Journal of Insect Physiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*)

Daniel R. Schmehl [a,*], Peter E.A. Teal [b], James L. Frazier [a], Christina M. Grozinger [a,1]

[a] Department of Entomology, Center for Pollinator Research, The Pennsylvania State University, University Park, PA, USA
[b] United States Department of Agriculture, Agricultural Research Service, Gainesville, FL, USA

ARTICLE INFO

Article history:
Received 5 July 2014
Received in revised form 19 September 2014
Accepted 6 October 2014
Available online xxxx

Keywords:
Honey bee
Pesticide
Nutrition
Stress
Transcription
Genomics

ABSTRACT

Populations of pollinators are in decline worldwide. These declines are best documented in honey bees and are due to a combination of stressors. In particular, pesticides have been linked to decreased longevity and performance in honey bees; however, the molecular and physiological pathways mediating sensitivity and resistance to pesticides are not well characterized.

We explored the impact of coumaphos and fluvalinate, the two most abundant and frequently detected pesticides in the hive, on genome-wide gene expression patterns of honey bee workers. We found significant changes in 1118 transcripts, including genes involved in detoxification, behavioral maturation, immunity, and nutrition. Since behavioral maturation is regulated by juvenile hormone III (JH), we examined effects of these miticides on hormone titers; while JH titers were unaffected, titers of methyl farnesoate (MF), the precursor to JH, were decreased. We further explored the association between nutrition- and pesticide-regulated gene expression patterns and demonstrated that bees fed a pollen-based diet exhibit reduced sensitivity to a third pesticide, chlorpyrifos. Finally, we demonstrated that expression levels of several of the putative pesticide detoxification genes identified in our study and previous studies are also upregulated in response to pollen feeding, suggesting that these pesticides and components in pollen modulate similar molecular response pathways.

Our results demonstrate that pesticide exposure can substantially impact expression of genes involved in several core physiological pathways in honey bee workers. Additionally, there is substantial overlap in responses to pesticides and pollen-containing diets at the transcriptional level, and subsequent analyses demonstrated that pollen-based diets reduce workers' pesticide sensitivity. Thus, providing honey bees and other pollinators with high quality nutrition may improve resistance to pesticides.

1. Introduction

Pollinators are critical to production of approximately 70% of our agricultural crops, particularly nutrient-rich fruits, vegetables and nuts (Eilers et al., 2011; Klein et al., 2007). However, populations of honey bees and other pollinators are in decline globally (González-Varo et al., 2013; Potts et al., 2010), with US beekeepers losing approximately 30% of their colonies each winter (vanEngelsdorp et al., 2012). These declines have been attributed to multiple factors, including pathogens, parasites, habitat loss and fragmentation, and intensive mono-cropping systems which lead to reduced floral resources and nutrition (Potts et al., 2010). In addition to these factors, there have been mounting concerns about the effects of pesticides (Council, 2007; Godfray et al., 2014; Sanchez-Bayo and Goka, 2014). Indeed, residues from over 120 different pesticides have been found in honey bee colonies in the US, with an average of six pesticides found in the stored pollen of these colonies (Mullin et al., 2010). Two pesticides in particular, fluvalinate and coumaphos, are the most prevalent (found in ~98% of the 749 colonies surveyed) and are found at the highest

Abbreviations: JH, juvenile hormone III; MF, methyl farnesoate; P450, cytochrome P450 monooxygenase; FDR, false discovery rate; CCE, carboxyl/cholinesterase; GST, glutathione-S-transferase; GO, gene ontogeny; qRT-PCR, quantitative real-time polymerase chain reaction; SDI, single drone inseminated; Qeq, queen equivalent; LC/MS–MS, liquid chromatography–mass spectrometry–mass spectrometry; GC/MS, gas chromatography–mass spectrometry; GSTD1, glutathione S-transferase D1; SODH2, superoxide dismutase.

* Corresponding author at: Entomology and Nematology Department, University of Florida, 970 Natural Area Drive, Steinmetz Hall, Gainesville, FL 32611, USA. Tel.: +1 (352) 273 3935.

E-mail addresses: danielrschmehl@ufl.com (D.R. Schmehl), peter.teal@ars.usda.gov (P.E.A. Teal), jff2@psu.edu (J.L. Frazier), cmgrozinger@psu.edu (C.M. Grozinger).

[1] Co-corresponding author.

http://dx.doi.org/10.1016/j.jinsphys.2014.10.002
0022-1910/

concentrations in hives, with maximum detection levels of 204 and 94 ppm in the wax, respectively (Mullin et al., 2010). More recently, (Berry et al., 2013) found coumaphos concentrations of 514 ppm following colony treatments of coumaphos (Checkmite+™) at the recommended label dose. These pesticides are commonly applied by beekeepers to control *Varroa* mites, a widespread and devastating parasite of honey bees (Anderson and Trueman, 2000; Rosenkranz et al., 2010). Since the half-life of fluvalinate and coumaphos is ~5 years in wax (Bogdanov, 2004), these pesticides can accumulate to unsafe levels in colonies (Haarmann et al., 2002) (the LD50 of coumaphos is 46.3 ppm, while that of fluvalinate is 15.86 ppm (Mullin et al., 2010)). Coumaphos, an organophosphate, inhibits acetylcholinesterase, while fluvalinate, a pyrethroid, targets the sodium channels of mites and insects (Eiri and Nieh, 2012). While there have been many studies examining the impacts of pesticides on the behavior and longevity of individual honey bees (Aliouane et al., 2009; Burley et al., 2008; Ciarlo et al., 2012; Collins et al., 2004; Decourtye et al., 2004, 2005, 2011; Eiri and Nieh, 2012; Frost et al., 2013; Haarmann et al., 2002; Henry et al., 2012; Pettis et al., 2004; Rinderer et al., 1999; Teeters et al., 2012; Williamson and Wright, 2013; Wu et al., 2011; Zhu et al., 2014), our understanding of the molecular and physiological mechanisms mediating these impacts, and the related pathways that convey resistance to these chemicals, remains limited.

While acute doses of pesticides can kill individual honey bees and colonies outright (reviewed in Atkins, 1992; Johnson et al., 2010), chronic exposure to low doses leads to sub-lethal effects in individual bees, which, in turn, may result in colony-level effects (reviewed in Johnson et al., 2010; Thompson and Maus, 2007). Honey bee colonies consist of a single reproductive female queen that lays all of the female eggs and the majority of unfertilized male eggs, tens of thousands of facultatively sterile female workers that perform all colony tasks (including feeding the developing larvae, building honeycomb, and foraging for food) and males (drones) (Graham, 1992). Sub-lethal effects of coumaphos and fluvalinate have been demonstrated in all three castes (queens, workers, and drones). Coumaphos and/or fluvalinate exposure can reduce learning, memory, and orientation in adult worker bees (Frost et al., 2013; Williamson and Wright, 2013), alter adult worker locomotion and feeding behavior (Teeters et al., 2012), and reduce larval longevity (Wu et al., 2011; Zhu et al., 2014). In drones, coumaphos and/or fluvalinate exposure reduce body weight and longevity (Rinderer et al., 1999), as well as sperm viability (Burley et al., 2008) which likely contributes to poor queen mating quality. In queens, coumaphos and/or fluvalinate exposure during development reduces adult queen weight (Haarmann et al., 2002; Pettis et al., 2004), the amount of stored sperm (Haarmann et al., 2002), and egg laying (Collins et al., 2004; Haarmann et al., 2002), and also disrupts ovary activation (Haarmann et al., 2002). At very high levels of coumaphos exposure, queen rearing is greatly inhibited (Collins et al., 2004; Pettis et al., 2004). Other pesticides (such as neonicotinoids) have similar effects (Aliouane et al., 2009; Ciarlo et al., 2012; Decourtye et al., 2004, 2005, 2011; Eiri and Nieh, 2012; Henry et al., 2012; Teeters et al., 2012; Williamson and Wright, 2013; Wu et al., 2011).

At the molecular level, exposure to pesticides can activate detoxification pathways (Boncristiani et al., 2012; Johnson et al., 2006, 2009b, 2012; Mao et al., 2011) and modulate expression of genes involved in immunity and behavioral maturation (Gregorc et al., 2012). However, the impacts of individual pesticides on expression of specific genes vary greatly among studies. Cytochrome P450 monooxygenases (P450s) are involved in xenobiotic detoxification (Claudianos et al., 2006; Johnson et al., 2006, 2009b), as well as hormone synthesis and metabolism (Claudianos et al., 2006; Helvig et al., 2004). Coumaphos and fluvalinate are known to activate P450 pathways both individually (Johnson et al., 2006; Mao et al., 2011) and synergistically in combination (Johnson et al., 2009b). However, in a recent study (Boncristiani et al., 2012) examining five different pesticides (including coumaphos and two other pesticides commonly applied to manage *Varroa* mites, thymol and formic acid) the only P450 to be upregulated was CYP6A514 by thymol; however, several other detoxification, immune, and developmental genes were either up or downregulated by thymol, coumaphos, and formic acid. Another study examining the effects of pesticides (including coumaphos and fluvalinate) on larval development (Gregorc et al., 2012), found no changes in P450 gene expression, but expression of several genes involved in immune function and behavioral maturation were significantly impacted. Finally, exposure to neonicotinoids results in reduced activity of the NF-κB immune signaling pathway and increased titers of Deformed Wing Virus (Nazzi et al., 2012), suggesting that honey bees compromised by pesticide exposure may be more susceptible to pathogen infection. While these studies quantified expression of specific candidate genes, the genome-wide expression responses to coumaphos and fluvalinate have not been examined.

At the physiological level, exposure to pesticides may impact endocrine pathways. The primary hormonal regulator of adult worker behavior is juvenile hormone III (JH), which is synthesized from methyl farnesoate (MF) (Huang et al., 1991; Robinson, 1987; Sullivan et al., 2000). Rising titers of JH drive behavioral maturation, the transition from nursing (brood care) to foraging in honey bee workers (Huang et al., 1991; Robinson, 1987; Sullivan et al., 2000). Bees exhibiting stress from *Nosema* infection, *Varroa* mites, viruses, anesthesia, injury, wax deprivation, and nutritional deprivation have all been observed to accelerate the transition to foraging (DeGrandi-Hoffman et al., 2010; Janmaat and Winston, 2000; reviewed in Tofilski, 2005, 2009; Toth and Robinson, 2005). This may, in turn, alter the social dynamics and organization of the colony, resulting in colony collapse through initiation of precocious foraging and reduced longevity of these foragers (Khoury et al., 2011; Thompson et al., 2007). However, a direct effect of pesticide exposure on physiological factors affecting behavioral maturation has not yet been demonstrated.

