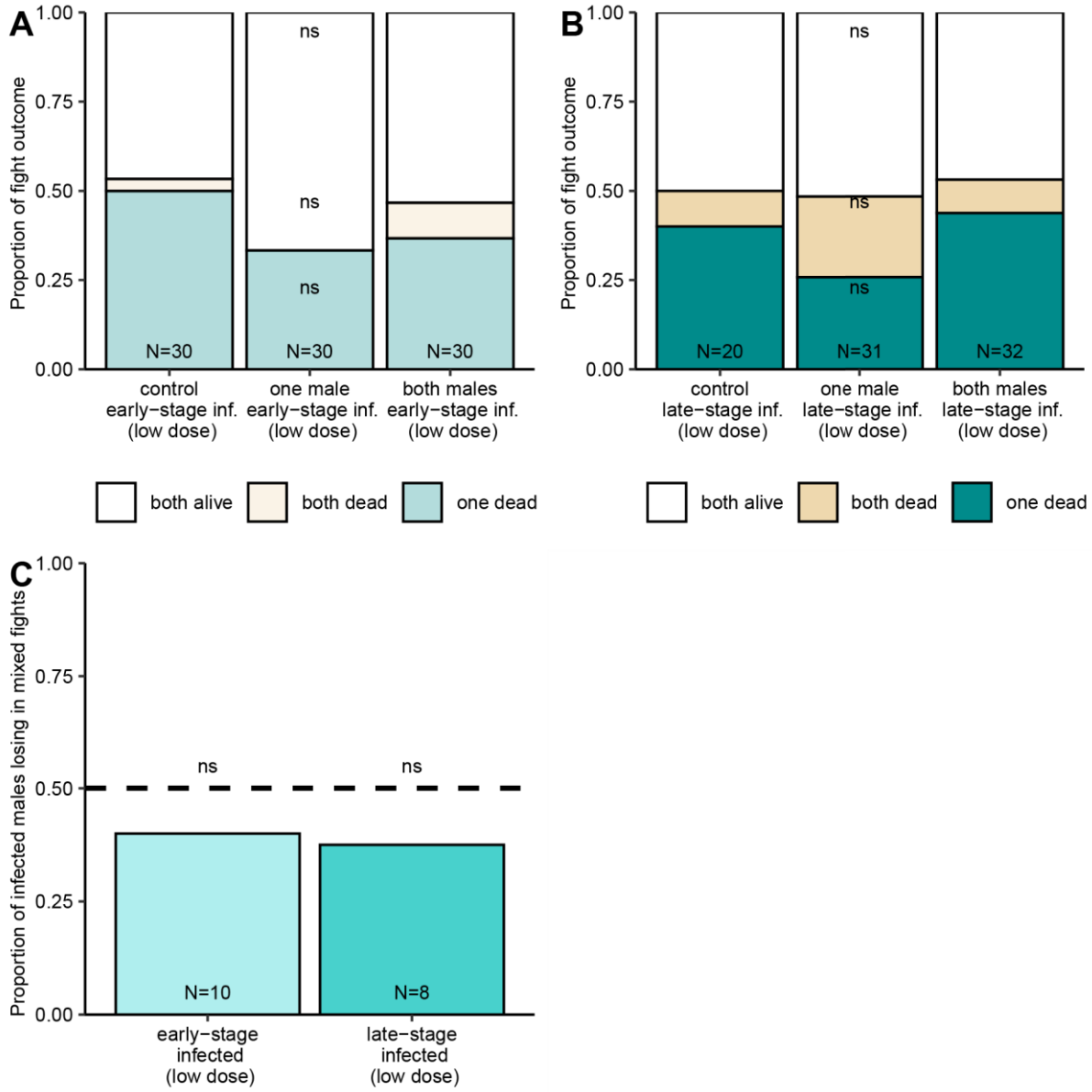

Figure 3.3. **Fight outcome depending on fight combination and the proportion of infected males losing in mixed fights (low dose).** We compared the fight outcome depending on the fight combination (A) at the early-stage of infection and (B) at the late-stage of infection of males exposed to a low dose of fungal spores. The proportion of fights that ended undecidedly with both males being alive are shown in white, cases where both males died are shown in beige and decided fights where only one male died are shown in turquoise. We found that the proportion of decided fights was not increased in mixed fights of early- as well as late-stage infected males. We further found that (C) early- or late-stage infected males are not more likely to lose against their sham-treated rivals than one would expect by chance. Bars indicate the proportion of cases in which the infected males (early-stage infected male represented in light blue and late-stage infected male represented in dark blue) were the losers in mixed fights. The dashed line indicates the proportion one would expect by chance. N denotes the total sample sizes per bar. ns=non-significant.

Early-stage infected males would not die without a fight, whereas late-stage infected males are already moribund when they encounter their rival

For both – early- and late-stage infected non-fighters – we found that *Metarhizium* induced mortality as expected in a dose-dependent manner. We found that early-stage infected males exposed to the high dose died significantly more (29% mortality) than sham-treated males (8% mortality) and males exposed to the low dose had an intermediate mortality (17%). This indicated that the low dose doubles their mortality risk, whereas the high dose makes early-

stage infected males approximately 3.5 times more likely to die (χ^2=9.033, d.f.=2, p=0.011, low vs. high p=0.233, control vs. low p=0.233 and control vs. high p=0.017, *Figure 3.4 A*) already within the first 24h after exposure.
Late-stage infected non-fighters at both dosages died significantly more than sham-treated males (χ^2=25.220, d.f.=2, p<0.001, high vs. low p=0.114, control vs. low p=0.011, control vs. high p<0.001, *Figure 3.4 B*). In more detail, 9% of the sham-exposed males, 36% of the late-stage infected males exposed to the low and 56% to the high dose had died. This illustrated that the infection per se increased their mortality risk four-fold in the low dose and even six-fold in the high dose.

We previously found that early- as well as late-stage infected males (exposed to the high dose) are in most of the cases the losers of mixed fights.
In late-stage infected males this can be explained by the fact that they would die anyway from the progressed infection - as their mortality when they were exposed to a rival only increased by 25% (mortality of non-fighters 56% vs. mortality of fighters 81%). Yet, the high mortality of early-stage infected males in the fights cannot be attributed to the early-stage infection alone as only 29% of the non-fighters died, whereas 85% of the early-stage infected males died in mixed fights, which means that a fight increased their mortality risk by an additional 56%. As *Cardiocondyla* males have a rather soft cuticle, it is likely that the spores that remained on the cuticle and were not groomed off, already started germinating - similar to reports in termites (Davis *et al.*, 2018) and possibly already penetrated the cuticle towards the end of the fight and activated the immune system. Overall, this suggested that the early-stage infected males might suffer from an immunity-fighting trade-off – a hypothesis that we have tested later on with immune gene expression analysis.

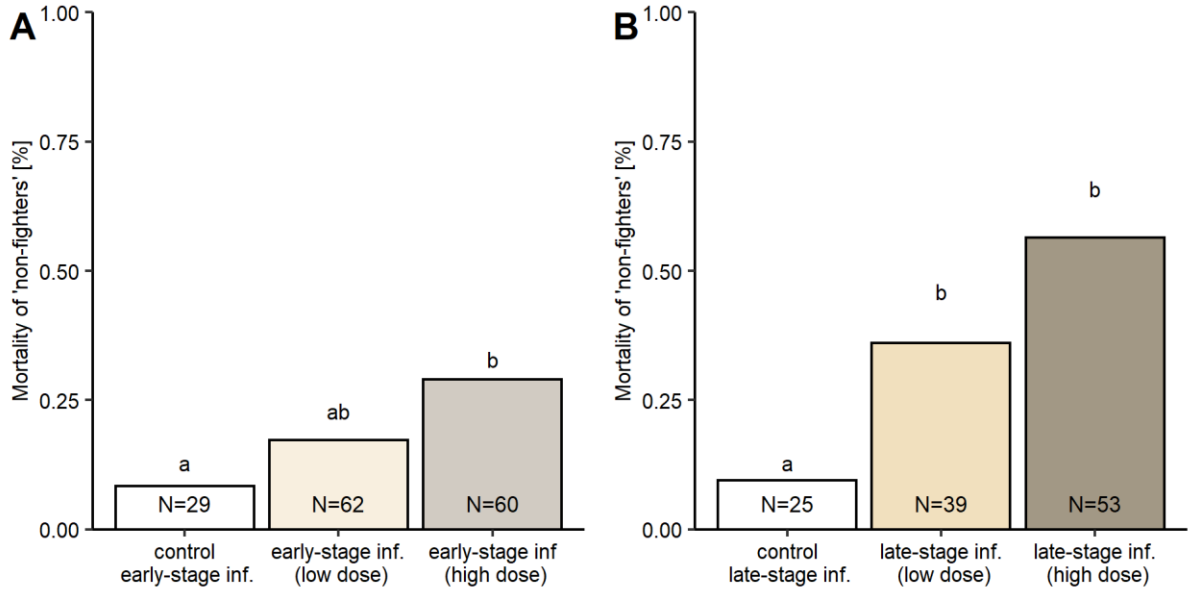

Figure 3.4. **Mortality risk of non-fighters.** We compared the survival of males that were (A) early-stage infected and exposed to 10^6 spores/ml (low, beige) and 10^9 spores/ml (high, brown) or (B) late-stage infected and exposed to 10^4 spores/ml (low, beige) and 10^6 spores/ml (high, brown) to the survival of their respective controls (sham-treated males; white). The bars represent the proportion of dead non-fighters after 24h in the presence of workers. We found that early-stage infected males exposed to the high dose died significantly more than sham-treated males, and males exposed to the low dose had an intermediate mortality. Late-stage infected non-fighters at both dosages died significantly more than sham-treated males, but the survival of males infected with the low and high dose did not differ. N denotes the total sample size per male treatment. Different letters indicate significance groups of all pairwise post hoc comparisons after Benjamini–Hochberg correction at α=0.05.

Early-stage infected males mount an immune response within the first 24h after exposure
To evaluate whether early-stage infected males invested in immune defense within the fighting period when spore exposed, we looked at the immune gene expression of three different genes. We analyzed the antimicrobial peptide defensin (Viljakainen and Pamilo, 2005, 2008), the *PPO*-activating factor *PPAF* (Gillespie *et al.*, 1997; Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008) and the transcription factor relish of the immune deficiency pathway (Myllymäki *et al.*, 2014; Sheehan *et al.*, 2020) - known to be involved in important antifungal defenses (details see material and methods).
We found that immune genes were not upregulated when the males were exposed to the low dose, but that males that were exposed to the high dose showed already a slight upregulation of immune genes after 12h and clear upregulation after 24h (*Figure 3.5*). This shows that *Metarhizium* acts, as expected, in a dose- and time-dependent manner, with a higher spore number leading to faster and more pronounced effects. The immune gene upregulation of males exposed to the high dose clearly demonstrates that the males have already invested into immune defense within the fighting period and supports the hypothesis that these males face a trade-off between immunity and fighting ability. Here, the cost of raising an immune response is only revealed by the additional stress of fighting, as the mortality of fighters (85% mortality), but not of non-fighters (29% mortality), was significantly increased over sham-treated males. This can explain why we found pronounced effects in early-stage diseased males, whereas no detectable effects on fight outcome and probability of losing a fight were found in males exposed to the low dose.

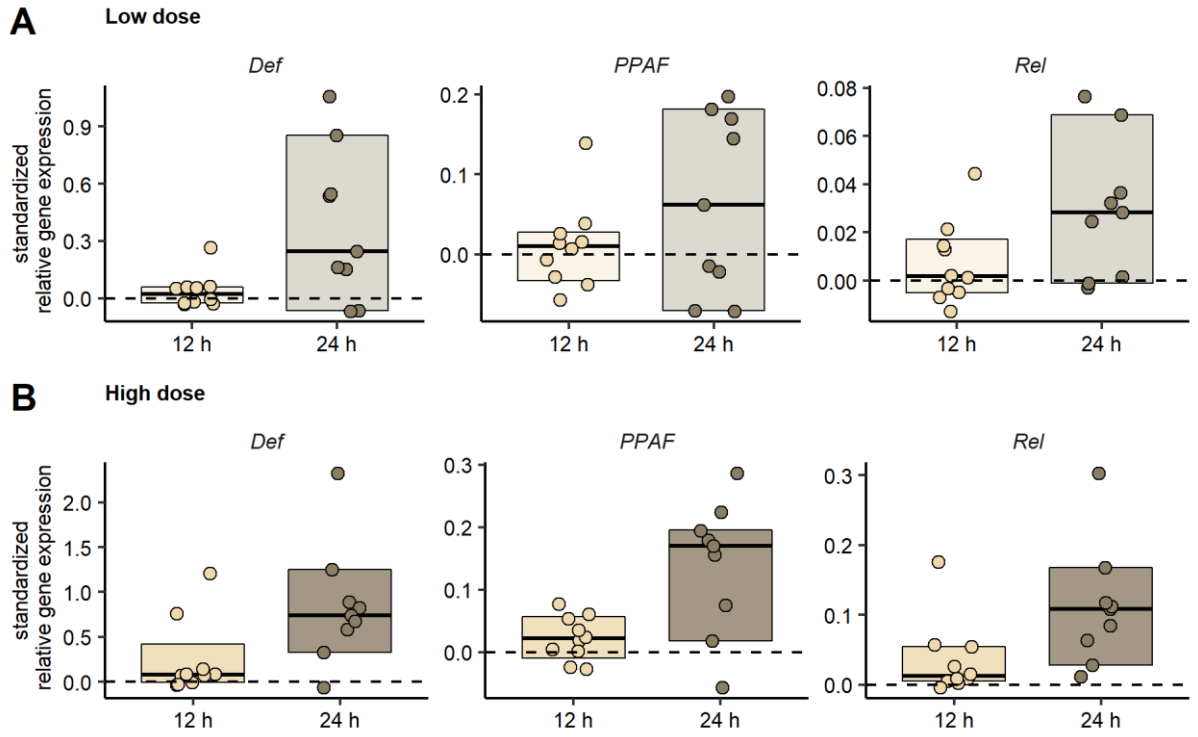

Figure 3.5. **Immune gene expression of males exposed to 1 x 10 ^{6} and 1 x 10^{9} spores/ml 12 and 24h post exposure.** Immune gene expression of males exposed to (A) 1 x 10 ^{6} and (B) 1 x 10^{9} spores/ml was measured 12 and 24h post exposure. Data points represent standardized gene expression values of each male (immune gene expression value per male - median of sham-treated males at 12 and 24h) grouped by time point at which immune gene expression was measured (12h: beige, 24h: brown). Boxes show the 95% confidence intervals and the median (dash). The dashed line represents the median immune gene expression of sham-treated males at each time point (=baseline). An overlap of the confidence interval with the baseline indicates that the immune genes are not differentially expressed, whereas a non-overlap of the confidence interval and the baseline indicates differential expression. Values above the baseline indicate an upregulation.

Fighting is costly

In a next step, we compared the survival of non-fighters to the survival of fighters which allowed us to make two more inferences: (i) how much more a fight on average increased the mortality risk of a male (independent of the rival's health status) and (ii) how much the mortality risk of a fighter increased depending on whether or not the rival had the same health status (see *Figure 3.6*).

In the following, we provide details on how the mortality risk of males was affected by being in a stressful combat situation. As the mortality risk of non-fighters already indicated, the effects are more pronounced in early- and late-stage infected males exposed to the high dose.
A fight of control males increased the mortality risk on average 3.2 times, whereas the risk increased 2.2 times for early-stage infected fighting males, but at the same time their "baseline" mortality (mortality risk of non-fighters) was already drastically increased. The mortality risk of control males was higher when they rivaled another control male (3.8-fold increase) compared to when facing an early-stage infected male as competitor (2.6-fold increase). This can be explained by the fact that they have a higher chance of winning in mixed fights as the infected rival is more likely to die.
For early-stage infected males an additional fight increased their mortality risk only about 2.2-fold, irrespective of the infection status of the rival (*Figure 3.6 A*). One would have expected early-stage infected males to have a higher chance of dying when they rival a healthy male, but an equally likely chance of dying when fighting another infected male. The high mortality in both males can be explained by the stress of fighting which causes them to die immediately. Taken together this confirms our hypothesis of an immunity-fighting ability trade-off.
In late-stage infected fights we found – consistent to control males of the early-stage infected fights – that the mortality risk of control males increased on average 3.4 times when they rivaled another male (irrespective of the other male's infection status). The additional stress of a fight was less pronounced for late-stage infected males as their mortality risk only increased roughly 1.25-fold when they had a rival present as their baseline mortality was already six-fold increased because of being infected (*Figure 3.6 B*). The fact that a fight increased the relative mortality of sham-treated control males much more, could indicate that infected males might already be too weak to engage actively in combat. A hypothesis that we subsequently have tested with behavioral scan sampling.

In control males of early- and late-stage infected males exposed to the low dose, fighting again increased their mortality risk on average 3.4 times which was consistent with results in our previously described control males in fight setups with the high dose.
Controls of the early-stage infected males had on average a three-fold higher mortality risk if they fight, whereas fighting had only a very slight negative effect on the survival probability of early-stage infected males exposed to a low dose when they rivaled another early-stage infected male as their morality risk was only increased 1.6–fold and not increased at all when rivalling a control male (*Figure 3.6 C*).
Consistent with our previous findings, a fight increased the mortality risk of healthy males on average 3.8 times. Healthy males were slightly worse off when fighting against a late-stage infected male with a low dose compared to when they rivaled another control male. In contrast, the mortality risk of late-stage infected fighters exposed to the low dose was not increased compared to non-fighting males (*Figure 3.6 D*). We speculate that the reason for the absence of increased mortality when with a rival that was also a late-stage infected male, could be that the males did not engage heavily in fighting, but rather avoided it. However, when they had a healthy combatant, the underlying cause could be different: it could be that these males have perceived that they were infected and are close to death and therefore invest all their resources

in the fight to secure mating opportunities thereafter, likely as a terminal investment strategy and fight their rival more violently, thereby compensating for their poor starting condition. We performed behavioral observations (scan sampling) to study the details of the fights.

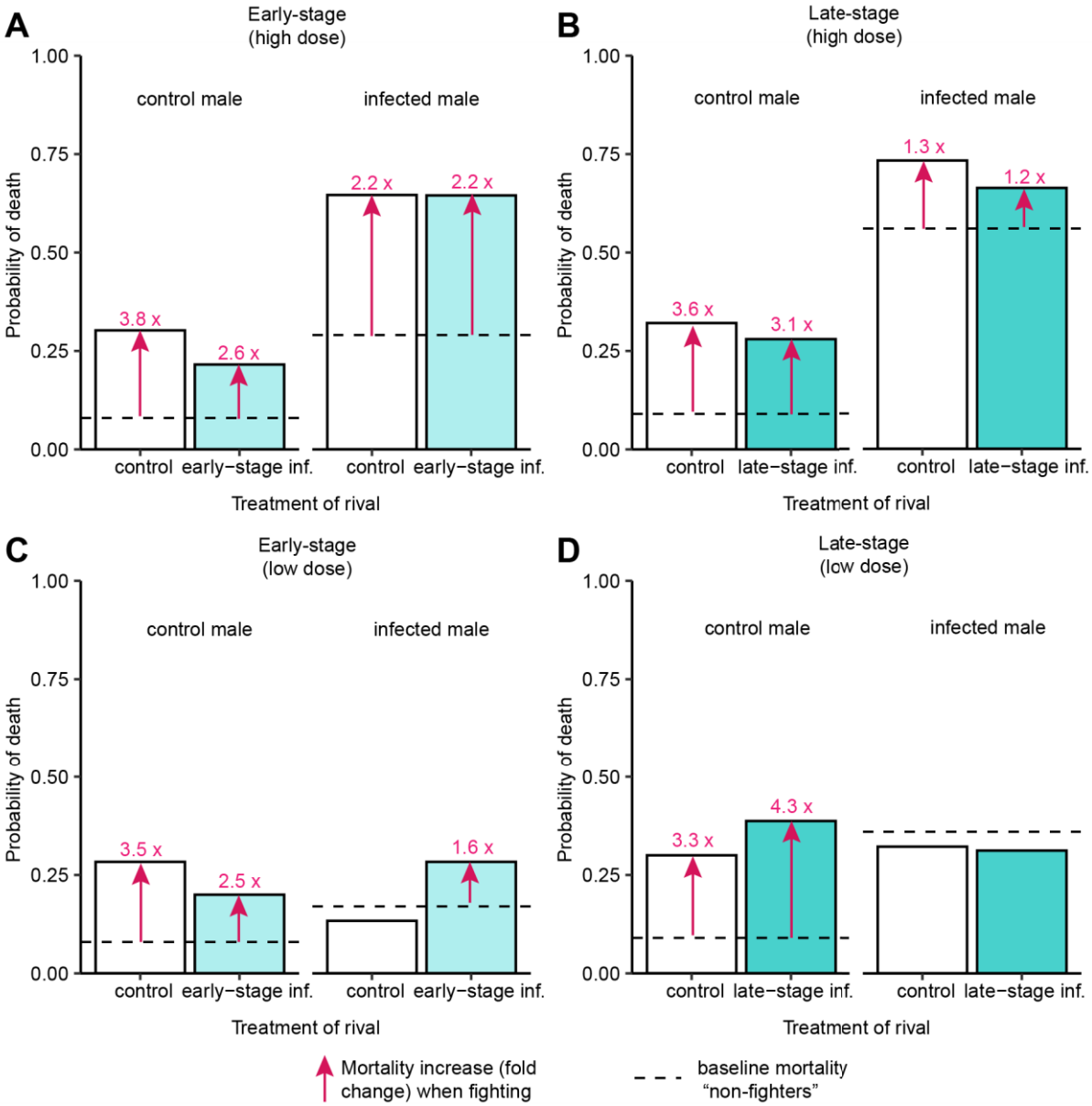

Figure 3.6. **Mortality risk of fighting males and non-fighters depending on their own and their rival's infection status.** This figure summarizes how much the mortality risk of fighting males is changed compared to non-fighters (=baseline mortality) at (A) early-stage infection (high dose), (B) late-stage infection (high dose), (C) early-stage infection (low dose) and (D) late-stage infection (low dose). Baseline mortality is represented by the dashed line. Pink arrows indicate the increase (fold change) in mortality when males fight compared to the baseline mortality. On the top of each graph the "focal" male is represented from whose perspective the mortality risk is inferred. The bars indicate the probability of death (mortality risk) of the focal male depending on its rival's treatment (sham-treated rival: white, early-stage infected rival in light blue and late-stage infected rival in dark blue).

Fight intensity is not affected by early-stage infection, but reduced at the late-stage of infection
We compared the fight intensity (as the number of aggressive interactions occurring per fighting pair) in the different fight combinations and found no difference for fights of early-stage infected males (χ^2=1.945, d.f.=2, p=0.454, *Figure 3.7 A*). Interestingly, there was a bimodal distribution - where some males seemed to fight very little, whereas others fought more often. In contrast, the fight intensity decreased in mixed fights when one male was late-stage infected and decreased even further when both rivals were late-stage infected (χ^2=19.695, d.f.=2, p<0.001; all pairwise comparisons p<0.026, *Figure 3.7 B*).

Worker aggression is increased when two late-stage infected males rival each other
Even though statistically only trending, worker aggression seemed to be slightly increased when both males were early-stage infected (χ^2=6.143, d.f.=2, p=0.093; post hoc: control early-stage inf. vs. one male early-stage inf. p=0.735, other combinations p=0.063, *Figure 3.7 C*). For males at the late-stage of infection, worker aggression was increased when there were two late-stage infected males rivalling each other (χ^2=14.591, d.f.=2, p=0.001, control late-stage inf. vs. one male late-stage inf. p=0.112, other combinations p<0.024, *Figure 3.7 D*).

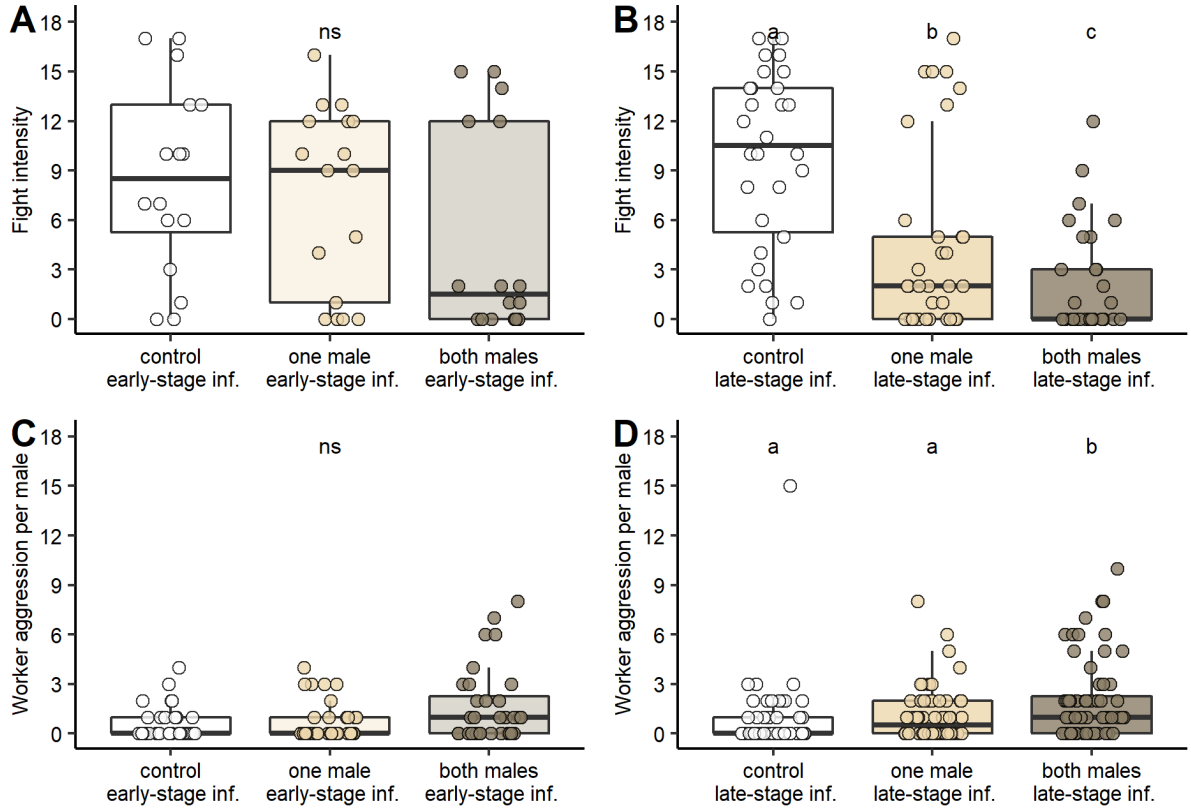

Figure 3.7. **Fight intensity and worker aggression depending on fight combination.** We analyzed the fight intensity (as numbers of aggressive interactions per fighting pair) in (A) early-stage infected and (B) late-stage infected males. Data points represent the number of aggressive interactions of a fighting pair grouped by fight combination (white: two sham-treated males, beige: one sham-treated and one infected male and brown: two infected males). Fight intensity of early-stage infected males was not affected, whereas fight intensity decreased in mixed fights of late-stage infected males and decreased even further in fights of two late-stage infected males. We also looked at worker aggression towards each male depending on the fight combination in (C) early-stage infected and (D) late-stage infected fights. Each data point represents the number of aggressive behaviors directed towards each male grouped by fight combination. Boxplots show median (bar), lower and upper hinges correspond to first and third quartiles (box) and "extremes" (whiskers; the upper whisker extends to the largest value if it is no greater than 1.5 times the interquartile rage from the hinge, and the lower whisker extends to the smallest value if it is no smaller than 1.5 times the interquartile range from the hinge).

Late-stage infected males are more aggressed by workers in mixed fights compared to healthy males

Next, we checked whether workers behave differently towards males with different health status by checking the aggressions directed towards each male in mixed fights.

We found that early-stage infected males compared to healthy males were not more aggressed by workers (χ^2=1.516, d.f.=1, p=0.327, *Figure 3.8 A*), whereas late-stage infected males were (χ^2=8.155, d.f.=1, p=0.005, *Figure 3.8 B*).

Male aggression is not affected by early-or late-stage infection

In mixed fights neither early- nor late-stage infected males showed differences in their aggressive behaviors (early-stage infected: χ^2=0.073, d.f.=1, p=0.787, *Figure 3.8 C*; late-stage infected: χ^2=0.178, d.f.=1, p=0.673, *Figure 3.8 D*), however with our scan sampling method, we could not differentiate which male initiated the fight and which male was only reacting passively to attacks by e.g. holding onto the rival.

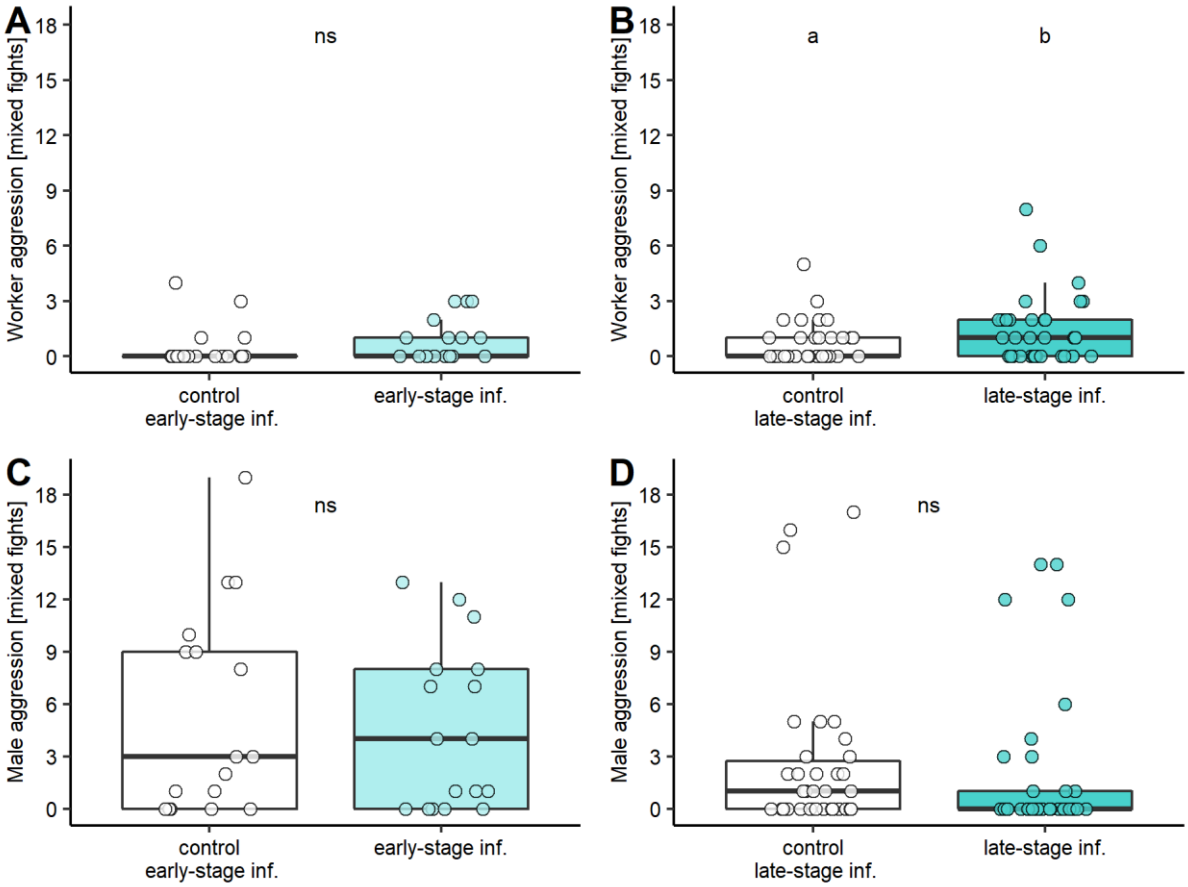

Figure 3.8. **Worker aggression towards males and male aggression performed depending on male infection status.** We analyzed whether males in mixed fights of (A) early-stage or (B) late-stage infected males are differently aggressed by workers depending on their infection status. Data points represent the number of received worker aggression per male grouped by their infection status (white: sham-treated, light blue: early-stage infected, dark blue: late-stage infected). We found that late-stage infected males were more aggressed than sham-treated males, whereas this was not the case for early-stage infected males. We further tested whether male aggression performed was affected by (C) early-stage or (D) late-stage infection. Data points represent the number of aggressive behaviors performed by each male. Neither early- nor late-stage infected males differed in their aggression compared to sham-treated males. Boxplots show median (bar), quartiles (box), and extremes (whiskers). Different letters indicate significance groups after Benjamini–Hochberg correction at α=0.05. ns=non-significant.

Worker aggression is not predictive of defeat in mixed fights

Next, we examined the role of worker aggression in decided mixed fights and checked whether aggressive interactions performed by workers towards the males impacted victory or defeat in a fight. For both early- and late-stage infected males, we found that worker aggression received did not affect whether a male lost or won a fight, but that male survival was only dependent on their infection status. Early- as well as late-stage infected males were more likely to die even without receiving any worker aggression (early-stage infected: worker aggression*male infection χ^2=2.187, d.f.=1, p=0.139, overall χ^2=16.312, d.f.=2, p=0.001, male infection χ^2=15.455, d.f.= 1, p<0.001, worker aggression χ^2=0.462, d.f=1, p=0.496, *Figure 3.9 A*; late stage infected: worker aggression*male infection χ^2=2.476, d.f.=1, p=0.116, overall χ^2=35.467, d.f.=2, p<0.001, male infection χ^2=31.163, d.f.=1, p<0.001, worker aggression χ^2=0.573, d.f=1, p=0.449, *Figure 3.9 B*).

Aggressive late-stage infected males increase their chance of victory

We further tested whether males that showed less aggressive behaviors themselves were more likely to lose a fight and found that sham-treated males (when they rivaled an early-stage as well as a late-stage infected male) were more likely to win irrespective of their aggressive behavior. Early-stage infected males could not increase their chance of victory by aggressive engagement (aggression performed*male infection χ^2=1.903, d.f=1, p=0.168, overall χ^2=16.812, d.f.=2, p=0.001, male infection χ^2=15.568, d.f.= 1, p<0.001, male aggression performed χ^2=0.963, d.f.=1, p=0.327, *Figure 3.9 C*), whereas late-stage infected males that showed more aggression, even though statistically only trending, were more likely to win (aggression performed*male infection χ^2=0.460, d.f.=1, p=0.498, overall χ^2=37.859, d.f.=2, p<0.001, male infection χ^2=32.284, d.f=1, p<0.001, male aggression performed χ^2=2.965, d.f.=1, p=0.085, *Figure 3.9 D*). However, it might be that the absence of the effect in early-stage infected males is due to the low sample size of only 14 mixed decided fights.

