BEHAVIORAL AND BIOCHEMICAL PROCESSING OF NATURAL AND SYNTHETIC XENOBIOTICS IN THE WESTERN HONEY BEE *APIS MELLIFERA*

BY

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DISSERTATION

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Abstract

As a eusocial insect, *Apis mellifera*, the western honey bee, accomplishes many tasks, including acquisition of food, defense against enemies, and reproduction, through division of labor. In this dissertation, I examined whether honey bees also exploit division of labor in the detoxification of natural and synthetic xenobiotics. I approached this question from a behavioral perspective by assessing the extent to which foragers can detect and avoid natural and synthetic xenobiotics, and from a biochemical perspective, by determining how detoxification capacity changes with temporal polyethism and task allocation and by assessing whether the toxicity of xenobiotics may be enhanced or ameliorated in the presence of co-occurring compounds.

From a biochemical perspective, sequencing the honey bee genome revealed that all major classes of detoxification enzymes are reduced in diversity relative to many other insect genomes, an observation that raised the possibility that honey bees may increase their biochemical versatility by adjusting detoxification activity according to age- and task-related division of labor. In this regard, while the contributions of cytochrome P450 monooxygenases to xenobiotic detoxification have been characterized to some extent, the role of carboxylesterases in detoxification of exogenous esters has not yet received attention. Using several natural esters as potential substrates, I investigated whether carboxylesterases, like some detoxifying P450s, vary in activity relative to caste differentiation and temporal polyethism.

From a behavioral perspective, I conducted a semi-field experiment to determine how free-flying foragers respond to natural and synthetic xenobiotics when alternate food is available. Some natural xenobiotics found in honey and beebread, derived from nectar and pollen respectively, have been shown to upregulate genes encoding proteins associated with detoxification and immunity and may thus potentially improve honey bee health. In contrast,

most synthetic organic compounds used in agriculture are associated with a diverse array of adverse physiological consequences and are regarded as significant factors contributing to population declines. Accordingly, I conducted a series of bioassays to determine if foragers display any ability to recognize and respond positively to potentially beneficial phytochemicals and/or to discriminate against harmful synthetic xenobiotics to reduce colony exposure to toxins.

Because certain phytochemicals—notably, some flavonols and phenolic acids—are almost invariably present in pollen irrespective of plant source, they are ubiquitous in the diet of honey bees. Just as folivorous insect species may come to rely on phytochemicals that are regularly encountered in their host plants for ecological and physiological functions, honey bees may also depend on some of these ubiquitous dietary phytochemicals and their absence from the diet may have effects that are as yet undetermined. One such physiological function played by these phytochemicals is upregulation of detoxification enzymes; their presence or absence may thus affect the toxicity of ingested xenobiotics. In order to clarify the impacts of common dietary phytochemicals on bees, I conducted a series of longevity assays with one-day-old adult honey bees to test if natural xenobiotics (phytochemicals from nectar) enhance honey bee worker longevity and detoxification capacity.

Finally, to characterize the likelihood that dietary phytochemicals may ameliorate toxicity of co-occurring pesticides during foraging under field conditions, I combined survivorship assays with flight performance assays using a flight treadmill in order to ascertain whether mortality may be reduced via phytochemical modification of energy-linked mitochondrial metabolism and energy production.

Chapter 1. Variation in ester metabolism with caste and temporal polyethism in *Apis mellifera*

I conducted a series of assays to determine whether the ability of adult females (workers) and males (drones) to metabolize aliphatic esters via carboxylesterases varies with age- and caste-related status. Both workers and drones are capable of esterase-mediated metabolism of four representative fatty acid esters and three monoterpenoid esters. Newly emerged workers have the lowest esterase activity; greater activity in nurses may relate to elevated consumption of phytochemically-rich pollen and honey for production of worker and royal jelly. High esterase activity in foragers may reflect frequent exposure to esters in nectar, pollen, and propolis. Low (and age-independent) esterase activity in drones is consistent with the fact that they neither forage for nor process nectar or pollen. These findings suggest that drones may be less tolerant of xenobiotics, including pesticides, that are detoxified in part by esterases.

Chapter 2. Honey bee behavioral responses to natural and synthetic xenobiotics

Little is known about whether honey bees have the behavioral capacity to differentiate among potential beneficial or detrimental substances during foraging. In this study, I tested the behavioral responses of foragers to nine naturally occurring phenolic acids and flavonoids frequently found in nectar, pollen, and propolis as well as three synthetic xenobiotices--two herbicides (atrazine and glyphosate) and three fungicides (boscalid, chlorothalonil, and prochloraz) frequently found as hive contaminants. In semi-field experiment, bees were offered a paired-choice between syrup feeders that differed in natural and synthetic xenobiotic content. Among the natural xenobiotics tested, foragers showed a consistent preference for quercetin at all five concentrations tested. Both visitation frequency and consumption were higher for the quercetin-treated feeder than the control feeder. In trials with 0.1 mM and 0.25 mM quercetin,

foragers collected 35% more syrup in the quercetin feeder than the control feeder. Naringenin at 100 ppm also triggered a similar preference response in bees; foragers collected 15% more syrup containing naringenin compared with the control feeder. These preference responses for certain natural dietary constituents may reflect a long evolutionary association between honey bees and angiosperms. Of pesticides eliciting a response, bees displayed a strong avoidance response to prochloraz at relatively high concentrations (i.e., 10 ppm and 100 ppm) and an increased preference at specific concentrations for glyphosate and chlorothalonil. Foragers, however, did not show a significant response to atrazine at the tested concentrations. This paradoxical preference or lack of an ability to differentiate and identify contaminated food may account for the relatively high frequency with which these pesticides are found as hive contaminants and thus may present a substantially greater risk factor for honey bee health than previously suspected.

Chapter 3. Impacts of dietary phytochemicals on honey bee longevity and detoxification capacity.

Feeding preferences demonstrated by honey bee foragers in behavioral assays for food containing certain ubiquitous phytochemicals, such as quercetin, suggests that such compounds may have beneficial effects on honey bee health. By the same token, some ubiquitous phytochemicals, such as *p*-coumaric acid, are known to have beneficial effects on health but are not detected or preferred by foragers. I conducted longevity assays to quantify the impacts of two ubiquitous phytochemicals, alone and in combination with pyrethroid pesticides and a dietary source of protein, on worker longevity. Both of the two dietary phytochemicals tested—*p*-coumaric acid and quercetin-- enhanced longevity of workers, a finding that reinforces the importance of naturally occurring phytochemicals in the diet of honey bees. Moreover, dietary

quercetin can ameliorate toxic effects of two pyrethroid insecticides, β-cyfluthrin and bifenthrin, when consumed together.

Chapter 4. Effects of a dietary phytochemical on honey bee foragers

4.1. Effects of a dietary phytochemical on fungicide suppression of flight performance in the honey bee Apis mellifera

Boscalid is a fungicide, frequently found as a hive contaminant, that interferes with fungal energy production, specifically via inhibiting succinate dehydrogenase in the mitochondrial complex II. Quercetin, a flavonol ubiquitous in honey and pollen, also may affect energy-linked mitochondrial metabolism in honey bees. In this study, the effects of dietary quercetin on levels of ATP in flight muscles of foragers as well as the effects of ingesting quercetin and boscalid together on forager flight performance were investigated. ATP levels in flight muscles of quercetin-treated foragers were higher than in foragers from a paired-control colony (37.46 ± 22.89 vs 10.29 ± 9.75 pmol/mg protein, respectively), as was the frequency of wing flapping (exercise intensity) (183.27 ± 2.93 Hz vs 171.65 ± 2.48 Hz). This finding confirms that consuming quercetin increases energy production per unit time and potentially facilitates faster flight. In contrast, foragers consuming boscalid alone exhibited the lowest frequency of wing flapping compared with paired hives treated with both boscalid and quercetin and a solvent-treated control hive (189.34 ± 2.36 Hz vs 201.31 ± 1.40 Hz vs 195.95 ± 1.82 Hz;). Thus, consuming quercetin can eliminate the adverse effects of boscalid on flight performance of foragers, a finding that reinforces the importance of naturally occurring phytochemicals in the diet of honey bees.

4.2. Dietary quercetin ameliorates toxic effects of insecticides in honey bee foragers

Consumption of certain flavonoids affects CYP450 gene expression, which may influence detoxification of pesticides and lead to synergistic or antagonistic interactions. One neonicotinoid (imidacloprid) and two pyrethroid (β-cyfluthrin and bifenthrin) pesticides were used to examine the effect of quercetin on detoxification capacity. Quercetin enhanced tolerance of the two tested pyrethroids but not imidacloprid at a concentration of 500 ppb. To identify sublethal effects that may not be detectable in a survivorship assay, a flight treadmill test was designed to examine the effects of interactions between quercetin and pesticides in flying foragers. The foragers that consumed quercetin simultaneously with pesticides increased the number of flight bouts, tolerated a higher accumulated pesticide dose and exhibited a delayed onset of paralysis relative to foragers treated with pyrethroids alone. In sum, quercetin enhances tolerance of foragers to these pyrethroid insecticides and may confer protection against toxicity of some pesticides.

Table of Contents

Chapter 1. Variation in ester metabolism with caste and temporal polyethism in *Apis mellifera*

Abstract

The honeybee *Apis mellifera* encounters aliphatic esters in pheromones, floral tissues, and pesticides; we investigated whether the ability of adult females (workers) and males (drones) to metabolize aliphatic esters via carboxylesterases varies with age- and caste-related status. Both workers and drones are capable of esterase-mediated metabolism of four representative phytochemical fatty acid esters and three phytochemical monoterpenoid esters. Newly emerged workers have the lowest esterase activity; greater activity in nurses may relate to elevated consumption of phytochemically rich pollen and honey for production of worker and royal jelly. High esterase activity in foragers may reflect frequent exposure to esters in nectar, pollen, and propolis. Low (and age-independent) esterase activity in drones is consistent with the fact that they neither forage for nor process nectar or pollen. These findings suggest that drones may be less tolerant of xenobiotics, including pesticides, that are detoxified in part by esterases.

Keywords:

division of labor, carboxylesterase-mediated metabolism, aliphatic esters, 1-octanol, 1-hexanol

Introduction

Relative to other insect genomes, the genome of *Apis mellifera*, the western honey bee, contains fewer genes in superfamilies associated with detoxification of xenobiotics (Claudianos *et al.* 2006), i.e., exogenous chemical substrates. This reduction is thought to result from the long evolutionary association of these pollinators with a eusocial lifestyle, in which division of labor, behaviorally mediated food processing, and learning might reduce the need for a large complement of detoxification genes. To date, much attention has been focused on honey bee cytochrome P450 monooxygenases, enzymes that functionalize toxins, including pesticides, via oxidation reactions so as to reduce their toxicity (Berenbaum and Johnson 2015). Esterases (EC 3.1.1.1), or carboxylesterases, however, comprise another major insect metabolic system for processing xenobiotics; the honey bee genome is similarly reduced in its inventory of genes encoding these enzymes (Claudianos *et al.* 2006; Oakeshott *et al.* 2010),but the impact of this reduction on honey bee biology has rarely been examined.

Esterases, hydrolytic enzymes that split esters into acids and alcohols, function in insects both in endogenous physiological processes (Campbell *et al.* 1998; Hinton and Hammock 2003; Oakeshott *et al.* 2010; Weirich and Wren 1976) and detoxification of xenobiotics. Of the 24 carboxyl/cholinesterase genes in the honey bee genome, the number associated with neuro/developmental functions is comparable to those in other insect groups. A single carboxylesterase gene (*Amjhe-like*) has been identified that contains the main functional motifs of insect juvenile hormone esterases; expression of this gene is upregulated by JH-III and suppressed by 20-hydroxyecdysone and its transcript levels during development fluctuate inversely with JH titers (Mackert *et al.* 2008). By contrast, the 8 carboxylesterase genes in the clade associated with xenobiotic metabolism is reduced compared with other genomes (e.g., half

the number seen in *Anopheles gambiae*). This reduction is surprising in view of the diversity of esters encountered by *A. mellifera*; these compounds are frequently found in nectar and floral fragrances and many honey bee pheromones contain ester moieties. Moreover, many synthetic organic insecticides contain esters and their detoxification by other insects is in many cases mediated by carboxylesterases (Alon *et al.* 2008; Anguiano *et al.* 2008; Claudianos *et al.* 1999; Cui *et al.* 2007; Devonshire and Field 1991; Field *et al.* 1999; Karunaratne *et al.* 1995; O'Brien *et al.* 1992; Oakeshott *et al.* 2010; Oakeshott *et al.* 1999; Pan *et al.* 2009; Saito and Hama 2000; Sogorb and Vilanova 2002; Suzuki *et al.* 1993; Vontas *et al.* 2000; Wheelock *et al.* 2005; Zhang *et al.* 2011).

In general, across all insects, comparatively little is known about the role played by carboxylesterase-mediated metabolism of dietary phytochemicals. In Lepidoptera, these enzymes are important in detoxification of plant-derived phenolic esters in papilionids (Lindroth 1989a; b) and of aliphatic esters in the oecophorid *Depressaria pastinacella (Zangerl et al. 2012)* . In *A. mellifera* to date, xenobiotic-metabolizing esterases have been examined in the context of their function in detoxification of tau-fluvalinate, an acaricide used inside hives to control the parasitic mite *Varroa destructor* (Johnson *et al.* 2006). The control of varroa mites has been one of main challenges to contemporary beekeeping. Varroa mites are parasites of bees that can cause mortality directly and can contribute to colony decline by transmitting a diversity of viruses, including deformed wing virus. For chemical control in-hive, a selective acaricide is usually preferred, because it should be more toxic to the mites than to the bees, which presumably owe their lower sensitivity to greater detoxification capacity. In the case of tau-fluvalinate, carboxylesterases are thought to act on the metabolite of tau-fluvalinate produced by Phase 1

detoxification mediated by cytochrome P450 monooxygenases (Mao *et al.* 2011; Pilling *et al.* 1995).

As a eusocial species, *Apis mellifera* displays age-related polyethism, or worker division of labor, whereby workers perform a sequence of tasks as they age, progressing through a series of duties inside the hive and culminating in several weeks of foraging for nectar, pollen, propolis and water outside the hive (Calderone 1998; Winston 1987). Caste and temporal polyethism thus results in differential likelihood of exposure to potential ester substrates for individual bees. Cell cleaning is the first task of honey bee workers, which typically occupies workers from one to three days after eclosion (Johnson 2010; Ribbands 1953; Seeley 1982; Winston 1987). In summer months, approximately four days after eclosion, worker bees become nurses responsible for feeding larvae, other nestmates, and the queen by producing glandular secretions for worker or royal jelly (Barker *et al.* 1959; Haydak 1970; Jung-Hoffman 1966; Michener 1974). These bees also comprise the queen's retinue (Allen 1960; Seeley 1982; Seely 1979; Winston and Punnett 1982) and contribute to distributing queen pheromonal signals, mainly mandibular gland secretions (Butler *et al.* 1973; Katzav-Gozansky *et al.* 2001; Lensky and Slabezki 1981; Slessor *et al.* 1988; Wossler and Crewe 1999), throughout the colony by palpating and licking the queen and later transferring queen pheromones to other workers (Kralj and Božič 2001; Naumann *et al.* 1991; Seely 1979). Between 12 and 21 days after eclosion, workers take on other hive duties outside the brood-zone (Calderone 1998; Johnson 2008; Seeley 1982; Siegel *et al.* 2013), including building comb, capping and trimming honey cells, and processing nectar into honey; at this stage, the hypopharyngeal glands produce carbohydrate-processing enzymes for honey production (Hrassnigg and Crailsheim 2005; Kubo *et al.* 1996; Ohashi *et al.* 1999; Pontoh and Low 2002; Takenaka *et al.* 1990). After circa 9 more days, 3 weeks after eclosion, bees progress

to tasks outside the hive as foragers and collect pollen, nectar, plant resins, and water to meet colony needs (Calderone 1998; Robinson 1992; Seeley 1995; Winston 1987).

The behavioral changes associated with age-related polyethism are accompanied by physiological and metabolic changes that equip workers to handle different colony tasks (Robinson 1992). Among changes associated with age and task are development and desorption of glands and changes in the composition of their secretions (Crailsheim and Hrassnigg 1998; Deseyn and Billen 2005; Fluri *et al.* 1982; Kubo *et al.* 1996), along with variation in vitellogenin production (Amdam and Omholt 2002), insulin/insulin-like growth factor signaling (Ament *et al.* 2008), juvenile hormone levels (Huang and Robinson 1996; Huang *et al.* 1991; Huang *et al.* 1994; Pankiw *et al.* 1998; Robinson 1985; Robinson 1987b; Robinson *et al.* 1989; Robinson *et al.* 1992; Schulz *et al.* 2002b; Sullivan *et al.* 2000) and biogenic amine production (Page and Erber 2002; Schulz *et al.* 2002a; Schulz and Robinson 1999; Schulz *et al.* 2002b; Spivak *et al.* 2003; Wagener-Hulme *et al.* 1999). The onset of foraging, characterized by repeated departures from the hive environment, is associated with an increased probability of encountering xenobiotics, particularly as workers fly through the external environment and search for phytochemical-rich nectar, pollen and plant resins. Whether this changing level of exposure to environmental xenobiotics is reflected by changes in the enzymatic capacity to process these xenobiotics has not previously been examined, although Mao *et al*. (2015) documented differential expression of CYP4G11 and CYP9Q genes in appendages of adult workers according to age and task performance.

Unlike their female hivemates, the main role of male (drone) bees is to inseminate queens (Winston 1987). They do not take care of brood or other hivemates nor do they participate in processing food, as nurse bees do, or gathering nectar and pollen, as do foragers. After they

eclose, they remain in the hive until they attain sexual maturity (in 6-9 days, on average) and then they fly out of the hive daily until they mate with a virgin queen and die. Based on the relatively low frequency with which they encounter xenobiotics, it seems likely that their detoxification capacity is low relative to adult females, although few direct measurements have been reported in the literature.

In this study, I examined how carboxylesterase activity varies with age or task in both female (worker) and male (drone) adults. I hypothesized that a division of metabolic capabilities should parallel division of labor in this eusocial species. In terms of potential substrates of carboxylesterases, I chose seven compounds that are representative of esters encountered by honey bees both within and outside the hive. Esters are frequent constituents of several honey bee pheromones; sting pheromones are particularly rich in these compounds (Blum and Fales 1988; Blum *et al.* 1978; Collins and Blum 1982) and production of esters, including isopentyl acetate, octyl acetate, 2-octenyl acetate, butyl acetate, and hexyl acetate, increase as workers age and peak when they are 30 to 40 days old (Allan *et al.* 1987). Many of these same aliphatic esters can be found in flowers (Knudsen *et al.* 1993; Metcalf and Kogan 1987), particularly species in the family Apiaceae (Borg-Karlson *et al.* 1994). Possibly due to their presence in nectar and pollen, aliphatic esters have been documented in honey (Chogovadze *et al.* 1973; Shimoda *et al.* 1996). As representatives of these aliphatic esters, I examined hexyl butyrate, octyl acetate, octyl butyrate, and butyl butyrate as potential substrates for honey bee esterases. I also chose three monoterpenoid esters that have been examined for their potential acaricidal activity (Fassbinder *et al.* 2002): myrtenyl acetate, thymyl acetate, and perillyl acetate. I used *in vitro* assays to determine whether esterase activity against these seven esters varies in a pattern consistent with the likelihood of encountering these compounds as a function of caste, age, and task and I also

compared esterase activity in honey bees and varroa mites against the acaricidal monoterpenoid esters.

Methods and Materials

Bees

Honey bees were collected from four naturally mated queen colonies, each with a population of *ca*. 40,000 workers (a two-story-hive), in an apiary at the University of Illinois Bee Research Facility in Urbana, IL in June-August, 2011. They were typical of North American populations of *Apis mellifera* [a mix of predominantly European subspecies]. Worker-capped brood combs were collected (usually two frames from the same hive per replicate) in early July and placed in a 35 ˚C incubator. Workers that emerged from the combs over each subsequent 24-h period were collected and removed from the brood comb cage for further experiments as a newly emerged worker group. Nurse bees were identified as workers whose heads were seen entering a cell containing a larva (Huang *et al.* 1994; Sakagami 1953). To identify and collect foragers, an obstruction was placed in front of the hive and those bees returning to the hive with pollen loads in their corbiculae were collected. To sample drones, four drone brood combs were collected from the same hive as well as from other three hives at the same apiary on the same day in June 2011.The drone brood combs were placed in a 35℃ incubator and checked daily to collect newly emerged drones. Approximately 600 to 1000 newly emerged drones were marked on the dorsal surface of the thorax with a spot of paint (Testor's Enamel) as they emerged every day for three days and the emerging marked drones were reintroduced to the hive from which workers were collected. After 10-14 days, the marked mature drones were recollected for further analysis.

Mites

Varroa mites (*Varroa destructor*) were collected from four colonies at the University of Illinois Phillips Tract research area apiary (Champaign County, IL) in July-August, 2011. The collecting method used was the modified "sugar shake" method from Macedo *et al.* (2002) and Johnson *et al.* (2010). After the queen was carefully removed from the hive, approximately 20,000 workers were shaken or brushed from 10 frames into a screened wooden box, which was then sealed. A tray was set up under the wooden box on the ground and 1 to 2 cups (236-472 ml) of powdered sugar were evenly applied through the screen onto the workers. Following 10-30 seconds of vigorous shaking of the box, mites coated with sugar fell through the screen onto the tray. The caged bees and the queen were then released back to their hive. Mites were cleaned with a paintbrush to remove residual sugar dust and collected for use in bioassays.

In vitro esterase activity assay

The enzyme assay used was a modified version of the assay used by Zangerl *et al.* (2012) to estimate esterase-mediated detoxification by a host-specific caterpillar of aliphatic esters in floral tissues of its hostplant. This method was well-suited to my objective of comparing activities of esterases against specific aliphatic and monoterpenoid esters both in bees of different developmental stages and castes and in varroa mites.

Bees, both workers and drones, were dissected in ice-cold dissection buffer [0.1 M sodium phosphate (pH 7.0), 0.25 M sucrose, 1% w/v polyvinylpyrrolidone, 1.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (Crankshaw *et al.* 1979)] under a dissecting microscope to obtain midguts; undigested food was removed and the guts were rinsed again in fresh buffer. Cleaned midguts from ten bees or whole bodies of 100 mites were pooled and homogenized in 10 ml of ice-cold grinding phosphate buffer [0.1 M

sodium phosphate, (pH 7.0), 0.25 M sucrose, 1% w/v polyvinylpyrrolidone, 1.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride and 5μL/L leupeptin]. One milliliter of homogenate was transferred into a 2-ml centrifuge tube to serve as a reaction tube and one milliliter of cold grinding buffer tube was set up as control. Two reaction tubes, reaction time 0 (Rt0) and reaction time 30 (Rt30), and two control tubes, control time 0 (Ct0) and control time 30 (Ct30), were prepared on ice at the same time. To all tubes, 10 μl of 1% of the tested aliphatic ester substrate in ethanol was added and the tubes were subsequently capped. Seven aliphatic substrates were tested: octyl acetate (oa) (ICN Pharmaceutical Inc.), octyl butyrate (ob) (ICN Pharmaceutical Inc., Cleveland, OH), hexyl butyrate (hb) (SAFC, France), butyl butyrate (bb) (Sigma-Aldrich, St. Louis, MO); (R)-myrtenyl acetate (ma) (Sigma-Aldrich, St. Louis, MO), (S)-perillyl acetate (pa) (Sigma-Aldrich, St. Louis, MO), and thymyl acetate (ta) (Sigma-Aldrich, St. Louis, MO). To Ct0 and Rt0 tubes, 500 μl of ethyl acetate were added prior to incubation, after which all tubes were gently mixed. To a separate set of tubes, 10 μl of S,S,S-tributyl phosphorotrithioate (DEF, Sigma-Aldrich, St. Louis, MO), a specific esterase inhibitor (Bernard and Philogène 1993; Jao and Casida 1974), was added at a final concentration in 65.67 mM to test for carboxylesterase activity of newly emerged workers, newly emerged drones and mites. All tubes were then incubated in a shaking water bath at 30°C for 30 min. After incubation, the tubes were returned to ice and 500 μl of ethyl acetate were added to the Rt30 and Ct30 tubes to terminate the reaction. Tubes were vortexed and then centrifuged at 14,000 rpm for two minutes to ensure complete separation of organic and aqueous layers. One microliter of the organic phase from each tube was analyzed with a gas chromatograph—mass spectrometer (Shimadzu QP2010 Plus, SHRXI-5MS capillary column, 30 m × 0.25 mm × 0.25 mm) in split-less mode for 1.5 minutes with an inlet temperature of 250ºC and helium as the

carrier. The initial oven temperature of 50ºC after 50 seconds was increased at 10ºC/min until a final temperature was reached of 250ºC for 5 minutes. The mass spectrometer was set to scan from 40 to 300 m/z. Peaks were identified by matching to the NIST08 spectral library. Amounts were quantified based on total ion chromatograms and compared to a product standard curve (1-hexanol (Sigma-Aldrich, St. Louis, MO), the metabolite of hexyl butyrate, and 1-octanol (Sigma-Aldrich, St. Louis, MO), the metabolite of octyl acetate and octyl butyrate, to obtain absolute values. Assays of each ester were replicated three to five times (biological replicates).

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics for Windows software (IBM Corp., Armonk, NY). One-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test were used to compare substrate disappearance and product appearance between different groups. For the data for which the assumption of homogeneity of variance was violated, the Kruskal–Wallis *H* test and Dunn's post-hoc method were applied instead.

Results

Four aliphatic esters known to occur as constituents of pheromones or food resources and three plant-derived esters with acaricidal activity were evaluated as possible substrates for esterases of *A. mellifera*. All compounds were metabolized in midgut enzyme preparations of workers and drones; in all cases, enzymatic metabolism of octyl acetate and octyl butyrate yielded 1-octanol as an alcohol product and metabolism of hexyl butyrate yielded 1-hexanol as an alcohol product (Fig. 1.1). The alcohol product of esterase metabolism of butyl butyrate, butanol, had a mass too small to be detected by my method. Worker task had a significant effect

on aliphatic ester metabolism rates (Fig. 1.1A) (oa: $F_{2,12} = 4.63$, $p = 0.032$, ANOVA; ob: $F_{2,10} = 3.88$, $p = 0.057$, ANOVA; hb: $F_{2,11} = 8.52$, $p = 0.006$, ANOVA; bb: $F_{2,11} = 24.13$, $p < 0.001$, ANOVA); older workers, i.e., nurses and foragers, metabolized these substrates at a higher rate than did newly emerged workers. Workers and drones performed differently with respect to these esters (Fig. 1.1A, B), but, in contrast with workers, drone age had no effect on rates of ester metabolism (Fig. 1.1B) (oa: $F_{1,8} = 3.22$, $p = 0.111$, ANOVA; ob: $F_{1,6} = 0.03$, $p = 0.869$, ANOVA; hb: $F_{1,8} = 0.03$, $p = 0.871$, ANOVA; bb: $F_{1,8} = 2.70$, $p = 0.139$, ANOVA).

Unexpectedly, rates of production of primary alcohol metabolites and disappearance rates of ester substrates were not consistent across all assays (Fig. 1.1C, D). Newly emerged bees produced significantly more 1-octanol metabolite from octyl acetate and octyl butyrate than did pollen foragers (1-octanol from oa: $H_2 = 10.55$, $p = 0.005$, Kruskal–Wallis H test; 1-octanol from ob: $F_{2,8} = 130.39$, $p < 0.001$, ANOVA) despite the fact that esterases of newly emerged workers displayed the lowest rate of ester consumption. Similarly, in hexyl butyrate assays, forager esterases consumed approximately 2 times greater amounts of substrate than did esterases of newly emerged bees, yet forager enzymes produced the smallest amount of hexanol product ($F_{2,11} = 13.86$, $p < 0.001$, ANOVA). In assays of octyl acetate and octyl butyrate, mature drones and newly emerged drones did not differ in rates of substrate consumption but production of metabolites declined with age (1-octanol from oa: $F_{1,6} = 12.86$, $p = 0.012$, ANOVA; 1-octanol from ob: $F_{1,5} = 1.09$, $p = 0.344$, ANOVA; 1-hexanol from hb: $F_{1,8} = 8.81$, $p = 0.018$, ANOVA). These inconsistencies in rates of substrate disappearance and product appearance suggest that other enzymes may be involved in esters and alcohol metabolism.

In addition to age or task, caste influences rates of ester consumption and metabolite production (Fig. 1.1 and 1.2). Ester consumption rate (Fig. 1.2 A, B) and metabolite production

(Fig. 1.2 C, D) differ between drones and workers when a general esterase inhibitor, DEF, is included in the assays. With octyl butyrate, hexyl butyrate, and butyl butyrate, both drones and workers displayed lower metabolism activity and reduced metabolite production in the presence of DEF (drone_ob: $F_{1,4} = 10.24$, $p = 0.033$, ANOVA; drone_hb: $F_{1,6} = 28.15$, $p = 0.002$, ANOVA; drone_bb: $F_{1,6} = 17.94$, $p = 0.005$, ANOVA; worker_ob: $F_{1,5} = 8.86$, $p = 0.031$, ANOVA; worker_hb: $F_{1,5} = 38.58$, $p = 0.002$, ANOVA; worker_bb: $F_{1,5} = 51.24$, $p < 0.001$, ANOVA; drone_1-octanol production from ob: $F_{1,5} = 7.18$, $p = 0.044$, ANOVA; drone_1-hexanol from hb: $F_{1,6} = 42.23$, $p <0.001$, ANOVA; worker_1-octanol production from ob: $F_{1,6} = 46.88$, $p < 0.001$, ANOVA; worker_1-hexanol from hb: $F_{1,6} = 45.47$, $p < 0.001$, ANOVA), consistent with the interpretation that metabolism of these butyric acid esters is mediated entirely by carboxylesterases. By contrast, adding the DEF inhibitor had no effect on octyl acetate consumption in either drones or workers (drone_oa: $F_{1,6} = 1.24$, $p = 0.307$, ANOVA; worker_oa: $H_1 = 0.2$, $p = 0.655$, Kruskal–Wallis *H* test) but the inhibitor had a caste-specific effect on metabolite production, with drones displaying an effect of inhibitor on 1-octanol production and workers displaying no effect of inhibitor on 1-octanol production (drone_1-octanol production from oa: $H_1 = 5$, $p = 0.025$, Kruskal–Wallis *H* test; worker_1-octanol production from oa: $F_{1,6} = 0.24$, $p = 0.640$, ANOVA). These disparate findings suggest that enzyme systems other than esterases may contribute to worker processing of aliphatic esters.

In terms of the monoterpenoid acetates, honey bees of all types were capable of greater metabolism of all four substrates than were *Varroa* mites (Fig. 1.3A, B,D). The only variation in honey bee esterase activity with caste or task was in metabolism of myrtenyl acetate, which was significantly higher in nurses than in newly emerged workers (Fig. 1.3A) (worker_ma: $F_{2,11} = 6.66$, $p = 0.013$, ANOVA; worker_pa: $F_{2,12} = 0.25$, $p = 0.786$, ANOVA; worker_ta: $F_{2,12} = 0.36$,

$p = 0.707$, ANOVA; drone_ma: $F_{1,10} = 0.33$, $p = 0.581$, ANOVA; drone_pa: $F_{1,10} = 0.06$, $p = 0.804$, ANOVA; drone_ta: $H_1 = 0.007$, $p = 0.935$, Kruskal–Wallis H test). The esterase inhibitor DEF affected only the metabolism of myrtenyl acetate in one-day-old workers (Fig. 1.3C) (ma: $F_{1,6} = 18.58$, $p = 0.005$, ANOVA; pa: $F_{1,6} = 0.02$, $p = 0.889$, ANOVA; ta: $F_{1,6} = 0.06$, $p = 0.820$, ANOVA). By contrast, the ability of *Varroa* mites to metabolize two of three monoterpenoid esters (myrtenyl acetate and perillyl acetate) was dramatically reduced in the presence of the inhibitor (ma: $F_{1,7} = 17.99$, $p = 0.004$, ANOVA; pa: $F_{1,6} = 15.57$, $p = 0.008$, ANOVA; ta: $H_1 = 0.56$, $p = 0.456$, Kruskal–Wallis H test), suggesting that the mites may rely to a greater extent than honey bees on esterases for metabolism of these substrates.