Finally, there is mounting evidence that diet can modulate expression of similar genes as pesticides and impact responses of honey bees to pesticides. Honey bees fed a diet of honey, pollen and propolis have elevated expression levels of the CYP6AS and CYP9Q P450 subfamilies (Johnson et al., 2012). Honey-fed bees demonstrated an increase in survival when fed aflatoxin B1 (Johnson et al., 2012), suggesting that diet reduces pesticide sensitivity. Additionally, feeding with *p*-coumaric acid (found in honey and pollen) induces expression of CYP9Q3 and significantly reduced coumaphos toxicity (Mao et al., 2013). Finally, nutritional deprivation increases honey bee susceptibility to pesticides (Wahl and Ulm, 1983).

Here, we examined the global gene expression responses of honey bee workers to chronic, sub-lethal exposure to coumaphos and fluvalinate, and used the resulting information to explore the molecular and physiological pathways that respond to and mediate sensitivity and resistance to pesticides. Unlike previous studies of the molecular effects of coumaphos and fluvalinate, we applied a genome-wide approach to investigate comprehensively the effects of these pesticides. Comparing our results to previous studies of global gene expression patterns in honey bee workers associated with immunity (Evans et al., 2006; Richard et al., 2012), behavioral maturation (Ament et al., 2011) and responses to different diet regimes (Ament et al., 2011) revealed that pesticide exposure significantly impacted all of these pathways. Subsequent experiments verified a strong link at the molecular level among nutrition- and pesticide-responsive pathways, as well as impacts of diet,

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

specifically pollen-based diets, on modulating worker bees' sensitivity to pesticide exposure.

2. Results

2.1. Effects of pesticide exposure on genome-wide gene expression patterns

One day old worker honey bees were caged (30 bees/cage, 12 cages per treatment) and fed 1:1 sucrose solution *ad libitum*. On the second day, they were fed untreated sucrose solution (control) or a sucrose solution mixed with 3% methanol (the solvent used to dissolve the pesticides), coumaphos (100 ppm) in methanol, or fluvalinate (100 ppm) in methanol *ad libitum*. Bees were collected after 7 days of treatment. Rates of food consumption (data not shown) and mortality (Kruskal–Wallis, chi-squared = 2.36; degrees of freedom = 3; $p = 0.5003$) were not significantly different between treatment groups.

We used honey bee whole genome microarrays to monitor genome-wide gene expression patterns in the abdomens from honey bees (5 pooled bees/cage, 6 cages/treatment) from the four treatment groups (sucrose, methanol, coumaphos, and fluvalinate). A total of 1118 unique transcripts (of the 12,483 transcripts printed on the microarrays) were significantly differentially expressed at a false discovery rate (FDR) of $p < 0.01$ across the four groups (see Supplementary Table S1 for the list of differentially expressed transcripts). A hierarchical clustering analysis of the 1118 significantly differentially expressed transcripts demonstrated that the two pesticide-treated groups have distinct expression patterns relative to the sucrose and methanol groups (Fig. 1).

Of these 1118 transcripts, 814 transcripts are significantly differentially expressed among the coumaphos and/or fluvalinate and the sucrose groups, while only 26 are significantly differentially expressed between the methanol and sucrose treatments (Fig. 2; see Supplementary Table S2 for lists of the differentially expressed transcripts). 566 and 131 transcripts are significantly regulated by only coumaphos and only fluvalinate, respectively, while 117 transcripts are significantly regulated by both pesticides relative to the sucrose control (Fig. 2). The observed overlap of significantly regulated genes between pesticide treatments was significantly greater than chance (Fisher's exact test; $p < 0.001$).

Gene ontology analysis identified several functional groups of genes whose expressions were significantly altered by pesticide exposure. Of the 814 transcripts significantly affected by exposure to either or both pesticides relative to the sucrose control, 576 had unique *Drosophila* orthologs with Flybase annotations and were used in this analysis. Sixteen functional categories were overrepresented ($p < 0.05$), including several involved in metabolism (including drug metabolism), cellular transport, cellular respiration, and development (Fig. 2, see Supplementary Table S3 for a complete listing of functional categories). Of these processes, only Citrate Cycle (modulated by coumaphos) and Lysine Degradation (modulated by both coumaphos and fluvalinate) survived the Benjamini correction ($p < 0.05$).

Expression of several genes involved in detoxification was significantly altered in response to pesticide exposure. Indeed, the gene with the largest change in expression in our study was CYP305D1, with expression 3.40-fold higher in coumaphos-treated samples relative to methanol controls. Its expression was not significantly regulated by fluvalinate treatment (0.95-fold). CYP305D1 is a member of the CYP2 clade of cytochrome P450s (P450s) and is traditionally associated with hormone function (Claudianos et al., 2006). Other P450s that were significantly upregulated by both coumaphos and fluvalinate treatment were

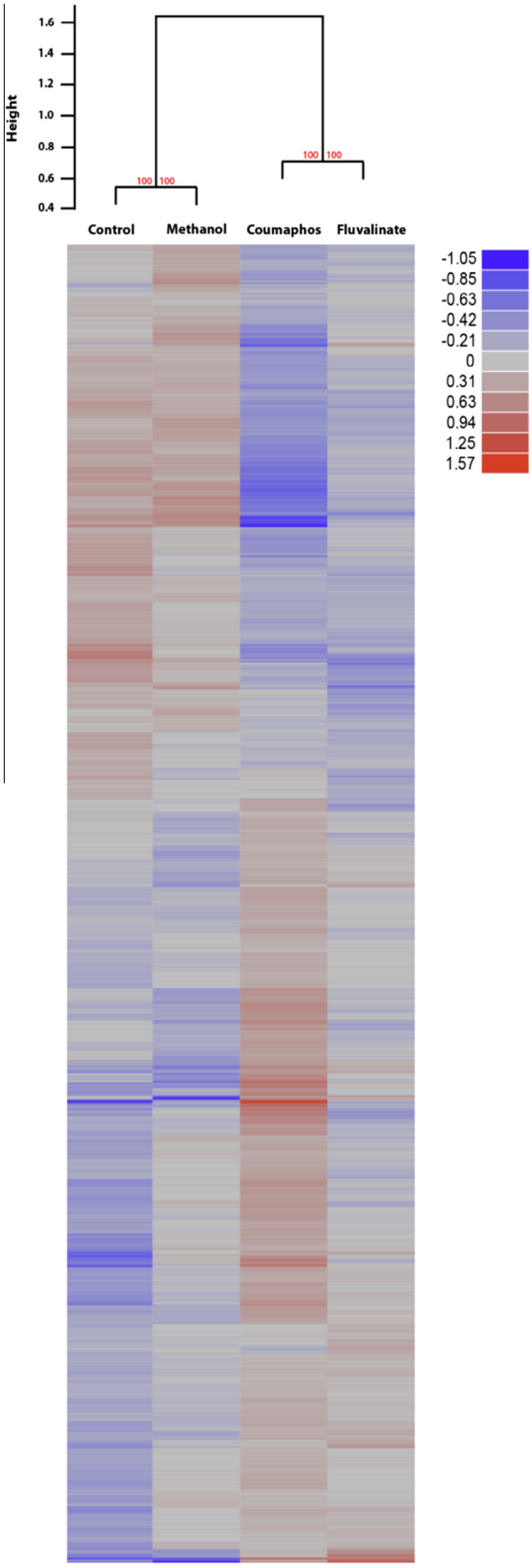

Fig. 1. Hierarchical clustering analysis of significantly regulated transcripts. Based on the expression levels of significantly regulated transcripts, coumaphos and fluvalinate treated bees clearly clustered separately from methanol and sucrose. This grouping is supported by an "approximately-unbiased" *p*-value of 100 and a bootstrap value of 100.

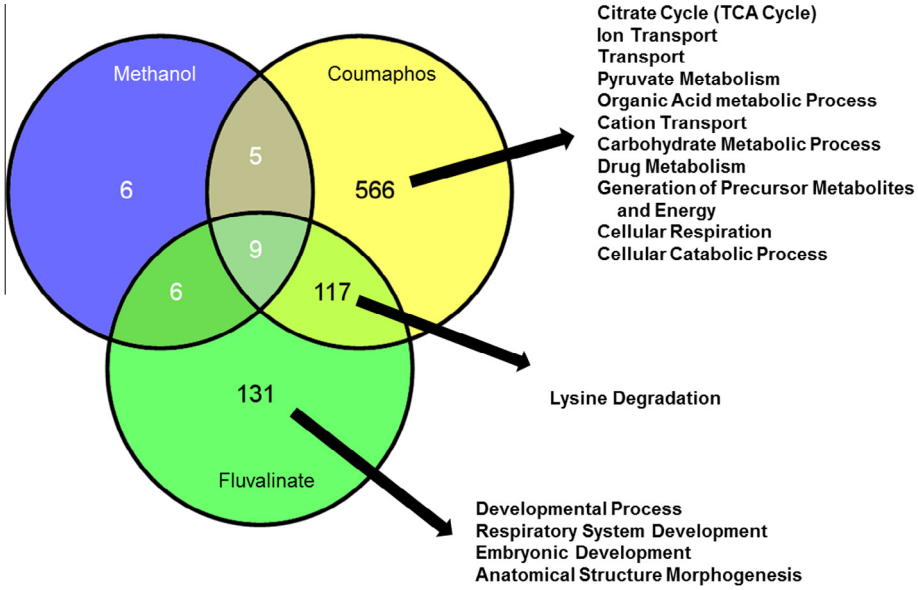

Fig. 2. Pairwise comparisons of the effect of treatment on transcript levels. Venn diagram of significantly regulated transcripts in the three treatment groups (methanol, coumaphos, and fluvalinate) relative to the sucrose control. Gene ontology analysis of the different sets of transcripts identified several over-represented ($p < 0.05$) functional categories (listed in black).

Table 1
The 1118 significantly regulated transcripts associated with pesticide exposure were compared to sets of transcripts associated with behavior and physiology in honey bee workers.

Transcript list	#sig. transcripts	All regulated transcripts (1118)	*p*-value (Fisher exact test)	Reference
Immune function "canonical"	192	22	0.070	Evans et al. (2006)
Immune function "acute"	302	44	**<0.001**	Richard et al. (2012)
Nurse/forager-associated	2641	429	**<0.001**	Ament et al. (2011)
Rich/poor diet-associated	3372	527	**<0.001**	Ament et al. (2011)

Significant *p* values are highlighted in bold.

CYP6AS3, CYP6AS4, and CYP9S1, all of which are members of the CYP3 clade, which has known functions including insecticide metabolism and resistance (Berenbaum, 2002; Feyereisen, 2005). In addition to these P450s, GB10854, a carboxyl/cholinesterase (CCE), and GSTD1, a glutathione-S-transferase (GST), were upregulated in response to coumaphos exposure. GB10854 may function in organophosphate detoxification (Claudianos et al., 2006; Johnson et al., 2009a), while GSTD1 is a Delta class GST (Claudianos et al., 2006). In other insect systems, delta class GSTs have the capability to metabolize organochlorine pesticides, such as DDT (dichlorodiphenyltrichloroethane), and organophosphate insecticides (reviewed in Claudianos et al., 2006). Interestingly, GSTD1 is the only Delta class GST identified in honey bees. Since coumaphos is an organophosphate, these enzymes may be directly involved in detoxifying this chemical.

