SUB-LETHAL EFFECTS OF PESTICIDE RESIDUES IN BROOD COMB ON WORKER HONEY BEES (*APIS MELLIFERA L.*)

By

JUDY YU WU

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JUDY YU WU find it satisfactory and recommend that it be accepted.

_____
Walter S. Sheppard, Ph.D., Chair

_____
Carol M. Anelli, Ph.D.

_____
John D. Stark, Ph.D.

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SUB-LETHAL EFFECTS OF PESTICIDE RESIDUES IN BROOD COMB ON WORKER HONEY BEES (*APIS MELLIFERA L*.)

Abstract

by Judy Yu Wu, M.S.
Washington State University
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Chair: Walter S. Sheppard

The European honey bee, *Apis mellifera* L., is an important pollinator for many agricultural crops. The United States Department of Agriculture estimates one-third of our diet comes from insect-pollinated plants. Of that, 80% are pollinated by honey bees (Thapa, *2006 J. Inst. Agric. Anim. Sci 27:1-23*). Unfortunately, honey bees are faced with challenges including mites, microsporidia, viruses, poor nutrition and exposure to pesticides.

Miticides have been used since the late 1980's in the U.S. to treat the problematic mite *Varroa destructor* (Varroidae) in bee colonies. Honey bees are often exposed to high frequent doses of miticides as beekeepers seek to counter the mite's rapidly developing resistance to chemical treatments (Elzen *et al.,1999 Apidologie 30: 17-19*; Elzen *et al. 2000 Apidologie 31: 437-441*). In addition, exposure to agrochemicals occurs during flight and foraging activities (Rortais *et al., 2005 Apidologie 36: 71-83*). Returning foragers may further contaminate nest-mates and

resources. Current risk assessments for pesticides examine lethality of adult bees to a single active ingredient in a laboratory setting. However, sub-lethal effects of pesticide on immature bees and effects from mixtures, used in the field, are not routinely used to assess the toxicity of pesticides during the registration process.

This study examined pesticide residue contamination found in brood comb and investigated sub-lethal effects of pesticide residues on larval survivability, adult longevity, and susceptibility to *Nosema ceranae (*Nosematidae) infection. Worker bees were reared in contaminated comb, containing high levels of pesticide residues, or in relatively "clean" comb within the same colony. Comparisons of survivability from egg to adult and larval development rates yielded lower survivability and evidence of delayed development for bees reared in contaminated combs. Adult longevity was. On average, four days shorter for bees reared in contaminated combs compared to control bees. In a field experiment, a significantly higher proportion of bees reared in contaminated comb were infected with *Nosema ceranae* spores at a younger age and with higher infections than bees reared in clean comb. This suggests early exposure to pesticide residues during development can have serious effects on larval survivability and subtle delayed effects in the adult stage.

TABLE OF CONTENTS

LIST OF TABLES

MANUSCRIPT ONE

MANUSCRIPT TWO

MANUSCRIPT THREE

LIST OF FIGURES

MANUSCRIPT ONE

MANUSCRIPT TWO

MANUSCRIPT THREE

Dedication

This thesis is dedicated to my mother and father who provided support and encouragement throughout my life

MANUSCRIPT ONE

ABSTRACT

Honey bees, *Apis mellifera* L., are beneficial insects, providing pollinating services for 130 crops and contributing $15 billion in added crop value in the US. Recently, pesticides have been implicated in the phenomenon, colony collapse disorder (CCD), responsible for major colony losses worldwide. Honey bee exposure to pesticides and contamination of resources can occur from agricultural and beekeeper-applied chemical treatments; therefore it is important to monitor the level of pesticide contamination in brood comb where larval bees develop. In our survey, we detected 62 different pesticides, 12 metabolites, and 1 synergist (piperonyl butoxide) from five categories of brood comb (n=98). Further analysis revealed significantly high levels of insecticide residues in comb sampled from dead colonies compared to comb sampled from live colonies (p=0.0338). Brood combs sampled from colonies identified with CCD symptoms, had significantly high levels of coumaphos, a common beekeeper-applied organophosphate acaricide (p=0.0008). While comb sampled from dead Pacific Northwest migratory colonies (PNW) and collaborative research colonies (CRC), had significantly high levels of fluvalinate, a common beekeeper-applied pyrethroid acaricide (p=0.0003). Further research on possible effects of pesticide residues in brood comb should be investigated given the gravity of pesticide contamination found in this survey.

Keywords Apis mellifera / pesticides / residues / brood comb

INTRODUCTION

The honey bee, *Apis mellifera* L. is widely regarded as an important pollinator, contributing $15 billion in added crop value for 130 crops, annually in the U.S. Beginning the winter of 2006-2007, U.S. beekeepers reported colony losses of 31.8% due to a phenomenon called colony collapse disorder (CCD). Symptoms of CCD include rapid colony decline, the presence of hives void of worker bees with only a few young bees to care for the brood and the queen present. Evidence of sick, dead, or dying bees were absent in and around the hives. Beekeepers also observed delayed robbing or complete avoidance of CCD colonies and hive equipment by common hive pests, such as small hive beetles (*Aethina tumida)* and wax moths (*Achroia grisella, Galleria mellonella)*. Currently, colony health continues to decline with estimated losses at 35.8 and 28.6% for 2007-2008 and 2008-2009, respectively [1]. Several factors have been implicated in the rapid decline of colony health, such as pesticide exposure, poor nutritional value of crop, low diversity and abundance of forage, and migratory stress. These factors along with numerous bee pests, pathogens, and viruses have all been identified as possible contributing factors of colony collapse disorder [2].

Pesticides have been widely used, in agricultural and non-agricultural settings, for many years. Market estimates of pesticide expenditure in the U.S. were reportedly over $11 billion, or about 35% of world market pesticide expenditures, in 2001. Worldwide, 5 billion pounds of pesticides were used annually, and over 20% or 1.2 billion pounds of active ingredients were used in the U.S. [3]. Heavy use and dependence on chemical treatments increases the risk of pesticide exposure and potential adverse effects on non-target organisms, such as honey bees, and their

environment. Chemical drift, persistent residues, leeching action, and contaminated water sources are known risks to foraging honey bees and are examined during risk assessments. Synergistic effects caused by chemical mixtures, effects of metabolites, and sub-lethal effects, however, are often overlooked and unaccounted for when assessing potential risks of pesticides to non-target organisms [4]. Acute poisoning may cause apparent physical effects, such as trembling motion, dizziness, uncoordinated movements, and feeding inhibition, while sub-lethal exposures may cause less apparent neurological, physical, behavioral and or developmental problems in honey bees. Honey bees and their products are effective biological indicators or environmental sentinels due to frequent exposure to environmental pollutants during foraging or flight activities [5]. Given the importance of honey bee pollination services there should be regular testing and monitoring of contamination on bees and their products. In order to investigate environmental risk pesticide residues on honey bees, we must first assess the contaminant exposure honey bees are faced with by examining the strength of the bees and the quality of bee products. A recent study examining pesticide levels in bees and bee products in more than a dozen U.S. states revealed astoundingly high levels of pesticide residues [6]. To compare, in this study, we focused mainly on migratory beekeeping operations based in the Pacific Northwest region, queen-producing operations and colonies that have succumb to CCD during almond pollination. We sampled brood comb to determine the level of contamination developing worker larvae are exposed to and established a baseline understanding of the level of contamination, specifically which, how many, and at what concentrations are pesticide residues found in honey bee comb according to the sample categories. We

also examined possible correlations between pesticide contamination in brood comb and colony strength.

MATERIALS AND METHODS

Sampling procedure

Standard Langstroth frames of brood comb (24.3 x 48.3 x 2.9-cm) were collected from Pacific Northwest beekeeping operations. A single comb sample was collected each from a separate colony. Comb samples were separated into five categories. The first category consisted of combs (n=24) from migratory operations based in the Pacific Northwest region (Washington, Montana, Oregon). These combs were selected from dead or failing colonies (PNW). The second group of combs (n=24) were sampled from collaborative research colonies (CRC) which were embedded within a migratory operation and monitored monthly for pests by Washington State University diagnostics lab. The third category of combs (n=19) were attained from six California queen-producing operations (QPO). Commercial foundation wax sheets (n=7), used by beekeepers to draw new comb, purchased from seven commercial suppliers (FWS) constitutes the fourth category. The last category of combs (n=24), originated from colonies suspected to have died from colony collapse disorder, were selected and provided by Dr. Jeff Pettis USDA-ARS Beltsville, MD (CCD).

Chemical analysis

Ninety-eight brood comb samples were sent to Roger Simonds USDA-AMS-National Science Laboratory, Gastonia, NC to be analyzed using QuEChERS method.

Pesticide residue extraction and analysis was accomplished using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS - Agilent 1100 LC equipped with a Thermo Quantum Discovery Max Triple Quadrupole Mass Spectrometer or equivalent), gas chromatography coupled with mass selective detection in electron impact mode (GC/MS-EI - Agilent 6890 GC equipped with a Agilent 5975 Mass Selective Detector in EI mode or equivalent), and gas chromatography coupled with mass selective detection in negative chemical ionization mode (GC/MS-NCI - Agilent 6890 GC equipped with a Agilent 5975 Mass Selective Detector in NCI mode or equivalent). Pesticide residues extracted from comb samples were quantified using matrix matched calibration standards of known concentrations prepared from neat standard reference material. Measurements were reported in nanograms of active ingredient per gram of wax (ng/g) or parts per billion (ppb). Identification of extracted residues was achieved through mass spectral comparison of ion ratios with standards, 171 of the most commonly used pesticides and their metabolites, of known identity. Limits of detection were low in the parts per billion (ppb).

Description of data set

Pesticide residue concentration and frequency of detection were considered for samples within five defined categories of brood comb. Mean residue concentrations were determined, for individual active ingredients and pesticide groups or classes, within each category. Insecticide and acaricide pesticides were grouped together due to the use of some active ingredients as either in different formulations. Residue concentrations are reported in parts per billion (ppb) or percent concentration. We

describe frequency of an active ingredient as the number of positive detections divided by the total number of detections within a category.

Statistical analysis

Statistical tests were conducted on mean pesticide content of a single active ingredient, such as coumaphos or fluvalinate, or a group of active ingredients of the same pesticide class or group, for example insecticide, fungicide, and metabolite groups. Frequencies of positive detections were determined but not used for statistically analysis. We used a linear mixed model one-way analysis of variance (ANOVA) to analyze the relationship between pesticide content and the category from which the comb was sampled. Category of comb was the independent variable while each brood comb sampled was treated as an experimental unit. Mixed proc procedure was used to account for the unequal variance in sample size between the five categories of comb. Likelihood ratio and type III tests were used to estimate the coefficient of the model. Indicators revealed that the effect (pesticide content and sample categories) was significant. Further multiple pairwise comparison procedures were performed using Fischer's least-significant-difference (LSD) test.

Statistical analysis was also performed on mean pesticide content and the binary response variable (dead or alive) representing colony strength at time of sampling. Overall insecticide, metabolite, and coumaphos oxon levels were found to be significant using one-way ANOVA procedure. Logistic regressions and Pearson correlation tests were performed to analyze maximum likelihood estimates for coumaphos, fluvalinate,

and overall insecticide content. Significance was determined at 5% (p-value ≤ 0.05). All tests were performed using SAS System, version 9.1.3.

RESULTS

Within 98 brood comb samples, residues of 62 different pesticides, 12 metabolites, and 1 synergist were found. The number of pesticides within a single comb ranged from 3 to 22, averaging 8.9 different residues in each sample. Insecticides (acaricides included) were the most frequent group of pesticide detected, constituting 51 to 70% of the total number of pesticide detected among the five categories of combs and an overall mean of 61% (Fig 1). Of the 38 different insecticides identified in the samples, 31 (82%) are categorized as either highly or moderately toxic to honey bees by U.S. Environmental Protection Agency (EPA) standards (table 1). The most frequent residue in combs consisted of two insecticide classes: pyrethroids or synthetic pyrethrin compounds targeting voltage-sensitive sodium channels, and organophosphates which are cholinesterase inhibitors. Fluvalinate, a pyrethroid acaricide used by beekeepers, contributed 98% of the overall pyrethroid concentration and was detected in 97% of the comb samples analyzed. The concentration of fluvalinate residue detected ranged from 130 to 92,600 ppb in individual combs. Coumaphos, an organophosphate acaricide applied by beekeepers, contributed 97% of the overall average organophosphate quantity among all categories and was detected in 99% of the combs. Individual samples varied in coumaphos residue levels from 60 to 226,000 ppb. Positive detection of fluvalinate and coumaphos, concurrently, was found in 96% of the total comb samples. Pesticide metabolites were

the second most commonly detected group of compounds after insecticides, averaging 15% of the total number of pesticide detections and ranging from 9 to 26% within the five categories of combs. Of the 12 different metabolites detected, coumaphos oxon was the most frequently found, present in 92% of all comb samples. Individual samples, positive for coumaphos oxon, ranged from 9 to 3,140 ppb. Fungicide residues, from 16 different active ingredients, constituted 11% of the total number of pesticide detections and were the third most frequently detected pesticide group in brood combs. Eight different herbicide residues were detected representing an average of 6.6% of the total pesticides detected across all categories. Only one synergist, piperonyl butoxide (PBO), was detected in two QPO samples, constituting 2 % of the total QPO pesticide residue profile.

Brood comb samples in categories

The number of different pesticides detected within each category was not significant. However, brood comb sampled from colonies with CCD had significantly higher insecticide levels than QPO and FWS combs ($p=0.0163$) but was not statistically different from PNW and CRC samples. PNW and CRC samples had significantly higher fluvalinate levels compared to the other categories of comb ($p=0.0003$). Commercial wax foundation or FWS samples had the lowest fluvalinate average (510 ppb), while combs sampled from CRC had the highest average (17,900 ppb) (Fig. 2). Combs from colonies suspected to have suffered from CCD had the highest average (29,730 ppb) coumaphos levels than the other categories ($p=0.0008$), while QPO brood comb had the lowest average (1,240 ppb) (Fig. 2). PNW and CCD samples had 54.2

and 45.8% of samples with concurrent detections of fluvalinate and coumaphos levels exceeding 3,500 ppb, a concentration in which fluvalinate residues have shown adverse sub-lethal effects [7]. In addition, PNW brood combs had the highest mean coumaphos metabolite contributions, (oxon and chlorferone) (578 ppb) while the overall metabolite residue level was significantly higher in QPO comb samples (p=0.0035) due to high concentrations of 2,4 dimethylphenyl formamide (DMPF) and 2,4 dimethylaniline, two major break-down products of amitraz, a beekeeper-applied triazapentadiene acaricide not currently registered in the U.S. for the control of *Varroa* mites in honey bee hives (Fig. 3). Fungicide residue levels were significantly higher in FWS samples (p=0.0063).

Brood comb samples from dead and live colonies

Collaborative research colonies were observed to be alive and strong when we first sampled brood combs. Pesticide residue analysis revealed surprisingly high levels of contaminates in CRC samples. Both fluvalinate and coumaphos residues were detected in 100% of the samples and fluvalinate levels, in particular, was significantly high (17,900 ppb). One year after the original sampling of combs, we found 67% of the CRC colonies had died. To examine a possible correlation between pesticide residue levels in brood comb and colony health we re-examined the combs based on whether the colonies were dead (n=62) or alive (n=29) at the time of sampling (commercial foundation wax sheets were not included in this grouping). Combs from dead colonies (colonies sampled after death) had significantly higher insecticide levels than combs from living colonies, while the number of different pesticides detected was not significant (p=0.0338). In fact, 50% of brood combs from live colonies had less than 9,000 ppb of

insecticide residues compared with 50% of comb sampled from dead colonies which had nearly twice the insecticide level, 17,000 ppb (Fig. 4). Fluvalinate and coumaphos residue levels were not significantly different in dead and live colonies ($p=0.429$, $p=0.059$). However, the proportion of samples with fluvalinate and coumaphos residue levels simultaneously exceeding 3,500 ppb was 33.9 % for brood comb sampled from dead colonies compared with 3.4% of combs sampled from live colonies (Fig. 4). Additionally, comb samples from living colonies had significantly lower levels of coumaphos oxon than combs from dead colonies ($p=0.0389$). Although fluvalinate and coumaphos levels were not significant ($p=0.429$, $p=0.0586$), Pearson's correlation coefficients revealed coumaphos and insecticide predictors were highly correlated (0.87912; $p<0.0001$) and fluvalinate was also correlated with the variable response, or dead colonies (0.32109; $p=0.0019$). Fluvalinate and coumaphos residues contribute a major portion of the total contamination in brood comb; however, there were many other insecticide, fungicide, and herbicide residues present. While there was no difference in the quantity of herbicide or fungicide residues between comb from dead and live colonies, there is still potential for interaction.

