Differential gene expression in *Acromyrmex* leaf-cutting ants after challenges with two fungal pathogens

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Abstract

Social insects in general and leaf-cutting ants in particular have increased selection pressures on their innate immune system due to their social lifestyle and monoclonality of the symbiotic fungal cultivar. As this symbiosis is obligate for both parties, prophylactic behavioral defenses against infections are expected to increase either ant survival or fungus garden survival, but also to possibly trade-off when specific infections differ in potential danger. We examined the effectiveness of prophylactic behaviors and modulations of innate immune defenses by a combination of inoculation bioassays and genome-wide transcriptomic studies (RNA-Seq), using an ant pathogen (*Metarhizium brunneum*) and a fungus-garden pathogen (*Escovopsis weberi*) and administering inoculations both directly and indirectly (via the symbiotic partner). Upon detection of pathogen conidia, ant workers responded by increasing both general activity and the frequency of specific defense behaviors (self-grooming, allo-grooming, garden-grooming) independent of the pathogen encountered. This trend was also evident in the patterns of gene expression change. Both direct and indirect (via fungus garden) inoculations with *Metarhizium* induced a general up-regulation of gene expression, including a number of well-known immune-related genes. In contrast, direct inoculation of the fungus garden by *Escovopsis* induced an overall down-regulation of ant gene expression whereas indirect inoculation (via the ants) did not, suggesting that increased activity of ants to remove this fungus garden pathogen is costly and involves trade-offs with the activation of other physiological pathways.

Keywords

Innate immunity; mutualism; RNA-Seq; entomopathogenic fungus; fungal crop parasite

INTRODUCTION

Social insect colonies are characterized by dense aggregations of individuals that are generally highly related. These characteristics facilitate the transmission of disease and thus make social insect colonies vulnerable to microbial parasites (Boomsma *et al.* 2005; Schmid-Hempel 2006). Among the social insects, the fungus-growing ants experience particular disease challenges, because they maintain clonal mutualistic fungus gardens (Mueller *et al.* 2010; Poulsen & Boomsma 2005) that also need to be kept free of disease (Bot *et al.* 2002; Poulsen *et al.* 2002). However, despite this high expected disease pressure, no specialized diseases of these ants are known and, although a variety of saprophytic weeds can be found in the fungus gardens, only one specialized garden disease, *Escovopsis*, appears to exist (Currie *et al.* 2003; Rodrigues *et al.* 2008; Yek *et al.* 2012*a*).

Ants with long-lived colonies have been under particularly strong selection to keep their nest environments free of disease, which may indirectly have inhibited the evolution of virulent specialized diseases (Boomsma *et al.* 2005; Hughes *et al.* 2008). However, how ants in general and leaf-cutting ants in particular achieve their highly efficient defenses against pathogens is only partly understood. Several defenses may have helped leaf-cutting ants to combat the spread of germs. First, these ants have unusually large metapleural glands (Hughes *et al.* 2010), which have many characteristics reminiscent of an additional cuticular immune system (Yek *et al.* 2012*b*). Second, most attine ants maintain actinomycete bacteria in specially adapted cuticular crypts and tubercles (Currie *et al.* 2006), which assist in the control of *Escovopsis* infections (Currie *et al.* 2003; Taerum *et al.* 2007; Yek *et al.* 2012*a*). Both these defenses are expressed by individual ants, but may become collective social defenses by the ways in which they are applied (e.g. Little

et al. 2006), and it is generally believed that collective defenses of this kind have very significant functions across the social insects (Cremer *et al.* 2007; Cremer & Sixt 2009).

The innate immune system of insects is generally very efficient in detecting and eliminating potentially harmful microorganisms. Pathogen recognition receptors bind and recognize non-self cells, which leads to the activation of signaling pathways such as Toll and IMD, resulting in the expression of antimicrobial effectors (Lazzaro 2008; Lemaitre & Hoffmann 2007; Levashina 2004). In non-social insects such as *Drosophila* fruit flies (Lemaitre & Hoffmann 2007) and *Anopheles* mosquitoes (Christophides *et al.* 2002) the availability of reference genomes has facilitated immune system studies in recent years, and to a lesser extent the same is true for honeybees (Weinstock *et al.* 2006). Initial analyses of ant genomes revealed that all the components of the innate immune system known from *Drosophila* are present (Gadau *et al.* 2011). Recent work has increasingly focused on the expression of multiple immune-genes responding to either pathogens or endosymbionts (Erler *et al.* 2011; Ratzka *et al.* 2011), but studies focusing on genome-wide expression responses including those of immune genes have not been attempted.

The present study exploits known individual and collective prophylactic defenses and the recently published reference genome of the leaf-cutting ant *Acromyrmex echinatior* (Nygaard *et al.* 2011; see also Suen *et al.* 2011 for a reference genome of the related leaf-cutting ant *Atta cephalotes*) to conduct a large-scale gene-expression study in large workers of *A. echinatior* leaf-cutting ants exposed to two types of pathogens: 1. the generalist fungus *Metarhizium brunneum* that infects ants, and 2. the specialized fungus *Escovopsis weberi* that infects fungus gardens. We administered inoculations both directly (targeting the known host) and indirectly (targeting the mutualistic partner). Due to the complex interactions between multiple defenses (e.g. metapleural

glands, actinomycete bacteria, individual and collective immune responses), we decided to conduct whole transcriptome analyses to capture overall gene-expression responses that likely represent all these defense mechanisms, rather than focusing on a limited subset of potentially relevant candidate genes. Whole transcriptome analysis also allows for a global comparison of expression levels across genes and thus removes any ascertainment bias that would emanate from an a-priori focus on known immune genes. Finally, whole transcriptome analysis has the potential to identify genes that were not previously known to be involved in these defenses. We supplemented our RNAseq analyses with a parallel set of inoculation experiments on ants from the same colonies to: 1. Monitor behavioral responses such as self-grooming, allo-grooming, garden-grooming and general level of activity of ants during the first hours after contact with fungal conidia to confirm that the ants experienced our experimental inoculations as significant challenges, and 2. Assess the likelihood of inoculations leading to persistent infections by measuring the extent to which ants died and the degree of fungus-garden mass-reduction in response to inoculations.

MATERIALS AND METHODS

Fungal inoculation experiments

Three representative colonies of *Acromyrmex echinatior*, collected in Gamboa, Panama in 2005 (Ae226B) and 2006 (Ae363 and Ae376), were used in the experiments. Colonies were kept under standardized conditions and were similar in worker number and fungus-garden mass (ca. 2 litres). To minimize age- or caste-specific variation, we used only major garden workers of approximately the same intermediate cuticular coloration (i.e. age). The entomopathogenic fungus *Metarhizium brunneum* (Bischoff *et al.* 2009; formerly *M. anisopliae*) that has a direct

negative and often lethal impact on *A. echinatior* workers (Baer *et al.* 2005; Hughes *et al.* 2002; Hughes & Boomsma 2004) and the specialized parasite *Escovopsis weberi* that attacks the fungal cultivar but not the *A. echinatior* workers (Reynolds & Currie 2004; Yek *et al.* 2012) were used as disease agents.

For each colony, we established five sub-colonies containing 10 major garden workers and 1 g of fungus garden each. The inoculation assays were set up as 2 by 2 factorial designs, with: 1. *Metarhizium* inoculation on ants (MbAnts), 2. *Metarhizium* on fungus garden (MbFungus), 3. *Escovopsis* on ants (EwAnts), 4. *Escovopsis* on fungus garden (EwFungus), and 5. Untreated ants and fungus garden (Controls) (Fig. 2f). For each inoculation, we used conidia taken from 1 cm^2 of fungal culture grown on potato dextrose agar (PDA), which corresponds to ca. 2.1 X 10^8 conidia/ml for *Metarhizium* and 9.2 X 10^7 conidia/ml for *Escovopsis*.

To verify whether our inoculations had indeed realized significant disease pressure challenges, we set up a parallel set of sub-colonies to monitor ant behaviours directly after inoculations as well as differential ant survival and changes in garden biomass over the following 10 days. From 10 minutes after inoculation until the start of the second hour, we recorded the frequencies of self-grooming, allo-grooming, garden-grooming, and immobility inside the fungus garden, during four observations periods of 10 minutes each with five minutes intervals between them. Grooming behaviors are characterized by licking forelegs with the mouth parts (Bot *et al.* 2001), upon which the ants start to remove fungal conidia from either their own body (self-grooming) or the body of a nestmate (allo-grooming). In contrast, "garden-grooming" starts with antennating a garden fragment, followed by grasping and lifting the fungal piece to pull it through the mouth parts (Bot *et al.* 2001). Ants that stayed motionless during the observation period were categorized as "immobile inside the fungus garden". Due to the relatively low

incidences of these behaviors, we pooled the four replicate observations in subsequent analyses. The cadavers of any ants that died during the experiment were surface sterilized, incubated and monitored for the appearance of *Metarhizium* conidiophores (see Yek *et al.* 2012*b* for further details).

