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Caste-specific expression of constitutive and *Beauveria bassiana* induced immunity in the ant *Formica exsecta* (Hymenoptera: Formicidae)

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Abstract

One of the distinctive features of eusocial insects is the production of separate castes, each of which specializes in different aspects of colony performance. As a consequence, queens, males, and workers follow different evolutionary trajectories, depending on their life histories and tasks within the colony. The short-lived males and long-lived queens only leave the colony for reproduction, whereas workers experience frequent contact with the surrounding environment. Yet, workers often perform tasks sequentially during their life. Younger workers tend brood, whereas older workers forage for food. Here, we examined the expression of nine candidate genes both at the constitutive level and following challenge with the entomopathogenic fungus *Beauveria bassiana* (VUILLEMIN, 1912) in males, young queens, nurses, and foragers of the ant *Formica exsecta* NYLANDER, 1846. We found no difference in the survival following an infection between queens and males or between nurses and foragers. However, we found clear caste- and worker class-specific differences in the response to *B. bassiana* in genes associated with immune functions. In queens and nurses, the expression of antifungal genes increased, which in the queens was coupled to reduced expression of genes not directly involved in immune responses. In contrast, in males and foragers, gene expression did not increase upon infection; instead the expression of antibacterial genes declined.

Key words: Gene expression, host-parasite interaction, infection, tolerance, life history, *Formica exsecta*.

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Introduction

One of the defining features of eusocial insects is the division of reproductive labor to separate castes (WILSON 1971, CRESPI & YANEGA 1995). Apart from fully reproductive males and queens, eusocial societies produce workers, which contribute to the common goods of the colony (such as nest building, defense, or foraging), at the expense of individual reproduction. These different roles are also reflected in the life history traits of each caste, such as longevity (CAREY 2001), morphology (MIURA 2005), and / or behavior (OSTER & WILSON 1979). For each caste, fitness is determined / maximized by the expression of different sets of these life history components, such as longevity in queens (KELLER 1993, CAREY 2001) or mating success and competitive sperm in males (BOOMSMA & al. 2005a). Given that selective pressures on worker traits act through the reproductive success of the queens (and thus through colony performance), worker fitness is reflected in traits that increase colony performance as a whole (FOSTER & al. 2006, HARPUR & al. 2014). However, life history traits are often subject to trade-offs, and thus, maximization is not possible for all traits. One such trait is the immune system, as immune defenses are often assumed to be costly (MORET & SCHMID-HEMPEL 2000, MCKEAN & al. 2008, VALTONEN & al. 2009). For example, fecundity can be negatively correlated with parasite resistance in *Drosophila melanogaster* MEIGEN, 1830 (MCKEAN & al. 2008), and oral exposure to bacteria has been shown to slow down development of *Trichoplusia ni* (HÜBNER, 1803) larvae (FREITAK & al. 2007). These costs of immunity can be split into maintenance costs when parasite pressure is low and the activation of an immune defense upon an infection. The optimal investment into constitutive and induced immune defenses can depend on life history (LEE 2006). Thus, depending on the life history strategy of a developmental stage, sex and / or caste, such trade-offs may result in differential expression of constitutive and induced immunity.

Life histories and the immune defenses in different castes of eusocial insects are intertwined (BOOMSMA & al. 2005b). For example, workers in most species spend the beginning of their adult life inside the relatively protective environment of the nest, and only later start to forage outside the colony (WILSON 1971, OSTER & WILSON 1979), at which point they encounter an array of challenges including exposure to different pathogens. Similarly, queens

spend most of their life, protected by the social immunity of the colony (CREMER & al. 2007). However, before colony foundation they face a solitary life stage, during which they search for a nest site, and they inevitably face a higher pathogen exposure (BAER & al. 2006, BRÜTSCH & al. 2014). By contrast, males rarely live long enough to be significantly affected by most pathogens (BAER 2003, BAER & al. 2005). Concomitantly, males often show weaker immune responses than queens (BAER & al. 2005), or workers (GERLOFF & al. 2003, VAINIO & al. 2004, BAER & SCHMID-HEMPEL 2006). Thus the differences in life histories, and divergent selection regimes that ensue, are associated with sex, caste and the role within the colony.

In this study we investigated the immune defenses, and the survival upon infection of unmated reproductives (queens and males) and two worker-classes (nurses and foragers) in the ant *Formica exsecta* NYLANDER, 1846. We measured gene expression of nine genes, which allowed us to process a larger set of samples (compared to a transcriptomics approach), and thus improved the confidence in the obtained results. The genes were chosen to represent immune defenses on a broad scale: We measured gene expression of six genes directly associated with immune defenses (Pro-Phenoloxidase, *PPO*; Hymenoptaecin, *Hyme*; LPS-binding protein, *LPS-bp*; Lysozyme C, *LysC*; Toll receptor, *Toll*; β-1,3-glucan-binding protein, *β1,3g*) covering the different pathways of insect immune defenses. The PPO pathway is directed against all forms of parasites, and can be activated through recognition proteins that bind to bacterial membrane components (e.g., LPS), or fungal cell wall components (e.g., β-1,3-glucan) (SÖDERHÄLL & CERENIUS 1998, BROWN & GORDON 2005). Recognition of fungal pathogens also activates the Toll pathway (BROWN & GORDON 2005). Hymenoptaecin is an antibacterial peptide, regulated through the Imd pathway (CASTEELS & al. 1993), whereas the antibacterial Lysozyme C appears not to be regulated by a specific immune pathway. In addition to these immune genes we measured the expression of three genes with a different main functionality but also show some immune functions (Arylphorin, *Aryl*; Vitellogenin, *Vg1*; Insulin-receptor 3, *IR3*). Arylphorin is a hemolymph storage protein with antimicrobial-binding properties (ZHU & al. 2009). The storage protein Vitellogenin has various functions in social insects (MORANDIN & al. 2014), including some immune functionality (AMDAM & al. 2004, SALMELA & al. 2015). The Insulin-pathway serves in the regulation of resource storage and consumption and has been shown to be involved in immune responses (WU & BROWN 2006, DIANGELO & al. 2009). We included these genes to also investigate genes that are usually not directly associated with immune responses. For simplicity we refer to these genes as pleiotropic genes in this manuscript. We measured the constitutive expression of these nine genes, as well as how the expression of these genes changed upon infection by the entomopathogenic fungus *Beauveria bassiana* (VUILLEMIN, 1912). Furthermore, to investigate the consequences of infection on each caste and worker-class (i.e., the susceptibility to infection by *B. bassiana*), we compared the survival of the ants upon infection.

Given that males are in general more frequently infected, and exhibit lower immune defenses (SCHMID-HEMPEL 2011), we expected males to have lower constitutive levels of expression in immune genes, as well as lower induced immune responses upon infection than queens. As a consequence, we predicted the males to suffer a higher mortality upon infection, compared to the queens. Upon infection, we predicted the queens to respond with an up-regulation of the immune genes. Thus, we expected males to weakly respond to the infection in the level of immune gene expression.

