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Differential gene expression of two extreme honey bee (*Apis mellifera*) colonies showing varroa tolerance and susceptibility

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Abstract

***Varroa destructor*, an ectoparasitic mite of honey bees (*Apis mellifera*), is the most serious pest threatening the apiculture industry. In our honey bee breeding programme, two honey bee colonies showing extreme phenotypes for varroa tolerance/resistance (S88) and susceptibility (G4) were identified by natural selection from a large gene pool over a 6-year period. To investigate potential defence mechanisms for honey bee tolerance to varroa infestation, we employed DNA microarray and real time quantitative (PCR) analyses to identify differentially expressed genes in the tolerant and susceptible colonies at pupa and adult stages. Our results showed that more differentially expressed genes were identified in the tolerant bees than in bees from the susceptible colony, indicating that the tolerant colony showed an increased genetic capacity to respond to varroa mite infestation. In both colonies, there were more differentially expressed genes identified at the pupa stage than at the adult stage, indicating that pupa bees are more responsive to varroa infestation than adult bees. Genes showing differential expression in the colony phenotypes were categorized into several groups based on their molecular functions, such as olfactory signalling, detoxification processes, exoskeleton formation, protein degradation and long-chain fatty acid metabolism, suggesting that these biological processes play roles in conferring varroa tolerance to naturally selected colonies. Identification of differentially expressed genes between the two colony phenotypes provides potential molecular markers for selecting and breeding varroa-tolerant honey bees.**

Keywords: honey bee, *Varroa destructor*, differential expression, DNA microarray.

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Introduction

The honey bee (*Apis mellifera*) plays an important role in the global agricultural economy by providing pollination services to crops, fruit trees and vegetables, as well as hive products directly for human consumption (Greenleaf & Kremen, 2006; vanEngelsdorp & Meixner, 2010). A healthy population of honey bees is essential for efficient pollination and honey production; however, like other insects, honey bees are subject to invasion by a wide range of parasites and pathogens (Genersch *et al*., 2010). Amongst these disease-causing agents, the ectoparasitic mite *Varroa destructor* is the greatest threat to beekeeping (Sammataro *et al*., 2000). This pest has been implicated in the death of millions of bee colonies by vectoring pathogenic viruses (Duay *et al*., 2003; Ryabov *et al*., 2014), leading to great economic losses and causing serious concern for apiculture.

Varroa destructor is a host-associated mite and lacks a free-living stage. The mother mite and her offspring feed on the haemolymph of pupae and adult bees, resulting in loss of nutrients and circulatory fluids (Sammataro *et al*., 2000), leading to decreased overall body weight and longevity, and eventually colony death (Amdam *et al*., 2004). In addition, the varroa mite also acts as a vector for spreading bacterial, fungal and viral pathogens within and amongst colonies (Davidson *et al*., 2003; Kanbar & Engels, 2003; Martin *et al*., 2012; Ryabov *et al*., 2014).

The host–parasite relationship between honey bees and varroa is complex, serving as an interesting model for studying the mechanisms used by social insects to defend themselves against parasites (Zakar *et al*., 2014). The Asian honey bee, *Apis cerana*, co-evolved with the varroa mite for centuries and thus possesses traits that enable it to tolerate varroa infestations with minimal harm (Rosenkranz *et al*., 2010). Hygienic behaviour, grooming behaviour and suppression of mite reproduction activities are possible mechanisms that the colonies use to defend against varroa infestation (Zakar *et al*., 2014). However, unlike the Asian honey bee, the western honey bee, *A. mellifera*, is more susceptible to varroa (Sammataro *et al*., 2000). Initial gene expression studies suggest that differences in physiology and behaviour, rather than in the immune response, might underlie varroa tolerance (Navajas *et al*., 2008). In addition, host tolerance to the mite may be characterized by different metabolic and nerve signalling processes (Zhang *et al*., 2010).

Functional genomics provides powerful tools to study host–parasite relationships in honey bees. Genomic resources developed by The Honey Bee Genome Project (The Honeybee Genome Sequencing Consortium, 2006) and new technologies in gene expression analysis offer an integrated and comprehensive resource for molecular research on the honey bee and varroa mite interaction (Robinson *et al*., 2006). The transcript profiles of two adult bee colony phenotypes, one with a high rate of hygienic behaviour and the other with a low rate of hygienic behaviour, were compared by DNA microarray (Le Conte *et al*., 2011). A comparison of gene expression between the western honey bee, *A. mellifera*, and the Asian honey bee, *A. cerana*, identified many differentially expressed genes that might be involved in metabolic processes (Zhang *et al*., 2010). Digital gene expression analysis on bee abdomens found that varroa parasitism could result in decreased metabolism, particularly inhibition of protein anabolism (Alaux *et al*., 2011). However, a comprehensive analysis of gene expression at the genomic level between phenotypically defined tolerant and susceptible honey bees to elucidate the molecular mechanisms of varroa tolerance is still lacking.

In this report, we describe the identification of a large number of genes that are clearly differentially expressed between two contrasting honey bee colonies, a varroa-susceptible phenotype (G4) and a varroa-tolerant phenotype (S88) selected from a large gene pool by a natural selection breeding programme. We separated the possible effects of colony phenotype and mite infestation during the comparison analysis, which provides a more detailed comparison of the genes differentially expressed in the different colony phenotypes in response to mite infestation. The differentially expressed genes were categorized into several functional groups based on their biological activities, such as olfactory signalling, detoxification processes, exoskeleton formation, protein degradation and fatty acid metabolism, indicating that these processes might be vital mechanisms underlying host tolerance to varroa infestation.

