Investigating the role of metapleural glands in social immunity and the role of co-founding in pathogen resistance in *Messor barbarus*

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1. Abstract

The risk of pathogen infection due to increased within-group transmission is theorised to be a major potential cost of group-living and eusociality. Many eusocial insects have evolved defence mechanisms to mitigate this risk and mount collective defences against pathogens. Social immunity describes the behaviours which are used to minimise pathogen spread within a colony, such as allo-grooming and waste management. Additionally, ants have developed chemical defences, such as the antimicrobial substance produced by the metapleural gland. This thesis investigated how the availability of metapleural glands and social immunity affect the survival probability of *Messor barbarus* and their response to an entomopathogenic fungus, *Metarhizium brunneum*. The results showed that the presence of fungal spores or Triton X on the cuticle increased self-grooming, and in the control treatment, an ant groomed for a longer period of time if they had a blocked gland. There was no difference in allo-grooming between individuals. Additionally, there was a high mortality of ants exposed to *M. brunneum*. More experiments are required to see whether the behaviours explored in this thesis are important or not in modulating the efficacity of the metapleural gland. There could be some other mechanism, potentially passive, involved in how the metapleural gland protects the ants from disease.

Colony-founding queens do not have access to the colony-wide defensive system, and new colonies suffer high rates of mortality. In a number of eusocial insects, queens join together and co-found a colony collectively. This thesis investigated how being exposed to *M. brunneum* and group type affects the founding of new colonies. The results showed that exposed single queens and unexposed previously paired queens produced the highest number of brood and adults comparatively. These findings could support a newly discovered phenomenon called "hygienic cannibalism" where a queen will reinvest nutrients back into egg production from eating infected larvae. The presence of an unrelated queen could be viewed as interacting with a foreign substance, as being exposed to either another queen or a pathogen produced a large number of brood and adults. Exposed previously paired queens had the disadvantage of both an immune response and energy lost due to fighting so produced a small number of brood and adults in comparison.

2. General Introduction

Group-living is a common phenomenon in nature, found in almost all animal taxa, ranging from simple mutual attractions between individuals, over temporary periods of parental care in family associations to permanent societies with complex social interactions and reproductive division of labour (Wilson, 1971; Costa, 2006; Meunier, 2015). The ecological success of group living species largely relies on the fitness benefits that social interactions provide to group members, for example: increased reproductive success; higher survival rates; enhanced foraging efficiency; and better protection against predators (Wilson, 1971; Krause & Ruxton, 2002; Kohlmeier *et al*., 2016). Group living can also come with major fitness costs such as: inbreeding (Chapman & Bourke, 2001); conflict (Ratnieks *et al.*, 2006); conspicuousness (Beauchamp, 2014); and importantly, the frequent interactions within the group settings can increase the risk of pathogen transmission and infection between individuals (Schmid-Hempel, 1998; Stroeymeyt *et al*., 2014; Theis *et al*., 2015). Consequently, the evolution of social living is associated with the development of mechanisms for group members to mitigate these risks and the potential for high rate of pathogenic spread through their group (Wilson, 1971; Schmid-Hempel, 1998; Kohlmeier *et al*., 2016).

Eusociality, the highest level of sociality, is characterised by overlapping adult generations, cooperative brood care and group members divided into reproductive and non-reproductive castes (Wilson, 1971; Andersson, 1984; Wilson & Hölldobler, 2005). This phenomenon is nearly confined to just insects, with examples ranging from ants (Herbers, 1984), termites (Thorne, 1997), bees (Cameron, 1993), wasps (Gadagkar, 1990), aphids (Chapman *et al*., 2008), thrips (Crespi, 1992) and beetles (Smith *et al*., 2018), but other examples outside of the insects include snapping shrimps (Chak *et al*., 2017) and two species of mammals, the naked mole rat (Jarvis, 1981) and the Damaraland mole rat (Burland *et al*., 2002). Eusocial groups gain greater ecological success by the robust and efficient division of labour by group members. Conflicts still occur within eusocial groups but there are key mechanisms within the groups which help maintain social cohesion, including: genetic homogeneity – resulting in high degrees of relatedness among group members (Keller & Chapuisat,

2001); colony size – the bigger the colony the less likely any one individual will become a replacement reproductive (Bourke, 1999); and benefits of group living over solitary breeding – selfish behaviours are selected against, as shown by worker policing, where worker-laid eggs are destroyed and aggressive behaviour is aimed at reproductive workers (Ratnieks & Visscher, 1989; Wenseleers & Ratnieks, 2006).

Due to the often low genetic diversity within eusocial groups, the close physical space and the frequent contact between individuals, the rate of transmission of a pathogen can be swift and cause eusocial groups to collapse and die (Schmid-Hempel, 1998; Altizer *et al*., 2003, Malagocka *et al.*, 2019). There are two different types of pathogens, micropathogens (bacteria, viruses, protozoa, fungi) and macropathogens (mites, nematodes, helminths). Micropathogens are small and have short generation times, with a very high rate of reproduction within a host body, compared to macropathogens which are larger and have longer generation times, with no or very slow reproduction within a host body, and living stages outside of a host (Anderson & May, 1981). Out of the micropathogens, bacteria, viruses and protozoa are usually transmitted orally, for example by sharing regurgitated food, compared to the spores of the fungi, which are mostly dispersed by the wind or rain and enter a host via the cuticle or other openings such as the trachea (Andreadis, 1987). Consequently, fungi are spread over a wider area and are often associated with soil, compared to the other micropathogens, which are deposited at sites visited by infected individuals (Boomsma *et al*., 2005).

Ants are one of the most ecologically dominant and ubiquitous insects on Earth. Their family, Formicidae, consists of 17 subfamilies and 334 genera, totalling about 13,500 described species to date (Borowiec *et al*., 2021). All ants are eusocial and have matriarchal colonies – the queens store the sperm which they use throughout their lives and all workers, the non-reproductive groups, are female. Workers are responsible for different tasks around the colony, including brood care, looking after the queen, nest building and foraging for resources (Hölldobler & Wilson, 1990; Keller & Chapuisat, 2001; Stuble *et al*., 2017). As ants are omnivorous feeders, they play an important role in ecosystem functions and can live in a wide range of habitats. Their nest sites, both on the ground and arboreally, contribute to seed dispersal, nutrient cycling and the scavenging of dead organisms. These nest sites also

expose ants to different pathogens and challenges, resulting in different life histories (Handel & Beattie, 1990; Hölldobler & Wilson, 1990; Astruc *et al.*, 2004).

The overall aim of this thesis is to evaluate the costs and benefits of eusociality through two different aspects of group living in ants – how immunity is modulated through shared anti-microbial secretions in a group of workers and how co-founding queens can impact colony development when challenged with a pathogen threat. Both are investigated in a species of ground-dwelling harvester ant, which store their food within the nest and so are expected to have particularly well-developed social immunity mechanisms.

3. Chapter 1: Metapleural Gland and Social Immunity

3.1. Introduction

Social insects have evolved both chemical and behavioural defences to chemically kill or physically remove pathogens contaminating their cuticles (Boomsma *et al*., 2005; Wilson-Rich *et al*., 2009; Tranter *et al.,* 2014). A common adaptation across ants is the metapleural gland which secretes antimicrobial compounds onto their cuticles (Hölldobler & Wilson, 1990). The secretion from these glands inhibits the growth of pathogens on the cuticle of the ant, resulting in adult workers with non-functional glands being more susceptible to pathogen infections (Blum, 1992; Bot *et al*., 2001; Tragust *et al*., 2013; Tranter *et al.,* 2014). The metapleural gland is located at the posterior end of the thorax of the ant (Figure 1). Some species of ant do not have metapleural glands, but in the species that have the gland, it is found on all workers and queens, with only a few species also having metapleural glands on their males (Hölldobler & Engel-Siegel, 1984; Yek & Mueller, 2011). The gland can be energetically costly to maintain (Poulsen *et al.,* 2002; Tranter *et al*., 2015) so metapleural gland investment varies between species, which in turn infers the strength of parasitic pressure in different species with different life histories (Hughes *et al*., 2002; Boomsma *et al*., 2005). The antimicrobial secretion can be spread over the body either passively or actively by grooming (Hölldobler & Wilson, 1990; Yek & Mueller, 2011).

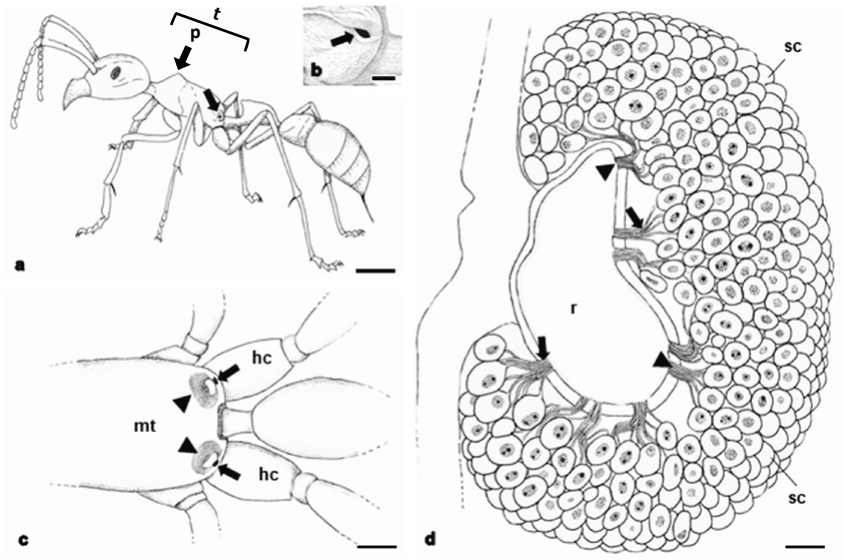


Figure 1. Schematic drawing of *Paraponera clavata* worker and its metapleural gland. (a), (b) and (c) detail of the metapleural gland opening (arrow) in the metathorax (mt), close to the hindcoxae (hc). In (a) detail of the thorax (t), the middle segment of an ant, and pronotum (p), a section of the ant behind its head. In (b) and (c) detail of the gland opening (arrow). (d) metapleural gland with secretory cells (sc), collecting canaliculi (arrows) clustered in the sieve-plates (arrowheads) opening into the reservoir (r). Bar: A = 0.3cm; B = 200µm. C = 0.1cm; D = 150µm. Figure and legend taken from Martins *et al*. (2022).

Individual disease defences ants possess include: physical – the cuticle providing a barrier to pathogens (Hölldobler & Wilson, 1990; Schmid-Hempel, 1998); physiological – the immune system's innate defences such as antimicrobial peptides and parasite encapsulation as well as the antimicrobial secretions of the metapleural gland (Cerenius *et al*., 2008; Otti *et al*., 2014; Tranter *et al*., 2014); and behavioural modifications – the suicidal, sickness behaviour of diseased individuals or the avoidance of infected colony members by healthy individuals (Oi & Pereira, 1993; Parker *et al*., 2011). These individual defences can all help with the collective overall colony defence because if a colony can accurately detect a pathogen early then this allows for either the avoidance of the threat in the first place or the prompt activation of early defence mechanisms, both of which can be useful in alleviating the inevitable cost of infection (Hart, 1990; Schmid-Hempel & Ebert, 2003; Wisenden *et al*., 2009). An example of this is shown when ants avoid infected colony members, as

demonstrated by the ant *Formica rufa*, who feed on nestmate carcasses. They avoid the cadavers of nestmates covered with contagious conidia, the infectious spores of the fungus, but remove or consume the infected dead workers who have died due to the fungus but are nonsporulating (Marikovsky, 1962; Oi & Pereira, 1993; de Roode & Lefèvre, 2012). It is unknown how these ants can accurately detect what stage of infection a diseased ant is in but a study conducted on bees by Conroy & Holman (2022) show that when a bee has a fungal infection, their cuticle hydrocarbon profile changes, alerting other bees to their infectious state. It is possible something similar is occurring in *Formica rufa* but no studies have so far addressed this. Additional avoidance behaviours by the colony include moving to a new nest site, for example, the ant *Solenopsis invicta* will move their colonies to a new nest site if their nest is contaminated with nematodes (Drees *et al*., 1992; Oi & Pereira, 1993). Insects can also perform avoidance behaviours against infected food sources because food sources can be a method of pathogen introduction to a colony, as many pathogens are trophically transmitted (de Roode & Lefèvre, 2012).

