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Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*)

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ABSTRACT

Honey bee (*Apis mellifera*) larvae reared *in vitro* were exposed to one of nine pesticides and/or were challenged with the parasitic mite, *Varroa destructor*. Total RNA was extracted from individual larvae and first strand cDNAs were generated. Gene-expression changes in larvae were measured using quantitative PCR (qPCR) targeting transcripts for pathogens and genes involved in physiological processes, bee health, immunity, and/or xenobiotic detoxification. Transcript levels for Peptidoglycan Recognition Protein (PGRPSC), a pathogen recognition gene, increased in larvae exposed to Varroa mites ($P < 0.001$) and were not changed in pesticide treated larvae. As expected, Varroa-parasitized brood had higher transcripts of Deformed Wing Virus than did control larvae ($P < 0.001$). Varroa parasitism, arguably coupled with virus infection, resulted in significantly higher transcript abundances for the antimicrobial peptides abaecin, hymenoptaecin, and defensin1. Transcript levels for Prophenoloxidase-activating enzyme (PPOact), an immune end product, were elevated in larvae treated with myclobutanil and chlorothalonil (both are fungicides) ($P < 0.001$). Transcript levels for Hexameric storage protein (Hsp70) were significantly upregulated in imidacloprid, fluvalinate, coumaphos, myclobutanil, and amitraz treated larvae. Definitive impacts of pesticides and Varroa parasitism on honey bee larval gene expression were demonstrated. Interactions between larval treatments and gene expression for the targeted genes are discussed.

1. Introduction

The Western honey bee, *Apis mellifera* L., provides pollination services to a diverse array of agricultural crop plants (Delaplane and Mayer, 2000). These services are at risk due to declining bee populations resulting from the exposure of bees to parasites, pathogens, and environmental chemicals, the latter including pesticides and other anthropogenic substances (vanEngelsdorp et al., 2009; Ellis et al., 2010). Honey bees are non-target organisms for most pesticide applications; nevertheless, they can be exposed to pesticides while collecting pollen and nectar from flowers, collecting resins from various plants, drinking water from rivers/lakes/ponds/etc., breathing, and during flight (if the pesticides are airborne) (Mullin et al., 2010; Gregorc and Ellis, 2011). Honey bees regularly forage 3–6 km from their colony (Visscher and Seeley, 1982; Seeley, 1985; Winston, 1987), representing a foraging area of 28–115 km^2 for a single colony. Consequently, honey bee exposure to pesticides *in vivo* likely is common. In fact, Mullin et al. (2010) found 121 different pesticides and metabolites in wax, bee and associated hive samples, including pollen, only a few of which are compounds used by beekeepers to control bee colony pests.

Honey bee foragers collect pollen and nectar from flowers to sustain the colony and support healthy brood development (Winston, 1987); thus pesticides in the environment potentially could be transmitted to immature bees (brood) through pollen, wax, or brood food contamination. For example, pollen is a primary food source of both adult and developing honey bees; as a result, an entire colony can be exposed to pesticides via pollen consumption (Chauzat et al., 2006; Villa et al., 2000). Exposure to pesticides can impact foraging honey bees (Vandame et al., 1995), shorten worker bee longevity (McKenzie and Winston, 1989), decrease the survival and weight of queen bees (Pettis et al., 2004), and affect colony vitality (Beliën et al., 2009), all among other effects documented in the literature. Sublethal effects of various pesticides on bees can lead to physiological changes (Papaefthimiou et al., 2002), changes in individual honey bee behavior (Weick and Thorn, 2002; Aliouane et al., 2009; Yang et al., 2008), and

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alterations in cellular physiology consistent with chemically induced stress (Gregorc and Bowen, 1999, 2000; Gregorc et al., 2004; Gregorc and Smodis Skerl, 2007; Smodis Skerl and Gregorc, 2010; Gregorc and Ellis, 2011).

Since honey bees can be exposed to multiple chemical agents simultaneously (Mullin et al., 2010), synergistic or antagonistic interactions among these pesticides or between pesticides and bee pests/pathogens also could play a role in bee and colony health (Johnson et al., 2009). For example, Alaux et al. (2010) published physiological evidence that the insecticide imidacloprid and the fungal pathogen *Nosema* can interact synergistically to affect bee health negatively, including physiological changes initiated by pesticide exposure that decreased bee tolerance toward *Nosema* infection. Similarly, Pettis et al., (2012) recently showed an increase in *Nosema* spore loads in colonies treated with imidacloprid.