Results

Differential gene expression in pupa and adult bees of two extreme colonies

A detailed description of the selection of the two contrasting colonies, the tolerant (S88) and the susceptible (G4), selected from a large diverse gene pool (approximately 50 000 colonies), is given in Robertson *et al*. (2014). The varroa infestation rates in white-eyed and dark-eyed pupae in sealed broods ($n = 500$) of G4 were 88 and 70%, respectively, and the rate in the adults was 67 mites per hundred bees. By contrast, the infestation rates in S88 were 44% in white-eyed pupae and 17% in dark-eyed pupae, and the rate in S88 adults was four mites per hundred bees. In July 2010, the infested brood score in G4 was 2.7 ± 2.0 mites per infected cell, whereas the score in S88 was 1.5 ± 1.0. The varroa-susceptible G4 colony collapsed and died in October 2010, 17 months after construction, whereas the varroa-tolerant S88 survived by natural selection, without synthetic miticide treatment, for more than 58 months. No swarming or supercedure events were observed during the study.

To identify molecular mechanisms underlying the defence of host bees against varroa parasitism, DNA microarray analysis in a loop design was employed to investigate genome-wide gene expression of the two extreme colony phenotypes. The susceptible and tolerant bees with and without varroa infestation at both pupa and adult stages were analysed for differential gene expression. At each developmental stage, four samples: susceptible with varroa mite infestation ($G4^+$), susceptible without varroa mite infestation ($G4^-$), tolerant with varroa infestation ($S88^+$) and tolerant without varroa mite infestation ($S88^-$) were arranged into a loop comparison (Fig. 1). This design maximizes direct pairwise comparisons between parasitized, nonparasitized, susceptible and tolerant bees. Amongst all the comparisons, the varroa infestation comparison and the honey bee colony comparison were considered to be the two main comparisons of the microarray analysis. The former compared differential gene expression of the honey bees with or without mite infestation within the same honey bee colony, including the susceptible colony with varroa mite infestation relative to the susceptible colony without varroa mite infestation ($G4^+/G4^-$), and the tolerant colony with mite infestation relative to the tolerant colony without mite infestation ($S88^+/S88^-$). The latter

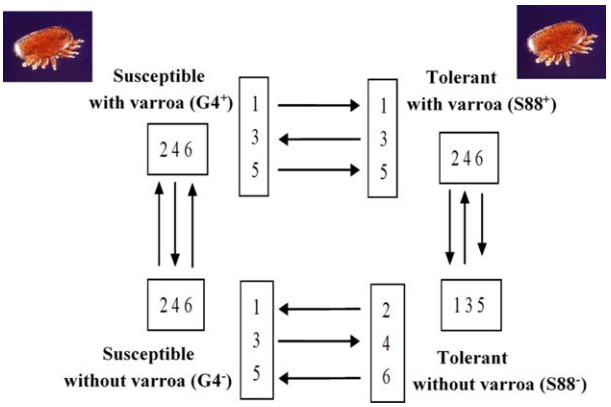

Figure 1. The loop design for the DNA microarray analysis. Two honey bee colonies differing in tolerance (S88 and G4), and infestation status (with and without varroa mites), at two developmental stages (pupal and adult), were employed in the microarray analysis. The pupa and adult honey bees were analysed separately. The arrangement of the samples followed the loop design for a 2*2 factorial experiment. The numbers 1–6 represent the biological replicates in each treatment group; three replicates were in the cyanine 3-dCTP dye and three replicates were in the cyanine 5-dCTP dye (Table S5).

compared differential gene expression between the two honey bee colonies, the tolerant colony with varroa mite infestation relative to the susceptible colony with varroa mite infestation ($S88^{+}/G4^{+}$), and the tolerant colony without varroa mite infestation relative to the susceptible without varroa mite infestation ($S88^{-}/G4^{-}$).

DNA microarray analysis of the bees at pupa stage 4 showed that there were 106 genes significantly differentially expressed in the varroa infestation comparison, whereas there were 126 genes that were differentially expressed between the two honey bee colony phenotypes [false discovery rate (FDR) $P < 0.05$ and fold-change >2, Fig. 2]. As shown in the Venn diagram, the largest difference in gene expression was observed in the colony comparison with mite infestation ($S88^{+}/G4^{+}$), in which 39 genes were up-regulated and 73 genes were down-regulated, indicating that varroa-tolerant and susceptible colonies responded to varroa infestation very differently (Fig. 2B). Another notable comparison at the pupa stage was $S88^{+}/S88^{-}$, the tolerant colony with and without mite infestation, in which 58 genes were up-regulated and 35 genes were down-regulated in expression (Fig. 2A). This was in contrast to the comparison of the susceptible colony with and without mite infestation ($G4^{+}/G4^{-}$) at the same pupa stage, in which only 14 genes were up-regulated and four genes were down-regulated, indicating that the tolerant colony S88 showed an increased capacity to mobilize gene expression in