Within a colony, the immunity of individual ants along with group-level responses can, together, create 'social immunity'. This term was coined by Cremer *et al.* (2007) and has been studied in a wide range of social insect defence experiments (Walker & Hughes, 2009; Hamilton *et al*., 2011; Konrad *et al*., 2012; López-Uribe *et al*., 2017; Cremer *et al*., 2018). Examples of social immunity behaviours include group members performing allo-grooming behaviours to remove pathogens and fungal spores from other members of the colony, and sharing antimicrobial secretions between themselves (Reber *et al*., 2011; Konrad *et al.*, 2012; Qiu *et al*., 2014). Additionally, waste management and moving dead ants to the rubbish pile helps with the removal of pathogens from the colony as a whole (Tranter *et al*., 2014). Social immunity can be important for the most vulnerable parts of the colony such as the developing brood, as they have yet to develop a fully functional physiological immune system or an antimicrobial-producing gland and they are unable to perform grooming behaviours, so they are particularly susceptible to pathogens (Hölldobler & Wilson, 1990; Lavine & Strand, 2002; Wilson-Rich *et al.,* 2008). This results in the brood having to rely more on social immunity and the donation of adult workers' antimicrobial secretions. Additionally, the nest substrate of a colony is also vulnerable to

contamination by pathogens. In order to keep the colony nest material hygienic, most workers spread antimicrobial secretions around the nest (Currie *et al*., 1999; Reber & Chapuisat, 2011; Tranter *et al*., 2014; Liu *et al*., 2019), with some ant species, such as *Formica paralugubris,* collecting antimicrobial substances from outside their nests, such as tree resin, which helps to stop bacterial and fungal growth on nest material (Chapuisat *et al.*, 2007).

To investigate how social immunity is modulated within a group, the study organism used was *Messor barbarus*, a harvester ant found in the Mediterranean region, which has a metapleural gland. Harvester ants collect and store seeds and grains in underground granaries, foraged from outside the colony (Hölldobler & Wilson, 1990). *M. barbarus* lives in a monogynous colony of about 8000 individuals (Cerdan, 1989; Jeanson *et al*., 2004) of varyingly sized ants, who have different roles within the colony – small workers are nurses and foragers, while large ants are soldiers, who protect the colony against predators and invaders, and also seed millers, crushing large seeds to make them easier for the smaller workers to use (Hölldobler & Wilson, 1990). The ants dig nests in soil which contain networks of interconnecting chambers, containing brood and workers, as well as their grain stores (Jeanson *et al*., 2004). *M. barbarus* are ecosystem engineers, influencing the surrounding vegetation, as they disperse plant seeds, impact seed banks by depleting seeds, cycle nutrients and modify the microclimate (MacMahon *et al*., 2000; Plowes *et al*., 2013; Steiner *et al*., 2018). Their grain stores can also change the physical, chemical and hydrological properties of the soil (Cammeraat *et al.*, 2002).

This study investigated how social immunity is modulated via the manipulation of the availability of the metapleural gland, by measuring resistance, via survival probability, of *M. barbarus* to a generalist fungal pathogen, *Metarhizium brunneum*. The duration and frequency of self- and allo-grooming of individuals within groups with different proportions of blocked and available metapleural glands were analysed, as well as their survival probabilities. Additional research investigated the likelihood of infection and survival probabilities, by comparing indirect exposure experiments, which allowed ants the choice of avoiding walking on contaminated filter paper, and direct exposure experiments, where spore solution was directly applied to the cuticle of an ant.

3.2. Materials & Methods

3.2.1. Study Organisms

Different combinations of four colonies of *M. barbarus* were used for the experiments. The colonies were kept at 26°C, 60% relative humidity and 12:12h photoperiod prior to experiments. The colonies were fed a diet of chia and grass seeds, cockroach cadavers, and 20% sucrose solution as well as readily available water. Out of the four colonies, there were two large colonies (>400 ants). One of these large colonies killed their queen, so for experimental purposes was classed as a queenless colony (QL). The other large colony did not kill their queen (Q). The remaining colonies were two smaller older colonies (≈300 ants). One of these colonies was used for *Experiment 1* (OQ) but using this colony reduced the number of ants available for experimentation so the other older colony (OQ2) was used in *Experiment 2*. All colonies were sourced from Ant Antics (https://www.antantics.wales).

During *Experiment 1*, there was a difference discovered in the colonies used and their colony level resistance. Workers from the queenless colony had a significantly lower survival rate after fungal exposure compared to the other colonies of origin, which had queens, regardless of the method used for exposure. As there was a significant difference between these colonies in the experiment, the queenless colony was not used in *Experiment 2*.

The pathogen used for the immune challenges was *Metarhizium brunneum*, a generalist fungal pathogen which paralyses and kills a wide range of insects and has been tested and used in a wide range of ant studies (Reber *et al.,* 2011; Tranter *et al*., 2014; Tranter *et al*., 2015; Scavetta *et al*., 2021). The fungal spores attach to the insects' cuticles and invade the body cavity, damaging hosts cells and producing toxins, which together ultimately kill the host (Vestergaard *et al*., 1999; Chouvenc *et al*., 2009). This is why individual defences such as physical, physiological and behavioural defences, as well as group defences such as allo-grooming are important for preventing death, by preventing penetration of the cuticle by the germinating spores of this lethal pathogen. Fungal conidia were harvested from freshly sporulating single strain sabouraud dextrose agar plates and suspended in 0.05% Triton X. Triton X has been used in multiple different ant studies to suspend *M. brunneum* spores,

(Tranter *et al*., 2014; Tranter *et al*., 2015; Scavetta *et al*., 2021) as the cuticle of an ant is hydrophobic but Triton X has the ability to wet this cuticle, allowing access inside the ant (Rostas & Blassmann, 2009). Spore concentration was then estimated using a Fast Read counting slide, and the required concentrations were made from this solution.

3.2.2. Experiment 1: How does the method of spore exposure affect survival probability?

Investigations were conducted into whether the time an ant spent walking on a piece of filter paper contaminated with fungal spores could predict the probability of death. This experiment also examined the effectiveness of an indirect method of spore exposure using a dosed response so that comparisons could be made between different concentrations of fungal spores. Additional investigations subsequently looked into the effect of pipetting the fungus directly onto the ant and what affect this had on the probability of the ant surviving. For comparison to take place between these two methods of spore exposure, time spent on fungus was used as a covariate to account for the behavioural aspect of the indirect method of spore exposure experiment.

To investigate an indirect method of spore exposure, a total of 142 ants from three different colonies (Q, OQ and QL) were used. From these colonies, ants were chosen that were similar in size. Individual ants were exposed to *M. brunneum* in a behavioural experiment, which consisted of ants walking over 90mm filter paper, half covered in different spore concentrations and half covered in 0.05% Triton X (Figure 2). The spore concentrations were labelled Stock, x8, x7, x6, x5 and were as follows respectfully: $1.75x10^9$; $1.75x10^8$; $1.75x10^7$; $1.75x10^6$; $1.75x10^5$ spores/ml suspended in 0.05% Triton X. A control was also used, consisting of two halves of filter paper covered in 0.05% Triton X. This environment provided a more natural scenario closer to what the ants may experience in the wild, as there would not naturally be a time when they would be constantly walking over fungus. The filter papers were constructed by cutting a new filter paper in half, then 300µl of fungal spores (or 0.05% Triton X for control) was pipetted onto one half of the filter paper, with 300µl of 0.05% Triton X

pipetted onto the other half. After the two halves of the filter paper had dried, they were stuck together with cello-tape and placed inside a fluoned Petri dish (Figure 2).

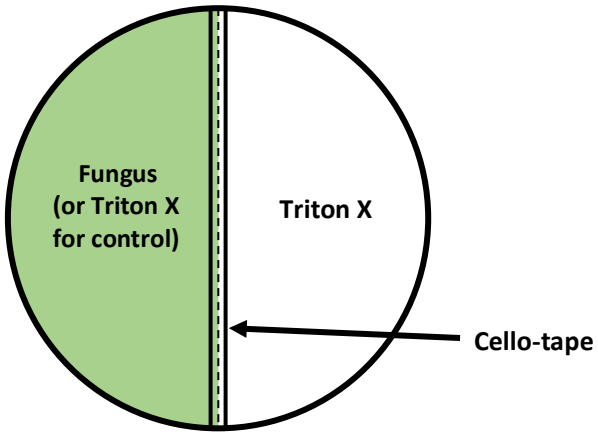


Figure 2. Experimental set up of the indirect method of spore exposure using a filter paper. The size of the filter paper and Petri dish were 90mm. Figure created by author.

Ants were placed inside the Petri dishes, each containing a different concentration of spores or control, and the lids were cello-taped down to prevent escape. Ant behaviour was observed for 15 minutes, with observations recorded as the time an ant spent on the fungal spore covered side of the filter paper out of the 15 minutes and for the control, one side of the filter paper was picked for the time study. After this, the ants were transferred into labelled 8oz plastic food storage pots, which contained a damp filter paper and a ball of cotton wool soaked in 20% sucrose solution. These ants were then checked daily and their day of death was noted for 15 days. After death, they were sterilised, following the sterilisation protocol of Lacey & Brooks (1997) and placed into a sterile Petri dish with a damp filter paper, to record fungus growth for confirmation of the cause of death. At the end of the experiment any surviving ants were humanely euthanised in a freezer.

To investigate a direct method of spore exposure, a total of 90 ants, also from the same colonies as above, were exposed to *M. brunneum* by a drop of spore solution. Ants were chosen from the colonies that were similar in size. The different concentrations of spores suspended in 0.05% Triton X solutions were the same as the indirect spore exposure experiment above. The ants were anaesthetised on ice, then 0.5µl of spore solution was pipetted onto the ants, onto their thorax (Figure 1), with

0.05% Triton X used as the control. The spore solutions were vortexed between pipetting to allow for even doses across ants. The ants were grouped together by colony and treatment, in groups of 5, and were placed in labelled 8oz plastic food storage pots, which contained a damp filter paper and a ball of cotton wool soaked in 20% sucrose solution. The method of sterilisation of ants after death was the same as the indirect spore exposure experiment above. At the end of the experiment any surviving ants were humanely euthanised in a freezer.

3.2.3. Experiment 2: How does the availability of metapleural glands in a group modulate social immunity?

Investigations were conducted into how the availability of the metapleural gland within a group affects ant behaviour and subsequent survival. A total of 600 ants were used in this experiment, from two different colonies (Q and OQ2). Ants were chosen from the colonies that were similar in size. To block the metapleural gland of half the ants (300), they were anaesthetised on ice, then a tiny drop of white nail varnish was applied to the opening of the metapleural gland, at the posterior of the thorax (Figure 1), using a 30µl micro-syringe. For the control, using the remaining 300 ants, they were anaesthetised on ice, then a small drop of nail varnish, using a 30µl micro-syringe, was placed on their pronotum (Figure 1). Nail varnish has been used in multiple ant studies to block metapleural glands and has been shown to be effective at fully blocking the gland (Poulsen *et al*., 2002; Graystock & Hughes, 2011; Tranter *et al.,* 2014; Tranter & Hughes, 2015). The nail varnish was checked daily and remained intact on all ants throughout the whole 14 days of the experiment.

