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Original Article

Induced immune responses in *Formica fusca* (Hymenoptera: Formicidae)

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Abstract

Parental immune experience can enhance offspring defence mechanisms towards prevalent pathogens in the surrounding environment. This process of inherited resistance from one generation to another is known as trans-generational immune priming (TGIP) in invertebrates. In sedentary and dense insect societies, such as ant colonies, TGIP can influence colony survival and fitness upon pathogen outbreaks. However, TGIP appears to depend on species and environmental stressors and therefore can vary in intensity, as well as in the molecular mechanisms leading to resistance. Here, we stimulated the immune system of queens of the ant *Formica fusca* (Linnaeus, 1758) by wounding or injecting dead conidia of the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912). The offspring were subsequently infected with *B. bassiana*, and the effect of this priming on survival was evaluated. Furthermore, we investigated whether immune challenge of the mother queen induces changes in the expression of immunity-related genes in queens themselves and their brood. We combined this information with measurements of offspring size and number. Larvae produced by untreated queens had a significantly higher mortality after infection with *B. bassiana*, whereas those produced by immune-primed queens survived no worse than unexposed ones. Adult worker offspring from sham-control mothers showed a protective effect of queen treatment, consistent with transgenerational immune priming. Thus, the effects of queen priming appear to manifest themselves slightly differently in larval and adult offspring. No differences were detected in offspring number or size, but immune gene expression levels showed changes, both in queens and their offspring.

Key words: Trans-generational immune priming, infection, gene expression, social insects, ants, fungal infection, *Formica fusca*, *Beauveria bassiana*.

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Introduction

Exposure to parasites and pathogens poses risks that can lead to reduced fitness if no countermeasures are deployed. Hence host-parasite interactions are characterized by adaptive behavioral and physiological responses by both counterparts (Cremer & al. 2007, Sadd & Schmid-Hempel 2009, Bos & al. 2015). The immune responses utilized by hosts against infections are manifold, but all carry a cost. As a consequence, trade-offs with other life-history traits arise (Zuk & Stoehr 2002, Schmid-Hempel 2005). The ability to predict pathogen threat in the environment facilitates optimal resource allocation and allows an organism to invest in defence mechanisms only under high risks of infection (Freitak & al. 2007, Mikonranta & al. 2014). Furthermore, in addition to maximizing personal fitness, preparing offspring for future exposure to pathogens is crucial. A well-known example of this phenomenon is the incorporation of antibodies into breast milk in vertebrate mothers and passing immunological memory from one generation to another (Grindstaff & al. 2003, Hasselquist & Nilsson 2009).

Invertebrates were long thought to rely only on innate defence mechanisms, as they lack antibodies, the carriers

of adaptive immunity in vertebrates (Little 2004, Pancer & Cooper 2006). However, research in the last decades has shown that invertebrates can gain resistance against pathogens they have been exposed to (Witteveldt & al. 2004, Sadd & Schmid-Hempel 2006, Roth & al. 2009, Rodrigues & al. 2010, Tidbury & al. 2011, McTaggart & al. 2012), and moreover, this can be passed on to their progeny (Huang & Song 1999, Little & al. 2003, Sadd & al. 2005, Roth & al. 2010, Yue & al. 2013, Freitak & al. 2014, Hernández-López & al. 2014, McNamara & al. 2014, Dubuffet & al. 2015, Fisher & Hajek 2015). This phenomenon has been coined immune priming, and trans-generational immune priming (TGIP) if the parental immune experience enhances offspring defences (Moret 2006, Freitak & al. 2009, Roth & al. 2009, Rodrigues & al. 2010, Pigeault & al. 2016). The immune response is triggered by immune elicitors, i.e., pathogen-associated molecular patterns, present on the cell walls of bacteria, fungi, and protozoans (Lemaitre & Hoffmann 2007). The presence of these molecules in the gut or the hemocoel has been shown to activate both local and systemic defence mechanisms within a single generation (Rolff & Reynolds 2009). Furthermore, the immune elicitors have recently been shown to be transferred from mother to eggs, which may lead to enhanced pathogen resistance in offspring (Freitak & al. 2014, Salmela & al. 2015). Thus, in the honey bee, *Apis mellifera* Linnaeus, 1758, the egg-yolk protein Vitellogenin transfers bacterial fragments to developing oocytes (Salmela & al. 2015), which has been hypothesized to directly transfer antimicrobial peptides to offspring. Immune-challenged meal worm *Tenebrio molitor* Linnaeus, 1758 females, were, for example, found to exhibit similar antimicrobial activity in their hemolymph as detected in their eggs (Zanchi & al. 2012).

Trans-generational immune priming has been found in some invertebrate species, but not in others (Voordouw & al. 2008, Vorburger & al. 2008, Linder & Promislow 2009, Pigeault & al. 2016). For example, in mosquitoes maternal infection lowers disease resistance in offspring infected with malaria (Vantaux & al. 2014) thus no TGIP has been detected is not demonstrated in this host-pathogen interaction. Strain-specific TGIP against the bacterium *Bacillus thuringiensis* Berliner, 1915 was detected by Roth & al. 2009 in the red flour beetle *Tribolium castaneum* (Herbst, 1797), whereas TGIP only confers protection towards Gram-positive, but not Gram-negative bacteria in the mealworm beetle *Tenebrio molitor* (see Dubuffet & al. 2015).

In addition to pathogens, also environmental stressors (e.g., temperature, diet, dehydration, injuries) affect the physiology of an organism, and can shape life-history trade-offs (Flatt & Heyland 2011). Indeed, immunity and stress responses are linked, as immune insults activate genes involved in stress adaptation (e.g., heat-shock proteins, cytochrome P450, defensin-2, thaumatin-1, apoD) (Adamo 2008, Altincicek & al. 2008, Freitak & al. 2012, Marshall & Sinclair 2012, Triggs & Knell 2012, Johnston & Rolff 2013). Hence, also wounding, could elicit priming in a manner similar to microbes, as cuticular damage increases the risk of pathogens entering via open wounds.

Social insects, such as ants, live in dense colonies (Bourke & Franks 1995, Cornman & al. 2012), which are sedentary for years, and local pathogen outbreaks need to be suppressed or the colony dies. Given that the worker offspring remain in the natal site, and hence face the same pathogens as their mother early in life, TGIP can be expected to play a major role in immune defences. To our knowledge TGIP has not been described in ants. However, within-generational immune priming against fungal parasites *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) has been investigated, but not detected in the ant *Formica selysi* Bondroit, 1918 (Reber & Chapuisat 2012), and demonstrated in queens of *Lasius niger* (Linnaeus, 1758) (Gálvez & Chapuisat 2014).

