Socijalni imunitet mravljih zajednica potaknut bakterijskom infekcijom

Bračić, Marko

University of Zagreb

Faculty of Science

Division of Biology

Marko Bračić

Social defences of ant colonies against bacterial infection

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This thesis had been conducted at the Institute of Science and Technology Austria (IST Austria), in Klosterneuburg, Austria, in collaboration with the Faculty of Science, University of Zagreb, Croatia, under the guidance of dr. rer. nat. Sylvia Cremer, Prof. and dr. sc. Duje Lisičić, Asst. Prof. The thesis was submitted for evaluation to Division of Biology, Faculty of Science, University of Zagreb in order to acquire the academic title Master of experimental biology.

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Faculty of Science
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Social defences of ant colonies against bacterial infection

Marko Bračić
Rooseveltov trg 6, 10 000 Zagreb, Croatia

In response to pathogen pressure, social insects evolved specific organisational, physiological and behavioural adaptations resulting in avoidance, control or elimination of parasites. Such collective action of individuals at the level of the whole colony is called **social immunity**; established primary on fungal "external" infections. However, **orally infecting parasites are poorly investigated**. Therefore, I here used an entomopathogenic bacterium *Bacillus thuringiensis* and ants, to test the influence of bacterial spore ingestion on ant survival, in order to establish a **novel model system** for exploring social immunity. However, spores from three different *B. thuringiensis* strains did not consistently induce mortality under used experimental setup. Given that mechanisms of social immunity may act before the parasite had established an infection, I further tested whether **behavioural changes** in *Linepithema humile* ants are observed after exposure to *B. thuringiensis*. Indeed, ***B. thuringiensis*-exposed ants self-groomed their gaster tip longer** then the control-exposed ants. *L. humile* have different gaster glands, which secretions consumed after spore ingestion might inhibit bacterial growth. Indeed, I found that the **gaster secretions inhibit *B. thuringiensis* growth**, indicating gaster tip self-grooming may serve as the first line of defence against orally induced parasite invasion of the colony, before the infection was even established within the host body.

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Keywords: social immunity, host-parasite interaction, gaster glands, orally infecting parasites, *Bt*

Supervisor 1: Dr. rer. nat. Sylvia Cremer, Prof.
Supervisor 2: Duje Lisičić, Ph.D., Asst. Prof.
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Socijalni imunitet mravljih zajednica potaknut bakerijskom infekcijom

Marko Bračić
Rooseveltov trg 6, 10 000 Zagreb, Hrvatska

Kao odgovor na patogene, zadružni kukci su razvili specifične organizacijske i fiziološke adaptacije te adaptacije u ponašanju, što je rezultiralo izbjegavanjem, kontrolom ili eliminacijom parazita. Takve grupne mjere koje poduzimaju pojedinci na razini čitave kolonije se nazivaju "**socijalni imunitet**"; primarno utvrđen na vanjskim gljivičnim infekcijama. Unatoč tome, **paraziti koji zaražavaju oralno, slabo su istraženi**. Stoga sam u svojim istraživanjima koristio entomopatogenu bakteriju *Bacillus thuringiensis* i mrave kako bih testirao utjecaj ingestije bakterijske spore na preživljavanje mrava, te postavio **novi modelni sustav** za istraživanje socijalnog imuniteta. Međutim, spore tri različita soja *B. thuringiensis* nisu dosljedno uzrokovale smrtnost u korištenom eksperimentalnom dizajnu. S obzirom da mehanizmi socijalnog imuniteta mogu djelovati prije nego što je parazit uopće započeo infekciju, istražio sam jesu li opažene promjene u ponašanju u *Linepithema humile* mravima, nakon izlaganja *B. thuringiensis*. **Mravi izloženi bakteriji su dulje čistili vrh zatka** nego kontrolna skupina. Nadalje, *L. humile* ima različite žlijezde u zatku čije sekrecije, konzumirane nakon ingestije spora, bi mogle inhibirati rast bakterija. Otkrio sam da sekreti zatka stvarno **inhibiraju rast *B. thuringiensis*,** što indicira da čišćenje vrha zatka može poslužiti kao prva linija obrane od oralno inducirane invazije kolonije od strane parazita, prije nego što se ona proširi po tijelu domaćina.

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Ključne riječi: socijalni imunitet, žljezde zatka, *Bt*, L. humile, paraziti

Voditelj 1: Prof. dr. rer. nat. Sylvia Cremer
Voditelj 2: Doc. dr. sc. Duje Lisičić
Ocjenitelji: Doc. dr. sc. Duje Lisičić, prof. dr. sc. Zlatko Liber, izv. prof. dr. sc. Ivana Maguire
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1 Table of Contents

2 Introduction

Group living has proven to be beneficial for the individual fitness in certain ecological conditions; in groups some pressures are easier to handle (Hamilton 1971; Wilson 1975). The first benefit on a long list is often increased protection from predators, which is an important driver of the evolution of sociality in many species (Davies et al. 2012). For instance, bees will be able to protect their colony from a hornet by covering its body, thereby increasing the temperature and killing the invader. Among many other possible benefits are increased feeding and reproductive efficiency, better competitiveness with other groups and species and higher survival rates (Davies et al. 2012), along with the ability of environment modification nicely represented with social thermoregulation (Wilson 1975). Considering these benefits, success of group living animals, as humans and social insects, is not a surprise.

By contrast, group living may also have its costs. For example, groups may experience within-group competition for resources (Molvar & Bowyer 1994) and mating partners or, they may in fact be easier to detect by predators and parasites, as they are spread out over a large area (Wilson 1971; Wilson 2003) which makes group living potentially more dangerous. However, in addition to competition and predation, closely living individuals also experience potential increased risk from parasites, here referring to parasites in a broad sense, including any type of organism entering the host and reducing its fitness, including bacteria, viruses, protozoans, helminths and fungi (Cremer et al. 2007; Meunier 2015). Harm from parasites poses considerable costs to the host fitness, because it decreases fertility, competitiveness and life-span (Sheldon & Verhulst 1996). Therefore, parasites burden is among the most important selection factor for many species, inducing fast evolutionary adaptations in both of the antagonists (Woolhouse et al. 2002). Parasite transmission will be limited by host density or frequency of interactions between individuals (Anderson & May 1979; Schmid-Hempel & Stauffer 1998; Fefferman et al. 2007; Wilson 2003; Rifkin et al. 2012), because conspecifics are usually the main sources of transmission (Freelander 1983). In contrast, in parasites that actively seek for the host this is not the case (Côté & Poulinb 1995). Moreover, related hosts will be more susceptible to the similar parasites (Cremer et al. 2007)**.** These conditions are regularly found in group living animals, especially in social insects; thousands of individuals live and interact closely together (Wilson 1971) and all have originated from the same or few different mothers. This represents an ideal environment for parasite transmission (Barthélemy et al. 2005). Therefore, parasites are expected to have major influences on insect societies.

Indeed, the whole social organization ranging from foraging behaviour (Feener 1988; Schmid-Hempel & Schmid-Hempel 1993; Schmid-Hempel & Stauffer 1998) to genetics (Bourke et al. 1995; Rosengaus et al. 2000) and colony life history (Sammataro et al. 2000; Rosengaus & Traniello 2001; Schmid-Hempel & Stauffer 1998; Morrison et al. 1999) is known to be under influence of parasites.

However, social insects are very successful in overcoming different ecological pressures, usually making themselves the most dominant species in various habitats (Wilson 1971); ants alone count for more than 14000 species (Ohio State University 2016). Moreover, argentine and fire ants for example, are considered to be one of the 100 most dangerous invasive species and efforts to control their spread did not yield promising results (Lowe et al. 2000). On the other hand, ants are important as ecosystem engineers, soil movers and system regulators, especially in the tropics (Wilson 1971). Considering their global success in spite of the parasite burden, efficient defence mechanisms are expected to be seen in ants and other social insects, as a result of host-parasite evolution.

Indeed, during this "arms race" social insects evolved impressive defence arsenal (Cremer et al. 2007; Schmid-Hempel 1998). Starting from their nest, which architecture influences pathogen spread, together with activity level of nestmates and their interactions (Pie et al. 2004). In addition to this spatial and network defences, workers will change their foraging behaviour in response to parasite; infected bees will change their flower choice compered to parasite-free bees (Schmid-Hempel & Stauffer 1998). Furthermore, evolution of bees' sociality is followed by production of progressively stronger antimicrobial compounds to battle parasites (Stow et al. 2007). In social, as in other insects, individual defences are also very important in protection against parasites, ranging from physical barriers such as the cuticle, gut and trachea, to physiological immune defences (Tsakas & Marmaras 2010). Immunity is traditionally viewed through scope of vertebrate adaptive immune system, however, more recent studies had challenged superiority of adaptive system. Now, innate immunity is considered frontline and organiser of defences that reduced the gap between vertebrates and solely innate insect's immunity (Schmid-Hempel 2005; Brown 2001). In addition to individual immunity, social insects have in parallel to the group living vertebrates, evolved sophisticated collective anti-parasites defences to counter strong selection pressure (Cremer et al. 2007). Ants, as well as primates groom their group members (allogrooming) to remove parasites (Hart 1990; Nunn & Altizer 2006; Schmid-Hempel 1998). Indeed, these group-level anti-parasite defences are

considered partially responsible for the global success of social insects and are called the "social immune system" (Schmid-Hempel 1998; Cremer et al. 2007).

Social immunity describes collective action of individuals at the level of the whole colony and results in avoidance, control or elimination of parasites (Cremer et al. 2007). Other authors use this term for parental care and non-eusocial animals (Cotter & Kilner 2010; Meunier 2015). These defensive behaviours can be prophylactic, when ant intakes e.g., tree resin for nest disinfection (Christe et al. 2003) or can be activated on demand when parasites are detected, as observed for allogrooming (Schmid-Hempel 1998) and disinfection of the nest or infected colony members (Tragust et al. 2013 a; Tragust et al. 2013 b). Due to the assumed higher costs of defence against parasites in energy and time (Sheldon & Verhulst 1996), animals need to balance well between the costs and benefits of social immunity. For example, hygienic behaviour can cause infection in the performing individual; risking potentially reduced fitness, but it can also benefit the colony fitness which could be compensated to that individual by gaining indirect fitness (Cremer et al. 2007).