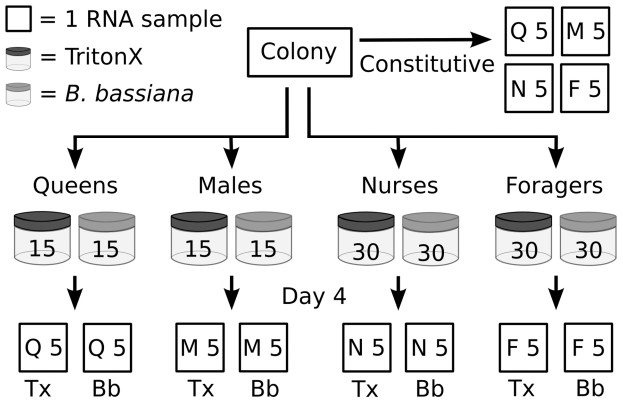

Fig. 1: Experimental setup. Overview on the experimental setup for each colony. Per caste / class and treatment (Constitutive, TritonX (Tx), Beauveria (Bb)) one RNA sample was collected (squares). The numbers in each square indicate the amount of individuals that were pooled per RNA sample. The numbers in the pots indicate the initial amount of individuals set up for the bioassay for each of the castes / classes (Q = Queens, M = Males, N = Nurses, F = Foragers).

Because the foragers in most *Formica* species are older individuals with shorter expected residual life span (LANGE 1967, ROSENGREN 1971), we expected lower constitutive levels of gene expression for the immune genes in these, as well as a lower response to the infection, due to immune senescence. As the nurses are in contact with the brood, and will be valuable to the colony as future foragers, we expected the nurses to respond strongly to the infection by up-regulating the immune genes. Owing to immune senescence, we expected foragers to tolerate the infection and not to change gene expression upon infection. As a consequence, we expected the foragers to show a higher mortality upon infection, compared to the nurses.

Material and methods

Sampling and bioassay: We collected queens and males (reproductives) from field colonies located on three islands in the Tvärminne-Archipelago (FI) (Furuskär: 59° 50' 00" N 23° 16' 05" E, Joskär: 59° 50' 42" N 23° 15' 21" E, Alören: 59° 50' 06" N 23° 15' 50" E), between the 8th and 21st of July 2013. In total, we collected 595 queens and 560 males from 22 colonies. Eleven of these colonies provided 385 individuals of both sexes, six colonies provided 210 queens only, and five colonies provided 175 males only. The workers (nurses and foragers) were collected on the 3rd of July 2015, from field colonies of the same islands. In total, we collected 1300 nurses and 1300 foragers from 20 colonies. Thirteen of these colonies were the same as those for the reproductives, whereas seven colonies had too few workers and were replaced by different colonies for sampling of the workers. Reproductives and indoor

workers (nurses) were collected by removing a part of the top of the mound and sampling from the nest interior. Outdoor workers (foragers) were collected from the foraging trails in the nest periphery.

The samples were brought to the laboratory and prepared for the experiments (Fig. 1). For queens or males we immediately pooled five individuals per colony and fixed them (directly cut into small pieces) in 300 µl Isol-RNA Lysis Reagent (5 Prime). For each worker-class five individuals per colony were pooled and fixed in a mix of 500 µl TRIsure (Bioline) and 150 µl 50mM EDTA, to prevent RNA degradation (VALLES & al. 2012). These samples were then stored at -80 °C until further processing to measure the constitutive gene expression, without any treatments. Thus, for constitutive gene expression, this resulted in a total of 17 RNA samples for the queens, 16 RNA samples for the males, and 20 RNA samples for each worker-class. For the bioassay, we divided the remaining individuals of each sex / worker-class into two groups, each destined for one of two treatments, and placed them into one of two Fluon® coated pots (Ø 7 cm, h: 5 cm) with plaster lining (Fig. 1). For queens and males, each pot per colony and treatment held 15 individuals, and for nurses and foragers each pot per colony and treatment held 30 individuals. The reproductives were kept separate from workers, given that during the mating flight queens and males are solitary also under natural conditions. To maintain humidity we added an open 1.5 ml tube filled with water and a piece of cotton in each pot; this maintained a relative humidity of ca. 70%. The ants were kept for at least one day without treatment in the pots and, for work flow logistics, colonies sampled on different days were grouped to be treated within a maximum of five days after collection.

At the initiation of the bioassay, we assigned one of the two prepared pots per colony and sex / worker-class to an infection treatment and the other to a control treatment. For the infection treatment we used spores of the entomopathogenic fungus *Beauveria bassiana* (strain KVL 03-90) suspended in Triton X-100 (Sigma-Aldrich). To expose the ants to the fungal spores we dipped all individuals separately for five seconds into a solution containing either ~ 1×10^8 spores / ml, or only Triton X-100 for the control treatment. For the next ten days we observed the ants daily for mortality and removed dead ants from the pots. On day four after the initiation of the bioassay, we fixed three reproductive individuals from each pot in 300 µl Isol-RNA Lysis Reagent and five worker individuals in a mix of 500 µl TRIsure and 150 µl 50mM EDTA (to prevent RNA degradation), to measure the induced gene expression. In pilot experiments the average incubation time of *B. bassiana* in *F. exsecta* was four days until the fungus started to show an effect on the survival of the ants. The samples were then stored at -80 °C until further processing for gene expression analysis. All ants were followed for a maximum of ten days, fed daily with ~ 200 µl Bhatkar-Whitcomb diet (BHATKAR & WHITCOMB 1970) and dead individuals were recorded and removed from the pots on a daily basis.

Gene expression analysis: We first homogenized the thawed samples in a total volume of 600 µl RNA isolation reagent (reproductives: Isol-RNA Lysis Reagent; workers: TRIsure) with two stainless steel beads using a TissueLyser (Qiagen). Subsequently we added 400 µl of the RNA isolation reagent and for the reproductives 150 µl chloroform (Sigma), or for the workers 150 µl 1-Bromo-5-Chloropentane (Sigma). After mixing we centrifuged the samples for 10 min at 13,000 rpm at 4 °C. We then transferred the upper, transparent phase, containing the RNA, to a new 1.5 ml tube and supplemented with 500 µl isopropanol (Sigma). After mixing, we let the suspended RNA precipitate over night at -20 °C, and then centrifuged the samples for 30 min at 13,000 rpm at 4 °C to sediment the RNA. After removal of the supernatant, we washed the pellet on ice with 500 µl 80% EtOH (Altia Oyj) and centrifuged for 10 min at 13,000 rpm at 4 °C. After drying the pellet we dissolved the RNA in autoclaved ddH_2O and the worker RNA in RNA storage solution (AMBION). We measured concentration and quality of the RNA photospectrometrically with a NanoDrop (PEQ-Lab) and eliminated possible DNA contamination by DNAse digest (DNase I, Rnase-free; ThermoScientific) before cDNA synthesis (iScript cDNA Synthesis Kit; Bio-Rad). For cDNA synthesis we used 1 µg RNA for each sample and afterwards diluted the resulting 20 µl cDNA in 80 µl autoclaved ddH_2O.