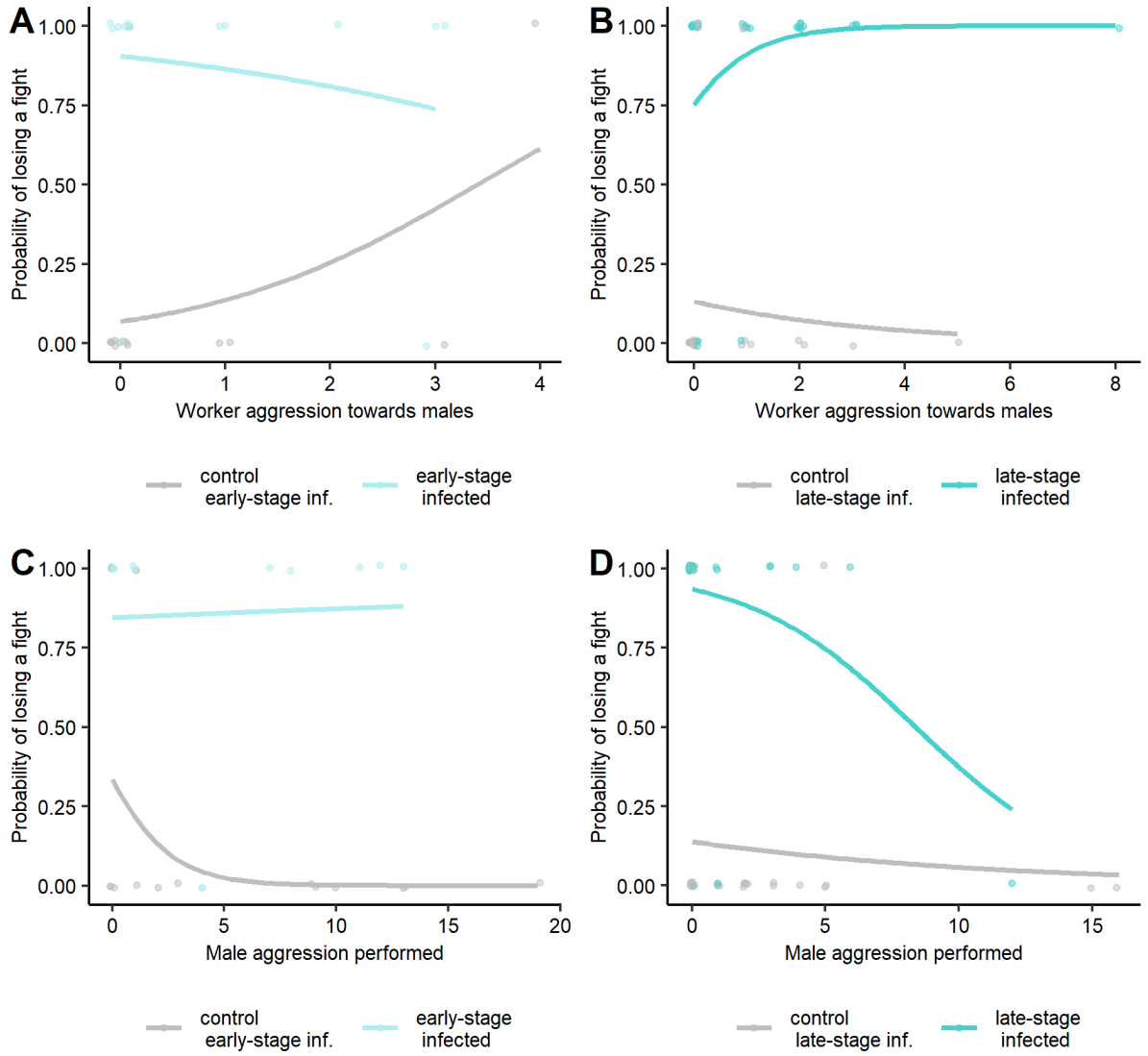

Figure 3.9. **The probability of losing a fight depending on worker aggression received and male aggression performed.** To determine whether worker aggression received and own infection status was predictive of winning or losing a fight, we analyzed decided mixed fights of (A) early-stage (N=14) and (B) late-stage infected males (N=25). Data points indicate individual males and lines represent logistic regressions for sham-treated (grey), early-stage infected males (light blue) and late-stage infected males (dark blue). For both early- and late-stage infected males, we found that worker aggression received did not affect whether a male lost or won a fight, but that male survival was only dependent on their infection status with the infected males being more likely to die. We further examined whether own infection status and aggression performed affected the fight outcome in (C) early-stage or (D) late-stage infected males. We found that early- as well as late-stage infected males were more likely to lose a fight, but late-stage infected males could increase their chance of winning by being more aggressive, whereas this was not the case in early-stage infected males.

Fighting with early-stage infected males – but not late-stage infected males – leads to spore transfer to the rival

Contagious early-stage infected males are not only a risk for their rival, but for the entire colony. In early-stage infected males exposed to the high dose, transmission was expected to occur in most cases. We checked this hypothesis by analyzing 20 decided mixed fights after 24h and found detectable transmission in 50% (10/20) of the cases (*Figure 3.10*) when workers were present. Individuals with the higher spore number were considered to be the initially exposed individuals. These individuals had on average still 21,837.5±6532.6 (mean±SE) after 24h, whereas their rival (in all of the cases the winner of the fight) had on average 1,036.4±448.4 spores on their body. This showed that fighting with a contagious early-stage infected male is indeed very risky for the winner. In the same way, we analyzed 28 decided mixed fights with late-stage infected males where spore transmission was not expected and found spore transmission only in one case.

Sanitary care performed by the workers is effective at the early-stage of infection

In order to determine how effective sanitary care by workers can operate at the early-stage of infection, we compared the proportion of detectable spore transmission of mixed decided fights with and without workers present. As stated above, we found spore transmission in 50% of the cases when workers were present. For comparison, we analyzed 16 decided mixed fights without workers present and found spore transmission in 81% (13/16) of the cases. This proves that social immunity operates effectively at the early-stage of infection before spores have germinated on the cuticle even at very high dosages (χ^2=3.763, d.f.=1, p=0.052).

Likewise, we analyzed 7 mixed decided fights at the late-stage of disease and could, as expected, not detect any spore transmission (*Figure 3.10*)

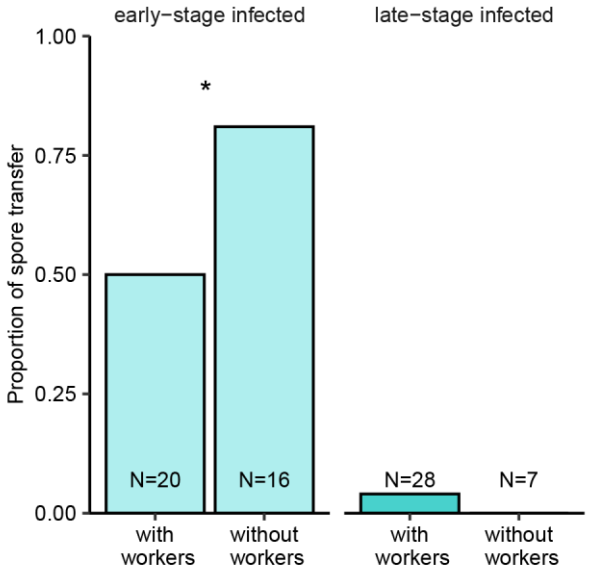

Figure 3.10. **Sanitary care and spore transmission in mixed decided fights of early- and late-stage infected males.** In order to determine how effective sanitary care performed by workers can operate at the (A) early-stage and (B) late-stage of infection, we compared the proportion of mixed decided fights in which we found detectable spore transmission when workers were absent or present. Bars represent the proportion of cases where spore transmission could be detected at the early-stage of infection (light blue) and the late-stage of infection (dark blue). We found that in the early-stage of infection sanitary care reduced the risk of detectable spore transmission. As expected, at the late-stage of infection spores were not transmitted anymore, irrespective of worker presence (except one positive case where spore transmission occurred). N represents the total sample sizes of analyzed fights. * denotes statistical difference (p=0.052).

3.5 Discussion

In summary, we found male-male competition to be strongly affected by infection and found more pronounced effects when the applied spore dose was high. Furthermore, we found that workers on the one hand take care of early-stage infected males by reducing their pathogen loads and thereby reduce the risk of disease development for the individual, but on the other hand workers also prevent transmission through the colony i.e. by preventing close contact of healthy individuals to contagious individuals or trying to remove fatally infected individuals from the colony.
Even though we did not find an effect of worker aggression on male mortality, we observed that in mixed fights late-stage infected males received more worker aggression than sham-treated males which could pinpoint to the fact that workers try to displace the males from the colony. Mixed fights at early- and late-stage of infection (when individuals were exposed to the high dose) ended mostly decidedly and in favor of the sham-treated male. This indicates that an incipient as well as progressed infection is costly and can negatively affect the fighting ability (Sheldon and Verhulst, 1996; Schwenke *et al.*, 2016; Barribeau and Otti, 2020). In late-stage infected males this can be explained by negative effects of the infection itself, whereas in early-stage infected males it is the combination of both – raising an immune response and being in a stressful situation of combat. Males exposed to the high dose appeared already lethargic and moved less than males exposed to the low dose after 12h, indicating starting sickness behavior (own observation). Thus, the fight situation uncovered the cost of the immune response and revealed that males exposed to a high amount of spores face an immunity-fighting ability trade-off. We found that *Cardiocondyla* males invested strongly in their immune system, since their immune system reacted strongly already within the first 24h after spore contact. The thin and rather soft cuticle of the males is probably responsible for the fact that we find a spore-specific immune reaction so early. Similar to reports in termites, the spores might already start penetrating the cuticle (Davis *et al.*, 2018). Males of other social Hymenoptera, e.g., in leafcutters (Baer *et al.*, 2005), the ant *Formica exsecta* (Vainio *et al.*, 2004), and bumble bees (Gerloff *et al.*, 2003), were found to invest very little in immune immunity. This can be explained by the usually short lifespan of males in social insects that typically only live up to several days (Stürup *et al.*, 2014) and the fact that haploid males are only hemizygous on the loci that affect resistance against parasites which makes them more vulnerable to disease (O´Donnell and Beshers, 2004; Ruiz-Gonzáles and Brown, 2006). The high investment into immune defense of wingless *C. obscurior* males is likely attributed to their relatively long lifespan up to several weeks or even months (Metzler *et al.*, 2016). It would be interesting in future experiments to compare the immune investment of wingless males to that of winged males, where one would expect that they invest less in immunity as they are short-lived and typically appear under stressful conditions (i.e. size reduction of the colony or temperature decrease) (Cremer and Heinze, 2003).

Overall, we found less pronounced effects in males exposed to a low dose – both early- and late-stage infected males did not have a disadvantage in fights and behaved similarly to sham-treated males. This demonstrates that *Metarhizium* clearly acts in a dose-dependent manner, with lower exposure dosages leading to milder symptoms compared to the same point in time after contact with a higher dose. As the immune gene expression of the early-stage infected males exposed to the low dose showed, the effect would likely be the same, just time-delayed. Fighting had only a slight negative effect on the survival probability of early-stage infected males exposed to a low dose, supporting our hypothesis that these males remain with such a minute spore number that they do not have to pay any costs yet, which is also underlined by the fact that we did not detect any behavioral differences compared to sham-treated males.

For males exposed to the high dose, the proportion of cases where both males died was increased when two early-stage infected males rivaled each other, whereas this was not the case for late-stage infected males. It could be that early-stage infected males perceive that they are infected or close to death and therefore invest all their resources in the fight and engage more violently to secure mating opportunities thereafter, likely as a terminal investment strategy (Clutton-Brock, 1984), whereas late-stage infected males are already too weak. This is supported by the fact that the fight intensity of early-stage infected males is not affected, whereas it decreased in mixed fights and decreased even further when two late-stage infected males were rivalling each other.

Interestingly, when two early-stage infected males fought, we found a bimodal distribution where some combatants seemed to fight very intensely, whereas others did not fight at all. It could be that males that did not fight so vigorously were the ones with more spores remaining on their cuticle, and these were therefore already further in the immunity-fighting ability trade-off. This again would support our hypothesis that infected males are already too weak to fight properly. Another explanation could be that they get less involved in fights as their chance of losing and dying the competition is high and they try to prevent additional injuries (that are often themselves lethal) and thereby avoid additional energetic costs of fighting (Arnott and Elwood, 2009). Retreating from a fight to minimize damage would be an optimal strategy for energetically or physically compromised males as the physical condition deteriorates with an increasing number of fights and progressing disease (Tidière *et al.*, 2017).

Furthermore, we found that worker aggression was increased when two late-stage infected males exposed to a high dose were rivalling each other, and that late-stage infected males are more aggressed by workers in mixed fights compared to sham-treated males. This could not be detected in early-stage infected males and in fights where males were exposed to the low dose. Aggression towards fatally infected colony members had been observed in other ant species against adult workers (Leclerc and Detrain, 2016) and brood (Pull *et al.*, 2018a), in order to protect the colony from pathogen spread.

In mixed decided fights of males exposed to the high dose, we did not find an effect of worker aggression towards the males: the probability of losing a fight was solely affected by the infection status. Surprisingly, late-stage infected males that showed more aggressive behaviors had a higher chance of winning – indicating that in some cases they are still able to terminally invest their resources in fighting – an effect that we had rather expected to find in early-stage infected males. Here, however, the absence of the effect might be attributed to the low sample size of only 14 fights (compared to 25 late-stage infected decided mixed fights).

We found that fighting with early-stage infected males - but not late-stage infected males - leads to spore transfer. In all of the observed cases, the spore transfer was to the winner of the fight. Typically the winner of a combat is able to monopolize mating with virgin queens in the nest. Consequently, depending on the amount of spores the winner contracted from its rival, there is a high risk for the virgin queens of being cross-contaminated during mating attempts, and by extension also the care-giving workers. As expected, pathogen-exposed individuals were excessively groomed by their healthy nestmates (Rosengaus *et al.*, 1998; Hughes *et al.*, 2002) as part of their social immune defense (Cremer *et al.*, 2007), protecting the colony.

We found that sanitary care by workers can prevent detectable spore transfer in 50% of the cases - even at very high pathogen doses – however, the average number of spores in these cases was still more than 1000 spores. Almost certainly, these males would not be able to mate anymore and are doomed to die, as further spore removal by the workers after more than 24h is unlikely, as spores have probably already begun to attach to the soft cuticle of males – similar to reports in termites (Davis *et al.*, 2018). Furthermore, this is still a considerable amount that the colony members have to deal with. As the grooming efficiency not only depends on the pathogen dose, but also on the number of individuals that remove the pathogen from the

contaminated individuals, the detrimental effects are reduced the more nestmates are available to perform sanitary care, so that higher worker numbers, e.g., in field populations, could buffer a large amount of spores better than the few workers in our experimental setups. As our technical detection threshold was limited to 25 spores, many more males could have been cross-contaminated with up to 25 spores, which could also have detrimental effects as already this amount forms a huge burden for *Cardiocondyla* males (see chapter 4). Even though we were not able to determine spore numbers of the early-stage infected males exposed to the low dose, spores can still get transmitted within the colony – most importantly to virgin queens. Even one spore can be enough to elicit long-lasting effects on the queen and her offspring (more details in chapter 4 where we studied the short- and long-term consequences of spore transfer during mating).
It is very likely that in early-stage infected males exposed to the low dose grooming by the workers can "rescue" the males. Spore removal could be so efficient that no spores remain on the males at all, or only a minute amount of spores that might only cause asymptomatic infections or even trigger the immune system and elicit protective immunization (Konrad *et al.*, 2012; Liu *et al.*, 2015).

In conclusion, our study revealed that late-stage infected males have a very high mortality that is caused by the established infection. Due to their impaired physical condition, they performed poorly against their rivals. However, in very rare cases, if they were not too weak yet, they could increase their chance of winning if they fought more violently.
In contrast, early-stage infected males would not have died yet in the absence of the additional stress of combat. Thus, the fight situation uncovered the cost of the immune response that the males have raised against the fungal spores within the fighting interval.
Surprisingly, we found a very high immune investment in males which was contrary to expectations in social Hymenoptera males. However, it pays off for them as they have a relatively long lifespan, continuous spermatogenesis, and can be the dominant male in a colony for several weeks, highlighting the importance of future reproduction for them.

Experimental work contribution in detail:
Experimental setups male fights: own 100%
Behavioral scan sampling with Elina Hanhimaki, Marta Gorecka: SM 50%
ddPCR for spore load determination: Elisabeth Naderlinger, Anna Grasse 100%
Experimental setups for immune gene expression: own 100%
ddPCR for immune gene expression: Jessica Kirchner 100%

4. Social immunity shapes sexual selection in ants

Photograph shows sexuals of the ant *Cardiocondyla obscurior* on a leaf.
Two males are fighting in the middle and two queens are next to them.
Photo credit: S. Metzler and R. Ferrigato

4. Social immunity shapes sexual selection in ants

4.1 Abstract

Mate choice is critical for the fitness of sexually reproducing individuals. In the context of disease, careful choice allows to prevent disease contraction during mating and may even allow to find a partner conveying disease resistance to the offspring. Social Hymenoptera only have a single mating period early in life to make these decisions. As mating typically occurs in massive aerial flights far away from the nest, it can rarely be studied in detail, except for species that mate inside the nest. We used the intra-nest mating ant *Cardiocondyla obscurior* and tested how exposure to the fungal pathogen *Metarhizium robertsii* affects mating behavior, survival and fitness of queens and males. We determined whether queens and males refuse risky mating attempts from contagious (early-stage infected) or symptomatic (late-stage infected) mating partners and observed if the workers may interfere. We found that mating success of infected queens and males was reduced, but mating with contagious partners still occurred. Workers performed sanitary care (removal of fungal spores by grooming), which reduced negative effects of the pathogen, but did not otherwise interfere in the matings. We further evaluated the long-term effect on queen survival and fertility, colony development and offspring immunocompetence when (i) she could contract disease from her potential mating partner or (ii) when her mating partner was sick (but not infectious anymore). We found that queens that contracted very low pathogen dosages had a reduced lifespan and produced less offspring, with the worker offspring being immunocompromised - this readily demonstrates the importance of social immunity and worker care in a colony and how strongly sexuals rely on pathogen defense mechanisms of the workers. Surprisingly, sharing the nest with a late-stage infected mating partner positively affected the offspring's immunocompetence as male offspring reacted more sensitively to challenge with the same fungal pathogen. Such transgenerational immune priming effects on the sexual offspring could ultimately lead to protection of the next generation of daughter colonies.

4.2 Introduction

Mate choice is critical for the fitness of sexually reproducing individuals. Selecting a superior mate may provide direct fitness benefits to the pair or have indirect effects on their offspring. As females typically invest more in reproduction than males do, they are mostly the choosy sex (Darwin, 1871).

There are several traits which females use to evaluate mate quality – e.g. body size, armament size, strength, fighting ability, or ornaments (Andersson and Iwasa, 1996). The latter can provide information about the intensity of transmissible parasites or resistance to infections (Hamilton and Zuk, 1982). In most species, health status is one of the most important assessments of a potential mating partner and is mainly used to successfully avoid parasitized partners. Mating with a parasitized or infectious partner not only implies a direct risk of disease contraction, but will also negatively affect both partners' fitness benefit (Able, 1996) and should therefore be avoided (Milinski and Bakker, 1990; Worden *et al.*, 2000; Landis *et al.*, 2015). As infected mates are likely to only have limited resources available, because infection and immune response are presumably costly, they will not be able to invest a lot into expensive secondary sexual attributes and will be perceived as less attractive (Hamilton and Zuk, 1982). Consequently, healthy mates are of higher quality and are perceived as more attractive due to their better immunocompetence (Rantala *et al.*, 2002).

In contrast to most animals, where males can increase their mating success by putting themselves on display and showing either their weapons, excessive courtships behaviors or costly ornaments in general, ants mate in aerial mating swarms which limits lengthy pre-copulatory choice processes (Helft *et al.*, 2015). During "scramble competition" between males during the mating flights, flight ability and overall vigor are presumably sufficient to assess male quality (Boomsma, 2013), whereas in species that rely on the detection of female pheromones ("female calling syndrome") (Hölldobler and Bartz, 1985), detection ability and physical condition to reach the female are used to evaluate the condition of a mating partner. In social insects mating only occurs within a short time frame early in their lives (Boomsma *et al.*, 2005; Boomsma, 2009). Therefore, sexuals rely on the sperm received mostly during a single mating event and use the obtained sperm to fertilize all female-destined eggs throughout their entire lives without ever mating again (Boomsma *et al.*, 2005; Boomsma, 2009). This lifelong partner-commitment and the lack of the possibility to change partner choice later in life, allows for the hypothesis that mate choice could be particularly important for social insect queens (Helft *et al.*, 2015).

As opposed to the just described "standard" mating procedures in ants, there are several ant species that mate on the ground, e.g., *Cataglyphis cursor* (Helft *et al.*, 2015, 2016) or even within their maternal nests, e.g., *Hypoponera* (Foitzik *et al.*, 2002) and *Cardiocondyla* species (Heinze *et al.*, 1998) in the presence of the workers. Mating in the shelter of the maternal nest not only allows for the possibility of mate choice, but gives workers the possibility to be involved in the process of mate choice as well (Franks and Hölldobler, 1987; Cronin *et al.*, 2011; Sunamura *et al.*, 2011). As typically workers in social insects are sterile and obtain indirect fitness by helping their relatives to reproduce (Bourke, 2011), workers should have an interest in that their queen mates with a good partner to increase their inclusive fitness. There is evidence in the ant *Cataglyphis cursor* (polyandrous) that workers can take part in sexual selection, where worker aggression directly influenced the access of different males to virgin queens. At the same time, virgin queens themselves did not seem to actively choose their mates (Helft *et al.*, 2015, 2016). Ants live in a pathogen-rich environment and are therefore at constant risk of pathogen contamination from their environment, which can be very costly.

Therefore, we hypothesize that workers should intervene when there is a high risk of death for the queen i.e. when the mating partner is contagious and close physical contact bears the risk

that pathogens get transmitted between the sexes. Accordingly, the queen herself should refuse mating attempts from males that put her at a high risk of contracting a harmful dose of the pathogen and workers should prevent that the queen succumbs from infection by (i) intervening in risky mating attempts and (ii) performing sanitary care to reduce the amount of external pathogens e.g. fungal spores by grooming.
However, the consequences of pathogen contraction are often dose-dependent, and can even result in a beneficial immune boost of individuals that contracted only a very low dose (Konrad *et al.*, 2012). In such a scenario it would be even favorable for the queen's survival after secondary pathogen contact, because the initial contact resulted in immunization - but might still come with the cost of reduced reproductive output as immune activation is costly (Fedorka *et al.*, 2016; Schwenke *et al.*, 2016). Apart from that, males should always engage in mating to maximize their fitness even at costs of their own survival (Cappa *et al.*, 2015; Heinze, 2016).
Furthermore, parental pathogen history may have transgenerational effects, namely, when parents vertically transmit immunological experience to their offspring, which protects them against future infection with the same pathogen. This phenomenon is termed transgenerational immune priming (TGIP) in invertebrates and can occur via both parents (Little and Kraaijeveld, 2004; Moret, 2006; Sadd and Schmid-Hempel, 2007; Roth *et al.*, 2018; Tetreau *et al.*, 2019). TGIP is expected to evolve in species where there is a high chance that the offspring encounters the same pathogens as the parents, for instance, ants that live in pathogen-rich environments, have low dispersal and overlapping parent-offspring generations (Sadd *et al.*, 2005; Cini *et al.*, 2020; Cole *et al.*, 2020; Casillas-Pérez *et al.*, 2022).
Maternal and paternal TGIP differ in their underlying mechanisms and their mode of protection. Maternal TGIP is mediated by either direct transfer of antimicrobial peptides or microbial elicitors to the developing egg mediated by the egg-yolk protein vitellogenin (Salmela *et al.*, 2015). Thereupon, the offspring mounts a specific and rather narrow immune response (reviewed in Vilcinskas, 2021). Besides this, epigenetic mechanisms – such as DNA methylation, histone acetylation and microRNA expression (Vilcinskas, 2021) - are also known to play an important role, where phenotypic changes that trigger a relatively non-specific immune response can be passed to the offspring via both parents. Based on the super-organismal structure of the ant colony, we expect that workers could have a role in immune priming in eusocial insects as well as they are responsible for colony maintenance and take care of the queen and the brood. It has been shown in honey bees that vitellogenin is also involved in the translocation of immune elicitors from the gut to the hypopharyngeal glands of workers (Harwood *et al.*, 2019) and from there the elicitors get incorporated into the royal jelly (Harwood *et al.*, 2021) and are horizontally transmitted among the nestmates. This provides colony-wide immune protection as a form of social immunity (Vilcinskas, 2021). The queen could have two different strategies where she could either prime (i) her sterile worker offspring which in turn would protect the current colony (Casillas-Pérez *et al.*, 2022) or (ii) her sexual offspring which could lead to the protection of the next generation of colonies (the next superorganism).

Aim of the study

We used the intra-nest mating tramp ant *Cardiocondyla obscurior* to study how exposure to the obligate killing fungal pathogen *Metarhizium robertsii* affects mating behavior and success of queens and males. We determined whether female and male sexuals refuse risky mating attempts from contagious (early-stage infected) or symptomatic (late-stage infected) mating partners and checked whether the workers interfere. Furthermore, we evaluated the long-term effects on queen survival and fertility, colony development and composition and finally the immunocompetence of the offspring (TGIP) when the potential mating partner was (i) either at an early-stage of infection where spore transmission was still possible or (ii) at a late-stage

of infection (when the male was sick, but no longer contagious to the queen). We checked offspring immune gene expression (with droplet digital PCR) of seven different genes that are involved in cellular and humoral immune defense of the innate immune response in ants and are mainly involved in the Toll - and prophenoloxidase *PPO* - pathway that are activated upon fungal infection (Lemaitre *et al.*, 1996; Gillespie *et al.*, 1997; Cerenius *et al.*, 2008; Cerenius and Söderhäll, 2021). The offspring was either (i) left untreated to measure the constitutive immune response or (ii) challenged with a fungal spore suspension to determine how the offspring reacts to the same pathogen that either of the parents has encountered (induced immunity).
We expected that the offspring with parental pathogen history – either an infected father or an infected mother that contracted spores from her mating partner – reacts more pronouncedly when challenged to the same pathogen as the parents have experienced. We further hypothesized that constitutive immunity could be altered, as this would protect the offspring even in the absence of a pathogen, despite constitutive mechanisms being costly to maintain as they consume energy to remain operative (Schwenke *et al.*, 2016, but see Armitage *et al.*, 2003).

4.3 Material and Methods

Experimental procedure
To assure controlled matings (virginity), we collected female (=virgin queens) and male sexuals as dark-pigmented "ready-to-hatch" pupae and reared them with two nurse workers originating from the same colony and 10% sucrose as food source. We standardized age to 3.5 - 5 days old when sexuals entered the experiments, since at this age their cuticle is fully sclerotized and they can survive the exposure, their immune system is fully developed and they are not too old yet for their mating window.
For all experiments, sexuals were exposed to either a conidiospore-suspension (hereafter referred to as spores) with a concentration of 5 x 10^6 spores/ml or 0.05% Tx as sham-treatment (control) and randomly assigned to the different treatment groups (details see below).

We set up matings at two different disease stages - either directly after pathogen exposure ("early-stage infected" sexuals) or when individuals had already an established infection ("late-stage infected" sexuals).
The mating pair was kept together for 72h, implying that all individuals progressed in their infection. In this way initially contagious individuals are at an early-stage of infection by the end of the mating interval, whereas late-stage infected individuals are moribund or die within the experimental interval. We use here the same terminology (early- vs. late-stage infected) as introduced in chapter 3, as the fight setups and the mating setups had started with the same conditions (freshly contaminated individuals and individuals that carried already internal infection). However, the disease progression is shorter in chapter 3, where the experimental interval was only 24h compared to 72h in the current mating setups.
We evaluated the survival of the mating pair, the mating success, and checked whether workers interfere in mate choice and whether they discriminate against early-or late-stage infected sexuals (inclusive sexual selection) (Sunamura *et al.*, 2011; Helft *et al.*, 2016). To test the effect of workers, we set up matings with only the mating pair present ("without workers" setups) or added five sham-treated nestmate workers ("with workers" setups) and checked mating success by dissection of the queens' spermathecae. In case workers would interfere in pre-copulatory mate choice, we expected to see a reduction in mating success in case of infection compared to matings without workers present.

Experimental design – short-term effects: mate choice, worker interference and mating success
Each mating pair consisted of a contagious (early-stage infected), respectively late-stage infected mating partner and a sham-treated individual or two sham-treated sexuals as control and was kept together for 72h.
Contagious early-stage infected sexuals were exposed to either 5 x 10^6 spores/ml or 0.05% Tx as sham-treatment and the experiments were immediately set up thereafter. When workers were added they were as well sham-treated with 0.05% Tx right before the experiment.
Similarly, late-stage infected sexuals were exposed to a spore suspension with a concentration of 5 x 10^6 spores/ml or 0.05% Tx and individually kept for 48h with 10% sucrose in isolation before the experiment so that infections could establish in the host. Workers were as well sham-treated with 0.05% Tx 48h before the experimental start and kept in groups of 35 individuals. The table below summarizes the sample sizes per mating combination (*Table 4.1, Figure 4.1*).

Table 4.1. Sample size per mating combination with and without workers present (early- and late-stage of infection). Control individuals were sham-treated with 0.05% Tx.

	Mating combination	**Without workers**	**With workers**
Early-stage infection	Early-stage control	33	33
	Early-stage infected queen	36	35
	Early-stage infected male	35	34
Late-stage infection	Late-stage control	14	18
	Late-stage infected queen	29	29
	Late-stage infected male	21	24

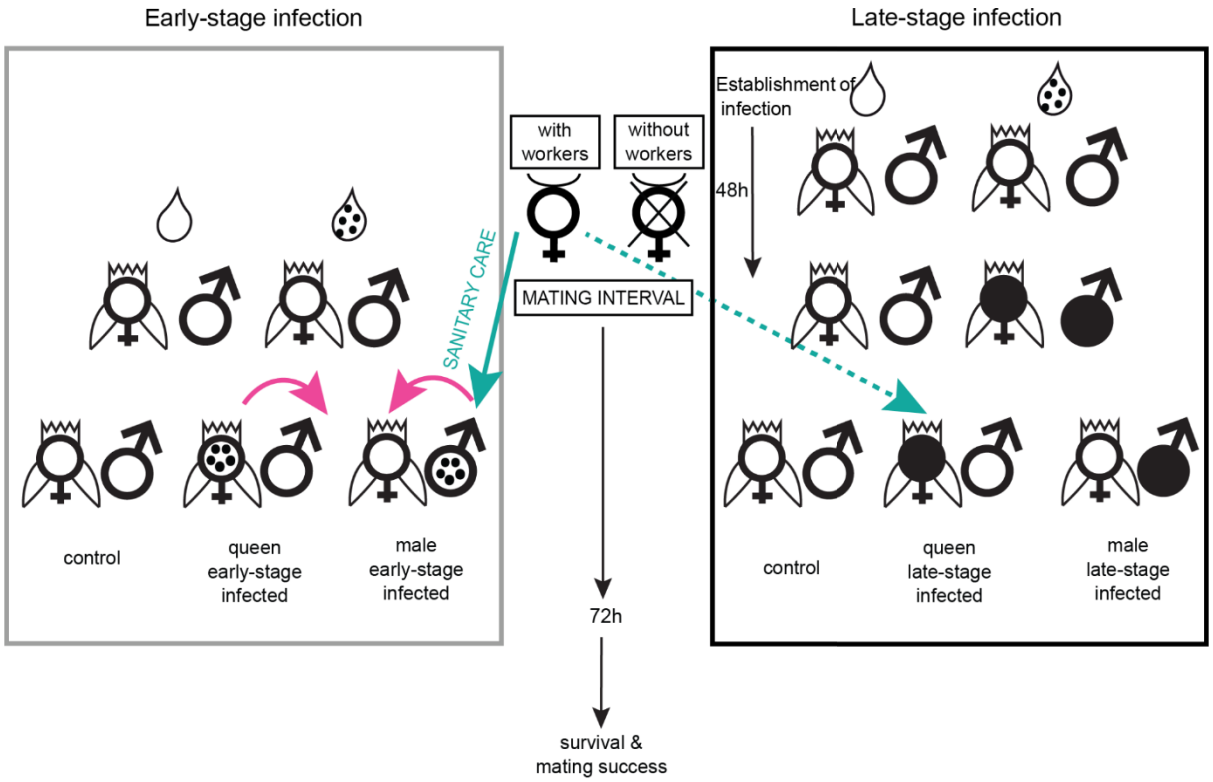

Figure 4.1. ***Experimental design – short-term effects: mate choice, worker interference and mating success.*** Each mating pair consisted of a contagious early-stage infected (mating combinations in grey box on the left) or late-stage infected mating partner (mating combinations in black box on the right) and a sham-treated individual or two sham-treated sexuals as control. The mating pairs were kept together for 72h with or without workers present. By comparing setups with and without workers, we were able to determine whether the workers intervene

in risky mating attempts with early- or late-stage infected sexuals (inclusive sexual selection). Individuals for early-stage infected mating combinations were exposed (either to 5 x 10^6 spores/ml or 0.05% Tx) and the experiment was directly set up thereafter (black dots indicate spores that can get transmitted from the exposed individual to the mating partner; indicated by the pink arrow). When workers were present they could perform sanitary care and groom off the spores of contagious individuals (indicated by the turquoise arrow) and can thereby hamper disease development for the individual and spore transmission through the colony), whereas this is not possible anymore when individuals are late-stage infected (indicated by dashed turquoise arrow). Late-stage infected individuals were exposed (either to 5 x 10^6 spores/ml or 0.05% Tx) and individually kept for 48h for the infections to establish (late-stage infected individuals are indicated in black). After 72h the survival and the mating status of queens that were still alive was determined by dissections of their spermathecae.

Determination of mating success

All female sexuals that were still alive at the end of the 72h mating period were dissected under a binocular microscope (magnification 100 x, Leica M125) to investigate whether they had been inseminated. Cases where it was not possible to reliably determine the mating status (eight cases in total) were included as unmated (therefore the proportion of successful matings is rather underestimated).

In brief, queens were killed by decapitation and dissected in 20 µl of Beadle solution (28.3 mM NaCl, 4.7 mM KCl, 2.3 mM $CaCl_2$). The spermatheca was isolated and transferred in a new 15µl droplet of Beadle solution and pierced to release the sperm. When queens were mated, sperm was clearly visible. The dissections were done blind to the treatment groups.

Natural queen cross-contamination from mating setup with a contagious male

To determine whether queens contract spores from a contagious mating partner, and whether this may lead to an active infection in the queen body (replication), we set up matings (as above) with untreated queens and kept them together for either 24h or 72h with males that were submerged in a spore-suspension (5 x 10^6 spores/ml) in the absence of workers (who otherwise may have groomed off spores from the males). Queens that were still alive at the end of the period were frozen and analyzed in pools (three pools of 15 queens each after 24h, and two pools of 41 and 42 queens after 72h) due to a very low expected spore transfer rate and to limitations of the sensitivity of our method. The spore concentration of the pools was determined with ddPCR (details hereafter). Our data are not sufficiently fine-grained to determine the proportion of queens that became cross-contaminated, but we found that – on average – 0.7 spores were transferred to each queen in the first 24h of a mating setup with a contagious, early-stage infected male (pool 1: 0.2 spores, pool 2: 1.3 spores, pool 3: 0.5 spores, average $\pm$ SD: 0.7 ± 0.5). This means that not every queen gets cross-contaminated, and their risk is presumably increased by their social interactions. After 72h, spore load was drastically increased to an average of fungal DNA that was equivalent to 81 spores per queen (pool 1: 18.3, pool 2: 143.6, average $\pm$ SD: 81 ± 63), indicating that the low number of spores transmitted, at least in some cases, caused successful infection and replication inside the queen's body. As spores are only transmissible (approximately) within the first 24h, the increased spore load cannot be explained by a longer period of spore transmission, but must have come from internal replication.