Recognizing that potential interactions between pesticides and bee pests/pathogens are poorly understood, our goal in the current project was to determine physiological responses of bees to chemical and biological threats by measuring gene expression after exposure to the ubiquitous parasitic mite *Varroa destructor* (Ellis and Munn, 2005) and a suite of pesticide threats. The tested pesticides (with pesticide class in parentheses) included two fungicides [myclobutanil (azole), chlorothalonil (substituted benzene)], two herbicides [simazine (triazine), glyphosate (phosphonoglycine)], and five insecticides/miticides [fluvalinate (pyrethroid), imidacloprid (nicotinoid), coumaphos (organophosphate), chlorpyrifos (organophosphate), amitraz (amidine)] and represent a range of modes-of-action and pesticide families. Three of these compounds (amitraz, fluvalinate, and coumaphos) are used often by beekeepers to control Varroa and/or small hive beetles (*Aethina tumida*) in colonies. The other chemicals are used commonly in agricultural settings and, with the exception of glyphosate, have been found as residues in honey bee colonies (Mullin et al., 2010). A second goal of our study was to perform parallel gene expression analyses for candidate genes involved with honey bee immunity, development, and pesticide detoxification. Gene candidates first were screened as a 47-gene array using quantitative PCR (qPCR; Evans, 2006). Promising candidates from this round then were screened across individual challenged bees and controls using qPCR.

2. Materials and methods

Experiments were conducted at the Honey Bee Research and Extension Laboratory at the University of Florida's Department of Entomology and Nematology (Gainesville, FL, USA) and at the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) Bee Research Laboratory in Beltsville, MD, USA.

2.1. Larval rearing procedure

Following the procedure outlined by Gregorc and Ellis (2011), one queen honey bee originating from a 10-frame, Langstroth-style production colony located in Gainesville, Florida was confined to a section of newly-drawn comb using a metal queen excluder cage ($\sim 10 \times 10 \times 3$ cm) at time $t = -12$ h. The caged queen and frame were returned to the center of the brood nest where worker bees could access and tend the queen. After 24 h of confinement, $t = 12$ h (Peng et al., 1992; Aupinel et al., 2005), we removed the queen from the cage and replaced the cage on the comb as before but this time for 108 h (from $t = 0$) to allow the eggs to hatch and larvae to reach an appropriate age for grafting. During this time, worker bees were able to access the comb to feed the developing larvae. At 108 h, we removed the test frame (now containing 36 ± 12 h old larvae) from the colony and took it to the laboratory.

At the laboratory, hatched worker (female) larvae were grafted into sterile, 96-well tissue culture plates (well volume = 0.32 mL, Fisher Scientific, Pittsburgh, PA, USA) (Evans, 2004; Aupinel et al., 2005; Gregorc and Ellis, 2011). Prior to grafting the larvae into plates, we provisioned individual wells in the plate with 20 μL of larval diet (Vandenberg and Shimanuki, 1987). The diet had a pH that ranged from 4.0 to 4.5 and consisted of 50% royal jelly (Glory Bee Foods, Eugene, OR, USA), 6% D-glucose (Fischer Chemical, Fair Lawn, NJ, USA), 6% D-fructose (Fischer Chemical, Fair Lawn, NJ), 37% double distilled water, and 1% yeast extract (Bacto™, Sparks, MD, USA) by volume (Aupinel et al., 2005). Prior to adding the diet to each cell, we pre-warmed it to 35 °C in an incubator (Percival Scientific Inc., Perry, IA, USA).

On consecutive days, we transferred larvae to a clean culture plate provisioned with fresh diet. The amount of artificial diet provided to each larva depended on the larva's age. We fed larvae 20 μL of diet at hours 108 and 132, 30 μL on hour 156, 40 μL on hour 180, and 50 μL on hour 204 (Vandenberg and Shimanuki, 1987; Aupinel et al., 2007). At 204 h post oviposition (larvae are 132 ± 12 h old), we transferred the larvae to a 48-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA, wells were 13 × 17 mm) because the growing larvae were too large to handle delicately in a 96-well plate. Throughout the study, trays containing larvae were incubated in the dark at 35 °C and ~96% RH (Peng et al., 1992).

At the 156 and 180 h feedings, larvae received specific pesticide concentrations mixed with the larval diet *ad libitum*. Nine treatment groups of 24 larvae per group were established. Each group was assigned one pesticide, and received concentrations of those pesticides as follows: 0.8 ppm chlorpyrifos, 200 ppm imidacloprid, 200 ppm amitraz, 100 ppm fluvalinate, 50 ppm coumaphos, 200 ppm myclobutanil, 200 ppm chlorothalonil, 200 ppm glyphosate, and 200 ppm simazine. Prior to administration to the larval diet, each pure pesticidal substance was diluted individually in an acetone solvent. The diet/pesticide combinations were prepared and stored in 1.5 mL snap-top plastic vials (Fisher Scientific, Pittsburgh, PA, USA). Two control groups of 24 larvae per group were included in the study. Larvae in the first control group were fed a mixture of diet plus acetone while larvae in the second group were fed only the untreated diet.