After the nail varnish treatment, the ants were left for 2 hours and then were split into groups of 5 within the same colony, and placed into an 8oz plastic food storage pot, with different numbers of ants with blocked and open metapleural glands as follows: 0/5 blocked glands; 1/5 blocked glands; 2/5 blocked glands; 3/5 blocked glands; 4/5 blocked glands; 5/5 blocked glands. Each group of 5 were then assigned a treatment: fungus; Triton X; or control. For the fungus and Triton X treatments either 0.5µl of 4.43×10^8 spores/ml suspended in 0.05% Triton X or 0.5µl of just 0.05% Triton X was pipetted onto the thorax (Figure 1) of each ant. The fungal solution was vortexed

between pipetting to allow for even doses across ants. For the control, ants were "pipetted" but with nothing in the pipette. After the treatment was applied, the ants were left for 10 minutes, then video-recorded using an iPhone 11 Pro for a further 10 minutes, from above, fitting two pots into the frame. The videos were later analysed to observe self-grooming and allo-grooming events and duration for all ants in each pot, with grooming events and duration analysed separately as they are metrics that could vary independently with different treatments. Self-grooming and allo-grooming events were classified according to behaviours described by Nilsson-Møller *et al.* (2018). After recording, the ants were then transferred into labelled 8oz plastic food storage pots which contained a damp filter paper and a ball of cotton wool soaked in 20% sucrose solution. These ants were then checked daily for 14 days and their day of death was noted. When an ant died it was taken out of the pot. At the end of the experiment any surviving ants were humanely euthanised in a freezer.

3.2.4. Statistical Analysis

Analyses were performed using the free analysis software R v.4.4.1 (R Development Core Team, 2024). For *Experiment 1*, the effect of condition (Stock, x8, x7, x6, x5, Control) and colony ID (Q, OQ, QL) and their interaction on ant survival after treatment was assessed using a survival model fitted using the survreg function from the Survival package v.3.8.3 (Therneau, 2024) on right censored data, with any deaths recorded as a "1" and ants surviving to the end of the experiment censored as "0", with percentage of time spent on fungus as a covariate. A Weibull distribution was fitted as an accelerated hazard function. The effect of condition (Stock, x8, x7, x6, x5, Control) and colony ID (Q, OQ, QL) on percentage of time spent on the fungus, as well as the effect of time spent on fungus and the ants day of death was modelled using linear mixed-effect models, implemented using the lmer function, in the lme4 package v.1.1.35.5 (Bates *et al*., 2015).

For *Experiment 2*, behavioural count data was analysed using generalised linear mixed-effect models, implemented using the glmer function and behavioural time data was analysed using linear mixed-effect models, implemented using the lmer function, both in the lme4 package v.1.1.35.5 (Bates *et al*., 2015). The effect of

treatment (fungus, Triton X, control), the metapleural gland state (blocked, open) or the proportion of ants with blocked metapleural glands within a pot (0-5) and their interactions on either the count of behaviour or time spent performing the behaviour was modelled. For all behavioural data, pot of ant origin, nested within colony of origin was fitted as a random effect, with a Poisson error distribution for the behavioural count data. Outliers were removed from the data set during analysis, with outliers being classed as 1.5 times the Inter Quartile Range (IQR) above the 75^{th} quartile. Ants would usually groom for an extended period of time if they were undisturbed by other ants interacting with them. Not all undisturbed ants groomed for a long time, which suggests that the reason for grooming in the first place was unresolved by the action of grooming in these ants. As ants would normally be in a colony and therefore frequently disturbed, it was considered that these extended grooming events could be safely disregarded as unrepresentative of typical grooming activity.

Additionally, for *Experiment 2*, the effect of treatment (fungus, Triton X), the metapleural gland state (blocked, open) or proportion of ants blocked within a pot (0-5) and their interaction on ant survival after treatment was assessed using a Cox proportional hazards model, implemented using the coxph function from the Survival package v.3.8.3 (Therneau, 2024) on right censored data, with any deaths recorded as a "1" and ants surviving to the end of the experiment censored as "0". The pot of ant origin, nested within colony of origin was fitted as a random effect using a frailty term.

P values were extracted from each model using log-likelihood ratio tests implemented using the Anova function in the Car package v.3.1.3 (Fox & Weisberg, 2019).

3.3. Results

3.3.1. Experiment 1: How does the method of spore exposure affect survival probability?

For the indirect spore exposure experiment, there was no effect of spore concentration on time spent in the treatment zone (χ^2 = 5.4, df = 6, *P* = 0.495; Figure 3a), no difference between colonies in how much time they spent in the treatment zone (χ^2 = 0.5, df = 2, *P* = 0.986; Figure 3a) and no interaction between spore

concentration and colony on time spent in the treatment zone (χ^2 = 2.8, df = 10, *P* = 0.986; Figure 3a). There was no relationship between the spore concentration and the probability of survival (χ^2 = 4.5, df = 5, *P* = 0.474; Figure 3b). However, there was a significant effect of colony on the probability of survival (χ^2 = 25.0, df = 2, *P* < 0.001; Figure 3c). There was no relationship between percentage of time spent on the fungus and the day of death (χ^2 = 0.001, df = 1, *P* = 0.970; Figure 3d).

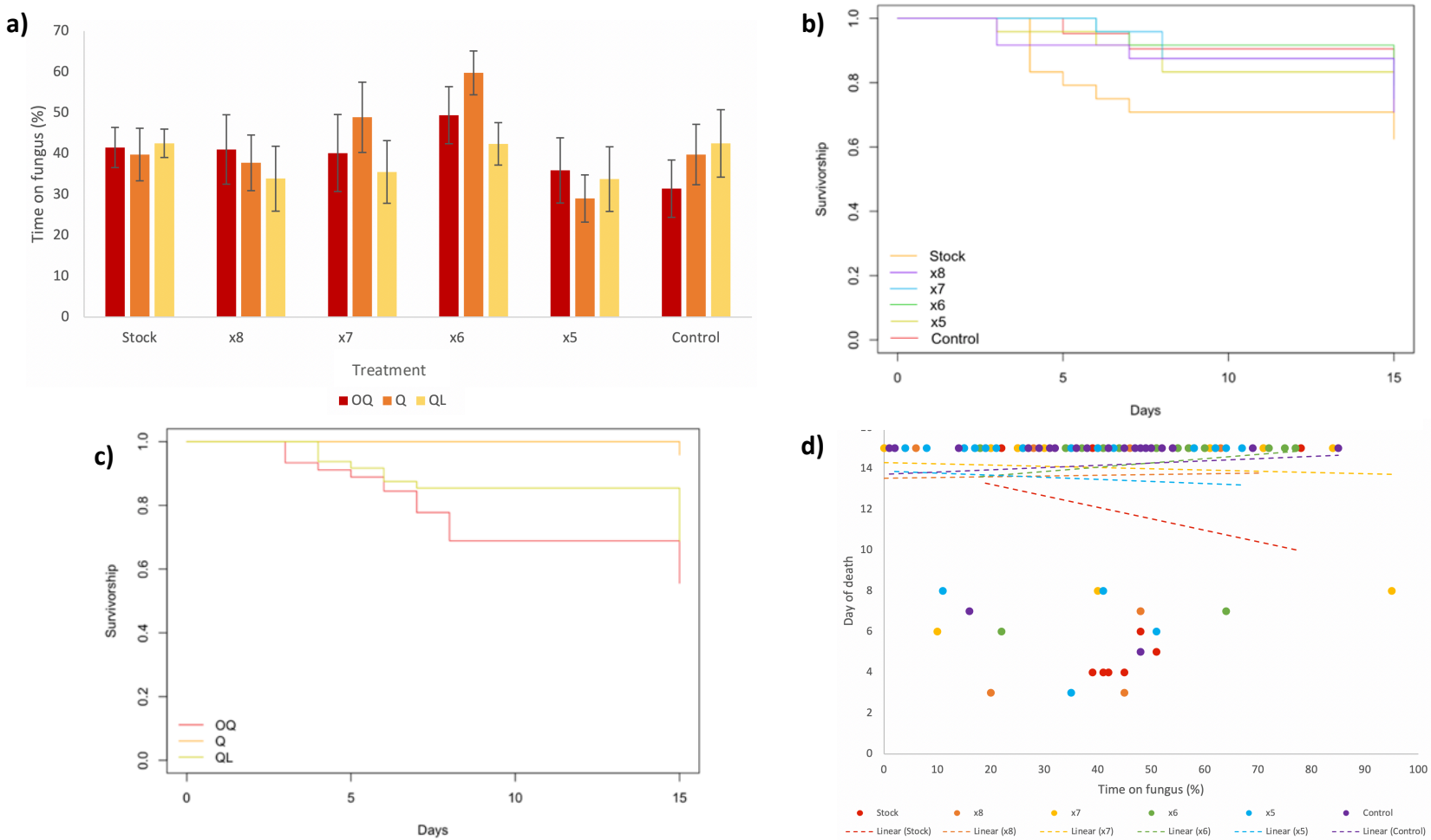


Figure 3. Data showing how colony and exposure to different concentrations of *M. brunneum* affect the behaviour and survival in *M. barbarus* harvester ants. a) percentage of time spent on the different spore concentrations, compared between the different colonies and spore concentrations. Error bars = ± standard error; b) survival plot for different spore concentrations; c) survival plot for the different colonies used; d) percentage of time an ant spent on the different spore concentrations and their day of death.

When the spores were applied directly to the ants, there was a significant effect of spore concentration on the probability of survival (χ^2 = 58.8, df = 5, P < 0.001; Figure 4a) as well as a significant effect of colony on the probability of survival (χ^2 = 20.6, df = 2, P < 0.001; Figure 4b). Due to these colony effects on the probability of survival, the queenless colony (QL) was not used in *Experiment 2*. As there was a strong effect of spore concentration on the probability of survival, when the spores were pipetted onto the ant, this method of spore application was used in *Experiment 2*.

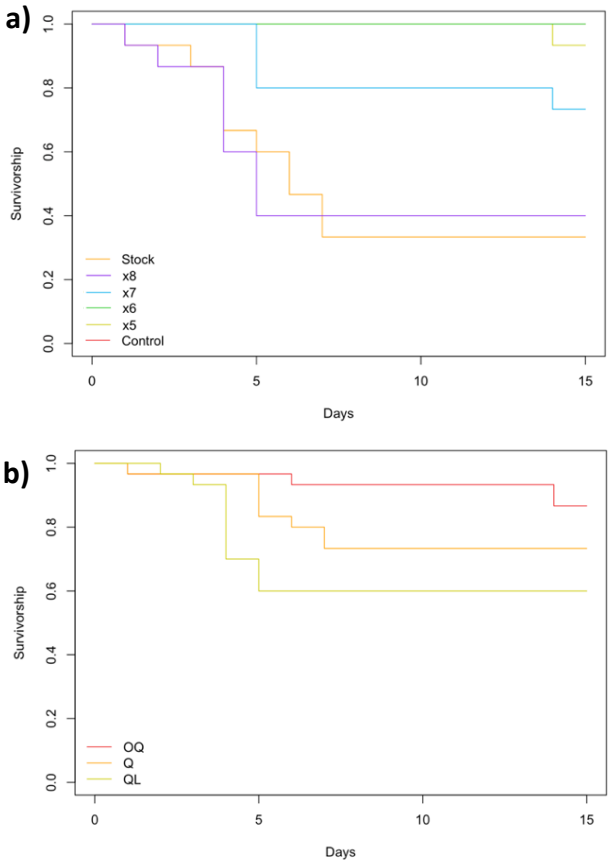


Figure 4. Data showing how colony and exposure to different concentrations of *M. brunneum* affect the survival in *M. barbarus* harvester ants. a) survival plot for different spore concentrations, when directly applied to the cuticle of the ant; b) survival plot for different colonies.

