Project title: Use of honey phytochemicals as honey bee "nutraceuticals" to boost pesticide detoxification

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Background: Honey bees (*Apis mellifera*) provide important pollination services to the U.S. agricultural enterprise, valued at more than $15 billion per year. However, colonies have been declining due to multiple stressors, including parasites, pests, diseases, pesticides, diet quality, and modern beekeeping practices, acting individually and in combination. Pesticides are among stressors with adverse health effects known to be influenced by certain aspects of diet quality (e.g., pollen protein). Although honey substitutes are economically important aids for enabling beekeepers to maintain bees over the winter, the absence of key phytochemicals in these substitutes may compromise immunity and detoxification. Through a series of studies in our laboratory (Mao et al. 2009, 2013, 2015, 2017), we have demonstrated that consumption of *p*-coumaric acid and quercetin, ubiquitous constituents of honey and beebread, upregulates cytochrome P450 detoxification genes in worker adults and larvae. Of particular significance is the upregulation by phytochemicals of P450s in the CYP9Q subfamily implicated in pesticide detoxification. We requested and received funds for "Follow-up research on a project which demonstrated a potential for practical application, but further study is advised"—to test if the upregulation of detoxification gene expression by dietary phytochemicals results in enhanced survival and longevity in the presence of pesticides

Objectives: To determine if ingestion of phytochemicals and concomitant upregulation of detoxification gene expression results in enhanced survival and longevity of honey bees in the presence and absence of pesticides.

Experimental approach:

We evaluated possible beneficial effects of phytochemical consumption in the presence and absence of ecologically relevant pesticides in two ways—by conducting longevity assays with adult bees to assess long-term benefits and by carrying out LD_{50} assays to assess short-term benefits. Phytochemicals tested were *p*-coumaric acid and quercetin for two reasons; first, because they are ubiquitous constituents of honey and beebread and, second, because we previously demonstrated that these two phytochemicals upregulate CYP9Q P450s, which are known to detoxify pesticides. In terms of pesticides tested, in 2016 we examined two pyrethroids—bifenthrin and beta-cyfluthrin—because they are in widespread use in U.S. crop production (including almond and other bee-pollinated crops). In 2017, we extended our study to examine an in-hive acaricide, tau-fluvalinate, and a neonicotinoid pesticide, imidacloprid. Tau-fluvalinate is a pyrethroid used to control *Varroa* mites and consequently is a ubiquitous hive contaminant (Mullin et al. 2010). Imidacloprid is a systemic pesticide used in many crops and bees in agroecosystems are challenged by its presence in nectar, pollen, beebread, and beeswax.

Results:

In 2016, we conducted assays of adult longevity on sugar syrup diets with and without a non-plant protein source (casein) in the presence and absence of quercetin and *p*-coumaric acid. We also tested the effects of casein, quercetin, and *p*-coumaric acid in the presence or absence of the two pyrethroid insecticides bifenthrin and beta-cyfluthrin. By the Cox proportional hazards

models (Cox model) analysis on the pooled results of 2975 caged honey bees, the survival analysis revealed that all tested experimental factors (protein, phytochemicals, and pesticides) affected the longevity of adult bees (Table 1 and 2). Dietary quercetin (HR = 0.82, $\chi^2 = 27.93$, $p < 0.01$), *p*-coumaric acid (HR = 0.91, $\chi^2 = 5.93$, $p = 0.015$), and casein (HR = 0.74, $\chi^2 = 66.31$, $p < 0.001$) were associated with extended lifespan (Table 1 and 2) whereas the two pyrethroid insecticides, 4 ppm bifenthrin and 0.5 ppm beta-cyfluthrin, reduced lifespan (HR = 9.17, $\chi^2 = 1741.640$, $p < 0.001$ and HR = 1.35, $\chi^2 = 42.157$, $p < 0.001$, respectively) as well reduced mean survival time by 12.63 and 1.89 days (−50.5% and −7.5%), respectively, of the tested bees (across Figure 1, 2, and 3). Dietary quercetin enhanced tolerance of both pyrethroids; *p*-coumaric acid had a similar effect trend (Figure 2 and 3), although of reduced magnitude. Casein in the diet appears to eliminate the life-prolonging effect of *p*-coumaric acid in the absence of quercetin.