"Experimental mimicry" of the spore transfer from contagious males

To be able to set up a control treatment, in which we could disentangle spore transfer by the male to the queen from other possible transfers (sperm, seminal fluids) or other cues elicited by e.g. male behavior, we used the above findings to test out, which dose we could directly apply to the queens to mimic an average spore transfer of 0.7 spores per queen that we determined is the natural rate of spore transmission when a queen was held together in a mating setup (without workers) with a contagious male for 24h in a controlled experimental way (independent of social interaction). We tested three different dosages (1 x 10^5, 5 x 10^5 and 1 x

10^6 spores/ml) in which we directly dipped the queens and performed ddPCRs on six pools of 20 queens for each dose (see details in *Table 4.2* below) - except for the concentration 1 x 10^5 spores/ml where we analyzed three pools of 20 and three pools of 40 individuals. It is important to mention that not all spores that are applied stick to the ant's cuticle because there is quite some loss to the environment and therefore more spores need to be initially applied.

We found that the average number of spores retrieved per queen was 0.6 for the 1 x 10^5 spores/ml dose, whilst dipping in 5 x 10^5 spores/ml led to an average of 3.1 spores per queen and dipping the queens in 5 x 10^5 spores/ml resulted in 8.6 spores per queen (*Table 4.2*). We therefore dipped the queens in a 1 x 10^5 spores/ml suspension to experimentally mimic the spore transfer during a mating setup with a contagious male in the absence of workers, which we used as the "queen infection control" in our long-term experiment (see below).

Table 4.2. Spore number per pool of queens exposed to 1 x 10^5, 5 x 10^5 and 1 x 10^6 spores/ml. Each pool consists of 20 queens each, except pools 4-6 of queens exposed to 1 x 10^5 spores/ml consist of 40 queens, but DNA extraction has failed in pool 4 (marked with *) and was therefore excluded. The last row shows the average spore number ± SD).

Pool	1 x 10^5 spores/ml	5 x 10^5 spores/ml	1 x 10^6 spores/ml
1	0.4	2.0	6.0
2	0.6	4.9	6.6
3	0.8	2.8	9.6
4	NA*	3.0	7.4
5	0.6	3.4	15.0
6	0.8	2.5	6.7
average	***0.6±0.2***	***3.1±1***	***8.6±3.4***

DNA extraction and ddPCR (ITS2 rRNA gene region)
For the DNA extraction, the samples were homogenized using a TissueLyser II (Qiagen) with a mixture of one 2.8 mm ceramic (Qiagen), five 1 mm zirconia (BioSpec Products) and ~100 mg of 425-600 µm glass beads, acid washed (Sigma-Aldrich) in 50 µl of nuclease-free water (Sigma). Homogenization was carried out in four steps (4 x 2 min at 30 Hz). After 2 min the tube racks were rotated to ensure uniform disruption and homogenization of all samples. DNA extraction was performed using the Qiagen DNeasy 96 Blood & Tissue Kit (Qiagen) following the manufacturer's instructions, with a final elution volume of 50 µl.

We used the QX200 Droplet DigitalTM PCR system (Bio-Rad), including the QX200 Droplet Generator, T100 Thermal Cycler and QX200 Droplet Reader (Bio-Rad) and followed the same protocol as described in "material and methods" of chapter 3.
For quantification of the fungal pathogen load we used the same primers and probe as published in Giehr *et al*. (2017) that bind to the *ITS2 rRNA* gene region of *Metarhizium* (Met-ITS2-Forward: 5′-CCCTGTGGACTTGGTGTTG-3′, Met-ITS2-Reverse: 5′- GCTCCTGTT-GCGAGTGTTTT-3′, Met-ITS2-Probe15' HEX- TGGCGGTCTCGCCGTGGCCC-BHQ1). The probe was designed based on GenBank sequence AY755505.1 using the software Primer3Plus (Untergasser *et al.*, 2007).
The *ITS2 rRNA* gene is a multi-copy gene, hence the obtained quantity does not correspond to the exact spore number. Therefore, we determined the number of *ITS2 rRNA* gene copies per spore by analyzing a dilution series for both the *mRFP1* and the *ITS2 rRNA* gene. Each dilution was run in seven technical replicates. We determined that 45 *ITS2* copies correspond to one *mRFP1* copy and thus to one spore (*Figure 4.2*).

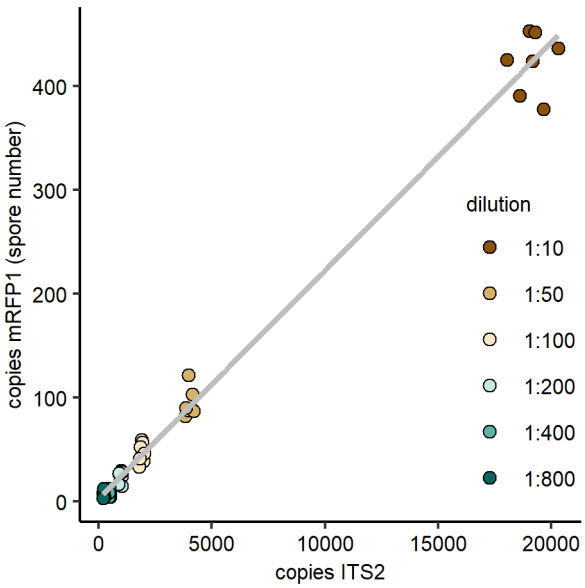

Figure 4.2. **Conversion factor from *ITS2* copy number to spore number (*mRFP1* copy number).** The *ITS2 rRNA* gene is a multi-copy gene and a ddPCR using primers for *ITS2 rRNA* is more sensitive than a ddPCR using the single copy gene *mRFP1* (detection threshold=25 spores). However, the copy number of the *ITS2 rRNA* gene does not correspond to the exact spore number. Therefore, we determined the number of *ITS2 rRNA* gene copies per spore by analyzing a dilution series (colors indicate different dilution factors) for both the *mRFP1* and the *ITS2 rRNA* gene. Each dilution was run in seven technical replicates (indicated by individual data points). The grey line represents a regression (regression coefficient 45=conversion factor).

Sperm viability

As our main objective was to determine queen and male fitness, we investigated whether males could still transfer viable sperm at the beginning of the mating interval, depending on the stage of the infection (early vs. late) when they were kept together with the queen.

Males were collected as pupae as described previously and exposed to fungal spores when they were approximately three days old. Contagious males and the respective controls were directly dissected after either dipping them in a spore suspension (5 x 10^6 spores/ml) or 0.05% Tx (sham-treated control: N=13, contagious: N=12).

Late-stage infected males and respective controls were equally collected and exposed at the age of approximately three days and kept in isolation for 48h and provided with 10% sucrose ad libitum before the dissections (sham-treated control: N=14, late-stage infected: N=17). As late-stage infected males consistently died within 72h of exposure, no later sperm measurements were possible.

To quantify sperm viability, males were killed by decapitation and their reproductive tract was dissected in 15 µl of semen diluent (188.3 mM sodium chloride, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine, 50 mM tris(hydroxymethyl/aminomethane), ph=8.7) (Paynter *et al.,* 2014) on a microscope slide. Seminal vesicles were transferred into a new droplet of 15 µl semen diluent, sperm was released and mixed carefully. Subsequently, the sperm was stained with the 'LIVE/DEAD Sperm Viability Kit' (Molecular Probes, Waltham, Massachusetts, USA). We added 2 µL of SYBR−14 working solution (Molecular Probes, L-7011; SYBR stock solution diluted 1:50 in semen diluent) to the sperm sample and incubated it in the dark for 5 min at room temperature. Thereafter, 0.5 µl of propidium iodide were added and incubated for further 5 min in the dark. Per ant male ~300 spores cells were counted without knowing the male's infection status and the proportion of live (green) and dead (red) sperm cells was determined by fluorescent microscopy (Leica DM 1000 LED, magnification 200 ×).

Long-term effects on queen survival, colony development and caste composition after being in contact with an early- or late-stage infected male

To determine the long-term consequences on queens that were kept together with a contagious early-stage infected or a late-stage infected male, we kept naïve queens with early- respectively late-stage infected males and their corresponding sham-treated controls in an experimental container (Ø25mm) without workers and fed them with 10% sucrose ad libitum for 72h. We checked the survival daily and removed dead males throughout the experiment to prevent spore transfer from sporulating cadavers to the queens. Only sexuals originating from the same stock colonies (six independent colonies) were paired.

All sexuals were collected as pupae, age-standardized and randomly assigned into the different treatment groups. Sexuals used for this experiment were slightly older than those used for the short term-experiment (~3.5-5 days) as a huge amount of sexuals was needed and we therefore had to increase the time window of the "hatching interval". Queens were between 3-4 days and males between 5-6 days old at the start of the experiment.

To be able to later determine whether potential differences of queens that were kept together with control or contagious males are due to spore transfer and a result of low-level infections or male-derived compounds, i.e., sperm or seminal fluid, and therefore paternal effects, we included another treatment group in our experiment where we manually applied the spores that they would have received during social interactions or mating with a contagious male by dipping them in 10^5 spores/ml after they were kept together with sham-treated males. After exposure these queens were kept individually for 48h. We refer to this group as "queen infection control" hereafter. Therefore, we sham-treated queens from the other treatment groups with 0.05% Tx and kept them individually for 48h as well. This allowed us to disentangle whether observed findings of being with a contagious male could have been explained merely by the spore transfer, excluding any other effects, e.g., caused by any social interaction. The table below summarizes the sample size per mating combination (*Table 4.3,* experimental setup see *Figure 4.3*).

Table 4.3. Sample size per mating combination of early- and late-stage infected sexuals and respective controls.

	Mating combination	**N**
Early-stage infection	Early-stage control	54
	Early-stage infected male	57
	Queen infection control	58
Late-stage infection	Late-stage control	59
	Late-stage infected male	58

After the mating and isolation period (on day six of the experiment), all queens were put into "nurse colonies" with 20 workers and five small larvae originating from the same stock colony as the queens. Setups were kept in experimental containers (Ø600mm) with plastered ground and a small indentation for the nest that was covered with red foil and fed with 10% sucrose and minced cockroach twice per week. The worker number was kept constant throughout by removing and replacing dead individuals once per week in order to control for an effect of colony size. We followed each colony until the last offspring had hatched after the queen's death. The first ten days, the colonies were checked daily and afterwards 2-3 times a week (total duration of the experiment was 33 weeks).

We evaluated colony success/failure, queen survival, colony composition (egg, larvae and pupae) and the quality of the offspring by means of immune gene expression in the absence or presence of a fungal challenge - possibly affected by parental pathogen history (see details below). During the course of the experiment, we found that an unexpectedly high number of queens exclusively produced male offspring, and were therefore likely unmated and simply only cohabited with the male. As mating status influences survival and egg-laying rate of *Cardiocondyla* queens (Schrempf *et al.*, 2005), we differentiated between "male-producing" colonies that only produced male offspring and "female-producing" colonies that produced female offspring (mainly workers, a few queens and occasionally males, more details in the result section of this chapter).

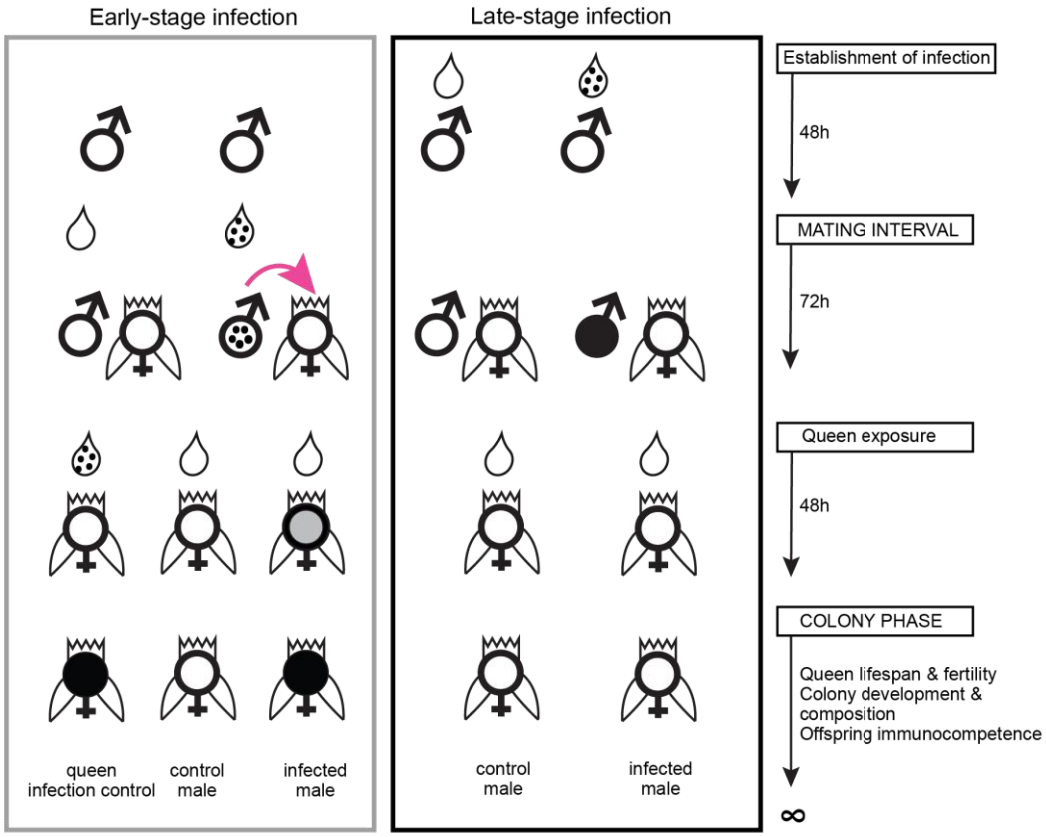

Figure 4.3. **Experimental design – long-term effects: queen survival and fertility, colony development, caste composition and offspring immunocompetence.** To evaluate the effect on queen survival and fertility, colony development, caste composition and offspring immunocompetence, we kept untreated queens either with a contagious early-stage infected male (grey box on the left) or a late-stage infected male (black box on the right) or a sham-treated male as control for 72h. The mating setups were performed without workers as they would otherwise groom off the spores from early-stage infected sexuals. As late-stage infected males had to be kept in isolation for 48h after they had been exposed to either to 5 x 10^6 spores/ml or 0.05% Tx for the infections to establish, we also kept untreated males for the early-stage infected mating setups in isolation before the matings were set up. Early-stage infected males were exposed (either to 5 x 10^6 spores/ml or 0.05% Tx) right before the mating setups (black dots indicate spores that can get transmitted from the exposed male to the queen; indicated by the pink arrow). Late-stage infected males are indicated in black. To be able to determine whether potential differences of queens that were kept together with sham-treated or contagious males are due to spore transfer and hence to disentangle changes resulting from low-level infections vs. male-derived compounds (i.e. sperm or seminal fluid) and therefore paternal effects, we included the "queen infection control". Here, we took queens that were reared with a sham-treated male and manually applied the spore number that they would have received during social interactions with a contagious male in the mating interval by submerging them in 10^5 spores/ml. We kept them individually for 48h so that they could develop an infection. To standardize time points, we sham-treated queens of the other treatment groups with 0.05% Tx and kept them individually for 48h as well. Queens that were kept together with a contagious male and could have contracted spores are represented in grey (before

they are sham-treated), indicating that at this time they could already carry established low-level infections. Afterwards, all queens were transferred into nurse colonies with 20 workers (at this time queens that were manually exposed, as well as queens that could have contracted spores naturally from the male, are indicated in black, as their infections were already established). We followed each colony until the last offspring had hatched after the queen's death and determined queen lifespan, fertility, colony development and caste composition and the immunocompetence of the offspring. The first ten days colonies were checked daily and afterwards 2-3 times a week.

Immune status of the offspring – transgenerational immune priming

We removed each offspring as pigmented pupa from its colony and placed it (i) in the case of sexual brood with two nurse workers and (ii) in the case of worker pupae with two "nurse" virgin queens originating from the same stock colony that could assist with the hatching as in *Cardiocondyla* the virgin queens take part in brood rearing as well (own observation, J. Heinze personal communication). All individuals were provided with 10% sucrose.

3-7 days post hatching, the offspring was isolated and randomly assigned to one of three treatment groups: the offspring was either (i) left untreated to measure the constitutive immune response or (ii) challenged with a fungal spore suspension (induced immunity) to determine how the offspring reacts to the same pathogen that either the mother or the male had encountered (father in case of insemination, otherwise only co-inhabitant). In the latter case, male and virgin queen offspring were exposed to 5 x 10^5 spores/ml and worker offspring to 5 x 10^6 spores/ml, to account for the higher susceptibility of the sexuals (please find details on the dosages used below). To test whether the induced immune response may have been affected by the solution in which spores were suspended (the surfactant 0.05% Tx), we also collected a smaller sample of individuals after sham-treatment with 0.05% Tx only.
After 48h all individuals were individually snap frozen in an 1.5 ml safe-lock Eppendorf tube containing a mixture of five 1 mm zirconia (BioSpec Products) and ~100 mg of 425-600 µm glass beads, acid washed (Sigma-Aldrich) and kept at -80 °C until further processing.
In more detail, we checked the relative gene expression (gene expression of the immune gene / gene expression of the housekeeping gene elongation factor 1-alpha (*EF1)* of genes that are involved in the Toll- and prophenoloxidase-pathway and are differentially expressed upon fungal infection. At the receptor level of the Toll-pathway, we analyzed persephone *(Psh)* and β-1,3-glucan-binding protein *(ß-1,3-GBP)* and the transcription factor dorsal (*Dor*) that activates the transcription of antimicrobial peptides (Silverman and Maniatis, 2001; Lemaitre and Hoffmann, 2007; Valanne *et al.*, 2011; Sheehan *et al.*, 2018).
We further tested the antimicrobial peptide defensin *(Def)* at the effector level that is known to have antifungal properties (Viljakainen and Pamilo, 2005, 2008). We also had a look at prophenoloxidase activating factor *(PPAF)* that activates prophenoloxidase *(PPO)* to its active form and is essential for the melanization response upon infection by a variety of pathogens, including entomopathogenic fungi (Gillespie *et al.*, 1997; Cerenius and Söderhäll, 2004, 2021; Cerenius *et al.*, 2008). Moreover, we also analyzed the inhibitor serine protease inhibitor 27 A *(Spn27A)* a serpin that (i) inhibits the activation of the *PPO* system (Nappi *et al.*, 2005) and (ii) whose increased gene expression also inhibits the Toll pathway (Hashimoto *et al.*, 2003). Lastly, we also looked at relish *(Rel)* a transcription factor of the immune deficiency (IMD) pathway whose induction results in the transcription and translation of antimicrobial peptides which are active against Gram-negative bacteria (Myllymäki *et al.*, 2014; Sheehan *et al.*, 2018) and in our case would indicate a general upregulation of immunity (spore-nonspecific). The table below shows the sample sizes per caste and immune gene (*Table 4.4*).

Table 4.4. Sample size per caste and type of immunity (constitutive and induced) depending on parental pathogen history for immune gene expression analysis. const=constitutive immunity (untreated offspring), sham=sham-treatment (offspring sham-treated with 0.05% Tx), induced=induced immunity (offspring received fungal challenge).

	Mating combination	**Type of immunity**	**Female-producers**			**Male-producers**
			Worker	Queen	Male	Male
Early-stage infected	Control	const	8	3	4	8
		sham	5	3	1	5
		induced	8	3	1	8
	Male infected	const	8	1	2	8
		sham	6	0	0	5
		induced	8	1	3	8
	Queen infected	const	8	1	1	8
		sham	5	0	0	5
		induced	7	1	1	8
Late-stage infected	Control	const	8	2	0	8
		sham	3	0	0	5
		induced	8	2	0	8
	Male infected	const	8	5	4	8
		sham	5	3	4	5
		induced	8	5	6	8

To measure the induced immunity, we originally aimed to apply a dose that would induce ~30% mortality (=lethal dose LD30) within the first week after exposure to trigger the immune system sufficiently, but not overwhelm it and determined the corresponding dosages per caste in a pilot experiment (details see below). We knew from a dose-response experiment in workers (not age-standardized, sham-treated N=62, fungus-exposed N=59) that a dose of 5 x 10^6 spores/ml corresponds to ~LD30. To match the same LD in sexuals, we determined in a pilot experiment that we would need to dip them in 5 x 10^4 spores/ml (sexuals 2.5-5 days old, N=41 for each treatment, except sham-treated virgin queens N=40, *Figure 4.4*). However, the spore number per individual this would correspond to, would have been so low that we would not have been able to assure that all exposed sexuals would have received spores. For this reason we increased the application to 5 x 10^5 spores/ml which corresponds to approximately three spores in virgin queens (details above).

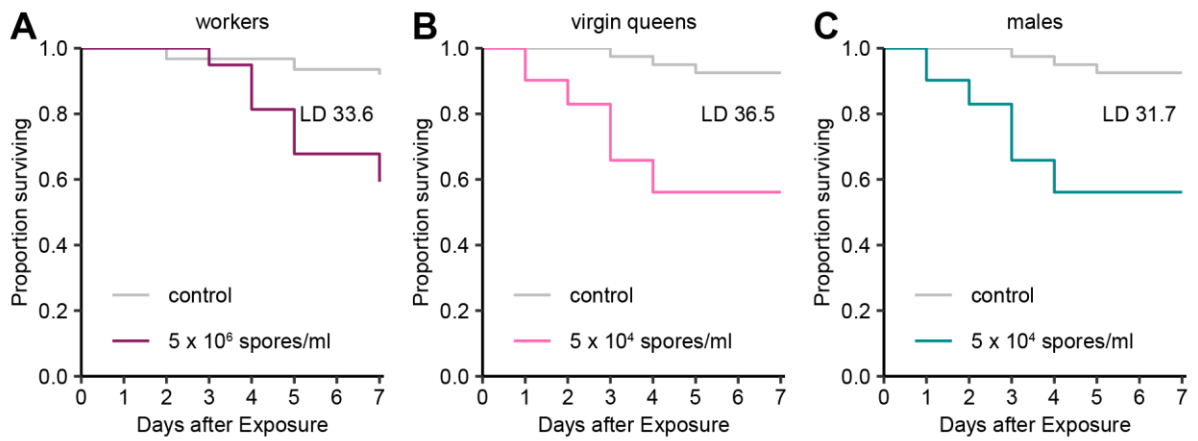

Figure 4.4. **Pilot experiment to determine an exposure dose that would induce 30% mortality.** To measure the induced immunity, we aimed to apply a dose that corresponds to a LD30 (within the first week after exposure) and therefore determined the corresponding dosages in (A) workers, (B) virgin queens and (C) males. All individuals were submerged in either a spore suspension or 0.05% Tx as sham-treatment and individually kept in plastered experimental containers with 10% sucrose for seven days. Workers were kept individually throughout the entire time interval, whereas queens and males received two nurse workers three days post exposure (after infections were already established) to care for them. The grey line represents the survival of sham-treated individuals and the colored line indicates spore exposure. To determine the LD, the survival of spore-exposed individuals was subtracted from the survival of sham-treated individuals on day seven.

RNA extraction, cDNA synthesis & ddPCR

RNA extraction, cDNA synthesis and ddPCR was performed as described in chapter 3 (material and methods).

We analyzed expression patterns of seven different immune genes (details below) in seven multiplex ddPCRs assays, each targeting one immune gene and the housekeeping gene *EF1*. The primers were designed and the protocols were established by J. Kirchner in her Bachelor's Thesis 2021. Primers were designed using Primer3Plus (Untergasser *et al.*, 2007) and "Multiple Primer Analyzer" (https://www.thermofisher.com) software according to the following sequence IDs of the *C. obscurior* genome Cobs.alpha.v.2.1 (Lukas Schrader, Institute for Evolution and Biodiversity, Münster, 2021, unpublished, *Table 4.5* and *Table 4.6*).

Table 4.5. Sequence IDs of genes used to design primers for the immune gene expression analysis.

Gene	ID in Cobs.alpha.v.2.1
EF1	COBS15396
ß-1,3-GBP	COBS08536
Psh	COBS10652
PPAF	COBS07801
Spn27A	COBS07793
Dor	COBS12321
Rel	COBS06226
Def	COBS15672

Table 4.6. Sequences of primers and probes used for immune gene expression in ddPCR.

Gene		Primer sequence (5´- 3´)
EF1	Forward	ATTGGAACAGTACCCGTTGG
	Reverse	CACCCTTCGGTGGGTTATTT
	Probe	[HEX]ACCTGGTATGGTCGTTACCTTTGCACCCGT[BHQ1]
ß-1,3-GBP	Forward	CGGCGAGAATATAACTGCGA
	Reverse	GGCACGTATTTCGATCTTGC
	Probe	[6FAM]TCGTTCTTGAATGTACGCGGCAGGCATTCT[BHQ1]
Psh	Forward	CTATCGTCGACACACGGAAA
	Reverse	CCAGCCAACAATTCCTGGTA
	Probe	[6FAM]TCGTGCAATGTCGACAACTCCGCAGGT[BHQ1]
PPAF	Forward	TGCTGCTCACTGTATCAAGG
	Reverse	TCTGTTTCAGTGTCGGTGTC
	Probe	[6FAM]ACTGGCGTCTGACCAGCGTCCGT[BHQ1]
Spn27A	Forward	CCGATAGTCGGCAATTGAAC
	Reverse	CGAAGTTAACAGCATCTGCG
	Probe	[6FAM]CGAACGACGCAGCCAGCCGCA[BHQ1]
Dor	Forward	GAGTGGAACGAGCGTTAAGT
	Reverse	GTATCTTCCCACCCTCGACT
	Probe	[6FAM]TGGCACCACGCGCGACTGTCTCAGAATTT[BHQ1]
Rel	Forward	ACGGATTTAGGATGGACACC
	Reverse	CTTGGTGGCTTCCTTCAACA
	Probe	[6FAM]TGCTCTCTTGTGCAGACTGGCGCAGA[BHQ1]
Def	Forward	ACGGGCCTACTTACGAATTG
	Reverse	CGCAAGCACTATGGTTGATG
	Probe	[6FAM]CGAAGAGGAGCCGTCACACCTGACGC[BHQ1]

Relative immune gene expression was calculated by dividing the value 'Copies/20µl Well' for the immune gene by the value 'Copies/20µl Well' of the housekeeping gene in the same well. The thresholds were set manually as follows: *EF1* 4000, *ß-1,3-GBP* 3000*, Psh* 6000*, PPAF* 1500, *Spn27A* 2000, *Dor* 3000, *Rel* 2000 and *Def* 4000.

Statistical analysis

All statistical analyses were carried out in the program 'R' version 4.0.3 (R Core Team 2020). Unless otherwise stated, we used a generalized mixed modelling approach ('lme 4' (Bates *et al.*, 2015)) in which the significance of the model predictors was estimated by comparing each model to a null model only containing the intercept and reduced models (containing all but the predictor of interest) using Likelihood Ratio (LR) tests (Bolker *et al.*, 2009). Likewise, in case of multiple predictors in our models, we tested whether the interaction of the predictors was significant by comparing models with the interaction term to models without the interaction using LR tests. When the interaction turned out not significant, the models were refitted and run without the interaction to achieve better estimates for the main effects. When the interaction revealed significance, the main effects were not interpreted, but we directly performed post hoc tests using the 'multcomp' package (Hothorn *et al.*, 2008) and corrected the p-values according to the Benjamini-Hochberg correction to protect against a false discovery rate of 5% and present adjusted p-values. To determine the significance of the predictors, we compared reduced models (containing all but the predictor of interest) to a model with all predictors included using LR tests. When multiple inferences were made, all overall p-values were

corrected using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) as well. We assessed model assumptions and checked whether residuals were normally distributed and if the model variances were homogeneous ('DHARMa', (Hartwig, 2021). We checked for overdispersion, assessed model stability and checked whether there were any influential data points (dfbetas). All logistic regressions were implemented as generalized linear mixed models (GLMMs) with binomial error distribution and logit-link function. Data for early- and late-stage infected sexuals were analyzed separately. If not stated differently all models included 'stock colony' as a random effect. All figures were made with the 'ggplot2' package (Wickham *et al.*, 2018).

Short-term effects

Survival, worker interference and mating success. To determine whether survival was affected by early- and late-stage infection and whether worker presence had an effect on survival, we ran a GLMM with binomial error term and logit-link function with survival as response and worker presence and infection status (and their interaction) as predictor. We ran one model for queens and one for males.

To determine the mating success of alive queens, we ran a GLMM with binomial error term and logit-link function with survival as response and worker presence and infection status (and their interaction) as predictor.

Pathogen effect on sperm viability. To test whether the sperm viability was affected by contamination or late-stage infection at the beginning of the mating interval, we ran a GLMM with binomial error structure and logit-link function on the proportion of alive sperm cells per ejaculate among all counted sperm cells per ejaculate (applying the cbind function to prevent calculation with proportion values and to account for sampling effort) and included 'male' and 'stock colony' as random effects.

Long-term effects

Queen survival in the acute phase of infection. To test whether queen survival differed between queens that were kept together with an early- or late-stage infected male compared to when queens were kept with sham-treated males before they entered the nurse colonies, we ran a GLMM with binomial error structure and logit-link function with survival as response and infection status of the male (paternal pathogen history) as predictor.

Colony failure and the proportion of female-and male-producing colonies. To determine whether the proportion of (i) colony failure (colonies that did not produce any offspring), (ii) female-producing and (iii) male-producing colonies was affected by male pathogen history, we ran three separate GLMMs with binomial error structure and logit-link function with either of the three categories as response and male pathogen history as predictor.

Queen lifespan in the chronic phase of infection. In order to evaluate whether queen lifespan was affected by male pathogen history, we again ran three separate negative binomial GLMM with queen lifespan as dependent variable and male pathogen history as independent variable as all GLMMs with Poisson error term revealed overdispersion. However, in one case – male-producing colonies where the queen cohabited with a late-stage infected male – the model assumptions where still not met after running a negative binomial GLMM, and therefore we ran a GLMM with Gaussian error structure and log transformed the queen lifespan to reach near normal distribution.

Average weekly number of eggs laid per queen. To determine whether queen egg-laying rate was affected by either mating or cohabiting with an early- or late-stage infected male, we calculated the average weekly number of eggs laid per queen. We ran a GLMM with Gaussian error structure and implemented the egg-laying rate as a response variable and male pathogen history as predictor.

Total number of pupae. To examine whether the total number of pupae produced was affected by male pathogen history, we ran negative binomial GLMMs as the data was overdispersed. We fitted the models with pupae number as dependent variable and male infection status as independent variable. As model assumptions were still not met for female-producing colonies at the early-stage of infection, we log transformed the pupae number to reach near normal distribution and ran a GLMM with Gaussian error structure.

Immune gene expression of male and worker offspring. To assess offspring quality by means of immune gene expression in the absence or presence of fungal challenge and to check whether the immunocompetence is affected by parental pathogen history, we analyzed male offspring of male-producing colonies and worker offspring of female-producing colonies (as we only had a sufficient sample size for these two categories).
We tested whether the relative immune gene expression (expression of the immune gene / expression of the housekeeping gene) differed between constitutive and induced immunity and depending on the paternal pathogen history and ran one GLMM per gene and modelled immune gene expression as a function of 'type of immunity' (constitutive vs. induced) and 'pathogen history' (early- or late-stage infected male vs. sham-treated male) and their interaction. We included 'experimental replicate' (as offspring originating from the same mother queen are not independent) and 'stock colony' as random effects. In order to get a better model fit, we log transformed the relative gene expression values to reach near normal distribution.

4.4 Results – short-term effects

Sanitary care by the workers rescues survival of early-stage infected sexuals
We found that the survival of early-stage infected sexuals was affected by their infection status and whether workers were present or not.
We found a non-significant trend (p=0.081) that queen survival was affected by the combined effect of worker presence and her own or her mating partner's health status (interaction worker presence*treatment: χ^2=5.020, d.f.=2, p=0.081, overall LR (without interaction): χ^2=35.798, d.f.=3, p<0.001; worker presence: χ^2=4.247, d.f.=1, p=0.039, treatment: χ^2=32.106, d.f.=2, p<0.001, details see *Figure 4.5 A*). Male survival, on the other hand, was affected by worker presence and health status, without an interaction between the two (worker presence*male treatment: χ^2=0.832, d.f=2, p=0.660, overall LR: χ^2=37.983, d.f=3, p<0.001, worker presence: χ^2=54.981, d.f.=1, p=0.026, treatment: χ^2=33.743, d.f.=2, p<0.001, *Figure 4.5 A*).
We found that exposure of queens and males decreased their survival drastically – queen survival was decreased by 25% and male survival by 23% compared to control individuals when workers were present and by 48% respectively 57% when there were no workers present. Workers groom off the spores of freshly contaminated individuals and thereby increase the survival of early-stage infected sexuals. Even though we found that social immunity (e.g. sanitary care) increased the survival of early-stage infected individuals, they still suffered from reduced survival compared to sham-treated individuals. Furthermore, there were only weak negative effects on survival of the other sex by cross-contamination of the mating partner, which we attribute to the low number of spores that are transferred. We found that on average only 0.7 spores are transmitted from an early-stage infected male to the queen (details see material and methods) without workers being present.

Social immunity is no longer beneficial at a late infection stage
In sexuals which already carried an established infection (late-stage infected), spores cannot be groomed off by the workers anymore (Walker and Hughes, 2009) and therefore we expected worker presence not to be beneficial anymore. All but one late-stage infected queen died within the mating interval, indicating that the queens infected with a relatively high dose of spores die and will not be able to reproduce. Therefore, late-stage infected queens were not included in future experiments. As the models did not converge because of complete separation, late-stage infected queens were excluded from statistical analysis. As expected, queen survival was not impaired when reared together with a late-stage infected male and also unaffected by worker absence or presence (worker presence*treatment: χ^2=0.042, d.f.=1, p=0.838, overall LR: χ^2=1.638, d.f.=2, p=0.441, *Figure 4.5 B*). Survival of late-stage infected males was also drastically reduced and not affected by worker presence (worker presence*treatment: χ^2=2.171, d.f.=2, p=0.338, overall LR: χ^2=60.914, d.f.=3, p<0.001, worker presence: χ^2=1.353, d.f.=1, p=0.245, treatment: χ^2=60.201, d.f.=2, p<0.001, *Figure 4.5 B*). Male survival dropped by 62% (when workers were present) and by 83% (without workers present), but was not reduced when they were kept together with a late-stage infected queen.

Early-stage infected sexuals suffer reduced mating success when sanitary care is prevented
We determined the mating status of queens that were still alive at the end of the mating interval, since only the queens that survived through disease and mating can give us insights into the long-term effects of the pathogen on colony fitness and development.
We found a marginally significant interaction (p=0.055) of worker presence and infection status of the sexuals, indicating that the spore removal by the workers can buffer the negative effect of the pathogen for the sexuals (worker presence*treatment: χ^2=5.810, d.f.=2, p=0.055,

overall (without interaction): χ^2=13.407, d.f.=3, p=0.004, worker presence: χ^2=5.693, d.f.=1, p=0.017, treatment: χ^2=7.641, d.f.=2, p=0.022, *Figure 4.5 C)*. In more detail, only half of the queens that were reared with an early-stage infected male without workers were mated, whereas the mating success was approximately 75% when workers were present, which corresponded to the mating success of healthy sham-treated sexuals. The drop in mating success cannot be explained by the males dying before they could mate, as only 50% of the males that survived beyond the mating interval did successfully mate and we know that males even at further stages of the infection were still able to mate successfully (details see below). Similarly, we found only 46% of the early-stage infected queens were mated when workers were absent. In both cases - when queens or males were contagious and workers were absent - the mating success was reduced by approximately 50%, which suggests that sexuals may refuse risky mating attempts that could lead to cross-contamination, but do not per se avoid mating with a contagious mating partner.
The mating success of early-stage infected sexuals in the presence of workers was not decreased, suggesting that workers do not discriminate against contagious mating partners, but perform sanitary care and thereby reduce the negative effects of a high pathogen load by grooming off the spores.

