THESIS

PLANT SECONDARY METABOLITES ENHANCE SURVIVAL AND PATHOGEN TOLERANCE IN THE EUROPEAN HONEY BEE: A STRUCTURE-FUNCTION STUDY

Submitted by

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ABSTRACT

Adequate nutrition is essential for European honey bee (*Apis mellifera*) colony growth, and productivity, yet foraging limitations resulting from factors such as habitat loss often lead to dietary deficiencies. Plant secondary metabolites are key constituents of floral nectar that support physiological processes in honey bees, however, these compounds are only available to bees with access to a diversity of floral resources.

Furthermore, the relationship between different classes of plant secondary metabolites and their function within honey bee diets requires further investigation. Using a structure-function framework, we evaluated whether four structurally similar plant secondary metabolites found in the nectar of common agricultural crops elicit comparable effects on honey bee survival and pathogen tolerance. The addition of plant secondary metabolites to artificial nectar solution enhanced median survival, in some cases more than doubling the lifespan of worker honey bees. Moreover, plant secondary metabolites demonstrated nutraceutical effects, and sometimes elicited medicinal effects on honey bees infected with *Nosema ceranae.* Our findings provide a platform to identify plant secondary metabolites which can augment current management techniques to support the long-term sustainability of the apiculture industry.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: PLANT SECONDARY METABOLITES ENHANCE HONEY BEE WORKER SURVIVAL; A STRUCTURE-FUNCTION RELATIONSHIP

INTRODUCTION

The chemical relationships between flowering plants and their pollinators are derived from an extensive co-evolutionary history. These chemical relationships sustain the long-standing mutualism between plants and pollinators through nutritive benefits to pollinators in return for pollination services (Bronstein 1994). Pollinators, such as the ubiquitous honey bee, are entirely dependent upon the macronutrients and micronutrients within floral nectar and pollen to satisfy their nutritional requirements (Seeley 1995). Therefore, the constituents of nectar and pollen, which include an assortment of proteins, lipids, and carbohydrates, as well as trace amounts of plant secondary metabolites are essential to honey bee nutrition (Brodschneider and Crailsheim 2010, Cane et al. 2011). The importance of macronutrients within the honey bee diet is well understood, yet, the functional significance of plant secondary metabolites on honey bee health has yet to be fully discovered (Erler and Moritz 2016).

Adequate nutrition is essential for maintaining normal honey bee colony growth and productivity, yet the nutritional requirements of a colony fluctuate over time in response to colony growth status and environmental conditions. Variation in nutritional needs throughout the year can be triggered by a number of natural factors including depletion of winter pollen stores, intensive brood rearing, and provisioning for times of resource scarcities during summer drought or overwintering (Mattila and Otis 2006a). The dietary deficiencies of a colony are mitigated by rigorous pollen and nectar collection by foragers, however, individual bees that have limited access to a balanced diet are poorly equipped to resist biotic and abiotic stressors. Malnourished,

stressed foragers are unable to effectively compensate for these dynamic dietary needs, and cannot efficiently contribute the enhancement of colony food storage (Brodschneider and Crailsheim 2010, Scofield and Mattila 2015). Therefore, the availability of high-quality floral resources is essential to resist environmental stressors, and satisfy colony foraging needs.

The value of floral resources is influenced by the assortment of macronutrients and micronutrient constituents within floral pollen and nectar (Seeley 1995, Di Pasquale et al. 2013). Each component of nectar and pollen supports specific physiological function in honey bees. Consequently, a broad variety of carbohydrates, lipids, proteins, and plant secondary metabolites is necessary to support healthy colonies. The proteins and amino acids found in pollen are essential to the development and growth of larvae and young workers, and enhance longevity whereas lipids, specifically Omega-3 and Omega-6 polyunsaturated fatty acids, support associative learning, cognitive performance, egg production, and wax production (de Groot 1953, Sagili and Pankiw 2007, Hendriksma and Shafir 2016, Avni et al. 2009, Arien et al. 2015, Vaudo et al. 2015). Alternatively, carbohydrates found in nectar fuel the high metabolic demands of foraging adult bees (Ricigliano et al. 2017). Floral resources containing a variety of proteins, lipids, and carbohydrates are considered to be of high nutritional value to honey bees, however, pollen and nectar also contain plant secondary metabolites. Plant secondary metabolites are known toxins to many insects and microbes, but their evolutionary persistence within floral nectar and pollen suggests they may have nutritive value within the honey bee diet (Richardson et al. 2015).

The evolution of plant secondary metabolites is rooted in defense from herbivory or disease, and like herbivores, pollinators can experience detrimental, even toxic effects from these compounds. Plant secondary metabolites found in non-reproductive plant tissues, as well as

floral nectar and pollen, include broad groups of compounds such as non-protein amino acids, alkaloids, terpenoids, and phenolics (Baker 1977). The consequences of exposure to these plant defense chemicals, while costly, may also provide benefits to bees (Adler 2000, Mayack 2009, Alaux et al. 2010, Manson et al. 2010, Hendriksma et al. 2014, Irwin et al. 2014, Richardson et al. 2015). A number of poisonous plant genera require their associated pollinators to adopt biochemical, physiological, and behavioral mechanisms to cope with toxic co-occurring plant secondary metabolites (Adler 2000, Gillespie and Adler 2013, Irwin et al. 2014, Masai Biller 2015, Palmer-Young et al. 2016). Studies have revealed that ingestion of plant secondary metabolites may reduce intensity of gut pathogen infections, and enhance immunity in bumble bees (Alaux et al. 2010, Manson et al. 2010, Hendriksma et al. 2014, Irwin et al. 2014, Richardson et al. 2015, Palmer-Young et al. 2016, 2017c). For example, consumption of gelsemine, a nectar alkaloid found in *Gelsemium sempervirens*, has shown to alleviate severity of *Crithidia bombii*, a gut pathogen which infects bumble bees (Manson et al. 2010). Other plant secondary metabolites including alkaloids, glycosides, phenolics, and terpenoids have also been demonstrated to reduce gut pathogen loading in artificially infected bees (Richardson et al. 2015). The immunocompetence health benefits bees receive from consumption of plant secondary metabolites may have facilitated key reliance upon plants which produce these compounds (Metabolhtes 2004, Pyke 2016, Abrahamczyk et al. 2017). Moreover, plants that produce a diversity of plant secondary metabolites within nectar and pollen may hold greater nutritional value.

Dietary diversity of pollen and nectar is desirable to honey bees, but plant secondary metabolites may be a stronger driving force in resource selection, thus reinforcing chemically mediated plant-pollinator relationships (Alaux et al. 2010). Polyfloral pollen and nectar are

nutritionally superior to single sources of macronutrients and micronutrients, as a variety of proteins, amino acids, and lipids support different functionalities. The same pattern may hold true to plant secondary metabolites. A broad array of dietary plant secondary metabolites supports the upregulation of essential gene expression, while a single phytochemical may have limited benefit to honey bees. Of the different plant secondary metabolites found in floral nectar, the role of p-coumaric acid has been explored at great lengths. In studies that describe the mechanisms behind the physiological benefits of phytochemical ingestion by honey bees, the functional role of p-coumaric acid has been identified to act upon the broad categories of gene upregulation and enzymatic function. The use of p-coumaric acid as a dietary additive not only upregulates cytochrome P450 genes encoding for antimicrobial peptides, but also upregulates the production of enzymes capable of metabolizing pesticides within the midgut of honey bees (Mao et al. 2013). Decreased toxicity and reduced probability of premature death from pesticide exposure are correlated with the consumption of p-coumaric acid (Liao et al. 2017). Furthermore, p-coumaric acid found within beebread and larval brood food influences gene down-regulation associated with ovary reduction in worker bees, and drives worker development and task group differentiation (Mao et al. 2015). While these studies indicate the benefits of a single phytochemical on bee health, further investigation in needed to identify other beneficial plant secondary metabolites to evaluate whether they possess comparable properties to p-coumaric acid.

Structurally similar plant secondary metabolites can elicit similar biological activities (Kelly et al. 2001). This structure-function relationship is demonstrated in a number of compounds found in floral nectar and pollen, and could serve as a means to identify plant secondary metabolites that have analogous properties to p-coumaric acid. p-coumaric acid is

classified as a phenolic acid, and is characterized by a hydroxyl (-OH) group attached to an aromatic ring (Zabka and Pavela 2013). Moreover, phenolic acids share the most antimicrobial, antioxidant activity of many plant secondary metabolites. For example, p-coumaric acid and gallic acid, two phenolic acids found in the nectar of common plants such as *Fragaria ananassa,* and *Rubus idaeus* have similar phenyl group configurations and equivalent biological functionalities in a variety of organisms. These two compounds are known to produce anti-diabetic, antioxidant, and anti-inflammatory activities in a number of insects and mammals (King and Young 1999, Abdel-Moneim et al. 2018). Given that p-coumaric acid has been established to produce beneficial effects in honey bees, it is probable that gallic acid could also have similar functionalities on honey bee physiology. Caffeine is another phenolic acid commonly present in the nectar of crop and non-crop plant species that shares molecular characteristics with p-coumaric acid. Specifically, the reactivity of a hydroxyl group on the phenyl ring within both compounds produces antioxidant behavior (Dejan et al. 2013). These comparable molecular structures yield similar antioxidant functionalities in other biological systems, suggesting they could elicit similar effects within honey bees. This structure-function relationship extends to p-coumaric acid and kaempferol. Similar to gallic acid and caffeine, these two plant secondary metabolites are key phenolic constituents found in *Rubus idaeus* honey that have been identified to limit antimicrobial activity of five Gram-positive bacteria (King and Young 1999). The presence of both compounds in honey bee products, together with their shared structural characteristics and functional similarities, indicate their potential importance within the honey bee diet. Using this structure-function framework, we identify whether a suite of biologically important plant secondary metabolites including p-coumaric acid, gallic acid, caffeine, and kaempferol, possess similar effects that enhance honey bee health.