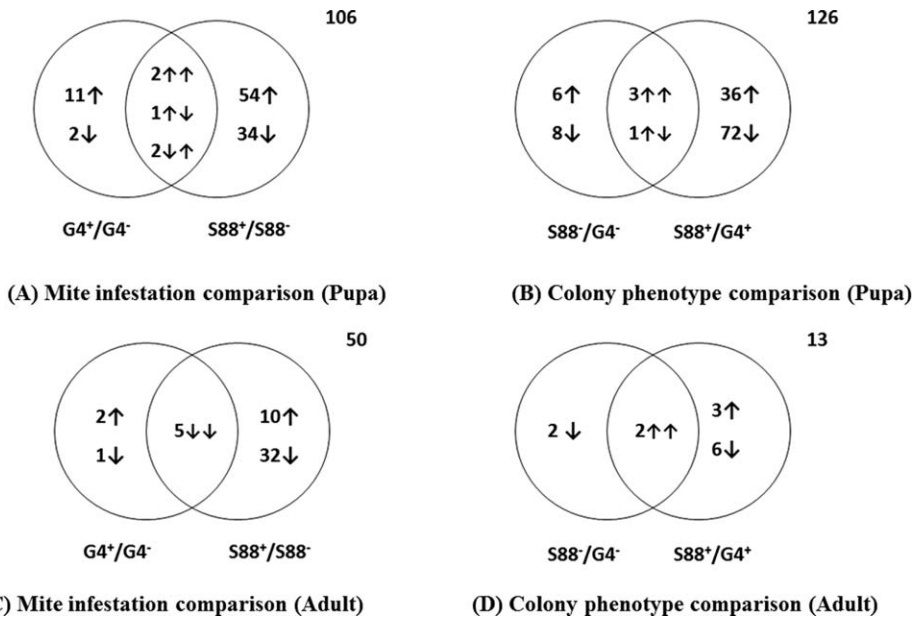

Figure 2. Venn diagrams showing the number of differentially expressed genes identified in mite infestation and colony comparisons at pupa and adult stages. (A) The mite infestation comparison (pupa): comparison between presence (+) and absence (–) of mites at the pupa stage. (B) The colony comparison (pupa): comparison between tolerant (S88) and susceptible (G4) colonies at the pupa stage. (C) The mite infestation comparison (adult): comparison between presence (+) and absence (–) of mites at the adult stage. (D) The colony comparison (adult): comparison between tolerant (S88) and susceptible (G4) colonies at the adult stage. $G4^{+}/G4^{-}$, the susceptible colony with varroa mite infestation ($G4^{+}$) relative to the susceptible colony without varroa mite infestation ($G4^{-}$); $S88^{+}/S88^{-}$, the tolerant colony with varroa mite infestation ($S88^{+}$) relative to the tolerant colony without varroa mite infestation ($S88^{-}$); $S88^{+}/G4^{+}$, the tolerant colony with varroa mite infestation ($S88^{+}$) relative to the susceptible colony with varroa mite infestation ($G4^{+}$); $S88^{-}/G4^{-}$, the tolerant colony without varroa mite infestation ($S88^{-}$) relative to the susceptible colony without varroa mite infestation ($G4^{-}$). ↑, up-regulation; ↓, down-regulation. The double arrow symbols in the intersection of the circles indicate that expression of a given gene was up-/down-regulated in different ways in the different comparisons.

Table 1. Comparison of expression differences of selected genes measured by DNA microarray analysis and real time quantitative PCR (qPCR)

Gene	Array $G4^+/G4^-$	qPCR $G4^+/G4^-$	Array $S88^+/S88^-$	qPCR $S88^+/S88^-$
GB14278	9.09	0.74	7.14	1.28
GB12600	2.78	14.29	0.31	0.20
GB19316	0.31	0.13	3.33	2.08
Gene	Array $S88^+/G4^+$	qPCR $S88^+/G4^+$	Array $S88^-/G4^-$	qPCR $S88^-/G4^-$
GB30203	0.33	0.07	2.20	2.91
GB14355	2.69	1.22	4.45	1.06
DB744987	3.40	33.22	2.02	19.13

Note: The numbers in the table represent the fold change in each comparison.

response to varroa mite infestation compared with the susceptible colony G4.

DNA microarray analysis at the adult stage showed that there were 50 genes that were differentially expressed in the mite infestation comparisons, whereas there were only 13 genes differentially expressed between the two honey bee colony phenotypes. Similar to the pupa stage, the $S88^+/S88^-$ comparison identified a larger number of differentially expressed genes, with 10 genes being up-regulated and 37 genes being down-regulated (Fig. 2C). By contrast, the $G4^+/G4^-$ comparison had only two genes that were up-regulated and six genes that were down-regulated, again indicating that the tolerant colony S88 had a higher capacity to alter gene expression in response to varroa mite infestation compared with the susceptible colony G4. In addition, in the comparison of colonies with mite infestation ($S88^+/G4^+$), five genes were up-regulated and six genes were down-regulated in expression, compared with the comparison of the colonies without mite infestation ($S88^-/G4^-$), in which only a total of four genes was differentially expressed (Fig. 2D). These results indicate that the different phenotypes respond to varroa mite infestation differently at the adult stage, with the tolerant phenotype being more responsive to mite infestation.

Validation of differential gene expression by quantitative PCR

To validate the microarray results, fold changes in two main comparisons of six highly differentially expressed genes were investigated by real-time quantitative RT-PCR. The results showed that all the comparisons of the six genes except for one comparison of *GB14278* ($G4^+/G4^-$) shared similar expression patterns. This suggests that DNA microarray analysis is reliable in profiling the transcripts of honey bees in response to mite infestation (Table 1).

Functional analysis of the differentially expressed genes

Gene ontology (GO) analysis was used to assign putative biological functions to the differentially expressed genes using the FlyBase orthologues as references. At the pupa stage, 74 out of 106 differentially expressed genes identified from the mite infestation comparison had identifiable fruit fly orthologues, and these genes clustered into 31 GO terms, whereas 85 out of 126 differentially expressed genes identified from the colony comparison had fruit fly orthologues and clustered into 31 GO terms (Table S1). At the adult stage, 35 out of the 50 differentially expressed genes identified from the mite infestation comparison had identifiable fruit fly orthologues and were clustered into 29 GO terms, whereas only five out of 13 differentially expressed genes identified from the colony comparison had fruit fly orthologues and these clustered into 18 GO terms (Table S2). Although these GO terms described a wide range of biological processes, molecular functions and cellular components, the highly differentially expressed genes (Table S3) could mainly be categorized into the following groups based on their molecular functions: olfactory signalling, detoxification processes, protein catabolism, lipid metabolism and exoskeleton formation (Table 2).