Twelve of the larvae from each group received only a pesticide treatment and were allowed to initiate the pre-pupal phase of development once they defecated. The remaining twelve larvae from each group received two adult female Varroa added to the wells after the larvae defecated. The mites were collected in their phoretic stage from honey bee colonies by placing ~300 worker bees in a 0.5 L glass jar, coating the bees with approximately 30 g of 10× confectionary sugar, shaking the jar lightly for 30 s, and collecting mites falling from the screened jar with a sieve. Eight live pupae from each pesticide group, challenged and not challenged with Varroa, were placed separately into 1.5 mL vials, 48 h after exposure of the challenged pupae to the mites. All vials with pupae were stored at −80 °C until molecular analyses were conducted.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from individual sampled pupae using Trizol reagent (Invitrogen, Carlsbad, CA) as described by Evans (2006). DNAse treatment was performed at 37 °C for 60 min followed by cDNA synthesis accomplished by adding 4 μL Superscript mix and incubating at 42 °C for 50 min. First strand cDNAs were generated from 8 μL of total RNA using a mix of 4 μL Superscript II Reverse Transcriptase (Invitrogen), 2 nmol of dNTP (deoxyribonucleotide triphosphate), and a composite of 2 nmol of poly dT-18 and 0.1 nmol of poly dT(12–18).

2.3. Parallel quantitative PCR screening for bee responses to pesticides and Varroa

Gene-expression assays were carried out in two stages. First, RNA levels of genes linked to immune-response traits, honey bee stress responses, bee development, and a subset of known honey bee pathogens were queried on pooled RNA samples in a 46-target format (Evans, 2006). Each pool consisted of equimolar RNA's derived from eight individual bees from each treatment and control group (those exposed to pesticides only or to pesticides and Varroa, and all relevant block controls). Primer pairs for these candidates were as in Evans (Evans, 2006), or as presented for new candidates in Table 1. Most primer pairs targeted products of 120–180 bp, with predicted annealing temperatures from 59.5 to 60.5 °C. Real-time PCR reactions for specific genes amplification were performed in 96-well plates using a BIO-Rad CFX-96 thermal cycler (Bio-Rad Corp.). Reaction mixtures consisted of 10 μL premixed ROX SYBR mix (Invitrogen), 20 μM of each gene-specific primer (forward and reverse), 2 μL cDNA template (reflecting the product of ~100 ng total RNA) and DEPC (diethylpyrocarbonate)-treated water to 20 μL. PCR reactions were conducted using a thermal program of 95 °C for 30 s, followed by 40 cycles denaturing at 95 °C 20 s, annealing at 60 °C 30 s and extension at 72 °C for 60 s. Fluorescence was measured during the annealing step.

Following this initial screening, cDNA's from each of the eight larvae from each treatment or control regime were screened individually for a subset of the primer targets. Quantitative-PCR reactions were carried out in 96-well plates as above. The subset of targets in this final screening included the pathogens found in the study population as well as immunity or stress-related genes that showed signs of differential expression during the pooled-bee analysis (Supplemental Fig. 1).

2.4. Data analyses

Fluorescence levels for qPCR were averaged during cycles 2–10, and this baseline was used in assigning a minimal fluorescence for later cycles above which amplified products were recognized. This CT (cycle threshold) value for each target was normalized for variance across samples in template amount using the dCT method and an internal honey bee transcript control (for actin, Evans, 2006).

Transcript abundances for pooled bee samples used to vet the entire set of candidate genes and pathogens were analyzed and visualized using cluster analyses (Ward's algorithm as presented by SAS JMP version 7.0, SAS Corp.). For the screens of individual larvae, analyses of variance were performed for each applied pesticide and/or Varroa application separately to eight larvae in each treatment group using the dCT values (i.e., each treated larvae was a replicate). Interactive effects of pesticide and Varroa exposure on honey bee larvae were tested for statistical significance using Mixed Model ANOVAs (SAS JMP version 7.0, SAS Corp.).

3. Results

3.1. Effects of Varroa parasitism on larval gene expression and pathogen targets

In the initial screening of 11 pathogen targets, only six showed positive signals by qPCR, including the RNA viruses Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV), and Black

Table 1
Primer pairs used for quantitative PCR of honey bee gene and pathogen transcripts.