OBJECTIVES

The primary goal of this study was to evaluate the structure-function relationship between structurally similar plant secondary metabolites that are abundant in floral nectar and pollen, and their biological function within honey bees. To validate this relationship, we compared survival probability of worker honey bees fed nectar solutions containing four structurally similar plant secondary metabolites that are common constituents of floral nectar and, we demonstrate potential for enhancing honey bee health. Moreover, we determined whether different concentration of the four plant secondary metabolites within nectar solution had measurable effects on worker honey bee survival. In pursuing this goal, we will assess the functional role of structurally similar plant secondary metabolites on honey bee physiology, and provide new tools for the enhancement of honey bee colony health.

EXPERIMENTAL DESIGN

To measure the effects of structurally similar plant secondary metabolites on survival probability of honey bees, we used worker honey bees of the same age-cohort from three hives in 2016 (n=171), and four hives in 2017 (n=391). Age-cohort bees were obtained using the method of Arathi and Spivak (2001), wherein honey bee queens from four experimental colonies were provided with empty, uniquely marked frames to lay eggs. The queen was caged to an empty frame for 24 hours to ensure that all eggs on the frame were laid on the same day (Arathi et al.

2000, Arathi and Spivak 2001, Arathi et al. 2006). The date when the queen was caged was marked on the frame, and marked frames containing late-stage pupae were removed from source colonies after 21 days. Frames with late stage pupae were placed in an incubator at 34°C and 50% humidity until the day of emergence (Arathi et al. 2000, Arathi and Spivak 2001, Arathi et al. 2006). Adult bees were color marked on the day of emergence using unique colors for different days to indicate age cohorts (Arathi et al. 2000, Arathi and Spivak 2001, Arathi et al. 2006). Marked bees were then reintroduced to source colonies, and collected after six days to begin the feeding assays.

FIGURE 1 - SINGLE USE CUP CAGES

For the feeding assays, nectar containing plant secondary metabolites and sucrose was created by adding treatment compounds to aqueous 20% sucrose solution until completely dissolved. Compounds tested included p-coumaric acid, gallic acid, kaempferol, and caffeine. Each compound was tested at three (25, 250, and 2500 ppm) concentrations (Table 1). Concentrations ranged from naturally occurring levels found in nectar (25 ppm), to extreme dosages (2500 ppm) (Adler 2000). Control treatment was aqueous 20% sucrose solution. New solutions were mixed every 10-12 days, and frozen at -8°C until use.

Same age-cohort worker bees were entered into the feeding assays at the ages of 8-10 days. Ten bees were randomly assigned to single-use cup cages in an incubator maintained at 34°C at 50% relative humidity (Figure 1) (Evans et al. 2009). Feeding syringes were used to administer nectar and bees had *ad libitum* access to phytochemical solutions. Syringes were

replaced every seven days to ensure freshness of solutions. The cages were monitored for dead bees daily. The date of mortality and number of dead bees for each treatment was recorded.

Statistical Analysis

Kaplan-Meier survival probability estimates were used to evaluate differences in worker bee survival between treatments and concentrations using the survival package in R Version 1.0.153 (Grambsch 2000). Best fit models were constructed using the survfit() function, and significant differences between treatment concentrations were detected using the surfdiff() function. Peto-Peto modified Gehan-Wilcoxon test comparisons, accounting for unequal variance and sample size, were used to compare differences in survival probabilities within treatments with the survdiff() function (Grambsch 2000). Graphics were generated using Microsoft Power BI. Significance was evaluated at alpha equal to 0.05.

RESULTS

Median survival of worker bees that received phytochemical supplementation with p-coumaric acid ($\chi^2 = 15.5$, df = 3, P = 0.001), gallic acid ($\chi^2 = 16.6$, df = 3, P = <0.001), kaempferol ($\chi^2 = 65.8$, df = 3, P = <0.001), and caffeine ($\chi^2 = 33.4$, df = 3, P = <0.001) was typically greater than bees that received control nectar solution (Table 2).

P-COUMARIC ACID

Peto and Peto pairwise comparisons of median survival within the p-coumaric acid feeding treatments showed increased median survival, with bees living 5 days longer in the 25 ppm treatment ($\chi^2 = 11$, df = 1, P = <0.001), and one day longer with the 250 ppm group ($\chi^2 = 3.1$, df = 1, P = 0.08), as compared to bees that received the control nectar solution. The highest dosage of p-coumaric acid, 2500 ppm, greatly extended median survival, with bees living 22

days longer or double that of bees provided the control solution ($\chi^2 = 9.2$, df = 1, P = 0.002) (Figure 2) (Table 3).

GALLIC ACID

Gallic acid supplementation improved median survival at concentrations of 250 ppm, with bees living one day longer than bees provided control nectar ($\chi^2 = 6.5$, df = 1, P = 0.01). Bees administered gallic acid at concentrations of 25 ppm and 2500 ppm demonstrated no significant effect on median survival, with increase of one day in median survival of the low dosage group ($\chi^2 = 0$, df = 1, P = 0.8), and a decrease in median survival by one day for the high dosage group ($\chi^2 = 5.5$, df = 1, P = 0.02) (Figure 3) (Table 3).

KAEMPFEROL

Dietary supplementation with kaempferol greatly enhanced median survival, with bees living 5 days longer with the 25 ppm solution ($\chi^2 = 7.7$, df = 1, P = 0.005), and 6 days longer with the 250 ppm solution ($\chi^2 = 10.7$, df = 1, P = 0.001). Similar to p-coumaric acid, bees that received the highest dose of kaempferol lived more than twice as long as the control bees, with a median survival of 47 days ($\chi^2 = 45.9$, df = 1, P = <0.001) (Figure 4) (Table 3).

CAFFEINE

Caffeine treatments showed that bees given nectar containing 25 ppm and 250 ppm of caffeine lived 4.5 and 6 days longer than bees that received control nectar ($\chi^2 = 13.8$, df = 1, P = <0.001; $\chi^2 = 10.4$, df = 1, P = 0.001). However, bees in the 2500 ppm caffeine treatment lived one day less than the control group ($\chi^2 = 2.8$, df = 1, P = 0.09) (Figure 5) (Table 3).

DISCUSSION

Our study supports the hypothesis the plant secondary metabolites p-coumaric acid, gallic acid, caffeine, and kaempferol, which have similar molecular structural components have

comparable beneficial functional effects on honey bee survival. The structure-function relationship used in this study provides a framework to identify plant secondary metabolites that enhance survival probability in honey bees. The elicited effects demonstrate the importance of plant secondary metabolites within the honey bee diet, as improved worker survival has important consequences for foraging performance as well as resource selection. Given the significant improvement in median survival, particularly with the highest concentrations of kaempferol, and p-coumaric acid supplementation, our findings may have implications for management practices.

The suite of phenolic acids tested in this study have been proven to stimulate antioxidant and antimicrobial activity in other biological systems, which could be mechanisms by which survival was enhanced (Abdel-Moneim et al. 2018). The addition of dietary plant secondary metabolites within supplemental feed may reduce oxidative stress that aging worker honey bees experience, and help bees combat microbial infections. The benefits of combating oxidative stress and microbial infections are exhibited through foraging performance and lifetime energy budget. The number of foraging trips, and consequently the length of peak foraging performance directly corresponds to honey bee worker survival (Maurizio and Hodges 1950, Visscher and Dukas 1997). Key physiological functions such as glycogen synthesis in flight muscles deteriorate after approximately 800km of foraging distance at peak performance (Neukirch 1982). Moreover, physiological deterioration dictates the probability a worker will reach the upper bounds of her lifetime energy budget. Foraging behavior accelerates aging, causes oxidative stress, and exposes honey bees to a host of microbial infections (Seeley 1998). The compounding impacts of these stressors results in a typical lifespan that extends three weeks, but with phytochemical supplementation the deleterious effects of external stressors could be

reduced, thus extending lifetime energy budget and length of peak foraging performance (Maurizio and Hodges 1950, Neukirch 1982).

While dietary supplementation is a necessary tool to promote healthy colony populations, long-standing patterns of pollinator-host plant interactions may be affected by limited phytochemical diversity. The availability of phytochemical diversity in nectar is ecologically significant to the chemical relationships between plants and their mutualistic pollinators. Diversity is a key indicator of nutritional quality, and similar to amino acids in pollen, phytochemical combinations and concentrations differ between plant species (Duffey and Stout 1996, Adler 2000). Pollen with a diversity of amino acids is more desirable to forager bees as it better satisfies nutritional requirements, therefore it could be expected that nectar with a diversity of plant secondary metabolites may also better satisfy these dietary requirements (Vaudo et al. 2015). However, access to diverse nectar plant secondary metabolites is only possible when diverse nutritional sources are present. When access to essential plant secondary metabolites is reduced or eliminated, the chemical relationships between plants and pollinators are weakened. Moreover, a reduction of chemical traits within a landscape may ultimately impact the robustness of plant-pollinator assemblages, and alter ecological interaction patterns between plants and their mutualistic pollinators.