Table 1. 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 (casein, phytochemicals, and pesticides) affected the longevity of the honey bees. Casein, *p*-coumaric acid, and quercetin had positive effects on caged worker longevity (with hazard ratios <1). The 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. 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 comparison symbols indicate results obtained by Cox proportional hazards model**. The blue comparison symbols indicate 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.

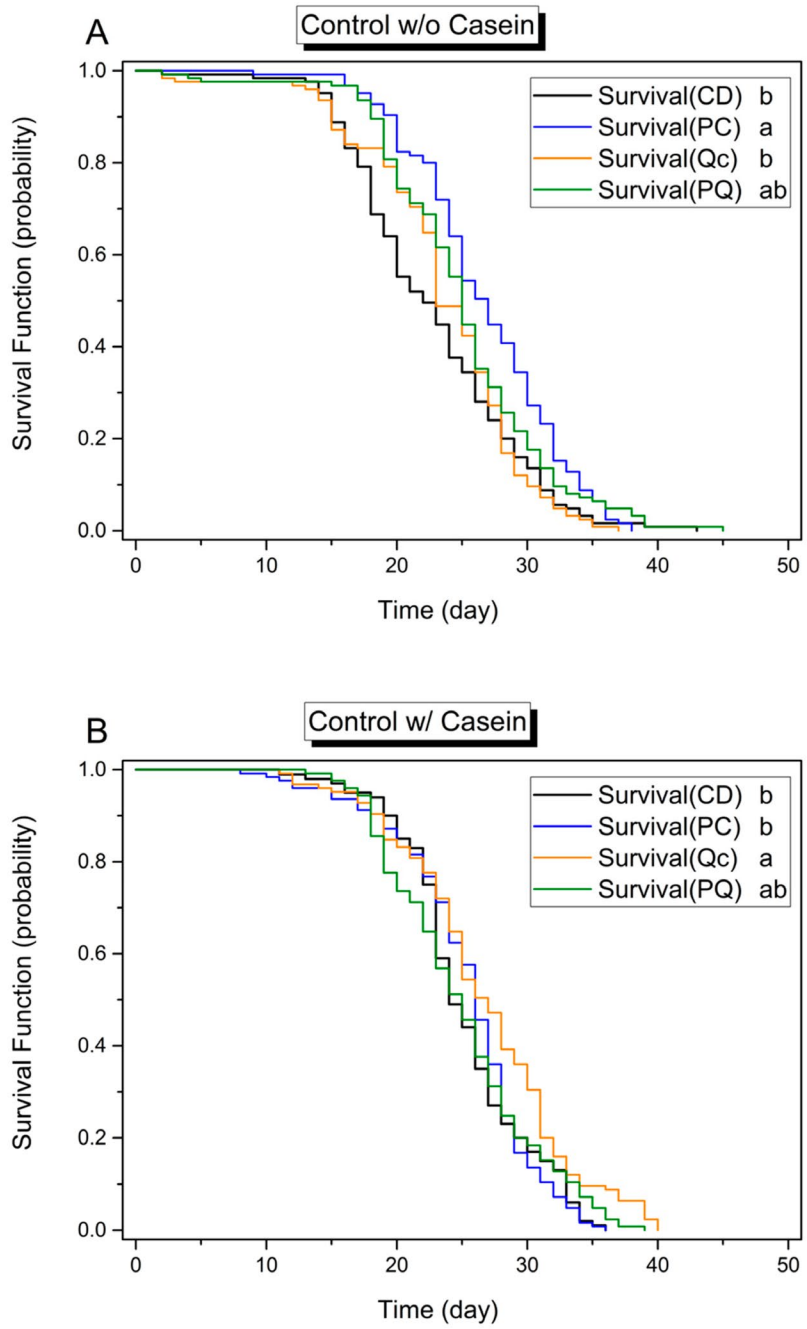

Figure 1. Kaplan–Meier plot of honey bee survival function on different diets with different phytochemical supplements. These diets were (**A**) protein-free or (**B**) protein-supplemented. CD, diet lacking phytochemicals; PC, diet containing 0.5 mM p-coumaric acid; Qc, diet containing 0.25 mM quercetin; PQ, diet containing 0.5 mM p-coumaric acid and 0.25 mM quercetin. (n = 100 for protein-rich and phytochemical-free diet group (CD Figure 1B), and n = 125 for the other groups). Different lower-case letters indicate statistical differences between treatments (log-rank paired test, $p < 0.0083$ after Bonferroni correction).