3.3.2. Experiment 2: How does the availability of metapleural glands in a group affect grooming response to a pathogen?

There was an effect of treatment (χ^2 = 65.8, df = 2, P < 0.001; Figure 5a) and the proportion of glands available (χ^2 = 11.3, df = 5, P = 0.036; Figure 5a) on time spent self-grooming but no interaction between the two (χ^2 = 11.3, df = 10, P = 0.333; Figure 5a). Ants self-groomed for longer periods of time when a fungus or Triton X treatment was pipetted onto them, compared to the control. There was an effect of treatment (χ^2 = 11.7, df = 2, P = 0.029; Figure 5b), the proportion of glands available (χ^2 = 16.9, df = 5, P = 0.005; Figure 5b) and an interaction between the two (χ^2 = 21.6, df = 10, P =

0.017; Figure 5b) on the number of self-grooming events which occurred. There was a complex interaction between the treatments and gland availablility (Figure 5b). Outliers, which accounted for 8% of all data points, were removed from the bar charts (Figure 5), with these outliers shown in the box plot (Figure 6).

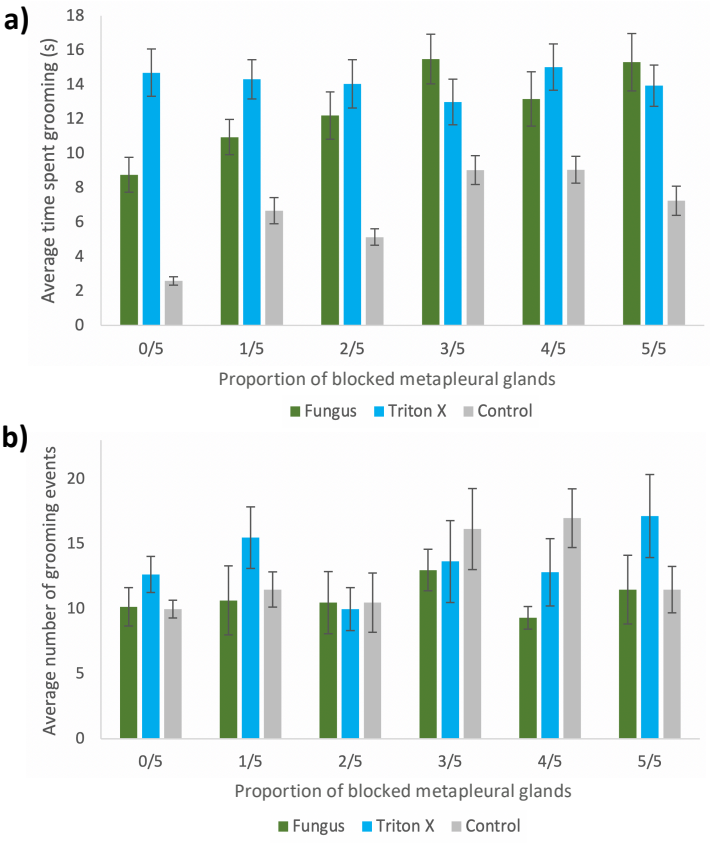


Figure 5. Data showing how exposure to *M. brunneum,* Triton X or control affect the behaviour in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. a) average time an ant spent self-grooming during the different treatments; b) the average number of self-grooming events which occurred across treatments. Outliers were removed if they were 1.5x the IQR above the 75th quartile. Error bars = ± standard error.

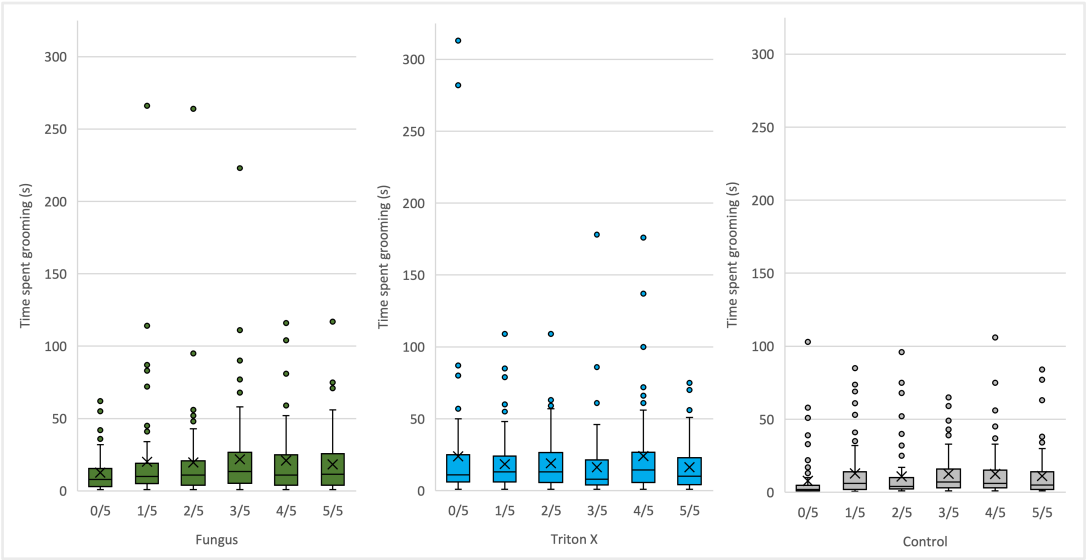


Figure 6. Data showing how exposure to *M. brunneum,* Triton X or control affect the behaviour in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. Time spent self-grooming across the treatments, showing the outliers, consisting of 8% of all data points, taken out of the previous bar charts (Figure 5).

There was no effect of treatment (χ^2 = 0.5, df = 2, *P* = 0.769; Figure 7a), the proportion of glands available (χ^2 = 2.7, df = 5, *P* = 0.742; Figure 7a) or interaction between the two (χ^2 = 5.6, df = 6, *P* = 0.467; Figure 7a) on time spent allo-grooming. There was also no effect of treatment (χ^2 = 1.5, df = 2, *P* = 0.483; Figure 7b), the proportion of glands available (χ^2 = 1.8, df = 5, *P* = 0.880; Figure 7b) or an interaction between the two (χ^2 = 3.2, df = 6, *P* = 0.781; Figure 7b) on the number of allo-grooming events which occurred.

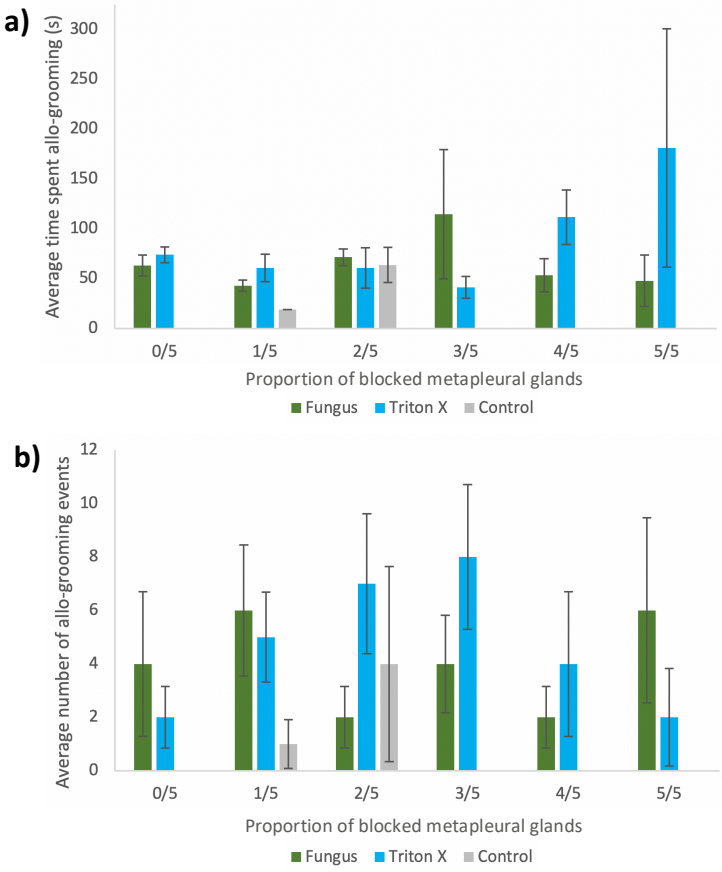


Figure 7. Data showing how exposure to *M. brunneum,* Triton X or control affect the behaviour in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. a) average time ants spent allo-grooming during the different treatments; b) the average number of allo-grooming events which occurred across treatments. No outliers were removed. Error bars = ± standard error.

There was an effect of treatment (χ^2 = 65.8, df = 2, *P* < 0.001; Figure 8a) but not the gland availability of the grooming ant (χ^2 = 0.1, df = 1, *P* = 0.737; Figure 8a) on time spent self-grooming. There was a significant interaction between both treatment and gland availability (χ^2 = 6.4, df = 2, *P* = 0.042; Figure 8a) on time spent self-grooming. There was an effect of treatment (χ^2 = 12.9, df = 2, *P* = 0.002; Figure 8b), but not gland availability of the grooming ant (χ^2 = 2.9, df = 1, *P* = 0.090; Figure 8b) on the number of self-grooming events which occurred. There was no significant interaction between both treatment and gland availability (χ^2 = 2.0, df = 2, *P* = 0.369; Figure 8b) on the number of self-grooming events which occurred. Outliers, which accounted for 8% of

all data points, were removed from the bar charts (Figure 8), with these outliers shown in the box plot (Figure 9).

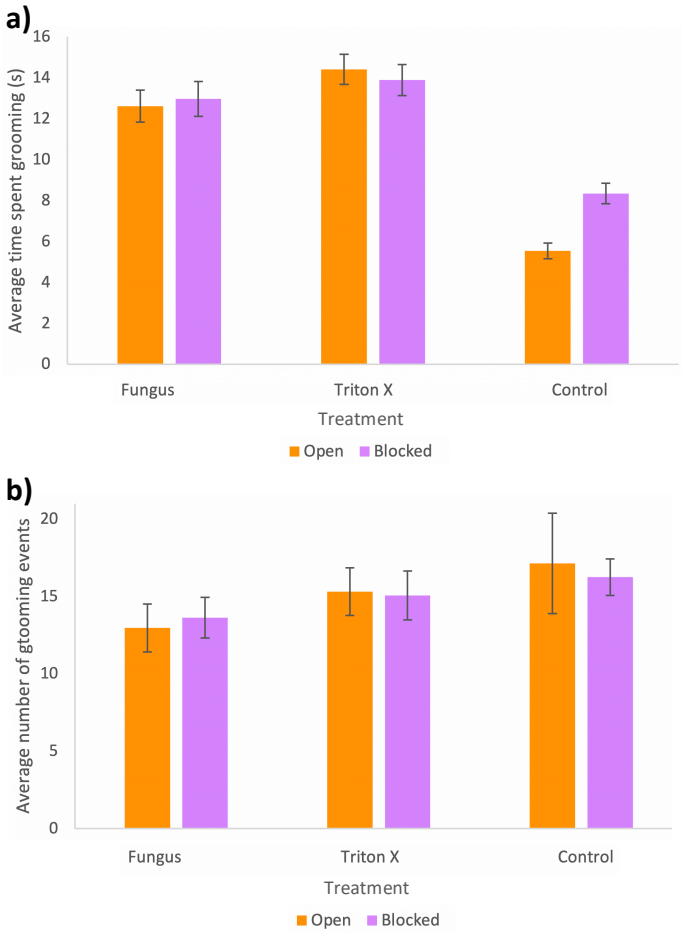


Figure 8. Data showing how exposure to *M. brunneum,* Triton X or control affect the behaviour in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. a) average time an ant spent self-grooming and whether that ant had a blocked or open metapleural gland; b) the average number of self-grooming events which occurred across treatments and whether the ant performing the self-grooming had a blocked or open metapleural gland. Outliers were removed if they were 1.5x the IQR above the 75th quartile. Error bars = ± standard error.