Quantitative real-time PCR (qRT-PCR) was used to examine expression of 10 candidate genes identified as differentially regulated in the microarray analysis: GSTD1, CYP9S1, GNBP3, GLD, SODH2, CG4398, NPC2, PTEN, SLS, and CYP305D1 (Supplementary Fig. 1). Four of the ten genes showed significant expression differences across the methanol, coumaphos and fluvalinate treatments, and all genes showed comparable trends in expression patterns as observed in the microarray study.

2.2. Comparative analyses of genes associated with pesticide exposure, behavioral, and physiological processes in honey bees

We examined the overlap between the 1118 significantly differentially expressed transcripts and the suites of genes associated with behavioral and physiological processes in honey bees identified in previous studies (Table 1; (Ament et al., 2011; Evans et al., 2006; Richard et al., 2012)). While there were large differences in the experimental designs among studies used in our comparative analyses, we compared these data sets to identify general trends within the data. A set of "canonical" immune genes was identified during annotation of the honey bee genome (Evans et al., 2006); 22 of these were also present in our significantly differentially expressed transcript list, and this overlap is not greater than expected by chance (Fisher's exact Test, $p = 0.070$). However a recent study examining acute, short-term (within 6 h) genome-wide transcriptional responses to immunostimulation (Richard et al., 2012) identified 302 significantly regulated transcripts; 44 of these were also significantly differentially expressed in our study, an overlap that is significantly greater than expected by chance ($p < 0.001$). Of the genes differentially expressed in the fat bodies of nurses and foragers (Ament et al., 2011), 429 transcripts overlapped with those from our study, which was significantly greater than expected by chance ($p < 0.001$). Of the genes differentially expressed in the fat bodies of bees fed a rich (pollen/honey) or poor (50% sucrose syrup) diet (Ament et al., 2011), 527 transcripts overlapped with those from our study, which was significantly greater than expected by chance ($p < 0.001$).

We next examined the directionality of overlap among these sets of genes (Table 2; (Ament et al., 2011; Evans et al., 2006; Richard et al., 2012)). Of the 814 transcripts that were significantly regulated in coumaphos and/or fluvalinate treated bees relative to sucrose, 500 transcripts were up-regulated by coumaphos and/or fluvalinate and 314 transcripts were down-regulated by

Table 2
Analysis of directional expression overlap among pesticide, physiology, and behavior associated transcripts.

Transcript list	#sig. transcripts	Coumaphos and/or fluvalinate upregulated (500 transcripts)	*p*-value (Fisher exact test	Coumaphos and/or fluvalinate downregulated (314 transcripts)	*p*-value (Fisher exact test)	Reference
Nursing-associated	1205	63	**0.003**	35	0.105	Ament et al. (2011)
Foraging-associated	1436	165	**<0.001**	44	**0.037**	Ament et al. (2011)
Up-regulated immune function	168	17	**<0.001**	5	0.366	Richard et al. (2012)
Down-regulated immune function	38	3	0.177	0	1.00	Richard et al. (2012)
Rich diet-associated	1486	181	**<0.001**	3	1.00	Ament et al. (2011)
Poor diet-associated	1860	26	1.00	167	**<0.001**	Ament et al. (2011)

Significant *p* values are highlighted in bold.

coumaphos and/or fluvalinate. We compared these directional transcript lists with lists of transcripts that were upregulated in nurses relative to foragers ("nursing associated"), upregulated in foragers relative to nurses ("foraging associated"), upregulated in rich diet relative to poor diet ("rich diet associated"), upregulated in poor diet relative to rich diet ("poor diet associated") and up- or down- regulated in bacteria-injected bees relative to controls ("immune function").

Transcripts upregulated by pesticide exposure overlapped significantly with both nursing and foraging associated transcripts, while transcripts downregulated by pesticides only significantly overlapped with foraging associated transcripts (Fisher's exact test, $p < 0.05$). Thus, there was no clear directionality in the expression patterns (e.g., expression patterns of pesticide-exposed bees did not resemble expression patterns in foragers), suggesting that exposure to these pesticides does not strongly accelerate or inhibit behavioral maturation. Transcripts upregulated by pesticide exposure overlapped significantly with upregulated immune transcripts ($p < 0.001$), suggesting that pesticide exposure upregulates immune function and/or triggers an immune response.

There was significant overlap in pesticide upregulated and rich diet associated transcripts, as well as in pesticide downregulated and poor diet associated transcripts ($p < 0.001$, see Supplementary Tables S4 and S5 for a list of these genes). A GO analysis of the 181 rich diet associated/pesticide upregulated transcripts (165 of which had unique *Drosophila* orthologs with Flybase annotations and were used in the analysis) revealed a significant overrepresentation of 10 categories, including transport and metabolism ($p < 0.05$, see Supplementary Table S6 for a complete list of these GO categories). A GO analysis of the 165 poor diet associated/pesticide downregulated transcripts (47 of which had unique *Drosophila* orthologs with Flybase annotations and were used in the analysis) revealed a significant overrepresentation of four categories, including respiratory system development, regulation of developmental process, anatomical structure morphogenesis, and metamorphosis ($p < 0.05$, Supplementary Table S7).

2.3. Effect of pesticide exposure on hormone levels

Our gene expression analyses revealed an effect of pesticide exposure on expression of sets of genes that are associated with nursing- and foraging-behavior. To further investigate the effects of pesticide exposure on physiological processes associated with behavioral maturation, we examined hemolymph titers of juvenile hormone III (JH) and its precursor methyl farnesoate (MF) in our treatment groups. As noted above, increasing titers of JH are associated with accelerated maturation. Levels were analyzed in sucrose, methanol, coumaphos and fluvalinate treated workers after 7 days of exposure. The total amount of JH did not differ between treatments (ANOVA, $F_3 = 0.4759$, $p = 0.7018$; data not shown). There was also no significant difference in MF levels between methanol (14.14 pg/μL ± 2.70) and sucrose (13.05 pg/μL ± 3.89) treatment groups (ANOVA, $F_1 = 0.2801$, $p = 0.6049$). However, the quantity of MF (Fig. 3) was significantly lower in both coumaphos (3.11 pg/μL ± 1.07) and fluvalinate (5.02 pg/μL ± 0.70) treated groups relative to methanol (ANOVA-Tukey HSD; $F_2 = 9.96$, $p = 0.0010$).

2.4. Impact of diet on sensitivity to pesticides

Given the strong correlation between gene expression changes induced by pesticide exposure and diet, we examined the effects of diet on survival of honey bees exposed to a third pesticide, chlorpyrifos. Chlorpyrifos (an organophosphate) is the third most prevalent and abundant pesticide detected in the hive, found at maximum levels of 890 ppb (Mullin et al., 2010). Chlorpyrifos is considerably more toxic to bees than coumaphos or fluvalinate (Mullin et al., 2010), with an LD50 of 3 ppm after a test duration of 72 h using our caged assays (data not shown). This high level of toxicity and rapid mortality allowed us to rapidly assess the impact of diet on pesticide sensitivity.

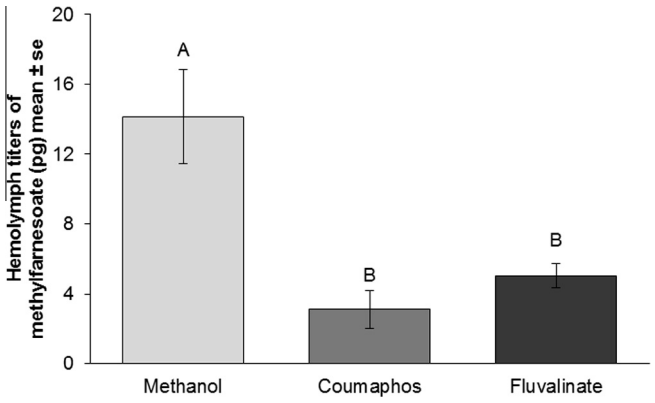

Fig. 3. Pesticide exposure reduces levels of methyl farnesoate in worker honey bees. Methyl farnesoate (MF) titers were measured in pooled samples of hemolymph collected from 3 to 5 bees treated with methanol ($n = 8$ samples), fluvalinate ($n = 8$ samples), and coumaphos ($n = 7$ samples). The total amount of MF was significantly lower in coumaphos- and fluvalinate-treated than methanol-treated bees (ANOVA-Tukey HSD; $F_2 = 9.96$, $p = 0.0010$, different letters denote significant differences in post hoc pairwise comparisons, $p < 0.05$).

Table 3
Effect of diet on honey bee survival under control and pesticide-exposed conditions.

Treatment	Diet	Mean survival (days)	Pollen long-term		Protein long-term		Sucrose		Pollen short-term	
			χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value
Pesticide	Pollen, long-term	10.71			42.15	<0.01	115.25	<0.01	73.25	<0.01
	Protein, long-term	8.81	42.15	<0.01			20.97	<0.01	2.76	0.10
	Sucrose	7.63	115.25	<0.01	20.97	<0.01			10.89	<0.01
	Pollen, short-term	8.28	73.25	<0.01	2.76	0.10	10.89	<0.01		
Sucrose	Pollen, long-term	15.58			0.37	0.54	3.42	0.06	1.98	0.16
	Protein, long-term	15.68	0.37	0.54			5.52	0.02	3.87	0.05
	Sucrose	15.93	3.42	0.06	5.52	0.02			0.37	0.54
	Pollen, short-term	15.78	1.98	0.16	3.87	0.05	0.37	0.54		

Significant *p* values are highlighted.

Honey bees were reared in cages (30 bees/cage, 6 cages/treatment) with four different diet regimes (sucrose, long-term soy protein, long-term pollen, short-term pollen). All cages were provided with 1:1 sucrose solution, and treatment cages received either a sucrose/soy protein paste or a sucrose/pollen paste throughout the experiment (long-term treatment) or a sucrose/pollen paste for 24 h prior to pesticide exposure, after which it was removed (short-term treatment). During a 16 day time period, half of the cages honey bees were not challenged with pesticides and had a mean survival time of greater than 15.5 days (Table 3 and Fig. 4). Interestingly, the intake of pollen or protein led to a reduction in honey bee longevity when the bees were assayed in the absence of pesticide exposure. The other half of the cages were challenged with chlorpyrifos (3 ppm) mixed into the sucrose solutions starting on day 5. In these groups, diet significantly impacted mean survival time (Table 3 and Fig. 5). Bees fed a long-term pollen diet ($n = 150$, mean survival time of 10.71 ± 0.25 days) lived significantly longer compared to those fed long-term protein (8.81 ± 0.22 days, χ^2 (DF = 3, $n = 180$) = 42.15, $p < 0.01$), short-term pollen (8.28 ± 0.16 days, χ^2 (DF = 3, $n = 180$) = 73.25, $p < 0.01$), or sucrose only (7.63 ± 0.14 days, χ^2 (DF = 3, $n = 150$) = 115.25, $p < 0.01$) diets. Therefore diet impacts survival during pesticide exposure as follows: long-term pollen >long-term protein = short-term pollen >sucrose only.

