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Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*

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ABSTRACT

Insects have a complex and highly successful immune system that responds specifically to different types of parasites. Different genotypes of a parasite species can differ in infectivity and virulence; which is important for host-parasite co-evolutionary processes, such as antagonistic, fluctuating selection. Such coevolution obviously requires a genetic basis, but little is known about how hosts immunologically respond to different genotypes. The common European bumblebee *Bombus terrestris* is infected by the highly prevalent trypanosome gut parasite, *Crithidia bombi*. Here we examined expression of 26 immunological and metabolic genes in response to infection by two clones of *C. bombi* and compared that with exposure to injection with a bacterial challenge. Exposure to the two clones of *C. bombi* elicits qualitatively different immune expression responses. Interestingly, infection with one clone results in up regulation of AMP's similar to bees given the bacterial challenge, while genes related to metabolism, signalling, and other effectors were similar between the two *Crithidia* exposures. Bees given different challenges were distinct enough to discern using linear discriminant analyses. We also found strong correlations, both positive and negative, among genes, which may shed light on how suites of genes are regulated and trade-offs in expression within this gene set.

1. Introduction

Parasites are an inescapable aspect of life and hosts are forced to interact with many different taxa of parasites, like viruses, bacteria, fungi, worms or parasitic arthropods (Windsor, 1998). Insect hosts recognize and respond to microbial invaders by initiating a number of interconnected immune pathways, in particular the Toll, Imd, JNK, and Jak/STAT pathways, culminating in the production of effector proteins such as antimicrobial peptides. Defense against larger parasites is by cellular encapsulation and the melanization response through the prophenol oxidase (PPO) cascade (for reviews see (Beckage, 2008; Cerenius and Söderhäll, 2004; Schmid-Hempel, 2011)). Some of these pathways are conserved across vast evolutionary time, such as the Toll pathway; this pathway is also important in developmental processes. Other pathways vary across insect species, like the Imd pathway, most of which is missing in pea aphids (Gerardo et al., 2010).

Regardless of the actual pathways, these defenses will require genes to be expressed to yield an effect. However, beyond some insect vectors of major human diseases and some well-studied model organisms (such as the honeybee and *Drosophila melanogaster*) relatively little is known about how insects immunologically respond to ecologically relevant parasites and, in particular, how gene expression might vary across host backgrounds and in response to different parasite genotypes. The issue is complicated by the fact that defense responses extend beyond the immune system. This includes modifications to behavioural and life-history traits, and the presence of symbionts, which can also play a role in determining resistance or the fitness loss after infection (Parker et al., 2011; Schmid-Hempel, 2011). Among organisms, socially living species as studied here, are faced with a double-edged sword of high density and high relatedness, both of which are conducive to parasite transmission, whereas cooperation, caste specialization, and spatial and behavioural structure ameliorate this risk. These adaptations to sociality may, in part, be responsible for the reduced immune system of honeybees relative to dipterans (Evans et al., 2006).

Different genotypes of a parasite differ in their infectivity and virulence (Carius et al., 2001; Ebert, 1994; Lambrechts et al., 2009, 2005; Sadd, 2011; Salvaudon et al., 2007) but remarkably little is known about whether hosts respond immunologically to different parasite genotypes in a discernibly different way. Examples of studies with this perspective include the crustacean *Daphnia*

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magna that only produces a cellular immune response when infected with specific strains of the castrating bacterium *Pasteuria remosa*, when these are able to infect that host genotype; presumably because these isolates are able to breach the gut and enter the hemocoel (Auld et al., 2012). Hymenopteran parasitoids with the immunosuppressive allele Ism^+ (for the *D. melanogaster* – *L. boulardi* system) prevent the encapsulation and melanization response of their host (reviewed in Nappi and Carton, 2001). Similarly, some lines of the human malaria causing parasite *Plasmodium falciparum* evade the *Anophales gambiae* TEP1 mediated immune response whereas another parasite genotype fails to evade this response leading to melanization of the parasite (Molina-Cruz et al., 2012). These examples represent cases in which there is parasite genetic variation that determines the success of evading the host immune system (parasitoid – *D. melanogaster* and, *P. falciparum* – *A. gambiae* systems) or when the parasite must travel across host tissues (*P. ramose* – *D. magna*, and *P. falciparum* – *A. gambiae* systems), thereby triggering different immune responses. Nevertheless, differential host genetic responses to different genotypes are an essential assumption of host-parasite coevolutionary scenarios, such as those assuming some kind of genotype–genotype interactions. These scenarios are of high relevance to, for example, problems of how genetic variation in host populations can be maintained (Hamilton, 1993; Peters and Lively, 1999).