Discussion

In this study, I have demonstrated that the progression of behavioral changes in workers is accompanied to some degree by changes in esterase-metabolizing abilities. In workers, generally, newly eclosed bees display the lowest level of esterase activity; activity levels increase as bees age and progress through nursing, and foraging tasks. The changing rate of ester metabolism in nurses and foragers may allow them to deal with exposure to xenobiotics associated with nectar and pollen processing and collecting or, in the case of foragers, to metabolize components of pheromonal signals associated with defense behavior outside the hive. The pattern of change, however, varies among substrates. To differentiate between the effects of age and task on esterase activities, experiments with single-cohort colonies may be necessary.

By contrast, drones, which display limited age-related changes in in-hive behaviors (basically restricted to eating independently and initiating mating flights), do not exhibit changes in esterase-metabolizing abilities over the course of their adult lives, despite the fact that the

onset of drone flight is associated with an age-related increase in juvenile hormone (Giray and Robinson 1996), indicative of other physiological changes associated with behavior. Although changes in JH titers are linked with changes in activity of juvenile hormone esterase (EC 3.1.1.59), this enzyme, which catalyzes the hydroxylation of juvenile hormone specifically, belongs to a different classes of esterases than do the carboxylesterase (EC 3.1.1.1) that catalyze exogenous carboxylic esters specifically and appear to be regulated differently as well.

The lack of a consistent correlation between substrate consumption and metabolite production suggests that the metabolites generated by esterase activity may undergo further metabolism by other enzyme systems. In this context, the metabolites, 1-hexanol in foragers and 1-octanol in both nurses and foragers, have specific behavioral effects; both compounds occur as constituents of alarm pheromone blends (Collins *et al.* 1989). Both Collins (1980) and Robinson (1987a) have shown that worker responses to alarm pheromone components increase with age, consistent with the concept that older workers have a higher probability of encountering and thus needing to process these compounds. Moreover, hexanol, hexanal, and octanal are components of floral odors (Buttery *et al.* 1982; Knudsen *et al.* 2004; Metcalf and Kogan 1987; Schiestl 2010), so foragers, again, have a higher probability of encountering these environmental chemicals and presumably have a greater physiological need to metabolize these compounds.

As for the use of ester-based acaricides in the hive, honey bees were better able to metabolize three tested monoterpenoid acetates than were varroa mites, which makes these three tested monoterpenoid acetates potentially selective acaricides. However, only the (R)-myrtenyl acetate appears to be a substrate for carboxylesterases; other enzyme systems are likely involved in metabolism of the other two substrates, (S)-perillyl acetate and thymyl acetate.

In sum, this study suggests that carboxylesterase activity should be included among the many biochemical capabilities of honey bees that vary according to the demands of the hive. Like the cytochrome P450s, the carboxylesterases contribute to metabolism of endogenous substrates, such as pheromones, and exogenous substrates, such as phytochemicals, and documenting in greater detail how patterns of expression of different genes in this enzyme class may shed light on how colonies adjust to changing needs and challenges.

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Figures

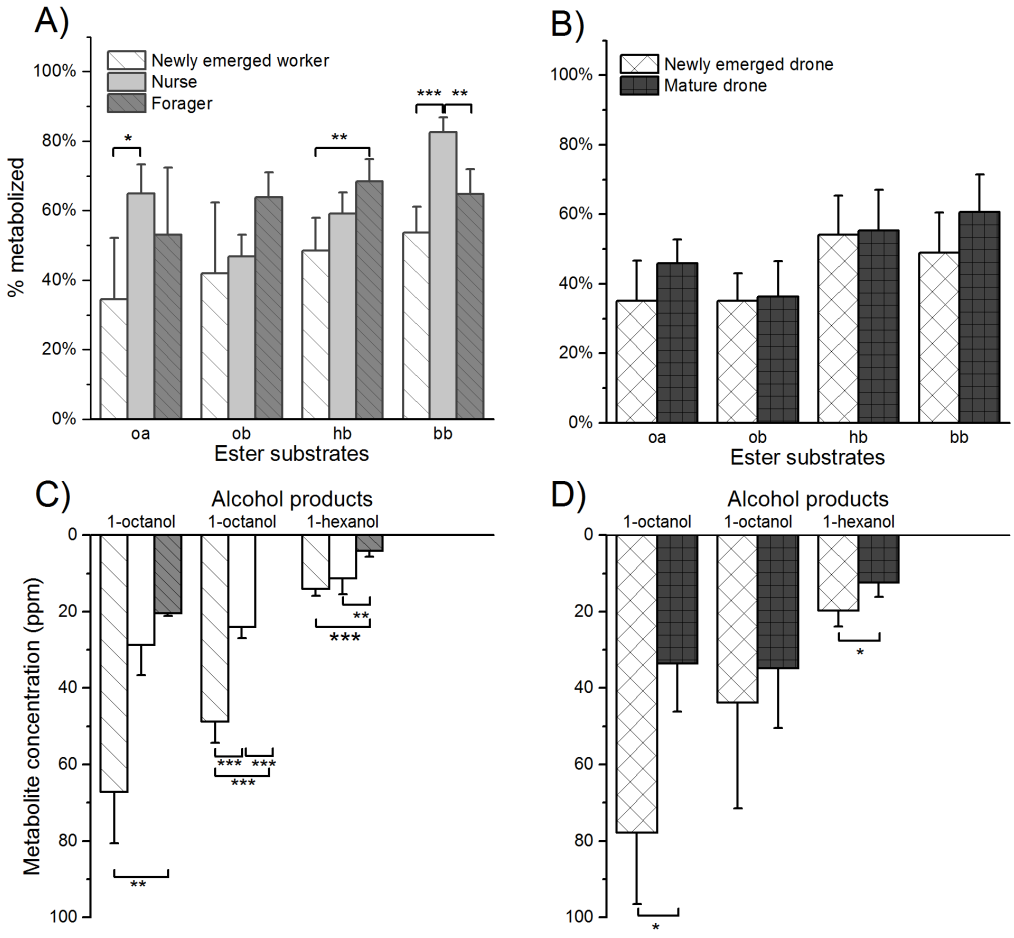

Figure 1.1. Ester substrate consumption and respective metabolite production (1-octanol produced from octyl acetate and octyl butyrate. and 1-hexanol produced by hexyl butyrate) by bees that differ by caste and age. A) Ester substrate metabolism by esterases of workers of different ages. B) Ester substrate metabolism by esterases of drones of different ages. C) Metabolites produced by esterases of workers. D) Metabolites produced by esterases of drones. (The mass of the primary metabolite of butyl buyrate was too small to be detected by our methods.) (oa: octyl acetate; ob: octyl butyrate; hb: hexyl butyrate; bb: butyl butyrate; *:$p < 0.05$, **: $p < 0.01$, ***:$p < 0.001$, using ANOVA with Tukey's HSD post-hoc test or using the Kruskal–Wallis *H* test with Dunn's post-hoc test)

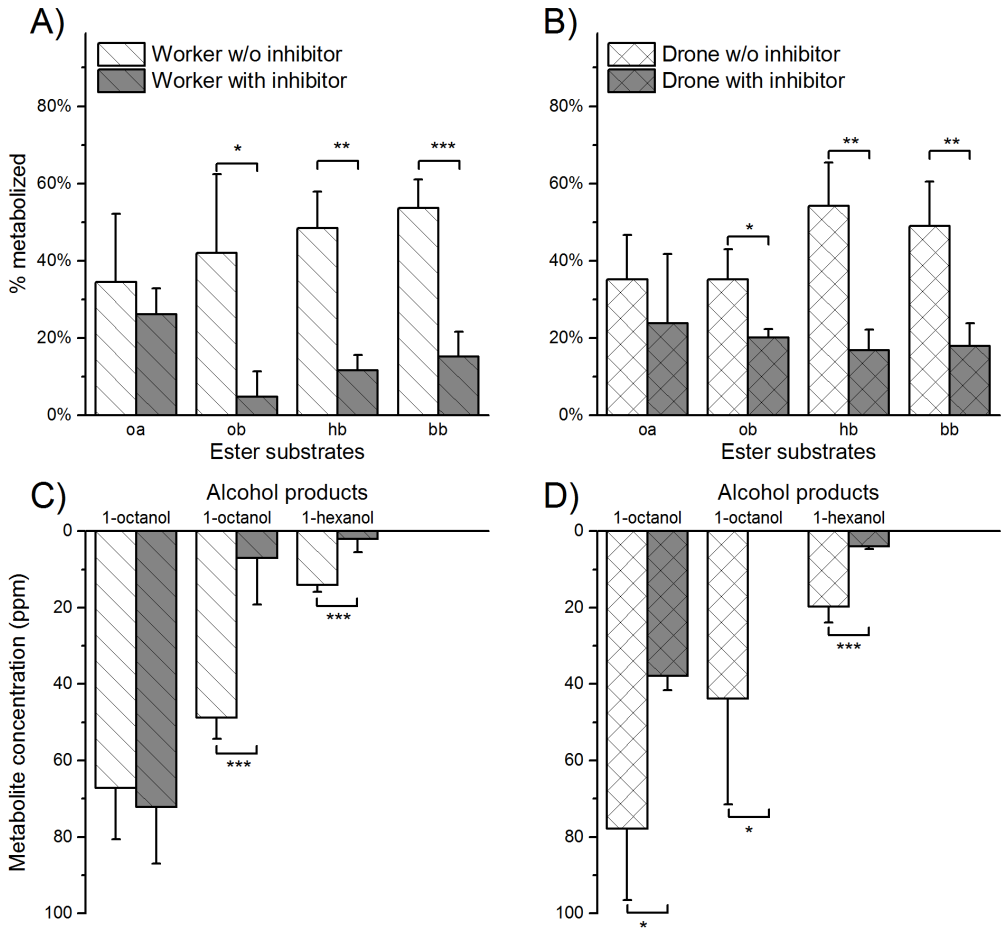

Figure 1.2. Impact of esterase inhibitor, DEF, on esterase activity and metabolite production in newly emerged drones and workers. A) Ester substrate consumption by esterases of workers with or without DEF inhibitor. B) Ester substrate consumption by esterases of drones with or without DEF inhibitor. C) Metabolite production by esterases of workers with or without DEF inhibitor. D) Metabolite production by esterases of drones with or without DEF inhibitor (substrate oa: octyl acetate; ob: octyl butyrate; hb: hexyl butyrate; bb: butyl butyrate; *:$p < 0.05$, **: $p < 0.01$, ***:$p < 0.001$, using ANOVA with Tukey's HSD post-hoc test or using the Kruskal–Wallis *H* test with Dunn's post-hoc test)

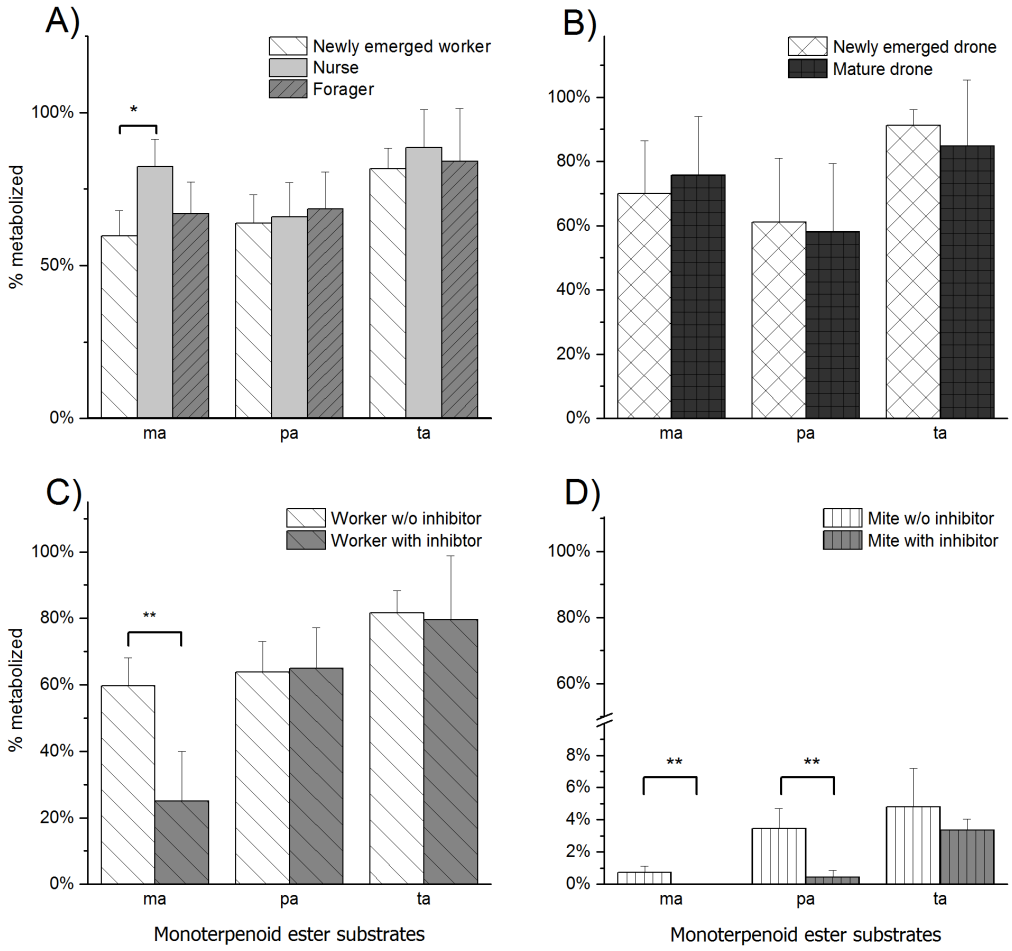

Figure 1.3. Monoterpenoid ester evaluation: monoterpenoid ester substrate consumption by bees that differ by caste and age and impact of esterase inhibitor, DEF, on esterase activity and metabolite production in worker honey bees and varroa mites. A) Monoterpenoid ester metabolism by esterases of workers of different ages. B) Monoterpenoid ester metabolism by esterases of drones of different ages. C) Monoterpenoid ester consumption by esterases of newly emerged workers with or without DEF inhibitor. D) Monoterpenoid ester metabolism by esterases of varroa mites with or without DEF inhibitor. (substrate ma: (R)-myrtenyl acetate; pa: (S)-perillyl acetate; ta: thymyl acetate; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, using ANOVA with Tukey's HSD post-hoc test or using Kruskal–Wallis *H* test with Dunn's post-hoc test)

References

Allan S.A., K.N. Slessor, M.L. Winston, G.G.S. King. (1987) The influence of age and task specialization on the production and perception of honey bee pheromones. J. Insect Physiol. **33**(12): 917-922.

Allen M.D. (1960) The honeybee queen and her attendants. Anim. Behav. **8**(3-4): 201-208.

Alon M., F. Alon, R. Nauen, S. Morin. (2008) Organophosphates' resistance in the B-biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) is associated with a point mutation in an *ace1*-type acetylcholinesterase and overexpression of carboxylesterase. Insect Biochem. Mol. Biol. **38**(10): 940-949.

Amdam G.V., S.W. Omholt. (2002) The regulatory anatomy of honeybee lifespan. J. Theor. Biol. **216**(2): 209-228.

Ament S.A., M. Corona, H.S. Pollock, G.E. Robinson. (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. Proc. Natl. Acad. Sci. USA **105**(11): 4226-4231.

Anguiano O.L., A. Ferrari, I.J. Soleno, M.C. Martinez, A. Venturino, A.M. Pechen de D'Angelo, C.M. Montagna. (2008) Enhanced esterase activity and resistance to azinphosmethyl in target and nontarget organisms. Environ. Toxicol. Chem. **27**(10): 2117-2123.

Barker S.A., A.B. Foster, D.C. Lamb, L.M. Jackman. (1959) Biological origin and configuration of 10-hydroxy-Δ2-decenoic acid. Nature **184**(4686): 634.

Berenbaum M.R., R.M. Johnson. (2015) Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. **10**: 51-58.

Bernard C.B., B.J. Philogène. (1993) Insecticide synergists: role, importance, and perspectives. J. Toxicol. Environ. Health **38**(2): 199-223.

Blum M.S., H.M. Fales. (1988) Eclectic chemisociality of the honeybee : A wealth of behaviors, pheromones, and exocrine glands. J. Chem. Ecol. **14**(11): 2099-2107.

Blum M.S., H.M. Fales, K.W. Tucker, A.M. Collins. (1978) Chemistry of the sting apparatus of the worker honeybee. J. Apic. Res. **17**(4): 218-221.

Borg-Karlson A.-K., I. Valterová, L.A. Nilsson. (1994) Volatile compounds from flowers of six species in the family Apiaceae: bouquets for different pollinators? Phytochemistry **35**(1): 111-119.

Butler C.G., R.K. Callow, C.G. Koster, J. Simpson. (1973) Perception of the queen by workers in the honeybee colony. J. Apic. Res. **12**(3): 159-166.

Buttery R.G., J.A. Kamm, L.C. Ling. (1982) Volatile components of alfalfa flowers and pods. J. Agric. Food Chem. **30**(4): 739-742.

Calderone N.W. (1998) Proximate mechanisms of age polyethism in the honey bee, *Apis mellifera* L. Apidologie **29**(1-2): 127-158.

Campbell P.M., J.G. Oakeshott, M.J. Healy. (1998) Purification and kinetic characterisation of juvenile hormone esterase from *Drosophila melanogaster*. Insect Biochem. Mol. Biol. **28**(7): 501-515.

Chogovadze S.K., G.L. Koblianidze, A.D. Dembitskii. (1973) Aromatic compounds in honey. Lebensmittel-Industrie **20**(5): 225-228.

Claudianos C., H. Ranson, R.M. Johnson, S. Biswas, M.A. Schuler, M.R. Berenbaum, R. Feyereisen, J.G. Oakeshott. (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. **15**(5): 615-636.

Claudianos C., R.J. Russell, J.G. Oakeshott. (1999) The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. Insect Biochem. Mol. Biol. **29**(8): 675-686.

Collins A.M. (1980) Effect of age on the response to alarm pheromones by caged honey bees. Ann. Entomol. Soc. Am. **73**(3): 307-309.

Collins A.M., M.S. Blum. (1982) Bioassay of compounds derived from the honeybee sting. J. Chem. Ecol. **8**(2): 463-470.

Collins A.M., T.E. Rinderer, H.V. Daly, J.R. Harbo, D. Pesante. (1989) Alarm pheromone production by two honeybee (*Apis mellifera*) types. J. Chem. Ecol. **15**(6): 1747-1756.

Crailsheim K., N. Hrassnigg. (1998) Adaptation of hypopharyngeal gland development to the brood status of honeybee (*Apis mellifera* L.) colonies. J. Insect Physiol. **44**(10): 929-939.

Crankshaw D.L., H.K. Hetnarski, C.F. Wilkinson. (1979) Microsomal NADPH-cytochrome *c* reductase from the midgut of the southern armyworm (*Spodoptera eridania*). Insect Biochem. **9**(1): 43-48.

Cui F., H. Qu, J. Cong, X.-L. Liu, C.-L. Qiao. (2007) Do mosquitoes acquire organophosphate resistance by functional changes in carboxylesterases? FASEB J. **21**(13): 3584-3591.

Deseyn J., J. Billen. (2005) Age-dependent morphology and ultrastructure of the hypopharyngeal gland of *Apis mellifera* workers (Hymenoptera, Apidae). Apidologie **36**(1): 49-57.

Devonshire A.L., L.M. Field. (1991) Gene amplification and insecticide resistance. Annu. Rev. Entomol. **36**(1): 1-21.

Fassbinder C., J. Grodnitzky, J. Coats. (2002) Monoterpenoids as possible control agents for *Varroa destructor*. J. Apic. Res. **41**(3-4): 83-88.

Field L.M., R.L. Blackman, C. Tyler-Smith, A.L. Devonshire. (1999) Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer). Biochem. J. **339 (Pt 3)**: 737-742.

Fluri P., M. Lüscher, H. Wille, L. Gerig. (1982) Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. J. Insect Physiol. **28**(1): 61-68.

Giray T., G.E. Robinson. (1996) Common endocrine and genetic mechanisms of behavioral development in male and worker honey bees and the evolution of division of labor. Proc. Natl. Acad. Sci. USA **93**(21): 11718-11722.

Haydak M.H. (1970) Honey bee nutrition. Annu. Rev. Entomol. **15**: 143-156.

Hinton A.C., B.D. Hammock. (2003) Juvenile hormone esterase (JHE) from *Tenebrio molitor*: full-length cDNA sequence, *in vitro* expression, and characterization of the recombinant protein. Insect Biochem. Mol. Biol. **33**(5): 477-487.

Hrassnigg N., K. Crailsheim. (2005) Differences in drone and worker physiology in honeybees (*Apis mellifera*). Apidologie **36**(2): 255-277.

Huang Z.-Y., G.E. Robinson. (1996) Regulation of honey bee division of labor by colony age demography. Behav. Ecol. Sociobiol. **39**(3): 147-158.

Huang Z.-Y., G.E. Robinson, S.S. Tobe, K.J. Yagi, C. Strambi, A. Strambi, B. Stay. (1991) Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. J. Insect Physiol. **37**(10): 733-741.

Huang Z.Y., G.E. Robinson, D.W. Borst. (1994) Physiological correlates of division of labor among similarly aged honey bees. J. Comp. Physiol., A **174**(6): 731-739.

Jao L.T., J.E. Casida. (1974) Esterase inhibitors as synergists for (+)-*trans*-chrysanthemate insecticide chemicals. Pestic. Biochem. Physiol. **4**(4): 456-464.

Johnson B.R. (2008) Within-nest temporal polyethism in the honey bee. Behav. Ecol. Sociobiol. **62**(5): 777-784.

Johnson B.R. (2010) Division of labor in honeybees: form, function, and proximate mechanisms. Behav. Ecol. Sociobiol. **64**(3): 305-316.

Johnson R.M., Z.Y. Huang, M.R. Berenbaum. (2010) Role of detoxification in *Varroa destructor* (Acari: Varroidae) tolerance of the miticide tau-fluvalinate. Int. J. Acarol. **36**(1): 1-6.

Johnson R.M., Z. Wen, M.A. Schuler, M.R. Berenbaum. (2006) Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entomol. **99**(4): 1046-1050.

Jung-Hoffman I. (1966) Die determination von Königin und Arbeiterin der Honigbiene. Zeitschr. Binenforschung **8**: 296-322.

Karunaratne S.H., J. Hemingway, K.G. Jayawardena, V. Dassanayaka, A. Vaughan. (1995) Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible *Culex quinquefasciatus* mosquitoes. J. Biol. Chem. **270**(52): 31124-31128.

Katzav-Gozansky T., V. Soroker, F. Ibarra, W. Francke, A. Hefetz. (2001) Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal? Behav. Ecol. Sociobiol. **51**(1): 76-86.

Knudsen J.T., L. Tollsten, L.G. Bergström. (1993) Floral scents—a checklist of volatile compounds isolated by head-space techniques. Phytochemistry **33**(2): 253-280.

Knudsen J.T., L. Tollsten, I. Groth, G. Bergström, R.A. Raguso. (2004) Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. Bot. J. Linn. Soc. **146**(2): 191-199.

Kralj J., J. Božič. (2001) Activity of attendants after licking and palpating the queen in honeybee (*Apis mellifera carnica*). Neth. J. Zool. **51**(4): 415-419.

Kubo T., M. Sasaki, J. Nakamura, H. Sasagawa, K. Ohashi, H. Takeuchi, S. Natori. (1996) Change in the expression of hypopharyngeal-gland proteins of the worker honeybees (*Apis mellifera* L.) with age and/or role. J. Biochem. **119**(2): 291-295.

Lensky Y., Y. Slabezki. (1981) The inhibiting effect of the queen bee (*Apis mellifera* L.) foot-print pheromone on the construction of swarming queen cups. J. Insect Physiol. **27**(5): 313-323.

Lindroth R.L. (1989a) Chemical ecology of the luna moth : Effects of host plant on detoxification enzyme activity. J. Chem. Ecol. **15**(7): 2019-2029.

Lindroth R.L. (1989b) Differential esterase activity in *Papilio glaucus* subspecies: absence of cross-resistance between allelochemicals and insecticides. Pestic. Biochem. Physiol. **35**(2): 185-191.

Macedo P.A., J. Wu, M.D. Ellis. (2002) Using inert dusts to detect and assess *varroa* infestations in honey bee colonies. J. Apic. Res. **40**(1-2): 3-7.

Mackert A., A.M. do Nascimento, M.M. Bitondi, K. Hartfelder, Z.L. Simões. (2008) Identification of a juvenile hormone esterase-like gene in the honey bee, *Apis mellifera* L. - expression analysis and functional assays. Comp. Biochem. Physiol., B: Biochem. Mol. Biol. **150**(1): 33-44.

Mao W., M.A. Schuler, M.R. Berenbaum. (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. USA **108**(31): 12657-12662.

Mao W., M.A. Schuler, M.R. Berenbaum. (2015) Task-related differential expression of four cytochrome P450 genes in honeybee appendages. Insect Mol. Biol. **24**(5): 582-588.

Metcalf R.L., M. Kogan. (1987) Plant volatiles as insect attractants. Crit. Rev. Plant Sci. **5**(3): 251-301.

Michener C.D. (1974) The social behavior of the bees: a comparative study. Harvard University Press, Cambridge.

Naumann K., M.L. Winston, K.N. Slessor, G.D. Prestwich, F.X. Webster. (1991) Production and transmission of honey bee queen (*Apis mellifera* L.) mandibular gland pheromone. Behav. Ecol. Sociobiol. **29**(5): 321-332.

O'Brien P.J., Y.A. Abdel-Aal, J.A. Ottea, J.B. Graves. (1992) Relationship of insecticide resistance to carboxylesterases in *Aphis gossypii* (Homoptera: Aphididae) from midsouth cotton. J. Econ. Entomol. **85**(3): 651-657.

Oakeshott J.G., C. Claudianos, P.M. Campbell, R.D. Newcomb, R.J. Russell. (2010) Biochemical genetics and genomics of insect esterases, pp. 229-301. *In*: Gilbert L.I. and Gill S.S. (Eds.), Insect pharmacology: channels, receptors, toxins and enzymes, Academic Press, London.

Oakeshott J.G., C. Claudianos, R.J. Russell, G.C. Robin. (1999) Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. Bioessays **21**(12): 1031-1042.

Ohashi K., S. Natori, T. Kubo. (1999) Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). Eur. J. Biochem. **265**(1): 127-133.

Page R.E., Jr., J. Erber. (2002) Levels of behavioral organization and the evolution of division of labor. Naturwissenschaften **89**(3): 91-106.

Pan Y., H. Guo, X. Gao. (2009) Carboxylesterase activity, cDNA sequence, and gene expression in malathion susceptible and resistant strains of the cotton aphid, *Aphis gossypii*. Comp. Biochem. Physiol., B: Biochem. Mol. Biol. **152**(3): 266-270.

Pankiw T., Z.Y. Huang, M.L. Winston, G.E. Robinson. (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.

Pilling E.D., K.A.C. Bromleychallenor, C.H. Walker, P.C. Jepson. (1995) Mechanism of synergism between the pyrethroid Insecticide λ-cyhalothrin and the imidazole fungicide prochloraz, in the honeybee (*Apis mellifera* L.). Pestic. Biochem. Physiol. **51**(1): 1-11.

Pontoh J., N.H. Low. (2002) Purification and characterization of β-glucosidase from honey bees (*Apis mellifera*). Insect Biochem. Mol. Biol. **32**(6): 679-690.

Ribbands C.R. (1953) The behaviour and social life of honeybees. London Dover Publ Inc, New York.

Robinson G.E. (1985) Effects of a juvenile hormone analogue on honey bee foraging behaviour and alarm pheromone production. J. Insect Physiol. **31**(4): 277-282.

Robinson G.E. (1987a) Modulation of alarm pheromone perception in the honey bee: evidence for division of labor based on hormonall regulated response thresholds. J. Comp. Physiol., A **160**(5): 613-619.

Robinson G.E. (1987b) Regulation of honey bee age polyethism by juvenile hormone. Behav. Ecol. Sociobiol. **20**(5): 329-338.

Robinson G.E. (1992) Regulation of division of labor in insect societies. Annu. Rev. Entomol. **37**(1): 637-665.

Robinson G.E., R.E. Page, Jr., C. Strambi, A. Strambi. (1989) Hormonal and genetic control of behavioral integration in honey bee colonies. Science **246**(4926): 109-112.

Robinson G.E., R.E. Page, Jr., C. Strambi, A. Strambi. (1992) Colony integration in honey bees: mechanisms of behavioral reversion. Ethology **90**(4): 336-348.

Saito T., H. Hama. (2000) Carboxylesterase isozymes responsible for organophosphate resistance in the cotton aphid, *Aphis gossypii* Glover (Homoptera: Aphididae). Appl. Entomol. Zool. **35**(1): 171-175.

Sakagami S. (1953) Untersuchungen über die Arbeitsteilung in einen Zwergvolk der Honigbienen: Beiträge zur Biologie des Bienenvolkes, *Apis mellifera* L., I. Japanese Jour Zool **11**: 117-185.

Schiestl F.P. (2010) The evolution of floral scent and insect chemical communication. Ecol. Lett. **13**(5): 643-656.

Schulz D.J., A.B. Barron, G.E. Robinson. (2002a) A role for octopamine in honey bee division of labor. Brain Behav. Evol. **60**(6): 350-359.

Schulz D.J., G.E. Robinson. (1999) Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. J. Comp. Physiol., A **184**(5): 481-488.

Schulz D.J., J.P. Sullivan, G.E. Robinson. (2002b) Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. Horm. Behav. **42**(2): 222-231.

Seeley T.D. (1982) Adaptive significance of the age polyethism schedule in honeybee colonies. Behav. Ecol. Sociobiol. **11**(4): 287-293.

Seeley T.D. (1995) The Wisdom of the Hive: the Social Physiology of Honey Bee Colonies. Harvard University Press, Cambridge.

Seely T.D. (1979) Queen substance dispersal by messenger workers in honeybee colonies. Behav. Ecol. Sociobiol. **5**(4): 391-415.

Shimoda M., Y. Wu, Y. Osajima. (1996) Aroma compounds from aqueous solution of haze (*Rhus succedanea*) honey determined by adsorptive column chromatography. J. Agric. Food Chem. **44**(12): 3913−3918.

Siegel J.A., M.K. Fondrk, G.V. Amdam, R.E. Page, Jr. (2013) In-hive patterns of temporal polyethism in strains of honey bees (*Apis mellifera*) with distinct genetic backgrounds. Behav. Ecol. Sociobiol. **67**: 1623-1632.

Slessor K.N., L.A. Kaminski, G.G.S. King, J.H. Borden, M.L. Winston. (1988) Semiochemical basis of the retinue response to queen honey bees. Nature **332**(6162): 354-356.

Sogorb M.A., E. Vilanova. (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. **128**: 215-228.

Spivak M., R. Masterman, R. Ross, K.A. Mesce. (2003) Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. J. Neurobiol. **55**(3): 341-354.

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

Suzuki K., H. Hama, Y. Konno. (1993) Carboxylesterase of the cotton aphid, *Aphis gossypii* GLOVER (Homoptera:Aphididae), responsible for fenitrothion resistance as a sequestering protein. Appl. Entomol. Zool. **28**(4): 439-450.

Takenaka T., H. Itoa, K. Yatsunami, T. Echigo. (1990) Changes of glucose oxidase activity and amount of gluconic acid formation in the hypopharyngeal glands during the lifespan of honey bee workers (*Apis mellifera* L.). Agric. Biol. Chem. **54**(8): 2133-2134.

Vontas J.G., G.J. Small, J. Hemingway. (2000) Comparison of esterase gene amplification, gene expression and esterase activity in insecticide susceptible and resistant strains of the brown planthopper, *Nilaparvata lugens* (Stål). Insect Mol. Biol. **9**(6): 655-660.

Wagener-Hulme C., J.C. Kuehn, D.J. Schulz, G.E. Robinson. (1999) Biogenic amines and division of labor in honey bee colonies. J. Comp. Physiol., A **184**(5): 471-479.