2.5. Examining the impact of diet on expression of pesticide-regulated candidate genes

The results of the microarray study revealed a significant overlap between genes whose expression was impacted by pesticide treatment (our study) and those impacted by diet (Ament et al., 2011). To examine further the relationship between diet and pesticide exposure on gene expression, we used qRT-PCR to measure expression of five pesticide-regulated candidate genes (CYP9S1, CYP9Q3, CYP305D1, GSTD1, and SODH2) in response to diets consisting of sucrose, sucrose/pollen, sucrose/soy protein, and honey, honey/pollen, honey/soy protein (Fig. 6). The pollen and honey used to create these diets were analyzed for chemical residues using GC–MS and LC–MS (which is capable of identifying ~170 chemical residues (Mullin et al., 2010)), and only trace levels of carbaryl (4.7 ppb) and pendimethalin (2.0 ppb) were found in the pollen and only coumaphos (1.0 ppb) was found in honey. These diets were therefore considered to be pesticide-free. With the exception of CYP9Q3, these genes were upregulated in response to coumaphos and/or fluvalinate in our microarray study. Additionally, previous studies demonstrated that expression of both CYP9S1 and CYP9Q3 were upregulated by honey feeding (Mao et al., 2011), while expression of SODH2 was upregulated in honey/pollen vs. sucrose fed bees (Ament et al., 2011).

Expression of CYP9S1 ($\chi^2 = 34.10$, $p < 0.0001$) and CYP9Q3 ($\chi^2 = 45.42$; DF = 5; $p < 0.0001$) were upregulated while expression of CYP305D1 ($\chi^2 = 33.1556$; DF = 5; $p < 0.0001$) was downregulated in bees fed a pollen diet (regardless of whether the carbohydrate source was sucrose or honey) relative to all other treatment groups (Fig. 6). Expression of SODH2 ($\chi^2 = 44.91$; DF = 5; $p < 0.0001$) was significantly upregulated in bees fed both pollen and soy protein diets, regardless of the carbohydrate source (Fig. 6). Expression of GST was not significantly affected by diet (Fig. 6).

3. Discussion

Our results demonstrate that chronic, sub-lethal exposure to two pesticides commonly applied to honey bee colonies causes large-scale changes in gene expression in abdominal tissues. In addition to modulating expression of several putative detoxifica-

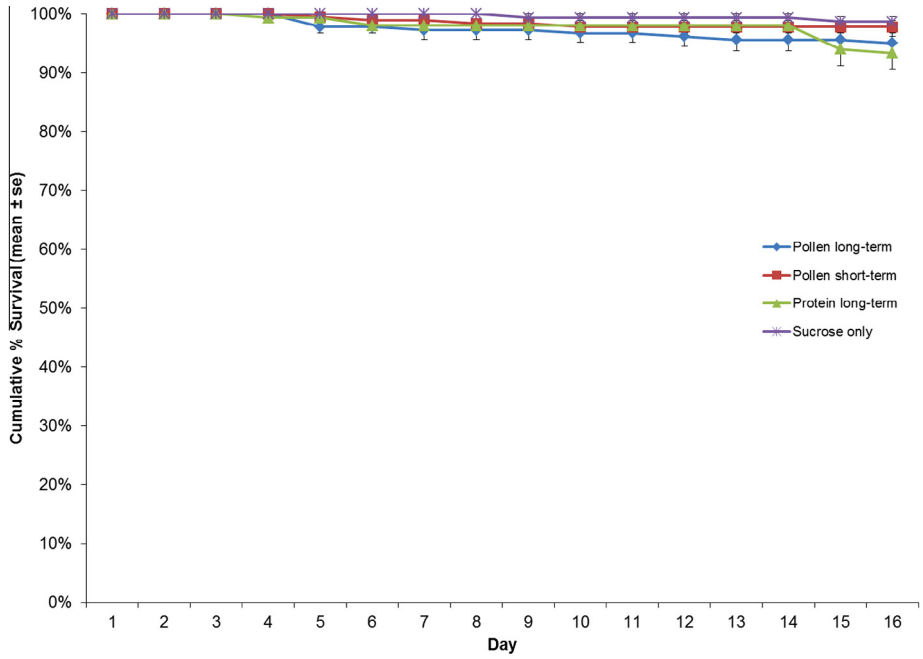

Fig. 4. Effect of diet on survival of honey bee workers in the absence of pesticide exposure. Cages were established with one of four diets for a period of 16 days. Mean survival time for bees treated with each of the diets was found to be greater than 15.5 days. There was a significant decrease in mean survival time of bees fed a long-term protein diet relative to bees fed a sucrose (Chi-squared (χ^2) = 5.52, p = 0.02) or short-term pollen (χ^2 = 3.87, p = 0.05) diet (see Table 3 for statistics).

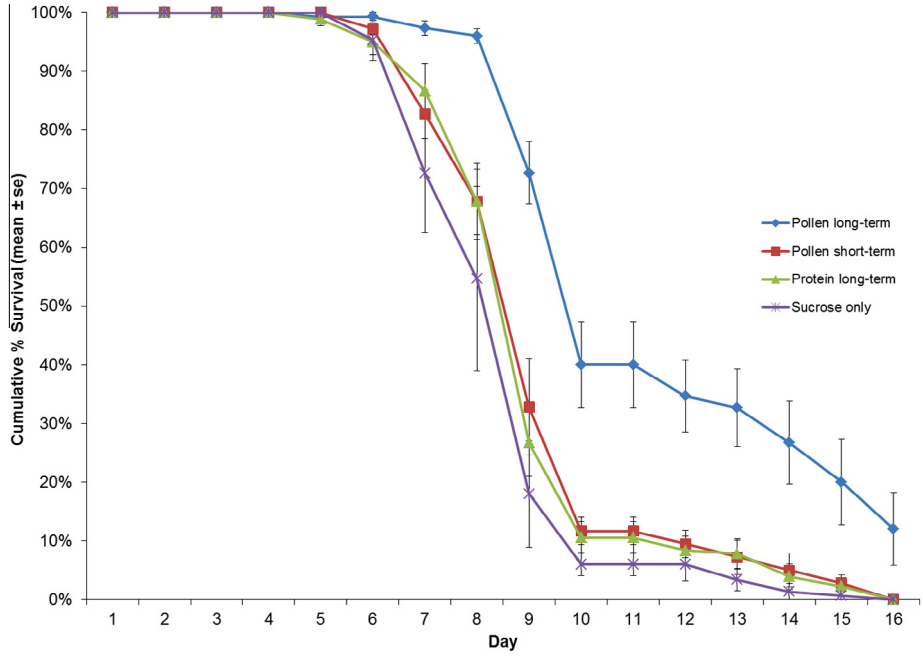

Fig. 5. Diet impacts survival time of bees exposed to pesticides. Cages were established with one of four diets for a period of 16 days. Honey bees were challenged with a daily chronic feeding of 3 ppm chlorpyrifos beginning on day five of the experiment. There were significant differences in mean survival time of bees exposed to the four diets, as follows: long-term pollen >long-term protein = short-term pollen >sucrose only (see Table 3 for statistics).

tion genes, pesticide exposure alters expression of genes involved in core physiological pathways, including behavioral maturation, immunity, nutrition, and metabolism. Interestingly, the effects of the two pesticides were largely overlapping, although the pesticides have different modes of action. Analysis of the impacts of pesticide exposure on endocrine profiles revealed that exposure did not alter levels of JH, a major regulator of behavioral maturation, but did significantly decrease levels of circulating MF, the precursor of JH. We validated the somewhat surprising association between genes whose expression is affected by diet and pesticide exposure by confirming the effect of diet (specifically, pollen and protein) on expression levels of a set of detoxification genes identified in our study. Finally, we demonstrated that resistance to a third pesticide, chlorpyrifos, increased with chemical and nutri-

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

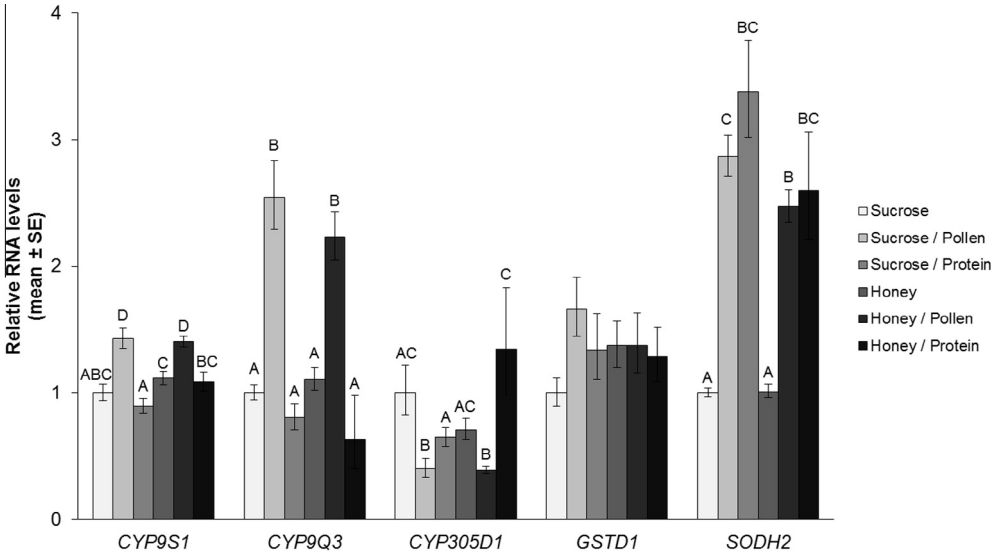

Fig. 6. Relative expression levels of selected candidate genes in response to diet. Cages of 30 one day old bees were fed one of six diets (sucrose, sucrose/pollen (wildflower), sucrose/protein (soy), honey, honey/pollen, honey/protein) for a seven day period. Abdomens were homogenized and RNA extracted. Samples were analyzed using qRT-PCR and relative amounts were calculated using the ΔΔCt method. Kruskal Wallis analyses revealed significant effects of treatment in CYP9S1 (chi-squared (χ^2) = 34.10; degrees of freedom (DF) = 5; $p < 0.0001$), CYP9Q3 ($\chi^2 = 45.42$; DF = 5; $p < 0.0001$), CYP305D1 ($\chi^2 = 33.1556$; DF = 5; $p < 0.0001$), and SODH2 ($\chi^2 = 44.91$; DF = 5; $p < 0.0001$). Subsequent posthoc pairwise comparisons were conducted to identify differentially regulated treatment groups and statistical differences ($p < 0.05$) are denoted by different letters.

tional complexity of the diet, with pollen-fed bees surviving significantly longer than bees fed soy protein or sucrose alone.