We here use the bumblebee, *Bombus terrestris*, as our host species. It is a primitively eusocial insect with colonies of up to a few hundred workers born from a singly mated queen. *B. terrestris* is commonly infected by a trypanosome gut parasite, *Crithidia bombi*, which infects *per os* and resides solely in the gut. The parasite reduces the ability of queens to successfully found new colonies (Brown et al., 2003) but is relatively avirulent in workers (Brown et al., 2000). Infected workers do, however, die sooner than uninfected bees under harsh conditions (Brown et al., 2000). Different genotypes of *C. bombi* vary in their ability to infect different host genotypes (Mallon et al., 2003; Schmid-Hempel, 2001) and this pattern is moderated by ecological factors (Sadd, 2011). QTLs for some defence mechanisms have been identified (Wilfert et al., 2007), but the genes that are responsible for this host-parasite matching are not known. However, previous studies have identified a number of genes that change in transcription upon infection by *C. bombi* (Riddell et al., 2009, 2011; Schlüns et al., 2010) and the application of a bacterial stimulus (Erler et al., 2011). To better understand the ecologically relevant variation in the defence responses, we explored the profile of gene expression changes of 26 genes in response to challenge by two different genotypes (clones) of *C. bombi*, or in response to a bacterial insult with *Escherichia coli*. We chose candidate genes that span the traditional insect immunological pathways and broad immunological categories including recognition, signalling, effector, reactive oxygen species (ROS), melanization and metabolic genes. The bacterial challenge was chosen to put the response to different genotypes of *C. bombi* into a broader context by comparing these responses to a standard bacterial challenge.

2. Materials and methods

2.1. Experimental procedure

We collected wild queens of *B. terrestris* from a site in Neunforn (TG), northern Switzerland in spring 2011. We immediately checked their feces for existing *C. bombi* and *Nosema bombi* (a microsporidian parasite which can be visually identified by the presence of spores in the gut and feces) infections, and allowed them to establish colonies in the lab. The colonies used here were free of existing *C. bombi* or *N. bombi* infection. We removed the callow workers (adults that emerged within the last 24 h and have not yet developed full pigmentation) daily from the two experimental colonies (named colony 1 and 41). We kept individuals separately and when these workers were 10 days old they were randomly allocated to one of five exposure conditions. Bees were kept in isolation to prevent any effects of variation in group size before treatment on immune activity (Ruiz-Gonzalez et al., 2009). The experimental treatments were being (a) fed with 10,000 cells of *C. bombi* clone A (internal code 08.091), (b) fed with clone B (code 08.226) – in each case mixed with 50% sugar-water; or (c) fed 50% sugar water without the parasite as a naïve sham treatment, (d) injected with 2 μL of a suspension of *E. coli* at a concentration of 10^8 cells per mL, or (e) injected with 2 μL of Ringer solution (control for the injection). Bees that were given either *Crithidia* clones or the naïve sham infection were starved for 2 h before being given their inoculum to encourage them to eat the inoculum. All bees ate the inoculum. 18 h after exposure, we snap-froze the bees in liquid nitrogen and stored them at −80 °C until further use. We immobilized bees given the bacterial or Ringers solution injections on ice for 30 min before injection. We injected the bees using pulled glass capillaries and administered the inocula between the abdominal tergites. 8 h after injection the bacteria- and Ringer-challenged bees were frozen and stored as above. We chose these time points based on previously published reports on gene expression after exposure (Erler et al., 2011; Riddell et al., 2011), and also on phenotypic assays of antibacterial activity and phenol oxidase (Korner and Schmid-Hempel, 2004). The immune expression response to *Crithidia* peaks 12 h post exposure (Riddell et al., 2011) and this expression response remains strong 18 h (Brunner et al., 2013). Bacterial challenge induces a more rapid response (Erler et al., 2011; Korner and Schmid-Hempel, 2004) than *Crithidia* infection so we used an earlier (8 h) time point to sample expression in response to this challenge. We treated four bees from each colony to each condition except for colony 41 exposed to *Crithidia* clone A, where one bee died before exposure and the bacterial treatment contained one additional bee. The sham-infected bees served as controls for the *Crithidia*-infected bees (both at 18 h post exposure), and the Ringers-injected group as a control for the bacteria-injected group (both at 8 h post injection). For analyses, the sham infected bees were used as the control for the *C. bombi* exposed bees as they received identical treatment except the presence of the parasite and similarly the bees injected with sterile Ringers solution acted as controls for the bacterially injected bees.

2.2. RNA preparation

We homogenized the abdomens before extraction with 0.5 g Zirkonium beads at 0 °C to −4 °C using an Omni Bead Ruptor 24 Homogenizer (OMNI International). We then extracted total RNA using Qiagen RNeasy Plus Mini extraction kits according to the manufacturer's instructions. We checked two to three samples from each extraction group on a 2100 Bioanalyzer (Agilent Technologies) with the RNA 6000 Nano Kit to confirm RNA integrity and quantified the RNA concentration of all samples with Nanodrop 8000 (Thermo Scientific). We transcribed the RNA to cDNA using Quantitect reverse transcription kits (Qiagen) including controls without reverse transcriptase (no-RT controls) to test for genomic contamination. All samples were checked using qPCR for reference genes to ensure that the no-RT controls amplified at least 10 cycles later, and thus contain less than 0.1% of the transcripts found in the RT samples.