Weirich G., J. Wren. (1976) Juvenile-hormone esterase in insect development: a comparative study. Physiol. Zool. **49**(3): 341-350.

Wheelock C.E., G. Shan, J. Ottea. (2005) Overview of carboxylesterases and their role in the metabolism of insecticides. J. Pestic. Sci. **30**(2): 75-83.

Winston M.L. (1987) The Biology of the Honey Bee. Harvard University Press, Cambridge.

Winston M.L., E.N. Punnett. (1982) Factors determining temporal division of labor in honeybees. Canadian Journal of Zoology **60**(11): 2947-2952.

Wossler T.C., R.M. Crewe. (1999) The releaser effects of the tergal gland secretion of queen honeybees (*Apis mellifera*). J. Insect Behav. **12**(3): 343-351.

Zangerl A.R., L.-H. Liao, T. Jogesh, M.R. Berenbaum. (2012) Aliphatic esters as targets of esterase activity in the parsnip webworm (*Depressaria pastinacella*). J. Chem. Ecol. **38**(2): 188-194.

Zhang J., J. Zhang, M. Yang, Q. Jia, Y. Guo, E. Ma, K.Y. Zhu. (2011) Genomics-based approaches to screening carboxylesterase-like genes potentially involved in malathion

resistance in oriental migratory locust (*Locusta migratoria manilensis*). Pest Manage. Sci. **67**(2): 183-190.

Chapter 2. Honey bee behavioral responses to natural and synthetic xenobiotics

Abstract

Honey bees (*Apis mellifera*) are eusocial insects whose foragers collect food to meet the needs of the entire colony and adjust their collecting behavior according to these collective needs. Under natural conditions, bee foods are typically rich in a diversity of naturally occurring floral phytochemicals. In contemporary agroecosystems, however, honey bees as managed pollinators also encounter a wide variety of pesticides as contaminants of floral tissues and thus are exposed to many natural and synthetic xenobiotics together as they forage. Whereas some common phytochemicals in bee foods up-regulate detoxification and immunity genes, thereby benefiting nestmates, in contrast, many synthetic pesticides encountered by bees in agricultural fields have adverse effects on bee health even at sublethal exposures. Little is known about if or how honey bees assess xenobiotic risk to protect the colony as they forage. In this study, I tested the behavioral responses of foragers to nine naturally occurring phenolic acids and flavonoids frequently found in nectar, pollen, and propolis, as well as five synthetic xenobiotics--two herbicides (atrazine and glyphosate) and three fungicides (boscalid, chlorothalonil, and prochloraz)--frequently found as hive contaminants. In semi-field free-flight experiments, bees were offered a choice between two paired sugar water feeders that differed in natural and synthetic xenobiotic content, or sugar water containing solvent as a control. Among the natural xenobiotics tested, foragers showed a consistent preference for quercetin at all five concentrations tested. Both visitation frequency and consumption were higher for the quercetin-treated feeder than the control feeder. In trials with 0.1 mM and 0.25 mM quercetin, foragers collected 35% more sugar water from the quercetin feeder than the control feeder. Naringenin at

100 ppm triggered a similar preference response in bees; foragers collected 15% more sugar water containing naringenin compared with the control feeder. These preference responses for certain natural dietary constituents may reflect a long evolutionary association between honey bees and angiosperms. As for responses to synthetic xenobiotics, in a free-flight assay, foragers did not show a significant response to most of the fungicides and herbicides tested. Of pesticides eliciting a response, bees displayed a strong avoidance response to prochloraz at relatively high concentrations (i.e., 10 ppm and 100 ppm) and an increased preference at specific concentrations for glyphosate and chlorothalonil. This paradoxical preference may account for the relatively high frequency with which these pesticides are found as hive contaminants, and thus may present a substantially greater risk factor for honey bee health than previously suspected.

Keywords:

secondary metabolite, synthetic xenobiotics, feeding preference, deterrence, behavioral response

Introduction

In a honey bee colony, foragers are the first members of the colony to encounter and evaluate potential food resources and to make decisions about whether materials are safe, nutritious and worth bringing back to the hive. Thus, the discriminative abilities and behavioral preferences of foragers have tremendous impact on the nutrition and health of the entire colony. Relative to other insect genomes, the *Apis mellifera* genome has an inventory of gustatory receptors that is strikingly reduced, with the 10 gustatory receptor genes (Grs) representing only 13-15% of those present in other insect genomes (Robertson and Wanner 2006). Despite this reduced inventory, honey bees still can identify certain natural and synthetic chemicals (de Sanchez *et al.* 2015; Kessler *et al.* 2015; Liu *et al.* 2015; Peng *et al.* 2015; Perry and Barron 2013).

Additionally, phytochemicals in nectar and pollen can both attract pollinators and repel inappropriate floral visitors (Adler 2000). In addition to its nutrient content, honey, the product of processed nectar, provides phytochemicals that can promote colony health in several ways. Gherman *et al.* (2014), e.g., demonstrated that nurse bees infected with *Nosema* preferentially consume sunflower honey, which has the highest antimicrobial activity among the four types of honeys offered as choices. Additionally, caffeine, an alkaloid found in nectar of plants in the Rutaceae and Rubiaceae, among others, can enhance memory in honey bees (Kessler *et al.* 2015). Moreover, phytochemicals in nectar and honey can confer other health benefits. For example, *p*-coumaric acid, a constituent of many honeys, upregulates both detoxification genes and immunity genes in larval and adult honey bees; bees consuming *p*-coumaric acid in sugar water were capable of 60% higher rates of metabolism of the organophosphate acaricide, coumaphos, than bees consuming sugar water alone (Mao *et al.* 2013; 2015).

Several studies have tested the ability of honey bees to detect phytochemicals and to change feeding behavior accordingly. Quinine, an alkaloid from several *Cinchona* species, is one of the most well-known phytochemicals that is detected and avoided by honey bees (de Sanchez *et al.* 2015). As well, some phenolic compounds in sugar water or nectar can enhance honey bee visitation (Liu *et al.* 2007; Liu *et al.* 2004; Singaravelan *et al.* 2005) whereas others deter feeding (Hagler and Buchman 1993; Liu *et al*. 2006, 2007a,b). Liu *et al.* (2006) speculated that foragers can estimate the amount of phenolics in pollen and change their foraging dynamics accordingly. Other examples include caffeic acid (Hagler and Buchman 1993), nicotine and caffeine (Singaravelan 2010), which at low concentrations induce feeding preferences. These findings collectively suggest that worker bees have the ability to evaluate food quality and use phytochemicals as cues to make foraging decisions, but the extent to which bees utilize certain phytochemicals, known to enhance colony health, as phagostimulants or deterrents, has not yet been systematically assessed.

In contrast with at least some phytochemicals, exposure to synthetic xenobiotics, such as agrochemicals, rarely if ever is beneficial; rather, pesticide ingestion is associated with a wide array of negative effects (Bernauer *et al.* 2015). Pesticides detected in honey and beebread in North American hives include insecticides, acaricides, fungicides and herbicides (Kiljanek *et al.* 2016; Mullin *et al.* 2010). Much attention of late has been focused, understandably, on pesticides that target arthropods, including insecticides and acaricides that contaminate hives. Neonicotinoids in particular have been shown to have a range of adverse effects on bees even at sublethal levels; notwithstanding, Kessler *et al*. (2015) demonstrated that honey bee foragers display a preference for sucrose solutions laced with neonicotinoid pesticides, absent any electrophysiological evidence that they can taste these compounds.

For their part, herbicides and fungicides have been comparatively understudied relative to the frequency with which they are documented as hive contaminants. Chlorothalonil is among the most frequently encountered contaminant in beehives, especially in wax and in pollen, where it has been found at levels up to 99 ppm (Mullin *et al.* 2010). The longstanding assumption has been that fungicides and herbicides, with relatively low acute toxicity relative to pesticides formulated to kill arthropods, are considered as safe for bees. Nonetheless, fungicide and herbicides can have unexpected and undesirable impacts on honey bees. The herbicide atrazine alters acetylcholinesterase activity in honey bees (Boily *et al.* 2013) and exposure to glyphosate interferes with navigation ability (Balbuena *et al.* 2015). Moreover, bees consuming food containing residues of the fungicide chlorothalonil experience higher rates of infection by the parasite *Nosema* (Pettis *et al.* 2012; Wu *et al.* 2012). and reduced queen body size, fewer workers and lower colony biomass (Bernauer *et al.* 2015). Chlorothalonil also synergizes tau-fluvalinate, a pyrethroid acaricide generally applied in beehives, to enhance toxicity to honey bees (Johnson *et al.* 2013). Furthermore, the phenomenon of "entombed pollen" (whereby bees seal off cells containing pollen with higher levels of fungicide) indicates that bees may by some means recognize the presence of contaminants in their hive (vanEngelsdorp *et al.* 2009); although foragers appear to bring fungicide-contaminated pollen into the hive, entombment suggests that nurse bees or other hive workers evaluate the pollen once it is in the hive and make the decision to cap off contaminated cells.

Complicating the assessment of how honey bees evaluate food quality with respect to its xenobiotic content is the fact that many of the behavioral studies to date have involved immobilization and/or force-feeding in no-choice assays. In laboratory tests, restrained bees can be induced to ingest toxic substances (e.g., quinine, salicin, amygdalin and L-canavanine) (de

Sanchez *et al.* 2015) and experience post-ingestion malaise or even death as a result (Ayestarán *et al.* 2010); in contrast, free-flying and freely-moving bees generally appear to detect and avoid toxic substances readily (Avarguès-Weber *et al.* 2010; Bermant and Gary 1966; de Sanchez *et al.* 2015; Rodríguez-Gironés *et al.* 2013). Even responses to sugar water concentration can differ between immobilized and free-flying bees (Wykes 1952). Moreover, forager preference responses to resources vary according to colony-level demand (Page *et al.* 1998). When foragers return from the field, they unload the nectar from their crop to receiver bees (or food storage bees) which, by taking up the nectar at different rates, signal to foragers that certain food resources are preferred (Seeley 1995). Thus, forager behavioral responses and decisions reflect not only an individual's assessment of foraging resources but also a forager's assessment of colony-level needs. Consequently, to understand forager behavioral responses to xenobiotics in natural situations, a free-flight assay of foragers that interact with hivemates is most likely to reflect natural behavior.

Accordingly, to characterize forager behavioral responses to xenobiotics when alternate food is available, I assessed their discriminatory behavior in free-flight assays in a semi-field setting. In these assays, free-flying bees from a functioning colony with nestmates present were allowed to choose between two identical feeders, one containing a test chemical in sugar water and the other containing sugar water and solvent as the control. This assay was used to compare honey bee foraging responses to natural phytochemicals and synthetic xenobiotics, found as common contaminants in U.S. beehives.

Methods and materials

Experimental animals

Experiments were performed with *Apis mellifera*, the western honey bee. Colonies used in assays were from several satellite apiary maintained by the University of Illinois Bee Research Facility located outside Urbana, IL. Colonies were relocated to the free-flight cage before use in the assay.

Bees for the acute toxicity pretest for free flight preference assay were collected from two hives in the same apiary. Foragers were collected at the colony entrance as they returned from foraging; five to seven foragers were placed in a small cage (12.7 cm × 5.1 cm) after collection and kept in the same cage for the assay to reduce handling stress. As a means of further reducing stress, cages were kept in the dark.

Standard five-frame colonies (containing ca. 4,000 worker bees with a naturally mated queen) were used for the free-flight preference assay in September-October, 2013 and June-August, 2014 at the University of Illinois Pollinatarium, located on the UIUC campus. Tested colonies were provided with a dish of ground bee pollen (Betterbee, Greenwich, NY) and a water feeder in front of their hives for the duration of the experiment. A hive inspection was done every two weeks to insure that the colony remained healthy and functioning normally. The colonies were replaced about every four weeks when foraging activity began to decline.

Chemicals

Two herbicides, atrazine(45330, Sigma-Aldrich, Milwaukee, WI) and glyphosate(45521, Sigma-Aldrich, Milwaukee, WI); and three fungicides, boscalid (33875, Sigma-Aldrich, Milwaukee, WI), chlorothalonil (36791, Sigma-Aldrich, Milwaukee, WI), and prochloraz (45631, Sigma-Aldrich, Milwaukee, WI), were obtained from Sigma-Aldrich.

Caffeine (C0750, Sigma-Aldrich, Milwaukee, WI) and three phenolic acids, caffeic acid (C0625, Sigma-Aldrich, Milwaukee, WI), cinnamic acid (C6004, Sigma-Aldrich, Milwaukee, WI), and *p*-coumaric acid (C9008, Sigma-Aldrich, Milwaukee, WI), as well as four flavonoids, chrysin (C80105, Sigma-Aldrich, Milwaukee, WI), naringenin (N5893, Sigma-Aldrich, Milwaukee, WI), pinocembrin (P5239, Sigma-Aldrich, Milwaukee, WI) and quercetin (Q4951, Sigma-Aldrich, Milwaukee, WI), were also purchased from Sigma-Aldrich. One flavonoid, galangin (50-908-908, Indofine Chemical Company, Inc., Hillsborough, NJ), was obtained from Indofine Chemical Company.

These five synthetic xenobiotics and nine natural xenobiotics were selected for testing because they are common contaminants of honey, pollen and propolis in U.S. hives (Mullin *et al*. 2010; Traynor *et al*. 2016). The natural phytochemicals were selected because they are known to up-regulate detoxification and immunity genes (Mao *et al.* 2013).

Free-flight preference assay

The acute toxicity of each chemical-containing sugar water at each concentration was tested in small indoor cages (12.7 cm × 5.1 cm, modified from BioQuip Products Inc. 2820D) before running the free-flight preference assay in the outdoor flight cage. This pre-test was conducted to ensure that the concentrations of the chemicals in our test did not cause acute toxicity. Foragers from a colony with a sister queen of the tested colonies were collected at the hive entrance when they returned from their foraging trip; five to seven foragers were collected and placed into a small cage, which was also used for running the tests for 48 hours. Tests of each concentration of each chemical were replicated five times. Only concentrations with no significant difference in mortality compared with the control group and with at least 80%

survival after 48 hours (e.g., Xavier *et al.* 2015), were considered as having no actual toxicity on bees and were used in the free flight preference assay.

In the free-flight preference assay, a large outdoor flight cage measuring 6 m × 20 m × 3 m was divided in half to yield two flight cages measuring 3 m × 20 m × 3 m. A standard five-frame colony was placed at the center of each flight cage. Artificial feeders with unscented 25% sugar water (w/v) were set up in two end corners of the flight cage equidistant from the hive (10 m). The artificial feeders had a feeder dish (14.75 × 14.75 cm with 24 1-mm-deep grooves that radiated from the center which allowed the bees to collect sugar water from the edge of the feeder), a 5 fl. oz. (147.87 ml) feeder cup (FC5-00090, 5.8 cm height × 7.1cm width, Solo Cup Operating Corporation), and a feeder cup cover. The feeder cup cover was the same size as the feeder cup and had an inner foil and an opaque gray outer layer made of tape. The foil was used to prevent chemical breakdown due to exposure to sunlight; the outer tape layer insured that the feeders appeared identical to the bees so as to prevent color cues from the different sugar water from influencing the bees' behavior.

Initially, the foragers were trained to the feeders for one or two days, after which the assays began. A trial was conducted as follows: first, 30 to 60 minutes with 25% sugar water feeders followed by 60 minutes with a 25% sugar water feeder with solvent (0.25% DMSO) vs. a treatment feeder containing 25% sugar water containing a test chemical in solvent. In order to minimize microenvironment and location effects, the locations of the control and treatment feeders were switched in the second 60 minutes. The same chemical with the same concentration was tested in both halves of the flight cages, and the treatment feeders were always placed in opposite corners of the cage (southwest vs. northeast or northwest vs. southeast) to reduce microenvironment (lights or wind) effects.

Every feeder containing the sugar water to be tested was weighed at the beginning and end of every experimental step to measure the consumption of sugar water. Visitation frequency at each feeder dish was recorded by a digital time-lapse camera with snapshots at one-minute intervals. Because our pretest showed that foragers generally take five to seven minutes to return to the feeder between two successive visiting, only the pictures recorded at 6-minute intervals were used to calculate the number of bees on the feeder dish.

Two herbicides (atrazine and glyphosate) and three fungicides (boscalid, chlorothalonil, and prochloraz) as well as one alkaloid (caffeine), three phenolic acids (caffeic acid, cinnamic acid, and *p*-coumaric acid), and five flavonoids (chrysin, galangin, naringenin, pinocembrin and quercetin) were tested. To make stock solutions, phenolic acids and flavonoids were dissolved in DMSO and caffeine was dissolved in water. Every tested sugar water diet was made fresh at the tested concentration from the chemical stock solution before a test. At least three concentrations were tested for each chemical. A naturally occurring concentration of a chemical was generally tested first. Next, a ten-fold higher concentration was tested, followed by a 100-fold higher concentration. Each chemical was tested three to 12 times at each concentration with two to four colonies (usually three replicates for each concentration in each colony and at two to three concentrations per colony). The final trial numbers varied because foraging was affected by varying weather and hive conditions. Low foraging frequency can occur during severe weather or when a hive is weak, which can bias results; accordingly, those low foraging trial data were discarded.

The amount of sugar water consumed from each chemical treatment feeder in two hours (one trial period) was divided by the amount of sugar water consumed from its paired control feeder to calculate the ratio as an index of preference. The sum of the number visiting each

chemical's treatment feeder in two hours was also divided by the sum of the number visiting its paired control feeder to calculate the ratio of visitation frequency. If the chemical treatment feeder and its paired control feeder were equally attractive to foragers, the ratio of sugar water consumption and the visitation frequency ratio should be equal to 1. A ratio higher than 1 indicates a preference for the test chemical, and a ratio lower than 1 indicates avoidance of the test chemical. Both the ratio of sugar water consumption and the ratio of visitation frequency were tested for normality and the mean values were tested by the one sample *t*-test using OriginPro software (ver. 9.0, OriginLab Corporation) to test if the mean of the ratio was equal to 1.

Results

Natural phytochemicals

Of all of the phytochemicals tested, at least one representative from each chemical class, albeit at varying concentrations, elicited a response indicative of either preference or avoidance (Table 1). Colony identity may have contributed to some of the variation in responses (data not shown). Caffeine, an alkaloid, was avoided by foragers according to both visitation frequency ratio at 1 ppm (one-sample $t_{(6)} = -2.568$, $p = 0.042$) and consumption ratio at 0.1 ppm (one-sample $t_{(8)} = -4.603$, $p = 0.002$). With respect to phenolic acids, evidence of discriminative behavior was found only for sugar water containing caffeic acid; foragers showed an avoidance response according to the visitation frequency ratio at 1ppm (one-sample $t_{(4)} = -2.908$, $p = 0.044$) but showed a preference according to the consumption ratio at the same concentration (one-sample $t_{(4)} = 23.522$, $p < 0.001$).

Among the five tested flavonoids, bees displayed a consistent preference response to quercetin at all five concentrations according to both visitation frequency (0.01 mM, one-sample $t_{(7)} = 3.162$, $p = 0.016$; 0.05 mM, one-sample $t_{(7)} = 7.146$, $p < 0.001$; 0.1 mM, one-sample $t_{(6)} = 2.586$, $p = 0.041$; 0.25 mM, one-sample $t_{(5)} = 2.961$, $p = 0.032$; 0.5 mM, one-sample $t_{(5)} = 5.396$, $p = 0.003$) and consumption ratios (0.01 mM, one-sample $t_{(7)} = 2.825$, $p = 0.026$; 0.05 mM, one-sample $t_{(7)} = 3.749$, $p = 0.007$; 0.1 mM, one-sample $t_{(6)} = 4.424$, $p = 0.004$; 0.25 mM, one-sample $t_{(5)} = 3.969$, $p = 0.011$; 0.5 mM, one-sample $t_{(5)} = 4.599$, $p = 0.006$). In 0.1 mM and 0.25 mM quercetin trials, foragers collected 35% more sugar water from the quercetin feeder than from the control feeder. Naringenin at 100 ppm also triggered a similar preference response in bees (one-sample $t_{(5)} = 3.955$, $p = 0.011$); foragers collected 15% more sugar water in the case of naringenin compared with the control feeder, but the visitation frequency ratio at this concentration did not show preference (one-sample $t_{(5)} = -0.021$, $p = 0.984$). With respect to chrysin and pinocembin, bees displayed an avoidance response to 0.1 ppm chrysin (one-sample $t_{(5)} = -2.676$, $p = 0.044$) and 1 ppm pinocembrin (one-sample $t_{(7)} = -3.539$, $p = 0.009$) according to the visitation frequency ratios but neither avoidance nor preference in consumption ratio was detected (0.1 ppm chrysin, one-sample $t_{(5)} = -0.419$, $p = 0.693$; 1 ppm pinocembrin, one-sample $t_{(7)} = 0.833$, $p = 0.432$).

Synthetic xenobiotics

In the free-flight preference test, boscalid, chlorothalonil, and prochloraz (all fungicides, as shown in Fig. 2.1A&C) as well as atrazine and glyphosate (both herbicides, as shown in Fig. 2.1B&D) were tested. Foragers did not show significantly different responses to the atrazine-sugar water according to either consumption ratios or visitation frequency ratios. As for glyphosate, foragers displayed a preference according to consumption ratio for 10 ppb

glyphosate-sugar water compared with control sugar water (one-sample $t_{(5)} = 3.289$, $p = 0.022$). At higher glyphosate concentrations, no differences in consumption ratios were detected. No difference in visitation frequency ratios was recorded at any of the tested concentrations.

In the tests with fungicides, foragers showed strong avoidance responses only to high prochloraz concentrations, i.e., 10 ppm (visitation frequency ratio, one-sample $t_{(5)} = -3.88$, $p = 0.012$; consumption ratio, one-sample $t_{(5)} = -5.801$, $p = 0.002$) and 100 ppm (visitation frequency ratio, one-sample $t_{(5)} = -13.616$, $p < 0.001$; consumption ratio, one-sample $t_{(5)} = -108.626$, $p < 0.001$). A preference for chlorothalonil was detected at 0.5 ppb, as indicated by both consumption ratios (one-sample $t_{(4)} = 3.504$, $p = 0.025$) and visitation frequency ratios (one-sample $t_{(4)} = 4.781$, $p = 0.009$). A similar preference for chlorothalonil at 50 ppb was evidenced by the consumption ratios (one-sample $t_{(4)} = 4.316$, $p = 0.012$) but not by the visitation frequency ratios (one-sample $t_{(4)} = 1.588$, $p = 0.188$). This preference for chlorothalonil on the part of the foragers may well explain its high frequency and abundance as a contaminant in beehives (Mullin *et al.*, 2010).

Discussion

Among all tested natural xenobiotics, foragers consistently show a preference response to quercetin according to both visitation frequency ratios and preference ratios at all tested concentrations. This clear predilection for quercetin under the conditions of the free-flight assay is indicative of biological significance to honey bees. Quercetin, one of most common phytochemicals in honey, is known to up-regulate detoxification and immunity genes in honey bees (Mao *et al*. 2013; in preparation)

Quercetin is also known for its antioxidant properties in mammals; it is likely that forager preference for quercetin is related to its potential health benefits for the colony. However, how quercetin is detected by honey bees is unclear; quercetin is a non-volatile constituent in pollen (Kaškonienė *et al.* 2015a; Kaškonienė *et al.* 2015b) and may thus be detectable by gustatory receptors. Some nectar phenolics can modulate gustatory responsiveness in the Asian honey bee, *Apis cerana* (Liu *et al.* 2007; Liu and Liu 2010). Thus, quercetin, a nectar phenolic, might alter gustatory receptor sensitivity in *A. mellifera* as well.

Forager responses to caffeine appear to be complex. Honey bee foraging and recruitment to sugar water feeders containing caffeine are stimulated at the concentrations at 25 and 100 ppm (Singaravelan *et al*., 2005; Couvillon *et al*., 2015). Due to the possible pharmacological effects of caffeine on honey bee neurons (Wright *et al.* 2013), the neuroactive effects of caffeine may be responsible for increasing foraging and recruitment, possibly for the benefit of the plant and to the detriment of the bee (Couvillon *et al.* 2015). In this study, honey bees avoided caffeine at low environmental concentrations (0.1 and 1 ppm) consistent with the report by Singaravelan *et al*. (2005) that caffeine is repellent to honey bee at high concentrations (150 and 200 ppm). An individual assay also demonstrated honey bee are more likely to reject sugar water augmented with caffeine (Wright *et al.* 2013). These findings indicate honey bees can detect and avoid caffeine in their food.

Sugar water contaminated with synthetic xenobiotics may have a discernible taste to bees. Foragers significantly avoided intake of prochloraz-sugar water at 10 ppm and 100 ppm, as evidenced by both visitation frequency ratios and consumption ratios. Nevertheless, our assays also show a significant preference for sugar water contaminated with certain fungicides and herbicides at least at some concentrations. The preference detected, however, is slight,

representing a difference of 1-5% between a treated feeder and a control feeder. It may be that only a subset of foragers can detect and respond behaviorally to these compounds; how they are detected, however, remains to be determined. De Brito Sanchez *et al*. (2011) have shown that taste perception of honey bees is more complex than assumed from the relatively low number of gustatory receptors. They suggest that there exist post-ingestive mechanisms in honey bees that might be as important as simple reflexive responses to chemicals, which may have been operative in our assays.

Another possible explanation for the observed preferences and aversions may be that they result not from assessment at the feeder by the forager but by nestmate responses in the hive. Foragers may collect contaminated sugar water and return to the hive, delivering it to receivers, or food storage bees, which may then ingest the compounds and experience post-ingestive malaise or well-being. These receiver bees may have some capacity to signal to foragers that certain food resources should be avoided or collected by the rate at which food is unloaded (Seeley 1995). Our experiments were not designed to detect social feedback, but other studies suggest that this mechanism may function in guiding forager behavior; foragers, for example, can remedy colony nutritional deficiencies by searching for complementary protein sources (Hendriksma and Shafir 2016).

If honey bees can perceive the presence of xenobiotics by gustation or any other means, another explanation of xenobiotic preference may be novelty-seeking behavior, which has been well-documented in both food scouts and nest scouts (Liang *et al.* 2012). Such novelty-seeking behavior allows discovery of new resources that can enhance colony fitness. A reward system in the brain of food scout foragers could act to insure a steady supply of adequate nutrition as floral community composition changes.

Even though there is no clear-cut preference for chemicals and concentrations, bees still show concentration-dependent choice patterns. Bees may well avoid a chemical in high concentrations that is preferred or ignored when present in low concentrations, such as prochloraz and naringenin, respectively. Singaravelan *et al*. (2005) found that relatively low concentrations of nicotine (2.5 ppm in 2.5-20 ppm assay and 0.5, 1 ppm in 0.5-5 ppm assay) elicited a significant feeding preference in honey bees. Köhler *et al*. (2012) observed similar dose-dependent preferences for nicotine at low concentrations but reported repellency at high concentrations. They also demonstrated that different sugar water concentrations may alter the behavioral response thresholds to nicotine. This synergistic interaction between xenobiotics and sugar suggests a possible explanation for some non-significant results in my natural xenobiotic preference assay.

Preferences for synthetic xenobiotics that are potentially detrimental can become problematical for honey bees when they are used as managed pollinators, particularly in orchard systems, where fungicides are often applied during the blooming season to prevent fungal diseases. In order to protect pollinators, fungicides are typically applied at night, under the assumption that the overnight interval is sufficient for avoiding adverse outcomes. However, in addition to the risk of direct exposure, this study suggests that residues that persist through the next day would in fact potentially make contaminated floral resources more attractive to honey bee foragers, thereby increasing the quantity of pesticide brought back to hives. Moreover, some fungicides and herbicides may interact not only with other agrochemicals (Johnson and Percel 2013) but also with phytochemicals; although there is abundant evidence that toxicity can be enhanced by combinations of xenobiotics (Berenbaum and Johnson 2015), how these

combinations affect foraging decisions has yet to be assessed, despite the implications for colony health.

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Table and Figure

Table 2.1. Foraging preference of foragers to natural phytochemical xenobiotics

Category	Chemical name	Concentration	*df* (trials)	Visitation frequency ratio[1]		Sugar water consumption ratio[1]			
				mean	± *SE*	*mean*	±	*SE*	
Alkaloid	Caffeine	0.1 ppm	8	0.99	± 0.04	0.93	±	0.02	**
		1 ppm	6	0.96	± 0.02 *	0.97	±	0.02	
		10 ppm	8	0.98	± 0.05	0.98	±	0.03	
Phenolic acid	Caffeic acid	0.1 ppm	5	0.97	± 0.04	0.96	±	0.02	
		1 ppm	4	0.91	± 0.03 *	1.08	±	0.00	***
		10 ppm	5	0.98	± 0.04	1.04	±	0.03	
	Cinnamic acid	5 ppb	4	1.22	± 0.14	1.11	±	0.09	
		50 ppb	1	1.11	± 0.08	1.21	±	0.09	
		5000 ppb	2	1.08	± 0.09	0.85	±	0.12	
	p-Coumaric acid	1 ppm	6	0.95	± 0.02	0.96	±	0.03	
		10 ppm	7	0.97	± 0.02	1.03	±	0.03	
		100 ppm	7	0.97	± 0.03	1.00	±	0.02	
Flavonoid	Chrysin	0.1 ppm	5	0.80	± 0.08 *	0.97	±	0.06	
		1 ppm	6	1.10	± 0.09	1.01	±	0.06	
		10 ppm	11	1.02	± 0.04	1.06	±	0.03	
	Galangin	0.1 ppm	5	0.95	± 0.09	1.08	±	0.05	
		1 ppm	5	1.08	± 0.04	1.12	±	0.05	
		10 ppm	5	1.00	± 0.05	1.00	±	0.02	
		100 ppm	5	1.11	± 0.05	0.95	±	0.02	
	Naringenin	0.1 ppm	8	1.05	± 0.15	1.08	±	0.10	
		1 ppm	11	0.92	± 0.05	1.01	±	0.04	
		10 ppm	11	1.01	± 0.07	1.00	±	0.03	
		100 ppm	5	1.00	± 0.10	1.15	±	0.04	*
	Pinocembrin	10 ppb	7	1.01	± 0.13	0.98	±	0.04	
		100 ppb	5	0.92	± 0.09	1.00	±	0.03	
		1000 ppb	7	0.82	± 0.05 **	1.04	±	0.05	
	Quercetin	0.01 mM	7	1.06	± 0.02 *	1.04	±	0.02	*
		0.05 mM	7	1.24	± 0.03 ***	1.17	±	0.05	**
		0.10 mM	6	1.20	± 0.08 *	1.35	±	0.08	**
		0.25 mM	5	1.26	± 0.09 *	1.37	±	0.09	*
		0.50 mM	5	1.18	± 0.03 **	1.17	±	0.04	**

[1] A ratio higher than 1 indicates a preference for the test chemical, and a ratio lower than 1 indicates avoidance of the test chemical. The asterisks indicate the means are significantly different from 1 (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one sample *t*-test).

