LETHAL AND SUBLETHAL EFFECTS OF IMIDACLOPRID AND AMITRAZ ON *Apis mellifera* LINNAEUS (HYMENOPTERA: APIDAE) LARVAE AND PUPAE

By

PATRICIA L. TOTH

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2009

To my parents

ACKNOWLEDGMENTS

I thank my advisor, Dr. Jamie Ellis, and the other members of my supervisory committee, Dr. Mike Scharf and Dr. Glenn Hall, for mentoring me. I thank my parents for all of their moral support. I thank Hannah O'Malley, Mike O'Malley, Meredith Cenzer, Renee Cole, and Sparky Vilsaint for technical support. Thanks go to the graduate students in the Honey Bee Research and Extension Laboratory for all of their help, support, and humor: Jason Graham, Eddie Atkinson, and Anthony Vaudo. Thanks go to Debbie Hall for all of her assistance throughout my entire program. I thank those at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry, especially Jerry Hayes and Tom Dowda, for their generous support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
NOED	No observable effect dose. The highest xenobiotic dose administered that does not produce an adverse affect on that organism.
Sublethal	A xenobiotic administered at a particular dose that does not kill a significant proportion of the experimental population.
Subchronic	A technique of administering a xenobiotic for 2 or more consecutive days, but not for the entire life of the test organism. This differs from chronic application, where a xenobiotic is applied to the organism throughout the organism's life.
Xenobiotic	A chemical found in an organism that is considered foreign and harmful to that organism.

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Patricia L. Toth

May 2009

Chair: James D. Ellis
Major: Entomology and Nematology

The population of feral and managed honey bee colonies in the United States has declined steadily since the mid 1950's. These declines have been attributed to a number of causes including, but not limited to, pesticide exposure, parasite vectored pathogens, arthropod bee pests, and various stressors. Investigators agree that bee declines more likely are due to a mixture of two or more of these causes, but few investigations have been conducted on interactions between these factors. Researchers have focused on testing the effects of various xenobiotics on honey bee workers, queens, and drones. Effects of these xenobiotics on honey bee brood have not been well-investigated. Herein, I fed bee larvae with various concentrations of imidacloprid and amitraz in an attempt to document lethal and sublethal effects of these chemicals on bee brood. Results indicated that larvae fed imidacloprid at 5, 10, 40, and 80 ppb or amitraz at 25, 50, 100, 200, and 400 ppb were significantly less likely to survive to adulthood. Larvae fed 25 and 400 ppb of amitraz were less likely to survive to the prepupal defecation stage. In general, treated bee mortality was greater during pupal development rather than during larval instar stages. I found no effects of imidacloprid or amitraz on larval time to defecation, larval weight at defecation, time to adult emergence, adult bee weight, or adult bee head weight. As a result of this investigation, several methods are discussed concerning topical treatment vs. oral

applications of imidacloprid and amitraz to developing larvae. In an attempt to better understand interactions among stressors, I wanted to develop a method for testing the susceptibility after pesticide exposure of treated larvae to depredation caused by the ectoparasite *Varroa destructor* (varroa mite). Results presented in this thesis provide important insights into the sublethal effects of chemicals on developing honey bee brood. This understanding ultimately can lead to better honey bee management and agricultural practices important for sustainable apiculture.

CHAPTER 1
OVERVIEW OF CURRENT HONEY BEE ASSOCIATED PROBLEMS

Economic Importance of Honey Bees

The European honey bee, *Apis mellifera* L., is an economically important insect not only for its production of honey and beeswax, but also because of its role as a pollinator. Pollination is important for the production of agricultural commodities such as food (grain, fruits, nuts, vegetables or domesticated animals), fuel, and drugs (Berenbaum et al. 2007). Honey bees are good pollinators because they are generalists, pollinating a wide variety of agricultural crops (Table 1-1) and native plants over a large foraging area throughout the year (Proctor et al. 1996, Delaplane and Mayer 2000). Additionally, pollination by honey bees often results in an increase in quantity and quality of crop yield (Delaplane and Mayer 2000).

The production of important crops that rely on honey bee pollination has increased steadily in recent years in response to increased human demand for these crops (National Agricultural Statistics Service (NASS) 1976-2008). This increased demand for food due to the exponential growth in the population of humans has increased the amount of managed honey bee colonies needed for crop pollination (Morse and Calderone 2000). In 2007, there were an estimated 2.44 million honey bee colonies in the U.S.[1] (NASS 1976-2008). Southwick and Southwick (1992) estimated the annual benefit of honey bee pollination to agriculture at between 1.6 and 8.3 billion USD. In 2000, the value attributed to honey bee crop pollination in the United States alone was estimated at 14.6 billion USD, a 57% increase from 1992 (Morse and Calderone 2000). The most recent study suggesting the benefits of insect pollinators in general on a worldwide scale estimated their value at 217 billion USD (Gallai et al.).

[1] Beekeepers with fewer than 5 colonies or colonies that did not produce honey were not counted in this survey.

Current Declines in Pollinator Populations

Pollination is important for plant reproduction and for maintaining plant genetic diversity (Delaplane and Mayer 2000). Due to the importance of pollinators, many researchers have investigated pollinator population dynamics. Native pollinators such as birds, bats, beetles, flies, and solitary bees have decreased in abundance for reasons including habitat fragmentation/destruction (Stephen 1955, Aizen and Feinsinger 1994) and the introduction of pesticides and parasites (Kevan 1975, Berenbaum et al. 2007). Monitoring programs led by the U.S. Department of Agriculture's National Agricultural Statistics Service (NASS) have documented the decline in managed honey bee colonies since 1947, making them the most significant example of pollinator decline in North America (Berenbaum et al. 2007, NASS 1976-2008) (Figure 1-1).

Factors believed to contribute to the decline of managed honey bee populations include the introduction of parasitic mites, mite resistance to acaricides, introduction of Africanized bees, pathogens, effects of pathogen-targeted antibiotics, pesticides, and many others (Johansen and Mayer 1990, Morse and Flottum 1997, Wilson et al. 1997). A sharp decline in managed colonies occurring from 1984-1996 corresponds to the introduction of two parasitic mite species, the tracheal mite, *Acarapis woodi* (Rennie), and the varroa mite, *Varroa destructor* Anderson and Trueman (DeJong 1997, Wilson et al. 1997). There are no established reasons for the managed bee declines occurring from 1947-1972. However, this decline corresponds with widespread, commercial use of first generation neurotoxic insecticides such as carbaryl, parathion, malathion, and diazinon (Johansen and Mayer 1990), among others.

Reasons for the most recent decline (from 2005 until the present) of managed honey bee colonies, referred to as "colony collapse disorder" or CCD, remain unknown. Beekeepers estimate the total colony losses due to CCD to be somewhere between 35% and 100% (Eccleston

2007, Stokstad 2007). Although the cause of CCD remains under investigation, researchers typically suggest the following, among others, as candidates: (1) pathogens (known or undiscovered), (2) pesticide use (both inside and outside of colonies), (3) stress (such as colony migration/transportation, overcrowding, and poor nutrition), (4) climate change, (5) lack of genetic diversity, or (6) poor nutrition (Eccleston 2007, Ellis 2007, Johnson 2007, Embrey 2008). Of particular interest to me is how pesticide use might affect immature honey bee development.

Pesticides used to suppress pest insect populations can affect non-target/beneficial insects, including pollinators. The yearly estimated cost of pollination losses due to pesticide exposure is $210 million USD (Pimentel 2005). To limit toxicity of pesticides to pollinators, regulations have been encouraged to avoid certain pesticide use during crop bloom or where bees are known to forage (Morse and Flottum 1997).

Additional measures were taken to protect pollinators by passing of the Federal Insecticide, Fungicide, and Rodenticide Act, which requires registered pesticides to be tested to determine the lethal dose (LD50) or concentration (LC50) on non-target insects (Desneux et al. 2007). However, legislation typically fails to consider sublethal effects that could be delayed, indirect, difficult to detect/quantify, or easily overlooked (Desneux et al. 2007). Parameters that have been shown to be influenced by sublethal doses of pesticides include developmental rate, metabolic activity, adult longevity, immunity, fecundity, and behavior (Desneux et al. 2007).

To date, investigations measuring sublethal effects of pesticides on honey bees have been quantified either biochemically or behaviorally. Bioassays performed using the proboscis extension response (PER) (Bitterman et al. 1983) typically are used to study sublethal effects of pesticides on honey bee behavior. Using this method, fipronil, deltamethrin, endosulfan, and prochloraz have been shown to decrease learning performance in bees (Decourtye et al. 2005).

Additional sublethal effects after treatment of adult bees with deltamethrin (700 ng/bee) include a decrease in food consumption, irregular spaced pupae in the comb, and a decrease in forager activity (Decourtye et al. 2004).

Investigations quantifying the effects of pesticides on bee brood are scarce, most likely because of difficulties associated with rearing larvae *in vitro* and/or difficulties controlling variability in field colonies. One investigation in which researchers were able to successfully rear bee larvae *in vitro* concluded that those larvae treated with dimethoate and carbofuran developed faster than those not treated, yet they failed to spin silk, suggesting that the larvae would not pupate (Davis et al. 2000).

It is important to focus on the sublethal effects of pesticides on bees, because bees are more likely to be exposed to low doses of pesticides from the environment rather than higher, acute doses. Sublethal doses of pesticides can be acquired by honey bees in the field as they forage for nectar, pollen (Chauzat et al. 2006), and perhaps water (Wauchope 1978). The theory of "flower constancy" states that honey bees are more likely to forage on the same species of flowers on which they had foraged in the past (Free 1963). Therefore, bees foraging on pesticide-treated plants will continue to be exposed to those plants over time.

Pesticides can contaminate nectar and pollen when directly sprayed on or near flowers frequented by bees, or when plants themselves are treated with systemic pesticides. Systemic pesticides are absorbed by a plant and then transported throughout the plant's vascular system. They are especially useful against sucking pests (Bennett 1957, Sicbaldi et al. 1997). Chemical residues from systemic pesticides are found not only on treated plants but also on neighboring vegetation due to pesticide drift even when non-honey bee pollinated crops are treated. For example, investigators found that fields sown with imidacloprid-dipped seeds resulted in residue

on bordering grass and flowers up to 4 days after sowing. Contamination occurs during the sowing process when imidacloprid residues escape through the fan drain of the planting drill (Greatti et al. 2003, Greatti et al. 2006).

Additional problems occur with pesticides that have long residual times. Residual time is the amount of time a pesticide can be found on a treated surface before it degrades. Residual times have been measured for a variety of pesticides and can last from 2 hours to 7 days (Johansen and Mayer 1990, Wang et al. 2008). Pesticide applicators who do not follow the label instructions and spray blooming crops risk an additional exposure to honey bee colonies.

Once honey bee foragers contact xenobiotic contaminants in the environment, they have the potential to return the material to their hive. Once in the hive, it is distributed to nurse bees and either stored or distributed to larvae as food (Johansen and Mayer 1990). For example, the highest levels of two pesticides, carbofuran and dimethoate, have been found in the adult honey bee crop, but smaller concentrations were also present in the hypopharyngeal gland (important in the production of worker and royal jelly) (Davis and Shuel 1988). The contents of the crop are shared between forager and nurse bees, facilitating the transfer of contaminated substances throughout the hive (Davis 1989). Once pesticides have been introduced into the hive they can be stored and deposited in a number of potential products such as honey, pollen, and beeswax. For example, chemicals that are hydrophilic (water soluble-such as systemic pesticides) are more likely to be dissolved in honey (Wallner 1999).

One such systemic pesticide is imidacloprid, a nicotinoid. It was chosen for testing here because of its mode of action, likelihood to be found in flowers due to its systemic properties, and its worldwide popularity. There are 584 registered products in the United States containing imidacloprid as an active ingredient, and these products are used on a variety of crops (Table 1-

2), structures, and landscapes for insect pest control (Kegley et al. 2008). Imidacloprid is applied primarily as a seed dressing or soil treatment but can be applied as a foliar treatment. Currently, imidacloprid has been banned in France where it was thought to have caused massive bee declines (Stokstad 2007). To date, it is registered for use in Cameroon, Madagascar, South Africa, Tanzania, Australia, India, New Zealand, the Philippines, Denmark, Finland, Germany, Hungary, Netherlands, Portugal, United Kingdom, Canada, and the United States (Kegley et al. 2008).

Imidacloprid functions by targeting the nicotinic acetylcholine receptor (nAChR) and acts as an agonist, resulting in an influx of sodium ions leading to neuroexcitation (Yu 2008). However, long term effects of imidacloprid on insects include primarily neuroinhibition (Nauen et al. 2001, Scharf 2003). Imidacloprid is considered highly toxic to honey bees regardless of bee race (Nauen et al. 2001). Using oral toxicity studies on adult bees, investigators have shown 17-50% mortality at doses greater than 3.1 ng AI (active ingredient) per bee and a contact LD50 of 42 to 104 ng AI per bee (Nauen et al. 2001). Even after imidacloprid is metabolized, its metabolites, such as olefin and 5-hydroxyimidacloprid, have toxic effects on honey bees comparable to those of the parent compound (Suchail et al. 2001).

Imidacloprid should be considered for possible sublethal effects on beneficial insects due to its persistence in plants long after initial application. Out of eleven different pesticides tested, imidacloprid had the longest residual time with 80% mortality for a parasitic wasp 7 days after treatment (Wang et al. 2008). Applications to citrus trees resulted in xylem fluid containing imidacloprid concentrations of 10g/L at 24 weeks post treatment (Castle et al. 2005). A soil half-life, which is the time it takes for half of the applied amount of a pesticide to degrade, is estimated at 8-48 days (Yu 2008). However, there was a mean estimate of 6 µg of imidacloprid

per kg of soil 1 to 2 years after soils were planted with maize, wheat, sunflower, or rape seeds dressed with imidacloprid (Bonmatin et al. 2003). Imidacloprid has been detected in sunflowers and corn at 1-11 μg of imidacloprid/kg of pollen (1-11 ppb) and 1-3 μg/kg (1-3 ppb), respectively (Bonmatin et al. 2003). Concentrations of imidacloprid found in pollen samples collected from honey bee colonies were reported to be between 2-6 μg/kg (2-6 ppb; Chauzat et al. 2006).

Acute sublethal doses of imidacloprid (100, 500, and 1000 ppb) have been found to disorient foragers and prevent/delay them from returning to the hive (Bortolotti et al. 2003). Bees treated with 100 and 500 ppb of imidacloprid experienced decreased mobility for up to one hour after a single dose (Medrzycki et al. 2003). Decourtye et al. (2004) reported that imidacloprid levels as low as 10 ng/bee resulted in decreased food consumption by foragers, decreased forager activity, reduced brood area, and reduced honey and pollen stores. These researchers also recorded irregular capping of brood cells by worker bees treated with imidacloprid.

In addition to being exposed to pesticides in agricultural and other settings, honey bees can be exposed to pesticides when beekeepers deliberately administer chemical treatments in colonies to control any number of hive pests. The primary example concerns beekeeper use of acaricides for control of the ecotoparasite *Varroa destructor* Anderson and Trueman (varroa). Treatment for varroa is important to beekeepers because varroa are considered the number one cause of honey bee colony deaths worldwide (DeJong 1997). The varroa's natural host is the Asian honey bee, *Apis cerana* Fabr, but the importation of managed *A. mellifera* colonies into Asia resulted in a host switch by varroa (DeJong 1997); varroa now successfully parasitizes *A. mellifera*. Unlike for *A. mellifera*, varroa are not lethal to *A. cerana* colonies due to bee cannibalism and removal of infested brood (Rath and Drescher 1990, Rath 1999), encapsulation

of infested brood (Koeniger 1987), grooming behavior (Rath 1999), and colony level absconding (Woyke 1976) in *A. cerana* colonies.

Varroa infestations cause colony death in *A. mellifera*, but the exact mechanism eliciting collapse is unknown (DeJong 1997). Varroa survive by feeding on adult and pupal hemolymph and reproducing inside capped brood. Even though varroa are large parasites (1.1 mm long) in relation to the size of their host, they seem to cause little damage to the bee exoskeleton when feeding (DeJong 1997). However, colony fitness could be limited due to reduced weight gain in worker (De Jong 1982) and drone pupae (Duay et al. 2003). Additionally, it is possible that varroa transmit honey bee viruses such as Kasmir bee virus (Shen et al. 2005) and deformed wing virus (Bowen-Walker et al. 1999), among others. Bee parasitization by the mite has been shown to lower the honey bee immune response by down-regulating immune related genes in moderately mite-infested bees (Gregory 2005) and possibly increasing the replication of varroa transmitted viruses (Yang and Cox-Foster 2005) resulting in the death of the colony. Varroa control in bee colonies is attempted using a limited number of different types of acaricides that typically are applied inside managed bee colonies.

Many acaricides are lipophilic and are distributed easily throughout the hive because they adhere to the cuticular wax layer of the honey bee exoskeleton. Their lipophilic properties make acaricides more likely to accumulate in beeswax as is the case for fluvalinate (Wallner 1999). Despite this, acaricides have been found in honey, as has been shown for coumaphos, an organophosphate (Rial-Otero et al. 2007). Pettis et al. (2004) investigated the sublethal effects of coumaphos on bees. They found that female bee larvae grafted into queen rearing cells constructed of coumaphos-impregnated beeswax were rejected by nurse bees resulting in 93% larval mortality. Of the surviving queens, those reared in coumaphos treated wax weighed less

than queens reared in coumaphos free wax (Pettis et al. 2004). The number of commercially available products for use against varroa is limited. Hence, varroa quickly developed resistance to most of the acaricides used against it in the U.S. (Oldroyd 2007).