The field relevant doses of coumaphos and fluvalinate are difficult to determine. Here, we fed bees a dose of 100 ppm, which is equivalent to concentrations found in wax (Berry et al., 2013; Mullin et al., 2010). The concentrations found in pollen and nectar are much lower (Mullin et al., 2010). However bees are primarily exposed to coumaphos and fluvalinate when beekeepers treat colonies with these chemicals to control *Varroa* mites, and during these treatments bees may be receiving 0.3–3.0 μg of either chemical per day (Haarmann et al., 2002; Johnson et al., 2009b), which is equivalent to the maximum possible daily dose our bees received (3 μg/bee/day). Notably, Berry et al. (2013) found lethal and sublethal effects of fluvalinate and coumaphos treatments on worker bees from colonies treated according to standard beekeeping practices, including reductions in survival and increases in the construction of queen supercedure cells. Thus, while our study clearly illustrates a strong transcriptional response of the bee to xenobiotics in their environment, additional studies are needed to examine correlated transcriptional, physiological and behavioral responses to a standard beekeeping dose in the field.

Chronic sub-lethal exposure to pesticides caused significant global changes in gene expression, with 8.96% of the 12,483 genes on the microarray differentially expressed. Although the two pesticides have different modes of action, there was significantly more overlap than expected by chance in the molecular responses they elicited, with 117 genes commonly regulated by both coumaphos and fluvalinate. Several detoxification genes were present in this group, including three P450s (CYP9S1, CYP6AS3, and CYP6AS4) from the CYP3 clade: this clade is commonly involved in insecticide detoxification (Claudianos et al., 2006). Interestingly, in a previous study, expression of these three P450s were not found to be significantly impacted by coumaphos and fluvalinate exposure (Mao et al., 2011) in the midgut. Despite the fact that the midgut is assumed to be the likely site of detoxification, our whole abdomen analysis may have increased the probability of detecting other detoxification pathways and suggests the involvement of additional abdominal tissues in pesticide metabolism.

Though there was significant overlap in gene expression responses to coumaphos and fluvalinate, it is clear that transcriptional responses to different pesticides can differ quite substantially. In our study, nearly 700 transcripts were regulated only by coumaphos or only by fluvalinate. When compared to another study which examined the transcriptomic effects of imidacloprid exposure (Derecka et al., 2013), expression of only 31 transcripts were significantly regulated by all three pesticides, though this overlap was significantly greater than expected by chance (Fisher's exact test; $p < 0.001$, see Supplementary Table S10 for a list of these transcripts). However, expression of three of these transcripts (which are associated with detoxification) were unaffected by exposure to imidacloprid or fipronil in a subsequent study (Aufauvre et al. (2014). Furthermore, neither CYP9Q3 nor CYP306A1, which were each found to be upregulated by pesticide exposure in previous studies (Boncristiani et al., 2012; Mao et al., 2011), were upregulated in our study. Thus, while there is evidence that pesticides can elicit substantial transcriptional responses and there can be significant overlap in the transcriptional responses to different pesticides, these responses are undoubtedly modified by genetics, environmental conditions, nutrition, and tissue sampled. With such complexity in the transcriptional response, it may be challenging to identify specific genes to serve as biomarkers of pesticide exposure in the field.

We found a significant overlap in the number of transcripts regulated by immunostimulation (Richard et al., 2012) and coumaphos and fluvalinate exposure. Note that the genes identified in Richard et al. significantly changed expression six hours after wounding, saline-injection, bead-injection, and/or bacterial-injection, and thus represent relatively general, short-term immune response genes. Thus, the significant overlap with pesticide exposure is surprising, but it suggests that pesticide-treated bees may be immunocompromised. Other studies have also demonstrated a downregulation of immune genes in bees exposed to the pesticide fipronil (Aufauvre et al., 2014). We did not find increased titers of several common pathogens (i.e., Deformed Wing Virus) in our treated samples. However, several other studies have demonstrated an increase of pathogen infection in bees exposed to

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

pesticides. For example, the NF-κB immune signaling pathway is modulated in response to clothianidin and imidacloprid (neonicotinoid) exposure, which in turn is correlated with increased Deformed Wing Virus replication (Nazzi et al., 2012). Additionally, exposure to fungicides and chlorpyrifos increased titers of Deformed Wing Virus and Blackened Queen Cell Virus (DeGrandi-Hoffman et al., 2013). Furthermore, bees exposed to imidacloprid were also found to be more susceptible to *Nosema* infection (Alaux et al., 2010a; Pettis et al., 2012). Adult bees reared on combs treated with a mixture of pesticide residues, including the two miticides coumaphos and fluvalinate, also were found to have a significantly greater *Nosema* infection than the control (Wu et al., 2012).

Our molecular and physiological data suggest that chronic exposure to coumaphos and fluvalinate may alter genes and physiological processes associated with behavioral maturation, but it is unclear if exposure accelerates or decelerates maturation. Pesticide exposure significantly impacted expression of a subset of genes involved in behavioral maturation, but there was no clear directional effect – for example, genes associated with nursing and foraging behavior were equally likely to be upregulated. Expression of vitellogenin, which is negatively correlated with behavioral maturation and serves as an excellent indicator of the bees' maturation state (Amdam and Omholt, 2003) was unaffected by our treatments. Similarly, hemolymph titers of JH, the major endocrine driver of behavioral maturation, were not affected. However, titers of MF, a precursor of juvenile hormone, were significantly reduced in pesticide-exposed bees. The role that MF plays in behavioral maturation is not known. MF has been found circulating in the hemolymph of insects from several different orders including honey bees (Teal et al., 2014) and can induce developmental and physiological effects in *Drosophila* (Harshman et al., 2010; Jones et al., 2010; Jones et al., 2013); thus, MF may function similarly to JH as a circulating hormone. We did not identify any increase in transcript abundance of common JH biosynthetic pathway genes (including *usp* and *hmgr*) in our microarray analysis, however one of the genes up-regulated by pesticide exposure in our study was CYP305D1, a P450 which may be involved in hormone biosynthesis (Claudianos et al., 2006; Helvig et al., 2004).

One of the most unexpected findings from our study was the significant overlap between pesticide-responsive and diet-responsive genes (Ament et al., 2011). Over a third of the genes up-regulated in response to pesticide exposure were also up-regulated in bees fed a rich diet (honey/pollen) versus a poor diet (sucrose). Subsequent analysis of the effects of different diets on expression of five pesticide-regulated candidate genes demonstrated complex effects of diet on these genes, with three genes significantly up-regulated in bees fed diets containing pollen diet (but not soy protein or honey), and one gene significantly up-regulated by diets containing protein, derived from either soy or pollen. Notably, while we found a slight but not significant up-regulation of three P450s (CYP9S1, CYP9Q3, and CYP305D1) in bees fed honey, previous studies found diets containing honey resulted in up-regulation of several P450s in the CYP6AS clade (Johnson, 2009; Johnson et al., 2012). Our results suggest there are suites of genes that are specifically activated in response to one or more of the myriad of chemicals found in pollen, including lipids, vitamins and minerals, and secondary plant compounds (Haydak, 1970), while expression of others are activated by proteins and/or amino acids. Indeed, *p*-coumaric acid, a structural component of the outer wall of pollen grains (Wehling et al., 1989), can upregulate CYP9Q3 expression (Mao et al., 2013). Some of these upregulated genes may be involved in detoxifying secondary plant compounds; for example, quercetin, a common secondary plant compound in honey and pollen, can be metabolized by CYP6AS1, CYP6AS3, CYP6AS4, and CYP6AS10 in *in vitro* enzymatic assays (Mao et al., 2009). However, it is also possible that these diet-activated genes are playing a larger role in metabolism. Ontology analysis of the set of genes upregulated by both rich diet and pesticide exposure revealed a number of metabolic processes that may be impacted, including ribonucleotide metabolic process, transport, organic acid metabolic process, acetyl-CoA catabolic process, organic acid biosynthetic process, monocarboxylic acid metabolic process, cellular respiration, ribonucleotide monophosphate metabolism, and mitochondrial transport. We did not, however, observe the downregulation of gluconeogenesis and glycolysis pathways as demonstrated in bees exposed to imidacloprid (Derecka et al., 2013), further suggesting that different pesticides are differentially affecting honey bee physiology.

The association between nutrition and pesticide regulated molecular pathways suggests that nutrition may modulate the impacts of pesticides on adult honey bees. Interestingly, while the inclusion of protein (from pollen or soy) in their diet slightly reduces the mean survival time of bees in the absence of pesticide exposure, protein-fed bees have significantly greater survival when exposed to pesticides than bees fed sucrose alone. Similar results have been observed in other studies: with one exception (Wang et al., 2014), protein consumption in honey bees and other social insects has been shown previously to reduce adult longevity (Altaye et al., 2010; Dussutour and Simpson, 2009, 2012; Pirk et al., 2010). However, when bees were challenged with the pesticide chlorpyrifos, protein/pollen consumption significantly lengthened honey bee longevity. The benefits of a protein diet in pesticide-exposed bees were highest for bees fed pollen throughout the course of the study, although bees fed pollen for only 24 h prior to pesticide exposure or bees fed soy protein also exhibited significantly greater survival times than bees fed only sucrose. Consumption of pollen from a diverse array of sources ensures proper nutrition, as pollen from different species of plants differs in nutritional content (Roulston and Cane, 2000). Pollen consumption is known to improve immune responses (Alaux et al., 2010b) and reduce pesticide sensitivity in honey bees (Archer et al., 2014; Wahl and Ulm, 1983). In other herbivores, induction of P450s through the consumption of small amounts of plant material can render the allelochemicals of the plant relatively harmless (reviewed in Glendinning, 2002). Pollen may have a similar priming response in the honey bee by triggering upregulation of P450s (as shown in our results) and thereby improved resistance to pesticide exposure. However, the fact that a soy protein based diet also improved resistance to pesticides, and a long-term pollen diet was more beneficial than a short-term pollen diet, suggests that the nutritional value of these diets also contributes to pesticide resistance.