Late-stage infected sexuals have drastically reduced mating success
Since all late-stage infected queens had died, they were excluded from the statistical analyses. As expected, worker presence (sanitary care) could not "rescue" the mating success of late-stage infected males anymore. Even though it seemed as if the mating success of late-stage infected males was reduced compared to sham-treated males, this was statistically not significant due to the low sample size of only 7-12 alive and mated queens (mating success*treatment: χ^2=0.098, d.f.=1, p=0.754, overall LR: χ^2=11.410, d.f.=2, p=0.005, workers: χ^2=0.388, d.f.=1, p=0.534, treatment: χ^2=11.104, d.f.=1, p=0.001, all pairwise comparisons p<0.086, except "with worker" control vs. "without worker" control p=0.924 and "with worker" inf. male vs. "without worker" inf. male p=0.590, *Figure 4.5 D*). There was no effect of worker presence on mating success, indicating that workers did also not discriminate against late-stage infected males.

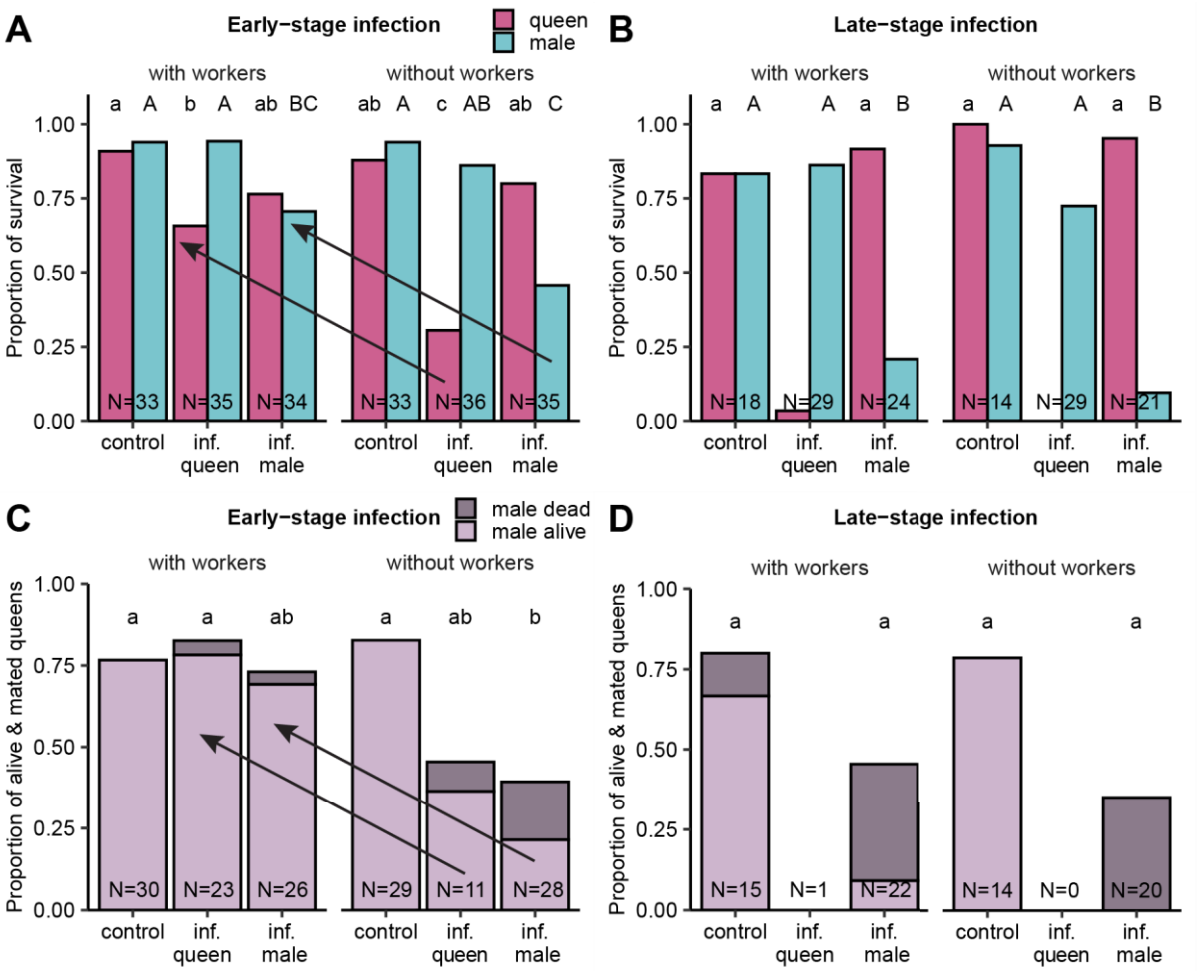

Figure 4.5. **Survival and mating success of early- and late-stage infected sexuals and the effect of workers.** We looked at the survival of sexuals depending on their own infection status and their partner's infection status at the (A) early-stage and (B) late-stage of infection and compared whether worker presence (left: workers present, right: workers absent) affected their survival. Bars represent the proportion of sexuals (pink: queens and blue: males) that are still alive at the end of the mating interval. N denotes the total sample size per mating combination. Similarly, we looked at the mating status of queens that were still alive at the end of the mating interval when sexuals were (C) at the early-stage and (D) at the late-stage of infection when workers were present or absent. Purple bars represent queens that were mated and the proportion of males that mated, but died afterwards, is shown in dark purple. N denotes the total sample size of alive queens per mating combination. We found that worker presence (sanitary care) is beneficial for survival as well as for mating success of early-stage infected sexuals (indicated by black arrows), whereas this was not the case when infections were already established. Different letters denote statistical differences after Tukey post hoc comparisons ($p<0.05$).

Sperm quality remains high at early- and late-stage of infection

We found that early-stage infected males (χ^2=0.012, d.f.=1, p=0.911) as well as late-stage infected males (χ^2=1.026, d.f.=1, p=0.311) were able to retain high sperm quality compared to sham-treated males and were able to transfer viable sperm to the queen (*Figure 4.6*) at the beginning of the mating interval. Unfortunately, it was not possible to measure sperm viability at later time points as all late-stage infected males died three days post exposure.

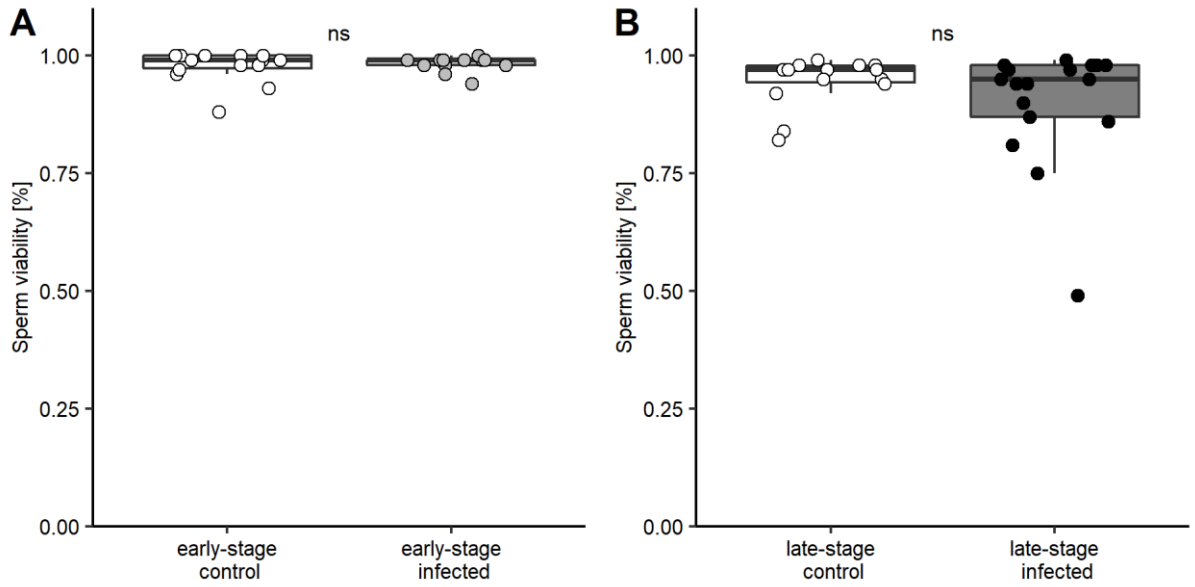

Figure 4.6. **Sperm viability of freshly contaminated and infected males 48h after spore exposure.** We measured sperm viability of (A) sham-treated (white) and freshly exposed (grey) males and (B) sham-treated (white) and infected (black) males 48h after spore exposure (these measurements correspond to the beginning of the mating interval). Data points represent the proportion of viable sperm cells per ejaculate of individual males (approximately 300 sperm cells were counted per male) grouped by their infection status. Boxplots show median (bar), lower and upper hinges correspond to first and third quartiles (box) and "extremes" (whiskers; the upper whisker extends to the largest value if it is no greater than 1.5 times the interquartile rage from the hinge, and the lower whisker extends to the smallest value if it is no smaller than 1.5 times the interquartile range from the hinge).

4.5 Discussion – short-term effects

We found that sexuals at both early and late stages of infection are still able to mate successfully, but their mating success is impaired. Sexuals at the early-stage of infection profit from sanitary care provided by the workers, i.e. spore removal, in terms of both survival and mating success.

The experiments revealed that queens were particularly susceptible to developing deadly infections when they were directly exposed. Late-stage infected queens have all died within the experimental interval and even the early-stage infected queens, both in the absence as well as in the presence of workers, had drastically reduced survival – which is why we performed subsequent experiments only with very low exposure dosages for queens. This indicates that queens seem to invest in reproduction rather than immunity. Therefore either the queens themselves or their workers should be selected for detection of contagious males.

Early-stage infected males only mated in 50% of the cases, which could either be explained by an immunity-reproduction trade-off (Schwenke *et al.*, 2016) that the males face due to raising an immune response (as it could be seen in chapter 3) or by the fact that queens actively avoided mating attempts from contagious males to avoid cross-contamination (Milinski and Bakker,

1990). Early-stage infected queens in the presence of workers were mated to the same extent as sham-treated queens, which was above 75% in both cases, whereas only 46% of the early-stage infected queens without workers were mated. It is likely that unmated queens were not perceived as attractive by the males anymore as they might have already been weakened from mounting an immune response. Alternatively, males could have avoided risky mating attempts. We found that even infected males at advanced stages of disease engaged in mating and were able to retain high sperm quality, likely as a terminal investment strategy (Clutton-Brock, 1984). However, their mating success was reduced with respect to infection and mating only occurred in 35-45% of the cases. This is comparable to the average mating success in a pilot study where we checked for the mating success of naïve sexuals within 48h, which was in the case of workers presence 35% (7/20) and in the case of worker absence 52.6% (10/19), which fits the data from our experiment reasonably well. This demonstrates that the mating success is not decreased by being infected, as males can still successfully mate even at the late stage of disease, but that their mating window is reduced.
We found no indication that workers discriminated against early- or late-stage infected mating partners – instead they performed social immunity whenever they could and thereby increased survival and mating success of the sexuals. We found that sexuals – queens even more than males – already suffered from the infection in a very short amount of time – likely because their cuticle is relatively soft and spores might penetrate the cuticle faster than in other ant species, rather similar to reports in termites where first germination was observed 10h post exposure (Davis *et al.*, 2018).
Furthermore, we confirmed that queens get cross-contaminated by an early-stage infected mating partner when there are no workers around and found that on average 0.7 spores got transferred within the first 24h which corresponds to an exposure dose of 1×10^5 spores/ml. We expect that such a minute dose might rather have a beneficial effect on the queen (e.g. immunization) as it was shown in *L. neglectus* workers where low-level infections did not lead to disease but provided a survival benefit upon later challenge with the same pathogen (Konrad *et al.*, 2012). However, depending on the context even very low pathogen dosages can have detrimental effects. Within the three days of the mating interval, this small dose had only very minor effects on queen survival, but there could still be long-term consequences on the survival and fitness of the queen and her offspring and on colony development.

We followed up on this by looking at the long-term effects of disease on the queen, the colony trajectories and checked whether there is transfer of immune protection to the offspring (i) when the queen could contract spores from an early-stage infected male and (ii) when she had a late-stage infected male as a potential mating partner and no spore transfer was possible.

4.6 Results – long-term effects

Being in contact with an early- or late-stage infected male does not increase queen mortality in the acute phase of disease
Queen survival in the acute phase of disease (the first five days of the experiment - mating interval and 48h of isolation; before they were put in their nurse colonies) was not affected by being together with an early- (χ^2=1.060, d.f.=1, p=0.303, *Figure 4.7 A*) or late-stage (χ^2=0.495, d.f.=1, p=0.482, *Figure 4.7 B*) infected male. This was expected given the low amount of spores transferred among sexuals (see above) and given that survival is dose-dependent (Hughes *et al.*, 2004a; Boomsma *et al.*, 2014)

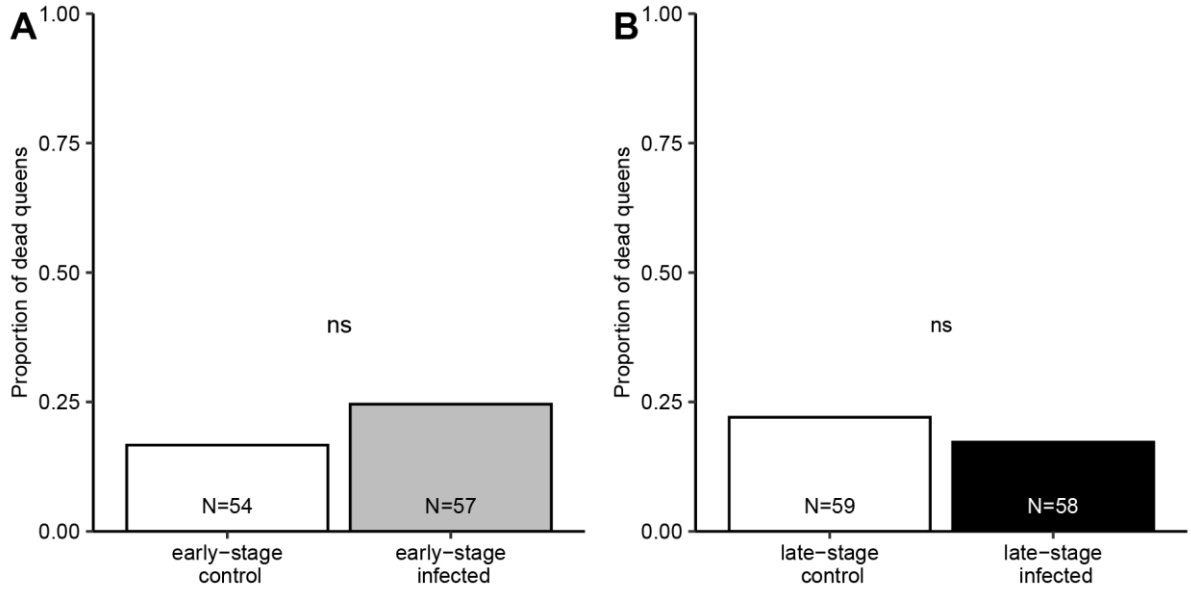

Figure 4.7. **Proportion of dead queens in the acute phase of infection.** Bars represent the proportion of dead queens that were kept together with (A) an early-stage infected (grey) or (B) a late-stage infected male (black). Their survival did not differ from that of queens kept together with sham-treated males (white). Queens were kept together with males for three days, sham-treated with 0.05% Tx and subsequently isolated for 48h before they entered the nurse colonies (on experimental day five). N denotes the total sample size per pathogen history.

High proportion of colony failure in all experimental setups and low proportion of mated queens

Colony failure – which means that colonies were left without any offspring – in this experiment was very high. This was true for queens of all mating groups. During the course of the experiment, we found that most of the colonies produced exclusively male offspring and that thus many more queens than expected from the short-term experiment were therefore likely unmated (Hölldobler and Wilson, 1990). Only a very small fraction (on average 10% of the set up colonies) of the colonies produced female offspring – workers and queens – and were therefore surely mated. This was again the same for all mating groups. Virgin and mated queens generally do not only differ in the castes that they are able to produce (as virgin queens can only produce haploid male offspring), but were previously found to also differ in lifespan and egg-laying rate (Schrempf *et al.*, 2005; Rueppell *et al.*, 2015) and were therefore not really comparable. Thus, we split the colonies into "male-producing" and "female-producing" colonies for further analysis. The statistical values are summarized in the table below (*Table 4.7, Figure 4.8*).

Table 4.7. Statistical values for the overall models of relative colony reproductive output depending on parental pathogen history (early- and late-stage of infection). Controls received a sham-treatment with 0.05% Tx. P-values are corrected for multiple testing according to the Benjamini-Hochberg procedure at α=0.05.

	Early-stage infected male	**Late-stage infected male**
No offspring	χ^2=0.028, d.f.=1, p=0.981	χ^2=0.148, df=1, p= 0.966
Female-producing	χ^2=0.127, d.f.=1, p=0.981	χ^2=0.010, df=1, p= 0.966
Male-producing	χ^2=0.001, d.f.=1, p=0.981	χ^2=0.002, df=1, p= 0.966

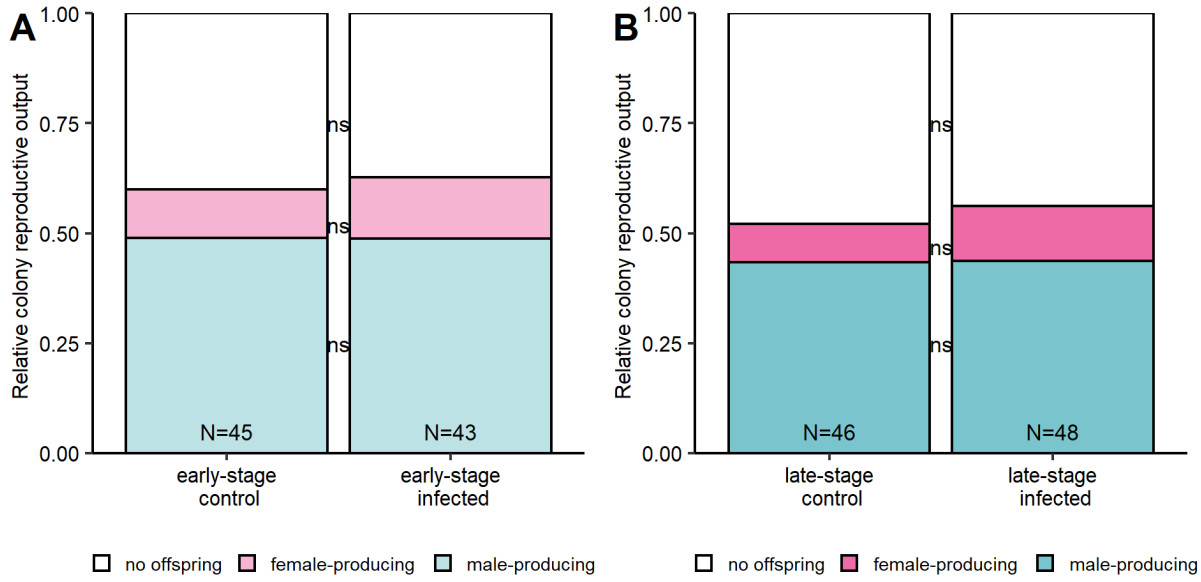

Figure 4.8. **Relative colony reproductive output.** We compared the proportion of queens that (i) failed to produce offspring (white), (ii) produced female offspring (pink) and (iii) produced exclusively male offspring (blue) when the queens were reared with an infected male or a respective control male at the (A) early-stage and (B) late-stage of infection. None of the proportions differed from each other depending on the parental pathogen history (indicated by ns=non-significant). N denotes the total sample size per pathogen history.

Queen survival is contingent both on offspring production and spore transfer during mating

We found that queens that failed to produce offspring died very early in all of the treatments, irrespective of whether they could have contracted spores or not. Male-producing queens that cohabited with an early- or late-stage infected male survived in both cases equally well as queens that were kept with sham-treated control males. In contrast, female-producing queens that have mated with an early-stage infected male had a higher mortality than queens that mated with a sham-treated partner. With the queen infection control we could show that this reduction is due to spore contraction during mating (early-stage male infected vs. queen infection control χ^2=0.393, d.f.=1, p=0.531). Queens that mated with a sham-treated male had a median lifespan of 117 days (interquartile range (IQR) 108-121) whereas queens that likely have contracted spores during mating had a median lifespan of 71.5 days (IQR 36-90.5), which indicated that they suffer a 39% reduction in their survival. The fact that we did not see this survival decrease in male-producing queens when they were together with early-stage infected males could be explained if these queens had less contact with the contagious male and were therefore not contaminated, implying that mating was the main source of contamination. As expected the survival of queens that mated with a late-stage infected male was not significantly different from the survival of queens that mated with sham-treated males (*Figure 4.9, Table 4.8*).

Table 4.8. Statistical values for the overall models of queen survival in the chronic phase of infection depending on pathogen history.

	Early-stage infected male	**Late-stage infected male**
No offspring	χ^2=0.967, d.f.=1, p=0.325	χ^2=0.280, d.f.=1, p=0.597
Female-producing	χ^2=3.653, d.f.=1, p=0.056	χ^2=0.330, d.f.=1, p=0.566
Male-producing	χ^2=0.842, d.f.=1, p=0.359	χ^2=1.353, d.f.=1, p=0.245

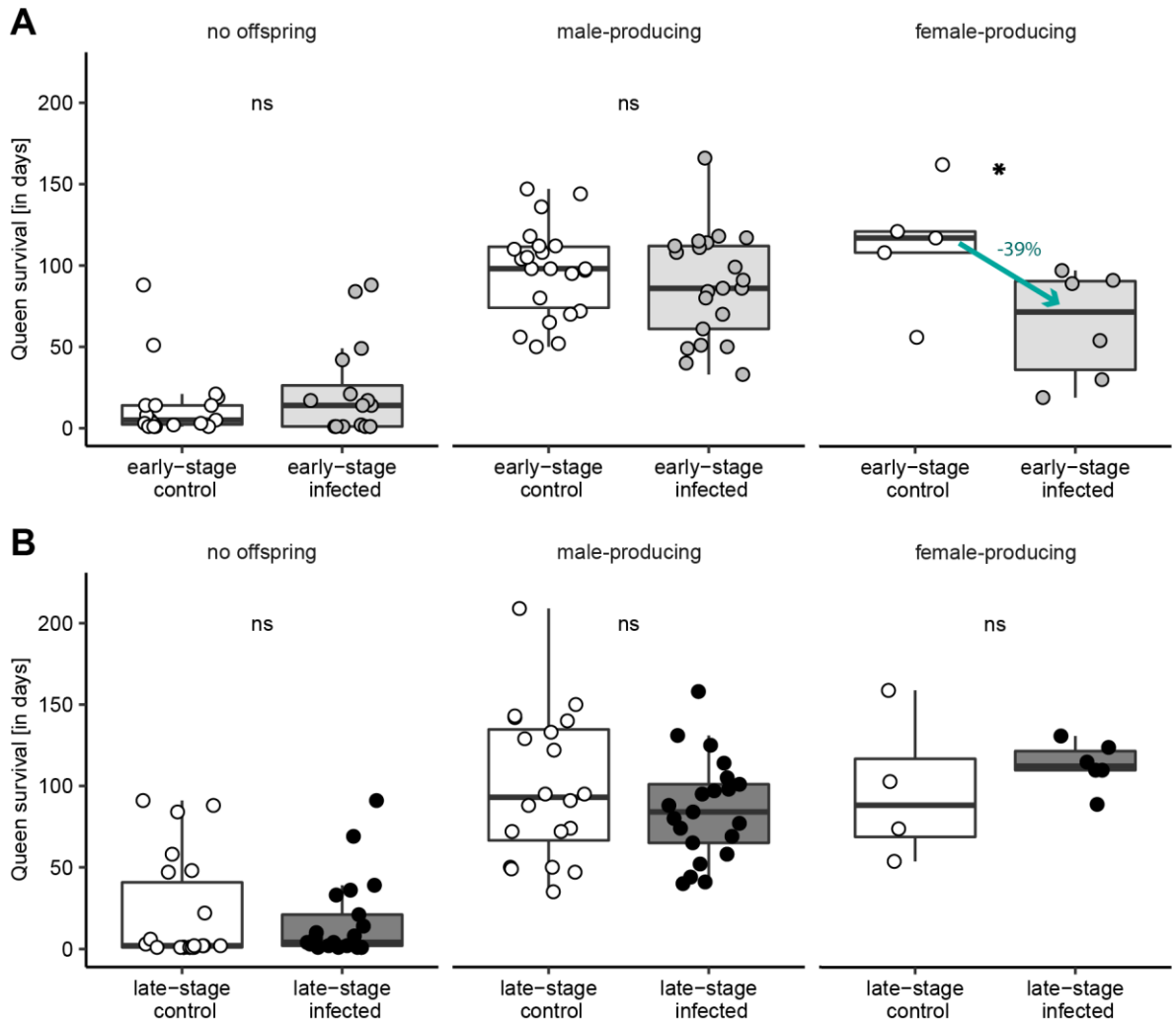

Figure 4.9. **Queen survival in the chronic phase of infection.** We compared the survival of queens that (i) failed to produce offspring (left), (ii) produced exclusively male offspring (middle) and (iii) produced female offspring and were reared together with (A) an early-stage infected male (grey) or a sham-treated male (white) and (B) a late-stage infected male (black) or a sham-treated male (white). Data points represent individual queens that are grouped by male pathogen history. Boxplots show median (bar), quartiles (box), and extremes (whiskers). Only queens that had surely mated with an early-stage infected male had reduced survival (their median lifespan was reduced by 39%; indicated by turquoise arrow; * indicates statistical significance p=0.056). All other comparisons revealed no significant differences. All queens that failed to produce offspring had a very short lifespan.

Queen egg-laying rate was not affected by male pathogen history, but pupal production was

Queen egg-laying rate (average number of eggs laid per queen and week) was not affected by the queen- or male pathogen history but was generally very low. These low average egg-laying rates were also influenced by the absence of egg laying in the first couple of weeks after the mating setups. In the highest reproductive phase (maximal average weekly egg number of queens that mated or cohabited with sham-treated males at the early- or late-stage of infection), female-producing queens laid a maximum of 4.1 eggs per week and male-producing queens a maximum of 1.3 eggs per week at the early-stage of infection. At the late-stage of an infection, female-producing queens laid a maximum of 1.4 egg per week and male-producing queens a maximum of 1.5 eggs per week.

The total number of pupae of male-producing queens was not different from the controls and hence not affected by male pathogen history, neither at the early- nor at the late-stage of infection. It was also unaffected in female-producing queens that mated with a late-stage infected male. However, female-producing queens that mated with an early-stage infected male

produced less pupae, presumably due to the transmission of spores (early-stage male infected vs. queen infection control χ^2=0.103, d.f.=1, p=0.748) and therefore to an infection (*Table 4.9, Figure 4.10*).

Table 4.9. Statistical values for the overall models of the average weekly number of eggs laid per queen and the total number of pupae produced depending on pathogen history.

		Early-stage infected male	**Late-stage infected male**
Egg-laying rate	Female-prod.	χ^2=0.073, d.f.=1, p=0.787	χ^2=0.948, d.f.=1, p=0.330
	Male-prod.	χ^2=0.507, d.f.=1, p=0.476	χ^2=0.002, d.f.=1, p=0.962
Number pupae	Female-prod.	χ^2=5.141, d.f.=1, p=0.023	χ^2=0.596, d.f.=1, p=0.440
	Male-prod.	χ^2=0.486, d.f.=1, p=0.486	χ^2=0.360, d.f.=1, p=0.548

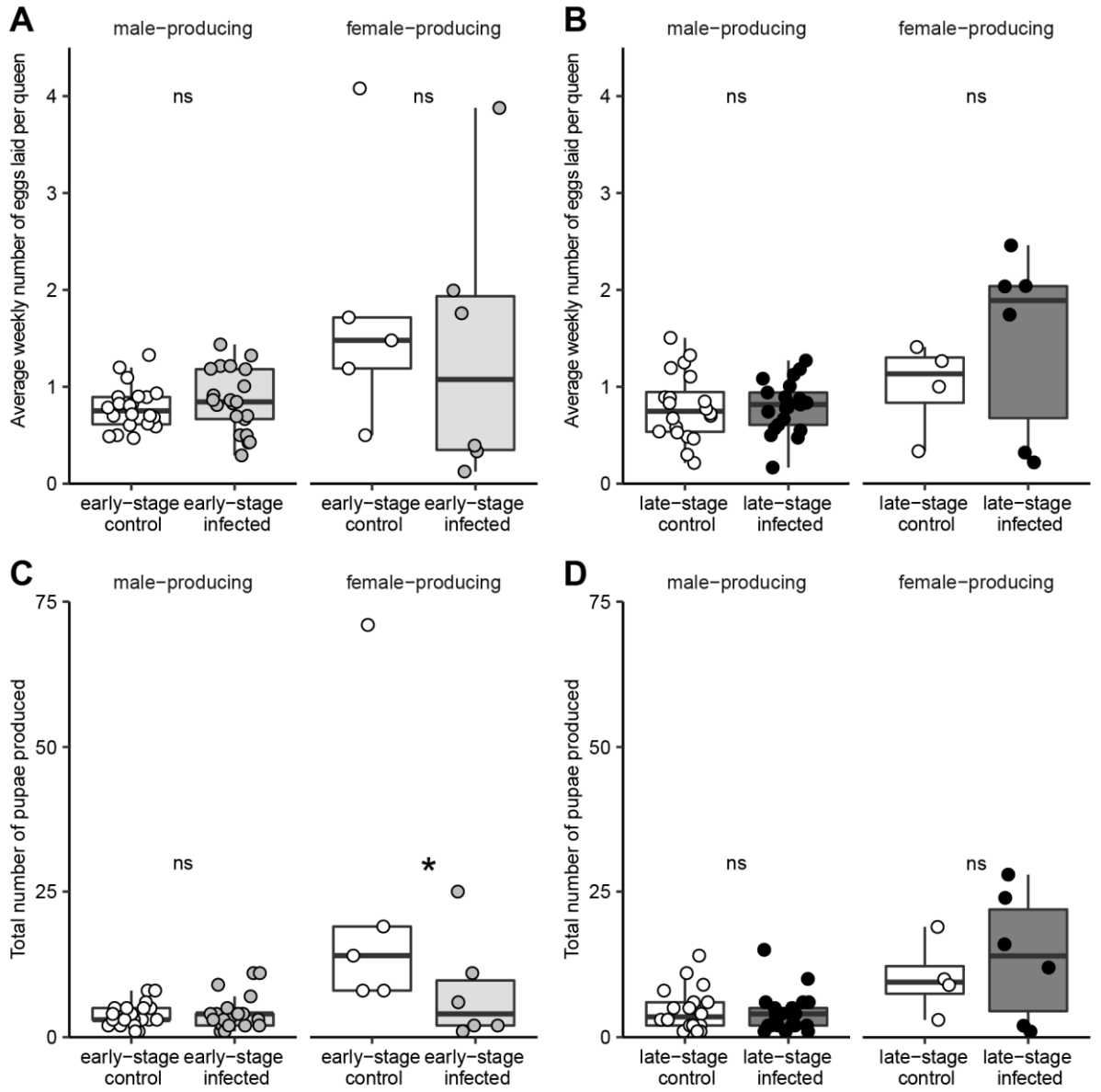

Figure 4.10. **Average weekly number of eggs laid per queen and the total number of pupae produced.** We compared the egg-laying rate (average weekly number of eggs laid per queen) of queens that cohabited or mated with (A) an early-stage infected (grey) or (B) a late-stage infected male (black) and compared it to queens that cohabited or mated with a sham-treated male (white). Data points represent the average number of eggs per queen per week. Egg-laying rate was not affected by parental pathogen history at either the early- or late-stage of

infection. We further looked at pupae production of queens that cohabited or mated with (C) an early-stage infected (grey) or (D) a late-stage infected male (black) and compared it to queens that cohabited or mated with a sham-treated male (white). Data points show the total pupae number of individual queens. We did not find an effect of pathogen history on pupae production – except for the case when queens had mated with an early-stage infected male. Here we found that queens produced less pupae (indicated by * p=0.023). Boxplots show median (bar), quartiles (box), and extremes (whiskers).

4.7 Discussion – long-term effects

Overall, we found a very high colony failure throughout all treatment groups, which could hint to the fact that the queens were very stressed by our experimental procedure. The experiment involved a lot of different handling steps, a sham-treatment with 0.05% Tx (respectively exposure to fungal spores) followed by 48h isolation and the transfer to the nurse colonies where we additionally disturbed the colonies by counting the brood the first ten days of the experiment daily. The contact with the surfactant 0.05% Tx and the absence of allogrooming and trophallaxis during the isolation phase of our experiment could have negatively affected queen physiology (Giehr and Heinze, 2018). A study in *Temnothorax crassispinus* queens also reported a negative effect after exposure to 0.05% Tx where queens refrained from laying eggs for several weeks (even up to 10 weeks) (Giehr and Heinze, 2018). Other studies in *C. obscurior* report that the average queen lifespan is approximately six months (Schrempf *et al.*, 2005) which is almost double as long as the queen lifespan in our experiments where queens survived approximately 3.4 months (median lifespan of queens mated with sham-treated males). However, the published lifespan is on a Brazilian population, whereas our experiments were performed with colonies collected from Tenerife. Even though it is unlikely, it could also be that the average life expectancy of queens differs between the two populations, which makes the above stated stress hypothesis the most likely explanation. Queens in our experiment were treated before their reproductive phase has begun, which may have made them even more vulnerable to handling stress as mating alone is associated with behavioral, physiological and immunological changes in ant queens (Castella *et al.*, 2009). We could not determine the mating status of queens that died without producing any offspring (as the mating status post mortem is often hard to determine as the tissue rapidly disintegrates and dead queens often get dismembered by the workers), but theoretically it is possible that they had been mated, but died because of a trade-off between reproduction and life-sustaining processes (Lochmiller and Deerenberg, 2000), induced by the sham-treatment and the additional handling stress. Based on the small number of female-producing colonies in this experiment, we were not able to obtain the same mating success rate which was >75% for sham-treated males and roughly 50% for the early- as well as the late-stage infected males in previous experiments (the short-term experiment with the same population). In the current experiment, only approximately 10% of the queens produced female offspring and were therefore surely mated (irrespective of queen or male treatment). We dissected seven male-producing queens that just recently had died and we therefore expected the spermatheca to be still well preserved and found that their spermathecae were indeed empty, supporting that male-producing queens are likely all unmated. The drastic drop in mating success in the current experiment could be a seasonal effect or an effect of the condition of the stock colonies from which the sexuals originated from. Overall, we have no clear indications of why they have not mated or whether those that mated had a very high probability of dying very quickly – especially not in the sham-treatment where mating success in previous experiments was very high. Future experiments should include a naïve control treatment to be able to disentangle possible effects of the sham-treatment from other influential factors like handling stress or seasonal effects.

We found that the queens that mated with an early-stage infected male had a 39% reduced lifespan (because of spore transmission), whereas queens that only cohabited with an early-stage infected male (and likely did not mate) did not have impaired survival. The effect of queen infections only became visible when queens were in their reproductive phase and were already producing eggs, which hints to a possible immunity-reproduction trade-off (Schwenke *et al.*, 2016) at a cost of their survival.
Even though we have not statistically tested the difference in lifespan and egg-laying rate between female-producing and male-producing queens (due to the very low sample size of female-producing queens), there is no visual indication of a large effect, opposite to a study in *C. obscurior* where mated queens lived 50% longer than virgin queens and had a three times higher egg-laying rate (Schrempf *et al.*, 2005). While the egg-laying rate of queens that mated with an early-stage infected male was not decreased, they produced less pupae which could indicate that the hatching success of the brood was reduced. It could either be that infected queens lay eggs of poor quality or that the workers treat these eggs differently. In line with this, eggs of infected *C. obscurior* queens were found to smell distinct from healthy queens' eggs (unpublished data with L. Masri and S. Cremer). Cohabiting and mating with a late-stage infected male did not have any effects on queen survival and colony development. However, it could still be the case that the quality of the offspring is altered depending on whether the queen herself or her mating partner/co-inhabitant was infected. Therefore, we proceeded to check the immune gene expression of the offspring.