Amitraz [1, 3-di-(2, 4-dimethylphenylimino)-2-methyl-2-azapropane], a formamidine insecticide, is a chemical used widely by beekeepers in the U.S. and Europe as an acaricide. I chose it for inclusion in this study because of the lack of research on its effects on honey bees. The lack of information may be because amitraz is not registered currently for use against varroa in the U.S. Unlike other acaricides, amitraz is shown to be unstable in honey and in beeswax, but instead it is broken down quickly into its metabolites (Lodesani et al. 1992, Martel et al. 2007, Rial-Otero et al. 2007). Within days, amitraz applied to plates containing beeswax was quickly degraded into its metabolites, one of which is 2-4-dimethyl-aniline (Wallner 1999).

Currently, there are 8 registered products containing amitraz in the U.S., which mainly are intended for use to control ticks, but can be used on organophosphate or carbamate-resistant insects such as the pea psylla and cotton bollworm (Kegley et al. 2008, Yu 2008). Amitraz is registered as an acaricide in most of Europe, but information is lacking on how it may affect honey bees negatively.

Concerning its *modus operandi*, amitraz acts on octopamine receptors as an agonist and can cause a varied degree of behavioral effects including reduced feeding and neurological effects such as neural excitation/inhibition (Matsumura 1975, Yu 2008). The half-life of amitraz in soil is approximately 2 days, which is short compared to other pesticides such as DDT (3-10 yrs), fipronil (18-308 days), and imidacloprid (8-48 days) (Yu 2008).

Compared to flumethrin and fluvalinate (both are acaricides), amitraz—applied topically—is considered to be moderately toxic to adult honey bees with an LD50 of 2.55 µg amitraz/mL of

acetone (255 ppb, Santiago et al. 2000). Santiago et al. (2000) described amitraz as being twelve times more potent as an acaricide (LD50: 1.7 pg of amitraz per mite) than insecticide (LD50: 2.55 µg of amitraz per bee) since varroa could be killed at much lower doses relative to bees. However, Santiago and colleagues did not investigate the potential sublethal effects of amitraz on adult bees or bee brood. There is a minimal amount of research that has been performed on toxicity and sublethal effects of amitraz on honey bee colonies, but such research is needed. Because amitraz is perceived by the beekeeping industry in the U.S. to be "safe" for bees, many beekeepers have misused it and/or overdosed their colonies. Consequently, it is vital that the effects of amitraz on bees be identified.

Introduction to Research

The purpose of this study was to investigate how sublethal doses of imidacloprid and amitraz affect immature bee development. Developing bee larvae were treated with various concentrations of one of the two test pesticides and then measured to quantify lethal and sublethal effects on larvae and puape (Chapter 2). I first had to ensure that my pesticides were not acutely lethal to bees by comparing treated and untreated larvae for the number of larvae that defecated, pupal survival (the number of individuals that survived pupal development of those that defecated as larvae), and adult bee emergence (total number of bees to eclose from those that were grafted as larvae). To measure sublethal effects, I looked for differences between treated and untreated larvae with regard to time to larval defecation (measured from egg hatch to defecation), larval weight at defecation, time to adult emergence (measured from egg hatch to adult bee emergence), adult bee weight, and adult bee head weight.

I also compared topical vs. oral administration of imidacloprid to bee larvae as well as attempted to develop a method for testing the susceptibility of treated larvae to depredation caused by varroa. My research methods for topically applying pesticides and my reasons for

choosing to administer the xenobiotic orally rather than topically are described in Appendices A and B. I introduced pesticide treated larvae into varroa infested colonies to determine the invasion rate of mites into cells containing pesticide-treated larvae (Appendix C). Results from my research suggest possible ways that certain pesticides could be affecting beneficial insects negatively without having direct effects (immediate, increased mortality; Chapter 3). This new understanding can lead to better management practices important for sustainable apiculture.

Table 1-1. Crops known to be pollinated by honey bees

Agave, alfalfa, allspice, almond, apple, asparagus, avocado, lima bean, fava bean, beet, blueberry, Brussels sprout, cabbage, canola, cantaloupe, carrot, cherry, clove, clover, cotton, cranberry, cucumber, guava, kiwifruit, lettuce, macadamia, onion, passion fruit, peach, pear, plum, raspberry, soybean, squash, strawberry, sunflower, tomato[a], watermelon

Proctor et al. 1996, Caron 1999, Delaplane and Mayer 2000, Caron 2001, Berenbaum et al. 2007

[a] limited to greenhouse production

Table 1-2. Crops most likely to be treated with imidacloprid in California in 2006

Apples, artichokes, bell peppers, blueberries, bok choy, broccoli, broccoli raab, brussel sprouts, cabbage, cantaloupe, cauliflower, celery, chicory, Chinese cabbage, cilantro, citrus, cotton, cucumbers, endive, grapefruit, grapes, head lettuce, kale, leaf lettuce, lemons, melons, oranges, pears, pecans, pomegranates, potatoes, pumpkins, spinach, strawberries, succulent beans, sugarbeets, tangelos, tangerines, tomatoes, and watermelons

Kegley et al. 2008

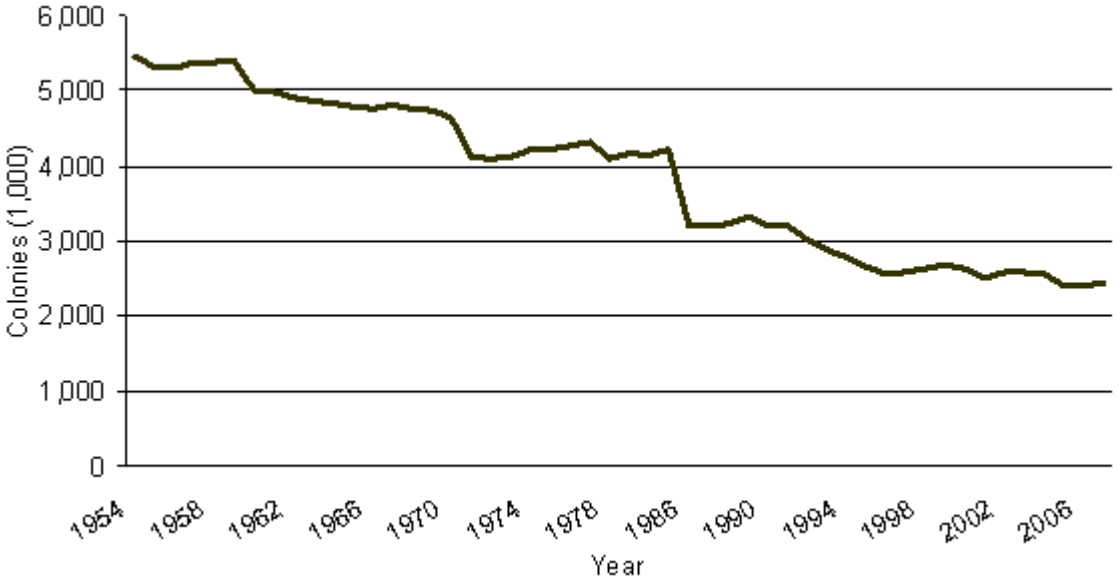

Figure 1-1. Number of honey bee colonies in the U.S. Numbers are based on the number of honey producing colonies per year (NASS 1976-2008, http://www.nass.usda.gov).

CHAPTER 2
TOXICITY AND SUBLETHAL EFFECTS OF IMIDACLOPRID AND AMITRAZ ON HONEY BEE LARVAE AND PUPAE

Honey bees are an important biological indicator species (Porrini et al. 2003), with a given colony's health potentially reflecting the health of its surrounding environment. Bees are known to forage up to 4.8 km for a total of 45 km^2 around their hives (Winston 1987). While foraging, bees can contact various agrochemicals such as insecticides, fungicides, and herbicides. In some instances, the sudden death of bee colonies has been attributed to pesticide use, and could indicate misuse/overuse of pesticides in a local area, non-point or point source environmental pesticide contamination, or any number of other environmental issues (Johansen and Mayer 1990). Consequently, it is important to understand how pesticides affect honey bee health in order to understand possible connections between pesticide exposure and honey bee health.

Despite the importance of understanding pesticide effects on bees, toxicity studies typically focus on acute mortality and behavioral/physiological effects of pesticides on adult honey bees. Pesticide effects on immature honey bees (larvae/pupae) remain understudied (Emmett and Archer 1980, Davis 1989, Davis et al. 2000, Aupinel et al. 2007). Therefore, I investigated 2 pesticides whose effects on honey bee brood are not well understood.

The first pesticide I tested was imidacloprid, a neonicotinoid that affects nicotinic acetylcholine receptors in the insect nervous system (Nauen et al. 2001). Imidacloprid acts in the early stages of poisoning by agonizing acetylcholine receptors, then in later stages by mild antagonism (Nauen et al. 2001). Imidacloprid, by volume, is one of the most abundant insecticides used globally. It is used to protect crops from sucking pests such as aphids, mealybugs, leafhoppers, whiteflies, and thrips (Elbert et al. 1991, Cox 2001). Imidacloprid's systemic properties allow it to be absorbed by plants and transported throughout the plant's vascular tissue, phloem and xylem (Bennett 1957, Sicbaldi et al. 1997). An example of

imidacloprid use is for control of the Asian citrus psyllid (*Diaphorina citri* Kuwayama, Homoptera: Psyllidae), which is responsible for vectoring citrus greening disease (Grafton-Cardwell et al. 2006). Honey bee foragers that visit citrus blooms have the potential to be exposed to imidacloprid.

In France, imidacloprid was thought to be linked to massive bee declines occurring from 1997-2000, which led to the ban of the pesticide in 1999 (Oldroyd 2007, Stokstad 2007). Researchers have not found direct evidence linking imidacloprid to honey bee declines in France.

The second pesticide I tested was amitraz. Amitraz is an octopamine agonist that stimulates intracellular second messenger signaling (Evans and Gee 1980, Hollingworth and Murdock 1980) within invertebrates. Typically, amitraz is used as an acaricide, but it can be used as an insecticide in other pest management programs (Yu 2008). Amitraz is of particular interest because it is used by beekeepers in Europe to control varroa (Marchetti et al. 1984). The harmful effects of amitraz on bees often are overlooked in the U.S. possibly because amitraz is not registered here for use within bee hives.

There is a lack of evidence suggesting that amitraz persists in the beeswax of treated hives (Wallner 1999, Martel et al. 2007, Rial-Otero et al. 2007); however, its metabolite, 2, 4-dimethylphenylformamide (DMPF), has been detected in commercial and recycled beeswax from Spain and France with amounts ranging from 0.5 to 35 mg/kg, or 500 to 35000 ppb (Korta et al. 2003). Additional research focusing on the detection of DMPF within bee colonies is underway (Frazier et al. 2008). Amitraz LD50s have been determined only for adult bees (Santiago et al. 2000). Consequently, there is a need to understand the direct effects of amitraz on bee brood as no such information exists.

I present data here resulting from my investigations into lethal and sublethal effects of imidacloprid and amitraz on developing honey bee brood when pesticides are delivered subchronically. Chronic effects result from longer term, slower exposure to small doses of pesticides over an organism's entire lifespan, while subchronic effects represent exposure only during part of an organism's life. Both exposure types are biologically relevant for honey bees because adult and larval bees are known to be exposed to pesticides (Davis 1989, Davis et al. 2000, Chauzat et al. 2006). Even though doses encountered in the field are small—2-6 ppb for imidacloprid found in pollen collected from foraging bees returning to the hive (Chauzat et al. 2006)—chronic effects can occur at doses $1/60^{th}$ of those eliciting acute effects (Decourtye et al. 2005).

I hypothesize that doses required to elicit lethal effects on bee brood are the same as those that lethally affect adult bees. Therefore, the doses I chose to test, 5-80 ppb for imidacloprid and 50-400 ppb for amitraz, are below the range of acute, lethal doses (LD50s) for imidacloprid and amitraz of 192 and 255 ppb (respectively) found in previous studies on adult bees (Santiago et al. 2000, Fischer and Chalmers 2007). For imidacloprid, I also chose doses that are relatively small (5-20 ppb) to represent levels (2-16 ppb) that bees may encounter in their environment (Chauzat et al. 2006, Krischik et al. 2007). For amitraz, I included doses above the known LD50 for adult bees as well as lower doses so that I could test concentrations believed to be sublethal. I hypothesized that bee brood would be as vulnerable to imidacloprid and amitraz as adult bees, since both adult and immature bees contain nicotinic acetylcholine and octopamine receptors, the targets of imidacloprid and amitraz respectively (Graham 1992, Chapman 1998).

I determined mortality by comparing data between chemically treated and untreated/acetone treated (control) larvae. I measured a number of variables to identify potential

sublethal effects of these xenobiotics on developing brood. I selected these variables because they were relatively easy to quantify in the laboratory without interrupting or preventing the individual bee's development.

Materials and Methods

General Rearing Procedure

I collected honey bee larvae from 8 commercial honey bee colonies maintained at the University of Florida's bee biology unit (Gainesville, FL; N 29° 37.632' W 082° 21.402'). To produce the test larvae, I confined the queen in each colony to a newly-drawn comb (no stored honey, pollen, or brood) using 2 different sized zinc queen excluder cages (96 × 106 × 21 mm or 165 × 130 × 24 mm, l × w × h) at time t = -12 h. I then returned the caged queen and frame to the center of the brood nest (Boot and Calis 1991). During this time, worker bees were able to access and tend the queen.

After 24 h, t = 12 h (Peng et al. 1992, Aupinel et al. 2005), I removed the queen from the cage and replaced the cage on the comb as before but this time for 108 h (from t = 0) to allow the eggs to hatch and larvae to reach an appropriate age for grafting. During this time, worker bees were able to access the comb to feed the developing larvae. At 108 h, I removed the test frames (now containing 36 ± 12 h old larvae) from the colonies and took them to the laboratory (Figure 2-1).

Each queen was confined to a single frame. As such, I grafted 42 larvae from a single frame (1 queen source) into a 96-well treated tissue culture plate (7 × 9 mm, BD Biosciences, Durham, NC), placing 1 larva per well. These tissue culture plates were used because they are the approximate size of a worker honey bee cell (5.2 mm diameter, Winston 1987). I repeated this procedure for each queen source (n = 8 queens × 42 larvae), using separate well plates for each queen's offspring. Prior to grafting the larvae into plates, I pipetted 20 µL of larval diet into

the bottom of each cell. The diet had a pH that ranged from 4.0-4.5 and consisted of 50% royal jelly (Glory Bee Foods, Eugene, OR), 6% D-glucose (Fischer Chemical, Fair Lawn, NJ), 6% D-fructose (Fischer Chemical, Fair Lawn, NJ), 37% double distilled water, and 1% yeast extract (Bacto™, Sparks, MD) by volume (Vandenberg and Shimanuki 1987). Prior to adding the diet to each cell, I pre-warmed it to 35°C in an incubator (Percival Scientific Inc, Perry, IA).

Each subsequent day, I transferred larvae to a clean culture plate provisioned with fresh diet. The amount of artificial diet provided to each larva depended on the larva's age. I fed larvae 20 µL of diet at hours 108 and 132, 30 µL on hour 156, 40 µL on hour 180, and 50 µL on hour 204 and thereafter (Aupinel et al. 2007). At 204 h post oviposition (larvae are 132 ± 12 h old), I transferred the larvae to a 48-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, wells were 13 × 17 mm) because the growing larvae were too large to handle delicately in a 96-well plate. Throughout the study, trays containing larvae were incubated in the dark at 35°C and ~96% RH (Vandenberg and Shimanuki 1987).

Honey bee larvae defecate only once after they finish feeding just prior to pupation (Winston 1987). The feces appear as orange/brown streaks in the brood food. Once the larvae defecated (216-240 h from t = 0, Figure 2-1), I moved them individually into clean tissue culture plates, covered them with the plate lid, and allowed them to pupate. Pupal rearing plates were incubated in the dark at 35°C and ~70% RH (Vandenberg and Shimanuki 1987) until each immature bee reached adulthood or died.

Pesticide Application

I initially was interested in investigating the effects of imidacloprid and amitraz on developing bees if a xenobiotic was ingested rather than received topically. I conducted

preliminary experiments using both topical and oral applications (Appendices A and B); however, after reviewing the preliminary data, I elected to apply the acaricide to the diet.

I diluted imidacloprid and amitraz in an acetone solvent and then mixed the stock solutions into the larval diet each day the diet was administered. This was done to limit potential amitraz degradation in the acidic diet (pH between 4.0-4.5). I created diets with 5, 10, 20, 40, or 80 ppb for imidacloprid and 25, 50, 100, 200, or 400 ppb for amitraz (ppb per the amount of food given to the larvae). I provided one of the resulting diets to each larva 4 times (at 132, 156, 180, and 204 h, Figure 2-1) during development. For both pesticides, I included 2 controls, one of acetone and a second with no acetone or pesticide application. For imidacloprid, n = 8 queens × 7 treatments (5 chemical doses and 2 controls) × 6 larvae/treatment, while for amitraz, n = 7 queens × 7 treatments (5 chemical doses and 2 controls) × 6 larvae/treatment. Offspring from one queen source did not contribute an acetone treatment for either pesticide, which reduced the acetone sample size to 6.