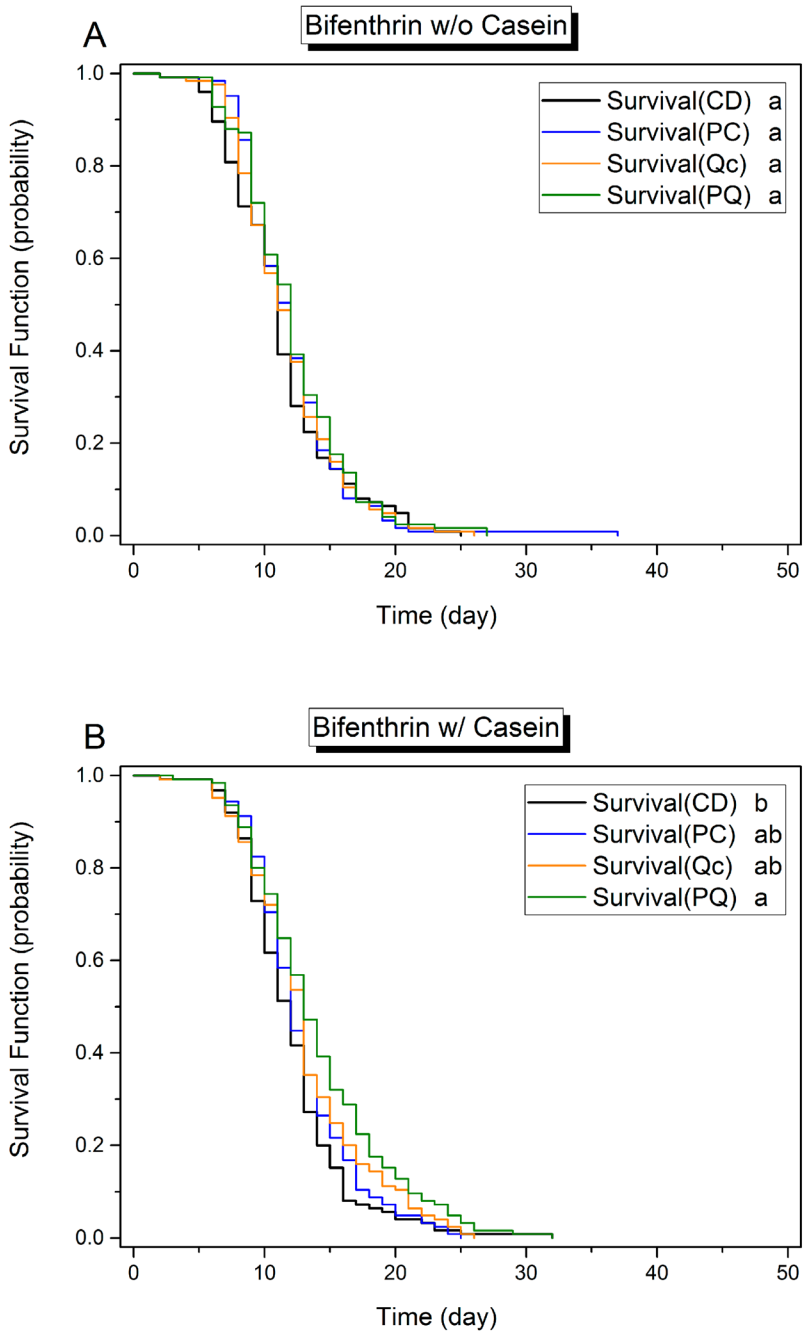

Figure 2. Kaplan–Meier plot of honey bee survival function on different diets with different phytochemical supplements and bifenthrin amendment. Theses diets were (**A**) protein-free or (**B**) protein-supplemented. CD, diet lacking phytochemicals; PC, diet containing 0.5 mM p-coumaric acid; Qc, diet containing 0.25 mM quercetin; PQ, diet containing 0.5 mM p-coumaric acid and 0.25 mM quercetin. (n = 125 for each group.) Different lower-case letters indicate statistical differences between treatments (log-rank paired test, $p < 0.0083$ after Bonferroni correction).