Locus	Forward primer	Reverse primer	Identifier
Abaecin	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	GB18323
AmEater	CATTTGCCAACCTGTTTGT	ATCCATTGGTGCAATTTGG	GB14645
AmNOS	TCCACTCGCAGGTACTTTCC	TCTGGAGGATCACCATTTCC	GB18010
AmVg	TCTTCCCCTTCGATAAACACA	GTTTCGGTGGGGAAACTGT	GB13999
ApidNT	TTTTGCCTTAGCAATTCTTGTTG	GTAGGTCGAGTAGGCGGATCT	GB17782
Basket	AGGAGAACGTGGACATTTGG	AATCCGATGGAAACAGAACG	GB16401
CactusB	TGGTTGTCGTGCCAATACAG	TGTTCCAGTGAAAACGCAAT	GB13520
Catalase	GTCTTGGCCCAAACAATCTG	CATTCTCTAGGCCCACCAAA	GB11648
CEst04	TTTTGGGCCACGTTTACTTC	CAAATCGGTGGGTGTCTTCT	GB13591
CYP4G11	CAAAATGGTGTTCTCCTTACCG	ATGGCAACCCATCACTGC	GB11973
Defensin1	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG	GB19392
Defensin2	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA	GB10036
Dorsal-1	AAATGGTTCGCTCGTAGCAC	TCCATGATATGAGTGATGGAAA	GB19537
Dscam	TTCAGTTCACAGCCGAGATG	ATCAGTGTCCCGCTAACCTG	GB11871
GCN5ProtSyn	GGACAACCACCTTTTGAACG	AGGAGCTTCCTCTGCACTGA	GB19492
GSTS3	TGCATATGCTGGCATTGATT	TCCTCGCCAAGTATCTTGCT	GB19254
Hexam10869	GGACAATTGGATCTGCTCGT	GTGTTGCTTCCGCTTTTCAG	GB10869
Hopscotch	TTGTGCTCCTGAAAATGCTG	AACCTCCAAATCGCTCTGTG	GB16422
Hymenopt	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT	GB17538
PGRPLC710	TCCGTCAGCCGTAGTTTTTC	CGTTTGTGCAAATCGAACAT	GB17188
PGRPSC	TAATTCATCATTCGGCGACA	TGTTTGTCCCATCCTCTTCC	GB19301
PPOact	GTTTGGTCGACGGAAGAAAA	CCGTCGACTCGAAATCGTAT	GB18767
Actin	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT	GB17681
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA	GB11132
A. apis	TCTGGCGGCCGGTTAAAGGCTTC	GTTTCAAGACGGGCCACAAAC	AY004344
A. woodi	TCAATTTCAGCCTTTTATTCAAGA	AAAACATAATGAAAATGAGCTACAA	EU190886
ABPV	ACCGACAAAGGGTATGATGC	CTTGAGTTTGCGGTGTTCCT	NC_002548.1
Bacteria	GAGAGTTTGATCCTGGCTCAG	CTACGGCTACCTTGTTACGA	M60313
BQCV	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA	NC_003784.1
DWV	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA	AY292384
FungI	GTTAAAAAGCTCGTAGTTG	CTCTCAATCTGTCAATCCTTATT	Zhou and Stanosz, 2001
IAPV	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	EU224279
KBV	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC	AY275710
M. Pluton	ACGCCTTAGAGATAAGGTTTC	GCTTAGCCTCGCGGTCTTGCGTC	X75752
N. apis	CAATATTTTATTGTTCTGCGAGG	TATATTTATTGTATTGCGCGTGCT	U26534.1
N. ceranae	CAATATTTTATTATTTTGAGAGA	TATATTTATTGTATTGCGCGTGCA	U26533.1
P. larvae	CGGGAGATGAGAAAACCAAT	CCGCAATCGTAAGCTGGTAT	DQ811780.1

Queen Cell Virus (BQCV), bacteria, fungi and the microsporidian parasite *Nosema ceranae* (Figs. 1A and 1B). The remaining pests/pathogens shown in Figs. 1A and 1B were not detected. These include *N. apis*, *Melissococcus pluton* (European foulbrood), *Paenibacillus larvae* (American foulbrood), *Ascosphaera apis* (chalkbrood), Acute Bee Paralysis Virus, Kashmir Bee Virus, and *Acarapis woodi* (tracheal mites). Universal primers for fungal and bacterial species showed variation in loads across the individually sampled larvae. Loads for DWV and IAPV were elevated in bees challenged with Varroa (Fig. 1A), an expected result given that Varroa is a potential vector of these and other honey bee RNA viruses (Chen and Siede, 2007). This result was confirmed for Deformed Wing Virus in individual larvae, whereby larvae exposed to mites had a 900-fold higher average load for DWV ($n = 113$ and 94 assayed bees; Fig. 2). Of larvae exposed to Varroa, 89% had measurable DWV loads verses 38% of those not exposed to mites. In contrast, Black Queen Cell Virus trended lower in mite-parasitized versus control individually tested larvae (Fig. 1A). Varroa parasitism resulted in significantly higher transcript abundances for the antimicrobial peptides abaecin, hymenoptaecin, and defensin1, and lower levels for Peptidoglycan Recognition Protein (PGRPSC, Fig. 1B). Mean PGRPSC expression for control larvae was 43-fold higher than for larvae exposed to mites (t ratio = 14.1; df = 177; $P < 0.01$, Fig. 2). Varroa presence also significantly decreased levels of the larval storage protein Hsp70a (Hexam), with a 415-fold higher transcript abundance in unchallenged prepupae (two tailed t-test, t-ratio = 19.9; df = 177; $P < 0.01$), and for the putative stress and xenobiotic protein Cyp4g11 (3-fold higher in unchallenged larvae, two tailed t-test, t-value = 6.59; df = 177; $P < 0.01$; Fig. 2). Differences in gene expression between the two control groups, larvae fed with a mixture of diet plus acetone or larvae fed only with untreated diet, were not detected. Results for both groups were pooled and are shown as control treated larvae.