The improvement of survival in honey bee workers is essential with the expansion of large-scale, ubiquitous land-use change such as agricultural intensification, increased monocultures, expanding loss of habitats with wild flowers, and increased commercialization of pollination services. Due to a high degree of floral fidelity and extensive monocultures in agricultural systems, commercial honey bee colonies may not receive the adequate mixture of plant secondary metabolites necessary for a balanced diet (Axel et al. 2011). While nutritional

deficiencies are traditionally addressed through management practices such as feeding with sucrose syrup and pollen, dietary supplementation with plant secondary metabolites is a novel tool that could be particularly important when access to quality nutritional resources is limited (Standifer 1977). Incorporating dietary plant secondary metabolites into the sugar syrup fed to bee colonies will allow bees to access these beneficial nutrients in addition to fulfilling their carbohydrate needs. Our findings suggest that supplemental dosages of p-coumaric acid and kaempferol are more nutritionally valuable than standard sugar syrup solution. Even though the lowest dosages of phytochemical concentrations used in this study best reflect concentrations naturally found in nectar, our results indicate that extreme concentrations, up to 2500 ppm, may have a greater impact on bee longevity. The efficacy of p-coumaric acid and kaempferol to extend honey bee survival indicates that more research is needed into the utility of plant secondary metabolites that share similar chemical structures with these molecules.

The structure-function framework used in this study is advantageous for the identification of plant secondary metabolites to advance our understanding of honey bee health and management. Plant secondary metabolites which are structurally similar to known beneficial compounds can be selectively tested to determine their functional significance in honey bee digestive, cognitive, and immune systems. Current research is underway to assess the benefits of structurally similar plant secondary metabolites on immune response of bees with pathogenic infections. Such findings would provide alternative methods for addressing a suite of problems threatening the long-term sustainability of the apiculture industry and the pollination services they provide for U.S. agriculture. As a result, the data presented here provide a basis for further research into the application of dietary phytochemical supplementation to promote honey bee colony health and productivity.

CHAPTER 2: THE MEDICINAL AND NUTRACEUTICAL VALUE OF PLANT SECONDARY METABOLITES WITHIN THE HONEY BEE DIET

INTRODUCTION

Honey bees have coevolved with a suite of natural enemies, however, malnutrition resulting from commercial management practices has reduced their ability to adequately combat pathogen infections. One such lethal pathogen that is quick to develop resistance against fungicides is *Nosema ceranae.* Synthetic fungicides have rapidly become insufficient in treating infections, but naturally occurring plant secondary metabolites found in nectar and pollen could serve as a supplemented defense mechanism against *Nosema ceranae*. Specifically, plant secondary metabolites that share structural characteristics with phenolic acids have been demonstrated to possess antifungal properties. Using a structure-function framework, we evaluate whether four structurally similar plant secondary metabolites found in the nectar of common agricultural crops elicit comparable nutraceutical effects on honey bee survival. Moreover, we assess the medicinal value of these compounds to treat *Nosema ceranae* infections. Dietary supplementation with p-coumaric acid, gallic acid, kaempferol, and caffeine elicited nutraceutical effects, whereas kaempferol and caffeine were effective in reducing pathogen infections. Our results offer not only nutraceutical tools to enhance honey bee health, but alternative methods of suppressing *Nosema ceranae* infections.

The mechanisms underlying how pollinators combat pathogen infections has significant ecological importance to the evolution of plant-pollinator mutualisms. Pollinators such as the honey bee are entirely reliant upon flowering plants to satisfy their nutritional requirements (Bronstein 1994, Erler and Moritz 2016, Pyke 2016). While an adequate assortment of

carbohydrates, lipids, and amino acids found in floral nectar and pollen support overall honey bee nutrition, a growing body of evidence suggests that plant secondary metabolites support immunocompetence (Seeley 1995, Di Pasquale et al. 2013) (Arien et al. 2015, Scofield and Mattila 2015). Although plant secondary metabolites in floral nectar are considered toxic constituents, the potential benefits to honey bees experiencing pathogen-induced stress may outweigh the harmful effects. This balance between the benefits to honey bees combating pathogen infections, and the fitness costs of consuming potentially toxic compounds is likely a contributing force in the evolution of chemical relationships between plants and their pollinators. Despite the important implications of these chemical interactions, further research is needed to evaluate the relationship between phytochemical compounds and their ability to alleviate pathogen infections in honey bees.

Many plant secondary metabolites are perceived to have evolved as plant defenses against herbivores and pathogens, yet despite their toxicity, these compounds may have subtle immunological benefits to honey bees. Remarkably, these toxic compounds can also be beneficial. Plant secondary metabolites which express long-term health promoting qualities, referred to as “functional foods”, are considered nutraceuticals. For example, the previous study highlights the nutraceutical benefit of p-coumaric acid, wherein healthy bees gained substantial increases in survival from long-term consumption. In contrast, plant secondary metabolites which act upon specific health problems, yet do not serve a nutritional role are considered medicinal (Briskin 2000, Leif Richardson 2016). One specific example of phytochemical medicinal utility has shown that bumble bees experimentally infected with a common gut pathogen, *Crithidia bombii,* will selectively forage for nectar containing iridoid glycosides to reduce infection severity. Yet, healthy bumble bees do not demonstrate this foraging behavior

(Leif Richardson 2016). Unlike nutraceutical or medicinal plant secondary metabolites, some compounds found in nectar simply reduce fitness, regardless of health status, and are simply toxic. Anabasine is an example of a toxic phytochemical as it demonstrates variable, often negative effects on both uninfected and infected bumblebees (Palmer-Young et al. 2017). These examples highlight the wide array of phytochemical properties, ranging from medicinal to toxic. Yet, there are few approaches to identify medicinal or nutraceutical compounds. Given the increase and frequency of pathogenic infections devastating the U.S. apiculture industry, there is a critical need to identify nutraceutical, and medicinal compounds within floral nectar.

Honey bees have co-evolved with a suite of natural enemies, however, evolutionarily derived defense mechanisms may not adequately provide protection against unfamiliar pathogens. Both European and Asian honey bees have adaptations to tolerate pathogens in which they share a common co-evolutionary history, but when a pathogen spreads between species, it can easily take advantage of ill-equipped hosts. For example, a common pathogen of the Asian honey bee, *Nosema ceranae*, does not share a co-evolutionary history with the European honey bee. This lack of exposure to *Nosema ceranae* leaves the European honey bee highly susceptible to infection, and poses a significant threat to population persistence.

Nosema ceranae is an obligate parasitic fungus which utilizes the fecal-oral route of transmission, wherein honey bees ingest metabolically inactive spores during foraging or cleaning activities (Chen 2008, Gisder et al. 2011). Ingested spores remain vegetative within the lumen of the midgut, but invade epithelial cells during their reproductive phase. Epithelial cells containing mature spores lyse, damaging the absorptive capacity of the midgut, and spreading millions of spores throughout the gut lumen. These spores are excreted, serving as a new infection source within and between colonies (Chen 2008, Fries et al. 1996). Infections impose

significant metabolic stress on hosts through epithelial cell destruction and energy robbing by the parasite (Mayack 2009, Jack et al. 2016). Resulting physiological impairments include underdeveloped glandular structures, acceleration of age polyethism, suppression of immune response, malabsorption of nutrients, energetic stress, and shortened lifespans (Antoine Lecocq 2016, Antúnez et al. 2009, Chen 2008, Jack et al. 2016, Mayack 2009). In addition, a number of behavioral functions deteriorate as a result of infection, including thermoregulation, learning and memory, homing ability, orientation, and foraging (Campbell et al. 2010, Dussaubat et al. 2013, Ptaszyńska et al. 2016). Furthermore, the consequences of infected queens are devastating on the colony level, as they experience insufficient ovary development resulting in poor worker production. Failure to maintain stable colony populations, due to lag in worker production will ultimately lead to queen supersedure or colony failure (Alaux et al. 2011b). Despite the profound effects infections impose on honey bees, a growing body of evidence suggests that select plant secondary metabolites could be used as a medical remedy against *Nosema ceranae.*

Plant secondary metabolites serve as an important defense mechanism by which honey bees can combat pathogen infections, however, few compounds with true medicinal properties have been identified (Manson et al. 2010). One such phytochemical which could possess medicinal properties is p-coumaric acid. As a dietary additive, p-coumaric acid upregulates cytochrome P450 genes encoding for antimicrobial peptides (Mao et al. 2013). Moreover, p-coumaric acid and other phenolic acids which share hydroxyl (-OH) aromatic-group configurations, demonstrate nutraceutical and medicinal benefits, stimulating anti-inflammatory, antioxidant, and antimicrobial activity in a variety of insects and mammals (Abdel-Moneim et al. 2018, (King and Young 1999, Kelly et al. 2001, Zabka and Pavela 2013, Abdel-Moneim et al. 2018). For example, p-coumaric acid and other phenolic acids including gallic acid, caffeine, and

kaempferol, have been established alternatives in the treatment against significant pathogenic filamentous fungi including *Fusarium*, *Penicillium*, and *Aspergillis* in food products (Zabka and Pavela 2013). This antifungal efficacy suggests that these compounds may be suitable treatments for honey bees with *Nosema ceranae* infections. The relationship between structurally similar plant secondary metabolites and their medicinal functions could offer novel approaches to the treatment of *Nosema ceranae.* We applied this structure-function framework to test the nutraceutical and medicinal properties of four structurally similar compounds in honey bees with and without *Nosema ceranae* infections.

OBJECTIVES

Our goal in this study was twofold. First, we hypothesized that the four structurally similar plant secondary metabolites commonly found in floral nectar had comparable nutraceutical effects. Nutraceutical benefits were assessed on enhancement of survival probability, wherein healthy and infected bees would both experience increases in longevity. Second, we hypothesized that the plant secondary metabolites tested would have medicinal function in honey bees. Plant secondary metabolites were deemed medicinal if they reduced infection intensity of honey bees experimentally infected with *Nosema ceranae*. By connecting the nutraceutical or medicinal function with plant secondary metabolites that share similar chemical structures, we can validate the structure-function framework by which other beneficial plant secondary metabolites can be easily identified.