Due to such social immunity, parasite entry into the colony is not straightforward. Similar to the animal body, colony has different "weak spots" where parasites can enter (for parallel between immune systems of organisams and colonies see Cremer & Sixt 2009). Firstly, queen of the newly-formed colony could bring parasites from her "mother colony" (vertical transmission) or during mating from other individual (horizontal transmission). As health of the mated queens could be crucial for successful establishment of the new colony, strategies to avoid vertical transmission are assumed. Indeed, queen avoidance by infected individuals and brood care are documented (Wang & Mofller 1970; Beattie et al. 1986). When colony is finally established, parasites can be transmitted by nestmates or conspecifics from other colonies (horizontal transmission). Danger is mostly coming from foragers that are usually the only ones leaving the nest and can potentially encounter parasites. In their environment, ants can encounter parasites through air, soil, food, or cadavers. To reduce this risk, foragers can avoid parasite rich areas (Mehdiabadi & Gilbert 2002). Moreover, entrance of individuals to the colony is highly controlled and infected individuals will be excluded or attacked (Cremer et al. 2007). Besides coming from the outside, parasites can enter the colony through the soil, which is abundant in parasites or via the air. Furthermore, parasites can accumulate in the colony during its life. To prevent epidemics, colony members can apply antimicrobial substances, either self-produced or collected form the environment. For the colony, significant problem is nestmate cadavers, which if infected with e.g., fungal spores, may pose threat to the healthy

nestmates (Kramm et al. 1982). As a consequence, many species move their cadavers to special isolated places in the colony ("graveyards") (Siebeneicher et al. 1992). Interesting case is in fungus growing ants, which have special waste chambers. In these chambers, older members of the colony are sorting potentially infectious waste in isolation from other nestmates (Hart & Ratnieks 2002; Ballari et al. 2007). Generally, age and cast separation is also prominent in other tasks. Young individuals are responsible for nursing tasks in the centre of the colony, while older workers are usually foraging (Schmid-Hempel & Schmid-Hempel 1993; Mersch et al. 2013). This behavioural compartmentalisation together with spatial one is predicted to prevent parasites from reaching the brood and the queen (Naug & Carazine 2002; Cremer et al. 2007; Stroeymeyt et al. 2014). Furthermore, nestmates across different species mechanically remove detected parasites from the body by grooming (Schmid-Hempel 1998) Moreover, in ants and wasps infectious particles are killed in special mouth compartments and are discarded outside of nest (Eisner & Happ 1962).

Mechanisms of social immunity in ants are mostly studied using parasites that infect via "external infections" (Tragust 2013a; Konrad et al. 2012) and several studies using bacterial injections into the insect haemolymph (Hamilton et al. 2011). Indeed, most of our knowledge about social immunity in ants is coming from studies that used entomopathogenic fungi. However, oral route of infection is a natural route of transmission for most other parasites, such as bacteria and viruses, and mechanisms against orally infecting parasites are poorly investigated in ants.

Therefore, here I used bacterial model as a novel system for exploring social immunity. For this purpose, I chose *Bacillus thuringiensis*, a Gram +, toxin and spore-forming entomopathogenic bacteria that naturally infects its hosts via the oral route (Raymond et al. 2016; Argôlo-Filho & Loguercio 2013). *B. thuringiensis* (*Bt*) is highly abundant in soil, water and phylloplane, but its role and epidemiology in the environment are not well understood (Raymond et al. 2016). During the sporulation phase, when nutrients become limited, *Bt* forms crystal endotoxins (Cry proteins) that, upon ingestion by susceptible host, dissolve in the gut, bind in a highly specific manner to receptors on epithelial cells and induces pore formation and cell burst (Nielsen-LeRoux et al. 2012). With this well-investigated mechanism, *Bt*-induced mortality is observed in various insect orders, usually with high specificity, such that certain *Bt* strains usually infect only certain insect orders, even species within the order (van Frankenhuyzen 2009). This specificity usually comes from specificity in Cry-protein binding, although synergism with bacteria and toxins in inducing mortality in

insects is observed (Tian et al. 2015). For this reason, *Bt* toxins are now commonly used as biopesticides and in GMO crops (Griffitts & Aroian 2005; Sanahuja et al. 2011). Since *Bt* successfully forms transmission stages in insects (Suzuki et al. 2004; Milutinović et al. 2015), ants could potentially encounter it while feeding on cadavers or by cannibalising other conspecifics (Milutinović et al. 2015). Alternatively, since *Bt* was found associated with plant roots and in the plants as well, ants may also come into contact with *Bt* by interacting with aphids and drinking their honeydew (Prabhakar & Bishop 2009). In the environment, a large variety of *Bt* strains is found that could all potentially interact with ants, but so far nothing is known about these interactions, nor is *Bt* being used to investigate host-parasite interactions in ant systems.

Therefore, my aim was to find a suitable *Bt* strain that will induce mortality in ants and enable further characterisation of host-parasite interactions in this novel system. Specifically, I intended to use such system to study physiological and behavioural components of ant defence against orally infecting bacteria, thereby providing new knowledge to the field of social immunity. In addition, I wanted to explore whether any costs are associated with defence against *Bt* in ants.

3 Materials and Methods

3.1 Ants

3.1.1 Species and collection sites

Lasius neglectus Van Loon, Boomsma & Andrásfalvy, 1990 is a polygynous and unicolonial species member of subfamily Formicinae (Boomsma et al. 1990). Formicinae can be distinguished due to node or scale-like petiole and acidopore, U-shaped or circular opening (Agosti & Collingwood 1987). *L. neglectus* is categorised as an invasive species in Europe and Asia, with assumed origin from the Middle East (Schultz & Seifert 2005). Ants used for the experiments in this study were collected in Seva El Montagna, Spain (2015).

Lasius niger (Linnaeus, 1758) is also a member of subfamily Formicinae, native to Holarctic with monogynous colonies with several hundred workers (Collingwood 1979). This species was collected in Harpenden, U.K. (2011).

Linepithema humile (Mayr, 1868), also known as Argentine ant and a member of Dolichoderinae, is characterized with specific slit-like gaster opening (Cardoso et al. 2012). It is a South American species with large invasive potential. This species has now spread around the globe from North America and Europe to Japan and Australia, where it established large supercolonies (Van Wilgenburg et al. 2010; Giraud et al. 2002). It is considered one of 100 most dangerous invasive species (Global Invasive Species Group, IUCN (www.issg.org) due to its negative impact on ecosystem (Human & Gordon 1996; Human & Gordon 1997; Holway 1998; Suarez et al. 1998; Sanders et al. 2001; Sanders et al. 2003), damage in agriculture (Markin 1970) and problems in urban environment (Vega & Rust 2001). This species was collected in Castel del´Aro (2011, 2016) and L´Escala, Spain (2016).

3.1.2 Maintenance of ant colonies in laboratory

Ant stock colonies were kept in plastic boxes (295x230x118mm, Lock & Lock, USA) with plastered floor to keep the moisture and plastic lid with opening for ventilation. Inside the box, smaller chambered boxes with transparent lid were provided and a red foil on the top to mimic natural nest chambers and provide darkness. Sides of the boxes were lined with Fluon® Aqueous Dispersions (AGC Chemicals Europe, U.K.) to prevent ants from climbing on the lid. Sterile 30% sucrose solution and water were provided all the time in plastic tubes plugged with cotton. In addition, ants were fed three times a week with homogenised cockroaches and honey. Additional water was occasionally poured onto the plaster to maintain humidity. Laboratory colonies were kept in incubators at a constant temperature of 27 ºC during summer, 15 ºC during

fall, 8 ºC during winter conditions, and 70% humidity. For *L. neglectus* and *L. niger*, incubation temperatures were varied according to seasonal changes to mimic natural conditions, whereas *L. humile* was kept under constant summer conditions, as the colonies reproduced throughout the year.

3.1.3 Ant marking for behavioural experiments

Ants were marked with a needle head dipped in a red dye (colour 006, Edding 751, Japan) on the middle of the gaster. Marking procedure was performed in different ways; some ants were cooled on ice for a few seconds to calm down, taken with forceps and between the fingers by the legs and marked. Other ants were calm, so cooling was not required. Finally, some ants were successfully marked without handling at all. I observed that blowing air lightly towards the ants makes some of them calmer; subsequent usage of this method resulted in less handling of ants. After marking, individual ants were placed individually in an empty 35 mm diameter (⌀) Petri dishes for starvation (see experimental design).

3.2 Bacteria

3.2.1 *Bacillus thuringiensis* strains

In this study, the susceptibility of ants to several *Bacillus thuringiensis* Berliner 1915 strains was tested in order to find a suitable strain for investigation of host-pathogen interactions in this novel system. The strains were chosen based on previous reports documenting their pathogenic activity towards several ant species; Payne al. 1997 showed that *B. thuringiensis* PS140E2 (*BtE2)* and *B. thuringiensis* PS86Q3 (*BtQ3)* induce mortality in several hymenopteran pests (including pharaoh ants) and Bulla et al. 2004 showed that strain *B. thuringiensis* UTD-001 has almost identical Cry toxin as *Bacillus thuringiensis tenebrionis* (*Btt*), which was found to be active against argentine, fire, carpenter and pharaoh ants. Both *BtE2* and *BtQ3* were ordered from the ARS Patent Culture Collection (Peoria, IL 61604) under the codes NRRL B-18812 and NRRL B-18765 respectively, whereas *Btt* was kindly provided by the research group of Joachim Kurtz at the University of Münster, Germany.

3.2.2 Spore preparation

Spores were prepared as described in Milutinović et al. 2013. Briefly, bacteria from the glycerol stocks (stored at -80°C) were plated on Luria broth (LB) agar and grown overnight at 30 °C. Before spore preparation, bacteria from glycerol stocks were plated freshly to prevent loss of pathogenicity by long-term storage and propagation on the LB agar plates. On the next

day, 3 ml of BT medium (w/V–0.75% bacto peptone (Sigma, United States), 0.1% glucose, 0.34% KH_2PO_4, 0.435% K_2HPO_4) was inoculated with three to five colonies with the addition of 15 µL of salt solution (w/V–2.46% $MgSO_4$, 0.04% $MnSO_4$, 0.28% $ZnSO_4$, and 0.40% $FeSO_4$, sterile-filtered) and 3.75 µL of 1M $CaCl_2 \times 2H_2O$ and was incubated overnight in an incubator at 30 °C with shaking (180 rpm). On the following day, 300 mL of BT medium was inoculated with 500 µL of the overnight bacterial suspension, 1.5 mL of salt solution and 375 µL of 1M $CaCl_2 \times 2H_2O$ and further incubated for a total of seven days in darkness again at 30 °C with shaking (180 rpm). On day four, another 1.5 mL of salt solution and 375 µL of 1M $CaCl_2 \times 2H_2O$ were added. After seven days the suspension was centrifuged at 3000xg for 15 minutes, supernatant discarded and 20 mL of phosphate buffered saline (PBS) was added. This procedure was repeated once more and spores were counted with a Thoma counting chamber (0.02 mm depth). Spore solution was stored for a maximum of five days at room temperature and protected from light until they were used in experiments. Before the experiments, spore solution was again centrifuged at 3000xg for 10 minutes and PBS was replaced with 10% sucrose solution.