DISCUSSION

Varroa mite problem

Varroa destructor (Anderson & Trueman) mites have been one of the most problematic pests since their introduction to the U.S. in the 1980s. The small external parasite feeds off bodily fluids or hemolymph of larval, pupal, and adult honey bees.

Puncture wounds in the integument of the host caused by feeding mites has been suggested as means of introduction of other pathogens. *Varroa* parasitism causes reduction of weight and longevity in bees while early infection can cause wing deformities, permanently inhibiting bees from flight. Beekeeper applied pesticide treatments including fluvalinate, coumaphos, amitraz, organic acids and essential oils such as formic acid and thymol, have been the primary control method for suppressing mite levels. However, mites have become increasingly resistant to registered pesticides such as Apistan (fluvalinate), Checkmite (coumaphos), and the currently unregistered amitraz [8-12]. Development of chemical-resistant mites is likely with sub-lethal concentrations of residues persisting in wax, especially when mites are in close proximity to contaminated wax for long periods of time [13]. In addition, cuticle layers shed by developing brood within comb cells may serve as a barrier protecting mites from concentrated exposure levels and potential effects from residual acaricides in wax, furthering the opportunity of developing resistance [14].

Beekeeper applied chemicals

High colony losses result from uncontrolled mite infestations. Development of chemical resistance to available acaricides develops rapidly, causing treatments to be ineffective and leads to concerns about overdosing, misuse of applications, and or improper timing and frequency of applications. The suggested control method to avoid or delay chemical resistance in mites, in the past, was alternating acaricide treatments of different pesticide classes and target sites [15]. Unfortunately, once in wax lipophilic pesticides residues do not easily degraded, become more stable, and accumulate over

time [16]. The risk of greater distribution of contaminants and measurable levels of residues in wax increases with higher concentrations, more frequent applications, and greater residual persistence of active ingredients applied to hives [17]. Comb analysis from this study illustrates the gravity of pesticide use and accumulation of residues in brood comb. Contamination of beekeeper applied pesticide residues, in wax, from other countries has revealed lower residue concentrations of fluvalinate (220 to 7,370 ppb) and coumaphos (648 to 5,000 ppb) [7, 13, 18-21]. To compare, in this study, CCD brood comb had over 4 times the average coumaphos level (29,730 ppb) and comb samples from PNW and CRC had over 3 times the fluvalinate levels (15,202 and 17,907 ppb, respectively). Concerns over potential effects of accumulating residues in beeswax are supported by studies that have reported adverse effects of sub-lethal exposure to pesticides in wax on honey bees [7, 12]. The extent of transfer of pesticides from comb wax to bees is not well studied; however, there are reports of residue levels as high as 0.1 µg of tau-fluvalinate per bee from exposure to a standard dose of Apistan from the previous year's treatment [7]. Toxicity testing on honey bees typically occurs on adult bees, although, all life stages of the honey bee are susceptible to pesticide exposure [22]. Immobile soft-bodied larvae are potentially more vulnerable than adult bees to exposure from contaminated comb during development. Studies on developing queen bees have shown significant queen weight and ovary reduction with fluvalinate and coumaphos levels in queen wax cells of 3,550 ppb and 50,000 ppb, respectively [7]. Queen cell rejection, up to 50%, was also observed when coumaphos was present in wax at 100,000 ppb [12]. Of the 92 samples positive for fluvalinate residue in this study, 65 (71%) had levels exceeding 3,550 ppb.

Chemical interactions

A majority of the pesticides were not at levels that would be considered dangerous to honey bees; however, when multiple pesticides occur in brood wax there is the potential for interactions that may increase the toxicity to exposed bees. The issue of pesticide residues in brood comb is further complicated by evidence of synergistic interactions between the most common beekeeper-applied acaricides. A recent study examined synergistic interactions between coumaphos and tau-fluvalinate and reported that a tau-fluvalinate pre-treatment of 1-3 µg increased toxicity of coumaphos by 3.4 fold. Conversely, coumaphos pre-treatment of 3 µg increased tau-fluvalinate toxicity by 4.4 fold, while a 10 µg pre-treatment increased fluvalinate toxicity by more than 32 times [23]. Only 1 % of the comb samples, in the current study, did not have both fluvalinate and coumaphos residues present. The high proportion of samples with fluvalinate and coumaphos residues, simultaneously exceeding 3,500 ppb, in brood comb sampled from dead colonies (33.9 % compared with 3.4% of live colonies) suggests potential for interaction effects, either additive or synergistic, occurring from the presence of both residues (Fig. 4). Additionally, potential contributions and interactions from other pesticide residues present are still unclear. Testing binary mixtures of organophosphate (OP) and carbamate (C) classes of pesticides on salmon reported additive and synergistic interactions. Most importantly, the frequency of interaction increased with greater exposure concentrations and the greatest synergism was observed when pesticides of the same class were combined, such as malathion (OP) combined with either diazinon (OP) or chlorpyrifos (OP) [24]. The tendency for interactions to occur at higher concentrations is of concern, given that chlorpyrifos,

diazinon, and malathion are three of the eleven organophosphate forms detected in honey bee brood comb. In addition to the organophosphate compounds, there were twelve different pyrethroid compounds, five neonicotinoid, six chlorinated hydrocarbon, and six carbamate contributions detected in brood comb (table 1).

Metabolites

Metabolites have the potential to cause honey bee harm. Some active ingredients are metabolized, in the animal system, into more toxic forms. Unfortunately, there is little known on the effects of metabolites in honey bee systems. As a result, accurate risk assessments cannot be made. Studies on the effects of chlorpyrifos, diazinon, and malathion metabolites on *Rana boylii* tadpoles reported oxon forms of chlorpyrifos, diazinon, and malathion to be more than 100 times, nearly 100 times, and about 10 times, respectively, more toxic then their parent forms [25]. While a direct comparison between larval frogs and honey bees can not be made, honey bees are at risk of exposure to all three of these chemical metabolites. Studies on effects of imidacloprid, a systemic neonicotinoid insecticide implicated in CCD, on honey bees led to discrepancies over the actual toxicity of imidacloprid. A study on imidacloprid seed treatments found negligible risks to honey bees while another study reported both temporary and lethal effects due to imidacloprid treated syrup fed to honey bees. Immediately after ingesting acute doses of imidacloprid, bees began to exhibit neurotoxic symptoms such as trembling and dizziness but recovered shortly after exposure. Chronic sub-lethal doses of imidacloprid, though, resulted in less immediate neurotoxic symptoms but greater mortality. In fact, 50% mortality occurred after 8 days

of exposure, an observation that is neglected by short-term acute toxicity tests. Upon further examination of imidacloprid metabolism in honey bees, rapid neurotoxic symptoms appear to be the effect of imidacloprid, the parent compound; while mortality was delayed, suggesting that the prolonged action was due to breakdown products of imidacloprid. As the honey bee metabolized the toxin, the chemical was breaking into compounds with greater toxicological consequences than the parent compound, imidacloprid [26, 27].

Fungicides

Some fungicide products do not have warning labels to protect pollinators and may be sprayed during the bloom when bees are actively foraging and most at risk of exposure. While not specifically targeting insects, a number of fungicides produce toxic interactions with other chemicals and cause harm [28]. Studies on the honey bee system show a particular class of fungicide, ergosterol biosynthesis inhibitors (EBI), when combined with pyrethroid insecticides, causes an increase in toxicity 10 to 100 fold [29]. Tebuconazole, an EBI fungicide, when combined with a cyano-substituted neonicotinoid thiacloprid insecticide, increased toxicity with uncoordinated movements, lethargy, and increased mortality of bees. Cyprodinil, a non-EBI class of fungicides, when combined with thiacloprid, also caused incoordination, but for a shorter period and mortality was only slightly increased compared to controls [30]. Pyrethroids have a repellent nature that, arguably, reduces or limits honey bee exposure, however, reduced repellency of pyrethroids, are reported, when combined with fungicides *in vitro* [31,32]. In response to beekeeper reports of high bee losses after pollinating crops sprayed with

particular fungicides, one-day old larvae were fed diets containing the fungicides captan (8.0 µg/bee), iprodione (0.5 µg/bee), and ziam (8.0 µg/bee) and significant mortality was found in brood fed fungicides [33]. Another study assessed toxicity of five fungicides to *Osmia lignaria* Say and *Apis mellifera* L. and found greater susceptibility of *O. lignaria* bees to fungicide poisoning than honey bees, reinforcing greater need to study effects of pesticides on other pollinators. Toxicity assessments, 72 hours after a single exposure dose on *O. lignaria* bees (122.5 µg /bee), were low enough to consider captan fungicide harmless (LD50contact = 269.7 µg/bee; LD50oral = 47.3 µg/bee) while assessments 7 days after the same exposure was 3 to 4 times more toxic, suggesting delayed toxicity [34]. The delayed effects, some would argue is due to sub-lethal interaction effects, interruption of nesting and foraging behavior of *O. lignaria* bees for several days post spraying, of tank mixtures containing fungicides, adjuvants, and fertilizers. Standard pre-mixing procedures, in which fungicides and insecticides are combined in large tanks and co-currently sprayed, are commonly used to reduce cost and time. This practice increase efficacy of the pesticide mixture but is unregulated and can increase the risk to bees and other pollinators. Risk to non-target organisms for tank mixtures can not be assessed accurately because toxicity tests, required for registration, focus on singular active ingredients in laboratory settings. There were 19 different fungicides identified, in this study, with several detected at relatively high levels, including iprodione (878 ppb), cyprodinil (838 ppb), and hydroxychlorothalonil (729 ppb), a degradation product of the active ingredient chlorothalonil. Chlorothalonil, an organochlorine fungicide, was the most frequently detected fungicide and residues constituted 37% of the total number of fungicide detections. Fungicide residues, in this

study, were detected more frequently in FWS samples (p=0.0063) with an average concentration of 773 ppb.

New foundation wax made from recycled comb

Commercial foundation wax sheets are typically made from recycled capping wax from extracted honey comb cells and can often be contaminated with pesticides residues. Honey bees produce wax to cap or cover honey cells ready for storage. During honey extraction, wax caps are removed to allow access to honey cells and wax caps are processed and recycled to make foundation wax sheets. High levels of fungicide residues in wax caps supports the prevalence of agricultural pesticide exposure of foraging bees and the need to better assess risk of fungicides, fungicide mixtures, and timing of sprays. A study in which wax was embedded with known amounts of pesticides was heated, melted, and processed to be recycled into foundation wax revealed acaricide residues to be on average 1.7 times higher then the original comb. Boiling contaminated wax for varying durations and temperatures also had no effect in reducing residue levels in old beeswax [16]. Analysis of foundation wax from other countries revealed contamination ranges of 200-3,500 ppb and 500-8,000 ppb for coumaphos and fluvalinate, respectively [17, 35-37]. In this study, foundation wax available for purchase from commercial suppliers, was positive for coumaphos residues on average 3,357 ppb (236 -12,500 ppb) and for fluvalinate on average 1,243 ppb (64 – 2620 ppb). In addition to coumaphos and fluvalinate, residues of 19 other insecticides, 7 fungicides, 4 herbicides, and 4 metabolites were detected in commercial foundation wax samples

Synergists

Synergists are substances added to pesticide formulas specifically to enhance the toxicity or efficacy of an active ingredient. This is accomplished by increasing absorbency and persistence, minimizing loss from evaporation and drift, or inhibiting detoxification by disrupting enzymatic activities in the target organism. Synergists lower costs to growers by reducing the amount of active ingredients required and are commonly used (though not regulated by the EPA). Piperonyl butoxide (PBO), for example, is found in many insect control products, from home and garden treatments to mosquito abatement programs [38]. Piperonyl butoxide, when combined with tau-fluvalinate increases the toxicity of tau-fluvalinate 980 fold, due to PBO inhibition of specific cytochrome P45O monooxygenase enzymes that would normally break down fluvalinate with little residual effect. Additionally, a four-fold increase in coumaphos toxicity occurs in the presence of PBO [39]. Tau-fluvalinate was detected in 95% of the comb samples analyzed in this study, while coumaphos residues were detected in 99% of combs sampled. Piperonyl butoxide was found in two combs sampled from queen producing operations (n=13) at 8,710 and 11,900 ppb. In the same comb, tau-fluvalinate was present at 4,620 and 16,400 ppb, respectively.

CONCLUSIONS

Any pesticide has the potential for adverse effects on non-target organisms and the environment. Dependency and heavy use of beekeeper-applied, agricultural, and urban pesticides, persistent residues, drift and leeching into water resources poses increased risks for honey bee poisoning and colony contamination. This study

illuminates the high contamination of brood comb by pesticide residues and thereby a constant source of chemical exposure for honey bees of all life stages and castes within the hive. Beeswax acts as a sink for lipophilic pesticides, stabilizing compounds and allowing residues to persist, even accumulate over time. The impact of contamination in comb on honey bees is still unclear however, analysis of samples revealed significantly high levels of insecticides in brood combs sampled from dead colonies. While this survey can not conclude that there is evidence for sub-lethal effects from residues in wax, it does suggest a correlation between the quantity of pesticides detected in brood comb and the ultimate fate of colonies living with chronic pesticide exposure. This correlation warrants further research specifically examining the effects of pesticide residues in comb on developing and adult honey bees. Many of the pesticide residues were detected infrequently or at low levels; therefore the analysis of brood comb, in this study, elucidates which chemicals are of concern and merit further examination. In addition to the importance of providing a relatively uncontaminated environment for honey bees to live and breed, honey bee products are being readily used worldwide in food storage, cosmetics, pharmaceuticals and apitherapy. Other industries also depend on the wholesome image honey bee products have maintained for years. The quality of beeswax, determined by the amount of measurable pesticide residues detected, should be assessed and monitored for human consumption and use. Standards and maximum residues limits for wax have been establish for beekeeper-applied acaricides in other countries and should be determined for the United States. In addition to beekeeper-applied pesticide standards, maximum residue limits should also be established for agrochemicals commonly detected in wax. Production of commercial foundation wax

sheets should be tested regularly for adulterations and practices in recycling old comb should be reduced or eliminated. Lastly, establishing a comb replacement schedule to remove old combs will reduce the amount of measurable pesticide residues and minimize chemical exposure to bees in the hive. Suppressing accumulation of residues in comb may delay mite resistance to treatments and may allow applied pesticides to be more effective to naïve mites. Reducing the concentration and frequency of beekeeper-applied pesticides would help ensure the quality of beeswax and bee products sold in the market and help protect honey bee health.

REFERENCES

1. VanEngelsdorp D, Hayes Jr. J, Underwood RM, Pettis J. 2010. A survey of honey bee colonies losses in the United States, fall 2008 and spring 2009. *J. Apic. Res* 49: 7-14.

2. VanEngelsdorp D, Evans J, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R, Tarpy DR, Pettis JS. 2009. Colony collapse disorder: a descriptive study. *PLosONE* 4: e6481.

3. U.S. Environmental Protection Agency. Pesticide industry sales and usage: 2000 and 2001 market estimates. (accessed May 2008) http://www.epa.gov/oppbead1/pestsales/01pestsales/market_estimates2001.pdf.

4. Lappé M, Bailey B. 2002. New considerations for evaluating pesticide impacts to endangered/threatened species. U.S. Fish and Wildlife Service and National Marine Fisheries Service report to the Environmental Protection Agency. Case No. C00-3150 CW: 1-64.

