

AMELIORATION OF ACUTE AND CHRONIC TOXICITY OF IMIDACLOPRID BY  
DIETARY PHYTOCHEMICALS IN HONEY BEES (*APIS MELLIFERA*)

BY

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THESIS

Submitted in partial fulfillment of the requirements  
for the degree of Master of Science in Entomology  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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## ABSTRACT

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, nutrition, and modern beekeeping practices. In particular, the neonicotinoid imidacloprid has been targeted as a potentially strong contributor to colony decline. Imidacloprid is a systemic pesticide, and honey bees are challenged by its presence in nectar, pollen, water, and beeswax. In the presence of imidacloprid, honey bees upregulate cytochrome P450 enzymes, which are implicated in their detoxification; because consumption of the phytochemicals quercetin and *p*-coumaric acid, common constituents of honey and beebread, also upregulates cytochrome P450 monooxygenases, the presence of these compounds in the diet of honey bees may ameliorate the toxic effects of imidacloprid. In order 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. Honey bee imidacloprid LC<sub>50</sub> values, the median lethal concentrations, were determined in the presence of field-realistic levels 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<sub>50</sub> of imidacloprid. However, at concentrations of 0, 15, and 45 ppb imidacloprid, *p*-coumaric acid significantly increased honey bee longevity. 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. These results indicate that these two phytochemicals may play an important role in detoxification activity 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.

## **ACKNOWLEDGEMENTS**

Research funds were provided by a grant from the National Honey Board. I would like to thank Ling-Hsiu Liao and Wen-Yen Wu for their support and assistance in the honey bee bioassays. Their initial guidance and help with the development of the methodology was incredibly useful, as well as their continued support when I had to troubleshoot any issues. I would also like to thank William Montag for his assistance in running the assay during the time periods that I was out of town. I also thank my advisor, May Berenbaum, for her advice and encouragement throughout the course of my master's degree. Her guidance, feedback, and edits were instrumental to my success in the entomology department. Finally, I'd like to thank my boyfriend Wade Coufal for being my biggest support during my time as a graduate student, especially during the most stressful periods. His unwavering encouragement gave me the motivation to always be a better student, teacher, and researcher here at University of Illinois.

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## Introduction

The western honey bee (*Apis mellifera* L.) provides important pollination services for agricultural crops in the United States valued at more than \$15 billion per year (Morse and Calderone 2000). They pollinate approximately 35% of major crops grown around the world (Klein *et al.* 2007). However, there have been annual losses in overwintering honey bee colonies in the USA at a rate of almost 30% per year (Steinhauer *et al.* 2016). These substantial losses have been a cause for concern as the pollination services provided by honey bees are vital for improving yields of major fruit, nut, and vegetable crops, such as almonds, apples, avocados, blueberries, cranberries, peaches, and many others (Delaplane and Mayer 2000). As a result, there has been a major investment into research to investigate the cause of these colony declines over the past decade.

The factors that contribute to colony decline are multifaceted and include parasites, pests, diseases, monoculture agriculture, pesticides, nutrition, and modern beekeeping practices (vanEngelsdorp *et al.* 2009). In particular, neonicotinoid pesticides such as imidacloprid have been a focus of attention because of their systemic activity and widespread use, resulting in residues consistently being found in nectar and pollen (Blacquièrè *et al.* 2012). They are effective against a wide variety of crop pests, including phloem-feeding insects. These pesticides function by acting as high-affinity irreversible agonists of the nicotinic acetylcholine receptor, resulting in neuron depolarization and eventual death (Bai *et al.* 1991).

Although there have been a large number of both laboratory-based and field-based experiments on the acute and chronic toxicity of imidacloprid on honey bees, there exists much debate on the reliability and interpretations of these studies (Cresswell 2011, Carreck and Ratneiks 2014, Chagnon *et al.* 2015). The fact that colony-level variation exists in honey bee

sensitivity to imidacloprid (Fairbrother 2014) means that an extensive sampling effort must be made across multiple colonies when estimating imidacloprid toxicity. However, multiple sub-lethal effects of imidacloprid have still been documented; imidacloprid can reduce foraging rate (Schneider *et al.* 2012), decrease learning (Decourtye *et al.* 2004), weaken the immune system (Pettis *et al.* 2012) and delay larval development, in addition to reducing adult longevity (Wu *et al.* 2011). These effects are often seen in laboratory experiments at field-realistic doses of less than 20 ppb, although imidacloprid residues have also been found to range as high as 222 ppb depending on the crop and timing of application (Blacquière *et al.* 2012).

Cytochrome P450 monooxygenases have been implicated in honey bee detoxification of several phytochemicals and pesticides, including neonicotinoids (Berenbaum and Johnson 2015; Manjon *et al.* 2018). Both larval and adult bees upregulate P450s in the CYP6AS and CYP9Q subfamilies when exposed to sublethal levels of imidacloprid (Derecka *et al.* 2013, DeSmet *et al.* 2017). Additionally, out of the 27 of 46 cytochrome P450 genes that make up the CYP3 clade, the only enzymes that metabolize imidacloprid include CYP9Q1, CYP9Q2, and CYP9Q3 (Manjon *et al.* 2018). Honey bees typically encounter imidacloprid as a contaminant in nectar, honey, or beebread, so the possibility exists that co-occurring dietary phytochemicals may alter behavioral or physiological responses to this toxicant.

Dietary phytochemicals play a major role in regulating detoxification enzymes in honey bees. The phenolic acid *p*-coumaric acid and the flavonol quercetin, both constituents of honey, pollen and propolis, upregulate members of the honey bee CYP9Q subfamily in adults and larvae (Mao *et al.* 2009, Mao *et al.* 2011). Specifically, quercetin upregulates CYP9Q1, CYP9Q2, and CYP9Q3 (Mao *et al.* 2017), while *p*-coumaric acid upregulates CYP9Q1 and CYP9Q3 (Mao *et al.* 2015). Quercetin also reduces the acute toxicity of fluvalinate (Johnson *et al.* 2012), and both

quercetin and *p*-coumaric acid increase longevity of honey bees in the presence of  $\beta$ -cyfluthrin and bifenthrin (Liao *et al.* 2017). All three of these pyrethroid insecticides are metabolized by members of the CYP9Q subfamily, suggesting that amelioration of pesticide toxicity by *p*-coumaric acid and quercetin is due to induction of these enzymes.

In this study, I assessed the acute and chronic toxicological effects of imidacloprid in the presence of the phytochemicals *p*-coumaric acid and quercetin. To assess the phytochemical effects on acute imidacloprid toxicity, one-day old bees were used to determine and compare LC<sub>50</sub> values with the addition of quercetin and *p*-coumaric acid individually or in combination. Additionally, to assess the effects of phytochemicals on chronic imidacloprid toxicity, one-day old bees were tested in a longevity bioassay with diets containing quercetin, *p*-coumaric acid, or both at a range of field-realistic values of imidacloprid. In view of evidence that these phytochemicals upregulate P450 genes encoding pesticide-metabolizing enzymes, consuming them should lead to decreased toxicity and increased longevity in honey bees exposed to imidacloprid on both a short-term and long-term basis.

## **Materials and Methods**

### *Honey Bee Colonies*

This experiment utilized three western honey bee colonies located in an apiary maintained by the University of Illinois at Urbana-Champaign in east Urbana (Champaign County, IL) during the summer of 2017. Frames of capped brood were taken from each colony and then moved into a dark incubator kept at 34°C and 50% humidity. After emergence within the incubator, the one-day old bees were swept off the frames and removed daily until there were sufficient numbers for at least one replicate from each colony. Estimates of the number of honey bees were made by sweeping them into a tray and weighing them on a scale while using the

approximate weight of 0.1 g/bee. Once a sufficient number of one-day-old bees emerged from the frames, they were transferred into closed clear plastic cups (9 oz./266 mL). Each cup had multiple ventilation holes and two larger holes for the insertion of food and water tubes. Tulle (#10271561, Celebrate It™ Occasions™, Irving, TX) was used to line opening of the cup before adding the top of the lid to create the completed experimental cages. Each cage contained 25 newly emerged day-old bees, as well as one-tenth of a strip of an artificial queen mandibular gland pheromone (DC-715, Mann Lake Ltd., Hackensack, MN) to mimic the pheromonal attributes of a queenright colony. The cages were immediately provisioned with the appropriate treatment diet and water and kept in a dark incubator room maintained at 34°C and 50% humidity for the duration of the experiment.