Here, we study the effect of maternal exposure to a pathogen on resistance against a fungal pathogen, *Beauveria bassiana*, in different developmental stages of offspring in the ant *Formica fusca* Linnaeus, 1758, a common species in southern Finland. This fungus is a generalist entomopathogen, used often in plant protection (Moura-Mascarin & Jarosnki 2016). Recently we have shown that *F. fusca* workers can self-medicate when infected with this fungus (Bos & al. 2015), and here we examine whether exposing *F. fusca* queens to dead *B. bassiana* conidia can lead to higher survival against the fungus in the offspring. We also measured changes in the expression of immunity related genes in queens and larval offspring. We hypothesize, that when queens are exposed to fungal conidia they upregulate their immune responses, and also come to immune prime their offspring. We predict that primed offspring will have higher survival upon infection. If immune priming is costly, we may expect lower survival in immune primed, non-infected individuals, compared to non-primed, non-infected individuals. In addition, the adult offspring of immune primed mothers may be smaller, compared to those produced by non-primed ones. It is thought that in some cases stress in mothers can lead to smaller offspring, possibly due to the trade-off between investing into offspring quality versus stress defenses (Gluckman & al. 2005). Furthermore, if TGIP occurs, we also predict immune related genes to be upregulated in pathogen challenged queens, as well as in their offspring. Activation of the immune system is resource demanding, and hence, its deployment can result in trade-offs with other physiological functions (Zuk & Stoehr 2002, Schmid-Hempel 2005).

Materials and Methods

Animals: Five polygyne (colonies contain multiple reproductive queens) colonies of the ant *Formica fusca* were collected on the Hanko peninsula (latitude: 59.841969°, longitude: 23.201824°) in Southwestern Finland in April 2015. Species identification was confirmed based on hair counts (Czechowski & al. 2002). All colonies were kept in climate chambers at + 4 °C, in closed buckets with the

Tab. 1: The total number of individuals, and the number of individuals per experimental nest (mean ± standard deviation in brackets) used in offspring survival experiments.

	F1 larvae		F1 adults	
	Control treatment	*B. bassiana* treatment	Control treatment	*B. bassiana* treatment
Fungal injection	39 (7.80 ± 2.86)	48 (9.60 ± 5.13)	79 (19.75 ± 5.25)	75 (18.75 ± 7.10)
Sham-control	31 (7.75 ± 3.95)	26 (6.50 ± 5.20)	123 (30.75 ± 20.30)	119 (29.75 ± 18.57)
Control	59 (14.75 ± 1.50)	64 (16.00 ± 2.94)	88 (22.00 ± 9.83)	90 (22.50 ± 10.15)

original nest material, to prevent the queens from initiating egg-laying until the start of the experiment. After three weeks the colonies were transferred to the experimental temperature of + 22 °C and housed in nests made of aerated concrete blocks (Siporex, H + H Finland Oy, 23 cm × 13 cm × 6 cm; Fig. S1, as digital supplementary material to this article, at the journal's web pages). Each block was enclosed in plastic covers to prevent the ants from escaping, and contained a cavity, which served as a brood chamber. The ants were fed Bhatkar-Whitcomb diet (Bhatkar & Whitcomb 1970), and the nests were moistened daily by filling the water reservoir surrounding the nest.

Queen treatments: Three experimental nests were established per field-collected colony, each with one queen, and ca. 300 workers across all treatments. The three experimental nests were each assigned to one of three treatments: 1) Fungal injection – the experimental treatment in which queens were injected with dead fungal conidia to elicit an immune response, 2) Sham-control – a control, in which queens were injected with phosphate buffered saline (1 × PBS) solution, and 3) Control, in which queens were left untreated (Fig. S2). In the fungal injection treatment, we used 2 µl autoclaved (heat killed) *B. bassiana* (strain KVL 03 - 90) spore solution (1×10^7 conidia / ml in 1XPBS). In order to ensure, that all the queens received the same concentration of conidia, the solution was vortexed before each injection. In the sham-control, we used 2 µl sterile 1XPBS. The injections were made on the dorsal side of the abdomen, between the third and fourth tergite, using a glass micro-capillary, two days after establishing the experimental nests. All the queens were briefly (ca. 5 min) placed in +4 °C before injection. After injection, the queens were returned to their respective nests, and allowed to oviposit in the presence of the workers. The untreated queens remained in their nests. Eggs laid during the first day were removed, in order to ensure that all the eggs in the nest were laid after queen treatment had taken effect. Ant queens have polytrophic ovaries, with continuous egg maturation and egg laying during the reproductive period (Chapman 1998, Khila & Abouheif 2010). This allows constant maternal modification of the offspring phenotype via environmental stimuli encountered by the queen. The 24 h window following the treatment excludes the eggs that matured prior to our treatments and were not part of the experiment.

Beauveria bassiana is the second most common generalist entomopathogenic fungus in several Finnish soil types (Husberg & al. 1988, Vänninen 1996), and commonly found in ant colonies (Reber & Chapuisat 2011). A Danish strain was used to mitigate possible effects of local adaptation between the host and the pathogen. This fungus strain we used is detrimental in our study species (Bos & al. 2015). We grew the fungus on PDA (potato Dextrose Agar) plates at + 23 °C for two weeks, and harvested conidia by using 1 × PBS, with 0.05% of TritonX-100. The conidia were counted with a Fuchs-Rosenthal Chamber (Hausser Scientific) under 400 × magnification. In order to ascertain that living conidia were absent from the injected aliquot, the spore solution was autoclaved, and an aliquot of 150 µl was plated out on PDA plates to check for absence of germination. Only 2% of the conidia germinated.

Tests of maternal priming – offspring survival: We tested the effect of maternal priming on the survival against infection with *Beauveria bassiana* both in offspring larvae, and adult offspring workers. We collected all larvae aged four to seven days, size category medium (Schultner & al. 2014), from each experimental nest (15 nests), and left the remaining larvae in the colonies to develop into adult workers. Larvae from each experimental nest were divided into two groups, each placed in a round pot (Ø 7 cm, H 5 cm), lined with plaster bottom, together with 40 workers from the original field colony. One of the pots was assigned as a control, whereas the larvae in the second pot were infected with *B. bassiana* as described below. The number of larvae obtained varied across queen treatments, hence sample sizes per colony and treatment differ (Tab. 1). Survival was monitored on a daily basis, and dead individuals removed from the pots. Larval mortality was determined by the loss of turgor and coloration in dead individuals.

All the old workers tending the emerging new workers were painted with enamel paint, which allowed us to distinguish between old and newly emerged adults. The newly emerged adults were left to mature for 3 - 7 days in the presence of old workers. All workers had reached their maximum coloration within 48 h, after which they were exposed to the same treatments as the larvae, except that no old workers were added to the pots (Tab. 1). Survival was monitored on a daily basis and dead individuals removed from the pots. We kept both the larvae, and the young workers in groups according to treatment, rather than singly. This allows testing for priming under more realistic conditions, and avoids excess mortality due to causes other than the treatments (e.g., starvation). Once

the tests on offspring workers started, the queens were stored in RNA-Later (Isol-RNA Lysis Reagent, 5 PRIME) for gene expression analyses.