Discussion

Varroa destructor is a serious pest that harms honey bees by sucking haemolymph from both adult bees and developing broods, as well as by transmitting pathogenic viruses. To investigate possible defence mechanisms that honey bees may use against varroa mites, a varroa-susceptible colony (G4) and a varroa-tolerant colony (S88) were selected from our natural selection breeding programme for DNA microarray analysis. This analysis identified a large number of genes that were differentially expressed when these two extreme honey bee colonies were compared in the presence/absence of varroa infestation. Functional classification of the genes obtained from this microarray comparison analysis revealed several biological processes that may play important roles in defining these contrasting honey bee colony phenotypes in response to varroa mite parasitism.

Comparisons between the tolerant and susceptible colony phenotypes

The comparisons of colonies at the pupa stage in the microarray analysis revealed that several genes encoding cuticle and apidermin proteins were highly expressed in the susceptible colony relative to the tolerant colony (Table 2). These proteins probably have effects on the composition and structure of the honey bee exoskeleton. Previous research has suggested that mites can exploit

Table 2. Selected differentially expressed genes identified by the DNA microarray analysis

Olfactory signalling			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB46227	–	0.42	Odorant binding protein 18
GB11092	–	0.32	Odorant binding protein 17
GB30365	–	0.41	Odorant binding protein 14
GB19453	–	0.38	Chemosensory protein 2
GB17702	–	2.4	Cadherin-87A
Mite infestation comparisons (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB30365	2.21	–	Odorant binding protein 14
GB11904	–	4.32	Putative odorant receptor 13a
GB14248	–	2.23	Putative odorant receptor 13a
GB13325	–	2.1	Chemosensory protein 6
GB11092	–	0.39	Odorant binding protein 17
GB17254	–	4.87	Neuronal nAChR Apisα7-2 subunit
GB12287	–	2.31	Neurogenic big brain protein
GB17702	–	2.12	Cadherin-87A
GB12853	–	2.11	Neural-cadherin
Colony phenotype comparisons (adult)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB16826	–	0.47	Odorant binding protein 16 precursor
GB30242	–	2.23	Odorant binding protein 3 precursor
Mite infestation comparisons (adult)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB10729	–	2.33	Putative odorant receptor 85b
GB14823	–	0.35	Neurotrimin
Cytochrome P450s			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB11754	–	0.31	Cytochrome P450-6A14
GB12136	–	4.08	Cytochrome P450-6A1
Mite infestation comparisons (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB11754	–	0.34	Cytochrome P450-6A14
GB12136	–	6.58	Cytochrome P450-6A1
Mite infestation comparisons (adult)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB19967	–	0.46	Cytochrome P450-9E2
GB19306	–	0.45	Cytochrome P450-6K1
GB14612	–	0.48	Cytochrome P450-6K1
Esterase			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB16889	–	3.41	Esterase E4

Table 2. *Continued*

Esterase			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
Mite infestation comparisons (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB16889	–	3.92	Esterase E4
Mite infestation comparisons (adult)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB16889	–	0.47	Esterase E4
Protein degradation			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB303790.29	–		Serine protease
GB179270.33	–		Serine protease
GB303780.4	–		Serine protease
GB11273–	0.41		Retinoid-inducible serine carboxypeptidase
GB18450–	0.44		Transmembrane protease serine 6
GB10646–	3.12		Trypsin-7
GB13489–	4.88		Serine protease 34
GB150182.11	–		Chymotrypsin inhibitor
Mite infestation comparisons (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB10646	0.48	–	Trypsin-7
GB13489	–	2.96	Serine protease 34
Lipid metabolism			
Colony phenotype comparisons (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB11723	–	6.88	Apolipoprotein D
GB18070	–	2.23	Acyl-CoA Δ11 desaturase
GB13246	–	0.47	Phospholipase A1
Mite infestation comparisons (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB11723	0.44	2.58	Apolipoprotein D
GB30529	–	3.04	Peroxisomal acyl A oxidase 1
GB11256	–	2.97	Pancreatic lipase 2
Mite infestation comparisons (adult)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB30529	2.71	–	Peroxisomal acyl-CoA oxidase 1
GB11969	–	0.34	Delta 11 acyl-CoA desaturase
GB17931	–	0.35	Fatty acyl-CoA reductase 1
GB12176	–	0.29	Elongation protein
GB13264	–	0.5	Elongation protein
GB19070	–	0.45	Elongation protein
GB12567	–	0.46	Long-chain fatty-acid-CoA ligase

Exoskeleton formation			
Colony phenotype comparison (pupa)			
Gene	$S88^-/G4^-$	$S88^+/G4^+$	Honey bee protein
GB30337	0.42	–	Endocuticle structural glycoprotein SgAbd-2
GB15203	–	0.18	Larval cuticle protein A3A
GB12600	–	0.14	Cuticle protein
GB19234	–	0.33	Tweedle motif cuticular protein 1
GB14193	–	0.4	Tweedle motif cuticular protein 2
GB30202	–	0.31	Apidermin 1
GB30203	2.2	0.33	Apidermin 3
Mite infestation comparison (pupa)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB14193	2.01	–	Tweedle motif cuticular protein 2
GB12600	2.74	0.31	Cuticle protein
GB12636	–	3.06	Apidermin 2
GB30337	–	2.02	Endocuticle structural glycoprotein SgAbd-2
GB30202	–	0.36	Apidermin 1
GB30203	–	0.23	Apidermin 3
Mite infestation comparison (adult)			
Gene	$G4^+/G4^-$	$S88^+/S88^-$	Honey bee protein
GB30202	–	7.45	Apidermin 1
GB30203	–	12.64	Apidermin 3