RNA isolation, library preparation and sequencing

Forty eight hours after inoculations, *A. echinatior* workers and fungus garden fragments were killed with liquid nitrogen and stored at -80˚C, after which RNA was isolated from the ants. RNA extractions and sequencing were replicated across the three experimental colonies (Ae226B, Ae363 and Ae376). For each of the three colonies and five treatments, 10 individuals were pooled and used for RNA extraction, giving a total of 15 RNA samples. The pooling of individuals was necessary in order to get enough RNA for construction of the sequencing libraries, and at the same time decrease the effect of individual variation on gene expression, so that error bars could indicate variation among colonies. RNA extractions of experimental samples from the same colony were carried out in parallel using the RNeasy Plant Mini Kit (Qiagen) with modifications from a standard protocol. Each sample (10 major *A. echinatior* workers, approximately 100 µg) was transferred to a 2 ml tube that contained 750 µl RLT buffer, 7.5 µl β-mercaptoethanol and 1 ceramic bead. The samples were disrupted in a Fastprep instrument at level 6 for 30 seconds, followed by centrifugation at 13.500g for 1 minute. The homogenized samples were then transferred to Qiashredder tubes, followed by centrifugation at 13.500 for 2 minutes.

The liquid phase was transferred to a phase lock tube that had been centrifuged at 13.500g for 30 seconds. 700 µl phenol/$CHCl_3$/iso-amy-alcohol (25:24:1) pH 8 was added to the phase

lock tube (5 Prime), and the resulting liquid was mixed by turning the tubes upside down for 1 minute. The tubes were incubated at room temperature for 3 minutes, after which they were centrifuged at 20.000g for 30 minutes in a cooling centrifuge at 20C. The upper phase was poured into a clean tube and half the volume 96% ethanol was added, thoroughly mixed and immediately transferred to a Qiagen column. The samples were further processed as described in the protocol, including an optional DNAseI incubation step. The integrity of the RNA samples was confirmed by agarose gel electrophoresis, and total RNA quantity and purity were determined spectrophotometrically. RNA-seq library construction and sequencing was carried out by BGI Hong Kong as described in Nygaard *et al.* (2011). The 200bp short-insert libraries were sequenced using Illumina HiSeq 2000 with 90 bp paired-end sequencing.

STATISTICAL ANALYSES

Inoculation assays

Survival of inoculated ants over 10 days was analyzed with a proportional-hazards regression model, with colonies, fungal treatments and their interactions as main effects. Post-hoc pairwise differences between colonies and fungal treatments were based on risk-ratio tests, with the significance level being Bonferroni adjusted to correct for multiple comparisons. The proportions of ants sporulating were compared using G-tests of goodness-of-fit for heterogeneity. Differences in the occurence of grooming behaviours were analyzed with one-way ANOVA, testing for differential effects of fungal treatments, with each experimental colony providing a data point consisting of the pooled observations. In the case of overall significance, post-hoc multiple comparison Tukey tests were performed to determine which fungal treatments had a significant effect. All statistical analyses were performed with JMP software (version 9.02, SAS Institute).

RNA-Seq analyses

Analysis of the RNA-Seq data was carried out with the Tuxedo pipeline (Bowtie, TopHat and Cufflinks programs) available through the web-based bioinformatics platform Galaxy (http://usegalaxy.org/). Sequence reads from five experimental samples (MbAnts, MbFungus, EwAnts, EwFungus and Controls) replicated over the three experimental colonies were first processed with FASTQ Groomer to convert the clean Illumina reads to the FASTQ format (Blankenberg *et al.* 2010). The reads of respective treatments were then aligned to the *A. echinatior* genome (Genome build 3.9, Nygaard *et al.* 2011) using TopHat (Version 1.5.0, parameters "-p 20") (Trapnell *et al.* 2009). We then used Cufflinks (Version 1.3.0, parameters "-q --no-update-check -I 300000 -F 0.100000 -j 0.150000 -p 8 -g") to measure gene expression for the transcripts in the test samples using the *Acromyrmex echinatior* transcriptome (GenBank accession number ERS032731) as a reference. Due to the presence of multiple treatments and replicates, we used Cuffmerge (Version 1.0.0, parameters "-o cm_output -p 4 -g") to merge the assemblies generated by Cufflinks using the *Acromyrmex echinatior* transcriptome as a reference. Finally, Cuffdiff (Version 1.3.0, parameters "-q -p 8 -c 10 --FDR 0.050000 -N -b") was used to identify genes for which the expression level was significantly different in a test sample relative to control (Trapnell *et al.* 2012). The distributions of significantly differentially expressed genes were compared using chi-square goodness-of-fit test, with the null hypothesis of computed genes from each experimental treatment having the same expression levels. Similarly, we examined the directionality of gene regulations for each experimental treatment with the null hypothesis of equal distribution of number of genes that were up- and downregulated. The profiles of these significantly differentially expressed genes were visualized with Venn diagrams, drawn using

free web-based software (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The correlations of direct (host) and indirect (non-susceptible symbiont) inoculations between pathogens (*Metarhizium* and *Escovopsis*) and targets (ants and fungus garden) were carried out using bivariate equal variance principal component analysis (PCA) on the expression levels of all genes differentially expressed in at least one of the compared treatments, relative to controls. A pseudocount of 0.1 was added to all expression values to allow expression change ratios for genes with zero expression in some treatments to be estimated.

Functional annotation of immune-related genes

We grouped immune-related genes into four categories on the basis of their known or inferred molecular functions: 'recognition genes' that encode pathogen surveillance proteins (e.g. peptidoglycan recognition proteins and Gram-negative binding proteins), 'signaling genes' that encode proteins in immune-related signaling pathways (e.g. Toll, IMD, JAK-STAT and JNK), 'effector genes' that encode proteins that directly inhibit pathogen growth and survival (e.g. antimicrobial peptides), and "stress-related genes" that have known functional roles in responses to external and biotic stimuli (Jesenberger & Jentsch 2002; Ratzka *et al.* 2011; Sackton *et al.* 2007). Any such broad functional classification is necessarily somewhat subjective, but serves the purpose of facilitating the overall interpretation of differences in gene expression.

The 375 significantly differentially expressed genes that we obtained after Cuffdiff computations were functionally annotated with Blast2GO (Conesa & Götz 2008). Genes that have functional notations related to immunity such as cell death, responses to stress, responses to external stimuli and responses to biotic stimuli were tentatively assigned to the respective immunity roles. ImmunoDB, a database containing immune-related gene families of sequenced

insects such as *Drosophila melanogaster*, *Anopheles gambiae,* and *Aedes aegypti* (Waterhouse *et al.* 2007) was also used to query our 375 differentially expressed genes. Furthermore, we added a list of well-annotated honeybee immune genes (Lumi Viljakainen, *personal communication*) in our query in the hope of identifying as many immune-related genes as possible. These queries were carried out mainly using the BLAST family of search functions (www.ncbi.nlm.nih.gov). The visualizations of immune-related genes in their respective experimental treatments and categories were created using the gplots package in R (http://www.R-project.org).

RESULTS

Survival and prophylactic responses to fungal inoculations

The survival of ants (Fig. 1a) differed significantly between the fungal treatments (Effect likelihood ratio (LR) $\chi^2 = 37.89$, df = 4, $P < 0.0001$), but there was no significant interaction between colonies and fungal treatment types (LR $\chi^2 = 10.89$, df = 8, $P = 0.2079$). Ants inoculated with *Metarhizium* consistently suffered higher mortality than control ants and ants inoculated directly (MbAnts) suffered significantly higher mortality than ants inoculated indirectly (MbFungus) (Wilcoxon $\chi^2 = 16.16$, df = 1, $P < 0.0001$). On the other hand, mortalities of ants inoculated directly or indirectly with *Escovopsis* (note that direct here refers to infection of the susceptible symbiotic partner, i.e. the fungal symbiont) did not differ significantly from those of control ants, confirming previous findings of *Escovopsis* not being harmful to ants (Reynolds & Currie 2004; Yek *et al.* 2012*b*). Direct inoculation of *Escovopsis* on the fungus garden (EwFungus) did not cause ants to suffer significantly higher mortality than did indirect inoculation (EwAnts) (Wilcoxon $\chi^2 = 0.83$, df = 1, $P = 0.3626$). As expected, infection treatments with *Escovopsis* and controls never produced sporulating cadavers, but 100% and 90% of the

respective ant cadavers from the direct and indirect inoculations with *Metarhizium* produced characteristic conidiophores after 10 days (G_{Het} = 97.60, df = 14, $P < 0.0001$; Fig. 1b). Survival patterns differed among the three colonies (LR χ^2 = 22.21, df = 2, $P < 0.0001$; Fig. 1a). Colony Ae363 appeared to be more robust compared to colonies Ae226B and Ae376B, as *Metarhizium*-induced mortality came later and with a lower proportion of dead ants sporulating (Fig. 1a-b).