4.8 Results – transgenerational protection

To measure immune gene expression, the offspring was age-standardized and either (i) left untreated to measure the constitutive immune response or (ii) challenged with a fungal spore suspension (induced immunity) to determine how the offspring reacts to the same pathogen as either the mother or the male (father in case of insemination, otherwise only co-inhabitant) experienced.
To test if the induced immune response may have been affected by the surfactant in which the spores were suspended, we also collected a smaller sample of individuals after sham-treatment with 0.05% Tx only. In case constitutive and induced immunity were significantly different from each other, we used sham-treated individuals (males and workers) originating from setups without any pathogen history (sham-treated males) to interpret whether the difference was caused by a reaction induced by 0.05% Tx or by the spores. Due to the small sample size, we did not include sham-treated individuals in the statistics.
As explained previously, we were limited in the number of female-producing colonies (between 4-6 female-producing queens per pathogen history) that mainly produced worker offspring and only very few sexuals. Consequently, we were not able to analyze sexual offspring of female-producing colonies, but only worker offspring. Therefore, it was not possible to disentangle caste-specific (queens and males vs. workers) from sex-specific (queens and workers vs. males) differences in immune gene expression, as we did not have a sufficient number of virgin queen offspring to compare it to worker offspring. Similarly, due to a low sample size in the colonies from mated queens, male offspring could only be analyzed from male-producing colonies. Consequently, we were not able to disentangle epigenetic effects from the queen (that would only come into play in haploid male offspring) and potentially interfering effects of mating with an early- or late-stage infected male.
For both male offspring (queens that cohabited with an early-stage infected male) and worker offspring (queens that mated with an early-stage infected male), we contrasted the pattern of immune gene expression with male and worker offspring of queens that were manually infected

(queen infection control) for the interpretation of the results and to determine whether the effects we found were due to maternal infection (see supplement, *Figure S 4.19* and *Figure S 4.20*).
An overview of all the samples in which immune gene expression was analyzed can be found in the supplement of this chapter.

Worker offspring of queens that have mated with an early-stage infected male are immunocompromised and non-reactive to fungal challenge
In worker offspring of queens that mated with an early-stage infected male immune gene expression was negatively affected by pathogen exposure history of the male in the case of *ß-1,3-GBP*, *Spn27A* and *Rel* - and even though statistically not significant this pattern could also be observed for the other genes (*Table 4.10*, *Figure 4.11*, for an alternative visualization see *Figure S 4.18*). Interestingly, we found lower immune gene expression, which might indicate that the immunocompetence of worker offspring is negatively affected by an infected mother (cross-contaminated from her mating partner). Moreover, we did not find a difference between constitutive and induced immunity, which indicated that workers did not react upon fungal challenge – however, in some genes we found that worker offspring originating from parents that did not have pathogen contact (i.e. sham-treated male), seemed to show increased gene expression after fungal challenge (even though this was statistically not significant), suggesting that the fungal dose might have been slightly too low, or our limited sample size was not large enough to pinpoint the effects. When comparing the worker offspring of queens that mated with an early-stage infected male to that of queens that we manually infected ourselves (queen infected control), it seemed that gene expression of the latter was slightly higher (*Figure S 4.19*), suggesting that there are no immunity costs in the offspring when the mother was infected. Manual spore application reduces the variation in the spore number a queen bears compared to what queens naturally receive from a mating partner, which would indicate that effect on the offspring immune gene expression might depend on the dose the queen was exposed to. Nonetheless, a larger sample size would be needed to confirm this effect.

Table 4.10. Statistical values for the overall models and main effects of worker offspring depending on parental pathogen history (early-stage infection). The table shows the raw p-values of the overall models and the adjusted p-values in a separate column (p-values were adjusted according to the Benjamini-Hochberg correction at α=0.05).

gene	interaction	overall model	adj. p	type of immunity	pathogen history
ß-1,3-GBP	χ^2=0.272, d.f.=1, p=0.602	χ^2=7.193, d.f.=2, p=0.027	p=0.064	χ^2=1.679, d.f.=1, p=0.195	χ^2=5.732, d.f.=1, p=0.017
Psh	χ^2=0.403, d.f.=1, p=0.526	χ^2=3.531, d.f.=2, p=0.171	p=0.110		
PPAF	χ^2=0.766, d.f.=1, p=0.381	χ^2=5.150, d.f.=2, p=0.076	p=0.133		
Spn27A	χ^2=1.265, d.f.=1, p=0.261	χ^2=8.994, d.f.=2, p=0.011	p=0.057	χ^2=95.303, d.f.=1, p=0.021	χ^2=4.246, d.f.=1, p=0.039
Dor	χ^2=1.330, d.f.=1, p=0.249	χ^2=3.990, d.f.=2, p= 0.136	p=0.190		
Rel	χ^2=0.333, d.f.=1, p=0.564	χ^2=8.251, d.f.=2, p=0.016	p=0.057	χ^2=2.338, d.f.=1, p=0.126	χ^2=6.235, d.f.=1, p=0.013
Def	χ^2=1.105, d.f.=1, p=0.293	χ^2=0.689, d.f.=2, p=0.708	p=0.709		

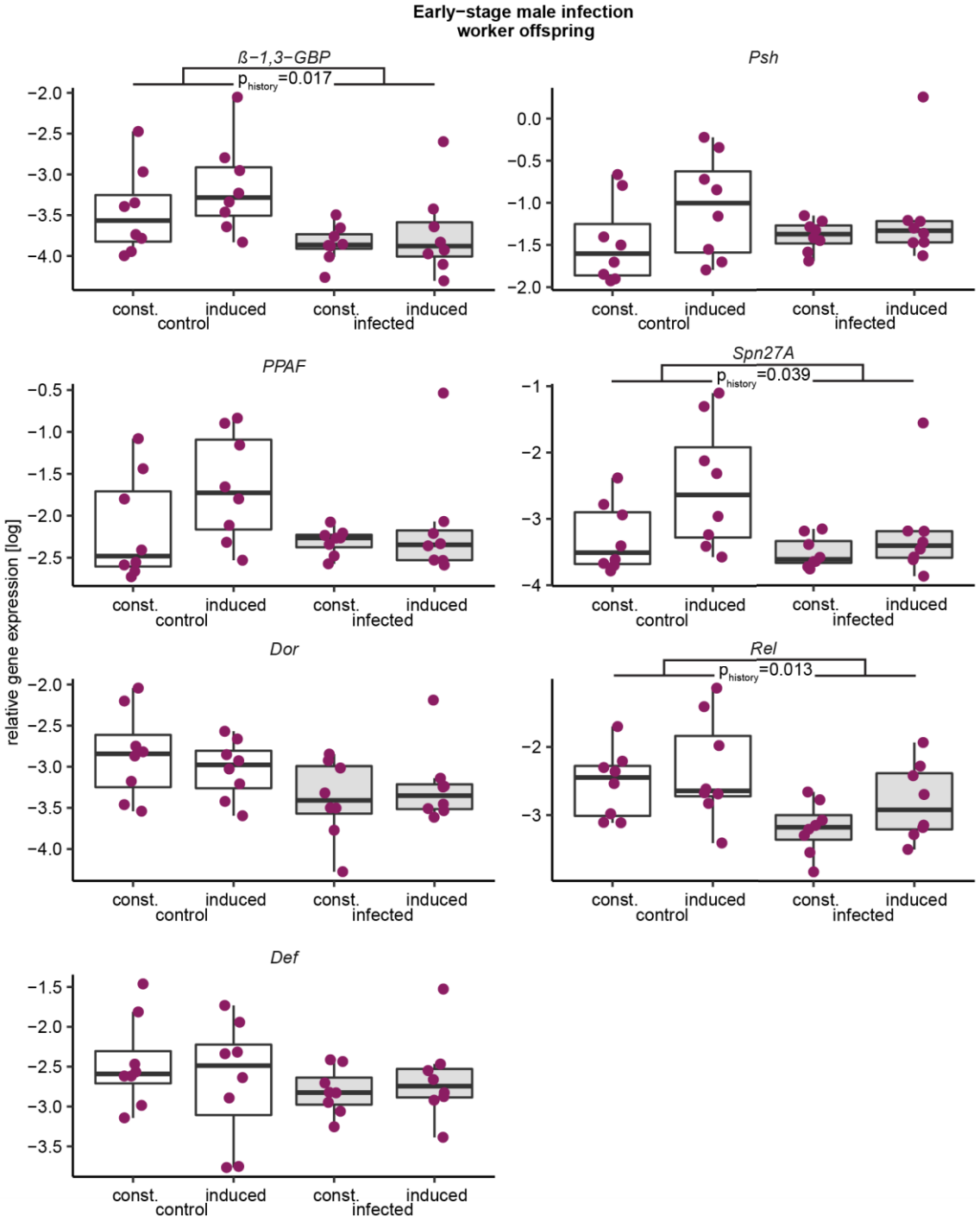

Figure 4.11. **Relative immune gene expression of worker offspring of queens that mated with an early-stage infected or a sham-treated male.** Data points (purple circles) indicate immune gene expression of individual workers grouped by their parental pathogen history (in white: worker offspring that originated from queens that have mated with a sham-treated male and in grey: worker offspring of queens that have mated with an early-stage infected male). Boxplots show median (bar), quartiles (box), and extremes (whiskers). Immune gene expression of worker offspring was not affected by fungal exposure, as immune gene expression of untreated worker offspring (constitutive immunity) was not different to immune gene expression of worker offspring that was exposed to fungal spores (induced immunity). In *ß-1,3-GBP*, *Spn27A* and *Rel* we found a significant effect of parental pathogen history, as worker offspring of queens that had mated with an early-stage infected male had lower immune gene expression compared to worker offspring where the queen had mated with a sham-treated male (indicated by solid bars above the plots). See also *Figure S 4.18*.

Male offspring of queens that only cohabitated with a sham-treated or early-stage infected male show an equal upregulation of immune genes after fungal challenge

All male offspring of queens that cohabited with an early-stage infected male or a sham-treated male showed an increase in immune gene expression after fungal challenge compared to males in which we measured constitutive immunity in all seven genes tested (*Table 4.11, Figure 4.12, Figure S 4.21* for an alternative visualization). For all genes, the immune gene expression of pathogen-challenged individuals was higher than the immune gene expression of sham-treated individuals, which indicates that the upregulation we find is a reaction to the pathogenic challenge with the spores and not simply a treatment effect (*Figure 4.13*). We initially hypothesized that these queens might have picked up fewer spores than queens that mated with early-stage infected males, but we found the same pattern when we looked at male offspring of queens that were manually infected, which suggests that the immunocompetence of males is not altered depending on parental pathogen history (more specifically, queen infection) (*Figure S 4.20*).

Table 4.11. Statistical values for the overall models and main effects of male offspring depending on parental pathogen history (early-stage infection). The table shows the raw p-values of the overall models and the adjusted p-values in a separate column (p-values were adjusted according to the Benjamini-Hochberg correction at α=0.05).

gene	interaction	overall model	adj. p	type of immunity	pathogen history
β-1,3-GBP	χ^2=0.792, d.f.=1, p=0.374	χ^2=14.249, d.f.=2, p<0.001	p=0.001	χ^2=14.119, d.f.=1, p<0.001	χ^2=0.471, d.f.=1, p=0.493
Psh	χ^2=0.247, d.f.=1, p=0.619	χ^2=6.945, d.f.=2, p=0.031	p=0.031	χ^2=6.675, d.f.=1, p=0.001	χ^2=0.033, d.f.=1, p=0.856
PPAF	χ^2=1.153, d.f.=1, p=0.283	χ^2=19.014, d.f.=2, p<0.001	p<0.001	χ^2=18.904, d.f.=1, p<0.001	χ^2=0.190, d.f.=1, p=0.663
Spn27A	χ^2=0.053, d.f.=1, p=0.818	χ^2=18.153, d.f.=2, p<0.001	p<0.001	χ^2=18.136, d.f.=1, p<0.001	χ^2=0.029, d.f.=1, p=0.865
Dor	χ^2=2.490, d.f.=1, p=0.115	χ^2=8.115, d.f.=2, p=0.017	p=0.200	χ^2=8.091, d.f.=1, p=0.004	χ^2=0.031, d.f.=1, p=0.859
Rel	χ^2=0.923, d.f.=1, p=0.337	χ^2=21.331, d.f.=2, p<0.001	p<0.001	χ^2=21.021, d.f.=1, p<0.001	χ^2=0.393, d.f.=1, p=0.531
Def	χ^2=1.083, d.f.=1, p=0.298	χ^2=20.832, d.f.=2, p<0.001	p<0.001	χ^2=20.767, d.f.=1, p<0.001	χ^2=0.123, d.f.=1, p=0.725

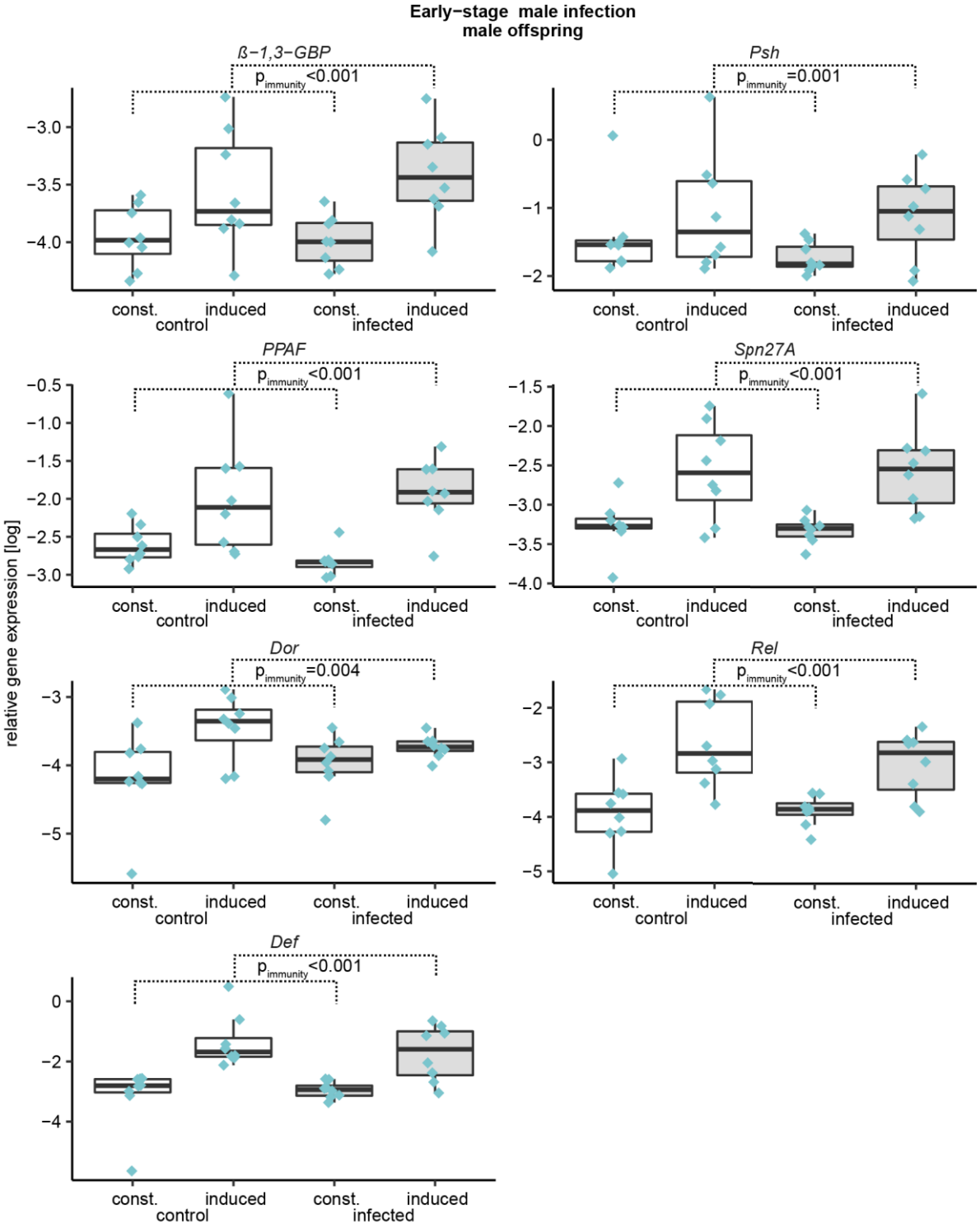

Figure 4.12. **Relative immune gene expression of male offspring of queens that cohabited with an early-stage infected or a sham-treated male.** Data points (blue diamonds) indicate immune gene expression of individual males grouped by their parental pathogen history (in white: male offspring that originated from queens that cohabited with a sham-treated male and in grey: male offspring of queens that cohabited with an early-stage infected male). Boxplots show median (bar), quartiles (box), and extremes (whiskers). Male offspring that were pathogen-challenged (induced immunity) had higher immune gene expression than untreated offspring (constitutive immunity) in all genes tested (indicated by dashed bars), but irrespective of their parental pathogen history. See also *Figure S 4.21.*

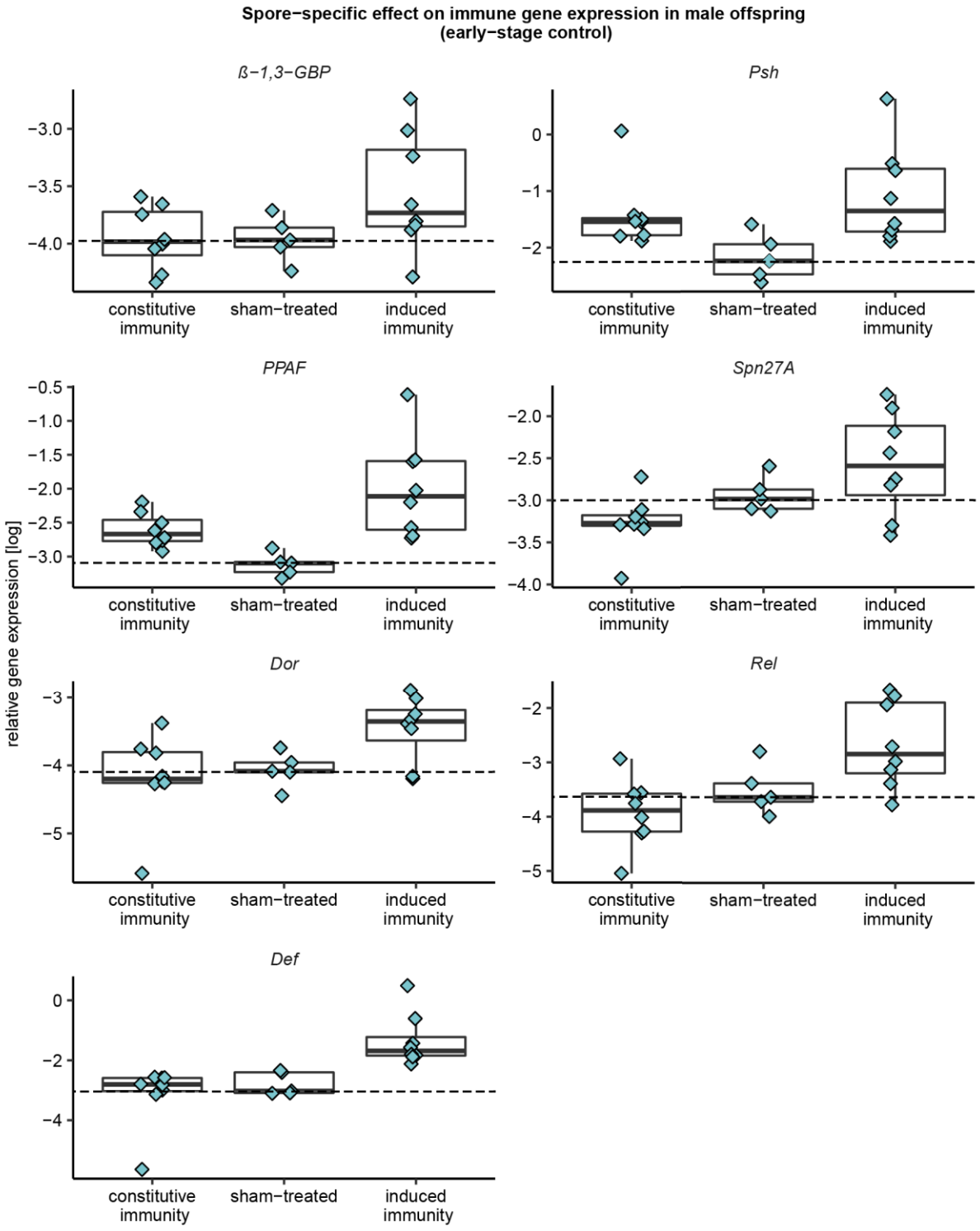

Figure 4.13. **Relative immune gene expression of male offspring of queens that cohabited with a sham-treated male.** Data points (blue diamonds) indicate immune gene expression of individual males grouped by the condition in which immune gene expression was measured (constitutive immunity: untreated, sham-treated: exposed to 0.05% Tx or induced immunity: exposed to fungal spores). Boxplots show median (bar), quartiles (box), and extremes (whiskers). The dashed line indicates the median immune gene expression of sham-treated males and was used to evaluate whether the upregulation in immune gene expression of spore-exposed males was induced by the spores or the surfactant in which the spores were submerged (0.05% Tx). No overlap indicates a spore-specific reaction of the immune genes.

Worker offspring of queens that mated with a sham-treated or late-stage infected male show an equal upregulation of immune genes after fungal challenge

All worker offspring of queens that had mated with a sham-treated or late-stage infected male showed an equal increase in immune gene expression after fungal challenge in 5/7 immune genes tested (except *Dor* and *Def, Table 4.12, Figure 4.14, Figure S 4.23* for an alternative visualization). The effect we observed is likely spore-induced, but this interpretation is limited as we only had three sham-treated worker samples to refer to (*Table 4.12*, *Figure 4.15*).

Table 4.12. Statistical values for the overall models and main effects of worker offspring depending on parental pathogen history (late-stage infection). The table shows the raw p-values of the overall models and the adjusted p-values in a separate column (p-values were adjusted according to the Benjamini-Hochberg correction at α=0.05).

gene	interaction	overall model	adj. p	type of immunity	pathogen history
β-1,3-GBP	χ^2=0.622, d.f.=1, p=0.430	χ^2=16.729, d.f.=2, p<0.001	p=0.002	χ^2=16.404, d.f.=1, p<0.001	χ^2=0.347, d.f.=1, p=0.556
Psh	χ^2=0.078, d.f.=1, p=0.780	χ^2=9.388, d.f.=2, p=0.009	p=0.021	χ^2=9.377, d.f.=1, p=0.002	χ^2=0.004, d.f.=1, p=0.950
PPAF	χ^2=0.057, d.f.=1, p=0.812	χ^2=14.91, d.f.= 2, p<0.001	p=0.002	χ^2=14.753, d.f.=1, p<0.001	χ^2=0.200, d.f.=1, p=0.655
Spn27A	χ^2=0.348, d.f.=1, p=0.555	χ^2=6.738, d.f.=2, p=0.034	p=0.048	χ^2=6.560, d.f.=1, p=0.010	χ^2=0.331, d.f.=1, p=0.565
Dor	χ^2=0.159, d.f.=1, p=0.690	χ^2=1.354, d.f.=2, p=0.508	p=0.577		
Rel	χ^2=0.004, d.f.=1, p=0.947	χ^2=7.309, d.f.=2, p=0.026	p=0.045	χ^2=6.922, d.f.=1, p=0.009	χ^2=0.480, d.f.=1, p=0.489
Def	χ^2=1.008, d.f.=1, p=0.315	χ^2=1.100, d.f.=2, p=0.577	p=0.577		

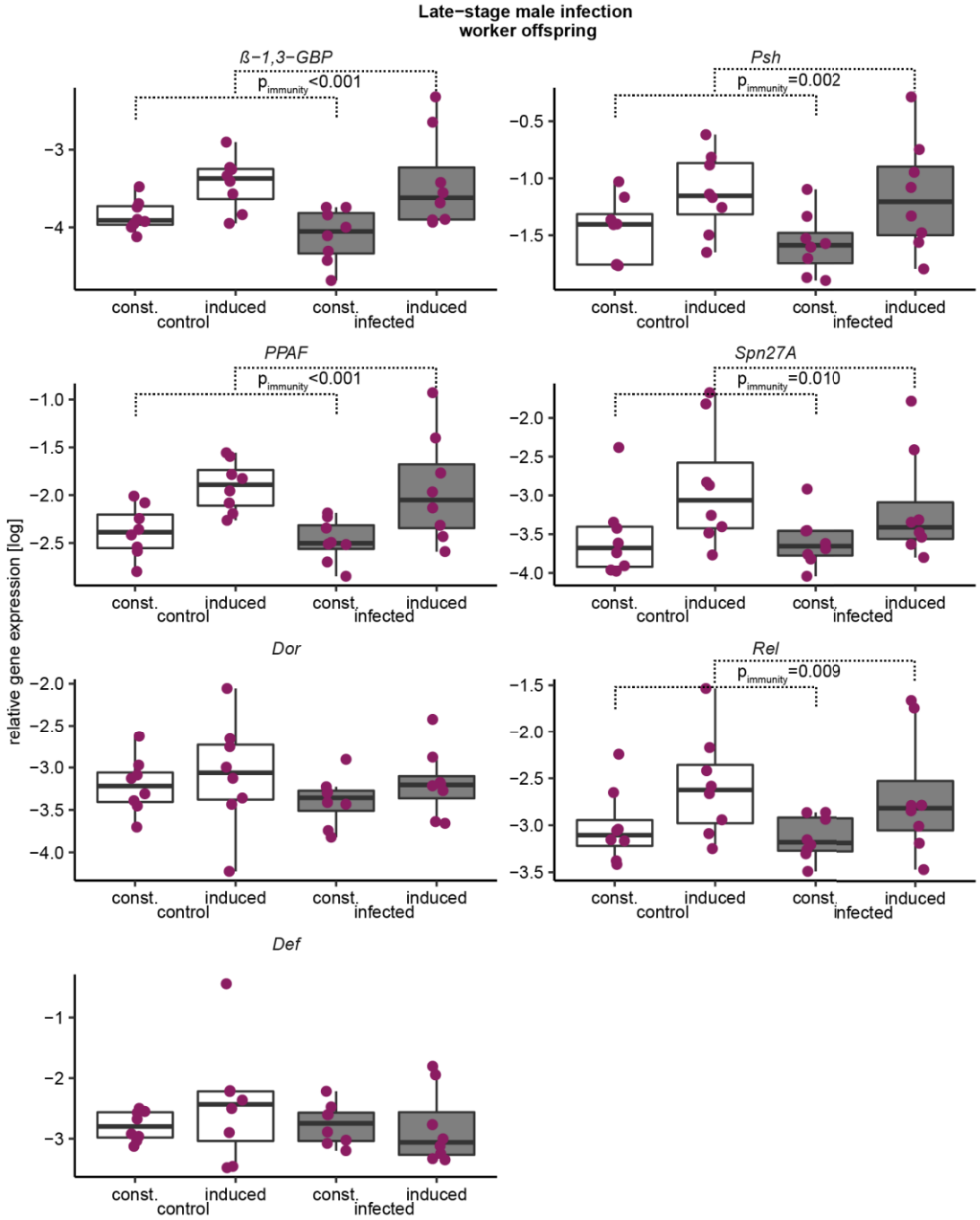

Figure 4.14. **Relative immune gene expression of worker offspring of queens that mated with a late-stage infected or a sham-treated male.** Data points (purple circles) indicate immune gene expression of individual workers grouped by their parental pathogen history (in white: worker offspring that originated from queens that mated with a sham-treated male and in black: worker offspring of queens that mated with late-stage infected male). Boxplots show median (bar), quartiles (box), and extremes (whiskers). Worker offspring that was challenged (induced immunity) had higher immune gene expression than untreated offspring (constitutive immunity) in 5/7 genes tested (indicated by dashed bars above the plot), but irrespective of their parental pathogen history. See also *Figure S 4.23.*

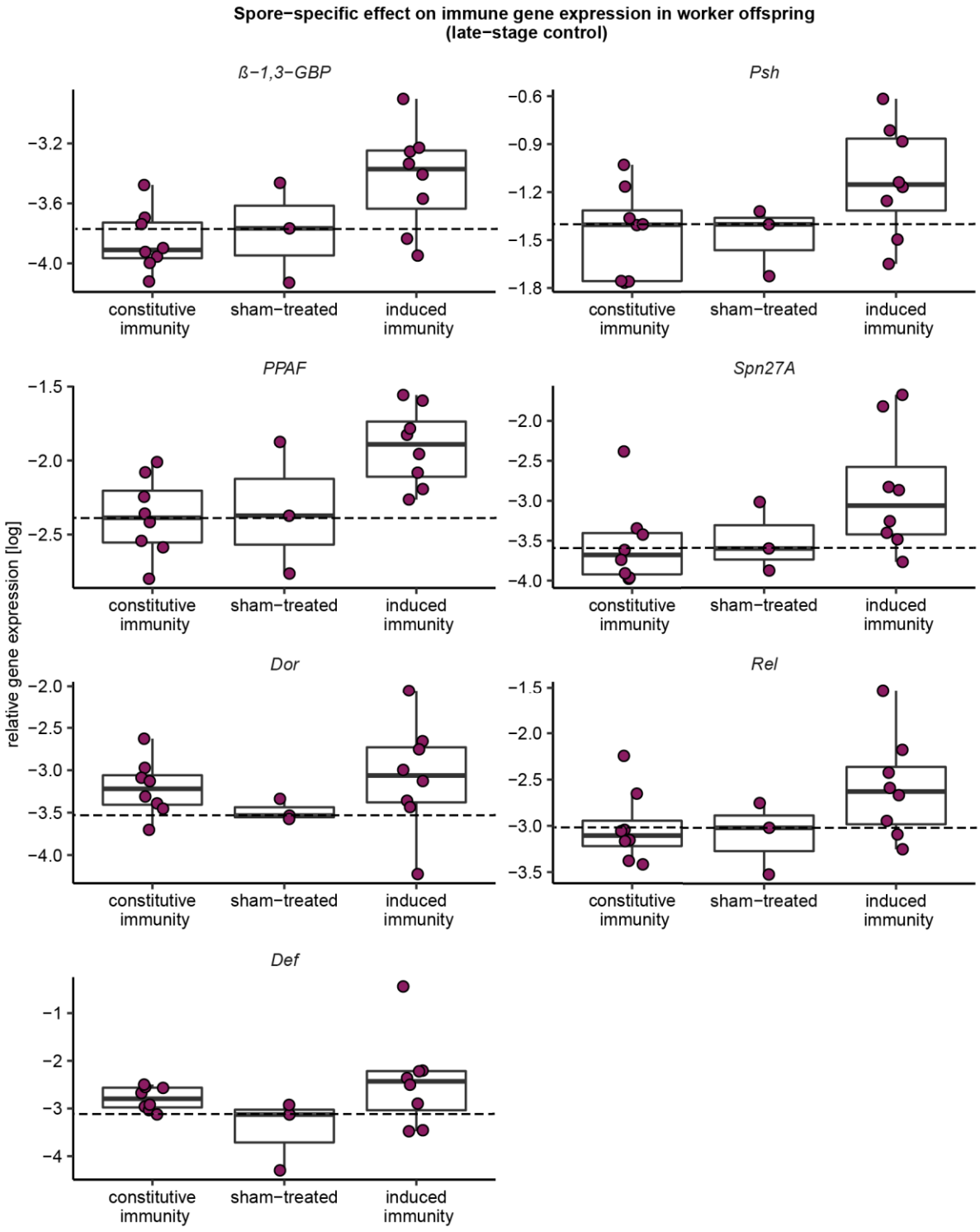

Figure 4.15. **Relative immune gene expression of worker offspring of queens that mated with a sham-treated male.** Data points (purple circles) indicate immune gene expression of individual workers grouped by the condition in which immune gene expression was measured (constitutive immunity: untreated, sham-treated: exposed to 0.05% Tx or induced immunity: exposed to fungal spores). Boxplots show median (bar), quartiles (box), and extremes (whiskers). The dashed line indicates the median immune gene expression of sham-treated workers and was used to evaluate whether the upregulation in immune gene expression of spore-exposed workers was induced by the spores or the surfactant in which the spores were submerged (0.05% Tx). No overlap indicates a spore-specific reaction of the immune genes.

Male offspring of queens that cohabited with a late-stage infected male show highest immune gene expression and react more sensitive to fungal spores

We found that queens that had contact with a late-stage infected male produced male offspring that were more sensitive to fungal challenge. We found that the combined effect of type of immunity (constitutive vs. induced) and pathogen history (sham-treated or late-stage infected male) affected gene expression, as indicated by a significant interaction term between these predictors in 4/7 genes tested (*PPAF*, *Spn27A*, *Rel* and *Def*, *Table 4.13, Figure 4.16*). Interestingly, male offspring of queens that cohabited with a sham-treated male seemed not to respond to a pathogen challenge as their immune gene expression after fungal challenge (induced immunity) did not differ from the immune gene expression of untreated males (constitutive immunity). However, male offspring of queens that cohabited with late-stage infected males reacted more pronouncedly to the fungal challenge, suggesting a higher sensitivity to pathogen challenge of male offspring of queens that had contact to a sick male.

