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THE TOXICODYNAMICS OF “INERT” AND “ALTERNATIVE” AGROCHEMICALS
AND THEIR IMPACTS ON HONEY BEES

A Dissertation in

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by

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Honey bees (*Apis mellifera*) are highly valued for the pollination services they provide to agricultural crops, but in recent years, beekeepers have experienced high yearly colony losses. Many researchers now believe that these declines are due in part to the effects of agrochemical pesticide use. However, the majority of pesticide research focuses on the effects of pesticide active ingredients on adult honey bees without consideration for the complexities of real world exposure. Pesticides are applied as formulated products composed of active and “inert” formulants, and these formulations are often tank mixed with inert adjuvants meant to enhance the spread and penetration of active ingredients. Many inerts are assumed by the US EPA to be biologically inert, despite growing evidence that they are toxic to many non-target organisms. Additionally, assessing the toxicity of agrochemicals to adult bees without consideration for their effects on developing larvae or potential interactions with other environmental stressors does not accurately represent their inherent risks. This dissertation explores the effects and environmental fate of chemicals thought to be harmless or of lower risk to bees, including the inert organosilicone adjuvant product, Sylgard® 309 (OSS), the inert pesticide formulant *N*-methyl-2-pyrrolidone (NMP), and the organophosphate alternative, novaluron. These experiments focus primarily on honey bee larvae, which are often overlooked in regulatory risk assessment. First, a study was conducted to investigate the toxicity of OSSs to honey bee larvae when combined with exposure to common viral pathogens such as black queen cell virus (BQCV). This exposure resulted in synergistic mortality related to decreased expression of a *Toll-7* homologue and
heightened BQCV titers. The dying brood exhibited symptoms similar to those observed in hives used for almond pollination during the 2014 and 2016 seasons. During almond pollination, tank mixes containing OSSs are often applied during bloom, and close proximity of hives from across the United States can facilitate the spread of viral pathogens. Therefore, the study described here represents a real-world scenario likely encountered by many commercial beekeepers. Next, the safety of the insect growth regulator formulation, Rimon® 0.83EC to honey bees was assessed. Residue studies in apple orchards were performed to monitor the fate of its active ingredient, novaluron, and its major co formulant, NMP, and the results indicate that NMP is likely to dissipate in the environment before foraging bees come into contact with it, but novaluron residues, which were detected at 3 ppm in pollen 24 hours after application, are more problematic. Concurrently run larval rearing experiments and a nucleus colony study demonstrated that novaluron is highly toxic to honey bee brood. These results suggest that applying novaluron to blooming flowers should be prohibited. Finally, age related differences in tolerances to NMP were investigated. In larval rearing experiments, NMP was chronically toxic to larvae at 100 ppm in diet, while adults are tolerant to significantly higher doses. Honey bees were found to detoxify NMP via a cytochrome P450 mediated pathway, and larvae may be more sensitive to this co-solvent because of a deficit of P450 enzyme activity in the fat body. This finding suggests that larvae may be more sensitive to some xenobiotics than adults, and highlights the importance of considering all members of a hive in honey bee risk assessment. This work seeks to address assumptions in current pesticide regulation and explores the possibility that the use of agrochemicals thought to be
inert or lower risk to pollinators may have unintended consequences for honey bee health. By considering multiple variables, including the susceptibility of larvae to agrochemicals, agrochemical environmental fate, and the influence of multiple stressors, these results are easily extrapolated to real-world scenarios and indicate that follow-up studies to characterize the persistence of these chemicals in hive environments are warranted.
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Chapter 1

Introduction

As generalist pollinators, honey bees add an estimated 15 billion dollars of value to the crops they pollinate in the United States alone\(^1\). However, since 2006, managed honey bee populations have been in decline\(^2,3\). Multiple factors including pesticides, pathogens, parasitism and poor nutrition have been implicated\(^4\), but at this time, no single element has been successfully shown to cause colony failure\(^5,6\). Scientists now believe that the phenomenon is a result of interacting stressors including pesticides, pathogens, parasites and poor nutrition\(^4,7,8\). Pesticides, however, are of particular importance due to their heavy use in agricultural settings.

Despite guidelines to protect pollinators from pesticides, honey bees are often exposed to pesticides through the residues that persist in pollen, nectar, comb wax or on the surface of the plants in the bees’ foraging environment\(^9,11\). The social nature of a honeybee colony can result in a systemic cascade as workers forage on a contaminated food source and return to supply the other workers, queen and developing larvae with pesticide laden food\(^12\). Concurrently, the use of pesticide products inside hives to control the population of *Varroa destructor* and other parasites can lead to a build-up of chemical residues in wax and food stores\(^13,14\). Therefore, studies examining the effects of single chemicals on adult bees may not accurately represent the risk to all members of a colony or the total chemical burden to which bees are exposed. In reality, bees are usually co-exposed to pesticides, inert formultants, adjuvants\(^15,16\) and other stressors including viral pathogens that can interact to elevate mortality across different honeybee life stages.

Historically, the U. S. Environmental Protection Agency’s (EPA) risk assessment requirements for pesticides has relied on a 48-hour contact toxicity test performed on adult honey
bees to assess the safety of these products to pollinating insects\textsuperscript{17,18}. Recently, this approach has come under criticism as pollinator populations have continued to decline\textsuperscript{19}. Among the reasons for this criticism is the concern that this test cannot accurately reflect the risks to honey bee larvae, queens, drones or the hive as a whole\textsuperscript{19}. Additionally, many newer pesticide classes such as systemic neonicotinoids exhibit higher oral toxicity than contact toxicity in insects, and are often encountered by honey bee foragers in pollen and nectar\textsuperscript{20}. In response to these concerns, the EPA has developed a tiered approach to pollinator risk assessment\textsuperscript{21}, and guidelines for assessing the oral toxicity of pesticides as well as the toxicity of pesticides to larvae have been put forward\textsuperscript{21,22}. Despite this significant progress, the proposed guidelines do not address the potential toxicity of inert pesticide formulants or adjuvants\textsuperscript{15,16}. Similarly, they do not address concerns over the use of previously registered products that are highly toxic to honey bee brood as lower risk alternatives to conventional pesticides.

The term “inert” is used by the EPA to refer to pesticide formulants or adjuvants that enhance the efficacy of active ingredients when they are added to a pesticide product or to a tank-mix respectively\textsuperscript{15,23}. These chemicals act as co-solvents, spreaders, penetration enhancers, and more, and while their moniker implies that they are biologically inactive, there is a growing body of work showing that not only do they significantly enhance the toxicity of active ingredients, but they sometimes exhibit their own toxic effects\textsuperscript{15,16,23-26}. This work will consider the toxicity of two common agrochemical inerts that have been demonstrated to be toxic to honey bees: N-methyl-2-pyrrolidone (NMP), and an organosilicone surfactant adjuvant\textsuperscript{24,25}. Additionally, the effects of novaluron, an insect growth regulator (IGR) considered by the EPA to be a lower risk alternative to more conventional organophosphate chemicals\textsuperscript{27}, on developing larvae will be examined. IGRs are a pesticide class used to control pest species by mostly targeting juveniles rather than adults\textsuperscript{28}, and recent work has shown that its use in alfalfa fields can result in increased mortality in alfalfa leaf cutter bee larvae and a decrease in the production of viable eggs\textsuperscript{29,30}. 
NMP is a small polar organic molecule that is highly soluble in water\textsuperscript{31}. In its pharmaceutical applications, it is often used as penetration enhancer, however, in its industrial applications, it is used as a solvent and cosolvent\textsuperscript{31,32}. As a pesticide inert, it is added to formulations to enhance the solubility of pesticide active ingredients, and although it is used at concentrations up to and potentially exceeding 50\%\textsuperscript{25}, it is exempt from residue tolerances and approved for pre-harvest applications to agricultural crops\textsuperscript{33}. Recently, the Pesticide Action Network has identified NMP as a “bad actor” chemical because of its reproductive toxicity to mammals\textsuperscript{34}, and a laboratory based study found NMP to be toxic to honeybee larvae at concentrations as low as 100 ppm\textsuperscript{25}.

Because of its uses in the electronics and petrochemical industries, its use in paint strippers, and its pharmaceutical applications\textsuperscript{31,32}, the toxicity of NMP to humans has been studied in model systems, and reproductive toxicity has been observed in rats following chronic dermal and inhalation exposure to NMP\textsuperscript{35-37}. Symptoms include decreased fetal body weight, increased fetal resorption and delayed ossification.

The metabolism of NMP has been extensively studied in order to evaluate potential biomarkers of exposure, and three primary hydroxylated and ketonic metabolites have been identified as products of a cytochrome P450 mediated pathway\textsuperscript{38}. In quantitative metabolic studies performed on humans or rats, the majority of NMP is recovered in the urine as 5-hydroxy-\textit{N}-methyl-2-pyrrolidone (5-HNMP), \textit{N}-methylsuccinimide (MSI), and 2-hydroxy-\textit{N}-methylsuccinimide (2-HMSI) or the parent compound\textsuperscript{38-42} with a small percentage of these compounds eliminated in the feces or transformed to CO\textsubscript{2} and eliminated via respiration\textsuperscript{39,40,43}. Although the mechanism of the observed toxicity has not been established, NMP has been shown to be significantly more toxic to developing fetuses than any of its three major metabolites, suggesting that the parent compound is primarily responsible for the reproductive toxicity\textsuperscript{44}.
Despite its use in agricultural settings, data pertaining to the effects of NMP exposure in invertebrates is strikingly limited. It has been shown to be toxic to the aquatic invertebrate *Daphnia magna*, with 50% of test populations dying after 48 hours of exposure to 1.23 mg NMP/L\(^4\), and under chronic exposure to 100 ppm NMP in diet for four days, artificially reared honey bee larvae experienced 50% mortality\(^2\). Studies have shown that under common wastewater treatment conditions, NMP degrades almost completely within 24 hours\(^4\), and in the gas phase, it can be transformed via photolysis to *N*-methylazetidine and other products\(^4\). This suggests that bioaccumulation of NMP is not likely, however it does not adequately address all concerns related to its agricultural uses. In particular, characterizing its risk to honey bees is dependent on its environmental fate in plant tissues, and on its presence and persistence in the honey bee hive\(^1\).

Another concerning class of inerts is the powerful nonionic surfactants known as organosilicone surfactants (OSSs). OSSs are used as agricultural spray-tank adjuvants to enhance the penetration and spread of active ingredients on agricultural crops by decreasing surface tension and increasing the uptake and translocation of active ingredients within plants\(^4\). Within a spray-tank, they can compose up to 1% of the total tank mix volume\(^2\). Because of their inert status, they are exempt from residue tolerances, and they can be used on organic or conventional crops without consideration for pollinating insects\(^3\).

Information on OSS use is limited, but worldwide OSS production in 2008 was estimated to be 1.3 billion pounds and yearly use has continued to increase\(^4\), while in California almonds, hundreds of thousands of pounds of OSS formulations are used every year, often during bloom\(^5\).

Annual California almond pollination is the largest pollination event in the United States, requiring over 60% of managed honey bee colonies in the country to be transported to the almond orchards during bloom\(^1\). Reports by beekeepers from 2014 and preliminary reports from 2016
describe colony die-offs characterized by healthy adults but with dying brood which are ejected from the hive as underdeveloped pupae. The hives, which face depleted populations, fail weeks later. The use of insect growth regulators, fungicides, and adjuvants have been suggested as possible causes.

OSS residues have been found in almond pollen and in pollen, nectar and wax samples from hives across the United States, and recent work has shown that OSS exposure can result in decreased learning ability in adult honey bees. These results confirm that OSS use can have unforeseen consequences to honey bees, but the effects of OSS on other members of the hive has not been previously explored. In light of the symptoms observed in declining colonies following recent almond pollination events, investigation into the effects of OSS’s on developing brood is warranted.

Finally, the status of the IGR novaluron as an organophosphate (OP) alternative may be somewhat misleading. OPs are a class of neurotoxic pesticides with considerable contact toxicity to adult honey bees, while novaluron has very low acute toxicity to adult honey bees when applied topically or ingested. In 2004, novaluron was approved as a lower risk alternative to organophosphate pesticides in apple trees, but the available records indicate that testing on pollinating insects has thus far only included adult honey bees. The major formulation of novaluron registered for use in apples is Rimon® 0.83EC, and the label permits its use during the apple blooming period. A warning within the text portion of the label cautions against using novaluron when pollinating insects are present because of potential effects on developing brood, but this is a direct contradiction to its prescribed use “from pink to petal fall.” The intent of the warning may have been to avoid spraying while pollinators are actively foraging, but this implies that novaluron residues will not persist in pollen and nectar at high enough concentrations to be problematic. Registrant reported residue studies suggest that novaluron can persist on foliage at concentrations as high as 18 ppm, but there is no available information on the persistence of
novaluron in pollen and nectar. This information as well as the effects of novaluron on the health of the hive as a whole should be considered when evaluating the safety of the use of novaluron in insect pollinated crops like apple trees.

This thesis will examine the hypothesis that inert and reduced risk alternative agrochemicals can have measurable consequences to the health of honey bees with particular focus applied to historically overlooked aspects of pollinator health including the consequences of pesticide exposure to developing brood, and the combined influence of other stressors such as viral pathogens. Although predicting real world consequences from controlled laboratory studies remains challenging, the experiments described here aim to address real world scenarios by considering the fate of chemicals in honey bee foraging environments, the influence of real world stressors, and whole colony dynamics. The objectives of this thesis in order of their presentation in this thesis are as follows:

- Examine the effects of organosilicone surfactant adjuvants on developing brood when brood are cross-exposed to common viral pathogens.
- Measure the presence and persistence of NMP and novaluron in apple pollen.
- Identify potential hazards of field relevant doses of novaluron and NMP to developing honey bee brood.
- Explore the hive level effects of novaluron and NMP exposure.
- Determine the metabolic detoxification pathway of NMP in honey bees and establish potential biomarkers of exposure.
- Explore the molecular basis for observed age related differences in the toxic effects of NMP.
- Make policy recommendations based on the findings of these experiments.


51. Wardell, G. Word from Wardell. *Project Apis m.*


55. U. S. Environmental Protection Agency. Rimon® 0.83EC Insecticide.


Chapter 2

An Inert Pesticide Adjuvant Synergizes Viral Pathogenicity and Mortality in Honey Bee Larvae

Author Contribution

Julia Fine performed the bioassays, and PCR in this chapter with some assistance provided by Tonilynn Baranowski. Experiments were designed by Julia Fine, Dr. Diana Cox-Foster, and Dr. Christopher Mullin. Statistical analysis was performed by Dr. Diana Cox-Foster and Julia Fine. The chamber used for the larval rearing was built by Randall McCullough, a senior research aide in Noll Laboratories.

Abstract

Colony losses following a major pollination event in the United States, almond pollination, have been characterized by brood mortality with specific symptoms, followed by eventual colony loss weeks later. In this study, we demonstrate that these symptoms can be produced by chronically exposing brood to both an organosilicone surfactant adjuvant (OSS) commonly used on many agricultural crops including wine grapes, tree nuts and tree fruits and exogenous viral pathogens by simulating a horizontal transmission event. Observed synergistic mortality occurred during the larval-pupal molt. Using q-PCR techniques to measure gene expression and viral levels in larvae taken prior to observed mortality at metamorphosis, we found that exposure to OSS and exogenous virus resulted in significantly heightened Black
Queen Cell Virus (BQCV) titers and lower expression of a Toll 7-like-receptor associated with autophagic viral defense (*Am18w*). These results demonstrate that organosilicone spray adjuvants that are considered biologically inert potentiate viral pathogenicity in honey bee larvae, and guidelines for OSS use may be warranted.

**Introduction**

Recent investigations have led scientists to believe that interacting stressors are the cause of the pollinator declines\(^4\). Because of the prevalence of honey bee pathogens and pesticide residues, the possibility of interactions between pesticides and viruses is of particular importance.