The occurrences of allo-grooming ($F_{2,4}$ = 6.00, P = 0.0100), garden-grooming ($F_{2,4}$ = 6.99, P = 0.0060), and immobility inside the fungus garden ($F_{2,4}$ = 13.18, P = 0.0005) were significantly different between fungal inoculation treatments. Ants spent significantly more time being immobile in the controls compared to any of the treatments (Fig. 2a). Ants also performed significantly more allo-grooming when fungal conidia were directly applied on them rather than indirectly via the fungus garden (Fig. 2b), and they performed more garden-grooming when fungal conidia were applied on the fungus garden rather than on the ants (Fig. 2c). There were no differences in the occurrences of self-grooming ($F_{2.4}$ = 2.36, P = 0.1228; Fig. 2d) and no significant changes in garden biomass before and after fungal treatments ($F_{2,4}$ = 1.18, P = 0.3766; Fig. 2e), although the inoculations of gardens induced somewhat elevated losses compared to controls and inoculations of ants. Thus, both pathogens induced the expected behavioral responses, but the *Escovopsis* inoculations were controlled by the ants and caused little or no lasting damage (in terms of garden loss), whereas the *Metarhizium* inoculations were 100% lethal for the ants.

Overall gene expression

In order to monitor gene expression changes in the ants after the fungal pathogen challenges, we performed RNA-seq analyses. Whole ants were used rather than specific body parts as many

different physiological processes could potentially be affected by the treatments (e.g. immune reactions, neurological processes, metabolic processes etc.), and we aimed to capture these overall effects. For each of the 15 RNA-seq samples about 10 million usable reads were generated, and the sequence and its mate pair could be mapped to the genome within an appropriate distance for more than 85 % of these (Table 1). Gene expression levels were measured in fragments per kilobase of exon per million fragments of mapped genome sequence (FPKM), a normalized measure of read-density that allows gene expression levels to be compared between samples (Mortazavi *et al.* 2008). This resulted in 24,330 genes in the MbAnts treatment, 22,215 genes in the MbFungus treatment, 21,653 genes in the EwAnts treatment, 21,660 genes in the EwFungus treatment, and 21,313 genes in the controls. To assess the dynamics of gene expression across the fungal treatments, we examined the differences in gene expression levels between each of the fungal treatments relative to the controls, and found in total 375 genes (ca. 2%) that appeared to show significant expression changes, i.e. *p* values significantly lower than the FDR (False Discovery Rates) after using Bonferroni corrections for multiple testing (Table S1).

The expression patterns of these genes in the different fungal treatments relative to the controls are summarized in Fig. 3. Overall, 172 genes were differentially expressed in MbAnts, 109 genes in MbFungus, 74 genes in EwAnts and 149 genes in EwFungus treatments, of which only 7 genes (1.9 %) overlapped for all the fungal treatments (Fig. 3a). Of these differentially expressed genes, 239 (64 %) were up-regulated (positive $\log_2$ values) and 144 (38 %) were down-regulated (negative $\log_2$ values) relative to controls (Fig. 3b-c). Note that these percentages do not add up to 100 %, because the same genes may be up-regulated in some treatments and down-regulated in others.

We found a statistically significant effect of fungal treatments on the distribution of genes in the up- and downregulated categories ($\chi^2 = 94.40$, df = 3, $P < 0.0001$). The MbAnts treatment caused significantly more genes to be up-regulated than down-regulated (141 vs. 31 genes; $\chi^2 = 70.35$, df = 1, $P < 0.0001$), whereas the opposite pattern was found in the EwFungus experiment (44 up-regulated vs. 105 down-regulated genes; $\chi^2 = 24.93$, df = 1, $P < 0.0001$). The two indirect inoculation experiments showed intermediate values, with MbFungus having about equal amounts of up- and down-regulated genes (57 vs. 52; $\chi^2 = 0.23$, df = 1, $P = 0.6320$) and EwAnts having more up- than down- regulated genes (50 vs. 24; $\chi^2 = 9.13$, df = 1, $P = 0.0025$). The least unique gene expression profile (i.e. sharing most genes with other treatments) was found in the EwAnts treatment, in which only 32 % (24 out of 74) of the genes were not found differentially expressed in any other experiment (Fig. 3a), consistent with expectation as this treatment should be least dangerous for the ant-garden symbiosis, and thus induce relatively non-specific responses.

Direct and indirect inoculations

Metarhizium inoculations onto the ants were administered either directly (MbAnts) or indirectly through the fungus garden (MbFungus). From the ants' perspective these two routes of infection represent a dosage difference as conidia present on the fungus garden can either be groomed off or avoided by the ants (Reber *et al.* 2011). Direct inoculations with *Metarhizium* (MbAnts) resulted in an overall up-regulation of differentially expressed genes with a median $\log_2$ value of 3.23, whereas indirect inoculations of *Metarhizium* via the fungus garden (MbFungus) resulted in a much lower degree of overall up-regulation (median $\log_2$ value 0.32) (Fig. 3b). Limiting the analyses to immune-related genes, direct inoculations with *Metarhizium* (MbAnts) likewise resulted in overall up-regulation of genes (median $\log_2$ value 2.47) while indirect *Metarhizium*

inoculation (MbFungus) resulted in a weaker up-regulation of genes (median $\log_2$ value 0.64) (Fig. 4).

Escovopsis inoculations of fungus gardens were also administered directly (EwFungus) or indirectly via the ants (EwAnts) and thus also represented dosage differences, as ant inoculations (EwAnts) will only partially be passed on to gardens because *Escovopsis* conidia will be removed by grooming and ants with *Escovopsis* conidia will avoid contact with the fungus garden (Currie & Stuart 2001). Direct inoculations with *Escovopsis* on fungus gardens (EwFungus) resulted in an overall down-regulation of differentially expressed ant genes (median $\log_2$ value -0.96), whereas indirect inoculations of *Escovopsis* through the ants (EwAnts) resulted in an overall up-regulation of differentially expressed genes (median $\log_2$ value 2.05), suggesting that the physiological responses of ants to these two *Escovopsis* treatments were very different (Fig. 3b-c). Limiting the analyses to immune-related genes resulted in a similar pattern (Fig. 4) although indirect inoculations of *Escovopsis* (EwAnts) gave a much weaker up-regulation of genes (median $\log_2$ value 0.27) than when all genes were taken into account. This suggests that *Escovopsis* infections do not particularly challenge the ant immune system, consistent with *Escovopsis* not being a pathogen of ants.

In order to compare gene expression patterns between treatments we plotted the $\log_2$ values for genes that had a significant expression change in at least one of the two compared treatments (Fig. 5; plotting the highlighted values in Table S1). As expected from the mortality patterns (Fig. 1a), direct and indirect *Metarhizium* inoculations were significantly positively correlated (ANOVA $F_{1,246} = 44.88$, $P < 0.0001$), whereas direct and indirect *Escovopsis* inoculations were not (ANOVA $F_{1,198} = 2.24$, $P = 0.1357$), and with only direct fungus-garden inoculation having a consistent down-regulation effect (Fig. 5a-b). Fig. 5a further indicates that

direct *Metarhizium* inoculations have a stronger up-regulating and weaker down-regulating effect than indirect inoculations, as the fitted correlation axis had a slope > 1. Focusing on comparisons between targets (Fig. 5c–d), inoculations on both ants and fungus garden were significantly positively correlated (ants, ANOVA $F_{1,208} = 54.12$, $P < 0.0001$; fungus garden ANOVA $F_{1,224} = 198.10$, $P < 0.0001$). Both *Metarhizium* and *Escovopsis* inoculations induced up-regulation of many expressed genes, but the degree of up-regulation was higher in the former than in the latter type of inoculation (slope correlation axes in Fig. 5c > 1). The opposite pattern was observed when comparing inoculations administrated via fungus gardens (Fig. 5d) where most differentially expressed ant genes were down-regulated. Also here the slope of the fitted correlation axis was > 1, consistent with *Escovopsis* being a direct pathogen of fungus gardens and *Metarhizium* not.