To determine nutraceutical effects of p-coumaric acid, gallic acid, kaempferol, and caffeine on survival of healthy honey bees and bees infected with *Nosema ceranae*. To evaluate the medicinal utility of p-coumaric acid, gallic acid, kaempferol, and caffeine to reduce spore concentration within the midgut of worker honey bees infected with *Nosema ceranae*.

EXPERIMENTAL DESIGN

To measure the nutraceutical and medicinal effects of structurally similar plant secondary metabolites on survival probability and infection intensity of honey bees, we used worker honey bees of the same age-cohort from five hives in 2017 (n=1000). Age-cohort bees were obtained using the method of Arathi and Spivak (2001), wherein honey bee queens from four experimental colonies were provided with empty, uniquely marked frames to lay eggs. The queen was caged to an empty frame for 24 hours to ensure that all eggs on the frame were laid on the same day (Arathi et al. 2000, Arathi and Spivak 2001, Arathi et al. 2006). The date when the queen was caged was marked on the frame, and marked frames containing late-stage pupae were removed from source colonies after 21 days. Frames with late stage pupae were placed in an incubator at 34°C and 50% humidity until the day of emergence (Arathi et al. 2000, Arathi and Spivak 2001, Arathi et al. 2006).

INOCULATION METHODS

Nosema ceranae spores for inoculation were obtained from stocks maintained by Dr. Mayack et al. (Mayack 2009) in the form of macerated intestinal tract suspension. The species of *Nosema* used was previously confirmed using multiplex PCR and electrophoresis method (Naug and Gibbs 2009). Fresh inoculum was produced by feeding 20 worker honey bees macerated intestinal tract suspension mixed with 50% sucrose. Inoculated bees were housed in single-use cup cages, provided 20% sucrose solution, and sacrificed after 10 days. Intestinal tracts were dissected, macerated into a composite sample, and vortexed for 30 seconds. Initial spore concentration was determined by counting using a hemocytometer. Inoculum solution containing 50% sucrose solution and *Nosema ceranae* spores in a concentration of $1x10^4$ spores per mL was used for infection inoculum. A 50% sucrose solution was used for the control inoculum.

On the day of emergence, bees were randomly assigned inoculation treatments. Bees were starved for two hours prior to individual feeding with experimental and control inoculum. Individual feeding was used to ensure exposure to a known quantity of spores, and to produce lower variation in infection level between bees (Fries et al. 2013). Bees were individually fed 5 µL of infection or control inoculum, and randomly assigned to feeding treatments according to their infection status. Bees were housed in single-use cup cages with access to feeding syringes containing phytochemical solutions, and placed in an incubator at 34°C and 50% relative humidity (Higes 2007). Feeding syringes were used to administer nectar to bees, ensuring they had *ad libitum* access to phytochemical solutions. Syringes were replaced every seven days to ensure freshness of solutions. The cages were monitored for dead bees daily. The date of mortality and number of dead bees for each treatment was recorded. Dead bees were frozen at -8°C to prevent degradation of midgut tissues until spore concentrations within individual bees were quantified using a hemocytometer.

FEEDING ASSAYS

For the feeding assays, nectar containing plant secondary metabolites and sucrose was created by adding treatment compounds to aqueous 20% sucrose solution until completely dissolved. Compounds tested included p-coumaric acid, gallic acid, kaempferol, and caffeine. Each compound was tested at three (25, 250, and 2500 ppm) concentrations (Table 1). Concentrations ranged from naturally occurring levels found in nectar (25 ppm), to extreme dosages (2500 ppm) (Adler 2000). Control treatment was aqueous 20% sucrose solution. New solutions were mixed every 10-12 days, and frozen at -8°C until use.

STATISTICAL ANALYSIS

Kaplan-Meier survival probability estimates were used to evaluate differences in worker bee survival between inoculation treatments using the survival package in R Version 1.0.153 (Grambsch 2000). Best fit models were constructed using the survfit() function, and significant differences between treatment concentrations were detected using the surfdiff() function. Peto-Peto modified Gehan-Wilcoxon test comparisons, accounting for unequal variance and sample size, were used to evaluate differences in survival probabilities within treatments with the survdiff() function (Grambsch 2000). Graphics were generated using Microsoft Power BI.

Kruskal-wallis rank sum tests were used to evaluate the medicinal effects of plant secondary metabolites, by determining significant differences in spore loading of worker bees using the kruskal.test() function (R Core Team 2017). Dunn's post-hoc test of multiple comparisons using the dunn.test() function was used to determine differences between spore loading in bees given different concentrations of the same phytochemical treatment(Dino 2017). Significance was evaluated at alpha equal to 0.05.

RESULTS

NUTRACEUTICAL EFFECTS

Nutraceutical properties were evaluated by comparing median survival between bees within the same infection treatments, fed control nectar solution or phytochemical compounds. All compounds tested in this study showed nutraceutical benefit, however, not all concentrations provided significant enhancement of survival.

P-COUMARIC ACID

Pairwise comparisons between median survival in healthy bees with and without p-coumaric acid supplementation show that p-coumaric acid has nutraceutical effects (χ^2=22, df

=3, P<0.001). Bees in the lowest dosage group lived six days more than bees provided control nectar solution (χ^2=17.7, df =1, P<0.001), while the 250 ppm and 2500 ppm groups demonstrated an increase in median survival by two days (χ^2=5.7, df =1, P=0.02) and 5 days (χ^2=10.2, df =1, P=0.001), respectively (Table 5) (Figure 6).

Bees infected with *Nosema ceranae* also displayed benefits from supplementation with p-coumaric acid, confirming the compound's nutraceutical activity. Similar to the healthy bees, infected bees given a lower dose of p-coumaric acid showed the greatest benefit. Median survival of bees given 25 ppm of p-coumaric acid increased by six days (χ^2=20.8, df =1, P= 0.01), while bees provided 250 ppm lived three days longer than bees provided control solution (χ^2=6.2, df =1, P=0.001). Moreover, survival in the highest dosage group increased by four days (χ^2=21, df =3, P<0.01) (Table 5) (Figure 6).

GALLIC ACID

The nutraceutical effect of gallic acid in healthy honey bees was significant only at 250 ppm (χ^2=8.4, df =1, P=0.004), with bees living two days longer than their control counterparts. While not significantly different from the control treatment, bees provided the lowest dose of gallic acid lived two days more (χ^2=0.6, df =1, P=0.4), and two days less at the highest dose (χ^2=3.3, df =1, P=0.7) (Table 5) (Figure 7).

Similar to the healthy bees, infected bees also received significant nutraceutical benefits from 250 ppm of gallic acid supplementation. Infected bees provided gallic acid at 250 ppm lived six days longer than bees receiving control solution (χ^2=18.6, df =1, P=0.01). Median survival of infected bees that received gallic acid at 25 ppm and 2500 ppm was not significantly different from infected bees that received the control solution (χ^2=3.3, df =1, P=0.7), (χ^2=0.2, df =1, P=0.7) (Table 5) (Figure 7).

KAEMPFEROL

Supplementation with kaempferol elicited the most striking differences in median survival. Healthy bees supplied kaempferol at 25 ppm and 250 ppm showed similar increases in longevity, with bees living six days (χ^2=7.4, df =1, P=0.006) and seven days (χ^2=19.2, df =1, P=0.001) longer than bees in the control group. The highest doses of kaempferol elicited the most prominent increase in survival, with bees living 19 days longer (χ^2=55.4, df =1, P=0.001), nearly twice as long as bees within the control group (Table 5) (Figure 8).

The median survival of infected bees given kaempferol was also longer than median survival of infected bees fed control nectar. The two highest dosage groups showed a median survival six days (χ^2=18.1, df =1, P= 0.001), and one day (χ^2=8.1, df =1, P=0.004) longer than the control group. The median survival of bees given the lowest dosage of kaempferol, 25 ppm, was not significantly different that of the control group (χ^2=2.3, df =1, P=0.1) (Table 5) (Figure 8).

CAFFEINE

Lower concentrations of caffeine had a positive effect on median survival, while extreme concentrations had no effect on bees with and without infections. Healthy bees provided caffeine at 25 ppm and 250 ppm showed an increase in median survival, living 5.5 days (χ^2=25.5, df =1, P=0.001), and seven days (χ^2=21.9, df =1, P=0.001) longer than bees that received control solution. However, bees that received 2500 ppm of caffeine did not show a significant difference in median survival compared to the control group (χ^2=1.4, df =1, P=0.2) (Table 5) (Figure 9).

The same patterns of survival were exhibited in the infected bees, with the 25 ppm and 250 ppm groups living three days (χ^2=39.6, df =1, P=0.001) and four days (χ^2=42.1, df =1,

P=0.001) longer than the control group. Moreover, the highest concentration had no effect on median survival (χ^2=0.2, df =1, P=0.6) (Table 5) (Figure 9).

MEDICINAL EFFECTS

Medicinal value was assessed by comparing infection intensity between bees fed control nectar solution or phytochemical compounds. All experimentally infected bees contained *Nosema ceranae* spores, indicating that our inoculation methods were successful. Moreover, no spores were detected in bees administered control inoculum solution, confirming there was no contamination during the execution of inoculation methods.

P-COUMARIC ACID AND GALLIC ACID

No significant differences in mean spore concentration were detected between experimentally infected bees fed control nectar solution (871.7±181.1 SD), p-coumaric acid (χ^2=4.28, df=3, P=0.23), and gallic acid (χ^2=2.38, df=3, P=0.49) (Figure 7) (Figure 10).