Note: The numbers in the table represent the fold change in each comparison. acyl-coA, acyl-coenzyme A; nAChR, nicotinic acetylcholine receptors.

differences in the cuticular composition of their host to reach a brood cell and start reproduction (Del Piccolo *et al*., 2010); this, in turn, may influence the water content of the honeybee and its survival (Annoscia *et al*., 2012). Therefore, higher expression of these cuticular genes in the susceptible phenotype could result in a composition and structure of cuticles that is more attractive to the varroa mite. In addition, a set of genes encoding odorant binding or chemosensory protein were highly differentially expressed when comparing colonies with mite infestation ($S88^+/G4^+$), whereas in the colonies without varroa infestation, these genes were not significantly different in expression between the colony phenotypes. This result indicates that the tolerant colony is more responsive to varroa mite presence in terms of perceiving the mites' odour and consequent reactions. Furthermore, several genes encoding cytochrome P450 and esterase involved in the detoxification process were up-regulated in the comparison of colonies with mite infestation ($S88^+/G4^+$). In an insecticide-tolerant strain of house flies, CYP6A1 was also highly expressed to the insecticide (Carino *et al*., 1994). High expression of these genes would provide the tolerant colony with an increased capacity to cope with possible toxic side effects of mite infestation or of miticides used to treat honey bee colonies against the mite infestation. Moreover, several genes involved in protein degradation were more highly expressed in the susceptible colony than in the tolerant colony. The fact that the varroa-susceptible colony showed a higher rate of protein catabolism when compared with the tolerant colony implies that higher levels of damage may be caused by varroa mites in susceptible bees.

Comparisons between the presence and absence of the varroa mite infestation

In the mite infestation comparisons at the pupa stage, out of nine olfactory signalling genes that were differentially expressed, only one gene was up-regulated in $G4^+$ relative to $G4^-$, whereas the rest were up-regulated in $S88^+$ relative to $S88^-$ (Table 2). The olfactory regulatory process in the insect brain is mainly cholinergic (Kreissl & Bicker, 1989). One gene, *GB17254*, encoding a neuronal nicotinic acetylcholine receptor, showed five times higher expression in $S88^+$ relative to $S88^-$. High expression of this gene would promote increased olfactory learning and memory in response to mite odour in the tolerant bees. This would be particularly important in the expression of varroa sensitive hygiene (VSH) behaviour, as the ability to detect and remove varroa mites from the brood would be increased (Tsuruda *et al*., 2012). In the same comparison, cadherin-87A (*GB17702*), neural-cadherin (*GB12853*) and neurogenic protein (*GB12287*) were also highly expressed in $S88^+$ relative to $S88^-$. High expression of these genes would be beneficial for healthy cell growth and differentiation in the tolerant phenotype (Parsons *et al*., 2010). All these indicate that the tolerant phenotype, S88, is more responsive to varroa mite infestation with respect to olfactory signalling processes than the susceptible phenotype, G4.

Of the differentially expressed cytochrome P450 genes at the pupa stage, the *P450-6A1* gene was up-regulated in $S88^+$ relative to $S88^-$. Interestingly, the esterase *E4* gene showed a similar expression pattern as the *P450-6A1* gene at the pupa stage, in which expression of the gene in $S88^+$ was approximately four times higher than in $S88^-$. However, at the adult stage, this gene was down-regulated in $S88^+$ relative to $S88^-$. Both cytochrome P450 and esterase are involved in detoxification processes. High expression of cytochrome P450-6A1 and esterase E4 in the tolerant pupa bees would help deal with possible toxic compounds generated by varroa infestation or stresses associated with the parasitism.

One differentially expressed peroxisomal acyl-coenzyme A (acyl-coA) oxidase 1 gene was identified in two of the mite infestation comparisons, $S88^+/S88^-$ at the pupa stage and $G4^+/G4^-$ at the adult stage. Peroxisomal acyl-CoA oxidase catalyses the reaction from acyl-CoA

to 2-trans-enoyl-CoA in the β-oxidation of very long chain fatty acids in the peroxisome. The increased expression in these two mite infestation comparisons indicates that, regardless of the colony phenotype, the mite infestation was able to cause enhanced oxidation of very long chain fatty acids. By contrast, six other genes involved in long chain fatty acid metabolism were differentially expressed in the adult mite infestation comparison. All these genes were down-regulated in the tolerant colony with the mite infestation, but not in the infested susceptible colony G4, indicating that the biosynthesis of long chain fatty acids was depressed in the tolerant colony as a result of the mite attack. The reason for this is currently unknown.