Each control larva or worker was dipped for 5 sec in double distilled water (ddH_2O) with 0.05% Triton X, whereas the individuals assigned to the fungal infection treatment were dipped in ddH_2O with 0.05% Triton X containing *Beauveria bassiana* at 1×10^7 viable conidia / ml. The viability of the conidia was confirmed by plating 150 µl of the spore solution on PDA plates, and incubated at 23 °C for 24 h, after which each plate was observed at 400 × magnification to monitor the germination of the conidia. Larval, and worker mortality were recorded for 12 and nine days, respectively, and dead individuals were removed daily. Dead adult individuals were discarded, as several experiments and pilot studies have implicated fungal infection as the cause of death in 100% of the cases. During the survival experiment, the ants were fed every day ad libitum with Bhatkar-Whitcomb diet, and water was supplied in an Eppendorf tube with a cotton plug to maintain constant humidity in the pots.

The effect of priming on queen fitness was measured by counting the total number offspring (larvae used for the first experiment + all eclosed F1 workers), produced during the 26 days the experiment lasted. This time frame corresponds to the first period of active egg-laying after hibernation, and is normally followed by a phase with low oviposition rates until resumed later in the season (personal observations). Counting egg-laying rates on a daily basis was not possible, as this would have caused too much disturbance to the experimental nests, with detrimental effects on productivity. By including mortality during brood development, our measure of queen fitness also includes potential costs of immune priming. To investigate whether queen treatment affected offspring size, the head width of the F1 adult workers was measured. Head width is commonly used as a proxy for the body size in ants and gives a better estimate than dry weight, as the latter is affected by feeding status. For this, all adult individuals were fixed in ethanol (96%) after the survival experiment. The heads were placed on millimeter paper and photographed (Nikon D3200, lens Sigma MACRO 105 mm F2.8 EX DG OS HSM, exposure 1 / 200, aperture F9, ISO400). The maximum width of the head was measured with the software package ImageJ 1.49 (Fig. S3) (Rasband 2012).

Tests of maternal priming – gene expression: In order to study gene expression and explain possible differences in the survival of larvae infected with fungus, we collected 5 larvae from each nest and treatment for gene expression at the time we set up the larval infection experiment. The queens from each experimental nest and treatment were fixed for gene expression analysis at the end of the rearing phase (26 days after the start of the experiment). It was not possible to collect them earlier as they were needed for brood production. Each individual was separately fixed in 300 µl of Isol-RNA Lysis Reagent (5 PRIME), cut into pieces, and stored at -80 °C until further analysis. We investigated the expression of nine immune genes: beta-1,3-glucan-binding protein (B1.3G), prophenoloxidase (PPO), P450, Serine / Threonine Kinase (STKin), Transferrin (Tfer), and Tyrosine-protein kinase (Tyro), Dual oxidase (DUOX), Lysozyme (Lys), and Vitellogenin 1 (Vg1). Lysozyme, Prophenoloxidase and Beta-1,3-glucan-binding protein are effector molecules interacting with pathogens (Gillespie & al. 1997, Söderhäll 1998, Yu & al. 2002, Jiang & al. 2004, Lemaitre & Hoffmann 2007, Cerenius & al. 2008, Bulmer & al. 2009). Dual oxidase generates hydrogen peroxide, and thus functions in the innate immune system as an antimicrobial defence (Ha & al. 2005). Protein kinases, such as the Serine / Threonine Kinase, are known to be involved in signal transduction in insect immunity (Chiou & al. 1998, Cerenius & al. 2008). Transferrin, P450, and Vitellogenin 1 were selected as they carry general immune-related functions (Yoshiga & al. 1997, 1999, Thompson & al. 2003, Altincicek & al. 2008, Salmela & al. 2015). RPS 9, a subpart of ribosomes functioning in protein synthesis, was added as an endogenous control. It is expressed constantly, and present in stoichiometric amounts in ribosomes (Wool 1996, Stelzl & al. 2015). The stability of the RPS9 Ct-values were evaluated using the standard deviation of their Ct-values as suggested in Silver & al. (Silver & al. 2006). It was selected because it showed the least amount of variation in expression across different developmental stages and treatments.

The oligonucleotide primers for the real-time qPCR were designed based on the *Formica fusca* transcriptome data (Morandin & al. 2016), using the online Primer-BLAST internet-based interface (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Untergasser & al. 2012). The primers were designed using the rules of highest maximum efficiency and sensitivity, in order to avoid the formation of self- and heterodimers, self-complementary, and hairpin structures. RNA was extracted using the phenol-chloroform method in accordance with the manufacturer's protocol. In short, the larval and queen samples were thawed on ice and homogenized (two 5 mm Ø stainless steel beads were added to each tube) using Tissuelyser (TissueLyser II Qiagen) at full speed for 30 seconds. After this 700 µl Isol-RNA Lysis Reagent (5 PRIME), and 150 µl chloroform (Sigma) were added, and the solution vortexed. The tubes were incubated for 5 minutes at room temperature (ca. 22 °C), 2 minutes on ice, and centrifuged for 15 minutes at 13,500 rpm at 4 °C for phase separation. The upper transparent phase was transferred into a new tube containing 500 µl isopropanol (Sigma). The samples were mixed well, and stored overnight at -20 °C to precipitate the RNA. The next day the samples were centrifuged at 13,500 rpm for 30 minutes at 4 °C. The isopropanol was removed, and the pellet was washed twice with 500 µl of 80% ethanol by centrifuging 10 minutes at 4 °C. After washing the pellet, it was let to air-dry, and dissolved in 40 µl distilled water. After that, the samples were vortexed and spun down. The extracted RNA was stored at -80 °C. The RNA quantity was determined photospectrometrically with a NanoDrop 2000 Spectrophotometer (ThermoSci-

entific). To remove any remains of genomic DNA that may interfere with amplification, all samples were treated with DNAse I (ThermoScientific), according to the manufacturer's protocol.

For cDNA synthesis and quantitative real-time PCR, 500 ng of cDNA-free RNA was converted into single-stranded DNA using iScript cDNA synthesis kit (BioRad) following the manufacturer's protocol. The PCR products were stored for gene expression analysis at -80 °C. Real time quantitative qPCR was performed on 384-well plates on a CFX384 Touch™ Real Time PCR Detection System (Biorad) using iQ™ SYBR® Green Supermix (Biorad). All the assays were run with two technical replicates using the following cycle conditions: 1. three minutes 95 °C, 2. 15 seconds 95 °C, 3. 45 seconds 58 °C, 4. go to step two for 39 times.