KAEMPFEROL

Infected bees provided kaempferol showed significantly reduced spore concentrations (χ^2=15.5, df=3, P<0.001). Spore concentrations in bees fed kaempferol at 25 ppm showed a 24.4% reduction in the number of spores with 668.1±297.9 per μL (χ^2=15.5, df=3, P=0.01). Bees in the 250 ppm group showed a similar pattern in spore reduction, with 23.3% fewer spores 666.8±322.6 per μL (χ^2=15.5, df=3, P<0.01) than bees fed control solution. However, the highest dose of kaempferol was least effective in reducing spore concentrations, with 19.8% fewer spores 698.6±314.9 per μL (χ^2=15.5, df=3, P=0.04) than the control solution (Figure 7) (Figure 10).

CAFFEINE

Caffeine at 25 ppm elicited an even greater reduction in mean spore concentration (χ^2=55.5, df=3, P<0.001), with bees experiencing a 69.9% fewer spores per μL of macerated gut solution 261.7±264.6 per μL (χ^2=55.5, df=3, P<0.01) as compared to the control group (871.7±181.1 SD). However, no significant differences were detected in bees fed higher concentrations of caffeine (Figure 7) (Figure 10).

DISCUSSION

Our findings reveal that p-coumaric acid, gallic acid, kaempferol, and caffeine elicit nutraceutical activity in honey bees, whereas kaempferol and caffeine have medicinal value in the treatment of *Nosema ceranae.* All phenolic acids tested in this study produced modest increases in median survival, with the exception of kaempferol and caffeine. The highest dosage of kaempferol elicited particularly strong effects, nearly doubling median survival. In contrast, the highest doses of caffeine had no effect, or reduced median survival. With the exception of caffeine supplementation at 2500 ppm, we conclude that p-coumaric acid, gallic acid, kaempferol, and low dosages of caffeine have nutraceutical value.

While all phytochemical compounds tested displayed nutraceutical benefits, only kaempferol and caffeine elicited medicinal effects. The lowest concentrations of kaempferol and caffeine, which best reflect levels naturally present in nectar, had the greatest effect in spore reduction (Adler 2000). The two phenolic acids exerted antifungal effects, reducing spore concentrations by up to 24.4% in kaempferol, and nearly 70% in caffeine. While kaempferol supplementation for infected bees did not reduce median survival, caffeine supplementation reduced host median survival by one day. These findings illustrate the tradeoffs between the beneficial and toxic effects of plant secondary metabolites. Although caffeine was highly

effective at reducing *Nosema ceranae* spores, it may have caused cell damage within the host, resulting in reduced survival probability. The method of antifungal effects exerted by kaempferol and caffeine is based on ability of each compound to affect the structural cellular integrity of *Nosema ceranae* spores (Zabka and Pavela 2013). Further investigation is needed to determine whether these compounds act on *Nosema ceranae* by acidification of pH, impairment the cellular ionic homeostasis of spores within the lumen of the honey bee midgut, or by other means (Zabka and Pavela 2013). Given the favorable effects of caffeine and kaempferol, these plant secondary metabolites could be effective tools in the treatment of pathogen infections. Despite the absence of medicinal utility in p-coumaric acid, and gallic acid, these compounds may possess medicinal properties that were not measured in this study.

The identification of nutraceutical and medicinal plant secondary metabolites is essential to the sustainability of honey bee populations. Interactions between pollinators and their natural enemies may be influenced by the availability of a diversity of plant secondary metabolites found in nectar. Only pollinators with access to these compounds are able to take advantage the medicinal qualities associated with phytochemical consumption. Not only limited access, but limited diversity of plant secondary metabolites within the landscape may also have ecological significance to host-pathogen interactions. The consumption of multiple plant secondary metabolites acquired from a breadth of floral nectar may have greater benefits to honey bees than consuming a single phytochemical compound. Consequently, landscapes with a diversity of nutraceutical and medicinal plant secondary metabolites have greater value to unhealthy bees, however, nectar containing compounds with these properties are rarely accessible to honey bees used for large-scale pollination services. These nutritional and medicinal deserts highlight a critical need for adequate management strategies to support honey bee health.

Management strategies to reduce pathogen infections in honey bees are needed to complement plant secondary metabolites present in agricultural landscapes. Commercially managed honey bees are especially susceptible to pathogen infections. Pathogens take advantage of the stressful conditions such as intensive transportation and exposure to large-scale mono-cropping systems which are often treated with pesticides and fungicides. These environmental stressors result in severe nutritional deficiencies and energetic stress, leaving honey bees with compromised immune function (Alaux et al. 2010). Current management practices used to mitigate stress and treat pathogen infections rely on both dietary supplementation with pollen and artificial nectar, as well as chemical fungicide treatments. Pathogens like *Nosema ceranae* are quick to develop resistance to synthetic fungicides, rendering them insufficient. Our results suggest that dietary supplementation with plant secondary metabolites could be used as a management tool to not only provide nutraceutical benefit, but to offer alternative methods of suppressing *Nosema ceranae.*

While further phytochemical feeding assays will be necessary to characterize the full extent of benefits elicited by the phytochemical compounds tested in this study, our data provide evidence that structure-function relationships can be used to identify nutraceutical and medicinal compounds within floral nectar. P-coumaric acid, gallic acid, kaempferol, and caffeine support a nutritionally balanced diet, while kaempferol and caffeine supplementation may offer new approaches for treating honey bees with *Nosema ceranae* infections.

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APPENDIX

TABLE 1- PHYTOCHEMICAL STRUCTURES AND FEEDING TREATMENT ASSAYS.

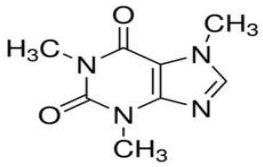
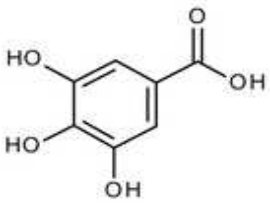
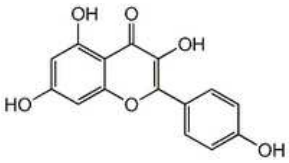
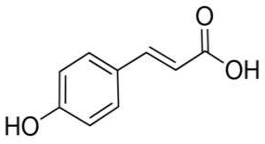
Compound	Molecular Structure	Chemical Structure	Concentration	Replicates	Total Number of Bees
p-Coumaric Acid	$C_9H_8O_3$		25 ppm	4	45
			250 ppm	4	35
			2,500 ppm	4	44
Gallic Acid	$C_7H_6O_5$		25 ppm	4	40
			250 ppm	4	39
			2,500 ppm	4	38
Kaempferol	$C_{15}H_{10}O_6$		25 ppm	4	30
			250 ppm	4	31
			2,500 ppm	4	31
Caffeine	$C_8H_{10}N_4O_2$		25 ppm	4	40
			250 ppm	4	41
			2,500 ppm	4	39
Control			20% Sucrose Solution	11	109

TABLE 1- KAPLAN-MEIER SURVIVAL CURVE DIFFERENCES

Treatment	X^2	df	P
p-Coumaric Acid	15.5	3	0.001
Gallic Acid	16.6	3	<0.001
Kaempferol	65.8	3	<0.001
Caffeine	33.4	3	<0.001

TABLE 2- PETO & PETO MULTIPLE TEST COMPARISONS

Treatment	Concentration	Median Survival (d)	X^2	df	P
p-Coumaric Acid - Control	25 ppm	27	11	1	<0.001
	250 ppm	23	3.1	1	0.08
	2500 ppm	44	9.2	1	0.002
Gallic Acid - Control	25 ppm	23	0	1	0.8
	250 ppm	23	6.5	1	0.01
	2500 ppm	19	5.5	1	0.02
Kaempferol - Control	25 ppm	27	7.7	1	0.005
	250 ppm	28	10.7	1	0.001
	2500 ppm	47	45.9	1	<0.001
Caffeine - Control	25 ppm	26.5	13.8	1	<0.001
	250 ppm	28	10.4	1	0.001
	2500 ppm	21	2.8	1	0.09

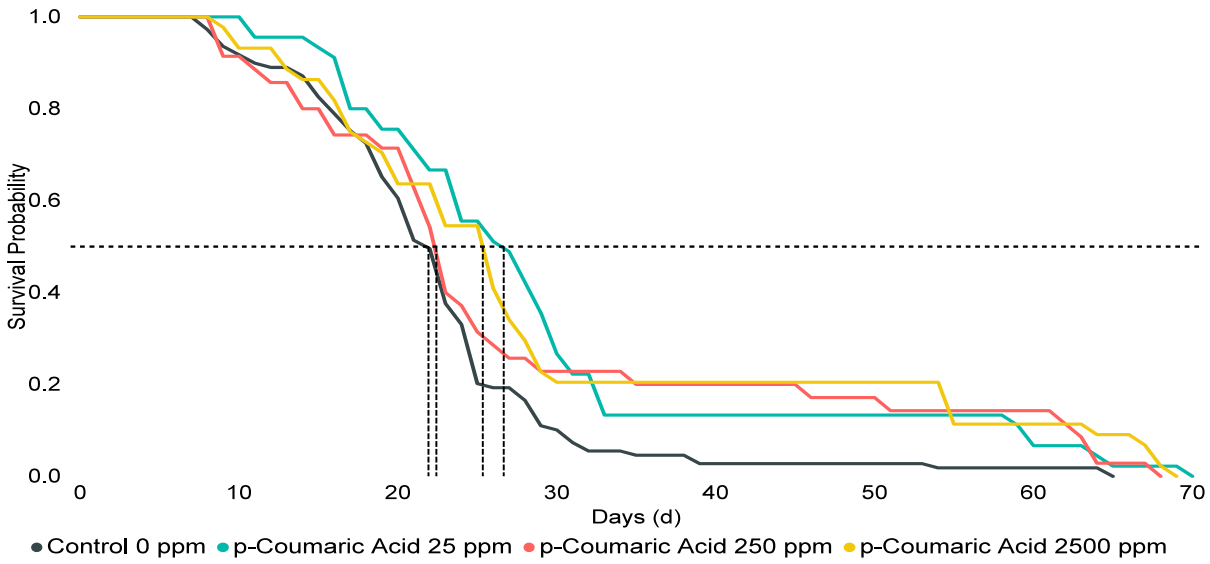


FIGURE 2 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR P-COUMARIC ACID TREATMENTS