3.2. Effects of pesticide exposure on gene expression and pathogen targets

Transcript levels for Prophenoloxidase-activating enzyme (PPOact), a key enzyme of the melanization reaction and a candidate immune protein, differed substantially across pesticide treatments. Significantly elevated or decreased transcript abundances are indicated in the Table 2. PPOact levels were especially high in bees treated with the fungicides myclobutanil and chlorothalonil, when compared to control, untreated bees and bees exposed to the insecticides chlorpyrifos, amitraz, and fluvalinate and the herbicides glyphosphate and simazine (Table 2). PGRPSC levels were decreased in bees exposed to the pyrethroid miticide fluvalinate, when compared to imidacloprid and chlorothalonil (Table 2). Hexameric storage protein Hsp70a was decreased in larvae treated with herbicides relative to the other compounds (Table 2). Deformed Wing Virus loads were relatively low in larvae exposed to glyphosate when compared to untreated, myclobutanil-treated, and imidacloprid-treated larvae (Table 2). Effects of pesticides were not seen on other tested genes.

There were no significant interactive effects between Varroa and pesticides on gene expression for the targeted genes or the 13 pathogen targets; i.e., the impacts of Varroa and pesticides were non-additive across treatments in terms of effects on gene expression for the targeted genes.

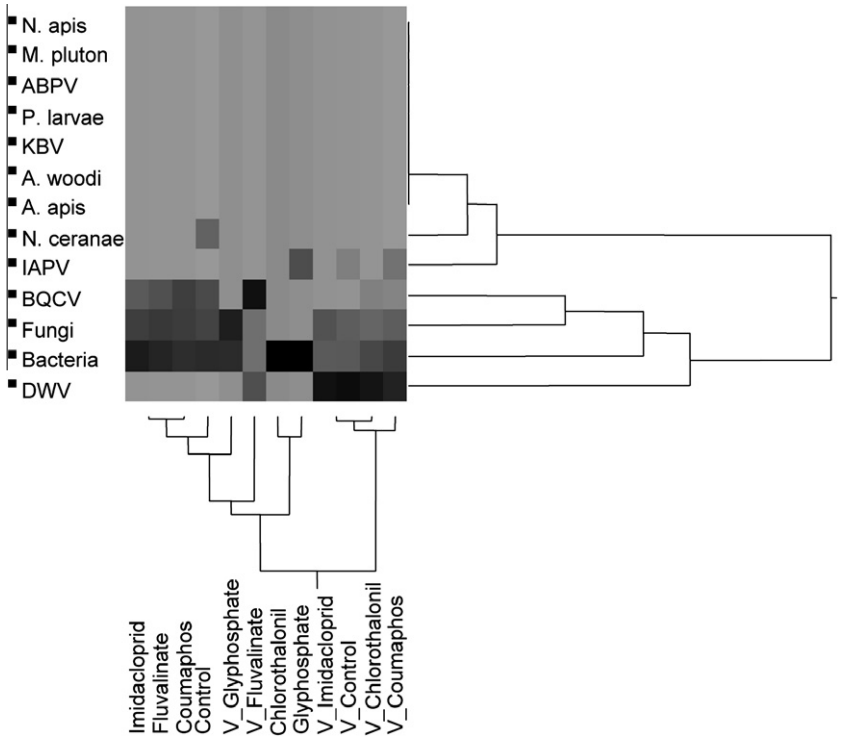

Fig. 1A. Pathogen targets using 13 primers gave positive signals for the RNA viruses Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV), and Black Queen Cell Virus (BQCV), and the microsporidian parasite *Nosema ceranae*, detected by qPCR. Universal primers for fungal (Fungi) and bacterial (Bacteria) species also were used and variation in transcripts levels across the sampled larvae of both fungi and bacteria are shown. Loads for DWV and IAPV were indicated with elevated levels in bees challenged with Varroa. Bee larvae were treated with different pesticides and challenged with Varroa (V); indications are given in bottom line description. Dark shading indicates elevated gene expression in comparison to light shading where gene expression was lower or not expressed in comparison to untreated, control larvae. The legend on the left indicates primer pairs for candidate pathogens applied to larvae and pupae exposed to pesticides and/or Varroa as shown in the bottom legend. Trees on both axes reflect geometric branch lengths of clustered samples (x-axis) or gene targets (y-axis), a measure of co-varying expression patterns (adjacent targets or treatments tend to have more similar expression patterns across the screened treatments/targets).