Just as an entire colony can be exposed to agrochemical residues in contaminated pollen, nectar or wax\(^5,7\), honey bees are host to numerous picorna-like RNA viruses with multiple routes of transmission\(^8,9\), and foraging bees can introduce viruses to naïve hives through drift or through shared floral resources\(^10\). The resulting systemic infection can be difficult to clear, and the presence and titers of these viruses vary as environmental conditions shift throughout the foraging season\(^11\). Recent work has shown that pesticides can negatively impact honey bee immunity, resulting in increased viral replication\(^12-16\); however, while attention has been given to the effects and persistence of pesticide “active” ingredients, little is known about the effects of pesticide adjuvants and other “inerts” in formulations\(^17\). Recently, a class of inert pesticide tank-mix adjuvants known as organosilicone surfactants (OSS) have been shown to be toxic to honey bee adults\(^18\).

As stated in Chapter 1, OSSs are a powerful class of nonionic surfactants used in agriculture as spray tank adjuvants to enhance the penetration and spread of active ingredients\(^17\). While there is little publicly available information regarding their toxicity and residual presence in the
environment, recent work has demonstrated that the residues of OSSs are encountered by foraging bees and persist in honeybee food and wax\textsuperscript{19}. Worldwide OSS production in 2008 was estimated to be 1.3 billion pounds and yearly use has continued to increase\textsuperscript{20}. In California almonds, hundreds of thousands of pounds of OSS formulations are used every year, often during bloom\textsuperscript{21}.

Over 60\% of the managed honey bee colonies in the country are transported to California almonds during bloom\textsuperscript{22}, and in 2014 and 2015, beekeepers reported colony declines following the event characterized by deformed and dying brood\textsuperscript{23}. Adjuvants were among the chemicals implicated in these losses\textsuperscript{23}. We hypothesize that the use of organosilicone adjuvants in combination with exposure to common viruses present in the hive environment and transmitted during pollination events can result in the interrupted development and brood mortality observed in hives used for almond pollination. Although there are limited data regarding the spread of diseases during almond pollination, shared floral resources and drift between colonies occurring more often due to the close proximity of the colonies\textsuperscript{24,25} creates a high-risk scenario for potential spread of viruses\textsuperscript{10}. Furthermore, we hypothesize that exposure to OSS treatments and an immune challenge will result in an inability to clear viral infections as evidenced by higher viral loads prior to pupation.

To test these hypotheses, we have examined the effects of chronic dietary exposure of Sylgard\textsuperscript{®} 309, an OSS spray adjuvant blend commonly used on almonds and many other crops, on developing larvae using a sterile \textit{in vitro} assay. Sylgard\textsuperscript{®} 309 is more uniform in composition than other spray adjuvants analyzed, being almost 80\% pure acetyl-capped trisiloxane surfactant (Fig. 2-1), and it is used in spray tanks at concentrations up to 1\%\textsuperscript{26}.

Records from the California Department of Pesticide Regulation (CADPR) from 2013 report a total use of approximately 0.3 lbs. per acre of OSS adjuvants in California almonds\textsuperscript{21,27}. Additional CADPR data referencing a major area of almond production, Stanislaus County,
indicate that Sylgard® 309 applications are commonplace during the late January to March bloom period (Fig. 2-2a), and that statewide organosilicone-surfactant use increased just prior to the onset of Colony Collapse Disorder and the observed increased colony losses in 2006 (Fig. 2-2b). 1,27.

Here, we report the results of an experiment that tested two factors, OSS chronic exposure and a single added viral exposure, and their interaction on the development of bees from larval to adult stage. To simulate a horizontal infectious event, the larvae were initially exposed to diet containing infectious viral material consisting of pathogens commonly found circulating in honey bee hives and foraging environments including Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV) Israeli Acute Paralysis Virus (IAPV) and Sacbrood Virus (SBV) or an equivalent volume of saline containing no infectious material. Chronic OSS exposure was assessed at 10 ppm in diet (v/v), a 1,000 fold dilution from a tank mix concentration of 1%, to simulate a potentially field relevant exposure rate. After the first day, all larvae received only 10 ppm OSS or control (Ctrl) diet according to treatment group. The larvae were monitored throughout development to adult eclosion for delayed development, mortality, and symptoms of abnormal pathology. Prior to pupation, quantitative real-time PCR (q-PCR) was used to compare virus titers and gene expression between groups to determine the underlying causes of mortality seen in the treatment groups.

Methods

Honey Bee Colonies

Three original colonies of *Apis mellifera ligustica* (hives 1, 2, and 3) and two splits (derived from hive 2 and 3) from a Pennsylvania State University apiary (University Park, PA...
16802) were selected based on relative health and surveyed for the presence of viruses. To ensure uniform age among larvae, the queen was caged on a frame for 24 hours and then excluded from the frame until it was collected for grafting three days later. The experiments commenced in June 2015, and the last frame was taken in late July 2015.

**Chemicals and Virus Inoculum**

Sylgard 309, lot no. 0006018786 was obtained from the Dow Corning Corp., Midland, MI, USA. It is not currently possible to obtain purified strains of honey bee viruses, therefore, a semi-purified virus solution was prepared using a protocol described in Singh 2010 *et al.*

Five adult bees (collected from colonies exhibiting symptoms of IAPV) were homogenized in 5 uL saline solution, and the solution was centrifuged to remove large debris and further purified using a 0.2 micron filter.

As measured using a Primerdesign™ Genesig® kit for the IAPV genome, the solution contained 940 genome equivalents of IAPV/µL of inoculum (see Supplementary Fig. 1 in Appendix A). However, the virus inoculum was composed of four known viruses and relative quantification data for the inoculum showed that IAPV was amplified roughly six cycles after both BQCV and DWV. SBV was amplified roughly four cycles following IAPV. Comparable parameters including similar melting temperatures and similar primer and amplicon sizes were used for all viruses; therefore, the differences in amplification times are likely to reflect the relative abundance of each virus.
Rearing

The larvae were grafted and reared using a modified version of a protocol described in Schmehl et al. 2016\textsuperscript{28}. This protocol, based on the rearing protocol developed by Crailsheim et al.\textsuperscript{29}, uses sterile technique to minimize the unintended exposure of the larvae to pathogens, and ensures stable and clean conditions during development. We modified the protocol by performing the grafting and feedings in a specialized sterile and heated box utilizing a commercial HEPA air filter fitted to vacuum tubing to generate sterile air flow and maintain positive air pressure in the chamber and a temperature/solid state relay to control the heating output of two 350-watt heating elements wired in parallel by referencing output air temperature (see Supplementary Fig. 2 in Appendix A). Exposure temperature of larvae was tightly controlled by use of an incubator or warm boxes to hold plates during observations.

Experimental Design

Within 24 hours of hatching, four plates of 24 larvae each were grafted onto larval diet. Two of these plates contained diet spiked with 10 ppm (v/v) Sylgard 309 (OSS) and two with an equivalent volume of Milli-Q purified water (Ctrl). This concentration was selected following an oral communication with an industry representative suggesting a thousand fold dilution from the tank mix concentration of 1\% as a field realistic dose of OSS. To one of the Ctrl plates (Ctrl+V) and one of the OSS plates (OSS+V), virus inoculum (approx. 47 copies of virus per larva) was added as a one-time exposure. An equivalent volume of saline was added to the Ctrl and OSS diets. Additionally, a positive control treatment group was present in each experimental replicate. See Supplementary Methods in Appendix A for details. Each treatment group was transferred to an incubator following grafting, and isolated from other groups in a humidity-controlled
desiccator. During larval development, OSS and OSS+V groups were fed a diet of 10 ppm OSS, and groups Ctrl and Ctrl+V were fed a diet containing an equivalent volume of Milli-Q® water. This experiment was repeated three times each for each of the three hives. Immature bees were photographed upon death and visible symptoms (Fig. 2-3) and date post-graft were recorded.

q-PCR

On day six of the larval rearing, 24 hours following the last scheduled feeding and immediately prior to transferring larvae to pupation plates, five larvae were removed from each plate and frozen at -80°C for future RNA extraction. Larvae selected were healthy in appearance and had consumed all of the provided diet. Quantitative real time polymerase chain reactions (q-PCR) were performed using primers for Israeli Acute Paralysis Virus (IAPV), Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV), Sacbrood Virus (SBV) and immune and detoxification genes, including 18-wheeler (Am18w), hymenoptaecin, defensin-1, and prophenoloxidase (see Supplementary Table 1 in Appendix A). Novel primer efficacy was verified by Sanger sequencing of cloned products. Three experimental replicates, one from each bio-replicate, were selected for analysis (see supplemental materials for details).

Statistical Analysis

Survivorship analysis (survival analysis and parametric regression), standard least squares regression, and contingency analysis were performed and graphs were prepared using JMP® Version Pro 12. Significance was evaluated using a 0.05 probability threshold. Survivorship analysis was performed across all hives and reps (n=171), and Wald tests were used to evaluate differences. Tukey’s HSD test or Student’s T-test were used to compare least squared
means of response variables between groups. Nominal logistic regression was evaluated using a
likelihood ratio test. All symptoms are displayed in Figure 2-3, but symptoms were simplified
into two categories, failed molt and other, for the analysis reported. See Supplementary Methods
in Appendix A for additional details.

Results

Larval Rearing – Physical Symptoms

The highest significant mortality was seen in larvae exposed to both OSS and the viral
inoculum (OSS+V) (Wald test, n=171, Wald chi square=6.73, p≤0.0095, df=1) with over 68.4%
(+/−4.3%) of the population failing before adult eclosion (Fig. 2-4). The highest number of
individuals lost in OSS+V occurred on day 10 which was the average day of pupation among
individuals in this group. Many of the individuals dying during this time were observed to have
attempted a molt, but died during the process with apparent melanization in the thorax and
abdomen regions and lack of eversion of imaginal discs (Fig. 2-3). The proportion of individuals
displaying this symptom at death was different between groups, (Nominal logistic regression,
n=171, X²=30.35, p < 0.0001, df=3, Fig. 2-5, see Table 2-1 for descriptions of symptoms).
Exposure to the viral inoculum was a significant predictor of this pathology (X²=12.37,
p<0.0004), and chronic exposure to OSS resulted in a positive trend toward higher numbers of
individuals exhibiting this symptom (X²=3.13, p<0.0769). Additionally, the two terms interact,
resulting in a similar positive trend (X²=3.13, p<0.0769).

In the larvae that successfully pupated, all treatment groups exhibited a significant delay
in pupal eclosion relative to Control larvae (Ctrl) (least squares regression, p≤0.0001, df=3, see
Supplementary Fig. 3 in Appendix A). Based on a post hoc comparison of the averages between groups, Ctrl larvae required the least time to complete development from larvae to pupae (9.8±0.05 days) followed by OSS exposed larvae (OSS) (0.25±0.05 day delay), larvae exposed to virus alone (Ctrl+V) (0.34±0.06 day delay), and OSS+V (0.53±0.07 day delay). OSS+V required a significantly longer time than Ctrl or Ctrl+V (post hoc Tukey HSD, p<0.05); but OSS+V, OSS, and Ctrl+V were not significantly different. The delay in development was reflected in the timing of adult eclosion as determined by the appearance of wings (df=3, p<0.0002). Relative to Ctrl, OSS, Ctrl+V and OSS+V experienced a 0.15±0.09, 0.43±0.10 and 0.6±0.14 day delay, respectively. As compared to Ctrl, the observed delays in adult eclosion were statistically significant in the treatments OSS+V and Ctrl+V (Tukey HSD, p<0.05; but these groups were not significantly different from each other). Time to adult eclosion did not differ between the treatments OSS and Ctrl. However, the amount of time required for development between pupation and adult eclosion was not significantly different between groups, indicating that the delay in adult eclosion was directly related to delayed pupation.

q-PCR

To test our second hypothesis, that the mortality observed in response to OSS and virus exposure together were related to elevated virus titers and compromised immunity, we examined the relative abundance of four honey bee viruses, Israeli Acute Paralysis Virus (IAPV), Black Queen Cell Virus BQCV DWV and SBV, and the expression of immune genes in larvae prior to pupation (see Supplementary Fig. 4 in Appendix A). We explored the effects and interactions of treatments and immune gene expression on the titers of each virus. Of the viruses examined here, BQCV titers were related to three predictors: exposure to virus, exposure to OSS, and the
expression of a Toll-like receptor gene (TLR) named 18-wheeler (Am18w) \(^3\) (least squares regression, n=9-10, \(R^2\) adj.=0.63, df=7, p<0.0001).

Exposure to OSS was a significant predictor of higher BQCV titers (p≤0.01134) and exposure to virus resulted in a positive trend toward higher viral titers (p≤0.08411). Additionally, the two terms interacted, resulting in significantly higher BQCV titers in treatment OSS+V (p≤0.00053) (Fig. 2-6, post hoc Tukey’s HSD, p<0.05). The third predictor, 18-wheeler, was positively correlated with BQCV (p<0.00001).

To further explore the three way interaction of BQCV with OSS and exposure to virus, the model was rerun with 18-wheeler as the response. Exposure to virus resulted in elevated 18-wheeler expression (p≤0.00008); but, exposure to OSS depressed this expression (post hoc Student’s T-test, p≤0.00001, Fig. 2-6). Virus exposure and OSS interacted (p≤0.00001), indicating that 18-wheeler expression was significantly higher in group Ctrl+V but was lowest in OSS+V (though not significantly different than OSS or Ctrl). These results suggest that 18-wheeler expression is upregulated by exposure to viral pathogens, but depressed following exposure to OSS.

In the larvae sampled, IAPV titers correlated with BQCV titers (linear regression, n=9-10, \(R^2\) adj.=0.63, df=3, p<0.0001). IAPV infection levels and exposure to OSS interacted to increase BQCV titers (least squares model, n=9-10, IAPV p≤0.0001, OSS: p≤0.0292, IAPV*OSS p≤0.0001).

Expression of 18-wheeler was positively correlated with several other immune genes, including prophenoloxidase (least squares regression model, p≤0.0001, df=1, \(R^2\) adj.=0.45), hymenoptaecin (p≤0.0017, df=1, \(R^2\) adj.=0.21), and defensin 1 (p≤0.0064, df=1, \(R^2\) adj.=0.16). Similar to 18-wheeler, the expression of these genes appears to be depressed in the OSS+V treated bees relative to those in bees from other treatments, but the expression levels for these immune genes are not by themselves significantly related to BQCV levels or related to treatments.
(see Supplementary Fig. 5 in Appendix A). The only measured immune gene whose gene expression is significantly correlated to BQCV and treatment interactions is 18-wheeler.

**Discussion**

The symptoms observed in our experiments allowed us to accurately identify patterns associated with organosilicone exposure and concurrent viral exposure that are most apt to be occurring during almond pollination. The high survivorship of larvae to adults in the Ctrl group allowed us to examine the impacts of chronic OSS exposure and early viral exposure (Fig. 2-4). OSS+V experienced a 44.5% increased mortality as compared to controls; whereas, OSS alone resulted in a 4.09% higher mortality and viral-exposure alone (Ctrl+V) had 21.6% higher mortality relative to Ctrl. If exposure to OSS and viruses had additive effects on mortality, a 25.7% loss relative to Ctrl would have been observed; however, the observed mortality with exposure to OSS and virus was nearly twice this, indicating a synergistic rather than additive interaction.

Interestingly, bees exposed to OSS and virus significantly affected the symptoms associated with death (Fig. 2-5). The deaths occurred immediately prior to the average day of pupation, and the death was most commonly characterized by a failure to evert the imaginal discs (failed molt, Fig. 2-3)\(^3\). These symptoms match those observed by beekeepers following almond pollination in 2014 and initial reports in 2016, as described by G. Wardell\(^2\). Hive losses were characterized by missing brood and “deformed and dying pupae”. Fungicides, insect growth regulators, and tank adjuvants applied at bloom were suggested as possible culprits.

