Comparative Biochemistry and Physiology, Part C 238 (2020) 108844

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effect of age on insecticide susceptibility and enzymatic activities of three detoxification enzymes and one invertase in honey bee workers (*Apis mellifera*)

Yu Cheng Zhu[a,*], Joel Caren[a], Gadi V.P. Reddy[a], Wenhong Li[a,b], Jianxiu Yao[a,c]

[a] USDA-ARS-JWDSRC, Southern Insect Management Research Unit, Stoneville, MS 38776, USA
[b] Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou, China
[c] Kansas State University, Manhattan, KS 66506, USA

ARTICLE INFO

Keywords:
Worker
Age
Aging
Detoxification enzymes
Insecticides
Invertase
Soluble protein

ABSTRACT

Honey bee is an economically important insect for honey production and pollination. Frequent exposure to toxic pesticides is one of the major risk factors causing the pollinator population decline. However, age effects of honey bees on pesticide susceptibility have been largely ignored and many researchers use bees of unknown age for assessing the risk of pesticides. Honey bee workers are known to go through physiological and behavioral changes in order to differentiate different phenotypes to perform specific duties over their natural lifetime of 6 weeks or longer. In this study, we provide multi-parameter evidences of unignorable age effects of honey bee workers and suggest using a standard bee age to produce reliable and comparable data when assessing variable and realistic situations of in-hive and field exposures to pesticides. Using honey bee workers aged 4- to 42-days old, we examined susceptibility of the bees to five different insecticides from five different classes and measured enzymatic activities of three major detoxification enzymes and an invertase involved in honey production. Results showed gradual increase of natural mortality and decrease of soluble protein content in bees over the age span from 4 days to 42 days. Significant increases of mortality after separate treatments of five different insecticides confirmed drastic age effects of bees over the assessed age span. As they aged, honey bees also showed a gradual increase of cytochrome P450 oxidase activity while still maintaining constant levels of two other detoxification enzymes (esterase and glutathione S-transferase) and an invertase responsible for honey production.

1. Introduction

Honey bee, *Apis mellifera* L, is a valuable social insect. A typical colony is composed of a single queen, a few hundred male drones, and approximately thirty thousand non-reproductive female workers. In addition to producing honey worth hundreds of millions of dollars, honey bees perform pollination which increases crop value by $12 billion annually in the United States (USDA-NASS, 2015; Calderone, 2012; Johnson and Corn, 2014).

Because foraging bees share certain ecosystems with crop pests, honey bees may be accidently exposed to insecticide sprays. Together with other factors (parasites, diseases, reduced habitats), wide commercialization of neonicotinoids has been blamed for honey bee population decline (Potts et al., 2010; Goulson et al., 2015). With the shift of crop pests from chewing to piercing insects in genetically modified crop systems (Greene et al., 1999; Bergé and Ricroch, 2010; Lu et al., 2008, 2010) and insecticide resistance development (Zhu et al., 2004; Zhu et al., 2012), honey bees and other pollinators face increasing risk of pesticide exposure while foraging. More than forty pesticides are currently recommended by extension specialists for the chemical control of row crop insects (Brandon and Robinson, 2017; Catchot et al., 2017; Krupke et al., 2017), particularly those which attack cotton, such as tarnished plant bugs and stink bugs (Catchot et al., 2017; Gore et al., 2012); these chemicals are usually applied using aerial or ground sprayers. Also, seed treatment with neonicotinoids has become routine and widely used. Recently, concerns have arisen regarding the toxicity to bees of airborne insecticide dust dispersed during planting (Marzaro et al., 2011) and of pesticide residues systemically transferred from seeds (treatment) to pollen (Stoner and Eitzer, 2012). Residues of more than 150 pesticides have been detected at various levels in wax, pollen, bee, or honey (Johnson et al., 2010; Mullin et al., 2010; Sanchez-Bayo and Goka, 2014).

* Corresponding author at: USDA-ARS-JWDSRC, 141 Experiment Station Road, Stoneville, MS 38776, USA.
E-mail address: yc.zhu@usda.gov (Y.C. Zhu).

https://doi.org/10.1016/j.cbpc.2020.108844
Received 24 May 2020; Received in revised form 30 June 2020; Accepted 3 July 2020
Available online 07 August 2020
1532-0456/ Published by Elsevier Inc.

It is clear that the honey bee worker is the only caste doing the pollination and producing honey. Workers stay inside the hive to perform in-hive duties, including cleaning, feeding larvae, and maintaining and expanding the hive structure. At two to three weeks, workers differentiate into foragers, who fly out to the fields to collect pollen and nectar. Foraging is substantially risky than performing in-hive duties. They may easily become prey to birds and other predators, and they may be incidentally or directly exposed to pesticide sprays, including herbicide sprays on wild flowers in early season and insecticide sprays on cotton in mid and late seasons. At the end of each foraging trip, the foragers can take pesticide-contaminated pollen and nectar back to their hives, where young workers and larvae are orally exposed to the contaminated food.

Previously we used a spray treatment method to simulate field exposure to examine acute toxicities of recommended foliar pesticides on honey bee workers (Zhu et al., 2017a). We also assessed long-term sublethal exposure to young bees by incorporating insecticides into a sucrose solution at residue level (Yao et al., 2018). Since workers typically live 3–6 weeks and become specialized to perform different duties and are therefore exposed to different environmental conditions at different ages (Page and Peng, 2001), we consistently used approximately one-week old workers for assessing pesticide toxicity (Zhu et al., 2015). However, many researchers use workers of mixed or unknown age (caught directly from hive frames) for testing the effects of pesticide on bees (Yu et al., 1984; Nauen et al., 2001; Suchail et al., 2001; Medrzycki et al., 2003; Bailey et al., 2005; Faucon et al., 2005; Laurino et al., 2013). Literature searches showed that the age effects on pesticide susceptibility had not been extensively studied. Limited research data (excluding resistance development) reportedly showed decreased susceptibility as insect age increases (Vincent and Lindcren, 1965; Yu and Hsu, 1993; Fine and Mullin, 2017). Much less attention has been paid to the increased susceptibility to pesticides as insects age (Qureshi et al., 1965). Limited examination on honey bee age effect on susceptibility to two insecticides showed increased sensitivity to one and reduced sensitivity to the other (Rinkevich et al., 2015). Therefore, whether there is a clear age effect on honey bee susceptibility to insecticides remained largely unknown. To better produce comparable data, we tested susceptibility of worker bees with a wide range of age (from 4 days old to 42 days old) to five different insecticides (representatives of five classes). We also examined age effects on major detoxification enzymes and honey-making invertase activity in workers.

