Xenobiotic detoxification pathways in honeybees
May R. Berenbaum¹ and Reed M. Johnson²

Address

¹University of Illinois at Urbana-Champaign, Department of Entomology, 505 S. Goodwin, Urbana, IL 61801-3795

²The Ohio State University, Department of Entomology, 1680 Madison Ave., Wooster, OH 44691-4114

Corresponding author: Berenbaum, May (maybe@illinois.edu)

Abstract

Relative to most other insect genomes, the western honey bee *Apis mellifera* has a deficit of detoxification genes spanning Phase I (functionalization), II (conjugation) and III (excretion) gene families. Although honeybees do not display across-the-board greater sensitivity to pesticides, this deficit may render them vulnerable to synergistic interactions among xenobiotics. Diet quality, in terms of protein and phytochemical content, has a pronounced influence on tolerance of toxic compounds. Detoxification gene inventory reduction may reflect an evolutionary history of consuming relatively chemically benign nectar and pollen, as other apoid pollinators display comparable levels of cytochrome P450 gene reduction. Enzymatic detoxification in the eusocial *A. mellifera* may be complemented by behaviors comprising a “social detoxification system,” including forager discrimination, dilution by pollen mixing, and colony food processing via microbial fermentation, that reduces the number or quantity of ingested chemicals requiring detoxification.

Keywords: carboxylesterase, cytochrome P450, eusociality, glutathione S-transferase, ABC transporter

Introduction

The fortuitously timed release of the genome of *Apis mellifera*, the western honeybee, in 2006 provided novel insights into the biology of this agriculturally critical species just as apicultural losses associated with what became known as Colony Collapse Disorder reached crisis proportions. Among the most striking findings was that the *A. mellifera* genome is deficient with respect to its inventory of all gene families associated with detoxification [1], a pattern that has for the most part held as more insect genomes have been sequenced (Table 1A). The genome also provided timely novel tools for characterizing the biochemical mechanisms underlying the detoxification of natural and synthetic xenobiotics, critical for evaluating the possible involvement of nutrition and diet in honeybee decline [2].

In general, xenobiotic detoxification involves the conversion of lipid-soluble substances to water-soluble, excretable metabolites. In Phase I detoxification, the toxin structure is enzymatically altered and rendered unable to interact with lipophilic target sites. Such functionalization is effected primarily by cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE), although other enzymes, including flavin-dependent monooxygenases [3] and cyclooxygenases can also contribute [4*]. Phase II reactions typically involve conjugation of products of Phase I detoxification for solubilization and transport. Glutathione-S-transferases (GST) are the principal Phase II enzymes, although they can also function as Phase I enzymes. Other Phase II enzymes in insects include glycosyltransferases, phosphotransferases, sulfotransferases, aminotransferases, and glycosidases [4*]. Nucleophilic compounds can be rendered hydrophilic by UDP-glycosyltransferases, although the natural substrates of these enzymes in herbivorous insects are unknown [5,6]. Phase III detoxification involves transport of Phase II conjugates out of cells for excretion. Among the proteins involved in this process are multidrug resistance proteins and other ATP-binding cassette transporters [7].

Phase I functionalization: cytochrome P450s

Genome-encoded functionalization of natural and synthetic xenobiotics in *A. mellifera* is attributable largely to the P450 superfamily [1], heme-thiolate enzymes that participate in both biosynthesis of endogenous signaling molecules and xenobiotic detoxification in insects [8]. Honeybees utilize P450s for detoxification of honey and pollen flavonoids [9], pyrethroid, organophosphate and phenoxypyrazole acaricides (fluvalinate, coumaphos, and fenpyroximate [10]), neonicotinoid insecticides [11], and mycotoxins (aflatoxin B1 and ochratoxin [12]). Despite this dependence on P450s, with 46 genes, the honeybee genome is reduced even in comparison with some other hymenopterans (Table 1B). The CYP2 and mitochondrial clans, with endogenous functions, are comparable in size with those of other insects whereas reductions in Clade 3 P450s, including the CYP6 and CYP9 families that carry out xenobiotic detoxification [8], are more pronounced. The greatest reduction is in the CYP4 clade; *A. mellifera* has only 12-14% of the number in other genomes (Table 1B). CYP4 genes in other insects have endogenous and exogenous roles [8]. This extreme reduction, also characteristic of the bumblebee *Bombus terrestris* [13, Table 1B], is not easily explained without greater knowledge of CYP4 functions.

Relative to *N. vitripennis*, a non-social parasitoid wasp, the subfamily CYP6AS is more diverse in honeybees (Table 1B). CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10 metabolize quercetin [9] and are upregulated by honey, pollen and propolis [14]. CYP6AS enzymes are not induced by their substrate quercetin [9], which is atypical of insect P450s [15]. CYP9Q1-3, however, are induced by their acaricides (tau-fluvalinate) and insecticide (cypermethrin and bifenthrin) substrates [15]. Genome-enabled surveys have identified P450 transcripts upregulated

by xenobiotics, although corresponding enzyme activities have not yet been characterized in many cases (Table 2). *CYP6AS14* is upregulated by the monoterpene thymol, used in-hive as an acaricide against varroa mites [16]. Acaricides coumaphos and fluvalinate upregulated *CYP6AS3*, *CYP6AS4* and *CYP9S1* in adult workers [17]; these P450s are involved in xenobiotic detoxification, although the CYP6AS enzymes do not metabolize these acaricides when expressed heterologously [9]. Coumaphos exposure also led to a 3.4-fold increase in expression of *CYP305D1*, a CYP2-clan P450 of unknown function [17].