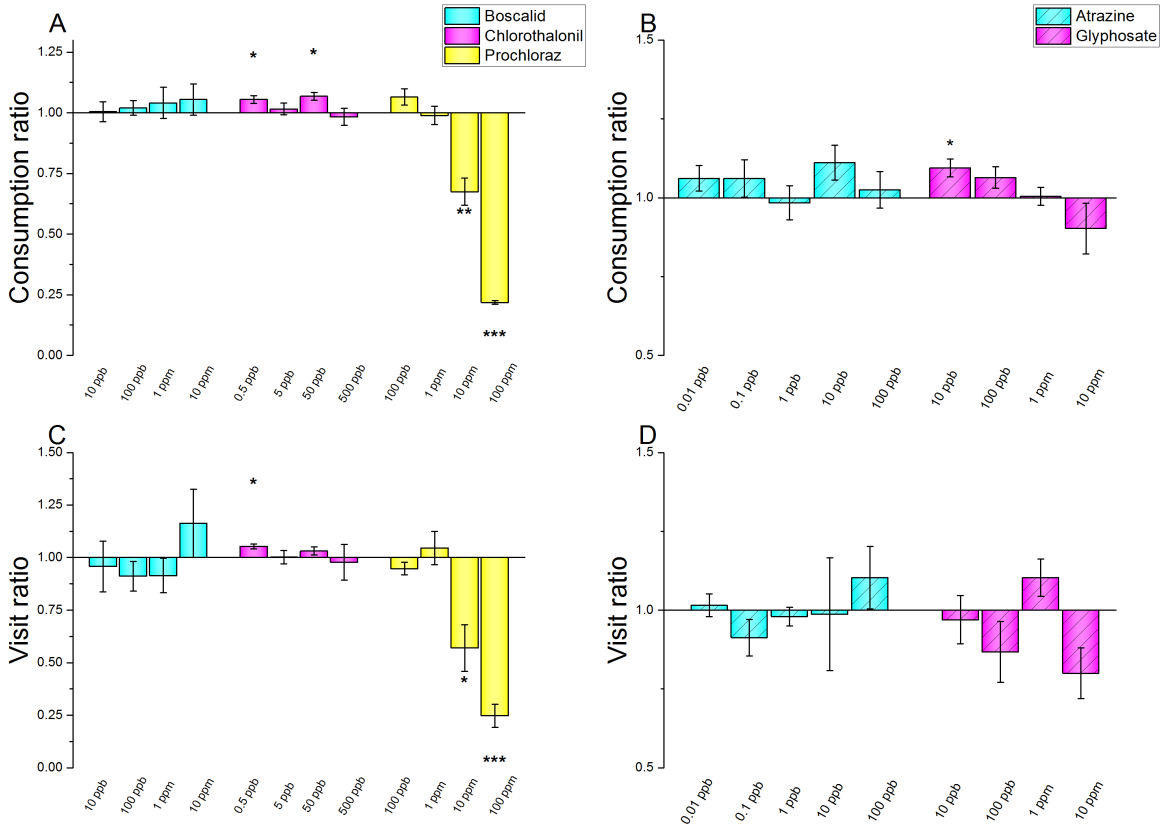

Figure 2.1. Ratios (mean ± SE) as preference indices of forager responses to selected synthetic xenobiotics, fungicides and herbicides. A) Consumption ratios for three fungicides-sugar water solutions in different concentrations. B) Consumption ratios for two herbicide-sugar solutions in different concentrations. C) Visitation ratios for three fungicide-sugar water solutions in different concentrations. D) Visitation ratios for two herbicide-sugar water solutions in different concentrations. A ratio higher than 1 indicates a preference for the test chemical, and a ratio lower than 1 indicates avoidance of the test chemical. The asterisks indicate the means are significantly different from 1 (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one sample *t*-test).

References

Adler L.S. (2000) The ecological significance of toxic nectar. Oikos **91**(3): 409-420.

Avarguès-Weber A., M.G. de Brito Sanchez, M. Giurfa, A.G. Dyer. (2010) Aversive reinforcement improves visual discrimination learning in free-flying honeybees. PLoS ONE **5**(10): e15370.

Ayestarán A., M. Giurfa, M.G. de Brito Sanchez. (2010) Toxic but drank: gustatory aversive compounds induce post-ingestional malaise in harnessed honeybees. PLoS ONE **5**(10): e15000.

Balbuena M.S.S., L. Tison, M.-L.L. Hahn, U. Greggers, R. Menzel, W.M. Farina. (2015) Effects of sublethal doses of glyphosate on honeybee navigation. J. Exp. Biol. **218**(Pt 17): 2799-2805.

Berenbaum M.R., R.M. Johnson. (2015) Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. **10**: 51-58.

Bermant G., N.E. Gary. (1966) Discrimination training and reversal in groups of honey bees. Psychon. Sci. **5**(5): 179-180.

Bernauer O.M., H.R. Gaines-Day, S.A. Steffan. (2015) Colonies of bumble bees (*Bombus impatiens*) produce fewer workers, less bee biomass, and have smaller mother queens following fungicide exposure. Insects **6**: 478-488.

Boily M., B. Sarrasin, C. Deblois, P. Aras, M. Chagnon. (2013) Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environ. Sci. Pollut. Res. Int. **20**(8): 5603-5614.

Couvillon M.J., H. Al Toufailia, T.M. Butterfield, F. Schrell, F.L. Ratnieks, R. Schürch. (2015) Caffeinated forage tricks honeybees into increasing foraging and recruitment behaviors. Curr. Biol. **25**(21): 2815-2818.

de Brito Sanchez M.G. (2011) Taste perception in honey bees. Chem. Senses **36**(8): 675-692.

de Sanchez M., M. Serre, A. Avarguès-Weber, A.G. Dyer, M. Giurfa. (2015) Learning context modulates aversive taste strength in honey bees. J. Exp. Biol. **218**(Pt 6): 949-959.

Gherman B.I., A. Denner, O. Bobiş, D.S. Dezmirean, L.A. Mărghitaş, H. Schlüns, R.F.A. Moritz, S. Erler. (2014) Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*. Behav. Ecol. Sociobiol. **68**(11): 1777-1784.

Hendriksma H.P., S. Shafir. (2016) Honey bee foragers balance colony nutritional deficiencies. Behav. Ecol. Sociobiol. **70**(4): 509-517.

Johnson R.M., L. Dahlgren, B.D. Siegfried, M.D. Ellis. (2013) Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS ONE **8**(1): e54092.

Johnson R.M., E.G. Percel. (2013) Effect of a fungicide and spray adjuvant on queen-rearing success in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. **106**(5): 1952-1957.

Kaškonienė V., P. Kaškonas, A. Maruška. (2015a) Volatile compounds composition and antioxidant activity of bee pollen collected in Lithuania. Chemical Papers **69**(2): 291-299.

Kaškonienė V., G. Ruočkuvienė, P. Kaškonas, I. Akuneca, A. Maruška. (2015b) Chemometric analysis of bee pollen based on volatile and phenolic compound compositions and antioxidant properties. Food Anal. Methods **8**(5): 1150-1163.

Kessler S.C., E.J. Tiedeken, K.L. Simcock, S. Derveau, J. Mitchell, S. Softley, A. Radcliffe, J.C. Stout, G.A. Wright. (2015) Bees prefer foods containing neonicotinoid pesticides. Nature **521**(7550): 74-76.

Kiljanek T., A. Niewiadowska, S. Semeniuk, M. Gaweł, M. Borzęcka, A. Posyniak. (2016) Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry-Honeybee poisoning incidents. J. Chromatogr. A **1435**: 100-114.

Köhler A., C.W.W. Pirk, S.W. Nicolson. (2012) Honeybees and nectar nicotine: deterrence and reduced survival versus potential health benefits. J. Insect Physiol. **58**(2): 286-292.

Liang Z.S., T. Nguyen, H.R. Mattila, S.L. Rodriguez-Zas, T.D. Seeley, G.E. Robinson. (2012) Molecular determinants of scouting behavior in honey bees. Science **335**(6073): 1225-1228.

Liu F., J. Chen, J. Chai, X. Zhang, I. X, H.E. D, D.W. Roubik. (2007) Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components. Funct. Ecol. **21**(1): 96-100.

Liu F., J. Gao, N. Di, L.S. Adler. (2015) Nectar attracts foraging honey bees with components of their queen pheromones. J. Chem. Ecol. **41**(11): 1028-1036.

Liu F.-L., X.-W. Zhang, J.-P. Chai, D.-R. Yang. (2006) Pollen phenolics and regulation of pollen foraging in honeybee colony. Behav. Ecol. Sociobiol. **59**(4): 582-588.

Liu F.L., W.J. Fu, D.R. Yang, Y.Q. Peng, X.W. Zhang, J.Z. He. (2004) Reinforcement of bee-plant interaction by phenolics in food. J. Apic. Res. **43**(4): 155-157.

Liu Y., F. Liu. (2010) Post-ingestive effect of plant phenolics on the feeding behaviour of the honeybee *Apis cerana*. Physiol. Entomol. **35**(2): 175-178.

Mao W., M.A. Schuler, M.R. Berenbaum. (2013) Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proc. Natl. Acad. Sci. USA **110**(22): 8842-8846.

Mao W., M.A. Schuler, M.R. Berenbaum. (2015) A dietary phytochemical alters caste-associated gene expression in honey bees. Sci. Adv. **1**(7): e1500795.

Mullin C.A., M. Frazier, J.L. Frazier, S. Ashcraft, R. Simonds, D. Vanengelsdorp, J.S. Pettis. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE **5**(3): e9754.

Page R.E., Jr., J. Erber, M.K. Fondrk. (1998) The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). J. Comp. Physiol., A **182**(4): 489-500.

Peng Y., S. Gillis-Smith, H. Jin, D. Tränkner, N.J. Ryba, C.S. Zuker. (2015) Sweet and bitter taste in the brain of awake behaving animals. Nature **527**(7579): 512-515.

Perry C.J., A.B. Barron. (2013) Honey bees selectively avoid difficult choices. Proc. Natl. Acad. Sci. USA **110**(47): 19155-19159.

Pettis J.S., D. vanEngelsdorp, J. Johnson, G. Dively. (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. Naturwissenschaften **99**(2): 153-158.

Robertson H.M., K.W. Wanner. (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. Genome Res. **16**(11): 1395-1403.

Rodríguez-Gironés M.A., A. Trillo, G. Corcobado. (2013) Long term effects of aversive reinforcement on colour discrimination learning in free-flying bumblebees. PLoS ONE **8**(8): e71551.

Seeley T.D. (1995) The Wisdom of the Hive: the Social Physiology of Honey Bee Colonies. Harvard University Press, Cambridge.

Singaravelan N., G. Nee'man, M. Inbar, I. Izhaki. (2005) Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. J. Chem. Ecol. **31**(12): 2791-2804.

vanEngelsdorp D., J.D. Evans, L. Donovall, C. Mullin, M. Frazier, J. Frazier, D.R. Tarpy, J. Hayes Jr, J.S. Pettis. (2009) "Entombed pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. J. Invertebr. Pathol. **101**(2): 147-149.

Wright G.A., D.D. Baker, M.J. Palmer, D. Stabler, J.A. Mustard, E.F. Power, A.M. Borland, P.C. Stevenson. (2013) Caffeine in floral nectar enhances a pollinator's memory of reward. Science **339**(6124): 1202-1204.

Wu J.Y., M.D. Smart, C.M. Anelli, W.S. Sheppard. (2012) Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to *Nosema* (Microsporidia) infection. J. Invertebr. Pathol. **109**(3): 326-329.

Wykes G.R. (1952) The preferences of honeybees for solutions of various sugars which occur in nectar. J. Exp. Biol. **29**(4): 511-519.

Xavier V.M., D. Message, M.C. Picanço, M. Chediak, P.A. Júnior, R.S. Ramos, J.C. Martins. (2015) Acute Toxicity and Sublethal Effects of Botanical Insecticides to Honey Bees. Journal of Insect Science **15**(1): 137.

Chapter 3. Impacts of dietary phytochemicals on honey bee longevity and detoxification capacity

Abstract

Because certain phytochemicals—notably, some flavonoids and phenolic acids-are almost invariably present in pollen irrespective of plant source, they are ubiquitous in the diet of *Apis mellifera*, the western honey bee. Just as folivorous insect species may evolve to rely on phytochemicals that are regularly encountered in their host plants for ecological and physiological functions, honey bees may also depend on some of these ubiquitous dietary phytochemicals, and their absence from the diet could have impacts on colony health. One such physiological function of phytochemicals is upregulation of detoxification enzymes, so their presence or absence may thus affect the toxicity of ingested xenobiotics. In order to clarify the impacts of common dietary phytochemicals on bees, we conducted a series of longevity assays with one-day-old adult honey bees to test if natural xenobiotics (phytochemicals from nectar) enhance honey bee worker longevity and detoxification capacity. One-day-old bees in the assay were maintained on a sugar syrup diet with or without supplemental casein as a phytochemical-free protein source and in the presence or absence two phytochemicals (quercetin and *p*-coumaric acid) as well as in the presence or absence of two pyrethroid pesticides, bifenthrin and β-cyfluthrin. Overall, according to the hazard ratio of the Cox model, diets extend life in the order casein>quercetin> *p*-coumaric acid>4 ppm bifenthrin>0.5 ppm β-cyfluthrin. Casein and two honey phytochemicals, the phenolic acid *p*-coumaric acid and the flavonol quercetin, contributed to prolonging the lifespan of bees. The presence of casein appears to eliminate the life-prolonging effect of *p*-coumaric acid when consumed without quercetin. Quercetin added to

the diet significantly enhanced tolerance of the two tested pyrethroids; *p*-coumaric acid had a similar effect trend although of considerably reduced magnitude. Collectively, these assays demonstrate that honey phytochemicals have a significant effect on honey bee longevity and stress resistance in the form of pesticide exposure; these findings suggest that certain apicultural practices that involve substituting sugar syrups for honey may have health impacts that have not previously been recognized.

Keywords:

honey bee, *Apis mellifera*, phytochemical, longevity, detoxification, nutrition

Introduction

Nectar and pollen, both raw and in their processed forms as honey and beebread, have long been considered as the principal natural sources of carbohydrate and protein, respectively, for honey bees. Contemporary beekeeping practices have led to the creation of substitutes or supplements for honey and pollen, notably sucrose or fructose for honey and soy flour diet for pollen (Brodschneider and Crailsheim 2010). However, phytochemicals clearly serve important functions beyond carbohydrate and protein nutrition for honey bees (Mao *et al.* 2009; Mao *et al.* 2013; 2015a) and their absence from dietary supplements or substitutes may have effects on honey bees that are as yet undetermined.

Among the phytochemicals present in honey from a diversity of nectar sources, the phenolic acid *p*-coumaric acid and the flavonol quercetin upon ingestion upregulate expression of a diversity of xenobiotic-metabolizing cytochrome P450 genes, including those encoding CYP9Q enzymes, in both adults and larvae (Mao *et al.* 2011; Mao *et al.* 2015b; Mao *et al.* submitted). When heterologously expressed in a baculovirus expression system, three members of the CYP9Q subfamily upregulated by quercetin, CYP9Q1, CYP9Q2, and CYP9Q3, metabolize quercetin as well as pyrethroid and organophosphate pesticides (Mao *et al.* 2011). In bioassays, Johnson *et al.* (2012) showed that quercetin can reduce toxicity of tau-fluvalinate, a broad-spectrum pyrethroid acaricide. Collectively, these findings strongly suggest that honey is more than a fuel source and that pollen is more than merely a protein source for the bees; the phytochemicals of honey and pollen appear to play an essential role in honey bee health, particularly in the presence of pesticides.

In addition to influencing detoxification capacity, phytochemicals may affect the lifespan of bees, as they are known to do in other organisms (Leonov *et al.* 2015; Si and Liu 2014).

Quercetin is an inducer of SirT1 (Davis *et al.* 2009; Dong *et al.* 2014; Lappalainen 2011), a member of the sirtuin family of proteins, considered as mediators of lifespan extension via the caloric restriction effect in many organisms (Wood *et al.* 2004). Honey bees (Rascón *et al.* 2012) and *Drosophila melanogaster* (Grandison *et al.* 2009; Lian *et al.* 2015) are among the insects known to exhibit the caloric restriction effect on lifespan (Altaye *et al.* 2010; Rascón *et al.* 2012); almost all known genes in the sirtuin family (i.e., *SirT1*, *Sir2*, *Sir4*, *Sir5*, *Sir6*, and *Sir7*) are represented in the honey bee genome. With respect to *p*-coumaric acid, Mao *et al*. (2015a) found that rearing larvae *in vitro* on a royal jelly diet with *p*-coumaric acid could reduce ovary development of adult bees. In view of the negative correlation between ovary development and survival rate in adult honey bees (Altaye *et al.* 2010), this finding suggests that consuming *p*-coumaric acid may increase survival and promote longevity, consistent with the caloric restriction effect. However, in at least one other study, higher vitellogenin concentrations and greater ovarian development in adult workers are correlated with increased longevity (Amdam *et al.* 2012; Corona *et al.* 2007). Thus, predicting the effects of consuming p-coumaric acid by adult workers is not straightforward.

As for quercetin, some circumstantial evidence links its presence to enhanced longevity in honey bees. Quercetin is a ubiquitous constituent of propolis, a hive sealant derived from plant resins that is typically rich in flavonols and other phenolics. In Brazil, Nicodemo *et al*. (2014)found that honey bee longevity is 6.6 % greater in hives with more propolis present; propolis typical of this region has been shown to be rich in quercetin, along with phenolic acids (Meneghelli *et al.* 2013).

To characterize the effect of phytochemicals in lifespan of honey bees, a series of longevity bioassays were carried out. One-day-old bees were provided with a sugar- casein

protein diet to standardize their nutrition. The sugar-casein protein diet was prepared with and without phytochemicals in four combinations (blank solvent control, *p*-coumaric acid, quercetin, and quercetin plus *p*-coumaric acid) to test the effects of these phytochemicals on longevity of honey bee workers. Because the absence of a queen may induce ovary development and egg-laying in workers, both of which affect longevity, we added a commercial queen pheromone strip to the cages to maintain workers in the sterile state. In addition, in order to determine whether phytochemicals alter the ability of honey bee workers to detoxify pesticides, an additional series of bioassays were conducted in which pesticides were added to the sugar/casein diets. Two pyrethroid insecticides, bifenthrin and β-cyfluthrin, were tested; both have been found as contaminants of wax, pollen, and bee bodies in North American hives (Mullin *et al*., 2010).

Materials and methods

Experimental insects

The experiment was conducted on western honey bees (*Apis mellifera*) kept in the apiary of the University of Illinois at Urbana–Champaign. In June 2016, three frames of capped brood were collected from a single naturally mated queen colony and then incubated in a dark room at 34°C to obtain newly emerged adult workers. These bees, collected within 24 hours of emergence, were introduced into small cages (9 oz./266 ml plastic cup with several ventilation holes and two feeding holes) in groups of 25 individuals. One-tenth of a strip of commercialized artificial queen mandibular pheromone (DC-715, Mann Lake Ltd., Hackensack, MN) was also introduced into each cage at the same time. Cages of newly emerged adult workers were prepared and randomly assigned evenly to three groups, a control and two pesticide treatment groups. The caged bees were immediately provided with water and diet, corresponding to their

treatment group. All bees used for this study were collected and prepared within a five-day period. One cage of bees in the control group was accidentally lost, so a total of 119 cages of 25 individual honey bee workers each were tested in this study.

Effects of dietary protein, phytochemicals and pesticides on longevity

During the experiment, the caged bees were kept in a dark room at 32.2°C with 50% relative humility. Each cage was equipped with a water feeder and a 50% (w/v) sucrose water-based diet feeder. The feeders were made by cutting a hole 6 mm in diameter on the top of a 2 ml micro-centrifuge tube. Bees could access water or food easily through the opening. Water was provided *ad libitum*; the water feeder was replaced every 5 days or whenever it appeared to be nearly empty. The diet feeders were replaced daily, just after the daily survival check of the caged bees. Approximately 1.5 ml sucrose water-based diet was used to fill each feeder in every cage; this amount was more than sufficient to feed all of the bees in each cage. The assay continued until all test subjects had died.

To determine the effects of pesticides on longevity, three types of amendments were made to the base diet: 4 ppm bifenthrin (N-11203, ChemService, Inc., West Chester, PA), 0.5 ppm β-cyfluthrin (N-11191, ChemService, Inc., West Chester, PA) and no amendments (control). The concentrations used for the tested pesticides concentration were based on the findings reported in Chapter 4. Within each pesticide treatment, two base diets were compared: protein-rich (protein:carbohydrate = 1:12, Altaye *et al*., 2010) and protein-free. Casein, an animal-derived protein product free from phytochemicals, was used here as a supplemental protein supplement, as it has been used in many insect artificial diets (Lee 2007). Within each base diet, phytochemical amendments were compared; these amendments included 0.5 mM *p*-coumaric acid (C9008, Sigma-Aldrich Co. LLC., St. Louis, MO) (PC), 0.25 mM quercetin (Q4951, Sigma-

Aldrich Co. LLC., St. Louis, MO) treatment (Qc), 0.5 mM *p*-coumaric acid and 0.25 mM quercetin-combined (PQ), and no phytochemicals (Control, CD). The phytochemical concentrations were based on previous work (Mao *et al.* 2015a). Consequently, there were 24 different treatments in each experimental replicate, and each treatment had five replicates (Table 1).

The protein-rich (casein) stock syrup was prepared by adding 25 g casein (C3400, Sigma-Aldrich Co. LLC., St. Louis, MO) into 600 g 50%(w/v) sucrose water. The pesticides and phytochemicals were first dissolved in dimethyl sulfoxide (DMSO; D128, Fisher Scientific International, Inc., Pittsburgh, PA) to make the 400× concentrated stock solutions. Finally, the sucrose water-based diets were prepared by adding 0.125 ml 400× phytochemical stock solutions into protein-free (casein$^-$) or protein-rich (casein) 50% syrups to make a total volume of 50ml. In addition, the unamended phytochemical-free control diet was prepared by adding 0.125 ml DMSO to protein-free or protein-rich 50% syrup for a volume of 50ml. As the result, all of the diets contained equal amounts of 0.25% DMSO.

Effects of dietary protein, phytochemicals and pesticides on diet consumption

Diet feeders were weighed individually before and after being made available to the bees to measure daily diet consumption. One additional cage, designated the evaporation cage, was established for each treatment in order to correct estimates of diet consumption for losses due to evaporation. The feeders in the evaporation cage were filled with the same tested syrup, to estimate as precisely as possible the evaporative characteristics of the diets being tested. The corrected lost weight of each feeder was then divided by the number of surviving bees in each cage to calculate diet consumption per bee per day.

Statistics

All statistical analyses were conducted using OriginPro 2016 software (OriginLab Corporation, Northampton, MA). The effects of treatment factors on bee survival were analyzed using the Cox proportional hazards model. Survival curves for each treatment group were obtained through the Kaplan-Meier estimator, and the difference between the curves was compared by the log rank test. The differences in the daily consumption per bee between treatment groups were analyzed by Kruskal-Wallis analysis of variance (ANOVA), and the post-hoc comparisons were performed by Mann-Whitney *U*-test with Bonferroni correction.

Results

Effects of dietary protein, phytochemicals and pesticides on survival

By the Cox proportional hazards models (Cox model) analysis on the pooled results of 2,975 caged honey bees, the survival analysis reveals that all tested experimental factors (protein, phytochemicals, and pesticides) affected the longevity of adult bees (Table 2 and Table 3). Overall, according to the hazard ratio of the Cox model, treatment diets affect longevity in a different level and in the order casein>quercetin> *p*-coumaric acid>4 ppm bifenthrin>0.5 ppm β-cyfluthrin. Casein, *p*-coumaric acid, and quercetin positively influenced longevity. Bees fed with casein experienced a lower hazard ratio of 0.739 ($\chi^2 = 66.306$, $P < 0.001$), as did bees fed with phytochemicals, with lower hazard ratios of 0.914 and 0.823 ($\chi^2 = 5.930$, $p = 0.015$ and $\chi^2 = 27.931$, $P < 0.01$) for *p*-coumaric acid and quercetin, respectively.

In contrast, two pyrethroid pesticides, bifenthrin and β-cyfluthrin, negatively affected worker longevity. Bifenthrin and β-cyfluthrin diets yielded higher hazard ratios of 9.171 and 1.345 ($\chi^2 = 1741.640$, $P < 0.001$ and $\chi^2 = 42.157$, $P < 0.001$), respectively, for the tested bees.

Effect of phytochemicals on longevity in the absence of pesticides

In the absence of pesticides (n=975)., the Cox model analysis reveals that protein and phytochemicals did not affect the longevity of adult bees. However, by making the PQ treatment as an independent covariance factor in the Cox model, all phytochemical treatments (PC, Qc, and PQ) enhanced the longevity of bees (PC: $\chi^2 = 9.76$, P=0.0018 < 0.01, hazard ratio = 0.75; Qc: $\chi^2 = 5.70$, P=0.017 < 0.05, hazard ratio = 0.80; PQ: $\chi^2 = 4.179$, P=0.04 < 0.05, hazard ratio = 0.83) but not the protein treatment ($\chi^2 = 3.07$, P=0.08). The results suggested the *p*-coumaric acid and quercetin together may have some synergistic effects. Regarding protein treatment, without pesticide stress, although casein supplementation did not extend longevity of bees by Cox model analysis, the cross-comparisons still show that casein supplementation enhanced longevity in some subgroups, such as in the phytochemical-free control subgroup (log rank test, $\chi^2 = 3.996$, $p = 0.046$), in the Qc subgroup (log rank test, $\chi^2 = 19.16$, P <0.001) and in the PC subgroup (log rank test, $\chi^2 = 7.10$, P=0.008<0.01) but not in the PQ subgroup.

p-Coumaric acid enhanced the longevity of bees (n=500) when they fed on the casein-free plus pesticide-free diet (Cox model, PQ not an independent covariable, $\chi^2 = 17.684$, $P < 0.001$, hazard ratio = 0.683). By the Kaplan-Meier estimator, bees consuming the casein-free diet survived longer in the presence of *p*-coumaric acid than in its absence (log rank test, $\chi^2 = 17.275$, $P < 0.001$), in the presence of quercetin than in its absence (log rank test, $\chi^2 = 17.767$, $P < 0.001$), and in the presence of both phytochemicals than in their absence (log rank test, $\chi^2 = 6.040$, $p = 0.014$) or in the presence of quercetin only (log rank test, $\chi^2 = 3.946$, $p = 0.047$) as well (Fig. 3.1A).

In contrast, when bees consumed a diet containing casein, the presence of phytochemicals in the diet did not have a significant effect on longevity in the Cox model. By the Kaplan-Meier

estimator, bees on pesticide-free casein-containing diet experienced the greatest longevity enhancement relative to the control diet with quercetin in the diet (log rank test, $\chi^2 = 7.444$, $p = 0.006$), with PC treatment (log rank test, $\chi^2 = 8.028$, $p = 0.005$) and PQ treatment (log rank test, $\chi^2 = 6.911$, $p = 0.009$) (Fig. 3.1B). However, diets containing both quercetin and *p*-coumaric acid did not experience longevity enhancement relative to those in the control treatment (log rank test, $\chi^2 = 0.236$, $p = 0.627$) or in the PC treatment (log rank test, $\chi^2 = 0.048$, $p = 0.827$) (Fig. 3.1B). Thus, while 0.25 mM quercetin in casein diet enhanced longevity of the caged bees, adding 0.5 mM *p*-coumaric acid may diminish the benefit of quercetin in a protein-rich diet.

Effect of phytochemicals on survival in the presence of pesticides

Casein supplementation improved the survival of caged honey bees in the presence of both pyrethroid pesticides; 4 ppm bifenthrin (n = 1,000; Cox model, $\chi^2 = 16.553$, $P < 0.001$, hazard ratio = 0.772) and 0.5 ppm β-cyfluthrin (n = 1,000; Cox model, $\chi^2 = 68.787$, $P < 0.001$, hazard ratio = 0.581). Further analyses revealed that quercetin improved survival (Cox model, $\chi^2 = 8.704$, $p = 0.003$, hazard ratio = 0.766) on the bifenthrin-containing casein diet group; on the β-cyfluthrin-containing casein-free diet group ($\chi^2 = 8.704$, $p = 0.003$, hazard ratio = 0.669) and the β- cyfluthrin-containing casein diet group ($\chi^2 = 16.603$, $P < 0.001$, hazard ratio = 0.692). However, the contribution of *p*-coumaric acid on lifespan of bees is trivial. It shows a positive effect on bifenthrin treated bees ($\chi^2 = 4.0318$, $P=0.0447 < 0.05$, hazard ratio = 0.881) but, further analysis of subgroups with casein and with casein-free diets, *p*-coumaric acid in the diet contributes only a positive trend that does not reach statistical significance.

Casein also had a positive effect on the longevity of bees consuming diets containing 4 ppm bifenthrin ($\chi^2 = 16.5529$, $P < 0.001$, hazard ratio = 0.7716). However, with further analysis by the Kaplan-Meier estimator, the presence of casein did not improve survival on diets

containing 4 ppm bifenthrin (log rank test, $\chi^2 = 1.724$, $p = 0.189$). Casein supplementation also did not have a detectable effect on longevity in any treatments containing *p*-coumaric acid (PC, casein- bifenthrin vs PC, casein bifenthrin; log rank test, $\chi^2 = 3.369$, $p = 0.066$). Conversely, supplementation with casein enhanced survival on all diets containing quercetin (Qc, casein-bifenthrin vs Qc, caseinbifenthrin; log rank test, $\chi^2 = 7.738$, $p = 0.005 < 0.05$) or containing both phytochemicals (PQ, casein-bifenthrin vs PQ, casein, bifenthrin; log rank test, $\chi^2 = 10.722$, $p = 0.001 < 0.05$).

On casein-free diets containing bifenthrin, phytochemical treatments had no significant effect on the survival curves (Fig. 3.2A). In contrast, on casein-supplemented diets containing bifenthrin, quercetin (Qc, casein, bifenthrin) enhanced survival over its CD subgroup (CD, casein, bifenthrin) (log rank test, $\chi^2 = 4.304$, $p = 0.038<0.05$), and its PQ subgroup (PQ, casein, bifenthrin) also showed prolonged survival over its CD subgroup (CD, casein, bifenthrin) (log rank test, $\chi^2 = 11.826$, $P < 0.001$) and its PC subgroup (PC, casein, bifenthrin) (log rank test, $\chi^2 = 6.405$, $p = 0.011$) (Fig. 3.2B).

Aside from the β-cyfluthrin-containing casein-free diet, bees in Qc treatments also experienced greater longevity than those in the control treatment (log rank test, $\chi^2 = 26.704$, $P < 0.001$) and PC treatment (log rank test, $\chi^2 = 24.937$, $P < 0.001$), and bees in the PQ treatment had better survival than those in the control treatment (log rank test, $\chi^2 = 4.039$, $p = 0.044$) (Fig. 3.3A). However, diets containing both phytochemicals yielded lower survival than diets containing quercetin alone (log rank test, $\chi2 = 13.020$, $P < 0.001$) (Fig. 3.3A), again demonstrating the adverse effects of the combination of *p*-coumaric acid plus 0.5 mM quercetin in a diet. With the β-cyfluthrin-containing casein diet, quercetin alone yielded enhanced longevity relative to the control (log rank test, $\chi^2 = 4.990$, $p = 0.026$) or diets containing both

phytochemicals (log rank test, $\chi^2 = 9.275$, $p = 0.002$); the diet containing both phytochemicals yielded greater longevity relative to bees on control diets (log rank test, $\chi^2 = 9.979$, $p = 0.002$) or diets containing *p*-coumaric acid alone (log rank test, $\chi^2 = 16.272$, $P < 0.001$) as well (Fig. 3.3B).

According to the Kaplan-Meier estimator, cross-comparisons between casein-free and casein-supplemented treatments in the presence of β-cyfluthrin revealed that bees consuming quercetin in casein-free diets (Qc, Casein$^-$, β-cyfluthrin) survived longer than bees consuming a casein-supplemented diet (Qc, Caseinβ-cyfluthrin) (log rank test, $\chi^2 = 0.029$, $p = 0.864$). Consuming diets supplemented with both quercetin and casein enhanced bee longevity to an even greater extent (Qc, caseinβ-cyfluthrin vs Qc, casein$^-$, β-cyfluthrin, log rank test, $\chi^2 = 5.095$, $p = 0.024 < 0.05$). Moreover, quercetin may reduce β-cyfluthrin toxicity; bees consuming diets containing both quercetin and β-cyfluthrin survived as well as those consuming unamended diets (QC, casein$^-$, β-cyfluthrin vs CD, casein$^-$, pesticide-free, log rank test, $\chi^2 = 1.483$, $p = 0.223$; QC, casein$^-$, β-cyfluthrin vs CD, casein, pesticide-free, log rank test, $\chi^2 = 0.774$, $p = 0.379$).