5. Porrini C, Sabatini AG, Girotti S, Ghini S, Medrzycki P, Grillenzoni F, Bortolotti L, Gattavecchia E, Celli G. 2003. Honey bees and bee products as monitors of the environmental contamination. *Apiacta* 38: 63-70.

6. Mullin CA, Frazier M, Frazier JL, Ashcraft S., Simonds R, vanEngelsdorp D, Pettis JS. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLOS one* 5: e9754

7. Haarmann T, Spivak M, Weaver D, Weaver B, Glenn T. 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. *J. Econ. Entomol.* 95: 28-35.

8. Baxter J, Eischen F, Pettis J, Wilson WT, Shimanuki H. 1998. Detection of fluvalinate-resistant Varroa mites in US honey bees. *Am. Bee J.* 138: 291.

9. Elzen PJ, Eischen FA, Baxter JR, Elzen GW, Wilson WT. 1999. Detection of resistance in US *Varroa jacobsoni* Oud. (Mesostimata: Varroidae) to the acaricide fluvalinate. *Apidologie* 30: 17-19.

10. Elzen PJ, Baxter JR, Spivak M, Wilson WT. 2000. Control of *Varroa jacobsoni* Oud. resistant to fluvalinate and amitraz using coumaphos. *Apidologie* 31: 437-441.

11. Elzen PJ, Westervelt D. 2002. Detection of Coumaphos resistance in *Varroa destructor* in Florida. *Am. Bee J.* 142: 291-292.

12. Pettis JS, Collins AM, Wilbanks R, Feldlaufer MF. 2004. Effects of coumaphos on queen rearing in the honey bee, Apis mellifera. *Apidologie* 35: 605-610.

13. Lodesani M, Pellacani A, Bergomi S, Carpana E, Rabitti T, Lasagni P. 1992. Residue determination for some products used against Varroa infestation in bees. *Apidologie* 23: 257-272.

14. Fries I, Wallner K, Rosenkranz P. 1998. Effects of Varroa jacobsoni from acaricides in beeswax. *J. Apic. Res.* 37: 85-90.

15. Eischen F. 1995. Varroa resistance to fluvalinate. *Am. Bee J.* 135: 815-816.

16. Bogdanov S, Kilchenmann V, Imdorf A. 1996. Acaricide residues in beeswax and honey. In Mizrahi A, Lensky Y, eds, *Bee Products*. Plenum Press, NY.

17. Wallner K. 1999. Varroacides and their residues in bee products. *Apidologie* 30: 235- 248.

18. Thrasyvoulou AT, Pappas N. 1988. Contamination of honey and wax with malathion and coumaphos used against the Varroa mite. *J. Apic. Res.* 27: 55-61.

19. Bogdanov S, Kilchenmann V, Imdorf A. 1998. Acaricide residues in some bee products. *J. Apic. Res.* 37: 57-67.

20. Lodesani M, Costa C, Serra G, Colombo R, Sabatini AG. 2008. Acaricide residues in beeswax after conversion to organic beekeeping methods. *Apidologie* 39: 324-333.

21. Chauzat MP, Carpentier P, Martel AC, Bougeard S, Cougoule N, Porta P, Lachaize J, Madec F, Aubert M, Faucon JP. 2009. Influence of pesticide residues on honey bee (Hymenoptera: Apidae) colony health in France. *Environ. Entomol.* 38: 514-523.

22. Rortais A, Arnold G, Halm M, Touffet-Briens F. 2005. Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. *Apidologie* 36: 71-83.

23. Johnson RM, Pollock HS, Berenbaum MB. 2009. Synergistic interactions between in-hive miticides in Apis mellifera. *Apiculture and Social Insects* 102: 474-479.

24. Laetz CA, Baldwin DH, Collier TK, Hebert V, Stark JD, Scholz NL. 2009. The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered pacific salmon. *Environ. Health Perspectives* 117: 348-353.

25. Sparlings DW, Fellers G. 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval Rana boylii. *Environmental Pollution* 147: 535-539.

26. Maus C, Cure G, Schmuck R. 2003. Safety of imidacloprid seed dressings to honey bees: a comprehensive overview and compilation of the current state of knowledge. *Bulletin of Insectology* 56: 51-57.

27. Suchail S, Guez D, Belzunces LP. 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in Apis mellifera*. Environmental Toxicology and Chemistry* 20: 2482-2486.

28. Brobyn PJ. 1999. Possible synergistic effects on honeybees of pyrethroids and fungicides: the UK regulatory consideration. In Belzunces LP, Pélissier C, Lewis GB, eds, *Hazards of pesticides to bees*. Institute National de la Recherche Agronomique, Avignon, France, pp 97-113.

29. Pilling ED, Jepson PC. 1993. Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pesticide Science* 39: 293-297.

30. Schmuck R, Stadler T, Schmidt HW. 2003. Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (Apis mellifera L, Hymenoptera). *Pest management science* 59: 279-286.

31. Rieth JP, Levin MD. 1989. The repellent effects of two pyrethroid insecticides on the honey bee. *J. Apicult. Res.* 28: 175-179.

32. Thompson H, Wilkins S. 2003. Assessment of the synergy and repellency of pyrethroid/fungicide mixtures. *Bulletin of Insectology* 56: 133-134.

33. Mussen EC, Lopez JE, Peng CYS. 2004. Effects of selected fungicides on growth and development of larval honey bees, Apis mellifera L. (Hymenoptera: Apidae). *Environ. Entomol.* 33: 1151-1154.

34. Ladurner E, Bosch J, Kemp WP, Maini S. 2005. Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera.* *Apidologie* 36: 449-460.

35. Bogdanvo S, Kilchenmann V. 1995. Acaricide residues in beeswax: long-term studies in Switerland. *Apidologie* 26: 319-321.

36. Wallner K. 1997. The actual beeswax quality in foundations from the market. *Apidologie* 28: 168-170.

37. Tremolada P, Bernardinelli I, Colombo M, Spreafico M, Vighi M. 2004. Coumaphos distribution in the hive ecosystem: case study for modeling applications. *Ecotoxicology* 13: 589-601.

38. Cox C. 2002. Insecticide synergist factsheet: piperonyl butoxide. *Journal of Pesticide Reform* 22: 12-20.

39. Johnson RM, Wen Z, Schuler MA, Berenbaum MR. 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. *Journal of Economic Entomology* 99: 1046-1050.

Table 1a. Characteristics of surveyed pesticides

Active ingredient	Purpose	s/ps	Chemical family	LD50/LC50	Toxicity	ppb/bee	%	Avgr	min	max	LOD
Azoxystrobin	FUNG	s	Strobilurin	>200			16.3%	2.4	2.0	36.0	2
Boscalid	FUNG	s	Carboxamide	100.0			10.2%	6.3	19.4	128.0	4
Chlorothalonil	FUNG		Chloronitrile	>100			37.8%	61.1	3.3	4170.0	1
Cyprodinil	FUNG	s	Anilinopyrimidine	113.0			25.5%	21.4	10.0	838.0	16
Dicloran	FUNG		Nirtoaniline	181.0			1.0%	0.0	*	2.5	1
Fenhexamid	FUNG		Hydroxyanilide	>200			1.0%	0.5	*	46.3	6
Flutolanil	FUNG	s	Benzanilide	-			7.1%	17.1	81.6	584.0	4
Hexachlorobenzene (HCB)	FUNG		Chlorinated hydrocarbon				1.0%	0.0	*	4.5	1
Iprodione	FUNG		Dicarboximide	>400			10.2%	17.4	37.6	297.0	20
Pyraclostrobin	FUNG		Strobilurin	>73.1			6.1%	2.0	9.0	46.1	15
Pyrimethanil	FUNG		Anilinopyrimidine	>100			7.1%	1.4	5.4	46.4	3
Quintozene (PCNB)	FUNG		Organochlorine	100.0			1.0%	0.0	*	4.8	1
Thiabendazole	FUNG	s	Benzimidazole	50.0			4.1%	0.2	1.6	7.6	4
Trifloxystrobin	FUNG	s	Strobilurin	200.0			4.1%	0.3	5.6	10.4	1
Triticonazole	FUNG	s	Triazole	>100			2.0%	4.5	27.4	409.0	10
Vinclozolin	FUNG		Dicarboximide	100.0			4.1%	0.1	1.1	1.4	1
Ethofumesate	HERB	s	Benzofuranyl alkylsulfonate	> 50,000			1.0%	2.0	*	200.0	5
Fluridone	HERB	s	aquatic	363.0			7.1%	0.4	1.2	10.6	50
Norflurazon	HERB	s	Fluorinated pyridazinone	>235			2.0%	0.1	5.0	5.6	6
Oxyfluorfen	HERB		Diphenyl ether	100.0			26.5%	1.2	1.1	26.5	1
Pendimethalin	HERB		Dinitroaniline	59.0			10.2%	1.8	5.8	63.0	6
Sethoxydim	HERB	s	Cyclohexadione	10.0	m	78000	1.0%	0.1	*	14.2	8
Tribufos (DEF)	HERB	SYN	Organophosphate	44.2			1.0%	0.0	*	4.2	2
Trifluralin	HERB		Dinitroaniline	100.0			7.1%	0.2	1.1	8.6	1
Aldicarb	INSECT	s	Carbamate	0.09	h	702	1.0%	0.2	*	20.0	4
Bifenthrin	INSECT		Pyrethroid	0.02	h	117	3.1%	0.2	4.8	12.0	1
Carbaryl	INSECT	ps	Carbamate	1.5	h	11700	2.0%	0.3	9.2	16.1	30
Carbofuran	INSECT	s	Carbamate	0.16	h	1248	1.0%	0.3	*	32.0	5
Chlorpyrifos	INSECT		Organophosphate	0.11	h	858	84.7%	7.1	1.0	49.7	1
Clothianidin	INSECT	s	Neonicotinoid	0.004	h	31.2	1.0%	0.4	*	35.0	20
Coumaphos	INSECT		Organophosphate	4.6	m	36114	99.0%	9193.6	63.5	226000.0	1
Cyfluthrin	INSECT		Pyrethroid	37.0			3.1%	0.2	3.6	7.9	2
Cyhalothrin total	INSECT		Pyrethroid	0.90	h	7020	4.1%	0.1	0.2	6.6	1
Cypermethrin	INSECT		Pyrethroid	0.03	h	195	19.4%	2.1	1.4	28.8	2
Diazinon	INSECT		Organophosphate	0.09	h	702	6.1%	0.6	1.4	24.4	1
Dicofol	INSECT		Chlorinated hydrocarbon	30.0			13.3%	3.4	1.7	240.0	1
Dinotefuran	INSECT	s	Neonicotinoid	0.02	h	179.4	1.0%	1.0	*	97.0	30
Diphenylamine	INSECT		Amine	-			3.1%	4.6	20.0	281.0	1
Endosulfan I	INSECT		Chlorinated hydrocarbon	7.0	m	54600	45.9%	2.8	1.0	80.9	1
Endosulfan II	INSECT		Chlorinated hydrocarbon	7.0	m	54600	37.8%	1.7	1.0	46.2	1

Active ingredient, purpose of use (FUNG, fungicide; HERB, herbicide; INSECT, insect/acaricide), systemic compound (s) or partially systemic (ps), synergist (SYN), chemical family, LD50/LC50 values for honey bees, toxicity category (h, highly toxic; m, moderately toxic to bees), *LD50/LC50 converted to ppb/bee, % positive detections, Avgr (average concentration of residue (ng/g)), minimum and maximum ranges of residue, LOD (limit of detection)

* ppb/bee (based on LD/LC50-bee and average fresh weight per bee (128 mg) (Pollinator protection: a bee & pesticide handbook. 1990. C.A Johansen, D.F. Mayer)

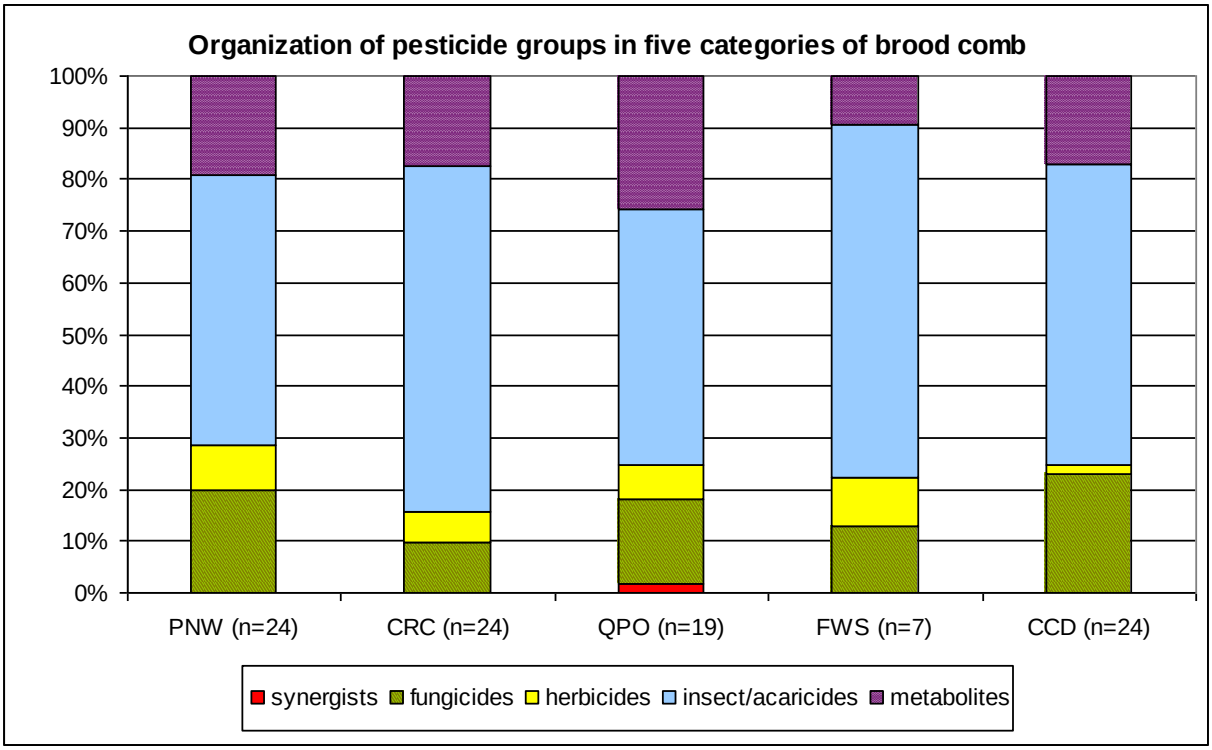
Table 1b. Characteristics of surveyed pesticides cont.