Differentially expressed immune-related genes

For functional annotation of immune-related genes, we focused on the 375 genes with significant expression changes (Fig. 3). Among these we identified 57 immune-related genes that were differentially expressed in one or more of the four treatments: 8 ‘recognition genes’, 36 ‘signaling genes’, 4 ‘stress-related genes’, and 9 ‘effector genes’ (Fig. 4, Table S2).

Direct inoculations with *Metarhizium* (MbAnts) resulted in an overall up-regulation of immune-related genes with a median $\log_2$ value of 2.47 (Fig. 4). This trend was experienced in all four gene categories, with only three genes being down-regulated, mainly from the signaling category (Fig. 4, Table S2). The indirect *Metarhizium* inoculation (MbFungus) likewise resulted in an overall up-regulation of immune-related genes (median $\log_2$ value 0.64) although this was mainly caused by up-regulation of effector genes, while signaling genes were slightly up-

regulated and recognition genes were unaffected (Fig. 4). Only 12 immune genes were found to be affected in the indirect *Escovopsis* inoculation experiment (EwAnts). Overall, there was a slight tendency towards up-regulation (median $\log_2$ value of 0.27) mainly caused by signaling genes, while stress response genes were unaffected and only one recognition and one effector gene were affected, the former being down-regulated and the latter being up-regulated (Fig. 4). Direct *Escovopsis* inoculation on fungus garden (EwFungus) was the only treatment resulting in an overall down-regulation of ant immune-related genes (median $\log_2$ value -0.60), a trend that was observed in three of the four gene categories, with only the effector gene category being up-regulated and for two genes only.

DISCUSSION

Our results show that inoculations with a deadly ant fungal pathogen solicit a series of immediate defense behaviors and lead to significant up-regulation of gene expression, both in general and for immune genes in particular, and more so when ants were inoculated directly rather than indirectly via their mutualistic fungus garden. However, inoculations with a fungus-garden pathogen did neither affect ant survival nor lead to significant garden loss, but resulted in significant down-regulation of gene expression in ants, quite possibly to enable them to meet the challenges as these inoculations did not ultimately result in lasting fungus-garden infection.

Survival and prophylactic responses to inoculations with fungal pathogens

Leaf-cutting ants are extremely effective in recognizing dangerous material, such as conidia of entomopathogenic fungi on themselves (Jaccoud *et al.* 1999) and on their fungus gardens (Currie & Stuart 2001). As soon as fungal conidia make contact with the ants' bodies or direct

environments, they trigger a rapid response in the form of increased activity such as self-grooming and activating otherwise immobile ants in the fungus garden (Fig. 2a & d). This is consistent with ants generally engaging in intensive prophylactic behaviors to rid themselves and nestmates of infectious fungal conidia (Currie & Stuart 2001; Reber *et al.* 2011). These prophylactic responses seem to be location specific, with conidia-inoculations on the ant cuticle (MbAnts and EwAnts) triggering more allo-grooming and conidia on the fungus garden fragments (MbFungus and EwFungus) triggering more garden-grooming (Fig. 2b-c).

Regardless of whether the *Metarhizium* conidia were directly infecting the ants or indirectly through the fungus garden, the inoculation doses were sufficient to cause 100% mortalities (Fig. 1a). We can thus infer that the immune systems of ants in the parallel experiment where we obtained gene expression data were indeed severely challenged. However, the mortality rate was significantly lower in the indirect treatment (MbFungus) than in the direct treatment (MbAnts) (Fig. 1a), and we would thus expect a less pronounced immune gene response in the indirect treatment. The correlation slope > 1 in Fig. 5a suggests that this was indeed the case. The inoculations with *Escovopsis* induced prophylactic responses (Fig. 2a-d) even though they are harmless for the ants (Fig. 1a-b) (See also Yek *et al.* 2012*b*), but these behaviors were highly efficient in preventing *Escovopsis* from becoming established, as garden masses were only slightly and not significantly affected (Fig. 2e). As the large workers in our experiments had very few actinomycetes on their cuticle, we believe that this mostly reflects that non-stressed colonies can control *Escovopsis* pathogens via efficient grooming and killing conidia in their infrabuccal pellets (Little *et al.* 2003). The harmlessness of *Escovopsis* conidia toward ants would make it reasonable to infer that the innate immune system of the ants was not severely challenged by our *Escovopsis* treatments. This is supported by our finding that the

expression of only very few immune genes was up-regulated by the application of *Escovopsis* conidia to either the ants or their fungus garden (Fig. 4).

Overall patterns of differentially expressed genes

Comparing treatments using different pathogens on the same target (Fig. 5c-d) revealed relatively similar slopes of gene expression change, but with up-regulation being typical for challenges of the ants and down-regulation for challenges of the fungus. A similar correlation was also observed when *Metarhizium* was used to inoculate either the ants or the fungus gardens (Fig. 5a), but the responses to treatments with *Escovopsis* on either the ants or the fungus gardens did not show any significant correlation (Fig. 5b). These findings suggest that the physiological responses of the ants are relatively unspecific, depending more on the target of inoculation than on the specific pathogen used. Finding little specificity may imply that even though *Escovopsis* is harmless to the ants, it still elicits an initial physiological response similar to the one induced by infection with the lethal pathogen *Metarhizium*. However, by comparing the changes in immune gene expression in the two experiments it became evident that the similarities are mostly due to expression patterns of signaling genes, while changes in effector gene expression were much more different. It seems, therefore, that only the upstream immune pathway genes are activated by *Escovopsis* inoculations, but that the downstream effector genes are not activated because *Escovopsis* is unable to harm the ants, and that natural selectionhas somehow established this fine-tuned response system.

Expression patterns of immune-defense genes

Our immune-related gene expression analysis showed that each of the immune response phases had representative genes that were differentially expressed across our inoculation treatments (Fig. 4; Table S2). Besides confirming the well-known genetic components involved in innate immunity, our differential gene expression data also generated a high number of uncharacterized (hypothetical) proteins that might function in cellular innate immunity. However, until further experimental evidence can be obtained, we refrain from including these putative immune-related genes in our discussion.

Immune responses begin with molecular recognition of microbial surface molecules producing immune signals. The up-regulation of ’recognition‘ genes observed in the direct *Metarhizium* inoculation treatment indicates that the innate immune system reacted to the successful penetration of the fungal conidia into the ants’ haemolymph. This response was less pronounced when *Metarhizium* infections happened more slowly via indirect inoculations of the garden.

Among the significantly up-regulated ‘recognition’ genes was a member of the *Dscam* (Down syndrome cell adhesion molecule) family ($\log_2$ fold change 0.53) and a ß-1,3-glucan-binding molecule ($\log_2$ fold change 1.39). The *Dscam* family contains a hyper-variable member (*Dscam-hv*) that can produce thousands of isoforms via alternative splicing, and together with ß-1,3-glucan-binding has been implicated in bacterial infection recognition in *Drosophila* (Armitage *et al.* 2012; Hoffmann 2003). The up-regulated *Dscam* gene in the present study is an ortholog of one of the other family members that is not believed to be hypervariable (Armitage *et al.* 2012) but may apparently still play a role in immune defence.

Immune signals produced by molecular recognition are modulated and/or transduced through four signaling pathways (Toll, IMD, JAK/STAT and JNK) before activating effector

mechanisms. Genes belonging to this 'signaling' category were found to be up-regulated after direct inoculations with *Metarhizium* (MbAnts) and to a lesser extent after inoculations with *Escovopsis* (EwAnts) and fungus-garden infections with *Metarhizium* (MbFungus). In contrast, inoculations of the fungus garden with *Escovopsis* caused a down-regulation of signaling genes, indicating that alternative signaling pathways may have been activated instead.

Current immunology theory suggests that fungal and bacterial challenges activate Toll and IMD pathways, whereas JAK/STAT and JNK pathways are activated after bacterial challenges and septic injury such as wounding, suggesting a correlation between cellular immunity and stress responses (Hoffmann 2003; Silverman *et al.* 2003). To elucidate any correlations between these stress and immune responses we functionally categorized an additional gene set as being 'Stress-related'. Only four such putative 'Stress-related' genes were identified, which seems to imply that stress reactions and immune responses were not correlated in our experimental design, contrary to immune challenge studies in other insects (Erler *et al.* 2011; Ratzka *et al.* 2011; Xu & James 2012). This could perhaps be due to the 'wounding' treatments that were customarily used to induce cellular innate immune responses in these studies, making it more likely to find correlations between stress and immune gene expression.