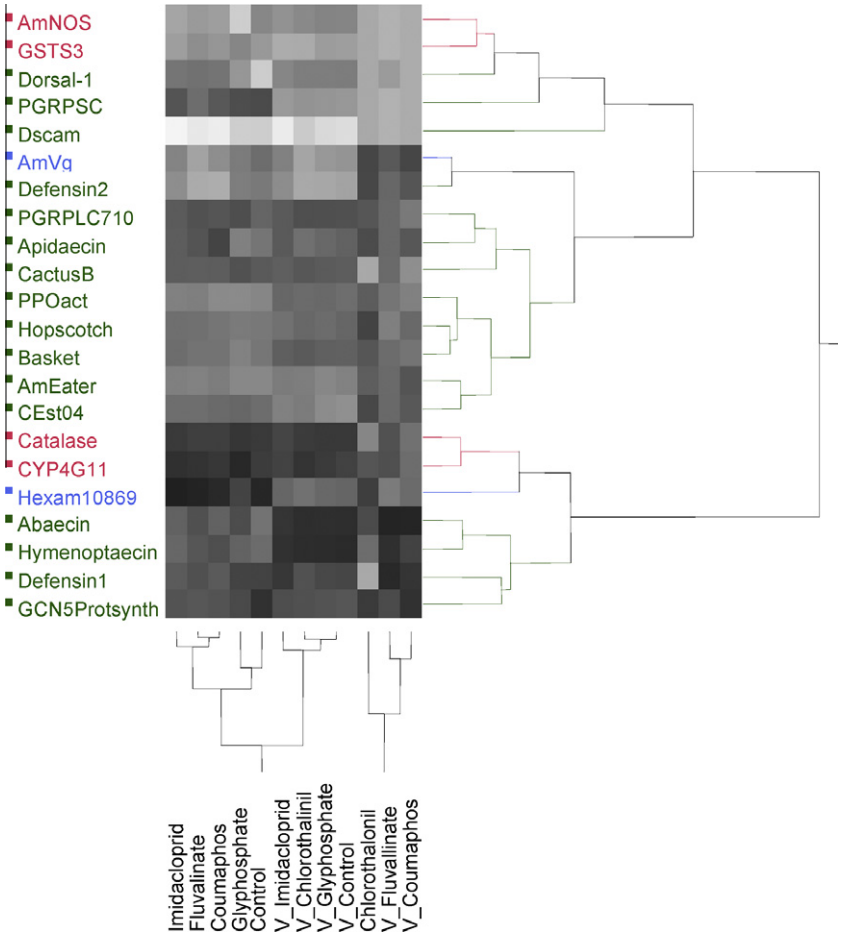

Fig. 1B. Transcript abundances for genes linked to immune-responsive traits (green font), honey bee stress responses (red font), and bee development (blue font) are shown, based on analyses of cDNA's obtained separately from eight bees treated with one of the selected pesticides and challenged/not challenged with Varroa. Primer pairs for these candidates were selected, or as new candidates shown in Table 1. Primer pairs for candidate genes are shown in the legend on the left side and larvae/pupae exposure to pesticides and/or Varroa parasitism is shown in the bottom legend. Dark shading indicates elevated gene expression in comparison to light shading where tested gene expression was lower or not expressed in comparison to control, untreated larvae. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

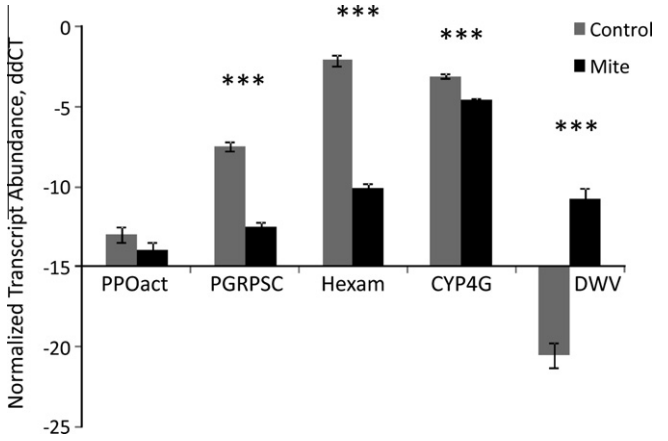

Fig. 2. Mean transcript abundance for selected genes in individual honey bee larvae reared in the incubator either treated with only Varroa or not treated with Varroa (control). Bars indicate standard deviation; asterisks indicate significantly higher transcript expressions for indicated genes in mite exposed larvae compared to untreated, control larvae. Statistical significance was determined using Mixed Model ANOVAs (SAS JMP version 7.0, SAS Corp.).

4. Discussion

Honey bees can be affected by exposure to environmental chemicals, including anthropogenic herbicides, insecticides, and fungicides and little is known about the long term impacts on honey bees of many of these toxins (Mullin et al., 2010; Johnson et al., 2010). Recent severe declines in some honey bee populations suggest that abiotic and/or biotic factors are presenting increasing challenges to honeybees (Ellis et al., 2010; Pettis and Delaplane, 2010). In this manuscript, we show changes in both disease load and transcriptional abundances for bees exposed to Varroa, their main parasite. BQCV appeared in a fraction of both Varroa-treated and untreated larvae, while DWV loads were sharply higher for bees exposed to mites. Varroa are a known vector of DWV (Yue and Genersch, 2005) and can induce stress or decrease immune responses in bee larvae. Consequently, both can lead to virus-associated collapse of honey bee colonies (Ball and Allen, 1988; Yang and Cox-Foster, 2007).