#### *Chemicals and Initial Treatment Preparation*

Technical-grade chemicals were used for all replicates of the experiment. DMSO (D128, Fisher Scientific International, Inc., Pittsburgh, PA), imidacloprid (PS2086, Sigma-Aldrich Co. LLC., St. Louis, MO), quercetin (Q4951, Sigma Aldrich Co. LLC., St. Louis, MO), *p*-coumaric acid (C9008, Sigma-Aldrich Co. LLC., St. Louis, MO), and casein (C3400, Sigma- Aldrich Co. LLC., St. Louis, MO) were all used to create treatment diets for the bioassays. All treatment components were dissolved in deionized water and stored at -20°C.

A stock solution of 50% (w/v) sucrose water was prepared by adding deionized water to 500 g of sucrose until a volume of 1000 mL was reached. All treatment diets were designed to provide a protein-rich diet of 1:12 protein to carbohydrate ratio (Altaye *et al.* 2010). Casein was used as the primary source of protein because of its phytochemical-free nature and use in previous artificial insect diets (Lee 2007), including honey bee diets (Liao *et al.* 2017). To prepare the treatment diets, 2.083 g of casein was placed into 50 mL Eppendorf tubes and filled

to 49.875 mL with the sucrose water stock solution. Stock solutions of imidacloprid and phytochemicals were then added to prepare all diets for use in both short-term and long-term assays.

#### *Effects of phytochemicals on honey bee imidacloprid LC<sub>50</sub> values*

The stock solutions of the phytochemicals and imidacloprid were created by first dissolving the chemicals in DMSO at a concentration of 400x greater than the intended amount. Treatment stock solutions without specific phytochemicals or imidacloprid were substituted with DMSO as the control solvent; to create final treatment solutions of 50 mL at the appropriate concentrations, 125 mL of the phytochemical and imidacloprid stock solutions were then added to the previously prepared 50 mL Eppendorf tubes. All quercetin-containing treatment diets were made up at a concentration of 0.25 mmol, and all *p*-coumaric acid-containing treatments were made up at a concentration of 0.50 mmol. Phytochemical concentrations were based on realistic levels representative of those typically encountered by honey bees in field situations (Mao *et al.* 2015, Mao *et al.* 2017). Imidacloprid-containing treatment diets were prepared at concentrations of 0, 5, 10, 15, 20, and 25 ppm. Imidacloprid concentrations used to determine the LC<sub>50</sub>s were chosen based on pilot trials conducted earlier to determine the general range of toxicity (data not shown). Overall, there were a total of 24 treatments with differing combinations of quercetin and *p*-coumaric acid at a range of imidacloprid concentrations (Table 1).

Each treatment diet was transferred to a 2-mL microcentrifuge tube with a 6-mm opening at the tip for the bees to access, which was inserted into a hole of the experimental cages. Diets were replaced every 24 hours for each cage. Another 2-mL microcentrifuge tube was also filled with deionized water and inserted into the second hole of the experimental cages for the honey bees to access *ad libitum*. Honey bees were checked for mortality every 24 hours over a 48-hour

time period. Honey bees which were immobile and unable to right themselves were considered to be dead. Three replicates from each of the three colonies were used for this experiment, for a total of 9 replicates of 216 cages and a total of 5,400 honey bees in this LC<sub>50</sub> bioassay.

#### *Effects of phytochemicals and imidacloprid on honey bee longevity*

The stock solutions of the phytochemicals and imidacloprid were prepared by first dissolving the chemicals in DMSO at a concentration of 400x greater than the intended amount. Treatment stock solutions without specific phytochemicals or imidacloprid were substituted with DMSO as the control solvent. To previously prepared 50-ml Eppendorf tubes, 125 mL of the phytochemical and imidacloprid stock solutions were added to produce final treatment solutions of 50 mL at the appropriate concentrations. All treatments containing quercetin were prepared at a concentration of 0.25 mmol, and all treatments containing *p*-coumaric acid were prepared at a concentration of 0.5 mmol. Treatments containing imidacloprid were prepared at concentrations of 0, 15, 45, 75, 105, and 135 ppb. These values represent a range of field-realistic concentrations of imidacloprid in agricultural settings (Wu-Smart and Spivak 2016). Overall, there were a total of 24 treatments with and without quercetin and/or *p*-coumaric acid with a range of imidacloprid concentrations (Table 2).

As before, all diets were transferred individually to 2 mL microcentrifuge tubes with a 6-mm opening at the tip for the bees to access, which was inserted into a hole in the experimental cages. Diets were replaced every 24 hours for each cage and an additional 2-mL microcentrifuge tube was filled with deionized water and inserted into the second hole of the experimental cages for the honey bees to access *ad libitum*. Water tubes were replaced every 5 days or when the volume of water fell below 1 mL. Cages were checked for mortality daily until the death of the last bee in each treatment. Three replicates from each of the three colonies were used for this

experiment, for a total of 9 replicates of 216 cages and a total of 5,400 honey bees in this longevity bioassay.

### *Statistical analysis*

All statistical analyses were performed in R version 3.1.1 (R Core Team, Vienna, Austria). For the LC<sub>50</sub> bioassays, Probit analysis was conducted using the “survival” statistical package to estimate the median lethal concentration needed to kill 50% of the honey bees over each 24-hour interval over 48 hours. The 95% confidence intervals for the corresponding LC<sub>50</sub> values were determined using Fieller’s method (Hoekstra 1991). Comparisons across treatments and colonies were made by analyzing the data for overlapping confidence intervals, in addition to performing pairwise LC<sub>50</sub> likelihood ratio tests (Wheeler 2006). The pairwise ratio comparisons were considered significant if their 95% confidence interval included a “1” between the upper and lower bounds.

For the longevity bioassays, survival curves were plotted using the Kaplan-Meier estimator. Pair-wise log rank tests, adjusted with the Bonferroni correction for each of the pairwise comparisons, were carried out to determine whether differences between curves were significant.

## **Results**

### *Effects of phytochemicals on honey bee imidacloprid LC<sub>50</sub> values*

Results from the analysis of overlapping confidence intervals showed that there were no significant differences between LC<sub>50</sub> values for imidacloprid after 24 hours on the different phytochemical treatment diets (Table 3). All of the pairwise likelihood ratio tests between the treatments resulted in 95% confidence intervals without a “1” within their upper and lower bounds, providing further evidence that there are no significant differences between these 24-

hour imidacloprid LC<sub>50</sub> values. All of these values ranged between 10.7 ppb – 11.3 ppb imidacloprid across the phytochemical treatments.

Similarly, no significant differences in imidacloprid LC<sub>50</sub> values were found between treatments after 48 hours (Table 4). No overlapping confidence intervals were found between the treatments, and likelihood ratio tests also results in confidence intervals without a “1” within their lower and upper bounds. All LC<sub>50</sub> values ranged from 5.8 ppb – 6.8 ppb imidacloprid across the phytochemical treatments.

#### *Effects of phytochemicals and imidacloprid on honey bee longevity*

Kaplan-Meier survivorship curves were plotted to determine if there was an overall significant effect of imidacloprid on honey bee longevity across the entire experiment (Figure 1). The log-rank test showed that bees feeding on diets containing imidacloprid lived significantly longer than honey bees on diets without imidacloprid ( $\chi^2 = 25.7$ ,  $p < 0.001$ ).