Phase I functionalization: carboxylesterases (CCE)

Carboxylesterases have both endogenous and exogenous functions. That CCEs in *A. mellifera* participate in xenobiotic metabolism is suggested by induction of carboxylesterase activity by organophosphate, neonicotinoid, pyrethroid, phenylpyrazole, and spinosyn pesticides [18]. With only 24 members, the *A. mellifera* CCE inventory is reduced relative to other insect genomes [1] (Table 1A). Ten of 13 major CCE clades are represented; only 8, however, are in the class involved in xenobiotic detoxification. Toxicity of cyfluthrin and tau-fluvalinate is enhanced by CCE inhibitors, consistent with carboxylesterase-mediated cleavage of the ester bond [19]; moreover, CYP9Q1-3 convert tau-fluvalinate to a metabolite suitable for CCE cleavage [15], providing further evidence of CCE involvement in pesticide tolerance. Carboxylesterase gene GB10854 (esterase E4-like) activity in xenobiotic metabolism is suggested by its upregulation in workers exposed to p-coumaric acid [20] and coumaphos [17].

Phase II conjugation: Glutathione S-transferases

Glutathione S-transferases contribute to Phase II detoxification of electrophilic xenobiotics via conjugation with glutathione (GSH), although they also participate in Phase I detoxification by binding and sequestering toxins (such as pyrethroids) directly [21]. Of the six major subclasses--sigma, omega, theta, zeta, delta and epsilon--delta and epsilon are largely restricted to insects and have been linked to xenobiotic detoxification. In these subclasses, the *A. mellifera* genome, with only ten ostensibly functional genes, is strikingly depauperate relative to other insects, but it contains comparable numbers of Sigma class GSTs. Inducibility by xenobiotics provides the only evidence of potential GST involvement in xenobiotic detoxification. The pyrethroid flumethrin induced GST activity in larvae, pupae and nurse bees but reduced activity in foragers [22] and workers consuming nectar and pollen of *Ziziphus jujuba* displayed elevated GST and P450 activities [23]. In the eastern honeybee *Apis cerana cerana*, the Sigma-class *AccGSTS1* was upregulated by phoxim, cyhalothrin and “acaricide” [24] and the Omega-class GST gene *GSTO2* was upregulated by cyhalothrin, phoxim, pyridaben and paraquat [25]. In both cases, upregulation may have been in response to oxidative damage caused by these pesticides. At the molecular level, coumaphos ingestion resulted in upregulation of *GSTD*, a Delta-class GST, which may, as in other insects, contribute to detoxification of this organophosphate [17].

Phase III transport: ABC/multidrug transporters

Phase III transporters, primarily the ATP-binding cassette (ABC) proteins, hydrolyze ATP and move a diversity of hydrophobic or conjugated products across lipid membranes and out of cells for excretion. These proteins are not well known in insects; this gene family has been annotated in only seven arthropod genomes to date [7]. Of these, *A. mellifera* has the smallest inventory, with 41 (~40% of the inventory in the *Tetranychus urticae* genome). The ABCB-FT (full transporter) subfamily comprising P-glycoproteins, the ABCC subfamily comprising multidrug resistance-associated proteins, and the ABCG FTs comprising pleiotropic drug resistance proteins have all been implicated in insect xenobiotic metabolism. ABC transporter involvement in honeybee xenobiotic detoxification is suggested primarily by synergism studies utilizing

inhibitors or by quantification of transcript levels. The toxicity of three neonicotinoids and two acaricides is synergized by the inhibitor verapamil, implicating multidrug resistance transporters in their detoxification. Prefeeding bees with oxytetracycline, an antibiotic frequently used in-hive to treat American foulbrood (*Paenibacillus larvae*), enhances toxicity of these acaricides, raising questions about beekeeping practices that entail simultaneous treatment for mites and bacterial diseases [26].

Interactions between xenobiotics

Anecdotal perceptions that honeybees are peculiarly sensitive to pesticides, bolstered by the genomic detoxification deficit, were dismissed by Hardstone and Scott [27], who compared published LD50 values for honeybees and other insects and documented the absence of across-the-board vulnerability. A more likely consequence of detoxification gene deficits may be increased susceptibility to interactions among toxicants, of particular significance in the context of recent documentation of massive contamination of hives by agricultural pesticides, averaging six pesticide detections per sample [28]. Indeed, workers reared as larvae in brood comb contaminated with multiple pesticide residues experienced reduced longevity relative to brood reared in less-contaminated comb [29].

Sterol biosynthesis-inhibiting fungicides (which inhibit fungal P450s) synergize insecticides and acaricides, presumably by inhibiting P450-mediated detoxification [10,30] (Table 3). In addition, tau-fluvalinate and coumaphos synergize other acaricides, possibly through competitive interactions at target sites or interference at P450 catalytic sites [30]; molecular models of CYP9Q1-3 show that both coumaphos and fluvalinate dock into the same catalytic pocket [15]. Combinations of fungicides, insecticides, acaricides and herbicides have all demonstrated some degree of interactive effects in bees [10, 31], though most of these interactions occur by undetermined mechanisms [30].