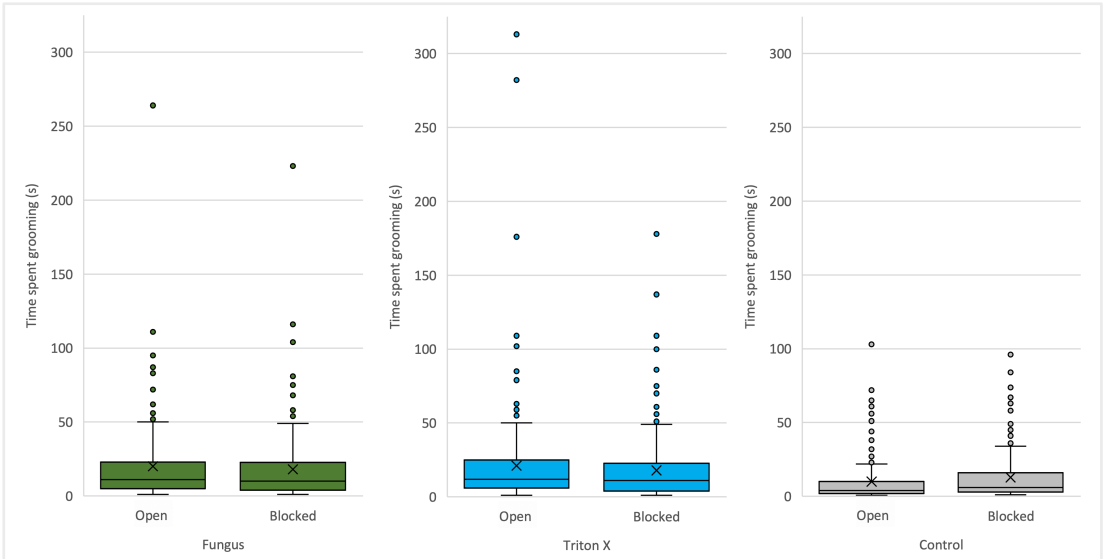


Figure 9. Data showing how exposure to *M. brunneum,* Triton X or control affect the behaviour in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. Time spent self-grooming across the treatments, showing the outliers, consisting of 8% of all data points, taken out of the previous bar charts (Figure 8).

3.3.3. Experiment 2: How does the availability of metapleural glands in a group affect survival probability?

There was no difference in the probability of survival of ants treated with control and Triton X, therefore results from hereon in only include Triton X as the control data (χ^2 = 6.286, df = 5, *P* = 0.280). Ants with blocked glands died at a higher rate than those with open glands (both treatments: χ^2 = 5.8, df = 1, *P* = 0.016; Figure 10a; fungus treatment: χ^2 = 21.5, df = 1, *P* < 0.001; Figure 10a). Additionally, there was an effect of treatment and the proportion of available glands within the pot (χ^2 = 3.7, df = 5, *P* = 0.004; Figure 10b & 10c) on probability of survival. All ants treated with fungus and placed in both 4/5 and 5/5 blocked pots died.

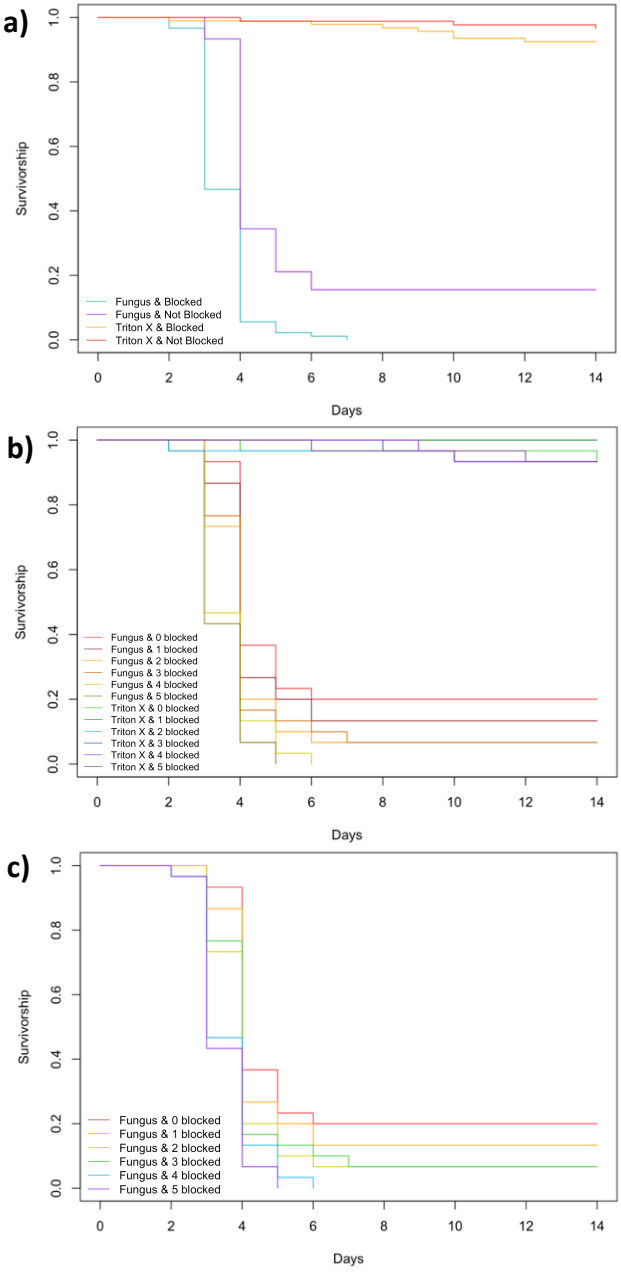


Figure 10. Data showing how exposure to *M. brunneum* or Triton X affect the survival in *M. barbarus* harvester ants, who either have their metapleural glands blocked or not. a) survival plot for fungus and Triton X treatment, comparing mortality between ants with a blocked metapleural gland and not blocked (open) metapleural gland; b) survival plot for fungus and Triton X treatment, comparing mortality between each pot of different proportion of ants with a blocked metapleural gland; c) survival plot for just fungus, emphasising the mortality between the pots of different proportions of ants with a blocked metapleural gland.

3.4. Discussion

Self-grooming duration and frequency occurred to a similar higher extent after the application of the spore suspension and the Triton X solution, compared to the control, which indicates a response to the presence of a foreign substance on the cuticle, instead of recognition of the pathogen. Some ants groomed for an extended period of time when undisturbed by interaction with other ants. Inside a colony, ants would be frequently disturbed, and therefore these extended grooming events are classed as unrepresentative of typical grooming activity. Outliers were removed as part of the results analysis and as they accounted for 8% of all data points, this gives confidence that the majority of ants were exhibiting behaviour that was consistent with that of a worker within a colony.

Within the pots for the fungus and control treatments, as an increasing proportion of ants had blocked metapleural glands, the duration of self-grooming increased. The duration of self-grooming in the Triton X treatment mostly stayed the same as metapleural gland availability decreased. Compared to the self-grooming results, there was no difference in duration or frequency of allo-grooming events due to either treatment or availability of metapleural gland. There were minimal allo-grooming occurrences overall, with similar results shown by Bos *et al*. (2019), where they found there was little or no allo-grooming, comparing twelve different ant species. Other studies with more allo-grooming data have suggested that the frequency of allo-grooming is influenced by the proportion of pathogen exposed verses clean workers, with the lowest frequency of allo-grooming occurring when the majority of workers in a group have been exposed to a pathogen (Reber *et al*., 2011; Cotazo-Calambas *et al*., 2022). Additionally, it has been shown that group size is directly proportional to the frequency of allo-grooming (Schmid-Hempel, 1998; Hughes *et al*., 2002; Okuno *et al*., 2012), and so the minimal allo-grooming occurrences in this study may be due to the small groups of five that the ants were placed in. This may be a group which is too small for ants to frequently perform colony cleaning behaviours such as allo-grooming.

When comparing the difference in self-grooming behaviours between ants with and without a blocked metapleural gland, there was no effect of the availability

of an individual's metapleural gland on the number of self-grooming events which occurred between treatments. Under the control treatment, there was an effect of an individual's gland availability on the duration of the self-grooming events – if ants had a blocked metapleural gland, they groomed for a longer period of time. For the fungus and Triton X treatments, there was no difference in the duration of self-grooming between ants with a blocked or open metapleural gland. Regardless of whether an ant had a blocked or open metapleural gland, the ants had the highest average time spent grooming when they were either treated with the spore solution or Triton X, compared to control. A study conducted by Graystock & Hughes (2011), compared species of ants who either did or did not have a metapleural gland. They observed that the species without a metapleural gland self-groomed substantially more compared to any other species, suggesting that more frequent self-grooming improves disease resistance. However, the results shown by Graystock & Hughes (2011) do not agree with the results found in this study because in the fungus and Triton X treatments, there was no difference in frequency or duration of self-grooming by ants with blocked and open metapleural glands (Figure 8). This may be due to the overall effect of the presence of a foreign substance increasing the duration and frequency of self-grooming for all ants treated with a substance. Additionally, the self-grooming events recorded were the removal of spores from the cuticle, not the grooming of the metapleural gland, which, if studied, may result in a difference in the frequency and duration of self-grooming of ants with blocked and open metapleural glands. The active grooming of the metapleural gland has been described as a method of actively accessing the antimicrobial substance, in multiple different ant species (Fernández-Marín *et al*., 2006). Tranter *et al*. (2015) showed that the rate of metapleural gland grooming differed between species, so a further study of *M. barbarus* metapleural gland grooming could be a next step in this research.

The results of this study demonstrated that the availability of the metapleural gland is an important element in ant survival when exposed to a fungal pathogen. Previous literature has shown that the metapleural gland is an important addition to the ants immune system response, and without it they are more likely to die from fungal pathogen exposure (Poulsen *et al*., 2002; Fernández-Marín *et al*., 2006; Tranter *et al*., 2015; Scavetta *et al*., 2021). This agrees with the results shown in this study,

where all ants in both groups of 4/5 and 5/5 blocked metapleural glands, treated with the spore suspension solution, died (Figure 10b & 10c). Additionally, every ant with a blocked metapleural gland treated with the spore suspension solution died, regardless of the glands available in the pot of origin (Figure 10a). This shows that despite the high duration and frequency of self-grooming that ants in the fungus treatment performed, this did not stop ants with blocked glands dying from the pathogen. Ants with open metapleural glands were observed to survive only when they were found in a pot with other ants with open metapleural glands. However, their chance of survival decreased when fewer open metapleural glands were available in the pot of origin (Figure 10c). This could suggest there may be a social immunity aspect occurring in the pots with more gland availability, such as unobserved allo-grooming occurring. Behavioural observations happened right at the beginning of the experiment so future work could look into observations throughout the experiment to see if allo-grooming may occur.