We selected 26 candidate genes to represent several pathways; these genes fall into five broad classes of response: recognition, signalling, effectors, metabolic and fat body activities (shortened to metabolic throughout), reactive oxygen species (ROS) and

melanisation. The full list of genes, including NCBI accession numbers and primers, can be found in Table 1. The genes were chosen based on previous studies in *B. terrestris* (Erler et al., 2011; Riddell et al., 2009; Schlüns et al., 2010) and other insect species (Aguilar et al., 2005; Radyuk et al., 2010; Rudenko et al., 2005; Vogel et al., 2011). We included 6 reference genes, based on previous studies, and then chose the reference genes that varied least across treatments and samples based on GeNorm analysis as references for the subsequent analyses. We developed new primers for most of these genes, using Primer3 (Rozen and Skaletsky, 2000) or Quantprime (Arvidsson et al., 2008) and preferentially selected primers that span exons to prevent amplification of genomic DNA. We designed the primers to be 20 ± 2 bp long and melting at 60 ± 1 °C, with a maximum 0.5 °C difference in melting temperature between the forward and reverse primers for a target. We tested the primers on multiple samples before use in this assay. All primers had amplification efficiency of between 1.9 and 2.1 and amplified reliably across samples. We used previously published primers for β-actin, hemomucin, ITPR, relish, RPL13, and vitellogenin (Table 1).

We measured gene expression of all 32 of our genes using a Biomark Fluidigm 96.96 Dynamic Array with EvaGreen DNA Binding Dye (Biotium) as a reporter following the manufacturer's instructions (Advanced Development Protocol 14, PN 100-1208B). Measurements were taken for each biological sample across three technical replicates, the average of which was used as the observed expression value (Ct) for each gene.

2.3. Analysis

We analysed these data using the delta–delta Ct (ddCt)-method normalized against the geometric mean of the three most stable reference genes, as determined by GeNorm within qbasePLUS (Biogazelle). These reference genes were elongation factor 1 α (ef1a: $M = 0.677$, CV = 0.304), ribosomal protein L13 (RPL13: $M = 0.742$, CV = 0.386), β-actin (ACTB: $M = 0.827$, CV = 0.246). We

Table 1
Gene and primer details. When no primer reference is given the primers were designed for this study.