Mortality was assed by measuring (1) number of larvae that defecated (expressed relative to the total number of treated larvae), (2) pupal survival (expressed relative to the total number of larvae that defecated), and (3) adult bee emergence (expressed relative to the total number of larvae treated). To measure sublethal effects, I measured the (4) time it took each larvae to defecate starting from oviposition (time to larval defecation), (5) larval weight immediately after defecation (larval weight at defecation), (6) time it took larvae to emerge as adults starting from oviposition (time to adult emergence), (7) amount each adult bee weighed just after emerging (adult bee weight) and (8) amount each adult bee head weighed immediately after bee emergence (adult bee head weight). I used an electronic, top-loading-bench scale with a draftshield to measure bee weights (Mettler-Toledo International Inc, Columbus, OH). To determine larval

mortality, I viewed each larva daily under a dissecting microscope (Fisher Stereomaster, Fisher Scientific, Pittsburgh, PA) to look for movement. If no movement was observed, I touched the larvae lightly with a grafting tool to encouragement movement. A larva was considered dead if it did not respond to 2 touches.

Statistical Analysis

Data for percentage of larvae that defecated (imidacloprid and amitraz), pupal survival (amitraz), and adult emergence (amitraz) were analyzed using the Kruskal-Wallis test for non-parametric data (Zar 1996) because the data were not normally distributed. Pupal survival (imidacloprid) and adult emergence (imidacloprid) data were normal and analyzed using a 1-way ANOVA with treatment serving as a main effect. Normality was assessed for all data using a Shapiro-Wilk test. Proportion data shown to be normally distributed were transformed prior to analyses using an arcsine square root transformation. However, the proportion data reported herein are the untransformed means. Where necessary, means were compared using Fisher's LSD tests. Homogeneity among variances was determined with Levene's test.

In order to compare mortality between larvae treated with any level of pesticide or no pesticide, I pooled the treatment data within a pesticide group. For example, to create the "pesticide treatment" group for imidacloprid, I pooled the data from larvae treated with all levels of imidacloprid (all ppb concentrations) and compared it to pooled data from larvae not treated with imidacloprid (untreated + acetone treated controls) using a 1-way ANOVA. Within treatment (pesticide or control), pooled means were grouped according to the stage (larva or pupa) in which the bee brood died in order to determine if pesticide-induced mortality was higher in the larval or pupal stage. I analyzed the data using a 1-way ANOVA.

Data for time to larval defecation, larval weight at defecation, time to adult emergence, adult bee weight, and adult bee head weight were analyzed for both pesticides using a 2-way

ANOVA recognizing treatment and queen source as main effects and treatment × queen source as the interaction term. The main effects were tested against treatment × queen source. I found a significant interaction for time to larval defecation (imidacloprid and amitraz) and larval weight at defecation (imidacloprid). Consequently, I analyzed these variables by queen source. Where necessary, means were compared using Fisher's LSD tests and differences accepted at $\alpha \leq 0.05$. All statistical tests were conducted using SAS (SAS Institute, 2008).

Results

Imidacloprid

Imidacloprid mortality data are reported in Table 2-1. Treatment did not affect the percentage of larvae that defecated ($\chi 2 = 3.2$; df = 6; $P = 0.78$) or pupal survival ($F = 10.55$; df = 6, 47; $P = 0.08$). There was, however, a treatment effect on adult bee emergence ($F = 2.80$; df = 6, 47; $P = 0.02$). Larvae fed a diet containing 5, 10, 40, or 80 ppb imidacloprid had lower rates of adult emergence when compared to control and acetone treated larvae.

Data were grouped within treatment type ("untreated" = control + acetone data; "imidacloprid treated" = 5 + 10 + 20 + 40 + 80 ppb data) according to bee developmental stage (larva or pupa) to compare mortality between larvae and pupae within imidacloprid treated and untreated groups. All data presented here and throughout are summarized as mean ± std.error (n). Mortality rates were statistically similar ($F = 3.20$; df = 1, 26; $P = 0.08$) for untreated pupae (24.5 ± 6.9 (14) % mortality) and untreated larvae (8.6 ± 2.4 (14) % mortality). In contrast, pupae developing from imidacloprid treated larvae (55.2 ± 4.8 (40) % mortality) experienced significantly higher mortality than did imidacloprid treated larvae (16.4 ± 3.1 (40) % mortality) ($F = 42.65$; 1, 78; $P < 0.01$). Mortality among imidacloprid treated and untreated larvae was similar ($F = 1.31$; 1, 52; $P = 0.26$). In contrast, pupae developing from imidacloprid treated

larvae experienced significantly higher mortality than those in the untreated group ($F = 11.64$; 1, 52; $P < 0.01$).

I did not find sublethal effects of imidacloprid on developing bees for time to larval defecation (Table 2-2), larval weight at defecation (Table 2-3), time to adult emergence, adult bee weight, or adult bee head weight (Table 2-4). There was a significant interaction between treatment and queen source for mean time to larval defecation ($F = 1.44$; df = 40, 216; $P = 0.05$), thus, I analyzed treatment by queen source for this variable (Table 2-2). Treatments differed significantly only among offspring from queen sources 106 and 112; however, no clear trends were apparent.

There was a significant interaction between treatment and queen source on larval weight at defecation ($F = 2.01$; df = 40, 215; $P < 0.01$), thus, I analyzed treatment by queen source for this variable. There were no significant treatment effects for queen sources 104, 110, 106, 101, 105, and 112 (Table 2-3). There were treatment effects for queen sources 108 and 115, but again, no clear trends were apparent.

There was no significant treatment × queen source interaction ($F = 1.42$; df = 33, 95; $P = 0.09$) or treatment effect ($F = 0.99$; df = 6, 95; $P = 0.43$) on the mean time to adult emergence (Table 2-4). There was a queen source effect ($F = 8.03$; df = 7, 95; $P < 0.01$) on this variable. Offspring from queen sources 104 (511.2 ± 2.4 (13) h), 108 (501.6 ± 2.4 (22) h), 110 (504.0 ± 2.4 (10) h), and 115 (508.8 ± 2.4 (22) h) took longer to develop than those from other queen sources (482.4 ± 2.4 (21) - 496.8 ± 2.4 (26) h).

There was no significant treatment × queen source interaction ($F = 0.47$; df = 25, 76; $P = 0.98$) or treatment effect ($F = 1.21$; df = 6, 76; $P = 0.31$) on adult bee weight. There was a significant queen source effect on adult bee weight ($F = 6.07$; df = 5, 76; $P < 0.01$). Adult

progeny from queen source 105 (87 ± 2 (20) mg) weighed significantly less than progeny from the other queen sources (95 ± 3 (9) - 104 ± 2 (25) mg). Furthermore, there was no significant treatment × queen source interaction ($F = 0.84$; df = 33, 94; $P = 0.70$), treatment effect ($F = 0.88$; df = 6, 94; $P = 0.51$), or queen source effect ($F = 1.51$; df = 7, 94; $P = 0.17$) on mean adult bee head weight (Table 2-4).

Amitraz

Amitraz mortality data are reported in Table 2-5. Treatment of larvae with amitraz had an effect on the percentage of larvae that defecated ($\chi^2 = 20.12$; df = 6; $P < 0.01$), pupal survival ($\chi^2 = 17.76$; df = 6; $P < 0.01$), and adult bee emergence ($\chi^2 = 20.85$; df = 6; $P < 0.01$). Larvae treated with 25 and 400 ppb of amitraz were less likely to defecate than were the untreated and acetone controls. Larvae fed 50, 200, and 400 ppb were less likely to pupate than were untreated and acetone controls. Larvae treated with any of the tested amitraz concentrations (25-400 ppb) were less likely to emerge as adults (Table 2-5).

To compare mortality between larvae and pupae within amitraz treated and untreated groups, data were further grouped within treatment type ("untreated" = control + acetone data; "amitraz treated" = 25 + 50 + 100 + 200 + 400 ppb data) according to bee development stage (larva or pupa). Pupae not treated as larvae experienced significantly higher mortality (38.7 ± 8.2 (13) % mortality) than untreated larvae (5.1 ± 2.2 (13) % mortality) ($F = 15.96$; df = 1, 24; $P < 0.01$). Likewise, pupae treated as larvae with amitraz experienced significantly higher mortality (70.3 ± 4.5% (35) mortality) than treated larvae (25.4 ± 4.3 (35) % mortality) ($F = 40.49$; df = 1, 68; $P < 0.01$). Treated larvae had significantly higher mortality than untreated larvae ($F = 8.84$; df = 1, 46; $P < 0.01$) and pupae treated as larvae had significantly higher mortality than pupae not treated as larvae ($F = 9.35$; df = 1, 46; $P < 0.01$).

I did not find sublethal effects of amitraz on developing bees for time to larval defection (Table 2-6), larval weight at defecation, time to adult emergence, adult bee weight, and adult bee head weight (Table 2-7). There was a significant interaction between queen source and treatment on time to larval defecation ($F = 1.52$; df = 34, 175; $P < 0.04$), so I analyzed the data by queen source. Despite this, no treatment related trends within queen source were apparent (Table 2-6).

There was no significant queen source × treatment interaction ($F = 1.28$; df = 34, 171; $P = 0.15$) or treatment effect ($F = 1.72$; df = 6, 171; $P = 0.12$) on larval weight at defecation, while there was an effect of queen source on this variable ($F = 11.54$; df = 6, 171; $P < 0.01$). Progeny from queen source 104 (112 ± 3 mg) weighed less at defecation than larvae from other queen sources (135 ± 3 to 151 ± 4 mg). In contrast, progeny from queen source 108 (151 ± 4 mg) weighed more than larvae from other queen sources (112 ± 3 - 143 ± 3 mg) (Table 2-7).

There was no significant queen source × treatment interaction ($F = 0.89$; df = 25, 52; $P = 0.62$) or treatment effect ($F = 1.01$; df = 6, 52; $P = 0.43$) on time to adult emergence (Table 2-7). I did find that offspring from queen source 110 (504.0 ± 4.8 (6) h) took longer to reach adulthood than offspring from all other queen sources (482.4 ± 2.4 - 501.6 ± 4.8) ($F = 3.02$; df = 6, 52; $P \leq 0.01$).

There was no significant interaction ($F = 0.33$; df = 19, 43; $P = 0.99$) or treatment effect ($F = 0.17$; df = 6, 43; $P = 0.98$) on adult bee weight. However, queen source did affect this variable with offspring from queen 106 (109 ± 3 (20) mg) weighing more than offspring from all other queens (95 ± 1 - 99 ± 2 mg) ($F = 2.91$; df = 4, 43; $P < 0.03$). Furthermore, I did not find a significant queen source × treatment interaction ($F = 0.36$; df = 25, 51; $P = 0.99$), treatment effect ($F = 0.45$; df = 6, 51; $P = 0.84$) or queen source effect ($F = 0.78$; df = 6, 51; $P = 0.59$) on adult bee head weight (Table 2-7).

Discussion

In general, "control" (untreated + acetone treated) larvae in the imidacloprid and amitraz tests had reduced adult emergence rates (imidacloprid test, 70%; amitraz test, 59%) compared to worker emergence rates typically observed under natural hive conditions (90-97%, Winston et al. 1981). My control results are similar to those of Aupinel et al. (2005) who showed that 90% of control larvae survived to defecate and 66% survived to adulthood. The relatively low number of adult bees to emerge under control conditions may be an indication of the amount of stress encountered by the immature bees during the artificial rearing procedure. This stress may have led to an enhanced effect, influencing the toxicity of the pesticide during pupal development. If stress caused enhanced effects of pesticides on bee development, then bees under more natural, unstressed conditions may experience less mortality at the tested pesticide doses. My results may suggest greater pesticide susceptibility in stressed bees.

Imidacloprid

Collectively, the data suggest that imidacloprid may have a delayed, lethal effect on honey bee pupae when larvae are fed low doses of imidacloprid under artificial conditions. In general, the only measurable effect was a reduced number of treated larvae that were able to develop into adults, which was lower in larvae treated with 5, 10, 40, and 80 ppb imidacloprid than in control larvae. The data presented here suggest that imidacloprid doses of 5, 10, 40, & 80 ppb, which are lower than the adult bee LD50 of 192 ppb (Fischer and Chalmers 2007) are lethal to brood. It generally is believed that the larval and pupal stages of insects are more susceptible to xenobiotics (Yu 2008). Eggs and pupae are contained in a protective casing and do not feed (Gullan and Cranston 2005), which likely results in reduced xenobiotic exposure. In my investigation, the larvae were fed the xenobiotic, but the effects were delayed until the pupal stage.

The results for imidacloprid were not dose dependent, meaning mortality did not increase as the tested pesticide dose increased. For example, I did not see a significant reduction in adult emergence at 20 ppb, but did for all other doses (5, 10, 40, and 80 ppb). Such an atypical distribution of mortality data are not reported in the literature because most data for determining LD50s are summarized graphically or only reported after probit analysis (Landis and Yu 1995, Yu 2008). Therefore, small discrepancies in dose-response data typically are not usually reported in the literature. Data further from the LD50 tend to "wobble" about the mean. Even though my data did not suggest dose dependency, this is not unexpected since greater variation occurs in data far from the LD50 (Suchail et al. 2000, Brandt et al. 2002). To determine dose dependency, I should test more imidacloprid doses to include a wider range and analyze the resulting data using probit analysis (Yu 2008).

The "pooled" data suggest that imidacloprid, though fed to developing larvae, was more lethal several days later to developing pupae. There are no known reasons for this delayed toxicity to occur, but bee larvae store their waste until defecation at which time metamorphosis begins (Winston 1987, Chapman 1998). It is possible that imidacloprid is more toxic during this metabolically active period because at this time, larval tissues are being broken down and reabsorbed (Winston 1987). The change from prepupa to pupae and then to adult is also an active period of hormone signaling. During pupal development, juvenile hormone is absent for the first time, which signals a change from an immature stage to the imago form (Chapman 1998, Gullan and Cranston 2005). At this time, there is also activity for ecdysone, eclosion hormone, and bursicon, which are all hormones that are associated with molting (Chapman 1998). Detoxification enzymes such as cytochrome P-450 monooxygenases (review by Feyereisen 1999, 2005) could be shunted to other metabolically important functions preventing

detoxification and clearance of xenobiotics stored within larvae. This could be particularly important with respect to honey bees because of their lack of xenobiotic detoxifying enzymes, such as cytochrome P-450 monooxygenases, when compared to other insects (Claudianos et al. 2006).

Imidacloprid did not elicit measurable sublethal or acutely lethal effects on developing honey bees at the doses I tested. I did not find that imidacloprid affected the rate at which immature bees develop even though it has been shown to affect the developmental rates of other insects. Abbott et al. (2008) found effects of imidacloprid on *Osmia lignaria* larvae, a solitary bee. They concluded that acute-sublethal doses (30 and 300 ppb) of imidacloprid mixed with pollen increased female larval developmental time (Abbott et al. 2008). To gain a more accurate assessment of the effects of imidacloprid on larval and pupal development rates, I should have measured larval mortality several times throughout each day.

Other sublethal effects could result from larvae eating food containing pesticides. For example, imidacloprid did reduce the number of larvae that reached adulthood, but some larvae still successfully pupated. Consequently, a number of larvae survived the treatment. It is possible that sublethal effects could manifest themselves in the surviving adults. These effects could include: disorientation, decreased learning ability, decreased longevity, decreased sperm viability, among other possibilities (Bitterman et al. 1983, Bortolotti et al. 2003, Medrzycki et al. 2003, Decourtye et al. 2004, Decourtye et al. 2005, Burley 2007), suggesting that the surviving adults could have been compromised in ways that were not considered in the current research.

Bee larvae in this study were affected by imidacloprid at doses that could be encountered in the environment. For example, Chauzat et al. (2006) found imidacloprid to be the most frequent pesticide found in pollen brought back to the nest by forager bees with concentrations

ranging from 1.1 - 5.7 µg/kg (ppb). Therefore, the effects I found could occur in managed bee colonies.

Results of this investigation suggest that risk assessment procedures for registering systemic pesticides be modified to include lethal effects on honey bee brood. Findings of the current study suggest that lethal levels of these xenobiotics may be lower than those lethal to adult bees. Therefore, current procedures may be underestimating pesticide toxicity to bees. Warnings have been placed on imidacloprid limiting its use during periods of flower bloom. As stated earlier, imidacloprid's long residual time in soil (Bonmatin et al. 2003) makes a short restriction of the pesticide non-beneficial because plants have been shown to acquire imidacloprid from the soil up to 2 years after treatment. Management practices limiting the use of systemic pesticides within areas important to bee pollination could be adopted. This remains a difficult task due to the large foraging range of honey bees (Winston 1987) and our heavy reliance on pesticides (annual global pesticide sales accumulate to ~33.6 billion USD; (Yu 2008).

Amitraz

As noted with imidacloprid, toxicity data collected on amitraz-fed larvae indicated that amitraz present in brood food from 25 to 400 ppb reduced larval survival to adulthood. Santiago et al. (2000) found that the LD50 for amitraz on adults bees is around 255 ppb. I did not test this level specifically, but I did bracket this dose by testing 200 and 400 ppb. I did not find amitraz effects on larval mortality at 200 ppb but did at 400 ppb; a lethal concentration similar to that observed previously for adults (Santiago et al. 2000).

Amitraz showed a more dose-dependent response compared to imidacloprid data for the percentage of treated larvae to emerge as adults. This may have occurred since I tested a larger range of doses for amitraz than I did for imidacloprid.