2. Methods and materials

2.1. Honey bee hives

Honey bee colonies were originally purchased from commercial bee keepers located in pine forest and pasture area near Perkinston and Magee, Mississippi (USA). These colonies were maintained prior to research in a managed bee yard inside the Mississippi Wildlife Management Area located approximately 5 km north Stoneville, Mississippi (USA). Over the course of the research, approximately 15% new colonies were purchased and added to the bee yard annually using package bees with Italian queens from commercial beekeepers near Little Rock, AR. Each hive was equipped with a bottom board oil trap (35 × 45 cm tray filled with vegetable oil) for monitoring and control of Varroa mite (*Varroa destructor* A.T.) and small hive beetle (*Aethina tumida*). To obtain bees (approximately 12,000) for conducting experiments, deep frames with more than 50% coverage of healthy, sealed brood were pulled from 5 to 7 colonies and transferred to a lidded container vented by large cutouts covered by mesh screen. The container was kept in a laboratory incubator (33 ± 0.5 °C; 60% ± 3 RH; no light). Each day, newly emerged worker bees were transferred in groups of 25 to cages made from a 500-ml round wide-mouth polypropylene jar (D × H: 9.3 × 10 cm) with a (3 × 8 cm) section of plastic foundation attached vertically to the inner side of the cage. Each of these cages had an 8.9 cm diameter (d) hole cut in the lid and covered with 3 × 3 mm-mesh metal screen to prevent escape. Caged bees were provided with one scintillation vial (20 ml) of 50% (W/V) sucrose solution and one vial (20 ml) of water placed upside down on the top of each cage. Two holes (1.6 mm) were drilled in each vial cap. Caged bees were maintained in incubators (the same conditions described in Section 2.1) before testing. Cages with more than five dead bees were not used for experimentation. Before the experiment was started, dead bees were counted and excluded from the total number of bees tested in each replicate.

2.2. Chemicals

Formulated insecticides imidacloprid (Advise® 2FL), acephate (Bracket®97), λ-cyhalothrin (Karate®), sulfoxaflor (Transform®), and oxamyl (Vydate®) were purchased from local agricultural chemical suppliers and stored in a refrigerator (6 ± 1 °C). These five insecticides represent five popular insecticide classes, including neonicotinoids, organophosphates, pyrethroids, sulfoximines, and carbamates, respectively, commonly recommended for major cotton insect control (Catchot et al., 2017). These representative insecticides were used for tests of honey bee susceptibility at various ages. Insecticide name, manufacturer, percentage of active ingredient (a.i.), spray treatment concentrations, field use (spray) concentrations of formulation, and mode of actions (IRAC, 2015) are listed in Table 1.

2.3. Bioassay on honey bees with insecticides at LC_{20}

Insecticide solution at concentration of LC_{20} was prepared by diluting each insecticide formulation with distilled water (d-H_2O). A d-H_2O only treatment was included as control. Each replicate consisted of one cage of 25 bees, all the same age, and five replicates were used for each treatment (either a pesticide or control), for a total number of 30 cages of bees. To evaluate contact toxicity, a modified Potter Spray Tower was used to deliver 500 μl of the pesticide solution or d-H_2O into each cage containing 30 bees. The sprayer was set at 10 psi with spray distance of 22 cm to ensure a uniform deposition of mist on bees and the inner surface of the container (Zhu et al., 2015). After spraying, caged bees were maintained in an incubator as described in Section 2.1. Mortality was recorded 48 h after treatment and surviving bees were collected for enzyme activity assays.

2.4. Enzyme activity assays

Enzyme activities of cytochrome P450 monooxygenase (P450), glutathione S-transferase (GST), Esterase (EST), and invertase were assayed using corresponding substrates in surviving bees from control-treatment after contact bioassays. Enzyme preparations and enzyme activity quantifications were processed according to the procedures of Zhu et al. (2017b).

2.5. Data processing and statistical analysis

Microsoft Excel was used to process bioassay and enzyme assay data. SigmaPlot (version 14) was used to perform dynamic (linear) regression analyses, test data normality, calculate slope, intercept, R^2 values and *t*-test parameters (P = 0.05) for each treatment, as well as create graphs by plotting mortality or enzymatic data against bee ages (days).

3. Results

3.1. Honey bee natural mortality and the effect of bee age on the susceptibility to five selected insecticides

Results of accumulative natural mortality in untreated honey bee

Table 1
Pesticide name, manufacturer, percentage of active ingredient (a.i.), spray concentration of formulation, and mode of action (MOA).

Chemical name (active ingredient)	Commercial name	Manufacturers	Active ingredient a.i.%	Spray concentration of formulation (mg/L)	Mode of action
Imidacloprid	Advise 2FL	Winfield Solutions LLC	21.4	274	Nicotinic acetylcholine receptor (nAChR) competitive modulators
Acephate	Bracket97	Winfield Solutions LLC	97	91	Acetylcholinesterase (AChE) inhibitors
λ-Cyhalothrin	Karate Z	Syngenta	22.8	273	Sodium channel modulators
Sulfoxaflor	Transform	Dow Agrosciences	0.5	162	Nicotinic acetylcholine receptor (nAChR) competitive modulators
Oxamyl	Vydate	DuPont	42	162	Acetylcholinesterase (AChE) inhibitors

workers kept in cages under laboratory incubator conditions over 42 days are shown in Fig. 1A. Bee mortality was below 3.3% in the first week and below 6% in the second week. Predicted accumulative mortality (based on a linear model) increased to 10% on day 21, 13.5% on day 28, 17% on day 35, and 20.2% on day 42. The regression of natural mortality vs. honey bee age fit the linear model with a slope equal to 0.4864 and R^2 equal to 0.8338 (Table 2).

Fig. 1B–F shows net mortality (initial dead bees excluded before spray treatment from the total number of bees used for each replicate, 25 bees/cage) 48 h after chemical treatment of bees of different ages at treatment. Bee mortality data points are plotted against corresponding bee ages. As shown in Fig. 1B, after spray-treatment with imidacloprid at the same concentration, younger bees exhibited lower mortality (36% in 7-day old bees) than older bees (57% in 21-day old bees, and 89.6% in 42-day old bees). The relationship between mortality and bee age was linearly correlated, with slope equal to 1.5225 and R^2 equal to 0.6697 (Table 2). This slope was substantially higher than the slope of natural mortality.