Nutrition and detoxification

Nutritional stress may be exacerbated in the presence of pesticides; upregulation of detoxification genes by imidacloprid is accompanied by down-regulation of genes associated with glycolysis and development [32**], indicative of an energy cost of detoxification. Additionally, nutrient quality can influence xenobiotic toxicity. Dietary protein quality and quantity are particularly important; pesticide sensitivity of adult bees depends at least in part on the quality of pollen consumed in the first ten days after eclosion, with bees consuming high-quality pollen displaying greater pesticide resistance than bees consuming inferior pollen or protein sources [33], especially with other stressors present (e.g., low temperature [34]). This mitigating effect of high-quality pollen explains in part seasonal differences in pesticide toxicity, with well-fed autumn bees less sensitive than early spring bees [33]. In a bioassay, workers consuming a pollen-based diet for 16 days displayed less sensitivity to chlorpyrifos relative to bees consuming sucrose alone [17**]. Pollen identity can also influence detoxification capacity, with different pollen diets associated with different levels of gut GST activity in bees with and without the gut parasite *Nosema ceranae* [34].

Beyond its nutritional content, the natural diet of honeybees contains phytochemicals that influence xenobiotic detoxification. A diet containing honey, pollen, or propolis added to sucrose upregulates *CYP6AS* genes, which encode enzymes that metabolize quercetin and other flavonoids, whereas a diet of sucrose or high-fructose corn syrup does not [14]. Constituents of honey, pollen, and propolis also upregulate *CYP9Q* genes, several of which encode enzymes that metabolize quercetin and acaricides [20**]. The most active inducers were *p*-coumaric acid, which in honey likely derives from pollen grains, and pinocembrin, and pinobanksin 5-methyl

ether, which likely derive from the propolis lining cells in which honey is stored. Fourteen xenobiotic-metabolizing P450 genes and a γ -glutamyltranspeptidase-1-like gene are upregulated by *p*-coumarate (Table 2). Adding *p*-coumaric acid to a sucrose diet enhanced detoxification of coumaphos by 60%, demonstrating its functional role [20**]. Schmehl et al. [17] also showed that a pollen diet upregulates many of the same detoxification-associated transcripts upregulated by coumaphos and fluvalinate, including *CYP9S1*, *CYP9Q3*, and *GSTD1* (Table 2). The evident importance of dietary phytochemicals in regulating detoxification raises questions about beekeeping practices involving replacing natural foods (honey, pollen) with artificial substitutes [35].

“Social detoxification system”?

Genome-level deficits in detoxification and immunity relative to other insects may be an evolutionary consequence of *A. mellifera* eusociality. In terms of immunity, bees display cooperative behavioral defenses against parasites and pathogens, including “social fever” to kill temperature-sensitive bacteria, collection of antimicrobial plant resins for propolis, and removal of diseased brood, that collectively comprise a “social immunity” system [36,37], which may have reduced reliance on genome-encoded physiological immunity. Whether social behaviors contribute comparably to xenobiotic detoxification, leading to or compensating for reduced detoxification gene inventories, has not yet received attention. Behavioral mechanisms that may reduce toxin exposure include selective foraging with detection and avoidance of toxins in nectar and pollen, dilution of toxins by mixing nectars and pollens, processing of nectar into honey, and culturing fungi and other microbes to convert pollen into beebread. These behaviors may significantly reduce the number and concentration of toxins requiring enzymatic processing—a “Phase 0” of sorts in the traditional scheme of xenobiotic detoxification (Figure 1).

The size and perennial nature of honeybee colonies necessitate nectar and pollen collection from a broad diversity of flower species; while bees encounter multitudinous environmental chemical signals, the extent to which they detect and utilize these signals prior to ingestion to avoid toxicity remains uncertain. Bees do collect pollen contaminated with pesticides [28], suggesting an inability to detect or avoid these chemicals, yet the phenomenon of “entombed pollen” (whereby bees seal cells containing pollen with high fungicide levels) suggests bees can recognize contaminants [38]. In terms of nectar, responses to nectar phytochemicals are idiosyncratic and dose-dependent, with bees avoiding certain substances but displaying paradoxical preferences for some neurotoxic compounds (e.g., caffeine [39]). Even if bees cannot taste and reject toxins, post-ingestive malaise allows bees to learn to avoid food cues associated with toxins with adverse physiological effects (e.g., amygdalin in almond nectar) [40**].

The diversity of floral resources utilized by *A. mellifera* also provides opportunities for mitigating nectar and pollen toxicity via mixing and dilution. This mechanism allows the generalist solitary bee *Osmia cornuta*, e.g., to utilize toxic pollen of *Ranunculus acris*, which becomes essentially nontoxic if consumed in a 50% admixture of pollens from other species [41*]. Whether collecting nectars from multiple floral sources similarly dilutes potential toxins has not yet been assessed.

The degree to which toxins are degraded by honey and beebread processing is also largely unexplored. Nectar phenolics decrease in concentration during honey “curing” at hive temperatures of 35°C [42] and incorporation of glucose oxidase into incipient honey may also reduce toxicity by converting glucose to gluconic acid and lowering honey pH. Beyond symbiotic gut microbes [43,44], bees benefit from mutualistic fungi in the hive in that beebread

is the product of fungal fermentation of pollen [45]. *Aspergillus* species dominate the fungal community during beebread “ripening” [46]. Many *Aspergillus* species are opportunistic plant pathogens that produce enzymes that degrade phytochemicals (e.g., Zhou et al [47]). In addition, glucose oxidase produced by *Aspergillus* has been linked to lignin degradation [48] and may thus be involved in phenolic detoxification. That associations with *Aspergillus* may be mutualistic is suggested by honeybee detoxification, rather than bioactivation (i.e., increase in toxicity after P450-mediated metabolism), of aflatoxins produced by these fungi [12]. Aflatoxin tolerance increases when bees consume honey rather than high-fructose corn syrup are also suggestive of adaptation to this mycotoxin [14]. As well, propolis, the resinous material used to line cells containing beebread, induces xenobiotic-metabolizing P450s and enhances survival in the presence of aflatoxin B1 [12], also suggestive of adaptation to cohabitation.