Comparison between pupa and adult stages

A larger number of genes showed differential expression at the pupa stage than at the adult stage. At the pupa stage, 126 genes were differentially expressed in the colony comparison. At the adult stage, however, only 63 genes showed differential expression amongst all the comparisons, with 50 of these showing differences in the colony comparison. The greater number of differentially expressed genes at the pupa stage indicates that pupae are more sensitive and responsive to varroa parasitism. Therefore, the pupa stage may be a critical period for detecting differentially expressed genes that can be used to distinguish the tolerant from the susceptible bees. There may be several reasons for this. At the pupa stage, the living space of a bee is limited to the sealed brood cell shared with the varroa mite. Within a sealed brood cell infected by the mite, the concentration of the odour emitted by the mite in the cell would be high, which may effectively induce expression of genes related to host defence against the pest (Del Piccolo *et al*., 2010). In addition, adult honey bees can carry out hygienic behavioural actions to remove varroa mites, whereas pupae are unable to actively eliminate mites (Ibrahim & Spivak, 2006). Furthermore, at the adult stage, defences against mites can be mounted at the group level, referred to as social immunity. The grooming behaviour amongst bees can effectively remove mites from adult bees (Peng *et al*., 1987). By contrast, without hygienic and grooming behaviours, the pupae have to rely on manipulation of gene expression to respond to mite parasitism.

Comparison between this study and previous similar studies

Microarray analysis has been used to look at changes in gene expression between varroa-tolerant and susceptible colonies screened by natural selection (Navajas *et al*., 2008), between *A. mellifera* and *A. cerana* (Zhang *et al*., 2010), and by analysing well-defined VSH phenotypes (Le Conte *et al*., 2011). Surprisingly, there were few changes in common between the previous studies and this study. It is difficult to compare these studies because of the variations in colony phenotypes used, selection environments and the tissues used for analysis. For example, the first microarray study (Navajas *et al*., 2008) used colonies from France in which the tolerant colonies survived varroa infestation without treatment for 11 years and the susceptible colonies survived for 5 years, showing a higher varroa population. In our experience varroa-susceptible colonies show very rapid varroa population growth during high brood production periods, with increases in deformed wing virus infections and decreases in life span (less than 2 years). In this study we examined two extreme colony phenotypes selected in Canada from a large diverse gene pool, where severe climatic conditions exist during winter months. The varroa-tolerant colony in this study survived five winters, showing good honey production and hive health. The microarray used for the analysis in Navajas *et al*. (2008) comprised a total of 4795 cDNAs, which is less than half of the probes in the microarray (13 440 distinct oligonucleotides representing 10 620 different genes) used in our study. The changes in gene expression levels between the two colonies analysed were minor in Navajas *et al*. (2008) and fewer differentially expressed genes were identified. We identified 232 differentially expressed genes, many of which showed multifold changes in expression (up to 12-fold by microarray and 14-fold by qRT-PCR) in our examination of transcripts from stage 4 pupa bee heads, whereas only 148 differentially expressed genes were identified from whole bee extracts in Navajas *et al*. (2008). We also examined changes in gene expression in adult bees collected from the brood nest, but changes were less significant. We identified seven transcripts with substantial increases of gene expression in the parasitized tolerant S88 phenotype pupae and one in the adult bees. This is consistent with the results of a high-resolution linkage analysis study of well-defined VSH+ and VSH– bees in which a number of the genes involved in olfaction, olfactory learning and vision processes in association with the VSH were identified within the mapped quantitative trait locus (QTL) (Tsuruda *et al*., 2012). The differences in the level of expression and number of differentially expressed genes found in this study can be explained by the extreme nature of the phenotypes investigated. In addition, all possible effects of colony phenotype and mite infestation, allowing more detailed comparisons of the genes differentially expressed in the different colonies in response to mite infestation, were investigated in our study.

Like previous studies (Navajas *et al*., 2008; Le Conte *et al*., 2011), we did not identify many differentially expressed genes involved in immune responses, which

could play a role in defence against parasite infestation. This consistent result indicates mechanisms other than immune response, such as olfactory signal transduction and detoxification processes might play more important roles in varroa tolerance. In this context, we note that the honey bee possesses only one-third of the number of immune response genes of other social insects (The Honeybee Genome Sequencing Consortium, 2006).

A complementary study on the same colony phenotypes using kinome arrays showed that varroa mite resistance and susceptibility are also reflected at the signal transduction level (Robertson *et al*., 2014). Distinct kinome profiles were observed between G4 and S88 bees at three developmental stages (pink-eyed, dark-eyed and adult). Kinome analysis also showed that differences in immune capabilities were not involved in varroa susceptibility. However, in the G4 pupae there was a trend toward the down-regulation of innate immune processes, which was not observed in the resistant, S88 phenotype. The mite-mediated immune suppression within the susceptible phenotype may reduce the ability of these bees to counter secondary viral infections. In the resistant phenotype mitogen-activated protein (MAP) signalling pathways were activated, consistent with genes involved in stress responses and detoxification processes.

The brain-specific gene expression profiles of two adult bee colony phenotypes, one with a high rate of hygienic behaviour (VSH+) and the other with a low rate of hygienic behaviour (VSH–) were compared in another DNA microarray study (Le Conte *et al*., 2011). Out of 39 genes identified, *GB16453* encoding fluoxetine resistant protein 6 and *GB30242* encoding odorant binding protein 3 were expressed at higher levels in VSH– compared with VSH+. In our study, *GB16453* was also found to be more highly expressed in $G4^+$ (susceptible colony with a low rate of VSH) compared with $S88^+$ (tolerant colony with a high rate of VSH) at the pupa stage, whereas at the adult stage *GB30242* was more highly expressed in adult $S88^+$ compared with $G4^+$. A comparison of gene expression between the western honey bee, *A. mellifera*, and the eastern honey bee, *A. cerana*, identified many differentially expressed genes that were involved in general metabolic processes (Zhang *et al*., 2010). Our study indicated that a number of genes involved in protein and lipid metabolism were highly differentially expressed in pupa bees in the resistant and susceptible colonies.