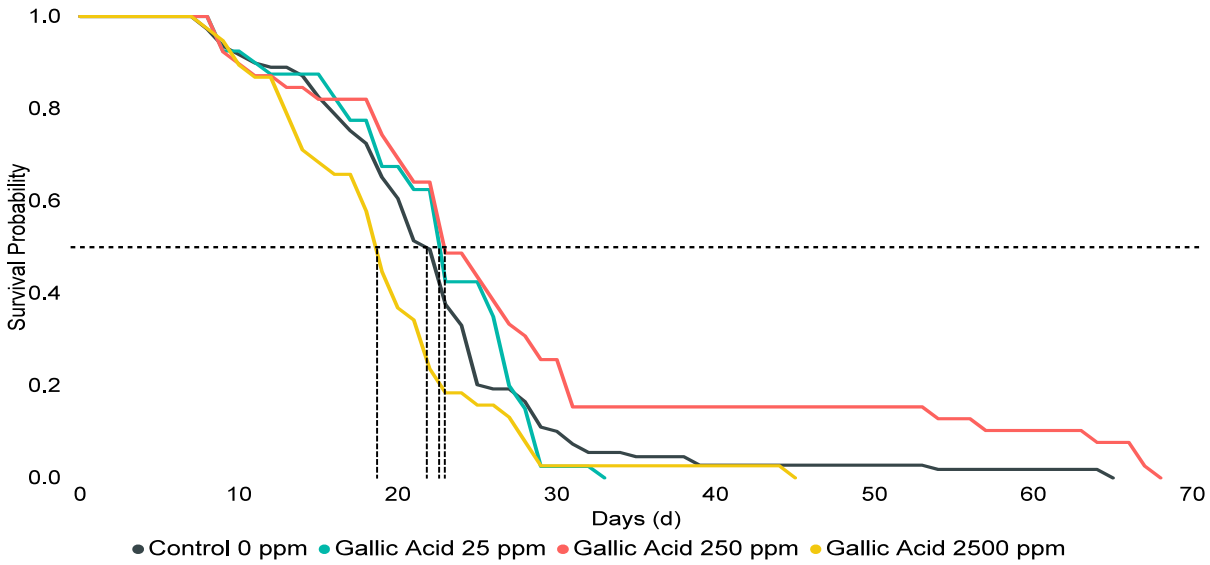


FIGURE 3 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR GALLIC ACID TREATMENTS

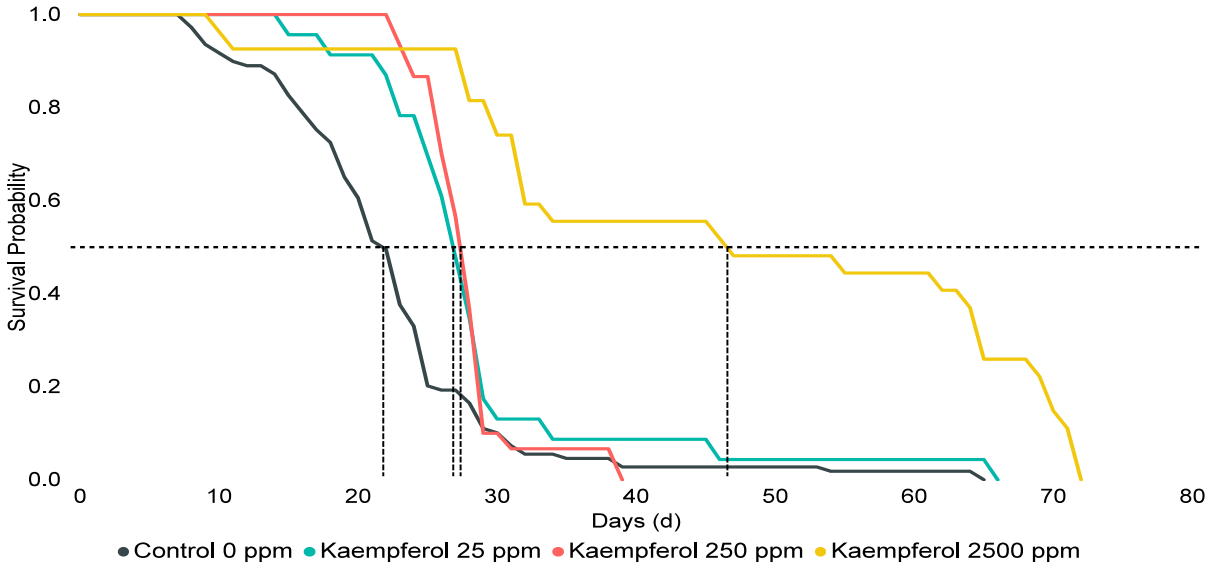


FIGURE 4 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR KAEMPFEROL TREATMENTS

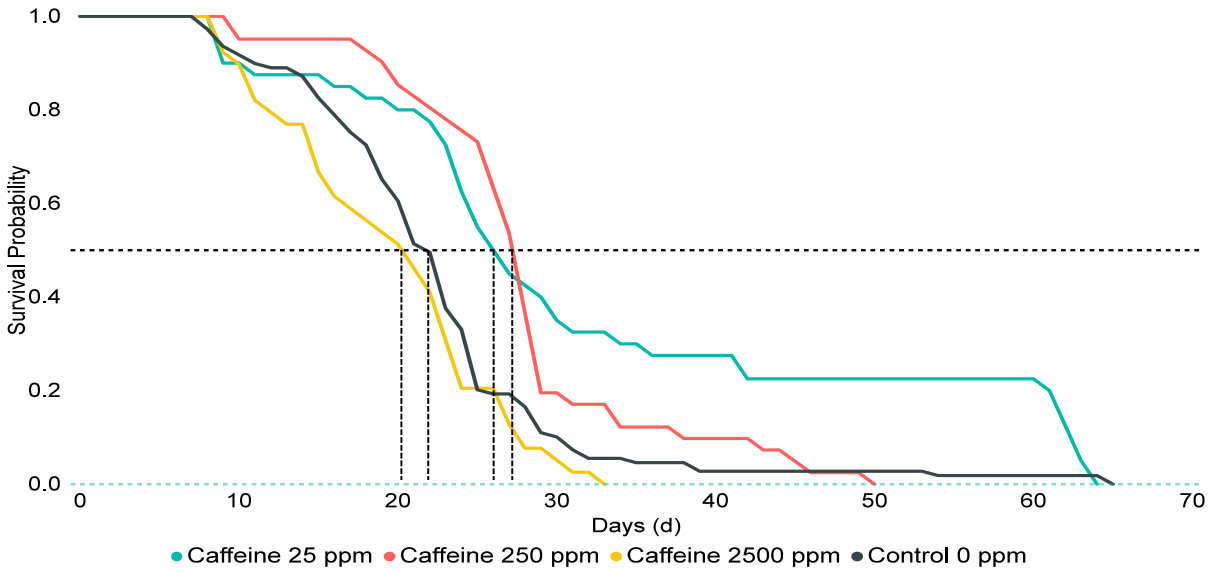


FIGURE 5 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR CAFFEINE TREATMENTS

TABLE 3- KAPLAN MEIER SURVIVAL CURVE DIFFERENCES

Treatment	Infection Status	X^2	df	P
p-Coumaric Acid	N	22	3	<0.001
p-Coumaric Acid	Y	26	3	0.009
Gallic Acid	N	14.8	3	0.002
Gallic Acid	Y	23.9	3	<0.001
Kaempferol	N	84.3	3	<0.001
Kaempferol	Y	21.7	3	<0.001
Caffeine	N	49.1	3	<0.001
Caffeine	Y	78.3	3	<0.001

TABLE 4- KAPLAN MEIER SURVIVAL CURVE DIFFERENCES COMPARED TO CONTROL

Treatment	Infection Status	Concentration (ppm)	Median survival (d)	X^2	df	P
Control	N	0	21			
	Y	0	16			
p-Coumaric Acid	N	25	27	17.7	1	<0.001
		250	23	5.7	1	0.02
		2500	26	10.2	1	0.001
p-Coumaric Acid	Y	25	22	20.8	1	0.001
		250	19	6.2	1	0.01
		2500	20	11.8	1	0.001
Gallic Acid	N	25	23	0.6	1	0.4
		250	23	8.4	1	0.004
		2500	19	3	1	0.09
Gallic Acid	Y	25	19	3.3	1	0.7
		250	22	18.6	1	<0.001
		2500	17.5	0.2	1	0.7
Kaempferol	N	25	27	7.4	1	0.006
		250	28	19.2	1	<0.001
		2500	40	55.4	1	<0.001
Kaempferol	Y	25	19	2.3	1	0.1
		250	22	18.1	1	<0.001
		2500	17	8.1	1	0.004
Caffeine	N	25	26.5	25.5	1	<0.001
		250	28	21.9	1	<0.001
		2500	21	1.4	1	0.2
Caffeine	Y	25	24	39.6	1	<0.001
		250	25	42.1	1	<0.001
		2500	17	0.2	1	0.6

TABLE 5- DIFFERENCES IN SPORE CONCENTRATION OF INFECTED WORKER BEE INTESTINAL TRACT GIVEN DIFFERENT LEVELS OF PHYTOCHEMICAL CONCENTRATION