When I pooled the amitraz treatments together according to developmental stage (larvae vs. pupae), I found that pupae fed amitraz as larvae had higher mortality than those fed as larvae on food containing amitraz. However, there was also an increase in mortality in the control groups, suggesting that variables other than the xenobiotic influenced pupal mortality. Since mortality increased in both untreated and treated groups, I specifically cannot conclude that mortality of amitraz-fed larvae is more likely to occur during pupation rather than while feeding in the larval stage.

Amitraz did not elicit measurable sublethal effects on immature bees in this investigation. However, amitraz may affect developing bees in ways not measured here. For instance, foramidines have been shown to cause decreased fecundity in the tobacco budworm, *Heliothis virescens* (F.); fall armyworm, *Spodoptera frugiperda* (J. E. Smith); southern armyworm, *S. eriddania* (Cramer); and pink bollworm, *Pectinophora gossypiella* (Saunders) (Wolfenbarger et al. 1974) as well as hyperexcitation and sterilization in ticks (Hollingworth 1976). I did not examine these variables. It is interesting to note that amitraz did not affect larval weight at defecation despite the fact that it has been shown to be a feeding deterrent in mites (Gladney et al. 1974) and lepidopteran larvae (Doane and Dunbar 1973).

Even though varroa mites can be lethal to bee colonies (DeJong 1997) and difficult to control due to acaricide resistance (Hillesheim et al. 1996, Spreafico et al. 2001); my data do not suggest that amitraz is an appropriate acaricide for varroa control.

Summary

Imidacloprid and amitraz were lethal to immature bees, limiting the number of bees that emerged as adults. Unlike for imidacloprid treated larvae, mortality during the pupal stage was dose-dependent for amitraz treated larvae. Imidacloprid and amitraz were lethal to immature bees, lowering the percentage of adult bees that emerged. Unlike for pupae mortality for larvae

treated with imidacloprid, pupae mortality for larvae treated with amitraz appeared to be dose-dependent. This difference could be explained by the wider range of amitraz doses I chose to test. A lack of dose dependency for imidacloprid may be explained by the idea that mortality responses at doses relatively close together tend to "wobble" about the mean, especially at the areas on the extremes of the LD50 curve.

No acutely lethal effects were observed for imidacloprid treated larvae. However, I did find an increase in larval mortality occurring for amitraz treated larvae, but mortality did not occur at all tested doses. Several reasons could lead to a difference in larval mortality. First, both chemicals have different physico-chemical properties; for instance, amitraz is lipophilic while imidacloprid is hydrophilic (Hollingworth 1976, Elbert et al. 1991). Secondly, imidacloprid and amitraz act on different receptors in the insect nervous system: acetylcholine and octopamine respectively (Yu 2008). Finally, I tested higher amitraz doses than imidacloprid ones in order to remain close to the adult LD50 values.

For imidacloprid, toxicity appeared to be delayed, mostly occurring during pupal development. The same could not be concluded for amitraz because there were acutely lethal effects on larvae. Delayed toxicity for amitraz could not be concluded because both untreated and treated larvae displayed increases in pupae mortality over that of larval mortality.

This research could have been improved by modifying methods to increase the percentage of adult bees to emerge under control, untreated conditions. This could be achieved by placing the prepupae laterally, and abdomen-first into smaller cells, thereby mimicking their natural orientation in the hive. Another modification I would have chosen to make would be to record the stage of larval or pupal development the treated bees reached prior to dying. Davis et al. (2000) showed that dimethoate and carbofuran treated larvae failed to spin silk, suggesting that

larvae would fail to molt from the prepupal to the pupal stage. In my study, this clearly was not the case as some larvae treated with either pesticide completed the prepupal stage.

Overall, the xenobiotics tested here appear to be detrimental to honey bee brood. Based on my findings I recommend that risk assessment tests on honey bees be changed to include effects on larval mortality and delayed effects on pupae when fed as larvae.

Table 2-1. Percentage of larvae that defecated, pupal survival, and adult bee emergence rates for larvae fed a diet containing imidacloprid.

Treatment	Larvae that defecated[a] (%)	Pupal survival[b] (%)	Adult bee emergence[c] (%)
Theoretical survival	na	na	93.5 ± 2.2
Untreated	91.2 ± 3.3 (8)a	76.7 ± 10 (8)a	70 ± 9.2 (8)a
Acetone	91.7 ± 3.7 (6)a	73.9 ± 10 (6)a	69.4 ± 11.7 (6)a
5	80 ± 6.8 (8)a	50.6 ± 8.5 (8)a	38.3 ± 5.2 (8)b
10	88.3 ± 7.4 (8)a	37.9 ± 8.3 (8)a	32.9 ± 8.4 (8)b
20	83.7 ± 7.6 (8)a	51.7 ± 12.2 (8)a	46.7 ± 12 (8)ab
40	81.7 ± 7.3 (8)a	43.5 ± 11.5 (8)a	37.5 ± 11.8 (8)b
80	84.2 ± 6.9 (8)a	40.4 ± 13.7 (8)a	30 ± 9.4 (8)b

Theoretical survival is the predicted number of eggs to survive to adulthood under natural conditions within the hive (Winston et al. 1981). Imidacloprid concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of queen sources whose offspring were tested. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests. [a] % of larvae that defecated = (the number of larvae that defecated/the total number of treated larvae) × 100. [b] % pupal survival = (the number of adult bees that emerged/the number of larvae that defecated) × 100. [c] % of adult bee emergence = (the number of adult bees that emerged/the total number of treated larvae) × 100.

Table 2-2. Mean time to larval defecation in hours for larvae fed diet containing imidacloprid.

	Queen source			
Treatment	101	104	105	106
Untreated	244.8 ± 9.6 (5)a	240 (6)	216 (5)a	228 ± 4.8 (6)a
Acetone	230.4 ± 4.8 (5)a	na	220.8 ± 4.8 (5)a	240 (6)ac
5	232.8 ± 4.8 (6)a	240 (5)	235.2 ± 4.8 (5)a	240 (6)a
10	235.2 ± 4.8 (6)a	240 (5)	232.8 ± 7.2 (6)a	240 (6)a
20	232.8 ± 4.8 (4)a	240 (6)	228 ± 7.2 (4)a	235.2 ± 7.2 (6)a
40	240 ± 7.2 (5)a	240 (4)	235.2 ± 4.8 (5)a	254.4 ± 4.8 (5)b
80	235.2 ± 4.8 (5)a	240 (6)	230.4 ± 4.8 (5)a	244.8 ± 4.8 (6)bc
ANOVA	$F = 0.64$; df = 6, 29; $P = 0.70$	F = na; df = 5, 26; P = na	$F = 1.65$; df = 6, 28; $P = 0.17$	$F = 3.23$; df = 6, 34; $P < 0.01$

Data are separated for treatment by queen source. Imidacloprid concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of queen offspring receiving a given treatment. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests.

Table 2-2. Continued

Treatment	108	110	112	115
Untreated	240 (6)a	220.8 ± 4.8 (4)a	230.4 ± 9.6 (5)a	240 (5)a
Acetone	240 (5)a	na	235.2 ± 4.8 (6)a	240 (5)a
5	240 (4)a	228 ± 12 (2)a	240 (5)a	249.6 ± 4.8 (5)a
10	240 ± 9.6 (5)a	228 ± 12 (2)a	240 (6)a	264 ± 9.6 (6)a
20	244.8 ± 4.8 (6)a	228 ± 12 (2)a	244.8 ± 4.8 (6)ac	244.8 ± 4.8 (5)a
40	240 (6)a	240 (2)a	235.2 ± 4.8 (6)a	244.8 ± 4.8 (4)a
80	240 (5)a	228 ± 12 (2)a	256.8 ± 4.8 (6)bc	244.8 ± 4.8 (5)a
ANOVA	$F = 1.90$; df = 6, 30; $P = 0.11$	$F = 0.44$; df = 5, 8; $P = 0.81$	$F = 3.08$; df = 6, 33; $P < 0.02$	$F = 1.95$; df = 6, 28; $P = 0.11$

Table 2-3. Mean larval weight at defecation for larvae fed a diet containing imidacloprid.

	Queen source			
Treatment	101	104	105	106
untreated	131 ± 4 (5)a	124 ± 3 (6)a	116 ± 3 (5)a	133 ± 6 (6)a
acetone	133 ± 8 (5)a	na	116 ± 7 (5)a	140 ± 5 (6)a
5	142 ± 10 (6)a	118 ± 4 (5)a	123 ± 2 (5)a	137 ± 3 (6)a
10	152 ± 7 (6)a	119 ± 6 (5)a	129 ± 5 (6)a	129 ± 4 (6)a
20	154 ± 11 (4)a	99 ± 9 (6)a	137 ± 11 (4)a	139 ± 10 (6)a
40	150 ± 13 (5)a	100 ± 17 (4)a	138 ± 4 (5)a	140 ± 12 (5)a
80	153 ± 16 (5)a	100 ± 15 (6)a	131 ± 9 (5)a	147 ± 4 (6)a
ANOVA	F = 0.82; df = 6, 29; P = 0.56	F = 1.35; df = 5, 26; P = 0.28	F = 2.09; df = 6, 28; P = 0.09	F = 0.67; df = 6, 34; P = 0.68

Data are separated for treatment by queen source. Defecation weights are given in milligrams. Imidacloprid concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of queen offspring receiving a given treatment. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests.

Table 2-3. Continued

Treatment	108	110	112	115
Untreated	138 ± 10 (6)ac	166 ± 4 (4)a	126 ± 3 (5)a	123 ± 4 (5)a
Acetone	147 ± 4 (5)ac	na	129 ± 4 (6)ab	152 ± 8 (5)bc
5	164 ± 4 (4)bc	148 ± 7 (2)a	145 ± 6 (5)bc	142 ± 4 (5)ac
10	150 ± 13 (5)ac	116 ± 2 (2)a	155 ± 4 (6)cd	147 ± 10 (5)bc
20	160 ± 6 (6)b	118 ± 63 (2)a	167 ± 8 (6)de	148 ± 7 (5)bc
40	180 ± 6 (6)b	154 ± 4 (2)a	163 ± 9 (6)ce	158 ± 3 (4)bc
80	189 ± 2 (5)d	163 ± 2 (2)a	152 ± 2 (6)ce	147 ± 8 (5)bc
ANOVA	$F = 5.58$; df = 6, 30; $P < 0.01$	$F = 1.07$; df = 5, 8; $P = 0.45$	$F = 6.46$; df = 6, 33; $P \leq 0.01$	$F = 2.40$; df = 6, 27; $P = 0.05$

Table 2-4. Mean time to adult emergence, adult bee weight, and adult bee head weight for larvae fed a diet containing imidacloprid.

Treatment	Time to adult emergence (h)	Adult bee weight[a] (mg)	Adult head weight[a] (mg)
Untreated	494.4 ± 2.4 (32)a	94 ± 2 (20)a	11.6 ± 0.2 (32)a
Acetone	494.4 ± 2.4 (24)a	97 ± 2 (23)a	11.4 ± 0.3 (23)a
5	499.2 ± 4.8 (18)a	94 ± 2 (14)a	11.1 ± 0.3 (18)a
10	499.2 ± 4.8 (15)a	101 ± 4 (12)a	12 ± 0.4 (15)a
20	501.6 ± 2.4 (22)a	101 ± 3 (19)a	11.7 ± 0.3 (22)a
40	496.8 ± 2.4 (17)a	99 ± 3 (16)a	11.6 ± 0.3 (17)a
80	506.4 ± 4.8 (14)a	101 ± 1 (9)a	11.4 ± 0.3 (14)a

Imidacloprid concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of individuals from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a]Measurements were taken just after adult bees emerged.

Table 2-5. Percentage of larvae that defecated, pupal survival, and adult bee emergence for larvae fed a diet containing amitraz.

Treatment	Larvae that defecated[a] (%)	Pupal survival[b] (%)	Adult bee emergence[c] (%)
Theoretical survival	na	na	93.5 ± 2.2
Untreated	95.2 ± 3.1 (7)a	60.5 ± 10 (7)a	59 ± 10.6 (7)a
Acetone	97.2 ± 2.8 (6)a	63.3 ± 14.4 (6)a	61.1 ± 14 (6)a
25	66.7 ± 11.5 (7)bc	47.5 ± 11.9 (6)ab	30.9 ± 8.5 (7)b
50	81.4 ± 7.2 (7)ab	25.5 ± 7.8 (7)bc	20 ± 4.9 (7)b
100	81.4 ± 11.3 (7)ab	45.2 ± 10.9 (7)ab	30.5 ± 5.7 (7)b
200	80 ± 7.1 (7)ab	28.6 ± 5.4 (7)bc	24.3 ± 4.9 (7)b
400	56.2 ± 7.2 (7)c	9.5 ± 9.5 (7)bc	4.8 ± 4.8 (7)c

Theoretical survival is the predicted number of eggs to survive to adulthood under natural conditions within the hive (Winston et al. 1981). Amitraz concentrations are in ppb. Data are mean ± std. error (n) where n = number of queen sources used to produce offspring for each replicate. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests. [a] % of larvae that defecated = (the number of larvae that defecated/the total number of treated larvae) × 100. [b] % pupal survival = (the number of adult bees that emerged/the number of larvae that defecated) × 100. [c] % of adult bee emergence = (the number of adult bees that emerged/the total number of treated larvae) × 100.

Table 2-6. Mean time to larval defecation (h) for larvae fed a diet containing amitraz.

	Queen source			
Treatment	101	104	105	106
Untreated	240. (6)a	216 (5)a	232.8 ± 4.8 (6)a	240 (6)a
Acetone	232.8 ± 4.8 (4)a	216 (6)a	235.2 ± 4.8 (6)a	247.2 ± 4.8 (6)ac
25	240 (5)a	220.8 ± 4.8 (5)a	240 ± 7.2 (5)a	256.8 ± 4.8 (4)bcd
50	240 (4)a	216 (5)a	235.2 ± 7.2 (6)a	240 (3)a
100	240 ± 4.8 (6)a	216 (6)a	228 ± 4.8 (6)a	264 (5)bd
200	230.4 ± 4.8 (5)a	216 (6)a	240 ± 7.2 (5)a	259.2 ± 4.8 (5)b
400	232.8 ± 12 (4)a	216 (3)a	240 ± 14.4 (4)a	264 (4)b
ANOVA	$F = 0.56$; df = 6, 27; $P = 0.76$	$F = 1.04$; df = 6, 29; $P = 0.42$	$F = 0.43$; df = 6, 31; $P = 0.85$	$F = 7.96$; df = 6, 26; $P < 0.01$

Treatment	108	110	112
Untreated	254.4 ± 4.8 (5)a	10.2 ± 4.8 (5)a	240 (6)a
Acetone	247.2 ± 4.8 (6)a	na	232.8 ± 4.8 (6)a
25	240 (4)a	na	230.4 ± 9.6 (5)a
50	252 ± 4.8 (6)a	247.2 ± 7.2 (3)a	232.8 ± 4.8 (6)a
100	256.8 ± 4.8 (4)a	240 (1)a	225.6 ± 4.8 (5)a
200	256.8 ± 7.2 (3)a	264 ± 14.4 (3)a	235.2 ± 7.2 (6)a
400	247.2 ± 7.2 (3)a	288 (1)a	223.2 ± 7.2 (3)a
ANOVA	$F = 1.02$; df = 6, 24; $P = 0.43$	$F = 2.17$; df = 4, 8; $P = 0.16$	$F = 0.73$; df = 6, 30; $P = 0.63$

Data are separated for treatment by queen source. Amitraz concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of queen source offspring receiving a given treatment. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests.

Table 2-7. Mean larval weight at defecation, time to adult emergence, adult bee weight, and adult bee head weight for larvae fed a diet containing amitraz.

Treatment	Larval weight at defecation (mg)	Time to adult emergence (h)	Adult bee weight[a] (mg)	Adult head weight[a] (mg)
Untreated	132 ± 3 (39)a	492 ± 2.4 (24)a	102 ± 3 (18)a	11.5 ± 0.3 (24)a
Acetone	139 ± 3 (34)a	492 ± 2.4 (21)a	101 ± 2 (20)a	12.1 ± 0.3 (21)a
25	139 ± 5 (27)a	492 ± 4.8 (13)a	99 ± 2 (11)a	11.9 ± 0.3 (13)a
50	135 ± 5 (33)a	480 (8)a	96 ± 4 (7)a	11.5 ± 0.4 (8)a
100	141 ± 5 (33)a	499.2 ± 4.8 (12)a	105 ± 5 (9)a	11.6 ± 0.4 (11)a
200	143 ± 5 (33)a	492 ± 4.8 (10)a	100 ± 4 (7)a	11.7 ± 0.3 (10)a
400	140 ± 6 (19)a	504 ± 24.0 (2)a	98 (1)a	13.0 ± 0.8 (2)a

Amitraz concentrations are shown in ppb. Data are mean ± std. error (n) where n = number of individuals pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] Measurements were taken just after adult bees emerged.