Additional experiments in this study looked at the likelihood of an ant becoming infected and subsequently dying, when different methods of spore exposure were used. The cuticle of an ant is hydrophobic but Triton X has the ability to wet this cuticle and allowing access inside the ant (Rostas & Blassmann, 2009). This means that ants will have different responses to wet (pipette drop) and dry (filter paper) treatments. Additionally, it has been shown that ants can detect fungal spores on a food source (de Roode & Lefèvre, 2012), so it is possible that they would avoid a fungal contaminated area. However, in this study it was shown that the ants spent no difference in time in the treatment zones and did not avoid the fungal contaminated side of the filter paper. Additionally, there was no correlation between the percentage of time spent on fungus and the day of death, with most ants surviving the full 15 days of the experiment. This contradicts previous studies which have shown a high mortality when fungus is introduced on a filter paper (Chapuisat *et al*., 2007; Reber *et al*., 2008; Reber *et al*., 2011). However, in this study the ants only had half the filter paper covered in fungal spores so this might have reduced the impact of the spore exposure compared to previous studies. Either a stronger concentration of fungal spores or a longer time exposed to the pathogen may be required to achieve the equivalent effect as that found in the literature. In comparison, there was a high

mortality of ants when a drop of spore suspension solution was pipetted directly to the cuticle, which agrees with previous literature (Graystock & Hughes, 2011; Okuno *et al*., 2012; Tranter *et al*., 2015; Tranter & Hughes, 2015). The advantages of applying the spore suspension solution directly to the cuticle is that there is more confidence of the known concentration of spores that the ants are exposed to, compared to exposure via walking over spore contaminated filter paper.

An additional interesting result from this experiment was a difference in the survival of workers from the different colonies for both exposure methods, where the ants from the queenless colony had a higher mortality rate compared to other colonies. This requires further investigation to see what effect having a queen within a colony might have on the immune response of her workers.

The results of Chapter 1 showed that: i) the presence of fungal spores or Triton X on the cuticle, as well as decreasing gland availability within a pot, increased self-grooming duration and frequency; ii) there was no difference in time spent allo-grooming or allo-grooming events between different treatments or gland availabilities; iii) groups with a lower proportion of available metapleural glands had a lower survival rate when exposed to a pathogen; iv) a direct method of spore application resulted in a lower ant survival rate compared to an indirect method. The next stage of this thesis was then to investigate how the impact of pathogen exposure affected the founding stages of colony development.

4. Chapter 2: Pathogen Resistance and Co-Founding Queens

4.1. Introduction

The life cycle of an ant colony can be split into three different parts: the founding (creating a colony), ergonomic (growing a colony) and reproductive stages. During the founding and ergonomic stages the colony consists of entirely female ants, until the reproductive stages, when males and immature virgin queens are produced (Oster & Wilson, 1978). In most species, the males and virgin queens from multiple colonies will fly away from their natal colony and take part in the nuptial flight, where they mate (Keller, 1991; Sommer & Hölldobler, 1995). Once a queen has been

inseminated, she finds a safe place to establish a colony, raising her brood by providing food from metabolising her own tissues (Waloff, 1957). Most social insects develop their nests underground, so founding queens are exposed to soil-borne pathogens such as entomopathogenic fungi (Keller *et al*., 2003; Reber & Chapuisat, 2011). If the queen has to expend resources on fighting an infection then this could affect the metabolising of her own tissues for food for her brood, affecting the overall survival of the founding colony (Waloff 1957; Pull *et al*., 2013). As well as diseases, developing colonies are vulnerable to a wide range of threats including predation and competition, with the mortality of emergent colonies estimated to be as high as 95% (Baer *et al*., 2006).

New queens can found colonies independently (haplometrosis), but it is also possible for them to co-found a colony together (pleometrosis) (Hölldobler & Wilson, 1990; Brütsch *et al*., 2017; Aron & Deneubourg, 2020). The study species *M. barbarus* has been recorded in literature as monogynous: all mature colonies have one reproductive queen, but during colony founding, it has been recorded that some species within the genus of *Messor* form pleometrotic groups (Rissing & Pollock, 1986; Helms-Cahan & Helms, 2012; Motro *et al*., 2017). These paired queens are often unrelated, an interesting phenomenon, as, under kin selection, it is expected that most cooperating individuals are related to each other (Queller & Strassmann, 1998). Other organisms apart from ant foundresses which form unrelated pairs include mongooses (Creel & Waser, 1994), manakins (McDonald & Potts, 1994), kingfishers (Reyer, 1984) and halictine bees (Kukuk & Sage, 1994). The advantage of founding a colony together can be: the production of more brood and at a higher rate than with solitarily founding queens (Rissing & Pollock, 1988); more efficient nest construction (Peeters & Andersen, 1989); earlier maturation of workers (Rissing & Pollock, 1988); queens losing less weight during the provisioning of the first brood (Waloff, 1957); and a higher rate of survival during the early stages of the founding period (Adams & Tschinkel, 1995; Cahan & Julian, 1999). Additionally, it is thought that co-founding is an evolutionary response to brood raiding as multiple queens can help give colonies advantages in brood raids – where invading ants steal the brood of another colony unrelated to themselves – or defending against invading queens from neighbouring colonies (Rissing & Pollock, 1987; Adams & Tschinkel, 1995; Balas & Adams, 1996).

However, these associations only last until the first workers emerge, at which point the queens duel, and the prevailing queen will injure and kill the loser. The winning queen can then build a larger colony with more initial workers than a single queen is able to produce on her own. The workers do not show aggression against the queens, until they fight, and then the workers target the injured queen. There is a risk to co-founding a colony, with a maximum cost of death for the loser, but a large benefit for the winner, being able to start a colony with many more workers than would otherwise be possible (Bernasconi & Strassmann, 1999; Pull *et al*., 2013; Aron & Deneubourg, 2020).

Ants display complete metamorphosis with their four life stages consisting of: egg, larvae, pupae (collectively called the brood), and adult. Their eggs are small, white and round and, for most harvester ant species, they take about two to four weeks to hatch into larvae, which are the immature, juvenile stage of an ant's life. The larvae do not have legs and have a larger, more oval shape compared to the eggs. Workers feed larvae regurgitated food and they require a lot of protein in order to grow significantly larger. As the larvae grow, they shed their exoskeletons and reach a new development stage, termed instar (Oster & Wilson, 1978). Most harvester ants have around three to four instars (Onoyama, 1982; Cassill *et al*., 2005). After the larvae have completed their final instar, they enter the pupal stage. During the pupal stage, many insects have a protective outer covering while they grow, known as a cocoon or pupal case. However, *M. barbarus* do not have a protective casing, allowing the observation of visibly growing legs and eyes during their pupal phase. Just before the pupae turn into an adult, they undergo eclosion, where the outer layer of the pupae hardens and becomes a light to dark brown colour (Oster & Wilson, 1978).

This study investigated the effect of pathogen exposure on the founding of colonies. The experimental nests were set up, with queens either being placed on their own or in a pair, and exposed to a fungal pathogen, *M. brunneum*, or a control. The production of brood and workers was monitored across the different treatments and group types, with the prediction that: paired queens will produce the most brood compared to single queens – as there are two queens producing brood, amongst other advantages; and exposed queens will produce less brood than unexposed queens – as exposed queens may have to expend more resources on an immune response.

4.2. Materials & Methods

4.2.1. Study organisms and experimental colony founding

A total of 30 *M. barbarus* queen ants were sourced from Ant Antics (https://www.antantics.wales). The queens were placed in experimental nests, either alone or in a pair (N = 10 replicates for each queen number category). Within a large tub, experimental nests consisted of: Falcon tubes (17.5 cm long, 1.5 cm diameter) containing water blocked by cotton wool; a 1.5 cm tube lid containing chia seeds, following the provider's instructions; and the small glass postal tubes the queens originally arrived in, covered with red acetate (absorption spectrum: 550-850nm, limiting light entering the tube). One postal tube was placed in single queen colonies and two were placed in paired queen colonies. After 53 days, the queens were also given a small piece of a cockroach cadaver, a source of protein, to help with larvae production.

4.2.2. Colony building and immune challenges

Queens were monitored for five days a week, for 85 days. This experiment investigated all life stages to see if there was any difference observed throughout a colony founding. Due to this, observations noted were as follows: number of eggs; number of larvae; number of pupae; number of pupae with eyes; number of light brown pupae; number of dark brown pupae; number of adult workers; and any queen death. As brood and adults moved around between each observation it was impossible to track the eggs through their life cycle so all counts were averages. After 25 days an immune challenge was introduced to the queens, using the generalist entomopathogenic fungus *M. brunneum*, the same pathogen as mentioned in *3.2.1. Study Organisms*, with the preparation method also the same. At the 25-day mark, only four groups had both queens still alive, with only one group showing pleometrosis, where the queens were found in the same tube together. This group was taken out of the experiment so the pleometrosis process could be observed but for the other 3 groups, the ants were split up, with the submissive queen taken out of the experiment (either injured or found in a suboptimum location such as the water

entrance). For the treatment, 5 solo and 5 previously paired queens were placed on spore covered 90mm filter paper, and 5 solo and 4 previously paired queens were placed on Triton X covered 90mm filter paper. The spore concentration was $2.69x10^7$ spores/ml suspended in 0.05% Triton X, whilst the filter paper ensured a sublethal challenge. Queens were placed on the filter paper, inside a 90mm Petri dish, for 8 minutes, and after exposure they were placed back into their original box. A previously paired queen which had been injured in a fight but had won, was randomly selected for the spore treatment. This queen died 9 days after the immune challenge so this queen has been taken out of the analysis, resulting in 5 single queens and 4 previously paired queens being analysed for both treatments.

4.2.3. Statistical Analysis

Analyses were performed using the free analysis software R v.4.4.1 (R Development Core Team, 2024). Count data was analysed using a negative binomial generalised linear mixed-effect model, implemented using the glmer.nb function, in the lme4 package v.1.1.35.5 (Bates *et al*., 2015). The effect of treatment (fungus or Triton X), group (single or paired) and day (1-85) and their interaction on the count measurements for the following: eggs; larvae; pupae; pupae with eyes; light brown pupae; dark brown pupae; and adult workers, was modelled. Day (1-85) was fitted as a random effect, to account for pseudoreplication that occurred due to the multiple counts on the same groups through time. P values were extracted from each model using log-likelihood ratio tests implemented using the Anova function in the Car package v.3.1.3 (Fox & Weisberg, 2019).

4.3. Results

4.3.1. How does being exposed to a pathogen and group type affect the founding of new colonies?

There was an interaction between treatment and group type (single or paired) (Figure 11; Table 1) on the following counts: eggs (χ^2 = 19.5, df = 1, P < 0.001); larvae (χ^2 = 10.8, df = 1, P = 0.001); pupae (χ^2 = 19.5, df = 1, P = 0.002); pupae with eyes (χ^2 = 6.9, df = 1, P = 0.009); light brown pupae (χ^2 = 19.1, df = 1, P < 0.001); and adults (χ^2 =

6.0, df = 1, *P* = 0.014). For exposed single queens, there were more counts of eggs compared to all other groups and treatments (Figure 11a). For exposed single and unexposed previously paired queens there were more counts of: larvae (Figure 11b); pupae (Figure 11c); and pupae with eyes (Figure 11d) compared to unexposed single and exposed paired queens. For unexposed paired queens there were more counts of light brown pupae (Figure 11e) and adults (Figure 11g) compared to all other groups and treatments. There was no interaction between treatment and group type for dark brown pupae (Figure 11f). For all counts, there was no interaction between treatment and day (Figure 12). There was a relationship between type of group and day for eggs (χ^2 = 6.1, df = 1, *P* = 0.014; Figure 12a, b, c, d; Table 1) but for all other counts there was no relationship.