Data analysis: All analyses were conducted in R 3.2.0. (R-Team 2013). To test if changes in offspring survival upon infection by *Beauveria bassiana* depends on maternal treatment, we analyzed both F1 larval, and adult worker survival in separate survival regressions with a Weibull distribution, using the survival (Therneau 2012), and multcomp (Hothorn & al. 2008) packages. In both survreg function models we used the day each individual died as a response variable, queen treatment (control, fungal injection, sham-control), and offspring treatment (double distilled water treatment, *B. bassiana* infection) as factors, including the interaction between the two. The data were censored to account for the fact that some individuals did not die during the experimental period. To account for within-colony dependencies we added colony as a frailty term, with a gamma distribution for each survival regression model (Therneau & al. 2003). As not all post-hoc comparisons are relevant (e.g., primed, exposed larvae vs. non-primed, non-exposed larvae), we used planned post-hoc comparisons with the aim to elucidate the specific effects that are indicative of immune priming. Thus, the fungal-injection and sham-control queen treatments were compared to the control queen treatment, and the offspring (both larval and adults) *B. bassiana* infection treatment to the double distilled water treatment. The comparisons were adjusted for false discovery rate (Benjamini & Hochberg 1995) to test for pairwise differences in survival among offspring, depending on queen treatment.

To test if the number of offspring produced in the experimental nests, and the size of adult offspring was affected by the maternal treatments, we analyzed both response variables separately in a mixed effects model, using the lmer function from the lme4 package (Bates & al. 2014). We included queen treatment as a fixed factor, and colony as a random effect. The full model was compared to a reduced model, without treatment as a factor (Field & al. 2012). Finally, to test whether queen treatment influences gene expression in queens and larvae, we first tested for collinearity among the genes with Bartlett's test (Field & al. 2012), and then analyzed each gene separately with linear models (lmer function from lme4 and lmerTest packages). The dCt values of each gene were used as dependent variables, treatment as a factor, colony as a random effect, and planned pairwise comparisons adjusted for false discovery rates (Benjamini & Hochberg 1995).

Results

Test of maternal priming: offspring survival: Brood production did not differ between queen treatments, nor did queen treatments affect offspring body size (Tab. 2). Overall, exposure to *Beauveria bassiana* significantly increased offspring mortality at both the larval, and the adult stages (Fig. 1A and B solid vs. dashed black lines, Tab. 3).

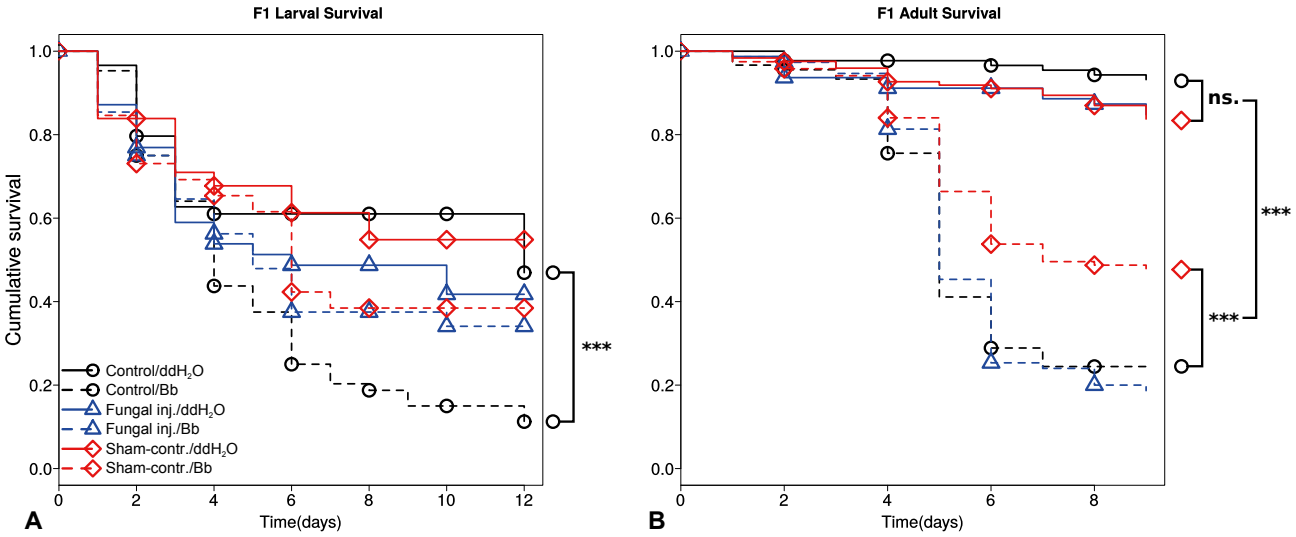

Fig. 1: Brood survival. The effect of queen treatment on F1 larval (A), and F1 adult worker (B) survival upon exposure to the fungus *Beauveria bassiana* (Bb). The queen treatments were: control queen treatment (Control, black / circles), fungal injection (Fungal Inj., blue / triangles), and sham-control (Sham-contr, red / diamonds). Solid lines indicate control treatments with double distilled water (ddH_2O), and dashed lines exposure to *B. bassiana* (Bb). The bars and stars show significant ($p < 0.001$) differences in the survival between treatments.

Tab. 2: The total number of offspring produced, and the size of F1 adults in each queen treatment. The average number of offspring produced and the mean F1 adults size ± standard deviation are given for each queen treatment, based on the results of the mixed effect model. df = degrees of freedom.

Queen treatment	Immune elicited	Sham-cont.	Control	X^2	df	p-value
Number of brood	98.75 ± 22.38	133.00 ± 73.28	147.50 ± 60.52	1.828	3,12	0.176
F1 adult size (mm)	0.832 ± 0.064	0.846 ± 0.066	0.828 ± 0.065	2.802	2,413	0.246

Tab. 3: Results from survival regression models for F1 larvae and F1 adults during the bioassay. Parameter estimates are given as β ± standard error (SE). The model compares each factor to the factor control treatment, which in the queen treatments is the treatment in which queens were left untreated, and in the brood treatments the double distilled water treatment.

	F1 larvae			F1 adults		
	β ± SE	z-value	p-value	β ± SE	z-value	p-value
Intercept	2.536 ± 0.211	12.154	< 0.001	3.445 ± 0.208	16.594	< 0.001
Queen Fungal inj.	-0.300 ± 0.249	-1.203	0.229	-0.407 ± 0.238	-1.713	0.087
Queen Sham-cont.	-0.134 ± 0.285	-0.468	0.640	-0.447 ± 0.221	-2.015	0.044
Brood fungus infection	-0.870 ± 0.205	-4.237	< 0.001	-1.497 ± 0.214	-6.983	< 0.001
Queen Fungal inj.: Brood fungus infection	0.739 ± 0.313	-2.363	0.002	0.372 ± 0.252	1.474	0.140
Queen Sham-cont.: Brood fungus infection	0.456 ± 0.370	-1.231	0.218	0.778 ± 0.239	3.261	0.003

Tab. 4: Results from the survival post-hoc contrasts for F1 larvae and F1 adults. Bb = fungus infection, ddH_2O = control double distilled water treatment. Control (Bb) vs. Fungus inj. (Bb) refers to fungus-infected brood from control queens, compared with (vs.) fungus-infected brood from fungal injected queens. Parameter estimates are given as β ± standard error (SE).