As target genes we chose six genes linked to immune responses. We chose the three antimicrobial peptides β-1,3-glucan-binding protein (*β1,3g*), Lipopolysaccharide-binding protein (*LPS-bp*) and Hymenoptaecin (*Hyme*), the enzyme Lysozyme C (*LysC*), and the two cascade molecules Pro-Phenoloxidase (*PPO*) and Toll receptor (*Toll*). In addition, we selected three genes that are not directly associated with immune responses, but have some immune functionality. For these we used the two storage protein coding genes Vitellogenin 1 (*Vg1*) and Arylphorin (*Aryl*), and the insulin receptor 3 (*IR3*). For simplicity we refer to these three genes as pleiotropic genes in this manuscript. A list of the primer sequences is provided in the supplementary table S1 (in Appendix S1, as digital supplementary material to this article, at the journal's web pages).

We designed qRT-PCR primers using the online Primer3 internet-based interface (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (UNTERGASSER & al. 2012). Primers were designed by the rules of highest maximum efficiency and sensitivity to avoid formation of self- and heterodimers, hairpins and self-complementarity. Gene-specific primers were designed on the basis of the sequences obtained from *F. exsecta* transcriptome (JOHANSSON & al. 2013). Q-RT-PCR was performed on 384-well plates on a CFX384 Touch™ Real Time PCR Detection System (Bio-rad) using iQ™ SYBR® Green Supermix (Bio-Rad), with an initiation of 3 min at 95 °C, 40 cycles of 15 sec at 95 °C for denaturation and 45 sec at 58 °C for annealing / extension, and a final step for 7 min at 95 °C. All the Q-RT-PCR assays were run using two technical replicates, which were assessed for consistency and possible outliers, and subsequently averaged before normalization. Outliers were assessed only in cases with a Ct-difference > 3 between the two technical replicates, and were only removed if the more likely value could be clearly identified (15 cases in total). Non-detects (no amplification signal within 40 qPCR cycles) were set to the maximal cycle number (i.e., Ct = 40), or removed in case the second technical replicate showed amplification.

Statistical analysis: We did not compare reproductives and workers statistically, because the two sets of samples

were collected in different years, and an improved protocol for gene expression was used in 2015 for the workers. To test for changes in survival upon infection by *Beauveria bassiana* we used survival regression (Weibull distribution), separately for the reproductives and the workers. For both models we used Nest as random effect, and Treatment (TritonX, Beauveria) and Caste (Queens, Males), or Class (Nurses, Foragers), as fixed factors. The interaction between Treatment and Caste/Class was included to test for a difference in the effect of Treatment between queens and males, as well as between nurses and foragers. Subsequently we ran post-hoc contrasts (adjusted for false discovery rates (BENJAMINI & HOCHBERG 1995) to test for pairwise differences in survival among castes / classes and treatments.

To test for differences in gene expression across castes / classes and treatments, we first performed Principal Component Analysis (PCA) on the inverted normalized Ct-values. We inverted the Ct-values because they are negatively correlated with the amount of mRNA (i.e., higher Ct-values indicate lower gene expression levels). Normalization of the Ct values was done with the NORMAgene algorithm, which does not require reference genes (HECKMANN & al. 2011). To simplify interpretation, we used separate PCAs for constitutive and induced gene expression. We first used unrotated PCAs for component selection and retained all components with an Eigenvalue > 1.0 (Tab. S2 in Appendix S1). We then rotated the scores of the retained components using oblique (oblimin) rotation. The rotated scores were then used as dependent variables in linear mixed effects models to test for an association of each cluster (PC) with Caste / Class differences (constitutive), as well as Caste / Class and Treatment differences (induced). For induced immunity we also performed planned post-hoc comparisons, where we compared for each PC the pairwise differences of infected against uninfected individuals (separately for each caste / class), and the pairwise differences between males and queens, or nurses and foragers (separately for the infected and uninfected status). Given that these were planned contrasts, we did this irrespective of the presence of a significant interaction (RUXTON & BEAUCHAMP 2008). Colony was added as a random factor. We here arbitrarily define the association of each gene with a principal component as strong (loading 0.67 - 1.0), moderate (loading 0.33 - 0.66), or weak (0.0 - 0.32).

We also ran gene-by-gene linear mixed effects models, with the inverted normalized Ct values as dependent variables, with separate models for constitutive and induced gene expression. For constitutive gene expression we specified Caste / Class as fixed factor and Colony as random factor. For induced gene expression we specified Treatment and Caste / Class as fixed factors, and Colony as random factor. Subsequently we ran post-hoc contrasts to investigate how each Caste / Class responded to the Beauveria-treatment (i.e., the pairwise differences in gene expression between TritonX and Beauveria, for each Caste / Class). In order to compare whether the response to infection differed between the Castes / Classes, we referred to the interaction terms of the mixed effects models.

For statistical analyses we used R 3.2.3 (R CORE TEAM 2015), with the packages survival (THERNEAU 2013) and multcomp (HOTHORN & al. 2008). For all statistical tests

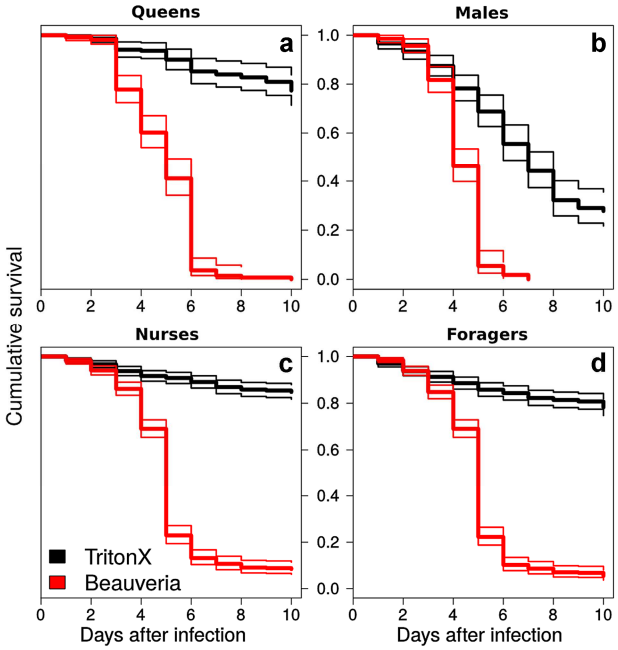

Fig. 2: Survival. Cumulative survival during the bioassay, separated by Caste / Class (panels a - d). The Triton-X treatment is shown in black and the Beauveria treatment is shown in red / gray. Thin lines indicate the 95% confidence intervals across all individuals.