3.2.3 General ant exposure assay

Unless otherwise stated, I exposed the ants to *Bt* spores as described here. Control group was exposed to 10% sucrose solution only. I used 35 mm (⌀) Petri dishes with plastered floor (by Life Science Facility, Institute of Science and Technology Austria) for moisture retention, in which a piece of red plastic foil was placed. The red foil provides space on which wet filter paper will be laid on. I arranged such dishes on plastic trays in a way that all treatments were always on the same tray, to exclude a possible batch effect due to trays. Moreover, I randomized and alternated the relative positions of the different treatments on the trays. Before I started with exposure, I moisturized the petri dishes with distilled water. However, moistening was not necessary when dishes were freshly prepared and wet. I then collected the required number of worker ants from the colony with exhauster (made in the laboratory using 50 mL Falcon tubes) and forceps into collecting Petri dish. In cases where there were enough ants in the colony, I put forceps in and let ants climb on it to avoid additional stress. Moreover, I collected ants a few minutes before I needed them, so they had time to calm down. Filters were dipped into sugar solution for the control group or *Bt* spore-containing sugar solution and were subsequently placed in moistened 35 mm (⌀) Petri dishes. I used forceps to handle filter paper, always mixed the solutions (as spores sink to the bottom of the beaker) and drained it on the edge of a 5 ml beaker to remove excessive amount of liquid before placing it on the red foil.

Next, I took the ants by the legs using forceps with pointed ends and added them to the Petri dishes. Trays were placed in warm room at constant temperature of 30ºC with 70% humidity and day/night cycle of 14/10 hours. After approximately 24 hours, or every 24 hours in longer experiments, mortality was checked. Final sample size was noted for each experiment after excluding the ants that had escaped or died due to the experimenter.

3.3 Experimental design

3.3.1 Influence of *B. thuringiensis* on mortality in ants

To explore the possible lethal effect of *Btt* strain on ants, I exposed *L. humile*, *L. niger*, and *L. neglectus* to $1x10^9$ mL^{-1} spore concentration of *Btt* as described above. I prepared 12 trays in total, each with 10 dishes of each ant species and both treatments, resulting in an end sample size: *Btt L. humile* 110, *Btt L. niger* 120, *Btt L. neglectus* 120; control *L. humile* 114, control *L. niger* 119 control *L. neglects* 118. Similarly, *L. humile* was exposed to$1x10^9$ mL^{-1} of *BtE2* strain**.** I set 4 trays with 25 dishes on each, resulting in an end same size 100 for each treatment. Because of the large sample size, experiment was performed with two additional experimenters. Survival was monitored for one day.

3.3.2 Dose response of *B. thuringiensis E2* strain

To test if mortality depends on the dietary spore concentration of *Bt*, I exposed *L. humile* to different concentrations of *BtE2* strain. I used $0.5x10^9$, $1x10^9$ and $2x10^9$ mL^{-1} spore concentrations. On each of the 7 trays, I had 14 dishes for each of the four treatments (including control). End sample size: $0.5x10^9$ 56, $1x10^9$ 57, $2x10^9$ 56 and control 57. I repeated this experiment six day later because spores in the first experiments were harvested one and a half day before experiment, which could have influenced spore infectivity. End sample size: $0.5x10^9$ 70, $1x10^9$ 70, $2x10^9$ 68 and control 69. Survival was monitored for one day in both experiments.

3.3.3 Dose response of *B. thuringiensis E2* strain with previous starvation

To ensure that ants indeed ingest the spore-containing diet in this experiment, they were starved before the exposure. For this, ants were collected from stock colony as described above and starved in 35 mm (⌀) empty Petri dishes for 6 hours at 30ºC, 70% humidity. After starvation I transferred the 35 mm (⌀) Petri dish with starved ant into a 50 mm (⌀) plastered dishes, containing empty space for 35 mm (⌀) dish. This design was chosen to avoid ant handling which would cause considerable stress. Here, I placed filter paper in the 35 mm (⌀) dish without plaster so there was no need for the red foil. To reduce stress even further, I left lid on 35 mm

(⌀) dish till finishing with the rest of the tray after placing filter paper. The ants still remaining on the lid were shaken off the lid and placed into 50 mm (⌀) dish. In this experiment, two concentrations of *BtE2* spores were used ($1x10^9$ and $2x10^9$ ml^{-1}). On each of the 8 trays I placed 10 dishes of every treatment, two different concentrations and control. End sample: $1x10^9$ 77, $2x10^9$ 76 and control 78 Because of the large sample size, experiment was performed with an additional experimenter. Survival was monitored for one day.

3.3.4 Mixed strain infection experiment

To test different strains and their combinations in the same experiment, I used procedure without starvation. *Bt* strains were either tested singly, as a mixture of two or a mixture of three strains in a fully-reciprocal design, resulting in following treatments and their combinations: *Btt*, *BtE2*, *BtQ3*, *Btt*/*BtE2*, *Btt*/*BtQ3*, *BtE2*/*BtQ3*, *Btt*/*BtE2*/*BtQ3*. Each solution was adjusted to the $1x10^9$ mL^{-1} of spores was used, with each strain contributing with the same amount. On each of the 10 trays, there were 7 dishes for each treatment. Sample size for each treatment was 70, except control where was 69. Because of the large sample size, experiment was performed with additional experimenter. Survival was monitored for 3 days without adding new diet.

3.3.5 Influence of social context, starvation and long-term exposure to *B. thuringiensis E2 strain* on ant survival

Here, I tested whether *BtE2* starts to influence ant survival later than three days after exposure, what was the longest period previously tested. In addition, after this long-term exposure, impact of starvation was explored to reveal potential costs of survival. Moreover, here I also exposed group of five nestmates to *Bt*, in contrast to previous experiments where ants were exposed only individually, socially isolated from their nestmates, what can effect digestion process. As a result, in same experiment I tested if ant survival, during 13-day *BtE2* exposure depends on social context in which they were reared and food availability after exposure (Fig. 1). In previous experiments, exposure to *Bt* or control solution was done just on the first day and it was not daily exchanged. In this case, solution to which ants were exposed on filter paper would gradually evaporate, thus, ants were probably not able to drink solution during whole experiment. Therefore, to ensure regular food source and treatment availability during whole experiment, freshly prepared spores (with a maximum age of 2 days after harvest) or control solution was added daily; this was done by completely removing the red foil and exchanging it with the new one, together with the moistened filter paper. After 10 days of constant exposure to *BtE2* or control solution, subsample of all treatment groups were starved to reveal potential hidden costs of defence against *BtE2*. For this, half of the trays with surviving

ants (trays 2, 3, 5, 8, 9) were kept as previously, whereas to other half (trays 1, 4, 6, 7, 10) only filter papers dipped in water were given. Mortality was monitored till day 13. Two colonies of *L. humile* were used; L´ Escala and Castel d´Aro, collected in April 2016. Ten trays were prepared, each containing all treatments. Ants that escaped or were accidentally killed by experimenter were excluded, resulting in the following sample size per treatment: individually reared 135 not starved and 128 starved in *BtE2*, 131 not starved and 128 starved in control; in group 156 not starved and 149 starved in *BtE2*, 155 not starved and 155 starved in control. Because of the large sample size, experiment was performed with an additional experimenter. Experiment was also prepared in two blocks, each containing all the treatments; block 1=trays 1-6, block 2=trays 7-10.

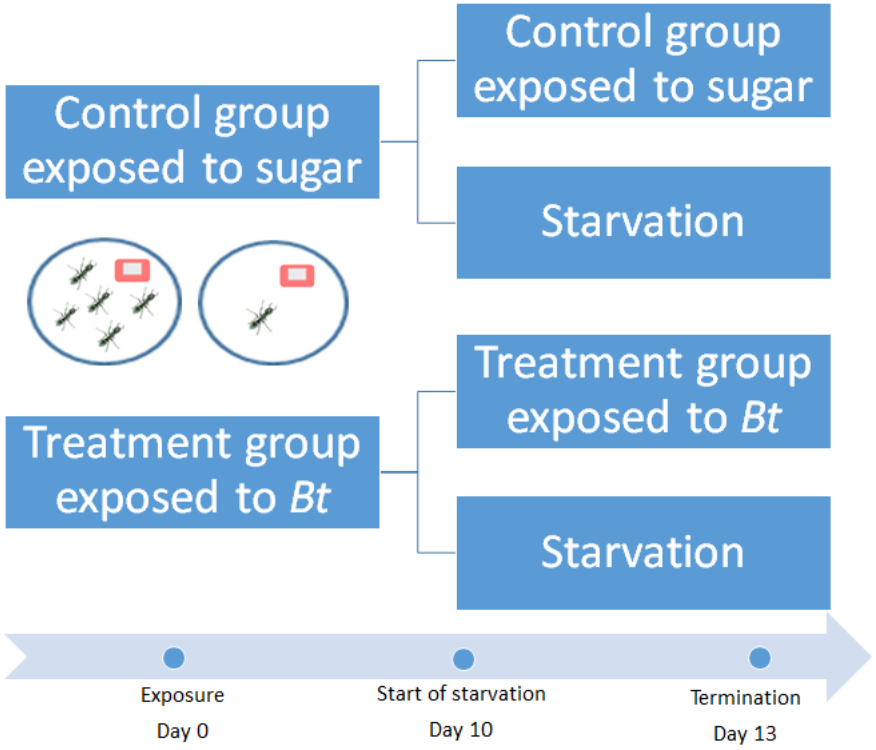

Figure 1. **Experimental design for testing influence of social context, starvation and long-term exposure to *BtE2* strain on ant survival**. I exposed ants to *BtE2* in a group of 5 nestmates, or individually and monitored survival for 13 days. Freshly prepared spores (with a maximum age of 2 days after harvest) or control solution was added daily by completely removing the red foil and exchanging it with the new one, together with the moistened filter paper. The influence of starvation after exposure to Bt on ant mortality was also tested. After 10 days of constant exposure to *BtE2* or control solution, half of the trays with surviving ants were kept as previously, whereas to other half only filter papers dipped in water were given. Mortality was monitored till day 13.