Across all the concentrations of imidacloprid in the overall experiment (Figure 2), a pairwise log-rank test showed that honey bees fed with *p*-coumaric acid lived significantly longer than bees that were fed on unamended diet ( $\chi^2 = 7.5$ ,  $p = 0.006$ ) after using the Bonferroni correction. However, all other pairwise comparisons between treatments over all concentrations of imidacloprid were not significant ( $p > 0.0083$ ).

In the absence of imidacloprid (Figure 3), honey bees consuming *p*-coumaric acid experienced greater longevity than bees consuming the control diet ( $\chi^2 = 13.0$ ,  $p = 0.003$ ). Additionally, bees consuming *p*-coumaric acid also experienced greater longevity than bees consuming diets containing both *p*-coumaric acid and quercetin ( $\chi^2 = 22.7$ ,  $p < 0.0083$ ). This same pattern was observed with quercetin; bees consuming diets containing this phytochemical also experienced greater longevity than bees consuming diets containing both *p*-coumaric acid

and quercetin ( $\chi^2 = 12.6$ ,  $p = 0.004$ ). However, all other pair-wise comparisons between diets lacking imidacloprid yielded no significant differences ( $p > 0.0083$ ).

At 15 ppb imidacloprid (Figure 4), bees consuming diets containing *p*-coumaric acid lived significantly longer than those on the control diet ( $\chi^2 = 8.4$ ,  $p = 0.004$ ), and honey bees consuming diets containing quercetin also lived significantly longer than bees consuming the control diet ( $\chi^2 = 9.2$ ,  $p = 0.002$ ) according to the results of a log-rank test with the Bonferroni correction. However, all other pairwise comparisons were not significantly different ( $p > 0.0083$ ).

At 45 ppb imidacloprid (Figure 5), bees consuming diets containing *p*-coumaric acid also experienced greater longevity than those on the control diet ( $\chi^2 = 7.8$ ,  $p = 0.005$ ). However, all other pairwise comparisons at this concentration of imidacloprid were not significant ( $p > 0.0083$ ).

At all higher concentrations of imidacloprid (75 ppb, 105 ppb, and 135 ppb), no significant pairwise comparisons were found (Figures 6-8) according to the results of log-rank tests with the Bonferroni correction ( $p > 0.0083$ ).

Additionally, no significant pairwise comparisons were detected between the different concentrations of imidacloprid on survival probability (Figure 9) according to the results of log-rank tests with the Bonferroni correction ( $p > 0.0033$ ). Over the entire experiment, higher concentrations of imidacloprid did not result in significantly lower survival probabilities.

## **Discussion**

Honey bees have two routes of exposure to imidacloprid: either exposure in the field as a result of foraging, or exposure within the hive from consumption of stored beebread and honey that contain imidacloprid residues (Blacquièrè *et al.* 2012). Additionally, imidacloprid has also

been shown to accumulate in beeswax, albeit at levels much lower than in a field setting (Mullin *et al.* 2010). As a result, honey bees in or near agricultural fields containing floral resources are constantly being challenged by the presence of imidacloprid from egg to adulthood. As larvae, they can potentially be reared in contaminated wax and fed honey or beebread laced with imidacloprid. Then, once they transition their behavior toward foraging as adults, they can encounter imidacloprid either topically by flying through planter dust or settling on contaminated surfaces, or orally in nectar, pollen, and water at even higher concentrations than seen within the hive (Blacquière *et al.* 2012). Therefore, both acute and chronic toxicity of imidacloprid are important factors to consider when evaluating their effects on honey bees.

In terms of acute toxicity of imidacloprid, consuming the phytochemicals *p*-coumaric acid and quercetin individually or in combination failed to provide any beneficial effects. In view of the fact that the presence of these phytochemicals has been previously been shown to upregulate detoxification genes and promote longevity in honey bees (Mao *et al.* 2015, Liao *et al.* 2017), it appears that this protective effect over imidacloprid must build up over a longer period of time than within the 24-48 hour timeframe used here for acute toxicity bioassays. However, this is not necessarily the case for all pesticides encountered by honey bees; the addition of quercetin to diet decreased the toxic effects of tau-fluvalinate within 24 hours (Johnson *et al.* 2012). Similarly, this pattern does not apply to all insects; quercetin reduced toxicity of lambda-cyhalothrin in the lepidopteran *Helicoverpa armigera* after 24 hours due to increased activity of cytochrome P450 enzymes (Chen *et al.* 2017). A possible explanation for the failure of phytochemicals to “rescue” bees from toxic effects of imidacloprid could relate to the fact that the toxicity of imidacloprid (and other neonicotinoids) manifests itself through a delayed reaction

compared to other pesticides (Suchail *et al.* 2001), and thus makes detection of amelioration of toxicity over a 24- to 48-hour assay unlikely.

The LC<sub>50</sub> of imidacloprid calculated in this study is considerably greater than values reported in other studies. Although LC<sub>50</sub> values ranging from 54 ppb (Suchail *et al.* 2000) to as high as 600 ppb after 48 hours (Suchail *et al.* 2001) were determined in other studies for honey bees, this study yielded an LC<sub>50</sub> value approximating 6 ppm, an order of magnitude higher than previously reported values. Discrepancies in these values could be attributed to colony-level differences in imidacloprid toxicity, as there does appear to be a strong colony effect regarding imidacloprid sensitivity (Fairbrother *et al.* 2014). This possibility is further supported by the fact that many studies have estimated LC<sub>50</sub> values for imidacloprid in honey bees that vary by a factor of more than 100-fold (e.g. Fischer and Chalmers 2007, Suchail *et al.* 2000). Insects such as *Musca domestica* also show large variation in imidacloprid toxicity, with LC<sub>50</sub> values differing by over 17-fold (Kaufman *et al.* 2007).

With respect to longevity, the fact that consuming a diet containing imidacloprid from 15 ppb – 135 ppb prolonged longevity relative to that of bees consuming unamended diet was completely unexpected. Although not previously reported, this enhanced longevity may reflect a tendency of bees exposed to pesticides to ingest greater quantities of diet and therefore survive for a longer period of time. Imidacloprid has been shown to be a feeding stimulant (Kessler *et al.* 2015, Dana 2017) and therefore treatments containing imidacloprid may have induced an increase in consumption rate. This feeding stimulant effect has also been seen with the pesticides bifenthrin and beta cyfluthrin (Liao *et al.* 2017), and the resulting increase in consumption of sugar and protein could lead to increased longevity despite the presence of the pesticide. Additionally, the incorporation of imidacloprid into the diet could have a hormetic effect, in

which the presence of imidacloprid actually enhances its own detoxification. For example, at low levels of imidacloprid, antioxidant and immunity genes are upregulated in honey bee workers (Chaimanee *et al.* 2016). Honey bee workers also have improved survival consuming sugar water when low levels of nicotine, a compound chemically similar to neonicotinoids, are added (Kohler *et al.* 2012). However, hormetic effects of pesticides, although known to exist, are not well understood (Guedes and Cutler 2014, Cutler and Rix 2015).

In contrast to the acute toxicity bioassay, ingesting *p*-coumaric acid significantly increased honey bee longevity in the chronic exposure bioassay. However, the beneficial effect of consuming either phytochemical appears to be manifested only at low concentrations of imidacloprid, ranging between 0 – 45 ppb and are undetectable at concentrations above 75 ppb. The fact that *p*-coumaric acid has the potential to increase honey bee longevity in the absence of pesticides has been documented previously (Liao *et al.* 2017), and this study provides further support for this phenomenon. However, in the presence of quercetin, the two phytochemicals interact antagonistically, such that *p*-coumaric acid significantly increases longevity in the absence of imidacloprid, but the effect is no longer significant with the addition of quercetin to the diet.

Quercetin itself can have both negative and positive effects on insect health. Although, as stated earlier, quercetin upregulates detoxification and immunity genes in honey bees (Mao *et al.* 2013), it can disrupt metabolism and energy production in larval honey bees (Mao *et al.* 2015) and interfere with the growth and development of other phytophagous insects such as the cotton bollworm (*Helicoverpa armigera*) in the absence of pesticides (Liu *et al.* 2015). The antagonism between quercetin and *p*-coumaric acid ingestion has been reported previously (Liao *et al.* 2017), and this interaction was also detected in treatments with lower concentrations of imidacloprid.