Class	Gene	Putative gene function (pathway)	NCBI accession	Forward primer	Reverse primer	Product size	Primer reference
Receptor	BGRP1	Recognition (Toll)	XM_003397996	AACGTGGAAGTCAAAGATGG	GCGAACGATGACTTGGTATT	206	
	BGRP2	Recognition (Toll)	XM_003394713	TAACTCCCTTTGGAAACACG	GGCGGTAAAATACTGAACGA	249	
	Hemomucin	Potential recognition receptor	XR_131963	AGCATTCCCAGATTTAGCACT	TAACAGTTGATTTCGGAGGTA	173	(Schlüns et al. 2010)
	PGRP-LC	Recognition (Imd)	XM_003396463	CAGCCACCTACGACAGATTT	GTACATTCCGCTTGTGTCCT	101	
	PGRP-S3	Recognition (Toll)	XM_003401893	CGTGAAGGAGCTCATACCAT	CCAGGACTCATAGTGGCTGT	200	
Signalling	Basket	Signalling (JNK)	XM_003402794	GGAACAAGATAATCGAGCAACTG	CTGGCTTTCAATCGGTTGTG	177	
	Hopscotch	Signalling (JAK/STAT)	XM_003401903	CACAGACTGAAGCAGGTTGA	CATATGGGTAATTTGGTGCC	353	
	Pelle	Signalling (Toll)	XM_003399470	TAAATCGACCTATGCAAGCC	GGGTATAGCTGCTTCTGCTG	107	
	Relish	Signalling (Imd)	XM_003399472	CAGCAGTAAAAATCCCCGAC	CAGCACGAATAAGTGAACATA	156	(Schlüns et al. 2010)
Effector	Abaecin	Antimicrobial peptide	XM_003394653	GCCACAATATGTGGAATCCT	ATGACCAGGGTTTGGTAATG	141	
	Apidaecin	Antimicrobial peptide	XM_003402966	CCCGACTAATGTACCTGCCA	GAAGGTGCGAATGTGTTGGA	131	
	Defensin	Antimicrobial peptide	XM_003395924	GTCTGCCTTTGTCGCAAGAC	GACATTAGTCGCGTCTTCTTCG	139	
	Ferritin	Iron transportation	XM_003393332	AAAGAATTGGACGCAAATGG	CAGCGAACTGATGTCCAAGA	259	
	Hymenoptaecin	Antimicrobial peptide (AMP)	XR_132450	TTCATCGTACTGGCTCTCTTCTG	AGCCGTAGTATTCTTCCACAGC	85	
	Lysozyme3	Bacteriolytic effector	XM_003394052	TATGGGCAAGAAGATTCGAC	GTGTACATCGTTCACGCATC	219	
	TEPA	Effector (JAK/STAT)	XM_003399699	GCGTTCTATGACCACCTGTT	TACAGGTTACTCCACAGCCC	212	
	Transferrin	Iron-binding, antibacterial	XM_003401163	CAATTTCTTCACCGCATCCT	CCTCGTTATTTGGCTTGCAT	131	
Melanisation	Catsup	Enzyme, melanin synthesis	XM_003398173	TTACCATGACGAGTCACCAA	ATGAGGAACCAAAGCATGAG	355	
	PPO	Prophenoloxidase, melanin synthesis	HM_142999	AGCGGCATAATACGTTGTGT	CCGAGGGATAGAAAGTCTCC	329	
	Punch	Enzyme, melanin synthesis	XR_131852	ATTGCCAGGACACTTTCAAC	TACAAGCTGGAAACGGAAAC	211	
	Serpin27a	Serine protease inhibitor (PPO)	XM_003392985	CCGATCATCCATTCGTATTC	ACCTGCACTTGATATCCCTG	164	
ROS	Jafrac	Peroxiredoxin, ROS regulation	XM_003401245	CTCACTTCAGTCACTTGGCA	GCCAGCAGGACATACTTCTC	290	
	Peroxiredoxin5	Peroxiredoxin, ROS regulation	XM_003394777	TCACACCAGGATGTTCCAAGAC	TTCTGCTCCGTGTTCTTTACCC	146	
Metabolic	APOIII	Apolipophorin III	XM_003402572	ATCAGGCTCAAACGAACATC	TTCGTTCACTTGTTGCTGAG	269	
	Cyp4g11	Cytochrome P450	XM_003399563	GAATGCGCAAAGAAGGTAGC	CGCTTTCCGCTCTTGTAATC	313	
	Vitellogenin	Metabolic, endocrinological functions	XM_003402655	GTGACAAGCGAAGAGACTATTATG	CCGTGTTATCTGGCGTGAC	154	(Li et al. 2010)
Reference	AK	Arginine kinase	AF_492888	CTGGACTCTGGTGTCGGTAT	GTCTTTTGGTGGATGCTTGT	129	
	ACTB	β-actin	FN_391379	TGACGCAGATTATGTTTGAA	AGCGTATAGCGAAAGTACAGC	77	(Hornáková et al. 2010)
	ef1α	Elongation factor 1 α	XM_003401944	GCTGGTGACTCGAAGAACAATC	GGGTGGTTCAACACAATAACCTG	74	
	ITPR	Inositol 1,4,5-trisphosphate	DQ_468668	TGCACGCAGACCAAGCGGAG	ACGTCTTCCTTCGCGTCAAACGG	190	(Erler et al. 2011)
	PLA2	Phospholipase A2	FN_391388	TATCTTTCAATGCCCAGGAG	GTCGTAACAAATGTCATGCG	129	Hornáková et al. 2010
	RPL13	Ribosomal protein L13	FN_391387	GGTTTAACCAGCCAGCTAGAAA	CTTCACAGGTCTTGGTGCAA	83	

then standardized the expression the genes of interest in the experimental samples (ddCt) to control bees given the relevant experimental controls (sham infected naïve bees for the *Crithidia* exposed bees and bees given the sterile injection for bacterially challenged bees). Thus, the expression analysed is relative to control bees that were treated identically to the experimental bees with the exception of the presence of parasite. We transformed the ddCt data using Yeo-Johnson transformations (MASS package (Venables and Ripley, 2002) in R (R Development Core Team, 2010)) to improve normality and homoscedasticity. We performed MANOVAs (R base package) on the ddCt of genes within the six classes (recognition, signalling, effectors, metabolic, ROS and melanisation), each time with colony and exposure as fixed effects, and Pillai's trace value as the test statistic. When MANOVA results were significant we explored the univariate ANOVA results for those genes and used Tukey's HSD (R base package) to assess significantly different levels. In cases where the MANOVA did not detect significant effects of our factors, we still examined the univariate ANOVA results but required that the critical *p*-value be reduced according to the number of genes tested. We also conducted linear discriminant analysis on the Yeo-Johnson transformed dCt values for individuals in all exposure groups (*Crithidia* A, B, sham, bacterial and Ringer-challenged) to assess differentiation across all genes and used leave one out cross validation to assess the predictive power of the gene expression values. Finally, we compared patterns of gene expression across genes by constructing a Spearman's rank correlation matrix (R base package) of the expression of every gene against every other gene. This included all five exposure groups, and produced a heatmap and clustering based on Euclidian distances among the correlation values.

3. Results

Bees altered their gene expression when exposed to our three challenges across all classes of genes except for the signalling and melanisation genes (Table 2). In particular, bacterial exposure upregulated expression of the receptor BGRP1, the signalling molecule hopscotch, the antimicrobial peptides (AMPs) abaecin, defensin and hymenoptaecin, the iron binding molecule ferritin, antibacterial lysozyme 3, the enzymes peroxiredoxin 5 and cytochrome P450; and downregulated expression of TEP-A. Strikingly, *C. bombi* exposure influenced expression differently depending on the clone. Clone A induced a 'bacteria-like' response in all of the antimicrobial peptides (Fig. 1A–D). Other genes, such as the other effectors, signalling, metabolic and ROS genes were similarly expressed between bees when given each clone of *Crithidia* (Fig. 1E–N). *C. bombi* clone B either did not induce a detectible increase in expression of immune genes (i.e., Fig. 1E–I, K and L) or showed a pattern of expression lower than the naïve controls (i.e., the AMPs Fig. 1A–D).