During beebread processing, bees may thus exploit the broad-spectrum detoxificative capacity of fungi, much like symbiotic yeasts associated with *Lasioderma serricorne* contribute to phytochemical detoxification [44]. Genomes of insect hosts often lose genes associated with biosynthesis of amino acids provided by their endosymbionts; an association with a symbiont contributing detoxification enzymes may similarly lead to losses in detoxification gene inventories in host genomes. The genome of *Atta echinator*, a leaf-cutter ant with symbiotic fungi, e.g., possesses fewer P450 genes than genomes of ants lacking such symbionts [49, 50]. P450 gene deficits in *A. mellifera* may similarly reflect dependence on behaviorally mediated detoxification processes, including mutualisms [51]. If such is the case, the widespread contamination of beehives by fungicides may have profound implications for bee health beyond direct fungicide toxicity [52**].

Implications for honey bee management

How honeybee behaviors influence or complement enzymatic processing of environmental toxins is of considerable importance in insuring the sustainability of apiculture and, consequently, the overall agricultural enterprise in the U.S.. The possibility that enzymatic detoxification in this eusocial species may be particularly vulnerable to synergistic interactions and may be complemented by behaviors, including forager avoidance of toxins that reduces intake and/or colony food processing activities that reduce toxicity, has profound implications for estimating risks of exposure and limits of tolerance for honeybees in U.S. agricultural landscapes, the vast majority of which are managed with the use of a diversity of pesticides.

Acknowledgements

We thank Dr. Terry Harrison for reviewing the manuscript and the editors for the invitation to contribute a manuscript on this topic and for their patience in waiting for its completion. Preparation of this review was supported by USDA Agriculture and Food Research Initiative 2010-03760

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

**of outstanding interest

- [1] Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG: **A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee.** *Insect Molec. Biol.* 2006, **15**: 615-636.
- [2] Berenbaum MR: **Bees in crisis: colony collapse, honey laundering, and other problems bee-setting American apiculture.** *Proc. Am. Phil Soc.* 2015, in press.
- [3] Sehlmeier S, Wang L, Langel D, Heckel DG, Mohagheghi H, Petschenka G, Ober D: **Flavin-dependent monooxygenases as a detoxification mechanism in insects: new insects from the Arctiids (Lepidoptera).** *PLoS ONE* 2010, **5(5)**: e10435
- [4] Xu J, Strange JP, Welker DL, James RR: **Detoxification and stress response genes expressed in a western North American bumble bee, *Bombus huntii* (Hymenoptera: Apidae).** *BMC Genomics* 2013, **14**:874.
- * These authors were first to construct a transcriptome for a eusocial hymenopteran other than *Apis mellifera* and in doing so shed light on the selective forces that led to a deficit of detoxification genes in *A. mellifera*. To insure a comprehensive transcriptome, mRNA was extracted from multiple life stages and all castes and the corresponding cDNA was subjected to pyrosequencing. Over 580 genes associated with detoxification and stress responses were identified and genome inventories compared among other insects. Similarities in numbers of P450s, CCEs, and GSTs with *A. mellifera* and with the solitary bee *Megachile rotundata* indicate that detoxification deficits are more closely associated with nectar and pollen feeding than they are with eusociality per se.
- [5] Heidel-Fischer H, Vogel H: **Molecular mechanisms of insect adaptation to plant secondary compounds.** *Current Opinion in Insect Science* 2014, **8**:1-7.
- [6] Ahn SJ, Vogel H, Heckel DG: **Comparative analysis of the UDP-glycosyltransferase multigene family in insects.** *Insect Biochem. Mol. Biol.* 2012, **42**: 133-147.
- [7] Dermaauw W, Van Leeuwen T: **The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance.** *Insect Biochem. Mol. Biol.* 2014, **45**: 89-110.
- [8] Feyereisen R: **Insect CYP genes and P450 enzymes.** In *Insect Molecular Biology and Biochemistry*. Edited by Gilbert, LI. Elsevier; 2012: 236-316.