The acephate treatment (Fig. 1C) also showed a drastic increase of bee mortality with increasing bee age (slope = 2.3717 and R^2 = 0.6264). It should be noted that acephate mortality reached a plateau of 100% when bees were 33 days old. Removing last two 100% data points (essentially decreasing the age range to 4–33 days old), the linear regression slope increased substantially to 3.1099 and R^2 slightly increased to 0.6567 (compared to those in Table 2 with no data point removed), indicating that older bees are more sensitive to acephate. Similarly, Fig. 1D–F also shows higher mortality in old bees than in young bees after separate treatments of three different insecticides (classes). The correlations were overall linear with slopes ranging from 1.933 to 2.4466, and R^2 between 0.762 and 0.9165 (Table 2).

3.2. Age effect of honey bee workers on three detoxification enzyme activities

Three major detoxification enzymes (cytochrome P450 monooxygenase [P450], glutathione S-transferase [GST], and esterase [EST]) showed different patterns of enzymatic changes over the range of bee ages from 4 days to 42 days (Fig. 2). P450 activity gradually increased with increasing honey bee age, with the oldest bees exhibiting approximately 4.4-fold higher levels than was detected in young bees (Fig. 2A). The relationship between P450 activity was linearly correlated with bee age, with the slope equal to 0.3413 and R^2 equal to 0.7212 (Table 2).

Unlike P450, both GST (Fig. 2B) and EST (Fig. 2C) activities showed no increasing or decreasing trend over the range of bee age from 4 to 42 days old. The correlation between GST activity and honey bee age was not significant ($P > 0.05$) with slope less than 0.2 and R^2 less than 0.008 (Table 2). Similarly, the correlation between EST activity and honey bee age was not significant ($P > 0.05$) with slope less than 0.03 and R^2 less than 0.006 (Table 2).

3.3. Age effect of honey bees on soluble protein content and invertase activity

The measurements of protein concentration (Fig. 3A) indicated that soluble proteins gradually decrease as honey bee workers age, by 22.56% over the age span from 4- to 42-days old. The significant correlation (Table 2: R^2 = 0.5913; Slope = −0.0126) indicates that 59.13% soluble protein content was negatively influenced by honey bee aging.

Plotting invertase (the honey-making enzyme) activity against bee age (Fig. 3B) revealed no particular trend. Invertase activity was insignificantly (Slope = −0.0449, $P > 0.05$; R^2 = 0.0075) influenced by bee age (Table 2).

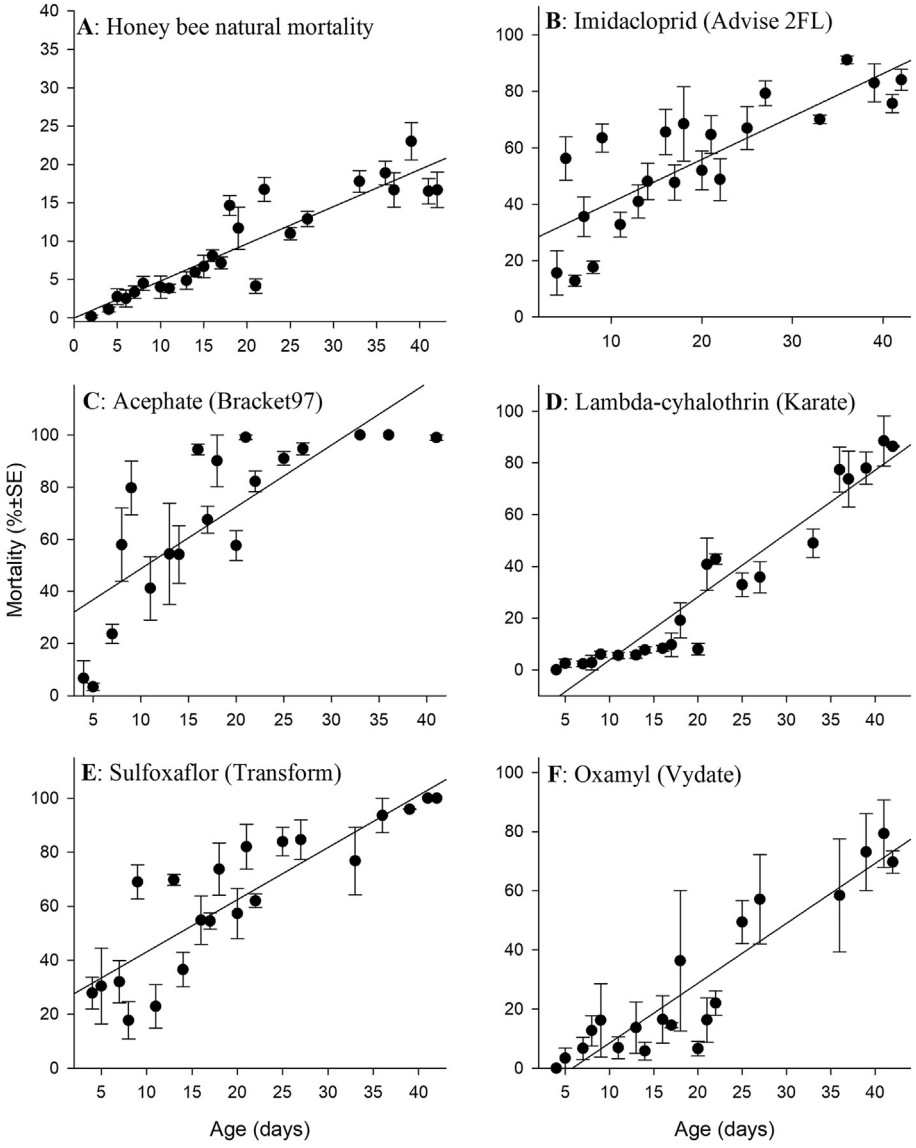

Fig. 1. A: Accumulated natural mortality in honey bee workers kept in laboratory conditions described in Section 2.1; B–F: 48-hour mortality after repeat treatments with five individual insecticides representing five different insecticide classes on workers at different ages (4-day to 42-day old).

Table 2
Summary of linear regression analyses of honey bee age vs. insecticide susceptibility, enzymatic activities, and protein content.