Table 1. Data showing how queen group type (single or paired), exposure to *M. brunneum* or not, and time taken affect the production of brood and adults of *M. barbarus* harvester ants. Generalised linear mixed-effect model P values, created using R v.4.4.1. Colours represent the following: green < 0.001; blue < 0.01; pink < 0.05.

Count	Treatment and Group	Treatment and Day	Group and Day
Eggs	< 0.001	0.108	= 0.014
Larvae	= 0.001	0.052	0.345
Pupae	= 0.002	0.671	0.789
Pupae with eyes	= 0.009	0.817	0.767
Light brown pupae (LB)	< 0.001	0.643	0.428
Dark brown pupae (DB)	0.065	0.887	0.903
Adults	= 0.014	0.432	0.247

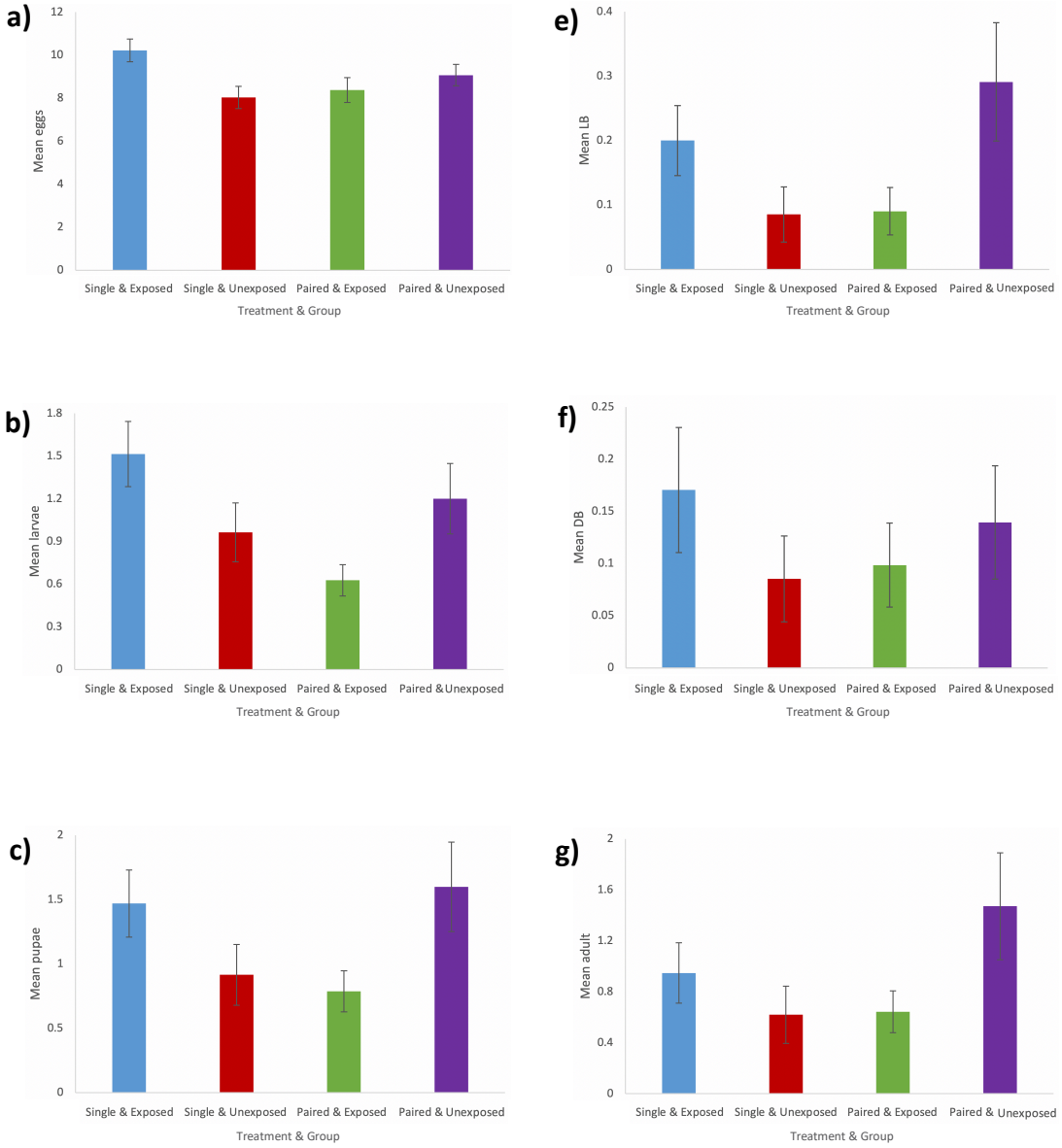
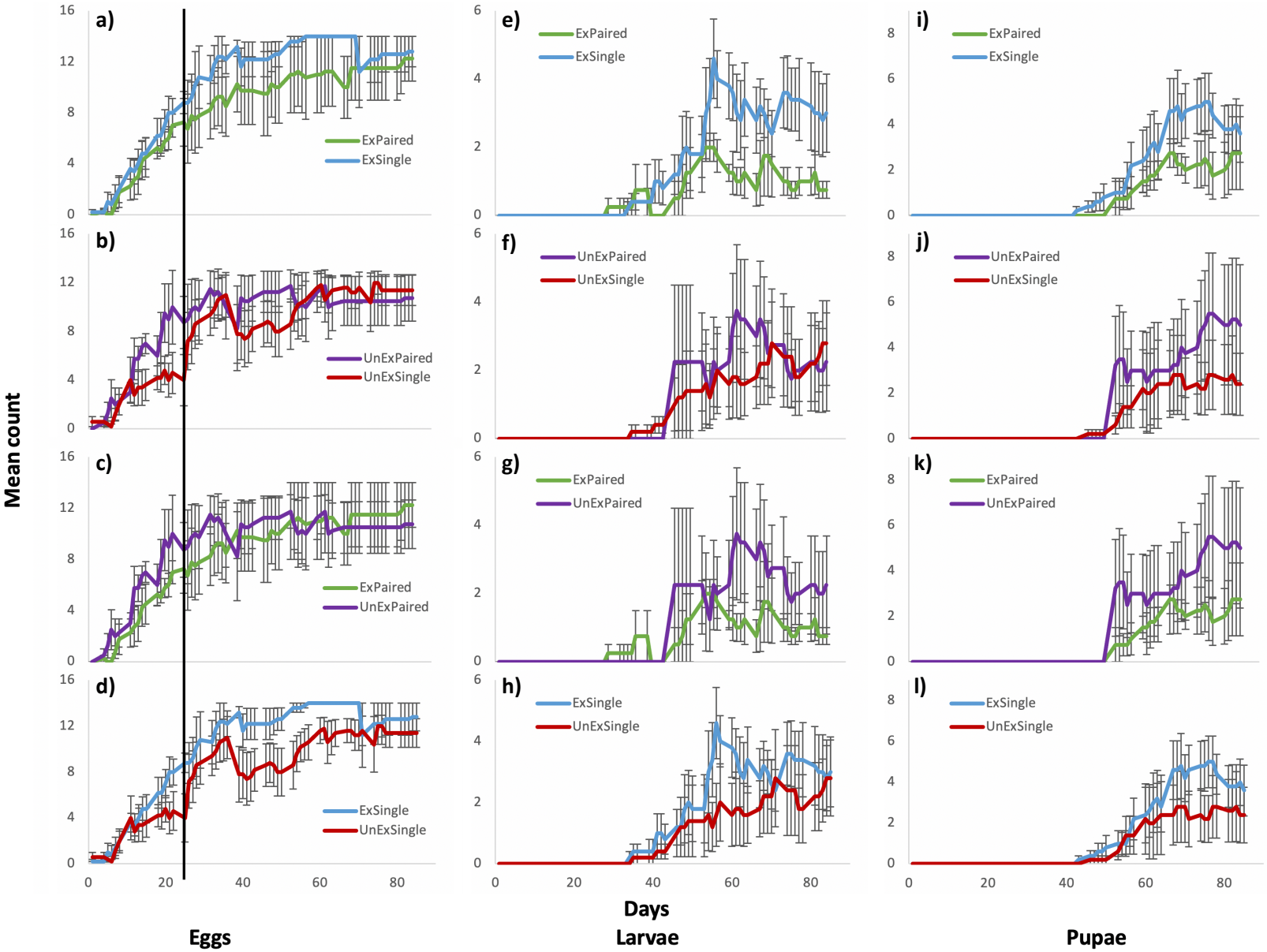


Figure 11. Data showing how queen group type (single or paired) and exposure to *M. brunneum* or not affects the production of brood and adults of *M. barbarus* harvester ants. Mean counts of: a) eggs; b) larvae; c) pupae; d) pupae with eyes; e) light brown (LB) pupae; f) dark brown (DB) pupae; g) adults, across replicates averaged over all days – comparing both single, paired, exposed and unexposed (control) queens. Error bars = ± standard error of variation between days.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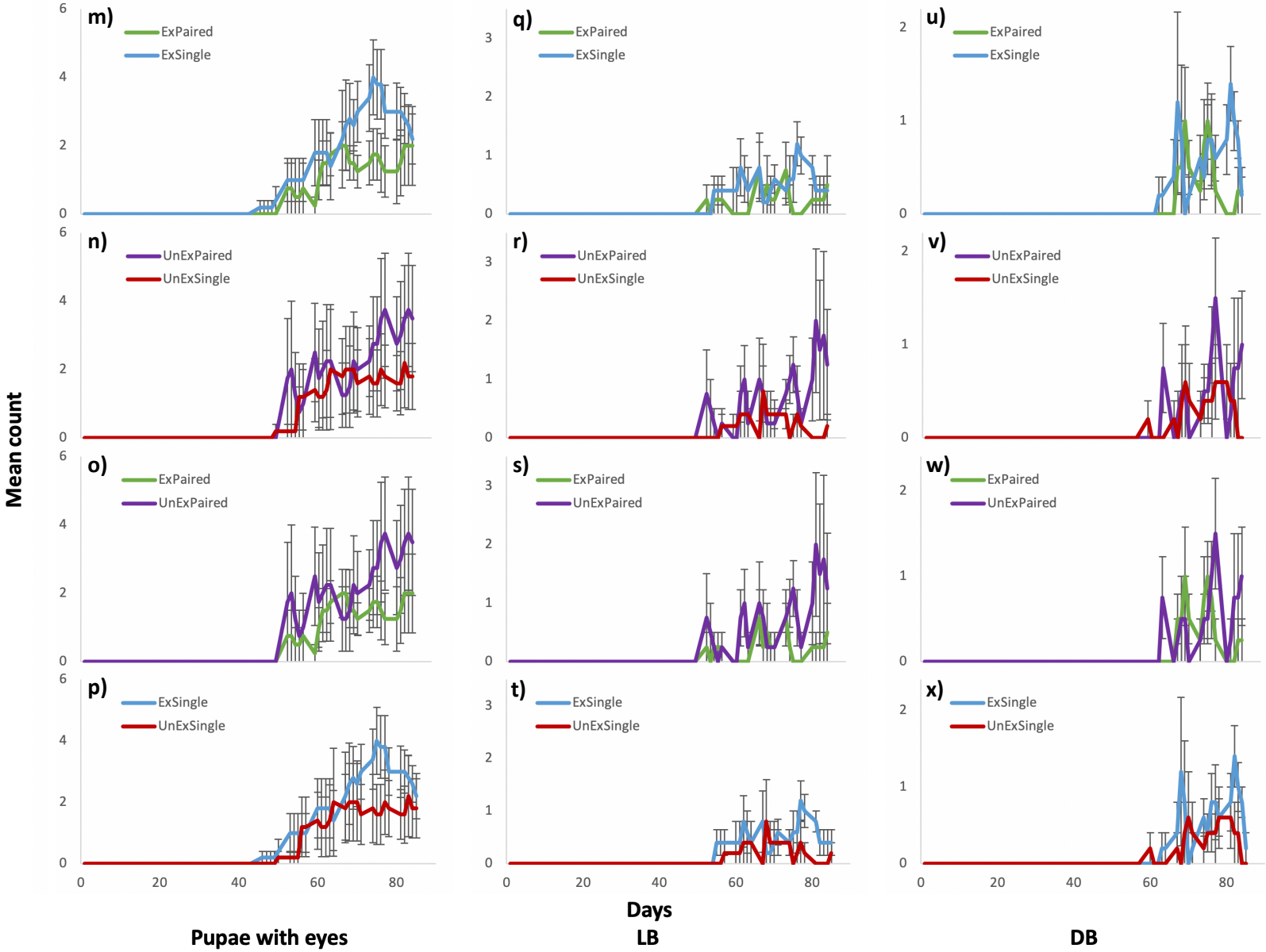
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s)
ExPaired
UnExPaired
w)
ExPaired
UnExPaired
p)
ExSingle
UnExSingle
t)
ExSingle
UnExSingle
x)
ExSingle
UnExSingle
Mean count
Days
Pupae with eyes
LB
DB