Our study demonstrates that chronic exposure to sub-lethal, field-relevant doses of two commonly used pesticides significantly impacts global gene expression patterns in adult honey bee workers. Several core pathways are affected, including those involved in immunity, behavioral maturation, nutrition and metabolism. However it is important to note that different life stages (larvae, pupae, nurses, foragers) likely have very different responses to pesticides (Schmehl et al., unpublished data). Pesticides are typically applied as formulations (which can be more toxic than the active ingredients alone (Johnson et al., 2010), and bees are typically exposed to mixtures of pesticides (Mullin et al., 2010), which may act additively, synergistically, or antagonistically (Biddinger et al., 2013; Johnson et al., 2009b; Zhu et al., 2014). Thus, the effects of field-applied pesticides on individual honey bees and colonies are complex. Future studies will be needed to determine if other pesticides (including chlorpyrifos) impact honey bee physiology similar to that of coumaphos and fluvalinate. Importantly, our study reveals a robust and significant overlap between transcriptional responses to diet and pesticides, and demonstrates that diet, specifically

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

pollen-based diets, can significantly reduce the sensitivity of bees to pesticide exposure. Thus, improving the complexity and nutritional value of the diet available to honey bees through optimized supplementary feeding or encouraging flowering plant diversity in agricultural fields may help buffer bee populations from the impacts of pesticide exposure and other stressors.

4. Materials and methods

4.1. Collections for microarray and hormonal assays

Worker bees were derived from one colony headed by a single-drone inseminated (SDI) Carniolan queen (Glenn Apiaries, Fallbrook, CA) and maintained using standard commercial apicultural practices at a Penn State University apiary located in State College, PA. Honeycomb frames containing emerging workers were removed from the colony and placed in an incubator overnight. Individual cages were constructed using two paired 100 mm × 20 mm Petri dish tops or bottoms (VWR, Radnor, PA) with a 15 cm × 30 cm piece of mesh metal screen formed into a cylinder. Holes for the pesticide feeders were punched into the plastic tops using a hot metal cork borer. Each cage was established using 30 newly emerged workers (<24 h old), along with one naturally mated Italian queen (BeeWeaver Apiaries, Austin, TX) and 1 mL 1:1 sucrose:water (w/v), and was placed in a dark environmental chamber at 35 °C and 50% relative humidity.

The following day (when the bees were <2 days old) cages were fed sub-lethal doses of pesticides or controls. 100 ppm fluvalinate (Chemservice-PS-1071, 95% purity) and 100 ppm coumaphos (Chemservice-PS-656, 99.5% purity) were dissolved in a 3% methanol/50% sucrose/water solution. Each cage received 1 mL of the following four treatments daily, to simulate a chronic, sub-lethal dose: fluvalinate, coumaphos, methanol (3% methanol/50% sucrose/water solution), and sucrose (50% sucrose/water solution). Pilot studies were performed to ensure these doses did not cause significant mortality, thereby confirming that these were indeed sublethal doses. Mortality was less than 5% in each cage and there were no significant differences (ANOVA $F_3 = 1.5707$, $p = 0.2277$) between treatments (data not shown). The selected concentration is consistent with levels found in the wax (coumaphos – up to 514 ppm, fluvalinate – up to 204 ppm) of US honey bee colonies (Berry et al., 2013; Mullin et al., 2010). Bees are primarily exposed during miticide treatments, and may receive 0.3–3.0 μg of either chemical per day (Haarmann et al., 2002; Johnson et al., 2009b), which is equivalent to the maximum daily dose our bees received (3 μg/bee). Each treatment group had 12 replicates, for a total of 48 cages. There was no significant difference in the volume of diet consumed between treatments (data not shown). The diet was replaced daily and mortality recorded daily during a course of 7 days.

After 7 days, five workers were removed from each cage and placed on ice to immobilize them for hemolymph extraction. The remaining workers were placed directly on dry ice and stored at −80 °C for further molecular analysis (see below).

4.2. Examining the impact of diet on expression of pesticide-regulated candidate genes

Here, we examined the impact of diet on expression of candidate genes identified in the microarray study. Cages were established as for the study examining the impact of pesticide exposure, but were modified to include an opening to introduce the pollen or soy protein diet. Cages received one of six treatment diets: sucrose only, honey only, sucrose/pollen, honey/pollen, sucrose/protein, and honey/protein. The sucrose (1:1 sucrose/water w/v) and honey (wildflower, YS Bee Farms, Illinois) treatments received no protein source during the course of the experiment. The pollen diet consisted of bee-collected wildflower pollen (collected at the hive entrance using pollen traps) from an organic farm near the eastern entrance to Cascades National Park, Oregon, mixed with sucrose solution (1:1) at an 1:1 w/v pollen/sucrose ratio to create a smooth, creamy texture. The protein diet consisted of soy protein isolate (NOW Sports, 90% soy protein) (Roulston and Cane, 2002) mixed at a 4:1 w/v protein/sucrose ratio to produce a consistency comparable to that of the pollen mixture. Since pollen and honey are known to contain large numbers of pesticides (Mullin et al., 2010), the honey and pollen were analyzed using LC/MS–MS and GC/MS–MS to confirm they were not contaminated (see results). Sucrose and honey (∼1.7 mL at each feeding) were replaced every 3 days, whereas the pollen and protein (∼0.5 g at each feeding) were replaced every 2 days. The food (sugar and protein) were provided *ad libitum* and not completely consumed before being replaced. Each treatment had six replicates, for a total of 36 cages. After 7 days, the workers were placed directly on dry ice and stored at −80 °C until qRT-PCR analysis. There was no mortality during the course of the experiment. See below for further information on the quantitative real-time PCR analysis.

4.3. Impact of diet on sensitivity to pesticides

We examined the impact of diet on the longevity of pesticide-exposed honey bees over a 16-day period. Cages were established as in the studies examining the impact of diet on pesticide-regulated genes. Cages were fed 1:1 sucrose solution daily in addition to one of four diets: pollen long-term, pollen short-term, protein long-term, or sucrose only (diets described above). The "long-term" diets were fed throughout the experiment and replaced every 2 days. The "short-term" diet was fed 24 h prior to initial pesticide exposure and then removed at the introduction of the pesticide. Half of the cages in each treatment group were fed pesticides while the other half received sucrose beginning on day five. We chose to begin pesticide feedings on day five to allow time for the nutritional value of the diets to have their impact on bee physiology. Five day old bees fed pollen have more lipid stores than bees which are pollen deprived (Toth et al., 2005), demonstrating that by this time point diet will have affected the physiology of the bees. Cages receiving the pesticide treatment were chronically fed chlorpyrifos in 1:1 sucrose solution at 3 ppm. Mortality was recorded daily for 16 days. Chlorpyrifos is an organophosphate (like coumaphos) and is the third most prevalent and abundant pesticide detected in the hive, with maximum levels of 890 ppb (Mullin et al., 2010). Chlorpyrifos is considerably more toxic to bees than coumaphos or fluvalinate (Mullin et al., 2010), with an LD50 of 3 ppm after a test duration of 72 h using our caged assays (data not shown). This high level of toxicity and mortality allows us to rapidly assess the impact of diet on pesticide sensitivity.

To determine differences in survival among our treatment groups, we conducted a Kaplan–Meier survival log-rank test (Kleinbaum and Klein, 2012) using diet and pesticide treatment as variables. Before performing our Kaplan–Meier analysis, we conducted a Cox regression proportional hazards model to confirm that the assumption of a linear hazard ratio between diets was met. Statistical analysis was performed using SPSS (v.21, IBM, Armonk, NY).

4.4. Microarray analysis

Six cages per treatment group were selected for microarray analysis to characterize pesticide-induced changes in gene expression. Sample preparation and microarray analysis were performed as in Niño et al. (2011) with slight modifications. After a period of

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

7 days, a pooled sample of five workers/cage were removed and whole abdomens were dissected from the bee. Whole abdomens were used for our analyses rather than the whole bee because the site of xenobiotic detoxification is located in the midgut (Mao et al., 2011), while abdominal fat bodies play a significant role in metabolism and immune response. We limited our analysis to the abdomens because additional tissues could have increased the signal to noise ratio of the expression patterns. Abdomens were extracted using QIAshredder (Qiagen, Valencia, CA) and an RNeasy RNA extraction kit (Qiagen). RNA was quantified using a Nanodrop 1000 (Thermo Scientific). 750 ng of RNA/sample were amplified using the Ambion MessageAMP II aRNA kit (Life Technologies, Grand Island, NY). Four micrograms of amplified RNA from each sample were labeled independently with Cy3 and Cy5 dyes (Kreatech, Amsterdam, Netherlands). Samples were hybridized to 24 microarrays (two samples/array) in a loop design with dye swaps incorporated. Whole genome microarrays containing 28,800 spotted oligos (including 12,483 paired oligos corresponding to honey bee transcripts) were purchased from the W.M. Keck Center for Functional Genomics at the University of Illinois, Urbana-Champaign. Samples were hybridized using the Maui mixer (BioMicro Systems, Salt Lake City, Utah) and scanned on an Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) using GENEPIX software (Agilent Technologies, Santa Clara, CA).

Analysis of the array data followed the protocol described in Richard et al. (2012). Spots with an intensity of less than 100 (the average array background for both dyes) were removed from the analysis. Transcripts present on less than 7 of the 24 arrays were excluded from further analysis. Expression data was log-transformed and normalized using a mixed-model ANOVA (proc MIXED, SAS, Cary, NC) with the following model:

$$Y = \mu + \text{dye} + \text{block} + \text{array} + \text{array} * \text{dye} + \text{array} * \text{block} + \epsilon$$

where Y is expression, dye, and block are fixed effects, and array, array * dye and array * block are random effects. Transcripts with significant expression differences between groups were detected by using a mixed-model ANOVA with the model:

$$Y = \mu + \text{treatment} + \text{spot} + \text{dye} + \text{array} + \epsilon$$

where Y represents the residual from the previous model; treatment, spot, and dye are fixed effects; and array is a random effect. p-values were corrected for multiple testing using a false discovery rate of <0.01 (proc MULTTEST, SAS).

The expression levels of all significantly regulated genes were normalized by calculating the average value across the treatment groups and subtracting this average from the normalized residual. Two-way hierarchical clustering analysis was performed using JMP 9 (SAS, Cary, NC). Approximately unbiased p values, bootstrap values, and Euclidean distances were calculated using R version 2.14.2 with 100,000 bootstrap replicates (http://www.r-project.org). All significantly regulated transcripts were annotated according to their *Drosophila* orthologs in Flybase (<http://flybase.org/>) when available. Gene ontology (GO) analysis was performed using DAVID version 6.7 (Dennis et al., 2003; Huang et al., 2008). The array data are available on the ArrayExpress website (http://www.ebi.ac.uk/arrayexpress/) according to MIAME standards under accession number E-MTAB-3025.

4.5. Validation of microarray results

To validate the microarray results using qRT-PCR for methanol, coumaphos, and fluvalinate, 200 ng of RNA from samples used in the microarray study was synthesized into cDNA using SuperScript® II Reverse Transcriptase (Life Technologies). Ten significantly regulated candidate genes were selected (see Supplementary Table S8 for a list of genes and primers). Expression levels of the selected candidate genes were determined using an ABI Prism® 7900 sequence detector with the SYBR Green detection method (Life Technologies). Triplicate reactions were performed for each of the samples and averaged together. The expression of each candidate gene was normalized to the geometric mean (Vandesompele et al., 2002) of the two housekeeping genes *actin* and *eIF-S8* (Grozinger et al., 2003; Huising and Flik, 2005), using the ΔCt method for relative quantification. Water and a no-enzyme control were included for each primer to ensure no contamination from DNA or primer dimers, and a dissociation curve was measured at the end of the qRT-PCR to confirm the presence of a single product.