Tab. 1: Results from survival regression models on differences in survival among Castes / Classes (Males vs. Queens, Foragers vs. Nurses) and Treatment (Beauveria vs. TritonX) during the bioassay. The relative difference in survival is given as the parameter estimates plus / minus standard error (β ± SE). Separate survival regressions were performed for reproductives and workers.

Reproductives, 2013	**β ± SE**	**z-value**	**p-value**
Caste	-0.59 ± 0.07	-9.02	< 0.0001
Treatment	-1.05 ± 0.06	-18.43	< 0.0001
Caste * Treatment	0.51 ± 0.07	7.27	< 0.0001
Workers, 2015			
Class	-0.169 ± 0.06	-2.80	0.00509
Treatment	-1.252 ± 0.05	-23.43	< 0.0001
Class * Treatment	0.139 ± 0.07	2.08	0.0372

we used a significance threshold of α = 0.05, and correction for false discovery rates where applicable. Variation is indicated as standard errors, unless specified otherwise.

Results

Survival: Infection by *Beauveria bassiana* significantly increased mortality for both queens and males:
$\beta[\text{Treatment}]_{\text{Queens}} = -1.05 \pm 0.06$, $z = -18.43$, $p < 0.0001$
$\beta[\text{Treatment}]_{\text{Males}} = -0.54 \pm 0.04$, $z = -13.40$, $p < 0.0001$
(see Figs. 2a, b, Tabs. 1, S2 in Appendix S1; complete data in Appendix S2), but the survival after infection did not differ between the two castes:
$\beta[\text{Caste}]_{\text{Beauveria}} = -0.08 \pm 0.05$, $z = -1.73$, $p = 0.0831$.
However, in the absence of an infection, queens survived significantly longer compared to males:

Tab. 2: Results from linear mixed effects models on the Caste / Class differences in scores of the selected Principal Components reflecting constitutive gene expression. The average difference between reproductives (Males vs. Queens) and workers (Foragers vs. Nurses) in PC scores is given as the parameter estimates plus / minus standard error (β ± SE). Separate PCAs were performed for reproductives and workers. All p-values within each caste-group were FDR corrected.

	Reproductives, 2013			**Workers, 2015**		
	β ± SE	**t-value**	**adj. p-value**	**β ± SE**	**t-value**	**adj. p-value**
PC1	-1.77 ± 0.15	-11.74	< 0.0001	-1.50 ± 0.19	-7.76	< 0.0001
PC2	-0.18 ± 0.31	-0.58	0.56	-0.78 ± 0.27	-2.89	0.0097
PC3	-0.43 ± 0.35	-1.21	0.47	-0.94 ± 0.30	-3.16	0.005
PC4	0.10 ± 0.36	0.27	0.79			

Tab. 3: Results from gene-by-gene linear mixed effects models on the difference in constitutive gene expression between the reproductive castes, or the worker-classes. The average difference between reproductives (Males vs. Queens) and workers (Foragers vs. Nurses) in constitutive gene expression (inverse Ct values) is given as the parameter estimates plus / minus standard error (β ± SE). All p-values within each caste-group were FDR corrected.

	Reproductives, 2013			**Workers, 2015**		
	β ± SE	**t-value**	**adj. p-value**	**β ± SE**	**t-value**	**adj. p-value**
Aryl	0.09 ± 0.36	0.25	0.8	-3.87 ± 0.35	-10.99	< 0.0001
Vg1	-7.06 ± 0.40	-17.47	< 0.0001	-4.49 ± 0.71	-6.35	< 0.0001
IR3	0.24 ± 0.29	0.84	0.53	-0.86 ± 0.33	-2.61	0.0173
PPO	-1.43 ± 0.29	-4.95	0.0001	-1.68 ± 0.34	-4.93	0.0005
Hyme	2.89 ± 0.80	3.6	0.0038	3.20 ± 0.81	3.94	0.0009
LPS-bp	-0.51 ± 0.34	-1.52	0.22	-1.65 ± 0.43	-3.89	0.0021
LysC	-0.71 ± 0.29	-2.44	0.0427	-0.72 ± 0.30	-2.43	0.0306
Toll	-0.07 ± 0.22	-0.32	0.8	-1.12 ± 0.39	-2.84	0.0112
β1,3g	1.37 ± 0.22	6.16	< 0.0001	-0.31 ± 0.21	-1.44	0.17

$\beta[Caste]_{TritonX} = -0.59 \pm 0.07$, $z = -9.02$, $p < 0.0001$,
which resulted in a significant statistical interaction of Treatment and Caste:
$\beta[Caste*Treatment] = -0.51 \pm 0.07$, $z = 7.27$, $p < 0.0001$.
Also in the workers, infection by *Beauveria bassiana* significantly increased mortality in both nurses and foragers:
$\beta[Treatment]_{Nurses} = -1.25 \pm 0.05$, $z = -23.43$, $p < 0.0001$
$\beta[Treatment]_{Males} = -1.11 \pm 0.05$, $z = -24.57$ $p < 0.0001$
(see Figs. 2c, d, Tabs. 1, S2 in Appendix S1). The survival after infection did not differ between the two worker-classes
$\beta[Class]_{Beauveria} = -0.03 \pm 0.03$, $z = -1.11$, $p = 0.27$,
but in the absence of an infection, nurses survived significantly longer than foragers
$\beta[Class]_{TritonX} = -0.17 \pm 0.06$, $z = -2.8$, $p = 0.0068$
$\beta[Class*Treatment] = 0.14 \pm 0.07$, $z = 2.08$, $p = 0.0372$.

Gene expression in reproductives: For the PCA of constitutive gene expression in males and queens the first four components had an Eigenvalue above one and explained 86% of the variation (Tab. S3 in Appendix S1; complete data in Appendix S2). Only PC1 showed a significant effect of caste, with significantly higher scores in queens than males (Tab. 2, Figs. S1a - d in Appendix S1). The loadings on PC1 showed a strong positive association with the genes *Vg1* (0.94) and *PPO* (0.82), a strong negative association with the gene *β1,3g* (-0.90) and a moderate negative association of the gene *Hyme* (-0.44). All other loadings showed only weak associations of the respective genes with PC1 (Tab. S4 in Appendix S1). This indicates that the genes *Vg1* and *PPO* were constitutively more expressed in queens, and the genes *β1,3g* and *Hyme* were more expressed in males. The gene-by-gene analysis of constitutive gene expression mirrored the outcome of the PCA; the pleiotropic gene *Vg1*, and the two immune genes *PPO* and *LysC*, were more expressed in queens than in males, whereas the two immune genes *Hyme* and *β1,3g* were more expressed in males than queens (Figs. 3b, d, e, g, j, Tab. 3). The caste difference in the expression of *LysC* was not captured by the PCA, but the direction of association was the same. None of the remaining genes were differently expressed between queens and males (Figs. 3a, c, f, h, Tab. 3).