Chronic OSS and early viral exposure during larval development were also found to effect pupal eclosion and metamorphosis (see Supplementary Fig. 2 in Appendix A). Delayed development is a common symptom of immune challenges in insects other than honey bees\(^32-36\).
The delay in pupal and adult eclosion observed in this study was related to viral infections. Insect pupation and immune function are energetically costly\textsuperscript{34,37}, and immune challenges can impair growth and development\textsuperscript{33,35,36}, perhaps resulting in the observed delay. This effect has not been previously documented in honey bees.

OSS exposure had impacts on immunity in honey bee larvae (Fig. 2-6). The higher BQCV titers associated with OSS treatment support our second hypothesis that OSS exposure can result in increased viral replication. Although our work did not look for active replication of the viruses, the significantly higher BQCV titers seen in OSS+V imply that replication of the virus in this group occurred at higher levels than in other groups. Almost all larvae, Ctrl or otherwise had some degree of infection, but introduction of exogenous virus in the 1\textsuperscript{st} instar stage resulted in significantly higher BQCV titers in OSS+V treated larvae. Elevated BQCV titers were only observed when OSS exposure was combined with a simulated viral exposure event. OSS was a significant predictor of lower expression of 18-wheeler, the homolog of \textit{Toll-7} in \textit{Apis mellifera}. \textit{Toll-7} is involved in autophagic viral defense in \textit{Drosophila}\textsuperscript{30,38} and is expressed in the fat bodies of \textit{Drosophila} larvae\textsuperscript{39}. This gene may have a similar function in honey bees. Previous studies examining changes in the honey bee transcriptome induced by viral infection have not found 18-wheeler to be significantly upregulated in infected bees, but these studies largely focus on the effects of other viruses on adults and pupae\textsuperscript{40-43}. This study found that 18-wheeler expression was positively correlated with viral titers in larvae prior to pupation. When larvae were subjected to viral exposure without OSS, 18-wheeler was significantly upregulated as an active immune response to viral infections.

Exposure to OSS and exogenous viruses resulted in higher BQCV titers and mortality, but the mechanisms underlying these combined impacts are not yet known. One hypothesized mechanisms is that OSS may have affected penetration of viral pathogens across the insect cuticle or peritrophic membrane. Adjuvants like OSS may enhance the penetration of pesticide active
ingredients into plant tissue through several mechanisms such as by increasing penetration through the stomatal pores or by becoming embedded in the plasma membrane and altering membrane fluidity to allow direct penetration of chemicals into the cell\textsuperscript{44,45}. The last of these mechanisms is poorly understood and has only been shown to occur under exposure to 100 ppm of a cationic surfactant\textsuperscript{45}. It is theorized that this activity is dependent on the polarity of the cationic surfactant\textsuperscript{45}. OSS’s are nonionic, and available literature has shown OSS facilitated membrane penetration to occur via the stomatal pores at high concentrations (300-1000 ppm)\textsuperscript{46,47}. Further research is needed to determine whether OSS’s can alter cellular membrane fluidity and how this might affect viral infections, but the low concentration of OSS used in this study suggests a different mechanism resulted in the observed mortality. Alternatively, the increased viral titers and mortality following combined OSS and viral exposure may be due to a “perfect storm” scenario: bees exposed to viruses required activation of immune pathways such as 18-wheeler to mediate the infection but concurrent OSS exposure depressed this pathway.

Transcriptomic studies have shown that pesticide exposure, poor diet, and Varroa parasitism can affect the expression of genes related to both nutritional regulation and immunity\textsuperscript{48,49}. Although more research is needed, it may be that these stressors impact a larger pathway such as the insect TOR pathway\textsuperscript{50}, and decreased immune function is a downstream consequence. We are currently performing experiments to examine the transcriptome of the larvae used in this study to identify pathways affected and correlated with the observed pathology.

Honey bee immune pathways engage in cross talk\textsuperscript{51} and the correlations observed between 18-wheeler and other immune genes may reflect this cross-talk. In \textit{Drosophila}, a Toll activated anti-fungal peptide gene \textit{Drosomycin} is moderately upregulated in response to viral infection but has no demonstrable role in viral defense. Other antimicrobial peptides representing the IMD and Jak-Stat pathways are not significantly affected by viral infection. In contrast, 18-wheeler has a 5 fold increase in expression in response to viral infection in \textit{Drosophila}\textsuperscript{38}. In honeybees,
hymenoptaecin and defensin are anti-microbial peptides active against bacterial pathogens\textsuperscript{52,53}, and prophenoloxidase is an enzyme precursor involved in encapsulation and melanization\textsuperscript{54}.

Although these other immune genes did not appear to be significantly affected by OSS and added viral exposure, correlative analysis suggests that they are affected indirectly by viral infection and by the activation or suppression of an immune pathway regulated by 18-wheeler (see Supplementary Fig. 5 in Appendix A).

BQCV was the only virus studied here that was significantly affected by the treatments. Few studies have reported impacts of BQCV on worker pupal development or colony survival, even though it is one of the most prevalent viruses detected in hives in the United States\textsuperscript{11,55-58} and its detection has significantly increased since the early 2000’s. In this work, the synergistic increase in mortality during development observed after treatment with OSS plus viral inoculum was associated with increased BQCV titers. BQCV is typically associated with death in the prepupal stage\textsuperscript{55,59}, which was also observed in this study. In contrast, SBV, which was not affected by treatment and was present only at low levels in the inoculum, produces death prior to pupation; the symptoms produced by SBV\textsuperscript{60} are inconsistent with the symptoms observed in this study. In brood, BQCV infection is thought to occur through horizontal transmission from infected nurse bees or though vertical transmission from infected queens\textsuperscript{61,62}. The virus, which replicates in worker and queen brood\textsuperscript{59,67}, causes death in developing bees\textsuperscript{59,62-64}, and although the majority of research has focused on its effects on developing queens, it has been shown that oral exposure to BQCV at high levels (10\textsuperscript{9} genome equivalents) and the neonicotinoid pesticide thiacloprid results in additive larval mortality and an increase in BQCV titers\textsuperscript{16}.

The relationship between BQCV and IAPV suggests that the co-infection of IAPV with BQCV may enhance BQCV infections. Likewise, the predictive relationship between IAPV titers and OSS exposure on BQCV suggest that the IAPV in the virus inoculum may have influenced the virulence of BQCV in larvae that had been immuno-compromised via OSS exposure. Recent
work reveals that exposure to different virus combinations results in different viral dynamics within the honey bee host\textsuperscript{65}, and co-infections are often a predictor of colony death\textsuperscript{66}. There is evidence that IAPV encodes an immune-suppressive protein and that the virus affects energy-related processes. Both of these effects could have consequences for the overall immune function of the host\textsuperscript{43,67}, perhaps resulting in the worsening of a co-infection. Indeed, IAPV infections have been suggested as a potential predictor of colony failure\textsuperscript{68} that may occur because of this putative immune-suppressive activity. This suggests that the virus may facilitate opportunistic infections. Therefore, we speculate that interactions between introduced and exogenous viruses may also have influenced the observed titers. Future studies involving cell lines and BQCV and IAPV isolates could elucidate this relationship.

The parallels between the symptoms observed in hives following almond pollination and the symptoms observed in this study are striking and support the hypothesis that OSS use in combination with other stressors can lead to the brood mortality observed following almond pollination. A recent survey of virus prevalence in hives before, during and after almond pollination found that the highest abundance of viral pathogens occurs following almond pollination, implying that infections are spread or worsened during the event\textsuperscript{57}. Of the viruses examined in this study, BQCV was one of the most commonly detected viruses in colonies used for almond pollination. Currently, hundreds of thousands of pounds of organosilicone adjuvant are used every year in California almond orchards alone\textsuperscript{21}. Sylgard\textsuperscript{®} 309 is one of the top three out of 45 different OSS products used on almonds while bees are pollinating\textsuperscript{27}; and though the issue is complicated by diversity of products and spray mix combinations, almond orchards are a major crop site where monitoring of OSS use should be considered. OSS’s are widely used in pesticide tank mixes applied to agricultural crops including wine grapes, tree nuts and tree fruits\textsuperscript{21}, and there are currently no restrictions on their use around pollinating insects\textsuperscript{17,69}. Potentially, other beneficial insects may experience similar symptoms in response to OSS
exposure, and the continued decline of pollinator populations suggests that OSS use be evaluated. Residue studies to assess the persistence of OSS in the environment should be considered to further characterize the risk of OSS adjuvants. Furthermore, many other agrochemical adjuvant classes and inert formulates that are currently unmonitored and understudied could have similar consequences, and evaluating their safety may be warranted.
References


60. White, G. F. Sacbrood. United State Department of Agriculture bulletin no. 431 (1917).


64. Laidlaw H. H. *Contemporary Queen Rearing*. (Dadant and Sons, 1979).


Figure 2-1. General organosilicone surfactant structure. For more details, see reference 25.

Sylgard® 309 is primarily R=acetyl, w = 0 with a variable polyethoxylate tail.

Figure 2-2 a. (Left) The percentage of OSS containing applications using Sylgard® 309 in Stanislaus Co. from 2001 to 2013. b. (Right) Tons of OSS used on California almonds from 2001 to 2013.

Figure 2-3. Common symptoms observed during larval rearing. Failed Adult Molt: pupae were unable to emerge from final molt successfully. Failed Molt: larvae failed to evert imaginal discs. Melanizing: Darkening internally or externally occurring in all stages. Other: No distinct symptoms could be observed, but mortality was assessed based on lack of spiracle movement, lack of growth or development for a prolonged period, or submersion in larval diet.
Figure 2-4. Survivorship graph of all groups. Parametric Survival significance indicated by letters, N parameters=1, df=1, OSS: p=0.0034, Ctrl+V: p=0.001, OSS+V: p=0.0095. "*" indicates mean day of pupation. See Supplementary Fig. S1 online for significance. "^" indicates mean day of adult eclosion.

Figure 2-5. Contingency analysis graph of survivorship and observed mortality symptoms (see Table 2-1), grouped by treatment. N=171, $X^2=30.35$, df=3, $p < 0.0001$. 
Figure 2-6. Expression or titers of 18-wheeler and BQCV according to treatment. Least squares regression, n=10-12, R^2 adj.=0.63, df=7, p<0.0001. Standard error indicated by error bars, Response variable significance in respective models indicated by “*”. BQCV: p≤0.00053, 18-wheeler: p≤0.00008.
Table 2-1. List and descriptions of symptoms used in contingency analysis in Fig. 2-5.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surviving</td>
<td>Survived and successfully eclosed as an adult bee</td>
</tr>
<tr>
<td>Other</td>
<td>No apparent symptoms other than arrested development and sometimes discoloration</td>
</tr>
<tr>
<td>Melanizing</td>
<td>Appearance of dark nodules or all over darkening of larvae not related to cuticle hardening</td>
</tr>
<tr>
<td>Failed molt</td>
<td>Appearance of a head capsule or other pupal characteristics, but the pupal molt is incomplete. May present with melanized nodules forming.</td>
</tr>
<tr>
<td>Failed adult molt</td>
<td>Failure to emerge from final molt</td>
</tr>
<tr>
<td>Early death</td>
<td>Submersion in diet, flattening, lack of spiracle movement or melanizing occurring prior to transfer to pupation plate</td>
</tr>
</tbody>
</table>
Chapter 3
The Field Residues and Effects of the Insect Growth Regulator Novaluron and Its Major Co-Formulant \(N\)-Methyl-2-Pyrrolidone on Honey Bee Reproduction

Author Contribution

Julia Fine performed the larval rearing bioassays and residue analysis. The field residue studies were designed by Julia Fine and implemented by Julia Fine, Ryan Reynolds and Donald Smith. The 2014 and 2015 nucleus colony field experiments were performed by Maryann Frazier, and Ryan Reynolds and designed by Maryann Frazier, Christopher Mullin, Ryan Reynolds and Julia Fine.

Abstract

Due to recent declines in honey bee populations, there is a need for field and laboratory studies to investigate threats to pollinator health. This study examines the hypothesis that the organophosphate alternative, Rimon\(^\circ\) 0.83EC, can have consequences to honey bee health by combining newly acquired field residue data, laboratory bioassays, and colony level feeding studies. Following label rate applications of Rimon\(^\circ\) 0.83EC to apple trees, average residue concentrations of the active ingredient, novaluron, were found to be 3.38 ppm in pollen. Residues of the major co-formulant in Rimon\(^\circ\) 0.83EC, \(N\)-methyl-2-pyrrolidone (NMP), were below the limit of detection in the field, but a greenhouse study described here found that NMP can persist in pollen for up to 7 days with average concentrations of 69.3 ppm. Concurrent larval
rearing studies found novaluron and NMP to be toxic to developing honey bees at doses as low as 100 ppb and 100 ppm respectively. Nucleus colony feeding studies found that chronic exposure to Rimon® 0.83EC at doses as low as 200 ppm (18.6 ppm novaluron) can result in interruptions to brood production that can last for up to 2 weeks after the feeding period. Taken together, these data indicate the use of Rimon® 0.83EC on blooming flowers is a significant threat to honey bee reproduction, and suggest the need for more strict usage guidelines.

**Introduction**

Recently, beekeepers have experienced colony declines following almond pollination characterized by a lack of brood in the hive, and while the ultimate cause of such declines has yet to be determined, there is concern that the use of insect growth regulators (IGRs) during bloom periods may be a contributing factor\(^1,^2\). IGRs are a pesticide class used to control pest species by mostly targeting juveniles rather than adults\(^3\). Primarily, IGRs inhibit the development of target species by acting as chitin synthesis inhibitors, ecdysone agonists, and juvenile hormone mimics\(^4\). Typically, these pesticides do not exhibit acute effects on adult bees, and numerous IGRs including the chitin synthesis inhibitors novaluron and diflubenzuron, and ecdysone agonist methoxyfenozide are registered as “reduced risk” or alternatives to conventional pesticides in various crops\(^5\). While chronic and subacute effects of IGRs on queens, drones and worker honey bees impacting egg production and pheromone communication have been noted\(^6,^7\), the acute risk to foraging adults is indeed lower\(^3\). However, IGRs can have considerable consequences for the larvae of pollinating insects\(^8-^11\). In order to accurately represent these risks, several factors must be considered. The IGR must persist in relevant environmental substrates at high enough concentrations and for a long enough period of time following its application that a foraging
pollinator will encounter it. Then, the foraging insect must provision its colony or brood with a sufficient dose to cause developmental effects. Depending on the pollinating species, the IGR in question, and the method of application, the outcome of using an IGR on insect pollinated crops may differ. For example, alfalfa leaf cutter bees exposed to alfalfa crops treated with label rates of novaluron applied as Rimon\textsuperscript{0} 0.83EC produced fewer viable offspring even up to two weeks after the initial application\textsuperscript{12}. Conversely, bumble bee colonies fed concentrations of novaluron equivalent to residues observed in corn pollen did not experience any observable brood loss\textsuperscript{13}.

Here, we examine the use of the IGR novaluron and its impacts on honey bees when it is applied as the pesticide product Rimon\textsuperscript{0} 0.83EC containing 9.3% novaluron.