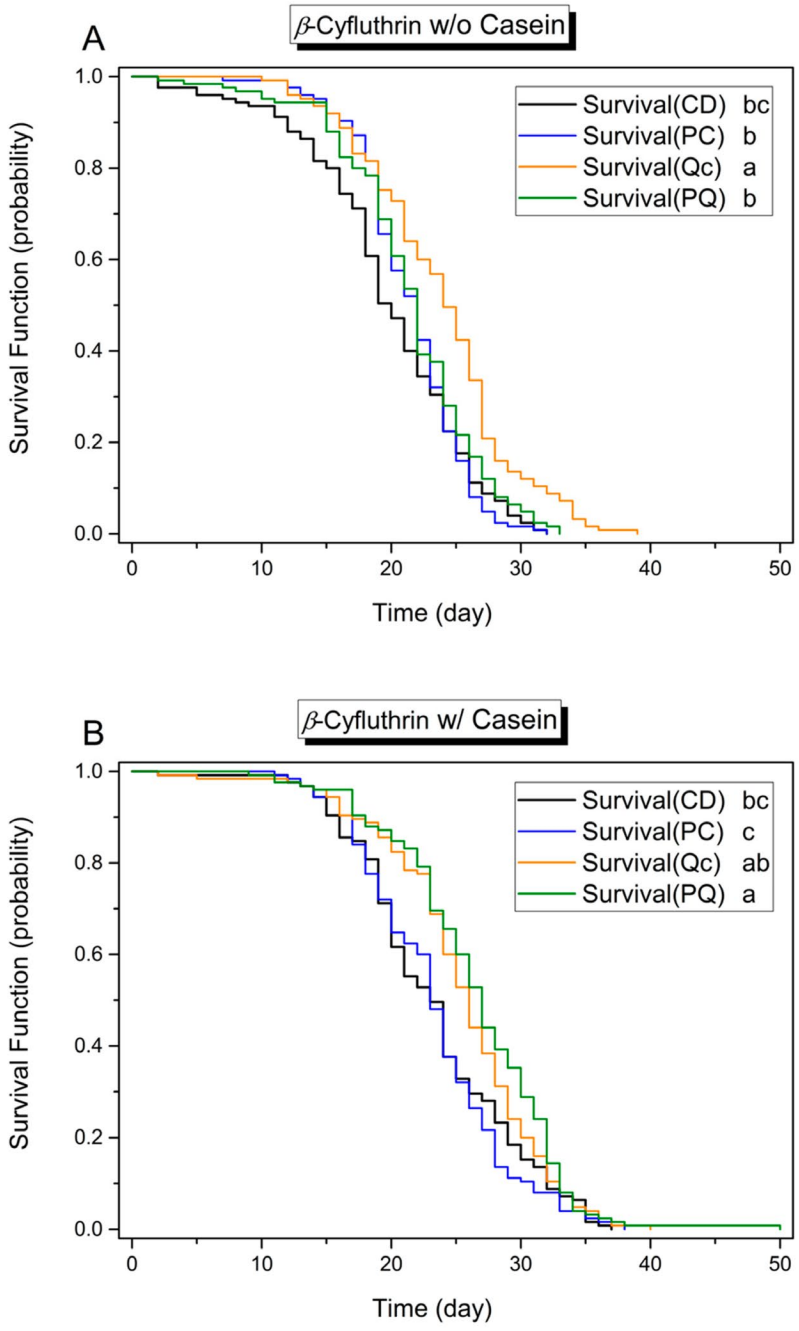

Figure 3. Kaplan–Meier plot of honey bee survival function on diets with different phytochemical supplements and β-cyfluthrin amendment. These diets were (**A**) protein-free or (**B**) protein-supplemented. CD, diet lacking phytochemicals; PC, diet containing 0.5 mM p-coumaric acid; Qc, diet containing 0.25 mM quercetin; PQ, diet containing 0.5 mM p-coumaric acid and 0.25 mM quercetin. (n = 125 for each group.) Different lower-case letters indicate statistical differences between treatments (log-rank paired test, $p < 0.0083$ after Bonferroni correction).

In 2017, we extended our 2016 studies and tested the interaction of these two phytochemicals with additional pesticides: the acaricide tau-fluvalinate and the neonicotinoid imidacloprid. We performed 24- and 48-hour LD_{50} assays on tau-fluvalinate in the presence and absence of quercetin and *p*-coumaric acid. and replicated the entire experiment three times. One-day-old bees were fed with a range of concentrations of phytochemicals for three days and then underwent topical treatments using a range of concentrations of tau-fluvalinate. Each phytochemical alone had no significant effect on the LD_{50} of tau-fluvalinate (Table 3). However, at 1.50 mM *p*-coumaric acid, 0.25 mM quercetin increases the tau-fluvalinate LD_{50} compared to 1.50 mM *p*-coumaric acid alone (from 1.757 to 2.779 ug/bee at 24 hours, and 1.401 to 2.343 ug/bee at 48 hours). The results were consistent with the findings of Johnson et al. (2012) that quercetin reduces the acute toxicity of tau-fluvalinate.

Table 3. Effects of phytochemicals on the 24-hour and 48-hour median lethal dose (LD_{50}, calculated by the probit model) of tau-fluvalinate to the honey bee