How these two phytochemicals interact in the presence of pesticides is as yet undetermined. These sublethal concentrations of imidacloprid may have a priming effect, as sublethal concentrations of another neonicotinoid, thiacloprid, increased metabolic detoxification expression in honey bees (Alptekin *et al.* 2016). As a result, quercetin metabolism could be favored rather than disrupted by the presence of low levels of imidacloprid, resulting in increased longevity. The possibility also exists that both phytochemicals as well as imidacloprid compete for access to the catalytic site of the same P450 enzyme for detoxification and thereby interact synergistically (Mao *et al.* 2011; Manjon *et al.* 2018).

The beneficial effect of both *p*-coumaric acid and quercetin on honey bee longevity are negligible at concentrations of imidacloprid at 75, 105, and 135 ppb. Despite the absence of impact on longevity of these sublethal doses of imidacloprid, at higher concentrations imidacloprid may still cause oxidative stress. The oxidative stress induced by imidacloprid has been well documented in vertebrates (e.g. Duzguner and Erdogan 2012, Sinan *et al.* 2013, Ge *et al.* 2015). Given that neonicotinoids have a much higher selectivity factor for nicotinic acetylcholine receptors in insects compared to vertebrates (Tomizawa and Casida 2005), the resulting oxidative stress is also likely to be much greater in insects than in vertebrates. Additionally, imidacloprid has been found to downregulate both antioxidant and immunity genes in honey bee queens (Chaimanee *et al.* 2016). Therefore, given that both *p*-coumaric acid and quercetin are antioxidants that can reduce oxidative stress (Alwasel *et al.* 2017, Dong *et al.* 2014) at low levels of imidacloprid, higher concentrations of imidacloprid with the concomitant oxidative stress associated with its toxicity may overwhelm the protective antioxidative effects of these two phytochemicals.

The benefits of consuming honey and beebread phytochemicals for honey bees may also stem from their role in P450 upregulation, and it is possible that the stress from imidacloprid exposure at high concentrations weakens their effect. Although P450 inhibitors piperonyl butoxide, triflumizole, and propiconazole do not have a significant effect of increasing the toxicity of imidacloprid (Isawa *et al.* 2004), cytochrome P450 enzymes still play the main role in their detoxification; the reduced sensitivity of imidacloprid to P450 detoxification compared to other neonicotinoids (Manjon *et al.* 2018) implies that upregulation of these genes is crucial to honey bee health. If imidacloprid cannot be efficiently metabolized, then increased upregulation of CYP9Q1-3 enzymes must make up for that deficiency. Because *p*-coumaric acid and quercetin differentially upregulate genes in the CYP9Q subfamily, they may be able to increase the metabolic capacity of honey bees to ameliorate the toxic effects of imidacloprid at low concentrations.

Quercetin and *p*-coumaric acid represent only two of a tremendous diversity of phytochemicals encountered by honey bees even over the course of a year in a single locality. The phytochemical composition of honey varies with the identity of plants from which nectar was collected to make the honey and consequently most honeys contain multiple phenolic acids and flavonols (Gheldof *et al.* 2002) as well as other constituents, including terpenoids, sulfur-containing compounds, and alkaloids (Jerković and Kuš 2017, Dübecke *et al.* 2011). Thus, at the same time bees ingest a diversity of pesticides due to hive contamination (Mullin *et al.* 2010), they also ingest complex mixtures of phytochemicals that may collectively have greater protective effects than have been detected here with just one phenolic acid (*p*-coumaric acid) and one flavonol (quercetin) (Mao *et al.* 2013). Poor nutrition has been a focus for concern because of the common beekeeping practice of provisioning colonies with honey and pollen substitutes.

For example, both sucrose and fructose (particularly in the form of high-fructose corn syrup) have frequently been used as honey substitutes in hives, and many pollen substitutes are also frequently used (Brodschneider and Crailsheim 2010). The absence of appropriate phytochemicals in these substitutes could affect how honey bees process synthetic and natural chemicals that they regularly encounter in contemporary agroecosystems.

## Figures and Tables

### Phytochemical Treatments

<u>Imidacloprid Concentrations (ppm)</u>	<b>Control (DMSO)</b>	<b>0.25 mmol quercetin</b>	<b>0.50 mmol <i>p</i>-coumaric Acid</b>	<b>0.25 mmol quercetin + 0.50 mmol <i>p</i>-coumaric Acid</b>
<b>0</b>	Treatment 1	Treatment 7	Treatment 13	Treatment 19
<b>5</b>	Treatment 2	Treatment 8	Treatment 14	Treatment 20
<b>10</b>	Treatment 3	Treatment 9	Treatment 15	Treatment 21
<b>15</b>	Treatment 4	Treatment 10	Treatment 16	Treatment 22
<b>20</b>	Treatment 5	Treatment 11	Treatment 17	Treatment 23
<b>25</b>	Treatment 6	Treatment 12	Treatment 18	Treatment 24

**Table 1.** Summary of the 24 different treatments used in the honey bee LC<sub>50</sub> bioassays. Each treatment was given to a cage of 25 bees, for a total of 600 bees per replicate. Three replicates from three hives were used, resulting in 9 replicates, 216 cages, and 5,400 bees used over the course of the imidacloprid LC<sub>50</sub> bioassay.

<u>Imidacloprid Concentrations (ppm)</u>	<b>Phytochemical Treatments</b>			
	<b>Control (DMSO)</b>	<b>0.25 mmol quercetin</b>	<b>0.50 mmol <i>p</i>-coumaric Acid</b>	<b>0.25 mmol quercetin + 0.50 mmol <i>p</i>-coumaric Acid</b>
<b>0</b>	Treatment 1	Treatment 7	Treatment 13	Treatment 19
<b>15</b>	Treatment 2	Treatment 8	Treatment 14	Treatment 20
<b>45</b>	Treatment 3	Treatment 9	Treatment 15	Treatment 21
<b>75</b>	Treatment 4	Treatment 10	Treatment 16	Treatment 22
<b>105</b>	Treatment 5	Treatment 11	Treatment 17	Treatment 23
<b>135</b>	Treatment 6	Treatment 12	Treatment 18	Treatment 24

**Table 2.** Summary of the 24 different treatments used in the honey bee longevity bioassays.

Each treatment was given to a cage of 25 bees, for a total of 600 bees per replicate. Three replicates from three hives were used, resulting in 9 replicates, 216 cages, and 5,400 bees used over the course of the imidacloprid longevity bioassay.