3.3.6 Behavioural changes induced after exposure to *B. thuringiensis E2*

For observation of behavioural response of worker ants to *Bt*, individual ants (focal ants) were exposed to *BtE2* and paired with unexposed nestmates for 15 minutes, during which their behaviour was recorded (Fig. 2). In order to follow interactions directed towards the focal nestmate and the ones directed from her towards other nestmates, ants exposed to *BtE2* or to control solution were marked as described above. After marking, these focal ants were placed in empty 35 mm (⌀) Petri dishes. I performed the same procedure with unexposed nestmates that were not marked and put them all on starvation at 30ºC and 70% humidity for about 10 hours. The next morning, I pipetted 0.5 µL of *BtE2* or the control solution (10% sucrose) on red foil and placed it in the dish with each marked ant. To ensure that all treatments were performed at the same time, I placed four foils with *BtE2* followed by four foils with sucrose. I marked the ants which I observed eating. I repeated this procedure, placing new and removing old foil two more times after approximately one hour. Only ants that drank during every exposure were taken for analysis of behaviour, or the ones which had drank fewer times, but their gasters were obviously enlarged. Each of the marked and exposed ants was added to the three starved nestmates in a new 35 mm (⌀) moisturized Petri dish with plastered floor. Note that the nestmates were starved for 3 hours longer then the focal ant. Immediately after focal ant was added to the group, I placed the Petri dish under a video camera to be recorded for few hours. The experiment was performed three times using the following colonies: 1) Colony 1: Castel d´Aro 2011a, 2) Colony 2: Castel d´Aro 2011b, 3) Colony 3: L´Escala 2016. The difference between the first and the second colony is that the second was obtained from a collaborator in Finland and had lived in a different conditions compared to the Colony 1; different food was offered, but they also just changed the season from Winter conditions to Summer conditions (2 weeks). The end sample size for each of the colonies was as follows: 1) *BtE2* 6, control 5; 2) *BtE2* 4, control 4; 3) *BtE2* 5, sugar 7.

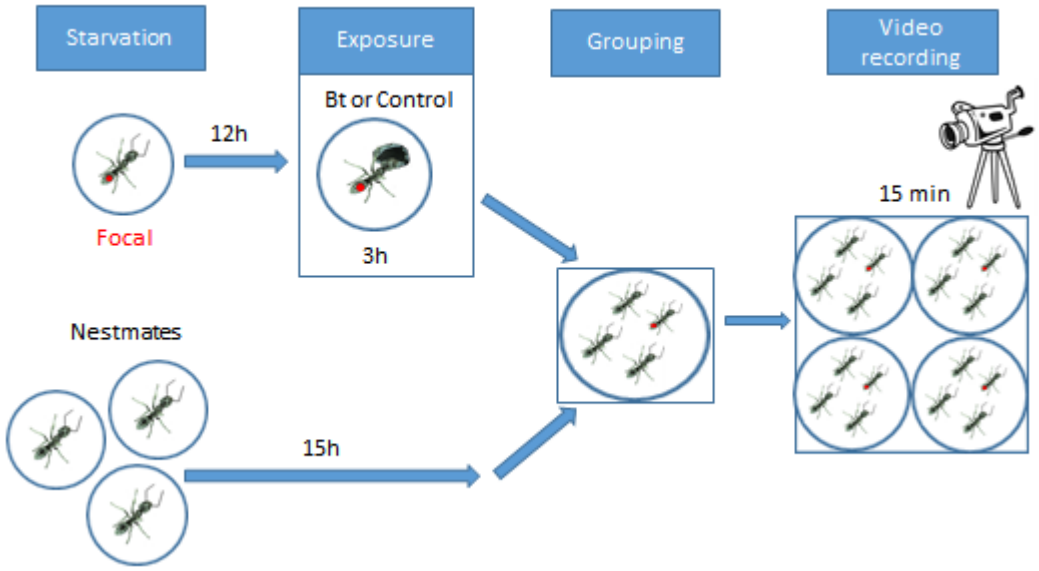

Figure 2. **Experimental design for testing induced behavioural changes after exposure to *BtE2*.** Marked ants were placed in empty Petri dishes. I performed the same procedure with unexposed nestmates that were not marked and put them all on starvation at 30ºC and 70% humidity for about 10 hours. The next morning, I pipetted 0.5 µL of *BtE2* or the control solution (10% sucrose) on red foil and placed it in the dish with each marked ant. I repeated this procedure, placing new and removing old foil two more times after approximately one hour. Each of the marked and exposed ants was added to the three starved nestmates in a Petri dish with plastered floor. Note that the nestmates were starved for 3 hours longer then the focal ant. Immediately after focal ant was added to the group, I placed the Petri dish under a video camera to be recorded and first 15 minutes later analysed.

To record videos, µEye 6 mm F 1.6 67709 (Edmound optics, Germany) cameras with StreamPix 5.0 software were used. On each of the four camera, I placed four Petri dishes (Figure 3). To code ant behaviours from the obtained videos (Table 1), I used Solomon Coder (beta 15.11.19) and was blinded during analysis. I coded 15 minutes of each video, after excluding first 15 seconds. After initial coding, I confirmed uncertain behaviours by zooming, image sharpening and colour inverting with editing tools in the VLC media player (2.2.1). I used Solomon Coder in-built analysis to obtain durations of different behaviours, which were copied to an Excel sheet and arranged for statistical analysis in JMP.

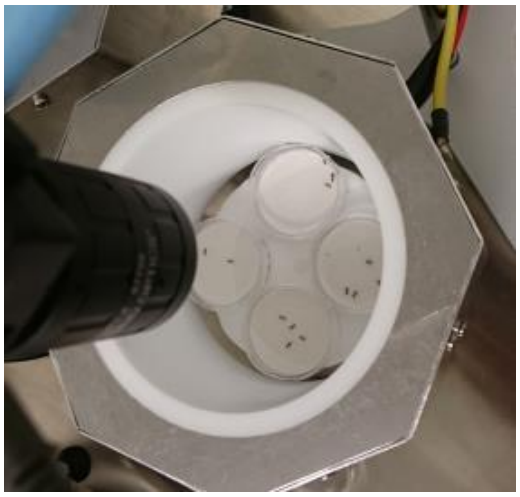

Figure 3. **Camera setup for recording behaviours.** To record videos, µEye 6 mm F 1.6 67709 (Edmound optics, Germany) cameras with StreamPix 5.0 software were used. On each of the four camera, I placed four Petri dishes.

Table 1. **Coded behaviours**

Behaviour	**Purpose**	**Description**
Self-grooming	Sanitary behaviour; self-cleaning	Focal ant: ✓ Cleans its body parts with mouthparts ✓ Brushes gaster with leg ✓ Still or tremble forward
Gaster tip self-grooming	Sanitary behaviour; unknown (see discussion)	Focal ant: ✓ Bends to reach gaster tip ✓ Performs grooming-like behaviour
Body allogrooming performed	Sanitary behaviour; mutual-cleaning	Focal ant: ✓ Grooms any body part of a nestmate except mouth
Focal allogrooming performed	Sanitary behaviour; mutual-cleaning	Focal ant: ✓ Grooms mouth parts of a nestmate
Body allogrooming received	Sanitary behaviour; mutual-cleaning	Nestmate: ✓ Grooms any body part of a focal ant except mouth
Mouth allogrooming received	Sanitary behaviour; mutual-cleaning	Nestmate: ✓ Grooms mouth parts of a focal ant
Trophallaxing	Mouth to mouth fluid transfer	Each ant involved: ✓ Slower antennal movement ✓ Mostly no head movement ✓ Head position that allows contact of mouth parts ✓ During separation sticky effect ✓ Can be just few seconds long

3.3.7 Antimicrobial properties of *L. humile* gaster secretions against *Bacillus thuringiensis E2*

In this experiment, I tested antimicrobial properties of gaster secretions of *L. humile* ants; they have four different glands with openings at their gaster tip (Hölldobler & Engel 1978). For this, ants were sacrificed by a 5-minute exposure to -80 ºC. I stabilized ants upside-down using a sponge with a slit, such that gaster opening was facing up and was easier to accesses with the capillary. Gaster was gently squeezed with forceps and emerged droplet was collected with a 0.5 µL capillary (Scruoprat, Germany). In the same capillary, droplets from 5 ants were pooled and then diluted in Millipore water to increase the volume and placed on ice. In total, 40 droplets, one from each ant, were diluted in 80 µL of Millipore water. Dilution was stored on 4 ºC for few hours before I pipetted it on LB agar plates with *BtE2* spores.

Six LB agar plates were prepared (by Life Science Facility, Institute of Science and Technology Austria) and stored at 4ºC. After testing different number of spores for optimal growth on the plates, I decided to use 100 µL of $2x10^8$ mL spore concentration, since this concentration resulted in a clear bacterial lawn on the plate. On each agar plate I placed 6 glass beads and then pipetted 100 µL of $2x10^8$ mL^{-1} spore concentration. Plates were shaken horizontally for a few seconds in all directions to spread the spores evenly. When the agar dried I pipped 10 µL of droplet dilution, Streptomycin as positive control (100 µg/mL) and ddH_2O as negative control. PBS in which spores are kept had been tested before and showed no influence on bacterial growth. I left the plates for one hour on room temperature to dry and then incubate them for 13 hours at 30 ºC.

3.4 Statistical analysis

All experiments were analysed with JMP statistical program (version 12 for Windows) together with my direct supervisor Dr. Milutinović (Table 2). To test the effect of treatment on survival in the first, second and third experiment (effect of *Btt* and *E2* exposure on ant survival, influence of spore concentration on ant mortality without and with previous starvation), a generalised linear model (GLM) was fitted with binomial distribution and logit link function using survival as response variable (0 = dead, 1 = alive). Bacterial treatments in the first experiment were analysed separately, since 3 ant species were used to test the influence of *Btt* and only one of *BtE2*. For *Btt*, model factors included treatment, ant species and treatment*species interaction. For *BtE2*, treatment was included as a model factor. In the second experiment, treatment, experiment and treatment*experiment interactions were used, whereas

for the analysis of the third experiment model factor was treatment. Because of the quasi-complete separation of *Btt* data in the first experiment and the data of the third experiment (spore concentration with starvation), the same model was fitted using Firth's bias-adjusted estimates (Firth 1993). The experiment testing the influence of *Bt* strains mixture on ant survival (experiment 4) was analysed using Cox proportional hazard model, since here, mortality was screened for 3 days. Following factors were included in the model: treatment, colony and treatment*colony interactions. Experiment 5 (influence of long-term exposure to *BtE2*, starvation and social context) was analysed using Cox proportional hazard model with following factors: Treatment (constant *Bt* diet, starvation after *Bt* diet, constant control diet, starvation after control diet), Colony, Block, Social context (Group vs. singly reared) and Treatment*Social context interaction. Vial number was included as a random factor. Generally, in each of the above experiments, experimental trays on which ant nests were kept were fitted as a random factor, except for the *BtE2* mortality data of the first experiment for which the number of trays per experimenter was too small. Also, in each experiment (except experiment 2), experimenter was included as a random factor in the model.