	F1 larvae			F1 adults		
Queen (Offspring) treatment	β ± SE	z-value	p-value	β ± SE	z-value	p-value
Control (ddH_2O) vs. Control (Bb)	0.869 ± 0.205	4.237	< 0.001	1.497 ± 0.214	6.984	< 0.001
Sham-cont. (ddH_2O) vs. Sham-cont. (Bb)	0.414 ± 0.309	1.337	0.412	0.719 ± 0.127	5.669	< 0.001
Fungal inj. (ddH_2O) vs. Fungal inj. (Bb)	0.131 ± 0.239	0.550	0.657	1.125 ± 0.158	7.137	< 0.001
Control (ddH_2O) vs. Sham-cont. (ddH_2O)	0.137 ± 2.855	0.468	0.657	0.447 ± 0.222	2.015	0.066
Control (Bb) vs. Sham-cont. (Bb)	-0.322 ± 0.247	-1.307	0.412	-0.331 ± 0.084	-3.911	< 0.001
Control (ddH2O) vs. Fungal inj. (ddH_2O)	0.299 ± 0.249	1.203	0.411	0.409 ± 0.238	1.714	0.111
Control (Bb) vs. Fungal inj. (Bb)	-0.439 ± 0.192	-2.281	0.101	0.037 ± 0.008	0.438	0.743
Sham-cont. (ddH_2O) vs. Fungal inj. (ddH_2O)	0.166 ± 0.293	0.568	0.656	-0.038 ± 0.173	-0.221	0.824
Sham-cont. (Bb) vs. Fungal inj. (Bb)	-0.116 ± 0.262	-0.444	0.657	0.367 ± 0.088	4.161	< 0.001

In larvae, we also found a significant interaction between queen treatment and brood treatment (Tab. 3). This was mainly due to the strong effect of fungal exposure in the offspring of control queens (Fig. 1A, solid vs. dashed lines, circles, Tab. 4). No significant effects of fungal exposure were found in the fungal injection or the sham-control groups (Fig. 1A, solid vs. dashed blue / triangles, and red lines / diamonds, respectively, Tab. 4). The absence of a significantly increased mortality is consistent with a TGIP effect in larvae, originating from maternal treatments, but not conclusive evidence, given that the survival of larvae produced by sham-injected or treated queens was not significantly higher than in the larvae produced by control queens.

In the adult F1 offspring, the responses were different. We found a significant increase in survival in the sham-control treatment, compared to the control, and a significant interaction between the sham-control and exposure to *B. bassiana* (Tab. 3). There were no significant differences in survival among the groups when left unchallenged (ddH_2O treatment), and all *B. bassiana* challenged groups showed significantly lower survival than their corresponding controls (Fig. 1B, solid vs. dashed lines, all symbols, Tab. 4). However, the offspring produced by

Tab. 5: The mean minus delta Ct-value ± standard deviation of each studied gene in queens and larvae in different queen treatments.

	Queens			Larvae		
	Treatment	**Sham-cont.**	**Control**	**Treatment**	**Sham-cont.**	**Control**
PPO	-8.651 ± 0.286	-9.517 ± 0.505	-9.484 ± 0.626	-7.810 ± 0.644	-7.934 ± 0.647	-7.323 ± 0.796
B1.3G	-6.784 ± 0.378	-6.344 ± 0.491	-6.776 ± 0.478	-6.147 ± 0.743	-6.341 ± 0.795	-5.879 ± 0.660
P450	-8.581 ± 0.084	-8.799 ± 0 682	-8.770 ± 0.372	-7.788 ± 1.253	-8.233 ± 0.913	-7.464 ± 1.048
Tfer	-6.489 ± 0.327	-6.408 ± 0.202	-6.506 ± 0.398	-5.370 ± 0.820	-5.585 ± 0.707	-5.248 ± 0.539
Tyro	-7.978 ± 0.502	-7.777 ± 0.364	-7.276 ± 0.726	-8.977 ± 0.551	-9.324 ± 0.424	-9.077 ± 0.408
STKin	-6.017 ± 0.168	-6.026 ± 0.496	-5.755 ± 0.218	-6.787 ± 0.473	-7.123 ± 0.468	-6.928 ± 0.476
LYS	-7.067 ± 0.470	-7.541 ± 0.427	-7.434 ± 0.302	-3.330 ± 1.209	-5.019 ± 0.737	-3.753 ± 1.426
Vg1	1.568 ± 0.484	1.594 ± 0.799	2.116 ± 0.494	-9.844 ± 1.999	-11.682 ± 1.622	-10.272 ± 1.458
DUOX	-9.161 ± 0.275	-9.038 ± 0.512	-9.039 ± 0.236	-10.052 ± 0.869	-10.106 ± 0.880	-9.451 ± 1.099

sham-control queens survived infection by *B. bassiana* significantly better than those produced by control, or fungal-injection queens (Fig. 1B, dashed red lines / diamonds vs. dashed black lines / circles and dashed blue lines / triangles, Tab. 4). This suggests a potential TGIP effect in sham-control queens, whereas no such effect was found in the adult offspring produced by fungal-injection queens – these survived no better than those produced by control queens, when exposed to *B. bassiana* (Fig. 1B, dashed blue lines / triangles vs. dashed black / circles, Tab. 4).

Test of maternal priming – gene expression: We found no significant collinearity in gene expression of queens (Bartlett test: $X^2 = 42.179$, $p = 0.221$), so we bypassed the principal coordinate analysis (PCA), and instead used linear models to assess expression in each gene separately. Following exposure to *Beauveria bassiana* two of the investigated genes, PPO, and Lysozyme, showed significant upregulation in fungal injection queens, compared to the sham-control queens, and one, PPO, compared to the control queens (Fig. 2, Tabs. 5 & 6). All comparisons were corrected for false discovery rate. None of the other genes, nor the house-keeping gene RPS9, showed any significant differences between the three treatments (Fig. 2, Tabs. 5 & 6).

In larvae we found significant collinearity in the expression patterns across genes (Bartlett test $X^2 = 318.515$, $p < 0.001$), but to retain comparability to the queen data, we used the same linear model approach also for larvae. All comparisons were corrected for false discovery rate. In larvae, two of the genes (Tyrosine-protein Kinase and Serine / Threonine Kinase) were upregulated in the offspring of fungus-injected queens, compared to those of sham-control queens, but not compared to control queens (Fig. 3A, B, Tabs. 5 & 6). By contrast, one of the investigated genes (PPO) was significantly downregulated in the larval offspring of fungus-injected queens, compared to both control, and sham-control queens (Fig. 3D, Tabs. 5 & 6). In two genes (B1.3G & P450) expression was down-regulated in the larval offspring of sham-control queens, but not in fungus-injected ones. Finally, both Vg1 and Lysozyme were downregulated in the larval offspring of sham-control queens, whereas expression in the offspring of fungus-injected queens was on par with the control. The genes DUOX and Transferrin were the only ones not to indicate significant differences in expression patterns (Fig. 3C, G, Tabs. 5 & 6).