The impacts of viral, fungal, and bacterial infections of insects can be reduced through host defenses (Lemaitre and Hoffmann, 2007). The antimicrobial host defense of *Drosophila* involves rapid synthesis of small-sized cationic peptides by the fat body (Bulet et al., 2002). These antimicrobial peptides are released into the open circulatory system where they attack invading

Table 2
Relative transcript levels for selected proteins in larvae reared in the incubator and exposed to pesticides. Only significantly elevated or decreased transcript abundances in treated larvae compared to untreated ones are indicated.

Target genes	Transcripts no.				
	Higher	Lower	Difference (dCT)	SE	*P*-value
PPOact	Imidacloprid	Acetone	3.936	1.63	0.017
PPOact	Imidacloprid	Amitraz	4.9315	1.68	0.004
PPOact	Coumaphos	Amitraz	3.58	1.49	0.009
PPOact	Myclobutanil	Chlorpyrifos	4.07	1.46	0.006
PPOact	Myclobutanil	Acetone	4.66	1.45	0.002
PPOact	Myclobutanil	Amitraz	5.65	1.51	0.0002
PPOact	Myclobutanil	Fluvalinate	4.05	1.51	0.008
PPOact	Chlorothalonil	Chlorpyrifos	4.58	1.71	0.008
PPOact	Chlorothalonil	Acetone	5.17	1.70	0.003
PPOact	Chlorothalonil	Amitraz	6.17	1.75	0.001
PPOact	Chlorothalonil	Fluvalinate	4.57	1.75	0.01
PPOact	Myclobutanil	Glyphosphate	4.11	1.44	0.005
PPOact	Chlorothalonil	Glyphosphate	4.62	1.69	0.007
PPOact	Myclobutanil	Simazine	3.78	1.49	0.01
PGRP	Chlorothalonil	Fluvalinate	1.96	0.86	0.02
PGRP	Imidacloprid	Fluvalinate	2.57	0.97	0.016
Hexam	Imidacloprid	Glyphosphate	2.42	1.00	0.017
Hexam	Fluvalinate	Glyphosphate	2.19	0.91	0.017
Hexam	Coumaphos	Glyphosphate	2.47	0.88	0.096
Hexam	Myclobutanil	Glyphosphate	2.12	0.89	0.018
Hexam	Imidacloprid	Simazine	3.2	1.03	0.002
Hexam	Amitraz	Simazine	2.5	0.94	0.007
Hexam	Fluvalinate	Simazine	2.98	0.945	0.002
Hexam	Coumaphos	Simazine	3.26	0.916	0.001
Hexam	Myclobutanil	Simazine	2.9	0.92	0.002
DWV	Myclobutanil	Chlorothalonil	5.6	2.35	0.018
DWV	Acetone	Glyphosphate	5.2	2.20	0.019
DWV	Imidacloprid	Glyphosphate	6.61	2.52	0.01
DWV	Myclobutanil	Glyphosphate	7.94	2.24	0.001

PPOact = Prophenoloxidase-activating enzyme, PGRP = Peptidoglycan Recognition Protein, Hexam = Hexameric storage protein, DWV = Deformed Wing Virus.

microorganisms. Transcription of these genes is activated after gram-positive bacterial and fungal infections (De Gregorio et al., 2001) and gram-negative bacterial infection (Gobert et al., 2003). PGRPs thus form a large group of conserved proteins present in insects and mammals which have in common a 160-amino acid–domain (Kang et al., 1998). Honey bees have components reflective of each of the major recognized insect immune pathways (Evans, 2006).

Exposure to parasitic Varroa, or arguably to viruses and other microbes carried by mites, is known to affect immunity traits in honey bees (Yang and Cox-Foster, 2007; Gregory et al., 2005). In this study, we provide novel insights into the expression of PGRPs in honey bee larvae by showing that pesticide treatments or simultaneous pesticide and mite treatments induce increased transcript levels for an apparent ortholog to *Drosophila* short-chain Peptidoglycan Recognition Protein PGRP-SC (PGRPSC 4300). PGRPs are pathogen recognition genes defined by a conserved 160-amino-acid domain (Aggrawal and Silverman, 2007). Our results show that elevated expression levels for PGRPs are associated with mite infestations and that the PGRP family member SC4300 can be associated with mite parasitism, and potentially with DWV pathology.