Also for induced gene expression the first four components of the PCA had an Eigenvalue above 1, and explained 85% of the total variation (Tab. S3 in Appendix S1). All four PCs indicated significant effects of caste, and three of the four PCs (PC1, PC3, PC4) showed a significant effect of treatment, as well as a significant interaction between caste and treatment (Tab. 4). PC1 captured most of the variation (37%) and mirrored the pattern for constitutive defense. The same genes that emerged as important in constitutive defense (*Vg1*, *PPO*, *LysC*) also emerged as important in induced defenses (*Vg1*: 0.91; *PPO*: 0.75; *LysC*: 0.94). In addition, *Toll* showed a strong positive association with PC1 (0.80), whereas the remain-

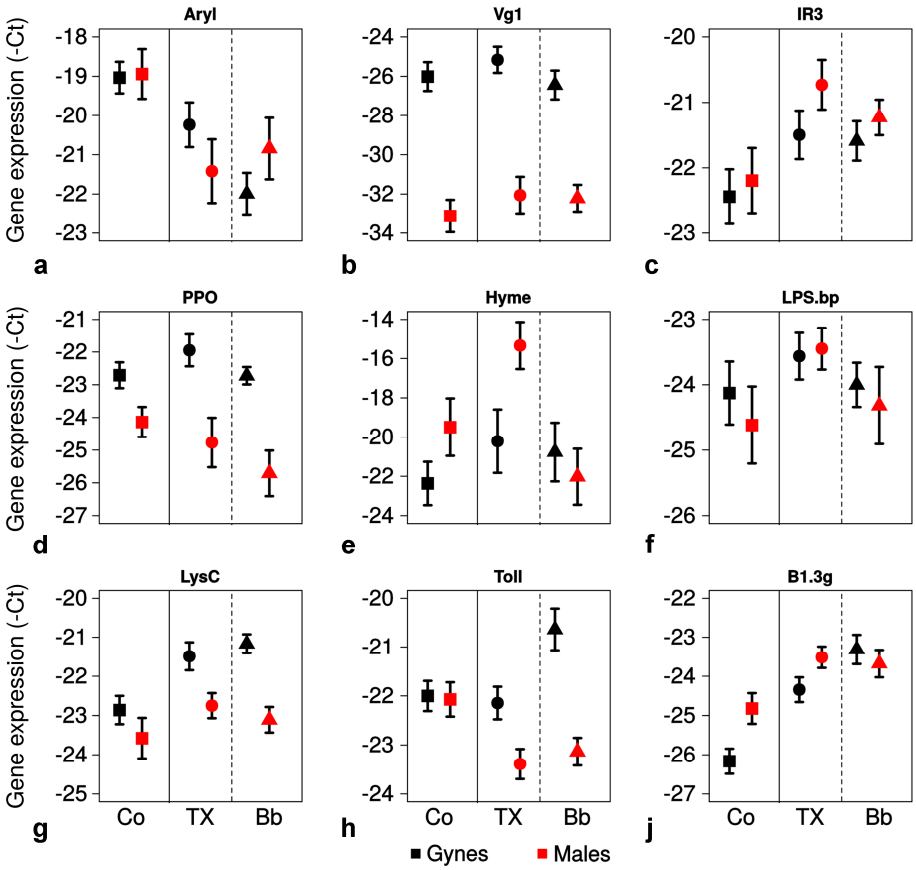

Fig. 3: Gene expression (reproductives). Gene expression levels for queens (black) and males (red / gray), shown as inverted Ct values (higher values indicate higher expression levels). Gene expression was measured for the constitutive state (Co – squares), four days after treatment with Triton-X (TX – circles), and four days after exposure to *Beauveria bassiana* (Bb – triangles). The top three genes (panels a - c) were classified as pleiotropic genes, and the lower six genes (panels d - j) were classified as immune genes. Error bars show the 95% confidence intervals across nests.

Tab. 4: Results from linear mixed effects models on the effect of Caste / Class, Treatment (Beauveria vs. TritonX) and the Caste / Class by Treatment interaction on the scores of the selected Principal Components reflecting induced gene expression. Parameter estimates are given as β plus / minus standard error (SE). Separate PCAs were performed for reproductives and workers. All p-values within each caste-group were FDR corrected.

	Caste / Class			Treatment			Interaction		
	β ± SE	**t**	**adj. p**	**β ± SE**	**t**	**adj. p**	**β ± SE**	**t**	**adj. p**
Reproductives, 2013									
PC1	-1.62 ± 0.12	-13.42	< 0.0001	0.30 ± 0.12	2.5	0.0338	-0.47 ± 0.17	-2.73	0.0127
PC2	0.69 ± 0.32	2.16	0.0353	-0.47 ± 0.31	-1.5	0.14	-0.58 ± 0.45	-1.27	0.2108
PC3	0.91 ± 0.27	3.34	0.002	1.71 ± 0.27	6.26	< 0.0001	-1.20 ± 0.39	-3.06	0.0067
PC4	-1.22 ± 0.29	-4.26	0.0002	-0.57 ± 0.28	-2.03	0.0656	1.93 ± 0.40	4.78	0.0001
Workers, 2015									
PC1	-0.07 ± 0.17	-0.41	0.68	1.61 ± 0.17	9.2	< 0.0001	-1.34 ± 0.25	-5.39	< 0.0001
PC2	-0.21 ± 0.14	-1.45	0.3	0.40 ± 0.14	2.86	0.006	-2.06 ± 0.20	-10.24	< 0.0001

ing genes only showed weak associations with PC1 (Tab. S3 in Appendix S1). PC2 explained 19% of the variation, and the loadings of two genes (*IR3* and *LPS-bp*) showed a strong positive association with PC2 (*IR3*: 0.86; *LPS-bp*: 0.87). PC3 and PC4 explained 18% and 12% of the variation, respectively. The loadings of *β1,3g* showed a strong positive association with PC3 (0.90), whereas *Aryl*, *PPO* and *Hyme* showed moderate negative associations with PC3 (*Aryl*: -0.39; *PPO*: -0.40; *Hyme*: -0.38). The loading of *Hyme* showed a strong negative association with PC4 (-0.82), that of *Aryl* showed a strong positive association with PC4 (0.83), whereas the remaining genes only showed weak associations with PC4 (Tab. S4 in Appendix S1).