Factor	Normality test	Intercept	Slope	Slope *t*-test	R^2
Natural mortality	Pass	−0.0471	0.4864	P < 0.0001	0.8338
Imidacloprid	Pass	25.475	1.5225	P < 0.0001	0.6697
Acephate	Pass	24.9385	2.3717	P < 0.0001	0.6264
λ-Cyhalothrin	Pass	−20.6572	2.4466	P < 0.0001	0.9165
Sulfoxaflor	Pass	23.7044	1.933	P < 0.0001	0.762
Oxamyl	Pass	−11.7551	2.0246	P < 0.0001	0.8603
P450 oxidase	Pass	1.9265	0.3413	P < 0.0001	0.7212
Glutathione S-transferases	Pass	135.36	0.18	P = 0.6794	0.0076
Esterases	Pass	22.03	0.0209	P = 0.727	0.0054
Protein content	Pass	2.1861	−0.0126	P < 0.05	0.5913
Invertase	Pass	15.05	−0.0449	P = 0.6871	0.0075

4. Discussion

In this study, we conducted multiple experiments in an attempt to clarify whether age effect exists and how the effect may influence the assessment of the impact of pesticides on honey bees. By using bioassays on honey bee workers, we found a gradual increase of natural mortality and decrease of soluble protein content over the age span from 4 days old to 42 days old. We also detected and confirmed age effects on honey bee susceptibility to five insecticides representing five insecticide classes. With the age effect, the susceptibility of worker bees to five insecticides drastically increased as bee age increased, resulting in a great up-shifting of mortality from 0–32% at 4 days old to 77–100%

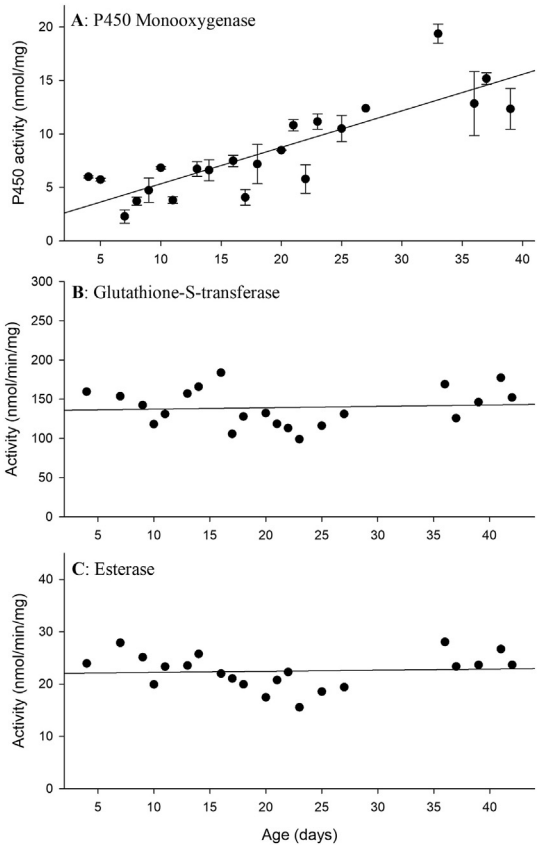

Fig. 2. Enzymatic activities of three major detoxification enzymes, cytochrome P450 monooxygenase (A), glutathione S-transferase (B), and esterase (C), assayed in honey bee workers at different ages from 4-day to 42-day old without any chemical treatment.

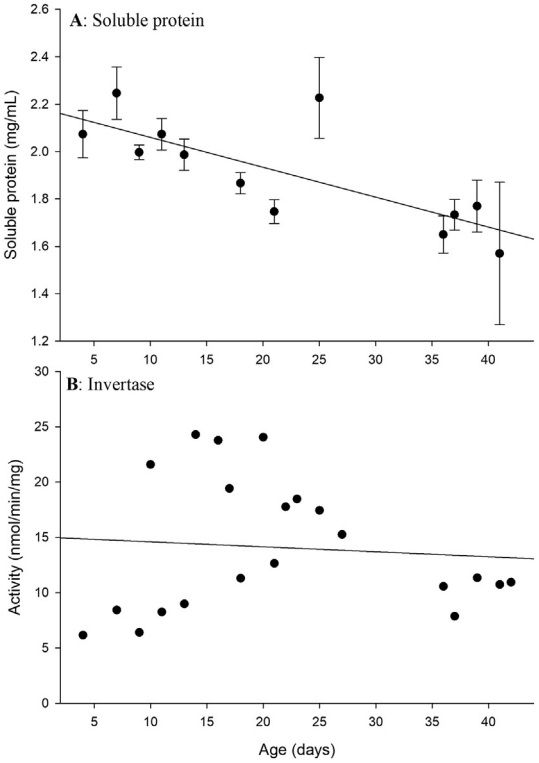

Fig. 3. A: Quantity of soluble proteins in honey bee workers (no chemical treatment) at different age range from 4-day to 42-day old. B: Invertase activities assayed in honey bee workers at different ages from 4-day to 42-day old without any chemical treatment.

at 42 days old. In addition, activities of three major detoxification enzymes, P450, GST, and esterase, and one honey-making enzyme, invertase, were examined in vitro. Only P450 showed gradual and substantial increase, while the other three enzymes remained constant or fluctuated insignificantly over the age range of 42 days. The discussions below provide potential insight into why and what is happening when honey bees are getting old.

4.1. Nutrition

Honey bee workers engage in age-correlated division of labor to perform within-colony activities, such as cleaning, food storage, rearing larvae, and attending the queen, during the first 2–3 weeks after eclosion and start foraging thereafter (Winston, 1987). As their duties shift from in-house nursing to field foraging, honey bee workers go through a substantial change of feeding preference to achieve a physiological and physical readiness to meet the requirements of their functional duties under variable environment conditions. Workers need different amounts of protein at different age for different duty functions (Crailsheim et al., 1992). Young bees start eating pollen soon after emergence (Haydak, 1970) and are additionally fed proteinaceous jelly by nurse bees (Crailsheim, 1991). They reach their maximal body protein content on the sixth day (Haydak, 1959). The pollen content, mainly and representatively in the midgut of workers (correlated with the developmental stage of the hypopharyngeal glands), is low in young bees, highest in about 9-day-old nurse bees and declines to minimal amounts in foragers at 3 or 4 weeks (Crailsheim et al., 1992). While transitioning from nurse bees to foragers (between 9- and 16 days), bees reduce pollen consumption but have still better developed hypopharyngeal glands than older bees (23–30 days). Although 23 day-old bees have nearly the same level of proteolytic activity as do one day-old bees, pollen is far more poorly utilized (Crailsheim et al., 1992). Lower ingestion of pollen and a higher rate of protein turnover in 23 day-old workers (Crailsheim, 1986) were the evidences of inter-adult ingestion of easily digestible jelly provided by nurses (Crailsheim, 1991).