Overall, and importantly for our basic question, colonies not only differed intrinsically, but also differed according to treatment (colony × treatment interaction, summary statistics can also be found in Table A.1). Notably, three effectors (ferritin, lysozyme 3, and TEP-A; Fig. 1E–G), prophenoloxidase (PPO, which is involved in the melanization response; Fig. 1N), the signalling genes hopscotch and basket (Fig. 1I and M), and two enzymes (cytochrome P450, and peroxiredoxin 5; Fig. 1K and L) were expressed differently upon challenge in the two colonies. Most of these interactions were driven by significant differences among the bacterial challenge and the *Crithidia* clone treatments. Bees given *C. bombi* clone B had higher expression values of TEP-A but lower expression of PPO if they are from colony 41 than if they are from colony 1 (Tukey's HSD $p = 0.01$, 0.03, respectively). Both peroxiredoxin 5 and PPO expression was lower in bees from colony 41 exposed to *C. bombi* clone B than bees from the same colony but given clone A (HSD $p = 0.014$, 0.026). The logfold-changes in expression for all of the significantly differentially expressed genes, and three genes that approached significance (PGRP-S3, basket, transferrin), are shown in Fig. 1 and Fig. A.1.

To gain a better insight in to the overall variation in the responses, we used a linear discriminant analysis to assess the clustering of all genes' expression from all samples. We found that the first two linear discriminant axes (LD1 and LD2, Fig. 2A, Table A.2) described 88% of the variation across samples, and that the samples segregated by treatment. The top absolute contributors (greater than the arbitrary cut-off value of 3) to these axes are the

Table 2
MANOVA results for each class of genes. Significant results are highlighted in boldface, results that approach significance are marked in italics.

Class	Factor	Df	Pillai	Approx F	Num df	Den df	P
Receptors	*Colony*	*1*	*0.462*	*2.408*	*5*	*14*	*0.0895*
	Exposure	**2**	**1.067**	**3.432**	**10**	**30**	**0.00424**
	Col∗Exp	2	0.717	1.675	10	30	0.13323
	Residuals	18					
Signalling	Colony	1	0.339	1.923	4	15	0.1588
	Exposure	*2*	*0.666*	*1.996*	*8*	*32*	*0.07929*
	Col∗Exp	**2**	**0.849**	**2.952**	**8**	**32**	**0.01368**
	Residuals	18					
Effectors	**Colony**	**1**	**0.716**	**3.462**	**8**	**11**	**0.03015**
	Exposure	**2**	**1.596**	**5.924**	**16**	**24**	**<0.00001**
	Col∗Exp	**2**	**1.242**	**2.459**	**16**	**24**	**0.0226**
	Residuals	18					
Metabolic	*Colony*	*1*	*0.341*	*2.764*	*3*	*16*	*0.075923*
	Exposure	2	0.932	4.949	6	34	0.000979
	Col∗Exp	2	0.89	4.547	6	34	0.001738
	Residuals	18					
ROS	Colony	1	0.065	0.592	2	17	0.5644
	Exposure	**2**	**0.635**	**4.188**	**4**	**36**	**0.006924**
	Col∗Exp	**2**	**0.49**	**2.924**	**4**	**36**	**0.034213**
	Residuals	18					
Melanisation	Colony	1	0.35	2.02	4	15	0.143
	Exposure	2	0.45	1.163	8	32	0.351
	Col∗Exp	*2*	*0.669*	*2.011*	*8*	*32*	*0.077*
	Residuals	18					