A nonparametric Kruskal–Wallis one-way ANOVA was performed for all multiple comparison statistics using JMP 9 (SAS, Cary, NC). Ordered letter differences were determined using a paired-difference Wilcoxon t-test.

4.6. Comparative genomic analyses

To determine the biological functions of the regulated genes, we compared our significantly regulated lists of genes with genes whose expression levels were significantly associated with behavioral maturation (Ament et al., 2011), immune function (Evans et al., 2006; Richard et al., 2012), and nutrition (Ament et al., 2011) in previous genome wide expression analyses. Additionally, we performed directional analyses with gene lists from (Ament et al., 2011), to determine if genes were similarly up- or down-regulated by diet and pesticide exposure. Comparisons between gene lists were performed using Venny (Oliveros, 2007;<http://bioinfogp.cnb.csic.es/tools/venny/index.html>). Significant overlaps in the gene lists was determined using a Fisher's exact test, using all the genes present on the microarray as a background list (Jim Lund, University of Kentucky, <http://nemates.org/MA/progs/overlap_stats.html>).

4.7. Characterization of the juvenile hormone and methyl farnesoate hemolymph titers

Hemolymph was collected from under the 4th abdominal segment of individual bees using a 10 μL pulled glass capillary tube. Hemolymph was pooled from 3 to 5 bees to yield a 10 μL sample for each cage and placed in a 1.7 mL Eppendorf tube and combined with 90 μL of methanol (HPLC grade). The tube and cap were wrapped with Teflon tape to prevent leaking. Samples were shipped to the USDA-ARS lab in Gainesville, FL for processing according to the protocols described in (Jones et al., 2010; Niño et al., 2012; Teal et al., 2000, 2014; Teal and Proveaux, 2006).

Across the four treatment groups collected for the microarray studies, eight samples were collected from sucrose, eight from methanol, seven from coumaphos, and eight from fluvalinate. Data were $\log_2$ transformed and analyzed using an ANOVA-Tukey HSD with treatment as a variable (JMP 9, SAS, Cary, NC).

4.8. Examining the impact of diet on expression of pesticide-regulated candidate genes

Whole abdomens from 3 bees/cage were homogenized using a FastPrep® FP120 (Thermo Scientific, Rockford, IL) for two-30 s cycles at a speed of 6.5 m/s. Samples were cooled on ice between cycles for 12 min. Each sample was transferred to a QIAshredder column (Qiagen) and centrifuged at 13,200g for 1.5 min. Lysate was removed and RNA was extracted with RNeasy® RNA extraction kit (Qiagen). DNA was removed from the product using a Turbo DNA-free kit (Life Technologies). RNA was quantified and cDNA synthesized as above. Expression levels of the selected candidate genes were determined as described above.

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

A total of 12 pooled samples (2 samples/cage) from each of the six nutrition treatments were analyzed to determine the relative expression of five candidate genes. We monitored expression levels of CYP305D1, CYP9S1, GSTD1, and notably CYP9Q3, which was previously shown to be upregulated by honey (Mao et al., 2011). We also examined expression levels of superoxide dismutase (SODH2); this gene was significantly upregulated by pesticide exposure in our microarray study, and in response to a rich diet (Ament et al., 2011), and may function in immunity (Luque et al., 1998; Richard et al., 2012) (see Supplementary Table S9 for a listing of the genes and associated primers).

A nonparametric Kruskal–Wallis one-way ANOVA was performed for all multiple comparison statistics using JMP 9 (SAS, Cary, NC). Ordered letter differences were determined using a paired-difference Wilcoxon *t*-test.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

D.R.S. conceived the study, participated in the design of the study, carried out the molecular genetic studies, comparative analyses, survival bioassays, and statistical analyses, and drafted the manuscript.

P.E.A.T. carried out the hormone analysis.

J.L.F. participated in the design of the study and provided critical feedback.

C.M.G. conceived the study, participated in the design and analysis of the study, and drafted the manuscript.

Acknowledgements

This project was funded by the United States Department of Agriculture – Agricultural and Food Research Initiative (USDA-AFRI Grant #2009-05207, awarded to Christina Grozinger and James Tumlinson).

We would like to thank James Tumlinson, Christopher Mullin and members of the Tumlinson and Grozinger labs for critical evaluations and discussions of the project and manuscript, Bernardo Niño for expert beekeeping assistance, Elina Lastro Niño and Holly Holt for assistance with experimental methodologies and statistical analysis, Tracey Baumgarten for assistance with the qRT-PCR studies, Mariam Khraibani for invaluable assistance with rearing and collections, and the Statistical Counseling Center at Penn State University.

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.2014.10.002.

References

Alaux, C., Brunet, J.-L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P., Le Conte, Y., 2010a. Interactions between Nosema microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Environ. Microbiol. 12, 774–782.

Alaux, C., Ducloz, F., Crauser, D., Le Conte, Y., 2010b. Diet effects on honeybee immunocompetence. Biol. Lett. 6, 562–565.

Aliouane, Y., el Hassani, A.K., Gary, V., Armengaud, C., Lambin, M., Gauthier, M., 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environ. Toxicol. Chem. 28, 113–122.

Altaye, S.Z., Pirk, C.W.W., Crewe, R.M., Nicolson, S.W., 2010. Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. J. Exp. Biol. 213, 3311–3318.

Amdam, G.V., Omholt, S.W., 2003. The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. J. Theor. Biol. 223, 451–464.

Ament, S.A., Chan, Q.W., Wheeler, M.M., Nixon, S.E., Johnson, S.P., Rodriguez-Zas, S.L., Foster, L.J., Robinson, G.E., 2011. Mechanisms of stable lipid loss in a social insect. J. Exp. Biol. 214, 3808–3821.

Anderson, D., Trueman, J., 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Exp. Appl. Acarol. 24, 165–189.

Archer, C.R., Pirk, C.W.W., Wright, G.A., Nicolson, S.W., 2014. Nutrition affects survival in African honeybees exposed to interacting stressors. Funct. Ecol. 28, 913–923.

Atkins, E., 1992. Injury to honey bees by poisoning. In: Graham, J.M. (Ed.). Dadant and Sons, Hamilton, IL, p. 1324.

Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., Blot, N., 2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. PLoS One 9, e91686.

Berenbaum, M.R., 2002. Postgenomic chemical ecology: from genetic code to ecological interactions. J. Chem. Ecol. 28, 873–896.

Berry, J.A., Hood, W.M., Pietravalle, S., Delaplane, K.S., 2013. Field-level sublethal effects of approved bee hive chemicals on honey bees (*Apis mellifera* L.). PLoS One 8, e76536.

Biddinger, D.J., Robertson, J.L., Mullin, C., Frazier, J., Ashcraft, S.A., Rajotte, E.G., Joshi, N.K., Vaughn, M., 2013. Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). PLoS One 8, e72587.

Bogdanov, S., 2004. Beeswax: quality issues today. Mol. Ecol. 10, 2095.

Boncristiani, H., Underwood, R., Schwarz, R., Evans, J.D., Pettis, J., vanEngelsdorp, D., 2012. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. J. Insect Physiol. 58, 613–620.

Burley, L.M., Fell, R.D., Saacke, R.G., 2008. Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. J. Econ. Entomol. 101, 1081–1087.

Ciarlo, T.J., Mullin, C.A., Frazier, J.L., Schmehl, D.R., 2012. Learning impairment in honey bees caused by agricultural spray adjuvants. PLoS One 7, e40848.

Claudianos, C., Ranson, H., Johnson, R., Biswas, S., Schuler, M., Berenbaum, M., Feyereisen, R., Oakeshott, J., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15, 615–636.

Collins, A.M., Pettis, J.S., Wilbanks, R., Feldlaufer, M.F., 2004. Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. J. Apic. Res. 43, 128–134.

Council, N.R., 2007. Status of Pollinators in North America. National Academies Press, Washington, DC.

Decourtye, A., Devillers, J., Aupinel, P., Brun, F., Bagnis, C., Fourrier, J., Gauthier, M., 2011. Honeybee tracking with microchips: a new methodology to measure the effects of pesticides. Ecotoxicology 20, 429–437.

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., Pham-Delègue, M.-H., 2004. Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. Ecotoxicol. Environ. Saf. 57, 410–419.

Decourtye, A., Devillers, J., Genecque, E., Menach, K.L., Budzinski, H., Cluzeau, S., Pham-Delegue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. Arch. Environ. Contam. Toxicol. 48, 242–250.

DeGrandi-Hoffman, G., Chen, Y., Huang, E., Huang, M.H., 2010. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). J. Insect Physiol. 56, 1184–1191.

DeGrandi-Hoffman, G., Yanping, C., Simonds, R., 2013. The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). Insects (2075–4450) 4, 71–89.

Dennis, G., Sherman, B., Hosack, D., Yang, J., Gao, W., Lane, H.C., Lempicki, R., 2003. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. 4, P3.

Derecka, K., Blythe, M.J., Malla, S., Genereux, D.P., Guffanti, A., Pavan, P., Moles, A., Snart, C., Ryder, T., Ortori, C.A., Barrett, D.A., Schuster, E., Stöger, R., 2013. Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. PLoS One 8, e68191.

Dussutour, A., Simpson, S.J., 2009. Communal nutrition in ants. Curr. Biol. 19, 740–744.

Dussutour, A., Simpson, S.J., 2012. Ant workers die young and colonies collapse when fed a high-protein diet. Proc. R. Soc. B Biol. Sci..

Eilers, E.J., Kremen, C., Greenleaf, S.S., Garber, A.K., Klein, A.-M., 2011. Contribution of pollinator-mediated crops to nutrients in the human food supply. PLoS One 6, e21363.

Eiri, D.M., Nieh, J.C., 2012. A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. J. Exp. Biol. 215, 2022–2029.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol. Biol. 15, 645–656.

Feyereisen, R., 2005. Insect cytochrome P450. Compr. Mol. Insect Sci. 4, 1–77.

Frost, E.H., Shutler, D., Hillier, N.K., 2013. Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. J. Exp. Biol. 216, 2931–2938.

Glendinning, J.I., 2002. How do herbivorous insects cope with noxious secondary plant compounds in their diet? Entomol. Exp. Appl. 104, 15–25.

Godfray, H.C.J., Blacquière, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., Raine, N.E., Vanbergen, A.J., McLean, A.R., 2014. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc. R. Soc. B Biol. Sci. 281.