Behavioural data obtained from the video analysis (experiment 6) were first tested for normal distribution (Shapiro-Wilk test). If the data were normally distributed, least squares method was used to analyse the influence of treatment on induced behaviours. For non-normally distributed data, residuals were saved after the model was fitted and tested for normal distribution. This was the case for behaviours *Self-grooming*, *Body allogrooming received* and *Mouth allogrooming received*, whereas *Gaster tip self-grooming* data were Log10 transformed and used in the same model. In the cases where saving residuals, Box-Cox or Log10 transformed data did not follow normal distribution (*Body allogrooming performed*, *Mouth allogrooming performed* and *Trophallaxing*, a nonparametric Wilcoxon test was used on the raw data. Three experiments were combined into one analysis and for the least Squares model the experiment was used as a random factor in the model.

4 Results

4.1 Influence of *B. thuringiensis* on mortality in ants

In this experiment, I tested two *Bt* strains for their ability to induce mortality in ants; *Bacillus thuringiensis tenebrionis* (*Btt*) and the *BtPS140E2* (*BtE2*), both of which have previously been shown to have pathogenic activity against ants (see materials and methods). The *Btt* strain was tested against three ant species; *L. neglectus*, *L. niger* and *L. humile* whereas *BtE2* only tested against *L. humile* since previous results from the Cremer group showed that the other three species are not susceptible to *BtE2* (B. Milutinović, unpublished results). Ants exposed to *Btt* spore-containing diet showed no difference in survival after 24h compared to control-exposed ants, and this was true for all three species tested (Fig. 4, Table 2, p=1.0). However, there was a difference in sensitivity to experimental handling across three species; both *L. niger* and especially *L. humile* showed substantial mortality due to the experimental procedure itself, whereas *L. neglectus* showed no mortality in either of the treatments (Fig. 4, Table 2, p=<.0001).

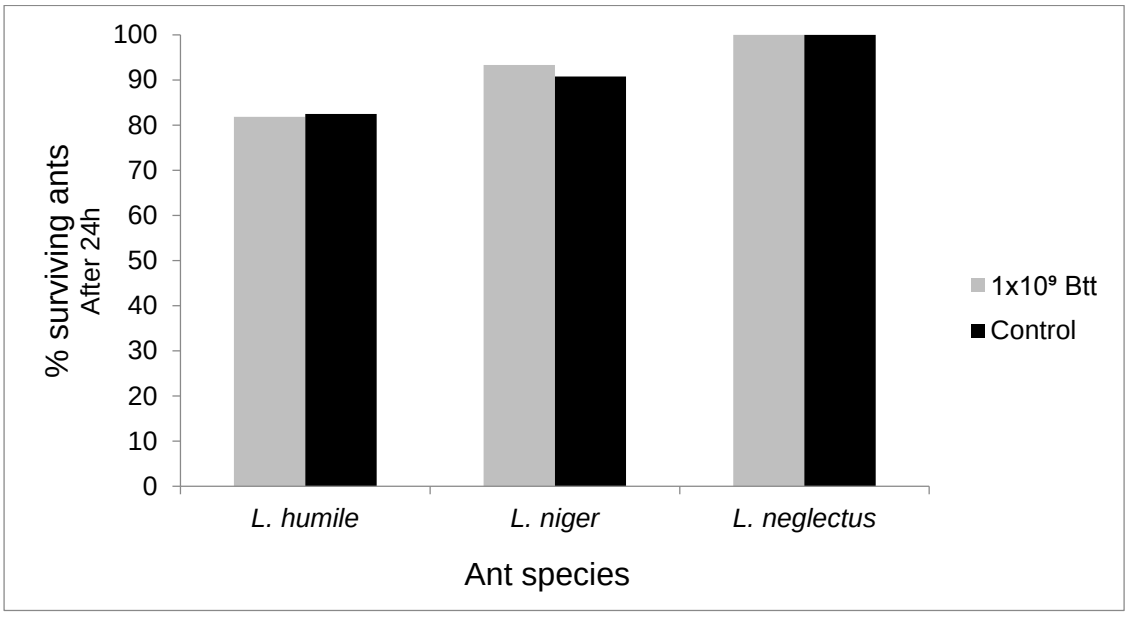

Figure 4. ***Btt*-induced mortality in ants**. Survival of workers of *L. humile*, *L. niger* and *L. neglectus* after constant exposure to *Btt* spore-containing diet. Survival was scored 24 hours after exposure.

However, a 24-hour exposure of *L. humile* to *BtE2* strain induced significantly different mortality compared to the control group (Fig. 5, Table 2, p=0.002). To test this further and to possibly obtain a stronger effect, I plan to test the influence of spore dose on survival of *L. humile* and focus only on the *BtE2* strain in my future experiment.

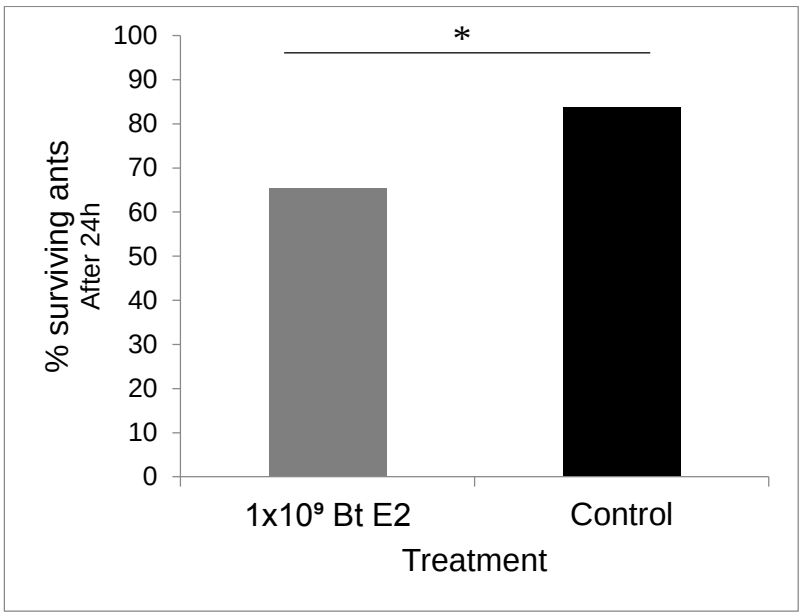

Figure 5. ***BtE2*-induced mortality in ants.** Survival of *L. humile* workers after constant exposure to *BtE2* spore-containing diet. Survival was scored 24 hours after exposure.

4.2 Dose response of *B. thuringiensis E2* strain

To determine if the effect observed with *BtE2* on *L. humile* is dose dependent, I tested three different concentrations; $0.5x10^9mL^{-1}$, $1x10^9mL^{-1}$ and $2x10^9mL^{-1}$. My results demonstrate that spore concentration did not have an influence on ant mortality. In fact, contradicting the previous experiment, I found no effect of *BtE2* treatment on ant survival compared to the control. This experiment was performed twice to confirm the results and indeed, the same results were obtained (Fig. 6, Table 2, p (treatment)=0.566). In addition, there was no difference between the two repeated experiments (Table 2, p=0.637). This suggests that mortality observed in the first experiment was potentially influenced by other uncontrolled factors and not by the treatment.

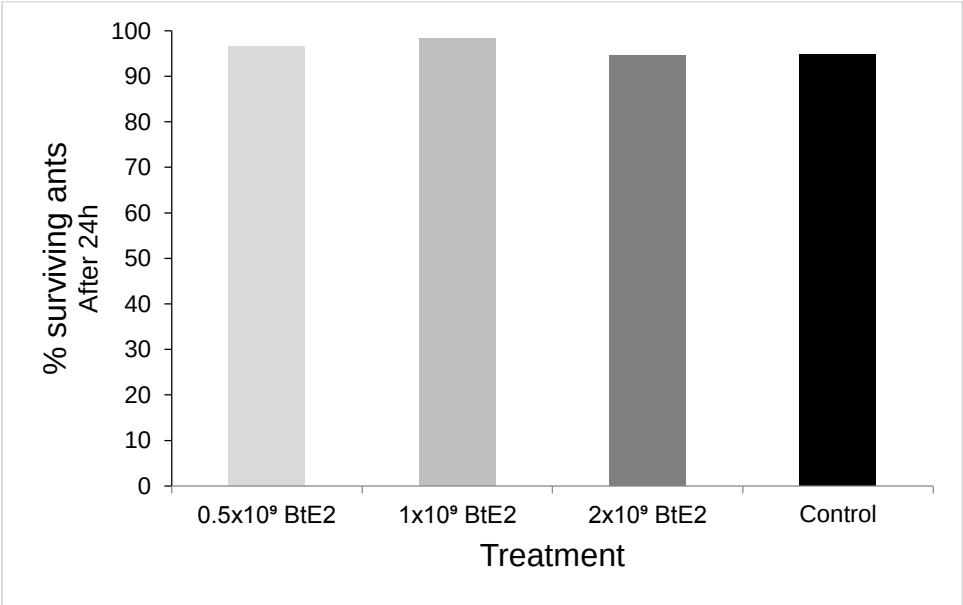

Figure 6. **Influence of dietary spore concentration on ant survival.** Worker ant survival of *L. humile* after constant exposure to 3 different concentrations of *BtE2* in their diet. Survival was scored 24 hours after exposure.

4.3 Dose response of *B. thuringiensis E2* strain with previous starvation

The absence of mortality in the previous experiment could also result from ants not even taking up the diet, either because they were not hungry, or as a consequence of possible avoidance of contaminated diet. Therefore, to ensure that ants indeed drink the spore-containing diet upon exposure, I starved *L. humile* for six hours after which they were exposed to *Bt* spore-containing diet. However, similarly to the previous experiment, no influence of treatment on ant mortality was observed (Fig. 7, Table 2, p=0.690). It is noteworthy that many starved ants, independent of the treatment, started drinking the offered diet immediately upon exposure (personal observation).