**24-Hour Imidacloprid LC<sub>50</sub>**

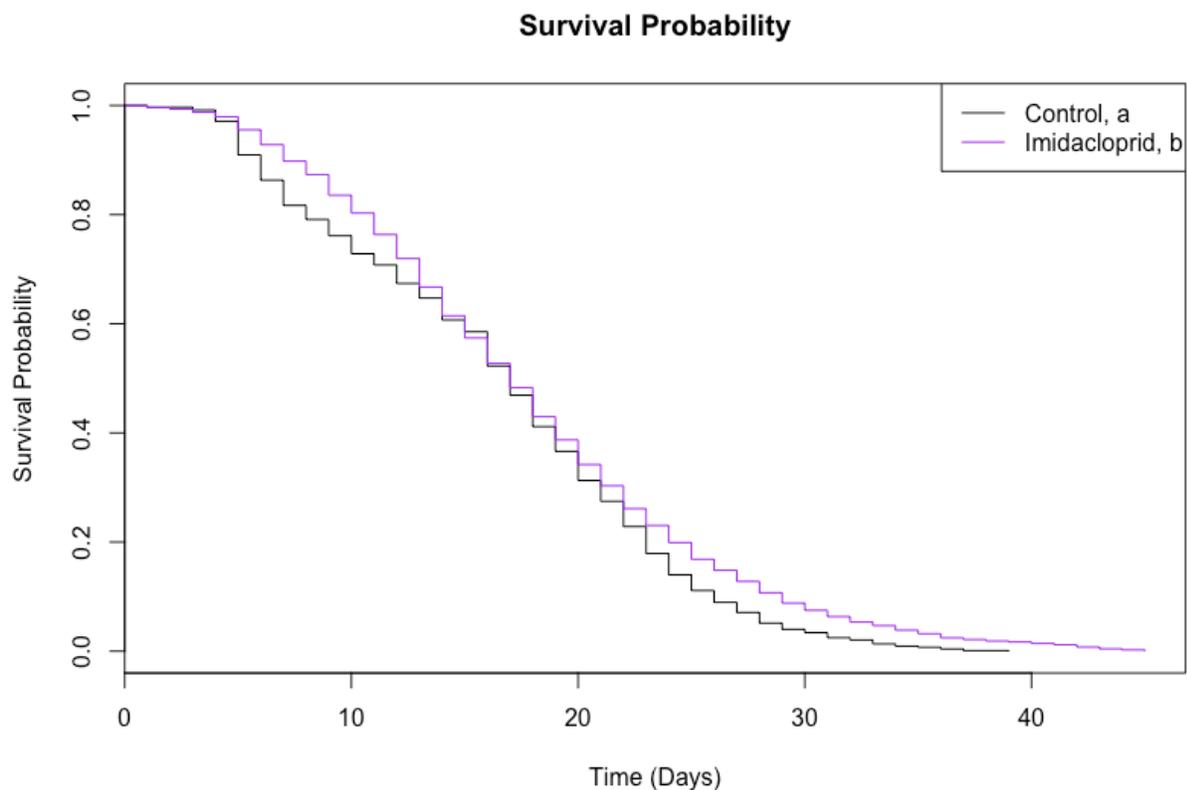
	<b>n</b>	<b>LD<sub>50</sub> (ppm)</b>	<b>95% Confidence Interval (ppm)</b>	<b>Standard Error (ppm)</b>
<b>Control (DMSO)</b>	2350	11.182	9.48 - 12.877	1.702
<b>0.25 mmol quercetin</b>	2350	11.273	9.808 - 12.738	1.465
<b>0.50 mmol <i>p</i>-coumaric Acid</b>	2350	10.695	9.408 - 11.952	1.287
<b>0.25 mmol quercetin + 0.50 mmol <i>p</i>-coumaric Acid</b>	2350	11.191	9.773 - 12.603	1.418

**Table 3.** Median-lethal concentration (LC<sub>50</sub>) values for imidacloprid containing different phytochemical treatments after 24 hours. n = total number of bees included in the bioassay, LC<sub>50</sub> = 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<sub>50</sub> confidence interval.

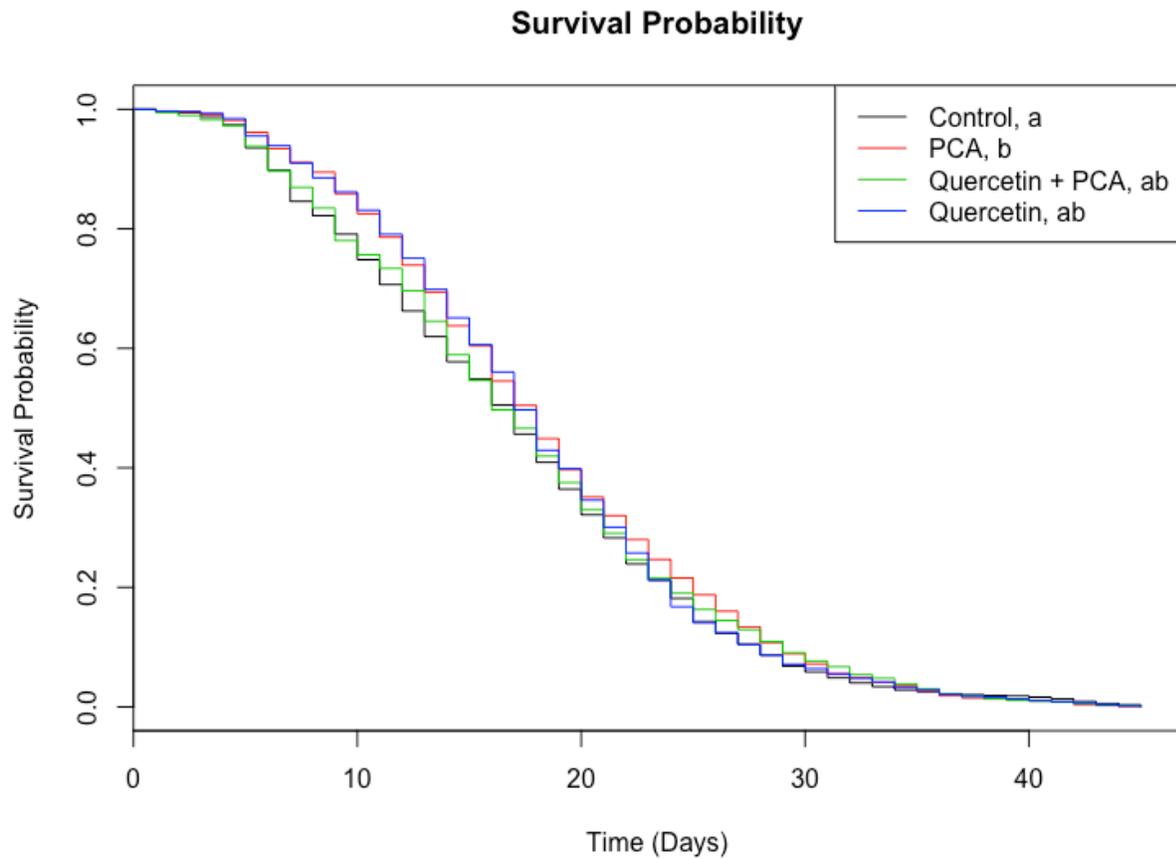
**48-Hour Imidacloprid LD<sub>50</sub>**

	<b>n</b>	<b>LD<sub>50</sub> (ppm)</b>	<b>95% Confidence Interval (ppm)</b>	<b>Standard Error (ppm)</b>
<b>Control (DMSO)</b>	2350	6.832	5.117 - 8.279	1.715
<b>0.25 mmol quercetin</b>	2350	6.246	4.976 - 7.346	1.270
<b>0.50 mmol <i>p</i>-coumaric Aci</b>	2350	5.831	4.25 - 7.143	1.581
<b>0.25 mmol quercetin + 0.50 mmol <i>p</i>-coumaric Acid</b>	2350	6.283	5.363 - 7.111	0.920