Immune effectors are required to target and neutralize the source of the immune signals. Across our inoculation treatments, effector genes were consistently up-regulated and most pronouncedly so in the MbAnts and MbFungus experiments (Fig. 4). Looking at the specific effector genes across the four treatments showed that the MbAnts and MbFungus treatments up-regulated three of the same effectors, while the EwAnts and EwFungus treatments had one common effector up-regulated. Despite the low number of genes, this might indicate a good

correlation between the pathogen used and the effector genes changing expression due to the inoculation.

The antimicrobial peptides (AMP) are the best studied effector molecules of insect immune systems. In our data, two AMPs (abaecin and hymenoptaecin) were up-regulated, and represented some of the most highly up-regulated genes in our data set, with $\log_2$ values ranging from 2.4 to 8.4, indicating ca. 5-300 fold change of differential expression (Table S2). Recent findings on hymenoptaecin by Ratzka *et al.* (2012) indicate that the three hymenoptaecin genes annotated in the *A. echinatior* genome probably should be merged into a single gene, consistent with the two hymenoptaecins identified in the present study having very similar expression patterns. The highly up-regulated abaecin and hymenoptaecin were mostly found after the *Metarhizium* infections, both in direct (MbAnts) and indirect inoculations (MbFungus), suggesting that abaecin and hymenoptaecin may in fact play an important role in the immune resoponse to *Metarhizium* infections. Abaecin and hymenoptaecin were first isolated in the honey bee (Casteels *et al.* 1990; Casteels *et al.* 1993) and are both characterized as antibacterial response peptides, but may in fact have a broader target spectrum as indicated by our results. Interestingly, the multi domain structure of the *A. echinatior* hymenoptaecin reported by Ratzka *et al.* (2012) may allow for multiple proteins to be expressed from the same gene by alternative splicing, each potentially targeting a different set of pathogens. In contrast, the antimicrobial peptide defensin (Viljakainen & Pamilo 2005) was not found to be significantly up-regulated after any of our inoculation treatments, which may indicate that defensin is not involved in immune responses towards fungal pathogens.

Fungus garden disease and ant immune response

Direct *Escovopsis* inoculations of fungus gardens induced a general down-regulation of gene expression in the ants, which was also evident when only immune genes were considered. This could be due to trade-offs in physiological pathways, for example inactivation of innate immunity towards insect pathogens so that other physiological pathways involved in neurological and locomotive processes could be prioritized because they play a key role in sustaining prophylactic behaviors (Fernández-Marín *et al.* 2009). Studies of innate immune systems of social insects (e.g. bumblebees, Mallon *et al.* 2003; honeybees, Weinstock *et al.* 2006; other ants, Viljakainen & Pamilo 2008) have shown that trade-offs between general and specific immune responses may occur. In leaf-cutting ants, innate phenoloxidase (PO) and ProPO immune responses have been documented (Armitage & Boomsma 2010) in addition to sex-specific encapsulation responses (Baer *et al.* 2005). A recent study in another leaf-cutting ant from the same area in Panama, *Atta colombica*, indicated that individual immune defenses are costly (Baer *et al.* 2006), and it is also known that *Escovopsis* infections in *Acromyrmex* can induce an increase in the abundance of cuticular actinomycete bacteria (Currie *et al.* 2003) at considerable metabolic cost (Poulsen *et al.* 2003). Almost none of the most downregulated genes in the experiment could be functionally annotated by our BLAST searches, which makes it difficult to understand the physiological implications of these findings. However, direct comparison of the GO categories of up and down regulated genes suggests an enrichment of downregulated genes involved in carbohydrate and lipid metabolic process (Table S1). This seems consistent with the hypothesis that *Escovopsis* conidia might induce significant changes in ant metabolic allocation patterns because they threaten to eliminate the vital fungus garden symbiont rather than being harmful for the ants themselves. A similarily pronounced down-regulation of genes was not observed when *Escovopsis* conidia were applied to the ants, or when *Metarhizium* conidia were

applied to the fungus garden. There was, however, a very strong correlation of gene expression patterns between the EwFungus and the MbFungus treatments (Fig. 5d), indicating that similar physiological responses may have taken place in the ant bodies, with *Escovopsis* inoculations on gardens soliciting stronger up- or down-regulations than *Metarhizium* inoculations on fungus gardens (slope in Fig. 5d > 1). The fact that the relatively drastic down-regulation of gene expression in the ants only happened in the EwFungus experiment suggests that it is a specific reaction to inoculation of the fungus-garden by *Escovopsis*, the occurrence of which might also be communicated by the fungus garden to the ants by yet unknown signals (North *et al.* 1999). Further targeted experiments will be needed to elucidate the possible mechanisms behind *Escovopsis* induced changes in gene regulation in *A. echinatior* fungus-farming ants.

In sum, our study shows that leaf-cutting ants have elaborate defense repertoires to counteract fungal infections of both major components of their symbiotic farming colonies, that these prophylactic defenses are expressed immediately, and that they can be efficient in keeping the fungal symbiont free from disease. Individual ants do succumb to infections with insect pathogenic fungi, but this will be unlikely to threaten colony survival unless conidia doses are massive.. We further show that the physiological responses by the ants depend on the nature of the inoculation challenges, and that trade-offs between the various defense mechanisms that are available to the ants may occur. These results are consistent with leaf-cutting ants having evolved multiple fine-tuned adaptations to cope with the common disease challenges of their natural environments.

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Data accessibility

Raw Illumina sequence reads were deposited at The Sequence Read Archive (SRA) with the accession number ERP002002 (http://www.ebi.ac.uk/ena/data/view/ERP002002). Raw data on fungal infection experiments were deposited in the Dryad depository (doi:10.5061/dryad.nb35n).

Author contributions

S.H.Y., J.B.B. and M.S. designed the study, analyzed the data and wrote the manuscript. S.H.Y. and M.S. performed the experiments.

Table 1. The samples used for RNA-seq, the number of high quality (clean) reads for each sample, and the number of clean reads that mapped to the genome within a proper distance of the mapped mate pair. The 15 sample IDs represent unique identification codes for the different treatments and colonies.

Sample ID	Treatment	Colony	Clean reads	Mapped and properly paired reads
D1	MbAnts	Ae363	12,431,574	11,401,788
D2	MbFungus	Ae363	12,138,055	11,305,658
D3	EwAnts	Ae363	11,180,006	10,322,488
D4	EwFungus	Ae363	11,853,276	10,914,108
D5	Control	Ae363	12,505,366	11,320,934
E1	MbAnts	Ae376	10,894,885	9,289,812
E2	MbFungus	Ae376	11,684,876	10,470,204
E3	EwAnts	Ae376	11,552,533	10,549,868
E4	EwFungus	Ae376	10,971,843	9,935,394
E5	Control	Ae376	10,226,125	8,938,540
F1	MbAnts	Ae226B	12,411,699	11,387,804
F2	MbFungus	Ae226B	12,050,785	10,535,894
F3	EwAnts	Ae226B	12,025,652	10,907,438
F4	EwFungus	Ae226B	12,079,257	10,655,842
F5	Control	Ae226B	12,328,936	11,047,388

Figures

Figure 1. (a) Survival curves of large *A. echinatior* workers in the three experimental colonies subjected to the experimental fungal treatments or controls: MbAnts (*Metarhizium brunneum* inoculations of ants), MbFungus (*Metarhizium* inoculations of fungus garden), EwAnts (*Escovopsis weberi* inoculations of ants), and EwFungus (*Escovopsis* inoculations of fungus garden). Controls had both unchallenged ants and fungus garden fragments. Survival of ants was monitored for 10 days following fungal inoculations. (b) The proportion of ant cadavers from the experimental treatments that showed characteristic *Metarhizium* sporulation three weeks after fungal inoculations. None of the *Escovopsis* inoculations or controls showed *Metarhizium* sporulation.