Novaluron is a benzoylphenylurea chitin synthesis inhibitor\textsuperscript{14} that is currently registered in the United States for use against coleopteran, hemipteran, and lepidopteran pests in a variety of crops including berries, cucurbit vegetables, fruiting vegetables, brassica vegetables, stone fruits, pome fruits, potatoes, strawberries and cotton. Currently, it is registered as an “organophosphate alternative” in pome fruits and as a “reduced-risk” pesticide in ornamentals\textsuperscript{5}. However, information pertaining to its presence and persistence in pollen and nectar in the honey bee foraging environment is limited, and recent studies have shown that it is toxic to alfalfa leaf cutter bee larvae\textsuperscript{11,12}. Additionally, the major novaluron formulation, Rimon\textsuperscript{0} 0.83EC, contains 40-50% of a powerful co-solvent, \textit{N}-methyl-2-pyrrolidone (NMP). NMP is considered to be a mammalian reproductive toxin\textsuperscript{15,16}, and preliminary studies have shown that it is toxic to honey bee larvae at doses as low as 100 ppm in diet\textsuperscript{17}. The work described in this chapter seeks to evaluate the hypothesis that novaluron and NMP persist in apple pollen following applications of Rimon\textsuperscript{0} 0.83EC to budding and blooming apple trees. Additionally, we will investigate the hypothesis that novaluron and NMP can affect honey bee reproductive success by evaluating the toxicity of formulated novaluron and NMP on developing larvae using an \textit{in vitro} larval rearing method, and
observing the effects of these agrochemicals on small nucleus colonies (nucs) following chronic feeding periods with Rimon® 0.83EC and equivalent concentrations of NMP.

Methods

Rimon® 0.83EC Field Application Study 2015 (Spray at Bud)

On May 1, 2015, 25 Red Delicious trees at Rock Springs Research Farm were selected for a field residue study of NMP using Rimon® 0.83EC (Chemtura Corporation, Middlebury, CT). The trees were divided into three blocks and randomly assigned to either Control (Ctrl Bud) or Rimon® 0.83EC (Rimon Bud) treatment groups with untreated buffer trees between all treated trees. A hand sprayer was used to apply the maximum label rate of Rimon® 0.83EC (50 fl. ozs./ac.) to Rimon Bud trees at bud (pink stage) and an equivalent volume of water was applied to Ctrl Bud trees. Two whole bud samples were collected from each tree 12 hours following the spray and pooled for analysis. Composite pollen samples (anthers) from 10 blooms per tree were collected at bloom on May 5th.

Rimon® 0.83EC Field Application Study 2016 (Spray at Bloom)

On May 27, 2016, four blooming Golden Delicious apple trees located on the Pennsylvania State University Fruit Research and Extension Center in Biglerville, PA were treated with a boom sprayer application of 30 fl. oz. Rimon® 0.83EC/A (Rimon Bloom). Composite pollen samples (anthers) from 50 blooms per tree were collected 24 hours later. Control pollen samples were collected from 3 nearby untreated trees (Ctrl Bloom).
Pilot NMP Residue Study

There is currently no information available regarding the persistence of NMP following its use in an agricultural setting. Therefore, a pilot study was undertaken to determine the likelihood of detecting NMP residues in the field. On February 16, 2015, Red Delicious apple tree branches were removed from trees located in the Rock Springs Research Farm and transported to the Pennsylvania State University Agricultural Sciences and Industry Building. There, the branches were placed in mason jars of fresh water inside of a growth chamber set at constant light, 65% relative humidity and 23.9°C. When the branches began to develop buds on March 3, the growth chamber was set to a night and day cycle (14hrs daylight), and the temperature was lowered to 15.6°C. The branches were divided into 3 groups as they budded in the following order: Control (Ctrl Pilot), NMP Bud and NMP Bloom. Buds in the Ctrl or NMP bud groups were treated when the buds had reached the “pink” stage, or when the buds appeared pink in color. Buds placed in the NMP Bloom group did not receive treatment until the flowers had fully opened. The treatments consisted of a spray to drip with aqueous solutions containing 0% NMP (Control Pilot) or 0.4% NMP (NMP Bud and NMP Bloom). This concentration of NMP (Sigma-Aldrich, St Louis, MO) was based on the Rimon® 0.83EC label recommended concentration for control of leafrollers in apple trees under 10 ft. in height. Flowers in the NMP Bud and Ctrl groups were collected upon bud burst and flowers in the NMP Bloom group were collected 2.5 hours or 24 hours after treatment. All flower samples from the pilot study were collected, pooled, and analyzed in groups of two. Application dates and collection dates were recorded for all samples.
Sample Preparation and Residue Quantification

Pollen samples were prepared by adding a volume of methanol (99.9% purity, Sigma-Aldrich, St Louis, MO) equivalent to 5 times the sample mass to flower anthers (1 g pollen/anthers in 5 mL methanol). For whole flower samples collected during the 2014 field application study and the NMP pilot residue study, the anthers of two pooled samples were removed and processed together. The mixture was vortexed and centrifuged at 5,000 rpm for 20 minutes, and 1 mL of the resulting supernatant was added to a Supel™-Que PSA 2 mL centrifuge tube (Bellefonte, PA). The tube was vortexed for 10 seconds and centrifuged at 10,000 rpm for 20 minutes, and the supernatant was collected for analysis. Surface residues were obtained by submerging the flower (minus the anthers) in methanol for 2 seconds. Pollen residues were quantified using analytical standards added in 10 µL methanol to 0.25 g corn pollen to create matrix standards, and percent recovery was calculated by comparing matrix detected peak areas to solvent calibration peak areas. Surface residues were quantified using a solvent calibration.

A Shimadzu LC-MS 2020 system with a 2.0 x 100 mm XR-ODS column (Shimadzu, Kyoto, Japan) was used for analysis of all samples. Numerous methods for the analysis of NMP and its metabolites exist\textsuperscript{18-20}, and the analysis reported here is based on methods described in Cohen et. al. 2007\textsuperscript{19} with adaptations to suit a single analyte and matrix specifications. The injection volume was 1 µL, and the quadrupole mass analyzer was operated using an electrospray ionization source in the positive mode with selective ion monitoring of 100 \(m/z\). The binary mobile phase consisted of (A) water and (B) methanol buffered with 0.1% formic acid (98% purity, Sigma Aldrich, St. Louis, MO, USA). Matrix and solvent calibration standards ranged from 0 to 2,500 ppb (vol./vol.) in methanol.

The same instrumentation was used for analysis of novaluron, and a method described by Qian et.al. 2012\textsuperscript{21} was adapted to suit instrument specifications. The injection volume was 1 µL,
and the quadrupole mass analyzer was operated using an electrospray ionization source in the negative mode with selective ion monitoring of 491 m/z. Matrix and solvent calibration standards ranged from 0 to 1,035 ppb (g/vol.) in methanol.

**Larval Rearing**

A small scale, dose range finding study was conducted in August 2015 to determine the chronic toxicity of Rimon® 0.83EC to honey bee larvae using 1st instar larvae grafted from a honey bee colony located in a Pennsylvania State University Apiary. Using a modified version of the protocol described in Schmehl et.al. 2016 (See Chapter 2 Methods and Appendix A), larvae were reared in an incubator until adult eclosion. Groups of 24 larvae each were exposed to doses of 100 ppb, 250 ppb and 1 ppm novaluron mixed into diet as Rimon® 0.83EC. NMP solvent control groups were administered corresponding doses of 537 ppb, 1.34 ppm and 5.37 ppm NMP. Finally, a positive control group was given 45 ppm dimethoate (Sigma-Aldrich, St Louis, MO) and a negative control group was given diet spiked with 10 µL of water.

Based on the NMP residues observed in the pilot study, a chronic dose of 100 ppm was chosen for a large scale larval rearing experiment (See Supplementary Figure 1 in Appendix B for small scale NMP dose range finding experiment results). Using 1st instar larvae taken from three colonies located in a Pennsylvania State University apiary, groups of 19 larvae were reared according to the modified version of the Schmehl et.al. 2016 protocol described in Chapter 2 and Appendix A under chronic exposure to 100 ppm NMP diluted in diet. Control groups were administered a corresponding dose of water. Because NMP is usually a solvent vehicle for other chemicals, a traditional positive control was not used. Larvae were monitored for developmental landmarks such as pupal and adult eclosion, and the time to mortality was recorded. This experiment was repeated three times each in three hives.
Nucleus Colony Field Study

Field studies using 5-frame nucleus colonies (nucs) established from new packages of bees were conducted in the summers of 2014 and 2015. Three pound packages (25 in 2014 and 27 in 2015) purchased from Spell Bee Co. (Braxley, GA) were established in standard equipment in early April of both years. All packages received a treatment of 25 mL 3.4% oxalic acid before installation and once installed the package queens were replaced with Varroa-resistant sister queens (Miksa Honey Farms, Groveland, FL). From these packages, 56 nucs were established in Jester’s EZ® corrugated plastic nuc boxes (Jester Bee Co., Mims, FL) (early July 2014, mid June 2015). Each nuc consisted of 1 pound of bees, one frame of emerging brood, and a mated sister queen (Miksa, FL) installed 24 hours after the nuc was established. For 7 days in 2014 and 10 days in 2015, the colonies were provisioned with approximately 473 mL of 50% sucrose solution delivered in a Mason jar and 100 g of a pollen substitute. Pollen substitutes were mixed according to product instructions [Mega Bee® (megabee.com) in 2014 and Ultra® Bee (Mann Lake, Hackensack, MN) in 2015] and delivered as a patty on top bars. Access to water was provided ad libitum via a second pint jar. Chemical treatments were delivered via the sucrose and pollen substitute and colonies where kept closed to prevent foraging during treatment application. Once the colonies had consumed the treated sucrose solution, untreated solution was provided and bees were permitted to feed ad libitum. In 2014, the experiment consisted of 8 groups of 7 nucs. In 2015, the number of treatment groups was decreased to 4 with 7 nucs each. This experiment will address the four repeated treatment groups, which were as follows: a positive and negative control in which the provisions were spiked with Actara (positive control, 25% neonicotinoid thiamethoxam, Syngenta), and water respectively, a group in which provisions were spiked with Rimon® 0.83EC, and a NMP group dosed with the amount of NMP corresponding to the approximate amount present in the Rimon® 0.83EC group (50%). In 2014,
the doses were as follows: Actara- 2 ppm, Rimon® 0.83EC – 1000 ppm (93 ppm novaluron) and NMP- 500 ppm. For 2015, lower doses of 0.4 ppm Actara, 200 ppm of Rimon® 0.83EC (18.6 ppm novaluron) and 100 ppm of NMP were used.

The treatments were applied on July 3, 2014 and June 18, 2015 (Day 1). After the conclusion of the treatment period (Day 7 in 2014 and 10 in 2015), colonies were opened and allowed to forage freely. Weight at time of treatment application, the weight of dead bees following the treatment period, and the colony weight (without dead bees) at the conclusion of treatment application were recorded for each nuc. In 2015, colony weight was assessed again on day 21. Queen egg laying was assessed in 2014 and 2015 by caging a queen on empty drawn comb. After 24 hours, the queen was removed, and the eggs were counted. These assessments took place on days 11 and 25 in 2014 and on day 14 in 2015. In 2015, foraging behavior was assessed by counting the number of bees returning to a hive over a period of three minutes. The assessment was performed once for each hive and then repeated twice between 12 and 4 pm on day 20. In both years, brood production was monitored but not quantified. Assessments were performed to identify open or capped brood on days 7, 14, 21 and 46 in 2014 and on days 10, 17, and 24 in 2015.

**Statistical Analysis**

Statistical analysis of and quantification of agrochemical residues was performed with Microsoft® Excel 2016. The concentration of analytes in a given sample was predicted based on a regression equation generated from matrix standards (See Supplementary Figures 2 and 3 in Appendix B). Survival Analysis, ANOVA, and Contingency Analysis were performed using JMP® Version Pro 12. Survivorship analysis was performed across all hives and replicates represented in the larval rearing studies, and log-rank significance tests between groups were used.
to evaluate significance. Contingency Analysis was used to identify matrix specific patterns in the number of positive versus negative detections and to evaluate the presence or absence of open brood in treated nucs. Significance was evaluated using a likelihood ratio test. Analysis of Variance (ANOVA) was used to identify trends or patterns associated with the concentrations of chemical residues observed over time and to identify significant treatment effects in the nucleus colony field study. Tukey’s Honest Significant Difference test (Tukey’s HSD) was used to determine significance between groups. Significance was evaluated using a 0.05 probability threshold, but a threshold of 0.1 was considered evidence of a noteworthy trend. In 2014, 5 Actara treated nucs were used for all evaluations due to early hive failure. For all other group in 2014, and all groups in 2015, n=7.

Results

Residue Studies

Pollen samples collected at bloom following the application of Rimon® 0.83EC to apple buds in 2015 were negative for novaluron and NMP. However, 12 hours following the initial application, average detections of NMP in pollen from unopened buds were 17.15+/-4.39 ppm (Rimon Bud 24 hr, n=4).

Novaluron was detected in all samples of pollen from the 2016 residue study in which blooming trees were treated with Rimon® 0.83EC. Average detection was 3.38+/-0.68 ppm (n=4). No NMP was detected in these samples.

In NMP Bud samples collected during the pilot study, NMP was detected in all pollen samples even up to 7 days after application (Fig. 3-1), however detections of NMP on the surface of the flowers were less frequent (chi-square=9.29, df=1, n=8; P=0.0023). There was no
significant relationship between the amount of time between application and sample collection and the concentration of NMP detected in NMP Bud samples. The highest concentration detected in this treatment group was 134 ppm, with average detections +/- standard deviation of 69.3 +/- 39.3 ppm (n=8). In NMP Bloom samples collected 24 hours after treatment, average detections were 35.7 +/- 15.1 ppm (n=2) with a high of 46.4 ppm. NMP residues (235 ppm) were highest in the single sample collected 2.5 hours after NMP application at bloom, and a negative trend was detected between time and residues in bloom treated flowers (F=116.2, df=2, n=3; P=0.0589).

All Control samples were negative for NMP or novaluron. See Supplementary Table 1 in Appendix B for a summary of all detections of NMP or novaluron. Percent recovery and R² for all methods are listed in Supplementary Table 2 in Appendix B. Figures 3-2 and 3-3 depict LC-MS chromatograms of NMP and novaluron respectively.

**Larval Toxicity Studies**

All larvae administered 1 ppm novaluron in diet were dead by day 3; all larvae administered 250 ppb were dead by day 5, and only 2 larvae administered 100 ppb novaluron survived to adulthood but eclosed with deformed wings and cuticle (chi-square=250.0, df=7, n=24; P≤0.0001, Fig. 3-4). The LT50 (median lethal time after initial exposure) of novaluron at 100 ppb was approximately 9 days, with the highest proportion of deaths occurring during the fifth instar stage, prior to pupation. Larvae administered formulation-equivalent doses of 537 ppb, 1.34 ppm or 5.37 ppm NMP in diet as a solvent control did not experience significant mortality compared to Ctrl larvae. Dimethoate exhibited toxic effects compared to Ctrl larvae (chi-square=53.59, df=1, n=24; p≤0.0001), and survivorship was significantly lower in novaluron treated groups relative to controls even at the lowest dose of 100 ppb (chi-square=27.73, df=1, n=24; P≤0.0001). Ctrl larvae experienced less than 21% mortality.
A dose of 100 ppm exhibited toxic effects on developing honey bees, with NMP exposed bees experiencing an increase in mortality greater than 15% relative to controls (chi-square=10.90, df=1, n=171; P≤0.0010, Figure 3-5). However, this effect was not uniform throughout all hives tested. Individually, only two of three hives experienced significant mortality (df=1, n=57; hive 1: chi-square=5.61, P≤0.0179; hive 2: chi-square=6.858, P≤0.0088; hive 3: chi-square=0.616, P≤0.4326). The highest mortality in an individual hive (roughly 20%) was recorded in hive 2. Larvae exposed to 100 ppm NMP also experienced a delay of pupation of approximately 0.42 days (F=35.8, df=259, n=171; P≤0.0001). Pupation was also influenced by the hive origins of the larvae, (F=8.86, df=259, n=171; p≤0.0468) but a Tukey’s Honest Significant Difference test did not detect any significant differences in the day of pupal eclosion between hives.