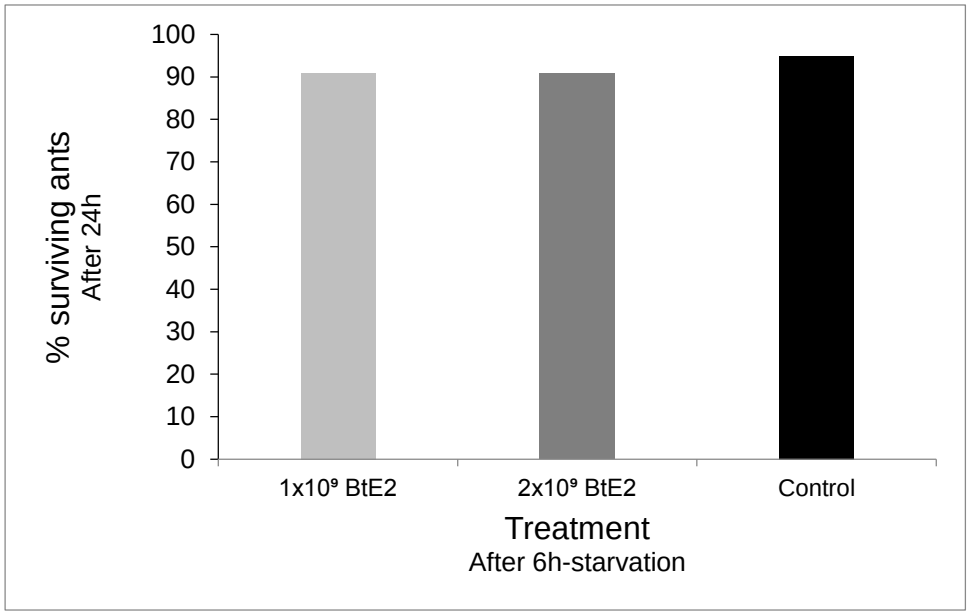

Figure 7. **Influence of dietary spore concentration on ant survival after starvation.** Survival of *L. humile* workers after constant exposure to three different concentrations of *BtE2* in their diet. Before the exposure ants were starved for 6 hours. Survival was scored 24 hours after exposure.

4.4 Mixed strain infection experiment

To further explore the relationship between *Bt* and *L. humile*, I exposed the ants to the mixture of various *Bt* strains in their diet, since such infections were often shown to induce larger mortality then the single strains (Raymond et al. 2013). For this, I again used the *Btt* and the *BtE2* strain, but I also included another strain, *Bt PS86Q3* (*BtQ3*), which was previously shown to have activity against hymenopterans (see materials and methods). Ants were exposed to the three strains alone, the mixture of two strains in a full reciprocal design and to the mixture of all three strains. This time, mortality was also screened for three days, to test whether it comes with a later onset in this system. Here as well, ants exposed to each strain alone, as well as to the different mixture combinations of the two or three strains, survived equally well as the ants in the control group (Fig. 8, Table 2, p=0.881).

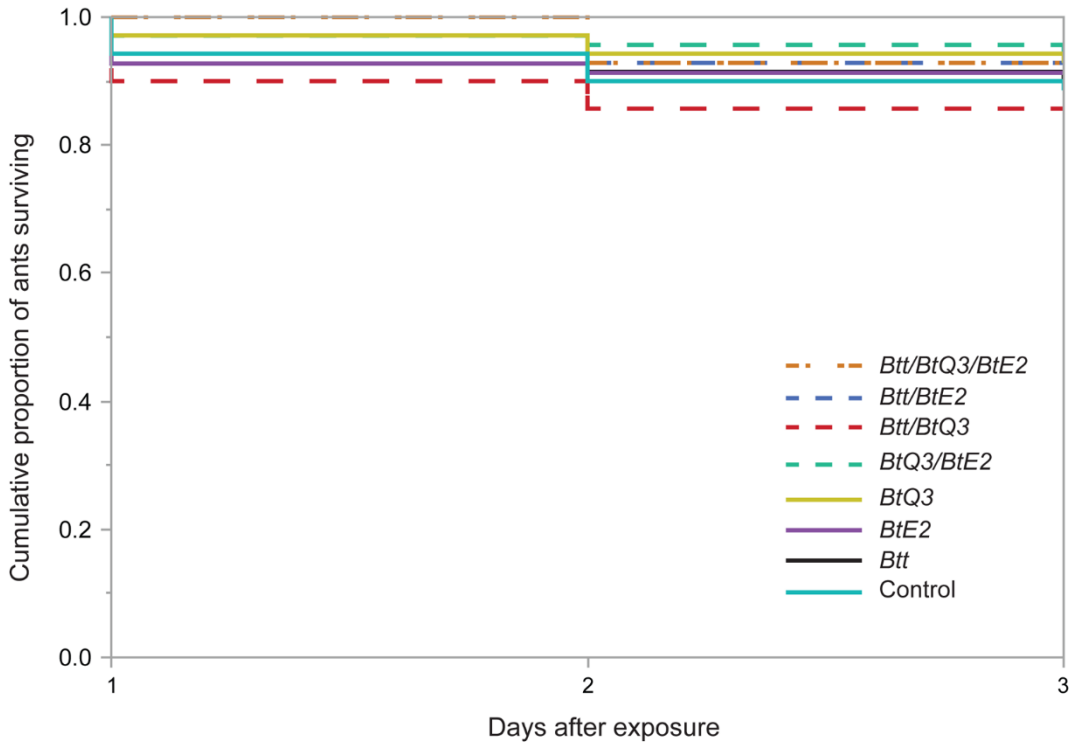

Figure 8. **Influence of mixture of *Bt* strains on ant survival.** Survival of *L. humile* workers after constant exposure to either single *Bt* strains or a mixture of two or three strains. Survival was monitored for three days.

4.5 Influence of social context, starvation and long-term exposure to *B. thuringiensis E2 strain* on ant survival

In this experiment, I addressed several questions. First, I tested whether longer exposure of ants to spores in their diet influence survival, since from the previous experiments, no constant influence was detected. For this, ant survival was monitored for 13 days (Fig. 1). At the same time, I wanted to test whether group- versus individual-rearing influences outcomes of exposure to spores. This is based on a recent work by Koto (Koto et al. 2015), which showed that in carpenter ants, only group-reared individuals actually properly ingest food, such that it may be possible that my individually-reared ants do not ingest *Bt* spores at all, or that the spores only arrive until the crop, as described for the carpenter ants. To test this, ants were exposed in groups or singly. Finally, I wanted to see whether there are any hidden survival costs after *Bt* exposure. It may be that, under ad libitum food resources, costs to infection will not be visible, as the hosts are able to compensate for the damage done by the parasites (Moret & Schmid-Hempel 2000). For this reason, after day 10, a subsample of ants was placed under starvation and survival was monitored until day 13 (Fig. 1). The results of such long-term experiment showed that again, there was no influence of treatment on survival, showing that longer exposures to spores do not induce mortality in ants (Fig. 9, Table 2, p=0.132). Although starved ants died faster (p=<.0001), no difference between *Bt*-exposed and control solution was observed (Fig. 9, Table 2, p=0.624).

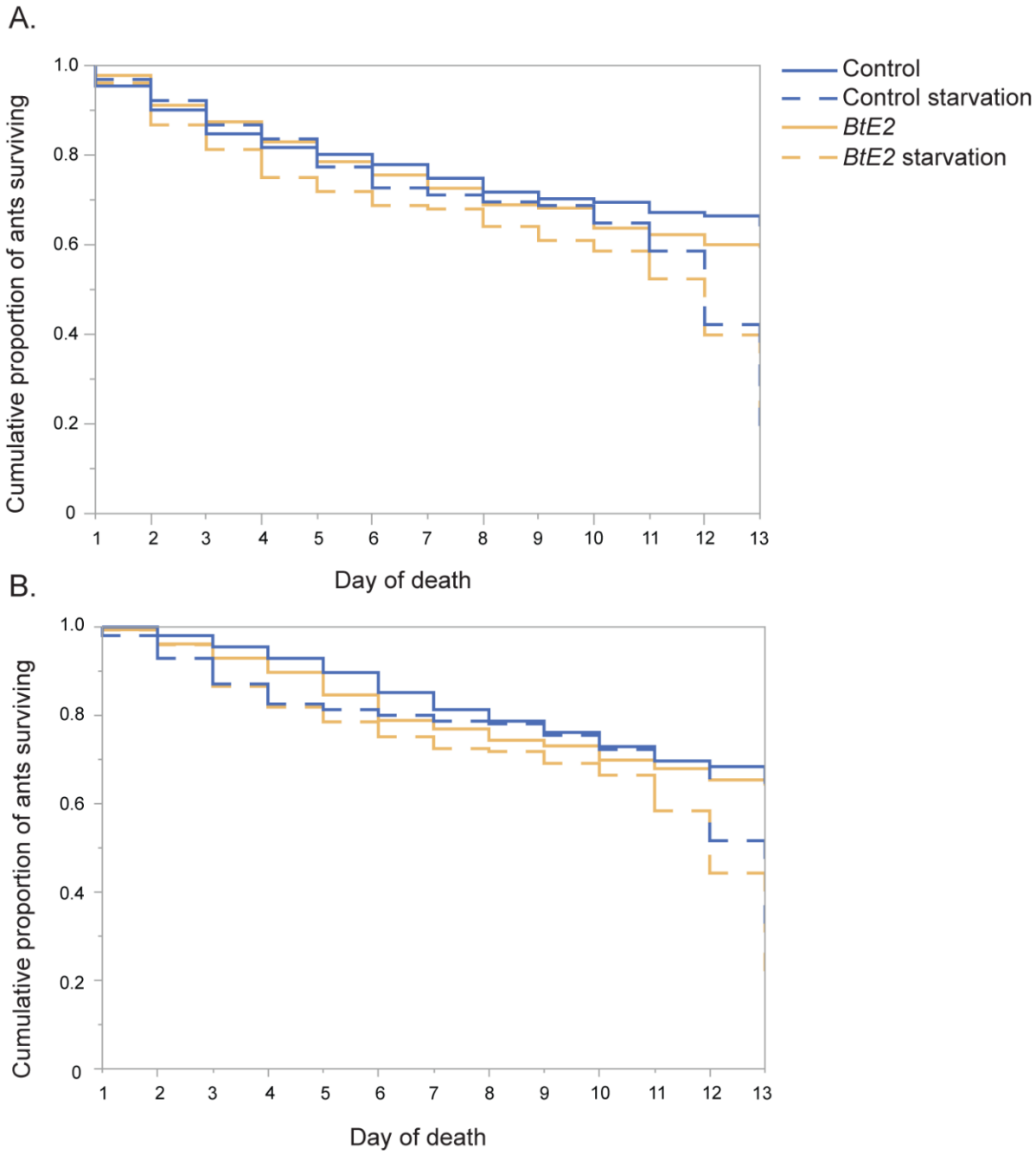

Figure 9. **Influence of social context (A. group, B. social isolation), starvation and long-term exposure to *BtE2* and on ant survival.** Survival of *L. humile* workers after constant exposure to *BtE2* strain. Survival was scored for 10 days after which a subset of ants was placed on starvation, whereas others were maintained on regular diet (*Bt* or sugar). Survival was further monitored until day 13.