Essential amino acids (EAAs) are used for growth and somatic maintenance in workers. Demand of for nutritional balance is age-dependent (Paoli et al., 2014). The nutritional optimum of young, queenless bees shifted from a proportion of EAAs-to-carbohydrates (EAA:C) of 1:50 towards 1:75 over a 2-week period. Workers exposed to queen mandibular pheromone (QMP) lived longer on diets high in EAA, and they prioritized their intake of carbohydrates over dietary EAAs, even overeating EAAs to obtain sufficient carbohydrates. Foragers required a diet high in carbohydrates (1:250) and had low survival on diets high in EAA as a function of age and caste. When young bees are not nursing brood and foragers are not flying, their nutritional needs shift towards a diet composed of largely/or only carbohydrates without any cost when they transit from within-hive duties to foraging (Paoli et al., 2014). Workers also continue to perform within-hive behavioral tasks as a function of their exposure to QMP. Bees near the queen remain more ‘nurse-like’ whereas bees exposed to less of QMP begin to behave like foragers (Pankiw et al., 1998). Those workers that have little or no exposure to the queen or her pheromones undergo substantial physiological changes orchestrated by juvenile hormone (Robinson, 1992). Furthermore, in the absence of the queen, workers lose the ability to digest protein after their eighth day post-eclosion (Moritz and Crailsheim, 1987; Paoli et al., 2014).

Based on the information above, we now have better understanding of how honey bee workers differentiate into different caste phenotypes and perform different duties, mainly nursing inside the hive and foraging outside the hive. Workers are quite different each other (types) based on changing food sources, receiving QMP, and encountering environmental hazards. They develop different behaviors, energy

demands, aging speed, and metabolic physiology, especially in the midgut. Many of these developments are age- and duty-related metabolisms that may result in accumulations of damaging ROS (reactive oxygen species). In previous studies, we used 7 ± 2-day old worker bees to simulate field exposure to insecticide spray. At this age, our bees would normally be nurse bees, but they may have differentiated into foragers according to Pankiw et al. (1998) and Paoli et al. (2014), because they had no contact with QMP and they fed almost exclusively on a carbohydrate diet. Mixed-age bees taken directly from the hive (Yu et al., 1984; and many other references cited in the Introduction) have no specific age range and may represent neither pure nurses nor pure foragers. Based on our results, therefore, using bees with known age is highly recommended for comparative examinations of the impact of pesticides on honey bees, in order to produce comparable and consistent research data.

4.2. Aging

Under our laboratory conditions, 20% of honey bee workers died after living in cages for 40 days. In a previous study, we also observed similar mortality rates after workers lived in cages for 34–41 days (Zhu et al., 2018). Our natural mortality data were well within the life span of 3–6 weeks reported by Page and Peng (2001). Under laboratory stable conditions, bees likely encounter substantially fewer environmental challenges and less nutritional diversity, but space restriction in a cage may be a stressor to the normally free-living social insect. If a bee died in a cage, survivors were observed to drag and move the dead bee around attempting to dispose of the dead body to keep their home clean. The other major contributing factor to bee death, however, is likely the aging – the time-related changes in all organisms that lead to senescence, or a progressive decline or deterioration of biological, physiological, psychological, behavioral, and social functions necessary for viability, responding to stress, fertility, and survival (Page and Peng, 2001; Jemielity et al., 2005; Sohal, 1986; Lucas and Keller, 2014; https://www.nia.nih.gov/about/aging-well-21st-century-strategic-directions-research-aging/understanding-dynamics-aging). Even when bees look healthy, 3-week-old bees may have endured an intrinsic age-related process of loss of viability and increase in vulnerability (Comfort, 1964). The environment also induces damage at various levels, e.g. damage to macromolecules, including DNA, proteins and lipids, tissues, and cells by oxygen radicals (widely known as free radicals), and some of this damage is not repaired and thus accumulates with time (Holmes et al., 1992). The damage may be modulated by juvenile hormone (JH) and vitellogenin mediated shutdown of the forager immune system (Fluri et al., 1982), which in turn leads to the cessation of vitellogenin synthesis with low zinc titers and decrease in the number and percentage of normal hemocytes for better immune defense in foragers (Jemielity et al., 2005). Other antioxidants include glutathione with the principal role in detoxifying potentially deleterious substances (including oxygen free radicals) and modulating the redox state of cells by eliminating those oxygen free radicals or their products, and amino acid transporting (Allen and Sohavl, 1986).

4.3. Detoxification and honey making enzymes

Over the age range of 42 days, honey bees also showed gradual increase of cytochrome P450 oxidase activity, while bees still maintained constant levels of other two detoxification enzymes (esterase and glutathione S-transferase [GST]) and an invertase for honey production. The major enzyme superfamilies responsible for the metabolism or detoxification of toxins are the cytochrome P450 monooxygenases (P450s), glutathione transferases (GSTs) and carboxylesterases (COEs) (Du Rand et al., 2015; Li et al., 2007). Honey bees' genome is characterized by a paucity of genes associated with detoxification, making them vulnerable to specific pesticides and especially to combinations of pesticides in real-field environments (Claudianos et al., 2006; Gong and Diao, 2017; Johnson et al., 2009; Johnson et al., 2012). Furthermore, adult honey bees do not contain a large amount of fat bodies for detoxification as well as storage of insecticides (Yu et al., 1984). Vincent et al. (1985) reported the induction of P450 was influenced by age. As aging of other insects, increased production of ROS is caused by the detoxification process, and increased energy metabolism induces oxidative and heat shock stress responses. ROS are generated as by-products of energy metabolism, and in insects, increased ROS production has also been associated with P450-mediated detoxification processes (Mutataliev et al., 2008). This finding may explain why, in this study, as honey bees became old, their cytochrome P450 activity gradually increased even without exposure to any insecticide. The increase of P450 activity may be an indication of increased energy demand and metabolic processes to overcome toxic ROS. This aging related increase of ROS metabolism may limit bee's ability to metabolize multiple toxins (including pesticides) simultaneously due to small capacity of detoxification in honey bee (Claudianos et al., 2006; Yu et al., 1984). However, Rinkevich et al. (2015) found a decreased sensitivity to phenothrin and associated the decrease of sensitivity to the increase of P450 activity in old bees. The insecticides they tested have not been used in our heavy crop production area, and the frequent exposure to phenothrin and naled in their urban environment may facilitate the detoxification of phenothrin in their local honey bee stock. Unlike P450, GST and esterase activities remained unchanged or fluctuated without trend over the tested age span of 4 days to 42 days. Lack of increase of these detoxification enzymes is an indication of a less important role of GST and esterase in detoxifying insecticides in honey bees (Johnson, 2015). Our previous study (Zhu et al., 2017a) of imidacloprid toxicity also provided similar evidence of less significant roles of GST and esterase in honey bee workers.