Active ingredient	Purpose	s/ps	Chemical family	LD50/LC50	Toxicity	ppb/bee	%	Avgr	min	max	LOD
Esfenvalerate	INSECT		Pyrethroid	0.41	h	3198	38.8%	7.7	1.0	215.0	1
Fenpropathrin	INSECT		Pyrethroid	0.05	h	390	3.1%	0.5	4.2	39.4	1
Fluvalinate	INSECT		Pyrethroid	0.88	h	6864	96.9%	10102.6	127.4	92600.0	1
Heptachlor	INSECT		Chlorinated hydrocarbon	0.53	h	4134	1.0%	0.0	*	3.1	4
Lindane	INSECT		Chlorinated hydrocarbon	<2	h	2106	2.0%	0.2	2.1	16.0	4
Malathion	INSECT		Organophosphate	0.27	h	2106	7.1%	1.1	2.4	51.6	4
Methidathion	INSECT		Organophosphate	0.24	h	1872	1.0%	0.2	*	17.7	10
Oxamyl	INSECT	s	Carbamate	0.38	h	2964	1.0%	0.2	*	22.0	5
Parathion methyl	INSECT		Organophosphate	0.11-0.24	h	858-1872	1.0%	0.1	*	7.7	2
Permethrin total	INSECT		Pyrethroid	0.029-0.16	h	226-1248	7.1%	38.0	83.3	1220.0	10
Phenothrin	INSECT		Pyrethroid	0.03	h	234	3.1%	0.9	19.5	44.8	10
Prallethrin	INSECT		Pyrethroid	0.028	h	218.4	1.0%	0.0	*	3.3	4
Propargite	INSECT	ps	Organosulfur	47.9			1.0%	26.9	*	2640.0	10
Pyrethrins	INSECT		Pyrethroid	0.022	h	171.6	7.1%	78.6	50.0	3570.0	50
Tetradifon	INSECT		mitochondrial ATP synthase inhibitor	11.0	m	85800	1.0%	0.1	*	9.6	1
Tetramethrin	INSECT		Pyrethroid	0.16	h	1248	4.1%	0.7	4.2	29.9	10
Thiacloprid	INSECT	s	Neonicotinoid	17.32			1.0%	1.2	*	113.0	8
Imidacloprid	INSECT	s	Neonicotinoid	0.02	h	185	1.0%	0.5	*	45.0	20
Phosalone	INSECT		Organophosphate	4.5	m	35100	5.1%	1.3	12.7	49.0	10
Pyriproxyfen	INSECT		Juvenile hormone mimic	100			1.0%	0.0	*	1.2	1
Fenpyroximate	INSECT	s	Phenoxypyrazole	15.8			4.1%	2.9	9.6	201.0	5
Thiamethoxam	INSECT	s	Neonicotinoid	0.024	h	187.2	1.0%	0.4	*	38.0	20
1-Naphthol		ps	Metabolite of carbaryl				1.0%	0.4	*	41.6	10
2,4 Dimethylaniline			Metabolite of amitraz				6.1%	33.8	77.0	1550.0	50
2,4 Dimethylphenyl formamide (DMPF)			Metabolite of amitraz				44.9%	137.9	18.5	3180.0	4
3-hydroxycarbofuran		s	Metabolite of carbofuran				1.0%	0.2	*	23.0	4
Carbendazim (MBC)		s	Metabolite of benomyl (benzimidazole) fungicide				13.3%	2.8	4.0	100.0	5
Chlorpyrifos methyl			Metabolite of chlorpyrifos				1.0%	0.0	*	1.2	1
Coumaphos oxon			Metabolite of coumaphos				91.9%	179.6	9.0	3140.0	5
Endosulfan sulfate			Metabolite of endosulfan				22.4%	1.2	1.0	60.0	1
Hydroxychlorothalonil			Metabolite of chlorothalonil (tetrachloroisophthalonitrile) fungicide				1.0%	7.4	*	729.0	50
Malathion Oxon			Metabolite of malathion				1.0%	0.2	*	22.0	
THPI		s	Metabolite of captan (dicarboximide) fungicide				7.1%	7.7	7.1	299.0	50
Chlorferone			Metabolite of coumaphos				5.1%	43.9	80.5	1890.0	50
Piperonyl butoxide	Synergist	SYN					2.0%	210.3	8710.0	11900.0	6

Active ingredient, purpose of use (FUNG, fungicide; HERB, herbicide; INSECT, insect/acaricide), systemic compound (s) or partially systemic (ps), synergist (SYN), chemical family, LD50/LC50 values for honey bees, toxicity category (h, highly toxic; m, moderately toxic to bees), *LD50/LC50 converted to ppb/bee, % positive detections, Avgr (average concentration of residue (ng/g)), minimum and maximum ranges of residue, LOD (limit of detection)

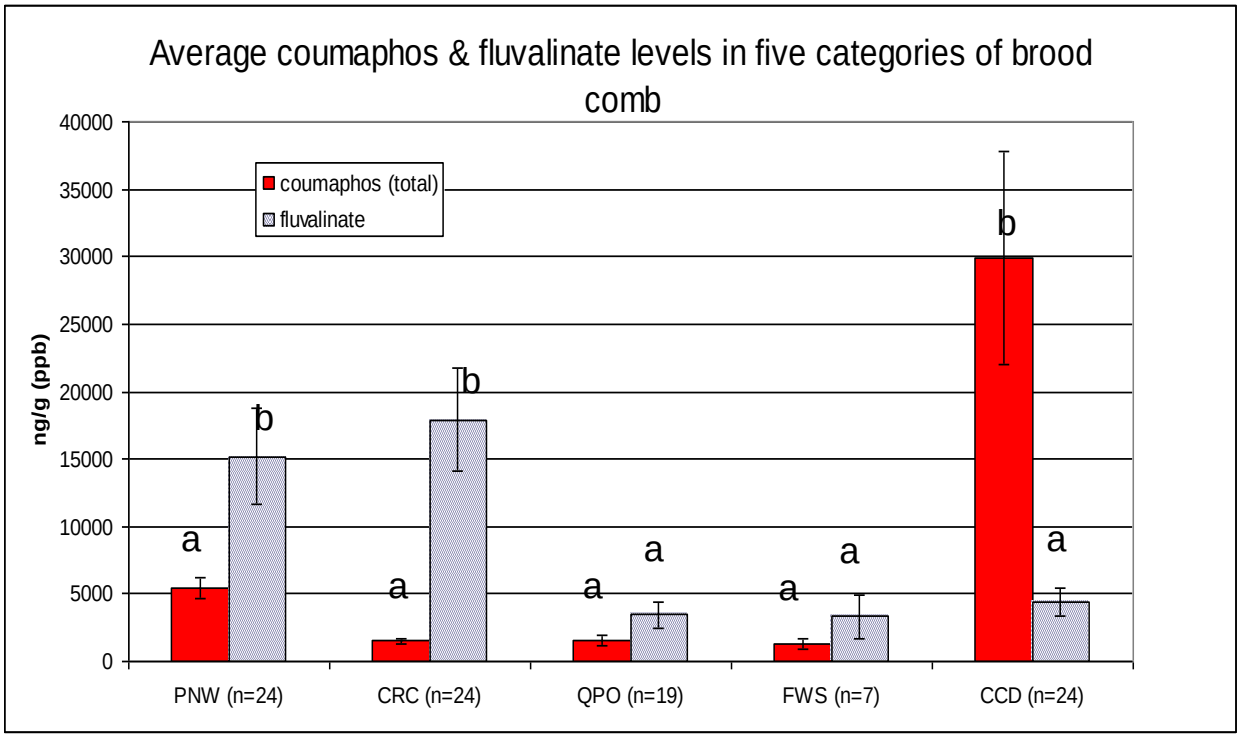
* ppb/bee (based on LD/LC50-bee and average fresh weight per bee (128 mg) (Pollinator protection: a bee & pesticide handbook. 1990. C.A Johansen, D.F. Mayer)

Fig 1. Residue contributions based on pesticide group in five categories of honey bee brood comb.



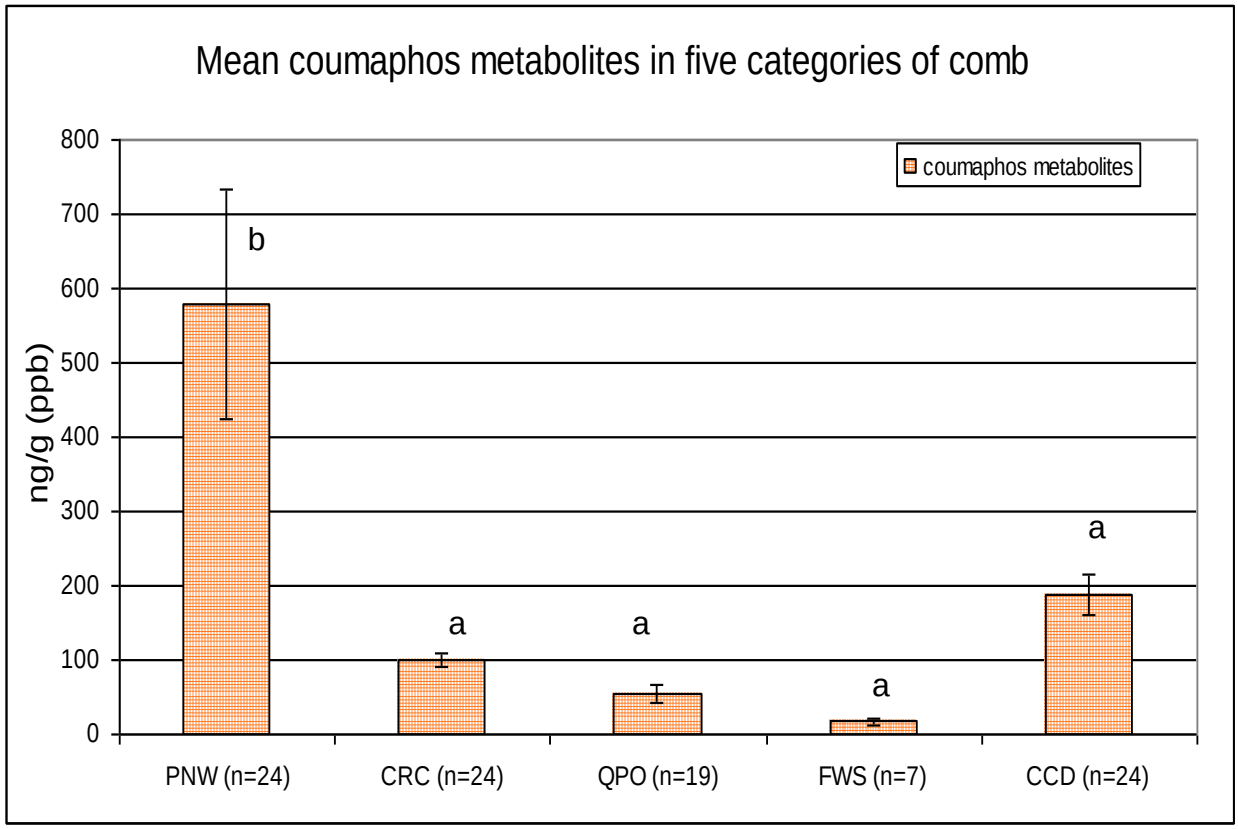
(PNW= migratory colonies in Pacific northwest, CRC= collaborative research colonies embedded in a migratory operation, QPO=California queen producing operation, FWS= commercial foundation wax sheets, CCD= colonies suspected to have died from colony collapse disorder).

Fig 2. Mean coumaphos and fluvalinate residue levels in five categories of brood comb.



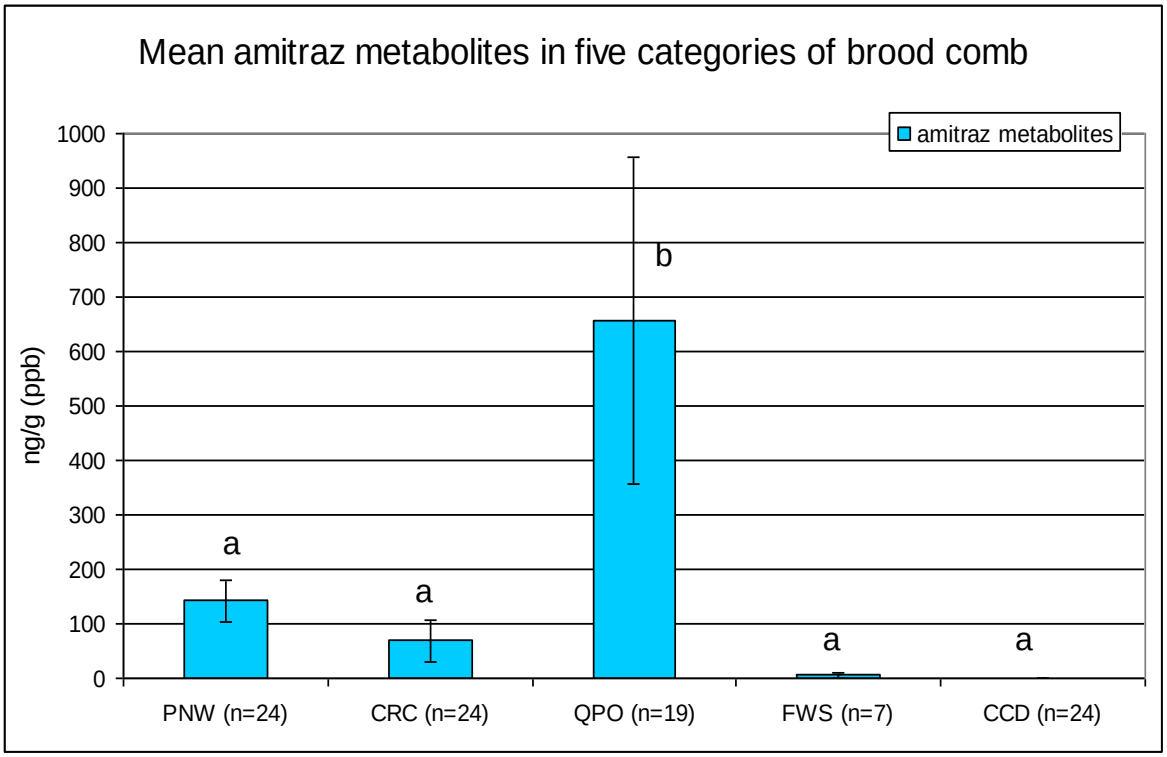
Statistical differences were detected by mixed procedure one-way analysis of variance (ANOVA) followed by t–tests, within a category, comparisons significant at $p \leq 0.05$ are indicated by different letters.

Fig 3. Mean coumaphos (oxon & chlorferone) metabolite contributions in five categories of brood comb.



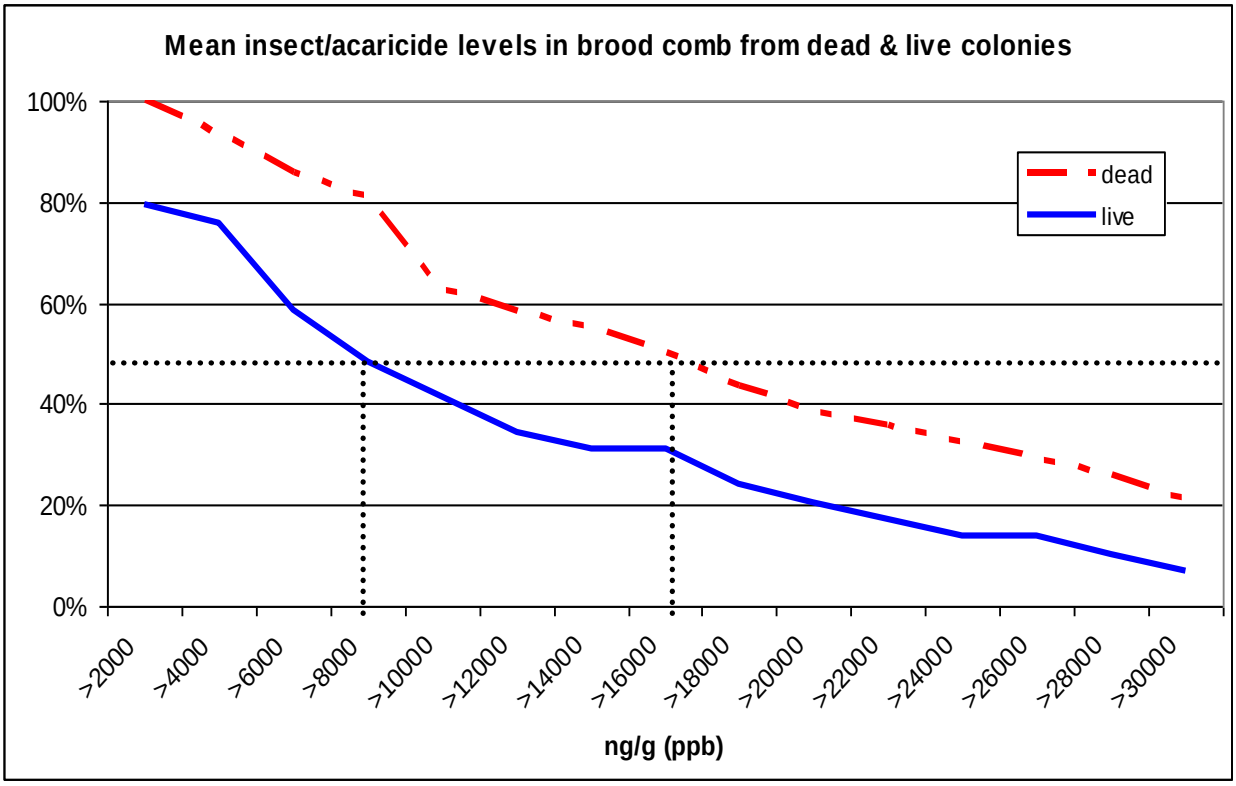
Statistical differences were detected by mixed procedure one-way analysis of variance (ANOVA) followed by t–tests, within a category, comparisons significant at $p \leq 0.05$ are indicated by different letters.