Table 4.13. Statistical values for the overall models and main effects of male offspring depending on parental pathogen history (late-stage infection). The table shows the raw p-values of the overall models and the adjusted p-values in a separate column (p-values were adjusted according to the Benjamini-Hochberg correction at α=0.05).

gene	interaction	overall model	adj. p	type of immunity	pathogen history
β-1,3-GBP	χ^2=1.692, d.f.=1, p=0.193	χ^2=1.393, d.f=2, p=0.498	p=0.581		
Psh	χ^2=0.848, d.f.=1, p=0.357	χ^2=14.012, d.f.=2, p<0.001	p=0.002	χ^2=12.406, d.f.=1, p<0.001	χ^2=2.718, d.f.=1, p=0.099
PPAF	χ^2=6.768, d.f.=1, p=0.009	χ^2=21.953, d.f=3, p<0.001	p<0.001		
Spn27A	χ^2=6.360, d.f.=1, p=0.012	χ^2=17.106, d.f=3, p<0.001	p=0.002		
Dor	χ^2=0.003, d.f.=1, p=0.956	χ^2=0.350, d.f.=2, p=0.840	p=0.840		
Rel	χ^2=6.504. d.f.=1, p=0.011	χ^2=8.476, d.f.=3, p=0.037	p=0.052		
Def	χ^2=5.437, d.f.= 1, p=0.020	χ^2=11.556, d.f.=3, p=0.009	p=0.016		

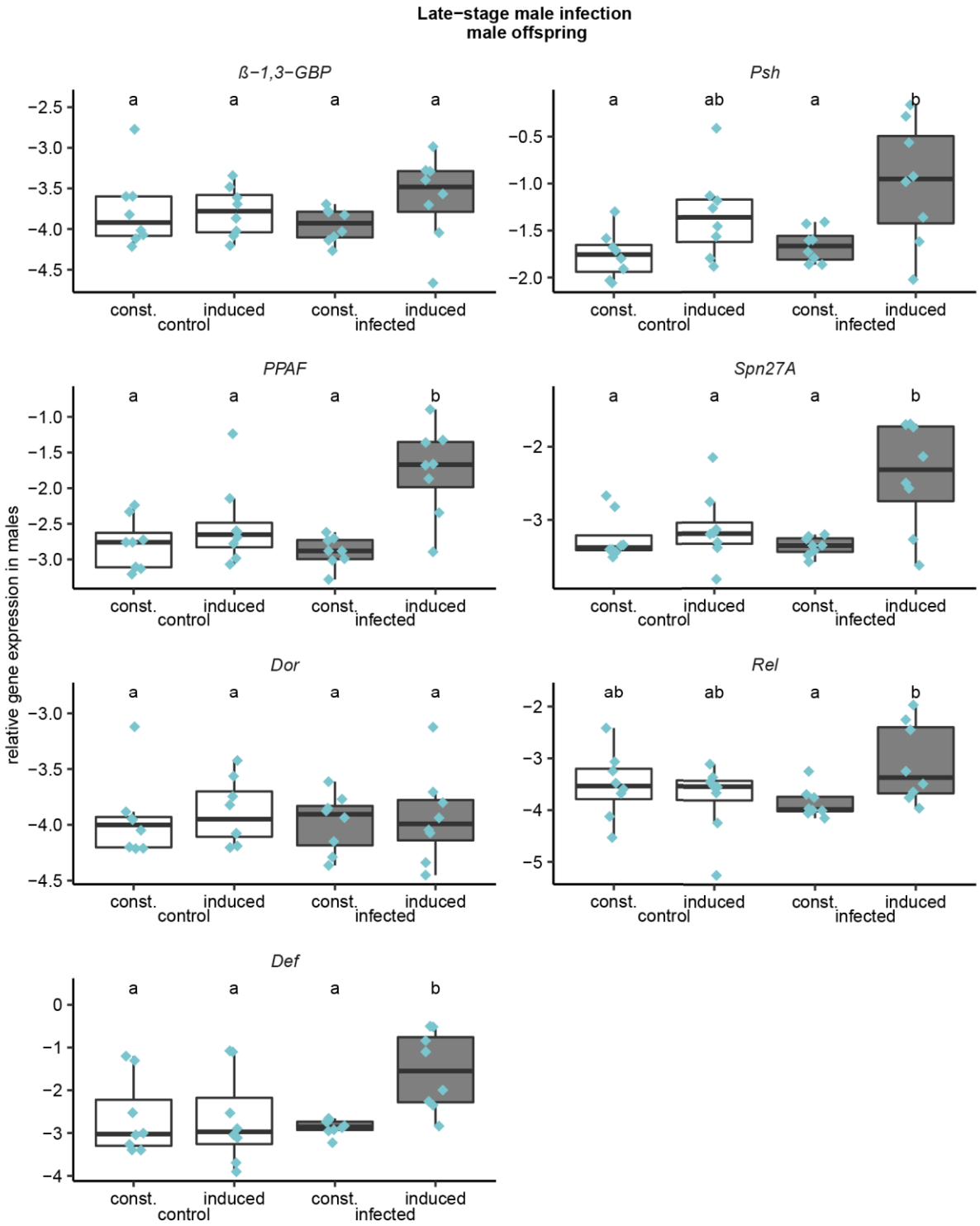

Figure 4.16. **Relative immune gene expression of male offspring of queens that cohabited with a late-stage infected or a sham-treated male.** Data points (blue diamonds) indicate immune gene expression of individual males grouped by their parental pathogen history (in white: male offspring that originated from queens that cohabited with a sham-treated male and in grey: male offspring of queens that cohabited with an early-stage infected male). Boxplots show median (bar), quartiles (box), and extremes (whiskers). In 4/7 genes tested (*PPAF*, *Spn27A*, *Dor* and *Def*) we found a significant interaction of type of immunity (constitutive immunity and induced immunity) and parental pathogen history. Male offspring of queens that cohabited with a late-stage infected male had higher immune gene expression after fungal challenge (induced immunity), indicating that they were more sensitive to fungal challenge. Different letters indicate significance groups of all pairwise post hoc comparisons after Benjamini–Hochberg correction at α=0.05. See also Figure *S 4.24*.

4.9 Discussion – transgenerational protection

In this study we have analyzed how offspring immunity is affected by parental pathogen history.
We found that worker offspring of queens that had mated with an early-stage infected male, from which they may have contracted spores, appeared to be immunocompromised (in *ß-1,3-GBP*, *Spn27A* and *Rel* - even though statistically not significant this pattern could also be observed in *Dor* and *Def)* compared to queens that have mated with a sham-treated male. Unexpectedly, we found no difference of constitutive and induced immunity, suggesting that the dose we have used might have been too low or the time point when we measured was too early, given that *Metarhizium* infects its host in a dose-dependent manner. However, the same dose has led to an upregulation of induced immunity in worker offspring when the queens were cohabiting with a late-stage infected male. Alternatively, it could also be that the queen produced offspring with an altered cuticle when she was infected herself or when she sensed that the male in her vicinity was contaminated with spores, which would make it harder for the spores to penetrate. A thicker or more melanized cuticle would make the offspring more tolerant to external pathogens or at least cause a delay for pathogen invasion (Barnes and Siva-Jothy, 2000).
As we found a difference between constitutive and induced immunity in worker offspring of queens that have mated with a late-stage infected male, we can at least conclude that theoretically we looked at genes that would be differentially expressed upon exposure, but we can currently not clearly verify that the upregulation we observed is driven by the spores or is a reaction to the surfactant 0.05%Tx - though the former is more likely.
However, in worker offspring of queen that mated with a late-stage infected male, we did not find an effect of paternal pathogen history, indicating that there might either be no protection transferred via a late-stage infected father or that mating with a late-stage infected male would have negative effects on the immunocompetence of the offspring, but gets compensated by a positive epigenetic effect of the mother that senses an infected partner (as we observed it in male offspring of queens that cohabited with a late-stage infected male). To be able to prove this hypothesis, we would need the male offspring of queens that have mated with a late-stage infected male and would expect these males to react more sensitively to fungal challenge as well. Unfortunately, we do not have any male offspring of queens that have mated with a late-stage sham-treated control male to compare the few male samples we got from queens that mated with late-stage infected males.
As we had only very few virgin queen offspring of mated queens, we were not able to disentangle whether we simply do not find protection in worker offspring, but would find it in queens, as it could be a strategy of the queen to protect specifically her sexual offspring and thus the next generation of colonies. In male offspring of queens that co-inhabited with early-stage infected males we did not detect any effects of pathogen history.
Unexpectedly, we found that queens cohabiting with a late-stage infected male produced male offspring that had increased investment into immune genes and reacted more pronouncedly to fungal challenge. This could either be induced by, e.g., immune effectors transferred from the late-stage infected male via trophallaxis to the queen or a consequence of a maternal epigenetic effect induced by the perception of a sick individual in her vicinity. The latter matches a study in *Drosophila* that found that mothers that cohabitated with a parasitic wasp produced offspring with better immunocompetence (Bozler *et al.*, 2020). A similar effect was reported in mice where mothers that were housed next to an infected conspecific had higher levels of serum corticosterone and increased kidney growth compared to mothers that were housed next to uninfected conspecifics (Curno *et al.*, 2009). Besides the effect on their own physiology, even their offspring was affected: the offspring of these mothers showed an accelerated immune

response to the same parasite and were less aggressive (Curno *et al.*, 2009). Both studies indicate that the mere sight of an infected individual is sufficient to alter offspring immunocompetence. There is further support in humans and canary birds that visual cues alone can alter the own immune function, as both studies reported that being near an infected individual compelled the immune system to preemptive action (Schaller *et al.*, 2010; Love *et al.*, 2021). Similarly, there was a study in *Tribolium castaneum* that showed that untreated beetles that were in a group of wounded conspecifics had reduced *Hsp90* gene expression from sharing a stressful environment (Peuß *et al.*, 2015).
In contrast to the increased sensitivity of male offspring in induced immunity when their mothers had contact with a late-stage infected male, we found no upregulation of immune gene expression after pathogen challenge in males whose mother had only contact to a healthy control male. It could either be that the fungal dose was too low (however, this is unlikely, as we found that male offspring in the early-stage setups showed an upregulation in immunity after fungal challenge) or that the presence of a healthy potential mate is inhibiting induced immunity in the male offspring – however this effect was absent for male offspring when the mother was cohabiting with a sham-treated male in the late-stage experiment. Theoretically, since there was no pathogen history in either case, we would have expected the same effect. The only difference between sham-treated males of the early- and the late-stage experiment is that they had been exposed to 0.05% Tx either 48h before the experimental start (late-stage experiment) or right before the experiment (early-stage experiment). Therefore, it could be that the males differed in their stress level as late-stage infected males were not only kept in isolation, but also received a sham-challenge in addition, whereas the early-stage infected males had at least a queen with them when they were sham-treated that could have groomed them.

Generally, it is very difficult to compare across different castes and sexes and the life-histories of queens, males and workers are very different. Queens and workers share the same sex, however queens monopolize reproduction in the colony, whereas workers are sterile and take care of the brood and the queen, forage for food and defend and maintain the colony. In contrast, males do not participate in colony activity and rather focus on fighting, mating and reproduction.
In this regard, observing different gene expression patterns between sexual offspring and workers would be very interesting, as it could hint to different mechanisms of how the current maternal colony and also daughter colonies are protected. However, to be able to draw such conclusions, one would need to minimize co-founding factors such as mating status, colony of origin, maternal and paternal infection status (ideally standardize pathogen load or at least reduce variation) and match the exposure dose of the offspring. Ideally, one would only use queens that are surely mated and produce sexuals as well as workers and determine maternal and paternal health status and compare only between parents with a similar pathogen load. However, as we are dealing with very tiny individuals with low pathogen loads, pathogen loads on an individual level might fall below the detection threshold of our molecular methods and would therefore be difficult to monitor. One alternative could be to determine the pathogen load in pools and compensate for occurring variability by using a larger sample size.
Furthermore, the spore dose applied to the offspring should be adjusted to a dose that induces the same mortality in sexuals and workers at a specific time point. As the workers' cuticle is harder than the cuticle of sexuals, it is likely that workers are less susceptible to fungal spores and that the spores take longer to penetrate the cuticle and activate the immune system. One would need to find a dose that is high enough to stimulate the immune system reliably, but at the same time is not so high that the immune system is overwhelmed. In the current setup worker and sexual offspring were not exposed to matching pathogen doses – workers were

exposed to a dose that would induce 30% mortality within a week, whereas sexuals were exposed to a lower spore number but this would cause a higher mortality (as we had to increase the dose that would induce the same morality in sexuals by factor 10 to ensure that every individual would receive some spores). Parental exposure dose could also be crucial, because in general priming responses are often not only pathogen-specific but also dose-specific (Pham *et al.*, 2007; Ng *et al.*, 2014). Effector molecules produced during the initial immune response might get degraded or depleted by the priming response itself, explaining why priming usually does not work as well at low doses (Cooper and Eleftherianos, 2017), likewise a dose that is too high might prevent a priming response in the host (Duneau *et al.*, 2016).
As the males in our experiment only need to survive until they have successfully mated – and we found that they are still able to mate when they are already moribund - the dosage in males would not be a limiting factor in future experiments and dropouts could be compensated with a high sample size. Ideally, the spore dose of male mating partners would be increased in future experiments (especially in the early-stage infected males), as this would reduce the variation in the number of spores that get transmitted during social interactions (so far only 0.7 on average were transmitted, meaning that not every queen got cross-contaminated in the current experiment). Apparently, however, queens are very vulnerable to spores (maybe already the surfactant) especially at the early-stage of colony foundation, which could make it difficult in future experiments to find the appropriate dose as queens seem to be easily overwhelmed. There were several studies that showed that mating triggers immunity in ant queens (Baer *et al.*, 2006; Castella *et al.*, 2009), which could explain why especially virgin queens might react so sensitively to low spore dosages. Pathogen resistance was found to be higher in mated and reproductive females in both social and solitary insects (Shoemaker *et al.*, 2006; Valtonen *et al.*, 2010; Gálvez and Chapuisat, 2014).
It is possible that *C. obscurior* queens in the field would be selected against if they have contracted a pathogen in their early-reproductive phase, as they can quickly be replaced by other virgin queens from the same maternal colony (Heinze, 2017).

To this end, besides having a high variation within individuals and a small sample size for all of our treatments tested, we are limited in the conclusions we can draw as we cannot compare the differences we find in male and worker offspring as they are (i) different castes, (ii) have a different sex, (iii) originated from queens with different mating status and (iv) were challenged with a different dosage of fungal spore suspensions.
Currently, it appears that (likely) infected queens produce (worker) offspring with lower immune investment, whereas queens that sense a potential pathogen risk may produce (male) offspring with increased investment in immunity. Another valuable addition to the existing experiments would be to test whether the transcriptional pattern of immune genes would also mirror translational evidence, e.g., by measuring antifungal activity or survival of the offspring.

4.10 Overall conclusion

In this study we examined the short-term and long-term effect of the obligate killing fungal pathogen *Metarhizium robertsii* on mating behavior and success of queens and males and the effect on the immunocompetence of the offspring.
We found that early-stage infected sexuals profit from sanitary care performed by workers as this increased their survival and their mating success, but workers did not discriminate against early- or late-stage infected individuals. In case workers were absent, we found that social contact (especially mating) with a contagious early-stage infected male was indeed risky for the queen due to spore contraction. Already the minute dosage determined in our experiments

(0.7 spores on average per queen) had several negative effects on the queen and her offspring: queens had a lifespan that was reduced by 39% and they produced less offspring with lower immunocompetence. We found that queens were 30% less mated when there were no workers present that could groom off spores from contagious males and themselves, indicating that the queens may have refused risky mating attempts or that males were not able to mate anymore, as they potentially faced a reproduction-immunity trade-off. Overall, queens were very vulnerable to spores, as their survival within the three days of mating interval – even in the presence of workers – was already reduced by 25%. All queens (except one) died during the mating interval when they were late-stage infected and even the low dose in the long-term experiment drastically reduced their survival. In summary, this shows that infection is very deleterious for queens.

Even though late-stage infected males were still able to mate successfully (they only had a reduced time window for it) with high sperm viability, it seems as they would not provide any protection to the worker offspring, as we found no difference in the immunocompetence of worker offspring depending on paternal pathogen history. Though, we found a positive effect of cohabitation with a late-stage infected male – likely based on a maternally transferred epigenetic effect when the queen perceived a late-stage infected male in her vicinity. Unfortunately, we could not analyze male offspring of mated queens, which could provide information on whether the positive effect of perception of a late-stage infected male is negated by mating with it. Overall, we found hints that there might be transgenerational protection to male offspring, but we would need to increase the sample size to be able to draw firm conclusions. In social species, eliciting an immune response upon mere contact to an infected conspecific, providing protection to both the individual and offspring, would benefit the entire colony while still allowing for close contact and social interactions.

Experimental work contribution in detail:
Experimental setups short-term effects: own 100%
Sperm viability with Jessica Kirchner (performed dissections): own 80%
Mating setups for spore transfer: own 100%
ddPCR for spore transfer with Anna Grasse: own 80%
Experimental setups long-term effects with Jennifer Robb and JK: own 70%
ddPCR for immune gene expression: JK 100%

4.11 Supplement

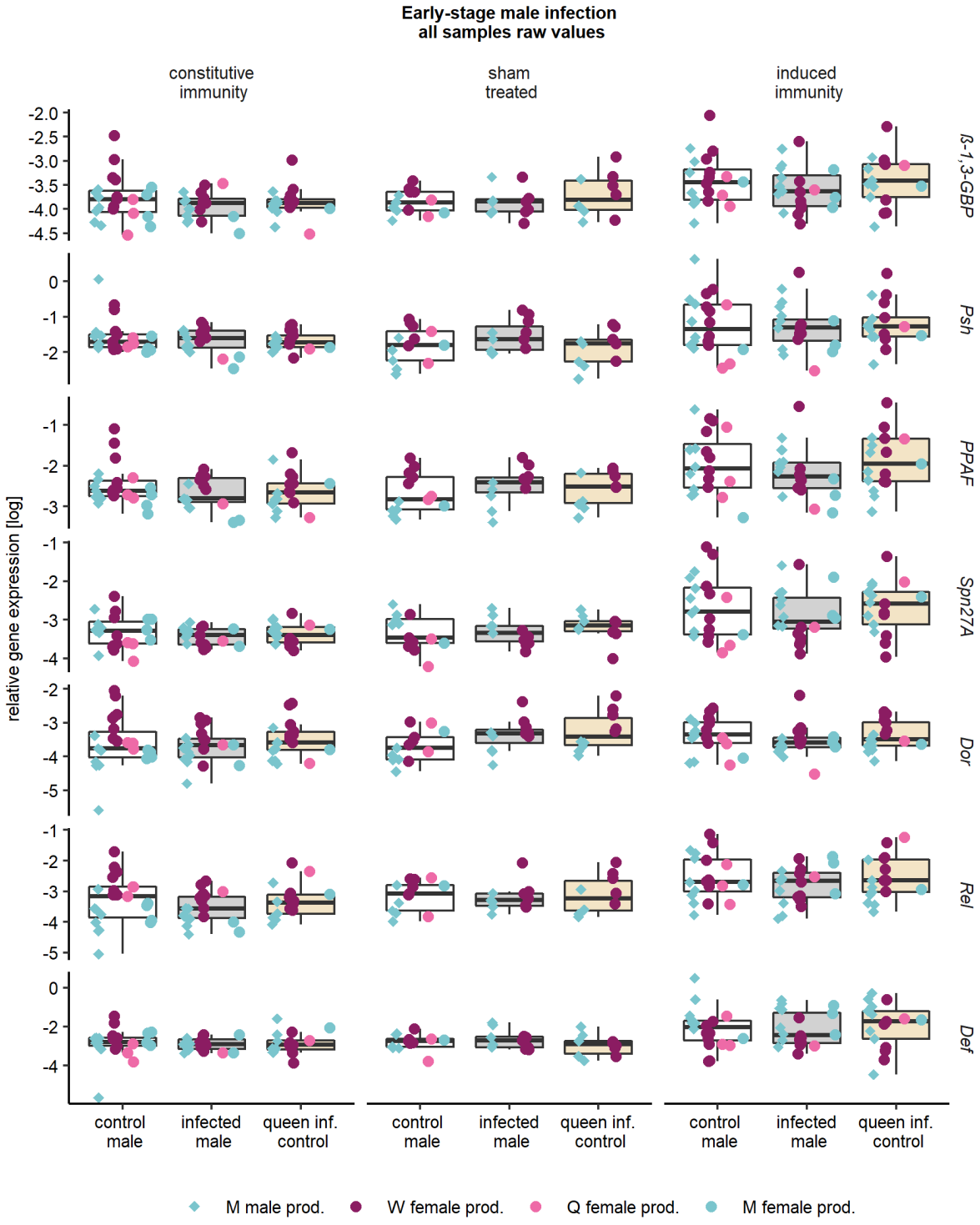

Figure S 4.17. **Overview of all offspring samples in which immune gene expression was measured (early-stage infection).** Data points indicate individuals in which immune gene expression was measured grouped by parental pathogen history (white: sham-treated males, grey: early-stage infected males and beige: queens that were manually exposed to fungal spores (queen infection control). Male offspring of male-producing queens ("M male prod.") are represented by blue diamonds. The offspring of female-producing queens ("female prod.") is represented by circles (purple: "W" worker offspring, pink: "Q" virgin queen offspring and blue: "M" male offspring). Boxplots show median (bar), quartiles (box), and extremes (whiskers).

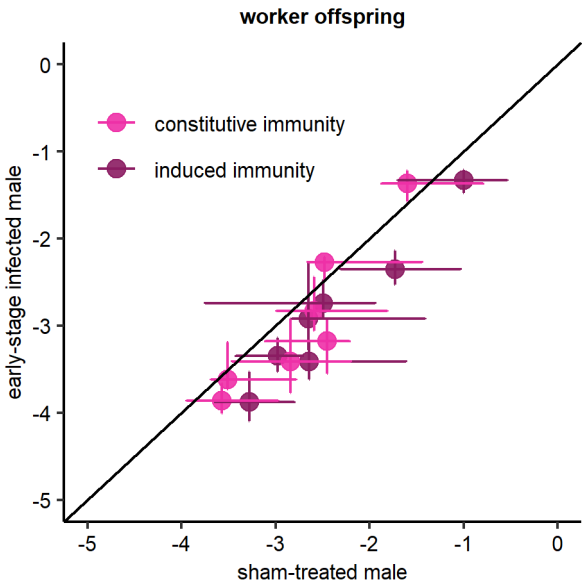

Figure S 4.18. **Average immune gene expression of worker offspring depending on parental pathogen history (early-stage infection).** Worker offspring of queens that mated with an early-stage infected male had lower immune gene expression levels (this applies to both induced and constitutive immunity) than worker offspring of queens that mated with sham-treated males. Data points represent the median in immune gene expression measured for constitutive (pink) and induced (purple) immunity. The error bars indicate 95% confidence intervals. The black line indicates a 1:1 ratio in immune gene expression of offspring with different parental pathogen histories. No or only very little overlap suggests that immune gene expression of worker offspring of queens that mated with a sham-treated control male is lower than that of queens that mated with an early-stage infected male.

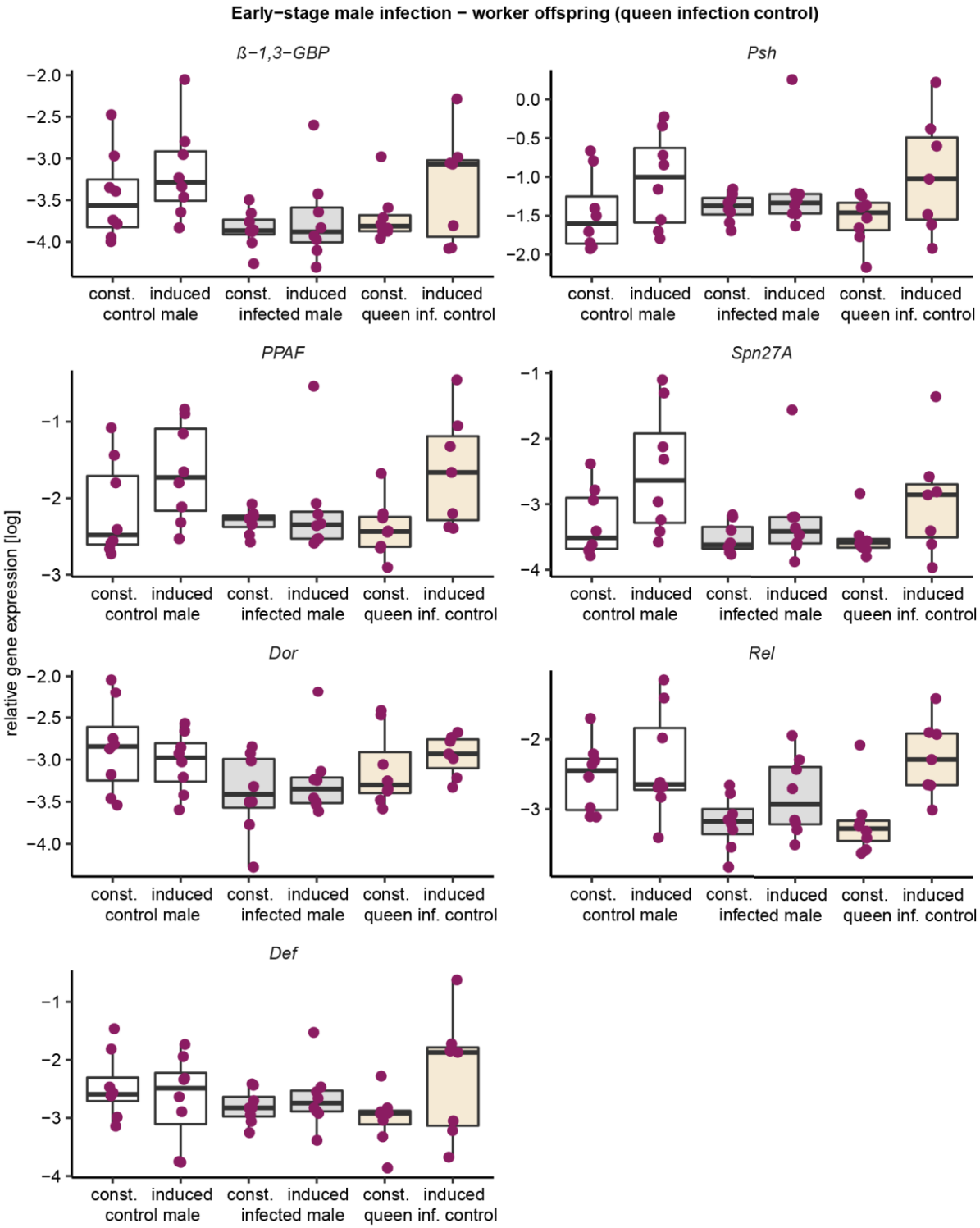

Figure S 4.19. **Relative immune gene expression of worker offspring – comparison to "queen infection control".** Data points (purple circles) indicate immune gene expression of individual workers grouped by their parental pathogen history (in white: sham-treated mating partner, in grey: early-stage infected mating partner and in beige: queens that mated with a sham-treated male and were manually exposed (queen infection control)). Boxplots show median (bar), quartiles (box), and extremes (whiskers). Comparing the worker offspring of queens that have mated with an early-stage infected male to the worker offspring of queens that we have manually infected ourselves, it sometimes looks as if these offspring would react slightly more pronouncedly to fungal challenge. Assuming that manual spore application reduced the variation in queen spore number (compared to what queens would have naturally received from a mating partner), this may indicate that the amount of spores received by the queen may be reflected in the immunity of the offspring.

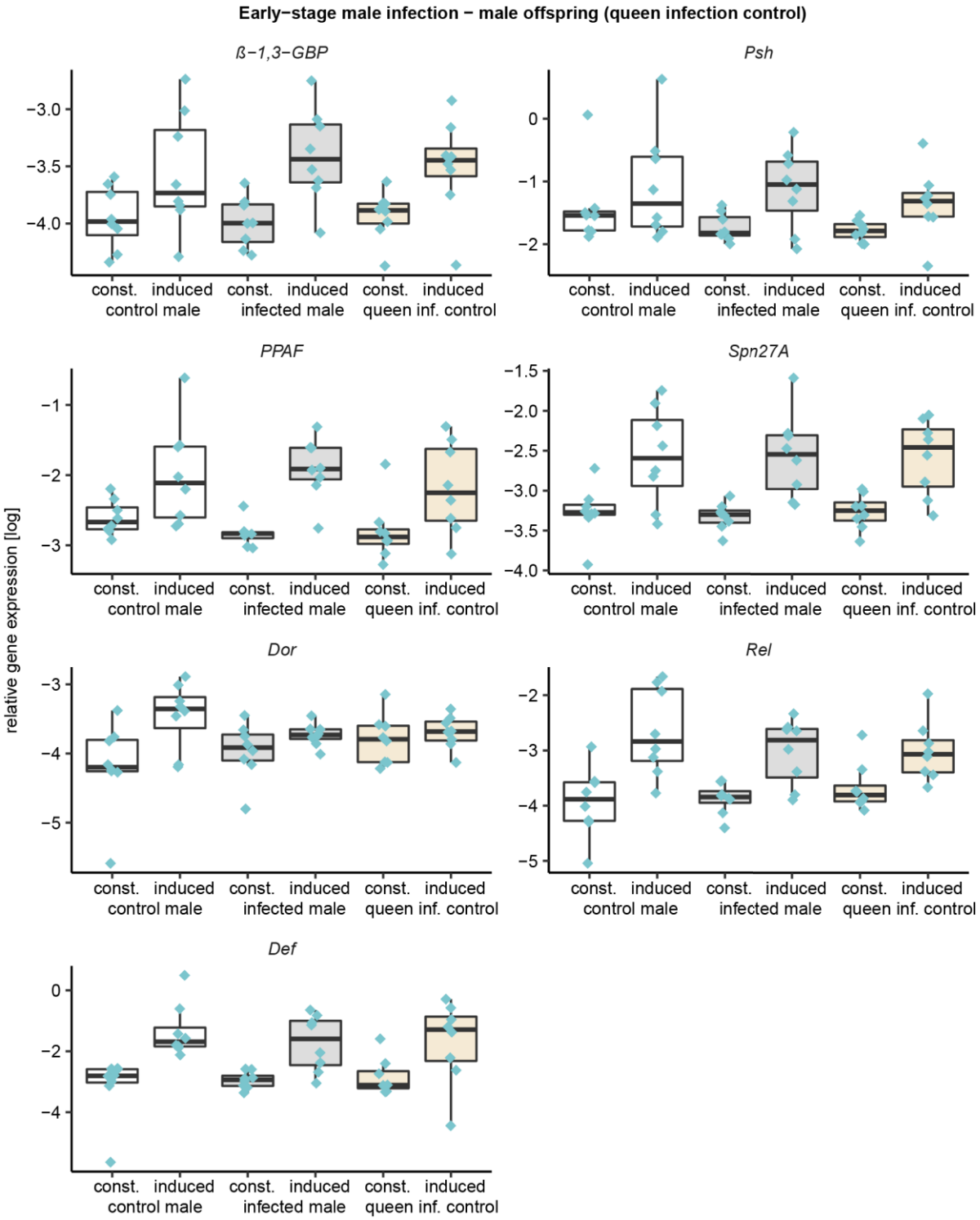

Figure S 4.20. **Relative immune gene expression of male offspring – comparison to "queen infection control".** Data points (blue diamonds) indicate immune gene expression of individual males grouped by their parental pathogen history (in white: sham-treated cohabitant, in grey: early-stage infected cohabitant and in beige: queens that cohabited with a sham-treated male and were manually exposed (queen infection control)). Boxplots show median (bar), quartiles (box), and extremes (whiskers). When comparing the male offspring of queens that cohabited with an early-stage infected male to those of queens that were manually exposed, it becomes apparent that the immune gene expression of both is very similar. From this, it seems that low-level infected queens do not prime their male offspring.

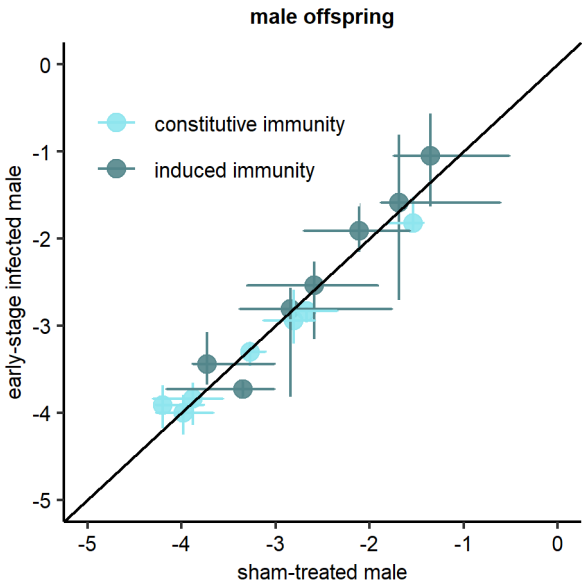

Figure S 4.21. **Average immune gene expression of male offspring depending on parental pathogen history (early-stage infection).** Data points represent the median in immune gene expression measured for constitutive (light blue) and induced (dark blue) immunity. The error bars indicate 95% confidence intervals. The black line indicates a 1:1 ratio in immune gene expression of offspring with different parental pathogen histories. The great overlap with the 1:1 ratio shows that parental pathogen history does not affect immune gene expression of male offspring.

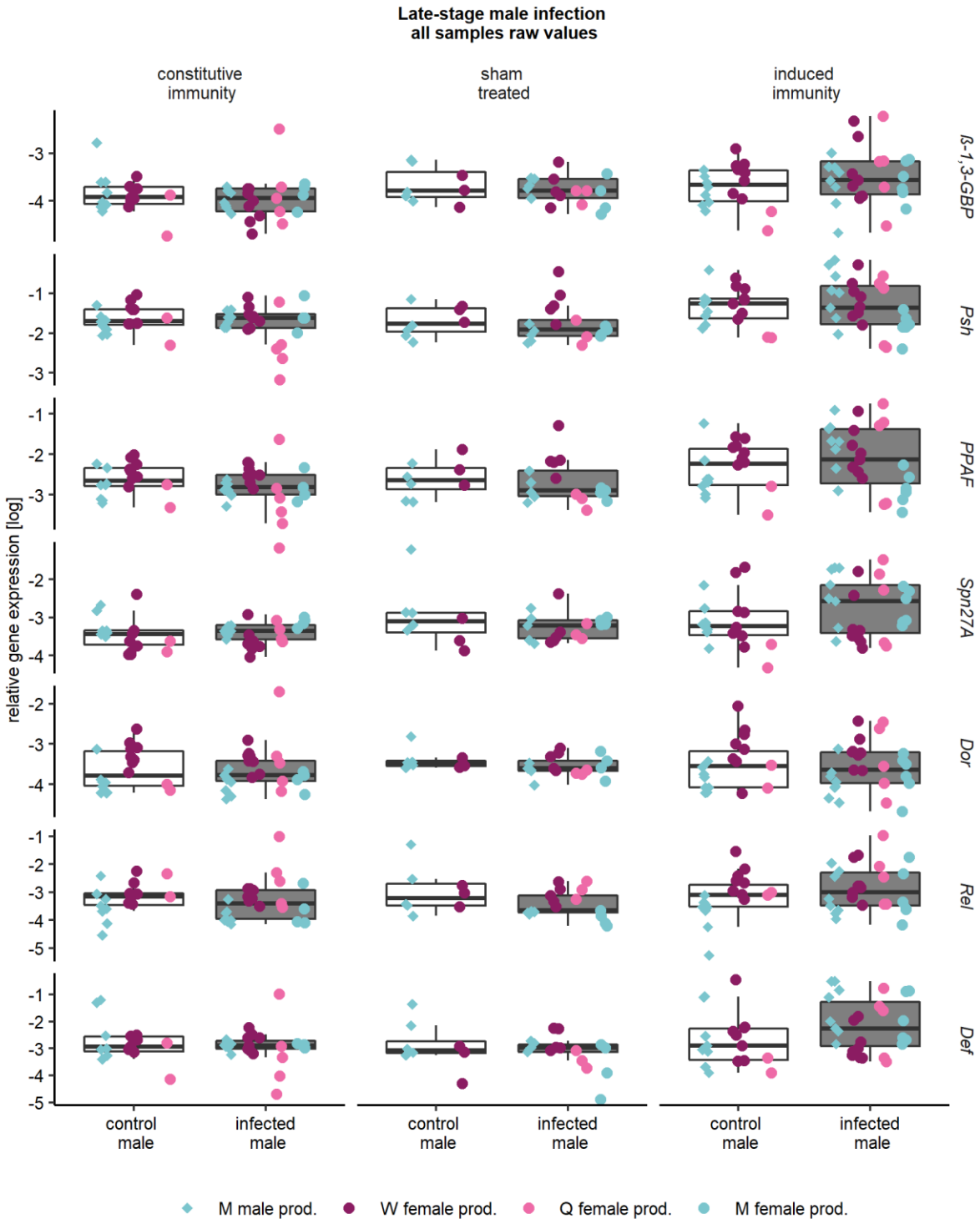

Figure S 4.22. **Overview of all offspring samples in which immune gene expression was measured (late-stage infection).** Data points indicate individuals in which immune gene expression was measured grouped by parental pathogen history (white: sham-treated males, black: late-stage infected males). Male offspring of male-producing queens ("M male prod.") are represented by blue diamonds. The offspring of female-producing queens ("female prod.") is represented by circles (purple: "W" worker offspring, pink: "Q" virgin queen offspring and blue: "M" male offspring). Boxplots show median (bar), quartiles (box), and extremes (whiskers).