4.4. Mechanisms of susceptibility change

Many age-related factors could influence the ability of honey bee workers to detoxify insecticides. Testing pyrethrum on the larvae of the sugar beet webworm (*Loxostege sticticalis* L.) showed that the same chemical with high toxicity against the first instar had no effect on 5th instar larvae. Most probably the reason lies in functional differences in the exoskeleton, through which the insecticide must pass before exerting its toxic effect. Chemical properties and fat content determined the permeability and the effect on insect (Pepper and Hastings, 1943). Protease activity is influenced by the age, sex, and feeding behavior of the insect. Insecticide treatment influences midgut digestive enzymes, and poor nutrition and low-nutrient diets have direct effects on primary biochemical and physiological systems (Vyjayanthi and Subramanyam, 2002). Overall chitin content increases as age increases. Subsequently, this may alter insecticide penetration and thus the toxicity of insecticides (Tsao and Richards, 1952). Physiological changes during aging often result in decreased or impaired locomotor behavior. Age-related locomotor deficits can be caused by neural or musculoskeletal degeneration. The age-related decrease in activity is often accompanied by tarsal (foot) abnormalities in aged individuals (Ridgel and Ritzmann, 2005). In one study, mortality in the control treatment was many times higher in the random-aged bees than in any of the known age groups. Bee mortalities from exposure to insecticide-treated surfaces showed sufficient differences (Mayland and Burkhar, 1970). The susceptibility of honey bees, as a function of age, to infestation by tracheal mites, *Acarapis woodi* Rennie, was highest in < 24 h old workers, and the frequency of infestation declined precipitously thereafter. Bees > 4 days old were rarely infested in colonies during active brood rearing (Gary et al., 1989).

4.5. Soluble protein

Soluble proteins, spherical in shape (Bailey, 2020), are present in the cytoplasm and organelles like mitochondria and the nucleus. Their

outer part contains hydrophilic amino acids which interact with the solvent and help solubilize the protein (Srere, 1984). Soluble proteins are associated with detoxification (Mouchès et al., 1987; Schlmeyer et al., 2010), immunity (Hultmark, 1993; Lavine and Strand, 2002), and digestion (Moritz and Crailsheim, 1987; Gillikin et al., 1992). In this study, we found a gradual decline (22.56%) of soluble proteins in honey bee workers over the age span of 4-days to 42-days old. Whether and how soluble protein decline influences bees' physiology and behavior has not been investigated before, and it would be very helpful to understand the impact and mechanisms of age-related decline of soluble proteins.

In summary, we addressed an important issue of whether the age of a test organism (honey bee workers in this case) should be standardized for laboratory examinations of the impact of pesticides on that organism. Although many questions remain unanswered, this study provided evidence of substantial increase of the susceptibility to five insecticides in honey bee workers and emphasized the necessity to use standard bee age to produce quality data in comparative analyses of the risk of pesticides to pollinators.

Funding

This research received no external funding.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to several anonymous journal reviewers and editors for their valuable comments and suggestions that improved earlier versions of this manuscript. The authors also appreciate Nathan Schiff of USDA Forest Service (Stoneville, MS) for providing space for a research bee yard, and Louisa Huang, Kenneth Adam Wells, and Narvetta Harvey for their field and laboratory assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture, Agricultural Research Service.

References

Allen, R.G., Sohavl, R.S., 1986. Role of glutathione in the aging and development of insects. In: Collatz, K.-G., Sohal, R.S. (Eds.), Insect Aging. © Springer-Verlag, Berlin Heidelberg (1986).

Bailey, R., 2020. Proteins in the cell. https://www.thoughtco.com/protein-function-373550.

Bailey, J., Scoott-Dupree, C., Harris, R., Tolman, J., Harris, B., 2005. Contact and oral toxicity to honey bees (Apis mellifera) of agents registered for use for sweet corn insect control in Ontario, Canada. Apidologie 36 (2005), 623–633. https://doi.org/10.1051/apido:2005048.

Bergé, J.B., Ricroch, A.E., 2010. Emergence of minor pests becoming major pests in GE cotton in China: what are the reasons? What are the alternatives practices to this change of status? GM Crops 1 (4), 214 ± 9. https://doi.org/10.4161/gmcr.1.4.13421. 2010. (PMID: 21844676).

Brandon, H., Robinson, E., 2017. Delta Agricultural Digest. Farm Press, Clarksdale, MS.

Calderone, N.W., 2012. Insect pollinated crops, insect pollinators and US agriculture. Trend analysis of aggregate data for the period 1992–2009. PLoS One 7, e37235. https://doi.org/10.1371/journal.pone.0037235. (2012).

Catchot, A., Allen, C., Cook, D., Dodds, D., Gore, J., Irby, T., Larson, E., Layton, B., Meyers, S., Musser, F., 2017. Insect Control Guide for Agronomic Crops. Insect Control Guide Committee, Mississippi State University Extension Service Publication 2471.

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

Comfort, A., 1964. Ageing: The Biology of Senescence. Routledge & Kegan Paul, London.

Crailsheim, K., 1986. Dependence of protein metabolism on age and season in the honeybee (Apis melltifer camica Pollm). J. Insect. Phvsiol. 32, 629–634.

Crailsheim, K., 1991. Interadult feeding of jelly in honeybee (Apis mellifera L.) colonies. J. camp. Physiol. 161B, 55–60.

Crailsheim, K., Schneider, L.H.W., Hrassnigg, N., Buhlmann, G., Brosch, U., Gmeinbauer, R., Schoffmann, B., 1992. Pollen consumption and utilization in worker honeybees (Apis mellifera Carnica): dependence on individual age and function. J. Insect Physiol. 38 (6), 409–419 (1992).

Du Rand, E.E., Smit, S., Beukes, M., Apostolides l, Z., Pirk, C.W.W., Nicolsom, S.W., 2015. Detoxification mechanisms of honey bees (Apis mellifera) resulting in tolerance of dietary nicotine. Sci. Rep. 5, 11779. https://doi.org/10.1038/srep11779. (2015).