Figure 2. Behavioral responses (a-d), garden loss (e), and overall design (f) in experiments testing the effects of direct (host) and indirect (via symbiont) *Metarhizium* and *Escovopsis* inoculations: (a) immobile ants inside the fungus gardens, (b) allo-grooming, (c) garden-grooming, and (d) self-grooming presented as boxplots, where the bottom and top represent the minimum and maximum number of occurrences and the middle band of the box the median. (e) Similar boxplots for percentage garden loss recorded at the end of the 10 day survival experiment (initial garden mass minus final garden mass). Photo credits: fungus garden Janni Larsen, and *Acromyrmex* worker Klaus Lechner.

Figure 3. Venn diagrams showing the overlapping profiles of genes differentially expressed after each of the inoculation treatments relative to controls: (*a*) overall profile of all 375 differentially expressed genes, (*b*) profile of the 239 up-regulated genes, and (c) profile of the 144 down-

regulated genes. Tables beside the Venn diagrams give the total number of genes (N) and the median, mean, and standard deviations (SD) across the three colonies of the $\log_2$ gene expression ratios for the respective fungal treatments. See Table S1 for the data on which these diagrams and summary statistics are based. Note that the numbers of (b) and (c) do not necessarily add up to the numbers in (a), because the same genes may be up-regulated in some treatments and down-regulated in others.

Figure 4. Expression profiles of immune-related genes in the categories,'recognition', 'signaling', 'stress-related', and 'effector', across the four fungal treatments relative to controls. The red/blue colors indicate the intensity of up (red) and down (blue) gene regulations. Hierachical ordering grouped MbAnts-EwAnts and MbFungus-EwFungus immune gene expressions as most similar to each other. The table at the bottom of the figure gives the median values of the $\log_2$ fold changes with bracketed figures indicating the number of significantly over- or under-expressed genes per category.

Figure 5. Scatter plots showing pairwise comparisons of gene expression levels in the different experiments, using only genes that showed significantly changed expression in at least one of the treatments. (a) direct and indirect *Metarhizium* inoculations, (b) direct and indirect *Escovopsis* inoculations, (c) both types of fungal conidia on ants, and (d) both types of fungal conidia on fungus garden fragments. Dotted horizontal and vertical black lines represent the null hypotheses of no change in expression (i.e. $\log_2$ value = 0). Red lines are the best-fit correlations based on the differentially regulated genes, with their correlation coefficient (r) and slope values given in

the scatter plots. Values along the axes represent $\log_2$ fold-changes of up-regulations (positive values) or down-regulations (negative values).

Supporting information

Additional Supporting Information may be found in the online version of this article.

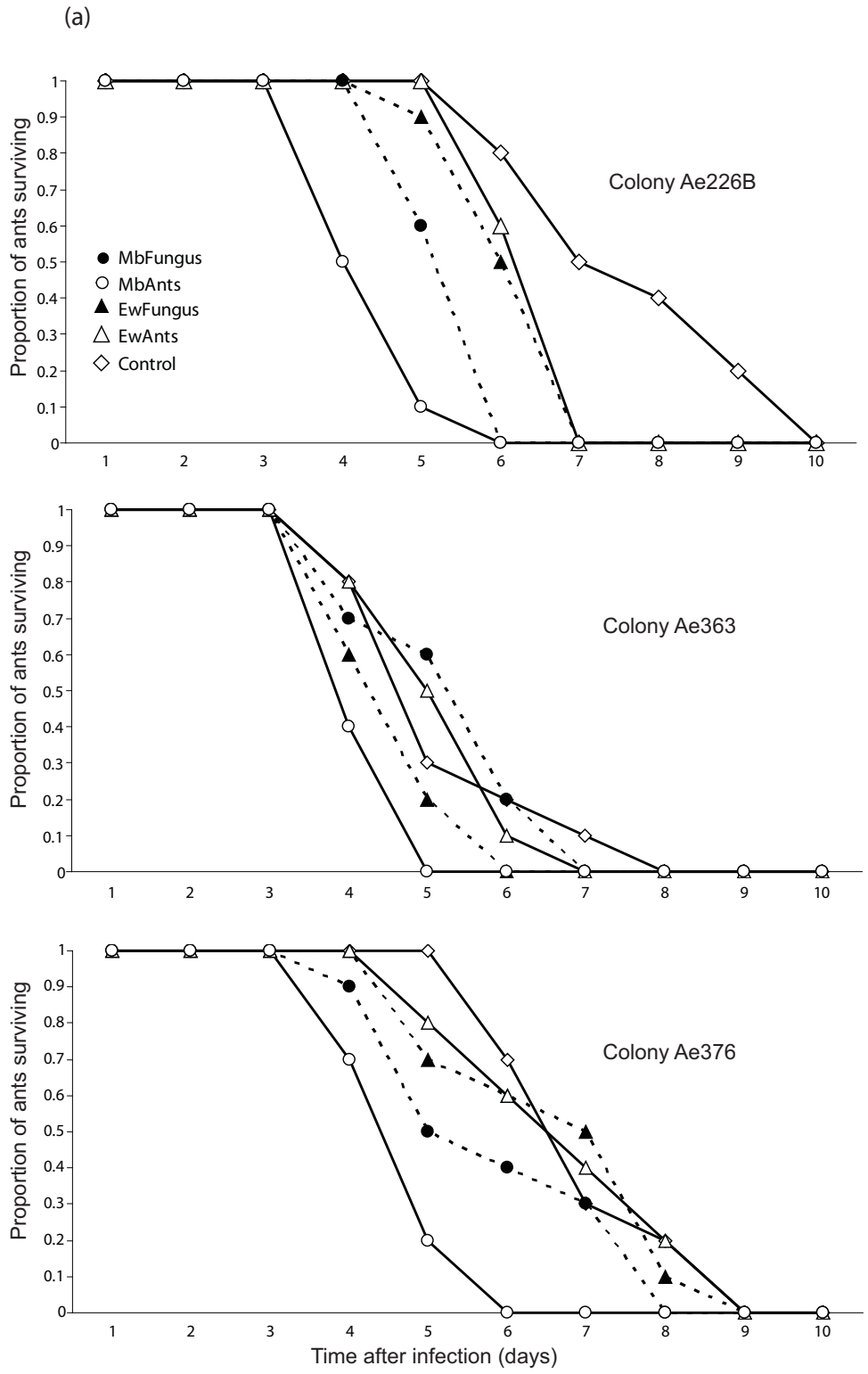
Table S1 Expression differences and Blast hits of differentially expressed genes.

Table S2 Expression differences of immune-related genes.

Table S3 Expression levels of differentially expressed genes.

Appendix S1. Methods and Materials. Section 1: Fungal inoculation experiments. Section 2: RNA extraction protocol.

Figure 1


(a)
Proportion of ants surviving
Colony Ae226B
MbFungus
MbAnts
EwFungus
EwAnts
Control
Colony Ae363
Colony Ae376
Time after infection (days)