Fig 4. Mean amitraz (2,4 dimethylaniline & 2, 4 dimethylphenyl formamide) metabolite contributions in five categories of brood comb.



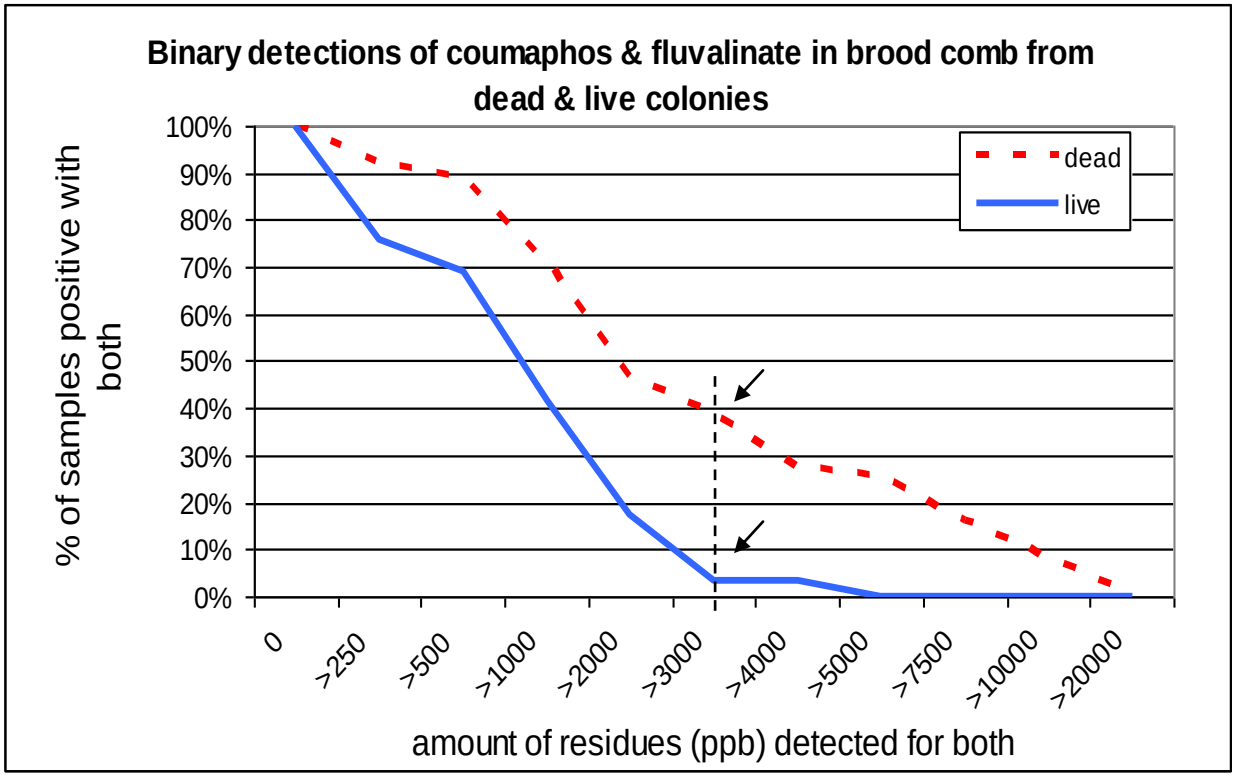
Statistical differences were detected by mixed procedure one-way analysis of variance (ANOVA) followed by t–tests, within a category, comparisons significant at $p \leq 0.05$ are indicated by different letters.

Fig 5. Mean insect/acaricide levels in brood comb from dead & live honey bee colonies.



The percent of samples, with insect/acaricide levels greater than the specified value, detected in brood comb from dead (n=62) and live (n=29) colonies. (At 50% positive detection, samples from dead colonies had over 2x the amount of residues than combs from live colonies).

Fig 6. Binary detections of coumaphos & fluvalinate in brood comb from dead & live honey bee colonies.



The number of samples with binary detections of fluvalinate & coumaphos residues at various concurrent levels. (34% of samples from dead colonies and 3% of from live colonies had fluvalinate and coumaphos each at 3,500 ppb).

MANUSCRIPT TWO

SUB-LETHAL EFFECTS OF PESTICIDE RESIDUES IN BROOD COMB ON WORKER HONEY BEE (*APIS MELLIFERA*) DEVELOPMENT AND LONGEVITY

ABSTRACT

Numerous surveys on bee products reveal high levels of pesticide residue contamination in honey bee comb. To study possible effects from pesticide exposure from contaminated brood comb, bees were reared in brood comb containing high levels of known pesticide residues versus brood comb relatively uncontaminated. Overall brood mortality was generally higher in bees reared in contaminated comb although there was no statistical difference. Comb age confounded early mortality in bees reared from newly drawn control comb compared to bees reared from old control comb or comb that had been used for only a few brood cycles. Pesticide residue migration and metabolism from comb containing high pesticide residues caused contamination of control comb after multiple brood cycles and provided insight on how quickly residues move through wax. Higher brood mortality and delayed adult emergence occurred after multiple brood cycles in contaminated control combs. In contrast, survivability increased in bees reared in treatment comb after multiple brood cycles when pesticide residues had been reduced in treatment combs due to residue migration into uncontaminated control combs, supporting comb replacement efforts. Chemical analysis after the experiment confirmed the migration of pesticide residues from treatment combs into previously uncontaminated control comb. Delayed development

was observed in bees reared from treatment comb containing high levels of pesticides particularly in the early stages (day 4 and 8) of worker bee development. Adult longevity was reduced by 4 days in bees exposed to pesticide residues in contaminated brood comb during development. These results suggest that sub-lethal effects of pesticide residues in brood comb can have serious effects on honey bee colony structure and health. (266 words)

INTRODUCTION

Managed honey bee (*Apis mellifera*) colonies in the United States have experienced high over-wintering losses and sudden crashes in colony population since the winter of 2006 (Van Engelsdorp et al. 2007). Over 60 contributing factors of the phenomenon known as colony collapse disorder (CCD) have been identified, including *Varroa destructor* mites, poor nutrition, pesticide exposure to both agrochemicals and beekeeper-applied chemicals, and various other pests and pathogens (Van Engelsdorp et al. 2009). Honey bee health decline and colony losses are not limited to the U. S. Many European studies attribute major recent bee losses to pesticide exposure, particularly, the class of neonicotinoid insecticides (Bonmatin et al. 2003; Ramirez-Romero et al. 2005; Chauzat et al. 2006). Studies from Spain focus mainly on the effects of *Nosema ceranae,* a microsporidian pathogen that targets the honey bee midgut, depriving infected bees of nutrients (Higes et al. 2009). There is some disagreement about which factors are more causative, and a few researchers have focused on interaction effects of combined factors. For example, the harmful effects of

pesticide exposure increases susceptibility to *Nosema ceranae* spore infection and vice versa (Ladas 1972; Alaux et al. 2009).

Honey bees are equipped with social behavioral and physiological defenses to protect against pests and pathogens, including grooming and other hygienic behaviors to remove mites and dead or diseased brood. Honey bees also collect and use propolis, a substance made from plant resins and wax that contains antibiotic, antiviral, and antifungal properties (Burdock 1998). Social immunity provides significant protection for honey bee colonies and as a result, individual honey bees are immunologically deficient and have only about half as many detoxifying enzymes as pesticide resistant insects (Claudianos et al. 2006). This deficiency increases the sensitivity of honey bees to pesticides and bacterial or viral toxins and reduces their ability to fight infections. Sensitivity to pesticides may also vary among honey bees due to varying individual detoxification capabilities (Suchail et al. 2001). Other factors such as age and nutrition also can influence pesticide sensitivity. Older forager bees are more susceptibility to pesticide exposure because of flight and foraging activity than younger bees that remain safely in the hive, while over-wintering adults are the most susceptible to pesticide exposure (Wahl and Ulm 1983, Rortais et al 2005). Honey bees fed high quality pollen are less susceptible to pesticide exposure than bees fed low quality pollen or pollen substitutes, due to protein deficiencies in low quality pollen (Wahl and Ulm 1983). Unfortunately, commercial beekeepers typically must provide pollen substitute to colonies during transport and seasonal dearth to maximize brood production prior to and during pollination services. Adult honey bees are also more susceptible to pesticides when reared at lower temperatures (33ºC) (Medrzycki et al.

2009), a consideration potentially contributing to stress associated with the transportation of honey bee colonies.

In this study we examined the sub-lethal effects of developmental exposure to pesticide residues in brood comb on worker bees. Worker bees were reared in brood comb containing high levels of known pesticide residues or brood comb relatively free of pesticide residues. Larval development from egg to adult emergence was monitored at days 4, 8, 12 and 19 and newly emerged adults from both treatments were subjected to cage longevity tests. Bees reared from treatment comb contaminated with high levels of pesticides exhibited delayed larval development and reduced adult longevity. We discuss implications of sub-lethal and indirect effects of pesticide residues in brood comb on colony health and structure.

MATERIALS & METHODS

Treatment combs

Frames of brood comb were sampled from migratory Pacific Northwest beekeeping operations using miticides and from colonies suspected to have failed from colony collapse disorder. Pesticide residue analyses for 171 of the most commonly used pesticides were performed on brood comb samples. Thirteen frames of brood combs positive for high levels of pesticide residues were cut into treatment blocks (11x11-cm), containing roughly 450 cells. The number of different pesticide residues detected in treatment combs ranged from 4 to 17, averaging 10. The total number of pesticides detected in all treatments was 39, including 7 fungicides, 2 herbicides, 23 insecticides (miticides included) and 7 metabolites (Table 1). The three most frequently

detected pesticide residues in treatment combs were the beekeeper applied miticides fluvalinate, coumaphos, and coumaphos oxon metabolite. Fluvalinate, a pyrethroid pesticide, was detected in treatment combs at levels as high as 24,340 ppb and averaged 6,712 ppb. Coumaphos and its oxon metabolite were detected at levels as high as 22,100 ppb and 1,850 ppb, averaging 8,079 ppb and 596 ppb, respectively. Control brood combs were newly drawn out from a single colony or sampled from feral colonies that tested negative for pesticide residue contamination. Coumaphos was the only residue detected in newly drawn out control combs (21 ppb).

Experimental design

Standard Langstroth frames, with the center area (22x11-cm) of the frame removed, were used as frame supports containing a pair of comb blocks, i.e., one control comb adjacent to a treatment comb block containing high pesticide levels (n=17). Three colonies of similar strength were used from May through August of 2008 and 2009 to host experimental frames supporting paired comb blocks. Hosting control and treatment combs within the same colony during larval development normalized possible effects of colony activity and quality of resources fed to brood, emphasizing potential effects of pesticide residue exposure from contaminated brood comb. Laying sister queens from each colony were caged for 24 hours over experimental frames, allowing access to both control and treatment combs. Queens were released the following day and excluded to the bottom box for the duration of the experiment. Frames containing a patch of 224 eggs on control and treatment blocks were photographed and frames with insufficient number of eggs were removed from the

experiment. The same patch of eggs was monitored for larval mortality on days 4, 8, 12, and 19 of development and photographs taken of larvae developing in control and treatment comb were mapped using Microsoft paint. On day 19, experimental frames containing pupae reared in control and treatment comb were incubated at 33±1ºC with push-in cages separating treatment blocks. Emergence of adult bees was recorded daily and bees were counted, tagged with Testor's enamel, and placed in a 1/8" mesh metal cage (11 x 9 x 5-cm). Worker bees were fed water and 50% sucrose syrup *ad libtum* and mortality was recorded daily. Some experimental frames (n=9), containing a pair of control and treatment comb blocks, were reused up to three times during the experiment. Experimental frame supports containing comb blocks that had not yet been used in the experiment (rep 1) were introduced to host colonies at the same time as other frames that had gone through multiple brood cycles (rep 2 & 3) to minimize seasonal effects of larval survivability during late summer months.

Measurements

To assess the sub-lethal effects of exposure to pesticide residues, biologically meaningful parameters were measured throughout the main stages of the honey bee life cycle. Egg eclosion, or successful hatching was measured at day 4; larval mortality and development time from egg to pupae were recorded at day 8; pupation was recorded at day 12 and 19; adult emergence rate was recorded on day 20 and continued daily until emergence was no longer observed; and adult longevity was recorded daily until all caged bees were dead. Observations of abnormal larval development and signs of disease or pest infection were also recorded. Taken

together, these life cycle parameters provide insight and enable assessment of exposure to sub-lethal pesticide residues in brood comb on honey bee colony health.

Statistical analysis

Pairwise comparisons with repeated measures were performed on larval mortality, adult longevity, and adult emergence rate of worker bees reared in relatively uncontaminated brood comb and brood comb containing high levels of pesticide residues. Comparisons of both treatments were made by sample day (4, 8, 12 and 19) and by the number of brood cycles (rep 1, 2, 3). Differences in pesticide analyses, specifically the number of pesticide residues and the levels, detected in control and treatment comb used multiple times were compared before and after the experiment. Statistical differences were detected by one-way analysis of variance (ANOVA) followed by paired two-tailed t-tests on control and treatment combs with significance determined at $p \leq 0.025$.

RESULTS

Brood effects

Larval mortality was generally higher in worker bees reared from comb containing high levels of mixed pesticide residues; however, there was no statistical difference in total larval mortality between bees reared in control and treatment combs (26 and 28.3%, respectively)(Fig 1). Evidence of delayed development at day 4 and 8 was observed in bees reared from four different combs containing high levels of

pesticide residues sampled from colonies suspected to have CCD (7 a-c). Confounding factors may have contributed to these results, one of which was the comb age affect from newly drawn out control combs. Brood pheromones, contained within previously molted larvae cuticle or exuviae residing in old comb cells, indicate brood presence to nurse bees (Berry and Delaplane 2001). Newly drawn comb lacks the presence of exuviae and thus pheromone cues. Significantly higher brood mortality was observed in eggs laid in control combs on day 4 than larvae on days 8, 12, and 19 ($p=0.0243$; $p=0.0005$; $p<0.0001$, respectively). Day four of worker bee development represents a critical time, when nurse bees must provide resources to newly hatched eggs or larvae will not survive. Furthermore, total survivability from egg to adult emergence was higher for bees reared in older control comb (78%) compared to bees reared in newly drawn controls (69%), although not statistically significant. Another factor in this experiment was the repeated use of experimental frames this allowed the unintended migration of pesticide residues in wax which reduced the difference in contamination between treatment and control combs and differences between treatment effects. Mortality was significantly higher in control bees reared from frames that were used in the experiment more than once and had experienced multiple brood cycles (Fig 2). Total larval mortality increased with repeated use of experimental frames in control combs from 13% through the first brood cycle (rep 1) to 28 and 39% through the second (rep 2) and third (rep 3) brood cycles, respectively (Fig 2). In fact, brood mortality in bees reared through the third brood cycle was significantly higher than in the first and second brood cycles ($p=0.023$; $p=0.048$, respectively). In treatment brood comb containing high levels of pesticide residues, overall mean larval mortality increased from 17 % to 37% then

decreased to 22% for the first, second, and third brood cycles respectively (Fig 4). Mortality for bees reared in treatment combs was significantly higher for the second brood cycle on day 8 of larval development than treatment bees reared in the first and third brood cycles (Fig 3).