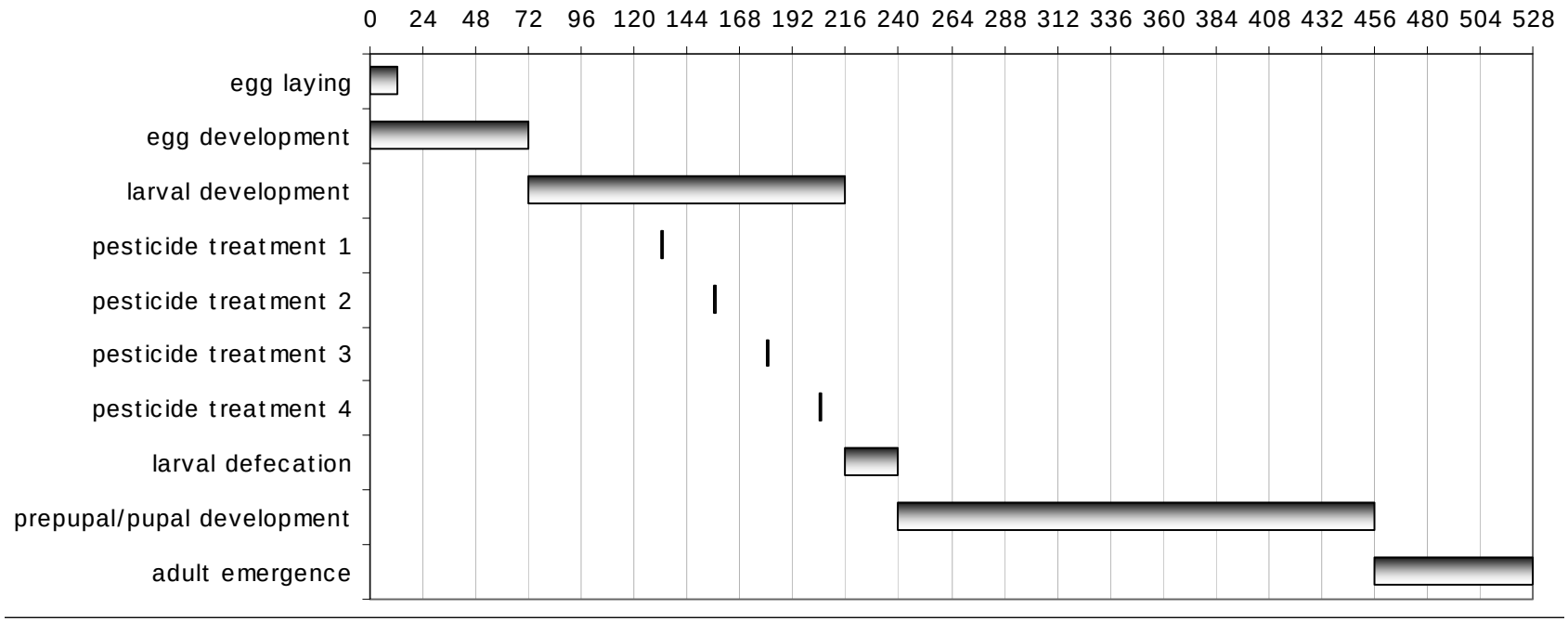

Figure 2-1. Laboratory bioassay: experimental timeline according to larval development in hours (0-528 h, x-axis). Pesticide treatment refers to the 4 time periods each larva was treated (at 132, 156, 180, and 204 h) with their corresponding pesticide treatment (imidacloprid or amitraz). The timeline does not illustrate that egg lying began at t = -12 h.

CHAPTER 3
SUMMARY

The research presented herein was conducted to gain insight into the effects of pesticides on immature stages of the honey bee, an important beneficial insect. Honey bee survival is important not only for the production of certain commodities such as honey and beeswax, but also for purposes of crop pollination. It has been estimated that one-third of the world's food production is dependent on bee pollination (Pimentel 2005). Therefore, any decline in honey bee populations potentially could affect our global food supply.

Beginning in 1950s, investigators began to notice a decline in managed honey bee populations (NASS 1976-2008). Parasitic mites were a major contributor to past bee population declines (Morse and Flottum 1997). Reasons for the most recent declines are unknown, but some researchers have suggested pesticides as possible causes, including neonicotinoids and acaricides, (Stokstad 2007).

Imidacloprid is a neonicotinoid and plant systemic insecticide that has been found in the pollen and nectar of plants visited by honey bees (Bonmatin et al. 2003, Chauzat et al. 2006, Greatti et al. 2006). Imidacloprid movement into the hive via foraging bees is the proposed mechanism by which honey bee larvae are exposed to plant systemic pesticides (Villa et al. 2000). To date, there has been no evidence to suggest that imidacloprid is responsible for the widespread losses of bees. However, it is important to understand the potential environmental and non-target effects of imidacloprid because it is, by volume, one of the most abundant pesticides used in the world (Kegley et al. 2008).

Previous research into the effects of pesticides on honey bees has focused primarily on adult bees (Bitterman et al. 1983, Bortolotti et al. 2003, Decourtye et al. 2004, Decourtye et al. 2005), leading investigators to suggest that quantities of imidacloprid found to be present in the

environment are not lethal to adult bees (Schmuck et al. 2001, Maus et al. 2003, Stadler et al. 2003). Despite known effects of imidacloprid, there is a lack of research concerning its effects on honey bee brood. The results from my research (Chapter 2) suggest that small quantities of imidacloprid in the ppb range are in fact lethal to bee brood. These levels are lower than reported LD50s for adult worker bees (Suchail et al. 2000, Nauen et al. 2001, Schmuck et al. 2001, Suchail et al. 2001).

Interestingly, the effects of imidacloprid on honey bee brood (presented in Chapter 2) were not dose-dependent at the doses I tested. In my investigation, imidacloprid killed developing brood at 5, 10, 40, and 80 ppb, but not at 20 ppb. Despite a lack of clear dose-dependent trends within the range I tested, the percentage of adult emergence at 20 ppb was 34% lower than that of the controls.

An increase in adult emergence at 20 ppb compared to 5 and 10 ppb does not insinuate a lack of dose-dependence. Mortality data typically are not reported in the literature, but are analyzed graphically using probit analysis procedure that covers a wider range of doses to produce the LD50 values that reported more commonly (Yu 2008). The range of doses I tested for imidacloprid in Chapter 2 (5-80 ppb) may seem large but is not compared to ranges likely to be tested to determine dose dependency, which may cover a range from ppb to ppt (parts per thousand, Figure 3-1). Natural variation in bee response to ppb doses close to one another (such as the ones tested in Chapter 2) are common and usually are overlooked, especially if they occur on the extreme ends of the dose-response curve (i.e. 0-10%, 90-100%, Figure 3-2). In a toxicity experiment verifying LD50s for imidacloprid on adult honey bees, Suchail et. al. (2000) plotted the log dose (ng/bee) against the percentage of bee mortality. Their graphs illustrate a “wobble”, or non-linear response, for percentage of bee mortality within a small range of doses, especially

on the lower and upper extremes of the response curve. However, across all tested doses, their graph depicts a generalized dose-dependent response. Had I wanted to determine an LD50 for imidacloprid, it would be necessary to test doses ranges that included doses > 192 ppb (Fischer and Chalmers 2007) and < 5 ppb. These doses probably would have bracketed the NOED (no observable effect dose). Once tested, this should lead to a better understanding of LD50 values for honey bee brood, which, in turn, can be used for risk assessment.

Even though I believe the results from my laboratory investigations were reliable, I hesitate to extrapolate my findings too far with regard to imidacloprid effects on brood in field colonies. For example, the *in vitro* rearing program clearly hindered bee development, decreasing adult emergence to 77% in the controls. As such, the rearing program itself could be a stressor that may have influenced the observed imidacloprid effects in this study. Consequently, it is possible that imidacloprid may not produce similar effects in field situations where brood rearing is optimized and background mortality is low (<5%; Winston et al. 1981) . However, if my results are an accurate indication of imidacloprid effects under natural, stressed hive conditions, then larval exposure to imidacloprid at levels as low as 5 ppb could result in a 45% reduction in the amount of brood in exposed colonies and up to 57% at 80 ppb.

Data for amitraz are equally interesting. Amitraz is not known to persist in treated honey bee colonies longer than 9 months post treatment (Lodesani et al. 1992, Korta et al. 2001) and was recorded to have degraded significantly within days after being applied to plates containing beeswax (Wallner 1999). Amitraz is thought to break down rapidly in acidic solutions such as honey, which is why it does not persist in hives. An amitraz metabolite, 2,4-dimethylphenylformamide (DMPF), has been detected at high levels in beeswax, but research is still in progress to identify DMPF persistence within bee colonies (Korta et al. 2003, Frazier et

al. 2008). Although, amitraz may be broken down quickly; its DMPF metabolite (or other unknown metabolites) could have similar effects on brood development. Future research should consist of measuring bee mortality when the bees are exposed to DMPF and amitraz stored in honey for several days or more.

In my research, I found that amitraz is lethal to honey bee brood (larvae and pupae) at doses lower than those found to be lethal to adults (Santiago et al. 2000). Consequently, beekeepers should not use this product as an acaricide. I believe that amitraz is a poor choice for varroa control based on 3 reasons. First, amitraz metabolites can be hazardous to mammals including humans (Hollingworth 1976, Yu 2008). Secondly, varroa have developed resistance to amitraz (Spreafico et al. 2001). Finally, my data suggest that amitraz could contribute to significant brood/pupa bee losses if concentrations ranging from 25-400 ppb are present in larval food. Larvae exposed to 25 ppb of amitraz in their food could result in an estimated 50% loss of bee brood, whereas larvae exposed to 400 ppb could result in an estimated 92% loss of brood.

Given more time, I would like to continue my investigations in field colonies. However, my initial field trials did not lead to conclusive results. This was due to a number of problems I encountered while administering pesticides to larvae in field colonies. I tried to administer the pesticide to larvae in two ways: via treated diet and by topical application. First, larvae died when I tried to administer small amounts of pesticide treated diet into their cells. I believe that the larvae drowned because the applied volume was too large (this method was not discussed in length). The biology of honey bees makes it difficult to deliver the correct amount of artificial diet to individual larva because bee larvae naturally are provisioned progressively (continuous small doses of food) by nurse workers (Winston 1987).

Therefore, I subsequently tried to apply the pesticide to each larva topically with the pesticide administered onto the larval cuticle in 1 μL of acetone (Appendix C). Unfortunately, I believe that the worker bees displayed hygienic behavior (Arathi et al. 2000) and removed the treated larvae because of an effect the solvent had on larval physiology. One possibility explaining the hygienic removal of the treated larvae could be that the application procedure stressed the treated larvae which, in turn, were removed by the worker bees. If pesticides are found in brood food and adult bees respond to the pesticide by aborting the brood, this could lead to significant honey bee population declines. Colony loss due to this probably is unrelated to CCD, because most colonies experiencing CCD have significant quantities of brood at colony death (Eccleston 2007, Ellis 2007, Johnson 2007, Embrey 2008).

Had the worker bees not aborted the larvae, topically applying the pesticides may have been the best method of pesticide delivery in the field. However, as seen for imidacloprid, contact LD50s typically are higher than oral LD50s (Table 3-1). It is well known that the insect cuticle is a formidable barrier to some toxins (Yu 2008). LD50s determined through contact, rather than by ingestion in the diet, would best estimate toxicities of xenobiotics that tend to accumulate in beeswax, such as acaricides (Wallner 1999).

For future studies, it will be important to determine a method for assessing pesticide effects on brood *in vivo*. This should help illuminate how environmental xenobiotics affect the entire colony and whether such chemicals can be linked to colony declines. Additional research should focus on the long term, sublethal effects of pesticides on bees. For example, a number of pesticide treated bees survived to adulthood in my study. It would be interesting to determine if these bees functioned normally after emergence.

Overall, my research indicates that acute sublethal pesticide doses for honey bee larvae can cause delayed mortality in honey bee pupae at concentrations lower than those shown to affect adult bees. A significant portion of the mortality experienced by the developing bees in Chapter 2 was manifested in the pupal stage, which is counterintuitive because it was the larvae that were fed a pesticide-containing diet. Based on my findings, I recommend that agrochemical safety registration studies be expanded to include immature bee toxicity assays. It is important that pupae be tested as well since in my study, pupae appeared to be more vulnerable to pesticides acquired during larval stages than the larvae themselves.

Table 3-1. Published contact and oral LD50 values for imidacloprid tested on adult honey bees.

Method	Contact LD50	Oral LD50/LC50	Source
Acute	14 ng/bee and 24 ng/bee	5 ng/bee	(Suchail et al. 2000)
Acute	49-102 ng/bee	41-81 ng/bee	(Nauen et al. 2001)
Acute	na	3.7-40.9 ng/bee	(Schmuck et al. 2001)
Acute	na	40-60 ng/bee	(Suchail et al. 2001)
Chronic	na	(0.01. 0.1, and 1 ppb)	(Suchail et al. 2001)
Acute	17.9 ng/bee	na	(Iwasa et al. 2004)

Each dose is categorized as either acute or chronic indicating the number of times each dose was administered to an adult bee.

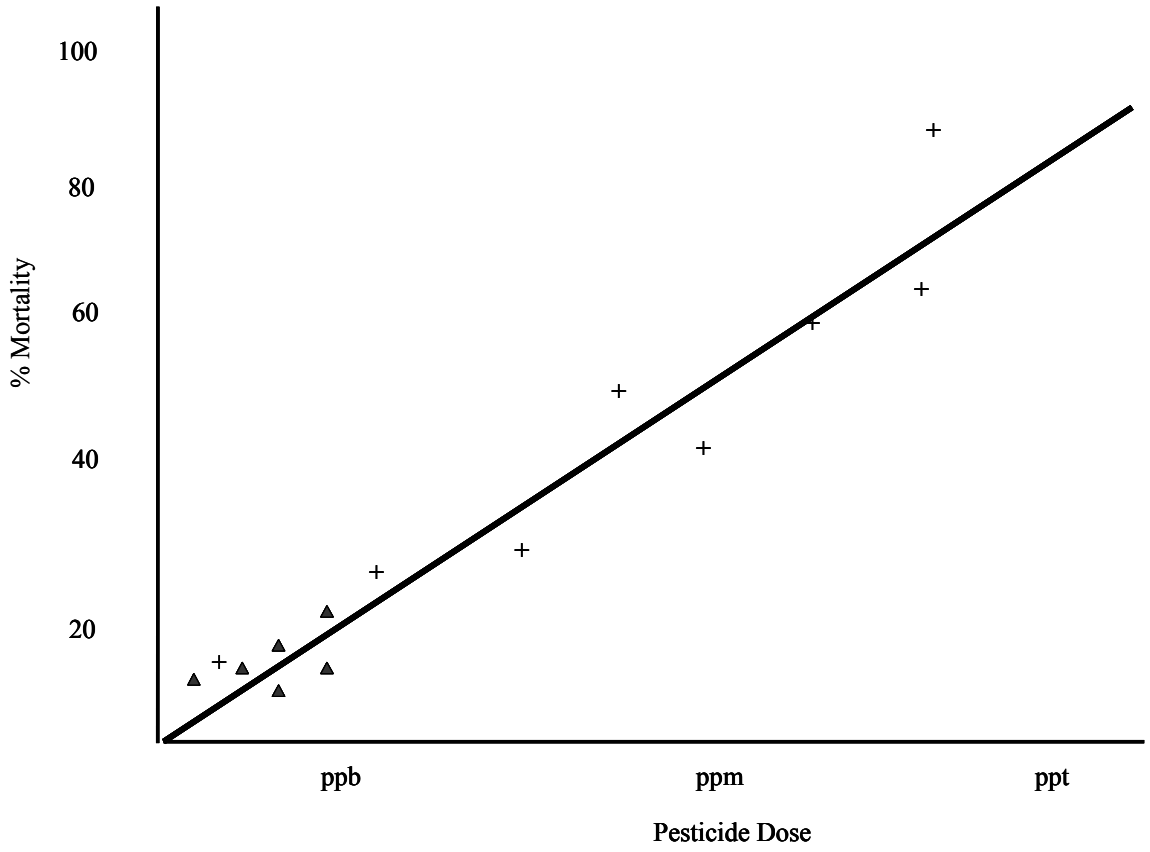

Figure 3-1. Hypothetical model depicting the range of imidacloprid doses tested in Chapter 2 (triangles, 5-80 ppb) and the range of doses likely to be tested in a full LD50 dose-response bioassay (plus signs, ppb-ppt).