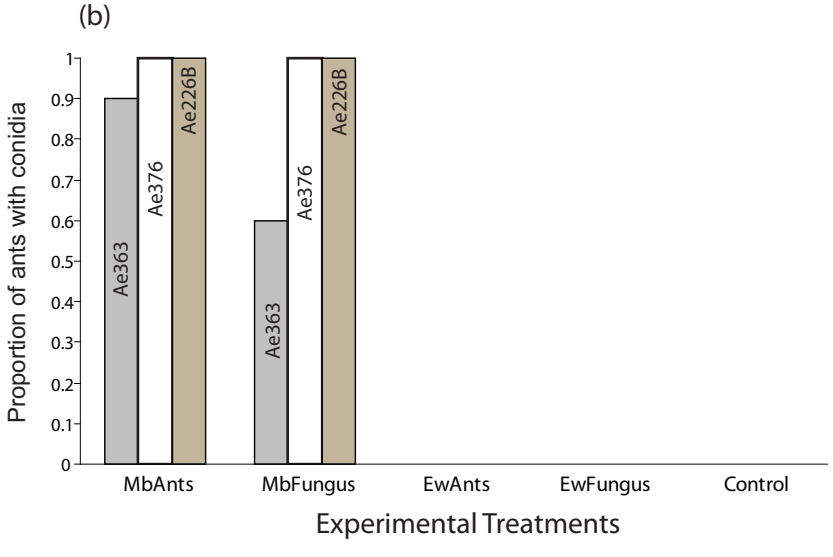
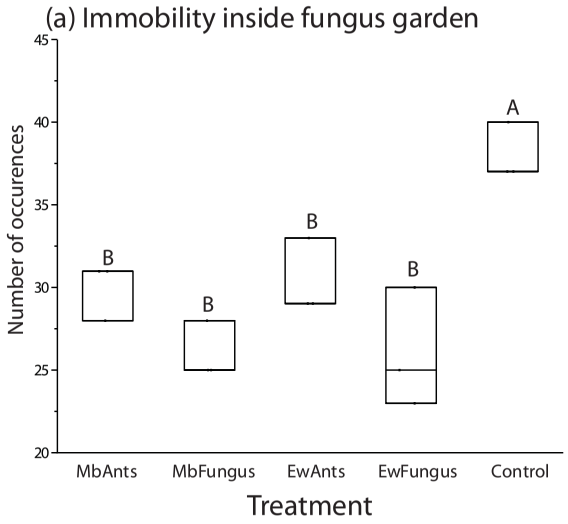
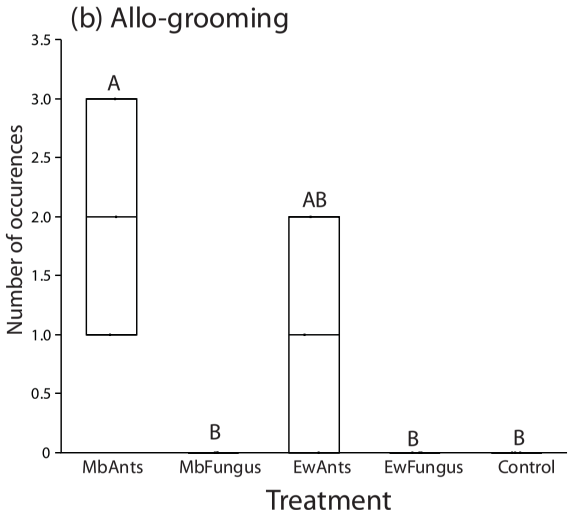
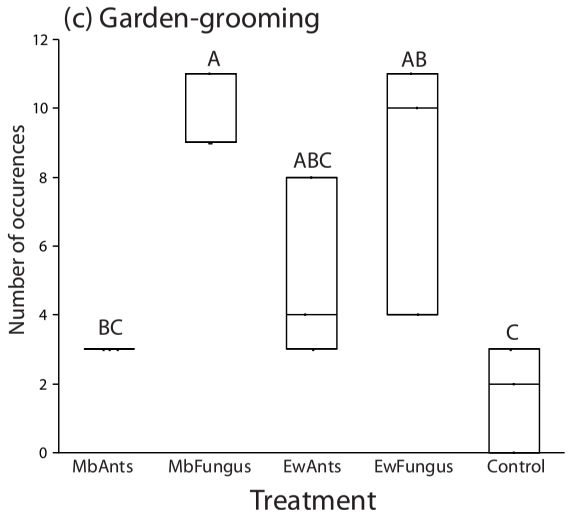
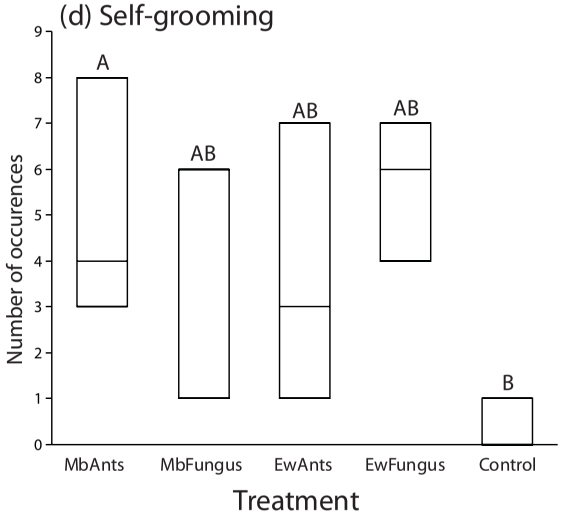

(b)
Proportion of ants with conidia
Ae363
Ae376
Ae226B
MbAnts
MbFungus
EwAnts
EwFungus
Control
Experimental Treatments

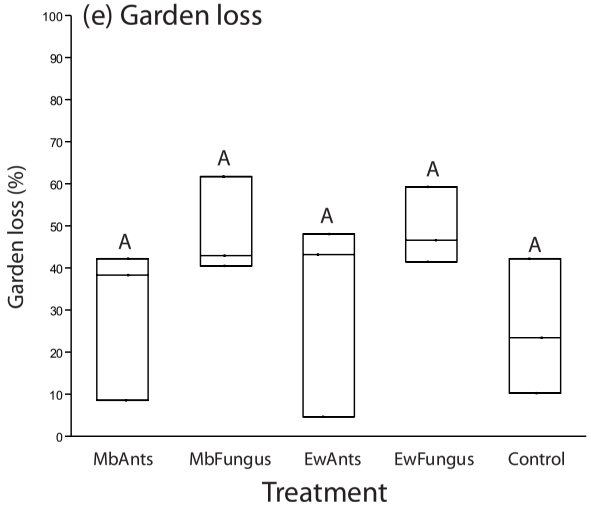
Figure 2


(a) Immobility inside fungus garden
Number of occurences
45
40
35
30
25
20
B
B
B
B
A
MbAnts
MbFungus
EwAnts
EwFungus
Control
Treatment


(b) Allo-grooming
Number of occurences
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
A
B
AB
B
B
MbAnts
MbFungus
EwAnts
EwFungus
Control
Treatment


(c) Garden-grooming
Number of occurences
12
10
8
6
4
2
0
BC
A
ABC
AB
C
MbAnts
MbFungus
EwAnts
EwFungus
Control
Treatment


(d) Self-grooming
Number of occurences
9
8
7
6
5
4
3
2
1
0
A
AB
AB
AB
B
MbAnts
MbFungus
EwAnts
EwFungus
Control
Treatment


(e) Garden loss
Garden loss (%)
100
90
80
70
60
50
40
30
20
10
0
A
A
A
A
A
MbAnts
MbFungus
EwAnts
EwFungus
Control
Treatment

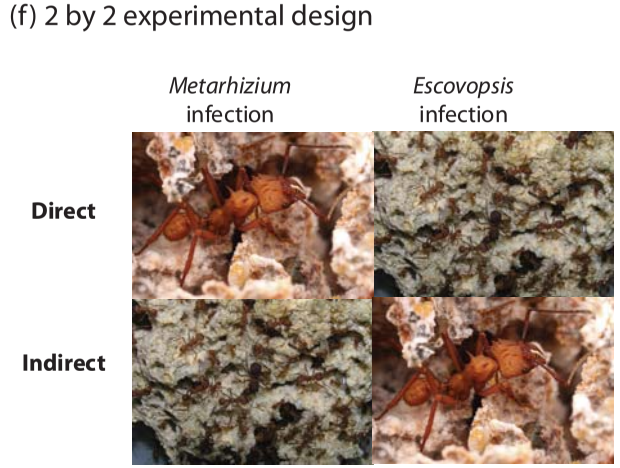
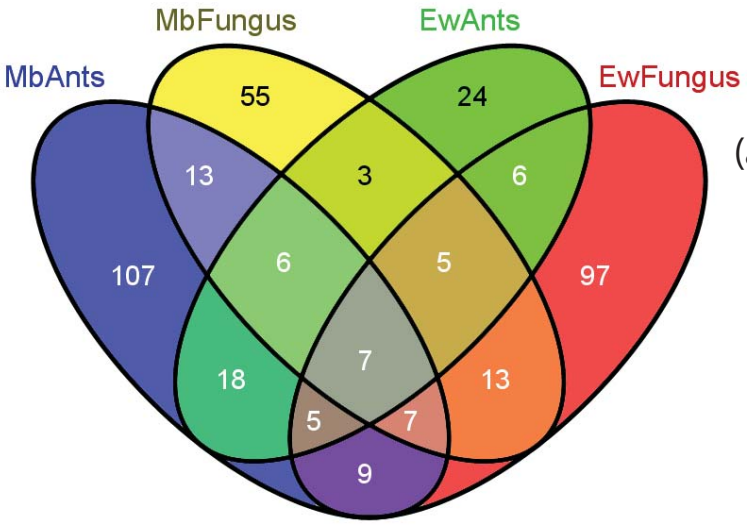
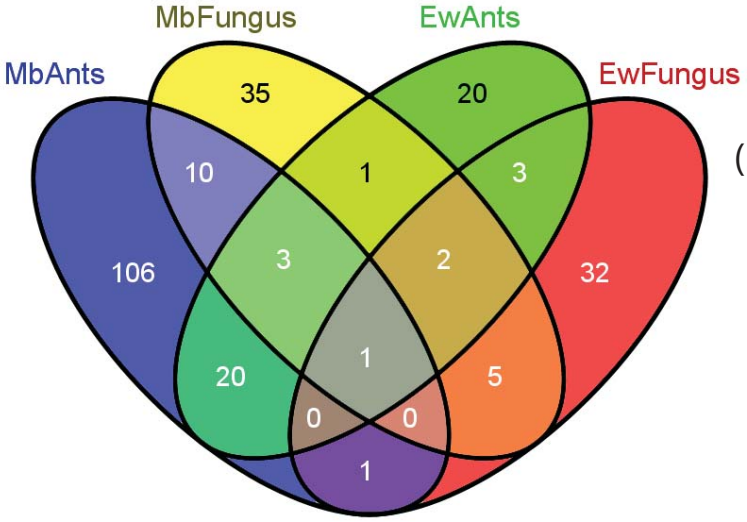