Effects of dietary protein, phytochemicals and pesticides on diet consumption

By Kruskal-Wallis ANOVA, pesticide amendment was the only factor with a significant effect ($\chi^2 = 10.255$, $p = 0.006$) on daily diet consumption per bee. Bees consuming the control diets lacking pesticides ingested less diet than did bees consuming diets containing bifenthrin (Mann-Whitney $U = 483$, $n_{control} = 39$, $n_{bifenthrin} = 40$, $p = 0.004$) and β-cyfluthrin (Mann-Whitney $U = 488$, $n_{control} = 39$, $n_{\beta\text{-cyfluthrin}} = 40$, $p = 0.004$); there was no significant difference between the two pesticide treatments (Mann-Whitney $U = 801$, $n_{bifenthrin} = n_{\beta\text{-cyfluthrin}} = 40$, $p = 0.996$) (Fig. 3.4). All other treatment factors or other subgroup combinations (e.g. phytochemical amendments to casein-free diet) did not have a significant effect in daily syrup consumption.

Discussion

Overall, the presence of a dietary protein prolongs the longevity of adult honey bees and the presence of pyrethroid pesticides reduces the longevity of honey bees. These findings are entirely consistent with past research (Brodschneider and Crailsheim 2010) and in and of themselves are not novel. What is novel, however, is the finding that two phytochemicals that are ubiquitous in the natural diet of honey bees can enhance longevity, despite the fact that they are not known to provide any strictly nutritional benefit. Quercetin ingestion also significantly enhanced tolerance of pyrethroids (bifenthrin, and beta-cyfluthrin) and survival rate of workers, as previously documented (Johnson *et al.* 2012).

In honey bees, quercetin is known to upregulate detoxification genes, including CYP9Q genes that detoxify pyrethroid pesticides (Mao *et al.* 2011; Mao *et al.* 2015b) and this effect may account for the protective effect against pesticides observed in this study. As well, as a powerful antioxidant (Boots *et al.* 2008), quercetin may reduce the toxic effects of pyrethroids by ameliorating the oxygen stress caused by pyrethroid pesticides (Banerjee *et al.* 2001; Dahlgren *et al.* 2012). With respect to longevity enhancement, in addition to its antioxidative properties, quercetin in honey bees may influence expression of potential longevity genes (sirtuin family) as it does in mammals (Davis *et al.* 2009; Dong *et al.* 2014; Lappalainen 2011) or expression of antioxidant enzymes associated with longevity as it does in other plant-feeding insects (Pritsos *et al.* 1988).

With respect to ameliorating pesticide toxicity, quercetin differs in its impact depending on the identity of the pesticide. On diets containing bifenthrin, quercetin yields a hazard ratio of 0.847 but the hazard ratio is only 0.6719 in β-cyfluthrin treatments. This apparent difference in efficacy may be a function of pesticide concentrations used in this study rather than toxicity per

se. The LC_{50} value for bifenthrin is 17 ppm as reported by Dai *et al*. (2010), which contrasts with the LD_{50} value of15 ng/bee reported by Mullin *et al*. (2010); in this study, bifenthrin was used at a concentration of 4 ppm (approximate 90.96 ng/bee/day). In contrast, β-cyfluthrin has a reported LD_{50} of 22 ng/bee (Mullin *et al.* 2010); β-cyfluthrin was used in this study at a concentration of 0.5 ppm (approximate 11.244 ng/bee/day). At this low level of stress, quercetin alone could rescue longevity even in the absence of dietary protein amendment.

Although *p*-coumaric acid amendment appeared to ameliorate effects of bifenthrin ingestion on longevity at the tested concentrations, the trend is not statistically significant. Notably, *p*-coumaric acid added to a diet lacking both casein and pesticide does significantly enhance longevity. In diets containing casein, however, the presence of *p*-coumaric acid may actually reduce longevity, although any such effect appears to be subtle. The mechanism underlying this apparent antagonism is open to speculation; given the fact that *p*-coumaric acid does upregulate a diversity of protein-encoding genes (Mao *et al.* 2013), its presence in the diet may alter protein utilization rates. Similarly, whereas diets lacking both pesticides and casein promote greater longevity when amended with both phytochemicals together, this effect of phytochemical amendment is not observed in diets containing casein. However, when pesticides are present in the diet, bees consuming diets containing both phytochemicals together generally experience greater longevity relative to bees on control diets.

Across all treatments, *p*-coumaric acid and quercetin, ubiquitous phytochemicals in the natural diet of honey bees, generally have a beneficial effect on honey bee longevity, particularly in the presence of pesticides. Notwithstanding, there is enough evidence of antagonistic interactions or negative effects that simply augmenting honey bee sugar substitutes with phytochemicals *ad libitum* is inadvisable without additional information on the mechanisms by

which phytochemicals can enhance longevity or ameliorate pesticide toxicity. The complexity of the social organization of honey bee colonies means that these phytochemicals may have effects that could operate only at the colony level. Mao *et al*. (2015a), e.g., reported that *p*-coumaric acid can alter expression of caste determination genes and Gao *et al*. (2010) found high concentrations of quercetin in diets may boost worker resistance to queen signals in the hive and lead to the production of laying workers. Clearly, these two phytochemicals, and possible other widely distributed constituents of pollens and nectars, have non-nutritive impacts on honey bee health that reflect the long evolutionary association between honey bees and flowering plants.

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Tables and Figures

Table 3.1. Summary of 24 different treatments

Protein	**Protein-free (casein)**				**Protein-rich (casein)**			
Phytochemical / Pesticides	Control (CD)	0.5 mM *p*-coumaric acid (PC)	0.25 mM quercetin (Qc),	0.5 mM *p*-coumaric acid and 0.25 mM quercetin (PQ)	Control (CD)	0.5 mM *p*-coumaric acid (PC)	0.25 mM quercetin (Qc),	0.5 mM *p*-coumaric acid and 0.25 mM quercetin (PQ)
Pesticide-free	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7	Treatment 8
β-Cyfluthrin	Treatment 9	Treatment 10	Treatment 11	Treatment 12	Treatment 13	Treatment 14	Treatment 15	Treatment 16
Bifenthrin	Treatment 17	Treatment 18	Treatment 19	Treatment 20	Treatment 21	Treatment 22	Treatment 23	Treatment 24

Table 3.2. Cox proportional hazards model analysis of effects of diet amendments on adult honey bee longevity

	df	Estimate	Standard error	χ^2	*P*	Hazard ratio
Casein	1	-0.30	0.04	66.31	0.000	0.739***
p-Coumaric acid	1	-0.09	0.04	5.93	0.015	0.914*
Quercetin	1	-0.20	0.04	27.93	0.000	0.823***
Bifenthrin	1	2.22	0.05	1741.64	0.000	9.171***
β-cyfluthrin	1	0.30	0.05	42.16	0.000	1.345***

All tested experimental factors (the casein, phytochemicals, and pesticides) affected the longevity of the honey bees. Casein, *p*-coumaric acid, and quercetin had positive effects on caged honey bee worker longevity (with hazard ratios <1). Two pyrethroid pesticides, bifenthrin and β-cyfluthrin, had negative effects on worker longevity (with hazard ratios >1.).
n=2,975 caged bees; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3.3. Summary of lifespan comparisons among honey bee workers consuming different diets by the Kaplan–Meier estimator and by the evaluation of Cox proportional hazards model

	Casein-free		**Casein-supplemented**
Overall		**<**[a]	
Pesticide-free diet[b]	PQ[c]=PC>CD=Qc	**=**	Qc>PC=CD=PQ
v			
β-cyfluthrin diet	Qc>PQ=PC≥ CD[d]	**<**	Qc=PQ>PC=CD
v			
Bifenthrin diet	PQ=Qc=PC=CD	**<**	PQ=Q>PC=CD

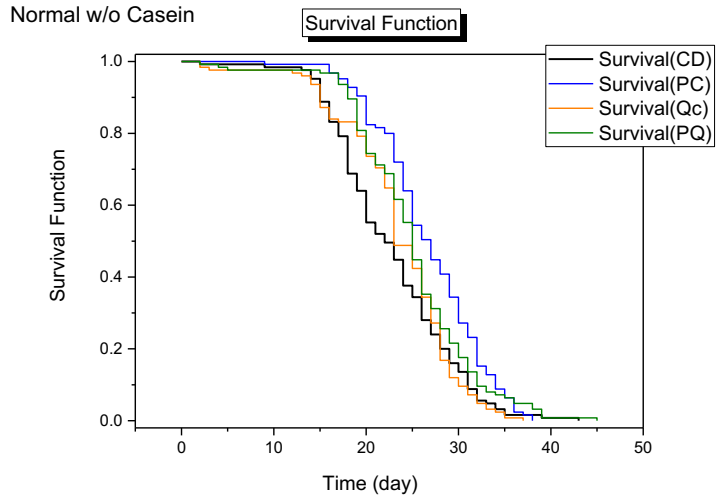
[a] The black bold and enlarged comparison symbols indicate the results obtained by Cox proportional hazards model. The blue comparison symbols in smaller fonts indicate the comparison results among phytochemical subgroups, as analyzed by Kaplan–Meier estimator and log rank test ('<' or '>' was regarded as statistically significant, $p < 0.05$; '=' indicated no significant difference)

[b] Pesticide treatment: Pesticide-free, 4 ppm bifenthrin or 0.5 ppm β-cyfluthrin; Casein treatment: casein-free, protein:carbohydrate =0:1; casein- supplemented, protein:carbohydrate = 1:12

[c] Phytochemicals subgroup: CD: 0.25% DMSO control syrup; PC: 0.5 mM *p*-coumaric acid; Qc: 0.25 mM quercetin; PQ: 0.5 mM *p*-coumaric acid and 0.25 mM quercetin-combined treatment.

[d] In this subgroup, according the analysis of Kaplan–Meier estimator and log rank test, PQ>CD and PC=CD.

A



B

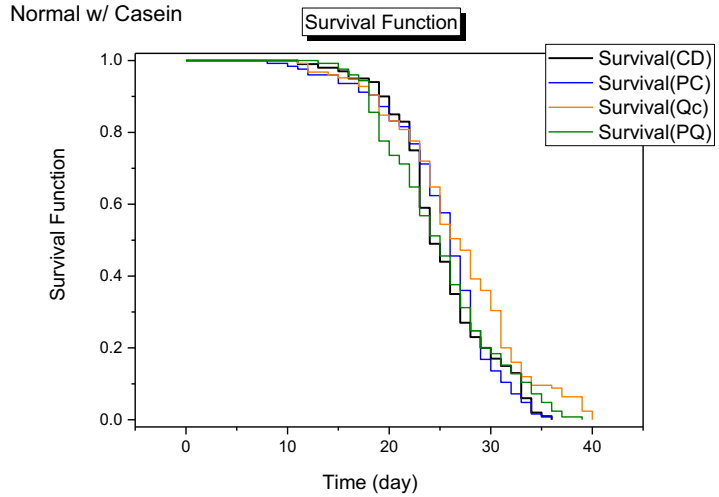
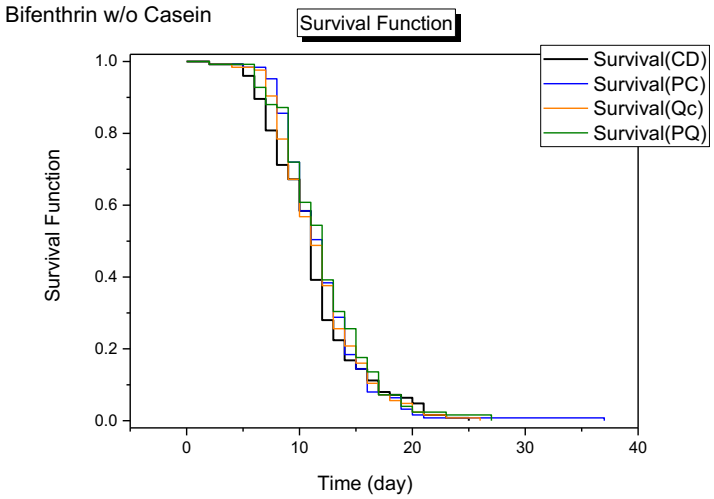


Figure 3.1. Kaplan–Meier plot of honey bee survival function on different diets with different phytochemical supplements. These diets were (A) protein-free or (B) protein-supplemented. CD, diet was phytochemical-free; PC, diet contained 0.5 mM *p*-coumaric acid; Qc, diet contained 0.25 mM quercetin; PQ, diet contained 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. (n = 100 for protein-rich and phytochemical-free diet group (subgroup CD in Fig. 3.1B), and n = 125 for the other groups.)

A



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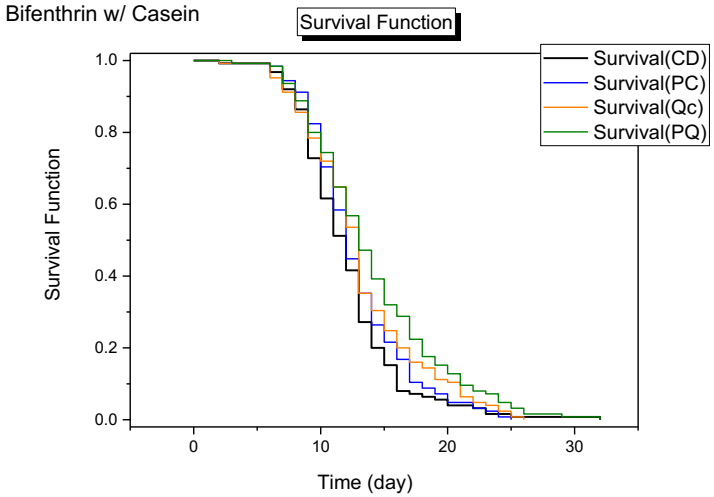
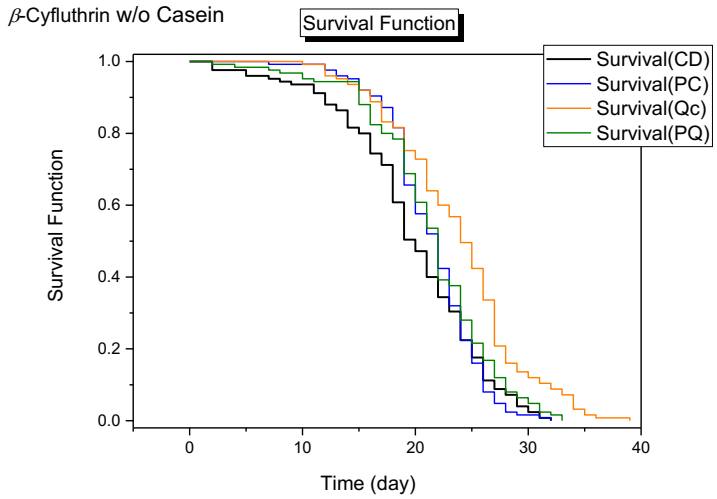


Figure 3.2. Kaplan–Meier plot of honey bee survival function on different diets with different phytochemical supplements and bifenthrin amendment. Theses diets were (A) protein-free or (B) protein-supplemented. CD, diet was phytochemical-free; PC, diet contained 0.5 mM *p*-coumaric acid; Qc, diet contained 0.25 mM quercetin; PQ, diet contained 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. (n = 125 for each group.)

A



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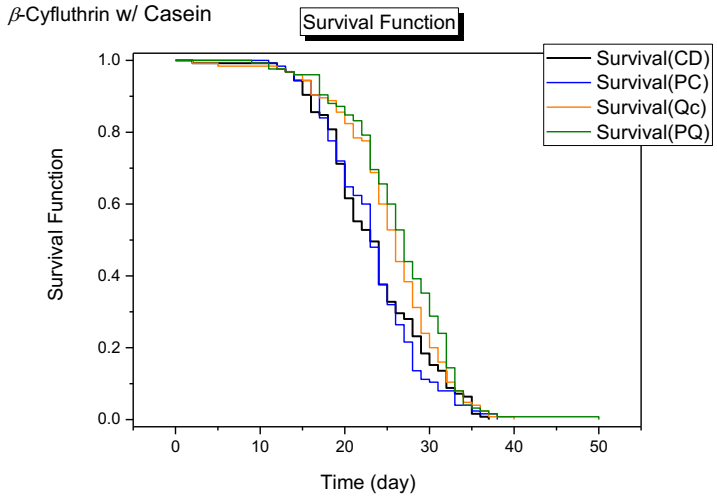

Figure 3.3. Kaplan–Meier plot of honey bee survival function on different diets with different phytochemical supplements and β–cyfluthrin amendment. These diets were (A) protein-free or (B) protein-supplemented. CD, diet was phytochemical-free; PC, diet contained 0.5 mM *p*-coumaric acid; Qc, diet contained 0.25 mM quercetin; PQ, diet contained 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. (n = 125 for each group.)

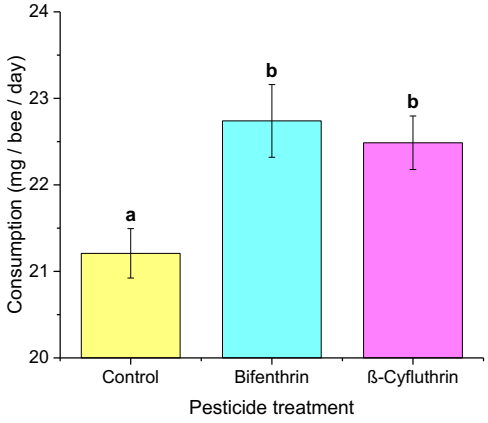

Figure 3.4. Mean ± SD milligrams of syrup diet consumption per bee per day over entire adult lifespan in cages containing different pesticide treatments. Different lower-case letters indicate significant differences (Kruskai-Wallis ANOVA, $\chi^2 = 10.255$, $p = 0.006$; post-hoc comparisons by Mann-Whitney *U*-test with Bonferroni correction, adjusted *alpha* = 0.017).

References

Altaye S.Z., C.W.W. Pirk, R.M. Crewe, S.W. Nicolson. (2010) Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. J. Exp. Biol. **213**(Pt 19): 3311-3318.

Amdam G.V., E. Fennern, H. Havukainen. (2012) Vitellogenin in honey bee behavior and lifespan, pp. 17-29. *In*: Galizia C.G., Eisenhardt D., and Giurfa M. (Eds.), Honeybee neurobiology and behavior. A tribute to Randolf Menzel, Springer.

Banerjee B.D., V. Seth, R.S. Ahmed. (2001) Pesticide-induced oxidative stress: perspectives and trends. Rev. Environ. Health **16**(1): 1-40.

Boots A.W., G. Haenen, A. Bast. (2008) Health effects of quercetin: from antioxidant to nutraceutical. Eur. J. Pharmacol. **585**(2-3): 325-337.

Brodschneider R., K. Crailsheim. (2010) Nutrition and health in honey bees. Apidologie **41**(3): 278-294.

Corona M., R.A. Velarde, S. Remolina, A. Moran-Lauter, Y. Wang, K.A. Hughes, G.E. Robinson. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc. Natl. Acad. Sci. USA **104**(17): 7128-7133.

Dahlgren L., R.M. Johnson, B.D. Siegfried, M.D. Ellis. (2012) Comparative toxicity of acaricides to honey bee (Hymenoptera: Apidae) workers and queens. J. Econ. Entomol. **105**(6): 1895-1902.

Dai P.L., Q. Wang, J.H. Sun, F. Liu, X. Wang, Y.Y. Wu, T. Zhou. (2010) Effects of sublethal concentrations of bifenthrin and deltamethrin on fecundity, growth, and development of the honeybee *Apis mellifera ligustica*. Environ. Toxicol. Chem. **29**(3): 644-649.

Davis J.M., E.A. Murphy, M.D. Carmichael, B. Davis. (2009) Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. Am. J. Physiol. Regul. Integr. Comp. Physiol. **296**(4): 7.

Dong J., X. Zhang, L. Zhang, H.X. Bian, N. Xu, B. Bao, J. Liu. (2014) Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPKα1/SIRT1. J. Lipid Res. **55**(3): 363-374.

Gao J., G. Zhao, Y. Yu, F. Liu. (2010) High concentration of nectar quercetin enhances worker resistance to queen's signals in bees. J. Chem. Ecol. **36**(11): 1241-1243.

Grandison R.C., R. Wong, T.M. Bass, L. Partridge, M.D.W. Piper. (2009) Effect of a standardised dietary restriction protocol on multiple laboratory strains of *Drosophila melanogaster*. PLoS ONE **4**(1): e4067.

Johnson R.M., W. Mao, H.S. Pollock, G. Niu, M.A. Schuler, M.R. Berenbaum. (2012) Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. PLoS ONE **7**(2): e31051.

Lappalainen Z. (2011) Sirtuins: a family of proteins with implications for human performance and exercise physiology. Res. Sports Med. **19**(1): 53-65.

Lee K.P. (2007) The interactive effects of protein quality and macronutrient imbalance on nutrient balancing in an insect herbivore. J. Exp. Biol. **210**(18): 3236-3244.

Leonov A., A. Arlia-Ciommo, A. Piano, V. Svistkova, V. Lutchman, Y. Medkour, V.I. Titorenko. (2015) Longevity extension by phytochemicals. Molecules **20**(4): 6544-6572.

Lian T., U. Gaur, D. Yang, D. Li, Y. Li, M. Yang. (2015) Epigenetic mechanisms of dietary restriction induced aging in *Drosophila*. Exp. Gerontol. **72**: 38-44.

Mao W., S.G. Rupasinghe, R.M. Johnson, A.R. Zangerl, M.A. Schuler, M.R. Berenbaum. (2009) Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp. Biochem. Physiol., B: Biochem. Mol. Biol. **154**(4): 427-434.

Mao W., M.A. Schuler, M.R. Berenbaum. (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. USA **108**(31): 12657-12662.

Mao W., M.A. Schuler, M.R. Berenbaum. (2013) Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proc. Natl. Acad. Sci. USA **110**(22): 8842-8846.

Mao W., M.A. Schuler, M.R. Berenbaum. (2015a) A dietary phytochemical alters caste-associated gene expression in honey bees. Sci. Adv. **1**(7): e1500795.

Mao W., M.A. Schuler, M.R. Berenbaum. (2015b) Task-related differential expression of four cytochrome P450 genes in honeybee appendages. Insect Mol. Biol. **24**(5): 582-588.

Meneghelli C., L.S.D. Joaquim, G.L.Q. Felix, A. Somensi, M. Tomazzoli, D.A. da Silva, F.V. Berti, M.B.R. Veleirinho, D.D.S. Recouvreux, A.C.D. Zeri, P.F. Dias, M. Maraschin. (2013) Southern Brazilian autumnal propolis shows anti-angiogenic activity: An *in vitro* and *in vivo* study. Microvasc. Res. **88**: 1-11.

Mullin C.A., M. Frazier, J.L. Frazier, S. Ashcraft, R. Simonds, D. Vanengelsdorp, J.S. Pettis. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE **5**(3): e9754.

Nicodemo D., E.B. Malheiros, D. De Jong, R.H.N. Couto. (2014) Increased brood viability and longer lifespan of honeybees selected for propolis production. Apidologie **45**(2): 269-275.

Pritsos C.A., S. Ahmad, S.M. Bowen, A.J. Elliott, G.J. Blomquist, R.S. Pardini. (1988) Antioxidant enzymes of the black swallowtail butterfly, *Papilio polyxenes*, and their response to the prooxidant allelochemical, quercetin. Arch. Insect Biochem. Physiol. **8**(2): 101-112.

Rascón B., B.P. Hubbard, D.A. Sinclair, G.V. Amdam. (2012) The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. Aging **4**(7): 499–508.

Si H., D. Liu. (2014) Dietary antiaging phytochemicals and mechanisms associated with prolonged survival. J. Nutr. Biochem. **25**(6): 581-591.

Wood J.G., B. Rogina, S. Lavu, K. Howitz, S.L. Helfand, M. Tatar, D. Sinclair. (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature **430**(7000): 686-689.

Chapter 4. Effects of a dietary phytochemical on honey bee foragers

4.1 Effects of a dietary phytochemical on fungicide suppression of flight performance in the honey bee *Apis mellifera*

Abstract

As a managed pollinator in agricultural fields, the honey bee *Apis mellifera* routinely encounters a broad diversity of pesticides, including fungicides, as contaminants of nectar and pollen. Because these pesticides are typically ingested by bees in their food, the potential exists for toxicological interactions between ingested pesticides and naturally occurring dietary phytochemicals. Boscalid is a fungicide that interferes with fungal energy production, specifically via inhibiting succinate dehydrogenase in the mitochondrial complex II. Quercetin, a polyphenolic flavonoid ubiquitous in pollen and honey, also may affect energy-linked mitochondrial metabolism. In this study, the effects of ingesting quercetin on levels of ATP in flight muscles of foragers as well as the effects of ingesting quercetin and boscalid together on forager flight performance were investigated. ATP levels in flight muscles of quercetin-treated foragers were higher than in foragers from a paired-control colony (37.46 ± 22.89 vs 10.29 ± 9.75 pmol/mg protein, respectively), as was the frequency of wing flapping (exercise intensity) (183.27 ± 2.93 Hz vs 171.65 ± 2.48 Hz). This finding confirms that consuming quercetin increases energy production per unit time and potentially facilitates faster flight. In contrast, foragers consuming boscalid alone exhibited the lowest frequency of wing flapping compared with paired hives treated with both boscalid and quercetin and a solvent-treated control hive (189.34 ± 2.36 Hz vs 201.31 ± 1.40 Hz vs 195.95 ± 1.82 Hz). Thus, consuming quercetin can

eliminate the adverse effects of boscalid on flight performance of foragers, a finding that reinforces the importance of naturally occurring phytochemicals in the diet of honey bees.

Keywords:

quercetin, boscalid, energy, ATP, flight performance

Introduction

Many fungicides target the basic cellular mechanisms of mitochondrial respiration (Yang *et al.* 2011). These include the new-generation common fungicides, such as succinate dehydrogenase inhibitors (SDHIs), which interfere with respiratory complex II (succinate dehydrogenase) in the mitochondrial electron transport chain. This enzyme complex is part of the adenosine triphosphate (ATP) production pathway and is shared among prokaryotic and eukaryotic organisms (Oyedotun and Lemire 2004); coupling of mitochondrial electron transport (in Complex I, III, and IV) and oxidative phosphorylation is crucial for energy production. Even partial inhibition of mitochondrial function can compromise growth and systemic responses to stress (Cloonan and Choi 2012; Galluzzi *et al.* 2012; Jones and Thompson 2007; Walker *et al.* 2014; West *et al.* 2011). Some subtle differences between plant and fungal mitochondrial respiration, such as the difference in sensitivity in some protein-binding sites, make this pathway an attractive target site for selective fungicides for control of plant pathogens.

Fungicides are usually considered safe for pollinators, with low acute toxicity to the western honey bee, *Apis mellifera*, in particular compared with other pesticides. In almond orchards, e.g., growers customarily spray fungicides during bloom (Atkins *et al.* 1981; Fell *et al.* 1983), which increases the likelihood that honey bees as managed pollinators in this crop system encounter them. Moreover, in bee hives and bee products, fungicides are among the most frequently encountered pesticide contaminants (Mullin *et al.* 2010; vanEngelsdorp *et al.* 2009). Despite expectations of selectivity, fungicides have been shown to have adverse effects on colonies including malnutrition, poor brood rearing, queen loss, reduced beneficial fungi, increased virus titers and elevated levels of infection by pathogenic microsporidian *Nosema* species (DeGrandi-Hoffman *et al.* 2013; DeGrandi-Hoffman *et al.* 2015; Pettis *et al.* 2013; Pettis

et al. 2012; Simon-Delso *et al.* 2014; Yoder *et al.* 2011a; Yoder *et al.* 2011b; Yoder *et al.* 2013; Zhu *et al.* 2014). Mussen (2008) reported high levels of mortality in the immature stages of honey bees during the weeks following the application of Pristine®--a formulated fungicide with two active ingredients, 25.2% boscalid (SDHI) and 12.8% pyraclostrobin (quinol oxidation inhibitor). The active ingredients inhibit respiratory subunits Complex II and Complex III (coenzyme Q),

Mortality of larvae associated with fungicide application, however, reportedly occurred only sporadically, which suggests that other factors might be involved. One possible factor that may contribute to sporadic mortality or to any acute adverse effects of respiratory inhibitor fungicides in bees (Campbell *et al.* 2016; DeGrandi-Hoffman *et al.* 2015; Johnson *et al.* 2013) is the relative rate of production and expenditure of energy in the mitochondria (Stammler *et al.* 2007; Yang *et al.* 2011). Although fungicides are known to target energy production pathways, in most studies to date honey bees are exposed while in a relatively static, resting state resulting from the use of small-cage feeding tests. The tested honey bees had little or no energy stress in such experiments. Metabolic rates from rest to flight can differ by 50-100-fold in insects (Beenakkers *et al.* 1984). Under little or no energy stress, honey bees in a resting state might overcome the acute disadvantage effect with certain alternative pathways. Because fungicides mainly inhibit energy production and respiration, the instantaneous energy demand in honey bees, for instance during flight, may be the key factor in the acute adverse effects of SDHI fungicides.

Flight is the most strenuous activity undertaken by insects (Harrison and Fewell 2002), requiring the highest rates of respiration as well as the highest rates of ATP hydrolysis in flight muscles (Candy *et al.* 1997; Wegener 1996). Therefore, flight activity can provide a useful metric for examining the effects of mitochondrial inhibitor fungicides on honey bees. Knowledge

of the effects of fungicides on energy production as well as flight in honey bees is limited. DeGrandi-Hoffman *et al.* (2015) found that bees fed with fungicide (Pristine®) -treated pollen had lower ATP concentrations in their flight muscles, which suggests fungicidal interference with mitochondrial respiration. Additionally, Campbell *et al.* (2016), in the first study associated with flight metabolic rates and fungicide exposure in honey bees, found that Pristine® inhibits mitochondrial function *in vitro* but does not affect CO_2 emission rates and thoracic temperatures during 1 min hovering flight in a small flight cage (8.5 cm 10.5 cm). These findings suggest that honey bees may have a means of protecting mitochondria against fungicide toxicity *in vivo* under these laboratory conditions. An alternative explanation is the existence of a compensatory energy-generating pathway in the honey bee during flight. Although both studies show that this fungicide has the potential to inhibit mitochondrial function, it is still not clear whether the resulting mitochondrial dysfunction affects flight ability in honey bees.

By consuming fungicides in contaminated food, honey typically ingest them in a matrix of the natural phytochemicals in their diet. Quercetin, a phytochemical ubiquitous in the honey bee diet, has demonstrable effects in a variety of systems on energy production that include reducing mitochondrial ATPase (Mao *et al.* submitted), inhibiting energy-linked reactions (Lang and Racker 1974), deterring ion-transport ATPases (Carpenedo *et al.* 1969) in cells *in vitro* and reducing ATP synthase activity (Chinnam *et al.* 2010; Dadi *et al.* 2009). In mammals, quercetin can increase brain and muscle mitochondrial biogenesis (Davis *et al.* 2009c), modulate mitochondrial functions in other tissues (Gibellini *et al.* 2015), escalate energy expenditures and increase exercise tolerance (Davis *et al.* 2009c). In humans, quercetin has also been utilized as a dietary supplement, particularly for athletes, due to its energy-boosting effect during exercise.

With respect to insects, at least one previous study suggests that quercetin ingestion can affect energy processing; in larval tobacco hornworms (*Manduca sexta*, Lepidoptera: Sphingidae), quercetin affects mitochondrial transhydrogenase (Vandock *et al.* 2012). More recently, RNA-Seq analyses of honey bee larvae (Mao *et al.* submitted) revealed that quercetin consumption leads to differential expression of mitochondrion-related genes, characterized by downregulation of the mitochondrial complex II and complex III genes. In view of the fact that both quercetin and SDHI fungicides individually have activity that alters ATP production in honey bees, there is a strong possibility that there may be additive or synergistic effects of consuming them together.

Because flight activity requires the preponderance of energy production in foraging bees, we hypothesized that quercetin might influence flight performance in bees due to its effect on cell respiration in flight muscles. We focused on the effects of oral consumption of quercetin and the common new-generation broad-spectrum SDHI fungicide boscalid on flight performance in honey bees. Boscalid, which has been used intensively since its registration in 2003 (Morton and Staub 2008), has been detected as a frequent contaminant in bee hives and in hive products that are consumed by bees (David *et al.* 2016; Mullin *et al.* 2010; Simon-Delso *et al.* 2014). As an inhibitor of the mitochondrial complex II (Stammler *et al.* 2007), it should directly affect production of ATP.