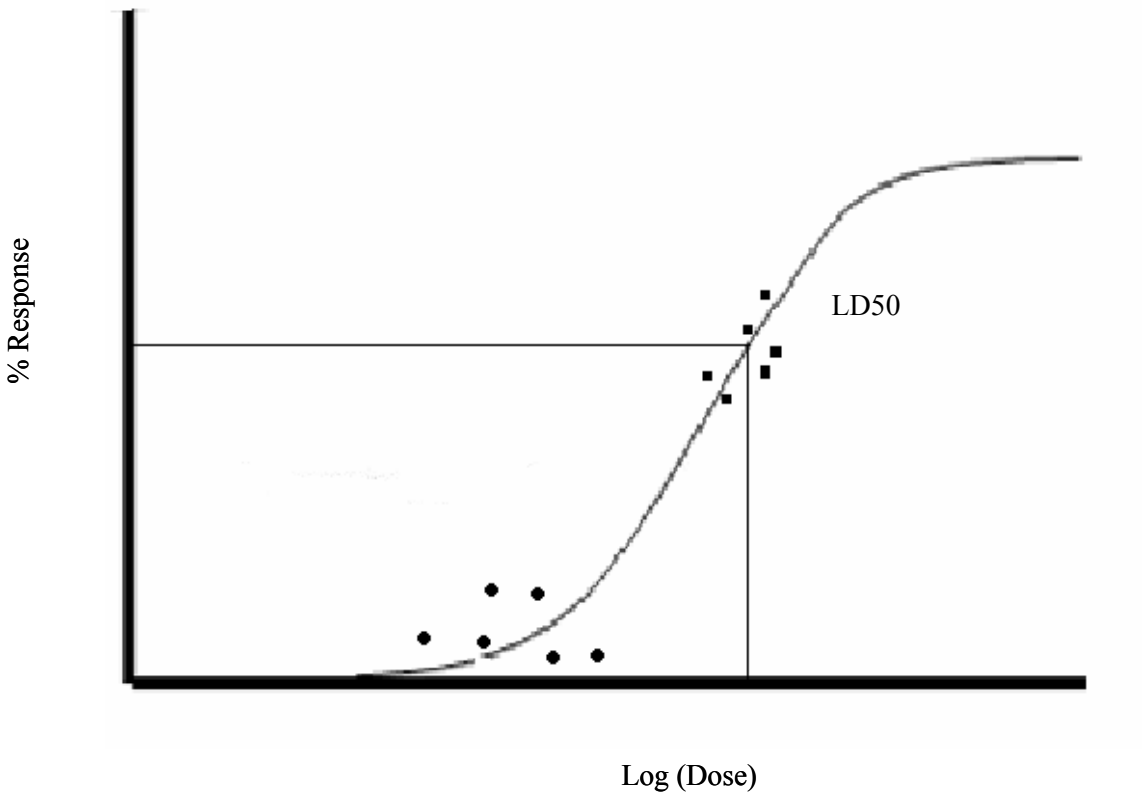

Figure 3-2. Hypothetical model illustrating a typical dose-response curve. Squares indicate mortality values close to the LD50. Larger variations or "wobble" are likely to occur at mortalities farther from the LD50 value (circles).

APPENDIX A
METHOD FOR TOPICAL APPLICATION OF SUBCHRONIC IMIDACLOPRID AND AMITRAZ DOSES TO HONEY BEE LARVAE

I investigated a method for topically applying imidacloprid and amitraz to developing bee larvae. I diluted both pesticides (imidacloprid and amitraz) in acetone (Fischer Chemical, Fair Lawn, NJ) to create 5 treatment stock solutions per pesticide. I stored the stock solutions at -20^{o}C in 4 mL-brown amber vials (FischerBrand, Fischer Scientific). For imidacloprid (Chem Service, West Chester, PA), I created 2.5, 5 , 10, 20, and 40 ppb treatment stock solutions while for amitraz (Chem Service, West Chester, PA), I created 15.62, 31.25, 62.5, 125, and 250 ppb treatment stock solutions. For both pesticides, I included two controls: (1) a 1 µl drop of acetone and (2) no pesticide/acetone application.

Following the general rearing procedure outlined in Chapter 2, I collected same-aged larvae from 5 different queen sources. Once the larvae were 156 h old, I applied a 1 µl drop of stock solution to the top of each larva using a Hamilton micro-applicator (Hamilton Company, Reno, NV). I repeated this procedure for 6 larvae × 7 treatments × 5 queen sources. For all treatments (5 levels of pesticide and 2 controls), I applied the doses once at hours 156, 180, 204, and 228. All developing larvae were maintained in the dark at 35^{o}C and ~96% RH (Vandenberg and Shimanuki 1987). Both imidacloprid (Zheng and Liu 1999) and amitraz (Hollingworth 1976) are stable at 35^{o}C.

I determined if doses of imidacloprid and amitraz were lethal to immature bees by comparing mortality data between chemically treated and control larvae. Mortality variables included number of larvae that defecated, pupal survival (number of larvae to emerge as adults from those that defecated), and adult emergence (total number of bees to reach adulthood from those that were grafted). I measured a number of variables to identify potential sublethal effects of these chemicals on developing brood. These measurements included time to larval defecation, larval weight at defecation, time to adult emergence, adult bee weight, and adult bee head weight.

Imidacloprid. Data for percent of larvae that defecated, pupal survival, and adult bee emergence are recorded in Table A-1. I did not find significant differences between treatments for percentage of larvae to defecate ($\chi^2 = 3.36$; df = 6; $P = 0.76$), pupal survival ($\chi^2 = 2.93$; df = 6; $P = 0.82$), or adult bee emergence ($\chi^2 = 4.09$; df = 6; $P = 0.66$).

Sublethal data are reported in Table A-2. There was no queen × treatment interaction ($F = 0.92$; df = 24, 107; $P = 0.58$) or treatment effect ($F = 1.52$; df = 6, 107; $P = 0.18$) for time to larval defecation. I did find a significant queen source effect on time to larval defecation ($F = 42.20$; df = 4, 107; $P < 0.01$) in which larvae from queen sources 112 and 103 (259.2 ± 2.4 (27) and 266.4 ± 2.4 (29) h, respectively) defecated later than larvae from queen sources 104 (223.2 ± 2.4 (24) h), 110 (230.2 ± 2.6 (27) h), and 114 (235.2 ± 2.4 (35) h).There was no queen × treatment interaction ($F = 0.71$; df = 24, 107; $P = 0.83$) or treatment effect ($F = 0.37$; df = 6, 107; $P = 0.90$) on larval weight at defecation. There was a significant effect of queen source on larval weight at defecation ($F = 4.65$; df = 4, 107; $P < 0.01$). Larvae from queens 103 (114.6 ± 3.8 (30) mg) and 114 (114.7 ± 3.1 (35) mg) were significantly heavier upon defecation than larvae from queen sources 110 (97.4 ± 2.7 (27) mg) and 104 (103.7 ± 2.5 (24) mg). I did not find a queen × treatment interaction ($F = 1.61$; df = 6, 6; $P = 0.29$), treatment effect ($F = 1.08$; df = 6, 6; $P = 0.46$), or queen source effect ($F = 3.77$; df = 4, 6; $P = 0.07$) for time to adult emergence. Finally, there was no queen × treatment interaction ($F = 2.56$; df = 6, 6; $P = 0.14$) or treatment effect ($F = 2.77$; df = 6, 6; $P = 0.12$) for adult bee head weight. Emerging adults from queen 112 had the

heaviest adult head weights (13.9 ± 1.6 (3) mg) compared to head weights from emerging adults from all queen sources 103 (10.6 ± 0.6 (6) mg), 104 (11.7 ± 1.0 (2) mg), 110 (10.1 ± 0.4 (2) mg), and 114 (10.5 ± 0.2 (10) mg) ($F = 7.22$; df = 4, 6; $P < 0.02$).

Amitraz. For amitraz treated larvae I found no significant differences between treatments for percent of larvae that defecated ($\chi^2 = 9.55$; df = 6; $P = 0.14$), pupal survival ($\chi^2 = 2.19$; df = 6; $P = 0.90$), or adult bee emergence ($\chi^2 = 3.00$; df = 6; $P = 0.81$) (table A-3). Data for sublethal effects on time to larval defecation, larval weight at defecation, time to adult emergence, and adult bee head weights are recorded in Tables A-4 and A-5. I did not find a queen × treatment interaction ($F = 0.97$; df = 24, 97; $P = 0.51$) or treatment effect ($F = 0.98$; df = 6, 97; $P = 0.44$) for time to larval defecation. I did find a significant effect of queen source ($F = 46.46$; df = 4, 97; $P < 0.01$). Larvae from queen sources 103 (261.6 ± 2.4 (23) h) and 112 (259.2 ± 2.4 (28) h) took significantly longer to defecate than larvae from queen sources 104 (228.0 ± 2.4 (24) h), 110 (228.0 ± 2.4 (25) h), and 114 (9.6 ± 0.1 (32) mg). I did not find a queen × treatment interaction ($F = 1.05$; df = 24, 98; $P = 0.41$), treatment effect ($F = 0.19$; df = 6, 98; $P = 0.98$), or queen source effect ($F = 0.19$; df = 4, 98; $P = 0.43$) for larval defecation weight. There was a significant queen × treatment interaction ($F = 5.47$; df = 6, 7; $P = 0.02$) for time to adult emergence. I analyzed time to larval emergence individually according to queen, but I could not detect any trends within the data because of the small sample sizes (Table A-5).There was no queen × treatment interaction ($F = 0.40$; df = 6, 7; $P = 0.86$), treatment effect ($F = 1.05$, df = 6, 7; $P = 0.47$), or queen source effect ($F = 0.15$; df = 4, 7; $P = 0.96$) for adult bee head weight.

The results from this investigation were not incorporated into the main body of my thesis because I felt the data produced during this test were unreliable. I believe the data to be unreliable for 4 reasons. First, the control larvae experienced a high mortality overall and results for untreated and acetone treated larvae were not consistent. Second, I believe that a considerable amount of the mortality larvae experienced while developing was caused by the method of applying the various treatments with the micro applicator. After some treatment applications, the larvae appeared to have been punctured by the applicator. Third, a large number of bees died while pupating. I believe this occurred due to my use of salt solutions to control the relative humidity. Once I switched to using a temperature and humidity controlled incubator, I had better success at rearing larvae. Finally, hydrophilic materials such as imidacloprid are not likely to easily cross the insect cuticle suggesting that the LD50 values for topically applied substances will be larger than orally applied ones (Yu 2008).

Initially, I chose to test topical treatment applications because this would have been the easiest method to replicate in the field (Appendix C). The inability to transfer laboratory experiments into the field is often termed the "lab-to-field dilemma" due to the difficulties that surround the process (Landis and Yu 1995). However, after I discovered the difficulties associated with topically applying pesticides, I decided to try applying the pesticides at larger doses once during larval development (Appendix B) rather than at smaller doses multiple times.

Table A-1. Percentage of larvae that defecated, pupal survival, and adult bee emergence for larvae treated topically with imidacloprid for 4 days.

Treatment	Larvae that defecated[a] (%)	Pupal survival[b] (%)	Adult bee emergence[c] (%)
Untreated	67 ± 9.4 (5)a	29.3 ± 14.4 (5)a	23.3 ± 11.3 (5)a
Acetone	67.3 ± 9.3 (5)a	6.7 ± 6.7 (5)a	3.3 ± 3.3 (5)a
2.5	69.3 ± 5.9 (5)a	19 ± 9.3 (5)a	14 ± 6.3 (5)a
5	73.3 ± 13.5 (5)a	26.7 ± 13.5 (5)a	13.3 ± 6.2 (5)a
10	76.7 ± 4.1 (5)a	12 ± 4.9 (5)a	10 ± 4.1 (5)a
20	76.7 ± 11.3 (5)a	10.7 ± 6.9 (5)a	10 ± 6.7 (5)a
40	62 ± 3.3 (5)a	10 ± 6.1 (5)a	6.7 ± 4.1 (5)a

Imidacloprid concentrations are in ppb. Data are mean ± std. error (n) where n = number of queen sources used. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] % of larval that defecated = (the number of larvae that defecated/the total number of treated larvae) × 100. [b] % pupal survival = (the number of adult bees that emerged/the number of larvae that defecated) × 100. [c] % adult bee emergence = (the number of adult bees that emerged/the total number of treated larvae) × 100.

Table A-2. Mean time to larval defecation, larval weight at defecation, time to adult bee emergence, and adult bee head weight for larvae treated topically with imidacloprid for 4 days.

Treatment	Time to larval defecation (h)	Larval weight at defecation (mg)	Time to adult bee emergence (h)	Adult bee head weight (mg)
Untreated	247.2 ± 4.7 (18)a	111.7 ± 3.3 (18)a	508.8 ± 4.1 (6)a	10.3 ± 0.4 (6)a
Acetone	244.8 ± 6.2 (20)a	107.5 ± 5.3 (19)a	480 (1)a	10.6 (1)a
2.5	247.2 ± 4.6 (20)a	107.6 ± 4.8 (20)a	508.8 ± 11.5 (4)a	10.8 ± 1.1 (4)a
5	240 ± 4 (22)a	104.9 ± 3.3 (22)a	508.8 ± 6 (4)a	12.9 ± 1.4 (4)a
10	247.2 ± 5 (23)a	110.3 ± 3.2 (23)a	520.8 ± 7.9 (3)a	10.8 ± 0.3 (3)a
20	240 ± 4.8 (22)a	111.6 ± 5 (22)a	520.8 ± 7.9 (3)a	10.6 ± 0.6 (3)a
40	237.6 ± 5.6 (17)a	105.9 ± 4.4 (18)a	528 ± 24 (2)a	11.6 ± 0.9 (2)a

Imidacloprid treatments are in ppb. Data are mean ± std. error (n) where n = number of individuals used, pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

Table A-3. Percentage of larvae that defecated, pupal survival, and adult bee emergence for larvae treated topically with amitraz for 4 days.

Treatment	Larvae that defecated[a] (%)	Pupal survival[b] (%)	Adult bee emergence[c] (%)
Untreated	80 ± 9.7 (5)a	17 ± 7.7 (5)a	14 ± 6.3 (5)a
Acetone	58.7 ± 3.7 (5)a	18.3 ± 13 (5)a	10 ± 6.7 (5)a
15.62	75.3 ± 7.3 (5)a	29 ± 9.5 (5)a	23.3 ± 8.5 (5)a
31.25	52 ± 5.6 (5)a	20 ± 13.3 (5)a	10 ± 6.7 (5)a
62.5	68.7 ± 13 (5)a	14 ± 9.8 (5)a	11.3 ± 7.8 (5)a
125	70 ± 9.7 (5)a	13.3 ± 8.2 (5)a	7.3 ± 4.5 (5)a
250	78 ± 7 (5)a	11.7 ± 7.3 (5)a	8 ± 4.9 (5)a

Amitraz concentrations are in ppb. Data are mean ± std. error (n) where n = number of queen sources used. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] % of larvae that defecated = (the number of larvae that defecated/the total number of treated larvae) × 100. [b] % of pupal survival = (the number of adult bees that emerged/the number of larvae that defecated) × 100 [c] % of adult bee emergence = (the number of adult bees that emerged/the total number of treated larvae) × 100.

Table A-4. Mean time to larval defecation, larval weight at defecation, and adult bee head weight for larvae treated topically with amitraz for 4 days.

Treatment	Time to larval defecation (h)	Larval weight at defecation (mg)	Adult bee head weight (mg)
Untreated	242.4 ± 103.2 (22)a	105.5 ± 3.1 (22)a	9.7 ± 1 (4)a
Acetone	244.8 ± 115.2 (17)a	105.7 ± 5.1 (17)a	9.3 ± 1 (3)a
15.62	244.8 ± 120 (22)a	105.5 ± 3.7 (22)a	11 ± 0.5 (7)a
31.25	237.6 ± 120 (15)a	103.7 ± 3.3 (15)a	11.1 ± 0.2 (3)a
62.5	240 ± 115.2 (19)a	103.7 ± 4 (19)a	10.1 ± 0.7 (3)a
125	240 ± 110.4 (19)a	100.7 ± 3.7 (20)a	12.1 ± 0.6 (2)a
250	240 ± 103.2 (18)a	104.8 ± 3.7 (18)a	8.6 ± 0.1 (2)a

Amitraz treatments are in ppb. Data are mean ± std. error (n) where n = number of individuals used, pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

Table A-5. Mean time to adult emergence in hours for larvae treated topically with amitraz for 4 days.

	Queen source				
Treatment	103	104	110	112	114
Untreated	528 (1)	528 (1)a	na	na	516 ± 12 (2)a
Acetone	600 (1)	na	na	na	492 ± 12 (2)a
15.62	na	492 ± 12 (2)a	480 (1)a	528 (1)a	504 (3)a
31.25	na	na	492 ±12 (2)a	528 (1)a	na
62.5	na	480 (1)a	na	552 (2)b	na
125	528 (1)	na	504 (1)a	na	na
250	na	528 (1)a	504 (1)a	na	na
ANOVA	F = na; df = 0, 0; P = na	F = 2.33; df = 3, 1; P = 0.44	F = 0.47; df = 3, 1; P = 0.76	F = infty; df = 2, 1; P < 0.01	F = 2.00; df = 2, 4; P = 0.25

Amitraz treatments are in ppb. Data are mean ± std. error (n) where n = number of individuals used, pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

APPENDIX B
METHOD FOR TOPICAL APPLICATION OF ACUTE IMIDACLOPRID DOSES TO HONEY BEE LARVAE

The purpose of this investigation was to develop a method for testing the effects of topically applying acute doses of imidacloprid to developing bee larvae. In appendix A, my findings suggested that larvae can be damaged by applying treatments using a micro applicator. As such, I hypothesize that larvae treated only once during their development would be more likely to survive treatment since their exposure to the applicator would be reduced. Consequently, I chose to use only one pesticide (imidacloprid) in the current study to determine if I could improve larval survivability. To accomplish this, I diluted imidacloprid in acetone (Fischer Chemical, Fair Lawn, NJ) to create 5 treatment stock solutions (5, 50, 100, 250, and 500 ppb imidacloprid/1 µl drop) and stored the solutions at -20^{o}C in 4 mL-brown amber vials (FischerBrand, Fischer Scientific). I increased the doses in this study over those used in Appendix A because I treated each larva only once.