(f) 2 by 2 experimental design
Metarhizium infection
Escovopsis infection
Direct
Indirect

Figure 3



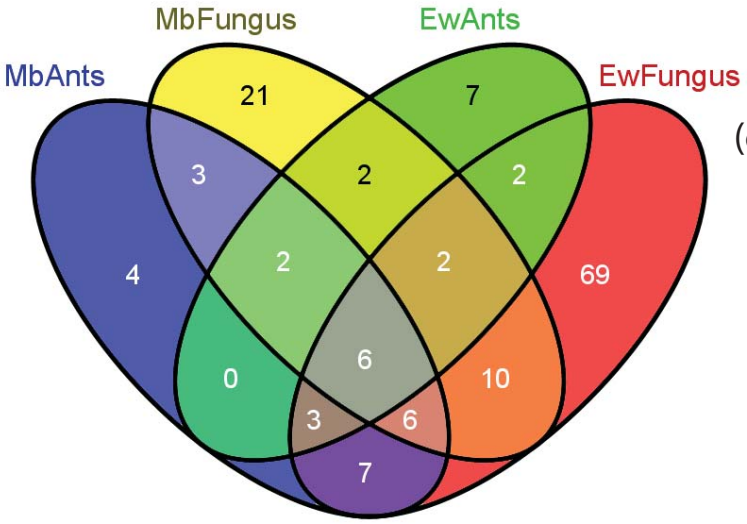
(a) overall profile of expressed genes

	MbAnts	MbFungus	EwAnts	EwFungus
Median	3.23	0.32	2.05	-0.96
Mean	3.63	-0.10	1.53	-1.67
SD	3.79	2.59	3.97	2.86
N	172	109	74	149



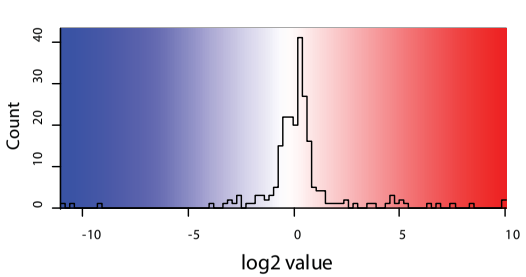
(b) profile of up-regulated genes

	MbAnts	MbFungus	EwAnts	EwFungus
Median	4.01	0.88	3.69	0.42
Mean	4.82	1.60	3.59	0.77
SD	3.06	1.53	2.45	1.15
N	141	57	50	44
Percentage	82%	52%	67%	30%



(c) profile of down-regulated genes

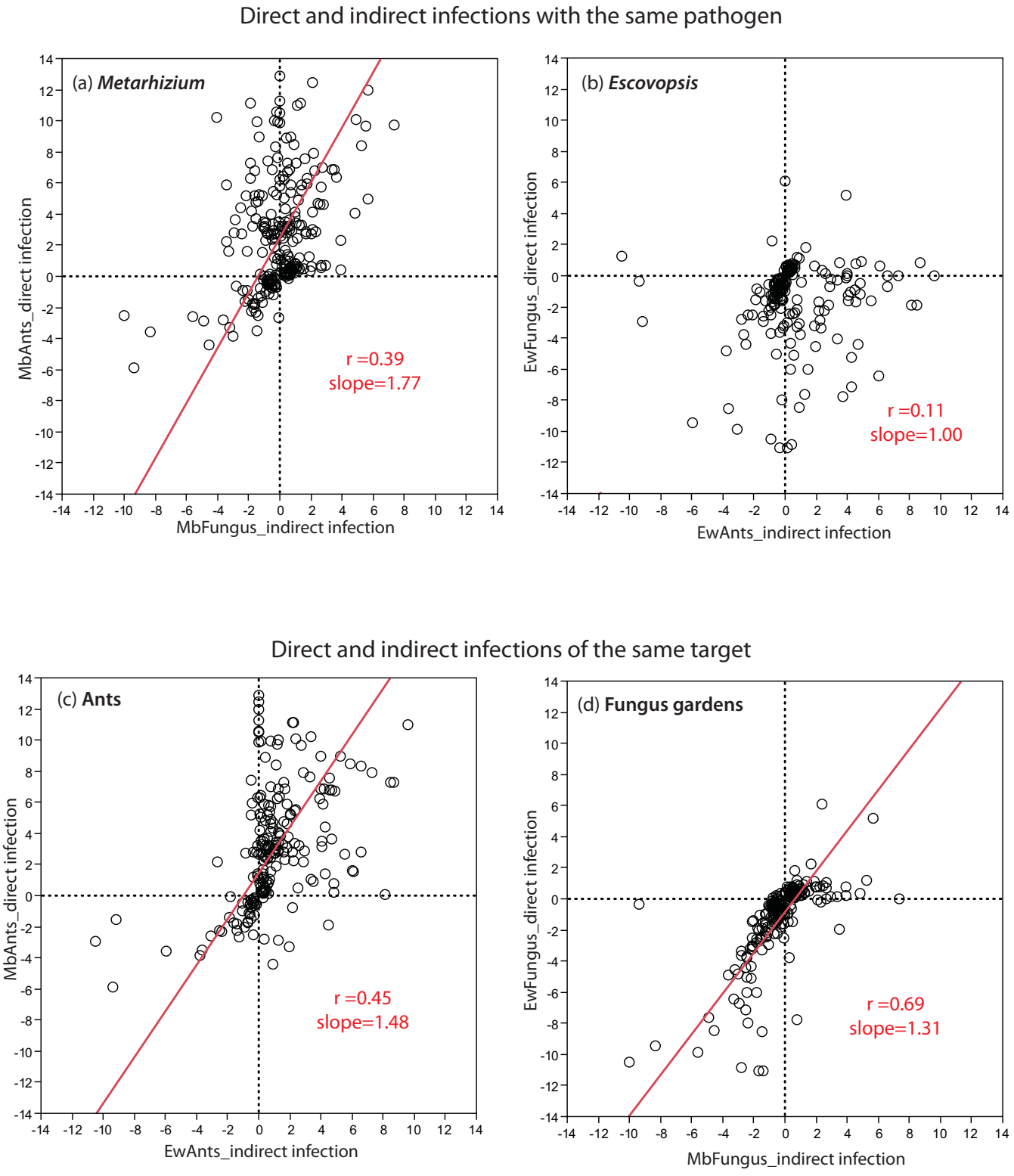
	MbAnts	MbFungus	EwAnts	EwFungus
Median	-1.67	-1.06	-1.71	-1.62
Mean	-1.79	-1.96	-2.75	-2.69
SD	1.14	2.20	3.01	2.74
N	31	52	24	105
Percentage	18%	48%	33%	70%



median $\log_2$value (gene number)

	EwFungus	MbFungus	EwAnts	MbAnts
Recognition	-0.90(2)	0(0)	-10.51(1)	4.58(5)
Signaling	-0.62(14)	0.37(13)	0.32(10)	0.89(12)
Response to stress	-0.16(2)	0.11(2)	0(0)	0.46(1)
Effector	0.32(2)	2.43(5)	0.28(1)	5.07(5)
Overall	-0.60(20)	0.64(20)	0.27(12)	2.47(23)

Figure 5


Direct and indirect infections with the same pathogen
(a) Metarhizium
MbAnts_direct infection
MbFungus_indirect infection
r =0.39
slope=1.77
(b) Escovopsis
EwFungus_direct infection
EwAnts_indirect infection
r =0.11
slope=1.00
Direct and indirect infections of the same target
(c) Ants
MbAnts_direct infection
EwAnts_indirect infection
r =0.45
slope=1.48
(d) Fungus gardens
EwFungus_direct infection
MbFungus_indirect infection
r =0.69
slope=1.31