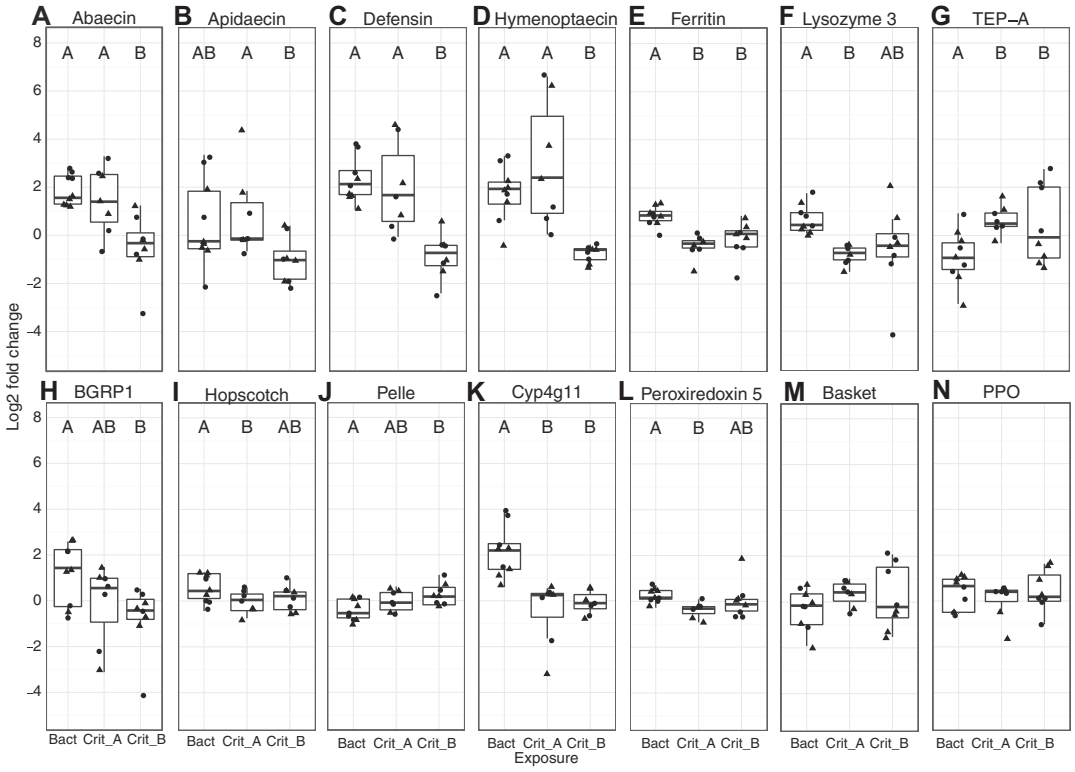

Fig. 1. (A–N) Logfold expression of genes in bees exposed to our three conditions, relative to their controls, and reference genes. Circles represent bees from colony 1, triangles from colony 41. Letters above denote significantly different groups based on Tukey's honestly significant differences test.

expression of the genes jafrac, serpin27a, PGRP-LC, pelle, hopscotch, basket, and BGRP1 (for axis LD1); and serpin27a, jafrac, hemomucin, and PGRP-LC (for axis LD2). The full table of these coefficients can be found in Table A.2. Furthermore, the linear discriminant function predicts each sample's group more often than expected by chance (with five groups, $p = 0.20$) in all exposure groups except for the bees given *C. bombi* clone A, which is similar in some responses to the bacterially challenged bees, and to the bees given clone B which is qualitatively similar to the naïve controls (Fig. 1).

The expression within functional groups of genes was correlated (Fig. 2B). The receptor PGRP-S3 was positively correlated with the putative receptor hemomucin, similarly, PGRP-LC, BGRP1, and BGRP2 expression was also positively correlated with one another. All antimicrobial peptides were strongly positively correlated with one another. The expression of the two reactive oxygen species associated enzymes peroxiredoxin 5 and jafrac are also positively correlated. Basket and TEP-A expression were negatively related to expression of PGRP-S3, apolipophorin III, peroxiredoxin 5, lysozyme 3, ferritin and PPO.

4. Discussion

Parasite genotypes vary in important aspects of their biology, such as in virulence (e.g., (Ebert, 1994) and infectivity (e.g., (Lambrechts et al., 2005)). Whereas such studies demonstrate variation among parasite genotypes in the phenotypic outcome of an infection, surprisingly little is known about how hosts respond immunologically via expression of genes to different parasite genotypes. The variable expression of appropriate host genes may be a key element in this context (Riddell et al., 2009). We were able to broadly survey gene expression in response to the ecologically important trypanosome gut parasite *C. bombi* and a classical bacterial challenge. A weakness of the approach is that it has to focus on a limited number of genes. However, this is more than compensated by the strength that it allows us to look at gene expression across a suite of genes, across backgrounds, and challenges. Specifically, we find that bees exposed to different clones of *C. bombi* mount qualitatively different gene expression profiles and, hence, are able to distinguish by expression of immune genes, in particular, between different genotypes of the same parasite. Moreover, exposure to *C. bombi* clone A induced a 'bacteria-like' immune response profile by some genes, as notably characterized by the antimicrobial peptides (Fig. 1A–D), but a *Crithidia*-consistent response for other genes, as in metabolic (Fig. 1K), signalling (Fig. 1I and J), signalling (Fig. 1H, Table A.1) and some effector genes (Fig. 1E–G, L, Table A.1). This similarity is also described by the linear discriminant analysis (Fig. 2A). Jafrac, serpin27a, pelle, hopscotch, basket PGRP-LC and BGRP1 all contribute to differentiation across the axis that separates the bees given the different parasite challenges. These suggest the involvement of multiple immune pathways and receptors. PGRP-LC activates the linked IMD and JNK pathway, which includes the MAP kinase basket, in *Drosophila* (Lemaitre, 2007; Tanji et al., 2007). JNK pathway activation also appears to be involved in wound repair as it linked to cytoskeletal remodelling (Boutros et al., 2002). Members of the JNK and JAK/STAT pathways also appear to be regulated in response to microbial challenge in *Drosophila* (Boutros et al., 2002). Beta-glucan receptor proteins, like BGRP1, induce the toll pathway that includes the signalling kinase pelle and eventually results in the production of antimicrobial pathways (Lemaitre, 2007).