Prophenoloxidases (PPOs) are key enzymes in the melanization reaction which, along with playing a critical structural role in the arthropod body, are prominent defense mechanism. Prophenoloxidase (PPO) in insects is implicated in the defense against microbes and wounding. Prophenoloxidase gene expression plays a critical role in restricting parasite development in insects (Zou et al., 2008; Wang et al., 2009). PO is synthesized in arthropods as an inactive zymogen, pro-phenol oxidase (proPO), which can be activated by proteolytic cleavage at a specific site near the protein's amino terminus (Kan et al., 2008). Wounding of an insect or exposure to certain microbial polysaccharides leads to activation of a protease that specifically activates proPO as the last step of a protease cascade. The reactions catalyzed by PO then can result in melanization of foreign organisms trapped in capsules or hemocyte nodules (Bidla et al., 2005). Pesticide-treated larvae showed increased transcript levels for PPOact after treatments with the pesticides imidacloprid, coumaphos, myclobutanil and chlorothalonil. Thus, our results show that PPOact transcript induction is a larval response to diet-acquired pesticides.

Enzymes from the cytochrome P450 (CYP) gene family in insects are involved in the synthesis and metabolism of hormones and pesticides and can also metabolize external substances, such as medications and environmental pollutants that are ingested (Feyereisen, 2005; Guengerich, 2008). CYPs have widespread and diverse functions in animals, often are inducible components of the detoxification systems of vertebrates, invertebrates and plants (Li et al., 2007), and are important in cellular metabolism (Willingham and Keil, 2004). Interestingly, the honey bee genome contains around half the CYP gene diversity of other genomes for non-social insects, suggesting compromised functionality (Claudianos et al., 2006) or the existence of CYPs with broader substrate specificity. Significant effects were observed in transcript down regulation for the Cyp4g11 gene by mite exposure. No pesticide treatment tested, with the exception of glyphosate, affected Cyp4g11 gene expression, but mite infestation together with associated DWV infection did. This finding suggests a putative role of CYPs in larval sensitivity to Varroa parasitism, but not all pesticides. Chemical treatments could be metabolized to some extent in larval tissues by cytochrome P450. Additionally, Varroa infestation can induce enzyme synthesis. Consequently, pesticide metabolism can occur sooner and the threshold before damage may increase. Members of the CYP4 family have also been associated with hormone biosynthesis and degradation (Feyereisen, 2005), putatively suggesting

Varroa may indirectly impact larval endocrinology. Further research will be required to substantiate P450 roles in larval responses to the physiological challenges tested here.

The expression pattern of the pathogen recognition gene Peptidoglycan Recognition Protein (PGRPSC 4300), Prophenoloxidases (PPOs) as key enzymes of the melanization reaction and defense mechanism of arthropods, and Hexameric storage protein are consistent with their role when honey bee larvae are exposed to pesticides and/or Varroa parasitism. The expression data obtained in treated larvae using quantitative PCR indicates that the levels of transcripts for these genes are significantly changed from those of the untreated larvae. The external influences such as pesticide treatment and/or exposure to Varroa appear to influence subclinical changes in developing larvae and effects should be further considered at the individual and colony levels.

In general, we found significant gene-expression changes following mite exposure, and less pronounced changes following exposure to exogenous chemicals. This results mimics that seen recently for adult bees in colonies subjected to chemical acaricides (Boncristiani et al., 2012), for which gene-expression changes with chemical exposure were variable but generally minor. Antimicrobial peptide transcripts increased upon mite exposure while a Peptidoglycan Recognition Protein, a Hexameric storage protein and a member of the cytochrome P450 family (Cyp4g11) decreased. Several genes showed changes in expression in response to different chemical challenges. Most notably, a candidate PPO-activating enzyme showed generally higher expression in larvae exposed to two fungicides, while a hexameric larval storage protein showed lower expression in bees presented with the herbicides glyphosate and simazine. Mites are important source of DWV in the field and also induce elevated levels of transcripts for Hsp70 in bee larvae. Recently, the Hsp70a gene was shown to respond significantly to juvenile hormone treatment (JH), which is known to play significant roles in honey bee larval development and worker temporal polyethism (Martins et al., 2010). Honey bee Hsp70 also possesses an apparent regulatory element that binds the nuclear receptor protein *Ultraspiracle* (Martins et al., 2010). More generally, insect hexamerins have also been shown to bind JH and play important roles in developmental physiology and sociobiology (Braun and Wyatt, 1996; Tawfik et al., 2006; Zhou et al., 2006a, 2006b; Scharf et al., 2007). Therefore, downstream impacts on JH signaling by Varroa, resulting from down-regulated Hsp expression, could have profound impacts on honey bee development and social behavior.

In conclusion, our data provide novel insights into understanding the genetic response of developing honey bees to Varroa and pesticides. These results highlight the potential effects of pesticides and varroa parasitism on transcriptional levels in individual bees and response mechanisms induced in honey bee larvae and pupae. Further tests should be performed in order to evaluate the potential threshold effects of synergistic agents and specific gene expression involved in individual honey bees and to study influence on bee longevity and potential effects on honey bee colonies. Additionally, functional analyses of candidate responsive genes identified here may reveal important insights into declining bee health.

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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.jinsphys.2012.03.015.

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