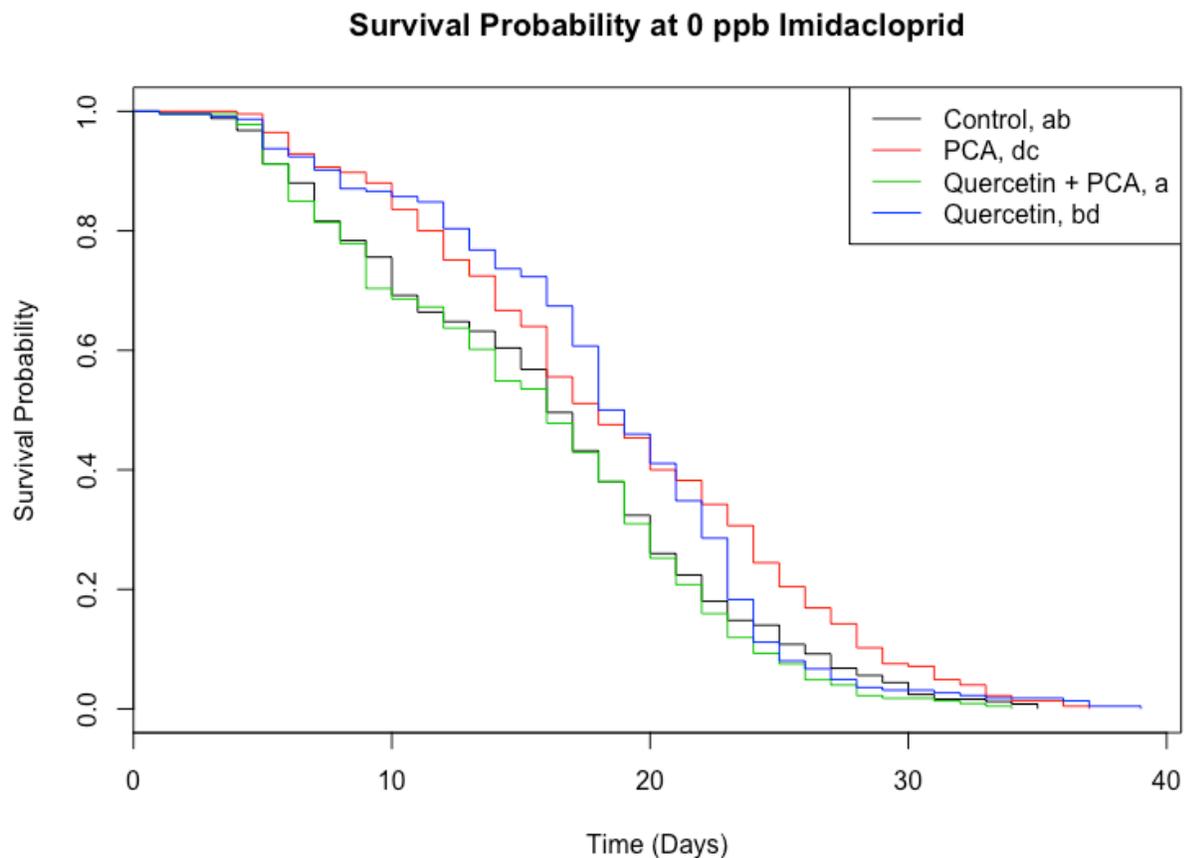
**Table 4.** Median-lethal concentration (LC<sub>50</sub>) values for imidacloprid containing different phytochemical treatments after 48 hours. n = total number of bees included in the bioassay, LC<sub>50</sub> = lethal concentration 50% calculated by the probit model, 95% Confidence Interval = confidence interval calculated using Fieller's method, standard error = standard error for the 95% LC<sub>50</sub> confidence interval.



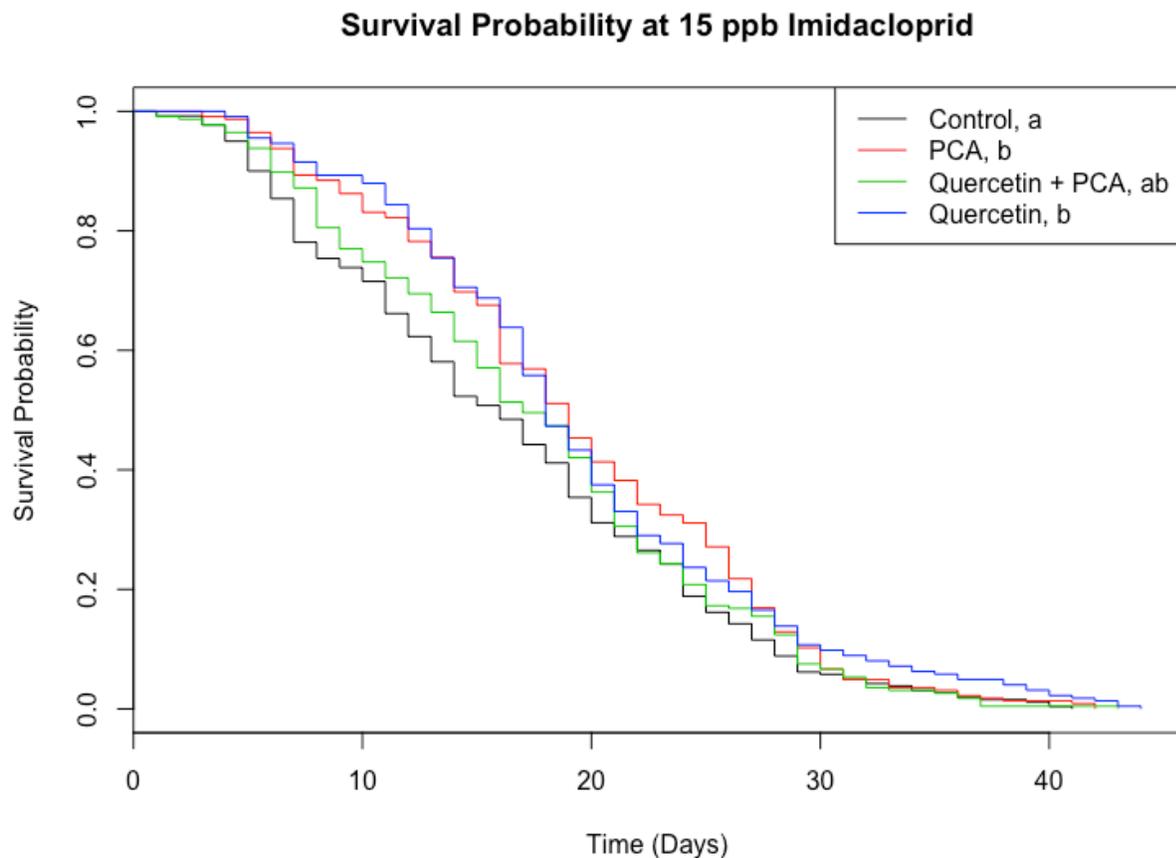
**Figure 1.** Kaplan-Meier plot of honey bee survival probability between those fed with a diet containing imidacloprid and those fed with a diet lacking imidacloprid across all of the phytochemical treatments. Concentrations of imidacloprid ranged from 15 – 135 ppb. Letters a and b represent significantly different groups,  $n = 900$  for the control group, and  $n = 4500$  for the imidacloprid group.



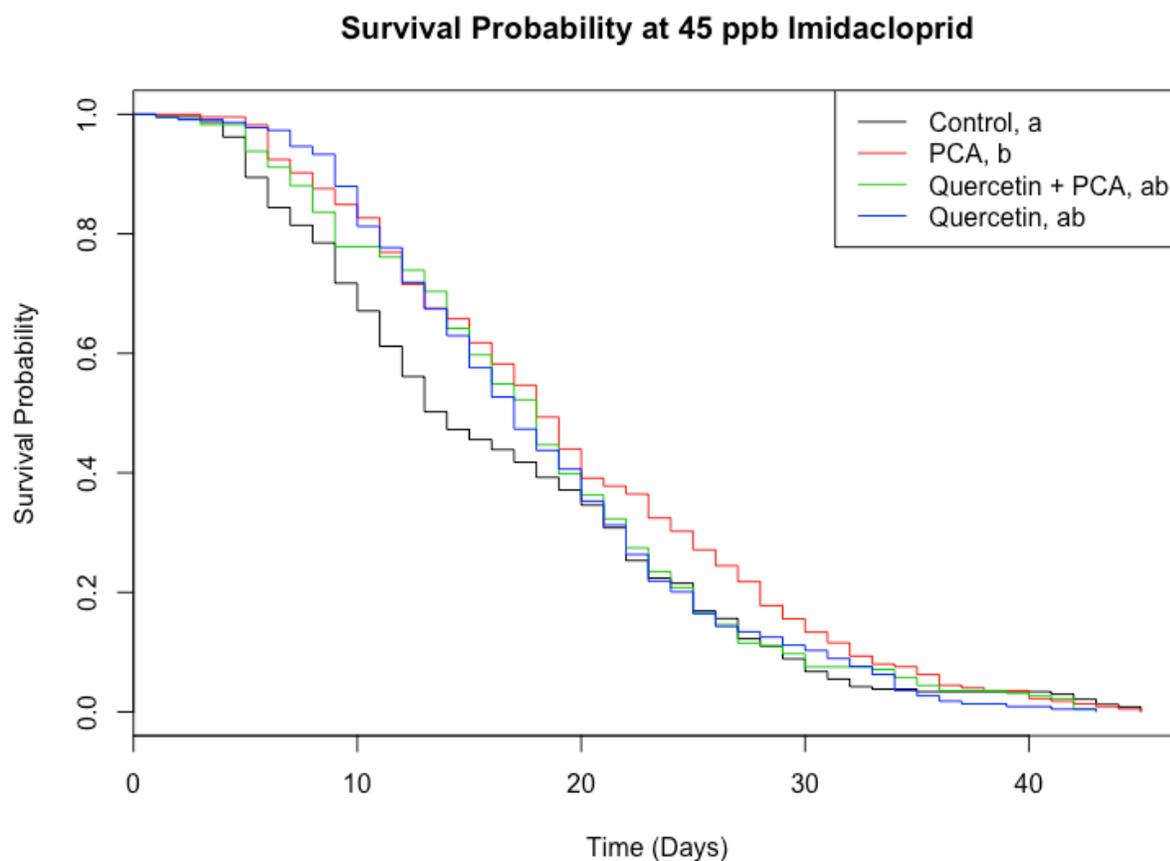
**Figure 2.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals across all concentrations of imidacloprid (0 – 135 ppb). The control diet lacked phytochemicals., whereas the *p*-coumaric acid PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.50 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. Letters a and b represent significantly different groups, and  $n = 225$  for each group.