Discussion

Here we show that exposing ant queens to immunity-related stress prior to egg laying can influence offspring responses to pathogens both at the larval and adult stage. Larvae produced by immune-primed queens survived infection with *Beauveria bassiana* no worse than unexposed ones, whereas those produced by untreated queens had the highest mortality. Once the larvae became adults, offspring from sham-control mothers showed a protective effect of queen treatment, consistent with transgenerational immune priming. Thus, the effects of queen priming appear to manifest themselves slightly differently in larval and adult offspring. We found no evidence for costs of reproduction following maternal challenges, as neither the total number of brood, nor the number and size of adult offspring produced, differed significantly between the treatments. We note, however, that the sample size is limited, which may have prevented the detection of such effects. The pattern of immunity-related gene expression indicated a clear activation of defense molecules, such as PPO and Lysozyme in challenged mothers. However, this effect was not transmitted to the larval offspring in full, as only Lysozyme showed up regulation.

The survival rate of larvae produced by either fungus-injected or sham-injected queens and exposed to *Beauveria bassiana* differed neither from the unexposed larvae produced by immune primed queens, nor from exposed larvae produced by unprimed queens, but was intermediate. This stands in contrast to the main prediction of TGIP, which holds that the survival rate of offspring from primed mothers should be higher than that of non-primed

Tab. 6: Results from the false discovery rate post hoc analysis on differences in gene expression between maternal treatments, both in queens and larvae. The average difference between treatment groups (fungal injected vs. control treated queens / sham-control vs. control queens / sham-control vs. fungal injected queens) in gene expression is given as parameter estimates ± standard error (β ± SE).

	Queens			F1 Larvae		
	β ± SE	z-value	p-value	β ± SE	z-value	p-value
Beta-1,3-glucan-binding protein (B1.3G)						
Fungal inj.vs. Control	0.081 ± 0.250	0.324	0.746	0.259 ± 0.206	1.259	0.312
Sham-cont. vs. Control	-0.359 ± 0.250	-1.438	0.226	0.458 ± 0.208	2.201	0.083
Sham-cont. vs. Fungal inj.	-0.440 ± 0.232	-1.896	0.174	0.198 ± 0.204	0.973	0.330
Prophenoloxidase (PPO)						
Fungal inj.vs. Control	-0.833 ± 0.321	-2.592	0.014	0.486 ± 0.199	2.445	0.022
Sham-cont. vs. Control	0.033 ± 0.321	0.104	0.918	0.604 ± 0.199	3.039	0.007
Sham-cont. vs. Fungal inj.	0.866 ± 0.303	2.859	0.013	0.118 ± 0.197	0.601	0.548
P450						
Fungal inj.vs. Control	-0.189 ± 0.307	0.615	0.808	0.311 ± 0.290	1.073	0.283
Sham-cont. vs. Control	0.029 ± 0.307	0.094	0.925	0.762 ± 0.292	2.607	0.027
Sham-cont. vs. Fungal inj.	0.218 ± 0.290	0.753	0.808	0.451 ± 0.286	1.575	0.173
Transferrin (Tfer)						
Fungal inj.vs. Control	-0.042 ± 0.121	-0.348	0.728	0.097 ± 0.187	0.519	0.604
Sham-cont. vs. Control	-0.123 ± 0.121	-1.019	0.701	0.324 ± 0.189	1.719	0.257
Sham-cont. vs. Fungal inj.	-0.081 ± 0.111	-0.727	0.701	0.227 ± 0.185	1.230	0.328
Tyrosine-protein kinase (Tyro)						
Fungal inj.vs. Control	0.680 ± 0.337	2.020	0.130	-0.107 ± 0.126	-0.848	0.396
Sham-cont. vs. Control	0.479 ± 0.337	1.423	0.232	0.243 ± 0.127	1.917	0.083
Sham-cont. vs. Fungal inj.	-0.201 ± 0.316	-0.637	0.524	0.350 ± 0.123	2.849	0.013
Serine/Threonine Kinase (STKin)						
Fungal inj.vs. Control	0.273 ± 0.212	1.290	0.296	-0.151 ± 0.120	-1.259	0.208
Sham-cont. vs. Control	0.282 ± 0.212	1.332	0.296	0.191 ± 0.121	1.581	0.171
Sham-cont. vs. Fungal inj.	0.009 ± 0.199	0.045	0.964	0.342 ± 0.118	2.890	0.012
Dual oxidase (DUOX)						
Fungal inj.vs. Control	0.122 ± 0.238	0.514	0.911	0.542 ± 0.303	1.791	0.110
Sham-cont. vs. Control	-0.001 ± 0.251	-0.005	0.996	0.622 ± 0.305	2.042	0.110
Sham-cont. vs. Fungal inj.	-0.124 ± 0.238	-0.519	0.911	0.080 ± 0.305	0.261	0.794
Lysozyme (LYS)						
Fungal inj.vs. Control	-0.286 ± 0.160	-1.791	0.110	-0.404 ± 0.362	-1.113	0.266
Sham-cont. vs. Control	0.108 ± 0.162	0.663	0.508	1.170 ± 0.365	3.201	0.002
Sham-cont. vs. Fungal inj.	0.394 ± 0.160	2.464	0.041	1.573 ± 0.362	4.345	<0.001
Vitellogenin 1 (Vg1)						
Fungal inj.vs. Control	0.549 ± 0.397	1.382	0.317	-0.437 ± 0.581	-0.752	0.452
Sham-cont. vs. Control	0.523 ± 0.418	1.250	0.317	1.226 ± 0.575	2.134	0.049
Sham-cont. vs. Fungal inj.	-0.027 ± 0.397	-0.067	0.947	1.663 ± 0.552	3.015	0.008