Candidate genes for marker-assisted selection of varroa resistance traits

Real time qRT-PCR was performed to validate the high differential expression of six candidate genes identified by DNA microarray analysis for marker-assisted selection in varroa mite resistance breeding. Of the six genes, four encode cytochrome P450 proteins. *GB12136* (cytochrome P450-6A1) and *GB14612* (cytochrome P450-6K1) had significantly higher expression in pupae of the tolerant colony with mite infestation ($S88^+$) compared with the susceptible colony (G4) with or without mite infestation. In particular, the expression level of *GB12136* was more than five times higher in $S88^+$ than in $G4^+$. The high expression of cytochrome P450-6A1 and cytochrome P450-6K1 in the S88 colony with mite infestation at the pupa stage might equip the tolerant bees with a better capacity to detoxify compounds generated or introduced by varroa infestation. By contrast, the two cytochrome P450 genes, *GB19306* (cytochrome P450-6K1) and *GB19967* (cytochrome P450-9E2), were more highly expressed in the tolerant colony at the adult stage without mites ($S88^-$) relative to the same colony with mites ($S88^+$), whereas at the pupa stage there was no significant difference in the expression levels of these two genes. The unique expression pattern indicates that this group of P450 genes might have distinct roles in bees responding to mite infestation.

GB16889, which encodes esterase E4, had significantly higher expression in the tolerant colony with mites ($S88^+$) relative to all the other samples. It was noted that the expression level was 10 times higher in $S88^+$ than that in the susceptible colony with mites ($G4^+$) at the pupa stage. However, at the adult stage this gene was more highly expressed in the tolerant colony without mites, $S88^-$. This expression pattern implies that the role of this gene varies with developmental stage. Nevertheless, at the pupa stage it might function in the detoxification of toxic esters introduced or generated by mite infestation.

The expression pattern of the gene *GB11723*, which encodes apolipoprotein D, was quite different from those of the genes involved in detoxification processes. At the pupa stage, this gene was significantly down-regulated in the susceptible colony with mites relative to the other samples. In particular, the expression level of this gene was 14 times lower in the susceptible colony with mites ($G4^+$) than that in the tolerant colony with mites ($S88^+$). However, at the adult stage an expression difference was observed only between the two colonies overall, regardless of the presence or absence of mites. The expression pattern at the pupa stage was consistent with the role of this gene in lipid transport, conferring a higher rate of lipid metabolism to the S88 colony, which may help to fight mite infestation.

Concluding remarks

In summary, this study employed a genome-wide DNA microarray to analyse differential gene expression at two different developmental stages of varroa-tolerant and

varroa-susceptible honey bee colony phenotypes selected from a Canadian breeding programme. Comparison of the microarray expression profiles revealed that nearly 300 genes were differentially expressed between the two extreme bee colonies in response to mite infestation. More differentially expressed genes were found at the pupa stage than at the adult stage, indicating that pupae are more responsive to varroa parasitism than adult bees. More differentially expressed genes were identified when comparing colonies than when comparing responses to mite infestation, regardless of the developmental stage. According to their molecular functions, the differentially expressed genes are classified into groups that are involved in olfactory signal transduction, detoxification processes, and protein and lipid metabolism as well as exoskeleton formation, implying that these processes may be critically involved in the defensive mechanisms of honey bees against varroa mite parasitism. Future investigation of host responses to multiple disease agents could be performed to identify comprehensive causes of bee colony death. This would offer a better understanding of disease pathogenesis in bees, including secondary infections and possible synergistic effects of more than one pathogen. In addition, examination of these highly differentially expressed genes identified by the DNA microarray analysis in a wide range of tolerant and susceptible colonies is critical for validation of the results obtained here. The long-term approach of selective breeding for varroa-tolerant honey bees is being explored through the use of different molecular techniques. In this regard, molecular markers developed based on the validated differential expression of key genes identified when comparing a wide range of tolerant and susceptible phenotypes would facilitate effective selection of productive, healthy bees tolerant to the varroa mite.

Experimental procedures

Honey bee colony phenotypes

The colony phenotypes used in this study were selected and characterized by the Saskatraz project (www.saskatraz.com; Robertson *et al.*, 2014). The Saskatraz natural selection apiaries are operated by Meadow Ridge Enterprises Ltd, Saskatchewan, Canada (52°11'N, 106°63'W). The tolerant colony S88 survived natural selection for 58 months with mild varroa infestation. By contrast, the susceptible colony died within 17 months of construction showing severe mite infestation. Samples for RNA extraction were collected from both adult and pupae stages between 22 and 23 September 2010 from the varroa-susceptible phenotype G4 and the varroa-tolerant phenotype S88. For pupa sampling, brood frames were removed from the hives and incubated in darkness at 32 °C and 80% humidity in the field laboratory at Meadow Ridge Enterprises Ltd or in the laboratory at the University of Saskatchewan. Capped brood cells were carefully opened; the eye cuticle colour of the brood was used to distinguish developmental stages of the pupae. Pupae at the dark-eye stage 4 were collected for this study from brood cells and frozen in liquid nitrogen before being stored at −80 °C. Pupae from cells infested with varroa were separated from non-infested ones before freezing. Young freshly emerged adult bees were captured on brood frames, and frozen in liquid nitrogen before being stored at −80°C. A bee was considered to be parasitized if there were varroa mite(s) attached to the bee, and bees with mites were separated from nonparasitized bees before freezing.