- [9] Mao W, Johnson RM, Rupasinghe S, Schuler MA, Berenbaum MR: **Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* Hymenoptera: Apidae.** *Comp. Biochem. Physiol. C: Toxicology and Pharmacology* 2009, **154**: 427-434.
- [10] Johnson RM, Dahlgren L, Siegfried BD, Ellis MD: **Acaricide, fungicide and drug interactions in honeybees (*Apis mellifera*).** *PLoS ONE* 2013, **8(1)**: e54092.
- **These authors performed a large number of laboratory bioassays with adult honey bees and convincingly demonstrated that pesticides assumed to be nontoxic to bees and routinely used in-hive to treat pathogens and parasites can interact synergistically to cause significant mortality. The documented diversity of synergistic interactions would in and of itself be breathtaking but the work takes on real-world urgency in view of the ubiquitous occurrence of contamination by multiple pesticides in U.S. beehives. This study provides some insight into the idea that bees, while not uniquely vulnerable to pesticide toxicity per se, may be especially at risk when exposed to toxins in combination. a possibility that should be considered in designing honey bee management practices.**
- [11] Iwasa T, Motoyama N, Ambrose JT, Roe RM: **Mechanism for the differential toxicity of neonicotinoid insecticides in the honeybee, *Apis mellifera*.** *Crop Protection* **23**: 371-378.
- [12] Niu, G., R.M. Johnson and M. R. Berenbaum. Niu G, Johnson RM, Berenbaum MR: **Toxicity of mycotoxins to honeybees and its amelioration by propolis.** *Apidologie* 2010, 10.1051
- [13] Sadd BM, Barribeau SM, Bloch G, de Graaf DC, Dearden P, Elisk CG, Gadau J, Grimmelikhuijzen CJP, Hasselmann M, Lozier JD, et al.: **The genomes of two key bumblebee species with primitive eusocial organisation.** *Genome Biology* 2015. In press.
- [14] Johnson RM, Mao W, Pollock HS, Niu G, Schuler MA, Berenbaum MR: **Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*.** *PLoS ONE* 2012, **7(2)**: e31051.
- [15] Mao W, Schuler MA, Berenbaum MR: **CYP9Q-mediated detoxification of acaricides in the honeybee (*Apis mellifera*).** *Proc. Natl. Acad. Sci. USA* 2011, **31**: 12657-12662.
- [16] Boncristianai H, Underwood R, Schwarz R, Evans JD, Pettis J, vanEngelsdorp D: **Direct effect of acaricides on pathogen loads and gene expression levels in honeybees *Apis mellifera*.** *J. Insect Physiol.* 2012, **58**: 613-620.
- [17] Schmehl DR, Teal PEA, Frazier JL, Grozinger CM: **Genomic analysis of the interaction between pesticide exposure and nutrition in honeybees (*Apis mellifera*).** *Journal of Insect Physiology* 2014, **71**:177-190.
- ** This study investigated the effects of pesticide exposure on gene expression. Extended exposure of adult bees to fluvalinate or coumaphos resulted in differential expression of over 1100 transcripts relative to bees that were not exposed to these acaricides, many of which were genes involved in detoxification, immunity, behavioral maturation, and nutrition. In addition, these investigators examined whether the quality and nature of the protein source in the diet influenced pesticide sensitivity and found via bioassay that bees consuming pollen were more**

tolerant of exposure to chlorpyrifos, an organophosphate, than bees consuming an artificial diet. The protective effects of a pollen diet may be attributable to the fact that pollen ingestion results in upregulation of many of the transcripts upregulated in response to pesticides, providing additional evidence of the importance of considering impacts of phytochemicals characteristic of the natural diet of honey bees in evaluating effects of pesticide exposure.

[18] Carvalho SM, Belzunces LP, Carvalho GA, Brunet J-L, Badiou-Beneteau A: **Enzymatic biomarkers as tools to assess environmental quality: a study of exposure of the honeybee *Apis mellifera* to insecticides.** *Environmental Toxicol. Chem.* 2013, **17(9)**: 2114-214.

[19] Johnson et al. 2006

[20] Mao W, Schuler MA, Berenbaum MR: **Honey constituents up-regulate detoxification and immunity genes in the western honeybee *Apis mellifera*.** *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110(22)**:8842-8846.

****Honey is known to upregulate detoxification genes in *Apis mellifera* but which specific constituents of honey were not identified until these authors fractionated honey extracts and identified three highly effective inducers that may not be derived from nectar per se—p-coumaric acid, which is a principal constituent of pollen cell walls, and pinocembrin and pinobanksin 5-methyl ether, common constituents of propolis. RNA-seq with the most effective inducer, p-coumaric acid, revealed that this universal constituent of bee foods upregulates transcripts of every class of detoxification gene and, surprisingly, transcripts of immunity-associated genes. This “nutraceutical” function of p-coumaric acid and other phytochemicals suggests that apicultural practices involving replacement of honey with sugar substitutes, including high-fructose corn syrup, may compromise the ability of bees to contend with pesticides and pathogens.**

[21] Kettermann AJ, Saisawang C, Wongsantichon J: **Insect glutathione transferases.** *Drug Metabolism Reviews* 2011, **43(2)**: 253-265.

[22] Nielsen SA, Brødsgaard CJ, Hansen H: **Effects on detoxification enzymes in different life stages of honeybees (*Apis mellifera* L. Hymenoptera: Apidae) treated with a synthetic pyrethroid (Flumethrin).** *Alternatives in Laboratory Animals* 2000, **28(3)**: 437-443.

[23] Ma W-Hm J-A Ahwn, Y Guo, Y-Y Zang, Y-Q Shao: **Effects of Zaohua disease on activities of digestive enzymes and detoxification enzymes in *Apis mellifera ligustica*.** *Acta Entomologica Sinica* 2011, **54**: 1076-1081

[24] Yan H, Jia H, Gao H, Guo X, Xu B: **Identification, genomic organization and oxidative stress response of a sigma class glutathione S-transferase (AccGSTS1) in the honeybee, *Apis cerana cerana*.** *Cell Stress and Chaperones* 2013, **18**:415-426.

[25] Zhang Y, Yan H, Lu W, Li Y, Guo X, Xu B: **A novel Omega-class glutathione S-transferase gene in *Apis cerana cerana*: molecular characterization of *GSTO2* and its protective effects in oxidative stress.** *Cell Stress Chaperones* 2013, **18**:503-516.