Following the general rearing procedure outlined in Chapter 2, I collected same-aged larvae from 3 different queen sources and grafted them into 96-well plates. When the larvae were 156 h old, I applied 1 µl of one imidacloprid stock solution onto the larva's exposed surface using a Hamilton micro-applicator (Hamilton Company, Reno, NV). I repeated this procedure for 6 larvae per stock solution for each queen's offspring. Furthermore, I included 2 controls for each queen, the first with a 1 µl drop of acetone and the second with no acetone or pesticide application. This also was repeated for 6 larvae per queen. To see if larvae of varying ages were more susceptible to topical imidacloprid applications, I repeated the entire procedure on larvae from ages 156, 180, 204, and 228 h. All developing larvae were maintained in the dark at 35^{o}C and ~96% RH (Vandenberg and Shimanuki 1987).

I determined if doses of imidacloprid were lethal by comparing mortality data between chemically treated and control larvae. Mortality variables included number of larvae that defecated, pupal survival (number of larvae to emerge as an adult from those that defecated), and adult emergence (total number of bees to reach adulthood from those that were grafted). I measured a number of variables to identify potential sublethal effects of imidacloprid on developing brood. These measurements included time to larval defecation, larval defecation weight, time to adult emergence, adult bee weight, and adult bee head weight.

Data for percent of larvae that defecated, pupal survival, and adult bee emergence are recorded in Tables B-1, B-2, & B-3. There was no significant day × treatment interaction (F = 0.96; df = 18, 56; P = 0.51) or treatment effect (F = 0.47; df = 6, 56; P = 0.83) on percent of larvae that defecated. There was a significant day effect in which larvae treated only at 84 h (79.4% ± 3.4) had lower percent larval defecation than larvae treated only at 132 h (94.4% ± 2.3) (F = 3.66; df = 3, 56; P = 0.02). Larvae treated at 108 h and 156 h were not different from those treated at 84 h and 132 h. There was no significant day × treatment interaction (F = 0.48; df = 18, 56; P = 0.96), day effect (F = 1.26; df = 6, 56; P = 0.29), or treatment effect (F = 0.11; df = 3, 56; P = 0.95) on pupation success. There was no significant day × treatment interaction (F = 0.45; df = 18, 56; P = 0.97), day effect (F = 1.08; df = 6, 56; P = 0.38), or treatment effect (F = 0.24; df = 3, 56; P = 0.87) on adult bee emergence.

Of the response variables tested, there were no significant sublethal effects of imidacloprid on treated larvae (Table B-4, B-5, B-6, & B-7). I did not find a day × treatment interaction (F = 0.73; df = 18, 307; P = 0.78), treatment effect (F = 0.28; df = 6, 307; P = 0.95) or day effect (F = 0.97; df = 3, 307; P = 0.41) for time to larval defecation. There was no day × treatment

interaction ($F = 1.04$; df = 18, 304; $P = 0.41$), treatment effect ($F = 1.45$; df = 6, 304; $P = 0.21$), or day effect ($F = 0.98$; df = 3, 304; $P = 0.42$) on larval weight at defecation. There was no day × treatment interaction ($F = 0.94$; df = 17, 62; $P = 0.54$), treatment effect ($F = 0.43$; df = 6, 62; $P = 0.86$), or day effect ($F = 0.39$; df = 3, 62; $P = 0.76$) for time to adult emergence. There was no queen × treatment interaction ($F = 1.54$; df = 17, 62; $P = 0.11$), treatment effect ($F = 1.33$; df = 6, 62; $P = 0.25$), or day effect ($F = 0.68$; df = 3, 62; $P = 0.57$) for adult bee head weight.

Results from this investigation were similar to those in Appendix A in that these data do not indicate the presence/absence of effects associated with treating developing larvae once with acute doses of imidacloprid. The primary reason for this is that there was high mortality among control larvae, indicating that the rearing procedure had not been perfected. I did not see differences between treated and untreated larvae. This was likely caused by the fact that some chemicals and solvents, especially hydrophilic ones, do not easily penetrate the insect cuticle (Yu 2008). I believe that this can be corrected by feeding larvae diet containing pesticides rather than topically applying the pesticide. Secondly, I will no longer use salt solutions to control humidity in the test chambers because I believe that better results can be achieved using a temperature and humidity controlled incubator.

Table B-1. Percentage of larvae that defecated for larvae treated topically with imidacloprid once during their development.

	Larvae that defecated[a] (%)			
Treatment	84	108	132	156
Untreated	66.7 (3)a	87.8 ± 6.2 (3)a	91.7 ± 8.3 (3)a	93.3 ± 6.7 (3)a
Acetone	72.2 ± 14.7 (3)a	83.3 ± 9.6 (3)a	100 (3)a	93.3 ± 6.7 (3)a
5	83.3 ± 9.6 (3)a	100 (3)a	94.4 ± 5.5 (3)a	88.9 ± 11.1 (3)a
50	94.4 ± 5.5 (3)a	69.4 ± 10 (3)a	88.9 ± 11.1 (3)a	83.3 ± 9.6 (3)a
100	72.2 ± 5.5 (3)a	77.8 ± 11.1 (3)a	100 (3)a	85.0 ± 7.6 (3)a
250	83.3 ± 9.6 (3)a	94.4 ± 5.5 (3)a	91.7 ± 8.3 (3)a	83.3 ± 16.7 (3)a
500	83.3 ± 9.6 (3)a	94.4 ± 5.5 (3)a	94.4 ± 5.5 (3)a	83.3 ± 9.6 (3)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] % of larvae that defecated = (the number of larvae that defecated/the total number of treated larvae) × 100.

Table B-2. Percentage of pupal survival for pupae treated topically as larvae with imidacloprid once during their development.

	Pupal survival[a] (%)			
Treatment	84	108	132	156
Untreated	33.3 ± 8.3 (3)a	32.8 ± 4.3 (3)a	50.0 ± 25.4 (3)a	38.9 ± 20 (3)a
Acetone	11.1 ± 11.1 (3)a	33.3 ± 33.3 (3)a	33.3 ± 33.3 (3)a	13.3 ± 13.3 (3)a
5	21.7 ± 11.7 (3)a	27.8 ± 20 (3)a	28.9 ± 19.7 (3)a	38.9 ± 13.9 (3)a
50	43.3 ± 23.3 (3)a	55.5 ± 29.4 (3)a	16.7 ± 16.7 (3)a	31.7 ± 9.3 (3)a
100	6.7 ± 6.7 (3)a	0 (3)a	6.7 ± 6.7 (3)a	30.5 ± 19.4 (3)a
250	20.0 ± 20 (3)a	27.8 ± 14.7 (3)a	48.9 ± 24.7 (3)a	33.3 ± 16.7 (3)a
500	57.8 ± 21.2 (3)a	36.7 ± 18.5 (3)a	24.4 ± 12.4 (3)a	48.3 ± 25.9 (3)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] % pupal survival = (the number of adult bees that emerged/the number of larvae that defecated) × 100.

Table B-3. Percentage of adult bee emergence for adult bees treated as larvae topically with imidacloprid once during their development.

	Adult bee emergence[a] (%)			
Treatment	84	108	132	156
Untreated	22.2 ± 5.5 (3) a	28.9 ± 4.4 (3) a	47.2 ± 26.5 (3) a	38.9 ± 20 (3) a
Acetone	11.1 ± 11.1 (3) a	33.3 ± 33.3 (3) a	33.3 ± 33.3 (3) a	13.3 ± 13.3 (3) a
5	16.7 ± 9.6 (3) a	27.8 ± 20.0 (3) a	27.8 ± 20.3 (3) a	36.1 ± 15.5 (3) a
50	38.9 ± 20.0 (3) a	33.3 ± 16.7 (3) a	11.1 ± 11.1 (3) a	25 ± 4.8 (3) a
100	5.5 ± 5.5 (3) a	0 (3) a	6.7 ± 6.7 (3) a	23.3 ± 14.5 (3) a
250	16.7 ± 16.7 (3) a	27.8 ± 14.7 (3) a	48.9 ± 24.7 (3) a	33.3 ± 16.7 (3) a
500	44.4 ± 11.1 (3) a	33.3 ± 16.7 (3) a	22.2 ± 11.1 (3) a	36.1 ± 15.5 (3) a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. [a] % of adult bee emergence = (the number of adult bees that emerged/the total number of treated larvae) × 100.

Table B-4. Mean time to larval defecation for larvae treated topically with imidacloprid once during their development.

	Time to larval defecation (h)			
Treatment	84	108	132	156
Untreated	10.1 ± 0.2 (10)a	9.9 ± 0.1 (12)a	9.8 ± 0.4 (10)a	10.2 ± 0.2 (13)a
Acetone	10.4 ± 0.1 (11)a	9.9 ± 0.2 (12)a	9.9 ± 0.3 (13)a	9.8 ± 0.2 (12)a
5	10.5 ± 0.3 (12)a	10.1 ± 0.2 (15)a	10 ± 0.1 (14)a	9.7 ± 0.2 (12)a
50	10 (14)a	9.9 ± 0.2 (9)a	10.3 ± 0.5 (12)a	9.7 ± 0.1 (11)a
100	10.3 ± 0.3 (11)a	10 ± 0.2 (12)a	10.1 ± 0.4 (13)a	9.8 ± 0.2 (12)a
250	9.9 ± 0.1 (12)a	9.8 ± 0.1 (14)a	9.7 ± 0.1 (11)a	10.1 ± 0.3 (7)a
500	9.9 ± 0.1 (13)a	10.1 ± 0.1 (13)a	10.1 ± 0.4 (14)a	10.2 ± 0.3 (11)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of individuals from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

Table B-5. Mean larval weight at defecation for larvae treated topically with imidacloprid once during their development.

	Larval weight of defecation (mg)			
Treatment	84	108	132	156
Untreated	117.8 ± 9.9 (10)a	124.4 ± 5.5 (12)a	117.2 ± 8.3 (10)a	123.6 ± 6.6 (13)a
Acetone	125.3 ± 10.6 (11)a	122 ± 6.9 (12)a	113.2 ± 5.8 (13)a	116.8 ± 6.6 (12)a
5	134.4 ± 6.4 (11)a	121.9 ± 5.4 (15)a	120.6 ± 4.5 (14)a	110.4 ± 5.4 (12)a
50	136.1 ± 2.8 (14)a	127.8 ± 5.4 (9)a	125.3 ± 9.1 (11)a	120.4 ± 5.6 (11)a
100	123.2 ± 6.2 (11)a	118.5 ± 6 (12)a	123.5 ± 6.5 (12)a	127.7 ± 6.2 (12)a
250	129.9 ± 5.5 (12)a	113.9 ± 6.1 (14)a	122.5 ± 4 (11)a	137.6 ± 9.3 (7)a
500	124.8 ± 6.7 (13)a	127.6 ± 6.6 (13)a	133.9 ± 5.2 (14)a	134 ± 9 (11)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of individuals pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

Table B-6. Mean time to adult emergence for larvae treated topically with imidacloprid once during their development.

	Time to adult emergence (h)			
Treatment	84	108	132	156
Untreated	504 ± 13.8 (3)a	504 (4)a	488 ± 8 (3)a	499.2 ± 4.8 (5)a
Acetone	504 (2)a	488 ± 8 (3)a	492 ± 12.0 (2)a	504 (2)a
5	504 (3)a	496. ± 8 (3)a	504 ± 13.8 (3)a	498 ± 6 (4)a
50	500.6 ± 3.4 (7)a	488 ± 8 (3)a	504 (1)a	488 ± 8 (3)a
100	504 (1)a	na	504 (1)a	512 ± 8 (3)a
250	496 ± 8 (3)a	492 ± 6.9 (4)a	500 ± 4 (6)a	504 ± 13.8 (3)a
500	488. ± 5 (6)a	504 (4)a	496 ± 8 (3)a	498 ± 6 (4)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of individuals from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

Table B-7. Mean adult bee head weight for adult bees treated topically as larvae with imidacloprid once during their development.

	Adult bee head weight (mg)			
Treatment	84	108	132	156
Untreated	12 ± 1.3 (3)a	10.2 ± 0.1 (4)a	10.3 ± 0.7 (3)a	11.5 ± 0.9 (5)a
Acetone	12.3 ± 0.4 (2)a	10.4 ± 0.6 (3)a	10.3 ± 1.3 (2)a	9.9 ± 0.7 (2)a
5	11.7 ± 0.1 (3)a	12.1 ± 0.4 (3)a	1.2 ± 0.5 (3)a	10.7 ± 0.2 (4)a
50	11.3 ± 0.4 (7)a	11.5 ± 0.5 (3)a	11.4 (1)a	9.6 ± 0.8 (3)a
100	13.1 (1)a	na	12.9 (1)a	11.9 ± 0.4 (3)a
250	19 ± 0.6 (3)a	9.4 ± 0.7 (4)a	11.9 ± 0.5 (6)a	11.4 ± 1.3 (3)a
500	10.4 ± 0.5 (6)a	10.6 ± 0.4 (4)a	11.1 ± 1 (3)a	11.5 ± 0.5 (4)a

Imidacloprid concentrations are in ppb and were administered to larvae either at hours 84, 108, 132, or 156 h. Data are mean ± std. error (n) where n = number of individuals pooled from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

APPENDIX C
METHOD FOR TESTING THE ATTRACTION OF FOUNDRESS VARROA MITES TO IMIDACLOPRID AND AMITRAZ TREATED LARVAE

Investigators have recognized many factors that could contribute to a decline in the overall health of honey bees. Such factors include pesticides, parasites, pathogens, Africanized bees, and other stressors such as transporting colonies for pollination services (Johansen and Mayer 1990, Morse and Flottum 1997). Unfortunately, most studies on bee decline focus on a single stressor in an effort to reduce experimental variation. This can lead one to ignore/overlook the possibility of interactions between multiple stressors affecting honey bee health.

In this study, I attempted to develop a method to investigate an interaction between 2 previously mentioned stressors: pesticides and parasites. More specifically, I measured how imidacloprid and amitraz affected larval honey bee attraction to varroa mites (*Varroa destructor* Anderson and Truemann), recognizing that pesticides and varroa may interact synergistically to kill larvae. The data I present in Chapter 2 provides the framework for the research presented in this chapter. In Chapter 2, I discussed how larval development is affected when larvae feed on a diet containing pesticides. I discovered that if larvae were treated with imidacloprid (5-80 ppb) or amitraz (50-400 ppb), they were less likely to survive to adulthood. In addition to the lethal and sublethal effects described in Chapter 2, pesticides may affect other aspects of honey bee larval life history including their susceptibility to parasites. I chose to use varroa mites in this investigation because they are considered the leading cause of honey bee colony deaths worldwide (DeJong 1997, Berenbaum et al. 2007, Board 2008) and a possible cause of current global declines in managed bee colonies (Stokstad 2007).

Varroa are a useful model for studying synergistic interactions between pesticides and bee pests on bee larvae because varroa life history overlaps with the honey bee brood cycle (Figure 3-1). Varroa reproduce only in brood cells, invading the cells 15 - 20 hours prior to the brood cell being capped by worker bees. Biological factors affecting varroa decision to invade brood cells have been studied (Fuchs 1990, Boot et al. 1994, Bienefeld et al. 1998, Beetsma et al. 1999, Piccirillo and De Jong 2004). However, there is a modicum of data on the effects of non-biological factors (such as larval pesticide stress) on mite invasion into brood cells. In one such investigation, Ellis and Delaplane (2001) did not find that colony treatment with Fumidil B® (fumagillin) and/or Terramycin® (oxytetracycline) influenced varroa invasion into brood cells. However, no similar studies exist on other chemical agents.

I chose to investigate imidacloprid and amitraz because both are associated closely with honey bee colonies—imidacloprid due to its systemic properties and widespread use (Elbert et al. 1991, Bonmatin et al. 2003, Chauzat et al. 2006, Yu 2008) and amitraz because it has been known to be applied directly into managed colonies by beekeepers in an effort to control varroa.

Same-aged larvae were collected following the methods outlined in Chapter 2. To produce the larvae, a queen was confined to a newly-drawn frame (no stored honey, pollen, or brood) using 1 of 2 different sized zinc queen excluder cages (96 × 106 × 21 mm; 165 × 130 × 24 mm) at time t = -12 h. The caged queen and frame were returned to the center of the brood nest (Boot and Calis 1991). After 24 h, t = +12 h (Peng et al. 1992, Aupinel et al. 2005), the queen was removed from the cage and the cage secured to the comb as before but this time for 132 h (from t = 0) to allow the eggs to hatch and larvae to grow large enough to treat (Figure 3-2). Worker bees exclusively were able to access the comb in order to feed the new larvae hatching from the eggs.

I treated the larvae with one of 2 different pesticides (amitraz or imidacloprid) at one of 5 different doses per pesticide. The tested imidacloprid doses were 100, 200, 400, 800, and 1600 ppb and amitraz doses were 500, 1000, 2000, 4000, and 8000 ppb. These doses were obtained by determining the specific quantity of pesticide received by a larva in 20 µL of diet during the oral toxicity test outlined in Chapter 2. For example, the lowest dose of amitraz mixed into larval diet was 25 ppb (Chapter 2). The lowest pesticide concentration in the current experiment was equal to 500 ppb. I calculated this by determining that 25 ppb of amitraz in 20 µL of larval diet is equivalent to 500 ppb in a 1 µL-drop. All pesticide doses were diluted in acetone to achieve the desired concentration. I included two control treatments for both pesticides, with one series of larvae receiving acetone only and the second series receiving nothing. I chose to use acetone as the pesticide solvent because it resulted in fewer larvae being removed from the combs by the bees when compared to bee removal of larvae treated with methanol or ethanol (Appendix D).