DNA microarray hybridization

Two honey bee heads of either dark-eye pupae or adult bees were separated from the bodies in liquid nitrogen for RNA extraction. The two bee heads were pooled and pulverized with a pestle with liquid nitrogen in a 2 ml plastic tube. The total RNA of each sample was isolated using RNeasy Plant Mini kits (Qiagen, Valencia, CA, USA) and treated with DNase (RNase free Dnase I, also Qiagen) as described by the manufacturer. RNA purity and integrity were checked by a spectrophotometer and agarose gel electrophoresis with 1% agarose gels. Six biological replicates were performed in each treatment group: the total sample size was 2 (heads) × 2 (stages) × 2 (varroa affliction status) × 2 (colonies) × 6 (replicates) = 96 bee heads.

DNA microarray hybridization was conducted at the Department of Entomology and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, on a custom basis. One μg of the total RNA from each sample was amplified using an Amino Allyl Message AmpII RNA Amplification kit (Ambion/Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. The amplified RNA sample was labelled with cyanine 3-deoxycytidine triphosphat (3-dCTP, Cy3, 532nm) and cyanine 5-dCTP (Cy5, 635 nm) fluorescent dyes, separately. Dye swaps were conducted between replicates to avoid the effects of dye bias. Labelled probes were hybridized to the bee whole-genome oligonucleotide arrays that were designed previously. Long oligos (70-mers) representing individual genes were synthesized and deposited on the arrays at the University of Illinois at Urbana-Champaign. Each array contains a total of 13 440 distinct oligonucleotides including an 'official gene set' of 10 620 oligos recommended by the Honey Bee Genome Sequencing Consortium, the oligos representing expressed sequence tags from other databases, and the honey bee viral pathogens (http://www.biotech.uiuc.edu/functionalgenomics/services-equipment/honeybeeoligo). Hybridizations were carried out at 42 °C overnight using Agilent hybridization cassettes (Santa Clara, CA, USA). Following incubation, slides were washed and fluorescence was measured on an Axon 4000B confocal laser scanner (Molecular Devices, Sunnyvale, CA, USA). Spot finding and image editing were performed using GenePix 6.1 software at the University of Illinois at Urbana-Champaign (Champaign, IL, USA).

Statistical analysis

Statistical analysis was performed using the R/Bioconductor package (R Core Team (2013) software, http://www.r-project.org/). For background subtraction, manually flagged spots were excluded, but auto-flagged spots were included. A print-tip loess

normalization was performed using $\log_2$-transformed values on each array to even out the green dye bias. A scale normalization was performed between all arrays so that the distributions of M-values [$\log_2$ (Cy5/Cy3)] was approximately the same for all spots. Subsequently, a mixed-model analysis of variance was fitted on the M-values that included a fixed term for dye (the same dyes always used), plus a random term for the duplicate spots per oligo. A Bayesian correction was used to moderate the variance for each oligo. The raw p-values were adjusted separately for each comparison using the false discovery rate method. The microarray data obtained met Minimum Information about Microarray Experiment standards (Brazma, A. *et al.*, 2001).

Functional analysis

BLAST searches of molecular databases at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) were carried out to identify homologies between probe cDNAs of interest and the honey bee genome, or genes from other organisms. GO analysis was used to explore the functional insights into differentially expressed genes using their corresponding FlyBase identification number. Each gene was assigned to the single 'best hit' match in BLASTX searches of fruit fly *Drosophila melanogaster* predicted proteins. GO functional terms and *Drosophila* gene GO annotations were downloaded from the GO website (www.geneontology.org, February 2012). Enrichment analysis was performed using GOTOOLBOX (http://genome.crg.es/GOToolBox/) through a hypergeometric test followed by the Benjamini & Hochberg false discovery rate adjustment. Functional clustering of the genes was also conducted in GOTOOLBOX using the unweighted pair group method with arithmetic algorithm with a Bonferroni correction for multiple testing. Only categories that had more than three genes were selected for further analysis.

Real time qRT-PCR

Six candidate genes identified by the DNA microarray analysis were chosen for real-time qRT-PCR analysis. Two housekeeping genes, *actin* and *ribosomal protein S5*, were used as internal references. Primers were developed using PRIMER3PLUS online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Table S4) and synthesized by Sigma-Aldrich (St. Louis, MO, USA, www.sigmaaldrich.com). One μg of a RNA sample used for the microarray analysis was reverse transcribed to the first-strand cDNA using qScript cDNA SuperMix (Quanta, Quanta BioScience, Gaithersburg, MD, USA). The reaction was heat-inactivated and diluted fivefold with water. Four μl of a diluted sample was used in a 16-μl real-time qRT-PCR reaction containing 4 μl each of the two primers (10 ng), and 8 μl SYBR Supermix (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 96-well plates in a CFX96 System (Bio-Rad), using the following thermocycling conditions: an initial denaturation at 95 °C for 30 s, 30-s annealing at 60 C and 30-s elongation at 72 C. For each sample, three biological replicates were performed. Data obtained by the ICYCLER software (Bio-Rad) were subsequently analysed with custom-designed spreadsheets. The relative expression ratios of target genes were calculated using the comparative CT method. General linear model univariate analysis and multiple comparisons were conducted using Duncan's post hoc test. A difference was regarded as statistically significant when the *P*-value was less than 0.05.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Enrichment analysis of gene ontology (GO) terms in the mite infestation comparison and colony comparisons at the pupa stage.

Table S2. Enrichment analysis of gene ontology (GO) terms in the mite infestation comparison and colony comparisons at the adult stage.

Table S3. Differentially expressed genes that were identified by the DNA microarray analysis.

Table S4. Genes and primers used in real-time quantitative PCR.

Table S5. Labelling of the samples on the DNA microarray.