Chemical analysis of comb

Comparisons of chemical analyses, performed on control and treatment combs before and after the experiment, confirmed pesticide residue transfer and contamination of control combs over 3 months time. Four additional new pesticide residues were detected in control comb, on average, compared to a reduction of 3 pesticide residues in treatment combs after the experiment Fig 5). The quantity or concentration of active ingredients also increased in control combs and decreased in treatment combs after the experiment, further evidence supporting pesticide residue transfer from contaminated areas of comb to uncontaminated areas (Fig 5). Fungicides were the only pesticide group that was detected at higher concentrations in treatment combs after the experiment than before the experiment, an increase that is not statistically significant averaging 280 ppb. Insecticides, including the 3 most frequently detected compounds (coumaphos, coumaphos oxon, and fluvalinate) initially in treatment combs, increased in concentration in control combs and decreased in treatment combs after the experiment. Concentrations for coumaphos oxon, fluvalinate and combined insecticides were significantly higher in control comb after the experiment than before ($p<0.025$; $p<0.01$; $p< 0.025$; respectively). High levels of metabolites were also detected in control combs after the experiment suggesting possible metabolism of active compounds as a

result of pesticide residues migrating through wax. Fluvalinate was significantly lower in treatment combs after the experiment than before ($p<0.025$). These results illustrate how quickly pesticide residues may diffuse through wax and potentially penetrate into honey stored in comb cells.

Adult emergence and longevity

Worker bees reared in relatively uncontaminated brood comb lived an average 4 days longer than bees reared in comb containing high levels of pesticide residues ($p<0.005$). Emergence time was affected by contamination of control comb after multiple brood cycles, resulting in a shift in the proportion of worker bees that emerged on days 19, 20, and 21 (Fig 6 a-c). During the first brood cycles, adult emergence was significantly higher on days 20 and 21 compared to day 22 for bees reared in control comb, in fact, forty-two and fifty-three percent of worker bees reared on control combs emerged on days 20 and 21, respectively ($p<0.0007$). To contrast, by the third brood cycle emergence on day 22 was no longer statistically different from day 20 and 21; only 2% of worker bees reared in contaminated control comb emerged on day 20; 80% of the bees emerged on day 21; and another 18% emerged on day 22 of larval development. To contrast, on the first brood cycle, emergence for bees reared in treatment combs was statistically different on each day (20, 21, 22). By the third brood cycle, however, there was no longer a statistical difference in adult emergence between day 20 and day 22 for bees reared in treatment comb (Fig. 6 a-c.) These data suggest that delayed emergence time for developing worker bees is an effect of pesticide residue exposure to contaminated brood comb.

DISCUSSION

Honey bees of all ages and castes are susceptible to effects from pesticide exposure (Rortais et al. 2005). Adult bees may be exposed to pesticides during flight and foraging; younger adults remain in the hive but may be exposed to incoming contaminated pollen and nectar; immature bees are immobile and remain in comb cells for up to 22 days of development and may be exposed to pesticide residues through contaminated comb cell walls or food sources. Queen bees may be exposed to pesticides by contact with contaminated bees, wax, and food. Egg laying and repeated contact of the abdomen to contaminated comb increases the risk of sub-lethal effects from pesticide residue exposure on queen bees. Pesticide exposure to bees can have adverse reproductive consequences such as reduced egg laying behavior, early supercedure, increased queen cell rejection, and reduced ovarian weight in queen bees (Haarman et al. 2002; Pettis et al. 2004).

Widespread pesticide use and contamination is illustrated in numerous surveys of pesticide residue detected in honey bee comb from around the world (Thrasyvoulou and Pappas 1988, Van Buren et al. 1992; Bogdanov et al. 1996, Wallner 1999, Chauzat and Faucon, 2007; Mullin et al. 2010). Honey bees are biological indicators, picking up chemicals and other pollutants from their surrounding environment. Pesticide analyses of bees and bee products provides information about the pesticide treatments being used in the hive and in surrounding field crops and can be essential information for assessment of potential risk to bees and, by extension, the safety of consumers and industries utilizing bee products such as honey, wax, or propolis (Porrini et al. 2003). The high level of pesticide contamination in brood comb is disconcerting and reflects the

urgent need for better management of pests, among both to growers and beekeepers, including safer alternative control methods.

Worker bees reared in comb containing high levels of pesticide residues had lower survivability than bees reared in relatively uncontaminated comb. Comb age was a factor in this experiment and brood mortality was higher in newly drawn control comb than older control comb sampled from feral colonies. Older brood comb, or comb which has had a few brood cycles, contains brood pheromones which attract nurse bees and increase larval survivorship. Unfortunately, *Varroa destructor* mites, serious external parasites of honey bees, are also attracted to the brood pheromones, exuviae, and other larval compounds absorbed into old brood comb (Piccirillo and De Jong, 2004; Free and Winder, 1983). Pesticide residues, bacteria, viruses and *Nosema spp.* spores are also contained and persist in old brood combs and can be a source of disease or pest transmission (Bailey and Ball, 1991; Gilliam, 1985). Even though initial larval survivability may be lower in bees reared in new comb, overall colony health in hives using old brood comb, is compromised by higher incidences of pests and pathogens and bacterial or viral infections (Berry and Delaplane 2001).

Pesticide residues accumulate in wax and may persist for years (Bogdanov et al. 1996). Contamination of control brood combs in this experiment, illustrated how quickly pesticide residues penetrate and migrate through brood comb wax. The presence of additional pesticide residues in control combs detected after the experiment confirmed pesticide residue transfer and contamination of control combs. High levels of metabolites also detected in control combs after the experiment suggests possible metabolism of active compounds as a result of pesticide residues migrating through

wax. In the paired comb blocks, detection of increasing mortality for bees reared in control blocks and decreasing mortality for treatment blocks, over time, suggests there were toxicological consequences, such as lower larval survivability and delayed emergence, from pesticide residue migrating in wax from contaminated treatment comb to relatively clean control comb (Fig 4). In addition, during the third brood cycle greater mortality was observed in worker bees reared in contaminated control comb versus treatment combs. This may have been caused by exposure to newly formed metabolites as pesticides migrated into control combs. Metabolites can be more harmful than parent compounds and can have delayed effects (Suchail et al. 2001; Sparlings and Fellers 2007). These results suggest that there are physiological consequences of pesticide contamination in brood comb to developing worker bees.

Brood effects of pesticide exposure

Sub-lethal effects of pesticides such as delayed adult emergence, observed in worker bees reared in contaminated control combs, may seem inconsequential. However, delayed adult emergence of bees provides a reproductive advantage for *Varroa* mites. A pregnant foundress mite will invade a comb cell occupied by a developing bee larva and lay four eggs in 30 hour intervals. The first eggs become male followed by multiple daughter mites. The most injurious effects of *Varroa* mites occur when the foundress and her multiple offspring are feeding on the hemolymph of a pupating bee, causing reductions in emergence weight and metabolic reserves and physical deformities on host bees (Bowen-Walker and Gunn 2001, Amdam et al. 2004). The third daughter mite only has a 13% chance of reaching maturity before the pupating

bee emerges from the cell, typically after 20 to 21 days of development. Mites that have not reached maturity do not have cuticles hardened to protect against emerging bees actively kicking and pushing themselves out from cells. The likelihood that the third daughter mite will successfully reach maturity and mate within the cell increases with delayed emergence of bees reared in contaminated comb. Drone-brood trapping and removal is an effective alternative mite control method because *Varroa* mites naturally prefer drone brood, which require a longer development period, 24 days from egg to adult emergence (Charriere et al. 2003; Calderone and Kuenen 2001; Calderone 2005). Delayed development of worker bees occurred in bees reared in treatment comb containing 17 different pesticides, including 9 systemic compounds and 5 neonicotinoid insecticides (Table 2). The normal growth pattern is expected to be uniform, as eggs were laid on both control and treatment comb within a 24 hour period. However, by day 4, 23% of eggs were unhatched in the treatment comb and by day 8, over 46% of remaining larvae reared in the contaminated treatment comb were small and their development visually stunted or delayed (Figs. 7a-c). Another three treatment combs, sampled from colonies suspected to have colony collapse disorder (CCD), had similar effects on egg hatching and development. An average of 19% of eggs laid in comb sampled from CCD colonies containing high levels of pesticides, remained unhatched on day 4 and 60-90% of unhatched eggs were removed by the next sampling date. Whether unhatched eggs are removed from cells by nurse bees or eventually hatch, but become developmentally delayed, high brood mortality places energetic stress on queens and increase the demand for brood production. In addition, “spotty” brood patterns have previously been used to indicate failing or poor queen quality. However,

this experiment illustrates that a spotty brood pattern can result from pesticide residue exposure in contaminated brood comb wax rather than poor queen quality. Efficiency in brood production is reduced when eggs or larvae remain in brood comb cells only to be later removed. Egg-laying efficiency is furthermore reduced when queen bees are unable to deposit eggs in a general area but, instead, must seek empty cells scattered throughout the hive (Mackensen 1950).

Adult longevity

Worker bees reared in treatment comb containing high levels of pesticide residues lived on average 4 days less than bees reared in relatively uncontaminated control combs in cage trials ($p<0.005$). Reduced adult longevity causes premature foraging activity by replacement bees, which further shortens hive duties, such as brood care, food processing and storage, queen care, and hygienic behavior. Combined effects from exposure to pesticide residue in brood comb, such as reduced adult longevity, increased brood mortality, higher fecundity of *Varroa* mites (due to longer development and emergence time of bees) and increased susceptibility to other pathogens such as *Nosema spp.* may lead to a decline in honey bee colonies (Ladas 1972; Alaux et al. 2009). Queens and worker bees may not be able to keep up with the demand for brood production and resources needed to sustain large populations of adult bees while beekeepers, researchers and other stakeholders may disagree on the primarily pest and pathogen contributing to colony collapse disorder and honey bee health decline worldwide, our findings suggest that one of the underlying commonalities is the problem of pesticide contamination and exposure in bees and bee products. This

study illustrates that sub-lethal effects from pesticide residues through developmental exposure of contaminated brood comb may be subtle and indirect but can have serious colony level consequences.

REFERENCES

Alaux C., Brunet, J., Dussaubat, C., Mondet, F.,Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P., Le Conte, Y. 2009. Interactions between Nosema microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Envirnomental microbiology. 12: 774-782.

Amdam GV, Hartfelder K, Norberg K, Hagen A, Omholt SW. 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? *J. Econ. Entomol.* 97: 741-747.

Bailey L and Ball BV. 1991. Honey bee pathology. Harcourt Brace Jovanovich; Sidcup, Kent, UK, p. 193.

Berry JA, Delaplane KS. 2001. Effects of comb age on honey bee colony growth and brood survivorship*. J. Apic. Res.* 40: 3-8

Bogdanov S, Kilchenmann V, Imdorf A. 1996. Acaricide residues in beeswax and honey. In Mizrahi A, Lensky Y, eds. *Bee Products*. Plenum Press, NY.

Bonmatin, J. M., I. Moineau, R. Charvet, M. E. Colin, C. Fleche, E. R. Bengsch. 2003. Fate of
imidacloprid in fields and toxicity for honeybees. *Environ Chem*.

Bowen-Walker PL and Gunn A. 2001. The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera)* emergence weights, water, protein, carbohydrate, and lipid levels. *Entomol. Exp. Appl.* 101: 207-217.

Burdock GA. 1998. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* 36: 347-363.

Calderone, N. W. 2005. Evaluation of drone brood removal for management of *Varroa destructor* (Acari: Varroidae) in colonies of *Apis mellifera* (Hymenoptera: Apidae) in the northeastern United States. *J. Econ. Entomol.* 98: 645-650.

Calderone, N. W., and L.P.S. Kuenen. 2001. Effects of western honey bee (Hymenoptera: Apidae) colony, cell, type, and larval sex on host acquisition by female *Varroa destructor*
(Acari: Varroidae). *J. Econ. Entomol.* 94: 1022-1030.

Charriere, J. D., A. Imdorf, B. Bachofen, and A. Tschan. 2003. The removal of capped drone brood: an effective means of reducing the infestation of varroa in honey bee colonies. *Bee World* 84: 117-124.

Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG. 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol. Biol*. 15: 615-636.

Chauzat, M. P., J. P. Faucon, A. C. Martel, J. Lachaize, N. Cougoule and M. Aubert. 2006.
Survey of pesticide residues in pollen loads collected by honey bees in France. *J. Econ. Entomol*. 99 (2): 253-262

Chauzat MP and Faucon JP. 2007. Pesticide residues in beeswax samples collected from honey bee colonies (*Apis mellifera* L.) in France. *Pest Manag. Sci.* 63: 1100-1106.

Free JB and Winder ME. 1983. Brood recognition by honey bee (*Apis mellifera*) workers, *Anim. Behav*. 31, 359-545.

Gilliam M. 1985. Microbes from apiarian sources: *Bacillus* spp. in frass of the greater wax moth, *J. Invertebr. Pathol*. 45, 218-224.

Haarmann T, Spivak M, Weaver D, Weaver B, Glenn T. 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. *J. Econ. Entomol.* 95: 28-35.

Higes M, Martín-Hernández R, Garrido-Bailón E, González-Porto AV, García-Palencia P, Meana A, del Nozal MJ, Mayo R, Bernal JL. 2009. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. *Environ. Microbiol. Reports*. 1: 110-113.

Ladas A. 1972. The influence of some internal and external factors upon the insecticide resistance of honeybee. *Apidologie.* 3: 55-78.

Mackensen O. 1951. Viability and sex determination in the honey bee (*Apis mellifera* L.). *Genetics* 36: 500-509.

Medrzycki P, Sgolastra F, Bortolotti L, Bogo G, Tosi S, Padovani E, Porrini C, Sabatini AG. 2009. Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides. *J. Apic. Res.*49: s52-s60.

Mullin CA, Frazier M, Frazier JL, Ashcraft S., Simonds R, vanEngelsdorp D, Pettis JS. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLOS one* 5: e9754

Pettis JS, Collins AM, Wilbanks R, Feldlaufer MF. 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. *Apidologie* 35: 605-610.

Piccirillo GA and De Jong D. 2004. Old honey bee brood combs are more infested by the mite *Varroa destructor* than are new brood combs. *Apidologie* 35: 359-364.

Porrini C, Sabatini AG, Girotti S, Ghini S, Medrzycki P, Grillenzoni F, Bortolotti L, Gattavecchia E, Celli G. 2003. Honey bees and bee products as monitors of the environmental contamination. *Apiacta* 38: 63-70.

Ramirez-Romero R, Chaufaux J and Pham-Delègue M. 2005. Effects of Cry1Ab protoxin,
deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee *Apis mellifera*, a comparative approach *Apidologie* 36: 601-11.

Rortais A, Arnold G, Halm M, Touffet-Briens F. 2005. Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. *Apidologie* 36: 71-83.

Sparlings DW, Fellers G. 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. *Environmental Pollution* 147: 535-539.

Suchail, S., Guez, D., and Belzunces, L.P. (2001). Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera. Environ. Toxicol. Chem.* 20: 2482-2486.

Thrasyvoulou AT, Pappas N. 1988. Contamination of honey and wax with malathion and coumaphos used against the Varroa mite. *J. Apic. Res.* 27: 55-61.

Van Buren NWM, Mariën J, Velthuis HHW, Oudejans R. 1992. Residues in beeswax and honey of Perizin, an acaricide to combat the mite *Varroa jacobsoni* Oudemans (Acari: Mesostigmata) *Environ. Entomol.* 21: 860-865.

Van Engelsdorp D, Underwood RM, Caron D, Hayes J Jr. 2007. An estimate of managed colony losses in the winter of 2006-2007: a report commissioned by the Apiary Inspectors of America. *Am. Bee J.* 147: 599-603.

Van Engelsdorp D, Evans J, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R, Tarpy DR, Pettis JS. 2009. Colony collapse disorder: a descriptive study. *PLosONE* 4: e6481.

Wahl O and Ulm K. 1983. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica. Oecologia.*59: 106-128.

Wallner K. 1999. Varroacides and their residues in bee products. *Apidologie* 30: 235-248.

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Fig. 1 Percent and total larval mortality for bees reared in control vs. treatment comb over time.