Phytochemical concentration		Median lethal dose (LD_{50}) of tau-fluvalinate					
		24-hour			48-hour		
p-Coumaric acid (mM)	Quercetin (mM)	LD_{50} (ug/bee)	95% CI* LowerBound	95% CI* UpperBound	LD_{50} (ug/bee)	95% CI* LowerBound	95% CI* UpperBound
0.00	0.00	2.380	1.9	2.9	2.054	1.6	2.5
0.00	0.25	2.271	1.8	2.7	2.125	1.7	2.6
0.00	1.25	2.592	2.1	3.1	2.441	2.0	3.0
0.50	0.00	2.226	1.8	2.7	2.010	1.5	2.5
0.50	0.25	2.516	2.0	3.1	1.887	1.4	2.4
0.50	1.25	2.586	2.1	3.1	2.090	1.6	2.6
1.50	0.00	1.757[a]	1.1	2.4	1.401[b]	0.9	1.9
1.50	0.25	2.779[a]	2.0	3.8	2.343[b]	1.7	3.0
1.50	1.25	2.270	1.6	3.0	1.967	1.4	2.5

Each treatment was tested in three replicate cages of honey bees, and each cage contained nine to ten honey bees; bees were treated topically with four doses of tau-fluvalinate (1.5, 2.5, 3.5 and 4.5 ug/bee) and an acetone control.

* CI: Confidence Interval

[a, b] the same superscript letter indicates a significate difference between the two LD_{50}

To determine the effect of quercetin and/or *p*-coumaric acid on honey bees in the presence of imidacloprid, both acute and chronic toxicity bioassays were conducted; three replicate experiments of three hives were carried out. Honey bee imidacloprid LC_{50} values, the median lethal concentrations, were determined in the presence of quercetin and/or *p*-coumaric acid. Additionally, longevity assays were conducted with one-day-old bees at varying field-realistic concentrations of imidacloprid in the presence of quercetin and/or *p*-coumaric acid. The phytochemicals had no significant effect on the LC_{50} of imidacloprid (Tables 4 and 5).