González-Varo, J.P., Biesmeijer, J.C., Bommarco, R., Potts, S.G., Schweiger, O., Smith, H.G., Steffan-Dewenter, I., Szentgyörgyi, H., Woyciechowski, M., Vilà, M., 2013. Combined effects of global change pressures on animal-mediated pollination. Trends Ecol. Evol. 28, 524–530.

Graham, J.M., 1992. The hive and the honey bee. Dadant & Sons.

Gregorc, A., Evans, J.D., Scharf, M., Ellis, J.D., 2012. Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and *Varroa* mites (*Varroa* destructor). J. Insect Physiol. 58, 1042–1049.

Grozinger, C.M., Sharabash, N.M., Whitfield, C.W., Robinson, G.E., 2003. Pheromone-mediated gene expression in the honey bee brain. Proc. Natl. Acad. Sci. 100, 14519–14525.

Haarmann, T., Spivak, M., Weaver, D., Weaver, B., 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. J. Econ. Entomol. 95, 28–35.

Harshman, L.G., Song, K.-D., Casas, J., Schuurmans, A., Kuwano, E., Kachman, S.D., Riddiford, L.M., Hammock, B.D., 2010. Bioassays of compounds with potential juvenoid activity on *Drosophila melanogaster*: Juvenile hormone III, bisepoxide juvenile hormone III and methyl farnesoates. J. Insect Physiol. 56, 1465–1470.

Haydak, M.H., 1970. Honey bee nutrition. Annu. Rev. Entomol. 15, 143–156.

Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., 2004. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. Proc. Natl. Acad. Sci. 101, 4024–4029.

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. Science 336, 348–350.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Natl. Protoc. 4, 44–57.

Huang, Z.-Y., Robinson, G.E., Tobe, S.S., Yagi, K.J., Strambi, C., Strambi, A., Stay, B., 1991. Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. J. Insect Physiol. 37, 733–741.

Huising, M.O., Flik, G., 2005. The remarkable conservation of corticotropin-releasing hormone (CRH)-binding protein in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor of insects and vertebrates. Endocrinology 146, 2165–2170.

Janmaat, A.F., Winston, M.L., 2000. The influence of pollen storage area and *Varroa jacobsoni*; Oudemans parasitism on temporal caste structure in honey bees (*Apis mellifera* L.). Insect Soc. 47, 177–182.

Johnson, R.M., 2009. Toxicogenomics of *Apis mellifera*. University of Illinois at Urbana-Champaign.

Johnson, R.M., Ellis, M., Mullin, C., Frazier, M., 2010. Pesticides and honey bee toxicity – USA. Apidologie 41, 312–331.

Johnson, R.M., Evans, J.D., Robinson, G.E., Berenbaum, M.R., 2009a. Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). Proc. Natl. Acad. Sci. 106, 14790–14795.

Johnson, R.M., Mao, W., Pollock, H.S., Niu, G., Schuler, M.A., Berenbaum, M.R., 2012. Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. PLoS One 7, e31051.

Johnson, R.M., Pollock, H., Berenbaum, M., 2009b. Synergistic interactions between in-hive miticides in *Apis mellifera*. J. Econ. Entomol. 102, 474–479.

Johnson, R.M., Wen, Z., Schuler, M., Berenbaum, M., 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entomol. 99, 1046–1050.

Jones, G., Jones, D., Li, X., Tang, L., Ye, L., Teal, P., Riddiford, L., Sandifer, C., Borovsky, D., Martin, J.-R., 2010. Activities of natural methyl farnesoids on pupariation and metamorphosis of *Drosophila* melanogaster. J. Insect Physiol. 56, 1456–1464.

Jones, G., Teal, P., Henrich, V.C., Krzywonos, A., Sapa, A., Wozniak, M., Smolka, J., Jones, D., 2013. Ligand binding pocket function of *Drosophila* USP is necessary for metamorphosis. Gen. Comp. Endocrinol. 182, 73–82.

Khoury, D.S., Myerscough, M.R., Barron, A.B., 2011. A quantitative model of honey bee colony population dynamics. PLoS One 6, e18491.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B 274, 303–313.

Kleinbaum, D.G., Klein, M., 2012. Kaplan–Meier survival curves and the log-rank test survival analysis. A Self-Learning Test. Springer, New York, Survival Analysis, pp. 55–96.

Luque, T., Hjelmqvist, L., Marfany, G., Danielsson, O., El-Ahmad, M., Persson, B., Jörnvall, H., Gonzàlez-Duarte, R., 1998. Sorbitol dehydrogenase of drosophila: gene, protein, and expression data show a two-gene system. J. Biol. Chem. 273, 34293–34301.

Mao, W., Rupasinghe, S.G., Johnson, R.M., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R., 2009. Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 154, 427–434.

Mao, W., Schuler, M., Berenbaum, M., 2011. CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). PNAS Proc. Natl. Acad. Sci. 108, 12657–12662.

Mao, W., Schuler, M.A., Berenbaum, M.R., 2013. Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proc. Natl. Acad. Sci. 110, 8842–8846.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., Pettis, J.S., 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS One 5, e9754.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., Della Vedova, G., Cattonaro, F., Caprio, E., Pennacchio, F., 2012. Synergistic parasite–pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog. 8, e1002735.

Niño, E.L., Malka, O., Hefetz, A., Teal, P., Hayes, J., Grozinger, C.M., 2012. Effects of honey bee (*Apis mellifera* L.) queen insemination volume on worker behavior and physiology. J. Insect Physiol. 58, 1082–1089.

Niño, E.L., Tarpy, D., Grozinger, C.M., 2011. Genome-wide analysis of brain transcriptional changes in honey bee (*Apis mellifera* L.) queens exposed to carbon dioxide and physical manipulation reproductive changes in honey bee queens. Insect Mol. Biol. 20, 387–398.

Oliveros, J.C., 2007. Venny. An interactive tool for comparing lists with Venn diagrams.

Pettis, J., vanEngelsdorp, D., Johnson, J., Dively, G., 2012. Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. Naturwissenschaften 99, 153–158.

Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35, 605–610.

Pirk, C.W.W., Boodhoo, C., Human, H., Nicolson, S.W., 2010. The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*)*. Apidologie 41, 62–72.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25, 345–353.

Richard, F.J., Holt, H., Grozinger, C., 2012. Effects of immunostimulation on social behavior, chemical communication and genome-wide gene expression in honey bee workers (*Apis mellifera*). BMC Genomics 13, 558.

Rinderer, T.E., Guzman, L., Lancaster, V.A., Delatte, G.T., Stelzer, J.A., 1999. *Varroa* in the mating yard. I. The effects of *Varroa jacobsoni* and *apistan* on drone honey bees. Am. Bee J. 139, 134–139.

Robinson, G.E., 1987. Regulation of honey bee age polyethism by juvenile hormone. Behav. Ecol. Sociobiol. 20, 329–338.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of *Varroa* destructor. J. Invertebr. Pathol. 103 (Supplement), S96–S119.

Roulston, T.a.H., RoulstonCane, J.H., 2002. The effect of pollen protein concentration on body size in the sweat bee *Lasioglossum zephyrum* (Hymenoptera: Apiformes). Evol. Ecol. 16, 49–65.

Roulston, T.H., Cane, J.H., 2000. Pollen nutritional content and digestibility for animals. Plant Syst. Evol. 222, 187–209.

Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees – a risk assessment. PLoS One 9, e94482.

Sullivan, J.P., Jassim, O., Fahrbach, S.E., Robinson, G.E., 2000. Juvenile hormone paces behavioral development in the adult worker honey bee. Horm. Behav. 37, 1–14.

Teal, P.E.A., Jones, D., Jones, G., Torto, B., Nyasembe, V., Borgemeister, C., Alborn, H.T., Kaplan, F., Boucias, D., Lietze, V.U., 2014. Identification of methyl farnesoate from the hemolymph of insects. J. Nat. Prod. 77, 402–405.

Teal, P.E.A., Proveaux, A.T., 2006. Identification of methyl farnesoate from in vitro culture of the retrocerebral complex of adult females of the moth, *Heliothis virescens* (Lepidoptera: Noctuidae) and its conversion to juvenile hormone III. Arch. Insect Biochem. Physiol. 61, 98–105.

Teal, P.E.A., Proveaux, A.T., Heath, R.R., 2000. Analysis and quantitation of insect juvenile hormones using chemical ionization ion-trap mass spectrometry. Anal. Biochem. 277, 206–213.

Teeters, B.S., Johnson, R.M., Ellis, M.D., Siegfried, B.D., 2012. Using video-tracking to assess sublethal effects of pesticides on honey bees (*Apis mellifera* L.). Environ. Toxicol. Chem. 31, 1349–1354.

Thompson, H.M., Maus, C., 2007. The relevance of sublethal effects in honey bee testing for pesticide risk assessment. Pest Manag. Sci. 63, 1058–1061.

Thompson, H.M., Wilkins, S., Battersby, A.H., Waite, R.J., Wilkinson, D., 2007. Modelling long-term effects of IGRs on honey bee colonies. Pest Manag. Sci. 63, 1081–1084.

Tofilski, A., 2009. Shorter-lived workers start foraging earlier. Insect. Soc. 56, 359–366.

Toth, A., Kantarovich, S., Meisel, A., Robinson, G.E., 2005. Nutritional status influences socially regulated foraging ontogeny in honey bees. J. Exp. Biol. 208, 4641–4649.

Toth, A.L., Robinson, G.E., 2005. Worker nutrition and division of labour in honeybees. Anim. Behav. 69, 427–435.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–11.

vanEngelsdorp, D., Caron, D.M., Hayes, J., Underwood, R., Henson, M., Rennich, K., Spleen, A., Andree, M., Snyder, R., Lee, K., Roccasecca, K., Wilson, M., Wilkes, J., Lengerich, E., Pettis, J., 2012. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J. Apic. Res. 51, 115–124.

Wahl, O., Ulm, K., 1983. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. Oecologia 59, 106–128.

Wang, H., Zhang, S.-W., Zeng, Z.-J., Yan, W.-Y., 2014. Nutrition affects longevity and gene expression in honey bee (*Apis mellifera*) workers. Apidologie 45, 618–625.

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002

Wehling, K., Niester, C., Boon, J.J., Willemse, M.T.M., Wiermann, R., 1989. p-Coumaric acid—a monomer in the sporopollenin skeleton. Planta 179, 376–380.
Williamson, S.M., Wright, G.A., 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. J. Exp. Biol. 216, 1799–1807.
Wu, J.Y., Anelli, C.M., Sheppard, W.S., 2011. Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. PLoS One 6, 1–11.
Wu, J.Y., Smart, M.D., Anelli, C.M., Sheppard, W.S., 2012. Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to *Nosema* (Microsporidia) infection. J. Invertebr. Pathol. 109, 326–329.
Zhu, W., Schmehl, D., Mullin, C., Frazier, J., 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PLoS One 9, e77547.

Please cite this article in press as: Schmehl, D.R., et al. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.10.002