In the absence of an infection the genes *Vg1*, *PPO*, *LysC*, *Aryl*, and *Toll* were significantly more expressed in queens than males, whereas the opposite was true for *β1,3g*, *Hyme*, and *IR3* (Fig. 3a - j, Tabs. 5, S5 in Appendix S1). After infection, the genes *Vg1*, *PPO*, *LysC*, and *Toll* remained more expressed in queens, whereas *Aryl* was more expressed in infected males than queens (Figs. 3a, b, d, g, h, Tabs. 5, S5 in Appendix S1). The genes *β1,3g*, *Hyme*, and *IR3*, which were male-biased in uninfected conditions, were not differentially expressed between infected queens and males (Figs. 3c, e, j, Tabs. 5, S5 in Appendix S1). The treatment-independent queen-bias of the genes *Vg1*, *PPO*, *LysC*, and *Toll* was also reflected in the strong

Tab. 5: Results from gene-by-gene linear mixed effects models on the effect of Caste / Class, Treatment (Beauveria vs. TritonX) and the Caste / Class by Treatment interaction on induced gene expression. Interaction indicates the Caste by Treatment interaction. Parameter estimates are given as β plus / minus standard error (SE). All p-values within each caste-group were FDR corrected.

	Caste/Class			Treatment			Interaction		
	β ± SE	**t**	**adj. p**	**β ± SE**	**t**	**adj. p**	**β ± SE**	**t**	**adj. p**
Reproductives, 2013									
Aryl	-1.15 ± 0.39	-2.96	0.0054	-1.76 ± 0.37	-4.76	0.0001	2.29 ± 0.53	4.31	0.0005
Vg1	-6.77 ± 0.45	-15.12	< 0.0001	-1.28 ± 0.43	-2.99	0.0108	1.11 ± 0.62	1.79	0.12
IR3	0.77 ± 0.22	3.48	0.0012	-0.09 ± 0.22	-0.42	0.68	-0.41 ± 0.32	-1.28	0.24
PPO	-2.86 ± 0.32	-9.04	< 0.0001	-0.78 ± 0.30	-2.61	0.0231	-0.17 ± 0.43	-0.39	0.7
Hyme	4.87 ± 0.94	5.18	< 0.0001	-0.56 ± 0.94	-0.59	0.63	-6.13 ± 1.35	-4.53	0.0003
LPS-bp	0.12 ± 0.24	0.51	0.61	-0.44 ± 0.23	-1.91	0.0939	-0.42 ± 0.33	-1.27	0.24
LysC	-1.26 ± 0.19	-6.49	< 0.0001	0.32 ± 0.19	1.7	0.13	-0.68 ± 0.27	-2.49	0.0317
Toll	-1.24 ± 0.23	-5.52	< 0.0001	1.51 ± 0.23	6.69	< 0.0001	-1.25 ± 0.32	-3.87	0.0006
β1,3g	0.83 ± 0.21	3.87	0.0004	1.03 ± 0.21	4.81	0.0001	-1.19 ± 0.31	-3.87	0.0006
Workers, 2015									
Aryl	-0.48 ± 0.27	-1.76	0.19	1.55 ± 0.27	5.68	< 0.0001	-2.30 ± 0.39	-5.91	< 0.0001
Vg1	-1.36 ± 0.40	-3.41	0.0108	0.20 ± 0.40	0.49	0.63	-2.74 ± 0.57	-4.81	< 0.0001
IR3	0.72 ± 0.25	2.92	0.0225	1.69 ± 0.25	6.83	< 0.0001	-2.55 ± 0.35	-7.22	< 0.0001
PPO	0.11 ± 0.29	0.39	0.77	0.48 ± 0.29	1.68	0.13	-2.58 ± 0.41	-6.26	< 0.0001
Hyme	0.97 ± 0.68	1.42	0.29	-0.36 ± 0.68	-0.52	0.63	-1.57 ± 0.97	-1.61	0.11
LPS-bp	0.11 ± 0.30	0.37	0.77	0.60 ± 0.30	1.98	0.0791	-2.10 ± 0.43	-4.89	< 0.0001
LysC	-0.06 ± 0.21	-0.3	0.77	0.89 ± 0.21	4.3	0.0001	-1.49 ± 0.29	-5.07	< 0.0001
Toll	-0.66 ± 0.32	-2.05	0.14	2.21 ± 0.32	6.82	< 0.0001	-1.57 ± 0.46	-3.41	0.0014
β1,3g	-0.07 ± 0.21	-0.33	0.77	1.70 ± 0.21	8.24	< 0.0001	-1.31 ± 0.29	-4.45	0.0001

positive association of these genes with PC1, which showed higher scores in queens than males, independent of treatment (Fig. S2a in Appendix S1, Tabs. 4, S6 in Appendix S1). As a response to infection, queens down-regulated *Aryl*, *Vg1*, and *PPO*, but up-regulated *LysC*, *Toll*, and *β1,3g*, compared to uninfected queens, whereas the remaining genes showed no significant change (Figs. 3a - j, Tabs. 5, S5 in Appendix S1). Males down-regulated *PPO*, *Hyme*, and *LPS-bp* upon infection, whereas the remaining genes remained unchanged (Figs. 3a - j, Tabs. 5, S5 in Appendix S1). These results were also reflected by PC3 and PC4, which showed similar sex-dependent effects of treatment (Figs. S2c, d in Appendix S1, Tabs. 4, S6 in Appendix S1). Taken together, both the analyses based on PCA scores and gene-by-gene comparisons point to overall caste differences, as well as significant differences in how gene expression patterns play out in males and queens following infection.

Gene expression in workers: For the PCA of constitutive gene expression three components were selected, which explained 75% of the variation (Tab. S3 in Appendix S1). All three components showed a significant effect of class, and the scores of all components were significantly higher in nurses than foragers (Figure S3a - c in Appendix S1, Tab. 2). The loadings on PC1 indicated strong positive associations with the genes *Aryl* (0.84) and *Vg1* (0.98), and a strong negative association with the gene *Hyme* (-0.73), those on PC2 showed strong positive associations with the genes *IR3* (0.80) and *β1,3g* (0.84), and those on *PC3* showed strong positive associations with the genes *LysC* (0.83) and *Toll* (0.81). The loadings of both PC2 and PC3 showed also moderate positive associations of these components with the genes *PPO* (0.53 and 0.42) and *LPS-bp* (0.58 and 0.37). The remaining loadings showed only weak associations (Tab. S4 in Appendix S1). This suggests that the genes *Aryl*, *Vg1*, *IR3*, *LPS-bp*, *LysC*, *Toll*, and *β1,3g* were more expressed in nurses than foragers, whereas only the gene *Hyme* was more strongly expressed in foragers. The gene-by-gene analysis largely agreed with the results from the PCA; nurses showed a higher expression of all genes except *Hyme* and *β1,3g* (Figs. 4a - j, Tab. 3), whereas the gene *Hyme* was constitutively more expressed in foragers. However, the gene *β1,3g* was not differentially expressed between nurses and foragers, although it was strongly associated with one of the PCs (Figs. 4e, j, Tab. 3).