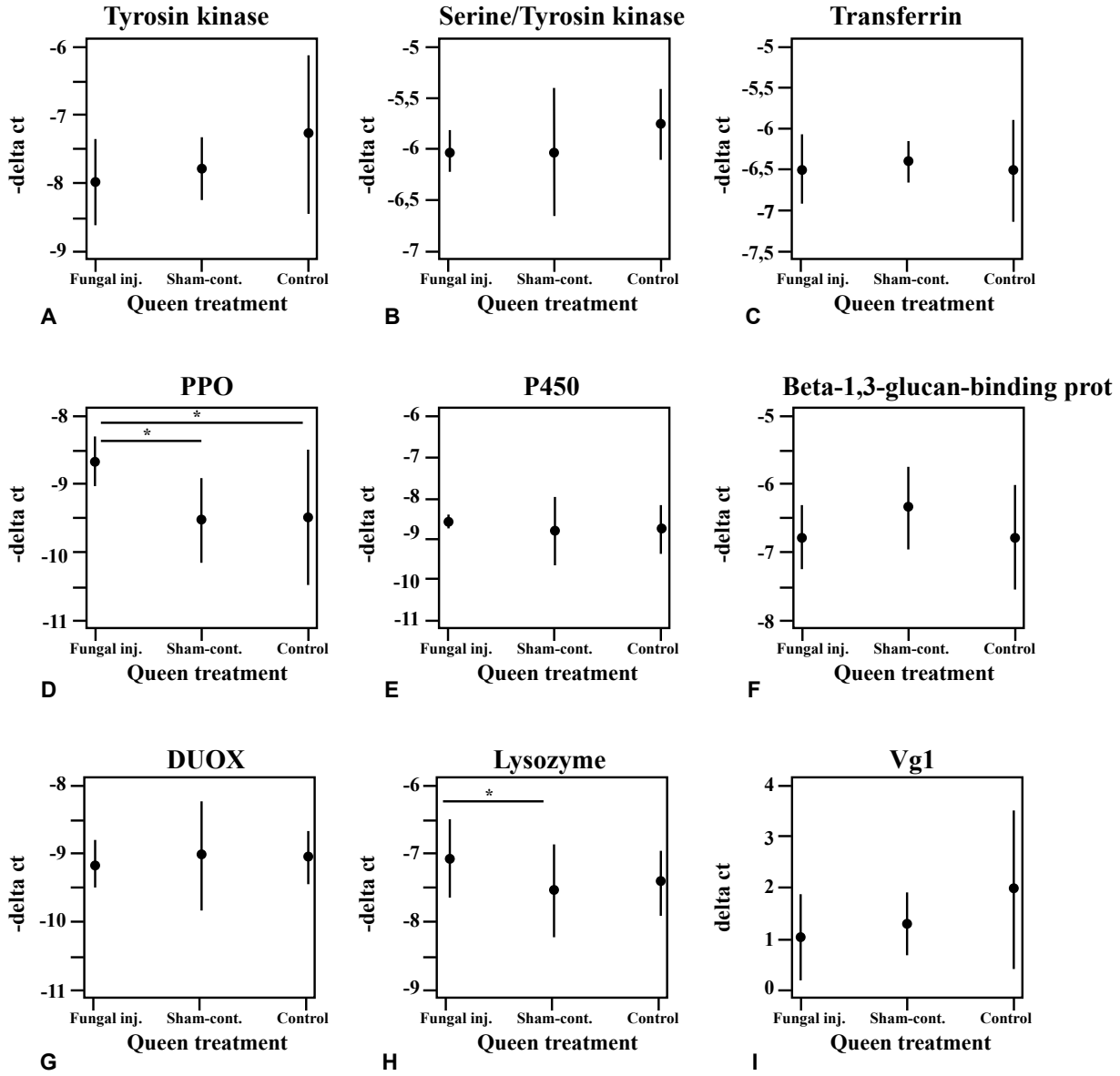

Fig. 2: Gene expression of the nine immune related genes in queens. The dots indicate the mean gene expression level shown as inverted Ct values (higher values indicate higher expression levels). Error bars show 95% -confidence intervals. The queen treatment abbreviations refer to Fungal inj. = fungus injected queens, Sham-cont. = sham-control queens, Control = control queens. The bars and stars show significant difference between the treatments (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

mothers (Hernández-López & al. 2014). However, the results also show that fungal infection did not decrease the survival rate of larvae produced by primed queens, but did so for unexposed larvae. One explanation may be that the priming effect is only partial, and may thus confer some fitness benefits. This may incur a selective advantage for priming even though the effect is not maximal. We also note, that the number of colonies included in the analysis is low, which limits the power of the analysis, especially if some of the queens have recently been exposed to pathogens in the wild. Our results, although inconclusive at present, show a good correlation with those found in the honeybee (*Apis mellifera*), in which offspring of queens injected with dead spores of bacterium *Paenibacillus larvae* showed higher resistance against the infection (Hernández-López & al. 2014).

Both queen treatments (sham and fungal injection) caused similar effects in larvae, seen as intermediate survival when challenged with *Beauveria bassiana*. This suggests, that injury itself may trigger up regulation of immune defenses in ants, like it has been demonstrated in the case of *Camponotus floridanus* (Buckley, 1866) (Ratzka & al. 2011). This is especially relevant in this case, given that fungal infections normally occur via ingrowth of hyphae, not conidia, as was the case during the priming phase. Injection results in an open wound in the cuticle, and can be considered equivalent to an injury under natural conditions. An opening in the cuticle, even if temporary,