Table 4. Median-lethal concentration (LC_{50}) for imidacloprid containing different phytochemical treatments after 24 hours.

	n	LC_{50} (ppm)	95% Confidence Interval (ppm)
Control (DMSO)	2350	11.182	9.48 - 12.877
0.25 mM quercetin	2350	11.273	9.808 - 12.738
0.50 mM *p*-coumaric Acid	2350	10.695	9.408 - 11.952
0.25 mM quercetin + 0.50 mM *p*-coumaric Acid	2350	11.191	9.773 - 12.603

Bees were provided with five concentrations of imidacloprid-containing treatment diets (5, 10, 15, 20, and 25 ppm) and a DMSO control.
n = a total number of bees included in the bioassay, LC_{50} = lethal concentration 50% calculated by the probit model, 95% Confidence Interval = confidence interval calculated using Fieller's method, standard error = standard error for the 95% LC_{50} confidence interval.

Table 5. Median-lethal concentration (LC_{50}) for imidacloprid containing different phytochemical treatments after 48 hours.

	n	LC_{50} (ppm)	95% Confidence Interval (ppm)
Control (DMSO)	2350	6.832	5.117 - 8.279
0.25 mM quercetin	2350	6.246	4.976 - 7.346
0.50 mM *p*-coumaric Acid	2350	5.831	4.25 - 7.143
0.25 mM quercetin + 0.50 mM *p*-coumaric Acid	2350	6.283	5.363 - 7.111

Bees were provided with five concentrations of imidacloprid-containing treatment diets (5, 10, 15, 20, and 25 ppm) and a DMSO control.
n = a total number of bees included in the bioassay, LC_{50} = lethal concentration 50% calculated by the probit model, 95% Confidence Interval = confidence interval calculated using Fieller's method, standard error = standard error for the 95% LC_{50} confidence interval.

In the test of long-term effects, however, at concentrations of 0, 15, and 45 ppb imidacloprid, *p*-coumaric acid significantly increased longevity (Figures 4, 5, and 6). Quercetin also significantly increased honey bee longevity at 15 ppb imidacloprid but had an antagonistic effect on *p*-coumaric acid in the absence of imidacloprid (Figure 5). These results indicate that these two phytochemicals may play an important role in detoxification activity of tau-fluvalinate and at low levels of imidacloprid but lose their protective effect at higher concentrations. This study thus reinforces the importance of considering the interactions between phytochemicals and other xenobiotics when evaluating honey bee health.