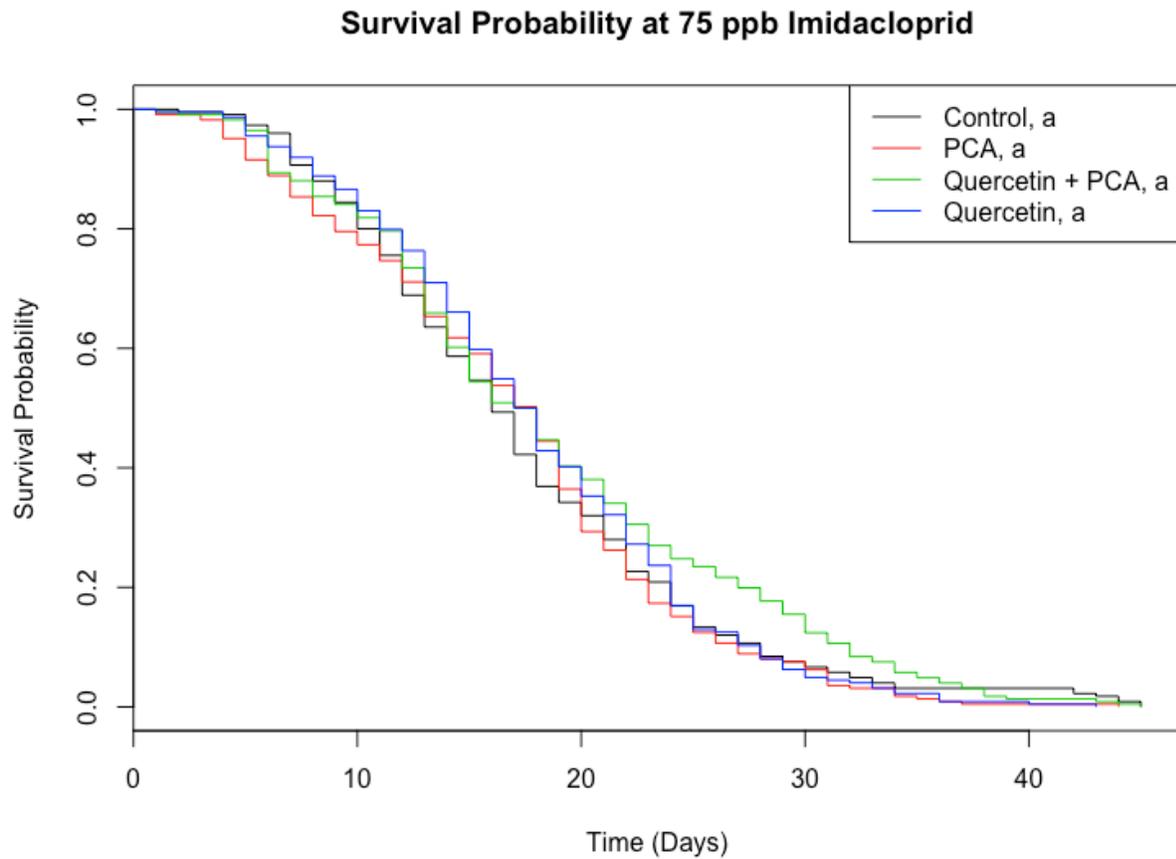
**Figure 3.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals but lacking imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. Letters a, b, c, and d represent significantly different groups, and  $n = 225$  for each group.



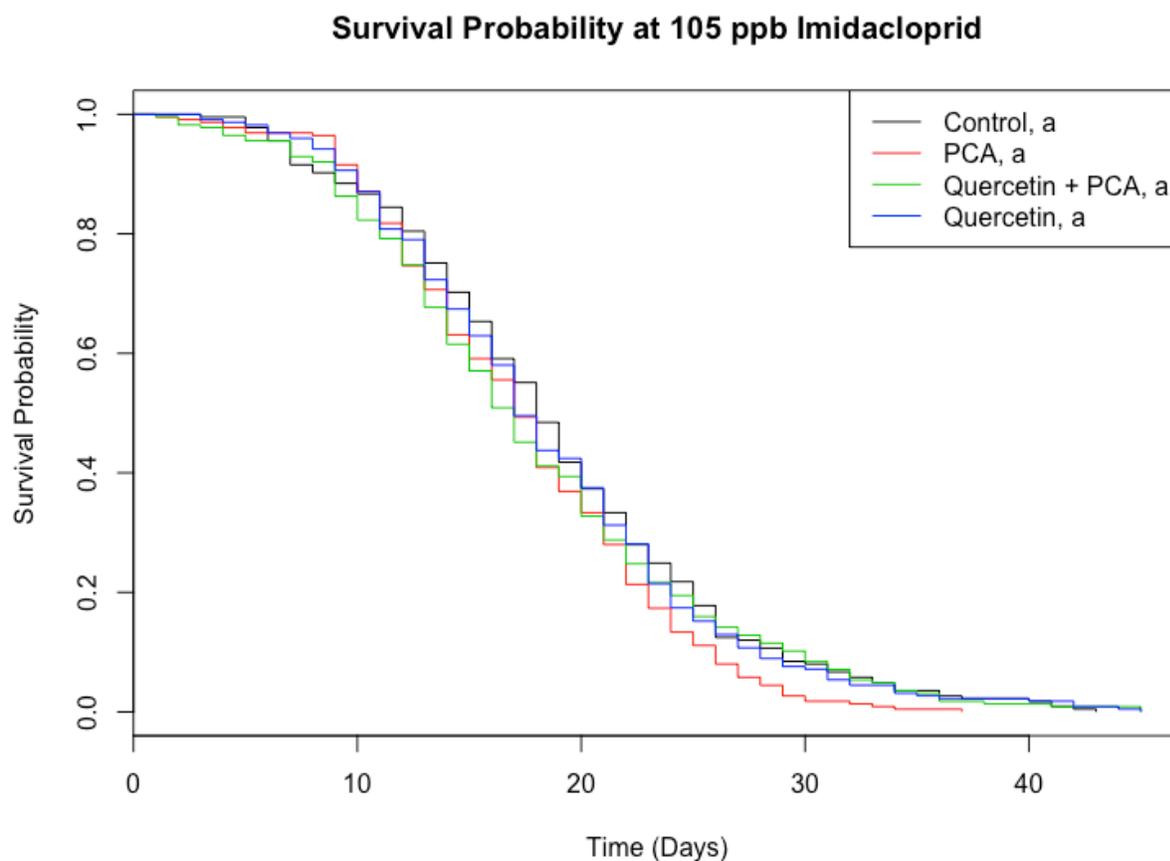
**Figure 4.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemical supplements and 15 ppb imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. Letters a and b represent significantly different groups, and n = 225 for each group.