- [26] Hawthorne DJ, Dively, GP: **Killing them with kindness? In-hive medications may inhibit xenobiotic efflux transporters and endanger honeybees.** *PLoS ONE* 2011, **6**: e26796.
- [27] Hardstone MC, Scott JG: **Is *Apis mellifera* more sensitive to insecticides than other insects?** *Pest. Management Science* 2010, **66**: 1171-1180.
- [28] Mullin CA, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS: **High levels of miticides and agrochemicals in North American apiaries: implications for honeybee health.** *PLoS ONE* 2010, **5(3)**: e9754.
- [29] Wu JY, Anelli CM, Sheppard WS: **Sub-lethal effects of pesticide residues in brood comb on worker honeybee (*Apis mellifera*) development and longevity.** *PLoS ONE* 2011, **6(2)**: e14720
- [30] Glavan G, Bozic J: **The synergy of xenobiotics in honeybee *Apis mellifera*: mechanisms and effects.** *Acta Biologica Slovenica* 2013, **56**:11-25.
- [31] Zhu W, Schmehl DR, Mullin CA, Frazier JL: **Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honeybee larvae.** *PLoS ONE* 2014, **9**: e77547.
- *Although synergistic interactions arising from binary mixtures of (ostensibly) nontoxic fungicides, acaricides, and insecticides have been demonstrated previously, these authors tested for interactions in all combinations. In fact, the toxicity of the binary mixture of the acaricide fluvalinate and the fungicide chlorothalonil is enhanced synergistically by the addition of the acaricide coumaphos. In addition, another striking finding reported in this paper is the toxicity of an ostensibly “inert” formulation solvent, N-methyl-2-pyrrolidone. Collectively, this study provides supportive evidence of the possibly heightened sensitivity of honey bees to combinations of chemicals as well as insights that should inform honey bee management practices.
- [32] Derecka K, Blythe MJ, Malla S, Genereux DP, Guffanti A, Pavan P, Moles A, Snart C, Ryder T, Ortori CA, Barrett DA, Schuster E, Stoeger R: **Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae.** *PLoS One* 2013, **8(7)**:e68191.
- **These authors effectively used whole transcriptome sequencing (RNA-Seq) of larvae to compare gene expression in honey bee colonies exposed to or free from low levels of imidacloprid, a widely used neonicotinoid, for 15 days. Among the 300 differentially expressed transcripts in pesticide-exposed larvae were expected upregulation of genes encoding detoxification enzymes; that this upregulation was coupled with downregulation of genes in glycolytic and sugar-metabolizing pathways is compelling evidence of an energetic and developmental cost of chronic pesticide stress and detoxification. This work adds a new dimension to evaluating impacts of pesticide exposure on colony health.
- [33] Wahl O, Ulm K: **Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*.** *Oecologia* 1983, **59**, 106–128.

[34] Archer CR, Pirk CWW, Wright GA, Nicolson SW: **Nutrition affects survival in African honeybees exposed to interacting stressors.** *Functional Ecology* 2014, **28(4)**:913-923.

[35] Johnson RM: **Honey bee toxicology.** *Annual Review of Entomology* 2015, **60**: 22.1–22.20

[36] Cremer S, Armitage S, Schmid-Hempel P: **Social immunity.** *Curr. Biol.* 2007, **17**: R693–R702.

[37] Wilson MB, Spivak M, Hegeman AD, Rendahl A, Cohen JD: **Metabolomics reveals the origin of antimicrobial plant resins collected by honeybees.** *PLoS ONE* 2013, **8(10)**: e77512

[38] vanEngelsdorp D, Evans JD, Donovan L, Mullin C, Frazier M, Frazier J, Tarpy DR, Hayes Jr. J, Pettis JS: **“Entombed pollen”: a new condition in honeybee colonies associated with increased risk of colony mortality.** *J. Invert. Pathol.* 2009, **101**:147-149.

[39] Irwin RE, Cook D, Richardson LL, Manson JS, Gardner DR: **Secondary compounds in floral rewards of toxic rangeland plants: impacts on pollinators.** *Journal of Agricultural and Food Chemistry* 2014, **62(30)**:7335-7344.

[40] Hurst V, Stevenson PC, Wright GA: **Toxins induce 'malaise' behaviour in the honeybee (*Apis mellifera*).** *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* 2014, **200(10)**:881-890.

******The degree to which honey bees can assess nectar or pollen quality and avoid natural or synthetic toxins while foraging is a critical question, particularly in view of the increasing use of systemic pesticides that can contaminate nectar. That honey bees can detect and avoid certain natural toxins has been demonstrated but it is also known that there are toxins that bees apparently cannot detect. In this important study, Hurst et al. determined that honey bees can learn to avoid toxins even if they cannot be tasted if they induce physiological symptoms of “malaise”, including toxin-induced locomotory difficulties. Although behavioral reactions to “malaise” is well known to influence food choice in mammals, demonstrating its existence in honey bees clarifies the importance of associative olfactory conditioning in reducing consumption of environmental toxins.

[41] Eckhardt M, Haider M, Dorn S, Mueller A: **Pollen mixing in pollen generalist solitary bees: a possible strategy to complement or mitigate unfavourable pollen properties?** *J. Anim. Ecol.* 2014, **83**:588-597.