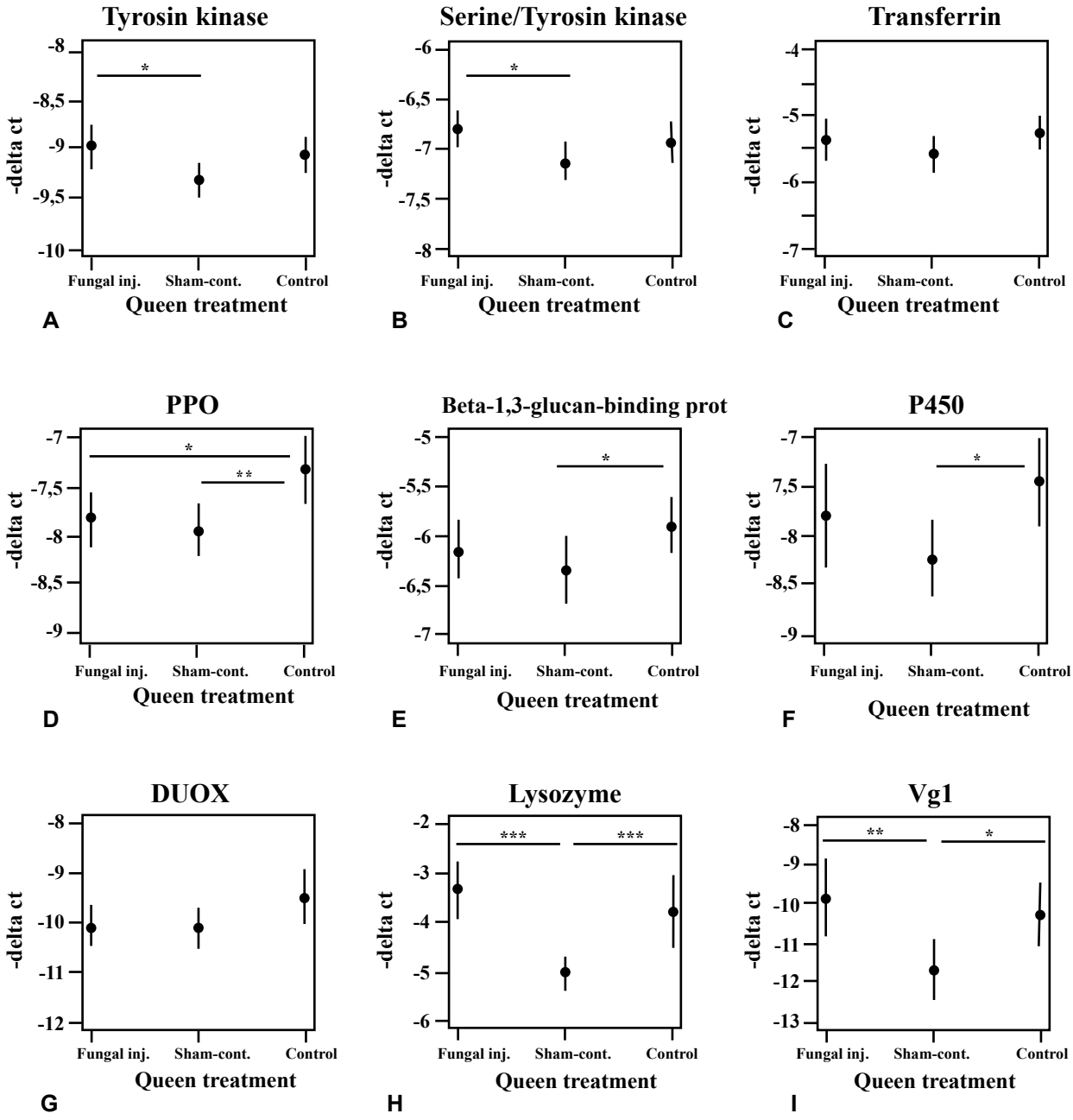

Fig. 3: Gene expression of the nine immune related genes in F1 larvae. The dots indicate the mean gene expression level shown as inverted Ct values (higher values indicate higher expression levels). Error bars show 95% -confidence intervals. The queen treatment abbreviations refer to Fungal inj. = fungus injected queens, Sham-cont. = sham-control queens, Control = control queens. The bars and stars show significant difference between the treatments (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

could create opportunities for pathogens in the environment to penetrate and infect the host. Indeed, even a sterile wounding can lead to up regulation of immunity in insects (Krautz & al. 2014), caused by the danger-signaling pathways, shared for responses dealing with wound healing and immunity. Wounding alone may be a strong enough stressor for ant queens to cause enhanced immunity in their offspring, given that the sheltered environment of the colony would under normal circumstances keep the queens well-guarded, and protected from mechanical damage.

Interestingly, we see differences in immunity also in the adult stage, but only in the offspring of sham-control queens, not in the offspring of fungus-injected queens. The benefit of priming was substantial, with a 24% increase in survival among the infected adult offspring of sham-control queens, compared to infected workers from control queens. Nonetheless, also the adult offspring of sham-control queens survived significantly less well than the unexposed adult offspring. The absence of a similar effect in the offspring of fungus-injected queens remains at this

point unexplained, but may be associated with the method of priming used in our study. Indeed, queens may have mounted a response simply following wounding, which would be a more general immune response, rather than a specific response tailored for a specific pathogen, such as BB. This may also explain the surprisingly long reach of the effect, and requires further investigation.

Both types of stressors can lead to immune priming, but the mechanisms that underpin the observed survival success may vary between the treatments, and become visible only at the adult stage. If injecting a large dose of dead fungal conidia leads to more specific transgenerational immune priming against fungal infections in the short term, then general wounding might up-regulate a more generic and longer lasting survival, in the case of infection. For example, if fungal priming leads to up regulation of costlier resistance mechanisms, such as the synthesis of high amounts of specific antimicrobial peptides (Lemaitre & Hoffmann 2007), or changes in the thickness and structure of the cuticula (Evison & al. 2016), at the larval stage, it may deplete the resources available for immune defense at the adult stage. In the case of injury alone (sham-control), the immune responses that confer priming may be more general, and less intense. If this leads to a lower absolute investment (e.g., production of AMPs, cuticular structure), then the priming effect could perhaps be sustained for longer, throughout the larval stage into the adult stage. Alternatively, it is possible, that maternal treatment leads to the transfer of specific antimicrobial peptides, which get depleted during the larval stage, and are therefore absent in the adult workers. However, this would not explain, why the priming effect from injury alone, would carry on beyond the larval stage.

Immune responses are costly (Schmid-Hempel 2011), and traded off against growth and development, yet, we found no significant differences in the numbers, or body size of adult workers produced in the different queen treatment groups. Although the raw data suggest, that control queens have more offspring, our modest sample size of queens (5 per treatment), and large variation in reproductive output of single queens would only allow us to statistically detect large effects, which is not the case here. However, given that individuals from fungus injected mothers did not show survival benefits against *Beauveria bassiana* at the adult worker stage, we hypothesize that there may be a trade-off between adult size, and immune defense. Thus, in order to achieve an optimal body size, immune primed workers may compromise their immunity, or rely on other defense mechanisms, such as cuticular integrity (Evison & al. 2016), or behavioral responses (Tragust & al. 2013). Thus, the costs of immune priming may not be reflected in the number and size of adult workers. However, the costs could have influenced the number of eggs laid and larvae hatched, but we were not able to measure these reliably.

Of the nine genes studied here, we found elevated expression of Prophenoloxidase in fungus-injected queens, compared to the other queen treatments. In Lysozyme, we found elevated expression levels in fungus-injected queens, compared to sham-control ones. This confirms that the immune system of the queens recognizes the injected fungal conidia as foreign particles, as both Prophenoloxidase and Lysozyme are effector molecules that interact with intruding pathogens (Söderhäll 1998, Lemaitre & Hoffmann 2007). One might expect, that the genes found to be up regulated in fungus-injected queens would also be up regulated in their offspring, as both are exposed to the same stressor. However, the only gene that showed consistent up regulation in both fungus injected queens, and their larvae, was Lysozyme. Prophenoloxidase, which was up regulated in fungus-injected queens, was down regulated in their larvae, compared to larvae from control queens. Several genes show similar expression patterns in queens across treatments, yet different expression patterns emerged across treatments in the offspring larvae. The different expression patterns seen in larvae suggest, that the two stressors experienced by egg-laying queens may activate different defense mechanisms in larvae, tailored to the type of immune elicitation their mothers received. This echoes the results found in the moth *Trichoplusia ni* (Hübner, 1803), that transgenerational immune priming is a complex process, in which gene expression may converge or diverge between parents and offspring (Freitak & al. 2009). Both in our study on *Formica fusca*, and that on *T. ni* (see Freitak & al. 2009) larval gene expression was measured before infection, but gene expression patterns may change following immune challenge. For example, in the moth *Manduca sexta* (Linnaeus, 1763) parental immune challenge did not affect the expression of immune genes of non-parasitized eggs, but when the eggs were parasitized, expression levels increased above that of parasitized eggs from challenged parents (Trauer-Kizilelma & Hilker 2015). In addition, detection of changes in gene expression is contingent on the candidate genes selected for the study. Studies based on the analysis of transcripts from RNA sequencing will give a more comprehensive picture of gene expression patterns, when built on the foundation of the immune priming assays, which we have laid out in this study.

In conclusion, we show for the first time, that queens of the ant *Formica fusca* are able to alleviate the mortality of their offspring following an immunological challenge. The underlying mechanisms to achieve this appear to be stressor-specific, and lead to differences in the immune responses in different life stages of the offspring. The results highlight the need to use a greater diversity of models in studies on insect immunity, as it is clear, that increases in host fitness can be mediated by different strategies in host-parasite interactions.

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