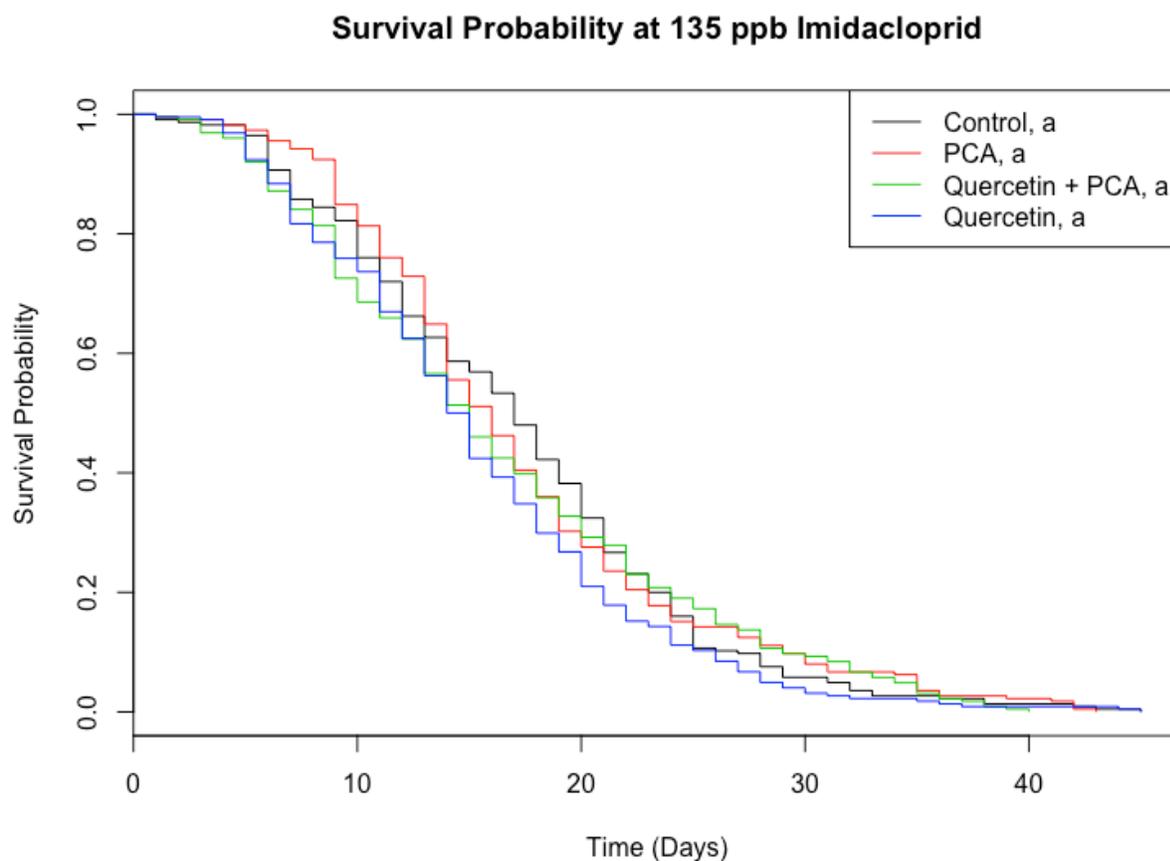
**Figure 5.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals and 45 ppb imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. Letters a and b represent significantly different groups, and  $n = 225$  for each group.



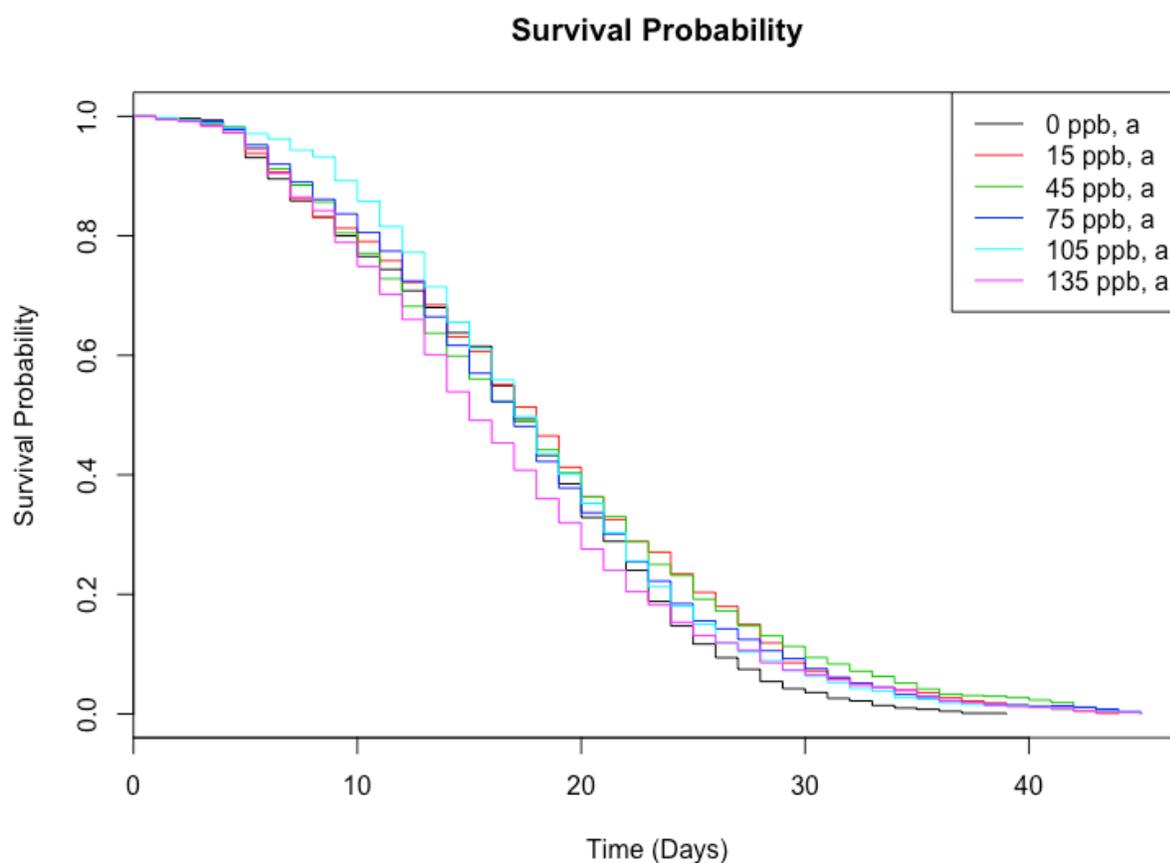
**Figure 6.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals and 75 ppb imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. The letter a represents groups that are not significantly different, and  $n = 225$  for each group.



**Figure 7.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals and 105 ppb imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. The letter a represents no significantly different groups, and  $n = 225$  for each group.



**Figure 8.** Kaplan-Meier plot of honey bee survival probability on diets containing phytochemicals and 135 ppb imidacloprid. The control diet did not contain any phytochemicals. The PCA diet contained 0.5 mmol of *p*-coumaric acid. The Quercetin + PCA diet contained both 0.5 mmol of *p*-coumaric acid and 0.25 mmol of quercetin. The Quercetin diet contained 0.25 mmol of quercetin. The letter a represents groups that are not significantly different, and n = 225 for each group.



**Figure 9.** Kaplan-Meier plot of honey bee survival probability on differing concentrations of imidacloprid across all phytochemical treatments. Each concentration group represents the amount of imidacloprid present in the diets fed to the honey bees. The letter a represents groups that are not significantly different, and  $n = 900$  for each group.

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