***** This study provides the first evidence that pollen generalists can utilize toxic pollen by combining it with more suitable pollen from other floral sources. Previous studies have shown that bees can benefit from combining pollens from multiple sources by virtue of consuming more nutritious protein, this study shows that generalized pollinators can ameliorate toxicity via behavioral means. Although the study was on the solitary megachilid *Osmia cornuta*, as the authors suggest, pollen mixing may in fact be a widespread strategy among pollen generalists.

[42] Liu FL, He JZ, Fu WJ: **Highly controlled nest homeostasis helps honeybees deactivate phenolics in nectar.** *Naturwissenschaften* 2005, **92**: 297-299.

- [43] Engel P, Martinson VG, Moran NA: **Functional diversity within the simple gut microbiota of the honey bee.** *Proc. Natl. Acad. Sci. USA* 2012, **109**: 11002-11007.
- [44] Hansen AK, Moran NA: **The impact of microbial symbionts on host plant utilization by herbivorous insects.** *Molecular Ecology* 2014, **23**: 1473-1486.
- [45] Gilliam M, Prest DB, Lorenz BJ: **Microbiology of pollen and bee bread: taxonomy and enzymology of molds.** *Apidology* 1989, **20**: 53-68.
- [46] Gilliam M: **Identification and roles of non-pathogenic microflora associated with honey bees.** *FEMS Microbiology Lett.* 1997, **155**: 1-10.
- [47] Zhou H, Wang C-Z, Ye J-H, Chen H-X, Tao R, Zhang Y-S: **Solid-state fermentation of *Ginkgo biloba* L. residue for optimal production of cellulase, protease and the simultaneous detoxification of *Ginkgo biloba* L. residue using *Candida tropicalis* and *Aspergillus oryzae*.** *European Food Research Technology* 2015, **240(2)**: 379-388.
- [48] Wong MC, Wong KH, Chen XD: **Glucose oxidase: natural occurrence, function, properties and industrial applications.** *Appl. Microbiol. Technol.* 2008, **78**:927-938.
- [49] Nygaard, S., G. Zhang, M. Schiott, C. Li, Y. Wurm, H. Hu, J. Zhou, L. Ji, F. Qiu, M. Rasmussen, H. Pan, F. Hauser, A. Krogh, C.J.P. Grimmelikhuijzen, J. Wang and J.J. Boomsma. 2011. **The genome of the leaf-cutting ant *Acromyrmex echinator* suggests key adaptations to advanced social life and fungus farming.** *Genome Research* 2011, **21**: 1339-1348.
- [50] Suen, G., C. Teiling, L. Li, C. Holt, E. Abouheif, E. Bornberg-Bauer, P. Bouffard, E.J. Caldera, E. Cash, A. Cavanaugh, O. Denas, E. Elhaik, M.-J. Fave and C.R. Currie. 2011. **The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle.** *PLoS Genetics* 2011, **7**: e1002007.
- [51] DeFine Licht HH, Schiott M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, Boomsma JJ: **Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts.** *Proc. Natl. Acad. Sci. USA* 2013, **110(2)**: 583-587.
- [52] Yoder JA, Jajack AJ, Rosselot AE, Smith TJ, Yerge MC, Sammartaro D: **Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honeybee, *Apis mellifera*, colonies.** *J. Toxicol. Environ. Health Part A* 2013, **76**: 587-600.
- **By analyzing samples of bee bread from colonies pollinating orchards that differed in frequency of fungicide use, these authors documented not only fungicide residues but the impact of those residues on the colonies. Honey bees depend on a diverse community of beneficial fungi for processing pollen into beebread; fungicide contamination, however, was associated with reductions in the numbers of fungal isolates. The disproportionate impact of fungicide residues on *Aspergillus* may be particularly detrimental to colony health. There are indications that altering the fungal community may increase colony susceptibility to fungal diseases such as chalkbrood.**

[53] Smith CR, Smith CD, Robertson HM, Helmkampf M, Zimin A, Yandell M, Holt C, Hu H, Abouheif E, Benton R, et al.: **Draft genome of the red harvester ant *Pogonomyrmex barbatus***. *Proc. Natl. Acad. Sci. U. S. A.* 2011, **108**:5667–5672.

Tables

	<i>Apis mellifera</i>	<i>Bombus huntii</i>	<i>Megachile rotundata</i>	<i>Nasonia vitripennis</i>	<i>Drosophila melanogaster</i>
P450	46	44	52	92	85
CCE	24	23	22	41	35
GST	10	11	9	16	38
UDP-GT	12	2	2	22	34
Total	92	80	85	171	192
Life history	Eusocial pollinator	Eusocial pollinator	Solitary pollinator	Solitary parasitoid	Solitary saprotroph
Order	Hymenoptera	Hymenoptera	Hymenoptera	Hymenoptera	Diptera

Table 1A. Genomic detoxification inventories for *Apis mellifera* and other species. Data for *Bombus huntii* and *Megachile rotundata* from [4*]; data for *Apis mellifera*, *Nasonia vitripennis* and *Drosophila melanogaster* from [6]

	<i>Apis mellifera</i>	<i>Bombus terrestris</i>	<i>Pogonomyrmex barbatus</i>	<i>Nasonia vitripennis</i>	<i>Drosophila melanogaster</i>
CYP3 clan	28	27	40	49	36
CYP4 clan	4	4	18	29	32
CYP2 clan	8	7	7	7	6
Mitochondrial	6	6	7	7	11
Total P450s	46	44	72	92	85
Life history	Eusocial pollinator	Eusocial pollinator	Eusocial seed-feeder	Solitary parasitoid	Solitary saprotroph
Order	Hymenoptera	Hymenoptera	Hymenoptera	Hymenoptera	Diptera