This study had two objectives. First, I quantified ATP production in flight muscles of foragers experiencing long-term exposure to quercetin and compared it to foragers exposed to a solvent control. I also set out to determine whether boscalid and quercetin synergistically affect the flight performance of bees. Several methods have been used for the study of insect flight behavior (Reynolds and Riley 2002). For laboratory studies, use of a flight mill is the most well

widely used and versatile technique (Attisano *et al.* 2015; Jones *et al.* 2016; Martí-Campoy *et al.* 2016). Krogh and Weis-Fogh (1952) first designed a roundabout, an umbrella-shaped device, for studying locusts flight. Chambers *et al*. (1976) subsequently modified the roundabout into a flight mill, which allowed measurement of flight behavior with a tethered flying insect in the laboratory. In honey bees, Luu *et al*. (2011) used four LCD monitors to design a virtual reality environment to study the flight of tethered bees. In this study, in order to standardize and evaluate flight performances of honey bees, a "flight treadmill" was designed, inspired by the system of Luu *et al*. (2011). The flight treadmill was constructed to compare the flight performance of foragers in the laboratory. Foragers were compared from four different treatment hives; a quercetin-treated hive, a boscalid-treated hive, a hive treated with both boscalid and quercetin, and a solvent-treated hive. Flight duration, flight frequency (wing flapping frequency) in real time, and total wing flapping were recorded and evaluated.

Materials and Methods

Experimental animals

Experiments were performed on western honey bees, *Apis mellifera*, kept in the University of Illinois apiaries near Urbana, IL, USA. The tested honey bees were collected from colonies split from the same colony in each experimental year. Initially, each tested colony was composed of 6,000 adult bees, one young larval brood frame and a newly introduced laying queen. These queens were naturally mated and were sisters. The test colonies were kept in separated and enclosed outdoor flight chambers (20 m × 3 m × 3 m), in the University of Illinois Bee Research Facility in Urbana, Illinois. Bees were restricted inside the chamber, where they were provided with water and 25% sucrose syrup feeders. Each colony was also provided with

pollen patties (dry pollen mix with 25% sucrose water, 2:1 (w/v)), which were placed on the top of frames and under the inner cover every week. Hives were checked regularly on a weekly basis to confirm general colony health. Honey bee foragers from each colony were trained to visit a syrup feeder, and foragers were caught as required for experiments.

Chemical treatment

Due to space limitation, two sets of colonies were tested in two consecutive years (2014 and 2015). The first set of experiments compared a quercetin-treated colony with a colony treated with solvent only (two colonies: control vs. quercetin), and the second set of experiments compared a boscalid-treated colony with a colony treated with both boscalid and quercetin and with a solvent-treated control colony (three colonies: control vs. boscalid vs. boscalid plus quercetin).

Quercetin (Q4951, Sigma-Aldrich, Milwaukee, WI) and boscalid (33875, Sigma-Aldrich, Milwaukee, WI) were dissolved in dimethyl sulfoxide (DMSO) (276855, Sigma-Aldrich, Milwaukee, WI) as stocks and kept at 4°C. Before use, the quercetin, boscalid, and boscalid-quercetin stock solutions as well as DMSO were incorporated into 25% sugar water into a final desired concentration (control: 0.25% DMSO; quercetin: 0.25 mM quercetin with 0.25% DMSO; boscalid: 10 ppm boscalid with 0.25% DMSO; boscalid with quercetin: 10 ppm boscalid with 0.25 mM quercetin and 0.25% DMSO). The sublethal concentration of boscalid was selected based on findings reported in Chapter 2 and in previous studies by other investigators (Johnson and Percel 2013; Mullin *et al.* 2010). The quercetin concentration was selected based on Mao *et al*. (submitted). Syrups were provided to each colony daily.

1. Effect of quercetin on foragers:

Foragers from a colony experiencing exposure to quercetin syrup (0.25 mM quercetin, 0.25% DMSO and 25% w/v sugar water) for six weeks as well as foragers from a hive fed only with solvent-syrup were collected at feeders during take-off. Each individual forager was positioned on the flight treadmill for flight ability tests. Details of the flight assay are provided in the section describing data collection and analysis of flight characteristics (vide infra).

During the flight test, each forager was fed 10 μL of 25% sucrose syrup with and without quercetin. The forager was then positioned to stand on a stick and subjected to a five-minute restoration period. Each forager was then entered in the flight treadmill arena again to test its flight ability. Two to four trials were conducted per honey bee to reduce inter-animal variability. Between each trial, the tested bee was also fed 10 μL of 25% sucrose syrup with and without quercetin and then held a five-minute restoration period. Wing-flapping frequencies and flight duration were recorded.

2. Effect of simultaneous boscalid - quercetin exposure:

Three hives were provided with three types of 25% syrup--solvent control, 10 ppm boscalid, or 10 ppm boscalid with 0.25 mM quercetin in 0.25% DMSO--for six weeks. Foragers were also collected from their feeder. Each individual forager was positioned on the flight treadmill for flight ability tests. The assay process was identical to that used in evaluating the flight performance of quercetin-treated foragers.

Measurement of proteins and ATP levels

Proteins and adenosine triphosphate (ATP) levels of the flight muscles of foragers were measured. Individual foragers were collected on the feeders when they finished feeding and immediately placed into liquid nitrogen. The thorax of each collected bee was placed on dry ice

and transferred to a 1.5 mL Eppendorf tube. The thoraces of three bees were ground together with an extraction buffer (5 mM Tris, pH 8.0, 20 mM EDTA, 0.5% Triton® X-100, 100 mM sodium orthovanadate) on ice. After grinding, the homogenates were placed in water at 95°C for one minute and returned to ice (Costa *et al.* 2013). The samples were centrifuged at maximum speed (20,238 × g) and the supernatant was collected to measure the protein and ATP levels in each sample. The total protein concentration of each sample was determined by the Bradford method (500-0201, Bradford Protein assay kit, Bio-Rad, Hercules, CA). The ATP level was measured with the CellTiter-Glo Luminescent Cell Viability Assay System (G7570, Promega Life Sciences, Madison, WI) according to the manufacturer's protocol. The bioluminescence signal was used to calculate ATP levels based on the ATP standard curve. Each experiment had a concurrent negative control, blank, and an ATP standard curve. The value expressed as picomoles ATP per milligram protein (pmol ATP /mg protein) was calculated from the ATP level to the total protein concentration of each sample. The ATP concentrations among treatments were compared by Mann–Whitney *U* test using OriginPro software (ver. 9.0, OriginLab Corporation, Northampton, MA).

Flight treadmill for testing the flight characteristics of foragers

A flight treadmill was set up to elicit steady flights of honey bee foragers. To elicit flight activity of insects, the tarsal reflex at the initiation of flight as well as visual stimuli and air flow stimuli during flight, were all needed. Two LCD monitors were arranged face-to-face in a vertical 36-degree V-shape arrangement to provide bees with visual and optical flow stimuli, from the lateral and ventral sides. Optical flow provided by each side monitor was a moving vertical black-and-white striped pattern generated by a custom-written LabVIEW program (National Instruments Corporation, Austin, TX); the horizontal spatial frequency of the pattern

was 0.016 cycles degree^{-1} and the speed of the optical flow was about 209.4 degrees s^{-1} as seen from the center point of the fixation position of the tested bee. Also, an in-line fan blower was set in front of the V-shaped monitors to provide a constant velocity air flow (3.6 m s^{-1}) stimulus. A metal rod was positioned vertically at ¾ of the longitudinal axis of the V-shaped "LCD valley". A small magnet was attached to the end of the rod. A bee equipped with a pre-glued iron plate was tethered to the magnet at the end of the rod. At this position, the tethered bee could perceive both visual and mechanical signal clues to stimulate flight. The tethered bees were then provided a 1.2-cm plastic stick to hold. Subsequently, the visual and air flow stimulus were triggered, then the stick was removed immediately from the honey bee, and the tarsal reflex initiated flight. During the flight treadmill tests, the room was set at a constant temperature (27℃) and relative humidity (50%).

Data collection and analysis of the flight characteristics

Each forager for the flight treadmill experiment was caught when it was feeding at a syrup feeder, placed in a vial and chilled down on ice for 15 minutes until it was incapable of moving and anesthetized. Hairs on the dorsal thorax of the bee were shaved using a razor blade, and a piece of carbon steel plate (2 × 2 × 0.127 mm^3) was glued on the middle of the notum of the bee using a cyanoacrylate adhesive (Loctite® Super Glue Gel, Henkel Corporation, Westlake, OH). Then the bee was set in a quiet humidified box for at least 30 minutes, considered as a restoration time-out, for recovery from stress before the first flight in the flight treadmill.

Without supplemental feeding, after a restoration time-out, the unstressed bee was placed on the flight treadmill for a depletion flight to exhaust her energy and stored sugar fuel. The "start" of a flight was designated as when continuous wing-flapping (> one minute) was triggered through a tarsal reflex. The "end" of a flight was designated as when the tested bee

stopped flapping wings and could not resume flight spontaneously and/or be triggered again in five successive attempts through triggering the tarsal reflex. Generally, a depletion flight required at least 20 minutes and could take as long as four hours. The experiments were conducted only on the individuals that could perform depletion flights lasting more than 20 minutes, in order to ensure that the tethering process and treatment did not harm the bee.

After the depletion flight, the tested bee was fed 10 μL of 25% sucrose syrup with the treatment chemical for its particular group. The bee was then positioned in the flight treadmill and allowed to hold on a stick to take a five-minute restoration rest. The stimulus of optic flow was turned off and the screens of the monitors were set to all-white during the resting period. After the five-minute restoration period, the stick was removed to trigger a tarsal reflex and the optic flow was started as well to induce another trial of flight of the bee. Three to five trials were conducted per honey bee to reduce inter-animal variability. Between each trial, the tested bee was also fed 10 μL of 25% sucrose syrup with the treatment chemical and then held a five-minute restoration period.

The flights were monitored with a webcam (Webcam C905, Logitech International S.A., Swiss), and the sound signal of wing-flapping was also monitored with the DL4YHF's Spectrum Lab (Audio Signal Analyzer, http://dl4yhf.darc.de/spectra1.html) in real-time. The sound of wing-flapping was recorded using a case-removed electric condenser microphone (standard 3.5 mm audio jacks, Creative Technology Ltd., Singapore) attached on the metal rod. The recorded signal was digitized at 32 kHz sampling rate and 16-bit depth by the built-in sound card in a laptop (Dell Latitude™ D620, Dell Inc., USA). The wing flapping frequency was estimated through measuring the fundamental frequency of the recorded humming sound. The fundamental frequency was measured every 0.1 second during a flight using custom-written LabVIEW

programs (National Instruments Corporation, USA). The searching band for the fundamental frequency was set between 0 to 320 Hz to avoid unnecessary interference from environmental high-frequency noise. Furthermore, because the sampling of the recording microphone was limited and the air blower fan produced a large amount of low-frequency noise, the measured fundamental frequencies equal to or lower than 50 Hz were also ignored. According to previous studies, the wingbeat frequency during honey bee flight varied from 122 Hz to 268 Hz, which can be affected by many factors, including temperature (Esch 1976), environment (Feuerbacher *et al.* 2003), age, and body mass (Vance *et al.* 2009). Generally, the wingbeat frequency of honey bee flight is reported at or near 200 Hz. Therefore, the duration of flight was calculated as the total time in a flight shown by the fundamental frequency to be higher than 50 Hz. The averaged wing flapping frequency was obtained from the average of the fundamental frequencies higher than 50 Hz, and the total wing flapping was calculated as one-tenths of a summation of the measured fundamental frequencies higher than 50 Hz during a flight. Data were tested for their normality, and equality of their means was compared by two-sample *t* test or by Mann–Whitney *U* test using the OriginPro software.

Results

In the ATP biochemical assay, the average value of ATP concentrations in flight muscles of foragers from the colony experiencing long-term dietary exposure to quercetin was 37.46 ± 4.67 pmol/mg protein (mean ± SE), which was ca. four times higher than concentrations from individuals from the solvent-control colony (10.29 ± 1.99 pmol/mg protein) (Fig. 4.1) ($U = 64$, $Z = -4.61$, $p = 4.06\text{E-}6 < 0.001$).

In the flight treadmill assays, typically, the wing flapping frequency of a forager speeds up from 0 Hz to her stable frequency in 5 to 20 seconds (Fig. 4.2A). After reaching stable wing flapping, the tested forager generally excreted within 1 to 3 minutes during flight. This could be due to her gut working on digesting the sugar water to fuel her flight or due to an effort to reduce her weight prior to taking flight. Foragers then remained in their stable wing flapping frequency (Fig. 4.2B) until they had exhausted their energy and stored sugar fuel. In the "end" of a flight, the tested bee stopped flapping wings and could not resume flight. The sound of wing flapping was not continuous (Fig. 4.2C). This wing flapping model characterized all bees in all tested groups. However, the mean values of wing flapping frequency were different between treatment groups. The foragers from the long-term dietary quercetin colony exhibited a higher wing flapping frequency (183.27 ± 2.93 Hz, mean ± SE) than did the foragers from the solvent control colony (171.65 ± 2.48 Hz, mean ± SE) (t = 3.026, df = 20, p = 0.007 < 0.01) in the same environment and with the same level of stimulation (Fig. 4.3A). However, the duration of each flight trial was not affected by long-term exposure to dietary quercetin (t = -1.196, df = 20, p = 0.246 > 0.05) (Fig. 4.3B), although there was a trend for foragers from the quercetin-treated colony to fly for a shorter period (1499.13 ± 46.65 s, mean ± SE) than did the foragers from the control colony (1589.61 ± 59.60 s, mean ± SE). Also, the total number of wing flaps per flight was not affected by the quercetin treatment (268592.86 ± 8365.25 vs 267909.08 ± 11280.10, mean ± SE; t = 0.049, df = 20, p = 0.962 > 0.05) (Fig. 4.3C).

In the boscalid-quercetin interaction assessment, foragers subjected to long-term dietary boscalid displayed reduced wing flapping frequencies (Boscalid: 180.85 ± 3.31 Hz; Control:190.98 ± 1.88 Hz, mean ± SE; Boscalid vs Control: U = 154, Z = 2.117, p = 0.034 < 0.05) (Fig. 4.4A). The boscalid-treated foragers displayed the lowest frequency of wing flapping

compared with foragers from the solvent control colony and the colony treated with boscalid plus quercetin (boscalid plus quercetin: 198.29 ± 2.04 Hz, mean ± SE). Consuming food containing both boscalid and quercetin appeared to increase wing-flapping frequency (boscalid plus quercetin vs. control: $U = 62$, $Z = -2.074$, $p = 0.038 < 0.05$; boscalid plus quercetin vs. boscalid: $U = 24$, $Z = -3.513$, $p = 4.426\text{E-}4 < 0.001$). These boscalid plus quercetin treated foragers displayed the highest wing flapping frequency among the three tested groups. Those on a solvent diet showed a mid-range wing-flapping frequency. In addition, neither the foragers from the colony treated with boscalid (1154.53 ± 46.34 s, mean ± SE) nor those from the colony treated with boscalid plus quercetin (1068.94 ± 27.92 s, mean ± SE) demonstrated any significant differences in terms of flight duration (control: 1154.53 ± 46.34 s) (Fig. 4.4B). However, I found that flight duration was significantly shorter in foragers that consumed both quercetin and boscalid than in those treated with boscalid only ($U = 164$, $Z = 2.553$, $p = 0.011 < 0.05$). Despite the difference in flight duration, the mean value of total number of wing flaps per flight was not affected by treatment with either boscalid or boscalid plus quercetin (Fig. 4.4C).

Discussion

This study is the first showing that boscalid exposure can have an adverse effect on flight ability of bees. Quercetin has been found to affect energy production and its related enzymes in *E. coli* (Chinnam *et al.* 2010; Dadi *et al.* 2009), in mammals(Davis *et al.* 2009c; Gibellini *et al.* 2015), and in other insects (Vandock *et al.* 2012). My study here demonstrated that quercetin has a positive effect on the energy production of honey bees. ATP concentrations in flight muscles in quercetin-treated foragers were significantly higher than those from the control colony, which suggests an effect of quercetin on mitochondria. The elevation of flight muscle ATP levels also

indicated that the quercetin-treated foragers had higher ATP stores in general and may also have had a higher metabolic rate. The results of this study of adult bees showed an opposite trend as compared with a study of the larval stage in honey bees performed by Mao *et al.* (submitted). They found out that dietary quercetin led to a difference in gene expression of mitochondria in honey bee larvae. The dietary quercetin resulted in down-regulation of complex II and complex III genes and overexpressed genes of some respiration-related enzymes. Their findings suggested that a lower mitochondrial function is expected when honey bee larvae are consuming a long-term quercetin diet. This opposite trend in the larval stage suggests that adult bees may have a compensatory pathway for modulation in energy metabolism. It is also important to note that the expression pattern of mitochondrial genes or respiration-related enzymes in adult honey bees on a quercetin diet may not be the same as in bee larvae. In both situations, it is conceivable that quercetin may influence the energy production and metabolism in different developmental stages of honey bees.

Insect flight is powered by ATP hydrolysis by aerobic metabolism. The high metabolic rates of insect flight are achieved by an effective control of ATP hydrolysis and regeneration (Wegener 1996). The fact that foragers from the quercetin-treated colony exhibited a higher average frequency of wing flapping suggests that the quercetin-treated foragers have a heightened energy production rate and may be capable of faster flight. In general, ATP concentration is under homeostasis (in a state of dynamic equilibrium) in cells (Atkinson and Walton 1967). For example, during flight, the ATP level of the migratory locust, *Locusta migratoria*, was not significantly affected (Wegener 1996). However, ATP storage in the flight muscles can provide sufficient energy for only a few seconds of flight. Most insects metabolize sugars as a means of powering the early stage of their flight but progressively turn to lipids as

fuel (Candy *et al.* 1997; Wegener 1996). In contrast, the flight of bees is entirely fueled by hexoses (Blatt and Roces 2001; Rothe and Nachtigall 1989).

This characteristic suggests that the power output of honey bees during flight mainly depends on the flux capacity of glycolytic and Krebs cycles enzymes in their flight muscles. Schippers *et al.* (2010) suggested that flight muscle metabolic development takes place early in the life of honey bees. Older foragers achieve increased flight metabolism by operating enzymes closer to the maximal flux capacity of related enzymes. It is conceivable that quercetin helps honey bees achieve increased flight intensity in a number of ways. First, quercetin may increase the amount of enzyme produced or enhance their maximum activity in younger bees. Second, quercetin may operate enzymes even closer to their maximum activity. More research is needed on the effects of quercetin on the physiological components of fight metabolism.

Boscalid is an SDHI, which inhibits complex II in the mitochondrial electron transport chain to kill fungi, has been shown to have adverse effects on pathogen respiration. Conceivably, dietary boscalid may have a negative effect on mitochondrial function in bees, which subsequently affects their flight ability. My study shows that boscalid exposure not only has effects *in vitro* or at a cellular level but also affects the flight performance of honey bees. I found that honey bees receiving dietary boscalid experienced a reduction in their wing-flapping frequencies. This reduction in flight performance, caused by boscalid, could be due to mitochondrial dysfunction in flight muscle but may also be a side effect of fungicide on digestion and gut function. Crailsheim (1988) has reported that honey bee workers held sugar water in their crop and sent it gradually to the midgut to absorb into the hemolymph and fuel their flight. Boscalid maybe contribute certain adverse effects on sugar absorption in the midgut. Moreover, DeGrandi-Hoffman *et al.* (2015) reported that Pristine®, which includes boscalid in its

active ingredient, can negatively affect feeding rates and protein digestion of honey bees, which may cause malnutrition in bees and consequently compromise their flight performance in the end.

Unexpectedly, those bees that consumed food containing both boscalid and quercetin appeared to display increased wing-flapping frequencies, which indicated that quercetin could have rescued honey bee foragers from the energy deficiencies caused by boscalid. These findings suggest that a compensatory change may exist in the mitochondria of bees that consumed boscalid-quercetin sugar water. However, *in vitro*, quercetin did not always support a positive effect on energetics in other systems, including isolated mammal mitochondria (Dorta *et al.* 2005; Trumbeckaite *et al.* 2006). *In vivo*, research findings are still mixed. In general, quercetin has positive effects. Quercetin is known to increase brain and muscle mitochondrial biogenesis as well as exercise tolerance and to rapidly enhance energy expenditure in rodents (Davis *et al.* 2009c). In humans, quercetin may influence exercise performance and muscle mitochondrial biogenesis (Kressler *et al.* 2011; Nieman *et al.* 2010). My results align with the *in vivo* studies that the flight performance of honey bees can be benefited by dietary quercetin. As well, they suggest that phytochemicals in the diet, such as quercetin, play a significant role in interacting with xenobiotics, such as boscalid, and hence influence exercise physiology of bees. Ketkar *et al.*(2015) have shown that pollen could relieve exercise-induced muscular stress in rats by improving impaired mitochondrial enzyme activity and down-regulating the myostatin expression. Moreover, it has also been suggested that quercetin could modulate some other pathways in mitochondria and cells (de Oliveira *et al.* 2016). These functions include not only energy-related oxidative respiration and ATP anabolism, but also other physiological functions such as anti-oxidation (Sikder *et al.* 2014; Silva *et al.* 2009), anti-inflammation (Boesch-Saadatmandi *et al.* 2012; Li *et al.* 2016; Middleton *et al.* 2000), mitochondrial biogenesis (Davis

et al. 2009c; de Oliveira *et al.* 2016; Nieman *et al.* 2010), mitochondrial membrane potential, intra-mitochondrial redox status(de Oliveira *et al.* 2016), and apoptosis (de Oliveira *et al.* 2016; Gibellini *et al.* 2015; Rayalam *et al.* 2008). Being able to modulate the metabolic pathways of quercetin may also protect mitochondria from damage caused by fungicides or other toxicants. Markham *et al.* (1987) reported that quercetin reduce the mortality and cytotoxic effect of T-2 mycotoxin in mice. In honey bees, Johnson *et al.* (2012) also observed that quercetin can diminish the toxicity of a pyrethroid miticide, tau-fluvalinate..

It is also worth noting that a high wing-flapping frequency of the quercetin-treated bees does not mean that the bees would necessarily fly faster or more efficiently during foraging trips in the field. In an additional test (data not shown here), I examined the duration of the foragers' trips between the hive and the feeder. The marked bees from the quercetin-treated hive and the control hive in the outdoor free flight chamber did not show a significant difference in their average foraging trip duration. The heightened wing flapping frequency of the quercetin-treated foragers in the flight treadmill was not paired with a similar increase in flight speed (shorter round trip durations) in the flight chamber. However, the distance between the feeder and the hive was about 20 meters. The average flight speed of honey bees is 3.3-5.1 $m \cdot s^{-1}$(Nachtigall *et al.* 1995) in a wind tunnel, which means that, in this outdoor flight chamber test, foragers could readily fly between the hive and the feeders in a few seconds. The distance may be too short to identify the differences in foraging trip duration or individual forager speed.

Additionally, the level of wing-flapping frequency of a forager is a neuronal feedback response to the stimulus of optical flow, airflow, and antennal position (Khurana and Sane 2016). The wing-flapping frequency response could be influenced by their previous flight experiences and their genomic background. In this study, all tested hives had almost the same foraging

environment. The effects of experience on wing-flapping frequency for these tested colonies were controlled, but possible effects of genomic background were not controlled. Although free-flight assays represent an improvement on small-cage studies, tests with freely-flying bees in a real field situation are optimal for disentangling possible confounding factors.

My findings also show that the duration per flight trial was shorter in bees that consumed both quercetin and boscalid compared with boscalid-treated foragers. This reduction in flight duration may reflect a trade-off between flight activity intensity (wing-flapping frequencies) and flight duration. Previous studies in other systems, however, have not consistently documented an effect of quercetin on the duration and intensity of exercise (Davis *et al.* 2009b; Kressler *et al.* 2011). Quercetin appeared to have a positive effect on mitochondrial oxidative respiration and ATP anabolism function but had a mixed, i.e., positive (Davis *et al.* 2009a; MacRae and Mefferd 2006; Nieman *et al.* 2010) and negative (Bigelman *et al.* 2010; Cheuvront *et al.* 2009; Cureton *et al.* 2009; Utter *et al.* 2009), effect on endurance exercise capacity. Additionally, Kressler *et al.* (2011) reported that, across different previous studies, quercetin provided a statistically significant positive effect on exercise performance but provided only a small benefit in endurance exercise performance of humans. The authors also stated that other factors may be associated with the effect of quercetin on endurance exercise performance in mammals.

Even though quercetin, boscalid and their synergistic interaction change the flight intensity level, the total number of wing flaps per flight was not affected by the quercetin, boscalid or the combined treatment. These results demonstrated that quercetin or boscalid did not change the utilization of sugar and hence did not improve flight endurance. This result is consistent with previous findings that, unlike other insects, honey bees store limited fat or glycogen (Panzenböck and Crailsheim 1997) and mainly depend on sugar in their digestive tract

for energy during flight (Blatt and Roces 2001). Thus, the same amount of sugar can fuel an equal amount of energy for flight and will not be affected by quercetin. Moreover, because fungicides could be applied in blooming season, the high concentrations of fungicides could simply pass through the body wall of foragers without being absorbed through the gut wall. DeGrandi-Hoffman *et al.* (2013) have shown that concentrations of boscalid and pyraclostrobin are much lower in bees than in their pollen diets, indicating that metabolic detoxification of fungicides might have occurred in the gut and caused a lower fungicide concentration in bees. Moreover, Campbell *et al.* (2016) have shown that Pristine® at levels of five ppm could strongly inhibit mitochondria *in vitro*, which implies that the concentration of fungicide during field application could affect bees. Therefore, the adverse effects of fungicide in honey bees may be noticeably more severe than expected in certain natural circumstances. In typical agricultural settings, foragers may benefit from the fact that they are exposed to fungicides along with nectar and pollen rich in quercetin and other phytochemicals, which is why they do not show signs of being affected by fungicide to the extent observed in the present study.

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Figures

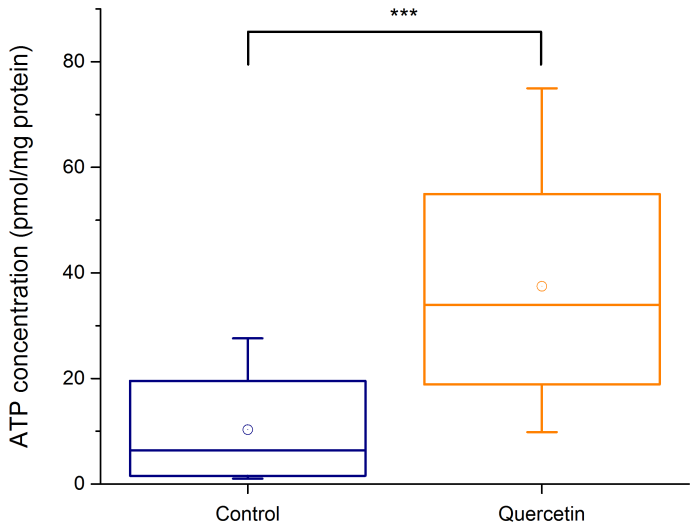

Figure 4.1. Box plots showing the level of ATP in the flight muscle of foragers from the quercetin-treated and control colony. The circle with a central point indicates the mean. The middle line of box shows the median value, the box delimits the 25th and 75th percentiles. The ends of the whiskers indicate the minimum and maximum of all of the data. The asterisk indicates significant difference between the two means (***: $p < 0.0001$, Mann–Whitney *U* test).

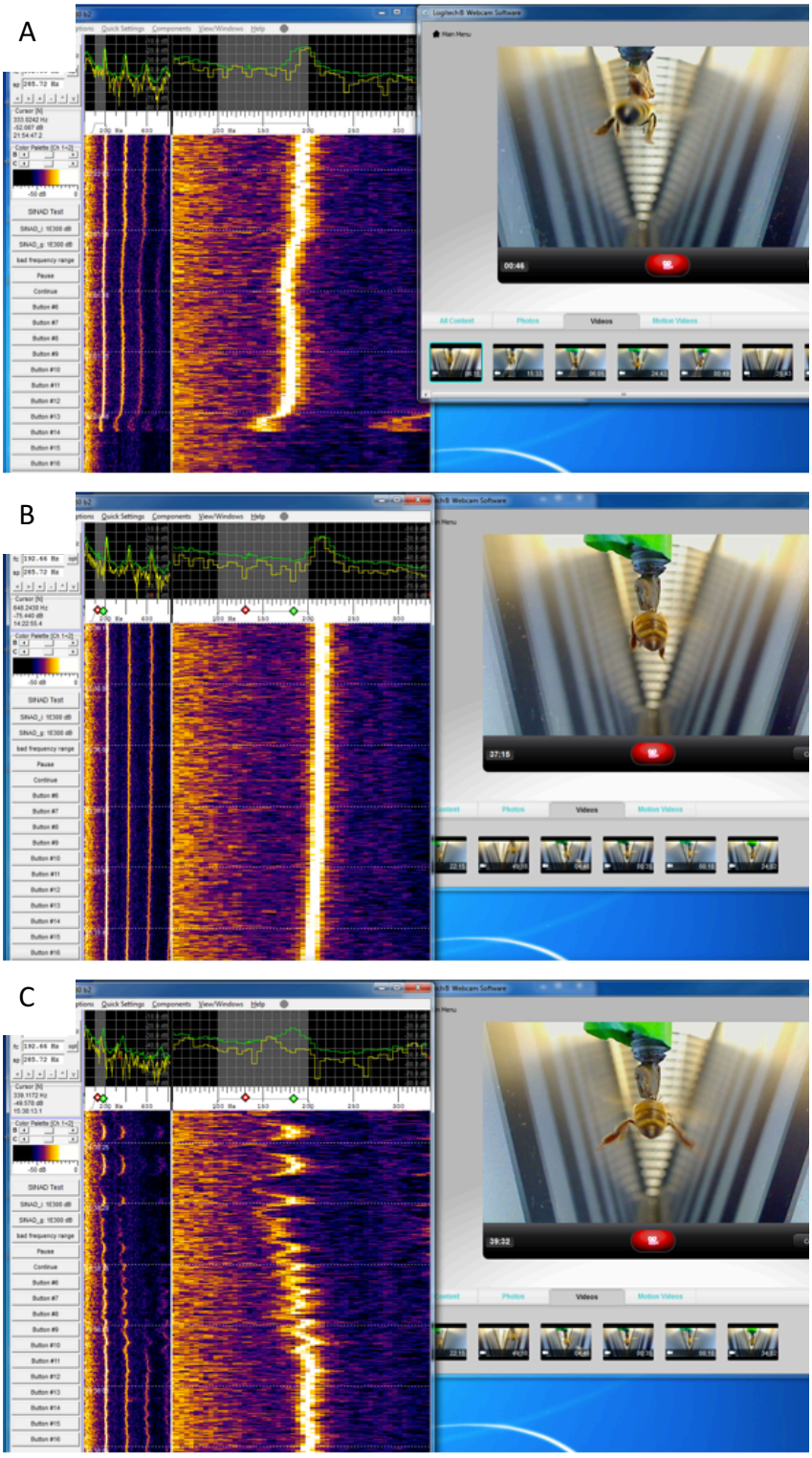

Figure 4.2. A typical flight treadmill assay in different flight stages. The left column represents wing- flapping frequency signal pattern in real time. The right column shows monitored image of bees on the flight treadmill arena. A) the beginning of flight stage. B) the stable flight stage. C) the end of flight stage.