Treatment	X^2	df	P
Gallic Acid	2.38	3	0.49
Caffeine	55.58	3	**< 0.001**
Kaempferol	15.55	3	**0.001**
p-Coumaric Acid	4.28	3	0.23

TABLE 6- DUNN'S POST-HOC PAIRWISE COMPARISONS OF MEAN SPORE CONCENTRATIONS

Treatment	Pairwise Comparisons	P
Caffeine	0 ppm - 25 ppm	**< 0.001**
Caffeine	0 ppm - 250 ppm	1
Caffeine	0 ppm - 2500 ppm	1
Kaempferol	0 ppm - 25 ppm	**0.011**
Kaempferol	0 ppm - 250 ppm	**0.008**
Kaempferol	0 ppm - 2500 ppm	**0.044**

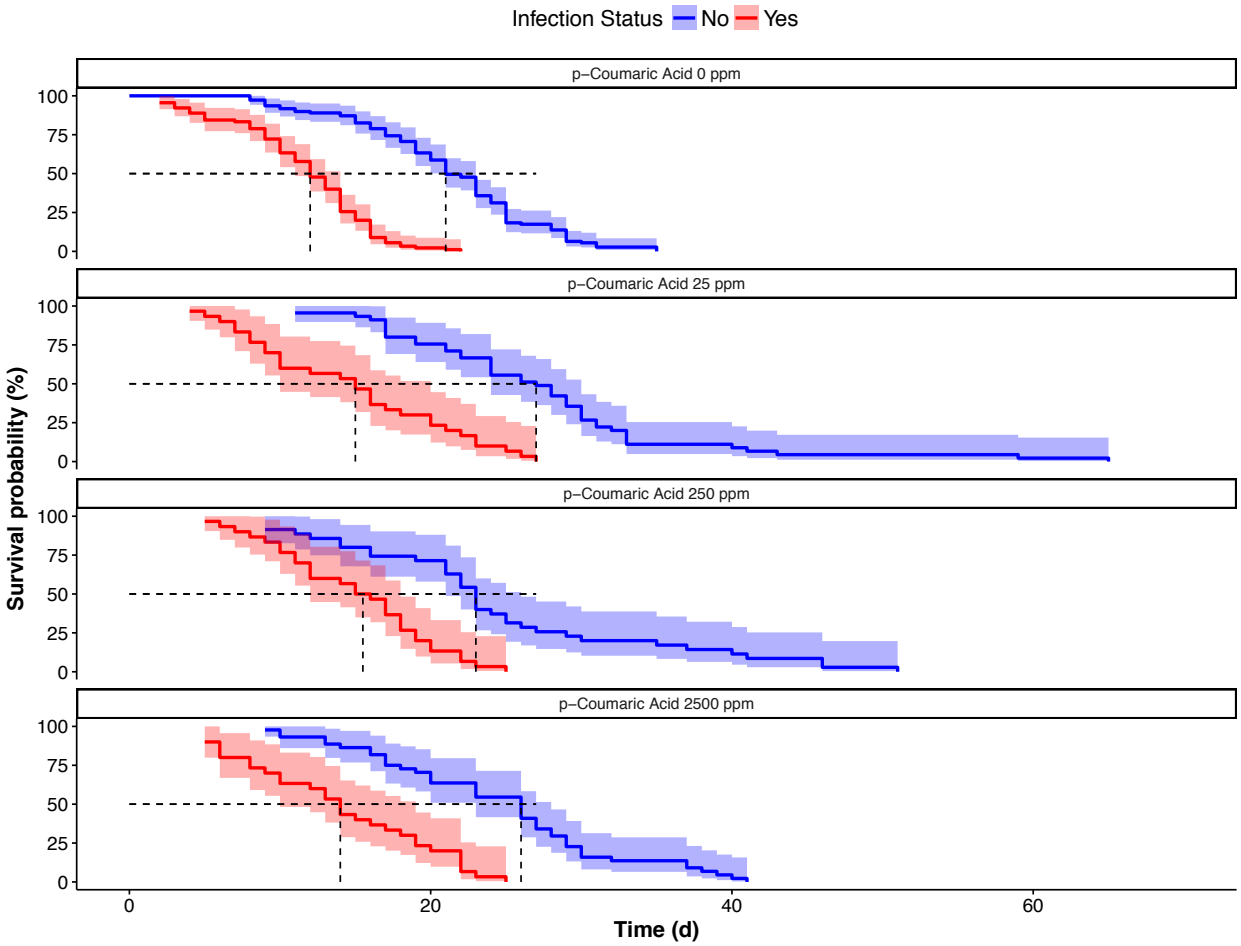


FIGURE 6 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR P-COUMARIC ACID TREATMENTS

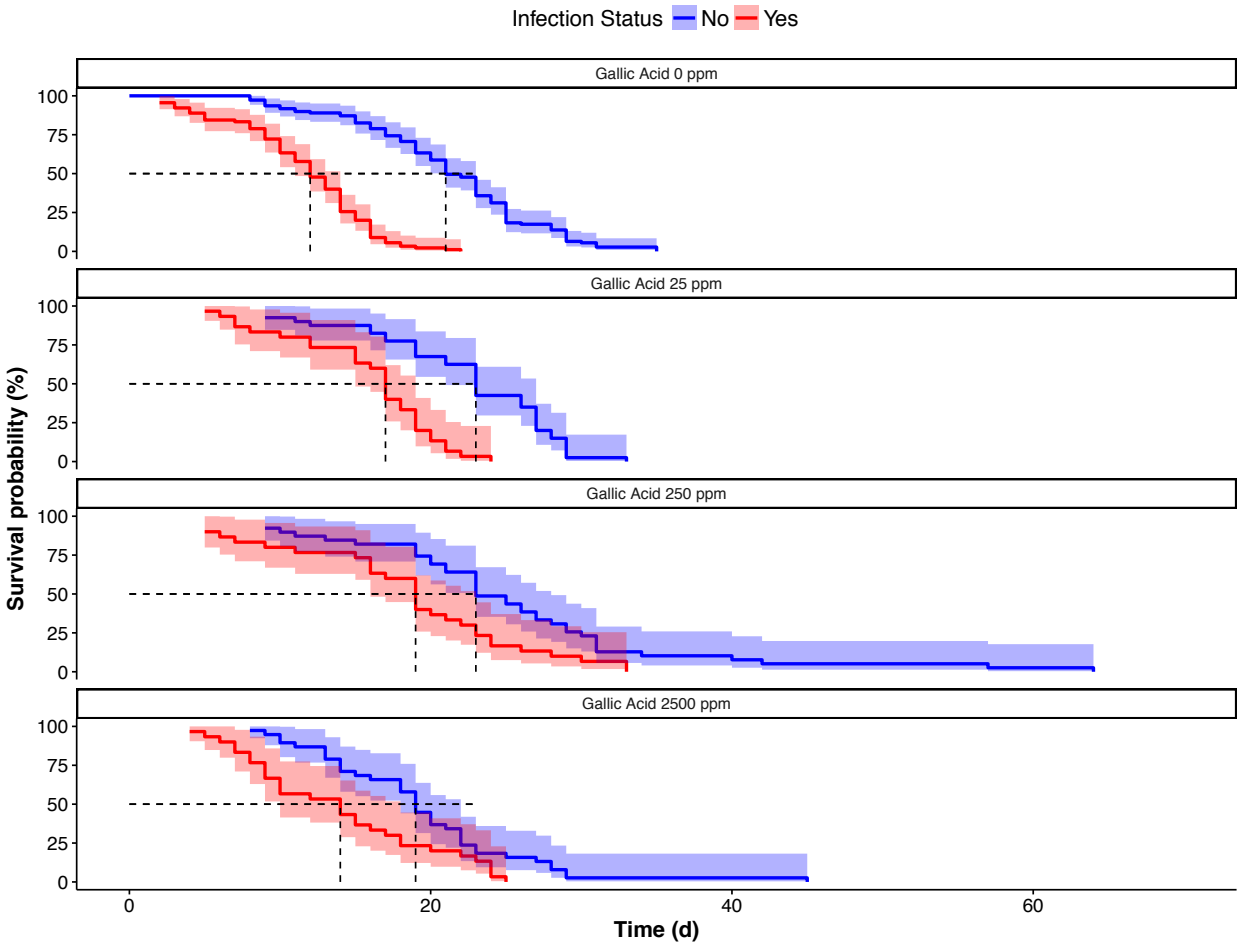


FIGURE 7 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR GALLIC ACID TREATMENTS