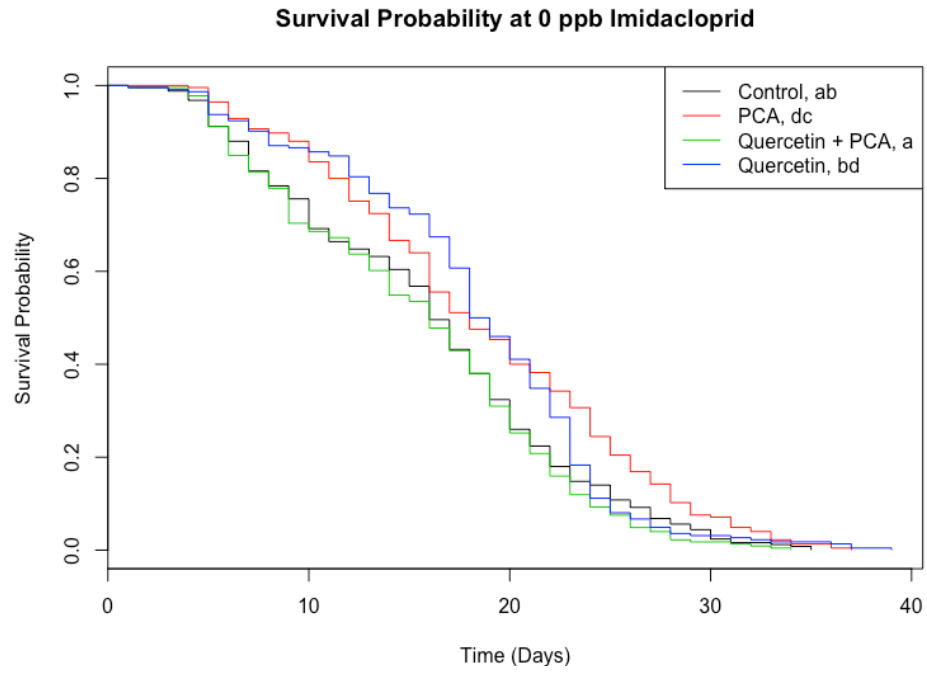

Figure 4. Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals but lacking imidacloprid. Control diet lacked phytochemicals. The PCA diet contained 0.5 mM *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. The Quercetin diet contained 0.25 mM quercetin. Letters a, b, c, and d represent significantly different groups, and n = 225 for each group.

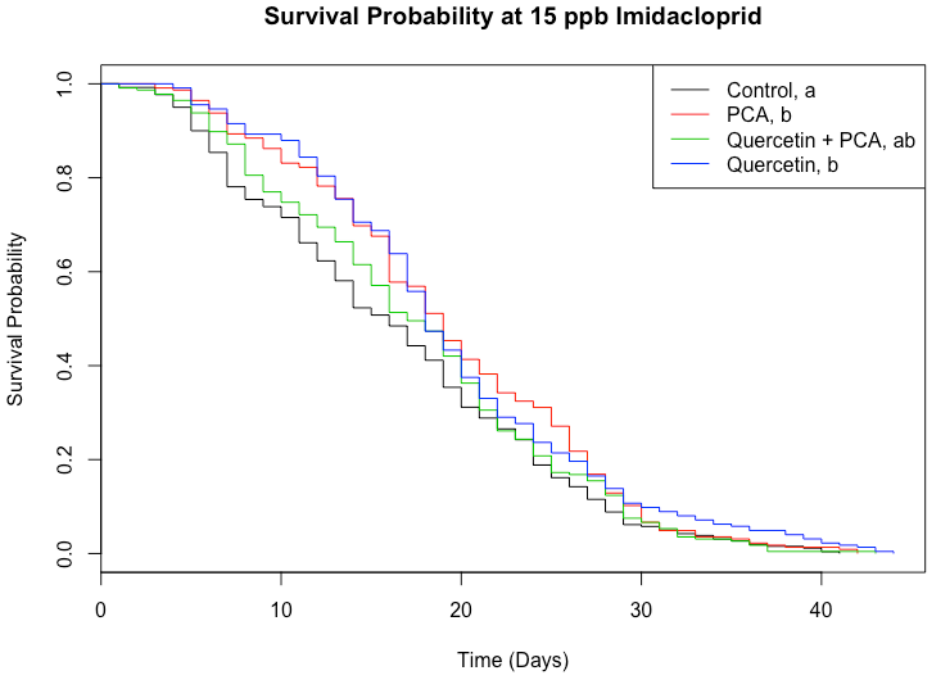

Figure 5. Kaplan-Meier plot of honey bee survival probability on diets containing phytochemical supplements and 15 ppb imidacloprid. Control diet lacked phytochemicals. The PCA diet contained 0.5 mM *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. The Quercetin diet contained 0.25 mM oquercetin. Letters a and b represent significantly different groups, and n = 225 for each group.