For the PCA of induced gene expression two components were selected, which together explained 56% of the variation (Tab. S3 in Appendix S1). Neither PC1 nor PC2 showed a significant effect of class, but both PC1 and

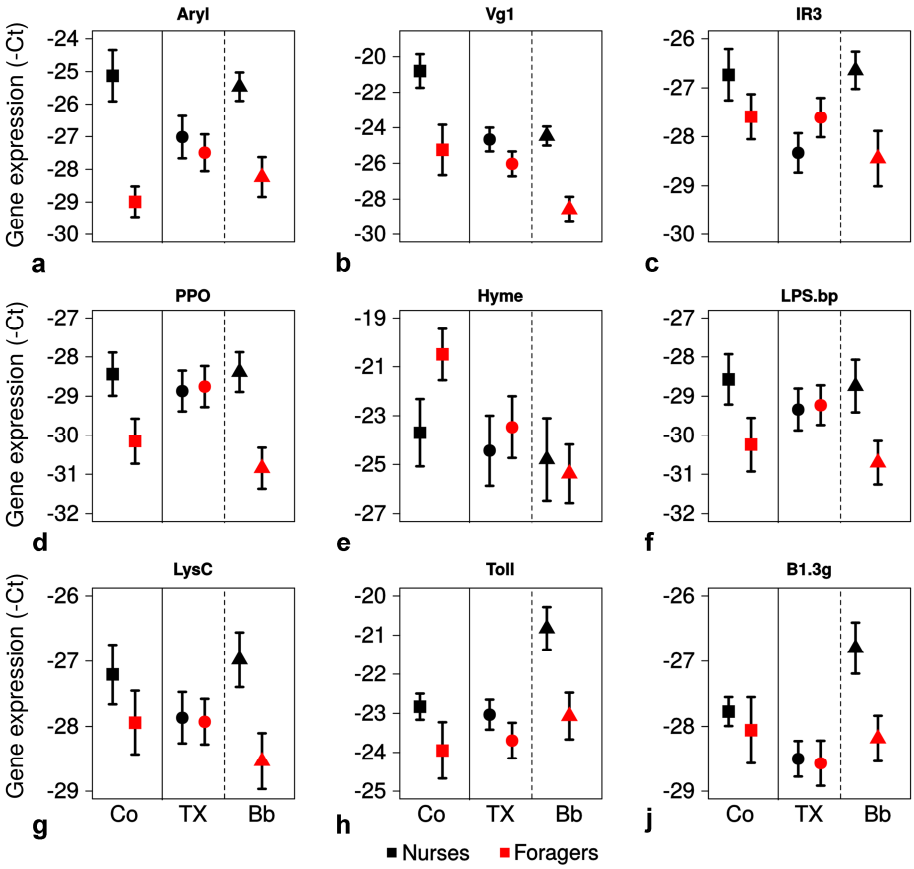

Fig. 4: Gene expression (workers). Gene expression levels for nurses (black) and foragers (red/gray), shown as inverted Ct values (higher values indicate higher expression levels). Gene expression was measured for the constitutive state (Co – squares), four days after treatment with Triton-X (TX – circles), and four days after exposure to *Beauveria bassiana* (Bb – triangles). The top three genes (panels a - c) were classified as pleiotropic genes, and the lower six genes (panels d - j) were classified as immune genes. Error bars show the 95% confidence intervals across nests.

PC2 showed significant effects of treatment and class by treatment interactions (Tab. 4). PC1 explained 41% of the variation and the loadings on PC1 showed positive associations for the genes *Toll* (0.73), *β1,3g* (0.93), *Aryl* (0.40), *IR3* (0.56) and *LysC* (0.64), as well as a moderate negative association for the gene *Hyme* (-0.60). PC2 explained 15% of the variation and the loadings on PC2 showed strong positive associations for the genes *Vg1* (0.84), *PPO* (0.69) and *LPS-bp* (0.67), and moderate positive associations for the genes *Aryl* (0.54), and *LysC* (0.32). This suggests that all the investigated genes were involved in the responses to infection, and that nurses and foragers responded differently to immune challenge.

In uninfected workers, only two genes were differentially expressed between the two worker-classes, which stands in contrast to constitutive gene expression. The gene *Vg1* was more expressed in uninfected nurses than foragers, whereas *IR3* was more expressed in uninfected foragers (Figs. 4b, c, Tabs. 5, S5 in Appendix S1). Upon infection, all genes were more expressed in infected nurses than foragers, except *Hyme* which was not differentially expressed between infected nurses and foragers (Figs. 4a - j, Tabs. 5, S5 in Appendix S1). This is also reflected by PC1 and PC2, which showed no significant differences between uninfected workers, but higher scores in infected nurses than foragers (Fig. S4a in Appendix S1, Tabs. 4, S6 in Appendix S1).As a response to infection, infected nurses showed higher expression of the genes *Aryl*, *IR3*, *LysC*, *Toll*, and *β1,3g* than uninfected ones, whereas infected foragers showed lower levels of *Aryl*, *IR3*, and *LysC*, but not for *β1,3g* or *Toll*, than uninfected ones; (Figs. 4a, c, g - j, Tabs. 5, S5 in Appendix S1). The genes *Vg1*, *PPO*, *Hyme*, and *LPS-bp* were not differentially expressed between infected and uninfected nurses, but were significantly down-regulated upon infection in foragers (Figs. 4b, d - f, Tabs. 5, S5 in Appendix S1), as was also reflected by the PCA (Fig. S4a in Appendix S1, Tabs. 4, S6 in Appendix S1).

Discussion

The aim of this study was to examine the caste-, and worker-class-dependent differences in immune responses and defense strategies against a fungal pathogen, *Beauveria bassiana*. We analyzed the survival of the ants upon infection, the constitutive expression level of nine genes, and the expression level of these genes in experimentally infected and uninfected (control-treated) ants. We found a higher baseline mortality for males compared to queens, and, among workers, for foragers compared to nurses. These differences were not found when the ants had been infected, although *B. bassiana* clearly reduced the survival of the ants. Nonetheless, as expected, the queens up-regulated immune genes upon infection, whereas males down-regulated gene expression across the genes. Similarly, as expected, nurses showed both higher constitutive levels of gene expression, and up-regulated immune genes upon infection, whereas foragers mostly down-regulated expression in several of the immune genes upon infection. These results may reflect immune senescence in males and foragers.