Faucon, J.P., Clément, A., Drajnudel, P., Mathieu, L., Ribiere, M., Martel, A.C., Zeggane, S., Chauzat, M.P., Aubert, M.F.A., 2005. Experimental study on the toxicity of imidacloprid given in syrup to honey bee (Apis mellifera) colonies. Pest Manag. Sci. 61, 111–125 (2005).

Fine, J.D., Mullin, C.A., 2017. Metabolism of N-methyl-2-pyrrolidone in honey bee adults and larvae: exploring age related differences in toxic effects. Environ. Sci. Technol. 51, 11412–11422. https://doi.org/10.1021/acs.est.7b03291. (2017).

Fluri, P., Lu, S.H., Wille, H., Gerig, L., 1982. Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honeybees. J. Insect Physiol. 28, 61Y68.

Gary, N.E., Page Jr., R.E., Lorenzen, K., 1989. Effect of age of worker honey bees (Apis mellifera) on tracheal mite (Acarapis woodi) infestation. Exp. Appl. Acarol. 7, 153–160.

Gillikin, J.W., Bevilacqua, S., Graham, J.S., 1992. Partial characterization of digestive tract proteinases from western corn rootworm larvae, Diabrotica virgiferd. Arch. Insect Biochem. Physiol. 19, 285–298 (1992).

Gong, Y., Diao, Q., 2017. Current knowledge of detoxification mechanisms of xenobiotic in honey bees. Ecotoxicology 26, 1–12. https://doi.org/10.1007/s10646-016-1742-7. 2017.

Gore, J., Catchot, A., Musser, F., Greene, J., Leonard, B.R., Cook, D.R., Snodgrass, G.L., Jackson, R., 2012. Development of a plant-based threshold for tarnished plant bug (Hemiptera: Miridae). J. Econ. Entomol. 105, 2007–2014.

Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347 (6229), 1255957.

Greene, J.K., Turnipseed, S.G., Sullivan, M.J., Herzog, G.A., 1999. Boll damage by southern green stink bug (Hemiptera: Pentatomidae) and tarnished plant bug (Hemiptera: Miridae) caged on transgenic Bacillus thuringiensis cotton. J. Econ. Entomol. 92, 941–944.

Haydak, M.H., 1959. Changes with age in weight and nitrogen content of honeybees. Bee Wld 40, 225–229.

Haydak, M.H., 1970. Honey bee nutrition. A. Rev. Ent. 15, 143–156.

Holmes, G.E., Bernstein, C., Bernstein, H., 1992. Oxidative and other DNA damages as the basis of aging: a review. Mutat. Res. 275 (3–6), 305–315.

Hultmark, D., 1993. Immune reactions in Drosophila and other insects: a model for innate immunity. Trends Genet. 9 (5), 178–183. MAY 01, 1993. https://doi.org/10.1016/0168-9525(93)90165-E.

IRAC, 2015. Mode of Action Classification Scheme. Insecticide Resistance Action Committee Version 8.0. 2015. Available online. https://www.irac-online.org/modes-of-action/, Accessed date: 19 August 2019.

Jemielity, S., Chapuisat, M., Parker, J.D., Keller, L., 2005. Long live the queen: studying aging in social insects. AGE 27, 241–248. https://doi.org/10.1007/s11357-005-2916-z. 2005.

Johnson, R.M., 2015. Honey bee toxicology. Annu. Rev. Entomol. 60, 415–434 2015.

Johnson, R., Corn, M.L., 2014. Bee health: background and issues for congress. Congressional research service 7-5700. 2014. Available online. https://fas.org/sgp/crs/misc/R43191.pdf, Accessed date: 19 August 2019.

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

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

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 (2012).

Krupke, C.H., Obermeyer, J.L., Bledsoe, L.W., 2017. Corn insect control recommendations – 2014. Purdue University extension publication E-219-W. https://extension.entm.purdue.edu/publications/E-219.pdf.

Laurino, D., Manino, A., Patetta, A., Porporato, M., 2013. Toxicity of neonicotinoid insecticides on different honey bee genotypes. Bulletin of Insectology 66 (1), 119–126 (2013).

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32 (10), 1295–1309. https://doi.org/10.1016/S0965-1748(02)00092-9. October 2002.

Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol. 52, 231–253 (2007).

Lu, Y.H., Qiu, F., Feng, H.Q., Li, H.B., Yang, Z.C., Wyckhuys, K.A.G., Wu, K.M., 2008. Species composition and seasonal abundance of pestiferous plant bugs (Hemiptera: Miridae) on Bt cotton in China. Crop Prot. 27, 465–472.

Lu, Y., Wu, K., Jiang, Y., Xia, B., Li, P., Feng, H., et al., 2010. Mirid bug outbreaks in multiple crops correlated with widescale adoption of Bt cotton in China. Science 328, 1151–1154. https://doi.org/10.1126/science.1187881. 2010.

Lucas, E.R., Keller, L., 2014. Ageing and somatic maintenance in social insects. Current Opinion in Insect Science 5, 31–36 2014.

Marzaro, M., Vivan, L., Targa, A., Mazzon, L., Mori, N., Greatti, M., et al., 2011. Lethal aerial powdering of honeybees with neonicotinoids from fragments of maize seed coat. Bulletin Insectology 64 (1), 119–126 (2011).

Mayland, P.G., Burkhar, C.C., 1970. Honeybee mortality as related to insecticide-treated