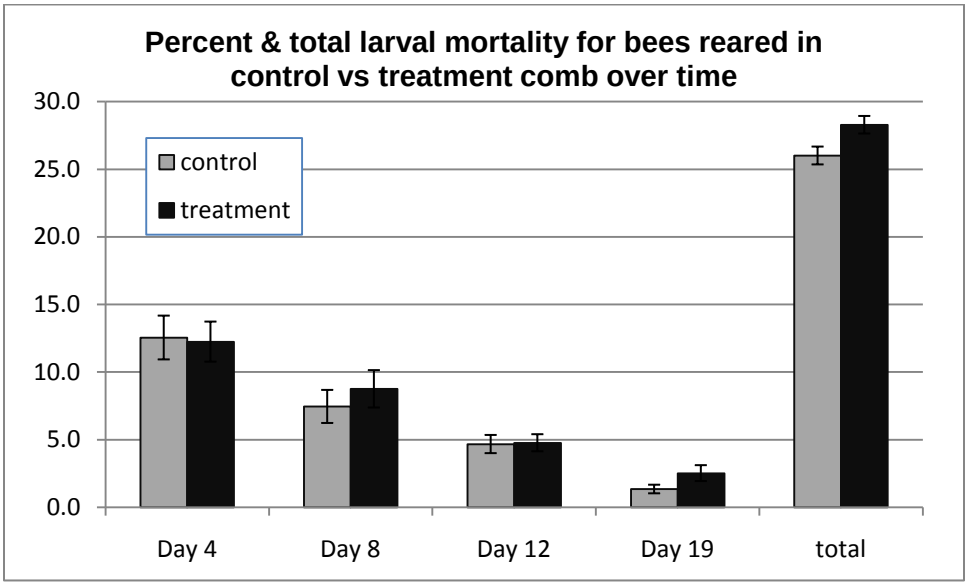

Fig. 2 Percent larval mortality each sample date for bees reared in control comb over multiple replications.

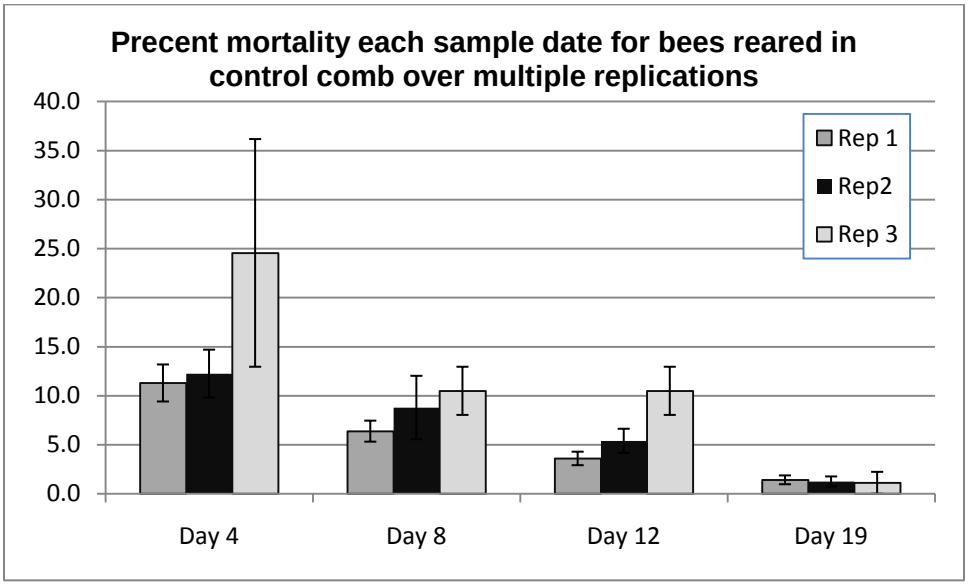

Fig. 3 Percent larval mortality each sample date for bees reared in treatment comb over multiple replications.

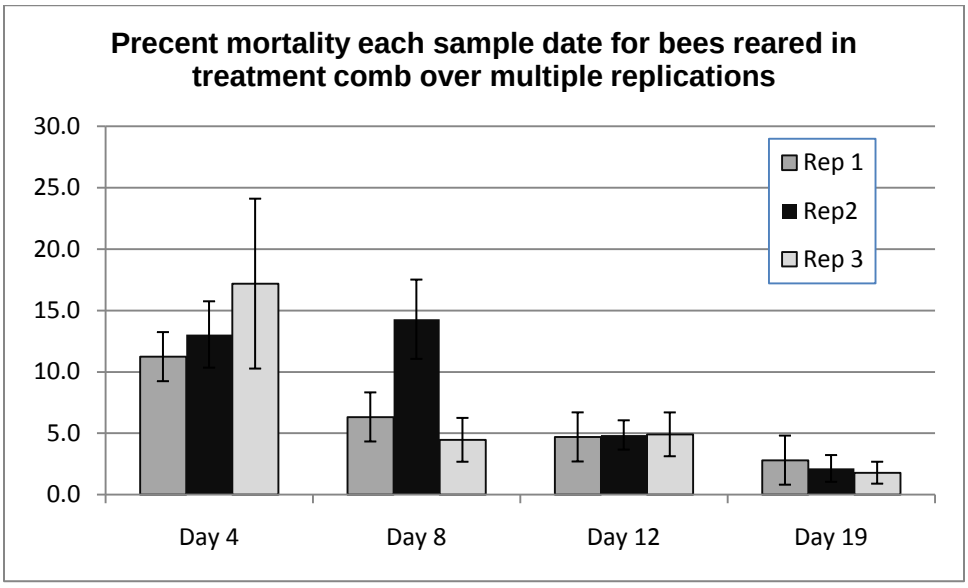

Fig. 4 Percent larval mortality in bees reared in control vs treatment comb over multiple replications.

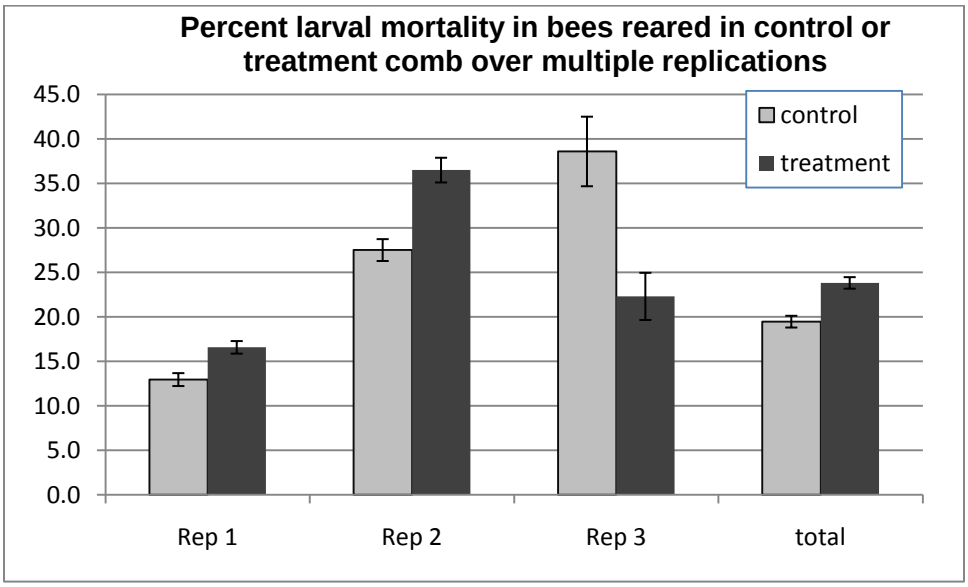

Fig. 5 Average quantity (ppb) differences between pre & post experiment analyses for control vs. treatment brood combs.

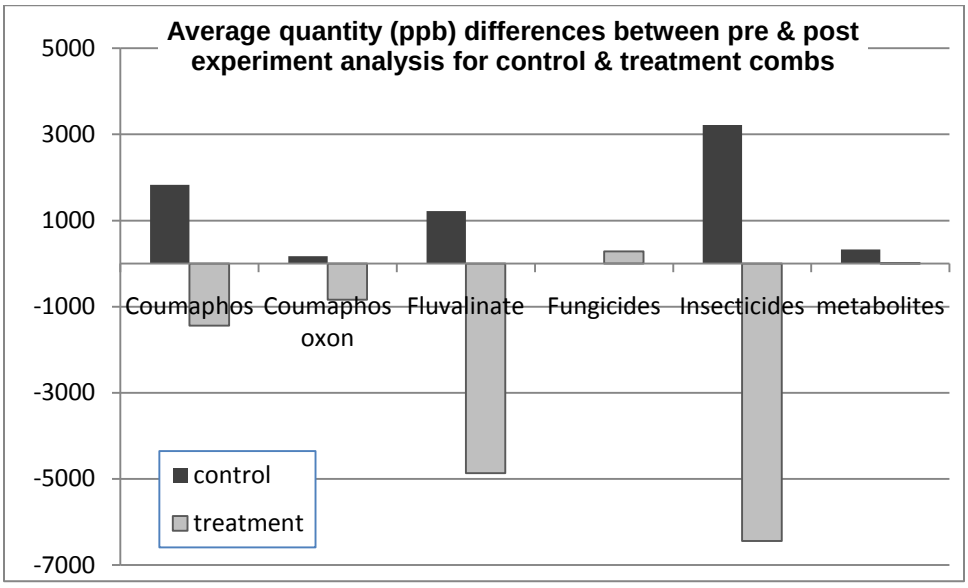

Fig. 6a Adult emergence for bees reared in control vs. treatment brood comb after the first brood cycle (Rep 1).

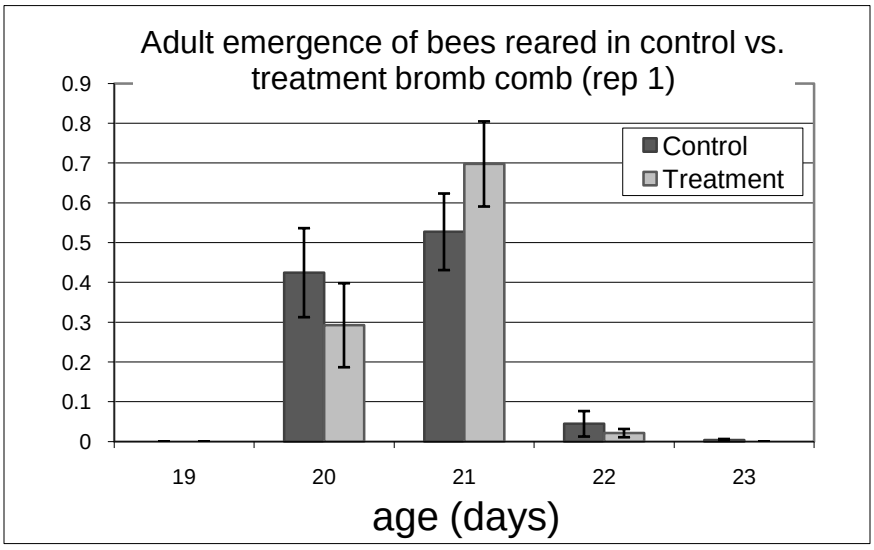

Fig. 6b Adult emergence for bees reared in control vs. treatment brood comb after the second brood cycle (Rep 2).

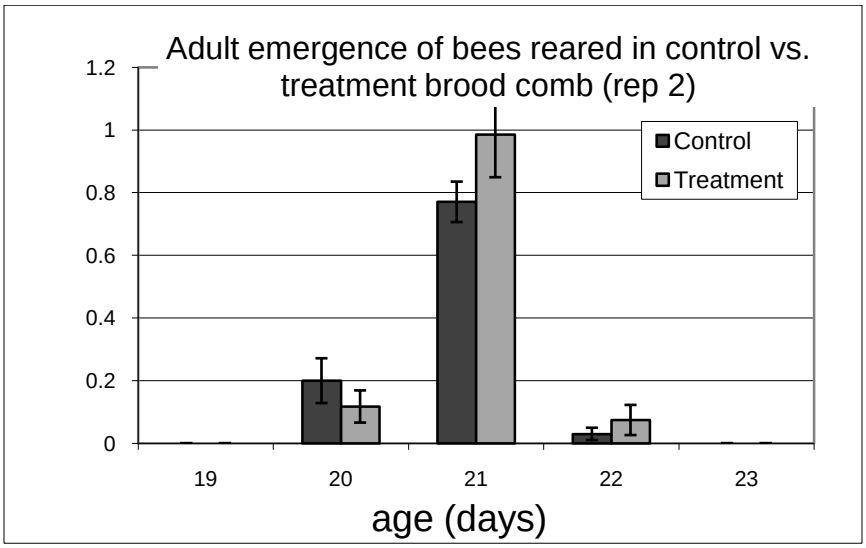

Fig. 6c Adult emergence for bees reared in control vs. treatment brood comb after the third brood cycle (Rep 3).

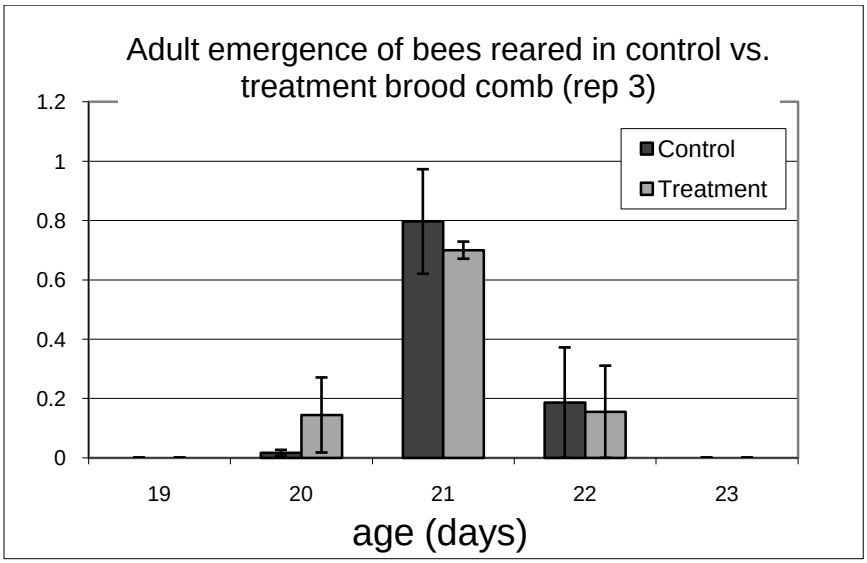

Fig. 7a Larval development of bees reared in control brood comb.

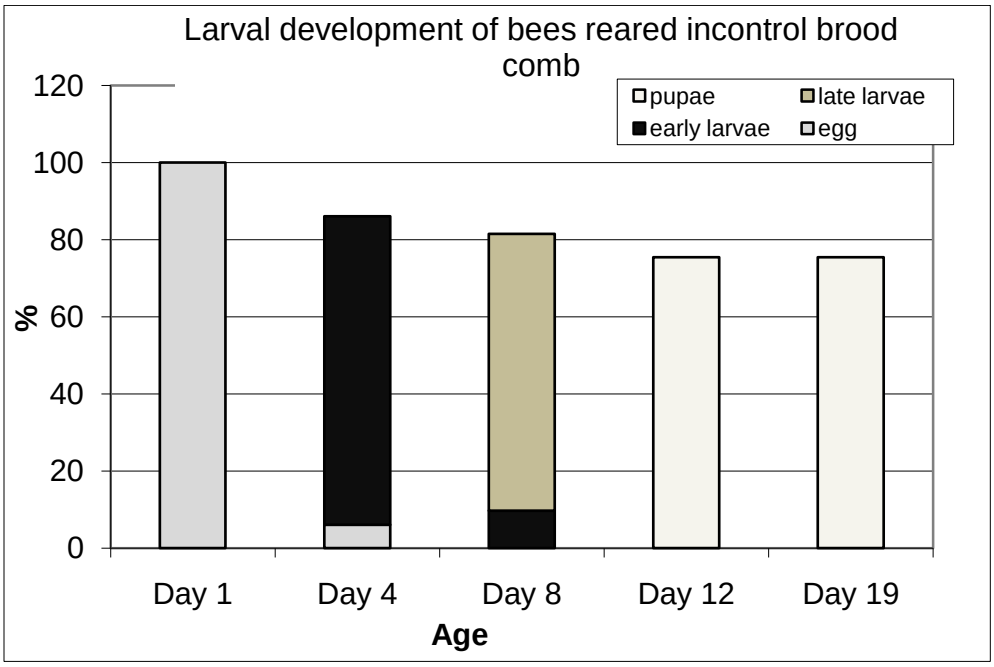

Fig. 7b Larval development of bees reared in brood comb containing 17 different pesticide residues.