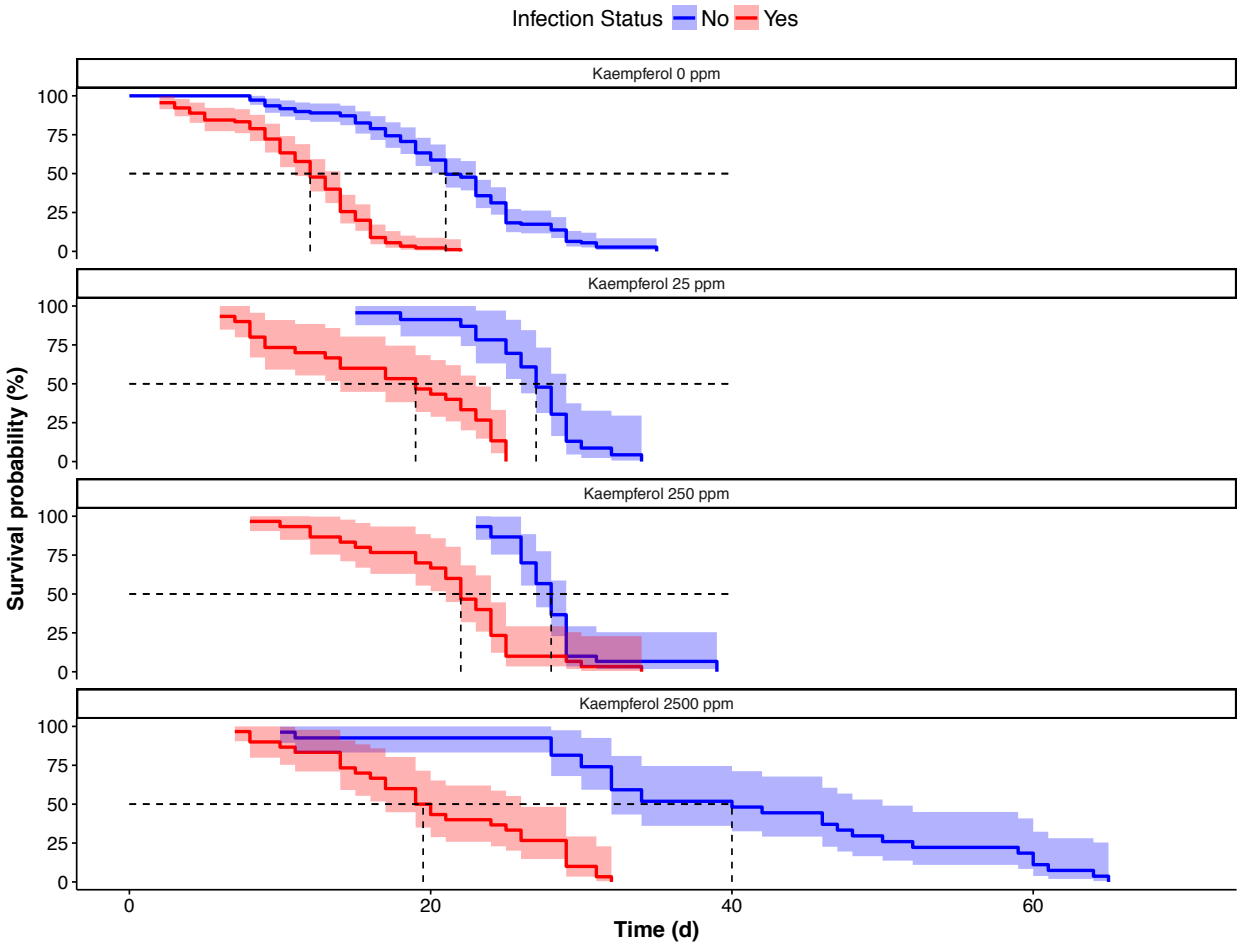


FIGURE 8 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR KAEMPFEROL TREATMENTS

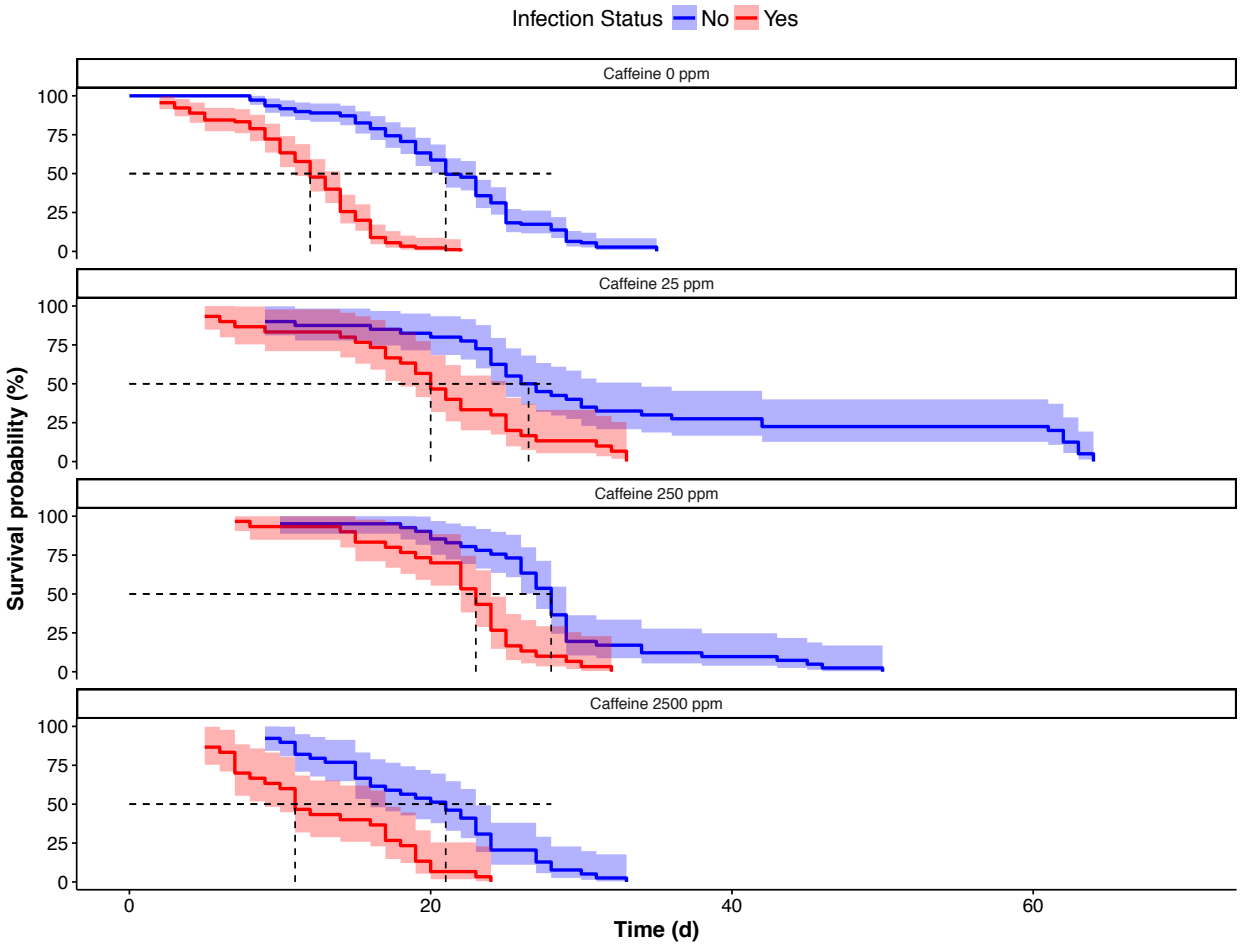


FIGURE 9 - KAPLAN-MEIER SURVIVAL PROBABILITY ESTIMATES FOR CAFFEINE TREATMENTS

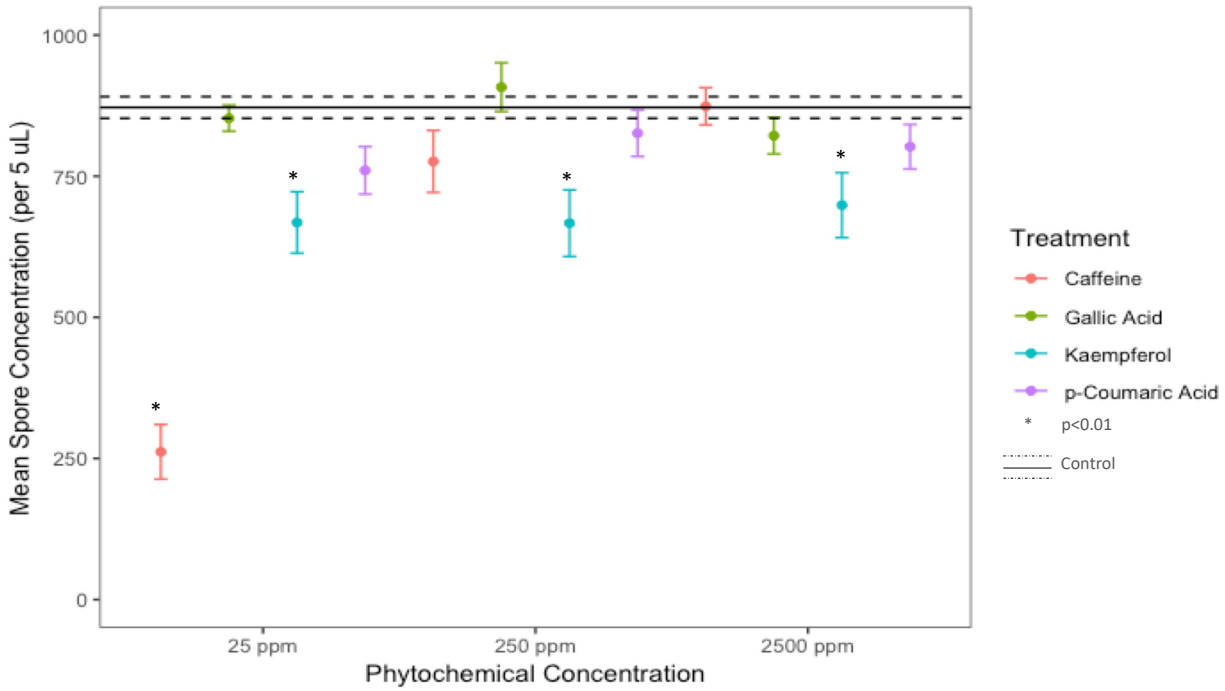


FIGURE 10 - AVERAGE SPORE CONCENTRATION PER 5UL OF MACERATED INTESTINAL TRACT SOLUTION OF INFECTED WORKER HONEY BEES SUPPLEMENTED WITH CAFFEINE, GALLIC ACID, KAEMPFEROL, AND P-COUMARIC ACID AT FOUR LEVELS OF CONCENTRATION