The similarity in the immune response of bees to an oral dose of *C. bombi* clone A and an internal injection of bacteria is surprising. One reason might be that the gut microbial community is important in determining susceptibility to *C. bombi* infections (Koch and Schmid-Hempel, 2011). It is conceivable that the *C. bombi*

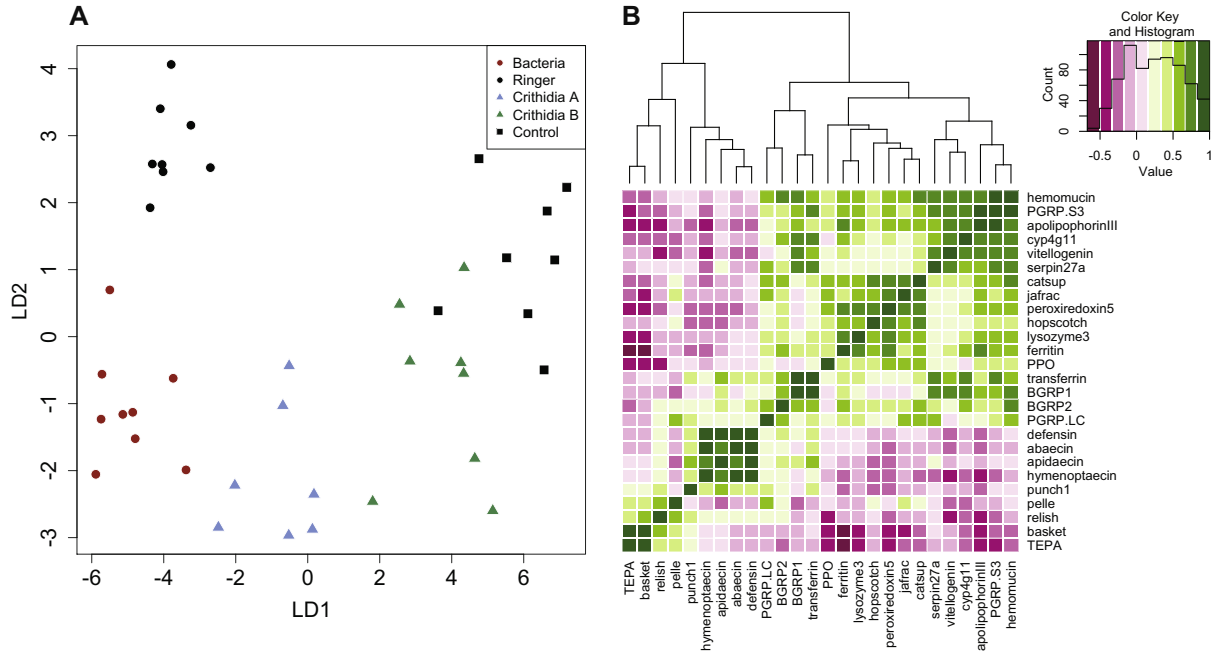

Fig. 2. (A) Linear discriminant analysis of the gene expression of bees according to exposure condition (see symbols). The top contributors to these axes are jafrac, serpin27a, PGRP-LC, pelle, hopscotch, basket, and BGRP1 (axis LD1), and serpin27a, jafrac, hemomucin, and PGRP-LC (axis LD2). Symbols indicate different treatments (infection by *Crithidia* genotype A or B, by bacteria, injection of Ringer, and naïve control). (B) Heatmap and cluster analysis of Spearman's rank correlation values among expression values for all genes. The tree is produced from the Euclidian distances of the correlation values. Color code, for the strength of the pairwise correlation coefficients, and the distribution of frequencies (histogram) of occurrence of these coefficients given in the insert. Green areas indicate pairs of genes that are synergistically expressed, red areas where pairs of genes are antagonistically expressed. (For interpretation of color in figure legend, the reader is referred to the web version of this article.)

inoculum interacts with this microbial community such that it either disturbs the gut homeostasis, or allows commensal gut bacteria to cross the gut barrier, resulting in a host antibacterial response. Variation in the gut microbiota itself, however, is unlikely to play a major role in describing our results as each colony, with its microbial community, was exposed to the same parasite treatments. The distinct patterns of immune responses produced by the two clones of *C. bombi* may suggest differences in either evasion or suppression of the host immune response and perhaps tolerance of the immune response itself. Clone A induced an immune response and to be successful it would need to be able to resist the produced effectors (e.g., AMPs). Clone B, in contrast, seemed to dampen the response such as in the expression of the effectors lysozyme, ferritin, and TEP-A (Fig. 1E–G), and appears to have actively suppressed the expression of AMPs (Fig. 1A–D). The expression of these key effectors is thought to be important for the control of *C. bombi* (Brunner et al., 2013; Riddell et al., 2009, 2011) and bees exposed to Clone B had lower expression of these AMPs than that of the naïve controls. Based on our results we anticipate that different *C. bombi* clones may have different strategies for interacting with the host immune response possibly including evading or suppression the host immune system.