**Nucleus Colony Field Study**

In 2014, no open or capped brood were observed in Rimon treated colonies until day 46, when brood was observed in a single colony (2014 day 21: chi square=30.29, df=3, n=3; p<0.0001, Fig. 3-6a). In 2015, no brood was observed for the duration of the study (2015 day 24: chi square=31.491, df=3; p<0.0001, Fig. 3-6b).

In both years, the weight of dead bees was significantly higher in the positive control group (Actara) following the treatment periods (2014: F=80.43, df=25; P<0.0001; 2015: F=14.32, df=27; P<0.0001). In 2014, Actara treated hive weights were significantly lower following treatments (F=5.951; df=25; P<0.0039). In 2015, hive weights changes did not differ significantly until day 21, when significantly more positive weight change was observed in Ctrl hives relative to all other treatments (F=7.856, df=27; p<0.0008, Fig. 3-7). In both years there
were no significant differences in the number of eggs laid by the queen in a 24 hour period observed between groups regardless of the amount of time that had elapsed since the treatment applications. The number of foragers observed in treatment groups on Day 20 was significantly higher in Ctrl treated hives than in Rimon or Actara treated hives in 2015 (F=4.093, df=27; P<0.0176, Fig. 3-8).

Discussion

Because the chemistry of novaluron and its major co-solvent in Rimon® 0.83EC are distinct, it is important to consider their environmental fate separately although they are applied together. NMP is a highly water soluble, penetrative, small molecule (MW = 99.13 g/mol\(^{-1}\)) that is often used to enhance movement of other chemicals across biological membranes\(^{23}\). Converely, the molecular weight of novaluron (MW = 492.71 g/mol\(^{-1}\)) is almost five times that of NMP, it is poorly soluble in water, and it has strong translaminar activity in plants\(^{24}\). NMP aids in dissolving novaluron in water and likely increases its penetration across cuticular and membrane barriers so that internal targets sites in pests can be attained.

When Rimon® 0.83EC was applied to blooming plants, average novaluron residue detections were 3.38+/-0.68 ppm, but the application rate used during this field trial was well below the maximum field rate of 50 fl. oz. for apples. Additionally, the US-EPA summarized data lists foliar residues of novaluron as high as 18.6 ppm\(^{25}\). Considering this, the doses used in the larval rearing study are likely to be below the residue levels in pollen collected by foragers from novaluron treated flowers, and the doses used in the field study can be considered a worst-case-scenario level of exposure. Similarly, the choice to use chronic assays may be representative of a worst case scenarios in which colonies do not have access to alternative sources of food.
Despite the much lower dose of Rimon® 0.83EC used in the 2015 nucleus colony field study compared to the 2014 study, Rimon® 0.83EC treated colonies still failed to rear brood for up to 24 days following the application of treatments. Decreases in hive weight gains and foraging activity observed in 2015 were likely caused by dwindling populations and a decreased need for resources related to the absence of brood. Based on the results of this study, it is evident that novaluron is highly toxic to honey bee larvae, but it is not entirely clear what caused the lack of brood observed in the field study. The results of the larval rearing study indicate that if larvae encountered residues of novaluron in royal jelly, pollen, or nectar at concentrations even 30 fold lower than the average concentration detected in pollen following applications to blooming flowers, they would not likely survive. However, only eggs were observed in these colonies until days 21 in 2014 and through day 24 in 2015, therefore residue testing of larval provisions was not possible. Because of the complete lack of brood, it is apparent that either the eggs hatched and the dying larvae were quickly removed, or the eggs were not viable and did not hatch at all. Queen egg laying was not affected at either dose of Rimon® 0.83EC, but reduced egg viability may have been a factor. Chitin synthesis inhibitors including novaluron have been shown to affect egg viability in other species, therefore it is probable that novaluron has an ovicidal and larvicidal effect in honey bees. Regardless, both honey bee queens and young larvae ingest only royal jelly, and the absence of larvae indicates that novaluron encountered in pollen and nectar provisions can be transferred to royal jelly. It has been suggested that the most toxic substances to honey bees are not likely to enter a hive because foraging bees collecting these toxic materials will die before returning with the contaminated food source. However, IGRs like novaluron that exhibit little to no toxicity to adult worker honey bees are an exception to this assertion.

The results of the residue studies indicate that under certain, controlled conditions, NMP can persist in pollen for up to 7 days, and novaluron residues can persist for at least 24 hours in
pollen if the formulation is applied to blooming flowers. NMP residues on the surface of bud sprayed flowers in the pilot study were detected relatively infrequently. The most likely causes of dissipation under the controlled conditions of the growth chamber are evaporation or photolysis. If either of these phenomena were indeed occurring it would also explain the high concentration of NMP in pollen 2.5 hours after application to blooming flowers, but the lower residues observed 21.5 hours later. Under experimental conditions, NMP photolyses to \( N \)-methylazetidine and other products\(^3\), and with a vapor pressure of 39-45 PA, some volatilization of NMP is expected\(^3\). However, when NMP was applied to buds in the pilot study, there was no apparent difference between residues in pollen collected 24 hours after application and residues in pollen collected up to 7 days later. These detections are evidence that NMP can penetrate floral tissue, and the persistence of NMP in the pollen for up to 7 days suggest that the petals of the closed bud act as a barrier to any conditions that might cause the dissipation of NMP. In the field, NMP was not detected in blooming flowers regardless of the timing of applications of Rimon\(^{\circledR} \) 0.83EC. This is not entirely surprising given the increase in uncontrollable variables associated with field studies\(^3\). According to the National Centers for Environmental Information\(^3\), State College and other townships surrounding the Rock Springs Research Farm experienced 1.8-4.8 mm of rain on the date of application in 2015, and Biglerville, PA experienced 10.2 mm on the date of application and 13.2 mm on the date of collection in 2016. NMP is highly water soluble\(^3\), and rainwater may have washed much of the pesticide formulant from the surface of the buds before NMP could penetrate into floral tissues. However, NMP was detected in the pollen of unopened flowers 12 hours following application of Rimon 0.83EC to buds in 2015. The fates of these residues were not determined in this work, but the systemic activity of agrochemicals is determined in large part by its solubility in plant xylem and phloem, and its ability to move across plant membranes\(^3\). NMP is miscible in octanol and water, and penetrates lipophilic biological membranes\(^3\). We speculate that residues of NMP may have been translocated within the apple
tree. Additionally, plant metabolism of NMP has not been described, and we did not attempt to
detect NMP or potential metabolites in plant matrices other than pollen, but more controlled
studies using whole plants in a greenhouse and more sensitive instrumentation could determine
the likelihood of this possibility. Regardless, these initial results indicate that NMP may persist
following agricultural applications and suggest that further study is needed to explore potential
risks to pollinating insects.

The results of the larval rearing studies indicate that exposure to 100 ppm NMP may be
harmful to developing honey bees and result in developmental delays, although these effects may
not be uniform throughout honey bee populations. The effects observed in the larval rearing
study are less dramatic than those reported by Zhu et.al. 201317, but several factors distinguish
this larval rearing study including the use of a sterile chamber and the increased number of
technical and biological replicates. For these reasons, this in vitro rearing study more accurately
reflects the isolated effects of NMP on developing larvae. The results of the nucleus colony field
study are difficult to relate to the in vitro rearing study of NMP treated larvae, and interpretation
is limited due to several variables. First, weight assessments were not quantitative in nature, and
the proportion of the colony containing brood, pollen, nectar or empty comb was not recorded.
Quantitative brood production monitoring was not performed, but could have identified treatment
effects similar to those observed in in vitro reared bees exposed to NMP. Similarly, sampling
royal jelly throughout the experiment could have determined the concentration of NMP that
larvae were exposed to in a hive setting. However, lower hive weight gain relative to Ctrl
colonies on day 21 in 2015 may suggest a decrease in brood success as observed in laboratory
studies.

The design of the field study was useful in detecting dramatic effects like the failure to
produce brood observed in Rimon treated colonies and the worker mortality observed in the
positive control (Actara) colonies, but more subtle effects such as the 15% larval mortality
observed in NMP treated, *in vitro* reared larvae may have been missed due to efforts to preserve the scale of the study. In this discussion, several changes to the nuclear colony field study methods have been suggested to increase the sensitivity of the design, but the practicality of these suggestions is not explored. Measurements of whole colony health such as hive weight, brood production, and foraging efficiency are thought to be advantageous because the holistic approach to colony health considers the ultimate consequences on the entire colony and the status of all members of the honey bee hive in realistic settings. However, numerous threats to honey bee health can influence hive health in the field, and there is some concern that efforts to control all variables in a field setting can decrease the reliability and limit the interpretation of the data.

The study presented here combines laboratory and field data in an effort to maximize understanding of the effects of novaluron and its major co-formulant, NMP. While several measurements could have aided in interpreting the data such as quantitative brood monitoring, we believe that this approach provides a more detailed representation of the consequences of Rimon® 0.83EC use around honey bees. In summary, although field study data can be extremely useful in detecting obvious changes in the health of a colony, quantitative determinations made in a controlled laboratory setting should be considered a more precise measurement of subtle impacts on honey bee health.
References


33. Winton, K. Integrating laboratory and field environmental fate studies: an introduction in *Pesticide Environmental Fate ACS Symposium Series*, 1-6 (American Chemical Society, 2002).


Figure 3-1: Summary of NMP concentrations in pollen at bloom when treatment was applied to budding flowers. No relationship could be detected between the time elapsed since treatment and the concentration in the pollen.
Figure 3-2: Chromatogram of 1 ppm NMP in pollen matrix.

Figure 3-3: Chromatogram of 0.75 ppm novaluron in pollen matrix.
Figure 3-4: Survivorship of novaluron (administered as Rimon® 0.83EC) treated bees. Equivalent NMP treatment groups not pictured.

Figure 3-5: Survivorship of larvae exposed to 100 ppm NMP in diet.
Figure 3-6: Contingency analysis of brood assessments. “+” = brood present. “−” = brood absent. a (left): 2014 day 21 brood assessments. Figure 3-6b (right): 2015 day 24 brood assessments.

Figure 3-7: Hive weight change relative to initial weight on day 21 of 2015. Letters indicate significant difference between groups (Tukey HSD). Standard error is represented by error bars.
Figure 3-8: Mean emerging forager counts on day 20 of 2015 nucleus colony field study. Letters indicate significant difference between groups (Tukey HSD). Standard error is represented by error bars.
Chapter 4

Detoxification of N-Methyl-2-Pyrrolidone in Honey Bee Adults and Larvae: Exploring Age Related Differences in Toxic Effects

Author Contribution

These experiments were designed by Julia Fine and Dr. Christopher Mullin. Julia Fine performed the bioassays, sample preparation and spectrometric analyses.

Abstract

In chronic feeding assays, the common agrochemical inert formulant, N-methyl-2-pyrrolidone (NMP) has been found to be at least 20 times more toxic to honey bee adults than to larvae, but the underlying cause of this difference is unknown. In other taxa, NMP is primarily detoxified via a cytochrome P450 mediated pathway. This study uses an LC-MS method to identify and quantify cytochrome P450 metabolites of NMP in adults and larvae following chronic exposure to 200 ppm of NMP, and explores the observed differences in cytochrome P450 metabolism of NMP by using a spectrofluorometric method to compare general cytochrome P450 enzyme activity. Oxidized NMP metabolites were identified in bees following a NMP feeding assay, suggesting that cytochrome P450 mediated metabolism is the main route of detoxification in larvae and adults, and that adults are able to metabolize a higher percentage of the administered NMP to its more polar metabolites. Additionally, higher microsomal levels of 7-ethoxycoumarin-O-deethylase activity in adult honey bee fat bodies suggests that the higher percentage of
unmetabolized NMP in larvae relative to adults may be due to lower cytochrome P450 activity in the larval fat bodies.

Introduction

The reproductive toxicity of NMP has been demonstrated in model rat systems\(^1\)-\(^3\) and *Daphnia magna*\(^4\), and the work presented in the Chapter 3 (see Fig. 3-5) and in previous work\(^5\) demonstrate that it is toxic to honey bee larvae at 100 ppm. Our pilot feeding studies have demonstrated that the No Observable Effects Limit (NOEL) of NMP is 0.2% for adult bees under chronic exposure for 9 days, which indicates that the toxic effects of NMP on honey bees are age dependent (0.5% NMP to Control (ctrl): chi-square=131.5, df=1, n=59; \(p \leq 0.0001\), Fig. 4-1). Because of the industrial applications of NMP and its use in pharmaceuticals\(^6\),\(^7\), a significant amount of work has been done to explore the metabolism of NMP in humans in order to evaluate biomarkers and establish tolerances\(^8\)-\(^11\). However, despite the extensive and unmonitored use of NMP in agrochemical products\(^4\),\(^12\), there is currently no available information pertaining to the metabolism of NMP in invertebrates. This information could help establish biomarkers for monitoring NMP exposure in nontarget organisms, and aid in predicting the consequences of NMP use on invertebrates. In this chapter, the metabolism of NMP in honey bees will be explored, and biomarkers for exposure will be evaluated.

Regardless of the route of exposure, NMP is primarily detoxified via hydroxylation in humans and rats\(^8\),\(^11\). In quantitative metabolic studies performed on humans or rats, the majority of NMP is recovered in the urine as 5-hydroxy-\(N\)-methyl-pyrrolidone (5-HNMP), \(N\)-methyl-succinimide (MSI), and 2-hydroxy-\(N\)-methylsuccinimide (2-HMSI) or the parent compound\(^8\),\(^11\),\(^13\),\(^14\) (Fig. 4-2) with a small percentage of these compounds eliminated in the feces or transformed to \(\text{CO}_2\) and eliminated via respiration\(^8\),\(^9\),\(^13\). The observed metabolites are suggestive
of a cytochrome P450 mediated pathway\textsuperscript{15}, and in humans, inhibition of Cyp2e1 in human liver microsomes significantly inhibits the transformation of NMP to one of the main metabolites and an exposure biomarker, 5-HNMP\textsuperscript{16}. We hypothesize that a similar pathway exists in honey bees, and that the observed difference in tolerance between adults and larvae is caused by differences in detoxification capacities.

Numerous analytical methods exist to identify and quantify NMP and its main metabolites\textsuperscript{13,17-19}. Here, a method described in Cohen \textit{et.al.} 2007\textsuperscript{18} is adapted to identify and quantify NMP and its hydroxylated and ketonic metabolites in whole body adult and larval honey bees following 6 days of chronic oral exposure to NMP at 200 ppm (vol./vol.) in diet using liquid chromatography coupled to a mass spectrometer (LC-MS). The presence and quantities of the metabolites generated and the remaining parent compound in adults and larvae were compared to determine if the lower No Observable Effects Level (NOEL) for NMP observed in larvae is related to their ability to metabolize NMP relative to their adult counterparts.

Additionally, general cytochrome P450 activity in \textit{in vitro} reared larvae were compared to activities in adults and larvae reared in a hive environment to determine if artificially rearing larvae influenced cytochrome P450 enzyme activity. Cytochrome P450 enzymes responsible for xenobiotic detoxification are upregulated in response to honey, pollen, and propolis constituents\textsuperscript{20,21}, and larvae reared in a hive begin to ingest pollen and nectar after the third day of development\textsuperscript{22}. The results of this experiment could identify a potential consequence of the artificial diet used to rear honey bees in laboratory settings.