At 132 h, the caged frames (now containing 60 ± 12 h old larvae) were removed from the colonies and taken to the lab to be treated. At the laboratory, I placed a sheet of transparency paper (Office Depot Inc, Delray Beach, FL) over the frame to cover the group of similar-aged larvae. I then taped the sheet down on 2 sides to hold it in place on the frame. Using a permanent marker, I marked 14 diagonal rows of 10 larvae per row on the transparency paper. This permitted me to use the paper as a "map" to find my treated larvae on subsequent days.

After marking the rows on the paper, I labeled each row 1-14 and then randomly assigned each row a treatment (5 doses of imidacloprid + 5 doses of amitraz + 4 controls = 14 treatments). I applied a given treatment to10 larvae in 1 µL droplets using a Hamilton micro-applicator (Hamilton Company, Reno, NV). To prevent the larvae from being damaged during treatment applications (see Appendix A & B), I did not touch the applicator to the larvae and instead "sprayed" the droplet into the cell. Once treated, the larvae were returned to their original colony until the following day's treatment. I treated all larvae consecutively for 3 days at 132 h, 156 h, and 180 h post oviposition and repeated this procedure for larvae from 9 queens. After the third treatment application, the larvae were returned to their original colonies. Varroa foundresses enter brood cells immediately before the cells are capped (Morse and Flottum 1997). Because cells containing larvae are capped by worker bees at ~204 h post oviposition (Winston 1987), I left treated larvae in the test colonies for 228 – 253 h. Following this period, I removed the treated comb sections from their respective hives, uncapped all remaining treated brood cells, removed the prepupae/pupae within, and counted the number of foundress varroa mites per cell.

To confirm varroa presence in test colonies, I estimated varroa populations in the colonies using varroa sticky-screens placed on top of the bottom board of the hive (Devlin 2001). I left the screens in the colonies for 2 days, after which I removed the screens and counted the varroa. This permitted me to determine if the test colonies were hosting similar varroa populations.

The effect of larval treatment history on the number of foundress mites per cell was analyzed using a 1-way ANOVA recognizing treatment as the main effect. All statistical tests were conducted using SAS (SAS Institute, 2008). I had a total of 2 rows for both controls (untreated and acetone) for both imidacloprid and amitraz, each row containing 10 larvae. Since both pesticide treatments were on the same frame, controls for both pesticides were pooled.

Although I treated 10 larvae per treatment (12 treatments) for each of 9 queens, I discovered that adding acetone to brood cells often caused the adult bees to abort the treated brood, regardless of the presence of pesticide (Appendix D). As such, I pooled like treatment data from each queen source to increase the sample size for the analysis. In summary: n = 9

queens × 12 treatments (5 imidacloprid, 5 amitraz, and 2 controls) × # larvae that were not aborted in each treatment.

Overall, I treated 1260 larvae with various doses of imidacloprid, amitraz, acetone, or nothing. Despite the volume of larvae treated, varroa mite invasion into brood cells was unaffected by the chemotherapeutic history of the host larvae ($F = 0.18$; df = 11, 211; $P = 0.99$; Table 3-1). There were no dead varroa present in any of the brood cells. Overall mite abundance was low (imidacloprid = 12; amitraz = 20 mites). This was calculated by summing the total number of mites found in every cell across all treatments.

Based on the method I used, I do not think testing varroa attraction to larvae would be achieved easily in the field. Furthermore, there is no conclusive evidence that suggests treatment of larvae with amitraz or imidacloprid influences varroa mite decision to invade worker brood cells. It is difficult to state with any certainty that imidacloprid and amitraz had no effect on varroa choice because overall, both pesticide groups contained low mite abundance. On average, tested colonies had 46.8 ± 16.0 (7) varroa/2 day sticky screen, mean ± std. error (n). The lack of dead mites in the cells suggests that even though the bee larvae were fed an insecticide/acaricide, lethal properties from the pesticide were not transferred through the pupae's hemolymph and fed on by mites resulting in mite death. The data also suggest that mites did not die as a result of contacting pesticide-laden food even though varroa foundresses are known to bury themselves in brood food until the cell is capped.

Difficulties in this experiment resulted from problems associated with administering pesticides to brood under field conditions. Past investigators used solitary bees, which mass provision their larvae, to successfully administer pesticide doses to individual larva because each larva was enclosed inside its own cell (Abbott et al. 2008). Consequently, the amount of pesticide consumed by the larva could be controlled. The difficulty with honey bees is that larvae are tended by worker bees who remove acetone-treated larvae from the hive. Why this occurred remains unknown.

Instead, future experiments testing my hypothesis could remain in the laboratory allowing the investigator to control for variables such as larval abortion. One way to test varroa attraction to pesticide treated brood in the lab would be to capture volatiles from treated brood and present them to varroa in controlled choice tests. A second method would be to rear and treat larvae in a laboratory as before and then release varroa onto the well plates, permitting them to invade cells at will. Such tests are not easily replicated in field colonies, but their results could indicate whether or not the chemotherapeutic history of larvae affects varroa invasion into brood cells.

Table C-1. Mean number of varroa found alive in capped cells containing either imidacloprid or amitraz fed larvae.

Treatment	Mite abundance
Untreated	0.14 ± 0.06 (73)a
Acetone	0.09 ± 0.05 (33)a
Imidacloprid 100	0.07 ± 0.07 (15)a
Imidacloprid 200	0.06 ± 0.06 (16)a
Imidacloprid 400	0.21 ± 0.11 (14)a
Imidacloprid 800	0.07 ± 0.07 (14)a
Imidacloprid 1600	0.10 ± 0.07 (19)a
Amitraz 500	0.12 ± 0.08 (16)a
Amitraz 1000	0.12 ± 0.12 (17)a
Amitraz 2000	0.13 ± 0.07 (23)a
Amitraz 4000	0.12 ± 0.08 (16)a
Amitraz 8000	0.14 ± 0.10 (14)a

Prepupae/pupae were removed from capped brood cells between 228-253 h and the number of varroa foundresses counted. Imidacloprid and amitraz doses are given in ppb/1 μL drop. Data are mean ± std. error (n) where n = number of capped cells pooled across all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$.

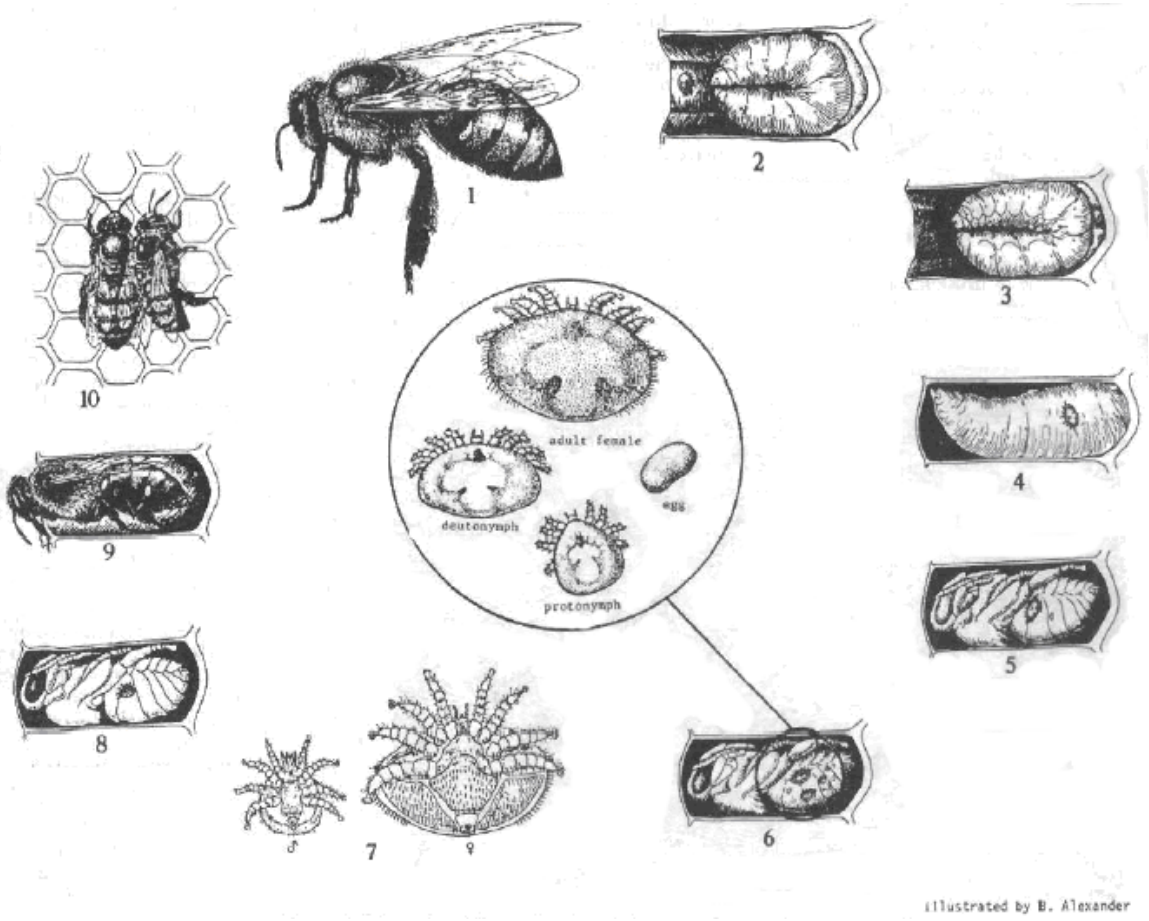

Figure C-1. Lifecycle of the varroa mite (modified from DeJong 1997) (1) A phoretic varroa foundress attaches to an adult bee from which she feeds on hemolymph. (2) The foundress enters a brood cell prior to cell capping (~5.5 day old larvae). (3) Mite submerges itself in the brood food at the bottom of the cell. (4) Mite feeds on prepupae hemolymph. (5) The mite foundress begins ovipositing 60 hours after the cell is capped and lays subsequent eggs every 30 minutes. The first egg laid is a male and all others are female. (6) Approximately 1 to 6 eggs develop to adults. (7) The males take 5-6 days to mature while female mites take 7-8 days to mature. (8) Female mites mate with the single male mite within the cell. (9) Adult female mites leave the cell attached to the adult bee. The male and any immature female mites are left in the cell to die. (10) Female mites can be transferred between adult bees. **DeJong, D. 1997**. Mites: varroa and other parasites of brood, pp. 284, figure 14.1. In R. A. Morse and K. Flottum (eds.), Honey bee pests, predators, and diseases, 3rd ed. A.I. Root Company, Medina, OH.

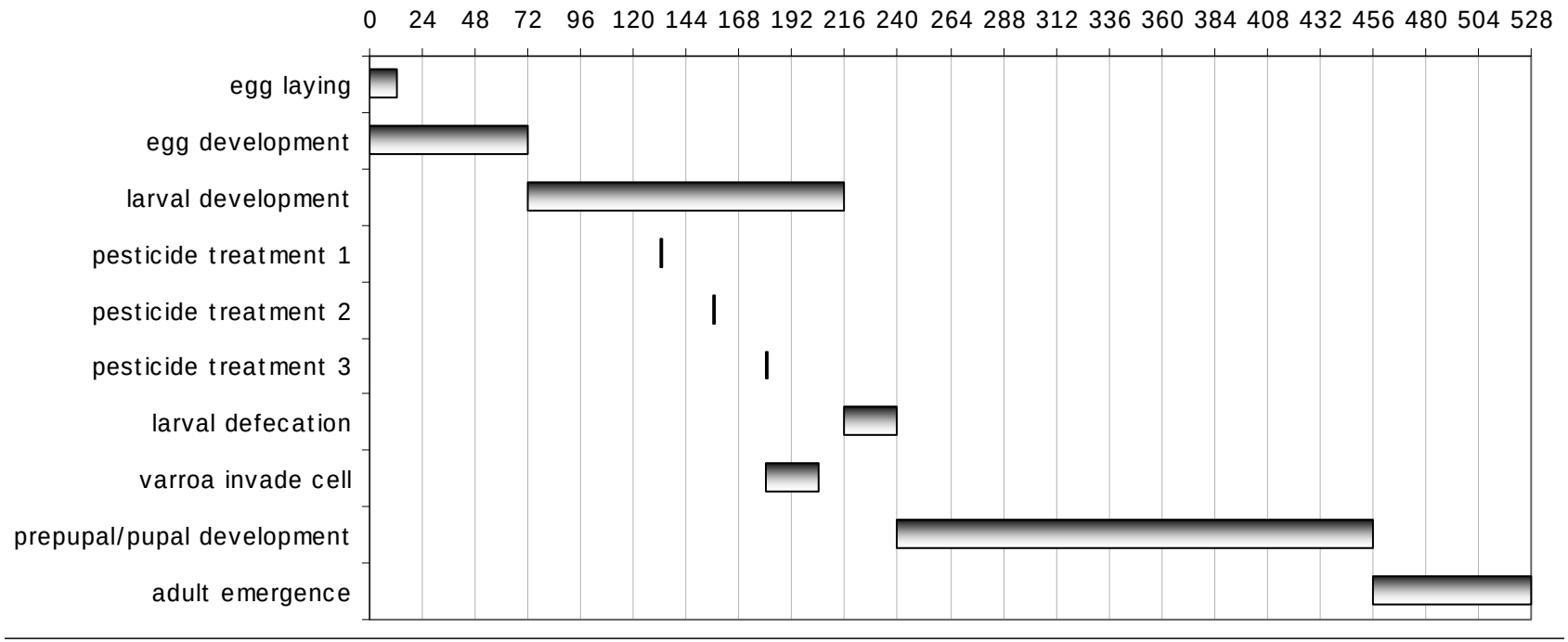

Figure C-2. Field bioassay: experimental timeline according to larval development in hours (0-528 h, x-axis). Pesticide treatment refers to the 3 time periods each larva was treated (at 132, 156, and 180 h) with their corresponding pesticide treatment (imidacloprid or amitraz). The timeline dose not illustrate that egg lying began at t = -12 h.

APPENDIX D
HYGIENIC BEHAVIOR OF ADULT HONEY BEES TOWARD LARVAE TREATED WITH DIFFERENT SOLVENTS

In the experiments presented in this thesis (Chapter 2, Appendices A, B, & C), imidacloprid and amitraz treatments were prepared by diluting the pesticides in an acetone solvent. Although acetone worked as a solvent in chapter 2, the *in vitro* study, there were problems with its use in Appendix C, the field study. When applying the two pesticides mixed in acetone to larvae in bee colonies, the worker bees regularly responded by removing (aborting) the treated larvae (hygienic behavior). In my general observation, I noticed that the bees were removing the acetone-only treated larvae but not the control (untreated) larvae. Consequently, I decided to investigate the effects of other solvents on bee removal of treated larvae in an effort to find a more suitable solvent to use in the field study. I limited my investigations to testing three other solvents: methanol, ethanol, and water. I did not expect water to be a good solvent for amitraz because amitraz is lypophilic (Hollingworth 1976) but maybe for imidacloprid since it is more hydrophilic (Elbert et al. 1991).

To test the effects of each solvent on bee removal of treated larvae, I treated larvae that weighed approximately 6 mg with one of 5 solvents: acetone, methanol, ethanol, water, or no solvent. I used a micro-applicator (Hamilton Company, Reno, NV) to treat larvae with 1 µL of test solvent: I treated 10 larvae per treatment. All treatments were on a single frame for each queen. After treatment, I returned the treated frame to its original colony. After 24 h, I brought the treated frame into the laboratory to count the number of larva present for each treatment. If a larva was missing it was assumed to have been aborted by worker bees. I analyzed the number of larvae bees aborted with treatment as my main effect in a Kruskal-Wallis test for non-parametric data. Data was analyzed in SAS (SAS Institute, 2008).

Results from this investigation did not show that solvent choice had any effect on the number of larvae aborted ($\chi^2 = 6.31$; df = 4; $P = 0.18$). Numerically, water caused the lowest abortion rate of all of the solvents (Table D-1). However, the failure of amitraz to dissolve in water only makes it an appropriate solvent for imidacloprid. Methanol, ethanol, and acetone all induced a larger number of aborted larvae. Even though there were no statistically significant differences between solvents detected in this investigation, the large number of larvae aborted from the field experiment (Appendix C) suggests that applying pesticides topically to larvae inside honey bee colonies is not an effective method for testing pesticides.

Table D-1. Percentage of larvae aborted according to solvent treatment applied topically. Abortion was determined by the number of empty cells that contained treated larvae 24 h earlier.

Solvent	Aborted larvae
Untreated	9.3 ± 9.3 (5)a
Acetone	37.8 ± 18.8 (5)a
Ethanol	51.5 ± 16.8 (5)a
Methanol	25 ± 16.1 (5)a
Water	6.4 ± 6.4 (5)a

Data are mean ± std. error (n) where n = number of individuals from all queen sources. Columnar means followed by the same letter are not different at $\alpha \leq 0.05$. Means were compared using Fisher's LSD tests.

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BIOGRAPHICAL SKETCH

I am a master's student in the University of Florida's Entomology and Nematology program. I received my bachelor's degree at West Virginia University with a major in biology and minor in geology. Afterwards, I was awarded an internship with the Conservancy of Southwest Florida where I studied sea turtle biology. I then became employed with the United States Geological Survey, Biological Resource Division, as an entomology research assistant. While at the USDA, I participated in a number of research projects involved in the control of invasive pests and conservation of native fauna. In the future, I plan to pursue a Ph.D. in ecology.