surfaces and bee age. J. Econ. Entomol. 63 (5), 1437–1439 1970.
Medrzycki, P., Montanari, R., Bortolotti, L., Sabatini, A.G., Maini, S., Porrini, C., 2003. Effects of imidacloprid administered in sub-lethal doses on honey bee behaviour. Laboratory tests. Bulletin of Insectology 56 (1), 59–62 (2003).
Moritz, B., Crailsheim, K., 1987. Physiology of protein digestion in the midgut of the honeybee (Apis mellifera L). J. Insect Physiol. 33 (12), 923–931.
Mouchès, C., Magnin, M., Bergé, J.B., de Silvestri, M., Beyssat, V., Pasteun, N., Georghiou, G.P., 1987. Overproduction of detoxifying esterases in organophosphate-resistant Culex mosquitoes and their presence in other insects. PNAS 84 (8), 2113–2116. https://doi.org/10.1073/pnas.84.8.2113. April 1, 1987.
Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdrop, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in north American apiaries: implications for honey bee health. PLoS One 5, e9754.
Murataliev, M., Guzov, V., Walker, F., Feyereisen, R., 2008. P450 reductase and cytochrome b5 interactions with cytochrome P450: effects on house fly CYP6A1 catalysis. Insect Biochem. Mol. Biol. 38, 1008–1015 (2008).
Nauen, R., Ebbinghaus-Kintscher, U., Schmuck, R., 2001. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in Apis mellifera (Hymenoptera: Apidae). Pest Manag. Sci. 57, 577–586 (2001).
Page Jr., R.E., Peng, C.Y.S., 2001. Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. Exp. Gerontol. 36, 695–711 2001.
Pankiw, T., Huang, Z.Y., Winston, M.L., Robinson, G.E., 1998. Queen mandibular gland pheromone influences worker honey bee (Apis mellifera L.) foraging ontogeny and juvenile hormone titers. J. Insect Physiol. 44 (7–8), 685–692.
Paoli, P.P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S.W., Simpson, S.J., Wright, G.A., 2014. Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. Amino Acids 46, 1449–1458. https://doi.org/10.1007/s00726-014-1706-2. 2014.
Pepper, J.H., Hastings, E., 1943. Age variations in exoskeletal composition of the sugar beet webworm and their possible effect on membrane permeability. J. Econ. Entomol. 36 (4), 633–634.
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 (6), 345–353.
Qureshi, A.H., Bond, E.J., Monro, H.A.U., 1965. Toxicity of hydrogen phosphide to the granary weevil, Sitophilus granarius, and other insects. J. Econ. Entomol. 58 (2), 324–331.
Ridgel, A.L., Ritzmann, R.E., 2005. Insights into age-related locomotor declines from studies of insects. Ageing Res. Rev. 4 (2005), 23–39.
Rinkevich, F.D., Margotta, J.W., Pittman, J.M., Danka, R.G., Tarver, M.R., Ottea, J.A., Healy, K.B., 2015. Genetics, synergists, and age affect insecticide sensitivity of the honey bee, Apis mellifera. PLoS One 10 (10), e0139841. https://doi.org/10.1371/journal.pone.0139841.
Robinson, G.E., 1992. Regulation of division-of-labor in insect societies. Annu. Rev. Entomol. 37, 637–665.
Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees – a risk assessment. PLoS One 9 (4), e94482.
Schlmeyer, S., Wang, L., Langgel, D., Heckel, D.G., Mohagheghi, H., Petschenka, G., Ober, Dietrich, 2010. Flavin-dependent monooxygenases as a detoxification mechanism in insects: new insights from the Arctiids (Lepidoptera). PLoS One 5 (5), e10435. https://doi.org/10.1371/journal.pone.0010435. (2010).
Sohal, R.S., 1986. The rate of living theory: a contemporary interpretation. In: Collatz, K.-G., Sohal, R.S. (Eds.), Insect Aging. Springer-Verlag, Berlin (1986).
Srere, P.A., 1984. Why are enzymes so big? Trends Biochem. Sci. 9, 387–390.
Stoner, K.A., Eitzer, B.D., 2012. Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (Cucurbita pepo). 2012. PLoS One 7, e39114.
Suchail, S., Guez, D., Belzunces, L.P., 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites Apis mellifera. Environ. Toxicol. Chem. 20 (11), 2482–2486 (2001).
Tsao, C.H., Richards, A.G., 1952. Studies on arthropod cuticle. IX. Quantitative effects of diet, age, temperature and humidity on the cuticles of five of insects. Annals Entomological Society of America 45, 585–599.
USDA-NASS, 2015. Honey National Agricultural Statistics Service (March 2015). Available online. https://www.nass.usda.gov/Publications/Todays_Reports/reports/hony0318.pdf, Accessed date: 19 August 2019.
Vincent, L.E., Lindcren, D.L., 1965. Influence of fumigation and age on carbon dioxide production of some stored-product insects. J. Econ. Entomol. 58 (4), 660–664.
Vincent, D.R., Moldenke, A.F., Farnworth, D.E., Terriere, L.C., 1985. Cytochrome P-450 in insects. 6. Age dependency and phenobarbital induction of cytochrome P-450, P-450 reductase, and monooxygenase activities in susceptible and resistant strains of Musca domestica. Pestic. Biochem. Physiol. 23, 171–181 (1985).
Vyjayanthi, N., Subramanyam, M.V.V., 2002. Effect of fenvalerate-20EC on sericigenous insects digestive enzymes in the nutritive physiology of silkworm, Bombyx mori L. Ecotoxicol. Environ. Saf. 53, 212d220. https://doi.org/10.1006/eesa.2002.2229. (2002).
Winston, M.L., 1987. The Biology of the Honeybee. Harvard University Press, Cambridge.
Yao, J., Zhu, Y.C., Adamczyk, J., 2018. Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures. J. Econ. Entomol. https://doi.org/10.1093/jee/toy140.
Yu, S.J., Hsu, E.L., 1993. Induction of detoxification enzymes in phytophagous insects: roles of insecticide synergists, larval age, and species. Arch. Insect Biochem. Physiol. 24, 21–32 (1993).
Yu, S.J., Robinson, F.A., Nation, J.L., 1984. Detoxication capacity in the honey bee, Apis mellifera L. Pestic. Biochem. Physiol. 22, 360–368 (1984).
Zhu, Y.C., Snodgrass, G.L., Chen, M.S., 2004. Enhanced esterase gene expression and activity in a malathion-resistant strain of the tarnished plant bug, Lygus lineolaris. Insect Bioch. Molec. Biol. 34, 1175–1186.
Zhu, Y.C., Guo, Z., He, Y., Luttrell, R., 2012. Microarray analysis of gene regulations and potential association with acephate-resistance and fitness cost in Lygus lineolaris. PLoS One 7 (5), e37586. https://doi.org/10.1371/journal.pone.0037586.
Zhu, Y.C., Adamczk, J., Rinderer, T., Yao, J., Danka, R., Luttrell, R., Gore, J., 2015. Spray toxicity and risk potential of 42 commonly used formulations of row crop presticides to adult honey bees (Hymenoptera:Aphidae). J. Econ. Entomol. 108 (6), 2640–2647.
Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017a. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (Apis mellifera). PLoS One 12 (5), e0176837.
Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017b. Feeding toxicity and impact of imidacloprid and mixtures with six representative pesticides at residue concentrations on honey bee physiology (Apis mellifera). PLoS One 12, e0178421. https://doi.org/10.1371/journal.pone.0178421. (2017).
Zhu, Y.C., Yao, J., Adamczyk, J., 2018. Long-term risk assessment on noneffective and effective toxic doses of imidacloprid to honeybee workers. J. Appl. Entomol. 143 (1-2), 118–128. https://doi.org/10.1111/jen.12572 9/25/2018.2019. 2019.