Because the specific enzyme involved in NMP detoxification in honey bees is not known, the transformation of 7-ethoxycoumarin (7-EC) to 7-hydroxycoumarin via 7-ethoxycoumarin-\textit{O}-deethylation in microsomal preparations was used to evaluate cytochrome P450 activity. This method, which involves the spectrofluorometric detection of the cytochrome P450 metabolite of 7-EC, umbelliferone, has been used to evaluate the activity of Cyp1, 2 and 3 families in
humans\textsuperscript{23}. The specific insect enzyme families involved in this transformation are not known, but this method has been used in arthropods including honey bees to successfully evaluate P450 enzyme activity\textsuperscript{24-26} and determine whether chemical exposure induces cytochrome P450 enzyme activity\textsuperscript{26}. However, the results of this experiment can be extrapolated beyond the scope of NMP detoxification. Currently, there is little information available regarding honeybee larvae and their ability to detoxify agrochemical xenobiotics. This assay has not been previously adapted for use on honey bee larvae, and these results will aid in understanding the detoxification capabilities of larvae versus other life stages by examining and comparing cytochrome P450 activity in adult and larval tissues.

**Methods**

**Chemicals**

HPLC grade methanol, 99.9% purity and formic acid, 98% purity for extraction and LC-MS analysis were purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical standards for NMP, MSI and 2-HMSI were purchased from Sigma Aldrich (St. Louis, MO, USA), and 5-HNMP was purchased from Toronto Research Chemicals (North York, ON, Canada). Supel\textsuperscript{TM}-Que PSA 2mL centrifuge tubes were purchased from Supelco Inc. (Bellefonte, PA). Potassium chloride, 99% purity, phenylmethylsulfonylfluoride (PMSF), soybean trypsin inhibitor, glycerol, 99% purity, Tris-hydrochloride, sodium phosphate, NADP, glucose-6-phosphate, magnesium chloride, glucose-6-phosphate dehydrogenase, 7-ethoxycoumarin, 99.5% purity and 7-hydroxycoumarin, 98% purity were purchased from Sigma Aldrich (St. Louis, MO, USA).
Honey Bees

Adult and larval honey bees were obtained from a single hive located in a Pennsylvania State University (University Park, PA) apiary. To avoid variation in the results of these experiments caused by seasonality, adults and larvae were obtained for their respective feeding assays within a single week.

Adult Feeding Assay

Adult nurse honey bees were obtained on June 27, 2016 from frames containing open brood and eggs. Using a low pressure aspirator, bees were transferred from a polypropylene mesh transfer cage into circular steel mesh cages assembled as described in Biddinger et al. 2013. Each cage held 20 bees each, and each experimental group consisted of 12 cages. After 2 hours, all bees were caged, and bees were permitted to feed *ad libitum* on a 50% aqueous sucrose solution (weight/vol.) containing either 200 ppm NMP (NMP-adults) or an equivalent amount of de-ionized water (Ctrl-adults). The bees were placed into 8/10 in. boxes in groups of four and kept in an incubator set at 34.5°C. To avoid trophylactic feeding, experimental groups were not mixed. Relative humidity was maintained at 75% by placing approximately 10 mL of a saturated NaCl solution in each box. Mortality was monitored daily, and dead bees were removed from the cages. Feeders were replaced every two days, and consumption was recorded by weighing the feeders before and after use. The experiment concluded on day 6, and the bees were either frozen for LC-MS analysis or prepared for cytochrome P450 activity measurement.
Larval Feeding Assay

Larvae were reared *in vitro* using the modified protocol from Schmehl *et al.* 2016 described in Chapter 2 and Appendix A Supplementary Methods. The queen bee was caged on an empty frame on July 2, 2015 and removed 24 hours later, having laid a sufficient amount of eggs. On July 6 (Day 0), 1st instar larvae were grafted from the frame into sterile queen cups containing artificial diet, and maintained at 34.5°C and 95% relative humidity in a Heratherm IMH750-S incubator (Thomas Scientific). Larvae were divided into two treatment groups consisting of 70 individuals each and kept in separate desiccators to prevent cross exposure from volatilizing NMP or metabolites. As in the adult feeding assays, experimental groups consisted of larvae chronically exposed to 200 ppm NMP in diet (NMP-larvae) and larvae exposed to an equivalent volume of de-ionized water (Ctrl-larvae). The experiment concluded on day 6, and the bees were either frozen for LC-MS analysis or prepared for cytochrome P450 activity measurement.

Hive Reared Larvae

After removing the queen from the frame used for larval rearing on July 2, she was placed on another empty frame and caged for 24 hours. She was released the following day, and the resulting larvae were reared by nurse bees in the hive until July 7th when they were removed and their tissues were prepared for cytochrome P450 enzyme activity measurement.

LC-MS Sample Preparation

Samples were pooled into groups of five adult or larval honey bees from the same cage or plate. Pooled samples were frozen with liquid nitrogen and homogenized using a mortar and
Metabolites and NMP in adult and larval matrices were quantified using analytical standards added in 10 µL methanol to 0.5 g homogenized adult or larval bodies to create matrix standards, and percent recovery was calculated by comparing matrix detected peak areas to solvent calibration peak areas. Methanol was added to the homogenates at a ratio of 1 g homogenate to 10 mL methanol. The mixture was vortexed, centrifuged at 3,000 rpm for 20 minutes, and 1 mL of the supernatant was added to a dispersive solid phase Supel™-Que PSA 2 mL centrifuge tube. The capsule was vortexed for 10 seconds and centrifuged at 12,000 rpm for 20 minutes, and the supernatant was analyzed via LC-MS. A Shimadzu LC-MS 2020 system with a 2.0 x 100 mm XR-ODS column (Shimadzu, Kyoto, Japan) was used for analysis of all samples. The analysis reported here is based on methods described in Cohen et al. 2007 with adaptations to suit the instrumentation and matrix specifications. The injection volume was 1 µL, and the quadrupole mass analyzer was operated using an electrospray ionization source in the positive mode with selective ion monitoring of 100 m/z (NMP), 116 m/z (5-HNMP), 114 m/z (MSI), and 130 m/z (2-HMSI). The binary mobile phase consisted of (A) water and (B) methanol buffered with 0.1% formic acid. Matrix and solvent calibrations ranged from 0 to 50 ppm in methanol (vol./vol. NMP/methanol, g/vol. other analytes/methanol).

Microsomal Preparations

Preparation of microsomal fractions of the fat bodies and midguts of adults and larvae and measurement of cytochrome P450 activity were performed according to Vidau et al. 2011 with minor modifications. Following the conclusion of the six day feeding study, all bees were anesthetized by chilling prior to tissue extraction. For adult preparations, 20 midguts were pooled for midgut samples and 10 abdomens (without the digestive tract, stinger, or venom sack) were pooled for fat body samples. For larval preparations, 10 midguts were pooled for midgut samples, and 10 of the remaining bodies were pooled for fat body samples. After dissections,
tissue samples were kept on ice before homogenization in 1 mL chilled extraction buffer (1% KCl, 50 mM Tris-HCl pH 7.6, 1mM EDTA, 1 mM PMSF, and 0.1 mg/mL soybean trypsin inhibitor). Homogenization was performed in a chilled glass tissue grinder with a polypropylene pestle. The homogenate was transferred to a 2 mL centrifuge tube and centrifuged at 12,000x g at 4°C for 20 minutes. The supernatant was transferred to a clean centrifuge tube and centrifuged at 20,000x g at 4°C for 8 hours. The resulting adult and larval microsomal pellets were resuspended in 200 and 50 µL of suspension buffer respectively (20% glycerol, 100nM sodium phosphate pH 7.4, 1 mM EDTA, and 1 mM PMSF) and frozen at -80°C until analysis.

7-Ethoxycoumarin-O-Deethylase Activity Measurement

10 µL of the suspended microsomal preparations were added to 90 µL of reaction buffer (100 nM Tris-HCl pH 7.4, 0.5 mM NADP+, 5 mM glucose 6-phosphate, 10 mM MgCl₂, 200 µM 7-ethoxycoumarin, and 1 U glucose-6-phosphate dehydrogenase). Following a 30 minute incubation period at 37°C while agitating, the reaction was quenched with 100 µL of 50/50 (vol./vol.) Tris base (pH 10) and acetonitrile and centrifuged at 3,000 rpm for 7 minutes to separate precipitated proteins. The formation of 7-hydroxycoumarin was measured using a SpectraMax® M5 plate reader to quantify fluorescence (λex 380 nm, λem 455 nm). A standard curve ranging from 0-560 nM umbelliferone in reaction buffer (without glucose-6-phosphate dehydrogenase) was used to quantify samples. Enzyme activity was normalized to protein concentration measured by the Bradford protein assay.

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**Statistical Analysis**

Statistical analyses of and quantification of NMP and its purported metabolites were performed with Microsoft® Excel 2016. The concentration of analytes in each sample was predicted based on a regression equation generated from matrix standards, and percent recovery was estimated by comparing matrix standard peak areas to solvent standard peak areas. Survival Analysis and Analysis of Variance (ANOVA) were performed using JMP® Version Pro 12. Log-rank significance was used to compare survivorship between treatment groups. ANOVA was used to identify treatment or tissue dependent differences in the metabolites produced or in enzyme activities. Tukey’s Honest Significant Difference tests (Tukey’s HSD) or Student’s T tests were used to identify significance between groups or tissues. Significance was evaluated using a 0.05 probability threshold.

**Results**

**Feeding Assays**

During both the adult and larval feeding assays, mortality was minimal. Adult and larval survivorship is depicted in Figure 4-3 and 4-4 respectively. There was no significant difference in the survivorship of NMP exposed individuals relative to control groups in both larvae and adults (Larvae: chi square-0.1444, df=1 log-rank significance: p=0.704, n=70; Adult: chi square-0.3835, df=1 log-rank significance: p=0.536, n=240). The differences in the masses of the feeders before and after use were used to estimate the average consumption of sucrose solution per adult bee. Consumption per cage every 2 days was divided by the number of bees in the cage minus bees that died during the period of measurement. The per bee consumption values estimated every 2 days were averaged, divided by 1.23 g/cm³ (the density of 50% sucrose
solution), and multiplied by 3 to account for the total duration of the experiment. Average consumption of diet per adult bee was estimated to be 0.15 mL. Based on this number, NMP consumption by individual NMP-adults was estimated to be 0.000030 mL or 0.3117 µmol. The possibility of treatment effects on consumption were estimated using a least squares regression model to predict the consumption of diet using the date of measurement and the treatment group. No significant differences were detected in the consumption of diet between NMP-Adults and Ctrl-Adults, but the date of measurement was negatively correlated with consumption of sucrose solution ($R^2_{adj}=0.27$, $F=22.32$, df=1, n=24; $p \leq 0.0001$).

Larvae were not sampled for analysis unless they had consumed all administered diet, therefore NMP consumption was assumed to be uniform between individuals. Based on the volume of the diet administered per larvae, total NMP consumption per larva was estimated to be 0.000032 mL or 0.3249 µmol.

**LC-MS Analysis of NMP and Metabolites of NMP**

Elution times and the percent recoveries for all analytes at varying concentrations are reported in Table 4-1. $R^2$ values of all calibration curves were above 0.96, and were therefore used to calculate the analyte concentration of samples. A signal to noise ratio of 3 was used to determine the limit of detection (LOD), and a signal to noise ratio of 6 and a linear response was used to evaluate and determine the limit of quantification (LOQ). System contamination was observed for NMP, therefore all positive detections below 1 ppm in solution were indistinguishable from a negative response. (See Figs. 4-5, 4-6, 4-7, and 4-8.)

The method showed reasonable recovery and reproducibility, particularly at NMP or metabolite concentrations at or above 10 ppm in solution. However, high percent recoveries
observed for several analytes may be due to the difficulties of reliably integrating broad peaks with matrix interferences (see Table 4-1).

Samples were run in triplicate and analyte concentrations were estimated based on matrix calibrations. Average detection for each sample was converted to the moles of analyte per individual by dividing the total moles of analyte by the number of bees in the pooled sample. The estimated consumption of NMP per adult or larva was used to estimate the percent of the total administered NMP represented by each analyte and the total percentage of the administered NMP recovered as the parent compound or the proposed metabolites (Table 4-2). The percentage of the administered NMP recovered as NMP was significantly higher in larvae than in adults (ANOVA, F=33.03, df=7, n=4; p≤0.0001, Student’s T-test, Fig. 4-9).

Of the metabolites examined here, the percentage of NMP recovered as MSI in both adults and larvae was significantly higher than other compounds (F=7.46, df=3; p=0.0028, n=3-8, Tukey HSD, Fig. 4-10). For this analysis, all detections below the LOQ were excluded. MSI was particularly difficult to detect, and matrix detections below 25 ppm (65% administered dose) were sometimes below the LOQ. Therefore, the samples with quantifiable MSI were assumed to reflect high but not abnormal levels of MSI.

**7-Ethoxycoumarin-O-Deethylation Activity**

Cytochrome P450 activity was compared between experimental groups and tissues by measuring 7-ethoxycoumarin-O-deethylation in microsomal preparations. No significant difference in activity was detected between hive reared larvae and *in vitro* reared larvae, and no increase in activity was observed in bees exposed to NMP versus Ctrl bees. Significantly higher cytochrome P450 activity was observed in the fat bodies of adult bees compared to the activity
observed in the fat bodies of larvae (F=12.4869, p=0.0002, DF=19, larvae: n=12, adults: n=8, Tukey HSD, Fig. 4-11).

Discussion

No significant mortality was observed in larvae or adults during the duration of the experiment. Adult survivorship is consistent with our previous results (Fig. 4-1) which have found a NOEL of 2,000 ppm for adults, and larval survivorship likely reflects the duration of the experiment. The survivorship of larvae reared under exposure to 100 ppm NMP described in chapter 3 did not begin to decline until after the six day feeding period.

The results of this study indicate that detoxification of NMP following ingestion by honey bees results in the production of metabolites observed in humans exposed to NMP. These metabolites are suggestive of a cytochrome P450 mediated pathway, and the high recoveries observed suggest that this is the primary detoxification pathway of NMP in honey bees. Furthermore, unlike in the mammalian taxa studied, MSI appears to be the dominant metabolite. In humans, 5-HNMP is the most abundant metabolite detected after NMP exposure followed by 2-HMSI. However, the usefulness of MSI as a biomarker may be hindered by the difficulty of detecting it at lower concentrations as observed in this study. However, more sophisticated instrumentation and different sample preparation may decrease the LOD to more acceptable levels.

In larvae, a significantly higher percentage of the administered NMP was recovered as untransformed NMP, which indicates that they are less able to detoxify the chemical relative to their nurse bee counter parts. However, larvae are smaller in size than adult bees for most of their development, and the obvious differences between adult and larval physiology make it difficult to
compare between the two groups. Therefore, cytochrome P450 activity normalized to protein concentration provides a more robust comparison between the tissues.

No difference was observed in cytochrome P450 activity between NMP exposed and Ctrl bees, and no difference was observed between larvae reared in vitro and larvae reared in the hive. However, this assay is designed to assess the activity of a broad range of cytochrome P450 isozymes, and increases in specific enzyme activity may not be represented here. Further work to identify specific enzymes involved in NMP detoxification and more precise measurement of targeted enzymes are needed to more accurately assess whether NMP induces cytochrome P450 enzyme expression. In humans, cytochrome P450 enzyme expression is regulated by nuclear receptors dependent mechanisms. Currently, the role of honey bee nuclear receptors in upregulating cytochrome P450 enzyme expression is unknown. However, specific cytochrome P450 enzymes upregulated by pollen and honey constituents have previously been identified in honey bees, therefore a more targeted approach such as reverse transcription RT-PCR could be used to more specifically address potential enzyme expression differences in artificially reared larvae.