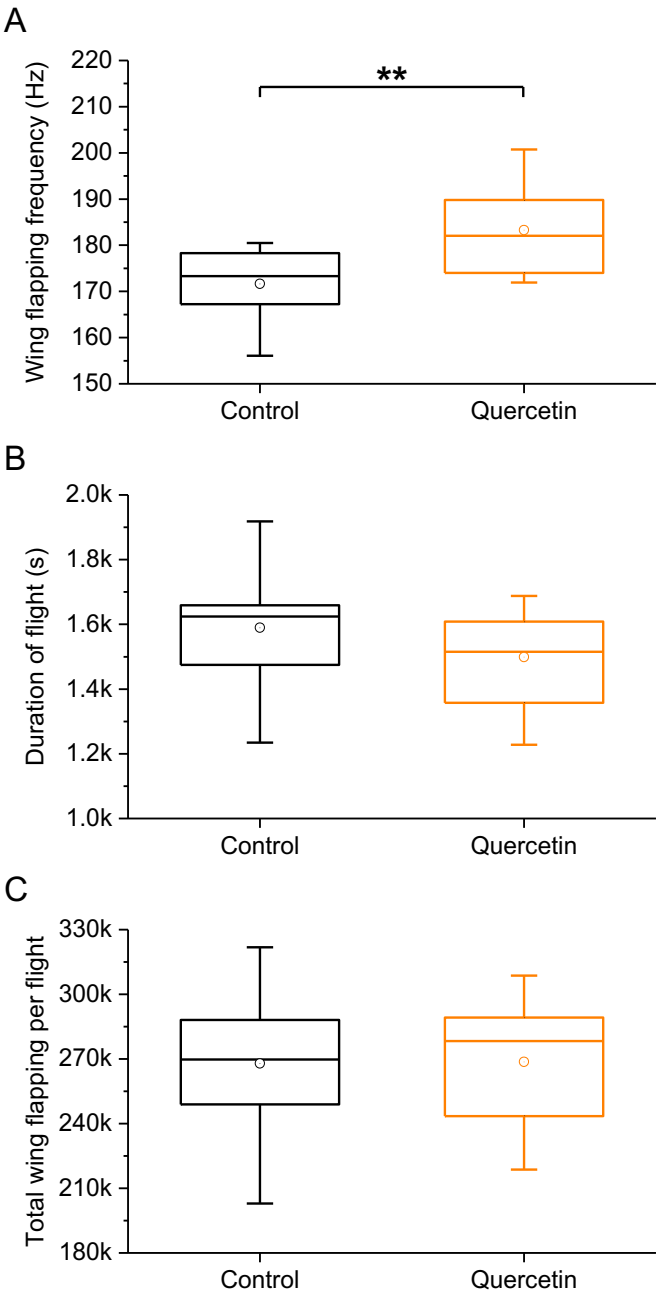

Figure 4.3. The effect of quercetin-treated foragers on the flight performance of foragers. A) the wing flapping frequency performance. B) duration of flight performance. C) total wing flapping per flight performance. In all charts (A, B and C), the circle with a central point indicates the mean. The middle line of box shows the median value, the box delimits the 25th and 75th percentiles. The ends of the whiskers indicate the minimum and maximum of all of the data. The asterisk indicates significant difference between the two means (**: $p < 0.01$, two-sample *t*-test).

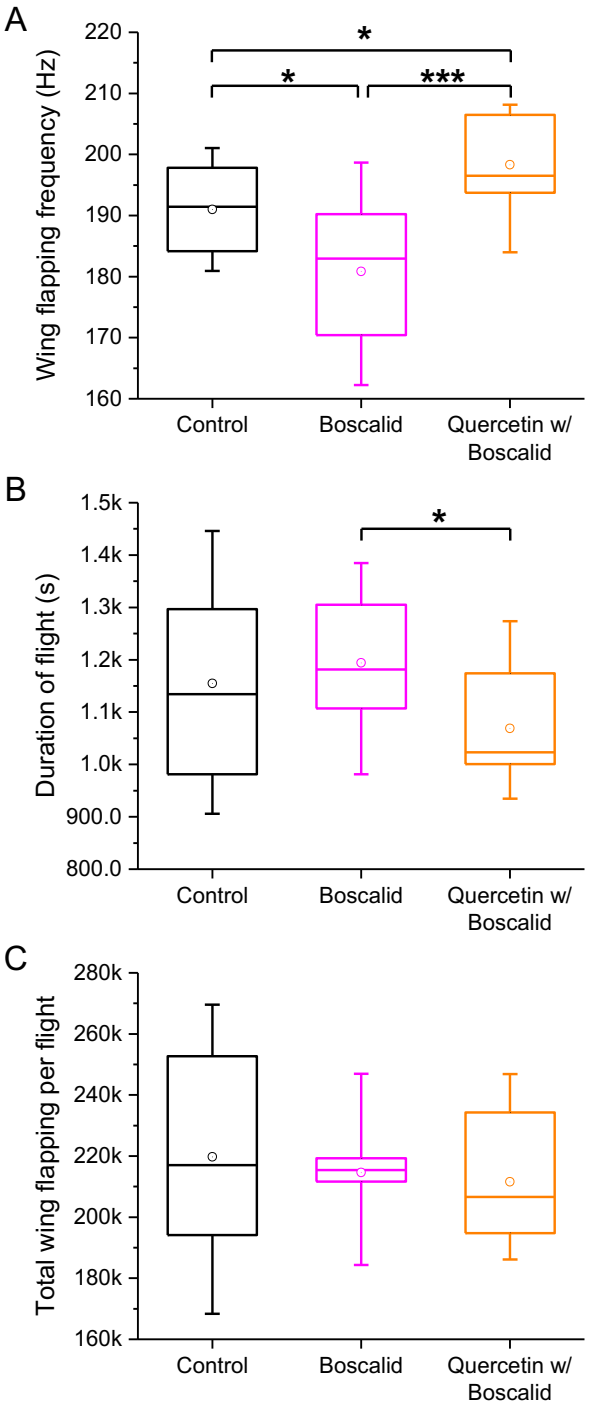

Figure 4.4. The effect of dietary boscalid, quercetin and their synergistic interaction on the flight performance of foragers. A) the wing-flapping frequency performance. B) duration of flight performance. C) total wing-flapping per flight performance. In all charts (A, B and C), the circle with a central point indicates the mean. The middle line of box shows the median value, the box delimits the 25th and 75th percentiles. The ends of the whiskers indicate the minimum and maximum of all of the data. The asterisk indicates significant difference between the two means (*: $p < 0.05$, ***: $p < 0.0001$, Mann–Whitney U test).

References

Atkins E.L., D. Kellum, K. Atkins. (1981) Reducing Pesticide Hazards to Honey Bees: Mortality Prediction Techniques and Integrated Management Strategies. Division of Agricultural Sciences, University of California, CA, USA.

Atkinson D.E., G.M. Walton. (1967) Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. **242**(13): 3239-3241.

Attisano A., J.T. Murphy, A. Vickers, P.J. Moore. (2015) A simple flight mill for the study of tethered flight in insects. J. Vis. Exp. **106**: e53377.

Beenakkers A.M.T., D.J. Van der Horst, W.J.A. Van Marrewijk. (1984) Insect flight muscle metabolism. Insect Biochem. **14**(3): 243-260.

Bigelman K.A., E.H. Fan, D.P. Chapman, E.C. Freese, J.L. Trilk, K.J. Cureton. (2010) Effects of six weeks of quercetin supplementation on physical performance in ROTC cadets. Mil. Med. **175**(10): 791-798.

Blatt J., F. Roces. (2001) Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): dependence on metabolic rate and *in vivo* measurement of maximal rates of trehalose synthesis. J. Exp. Biol. **204**(Pt 15): 2709-2716.

Boesch-Saadatmandi C., A.E. Wagner, S. Wolffram, G. Rimbach. (2012) Effect of quercetin on inflammatory gene expression in mice liver *in vivo* – role of redox factor 1, miRNA-122 and miRNA-125b. Pharmacol. Res. **65**(5): 523-530.

Campbell J.B., R. Nath, J. Gadau, T. Fox, G. DeGrandi-Hoffman, J.F. Harrison. (2016) The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. J. Insect Physiol. **86**: 11-16.

Candy D.J., A. Becker, G. Wegener. (1997) Coordination and integration of metabolism in insect flight. Comp. Biochem. Physiol., B: Biochem. Mol. Biol. **117**(4): 497-512.

Carpenedo F., C. Bortignon, A. Bruni, R. Santi. (1969) Effect of quercetin on membrane-linked activities. Biochem. Pharmacol. **18**(6): 1495-1500.

Chambers D.L., J.L. Sharp, T.R. Ashley. (1976) Tethered insect flight: A system for automated data processing of behavioral events. Behav. Res. Meth. Instrum. **8**(4): 352-356.

Cheuvront S.N., B.R. Ely, R.W. Kenefick, B.B. Michniak-Kohn, J.C. Rood, M.N. Sawka. (2009) No effect of nutritional adenosine receptor antagonists on exercise performance in the heat. Am. J. Physiol. Regul. Integr. Comp. Physiol. **296**(2): R394-R401.

Chinnam N., P. Dadi, S. Sabri, M. Ahmad, M. Kabir, Z. Ahmad. (2010) Dietary bioflavonoids inhibit *Escherichia coli* ATP synthase in a differential manner. Int. J. Biol. Macromol. **46**(5): 478-486.

Cloonan S.M., A.M.K. Choi. (2012) Mitochondria: commanders of innate immunity and disease? Curr. Opin. Immunol. **24**(1): 32-40.

Costa A.C., S.H. Loh, L.M. Martins. (2013) *Drosophila Trap1* protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. Cell Death Dis. **4**: e467.

Crailsheim K. (1988) Regulation of food passage in the intestine of the honeybee (*Apis mellifera* L.). J. Insect Physiol. **34**(2): 85-90.

Cureton K.J., P.D. Tomporowski, A. Singhal, J.D. Pasley, K.A. Bigelman, K. Lambourne, J.L. Trilk, K.K. McCully, M.J. Arnaud, Q. Zhao. (2009) Dietary quercetin supplementation is not ergogenic in untrained men. J. Appl. Physiol. **107**(4): 1095-1104.

Dadi P.K., M. Ahmad, Z. Ahmad. (2009) Inhibition of ATPase activity of *Escherichia coli* ATP synthase by polyphenols. Int. J. Biol. Macromol. **45**(1): 72-79.

David A., C. Botías, A. Abdul-Sada, E. Nicholls, E.L. Rotheray, E.M. Hill, D. Goulson. (2016) Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. Environ. Int. **88**: 169-178.

Davis J.M., C.J. Carlstedt, S. Chen, M.D. Carmichael, E.A. Murphy. (2009a) The dietary flavonoid quercetin increases VO(2max) and endurance capacity. Int. J. Sport Nutr. Exerc. Metab. **20**(1): 1-13.

Davis J.M., E.A. Murphy, M.D. Carmichael. (2009b) Effects of the dietary flavonoid quercetin upon performance and health. Curr. Sports Med. Rep. **8**(4): 206-213.

Davis J.M., E.A. Murphy, M.D. Carmichael, B. Davis. (2009c) Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. Am. J. Physiol. Regul. Integr. Comp. Physiol. **296**(4): 1071–1077.

de Oliveira M.R., S.M. Nabavi, N. Braidy, W.N. Setzer, T. Ahmed, S.F. Nabavi. (2016) Quercetin and the mitochondria: A mechanistic view. Biotechnol. Adv. **34**(5): 532-549.

DeGrandi-Hoffman G., Y. Chen, R. Simonds. (2013) The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). Insects **4**(1): 71-89.

DeGrandi-Hoffman G., Y. Chen, E. Watkins Dejong, M.L. Chambers, G. Hidalgo. (2015) Effects of oral exposure to fungicides on honey bee nutrition and virus levels. J. Econ. Entomol. **108**(6): 2518-2528.

Dorta D.J., A.A.A. Pigoso, F.E.E. Mingatto, T. Rodrigues, I.M. Prado, A.F.F. Helena, S.A.A. Uyemura, A.C. Santos, C. Curti. (2005) The interaction of flavonoids with mitochondria: effects on energetic processes. Chem.-Biol. Interact. **152**(2-3): 67-78.

Esch H. (1976) Body temperature and flight performance of honey bees in a servo-mechanically controlled wind tunnel. J. Comp. Physiol., A **109**(3): 265-277.

Fell R.D., E.G. Rajotte, K.S. Yoder. (1983) Effects of fungicide sprays during apple bloom on pollen viability and honey bee foraging. Environ. Entomol. **12**(5): 1572-1575.

Feuerbacher E., J.H. Fewell, S.P. Roberts, E.F. Smith, J.F. Harrison. (2003) Effects of load type (pollen or nectar) and load mass on hovering metabolic rate and mechanical power output in the honey bee *Apis mellifera*. J. Exp. Biol. **206**(Pt 11): 1855-1865.

Galluzzi L., O. Kepp, G. Kroemer. (2012) Mitochondria: master regulators of danger signalling. Nat. Rev. Mol. Cell Biol. **13**(12): 780-788.

Gibellini L., E. Bianchini, S. De Biasi, M. Nasi, A. Cossarizza, M. Pinti. (2015) Natural compounds modulating mitochondrial functions. Evid. Based Complement. Alternat. Med. **2015**: e527209.

Harrison J.F., J.H. Fewell. (2002) Environmental and genetic influences on flight metabolic rate in the honey bee, *Apis mellifera*. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. **133**(2): 323-333.

Johnson R.M., L. Dahlgren, B.D. Siegfried, M.D. Ellis. (2013) Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS ONE **8**(1): e54092.

Johnson R.M., W. Mao, H.S. Pollock, G. Niu, M.A. Schuler, M.R. Berenbaum. (2012) Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. PLoS ONE **7**(2): e31051.

Johnson R.M., E.G. Percel. (2013) Effect of a fungicide and spray adjuvant on queen-rearing success in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. **106**(5): 1952-1957.

Jones H.B.C., K.S. Lim, J.R. Bell, J.K. Hill, J.W. Chapman. (2016) Quantifying interspecific variation in dispersal ability of noctuid moths using an advanced tethered flight technique. Ecol. Evol. **6**(1): 181-190.

Jones R.G., C.B. Thompson. (2007) Revving the engine: signal transduction fuels T cell activation. Immunity **27**(2): 173-178.

Ketkar S., A. Rathore, A. Kandhare, S. Lohidasan, S. Bodhankar, A. Paradkar, K. Mahadik. (2015) Alleviating exercise-induced muscular stress using neat and processed bee pollen: oxidative markers, mitochondrial enzymes, and myostatin expression in rats. Integr. Med. Res. **4**(3): 147-160.

Khurana T.R., S.P. Sane. (2016) Airflow and optic flow mediates antennal positioning in flying honeybees. eLife **5**: e14449.

Kressler J., M. Millard-Stafford, G.L. Warren. (2011) Quercetin and endurance exercise capacity: a systematic review and meta-analysis. Med. Sci. Sports Exerc. **43**(12): 2396-2404.

Krogh A., T. Weis-Fogh. (1952) A roundabout for studying sustained flight of locusts. J. Exp. Biol. **29**(2): 211-219.

Lang D.R., E. Racker. (1974) Effects of quercetin and F1 inhibitor on mitochondrial ATPase and energy-linked reactions in submitochondrial particles. Biochim. Biophys. Acta **333**(2): 180-186.

Li Y., J. Yao, C. Han, J. Yang, M. Chaudhry, S. Wang, H. Liu, Y. Yin. (2016) Quercetin, inflammation and immunity. Nutrients **8**(3): e167.

Luu T., A. Cheung, D. Ball, M.V. Srinivasan. (2011) Honeybee flight: a novel 'streamlining' response. J. Exp. Biol. **214**(Pt 13): 2215-2225.

MacRae H.S., K.M. Mefferd. (2006) Dietary antioxidant supplementation combined with quercetin improves cycling time trial performance. Int. J. Sport Nutr. Exerc. Metab. **16**(4): 405-419.

Markham R.J.F., N.P. Erhardt, V.L. Dininno, D. Penman, A.R. Bhatti. (1987) Flavonoids protect against T-2 mycotoxins both *in vitro* and *in vivo*. J. Gen. Microbiol. **133**(6): 1589-1592.

Martí-Campoy A., J.A. Ávalos, A. Soto, F. Rodríguez-Ballester, V. Martínez-Blay, M.P. Malumbres. (2016) Design of a computerised flight mill device to measure the flight potential of different Insects. Sensors **16**(4): e485.

Middleton E., Jr., C. Kandaswami, T.C. Theoharides. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. **52**(4): 673-751.

Morton V., T. Staub. (2008) A short History of fungicides, APSnet Feature [online]. DOI: 10.1094/APSnetFeature-2008-0308

Mullin C.A., M. Frazier, J.L. Frazier, S. Ashcraft, R. Simonds, D. VanEngelsdorp, J.S. Pettis. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE **5**(3): e9754.

Mussen E. (2008) Fungicides toxic to bees? From the U.C. Apiaries **17**: 1-3.

Nachtigall W., U. Hanauer-Thieser, M. Mörz. (1995) Flight of the honey bee VII: metabolic power versus flight speed relation. J. Comp. Physiol., B **165**(6): 484-489.

Nieman D.C., A.S. Willia, A.R. Shanely, F. Jin, S.R. McAnulty, T.N. Triplett, M.D. Austin, D.A. Henson. (2010) Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. Med. Sci. Sports Exerc. **42**(2): 338–345.

Oyedotun K.S., B.D. Lemire. (2004) The quaternary structure of the *Saccharomyces cerevisiae* succinate dehydrogenase: homology modeling, cofactor docking, and molecular dynamics simulation studies. J. Biol. Chem. **279**(10): 9424-9431.

Panzenböck U., K. Crailsheim. (1997) Glycogen in honeybee queens, workers and drones (*Apis mellifera carnica* Pollm.). J. Insect Physiol. **43**(2): 155-165.

Pettis J.S., E.M. Lichtenberg, M. Andree, J. Stitzinger, R. Rose, D. vanEngelsdorp. (2013) Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS ONE **8**(7): e70182.

Pettis J.S., D. vanEngelsdorp, J. Johnson, G. Dively. (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. Naturwissenschaften **99**(2): 153-158.

Rayalam S., M.A. Della-Fera, C.A. Baile. (2008) Phytochemicals and regulation of the adipocyte life cycle. J. Nutr. Biochem. **19**(11): 717-726.

Reynolds D.R., J.R. Riley. (2002) Remote-sensing, telemetric and computer-based technologies for investigating insect movement: a survey of existing and potential techniques. Comput. Electron. Agric. **35**(2-3): 271-307.

Rothe U., W. Nachtigall. (1989) Flight of the honey bee. J. Comp. Physiol., B **158**(6): 739-749.

Schippers M.-P., R. Dukas, G.B. McClelland. (2010) Lifetime-and caste-specific changes in flight metabolic rate and muscle biochemistry of honeybees, *Apis mellifera*. J. Comp. Physiol., B **180**(1): 45-55.

Sikder K., S.B. Kesh, N. Das, K. Manna, S. Dey. (2014) The high antioxidative power of quercetin (aglycone flavonoid) and its glycone (rutin) avert high cholesterol diet induced hepatotoxicity and inflammation in Swiss albino mice. Food Funct. **5**(6): 1294-1303.

Silva M.M., M.R. Santos, G. Caroço, R. Rocha, G. Justino, L. Mira. (2009) Structure-antioxidant activity relationships of flavonoids: a re-examination. Free Radical Res. **36**(11): 1219-1227.

Simon-Delso N., G. San Martin, E. Bruneau, L.-A. Minsart, C. Mouret, L. Hautier. (2014) Honeybee colony disorder in crop areas: the role of pesticides and viruses. PLoS ONE **9**(7): e103073.

Stammler G., H. Brix, B. Nave, R. Gold, U. Schoefl. (2007) Studies on the biological performance of boscalid and its mode of action, pp. 45-51. *In*: Dehne H., Deising H., Gisi U., Kuck K., Russell P., and Lyr H. (Eds.), Modern Fungicides and Antifungal Compounds V: 15th International Reinhardsbrunn Symposium, May 6-10, 2007, Friedrichroda, Germany.

Trumbeckaite S., J. Bernatoniene, D. Majiene, V. Jakštas, A. Savickas, A. Toleikis. (2006) The effect of flavonoids on rat heart mitochondrial function. Biomed. Pharmacother. **60**(5): 245-248.

Utter A.C., D.C. Nieman, J. Kang, C.L. Dumke, J.C. Quindry, S.R. McAnulty, L.S. McAnulty. (2009) Quercetin does not affect rating of perceived exertion in athletes during the Western States endurance run. Res. Sports Med. **17**(2): 71-83.

Vance J.T., J.B. Williams, M.M. Elekonich, S.P. Roberts. (2009) The effects of age and behavioral development on honey bee (*Apis mellifera*) flight performance. J. Exp. Biol. **212**(Pt 16): 2604-2611.

Vandock K.P., M.J. Mitchell, C.F. Fioravanti. (2012) Effects of plant flavonoids on *Manduca sexta* (tobacco hornworm) fifth larval instar midgut and fat body mitochondrial transhydrogenase. Arch. Insect Biochem. Physiol. **80**(1): 15-25.

vanEngelsdorp D., J.D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B.K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D.R. Tarpy, J.S. Pettis. (2009) Colony collapse disorder: a descriptive study. PLoS ONE **4**(8): e6481.

Walker M.A., S. Volpi, K.B. Sims, J.E. Walter, E. Traggiai. (2014) Powering the immune system: mitochondria in immune function and deficiency. J. Immunol. Res. **2014**: e164309.

Wegener G. (1996) Flying insects: model systems in exercise physiology. Experientia **52**(5): 404-412.

West P.A., G.S. Shadel, S. Ghosh. (2011) Mitochondria in innate immune responses. Nat. Rev. Immunol. **11**(6): 389-402.

Yang C., C. Hamel, V. Vujanovic, Y. Gan. (2011) Fungicide: modes of action and possible impact on nontarget microorganisms. ISRN Ecol. **2011**: e130289.

Yoder J.A., B.Z. Hedges, D.J. Heydinger, D. Sammataro, G. DeGrandi-Hoffman. (2011a) Differences among fungicides targeting the beneficial fungi associated with honey bee colonies, pp. 181-192. *In*: Sammataro D. and Yoder J.A. (Eds.), Honey Bee Colony Health: Challenges and Sustainable Solutions, CRC Press.

Yoder J.A., D.J. Heydinger, B.Z. Hedges, D. Sammataro, J. Finley, G. DeGrandi-Hoffman, T.J. Croxall, B.S. Christensen. (2011b) Fungicides reduce symbiotic fungi in bee bread and the beneficial fungi in colonies, pp. 193-214. *In*: Sammataro D. and Yoder J.A. (Eds.), Honey Bee Colony Health: Challenges and Sustainable Solutions, CRC Press.

Yoder J.A., A.J. Jajack, A.E. Rosselot, T.J. Smith, M.C. Yerke, D. Sammataro. (2013) Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, *Apis mellifera*, colonies. J. Toxicol. Environ. Health, A **76**(10): 587-600.

Zhu W., D.R. Schmehl, C.A. Mullin, J.L. Frazier. (2014) Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PLoS ONE **9**(1): e77547.

4.2 Dietary quercetin ameliorates toxic effects of insecticides in honey bee foragers

Abstract

The diet of the honey bee *Apis mellifera*, nectar and pollen, as well as honey and beebread, respectively, is by nature rich in phytochemicals. Thus, honey bees that forage for and ingest floral resources contaminated by insecticides consume these toxins within a matrix of phytochemicals, raising the possibility of synergistic or antagonistic interactions between natural and synthetic compounds. Quercetin is a common and abundant flavonoid in honey and beebread that is known to induce multiple genes encoding xenobiotic-metabolizing cytochrome P450 monooxygenases. In this study, we examined the effect of quercetin-insecticide interactions on honey bee foragers. Toxicity of two pyrethroid pesticides (β-cyfluthrin, and bifenthrin) and one neonicotinoid pesticide (imidacloprid) was tested in the presence and absence of quercetin. Bees confined individually were tested both to control the amount of food ingested and to prevent trophallaxis (sharing food with nestmates). I developed two assays to evaluate toxicity—a conventional test of survivorship and a "flight treadmill" assay, to compare responses of bees in resting and active states. In the survivorship assay, quercetin enhanced tolerance of two tested pyrethroids (500 ppb β-cyfluthrin and 4 ppm bifenthrin) but not 50 ppb imidacloprid. In the flight treadmill test, foragers that consumed a sugar syrup diet to which the pyrethroid insecticide bifenthrin (1 ppm) or β-cyfluthrin (250 ppb) with quercetin was added exhibited higher tolerance to the pesticides than foragers treated with pyrethroids alone. These quercetin-fed foragers also engaged in a greater number of flight bouts and displayed a longer period of exposure prior to the onset of paralysis. As in the survivorship assays, imidacloprid toxicity was not ameliorated by quercetin in the flight treadmill activity assay; rather, ingestion of quercetin in the presence of

15 ppb imidacloprid enhanced the toxicity of the pesticide. In sum, the ubiquitous phytochemical constituent of the diet of *A. mellifera* can enhance forager tolerance of some, but not all, pesticide contaminants of flora foods.

Keywords:

quercetin, sublethal effects, synergism, pyrethroid, neonicotinoid

Introduction

Quercetin, a flavonol, and its glycosides are among the most widely distributed and abundant plant secondary metabolites (Biesaga and Pyrzynska 2009). Ecologically, it plays a diversity of roles in mediating interactions between plants and herbivorous insects (Simmonds 2001; Simmonds 2003) . Depending on concentration, it can act as either a feeding stimulant or deterrent for a diversity of lepidopterans; as well, its effects on feeding behavior in herbivores from several orders can be influenced by both the pattern of glycosylation and the presence of co-occurring phytochemicals. Quercetin can function as a defensive allomone, reducing fitness in erstwhile consumers in a variety of ways, including reducing growth rates (Elliger *et al.* 1980; War *et al.* 2013), survivorship (Lindroth and Peterson 1988) and egg hatch (Sharma and Sohal 2013), or as a kairomone, decreasing mortality (Lindroth and Peterson 1988).

Among the physiological impacts of quercetin that may have beneficial effects on consumers is its ability to alter detoxification enzyme activity. In lepidopterans in particular, quercetin ingestion increases the catalytic activity of cytochrome P450 monooxygenases (Liu *et al.* 2006; Rose *et al.* 1991) and upregulates P450 gene expression (Li *et al.* 2004; Wang *et al.* 2015; Zhang *et al.* 2012). Liu *et al*. (2015) showed that, in addition to acting as an inducer of two P450 genes in the caterpillar *Helicoverpa armigera*, quercetin ingestion also functioned as a repressor of six P450 genes, including CYP6B and CYP9A subfamily members known to play a role in xenobiotic metabolism. In the domestic silkworm *Bombyx mori (*Lepidoptera: Bombycidae), quercetin consumption increased the activity levels of all three superfamilies of Phase 1 detoxification enzymes—cytochrome P450 monooxygenases, carboxylesterases, and glutathione-S-transferases—and upregulated expression of at least two P450 genes and a gene encoding a glutathione-S-transferase (Zhang *et al.* 2012).

Because quercetin is ubiquitous in nectar and pollen, it is potentially of ecological importance in mediating interactions between plants and their pollinators. In the honey bee *Apis mellifera*, although quercetin is metabolized by enzymes in the CYP6AS and CYP9Q subfamilies (Mao *et al.* 2009; 2011), members of which are known to detoxify pesticides, in developing larvae it upregulates expression of seven P450s at both low and high concentrations, two P450s at low concentrations (*CYP6AS17* and *CYP9R1)*, and five (*CYP6AS1*, *CYP9Q1*, *CYP9Q2*, and *CYP9Q3*) at high concentrations (Mao *et al*. in review). The phenolic acid p-coumaric acid, which is found in both honey and beebread, also upregulates P450 gene expression and when consumed along with the pesticides coumaphos and tau-fluvalinate enhances detoxification (Mao *et al.* 2013). Quercetin may act in a similar matter, enhancing resistance to pesticides by upregulating expression of xenobiotic-metabolizing P450 genes and boosting catalytic activity; indeed, dietary quercetin reduced the toxicity of the pyrethroid acaricide tau-fluvalinate to honey bees (Johnson *et al.* 2013).

In this study, we examined the effect of quercetin on pesticide toxicity in two types of assays; for both types of assays, two pyrethroids (β-cyfluthrin, and bifenthrin) and one neonicotinoid (imidacloprid) were used. To carry out conventional survivorship tests, we fed the foragers individually with quercetin and one insecticide together. Individual feeding was carried out both to insure that all individuals tested consumed identical amounts of tested chemicals and to prevent trophallaxis (social sharing of food). Differences in survivorship among groups were evaluated and compared to the control group.

Although lethality is a clear endpoint for assessing potential synergistic or antagonistic interactions between quercetin and pesticides, with a social species such as *A. mellifera*, sublethal effects of pesticides may have consequences for the colony. Although sublethal effects

of pyrethroids and neonicotinoids on honey bees are well documented (Desneux *et al.* 2007; Johnson 2015), little is known about how dietary phytochemicals may affect responses short of outright mortality—whether, for example, quercetin may ameliorate sublethal effects as effectively as it ameliorates lethal effects. In a field situation, paralysis or even just flight impairment can lead to a lethal result for foragers. Moreover, pesticide detoxification may come with energetic costs, which could compromise flight activity, the most energy-consumptive activity carried out by bees. By altering cellular respiration and energy metabolism, quercetin, in combination with other toxins, may influence flight performance of bees in a way that would not be detectable in a conventional survivorship assay. In this study, we designed a "flight treadmill" for bees to examine the sublethal effects of simultaneous ingestion of quercetin and pesticides on forager flight performance.

Materials and Methods

Experimental insects

Western honey bees (*Apis mellifera*) were used in the experiments. The naturally mated queen hives from which bees were sampled for all tests were managed without the use of synthetic pesticides for about one year in University of Illinois apiaries near Urbana, Illinois, USA. All the tested bees were foragers collected at a syrup feeder near the colony entrance as they returned from foraging. Each group of 25-30 bees was placed in a vial measuring 12.7 cm in length and 5.1cm in diameter, which was then chilled down on ice for 15 minutes until the bees were immobile.

Survivorship test

Each forager was inserted in a tube such that its head remained outside the tube. The bees were allowed at least 30 minutes to recover from chilling. After the chilling recovery period, they were fed with 20 µl 50% sugar water (w/v) and placed in a quiet humidified box (35°C and 50% relative humidity) for one night to recover from the stress of handling.

For the conventional toxicity tests, foragers were randomly assigned to a feeding group. Each forager was forced to consume 15 µl of 50% sugar water (w/v) containing either a pesticide, a pesticide and quercetin (Q; Q4951, Sigma-Aldrich Co. LLC., St. Louis, MO), or only a dimethyl sulfoxide solvent control (DMSO; D128, Fisher Scientific International, Inc., Pittsburgh, PA). This procedure was repeated three times at four-hour intervals. After the three feeding bouts, each forager was provided *ad libitum* sugar water for one minute every four hours, during which time the forager could consume as much sugar water as desired. Survivorship in each tested pesticide group was recorded every four hours.

Three pesticides – 50 ppb imidacloprid (IMI; 37894, Sigma-Aldrich Co. LLC., St. Louis, MO), 500 ppb ß-cyfluthrin (CyF; 46003, Sigma-Aldrich Co. LLC., St. Louis, MO), and bifenthrin (Bif; N-11203, ChemService, Inc., West Chester, PA) (4 ppm as well as 2 ppm) – were tested in the presence and absence of quercetin. The pesticide concentrations were based on the resultd of the flight treadmill experiment. I selected the highest sublethal concentrations that began to show possible interactivity with quercetin. Two control groups (DMSO control and quercetin-alone control) were also tested. There were total of 10 tested groups and each group contained 40 to 60 foragers, except for the 2 ppm bifenthrin group, which had only 19.

The number of bees surviving in each group was pooled every four hours for the sake of comparison. We calculated survival for each diet treatment by Kaplan–Meier estimates and

tested differences in survival between treatments using log-rank, Breslow, and Tarone-Ware tests (OriginPro 2016. Sr2, OriginLab Corporation, Northampton, MA). The Breslow test is more sensitive to differences in survival occurring earlier in the trials, while log-rank test is more sensitive to differences that occur later (Allison 2010) and the Tarone-Ware test is intermediate in sensitivity.