4.6 Behavioural changes induced after exposure to *B. thuringiensis E2*

No mortality was observed after exposure to *Bt* in the previous experiments. However, due to the mechanisms of social immunity (described in the introduction section), it may be possible that ants in some way even prevent *Bt* from establishing an infection in the body by performing specific behaviours, different than the unexposed ants. To test this, I analysed duration changes of certain behaviours after exposure to *BtE2,* which could indicate their importance for *L. humile* against *Bt*. Following behaviours were scored for the focal ant; self-grooming, time spent grooming other nestmates (allogrooming; separately mouth parts and the rest of the body), trophallaxing or being groomed (separately mouth parts and the rest of the body) (Table 1). Behaviour of ants exposed to *BtE2* was not significantly different compared to control ants for behaviours self-grooming (Table 2, p=0.133), being groomed by others (Table 2, p (grooming mouth parts)=0.65, p (grooming body=0.902), trophallaxing (Table 2, p=0.284), allogrooming body and mouth parts of the unexposed nestmates (Table 2, p (grooming mouth parts)=0.103, p (grooming body=0.402)) (Fig. 10). However, one of the behaviours performed by the *BtE2* focal nestmates changed; gaster tip self-grooming was significantly longer compared to controls (Table 2, p=0.014).

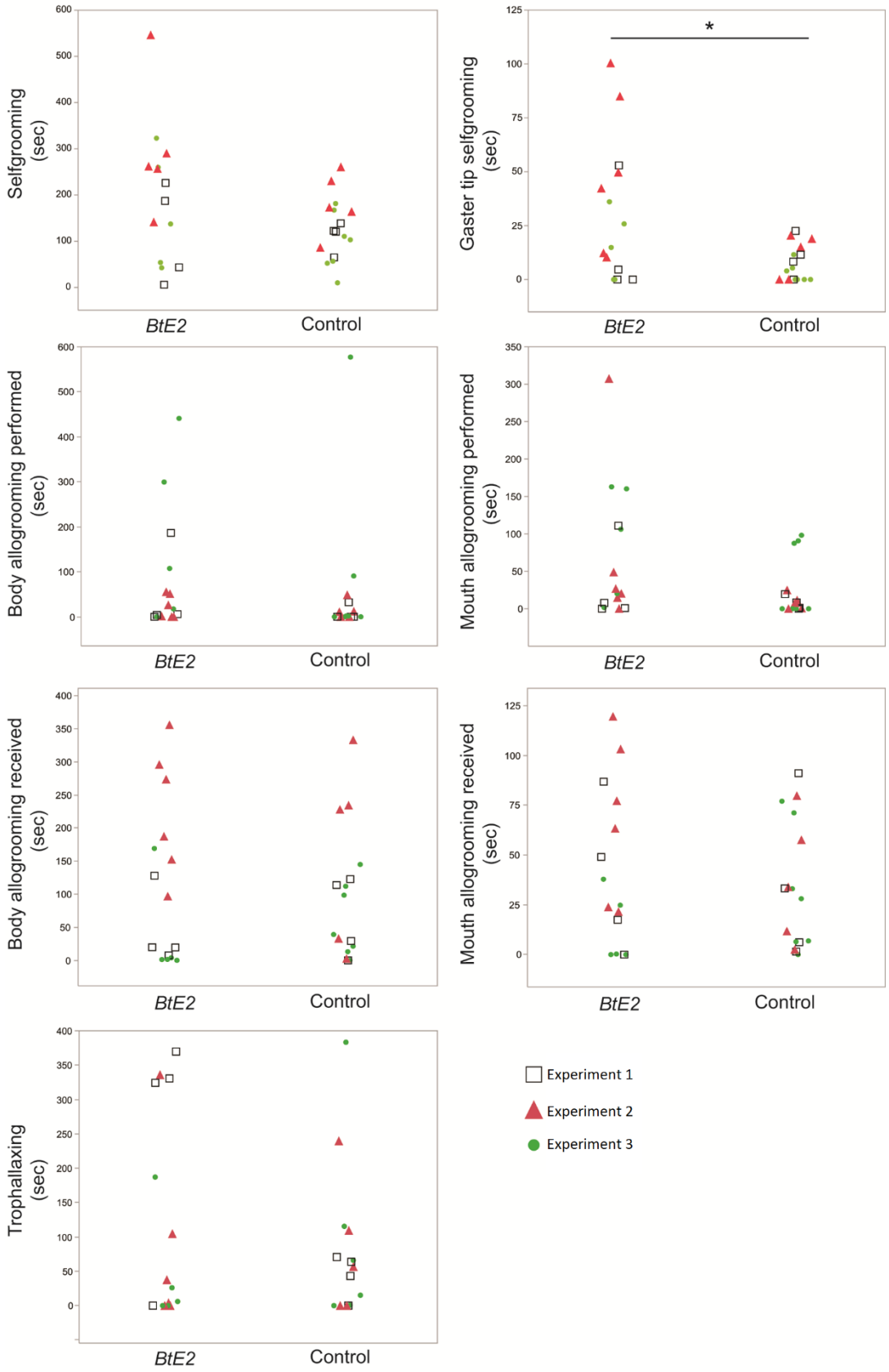

Figure 10. **Change in behaviours after exposure to *BtE2*.** Change in behaviours directed towards and from the focal ant to the unexposed nestmates after exposure to *BtE2* or sugar solution (control) measured in three experiments. Behaviours were scored from three experiments for 15 minutes.

4.7 Antimicrobial properties of *L. humile* gaster secretions *against B. thuringiensis E2*

Since *L. humile* have poison glands in their gaster with opening at their gaster tip (Hölldobler & Engel 1978), I further analysed if gaster tip secretions have inhibitory effect on bacterial growth. Indeed, a clearly visible inhibition zone with no bacterial growth was visible in each of the six plates (here shown only one example) at the place where extracted secretion from the gaster was pipetted (Fig. 8).

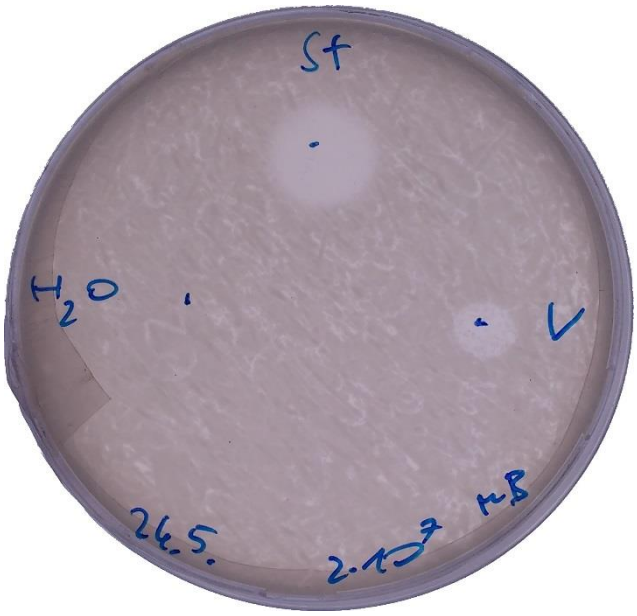

Figure 11. **Inhibitory effect of gaster tip secretions on *BtE2* bacteria.** A droplet of *L. humile* gaster tip secretions were isolated and pipetted on LB agar plate where *BtE2* spores had been plated. After the overnight incubation, bacteria-free zone was observed at the point where droplet was pipetted, indicating inhibited bacterial growth (V). St: Streptomycin control, H2O: water control.

Table 2 ***Statistical analysis***

Influence of *Bt*	χ^2	*p*	*d.f.*
A. Overall model- ***Influence of Btt***	*60.583*	***<.0001***	*5*
Treatment	*0*	*1.000*	*1*
Species	*60.105*	***<.0001***	*2*
*Treatment*Species*	*0*	*1.000*	*2*
Contrasts			*2*
L. neglectus vs. L. humile	*60.002*	***<.0001***	
L. neglectus vs. L. niger	*24.669*	***<.0001***	
L. humile vs. L. niger	*9.640*	***0.002***	
B. Overall model - ***Influence of BtE2***	*90.854*	***0.002***	*1*
Dose response	χ^2	*p*	*d.f.*
Overall model	*2.530*	*0.924*	*7*
Treatment	*2.030*	*0.566*	*3*
Experiment	*0.221*	*0.637*	*1*
*Treatment*Experiment*	*0.415*	*0.937*	*3*
Dose response with starvation	χ^2	*p*	*d.f.*
Overall model	*0.740*	*0.690*	*2*
Mixed strain infection	*Likelihood ratio*	*p*	*d.f.*
Overall model	*4.463*	*0.881*	*15*
Treatment	*4.463*	*0.566*	*7*
Colony	*0.212*	*0.637*	*1*
*Treatment*Colony*	*4.545*	*0.937*	*7*
Longterm exposure, social context and costs	*Likelihood ratio*	*p*	*d.f.*
Overall model	*62.673*	***<.0001***	*9*
	χ^2	*p*	*d.f.*
Treatment (Bt, Sugar, Bt starvation, Sugar starvation)	*104.592*	***<.0001***	*3*
Colony	*5.602*	***0.018***	*1*
Block	*5.556*	***0.019***	*1*
Social context	*5.491*	***0.020***	*1*
*Treatment*Social context*	*1.265*	*0.737*	*1*
Behavioural analysis	*F*	*p*	*d.f.*
1. Self-grooming	*3.229*	*0.083*	*1*
2. Gaster tip self-grooming	*6.846*	***0.014***	*1*
	χ^2	*p*	*d.f.*
3. Body allogrooming performed	*2.148*	*0.142*	*1*
4. Mouth allogrooming performed	*3.973*	***0.046***	*1*
5. Trophallaxing	*0.032*	*0.856*	*1*
	F	*p*	*d.f.*
6. Body allogrooming received	*0.049*	*0.826*	*1*
7. Mouth allogrooming received	*0.257*	*0.615*	*1*

5 Discussion

The aim of this study was to investigate host-pathogen interactions using a novel model system of *B. thuringiensis* bacteria and ant societies, thereby providing additional knowledge to the field of social immunity. *Bt* infects its host naturally via the gut, by ingestion of spores and their toxins (Porcar et al. 2008) which is in contrast to majority of studies performed so far in ants, relying mostly on "external" infections induced by fungal parasites.