The finding that larvae exhibit lower cytochrome P450 enzyme activity in fat body tissue relative to adults is highly significant, and may be the underlying cause of the differences in the susceptibility of larvae and adults to NMP and the higher percentage of untransformed NMP found in larvae. The role of the insect fat body in detoxification has been established in numerous taxa, and its high degree of contact with the hemolymph suggests a role in detoxifying xenobiotics found therein. Toxins introduced by contact exposure penetrate through the insect cuticle to the hemolymph, and would therefore be likely to be detoxified by enzymes of the fat body. In contrast, the cytochrome P450 enzymes of the midgut may be likely to detoxify xenobiotics introduced by ingestion. When considering the results of this study, it is important to consider pharmacological uses of NMP in enhancing drug penetration across
biological membranes\textsuperscript{6,37}. It is furthermore necessary to consider that unlike adult honey bees, larvae are immersed in diet as they ingest it. These results suggest that larval and adult honey bees exhibit similar cytochrome P450 enzyme activity in the midgut when larvae are 7 days old, but the lower activity observed in the larval fat body tissues may render them more susceptible to xenobiotics that are likely to penetrate the larval cuticle and enter the hemolymph.

Currently, the majority of required testing for pesticide products focuses on adult honey bees rather than larvae\textsuperscript{38}. Guidelines exist for testing effects of agrochemicals on larvae\textsuperscript{37}, and depending on the compound in question, these tests may be required or desirable before a product makes it to market. Information relating to the detoxification capabilities of larvae compared to adults could help to predict when these tests are warranted. As a penetrant, NMP is likely to move through insect cuticle and into the hemolymph, which this work suggests can lead to toxic effects in larvae at lower exposure levels than in adults. If oral exposure is a risk to larval honey bees, contact exposure should always be assumed, and it may be wise to consider this when assessing the effects of compounds with high contact toxicity. Furthermore, NMP enhances the penetration of other compounds across biological membranes\textsuperscript{6,37}, and if NMP is present in the diet, it may increase the penetration of other chemicals across the cuticle.

This work focuses on the activity of cytochrome P450 enzymes at a particular point in larval development, but the utility of exploring differences in detoxification enzyme activity across ages of honey bees is clear. It may be beneficial to further explore this by considering the activity of other enzymes involved in detoxification such as glutathione S-transferases and carboxylesterases\textsuperscript{15}. Similarly, comparing other age groups such as younger larvae or older worker bees may provide insight into age related toxic effects.

The finding that NMP is detoxified via a cytochrome P450 mediated pathway could be further validated by incubating NMP with honey bee microsomal preparations and measuring the metabolites generated. \textit{In vivo} inhibition of cytochrome P450 enzymes with piperonyl butoxide
(PBO) was attempted in our earlier experiments, but this approach is limited by the insolubility of PBO in aqueous diet. In the future, PBO could be used to directly inhibit cytochrome P450 enzymes in microsomal preparations to confirm their involvement in generating the observed metabolites. Enzyme inhibition using monoclonal antibodies as described in Ligocka et. al. 2003 or gene knock-down via RNA interference could identify which specific enzymes are involved in this pathway. Once identified, reverse transcription RT-PCR could be used to detect induction in the enzymes involved in NMP detoxification.

To further validate the proposal that the higher sensitivity of honey bee larvae to NMP is associated with the lower cytochrome P450 activity observed in larvae, distribution of NMP in adult and larval honey bees following exposure could be measured. If the concentration of NMP detected in the hemolymph were higher in larvae than adults, this would support the hypothesis that lower cytochrome P450 activity in the larval fat body is related to the observed sensitivity.

References


Figure 4-1: Adult survivorship during NMP chronic feeding study. NOEL=0.2% (0.5% NMP to Control (ctrl): chi-square=131.5, df=1, n=59; p≤0.0001).

Figure 4-2: Metabolites generated during cytochrome P450 metabolism of NMP in rats and humans.
Figure 4-3: Survivorship of adult bees chronically exposed to 200 ppm NMP.
Figure 4-4: Larval survivorship under chronic exposure to 200 ppm NMP in diet.

Figure 4-5: Chromatogram of 40 ppm NMP in methanol in adult matrix.
Figure 4-6: Chromatogram of 40 ppm 5-HNMP in methanol in adult matrix.

Figure 4-7: Chromatogram of 40 ppm MSI in methanol in adult matrix.
Figure 4-8: Chromatogram of 40 ppm 2-HMSI in methanol in adult matrix.

Figure 4-9: Mean percentage of NMP recovered as NMP in larvae vs. adults. Error bars represent standard error. Significance indicated by “*”, F=33.03, df=7, n=4; p<0.0001, Student’s T-test.
Figure 4-10: Percentage of administered NMP recovered as NMP or metabolites. Error bars represent standard error. Significance indicated by “*”, F=7.46, df=3; p=0.0028, n=3-8, Tukey HSD.
Figure 4-11: Tissue and life stage specific 7-ethoxycoumarin-\(O\)-deethylase activity. Letters indicate significant differences between groups.
Table 4-1: Average percent recoveries ± SD of NMP and its metabolites at varying concentrations in larval or adult matrices, and respective LC retention times.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>NMP-Adults</th>
<th>NMP Larvae</th>
<th>HNMP-Adults</th>
<th>HNMP-Larvae</th>
<th>MSI-Adult</th>
<th>MSI Larvae</th>
<th>HMSI Adult</th>
<th>HMSI Larvae</th>
<th>Retention Time (minutes)</th>
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</thead>
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<tr>
<td>1</td>
<td>-12.49</td>
<td>&lt;LOD</td>
<td>neg. 21.8+-</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>5.5-6.5</td>
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<tr>
<td>5</td>
<td>82.8+-/7.8</td>
<td>91.8+-/6.5</td>
<td>111.3+-/21.8</td>
<td>111.0+-/28.5</td>
<td>104.8</td>
<td>71.37</td>
<td>109.8+-/23.2</td>
<td>162.7</td>
<td>104.3+-/6.4</td>
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<tr>
<td>10</td>
<td>118.2+-/21.8</td>
<td>119.0+-/24.7</td>
<td>110.0+-/28.5</td>
<td>108.2+-/30.8</td>
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<td>25</td>
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<td>114.3+-/23.2</td>
<td>112.1+-/10.5</td>
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<td>93.8+-/18.1</td>
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<td>84.8+-/9.3</td>
<td>133.6</td>
<td>97.2+-/51.8</td>
</tr>
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</table>

Table 4-2: Percentage of total administered NMP recovered (%Total Dose Recovered), and percentages of administered NMP recovered as parent compound or metabolites in adult and larval samples. Percent recoveries were based on average recoveries from pooled samples.

Analyte concentrations below limit of quantification (LOQ) are considered positive detections, but are not represented in the total percentage of NMP recovered. Limit of quantification=LOQ.

<table>
<thead>
<tr>
<th></th>
<th>% NMP</th>
<th>% 5-HNMP</th>
<th>% MSI</th>
<th>%2-HMSI</th>
<th>% Total Dose Recovered</th>
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</thead>
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<tr>
<td>Adult #1</td>
<td>18.50</td>
<td>≤LOQ</td>
<td>70.03</td>
<td>≤LOQ</td>
<td>94.66</td>
</tr>
<tr>
<td>Adult #2</td>
<td>8.97</td>
<td>6.13</td>
<td>≤LOQ</td>
<td>≤LOQ</td>
<td>18.00</td>
</tr>
<tr>
<td>Adult #3</td>
<td>6.43</td>
<td>9.03</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
<td>15.13</td>
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<tr>
<td>Adult #4</td>
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<td>8.70</td>
<td>≤LOQ</td>
<td>≤LOQ</td>
<td>18.20</td>
</tr>
<tr>
<td>Larva #1</td>
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<td>27.15</td>
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<td>77.09</td>
</tr>
<tr>
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<td>10.27</td>
<td>108.08</td>
</tr>
<tr>
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<td>&lt;LOD</td>
<td>≤LOQ</td>
<td>12.51</td>
<td>64.51</td>
</tr>
<tr>
<td>Larva #4</td>
<td>36.19</td>
<td>&lt;LOD</td>
<td>≤LOQ</td>
<td>10.69</td>
<td>46.88</td>
</tr>
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</table>
Chapter 5
Conclusion

In light of recent declines in pollinator populations\textsuperscript{1,2}, it is important to fully evaluate the fate and effects of pesticides and their formulants when they are used in honey bee foraging environments. The research presented in this thesis demonstrates potential hazards to honey bee health associated with the use of two pesticide inerts, $N$-methyl-2-pyrrolidone (NMP) and an organosilicone surfactant adjuvant (OSS), and an organophosphate alternative, novaluron. The terminology used to describe these chemicals implies that they are completely without consequence to non-target organisms, or that they are a safer alternative to more conventional pesticides, however, the findings described in this thesis challenge these implications. An OSS Sylgard\textsuperscript{®} 309 was found to enhance the pathogenicity of a viral pathogen in honey bee larvae, novaluron was shown to interrupt brood cycles in colonies, and chronic NMP exposure can lead to larval mortality.

OSSs are widely used in agricultural settings as spray-tank adjuvants\textsuperscript{3,4}, however, California is one of the few states where records of pesticide product use are public due to mandatory reporting\textsuperscript{5}. Simultaneously, almond pollination in California is the largest pollination event in the country\textsuperscript{6}, and because of the high number of colonies transported to California by migratory beekeepers, any potential consequences of pesticide use in almonds could severely impact honey bee populations.

Here, chronic exposure to an OSS following a single infectious event was shown to synergistically enhance Black Queen Cell Virus (BQCV) associated mortality. Chronic, asymptomatic viral infections are extremely common in honey bee hives, and it is likely that a
developing larva will encounter viral pathogens through vertical or horizontal transmission\(^7\). During pollination events, infection frequency may increase. Honey bee viruses can be transmitted to naïve hosts through shared floral resources\(^8\), and monofloral diets, and low quality pollen may decrease immune function in honey bees\(^9,10\). Numerous hives foraging on one available food source could create a scenario where viruses will be transmitted from one hive to another through foragers. The subsequent transmission of viruses from foragers to nurse bees, larvae, queen and drones could result in a systemic infection worsened by limited nutritional resources\(^7,9,10\). The movement of chemicals through a hive is thought to follow a similar pattern\(^11\). Pesticide formulates like OSS will be encountered by foraging bees and spread through the hive in pollen and nectar and accumulating in wax\(^12-14\). The overlapping symptoms observed in hives following almond pollination and the work presented in Chapter 2 suggests that a phenomenon similar to the described scenario is occurring\(^15\). OSS residues have already been detected in honey bee colonies and in almond pollen, which implies that OSS’s are encountered by foraging bees during almond pollination, and the residues can persist in the hive environment\(^16,17\). Chemicals such as fungicides, insect growth regulators, and other adjuvants have also been suggested as possible causes of colony declines following almond populations\(^15\), but OSSs like Sylgard\(^\circledR\) 309 generally compose a greater proportion of the spray tank volume than fungicide or insecticide active ingredients\(^4\). However, information pertaining to their environmental fate and metabolism is limited. Fungicide residues, which are often among the most frequently detected chemicals in pollen and other hive matrices, have been detected well within the part per million range in pollen\(^18-20\). Therefore, it is likely that further investigation will reveal that OSS residues can persist in the hive at a concentration comparable to the dose used in this study.

Future work to determine the fate of OSS in the foraging and hive environments as well as identifying and quantifying the pathogens present and the expression levels of 18-wheeler in OSS exposed hives could determine if OSS exposure is related to the observed brood losses.
BQCV is one of the most common viruses detected in honeybee colonies used for almond pollination\textsuperscript{21}, therefore larval exposure to this pathogen is highly likely. However, identifying and quantifying the proportion of infected larvae in hives used for almond pollination could help researchers understand its role in colony declines.

The identification of 18-wheeler as an immune gene\textsuperscript{22,23} that is differentially expressed in response to OSS exposure in virus exposed larvae not only presents a potential mechanism for the observed synergistic interaction between OSS and viral pathogens, it may also lead to the description of an immune pathway that has not previously been explored in honey bees. The majority of research to identify immune pathways in honey bees has focused on adult insects, however, the unique physiology and lifestyle of many larval insects compared to their adult counterparts\textsuperscript{24} suggest that there may be developmentally related differences in expression and expression patterns of immune genes. Most research exploring age related immune function in insects has thus far focused on differences between younger and older adult insects\textsuperscript{25,26}, but comparative research on honey bee larvae and adults may aid in predicting and mitigating threats to honey bee health. More work is needed to determine the role of 18-wheeler in antiviral immunity in honey bee larvae, but these initial findings are promising evidence of its importance.

The finding that novaluron is highly toxic when administered directly to honey bee larvae is not surprising given its mode of action as a chitin synthesis inhibitor\textsuperscript{27}, however, the residual toxicity observed in nucleus colonies weeks after the initial exposure period is problematic. The observations made in these hives indicate that novaluron was being transferred from the administered pollen and sucrose solution to the royal jelly at high enough concentrations to affect the health of the larvae, egg viability, or both. The details of chitin synthesis inhibition caused by novaluron exposure are not well described, but inhibiting chitin synthesis in adult female insects can decrease egg viability\textsuperscript{28}. This may be related to the weakened structure of the embryonic cuticle, or the weakened structure of the egg itself\textsuperscript{29,30}. More targeted studies designed to assess
effects on queen egg laying and the structure and chitin composition of the eggs laid could determine how novaluron was influencing brood production.

The dose of novaluron administered to the experimental colonies was roughly five times higher than the concentration detected in apple pollen following applications to blooming flowers. However, it is not clear how much these concentrations were influenced by precipitation and the conservative application rates. Furthermore, the concentration in nectar was not assessed. Performing hive level assessments at lower concentrations and for shorter periods of time could determine the toxic dose range of novaluron and the amount of time needed for recovery. Additionally, field studies using hives placed directly in orchards treated with novaluron could provide a more realistic depiction of the hazards of novaluron use.

The classification of novaluron as an organophosphate alternative implies that its use presents a lower risk to non-target organisms like honey bees compared to conventional pesticides like organophosphates, and depending on the use pattern, this may be the case. Organophosphate insecticides are highly toxic to honey bees, and can potentially kill foraging bees before they return to the hive\textsuperscript{31}. However, the findings of Chapter 3 demonstrate that using novaluron according to label recommendations can expose bees to a chemical that disrupts brood production for weeks after the initial exposure period. Moreover, exposure to relatively high doses of novaluron is not likely to kill a foraging adult bee before it returns to the hive with contaminated food\textsuperscript{27}, increasing the likelihood that the brood and the queen will be exposed. No residues were detected in pollen following applications to closed buds, and therefore amending the Rimon\textsuperscript{®} 0.83EC label to exclude the bloom period from the recommended application timings would reduce confusion and greatly increase the safety of novaluron to honey bees.

The effects of NMP on \textit{in vitro} reared brood were less dramatic than those seen in previous experiments. This may be due to the increased sterility and environmental stability offered by the new rearing method. While the results reported here should be considered to be a
more accurate description of the isolated effects of NMP on brood, the mortality reported by Zhu et. al. should be considered a worst case scenario perhaps caused by different physiological traits or compounding stressors. The nucleus colony study described in Chapter 3 was not designed to identify small effects on brood success, and suggestions for developing a more conclusive study including more quantitative measurements of colony health and success have been made. However, residue testing of royal jelly and other hive matrices may provide a simpler way to predict the outcome of NMP exposure. Similarly, the conflicting results of field and growth chamber residue studies with NMP call for further testing of NMP to accurately quantify residues in pollen and nectar when it is applied as a formulant to different crops. The pilot study indicates that NMP can persist in pollen for days after the initial application, but the field residue studies suggest that this persistence is situationally dependent. Understanding the fate of chemicals in the hive and foraging environment is important when interpreting laboratory bioassay results such as these. Greenhouse experiments using a variety of plants as well as tissue specific testing to quantify NMP and potential metabolites could determine if and how NMP is transported throughout plant tissue.