So far, three studies of *B. terrestris* expression upon parasite exposure found a variety of responses. Erler et al. (2011) also found that bacterial exposure increased expression of a number of antimicrobial peptides. TEP-A was downregulated relative to naïve controls in both sterile-challenged, and bacterial-challenged bees in the same study. We found that TEP-A was marginally downregulated in bees injected with bacteria compared to sterile-injected bees, but could not compare these values to naïve bees. Erler et al. (2011) also described various temporal patterns in expression of immunological genes that we did not explore. Consistent with the results here, Riddell et al. (2009) found strong upregulation of abaecin, defensin and hymenoptaecin and that this expression varied across colonies and depending on the genotype of *C. bombi* used. Schlüns et al. (2010), however, found significant effects of both colony and infection and their interaction on the expression of the putative receptor hemomucin and the signalling protein relish, whereas neither of these genes varied in the current experiment. *B. terrestris* colonies vary considerably in immunological gene expression, as seen here and elsewhere. Some of the differences among studies may be explained by these differences among colonies.

Macroscopically and from standard infection experiments, different bumblebee colonies are susceptible to different clones of *C. bombi*. Bumblebee genes that differ in expression upon infection with two clonal isolates of *C. bombi* could give hints as to what determines this host-parasite specificity. This includes both traditional immunological genes such as effectors but also genes related to nutrition. Diet plays an important role in this system as infection with *Crithidia* alters feeding behaviour (Otterstatter et al., 2005), access to protein-rich pollen increases *Crithidia* growth within the gut (Logan et al., 1999), and the quality of sugar-water changes the dynamics of host-parasite genetic susceptibility (Sadd, 2011). Here we found that colonies differed in their expression of metabolic genes upon immune challenges. The differences in expression of these metabolically important genes across our clonal *Crithidia* isolates could indicate which genes are involved in the interaction between the genotypes of hosts and parasites and nutritional environment. In an earlier and important study, Riddell et al. (2009) found that some antimicrobial peptides differ in expression across colonies given *C. bombi* from different source colonies. With our combinations of colonies and clones of *C. bombi* we did not see an interaction between host colony and treatment type for antimicrobial peptides, but we did see such interactions with other effectors (TEP-A, lysozyme 3, ferritin), prophenoloxidase which leads to the melanisation response, and peroxiredoxin 5 which controls ROS and is vital for gut immunity (Ha et al., 2005). It is very possible that complex combinations of both antimicrobial peptides and other effectors are responsible for this host-parasite genotype-

by-genotype interaction, and indeed, that other genes, such as cytochrome p450, are involved in moderating these responses to produce genotype-by-genotype-by-environment interactions as seen when resources are limiting (e.g., (Sadd, 2011)).

By surveying a broad suite of genes we are also able to detect patterns of coexpression. These genes were expressed in a strikingly non-random fashion as represented by clades in Fig. 2B. On a broad scale, the expression of these genes fell into two major clades; the positively 'attractive' clade in which most genes are positively correlated with one another, and a smaller 'repulsive' clade in which the expression of these genes were generally associated with down-regulation of other genes. Within these broad categories there were finer, and stronger, expression association. Antimicrobial effectors were all expressed in concert but either negatively or very weakly correlated with expression of most other genes. The expression of some genes was almost universally 'repulsive' such as the effector TEPA and the signalling molecule basket, which are negatively correlated with the expression of most other genes. The receptors (BGRPs and PGRP-LC) were also tightly associated with one another. Metabolism related genes (vitellogenin, apolipophorin III, cytochrome P450) cluster together and are correlated positively with the majority of the genes surveyed here. Other positively correlated genes that transcend broad classes include a cluster containing ROS (peroxiredoxin, jafrac), effector (lysozyme, ferritin), and melanisation (hopscotch, PPO) genes. These patterns of concerted and antagonistic expression may suggest functionally related groups of immunological genes that function together (e.g., AMPs) and functions that are traded-off against one another (e.g., ferritin with TEPA, or hymenoptaecin with metabolic genes).

In most bumblebee species the queens mate singly, which, combined with the haplodiploid genetics of hymenopterans, results in workers that are highly related to one another (75% on average). Despite this high genetic similarity there is high variability in *B. terrestris* gene expression to parasite challenges (Riddell et al., 2009). Here, we also see strong variation in gene expression for many genes, even when bees are highly related and age-controlled (to the day). This diversity in response is intriguing, but it is as yet unclear if this variation is adaptive. Nevertheless, the diversity of the kind discovered here is a potentially important pre-condition that specific interactions between host and parasites can take any effect in evolutionary terms. This possibility remains an important point in the vivid discussion about the significance of antagonistic co-evolution for the maintenance of biological diversity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.09.014.

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