Bt bacteria are usually very specific to their hosts (van Frankenhuyzen 2013). Some strains have a general activity against lepidopteran or coleopteran insects, but also within certain insect orders, larger specificity is found such that some strains infect only specific families or even only certain species within a family (van Frankenhuyzen 2013; Crickmore 2006; Milutinović et al. 2013). Therefore, it was crucial for this study to find a *Bt* strain that is able to infect ants. I chose *Bt* strains used in this study based on previous reports that showed activity against several ant species, including *L. humile*. For example, Payne et al. 1997 showed that *BtE2* and *BtQ3* strain induce considerable mortality in argentine ants and Bulla et al. 2004 showed activity of *Btt* strain toxins on fire ants. However, I could not confirm these results under used experimental conditions. Neither did the *BtQ3*, *Btt* nor *BtE2* strain induce mortality in ant species tested (Fig. 4, Table 2), although results for the *BtE2* strain seemed to be more variable (Fig. 4 and Fig. 5) suggesting a small, but inconsistent effect. I further tried to increase morality induced by the *BtE2* strain by exposing the ants to three different doses, however no difference in mortality was observed, even when the ants were starved before the exposure to ensure they were indeed ingesting contaminated diet (Fig. 6, Fig. 7 and Table 2). Similarly, when ants were exposed to a *Bt* strains mixture, which was shown to increase mortality in other insect hosts (Raymond et al. 2013), no mortality was observed in this host-pathogen system and under these experimental conditions (Fig. 8) It is difficult to speculate about possible reasons for the observed discrepancies between the reported patent (Payne 1997) and my results; one reason may be because different supercolonies were used (Giraud et al. 2002).

A recent study on carpenter ants reported an interesting observation that social living, compared to isolation, strongly influences ant metabolic rate, food processing and hence, the survival (Koto et al. 2015). The ants that had been reared in isolation did not ingest their food completely, but have rather stored it in the crop, which, in the combination with higher activity, induced also higher mortality of the singly infected ants. I suspected that something similar might be occurring in this system as well; it may be possible that ants when isolated, do not

ingest their *Bt*-containing diet and hence therefore survive. However, not even after a constant exposure to *Bt* spores did the ants show differences in survival between the group-reared and isolated ants (Fig. 9). The ants must have been somehow able to use the sugar from the *Bt*-contaminated diet, as they only start rapidly dying after placed on complete starvation (Figure 9, Table 2, p=<.0001). Usually, costs are expected for host-pathogen interactions (Sheldon & Verhulst 1996), which I assumed will appear visible during the long term exposure; ants that constantly ingest spores should use more resources for defence, which could have manifested in a decrease survival. However sometimes, hidden costs might be at play which would only be visible upon food deprivation (Moret & Schmid-Hempel 2000). For this reason, I starved the ants after longer exposure to *Bt*, in order to see whether such potential costs might exist. However, I did not observe any difference in mortality between *Bt* and control-starved ants, nor did this depend on the social context (Fig. 9, Table 2, p (Treatment*Social context)=0.737.

As mentioned previously, social immunity is very effective in preventing potential epidemiological outbreaks in insect societies and often it acts even before parasites have managed to enter the colony or the host body (Cremer et al. 2007). Therefore, I suspected that in this specific system, *Bt* may in some way be hindered from even establishing an infection in ants and inducing mortality. Indeed, my results indicate that, although *Bt* has no effect on ant mortality, ants seem to be able to detect it or some of its components, thereby changing their specific behaviour. I found that ants groomed their own gaster tip significantly longer after exposure to *BtE2* then the ants that were offered only sugar (Fig. 10). This behaviour is similar to the previously observed behaviour in *L. neglectus* ants, where workers were found to take-up the self-produced poison by self-grooming the acidopore at the gaster tip and applying it to the fungus-exposed brood (Tragust et al. 2013a). In this way, majority of fungal spores that had remained after grooming had been inactivated, as the poison was further found to inhibit the spore germination (Tragust et al. 2013a). The major component of the poison in *L. neglectus* was found to be formic acid, which seemed to have a major effect, although several hydrocarbons from the Dufour gland showed an additive inhibitory effect as well. Similar to this system, *L. humile* may also take-up secretions from its gaster tip and by doing this, inhibit bacterial growth and prevent infection from developing. Indeed, I found that isolated secretions have antimicrobial properties against *BtE2* (Fig. 11). This is to the best of my knowledge the first report on such activity, as majority of studies on *L. humile* gland secretions, focused on their role in trail pheromones (Robertson et al. 1980; Reid et al. 2012). The composition of the inhibitory droplet is at the moment unclear, as *L. humile* have four different glands in their

gaster (Fig. 12) and all four may have contributed to the droplet composition upon isolation (see materials and methods). Upon isolation, gasters of ants are slightly squeezed, which allows the formation of a droplet which is then collected for the assay. Further research, where individual glands will be dissected and tested in a similar assay is needed. Moreover, I cannot say which of the gland the ants really use upon grooming; this requires further research by e.g., analysing the mouth or crop content after the observed behaviour and comparing it to the individual gland content using gas chromatography-mass spectrometry methods (Tragust et al. 2013a).

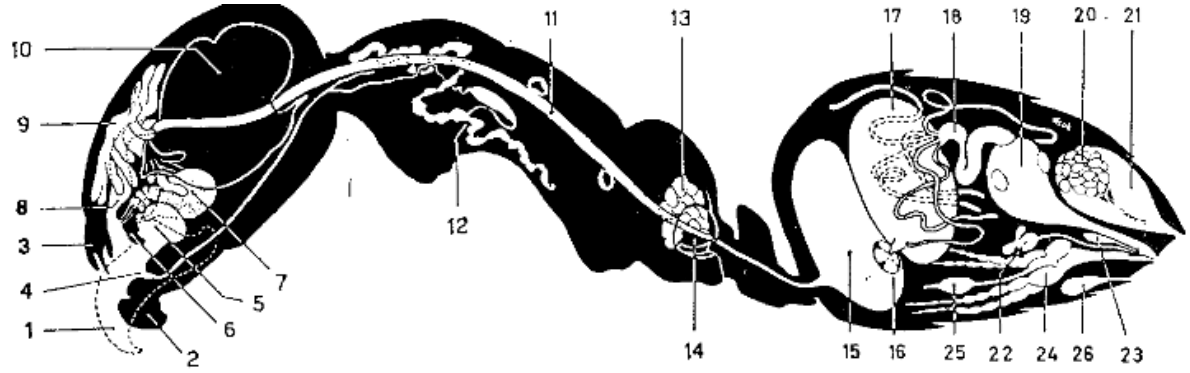

Figure 12 **Four different gaster glands in *L. humile.***
20: Anal gland, 22: Poison gland, 23: Dufour gland, 26: Pavan gland
(from Pavan & Ronchetti 1955)

Other behaviours tested; self-grooming body, allogrooming other nestmates and trophallaxing, were not statistically different from controls (Fig. 10, Table 2). Self- and allogrooming were often observed increased in fungus-exposed ants (Hughes et al. 2002) where the fungus is applied topically on the cuticle and can be removed by these specific behaviours. Since *Bt* is here applied orally, it is perhaps not surprising that no change in self-grooming was observed. However, I did expect the trophallaxis to show changes compared to controls, either in reduced or increased direction. For example, it may be that ants who took up a lot of *Bt* spores try to "dilute" the ingested spores which would make such reduced concentration easier to combat. Similarly, low level infections have been shown to act immunostimulatory, providing protection upon lethal challenge with high dose of fungal spores (Konrad et al. 2012). It may be possible that here as well, exposed nestmates contribute to immunisation of their unexposed nestmates in the same way. Alternatively, they may not give, but rather receive liquids from

other nestmates that would act inhibitory on bacteria or would aid immunity of the focal ant as was shown in *Serratia marcescens* Bizio, 1823 immunized *Camponotus* ants (Hamilton et al. 2011). Whereas all of the above hypotheses assume an increase in trophallaxis behaviour of the *Bt*-exposed ants, it could also be possible that the ants would reduce their trophallaxis with other nestmates, to prevent the pathogen from spreading within the colony; however, these hypotheses require further research. Note that, in this experiment, the focal ant and the nestmates were starved before the filming to ensure firstly, that the focal ant would take up the spore-containing diet but also to induce the trophallaxing behaviour in others, since this is a rather rare event. However, it would be interesting to see whether changes in trophallaxis are observed when only e.g., focal ant was starved, to see whether others would be so eager to exchange liquids if they are not thirsty as they were in this experiment.

Coming back to the costly defences against parasites (Sheldon & Verhulst 1996), my results indicate that *L. humile* may be taking up its secretions from one or a mixture of its glands which they may use to inhibit bacterial growth or even spore germination after ingestion. The production of this inhibitory substance should be very costly, but I observed no costs for survival during the long term exposure. I would speculate that costs should be visible elsewhere; e.g., those ants might be worse in performing other tasks or use up the nutrients of the colony which could all affect the colony fitness. However, although costly, such behaviours may prove beneficial for the whole colony. This study indicates that *L. humile* ants respond to *Bt* spore-ingestion by mounting specific behaviours which may prove as the first line of defence against parasite invasion of the colony, or before the infection managed to form within the host body. Future research is needed to find out whether ants react also by mounting specific immune defences and how costly it is to use and produce the poison, i.e., inhibitory substances from the gaster secretions.

6 Conclusion

- Ant survival was uninfluenced by exposure to *Bt*; when testing different strains or their mixtures, dietary spore concentrations, different exposure time or the exposure in groups compared to socially-isolated ants. Similarly, the expected hidden survival costs of defence against *Bt* were also not visible upon starvation, suggesting *Bt* might be hindered from even establishing an infection in the ant host.
- Indeed, *Bt* exposed ants self-groom their gaster tip longer then control-exposed ants, suggesting presence of an active defence against the parasite. This potential uptake of novel detected antibacterial properties of gaster tip secretions, could be the first line of defence against orally infecting parasite.
- Further research is needed to directly confirm which of the glands from *L. humile* has antimicrobial properties and whether or not ants indeed use specific gland secretions against *Bt*. Furthermore, to place this behaviour in social immunity context, the effect of exposed individual should be investigated on other nestmates, specifically testing physiological and/or behavioural changes.

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