Table 1B. Cytochrome P450 inventories for *Apis mellifera* and other species by clan detoxification functions are concentrated in CYP3 clan (families CYP6 and CYP9) (data from [4], [53])

Table 2. Xenobiotic genes upregulated in honeybee workers after exposure to xenobiotics

Group	Gene name	Fold	Inducer	Ref
Phase I	CYP6AS2	2.70	p-coumarate	[20]
	CYP6AS3	3.11	p-coumarate	[20]
	CYP6AS3	1.70	imidacloprid	[32]
	CYP6AS3		coumaphos	[17]
	CYP6AS3		fluvalinate	[17]
	CYP6AS4	2.60	p-coumarate	[20]
	CYP6AS4	1.9	imidacloprid	[32]
	CYP6AS4		coumaphos	[17]
	CYP6AS4		fluvalinate	[17]
	CYP6AS5	2.55	p-coumarate	[20]
	CYP6AS14	2.00	imidacloprid	[32]
	CYP6AS15	1.40	imidacloprid	[32]
	CYP6AR1	1.50	imidacloprid	[32]
	CYP6BD1	1.92	p-coumarate	[20]
	CYP9Q3	2.55	p-coumarate	[20]
	CYP9R1	1.60	imidacloprid	[32]
	CYP9S1	1.40	imidacloprid	[32]
	CYP9S1		coumaphos	[17]
	CYP9S1		fluvalinate	[17]
	Esterase FE4	1.47	p-coumarate	[20]
	GB10854 (carboxylesterase)		coumaphos	[17]
Phase II	Gamma-glutamyltransferase 1	1.50	p-coumarate	[20]
	Glucuronosyltransferase 1	1.46	p-coumarate	[20]
	Glucuronosyltransferase 2	1.81	p-coumarate	[20]
	GSTD1		coumaphos	[17]
Phase III	Multidrug resistance-associated protein4	1.60	p-coumarate	[20]
	Multidrug resistance-associated protein1	1.80	p-coumarate	[20]
Unknown	CYP305D1	3.40	coumaphos	[17]

Table 3. Pesticide combinations with enhanced toxicity in *Apis mellifera*

(underlined compounds detected in 10% or more of wax samples in [28]; references in brackets)

Interactions between in-hive acaricides used by beekeepers

<u>coumaphos</u>	<u>tau-fluvalinate</u>	[10**]
thymol	<u>coumaphos</u>	[10**]
	<u>tau-fluvalinate</u>	
amitraz	<u>coumaphos</u>	[10**]
	fenpyroximate	
	<u>tau-fluvalinate</u>	
fenpyroximate	<u>coumaphos</u>	[10**]
	<u>tau-fluvalinate</u>	
oxalic acid	amitraz	[10**]
	<u>tau-fluvalinate</u>	
	fenpyroximate	
	thymol	

Interactions between in-hive acaricides and fungicides

<u>coumaphos</u>	flumethrin	[30], [10**]
	carbendazim	
	difenconazole	
	flusilazole	
	prochloraz	
	propiconazole	
	tebuconazole	
	thiophanate-methyl	
	<u>chlorothalonil</u>	[31*]
	<u>chlorothalonil+fluvalinate</u>	[31*]
<u>tau-fluvalinate</u>	carbendazim	[10*],[30]
	difenconazole	
	flusilazole	
	prochloraz	
	propiconazole	
	tebuconazole	
	thiophanate-methyl	
	myclobutanil	
	metconazole	
	fenbuconazole	
	<u>chlorothalonil</u>	[31*]
fenpyroximate	prochloraz	[10*]
tau-fluvalinate	<u>boscalid</u>	[10*]
	pyraclostrobin	
fenpyroximate	pyraclostrobin	[10*]
<i>Interactions between in-hive acaricides and insecticides</i>		
coumaphos	dialios	[30]
	dimethoate	
	ethyl parathion	

Interactions between pyrethroid insecticides and fungicides

deltamethrin	difenoconazole+carbendazim	[30]
	prochloraz	
	prochloraz+	
	difenoconazole 850	
lambda-cyhalothrin	difenoconazole	[30]
	flusilazole	
	prochloraz	
	propiconazole	
	tebuconazole	
	thiophanate-methyl	
	<u>chlorothalonil</u>	
alpha-cypermethrin	difenoconazole	[30]
	flusilazole	
	prochloraz	
	propiconazole	
	tebuconazole	
	<u>chlorothalonil</u>	

Interactions between neonicotinoid insecticides and fungicides

acetamiprid	epoxiconazole	[30]
	propiconazole	
	triadimefo	
	triflumizole	
	uniconazole-P	
thiacloprid	prochloraz	[30]
	propiconazole	
	ebuconazole	
	triflumizole	
	cyprodinil	
	tolyfluanid	
imidacloprid	propiconazole	[11]
	triflumizole	

Interactions between herbicides and fungicides

<u>atrazine</u>	carbaryl	[30]
-----------------	----------	------

Figure 1. Schematic of xenobiotic detoxification processes in *Apis mellifera*, with enzymes associated with each phase. “Phase 0” is proposed for behavioral processing of food resources by honeybees that eliminate (or reduce the concentration of) toxic compounds prior to consumption

Figure
Figure 1