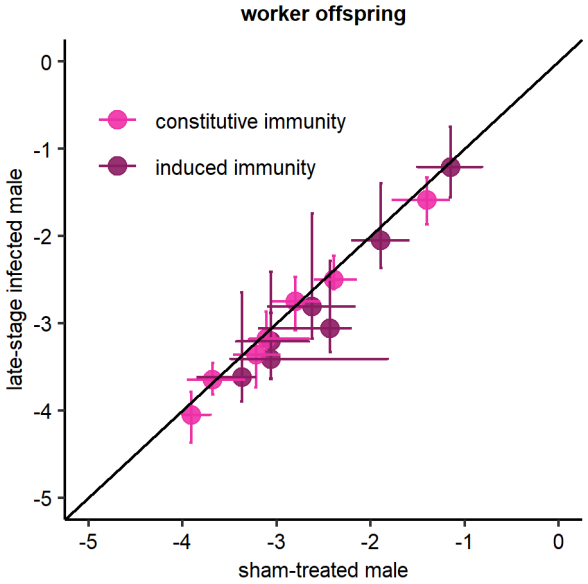

Figure S 4.23. **Average immune gene expression of worker offspring depending on parental pathogen history (late-stage infection).** Data points represent the median in immune gene expression measured for constitutive (pink) and induced (purple) immunity. The error bars indicate 95% confidence intervals. The black line indicates a 1:1 ratio in immune gene expression of offspring with different parental pathogen histories. The great overlap with the 1:1 ratio shows that parental pathogen history does not affect immune gene expression of worker offspring.

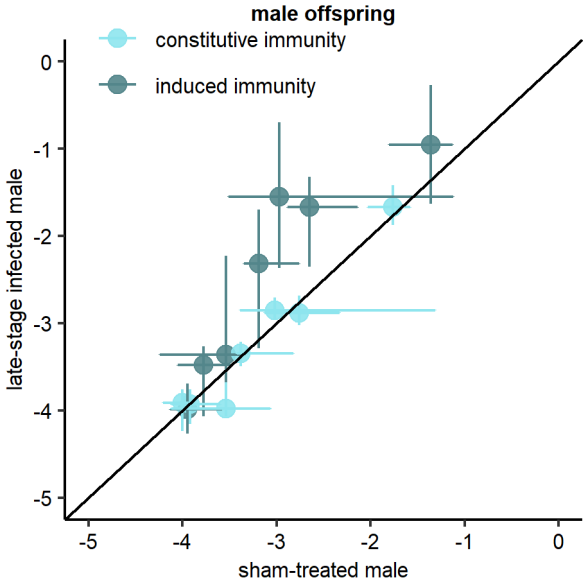

Figure S 4.24. **Average immune gene expression of male offspring depending on parental pathogen history (late-stage infection).** Data points represent the median in immune gene expression measured for constitutive (light blue) and induced (dark blue) immunity. The error bars indicate 95% confidence intervals. The black line indicates a 1:1 ratio in immune gene expression of offspring with different parental pathogen histories. Male offspring of queens that cohabited with a late-stage infected male had higher immune gene expression after fungal challenge (induced immunity), indicating that they were more sensitive to fungal challenge.

5. Conclusions

Social immunity is a crucial part of colony life, as it allows the ants to thrive in a pathogen rich environment (Ugelvig and Cremer, 2007; Cremer *et al.*, 2018). Grooming – one of the most important sanitary care measures - is very effective in reducing the risk of infection for the individual that just recently came in contact to an external pathogen e.g. fungal spores (Reber *et al.*, 2011), but at the same time close contact to contagious individuals increases an individual's own risk of low-level infection (Konrad *et al.*, 2012). These socially acquired low-level infections were found to have a protective effect after a secondary encounter with the same fungal strain (Ugelvig and Cremer, 2007; Konrad *et al.*, 2012), but can also be costly e.g. when the secondary pathogen encounter was with a very dissimilar pathogen (Konrad *et al.*, 2018).

In the **second chapter**, we dissected this benefit-cost axis by using phylogenetically-distant fungal strains of *M. brunneum* and *M. robertsii* in *L. neglectus*. We found that cross-protection is possible when the second pathogen is of the same species (species-level protection), whereas different species of the same genus as secondary challenge did neither induce a protection nor a cost, as known by the detrimental effects of secondary challenge with a different genus (Konrad *et al.*, 2018) where ants developed harmful superinfections. Such detrimental consequences have positive feedback and fueled the evolution of host defenses in a way that ants can recognize the threat and alter their social immunity behavior towards nestmates contaminated with a very different fungus. Ants continued to care for contaminated nestmates, but in a risk-averse manner where grooming was replaced by less risky disinfection by application of formic acid that does not require close contact (Konrad *et al.*, 2018). There might be a dual cause that can explain why low-level infections can be cross-protective, neutral or sometimes even deleterious when the host encounters a secondary threat, in dependence on the phylogenetic difference between the first and second pathogen. It could either result from (i) a too specific immunity that is formed against the first fungal strain, making the secondary infection challenging for the host (either because there are not enough resources for a new response or the immune system gets directed into one direction that doesn't help in the second challenge or (ii) fungal competition within the host, where two very different fungi compete for host resources by e.g. investing into rapid growth (Ben-Ami *et al.*, 2008, 2011).
Interestingly, we found that for the immune protection to occur, the first low-level infecting strain has to have lower virulence than the second strain and at the same time elicit higher immune gene expression. It could be that highly virulent strains overwhelm the immune system and that the host activates survival mechanisms instead of forming a priming response. This is further corroborated by the lack of a strong upregulation of immune genes upon low-level infection with more virulent strains, although we note that we only tested a very small subset of immune genes. To verify this hypothesis, a broader analysis would be necessary, including e.g. priming with a higher dose of the less virulent strains, to see if the beneficial protective effect would disappear, or with a lower dose of the more virulent strains. Although very speculative, pathogen load within the host was shown to be the key determinant of host immune gene expression (Tate and Graham, 2017) such that careful experiments controlling for pathogen load within the host should prove valuable in elucidating this. Overall, we showed that social immunization is probably shaped by a combination of within-host pathogen interactions and an activation of the host immune system.

In the **third and fourth chapters**, I studied how social immunity shapes other important colony traits such as mating behavior. So far, the influence of social immunity on sexual selection has not been addressed, but infection and disease signals have utmost importance on the fitness of

individuals. Recognizing and avoiding sick individuals is universal and can be found across all animal taxa by means of various cues. Fish and birds mainly rely on visual cues where infection often correlates with poor physical condition (often a loss of bright coloration) (Hamilton and Zuk, 1982; Milinski and Bakker, 1990; Poulin, 1994; Stephenson *et al.*, 2018) and even humans can identify sick individuals by examining facial photos (Axelsson *et al.*, 2018). Furthermore, auditory cues are used e.g. by vampire bats where individuals challenged with immunogenic lipopolysaccharide reduce contact calling rates (Stockmaier *et al.*, 2020). However, also sickness behaviors (i.e. lethargy and reduced social interactions) (Hart, 1990; Kent *et al.*, 1992; Dantzer, 2001) or olfactory signals can be universal cues for recognizing infected conspecifics. The latter is especially relevant in social insects such as ants, which heavily rely on chemical communication: here, fungus-infected ant pupae were shown to produce chemical cues that triggered hygienic behaviors in adult ants (Pull *et al.*, 2018a).
Recognizing sick individuals and avoiding mating with them will increase the likelihood of siring healthy offspring, so these traits should be under strong selection in species with overlapping generations, such as eusocial insects.
C. obscurior is a particularly good model to test the influence of social immunity on sexual selection, as mating occurs within the colony where workers are present and can perform defensive behaviors and males are fighting for mating. Workers can on the one hand take care of individuals that just recently came in pathogen contact e.g. by grooming off external pathogens, like fungal spores, and therefore reduce the risk of the contagious individual to develop a fatal infection and on the other hand prevent close contact of healthy individuals to contagious nestmates and thereby hamper disease transmission through the colony. Social immunity can therefore also relieve possible fecundity-immunity trade-offs of the sexuals (Calleri *et al.*, 2006; Schwenke *et al.*, 2016; Cole *et al.*, 2018; Casillas-Pérez *et al.*, 2022).

In the **third chapter**, I studied how different stages of disease influence male-male competition and the role of social immunity. Males with symptomatic infections were worse off in fights against healthy rivals as they were weakened by the progressing infection. Unexpectedly, males that were just freshly contaminated with fungal spores performed equally poorly. I found that this was due to an immunity-fighting trade-off – a hidden cost of mounting an immune response that would not have become apparent if the males were not exposed to the additional stress of rivalling an opponent. The fact that immune genes were already upregulated within the first 24h indicates that males invest more in immunity than expected for a social Hymenopteran male (Gerloff *et al.*, 2003; Vainio *et al.*, 2004; Baer *et al.*, 2005) – which can be attributed to their relatively long lifespan and the fact that they can be the dominant male in a colony for several weeks. Fighting is not only energetically costly for both opponents, but also bears the risk of cross-contamination when fighting against a contagious rival. I could show that even at extremely high pathogen doses, sanitary care performed by the workers can buffer the negative effect of disease transmission through the colony, as cross-contamination of the rival was only detectable in half of the cases. Even though social immunity proved to be very efficient, the risk of spore transmission, e.g. during mating remains, as typically the winner of a fight is the one that monopolizes mating with newly emerging virgin queens.

In the **fourth chapter**, I further investigated the effect of different stages of infection on reproductive success of queens and males. I found that even though the mating success of early- as well as late-stage infected individuals was reduced when workers were absent, risky mating attempts where pathogen transfer could happen were not per se avoided. It could either be that healthy mating partners refused risky mating attempts or that infected individuals were physically not able to mate anymore. In any case, social immunity operated effectively and could rescue the success of contagious sexuals who otherwise might suffer from fatal infections. As already very low pathogen dosages had several negative effects on the queen

and her offspring: queens had a 39% lifespan reduction and they produced less offspring with lower immunocompetence – this readily demonstrates the importance of social immunity and worker care in a colony and how strongly sexuals rely on pathogen defense mechanisms of the workers.
Surprisingly, I found a positive effect of cohabitation with a late-stage infected male – likely based on a maternal epigenetic effect when the queen perceived a late-stage infected male in her vicinity. In this case, male offspring reacted more sensitively upon fungal challenge compared to male offspring without parental pathogen history. Priming the local males could ultimately lead not only to protection of the current colony when they in turn protect their worker offspring, but also to the next generation of superorganisms in case virgin queen offspring gets protected. As virgin queens are able to produce primed male offspring at mere contact to an infected conspecific, the risk of pathogen transmission during social interactions, e.g. mating, is reduced and represents an effective way how several queens in the colony could protect their offspring at the same time.

6. References

Abe, J., Kamimura, Y. and Shimada, M. (2005) 'Individual sex ratios and offspring emergence patterns in a parasitoid wasp, *Melittobia australica* (Eulophidae), with superparasitism and lethal combat among sons', *Behavioral Ecology and Sociobiology*, 57(4), pp. 366–373. doi:10.1007/s00265-004-0861-y.

Able, D.J. (1996) 'The contagion indicator hypothesis for parasite-mediated sexual selection', *Proceedings of the National Academy of Sciences of the United States of America*, 93(5), pp. 2229–2233. doi:10.1073/pnas.93.5.2229.

Adamo, S.A., Roberts, J.L., Easy, R.H. and Ross, N.W. (2008) 'Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets', *Journal of Experimental Biology*, 211(4), pp. 531–538. doi:10.1242/jeb.013136.

Alexander, J. and Stimson, W.H. (1988) 'Sex hormones and the course of parasitic infection', *Parasitology Today*, 4(7), pp. 189–193. doi:10.1016/0169-4758(88)90077-4.

Anderson, R.M. and May, R.M. (1985) 'Vaccination and herd immunity to infectious diseases', *Nature*, 318(6044), pp. 323–329. doi:10.1038/318323a0.

Andersson, M. and Iwasa, Y. (1996) 'Sexual selection', *Trends in Ecology and Evolution*, 11(2), pp. 53–58. doi:10.1016/0169-5347(96)81042-1.

Armitage, S.A.O., Thompson, J.J.W., Rolff, J. and Siva-Jothy, M.T. (2003) 'Examining costs of induced and constitutive immune investment in *Tenebrio molitor*', *Journal of Evolutionary Biology*, 16(5), pp. 1038–1044. doi:10.1046/j.1420-9101.2003.00551.x.

Arnott, G. and Elwood, R.W. (2009) 'Assessment of fighting ability in animal contests', *Animal Behaviour*, 77(5), pp. 991–1004. doi:10.1016/j.anbehav.2009.02.010.

Avitsur, R. and Yirmiya, R. (1999) 'The immunobiology of sexual behavior: Gender differences in the suppression of sexual activity during illness', *Pharmacology Biochemistry and Behavior*, 64(4), pp. 787–796. doi:10.1016/S0091-3057(99)00165-3.

Axelsson, J., Sundelin, T., Olsson, M.J., Sorjonen, K., Axelsson, C., Lasselin, J. and Lekander, M. (2018) 'Identification of acutely sick people and facial cues of sickness', *Proceedings of the Royal Society B: Biological Sciences*, 285(1870), 20172430. doi:10.1098/rspb.2017.2430.

Backwell, P. and Jennions, M.D. (2004) 'Coalition among male fiddler crabs', *Nature*, 430, p. 417. doi:10.1038/430417a.

Baer, B., Armitage, S. A O. and Boomsma, J.J. (2006) 'Sperm storage induces an immunity cost in ants', *Nature*, 441(7095), pp. 872–875. doi:10.1038/nature04698.

Baer, B., Krug, A., Boomsma, J.J. and Hughes, W.O.H. (2005) 'Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection', *Insectes Sociaux*, 52, pp. 298–303. doi:10.1007/s00040-005-0809-x.

Barnes, A.I. and Siva-Jothy, M.T. (2000) 'Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): Cuticular melanization is an indicator of investment in immunity', *Proceedings of the Royal Society B: Biological Sciences*, 267(1439), pp. 177–182. doi:10.1098/rspb.2000.0984.

Barribeau, S.M. and Otti, O. (2020) 'Sexual reproduction and immunity', in *eLS*. John Wiley & Sons, Ltd: Chichester, pp. 1–10. doi:10.1002/9780470015902.a0028146.

Barribeau, S.M., Schmid-Hempel, P. and Sadd, B.M. (2016) 'Royal decree: Gene expression in trans-generationally immune primed bumblebee workers mimics a primary immune response', *PLOS ONE*, 11(7), pp. 1–13. doi:10.1371/journal.pone.0159635.

Bates, D., Mächler, M., Bolker, B.M. and Walker, S.C. (2015) 'Fitting linear mixed-effects models using lme4', *Journal of Statistical Software*, 67(1). pp. 1-48. doi:10.18637/jss.v067.i01.

Baxter, C.M., Barnett, R. and Dukas, R. (2015) 'Aggression, mate guarding and fitness in male fruit flies', *Animal Behaviour*, 109, pp. 235–241. doi:10.1016/j.anbehav.2015.08.023.

Ben-Ami, F., Mouton, L. and Ebert, D. (2008) 'The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system', *Evolution*, 62(7), pp. 1700–1711. doi:10.1111/j.1558-5646.2008.00391.x.

Ben-Ami, F., Rigaud, T. and Ebert, D. (2011) 'The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies', *Journal of Evolutionary Biology*, 24(6), pp. 1307–1316. doi:10.1111/j.1420-9101.2011.02264.x.

Benjamini, Y. and Hochberg, Y. (1995) 'Controlling the false discovery rate: A practical and powerful approach to multiple testing', *Journal of the Royal Statistical Society : Series B (Methodological)*, 57(1), pp. 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x.

Bischoff, J. F., Rehner, S. A., and Humber, R. A. (2006) '*Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex', *Mycologia*, *98*(5), pp. 37-745. doi: 10.1080/15572536.2006.11832645

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H. and White, J.S.S. (2009) 'Generalized linear mixed models: a practical guide for ecology and evolution', *Trends in Ecology and Evolution*, 24(3), pp. 127–135. doi:10.1016/j.tree.2008.10.008.

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B. and Sorci, G. (2003) 'Assessing the cost of mounting an immune response', *The American Naturalist*, 161(3), pp. 367–379. doi:10.1086/346134.

Boomsma, J.J. (2009) 'Lifetime monogamy and the evolution of eusociality', *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364(1533), pp. 3191–3207. doi:10.1098/rstb.2009.0101.

Boomsma, J.J. (2013) 'Beyond promiscuity: Mate-choice commitments in social breeding', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1613), 20120050. doi:10.1098/rstb.2012.0050.

Boomsma, J.J., Baer, B. and Heinze, J. (2005) 'The evolution of male traits in social insects.', *Annual Review of Entomology*, 50, pp. 395–420. doi:10.1146/annurev.ento.50.071803.130416.

Boomsma, J.J. and Gawne, R. (2018) 'Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation', *Biological Reviews*, 93(1), pp. 28–54. doi:10.1111/brv.12330.

Boomsma, J.J., Jensen, A.B., Meyling, N. V. and Eilenberg, J. (2014) 'Evolutionary interaction

networks of insect pathogenic fungi', *Annual Review of Entomology*, 59, pp. 467–485. doi:10.1146/annurev-ento-011613-162054.

Bordoni, A., Dapporto, L., Tatini, I., Celli, M., Bercigli, M., Barrufet, S.R., Perito, B. and Turillazzi, S. (2018) 'Trans-generational immunization in the acrobat ant *Crematogaster scutellaris*', *Biology Letters*, 14(4), pp. 10–13. doi:10.1098/rsbl.2017.0761.

Bourke, A.F.G. (2011) 'The validity and value of inclusive fitness theory', *Proceedings of the Royal Society B: Biological Sciences*, 278(1723), pp. 3313–3320. doi:10.1098/rspb.2011.1465.

Bozler, J., Kacsoh, B.Z. and Bosco, G. (2020) 'Maternal priming of offspring immune system in *Drosophila*', *G3: Genes, Genomes, Genetics*, 10(1), pp. 165–175. doi:10.1534/g3.119.400852.

Briffa, M. and Elwood, R.W. (2004) 'Use of energy reserves in fighting hermit crabs', *Proceedings of the Royal Society B: Biological Sciences*, 271(1537), pp. 373–379. doi:10.1098/rspb.2003.2633.

Brunner-Mendoza, C., Reyes-Montes, M. D. R., Moonjely, S., Bidochka, M.J. and Toriello, C. (2019) 'A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico', *Biocontrol Science and Technology*, 29(1), pp. 83–102. doi:10.1080/09583157.2018.1531111.

Buckling, A. and Brockhurst, M.A. (2008) 'Kin selection and the evolution of virulence', *Heredity*, 100(5), pp. 484–488. doi:10.1038/sj.hdy.6801093.

Calleri, D. V., Rosengaus, R.B. and Traniello, J.F.A. (2006) 'Disease and colony establishment in the dampwood termite *Zootermopsis angusticollis*: Survival and fitness consequences of infection in primary reproductives', *Insectes Sociaux*, 53(2), pp. 204–211. doi:10.1007/s00040-005-0859-0.

Candolin, U. (1999) 'The relationship between signal quality and physical condition: Is sexual signalling honest in the three-spined stickleback?', *Animal Behaviour*, 58(6), pp. 1261–1267. doi:10.1006/anbe.1999.1259.

Cappa, F., Beani, L., Cervo, R., Grozinger, C. and Manfredini, F. (2015) 'Testing male immunocompetence in two hymenopterans with different levels of social organization: "live hard, die young?"', *Biological Journal of the Linnean Society*, 114(2), pp. 274–278. doi:10.1111/bij.12427.

Casillas-Pérez, B., Pull, C.D., Naiser, F., Naderlinger, E., Matas, J. and Cremer, S. (2022) 'Early queen infection shapes developmental dynamics and induces long-term disease protection in incipient ant colonies', *Ecology Letters*, 25(1), pp. 89-100. doi:10.1111/ele.13907

Castella, G., Christe, P. and Chapuisat, M. (2009) 'Mating triggers dynamic immune regulations in wood ant queens', *Journal of Evolutionary Biology*, 22(3), pp. 564–570. doi:10.1111/j.1420-9101.2008.01664.x.

Cerenius, L., Lee, B.L. and Söderhäll, K. (2008) 'The proPO-system: pros and cons for its role in invertebrate immunity', *Trends in Immunology*, 29(6), pp. 263–271. doi:10.1016/j.it.2008.02.009.

Cerenius, L. and Söderhäll, K. (2004) 'The prophenoloxidase-activating system in invertebrates', *Immunological Reviews*, 198, pp. 116–126. doi:10.1111/j.0105-2896.2004.00116.x.

Cerenius, L. and Söderhäll, K. (2021) ‘Immune properties of invertebrate phenoloxidases’, *Developmental and Comparative Immunology*, 122, 104098. doi:10.1016/j.dci.2021.104098.

Cini, A., Bordoni, A., Cappa, F., Petrocelli, I., Pitzalis, M., Iovinella, I., Dani, F.R., Turillazzi, S. and Cervo, R. (2020) ‘Increased immunocompetence and network centrality of allogroomer workers suggest a link between individual and social immunity in honeybees’, *Scientific Reports*, 10, 8928 (2020). doi:10.1038/s41598-020-65780-w.

Clutton-Brock, T.H. (1984) ‘Reproductive effort and terminal investment in iteroparous animals’, *The American Naturalist*, 123(2), pp. 212–229.

Clutton-Brock, T.H., Albon, S.D., Gibson, R.M. and Guinness, F.E. (1979) ‘The logical stag: Adaptive aspects of fighting in red deer (*Cervus elaphus L.*)’, *Animal Behaviour*, 27, pp. 211–225. doi:10.1016/0003-3472(79)90141-6.

Cole, E.L., Empringham, J.S., Biro, C., Thompson, G.J. and Rosengaus, R.B. (2020) ‘Relish as a candidate marker for transgenerational immune priming in a dampwood termite (Blattodae: Archeotermopsidae)’, *Insects*, 11(3), 149. doi:10.3390/insects11030149.

Cole, E.L., Ilieş, I. and Rosengaus, R.B. (2018) ‘Competing physiological demands during incipient colony foundation in a social insect: consequences of pathogenic stress’, *Frontiers in Ecology and Evolution*, 6, 103. doi:10.3389/fevo.2018.00103.

Cooper, D. and Eleftherianos, I. (2017) ‘Memory and specificity in the insect immune system: Current perspectives and future challenges’, *Frontiers in Immunology*, 8, 539. doi:10.3389/fimmu.2017.00539.

Cremer, S., Armitage, S.A.O. and Schmid-Hempel, P. (2007) ‘Social immunity.’, *Current Biology*, 17(16), pp. R693–R702. doi:10.1016/j.cub.2007.06.008.

Cremer, S., D’Ettorre, P.D., Drijfhout, F.P., Sledge, M.F., Turillazzi, S. and Heinze, J. (2008a) ‘Imperfect chemical female mimicry in males of the ant *Cardiocondyla obscurior*’, *Naturwissenschaften*, 95, pp. 1101–1105. doi:10.1007/s00114-008-0430-8.

Cremer, S. and Heinze, J. (2002) ‘Adaptive production of fighter males: Queens of the ant *Cardiocondyla* adjust the sex ratio under local mate competition’, *Proceedings of the Royal Society B: Biological Sciences*, 269(1489), pp. 417–422. doi:10.1098/rspb.2001.1892.

Cremer, S. and Heinze, J. (2003) ‘Stress grows wings: Environmental induction of winged dispersal males in *Cardiocondyla* ants’, *Current Biology*, 13(3), pp. 219–223. doi:10.1016/S0960-9822(03)00012-5.

Cremer, S., Pull, C.D. and Fürst, M.A. (2018a) ‘Social Immunity: Emergence and evolution of colony-level disease protection’, *Annual Review of Entomology*, 63, pp. 105–123. doi:10.1146/annurev-ento-020117-043110.

Cremer, S. and Sixt, M. (2009) ‘Analogies in the evolution of individual and social immunity.’, *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364(1513), pp. 129–142. doi:10.1098/rstb.2008.0166.

Cremer, S., Sledge, M.F. and Heinze, J. (2002) ‘Chemical mimicry: Male ants disguised by the queen’s bouquet’, *Nature*, 419(6910), p. 897. doi:10.1038/419897a.

Cremer, S., Suefuji, M., Schrempf, A. and Heinze, J. (2012) ‘The dynamics of male-male competition in *Cardiocondyla obscurior* ants’, *BMC Ecology*, 12(7), pp. 11–15. doi:10.1186/1472-6785-12-7.

Cremer, S., Ugelvig, L. V, Drijfhout, F.P., Schlick-Steiner, B.C., Florian, M., Seifert, B., Hughes, D.P., Schulz, A., Petersen, K.S., Stauffer, C., Kiran, K., Espadaler, X., Ettorre, P. and Aktac, N. (2008b) 'The evolution of invasiveness in garden ants', *PLOS ONE*, 3(12), e3838. doi:10.1371/journal.pone.0003838.

Cronin, A.L., Molet, M., Doums, C., Monnin, T. and Peeters, C. (2013) 'Recurrent evolution of dependent colony foundation across eusocial insects', *Annual Review of Entomology*, 58, pp. 37–55. doi:10.1146/annurev-ento-120811-153643.

Cronin, A.L., Monnin, T., Haussy, C. and Doums, C. (2011) 'Opportunities for mate choice in the fission-performing ant *Cataglyphis cursor*', *Ecological Entomology*, 36(4), pp. 522–525. doi:10.1111/j.1365-2311.2011.01291.x.

Curno, O., Behnke, J.M., McElligott, A.G., Reader, T. and Barnard, C.J. (2009) 'Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy', *Proceedings of the Royal Society B: Biological Sciences*, 276(1659), pp. 1047–1054. doi:10.1098/rspb.2008.1612.

Dantzer, R. (2001) 'Cytokine-induced sickness behavior: mechanisms and implications', *Annals of the New York Academy of Sciences*, 933(1), pp. 222-234. doi:10.1111/j.1749-6632.2001.tb05827.x.

Darwin, C. (1871) '*The descent of man, and selection in relation to sex*' (Reprint. Princeton University Press, Princeton, 2008)

Davis, H.E., Meconcelli, S., Radek, R. and McMahon, D.P. (2018) 'Termites shape their collective behavioural response based on stage of infection', *Scientific Reports*, 8, 14433 (2018). doi:10.1038/s41598-018-32721-7.

Ditchkoff, S.S., Lochmiller, R.L., Masters, R.E., Hoofer, S.R. and Van Den Bussche, R.A. (2001) 'Major-histocompatibility-complex-associated variation in secondary sexual traits of white-tailed deer (*Odocoileus virginianus*): Evidence for good-genes advertisement', *Evolution*, 55(3), pp. 616–625. doi:10.1111/j.0014-3820.2001.tb00794.x.

Duneau, D., Ebert, D. and Du Pasquier, L. (2016) 'Infections by *Pasteuria* do not protect its natural host *Daphnia magna* from subsequent infections', *Developmental and Comparative Immunology*, 57, pp. 120–125. doi:10.1016/j.dci.2015.12.004.

Eisenegger, C., Haushofer, J. and Fehr, E. (2011) 'The role of testosterone in social interaction', *Trends in Cognitive Sciences*, 15(6), pp. 263–271. doi:10.1016/j.tics.2011.04.008.

Espadaler, X., Rey, S. and Bernal, V. (2004) 'Queen number in a supercolony of the invasive garden ant, *Lasius neglectus*', *Insectes Sociaux*, 51(3), pp. 232–238. doi:10.1007/s00040-003-0732-y.

Espadaler, X., Tartally, A., Schultz, R., Seifert, B. and Nagy, C. (2007) 'Regional trends and preliminary results on the local expansion rate in the invasive garden ant, *Lasius neglectus* (Hymenoptera, Formicidae)', *Insectes Sociaux*, 54(3), pp. 293–301. doi:10.1007/s00040-007-0944-7.

Evans, H.C. (1974) 'Natural control of arthropods, with special reference to ants (Formicidae), by fungi in the tropical high forest of Ghana', *The Journal of Applied Ecology*, 11(1), pp. 37-49. doi:10.2307/2402003.

Fang, W., Pei, Y. and Bidochka, M.J. (2006) 'Transformation of *Metarhizium anisopliae* mediated by *Agrobacterium tumefaciens*', *Canadian Journal of Microbiology*, 52(7), pp. 623–

626. doi:10.1139/W06-014.

Farji-Brener, A.G., Elizalde, L., Fernández-Marín, H. and Amador-Vargas, S. (2016) 'Social life and sanitary risks: Evolutionary and current ecological conditions determine waste management in leaf-cutting ants', *Proceedings of the Royal Society B: Biological Sciences*, 283(1831), 20160625. doi:10.1098/rspb.2016.0625.

Fedorka, K.M., Kutch, I.C., Collins, L. and Musto, E. (2016) 'Cold temperature preference in bacterially infected *Drosophila melanogaster* improves survival but is remarkably suboptimal', *Journal of Insect Physiology*, 93, pp. 36–41. doi:10.1016/j.jinsphys.2016.08.005.

Foitzik, S., Heinze, J., Oberstadt, B. and Herbers, J.M. (2002) 'Mate guarding and alternative reproductive tactics in the ant *Hypoponera opacior*', *Animal Behaviour*, 63(3), pp. 597–604. doi:10.1006/anbe.2001.1945.

Franks, N.R. and Hölldobler, B. (1987) 'Sexual competition during colony reproduction in army ants', *Biological Journal of the Linnean Society*, 30(3), pp. 229–243. doi:10.1111/j.1095-8312.1987.tb00298.x.

Fuchs, S., Sundström, L., Bos, N., Stucki, D. and Freitak, D. (2018) 'Induced immune responses in *Formica fusca* (Hymenoptera: Formicidae)', *Myrmecological News*, 28, pp. 53–66. doi:10.25849/myrmecol.news.

Gálvez, D. and Chapuisat, M. (2014) 'Immune priming and pathogen resistance in ant queens', *Ecology and Evolution*, 4(10), pp. 1761–1767. doi:10.1002/ece3.1070.

Gerloff, C.U., Ottmer, B.K. and Schmid-Hempel, P. (2003) 'Effects of inbreeding on immune response and body size in a social insect, *Bombus terrestris*', *Functional Ecology*, 17(5), pp. 582–589. doi:10.1046/j.1365-2435.2003.00769.x.

Giehr, J. and Heinze, J. (2018) 'Queens stay, workers leave: Caste-specific responses to fatal infections in an ant', *BMC Evolutionary Biology*, 18(1), pp. 1–11. doi:10.1186/s12862-018-1320-0.

Gillespie, A.T. and Claydon, N. (1989) 'The use of entomogenous fungi for pest control and the role of toxins in pathogenesis', *Pesticide Science*, 27(2), pp. 203–215. doi:10.1002/ps.2780270210.

Gillespie, J.P., Kanost, M.R. and Trenczek, T. (1997) 'Biological mediators of insect immunity', *Annual Review of Entomology*, 42, pp. 611–643. doi:10.1146/annurev.ento.42.1.611.

Graystock, P. and Hughes, W.O.H. (2011) 'Disease resistance in a weaver ant, *Polyrhachis dives*, and the role of antibiotic-producing glands', *Behavioral Ecology and Sociobiology*, 65(12), pp. 2319–2327. doi:10.1007/s00265-011-1242-y.

Greenough, P.R. (1980) 'Variolation and vaccination in South Asia, c. 1700-1865: A preliminary note', *Social Science and Medicine*, *Part D: Medical Geography*, 14(3), pp. 345-347. doi:10.1016/0160-8002(80)90047-7.

Griffin, A.S., West, S. A. and Buckling, A. (2004) 'Cooperation and competition in pathogenic bacteria', *Earth*, 430(7003), pp. 1024–1027. doi:10.1038/nature02802.1.

Hajek, A.E. and St Leger, R.J. (1994) 'Interactions between fungal pathogens and insect hosts', *Annual Review of Entomology*, 39(1), pp. 293–322. doi:10.1146/annurev.ento.39.1.293.

Hakala, S.M., Seppä, P. and Helanterä, H. (2019) 'Evolution of dispersal in ants (Hymenoptera: Formicidae): a review on the dispersal strategies of sessile superorganisms', *Myrmecological News*, 29, pp. 35–55. doi:10.25849/myrmecol.news.

Hamilton, C., Lejeune, B.T. and Rosengaus, R.B. (2011) 'Trophallaxis and prophylaxis: social immunity in the carpenter ant *Camponotus pennsylvanicus*', *Biology Letters*, 7(1), pp. 89–92. doi:10.1098/rsbl.2010.0466.

Hamilton, W.D. and Zuk, M. (1982) 'Heritable true fitness and bright birds: A role for parasites?', *Science*, 218(4570), pp. 384–387. doi:10.1126/science.7123238.

Hart, B.L. (1988) 'Biological basis of the behavior of sick animals', *Neuroscience and Biobehavioral Reviews*, 12(2), pp. 123–137. doi:10.1016/S0149-7634(88)80004-6.

Hart, B.L. (1990) 'Behavioral adaptations to pathogens and parasites: Five strategies', *Neuroscience and Biobehavioral Reviews*, 14(3), pp. 273–294. doi:10.1016/S0149-7634(05)80038-7.

Hartig, F. (2020) 'DHARMa: Residual Diagnostics for Hierarchical Regression Models', *The Comprehensive R Archive Network*, pp. 1–26.

Harwood, G., Amdam, G. and Freitak, D. (2019) 'The role of Vitellogenin in the transfer of immune elicitors from gut to hypopharyngeal glands in honey bees (*Apis mellifera*)', *Journal of Insect Physiology*, 112, pp. 90–100. doi:10.1016/j.jinsphys.2018.12.006.

Harwood, G., Salmela, H., Freitak, D. and Amdam, G. (2021) 'Social immunity in honey bees: royal jelly as a vehicle in transferring bacterial pathogen fragments between nestmates', *Journal of Experimental Biology*, 224(7), pp. 1–8. doi:10.1242/jeb.231076.

Hashimoto, C., Kim, D.R., Weiss, L.A., Miller, J.W. and Morisato, D. (2003) 'Spatial regulation of developmental signaling by a serpin', *Developmental Cell*, 5(6), pp. 945–950. doi:10.1016/S1534-5807(03)00338-1.

Hawley, D.M., Gibson, A.K., Townsend, A.K., Craft, M.E. and Stephenson, J.F. (2021) 'Bidirectional interactions between host social behaviour and parasites arise through ecological and evolutionary processes', *Parasitology*, 148(3), pp. 274–288. doi:10.1017/S0031182020002048.

Heinze, J. (2016) 'The male has done his work - The male may go', *Current Opinion in Insect Science*, 16, pp. 22–27. doi:10.1016/j.cois.2016.05.005.

Heinze, J. (2017) 'Life-history evolution in ants: The case of *Cardiocondyla*', *Proceedings of the Royal Society B: Biological Sciences*, 284(1850), 20161406. doi:10.1098/rspb.2016.1406.

Heinze, J., Cremer, S., Eckl, N. and Schrempf, A. (2006) 'Stealthy invaders: the biology of *Cardiocondyla* tramp ants', *Insectes Sociaux*, 53(1), pp. 1–7. doi:10.1007/s00040-005-0847-4.

Heinze, J. and Delabie, J. (2005) 'Population structure of the male-polymorphic ant *Cardiocondyla obscurior*', *Studies on Neotropical Fauna and Environment*, 40(3), pp. 187–190. doi:10.1080/01650520500175250.