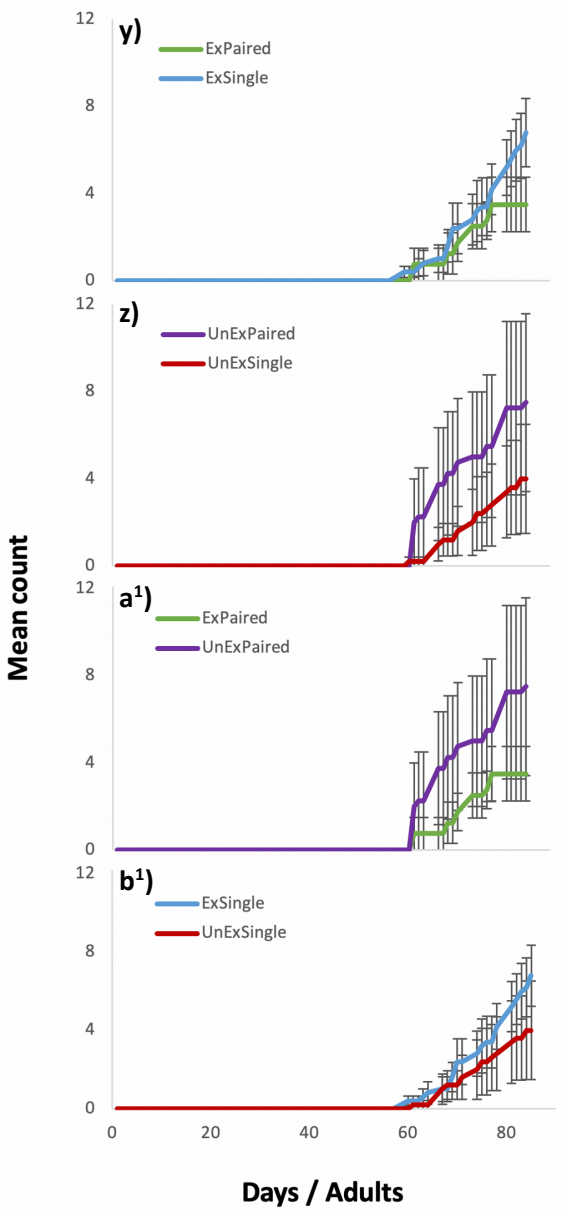

Figure 12. Data showing how queen group type (single or paired), exposure to *M. brunneum* or not and time taken affect the production of brood and adults of *M. barbarus* harvester ants. Change in mean (± standard error) counts over time of: eggs (a, b, c, d); larvae (e, f, g, h); pupae (i, j, k, l); pupae with eyes (m, n, o, p); light brown (LB) larvae (q, r, s, t); dark brown (DB) larvae (u, v, w, x); and adults (y, z, a^1, b^1), in the treatment groups: exposed (a, e, i, m, q, u, y); control (b, f, j, n, r, v, z); paired (c, g, k, o, s, w, a^1); and single (d, h, l, p, t, x, b^1). The key labels ExPaired and ExSingle represent the exposed treatments and UnExPaired and UnExSingle represent the control treatments, respectively. The vertical line on the egg plots represents the day of fungus exposure. Error bars = ± standard error.

4.4. Discussion

The results of this study suggest that single exposed queens produce the most eggs, larvae and pupae as part of their colony building. Previously paired unexposed (control) queens also produce more larvae and pupae as well as light brown pupae and adults than either unexposed single queens or exposed previously paired queens. The exposed single queen result contradicts a previous similar study conducted by Pull *et al*. (2013) which showed that exposed single and paired queens produced less brood and workers compared to their controls. Their results were opposite to this study, where their single exposed queens produced the least amount of brood and workers out of all treatments and types of grouping, which suggests that there is

potentially a trade-off between reproduction and an immune response. However, a recent study conducted by Bizzell & Pull (2024) has suggested that a queen can eat and destroy her larvae if they are infected, in “hygienic cannibalism”. In this study, they found that queens which consumed their infected larvae laid an additional 55% more eggs than non-cannibalising control queens. During filial cannibalism, the queen is reinvesting recouped nutrients back into egg production, important in colony founding as queens do not forage for resources (Bernasconi & Strassmann, 1999). Larval growth represents a return on the queens’ investment on generating additional eggs, so as a queen consumes larvae, she could correspondingly produce more eggs creating a proportionally higher number of larvae. Even if she eats some of those larvae again, there could still be excess larvae left over to grow her brood bigger (Bizzell & Pull, 2024). It is possible that this phenomenon is occurring in both in the single exposed queens and unexposed previously paired queens. The presence of an unrelated queen could be viewed as a foreign substance, which could consequently be causing hygienic cannibalism to occur, unobserved, hence why the exposed single queen and the unexposed previously paired queen results are so high. The exposed previously paired queens had the disadvantage of both an immune response and energy lost due to fighting, which could be why they did not show this effect and only produced a small number of brood and adults in comparison. Additionally, there was more variation observed between the two previously paired treatment groups, which could mean the queens had produced lower or more variable quality workers. Future experiments could investigate the measure of resistance in resulting workers and what effect being paired and an immune challenge might have on this resistance.

This experiment was adapted from Brütsch *et al*. (2017) looking into social immunity in queens. However, unlike the *Lasius niger* used in Brütsch *et al*. (2017) and Pull *et al*. (2013), when paired together, *M. barbarus* did not show co-founding with other queens. Within the first two weeks, six queen fights had occurred, leaving only four paired queens left. Out of these four paired queens, only one group were found in the same tube together, interacting with one another. Queens which had been in fights bore injuries, and one injured queen, which had survived her fight, died 9 days after being exposed – the fight had weakened her and this may have resulted in her being unable to defend herself against the exposure to the pathogen. No published

data has looked into colony founding of previously paired queens, but the results shown for the previously paired queens in this study are similar to that found with co-founding queens in Pull *et al*. (2013) and Teggers *et al.* (2021). This suggests there is an advantage for queens which had previously formed co-founding groups, with these previously paired queens being able to produce a large brood, if not exposed to a fungal pathogen.

There could be a few different reasons why the queens did not co-found in this study. To pair the queens together, two queens were randomly selected and placed in the same experimental nest. It may be that if the queens were allowed to form their own associations, the co-founding may have been more successful. However, this is unlikely as co-founding appears to be random and opportunistic (Sommer & Hölldobler, 1995; Aron *et al*., 2009). Another possible reason may be the set-up of the experimental nest. In both Pull *et al*. (2013) and Teggers *et al.* (2021) where successful co-founding occurred with queens, their experimental set up consisted of mini plaster of Paris colonies and large chambers, with choices for where the queens could end up and space for queens to roam together. This could be similar to what queens may find or make in the wild for nest sites. Additionally, it may be possible that *M. barbarus* does not form these associations in nature. However, the one successful co-founding pair in this study, which was taken out for observation, suggests that it is possible for this species to co-found.

An interesting observation from this study was the variability of the time it took queens to found a colony. Two queens, a single queen and a previously paired queen, both in the control group, never produced any larvae. Additionally, there were four queens, one from each group and treatment, which took around 80 days to produce their first worker, compared to other queens which had 14 or 15 workers by the end of the experimental period. It was interesting to observe that the colony building timeline was so variable between queens. Size and weight data were not collected as part of this experiment, so this variability may be a result of different sized queens, with larger queens producing more brood, as shown in Szabó *et al.* (2023); previous studies have demonstrated that some species of queens additionally use their body reserves to raise their brood (Waloff, 1957; Johnson, 2002; Brown & Bonhoeffer, 2003).

The results of Chapter 2 showed that: i) when exposed to an immune challenge, single exposed queens and previously paired unexposed queens produced the most brood and adults out of all the treatments and groups; ii) when exposed to an immune challenge, single unexposed queens and previously paired exposed queens had a low number of brood and adults compared to the other treatments and groups; iii) *M. barbarus* did not successfully co-found in the environmental conditions in this experiment.

5. Conclusions

Experiments were conducted on how the availability of metapleural glands as well as social immunity affects the survival probability of ants and their response to a pathogen. This study showed that duration and frequency of self-grooming for ants exposed to the fungus and Triton X treatment occurred to a similar higher extent, compared to the control. Within the fungus and Triton X treatments, there was no difference in self-grooming duration or frequency between either blocked or open metapleural glands. This suggests that the ants are responding to a foreign substance on their cuticle, rather than pathogenic spores. There was also minimal allo-grooming which occurred in all treatments, similar results shown in a study conducted by Bos *et al.* (2019). The experiments conducted were unable to distinguish whether this is due to treatment or experimental design so using larger groups of ants or different proportions of exposed to clean workers within a group may allow further investigation into the effects of allo-grooming in more detail (Reber *et al*., 2011; Okuno *et al*., 2012). Other future studies can also look at the frequency and duration of specific metapleural gland grooming over regular antennae grooming.

In this thesis, experiments investigating the likelihood of infection due to different spore application methods were conducted. The low mortality of ants when exposed indirectly to fungal spores contradicted previous studies which showed high mortality when using this indirect filter paper method (Chapuisat *et al*., 2007; Reber *et al*., 2008; Reber *et al*., 2011), which may simply be due to the experimental design, with only half the filter paper covered in spores, or there may be something of interest requiring additional investigation. Further experiments could investigate this indirect

method and see whether a higher fungal spore concentration would increase ant mortality when only half the filter paper is covered in spores, and also whether this would affect ant behaviour and result in avoidance of the contaminated side. Additionally, the interesting result of a difference in survival of workers from the different colonies for both exposure methods requires further investigation to see what effect having a queen within a colony might have on the immune response of her workers.

Experiments also investigated the effect of pathogen exposure on colony founding, with results differing from a previous study conducted by Pull *et al*. (2013), as single exposed queens and previously paired unexposed queens produced the most brood compared to other treatments and group types. Further work could look into whether this may be due to "hygienic cannibalism", as recently described by Bizzell & Pull (2024). To determine this, subsequent experiments could look into tracking the different life stages of a colony founding in more depth, as this could provide more information about the differences observed between the treatments and groups in this thesis. Additional experiments could investigate whether a prior pairing with another queen influences the subsequent amount of brood a previously paired queen then goes onto produce, as the previously paired queen data from this study was similar to that of co-founding queens in previous studies (Pull *et al*., 2013; Teggers *et al.,* 2021). Future *M. barbarus* colony founding studies may benefit from research into the co-founding habits of *M. barbarus* in nature, and how often this phenomenon occurs in the wild.

6. References

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