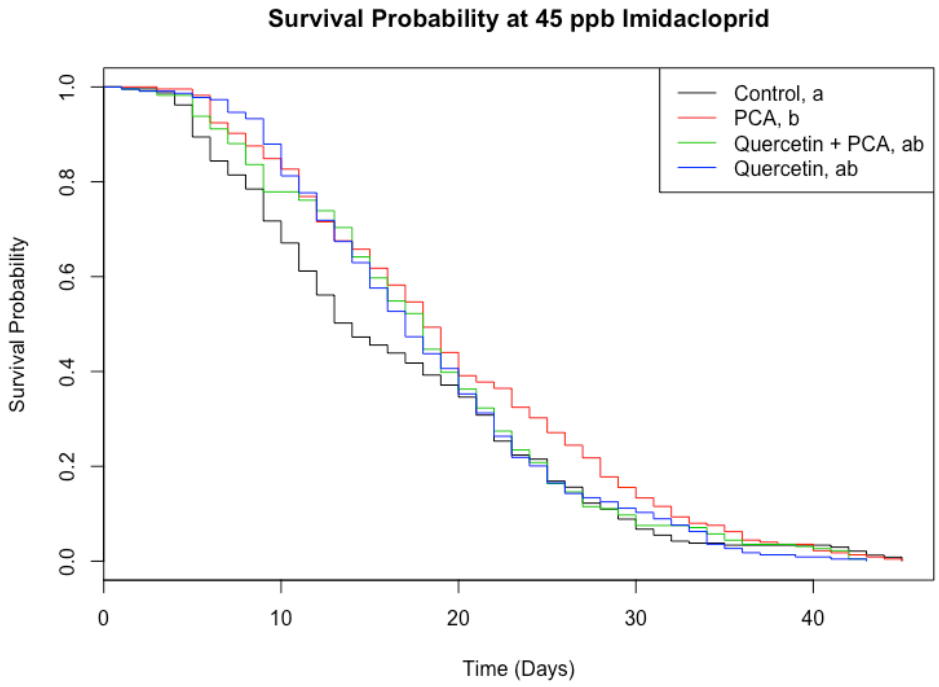

Figure 6. Kaplan-Meier plot of bee survival probability on diets containing phytochemicals and 45 ppb imidacloprid. Control diet lacked phytochemicals. The PCA diet contained 0.5 mM *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mM *p*-coumaric acid and 0.25 mM quercetin. The Quercetin diet contained 0.25 mM quercetin. Letters a and b represent significantly different groups, and n = 225 for each group.

Summary

Collectively, the work supported by the National Honey Board has yielded evidence that dietary phytochemicals influence honey bee longevity and responses to pesticide stress. Our findings can potentially inform decision-making by beekeepers about substituting sugar syrups for honey or yeast/soy flour patties may thus have hitherto unrecognized impacts on adult bee health.

Publications resulting from National Honey Board support

A portion of this work was published in the journal *Insects*, in a special issue edited by Jay Evans devoted to studies of interacting factors contributing to bee stress:

Liao, L-H, W-Y Wu and M R Berenbaum, 2017. Impacts of dietary phytochemicals in the presence and absence of pesticides on longevity of honey bees (*Apis mellifera*). *Insects* 2017 8, 22: doi:10.3390/insects8010022.

The acute and chronic toxicity assays involving imidacloprid were carried out as part of the masters degree program of graduate student Michael Wong and can be found in his thesis:

Wong, M., 2018. Amelioration of acute and chronic toxicity of imidacloprid by dietary phytochemicals in honey bees (*Apis mellifera*). Master's thesis, University of Illinois at Urbana-Champaign.

The thesis work is being prepared for submission to a journal for publication.

References

Mao, W., R. M. Johnson, S. Rupasinghe, M. A. Schuler and M. R. Berenbaum, 2009. Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp Biochem Physiol C: Toxicology and Pharmacology 154: 427-434.

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

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

Mao, W, MA Schuler and MR Berenbaum, 2013. Honey constituents upregulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proceedings of the National Academy of Sciences 110: 8842-8846.

Mao, W., M.A. Schuler and M.R. Berenbaum, 2015. A dietary phytochemical alters caste determination gene expression in honeybees. Science Advances 1(7): e1500795

Mao, W., M.A. Schuler and M.R. Berenbaum, 2017. Disruption of quercetin metabolism by fungicide affects energy production in honey bees (*Apis mellifera*). Proc. Natl. Acad. Sci. USA 114: 2538-2543.

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