Although NMP is considered to be a reproductive toxin in mammalian model systems, and this work demonstrates its toxicity to larvae, the mechanism of the observed toxicity in rats or honey bees is unknown. Most recently, NMP has been investigated as a potential anti-inflammatory drug to combat osteoporosis. It has been shown that NMP inhibits the nuclear factor kappa B signaling pathway by acting as an epigenetic modifier, though it does not appear to affect mammalian developmental pathways through this mechanism. As a penetration enhancer, NMP interacts with aromatic, planar compounds through π-π interactions. These interactions are important for many biological functions including stabilizing DNA and RNA structures, and tertiary protein structure folding, and the introduction of large amounts of NMP has the potential to affect these processes. Similarly, NMP increases cell membrane fluidity.
Membrane homeostasis can influence numerous cellular processes, including membrane signaling and diffusion, and changes in this property will result in unforeseen consequences\textsuperscript{44}.

The lower cytochrome P450 activity in larvae and higher percentage of untransformed NMP in larvae are the likely causes of the higher mortality seen in larvae exposed to NMP compared to adult honey bees in Chapter 4. In mammals, NMP has been shown to be more toxic than its metabolites, 5-hydroxy-\textit{N}-methyl-2-pyrrolidone (5-HNMP), \textit{N}-methylsuccinimide (MSI) and 2-hydroxy-\textit{N}-methyl succinimide (2-HMSI)\textsuperscript{45}. Although the toxicity of each metabolite to honey bees was not investigated here, the finding that NMP is metabolized less effectively in larvae than adults supports the conclusion that NMP is the primary cause of the observed symptoms and not a metabolite.

This work demonstrates the potential for NMP to negatively impact honey bee health, but the likelihood of honey bees encountering NMP at the concentration used in Chapter 3 is not adequately explored here. However, the finding that 7-ethoxycoumarin-\textit{O}-deethylation is lower in fat bodies and residual non-gut tissues of larvae relative to adults is highly significant. Historically, pesticide risk assessments for pollinators have relied heavily on contact toxicity assays performed on adults\textsuperscript{31,46}. The lower cytochrome P450 activity in the fat bodies of larvae may translate to an increased susceptibility to contact toxins. Because larvae are in contact with wax and diet, pesticide residues in these matrices may affect their survivorship\textsuperscript{47,48}. This highlights the need for required toxicity assessments to be performed on larvae rather than adults alone.

The EPA’s proposed amendments to risk assessment requirements for pollinators makes great efforts to ensure that pollinator health is inclusively evaluated before a pesticide can be registered for use\textsuperscript{47}. However, the research presented here highlights several areas that still require improvement. High volumes of pesticide inerts are used largely without monitoring or residue tolerances, and there is little to no available data supporting their safety\textsuperscript{4,48,49}. This work
adds to the growing body of research indicating that inert formulants can have measurable consequences to non-target organisms. Likewise, the decision to identify an insect growth regulator (IGR) as a reduced risk alternative to conventional pesticides should be based on both adult and larval toxicity data. These findings suggest that these reduced-risk insecticide labels and any associated exemptions be reevaluated and reconsidered, and that guidelines and limitations need to be imposed on the use of inert agrochemicals. Furthermore, if there is unpublished data that has been generated by industry to support the safety of inert or reduced risk chemicals, it should be made publicly available. This would aid in the scientific community’s evaluation of risk and would facilitate trust between academics and private industry.

The governing principle of toxicology dictates that it is the dose that makes the poison, but a simplistic view of this adage can lead to misconceptions and inappropriate regulatory decisions. This work highlights the importance of evaluating the impacts of xenobiotics on pollinator health at multiple levels by considering age-related differences in exposure and tolerance, interactions between multiple stressors, and the effects on whole colony dynamics. Recently, industry affiliates have endorsed the view that colony health may be protected by filtering effects occurring when honey bees encounter acutely toxic substances in the field and die before returning to the hive. They have further argued that residues that do enter the hive will not affect the brood or queen because they will be filtered from the royal jelly by nurse bees. These effects, they argue, are grounds for increased residue tolerances\textsuperscript{50,51}. However, this view is based on limited data and largely untested assumptions, and does not consider the nuances of honey bee biology and hive dynamics. The EPA has made significant progress in their attempts to rectify this oversight, but accurate understanding of the toxicological risks posed by pesticides and chemical formulants to pollinators will require cooperative efforts and transparency between researchers from all sectors.
References


15. Wardell, G. *Word from Wardell.* *Project Apis m.*


Appendix A

Chapter 2 Supplementary Methods, Figures, and Tables

Supplementary Methods

Honey Bee Larval Rearing

Hives used in this experiment were selected based on low prevalence of viruses in nurse bees (five from each of 5 hives, total N=25) taken within 2 weeks of the first grafting from each hive. All hives and the virus inoculum were negative for SBV. IAPV was detected in one individual in hive 1, BQCV was detected in one individual in a hive 2 split, and DWV was detected in 3 individuals in the hive 2 split. Based on the relatively low levels of the viruses of interest detected in this screening, these hives were deemed suitable for this work.

Larvae were reared according to a modified protocol described in Schmehl et al. 2016. Because a sterile environment is critical when performing the initial grafting and subsequent feedings, a sterile, temperature controlled cabinet was designed and assembled by Randall A. McCullough, Senior Research Aide in Noll Laboratories at Pennsylvania State University. The 91.5 cm wide, 61 cm tall and 61 cm deep frame was assembled using 80/20® Quick Tube material and covered in Frost King® shrink film window insulation. A Plexiglass sash with adjustable height served as the opening through which grafting and feeding manipulations were performed. The chamber was placed on an aluminum covered surface and was sterilized with ethanol and a bleach solution before and after each use. To ensure sterile air flow throughout the chamber, a commercial HEPA air filter was fitted to vacuum tubing used to generate positive air pressure in the cabinet. To control the heat of the filtered air, a temperature/solid state relay was
used to control the heating output of two 350 watt heating elements wired in parallel by referencing output air temperature. An image of the chamber is shown in Figure 2. Aside from the use of the sterile chamber, the protocol described in Schmehl et al. 2016 \(^1\) was followed.

Grafting commenced on June 1, 2015 and the final round was performed on July 5, 2015. Supplementary Figure 6 depicts the arrangements and relationships of the hives used in this study and the exact dates that each grafting was performed. Due to the unpredictable nature of honeybee hives as a model organism, splits with sister queens were used as replacement colonies in the event of queen death or absconding. Colonies 2 and 3 both experienced queen death during the course of the experiment, and a sister colony (split from the original colony with a daughter queen) was used to complete the remaining replicates. While we acknowledge that this is an imperfect replication, we believe this solution to be the most practical and effective. Total control of the genetic heritage of the brood used in these experiments is beyond the scope of this research and may not be of practical importance for beekeepers.

**Positive Control**

Because the toxicity of OSS to larvae was unknown and the mode of action has not been previously explored, we did not use a traditional positive pesticide control such as dimethoate. Instead, we administered a chronic dose of OSS at 100 ppm v/v to larvae in diet (OSS-100). Based on log-rank significance tests, the survivorship of OSS-100 (56% adult eclosion) was significantly lower than Ctrl or OSS (10 ppm), higher than OSS+V(10 ppm) but not significantly different than Ctrl+V (\(p\leq0.000, 0.002, 0.002, \text{ and } 0.947 \text{ respectively} \)).
RNA Extraction

Individual larvae were manually homogenized using a plastic pestle and RNA extraction was carried out using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s method. Homogenized larvae were vortexed with 0.5 mL Trizol and incubated for 3 minutes at room temperature before centrifugation. Precipitated RNA was purified using the RNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA). Samples were selected based on the purity of extraction (260 nm/280 nm absorbance ratios above 1.8). Ctrl samples were comprised of 3 larvae from hive 1, 2 from hive 2, and 4 from hive 3 for a total N=9. Ctrl+V samples were comprised of 3 larvae from hive 1, 3 from hive 2, and 4 from hive 3 for a total N=10. OSS samples were comprised of 3 larvae from hive 1, 3 from hive 2, and 4 from hive 3 for a total N=10. OSS+V samples were comprised of 3 larvae from hive 1, 3 from hive 2, and 3 from hive 3 for a total of N=9.

RT-PCR

CDNA was synthesized using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI) using the protocol described in Cox-Foster et al. 2007. Rt-PCR was performed using Sybr® Green Master Mix (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. For actin, IAPV, DWV, and SBV, the following thermal profile was used: 8 minutes at 94°C, 35 cycles of 1 minute at 94°C, 55 seconds at 51.5°C, and 1 minute 25 seconds at 72°C, and an extension step of 10 minute at 72°C. For BQCV, the thermal profile used was as follows: 8 minutes at 94°C, 38 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute 15 seconds at 72°C, and an extension step of 10 minute at 72°C.
qRT-PCR

QRT-PCR was performed using Power Sybr® Green Master Mix (Life Technologies, Carlsbad, CA) with a reaction volume of 20 µL composed of 10 µL master mix, 5 µL nuclease free water, 2 µL each of forward and reverse primers, and 1 µL cDNA template. The following thermal profile was used: 2 minutes at 50°C, 10 minutes at 95°C, 40 reps of 15 seconds at 95°C and 1 minute at 60°C, and a dissociation step of 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. The temperature at which each product dissociated was used to ensure that the correct product was amplifying. To verify the novel primer set used to quantify the expression of 18-wheeler, a new reverse primer was used to generate a longer product containing the original product used for q-PCR. Following gel purification, the product of this primer set was validated by Sanger sequencing of the subcloned purified product (pGEM®T Vector System, Promega Corporation, Madison, WI). All PCR primers are listed in Supplementary Table 1.

Data Analysis

All expression and virus data were normalized to actin, an established and reliable endogenous control, and expression was calculated according to the formula $2^{-\Delta Ct}$. The $\Delta Ct$ was calculated by subtracting the Ct (or cycle time at which a fluorescent signal is amplified above a threshold) of actin from the Ct of the gene or virus of interest. The $\Delta Ct$ method was used instead of the $\Delta \Delta Ct$ method, which compares the $\Delta Ct$ of a treatment group to a control group, in order to facilitate comparisons of individual data points. The number of samples used in each group are as follows: Ctrl n=10, OSS n=11, Ctrl+V n=11, OSS+V n=12.
Absolute quantification of IAPV in the virus inoculum was performed using Microsoft Excel 2010®. The calibration curve used is shown in Supplementary Figure 1. Statistical analysis was accomplished using JMP Pro 12® (SAS Institute, Cary, NC).

Regression analysis was performed to determine whether seasonality affected the outcome of the experiment, using day of grafting to predict day of death. No significant relationship was found (n=168, $R^2\text{adj.}=0.000215$, df=682, $p<0.2846$). To identify hive effects, a least squares regression model was fit to each gene or virus of interest in response to hive source. IAPV and BQCV titers and 18-wheeler expression were significantly predicted by hive (least squares regression, hive 1 and 2: n=12, hive 3: n=15; IAPV: $R^2\text{adj.}=0.290$, df=38, $p<0.0008$; BQCV: $R^2\text{adj.}=0.113$, df=38, $p<0.0433$; 18-wheeler: $R^2\text{adj.}=0.298$, df=38, $p<0.0006$).

According to a post hoc Tukey’s HSD test, the highest titers of IAPV and the highest expression of 18-wheeler were observed in hive 2. Titers of BQCV were found to be significantly higher in hive 2 compared to hive 1, and titers in hive three were intermediate according to a Tukey’s HSD test. When hive was used to predict the day of death of the larvae using a standard least squares model, individuals in hives 2 and 3 were found to die significantly sooner than individuals in hive 1. This data demonstrated that viral titers and mortality varied between individuals from different hives in a similar manner. The increased expression of 18-wheeler and the higher IAPV titers in hive 2 suggests that this gene is an important player in defense against these viruses.

References

4. Shen, M., Cui, L., Ostiguy, N. & Cox-Foster, D. L. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood


Supplementary Figure 1. Absolute quantification curve for Israeli Acute Paralysis Virus (IAPV), used to determine the IAPV in viral inoculum. Ct = cycle time, Log concentration = log of copy number in q-PCR reaction.

Supplementary Figure 2: An image of the sterile chamber used for grafting, feeding and mortality assessments.
Supplementary Figure 3. Mean day of pupation after hatching for larvae following treatment (Control (Ctrl), OSS treated larvae (OSS), Added viral exposure (Ctrl+V), and OSS treatment/Added viral exposure (OSS+V)). (Least squares regression, p≤0.0001, df=3. Tukey HSD significance indicated by letters.)
Supplementary Figure 4. Average relative titers for Black Queen Cell Virus (BQCV), Israeli Acute Paralysis Virus (IAPV), Deformed Wing Virus (DWV), and Sacbrood Virus (SBV) for all treatment groups (Control (Ctrl), OSS treated larvae (OSS), Added viral exposure (Ctrl+V), and OSS treatment/Added viral exposure (OSS+V)). Viral titers in each bee were normalized to beta-actin levels in that bee. (Significance tested using least squares regression, n=10-12. Model using BQCV: R^2 adj.=0.63, df=7, p<0.0001). Tukey HSD significance indicated by “*”. 
Supplementary Figure 5. Relative expression of immune genes in day 6 larvae following treatment (Control (Ctrl), OSS treated larvae (OSS), Added viral exposure (Ctrl+V), and OSS treatment/Added viral exposure (OSS+V)). Tukey HSD significance indicated by “*”.

Supplementary Figure 6. Arrangement of hives and dates of grafting used in this study.
Supplementary Table 1: All PCR primers used in this study.

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Appendix B
Chapter 3 Supplementary Figures and Tables

Supplementary Figure 1: Mortality of larvae during NMP dose range finding larval rearing experiment in October 2013.
Supplementary Figure 2: Example of NMP matrix calibration in pollen. $R^2=0.99$. 
Supplementary Figure 3: Example of novaluron matrix calibration in pollen. $R^2=0.98$.

Supplementary Table 1: Summary of all residue detections in Chapter 3. LOD=limit of detection, LOQ=limit of quantification, NA=not applicable.

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<tr>
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<td>NA</td>
</tr>
<tr>
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<td>&lt;LOD</td>
<td>NA</td>
</tr>
<tr>
<td>2015 Field Study-24 hr buds</td>
<td>Ctrl Bud</td>
<td>NMP</td>
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<td>&lt;LOD</td>
<td>NA</td>
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<tr>
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<td>Ctrl Bud</td>
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<td>NA</td>
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<td>4.07+/-0.69</td>
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<td>3.46+/-0.49</td>
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Supplementary Table 2: Average percent recoveries and R$^2$ for matrix calibrations used in Chapter 3.

<table>
<thead>
<tr>
<th>Study</th>
<th>Analyte</th>
<th>Conc. (ppb)</th>
<th>Average % Recovery +/- std dev</th>
<th>Model R$^2$</th>
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<td>137.9 +/- 7.3</td>
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<tr>
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<td>128.8 +/- 15.1</td>
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<td>Novaluron</td>
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<td>111.9 +/- 7.6</td>
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<td></td>
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</tbody>
</table>
VITA

Julia Diane Fine

Education

- PhD in Entomology, 2017
  Pennsylvania State University
  Dissertation: The Toxicodynamics of “Inert” and “Alternative” Agrochemicals and Their Impacts on Honey Bees
  Adviser: Dr. Christopher A. Mullin

- B.S. in Biology, 2010
  The University of Akron

Professional Experience

- Senior Spectroscopy Technician, PolyOne, Polymer Diagnostic Incorporated - 2012
- Laboratory Technician, Lubrizol Measurement Sciences - 2008-2012

Publications

- Fine, JD, Cox-Foster, DC, Mullin, CA (2016) An Inert Pesticide Adjuvant Synergizes Viral Pathogenicity and Mortality in Honey Bee Larvae. Scientific Reports accepted.

Awards

- College of Agricultural Sciences Graduate Student Competitive Grant-$2,500, 2015
- ACS AGRO Division 2015 Educational Award, 2015
- Yendol Award, 2015
- Langstroth Fellowship, 2013
- Sigma Xi Grant in Aid of Research -$500.00, 2013
- Graham Endowed Fellowship, 2012