Heinze, J. and Hölldobler, B. (1993) 'Fighting for a harem of queens: Physiology of reproduction in *Cardiocondyla* male ants', *Proceedings of the National Academy of Sciences of the United States of America*, 90(18), pp. 8412–8414. doi:10.1073/pnas.90.18.8412.

Heinze, J., Hölldobler, B. and Yamauchi, K. (1998) 'Male competition in *Cardiocondyla* ants',

Behavioral Ecology and Sociobiology, 42(4), pp. 239–246. doi:10.1007/s002650050435.

Heinze, J. and Schrempf, A. (2012) 'Terminal investment: Individual reproduction of ant queens increases with age', *PLOS ONE.*, 7(4), p. e35201. doi:10.1371/journal.pone.0035201.

Helft, F., Doums, C. and Monnin, T. (2016) 'No evidence of pre-copulatory mate choice by gynes in the facultatively parthenogenetic ant *Cataglyphis cursor'*, *Insectes Sociaux*, 63(1), pp. 199–201. doi:10.1007/s00040-015-0444-0.

Helft, F., Monnin, T. and Doums, C. (2015) 'First evidence of inclusive sexual selection in the ant *Cataglyphis cursor*: worker aggressions differentially affect male access to virgin queens', *Ethology*, 121(7), pp. 641–650. doi:10.1111/eth.12376.

Hernandez, C.E.M., Guerrero, I.E.P., Hernandez, G.A.G., Solis, E.S. and Guzman, J.C.T. (2010) 'Catalase over expression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*', *Applied Microbiology and Biotechnology*, 87(3), pp. 1033–1044. doi:10.1007/s00253-010-2517-3.

Hernández López, J., Riessberger-Gallé, U., Crailsheim, K. and Schuehly, W. (2017) 'Cuticular hydrocarbon cues of immune-challenged workers elicit immune activation in honeybee queens', *Molecular Ecology*, 26(11), pp. 3062–3073. doi:10.1111/mec.14086.

Hillgarth, N. and Wingfield, J.C. (1997) 'Testosterone and immunosuppression in vertebrates: implications for parasite-mediated sexual selection', In *Parasites and Pathogens* Springer, Boston, MA, pp. 143–155. doi:10.1007/978-1-4615-5983-2_7.

Hölldobler, B. and Wilson, E.O. (1990) *The ants.* Harvard University Press.

Hölldobler, B. and Bartz, S. (1985) 'Sociobiologie of reproduction in ants', *Fortschritte der Zoologie (Stuttgart)*, 31, pp. 237–257.

Hothorn, T., Bretz, F. and Westfall, P. (2008) 'Simultaneous inference in general parametric models', *Biometrical Journal*, 50(3), pp. 346–363. doi:10.1002/bimj.200810425.

Hughes, W.O.H., Eilenberg, J. and Boomsma, J.J. (2002) 'Trade-offs in group living: transmission and disease resistance in leaf-cutting ants' *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269(1502), pp. 1811–1819. doi:10.1098/rspb.2002.2113.

Hughes, W O.H., Petersen, K.S., Ugelvig, L. V., Pedersen, D., Thomsen, L., Poulsen, M. and Boomsma, J.J. (2004a) 'Density-dependence and within-host competition in a semelparous parasite of leaf-cutting ants', *BMC Evolutionary Biology*, 4, pp. 1–12. doi:10.1186/1471-2148-4-45.

Hughes, W.O.H., Thomsen, L., Eilenberg, J. and Boomsma, J.J. (2004b) 'Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*', *Journal of Invertebrate Pathology*, 85(1), pp. 46–53. doi:10.1016/j.jip.2003.12.005.

Hunt, J., Breuker, C.J., Sadowski, J.A. and Moore, A.J. (2009) 'Male-male competition, female mate choice and their interaction: Determining total sexual selection', *Journal of Evolutionary Biology*, 22(1), pp. 13–26. doi:10.1111/j.1420-9101.2008.01633.x.

Lenoir, J. C., Schrempf, A., Lenoir, A., Heinze, J. and Mercier, J. L. (2007). 'Genetic structure and reproductive strategy of the ant *Cardiocondyla elegans*: strictly monogynous nests invaded by unrelated sexuals', *Molecular Ecology*, 16(2), pp.345-354. doi:10.1111/j.1365-

294X.2006.03156.x.

Jamieson, A.M., Pasman, L., Yu, S., Gamradt, P., Homer, R.J., Decker, T. and Medzhitov, R. (2013) 'Role of tissue protection in lethal respiratory viral-bacterial coinfection', *Science*, 340(6137), pp. 1230–1234. doi:10.1126/science.1233632.

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M. (2007) *Immunobiology: the immune system in health and disease*. 7th Edition, Garland Publishing, New York.

Jenner, E. (1798) '*An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox*', https://archive.org/details/b24759247/page/n7/mode/2up.

Kappeler, P.M., Cremer, S. and Nunn, C.L. (2015) 'Sociality and health: Impacts of sociality on disease susceptibility and transmission in animal and human societies', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1669), 20140116. doi:10.1098/rstb.2014.0116.

Keller, S., Kessler, P. and Schweizer, C. (2003) 'Distribution of insect pathogenic soil fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metharhizium anisopliae*', *BioControl*, 48(3), pp. 307–319. doi:10.1023/A:1023646207455.

Kemp, D.J. and Wiklund, C. (2001) 'Fighting without weaponry: A review of male-male contest competition in butterflies', *Behavioral Ecology and Sociobiology*, 49(6), pp. 429–442. doi:10.1007/s002650100318.

Kent, S., Bluthé, R.M., Kelley, K.W. and Dantzer, R. (1992) 'Sickness behavior as a new target for drug development', *Trends in Pharmacological Sciences*, 13, pp. 24–28. doi:10.1016/0165-6147(92)90012-U.

Kinomura, K. and Yamauchi, K. (1987) 'Fighting and mating behaviors of dimorphic males in the ant - *Cardiocondyla wroughtoni*', *Journal of Ethology*, 5(1), pp. 75–81. doi:10.1007/BF02347897.

Konrad, M., Grasse, A. V, Tragust, S. and Cremer, S. (2015) 'Anti-pathogen protection versus survival costs mediated by an ectosymbiont in an ant host.', *Proceedings of the Royal Society of London B: Biological Sciences*, 282(1799), 20141976. doi:10.1098/rspb.2014.1976.

Konrad, M., Pull, C.D., Metzler, S., Seif, K., Naderlinger, E., Grasse, A. V. and Cremer, S. (2018) 'Ants avoid superinfections by performing risk-adjusted sanitary care', *Proceedings of the National Academy of Sciences*, 115(11), pp. 2782–2787. doi:10.1073/PNAS.1713501115.

Konrad, M., Vyleta, M.L., Theis, F.J., Stock, M., Tragust, S., Klatt, M., Drescher, V., Marr, C., Ugelvig, L. V. and Cremer, S. (2012) 'Social transfer of pathogenic fungus promotes active immunisation in ant colonies', *PLOS Biology*, 10(4), e1001300. doi:10.1371/journal.pbio.1001300.

Kurtz, J. (2005) 'Specific memory within innate immune systems', *Trends in Immunology*, 26(4), pp. 186–192. doi:10.1016/j.it.2005.02.001.

Kurtz, J. and Franz, K. (2003) 'Evidence for memory in invertebrate immunity', *Nature*, 425(6953), pp. 37–38. doi:10.1038/425037a.

Kurze, C., Jenkins, N.E. and Hughes, D.P. (2020) 'Evaluation of direct and indirect transmission of fungal spores in ants', *Journal of Invertebrate Pathology*, 172, 107351. doi:10.1016/j.jip.2020.107351.

Landis, S.H., Sundin, J., Rosenqvist, G., Poirier, M., Jørgensen, G.Ø. and Roth, O. (2015) 'Female pipefish can detect the immune status of their mates', *Behavioral Ecology and Sociobiology*, 69(12), pp. 1917–1923. doi:10.1007/s00265-015-2004-z.

Leclerc, J.B. and Detrain, C. (2016) 'Ants detect but do not discriminate diseased workers within their nest', *Science of Nature*, 103(7), 70 (2016). doi:10.1007/s00114-016-1394-8.

Lemaitre, B. and Hoffmann, J. (2007) 'The host defense of *Drosophila melanogaster*', *Annual Review of Immunology*, 25, pp. 697–743. doi:10.1146/annurev.immunol.25.022106.141615.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. and Hoffmann, J.A. (1996) 'The dorsoventral regulatory gene cassette spätzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults', *Cell*, 86(6), pp. 973–983. doi:10.1016/S0092-8674(00)80172-5.

Little, T.J. and Kraaijeveld, A.R. (2004) 'Ecological and evolutionary implications of immunological priming in invertebrates', *Trends in Ecology & Evolution*, 19(2), pp. 58–60. doi:10.1016/j.tree.2003.11.011.

Liu, L., Li, G., Sun, P., Lei, C. and Huang, Q. (2015) 'Experimental verification and molecular basis of active immunization against fungal pathogens in termites.', *Scientific Reports*, 5, 15106. doi:10.1038/srep15106.

Liu, P., Wei, J., Tian, S. and Hao, D. (2017) 'Male-male lethal combat in the quasi-gregarious parasitoid *Anastatus disparis* (Hymenoptera : Eupelmidae)', *Scientific Reports*, 7, 11875. doi:10.1038/s41598-017-11890-x.

Lochmiller, R.L. and Deerenberg, C. (2000) 'Trade-offs in evolutionary immunology: Just what is the cost of immunity?', *Oikos*, 88(1), pp. 87–98. doi:10.1034/j.1600-0706.2000.880110.x.

Van Loon, A.J., Boomsma, J.J. and Andrasfalvy, A. (1990) 'A new polygynous *Lasius* species (Hymenoptera; Formicidae) from central Europe', *Insectes Sociaux*, 37(4), pp. 348–362. doi:10.1007/bf02225997.

Love, A.C., Grisham, K., Krall, J.B., Goodchild, G., C. and DuRant, S.E. (2021) 'Perception of infection: disease-related social cues influence immunity in songbirds', *Biology Letters*, 17(6), 20210125. doi:10.1098/rsbl.2021.0125.

Ma, C. and Kanost, M.R. (2000) 'A β1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade', *Journal of Biological Chemistry*, 275(11), pp. 7505–7514. doi:10.1074/jbc.275.11.7505.

Masri, L. and Cremer, S. (2014) 'Individual and social immunisation in insects', *Trends in Immunology*, 35(10), pp. 471–482. doi:10.1016/j.it.2014.08.005.

Mazur, A. and Booth, A. (1998) 'Testosterone and dominance in men', *Behavioral and Brain Sciences*, 21(3), pp. 353–397. doi:10.1017/S0140525X98001228.

McKinney, H.H. (1929) 'Mosaic diseases in the Canary Islands, West Africa and Gibraltar', *Journal of Agricultural Research*, 39(8), pp. 557–578

Medina-Gómez, H., Farriols, M., Santos, F., González-Hernández, A., Torres-Guzmán, J.C., Lanz, H. and Contreras-Garduño, J. (2018) 'Pathogen-produced catalase affects immune priming: A potential pathogen strategy', *Microbial Pathogenesis*, 125, pp. 93–95. doi:10.1016/j.micpath.2018.09.012.

Merle, C.S., Cunha, S.S. and Rodrigues, L.C. (2010) 'BCG vaccination and leprosy protection: Review of current evidence and status of BCG in leprosy control', *Expert Review of Vaccines*, 9(2), pp. 209–222. doi:10.1586/erv.09.161.

Metzler, S., Heinze, J. and Schrempf, A. (2016) 'Mating and longevity in ant males', *Ecology and Evolution*, *6*(24), pp. 8903-8906. doi:10.1002/ece3.2474.

Milinski, M. and Bakker, T.C.M. (1990) 'Female sticklebacks use male coloration in mate choice and hence avoid parasitized males', *Nature*, 344(6264), pp. 330–333. doi:10.1038/344330a0.

Milutinović, B. and Kurtz, J. (2016) 'Immune memory in invertebrates', *Seminars in Immunology*, 28(4), pp. 328–342. doi:10.1016/j.smim.2016.05.004.

Moret, Y. (2006) ''Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*', *Proceedings of the Royal Society B: Biological Sciences*, 273(1592), pp. 1399-1405. doi:10.1098/rspb.2006.3465.

Moret, Y. and Siva-Jothy, M.T. (2003) 'Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*', *Proceedings of the Royal Society B: Biological Sciences*, 270(1532), pp. 2475–2480. doi:10.1098/rspb.2003.2511.

Myllymäki, H., Valanne, S. and Rämet, M. (2014) ' The *Drosophila* Imd Signaling Pathway ', *The Journal of Immunology*, 192(8), pp. 3455–3462. doi:10.4049/jimmunol.1303309.

Nagy, C., Tartally, A., Vilisics, F., Merkl, O., Szita, É., Szél, G., Podlussány, A., Rédei, D., Cssz, S., Pozsgai, G., Orosz, A., SzöÉnyi, G. and Markó, V. (2009) 'Effects of the invasive garden ant, *Lasius neglectus* Van Loon, Boomsma & András-Falvy, 1990 (Hymenoptera: Formicidae), On arthropod assemblages: Pattern analyses in the type supercolony', *Myrmecological News*, 12, pp. 171–181.

Nappi, A.J., Frey, F. and Carton, Y. (2005) '*Drosophila* serpin 27A is a likely target for immune suppression of the blood cell-mediated melanotic encapsulation response', *Journal of Insect Physiology*, 51(2), pp. 197–205. doi:10.1016/j.jinsphys.2004.10.013.

Ng, T.H., Chiang, Y.A., Yeh, Y.C. and Wang, H.C. (2014) 'Review of Dscam-mediated immunity in shrimp and other arthropods', *Developmental and Comparative Immunology*, 46(2), pp. 129–138. doi:10.1016/j.dci.2014.04.002.

O´Donnell, S. and Beshers, S.N. (2004) 'The role of male disease susceptibility in the evolution of haplodiploid insect societies', *Proceedings of the Royal Society B: Biological Sciences*, 271(1542), pp. 979–983. doi:10.1098/rspb.2004.2685.

Owen-Ashley, N.T., Turner, M., Hahn, T.P. and Wingfield, J.C. (2006) 'Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*)', *Hormones and Behavior*, 49(1), pp. 15–29. doi:10.1016/j.yhbeh.2005.04.009.

Paynter, E., Baer-Imhoof, B., Linden, M., Lee-Pullen, T., Heel, K., Rigby, P., and Baer, B. (2014). 'Flow cytometry as a rapid and reliable method to quantify sperm viability in the honeybee *Apis mellifera*', *Cytometry Part A*, *85*(5), pp. 463-472. doi: 10.1002/cyto.a.22462

Paris, C.I. and Espadaler, X. (2009) 'Honeydew collection by the invasive garden ant *Lasius neglectus* versus the native ant *L. grandis*', *Arthropod-Plant Interactions*, 3(2), pp. 75–85. doi:10.1007/s11829-009-9057-8.

Pechinger, K., Chooi, K.M., Macdiarmid, R.M., Ziebell, H. and Harper, S.J. (2019) 'A new era for mild strain cross-protection', *Viruses*, 11(7), pp. 1–17. doi:10.3390/v11070670.

Pedras, M.S.C., Irina Zaharia, L.I. and Ward, D.E. (2002) 'The destruxins: Synthesis, biosynthesis, biotransformation, and biological activity', *Phytochemistry*, 59(6), pp. 579–596. doi:10.1016/S0031-9422(02)00016-X.

Peuß, R., Eggert, H., Armitage, S.A.O. and Kurtz, J. (2015) 'Downregulation of the evolutionary capacitor Hsp90 is mediated by social cues', *Proceedings of the Royal Society B: Biological Sciences*, 282(1819). doi:10.1098/rspb.2015.2041.

Pfaffl, M.W. (2001) 'A new mathematical model for relative quantification in real-time RT–PCR', *Nucleic Acids Research*, 29(9), e45. doi:10.1093/nar/29.9.e45.

Pham, L.N., Dionne, M.S., Shirasu-Hiza, M. and Schneider, D.S. (2007) 'A specific primed immune response in *Drosophila* is dependent on phagocytes', *PLOS Pathogens*, 3(3), e26. doi:10.1371/journal.ppat.0030026.

Poulin, R. (1994) 'Mate choice decisions by parasitized female upland bullies, *Gobiomorphus breviceps*', *Proceedings of the Royal Society B: Biological Sciences*, 256(1346), pp. 183–187. doi:10.1098/rspb.1994.0068.

Pull, C.D., Brown, M.J., Grasse, A. V, Schmitt, T., Wiesenhofer, F., Tragust, S., Cremer, S. and Ugelvig, L. V (2018a) 'Destructive disinfection of infected brood prevents systemic disease spread in ant colonies', *eLife*, 7, pp. 1–29. doi:10.7554/elife.32073.

Pull, C.D. and Cremer, S. (2017) 'Co-founding ant queens prevent disease by performing prophylactic undertaking behaviour', *BMC Evolutionary Biology*, 17(219), pp. 1–10. doi:10.1186/s12862-017-1062-4.

Pull, C.D., Metzler, S., Naderlinger, E. and Cremer, S. (2018b) 'Protection against the lethal side effects of social immunity in ants', *Current Biology*, 28(19), pp. R1139–R1140. doi:10.1016/j.cub.2018.08.063.

Quan, F.-S., Huang, C., Compans, R.W. and Kang, S.-M. (2007) 'Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus', *Journal of Virology*, 81(7), pp. 3514–3524. doi:10.1128/jvi.02052-06.

Rantala, M.J., Jokinen, I., Kortet, R., Vainikka, A. and Suhonen, J. (2002) 'Do pheromones reveal male immunocompetence?', *Proceedings of the Royal Society of London B: Biological Sciences*, 269(1501), pp. 1681–5. doi:10.1098/rspb.2002.2056.

Rantala, M.J. and Kortet, R. (2004) 'Male dominance and immunocompetence in a field cricket', *Behavioral Ecology*, 15(2), pp. 187–191. doi:10.1093/beheco/arg103.

R Core Team. (2020) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.'

Reber, A. and Chapuisat, M. (2012) 'Diversity, prevalence and virulence of fungal entomopathogens in colonies of the ant *Formica selysi*', *Insectes Sociaux*, 59(2), pp. 231–239. doi:10.1007/s00040-011-0209-3.

Reber, A., Purcell, J., Buechel, S.D., Buri, P. and Chapuisat, M. (2011) 'The expression and impact of antifungal grooming in ants', *Journal of Evolutionary Biology*, 24, pp. 954–964. doi:10.1111/j.1420-9101.2011.02230.x.

Roberts, D.W. and St Leger, R.J. (2004) '*Metarhizium* spp., Cosmopolitan insect-pathogenic fungi: Mycological aspects.', *Advances in Applied Microbiology*, 54(1), pp. 1–70. doi:10.1016/S0065- 2164(04)54001-7.

Rojas, M.G., Elliott, R.B. and Morales-Ramos, J.A. (2018) 'Mortality of *Solenopsis invicta* workers (Hymenoptera: Formicidae) after indirect exposure to spores of three entomopathogenic fungi', *Journal of Insect Science*, 18(3): 20, pp. 1-8, doi:10.1093/jisesa/iey050.

Rosengaus, R.B., Malak, T. and Mackintosh, C. (2013) 'Immune-priming in ant larvae: social immunity does not undermine individual immunity', *Biology Letters*, 9(6), 20130563. doi:10.1098/rsbl.2013.0563.

Rosengaus, R.B., Maxmen, A.B., Coates, L.E. and Traniello, J.F.A. (1998) 'Disease resistance: A benefit of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera: Termopsidae)', *Behavioral Ecology and Sociobiology*, 44(2), pp. 125–134. doi:10.1007/s002650050523.

Rosengaus, R.B., Traniello, J.F.A., Chen, T., Brown, J.J. and Karp, R.D. (1999) 'Immunity in a social insect', *Naturwissenschaften*, 86(12), pp. 588–591. doi:10.1007/s001140050679.

Roth, O., Beemelmanns, A., Barribeau, S.M. and Sadd, B.M. (2018) 'Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution', *Heredity*, 121(3), pp. 225–238. doi:10.1038/s41437-018-0101-2.

Roth, O. and Kurtz, J. (2009) 'Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda)', *Developmental and Comparative Immunology*, 33(11), pp. 1151–1155. doi:10.1016/j.dci.2009.04.005.

Roth, O., Sadd, B.M., Schmid-Hempel, P. and Kurtz, J. (2009) 'Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*', *Proceedings of the Royal Society B: Biological Sciences*, 276(1654), pp. 145–151. doi:10.1098/rspb.2008.1157.

Rothenbuhler, W.C. and Thompson, V.C. (1956) 'Resistance to American foulbrood in honey bees. I. Differential survival of larvae of different genetic lines.', *Journal of Economic Entomology*, 49(4), pp. 470–475. doi:10.1093/jee/49.4.470.

Rueppell, O., Königseder, F., Heinze, J. and Schrempf, A. (2015) 'Intrinsic survival advantage of social insect queens depends on reproductive activation', *Journal of Evolutionary Biology*, 28(12), pp. 2349–2354. doi:10.1111/jeb.12749.

Ruiz-Gonzáles, M.X. and Brown, M.J. (2006) 'Males vs workers : testing the assumptions of the haploid susceptibility hypothesis in bumblebees', *Behavioral Ecology and Sociobiology*, 60, pp. 501–509. doi:10.1007/s00265-006-0192-2.

Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R. and Schmid-Hempel, P. (2005) 'Trans-generational immune priming in a social insect', *Biology Letters*, 1(4), pp. 386–388. doi:10.1098/rsbl.2005.0369.

Sadd, B.M. and Schmid-Hempel, P. (2006) 'Insect immunity shows specificity in protection upon secondary pathogen exposure', *Current Biology*, 16(12), pp. 1206–1210. doi:10.1016/j.cub.2006.04.047.

Sadd, B.M. and Schmid-Hempel, P. (2007) 'Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees', *Current Biology*, 17(24), pp. 1046–1047. doi:10.1016/j.cub.2007.11.007.

Sadd, B.M. and Schmid-Hempel, P. (2009) 'A distinct infection cost associated with trans-generational priming of antibacterial immunity in bumble-bees', *Biology Letters*, 5(6), pp. 798–801. doi:10.1098/rsbl.2009.0458.

Salmela, H., Amdam, G. V. and Freitak, D. (2015) 'Transfer of immunity from mother to offspring is mediated via egg-yolk protein vitellogenin', *PLOS Pathogens*, 11(7), pp. 1–12. doi:10.1371/journal.ppat.1005015.

Schaller, M., Miller, G.E., Gervais, W.M., Yager, S. and Chen, E. (2010) 'Mere visual perception of other people's disease symptoms facilitates a more aggressive immune response', *Psychological Science*, 21(5), pp. 649–652. doi:10.1177/0956797610368064.

Schmid-Hempel, P. (2003) 'Variation in immune defence as a question of evolutionary ecology', *Proceedings of the Royal Society of London B: Biological Sciences*, 270(1513), pp. 357–366. doi:10.1098/rspb.2002.2265.

Schmid-Hempel, P. (2011) '*Evolutionary Parasitolgy: The integrated study of infection, immunology, ecology and genetics*', Oxford University Press.

Schmid-Hempel, P. (2017) 'Parasites and their social hosts', *Trends in Parasitology*, 33(6), pp. 453–462. doi:10.1016/j.pt.2017.01.003.

Schnell, A.K., Smith, C.L., Hanlon, R.T. and Harcourt, R. (2015) 'Giant Australian cuttlefish use mutual assessment to resolve male-male contests', *Animal Behaviour*, 107, pp. 31–40. doi:10.1016/j.anbehav.2015.05.026.

Schrempf, A., Heinze, J. and Cremer, S. (2005) 'Sexual cooperation : mating increases longevity in ant queens', *Current Biology*, 15(3), pp. 267–270. doi:10.1016/j.

Schwenke, R.A., Lazzaro, B.P. and Wolfner, M.F. (2016) 'Reproduction-immunity trade-offs in insects', *Annual Reviews of Ethology*, 61, pp. 239–256. doi:10.1146/annurev-ento-010715-023924.

Seifert, B. (2000) 'Rapid range expansion in *Lasius neglectus* (Hymenoptera, Formicidae) - An Asian invader swamps Europe', *Deutsche Entomologische Zeitschrift*, 47(2), pp. 173–179. doi:10.1002/mmnd.4800470206.

Seppälä, O., Karvonen, A., Rellstab, C., Louhi, K.-R. and Jokela, J. (2012) 'Reciprocal interaction matrix reveals complex genetic and dose-dependent specificity among coinfecting parasites', *The American Naturalist*, 180(3), pp. 306–315. doi:10.1086/666985.

Sheehan, G., Farrell, G. and Kavanagh, K. (2020) 'Immune priming: the secret weapon of the insect world', *Virulence*, 11(1), pp. 238–246. doi:10.1080/21505594.2020.1731137.

Sheehan, G., Garvey, A., Croke, M. and Kavanagh, K. (2018) 'Innate humoral immune defences in mammals and insects: The same, with differences?', *Virulence*, 9(1), pp. 1625–1639. doi:10.1080/21505594.2018.1526531.

Sheldon, B.C. and Verhulst, S. (1996) 'Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology', *Trends in Ecology and Evolution*, 11(8), pp. 317–321. doi:10.1016/0169-5347(96)10039-2.

Shoemaker, K.L., Parsons, N.M. and Adamo, S.A. (2006) 'Mating enhances parasite resistance in the cricket *Gryllus texensis*', *Animal Behaviour*, 71(2), pp. 371–380. doi:10.1016/j.anbehav.2005.05.007.

Silverman, N. and Maniatis, T. (2001) 'NF-κB signaling pathways in mammalian and insect innate immunity', *Genes and Development*, 15(18), pp. 2321–2342. doi:10.1101/gad.909001.

Steinwender, B.M., Enkerli, J., Widmer, F., Eilenberg, J., Thorup-Kristensen, K. and Meyling, N. V (2014) 'Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem', *Journal of Invertebrate Pathology*, 123, pp. 6–12. doi:10.1016/j.jip.2014.09.002.

Stephenson, J.F., Perkins, S.E. and Cable, J. (2018) 'Transmission risk predicts avoidance of infected conspecifics in *Trinidadian* guppies', *Journal of Animal Ecology*, (87), pp. 1525–1533. doi:10.1111/1365-2656.12885.

Stockmaier, S., Bolnick, D.I., Page, R.A., Josic, D. and Carter, G.G. (2020) 'Immune-challenged vampire bats produce fewer contact calls', *Biology Letters*, 16(7), 20200272. doi:10.1098/rsbl.2020.0272.

Stockmaier, S., Stroeymeyt, N., Shattuck, E.C., Hawley, D.M., Meyers, L.A. and Bolnick, D.I. (2021) 'Infectious diseases and social distancing in nature', *Science*, 371(6533), eabc8881. doi:10.1126/science.abc8881.

Stürup, M., Baer, B. and Boomsma, J.J. (2014) 'Short independent lives and selection for maximal sperm survival make investment in immune defences unprofitable for leaf-cutting ant males', *Behavioral Ecology and Sociobiology*, 68, pp. 947–955. doi:10.1007/s00265-014-1707-x.

Sun, Q. and Zhou, X. (2013) 'Corpse management in social insects', *International Journal of Biological Sciences*, 9(3), pp. 313–321. doi:10.7150/ijbs.5781.

Sunamura, E., Hoshizaki, S., Sakamoto, H., Fujii, T., Nishisue, K., Suzuki, S., Terayama, M., Ishikawa, Y. and Tatsuki, S. (2011) 'Workers select mates for queens: a possible mechanism of gene flow restriction between supercolonies of the invasive Argentine ant', *Naturwissenschaften*, 98(5), pp. 361–368. doi:10.1007/s00114-011-0778-z.

Tamura, K., Stecher, G., and Kumar, S. (2021). 'MEGA11: molecular evolutionary genetics analysis version 11', *Molecular Biology and Evolution*, 38(7), pp. 3022-3027. doi: 10.1093/molbev/msab120.

Tassetto, M., Kunitomi, M. and Andino, R. (2017) 'Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in *Drosophila*', *Cell*, 169(2), pp. 314-325. doi:10.1016/j.cell.2017.03.033.

Tate, A.T. and Graham, A.L. (2017) 'Dissecting the contributions of time and microbe density to variation in immune gene expression', *Proceedings of the Royal Society B: Biological Sciences*, 284(1859), 20170727. doi:10.1098/rspb.2017.0727.

Tetreau, G., Dhinaut, J., Gourbal, B. and Moret, Y. (2019) 'Trans-generational immune priming in invertebrates: Current knowledge and future prospects', *Frontiers in Immunology*, 10, p. 1938. doi:10.3389/fimmu.2019.01938.

Therneau, T.M. (2015) 'coxme: Mixed Effects Cox Models', *The Comprehensive R Archive Network,* pp. 1-14.

Tidière, M., Lemaître, J.F., Pélabon, C., Gimenez, O. and Gaillard, J.M. (2017) 'Evolutionary allometry reveals a shift in selection pressure on male horn size', *Journal of Evolutionary Biology*, 30(10), pp. 1826–1835. doi:10.1111/jeb.13142.

Tragust, S., Mitteregger, B., Barone, V., Konrad, M., Ugelvig, L. V. and Cremer, S. (2013a) 'Ants disinfect fungus-exposed brood by oral uptake and spread of their poison', *Current Biology*, 23(1), pp. 76–82. doi:10.1016/j.cub.2012.11.034.

Tragust, S., Ugelvig, L. V., Chapuisat, M., Heinze, J. and Cremer, S. (2013b) 'Pupal cocoons affect sanitary brood care and limit fungal infections in ant colonies', *BMC Evolutionary Biology*, 13(1), pp. 1–10. doi:10.1186/1471-2148-13-225.

Traniello, J.F.A., Rosengaus, R.B. and Savoie, K. (2002) 'The development of immunity in a social insect: Evidence for the group facilitation of disease resistance', *Proceedings of the National Academy of Sciences of the United States of America*, 99(10), pp. 6838–6842. doi:10.1073/pnas.102176599.

Tranter, C. and Hughes, W.O.H. (2015) 'Acid , silk and grooming : alternative strategies in social immunity in ants ?', *Behavioral Ecology and Sociobiology*, 69(10), pp. 1687–1699. doi:10.1007/s00265-015-1980-3.

Tsutsui, N.D. and Suarez, A. V. (2003) 'The colony structure and population biology of invasive ants', *Conservation Biology*, 17(1), pp. 48–58. doi:10.1046/j.1523-1739.2003.02018.x.

Ugelvig, L. V. and Cremer, S. (2007) 'Social prophylaxis: group interaction promotes collective immunity in ant colonies', *Current Biology*, 17(22), pp. 1967–1971. doi:10.1016/j.cub.2007.10.029.

Ugelvig, L. V, Kronauer, D.J.C., Schrempf, A., Heinze, J. and Cremer, S. (2010) 'Rapid anti-pathogen response in ant societies relies on high genetic diversity', *Proceedings of the Royal Society B: Biological Sciences*, 277(1695), pp. 2821–2828. doi:10.1098/rspb.2010.0644.

Ulrich, Y. and Schmid-Hempel, P. (2012) 'Host modulation of parasite competition in multiple infections', *Proceedings of the Royal Society B: Biological Sciences*, 279(1740), pp. 2982–2989. doi:10.1098/rspb.2012.0474.

Untergasser, A., Nijveen, H., Rao, X. and Bisseling, T. (2007) 'Primer3Plus , an enhanced web interface to Primer3', *Nucleic Acids Research*, 35, pp. 71–74. doi:10.1093/nar/gkm306.

Vainio, L., Hakkarainen, H., Rantala, M.J. and Sorvari, J. (2004) 'Individual variation in immune function in the ant *Formica exsecta*; effects of the nest, body size and sex', *Evolutionary Ecology*, 18(1), pp. 75–84. doi:10.1023/B:EVEC.0000017726.73906.b2.

Valanne, S., Wang, J.-H. and Rämet, M. (2011) 'The *Drosophila* toll signaling pathway', *The Journal of Immunology*, 186(2), pp. 649–656. doi:10.4049/jimmunol.1002302.

Valtonen, T.M., Viitaniemi, H. and Rantala, M.J. (2010) 'Copulation enhances resistance against an entomopathogenic fungus in the mealworm beetle *Tenebrio molitor*', *Parasitology*, 137(6), pp. 985–989. doi:10.1017/S0031182009992009.

Vilcinskas, A. (2021) 'Mechanisms of transgenerational immune priming in insects', *Developmental and Comparative Immunology*, 124, 104205. doi:10.1016/j.dci.2021.104205.

Viljakainen, L. and Pamilo, P. (2005) 'Identification and molecular characterization of defensin gene from the ant *Formica aquilonia'*, *Insect Molecular Biology*, 14(4), pp. 335–338. doi:10.1111/j.1365-2583.2005.00564.x.

Viljakainen, L. and Pamilo, P. (2008) 'Selection on an antimicrobial peptide defensin in ants', *Journal of Molecular Evolution*, 67(6), pp. 643–652. doi:10.1007/s00239-008-9173-6.

Vojtek, I., Buchy, P., Doherty, T.M. and Hoet, B. (2019) 'Would immunization be the same without cross-reactivity?', *Vaccine*, 37(4), pp. 539–549. doi:10.1016/j.vaccine.2018.12.005.

Walker, T.N. and Hughes, W.O.H. (2009) 'Adaptive social immunity in leaf-cutting ants', *Biology Letters*, 5(4), pp. 446–448. doi:10.1098/rsbl.2009.0107.

West, S.A., Murray, M.G., Machado, C.A., Griffin, A.S. and Herre, E.A. (2001) 'Testing Hamilton's rule with competition between relatives', *Nature*, 409(6819), pp. 510–513. doi:10.1038/35054057.

Westra, E.R., Van houte, S., Oyesiku-Blakemore, S., Makin, B., Broniewski, J.M., Best, A., Bondy-Denomy, J., Davidson, A., Boots, M. and Buckling, A. (2015) 'Parasite exposure drives selective evolution of constitutive versus inducible defense', *Current Biology*, 25(8), pp. 1043–1049. doi:10.1016/j.cub.2015.01.065.

Wheeler, D. (1911) 'The ant-colony as an organism', *Journal of Morphology*, 22, pp. 307–325. doi:10.1002/jmor.1050220206.

Wickham, H., Chang, W., Henry, L., Pedersen, T.L., Takahashi, K., Wilke, C., Woo, K., Yutani, H., Dunnington, D., Revelle, W. and Therneau, T.M. (2018) 'ggplot2: create elegant data visualisations using the grammar of graphics'. R package version 3.3.3

Wilson-Rich, N., Spivak, M., Fefferman, N.H. and Starks, P.T. (2009) 'Genetic, individual, and group facilitation of disease resistance in insect societies', *Annual Review of Entomology*, 54, pp. 405–423. doi:10.1146/annurev.ento.53.103106.093301.

Worden, B., Parker, P. and Pappas, P. (2000) 'Parasites reduce attractiveness and reproductive success in male grain beetles.', *Animal Behaviour*, 59(3), pp. 543–550. doi:10.1006/anbe.1999.1368.

Yamauchi, K. and Kawase, N. (1992) 'Pheromonal manipulation of workers by a fighting male to kill his rival males in the ant *Cardiocondyla wroughtonii*', *Naturwissenschaften*, 79(6), pp. 274–276. doi:10.1007/BF01175395.

Zuk, M. and Stoehr, A.M. (2002) 'Immune defense and host life history', *The American Naturalist*, 160(S4), pp. S9–S22. doi:10.1086/342131.

Zuk, M., Thornhill, R., Ligon, J.D. and Johnson, K. (1990) 'Parasites and mate choice in red jungle fowl', *Integrative and Comparative Biology*, 30(2), pp. 235–244. doi:10.1093/icb/30.2.235.