Flight treadmill for testing the flight characteristics of foragers

A flight treadmill was set up to elicit steady flights of honey bee foragers. Three components combine to maintain steady flight in bees: the tarsal reflex at the initiation of flight, visual stimuli and air flow stimuli. Two LCD monitors were arranged face-to-face in a vertical 36-degree V-shape arrangement to provide bees with optical flow stimuli, from the lateral and ventral sides. Optical flow provided by each side monitor was a moving vertical black-and-white striped pattern generated by a custom-written LabVIEW program (National Instruments Corporation, Austin, TX); the horizontal spatial frequency of the pattern was 0.016 cycles degree^{-1} and the speed of the optical flow was about 209.4 degrees s^{-1} as seen from the center point of the fixation position of the tested bee. Also, an in-line fan blower was set in front of the V-shaped monitors to provide a constant velocity air flow (3.6 m s^{-1}) stimulus. A metal rod was positioned vertically at three-fourths of the longitudinal axis of the V-shaped "LCD valley". A small magnet was attached to the end of the rod. A bee equipped with a pre-glued carbon steel plate was tethered to the magnet at the end of the rod. At this position, the tethered bee could perceive both visual and mechanical signal cues to stimulate flight. The tethered bees were then provided with a 12-mm diameter round plastic stick to hold. Subsequently, the visual and air flow stimuli were triggered, then the stick was removed immediately from the honey bee and the

tarsal reflex initiated flight. During the flight treadmill tests, the room was kept at a constant temperature (27℃) and relative humidity (50%).

Setting of the sub-lethal effects of short-term exposure to dietary insecticides and quercetin on flight treadmill (Fig. 4.5)

Each forager for the flight treadmill experiment was also caught at a syrup feeder, placed in a vial and chilled down on ice for 15 minutes until she was incapable of moving. Hairs on the dorsal thorax of the bee were shaved using a razor blade, and a piece of carbon steel plate (2 * 2 * 0.127 mm^3) was glued on the middle of the notum of the bee using a cyanoacrylate adhesive (Loctite® Super Glue Gel, Henkel Corporation, Westlake, OH). Then the bee was set in a quiet humidified box for at least 30 minutes, considered as restoration time-out, for recovery from stress before the first flight in the flight treadmill.

Depletion flight: Without supplemental feeding, after a restoration time-out, the unstressed bee was placed on the flight treadmill for a "depletion flight" to exhaust her energy and stored sugar fuel. The "start" of a flight was designated as when continuous wing-flapping (longer than one minute) was triggered through a tarsal reflex. The "end" of a flight was designated as when the tested bee stopped flapping wings and could not resume flight spontaneously and/or be triggered to fly again in five successive attempts through triggering the tarsal reflex.

Preflight: After the depletion flight, the tested bee was fed 10 μL of 0.25% DMSO 25% sucrose syrup and allowed to hold on a stick to take a five-minute restoration rest on the round plastic stick. After the five-minute restoration period, the stick was removed to trigger a tarsal reflex and the optic flow was started as well to induce another trial of flight of the bee. Between each trial, the tested bee was also fed 10 μL of 25% sucrose syrup with the treatment chemical and then held for a five-minute restoration period. Each forager was subjected to three rounds of "pretest-

flight" to make sure her physical condition was good. Generally, a flight required about 15 minutes of continuous wing-flapping. The experiments were conducted only on the individuals that could perform a preflight lasting more than 10 minutes each time.

Exhausted flight test: After three pretest-flight rounds, foragers were fed 10 μL tested syrup (with a sub-lethal concentration of an insecticide or with an insecticide plus 0.25 mM quercetin in 0.25% DMSO 25% sucrose syrup). The foragers were allowed to rest for five minutes and then subjected to a flight test. Between each flight test interval, bees were fed with same tested syrup and permitted a five-minute rest until they displayed paralysis or dysfunction due to insecticide toxicity. If bees showed no impairment after 10 hours, the testing was stopped.

Data collection: Three insecticides – bifenthrin, β-cyfluthrin, and imidacloprid – were tested at sublethal concentrations. Each forager was tested with only one insecticide at one concentration. Foragers fed syrup with quercetin or without quercetin were tested in turn. If the total flight bout number before paralysis set in between the quercetin-treated forager and the quercetin-free forager was not different, the concentration of the tested insecticide would be reduced for the next individual to be flight-tested.

The duration before paralysis was calculated as the total time after consumption of the first dose of pesticide syrup. The time included the total duration of all flights as well as the five-minute rest intervals. The total number of flight bouts undertaken by a forager tested individually and the accumulated tested pesticide dose consumed by each individual tested were recorded and compared between treatments with or without quercetin.

Results

In survival tests, quercetin alone did not affect the mortality of foragers compared with those in the sugar water control group (Fig. 4.6). By contrast, quercetin did enhance tolerance of the two pyrethroids, β-cyfluthrin (Fig. 4.7) and bifenthrin (Fig. 4.8&4.9). Survival was significantly higher for foragers that ingested 500 ppb β-cyfluthrin plus 0.25 mM quercetin (mean estimate = 66.01 ± 4.33 hours) than it was for the foragers that ingested 500 ppb β-cyfluthrin alone (mean estimate = 42.94 ± 4.57 hours) (Fig. 4.7). The mean survival time of bees that ingested quercetin plus β-cyfluthrin was longer than that of the bees fed only β-cyfluthrin in sugar water by 23 hours.

Bifenthrin appeared to interact with quercetin in a similar way. The survival of bees ingesting 4 ppm bifenthrin in sugar water (mean estimate = 43.07 ± 3.48 hours) was significantly worse than that of bees ingesting 4 ppm bifenthrin and 0.25 mM quercetin in sugar water simultaneously (mean estimate = 56.78 ± 3.51 hours) (Fig. 4.8). The ingestion of quercetin simultaneously with 4 ppm bifenthrin enhanced the mean survival time of bees by 13.71 hours compared with survival time of bees ingesting only 4 ppm bifenthrin.

In the 2-ppm bifenthrin test, the survival of bees ingesting 2 ppm bifenthrin in sugar water (mean estimate = 36.58 ± 3.57 hours) was significantly worse than that of bees ingesting 2 ppm bifenthrin and 0.25 mM quercetin in sugar water simultaneously (mean estimate = 47.42 ± 0.56 hours) in 48 hours (Fig. 4.9). However, the survival curves of the two groups converged and crossed after 72 hours, which means that the effect of quercetin ingestion may have been diminished. The survival of honey bees ingesting 2 ppm bifenthrin (mean estimate = 63.53 ± 8.72 hours) and that of honey bees ingesting both bifenthrin and quercetin (mean estimate = 79.32 ± 4.26 hours) were not significantly different in the entire 93-hour observation.

In contrast, the survival of foragers in the 50 ppb imidacloprid group (mean = estimate 67.18 ± 4.34 hours) was not significantly different from that of foragers in the 500 ppb imidacloprid plus quercetin group (mean estimate = 59.24 ± 4.30 hours) (Fig. 4.10). Ingestion of quercetin and imidacloprid together actually appeared to increase the onset of mortality. In the first 24 hours, the two survival curves crossed in several cases. After 24 hours, the curves diverged.

In the exhausted flight test, with short-term chemical exposures, quercetin also rescued bees from effects of synthetic pyrethroid insecticides (Table 1). When 1 ppm bifenthrin or 250 ppb β-cyfluthrin was added to a sugar syrup diet, the foragers that consumed quercetin simultaneously exhibited higher tolerance of the pesticideand could fly longer before paralysis set in than foragers treated with pyrethroid alone.

The average value of the time to the onset of paralysis of foragers from the group ingesting 1 ppm bifenthrin plus dietary quercetin was 439.0 ± 193.4 minutes (mean ± SD), which was ca. four times longer than concentrations from individuals from the pesticide alone groups (118.0 ± 14.7 minutes). As well, the flight bout number and accumulated dose of bifenthrin of foragers from bifenthrin plus dietary quercetin group were also higher than they were for the foragers of the β-cyfluthrin control group (4.0 ± 0.0 vs. 9.1 ± 2.8 bouts; 40.0 ± 0.0 vs. 97.1 ± 36.1 ng). Additionally, in the 250 ppb β-cyfluthrin flight test, the foragers from dietary quercetin plus β-cyfluthrin group also displayed a longer tolerance duration, an increased flight bout number and a higher accumulated dose of β-cyfluthrin than did the foragers of β-cyfluthrin control group (137.9 ± 98.5 vs. 512.5 ± 68.1minutes; 4.4 ± 2.7 vs. 12.0 ± 2.4 bouts;11.1 ± 6.8 vs. 30.0 ± 6.1 ng). Nevertheless, this rescue effect of quercetin appears only at relatively low sublethal concentrations of pyrethroids. At a relatively higher pesticide concentration, all tested

syrup did not show a different effect of quercetin on the duration before the onset of paralysis or on the number of flight bouts undertaken by foragers.

This rescue effect of quercetin, however, did not occur at all with imidacloprid, a neonicotinoid. Imidacloprid and quercetin actually appeared to interact synergistically. When 15 ppb imidacloprid was added to a sugar syrup diet, the foragers that consumed quercetin simultaneously experienced greater toxicity. These foragers flew for a shorter duration before paralysis set in, undertook fewer flight bouts and consumed a lower accumulated intake dose than did the foragers treated with 15 ppb imidacloprid alone (256.7 ± 98.93 vs. 182.7 ± 6.549 minutes; 8.0 ± 2.45 vs. 5.7 ± 0.47 bouts; 1.5 ± 0.552 vs. 1.1 ± 0.225 ng).

Discussion

Quercetin is known to have a diversity of behavioral and physiological effects on insect herbivores that consume leaves, stems and seeds; among these effects is amelioration of toxicity of co-occurring compounds. In my study, we have documented that this protective effect of quercetin also exists in the pollinator *A. mellifera*. Honey bee food is rich in both phenolic acids, such as p-coumaric acid, and flavonols, such as quercetin, and these phytochemical classes appear to be important dietary constituents that offer benefits beyond nutrition per se (Mao *et al*. 2011, 2013). In my study, conventional survival tests showed that quercetin ingestion enhanced the tolerance of foragers to β-cyfluthrin (Fig. 4.8) and bifenthrin (Fig. 4.9 and Fig. 4.10), both of which are frequent hive contaminants (Mullin *et al.* 2010). A similar beneficial interaction between pesticides and quercetin was detectable in the exhausted flight tests. Foragers consuming bifenthrin plus quercetin exhibited tolerance for a longer time period, undertook a greater number of flight bouts and tolerated a higher accumulated dose of bifenthrin than did the

foragers consuming β-cyfluthrin in the absence of quercetin. The likely mechanism underlying these beneficial effects may be due to the ability of quercetin to upregulate a high proportion of genes that encode P450s in the CYP6AS and CYP9 families, both of which are known to contribute to xenobiotic detoxification (Mao *et al*. 2011, 2013, in review).

As well, although no systematic survey has been conducted, at least some xenobiotics themselves upregulate many of the same P450 genes that are induced by quercetin, suggestive of toxicological complementarity as a mechanism underlying the protective effects of quercetin if in fact these P450s metabolize the inducers. Several CYP6AS genes are upregulated by extracts of honey, pollen and propolis (Johnson *et al.* 2012); as well, CYP6AS14 is upregulated by the monoterpene thymol, used in-hive as an acaricide against varroa mites; two other acaricides, coumaphos and tau-fluvalinate, upregulate CYP6AS3, CYP6AS4, and CYP9S1 in adult workers (Boncristiani *et al.* 2012); and imidacloprid upregulates CYP6AS3, CYP6AS4, CYP6AS14, CYP6AS15, CYP6AR1, CYP9R1 and CYP9R1 in honey bee larvae (Derecka *et al.* 2013). With regard to CYP9, CYP9Q enzymes also metabolize and are induced by acaricides (tau-fluvalinate) as well as insecticides (cypermethrin and bifenthrin) (Mao *et al.* 2011).

Two of the tested pyrethroid pesticides have also been known to cause oxidative stress (Jin *et al.* 2014; Sadowska-Woda *et al.* 2010; Syed *et al.* 2015). The anti-oxidative characteristics of quercetin (Chanput *et al.* 2016) may be another mechanism by which quercetin ingestion allows bees to overcome pyrethroid toxicity. Sadowska-Woda *et al*. (2010) demonstrate that flavonoids quercetin and rutin can alleviate the effects of bifenthrin-induced oxidative stress in an *in vitro* system; these chemicals may have similar effects in intact organisms as well.

It must be noted that quercetin does not invariably protect against pesticide toxicity. In this study, the exhausted flight test of imidacloprid-quercetin interaction revealed that 15 ppb

imidacloprid in the diet interacted synergistically interaction with quercetin such that toxicity was enhanced by ingesting these two compounds together. By contrast, the survival of foragers in the 50 ppb imidacloprid group did not show a significant difference with the group where imidacloprid was ingested with quercetin. In the first 24 hours, the survival curves crossed on several occasions and then diverged. The survival was worse in the imidacloprid plus quercetin group (no statistical difference) than in the imidacloprid-alone group. Both the survival test result and the exhausted flight results are suggestive of a form of competitive inhibition at the catalytic site of detoxification enzymes.

Further research could also attempt to find precise chemical concentrations or a response curve with multiple concentrations in order to examine the response and interaction between phytochemicals and pesticides. The concentration of quercetin used in the study was 0.25 mM (ca. 76 ppm), which is much higher than in natural nectar, but it approximates the concentrations of quercetin (or total flavonols) in honey (Jantakee and Tragoolpua 2015) and in bee pollen (Kaškonienė *et al.* 2015). Whether other co-occurring flavonoids have similar protective effects against pesticides has yet to be determined. Also remaining to be characterized is the effect of chronic ingestion of quercetin in the presence and absence of other flavonoids. The assays in this study involved delivery of a single acute dose; under natural conditions in North America, however, honey bees are continually exposed to quercetin not only in nectar, honey, pollen, and beebread but also in propolis (Ghisalberti 1979; Zhang and Hu 2009), the resinous material prepared by bees by collecting and processes exudates of plants, particularly in the Salicaceae (Montenegro *et al.* 2001). These materials are rich in quercetin and other flavonoids, some of which may share the ability to induce detoxification enzymes and general protect against toxicity.

If such is the case, the findings of this study could have important implications for the apiculture industry.

Acknowledgments

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Figures and Tables

Table 4.1. Flight treadmill experiment testing effects of short-term exposure to dietary quercetin and insecticides

Insecticides	concentration (ppb)	treatment	n	Time to paralysis Mean±SD (min)	Flight bout Mean±SD (n)	Accumulated dose Mean±SD (ng)
Bifenthrin (Bif)	1,000	Bif	3	118.0 ± 14.7	4.0 ± 0.0	40.0 ± 0.0
		Bif w/ Q	7	439.0 ± 193.4	9.1 ± 2.8	97.1 ± 36.1
	10,000	Bf	1	99.0	3.0	300.0
		Bif w/ Q	1	100.0	3.0	300.0
β-Cyfluthrin (CyF)	250	CyF	7	137.9 ± 98.5	4.4 ± 2.7	11.1 ± 6.8
		CyF w/ Q	6	512.5 ± 68.1	12.0 ± 2.4	30.0 ± 6.1
	400	CyF	2	147.5 ± 108.5	4.5 ± 2.5	18.0 ± 10.0
		CyF w/ Q	1	503.0	13.0	53.0
	500	CyF	2	68.0 ± 2.0	2.0 ± 0.0	10.0 ± 0.0
		CyF w/ Q	1	342.0	8.0	40.0
	1,000	CyF	2	26.0 ± 1.0	1.5 ± 0.5	15.0 ± 5.0
		CyF w/ Q	2	104.5 ± 21.5	3.0 ± 1.0	30.0 ± 10.0
Imidacloprid (Iml)	15	Iml	3	256.7 ± 98.93	8.0 ± 2.45	1.5 ± 0.552
		Iml w/ Q	3	182.7 ± 6.549	5.7 ± 0.47	1.1 ± 0.225
	25	Iml	1	57.0	2.0	0.8
		Iml w/ Q	1	84.0	3.0	1.0
	50	Iml	1	81.0	3.0	3.0
		Iml w/ Q	1	98.0	3.0	7.5
	100	Iml	1	86.0	2.0	4.0
		Iml w/ Q	3	20.3 ± 15.33	1.3 ± 0.47	7.3 ± 3.771

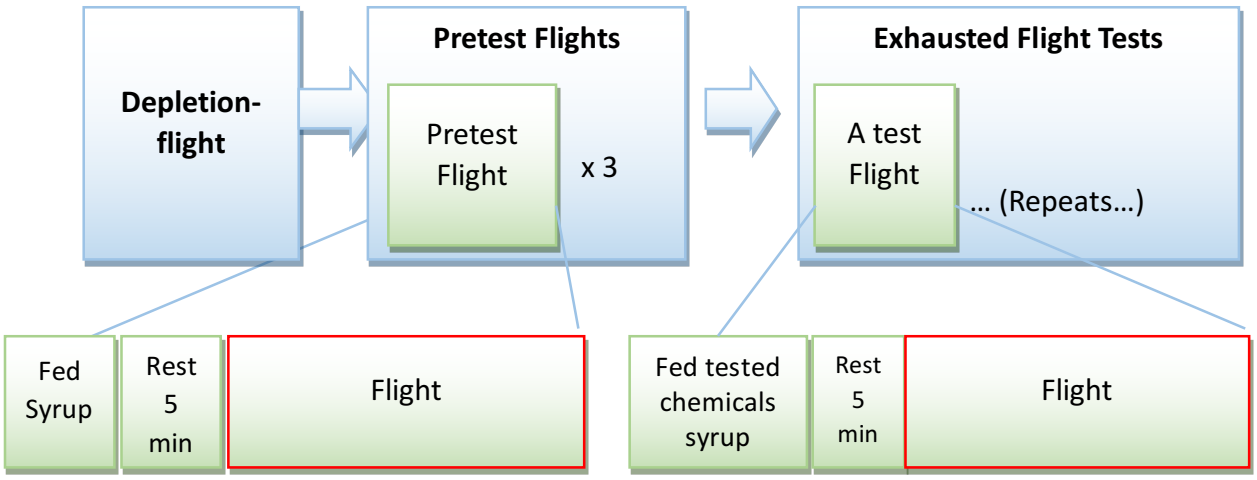

Figure 4.5. The exhausted flight test design and steps. There are three main test components. Depletion-flight: Without supplemental feeding, a forager was placed on the "flight treadmill" for a depletion-flight in order to exhaust her energy stores before a test. Pretest Flights: After the depletion-flight, each forager was fed with 10 µL of 25% sucrose syrup and then held for 5 minutes to recover. Each forager was subjected to 3 rounds of "pretest-flight" to make sure her physical condition was good. Exhausted flight test: After 3 pretest-flight rounds, bees were fed 10 µL of 25% insecticide sugar water with/without quercetin and then subjected to a flight test. Between each flight test interval, bees were fed with same insecticide sugar water solution until they displayed paralysis or dysfunction due to insecticide toxicity.

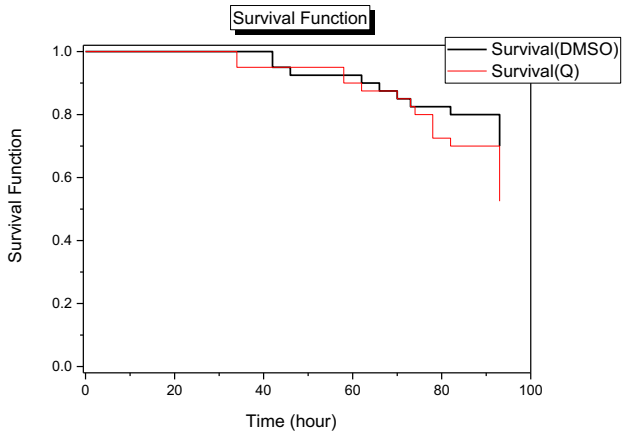

Figure 4.6. Effect of 0.25 mM quercetin on the survival of honey bees (no significant difference; $n_{DMSO} = 40$, $n_Q = 40$; Log rank test, $\chi^2 = 1.232$, $P = 0.267$; Breslow test, $\chi^2 = 0.894$, $P = 0.344$; Tarone-Ware test, $\chi^2 = 1.038$, $P = 0.308$).

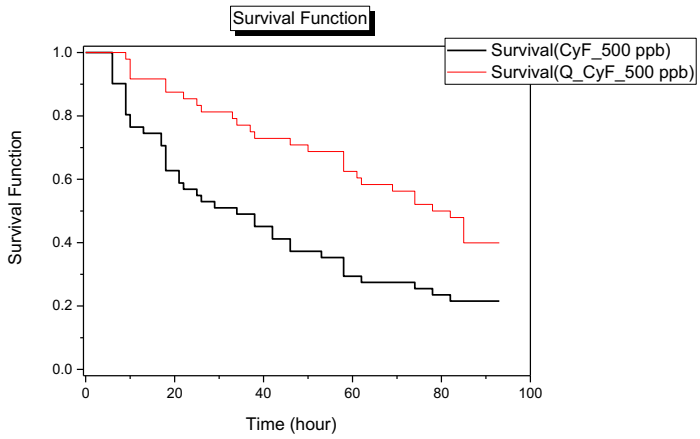

Figure 4.7. Survival of honey bees ingesting 500 ppb β-cyfluthrin with and without quercetin. (significant difference; $n_{CyF} = 51$, $n_{Q\_CyF} = 48$; Log rank test, $\chi^2 = 9.706$, $P = 0.002 < 0.01$; Breslow test, $\chi^2 = 12.484$, $P < 0.001$; Tarone-Ware test, $\chi^2 = 11.503$, $P < 0.001$).

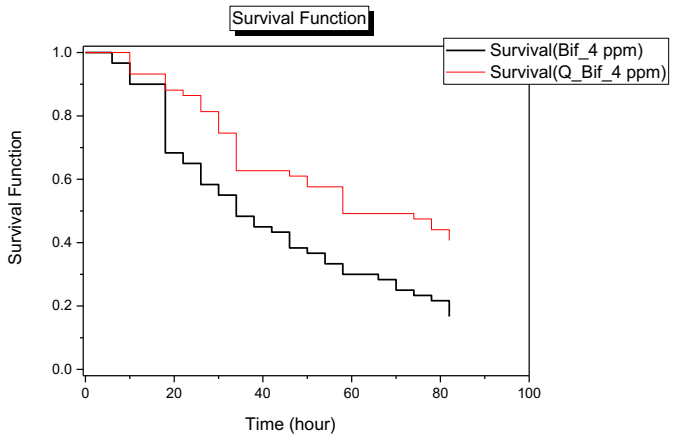

Figure 4.8. Survival of honey bees ingesting 4 ppm bifenthrin with and without quercetin. (significant difference; $n_{\text{Bif\_4ppm}} = 60$, $n_{\text{Q\_Bif\_4ppm}} = 59$; Log rank test, $\chi^2 = 9.697$, $P = 0.002 < 0.01$; Breslow test, $\chi^2 = 8.909$, $P = 0.003 < 0.01$; Tarone-Ware test, $\chi^2 = 9.404$, $P = 0.002 < 0.01$;).

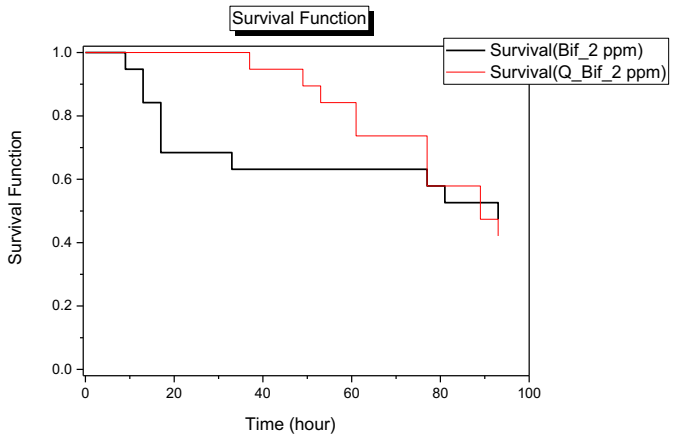

Figure 4.9. Survival of honey bees ingesting 2 ppm bifenthrin with and without quercetin. (There is no significant difference when the observation is ended at 93 hours; $n_{Bif\_2ppm} = 19$, $n_{Q\_Bif\_2ppm} = 19$;; Log rank test, $\chi^2 = 0.023$, $P = 0.878$; Breslow test, $\chi^2 = 0.396$, $P = 0.529$; Tarone-Ware test, $\chi^2 = 0.153$, $P = 0.696$. But there is significant difference if the observation is ended at 48 hours, Log rank test, $\chi^2 = 5.904$, $P = 0.015 < 0.05$; Breslow test, $\chi^2 = 6.206$, $P = 0.013 < 0.05$; Tarone-Ware test, $\chi^2 = 6.064$, $P = 0.014 < 0.05$).

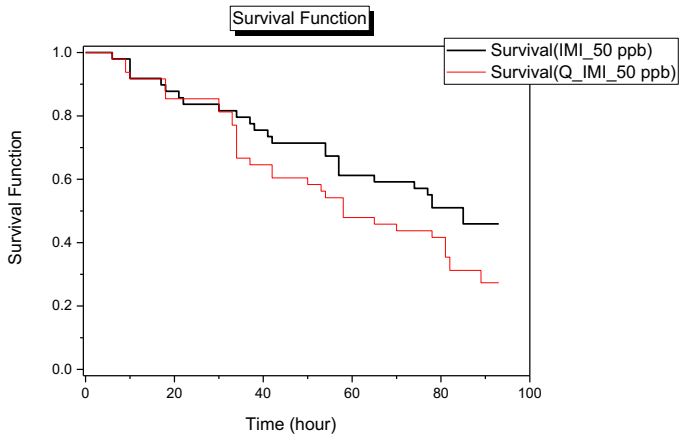

Figure 4.10. Survival of honey bees ingesting 50 ppb imidacloprid versus survival of honey bees ingesting both 50 ppb imidacloprid and quercetin. (no significant difference; $n_{IMI\_50ppb} = 49$, $n_{Q\_IMI\_50ppb} = 48$; Log rank test, $\chi^2 = 3.065$, $P = 0.080$; Breslow test, $\chi^2 = 2.353$, $P = 0.125$; Tarone-Ware test, $\chi^2 = 2.723$, $P = 0.099$).

References

Allison P.D. (2010) Survival analysis using SAS: a practical guide. SAS Institute, Inc, Cary, N.C.

Biesaga M., K. Pyrzynska. (2009) Analytical procedures for determination of quercetin and its glycosides in plant material. Crit. Rev. Anal. Chem. **39**(2): 95-107.

Boncristiani H., R. Underwood, R. Schwarz, J.D. Evans, J. Pettis, D. vanEngelsdorp. (2012) Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. J. Insect Physiol. **58**(5): 613-620.

Chanput W., N. Krueyos, P. Ritthiruangdej. (2016) Anti-oxidative assays as markers for anti-inflammatory activity of flavonoids. Int. Immunopharmacol. **40**: 170-175.

Derecka K., M.J. Blythe, S. Malla, D.P. Genereux, A. Guffanti, P. Pavan, A. Moles, C. Snart, T. Ryder, C.A. Ortori, D.A. Barrett, E. Schuster, R. Stöger. (2013) Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. PLoS ONE **8**(7): e68191.

Desneux N., A. Decourtye, J.-M. Delpuech. (2007) The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. **52**(1): 81-106.

Elliger C.A., B.C. Chan, A.C. Waiss. (1980) Flavonoids as larval growth inhibitors - structural factors governing toxicity. Naturwissenschaften **67**(7): 358-360.

Ghisalberti E.L. (1979) Propolis: a review. Bee World **60**(2): 59-84.

Jantakee K., Y. Tragoolpua. (2015) Activities of different types of Thai honey on pathogenic bacteria causing skin diseases, tyrosinase enzyme and generating free radicals. Biol. Res. **48**: 4.

Jin Y., X. Pan, Z. Fu. (2014) Exposure to bifenthrin causes immunotoxicity and oxidative stress in male mice. Environ. Toxicol. **29**(9): 991-999.

Johnson R.M. (2015) Honey bee toxicology. Annu. Rev. Entomol. **60**: 415-434.

Johnson R.M., L. Dahlgren, B.D. Siegfried, M.D. Ellis. (2013) Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS ONE **8**(1): e54092.

Johnson R.M., W. Mao, H.S. Pollock, G. Niu, M.A. Schuler, M.R. Berenbaum. (2012) Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. PLoS ONE **7**(2): e31051.

Kaškonienė V., G. Ruočkuvienė, P. Kaškonas, I. Akuneca, A. Maruška. (2015) Chemometric analysis of bee pollen based on volatile and phenolic compound compositions and antioxidant properties. Food Anal. Methods **8**(5): 1150-1163.

Li X., J. Baudry, M.R. Berenbaum, M.A. Schuler. (2004) Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. Proc. Natl. Acad. Sci. USA **101**(9): 2939-2944.

Lindroth R.L., S.S. Peterson. (1988) Effects of plant phenols of performance of southern armyworm larvae. Oecologia **75**(2): 185-189.

Liu D., Y. Yuan, M. Li, X. Qiu. (2015) Effects of dietary quercetin on performance and cytochrome P450 expression of the cotton bollworm, *Helicoverpa armigera*. Bull. Entomol. Res. **105**(6): 771-777.

Liu X., P. Liang, X. Gao, X. Shi. (2006) Induction of the cytochrome P450 activity by plant allelochemicals in the cotton bollworm, *Helicoverpa armigera* (Hübner). Pestic. Biochem. Physiol. **84**(2): 127-134.

Mao W., S.G. Rupasinghe, R.M. Johnson, A.R. Zangerl, M.A. Schuler, M.R. Berenbaum. (2009) Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp. Biochem. Physiol., B: Biochem. Mol. Biol. **154**(4): 427-434.

Mao W., M.A. Schuler, M.R. Berenbaum. (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. USA **108**(31): 12657-12662.

Mao W., M.A. Schuler, M.R. Berenbaum. (2013) Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proc. Natl. Acad. Sci. USA **110**(22): 8842-8846.

Montenegro G., R.C. Peña, G. Avila. (2001) Botanical origin and seasonal production of propolis in hives of Central Chile. Bol. Bot. Univ. São Paulo **19**: 1-6.

Mullin C.A., M. Frazier, J.L. Frazier, S. Ashcraft, R. Simonds, D. Vanengelsdorp, J.S. Pettis. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE **5**(3): e9754.

Rose R.L., F. Gould, P.E. Levi, E. Hodgson. (1991) Differences in cytochrome P450 activities in tobacco budworm larvae as influenced by resistance to host plant allelochemicals and induction. Comp. Biochem. Physiol. B **99**(3): 535-540.

Sadowska-Woda I., D. Popowicz, A. Karowicz-Bilińska. (2010) Bifenthrin-induced oxidative stress in human erythrocytes *in vitro* and protective effect of selected flavonols. Toxicol. In Vitro **24**(2): 460-464.

Sharma R., S.K. Sohal. (2013) Bioefficacy of quercetin against melon fruit fly. Bull. Insectol. **66**(1): 79-83.

Simmonds M.S. (2001) Importance of flavonoids in insect--plant interactions: feeding and oviposition. Phytochemistry **56**(3): 245-252.

Simmonds M.S. (2003) Flavonoid-insect interactions: recent advances in our knowledge. Phytochemistry **64**(1): 21-30.

Syed F., P.J. John, I. Soni. (2015) Neurodevelopmental consequences of gestational and lactational exposure to pyrethroids in rats. Environ. Toxicol. **Advanced-Online**: 1-10.

Wang R.-L.L., C. Staehelin, Q.-Q.Q. Xia, Y.-J.J. Su, R.-S.S. Zeng. (2015) Identification and characterization of CYP9A40 from the tobacco cutworm moth (spodoptera litura), a cytochrome P450 gene induced by plant allelochemicals and insecticides. Int. J. Mol. Sci. **16**(9): 22606-22620.

War A.R., M.G. Paulraj, B. Hussain, A.A. Buhroo, S. Ignacimuthu, H.C. Sharma. (2013) Effect of plant secondary metabolites on legume pod borer, *Helicoverpa armigera*. J. Pest Sci. **86**(3): 399-408.

Zhang, F.-l. Hu. (2009) Flavonoids in propolis. Nat. Prod. Res. Dev. **21**(6): 1084-1090.

Zhang Y.E., H.J. Ma, D.D. Feng, X.F. Lai, Z.M. Chen, M.Y. Xu, Q.Y. Yu, Z. Zhang. (2012) Induction of detoxification enzymes by quercetin in the silkworm. J. Econ. Entomol. **105**(3): 1034-1042.