Surprisingly, survival after infection did not differ significantly between queens and males, although males showed significantly lower levels of gene expression following infection, whereas the opposite was true for the queens. This stands in contrast to our expectations, and the frequent observation that males are more susceptible to pathogens than

females (SCHMID-HEMPEL 2005), yet, agrees with an extensive study on nine ant species, which also found no differences in the susceptibility to *Beauveria bassiana* between males and queens (HO & FREDERICKSON 2014). Similarly, nurses and foragers did not show a difference in susceptibility to infection, although nurses mostly up-regulated and foragers mostly down-regulated gene expression upon infection. The absence of a difference in survival upon infection, between males and queens, and between nurses and foragers may, however, have originated from the high dosage. This led to a high overall mortality, and may have reduced the resolution of the statistical analysis. Indeed, the difference in mortality between males and queens following infection was close to significance ($p = 0.0831$), with males being slightly more susceptible.

The PCA and the gene-by-gene analysis were largely congruent, and both indicated generally higher constitutive gene expression levels in queens than males, as well as in nurses compared to foragers. Two of the three pleiotropic genes showed no significant overall differences in expression between males and queens, but, not unexpectedly, the egg-yolk-protein Vitellogenin was more expressed in queens than in males. Vitellogenin is commonly more expressed in females given its role in oogenesis (VALLE 1993). In addition to its conserved functions, it may also be influenced by regulatory functions in immune defense and longevity in social insects (AMDAM & al. 2004). In the workers, all pleiotropic genes were constitutively less expressed in foragers than nurses. The higher expression of Vitellogenin may also be linked to increased ovarian activity in the nurses. Indeed, in many species of social insects young workers lay eggs, which can be trophic and used as nutrients, or fertile and develop into worker-laid males (HELANTERÄ & SUNDSTRÖM 2007). The differential expression of Vitellogenin between the worker-classes may also be linked to the reproductive ground plan hypothesis, which states that the division of labor in social insects derived from conserved reproductive regulatory networks (AMDAM & al. 2004, KAPHEIM & JOHNSON 2017). Alternatively, this reflects an age-dependent decrease in resource storage and reduced resource consumption in foragers (TOTH & ROBINSON 2005, DUSSUTOUR & al. 2016).

Although the constitutive gene expression was generally higher in queens than in males, two immune genes – Hymenoptaecin and β-1,3-glucan-binding protein – were constitutively more expressed in males than in queens. This agrees with results obtained for the ant *Atta vollenweideri* FOREL, 1893 (KOCH & al. 2013), and the bumblebee *Bombus terrestris* (LINNAEUS, 1758) (COLGAN & al. 2011), in which the antimicrobial peptide Hymenoptaecin also was more expressed in males than in queens. A similar pattern also emerged in the workers, in which Hymenoptaecin was constitutively more expressed in foragers than in nurses. Such a forager-bias in Hymenoptaecin was also found in honeybees (LIU & al. 2011, VANNETTE & al. 2015), but, to our knowledge, this has not been investigated in other ants.

With respect to the induced immune responses, the PCA and the gene-by-gene analysis were again congruent, and indicated an induction of antifungal defenses (via *Toll* and *β1,3g*) in queens and nurses, but not in males or foragers. Given that the β-1,3-glucan-binding protein induces the Toll pathway, it is not surprising that these genes were co-regulated upon infection (BROWN & GORDON 2005). Furthermore, in uninfected reproductives, the differences in gene expression between males and queens largely mirrored the pattern found for constitutive expression levels. However, in control-treated workers the differences were much less pronounced, or absent. This suggests that gene expression in workers is rapidly adjusted to current conditions, in this case the laboratory environment, and that the physiological status of the foragers and nurses converge under similar conditions (DUSSUTOUR & al. 2016). The results also revealed striking differences in how the two reproductive castes, and the worker-classes responded upon fungal infection. Both queens and nurses up-regulated the two antifungal genes Toll receptor and β-1,3-glucan-binding protein, but only the queens down-regulated the two pleiotropic genes Arylphorin and Insulin receptor 3, as well as the immune gene Pro-Phenoloxidase, whereas no genes were down-regulated in the nurses. Given that Arylphorin and the Insulin pathway are involved in resource storage and consumption, this is consistent with the expectation that queens face a trade-off between investment in immune defenses and resources needed for maintenance of metabolic functions. The nurses do not appear to face such a trade-off, perhaps because they remain in their social environment with nutritional and social support, and will not embark on a nuptial flight, followed by a solitary (colony founding) stage (CREMER & al. 2007), and be exposed to environmental hazards surviving only on their stored fat resources (BAER & al. 2006, BRÜTSCH & al. 2014). In contrast to queens and nurses, males and foragers did not respond to infection via up-regulation of the antifungal genes, but down-regulated gene expression in general.

In males, two antibacterial genes, Hymenoptaecin and LPS-binding protein, and the general immune gene Pro-Phenoloxidase were down-regulated following infection. In the foragers all immune genes, except the two antifungal genes (Toll and β-1,3-glucan-binding protein) were less expressed upon infection, whereas the expression of the antifungal genes remained unchanged. This indicates that both males and foragers respond to infection by selectively down-regulating the expression of genes, which are not directly involved in defense against the offending pathogen. By contrast, queens and nurses respond by up-regulating genes involved in antifungal defenses. This suggests that males and foragers respond by saving resources, whereas queens and nurses respond by mobilizing resources for defense, and suggests that males and foragers invest in general less in immune defenses than queens and nurses (HELFT & al. 2012, KOCH & al. 2013). Given the extensive variation in residual life expectancy between males and queens, and between nurses and foragers (PAMILO 1991, TOFILSKI 2002), this may also be an important factor in mediating patterns of immune defenses in both the reproductive castes, and in different cohorts of workers. This is also consistent with the observed male- and forager-bias in the Imd pathway related gene Hymenoptaecin, which may reflect a dependency of the Imd pathway on age related life histories (MYLLYMÄKI & al. 2014).

In conclusion, our results highlight the role of caste- and worker-class-specific life histories in the mediation of immune defenses, and the way these are visible in patterns of gene expression. Both males and foragers responded to infection by maintaining the expression levels in critical

genes at the expense of presumably non-critical genes. This is what is expected, as males have no further role beyond mating and the foragers have reduced life expectancy owing to a high extrinsic rate of mortality (WILSON 1971, OSTER & WILSON 1979). Queens and nurses clearly mounted a response against the fungus, but the queens did so at the expense of pleiotropic gene expression. The absence of a similar trade-off between pleiotropic genes and immune genes in nurses may be due to the social environment, which the queens are devoid of during their nuptial flight and colony founding stage (ARMITAGE & BOOMSMA 2010). Our study highlights differences in the investment in immune functions between the reproductive castes, as well as workers of different age and tasks in a natural environment. These similarities are consistent with different life history trajectories between males and queens, as well as workers of different age.

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Author contribution

DS conducted the experimental work. DS performed the laboratory work (qPCR), and statistical analysis of the survival and qPCR data. The study is based on an original idea by LS and DF, later modified by all authors. All authors also contributed to the study design and manuscript preparation.

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