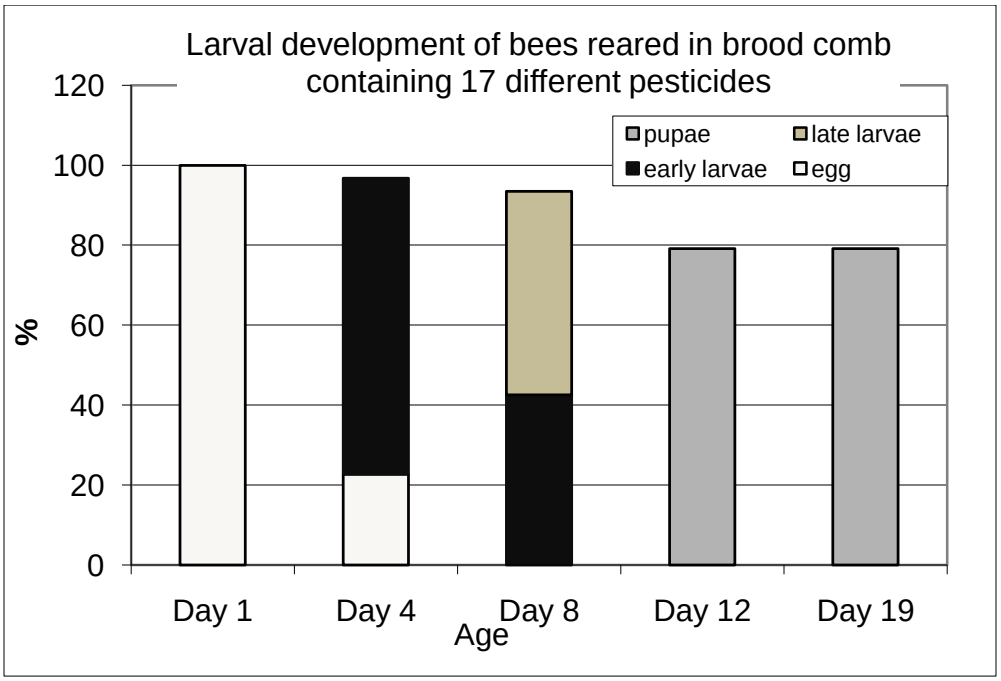

Fig. 7c Worker brood reared in brood comb containing 17 different pesticides at day 8 of development. Left: delayed growth. Right: normal development.

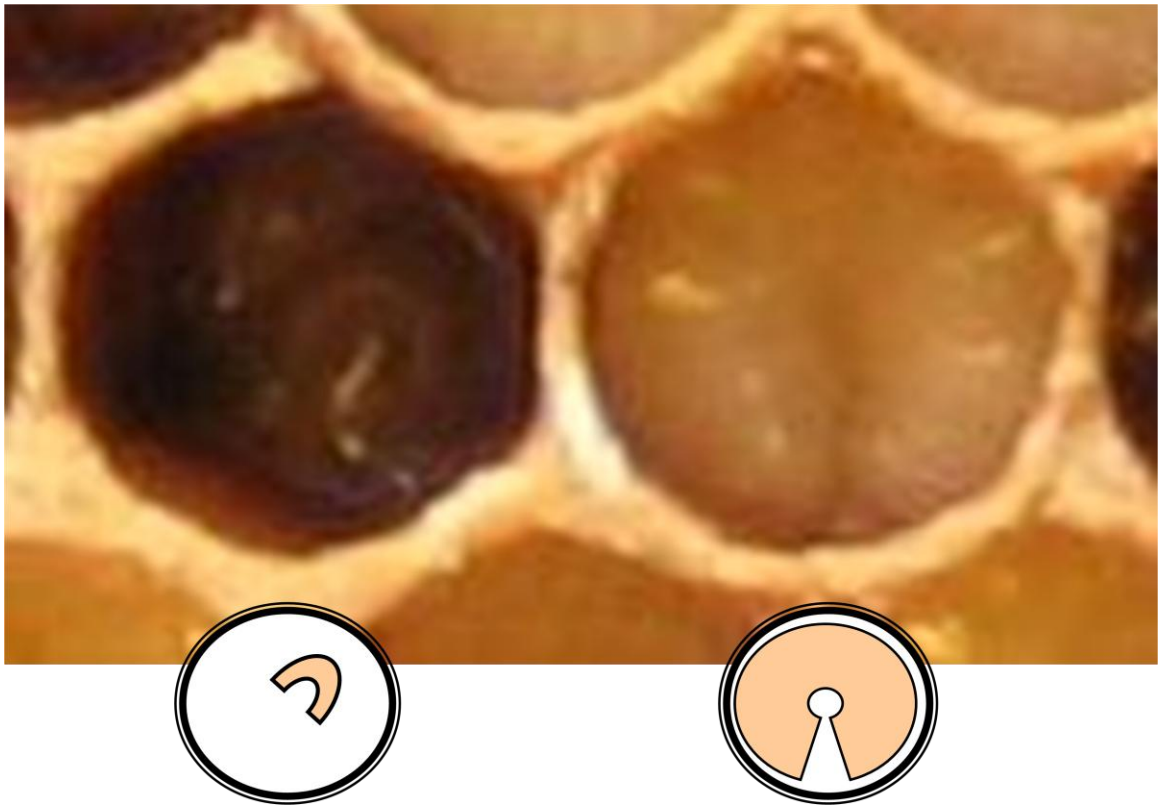

Table 1. Pesticide residues detected in treatment combs (n=13) used to rear treatment worker bees in experiments.

Active ingredient	Chemical Family	Purpose of use	Toxicity honey bee	Average (ng/g)	% detected	min	max	LOD
2,4 Dimethylphenyl formamide (DMPF)		metabolite		145	15	142	147	4
3-hydroxycarbofuran		metabolite		23	8	*	23	4
Aldicarb	Carbamate	INSECT	High	20	8	*	20	4
Azoxystrobin	Strobilurin	FUNG		19	38	5	29	2
Boscalid	Carboxamide	FUNG		35	15	35	64	4
Carbendazim (MBC)		metabolite		21	31	4	48	5
Carbofuran	Carbamate	INSECT	High	32	8	*	32	5
Chlorothalonil	Chloronitrile	FUNG		17	62	4	66	1
Chlorpyrifos	Ogranophosphate	INSECT	High	8	62	3	15	1
Clothianidin	Neonicotinoid	INSECT	High	35	8	*	35	20
Coumaphos	Ogranophosphate	INSECT	Mod	8079	100	281	22100	1
Coumaphos oxon		metabolite		596	100	10	3140	1
Cyfluthrin	Pyrethroid	INSECT	Low	43	17	8	79	2
Cypermethrin	Pyrethroid	INSECT	High	2	8	*	2	2
Cyprodinil	Anilinopyrimidine	FUNG		27	31	13	61	16
Diazinon	Ogranophosphate	INSECT	High	1	15	1	2	1
Dicofol	Chlorinated hydrocarbon	INSECT	Low	6	23	4	8	1
Dinotefuran	Neonicotinoid	INSECT	High	97	8	*	97	30
Diphenylamine	Amine	INSECT		151	23	20	281	1
Endosulfan 1	Chlorinated hydrocarbon	INSECT	Mod	2	54	1	4	1
Endosulfan II	Chlorinated hydrocarbon	INSECT	Mod	2	38	1	5	1
Endosulfan sulfate		metabolite		1	31	1	2	1
Esfenvalerate	Pyrethroid	INSECT	High	5	46	1	12	1
Fenhexamid	Hydroxyanilide	FUNG		46	8	*	46	6
Fenpropathrin	Pyrethroid	INSECT	High	7	8	*	7	1
Fluvalinate	Pyrethroid	INSECT	High	6712	100	164	24340	1
Imidacloprid	Neonicotinoid	INSECT	High	45	8	*	45	20
Iprodione	Dicarboximde	FUNG		283	8	*	283	20
Malathion oxon		metabolite		22	8	*	22	4
Norflurazon	Fluorinated pyridazinone	HERB		5	8	*	5	6
Oxamyl	Carbamate	INSECT	High	22	8	*	22	5
Oxyfluorfen	Diphenyl ether	HERB		2	23	1	2	1
Permethrin total	Pyrethroid	INSECT	High	103	8	*	103	10
Phosalone	Ogranophosphate	INSECT	Mod	32	8	*	32	10
Pyrethrins	Pyrethroid	INSECT	High	229	8	*	229	50
Thiacloprid	Neonicotinoid	INSECT	Low	113	8	*	113	8
Thiamethoxam	Neonicotinoid	INSECT	High	38	8	*	38	20
THPI		metabolite		96	15	93	99	50
Vinclozolin	Dicarboximde	FUNG		1	8	*	1	1

Toxicity category for honey bees: High; LD50 ≤2 µg/bee = highly toxic; Mod; LD50 2-11 µg/bee = moderately toxic; minimum and maximum ranges, LOD; limit of detection.

Table 2. Pesticide residues contained in treatment brood comb with observed delayed development of worker honey bees

Pesticides	Chemical family	Systemic	Toxicity honey bee	(ng/g) ppb	LOD
3-hydroxy-carbofuran	metabolite	Systemic		23	4
Aldicarb	Carbamate	Systemic	High	20	4
Carbofuran	Carbamate	Systemic	High	32	5
Chlorothalonil	Fungicide		---	4	1
Clothianidin	Neonicotinoid	Systemic	High	35	20
Coumaphos	Organphosphate		Moderate	22100	1
Coumaphos oxon	metabolite			1850	5
Cyfluthrin	Pyrethroid		High	7.9	2
Dinotefuran	Neonicotinoid	Systemic	High	97	30
Diphenylamine	Amine		---	281	1
Endosulfan 1	Organochlorine		Moderate	1	1
Fluvalinate	Pyrethroid		High	164	1
Imidacloprid	Neonicotinoid	Systemic	High	45	20
Malathion Oxon	metabolite			22	4
Oxamyl	Carbamate	Systemic	High	22	5
Thiacloprid	Neonicotinoid	Systemic	High	113	8
Thiamethoxam	Neonicotinoid	Systemic	High	38	20

Toxicity category for honey bees: High; LD50 ≤2 µg/bee = highly toxic; Mod; LD50 2-11 µg/bee = moderately toxic; LOD; limit of detection.

MANUSCRIPT THREE

Increased susceptibility to *Nosema* spore (Microsporidia) infection in honey bees (*Apis mellifera*) reared from brood combs containing high levels of pesticide residues

ABSTRACT

Nosema ceranae and pesticides are factors contributing to honey bee health decline. Bees reared from brood comb containing high or low levels of pesticide residues were placed in common colony environments. Treatment colonies were inoculated with *N. ceranae* spores weekly and, subsequently, workers were sampled from treatment and control colonies and analyzed for *Nosema* spores. A higher proportion of bees reared from contaminated comb were infected with *N. ceranae* and spore infectivity occurred at a younger age then compared to bees reared from control comb. These data suggest that developmental exposure to pesticides in brood comb increases the susceptibility of bees to *Nosema* spore infection. (105 words)

SHORT COMMUNICATION

Nosema ceranae is an obligate intracellular parasite that infects the honey bee midgut, robbing nutrients and causing energetic stress (Higes et al. 2006, Mayack and Naug 2009). Spore infection is considered highly pathogenic and widespread in European honey bees (*Apis mellifera*). *Nosema ceranae* spores have been found in honey bee samples collected as early as 1995 in the United States and *N. ceranae* infection was recently identified as a potential contributing factor in the phenomenon

known as colony collapse disorder, CCD (Chen et al. 2008, van Engelsdorp et al. 2009). Transmission and distribution of *Nosema ceranae* in the hive is still unclear, although high spore levels are generally found in older foraging bees than in young adult bees (Higes et al. 2008). Infected bees have difficulty absorbing nutrients and may consume more resources to compensate for deficiencies (Naug and Gibbs 2009). Forager bees collected from infected colonies and allowed to feed on sucrose *ad libitum* showed no difference in mortality from uninfected bees, while infected bees with restricted food excess had significantly lower survivability and shortened longevity (Mayack and Naug 2009). Colonies infected with *Nosema* spores typically have reduced nectar and pollen stores and reduced colony population, possibly due to insufficient resources resulting from compensatory feeding or less productive foraging by infected bees. In addition to behavioral changes, *Nosema ceranae* infection can suppress individual and social immunity in honey bees leaving bees more vulnerable to other pest and pathogen invasions (Antúnez et al. 2009).

Pesticide exposure has also been identified as a potential factor in CCD and some studies have reported high levels of pesticide residues in bees and bee products, such as bee bread, honey, and wax comb (Bogdanov et al 1996, Mullin et al. 2010). Pesticide exposure can have sub-lethal effects on honey bees, impairing memory, communication, and flight navigation, which are important aspects of foraging and social organization (Haynes 1988, Bortolotti 2003, Desneux et al. 2007). Exposure to pesticides during queen bee development yielded weight and ovary reduction, increased queen cell rejection, early supercedure, and reduced egg-laying (Haarmann 2002). Other studies have shown increased susceptibility to *Nosema ceranae* infections

in adult bees fed imidacloprid, a neonicotinoid insecticide (Alaux et al. 2009). Conversely, adult bees artificially infected with *Nosema* spores were more susceptible to DDT and trichlorfon, an organochlorine and an organophosphate insecticide, respectively (Ladas 1972).

In this study, we examined potential effects of developmental exposure to pesticide residues and subsequent susceptibility to *Nosema ceranae* spore infection in adult worker honey bees. Two full-sized colonies (numbered 295 and 103) were monitored for *N. ceranae* infection status weekly by regular sampling of forager bees, collected on outer food frames. *N. ceranae* spore inoculant (50 million spores in 10 ml of 50% sucrose syrup) was fed to colony 103 each week, while 10 ml of 50% syrup (without spores) was fed to colony 295.

Two brood comb frames (labeled Y and G) from separate apiaries were sampled and analyzed for pesticide residues. The brood combs were screened for 171 of the most commonly used pesticides, including several pesticide metabolites. Comb Y was positive was 10 different pesticide residue detections, including 7 insecticides and 3 metabolites. In comb G there were 4 insecticides, 1 fungicide, and 2 metabolites, totaling 7 detections (Table 1). A single comb source was used for control treatments (paired with either comb Y or G). The control comb was positive for one pesticide; coumaphos residues at 20.9 ppb. Treatment comb frames were cut into blocks (11x11-cm) containing roughly 450 cells. A standard Langstroth frame, with the center area (22x11-cm) removed, was used as a frame support for a pair of treatment comb blocks, one control comb adjacent to either Y or G comb blocks.

The two experimental frame supports, containing paired block treatments (comb Y/control; comb G/control) were kept in a single colony, under the same environmental conditions. A laying queen was caged over each frame containing the pair of comb blocks for 24 hours. Eggs were monitored through development at day 4, 8, 12, and 19. Paired block treatments were separated with push-in cages and incubated at day 19 at 30-33ºC. Newly emerged adult workers reared from brood combs (Y & G) with high levels of known pesticide residue levels (n=547) and from relatively uncontaminated control comb (n=468) were tagged with Testor's enamel and placed into colony 295 with normal *Nosema* spore levels (no inoculants) and colony 103 where *Nosema* spore inoculants (50 mil spores) were added each week. Twenty painted bees from each treatment were sampled from both colonies at week 2, 3, and 4 post-release. Sampling error for week 4 was large due to the limited number of painted bees that remained available from each treatment. A total of 178 bees were sampled from colony 295 (controls = 87, comb Y & G = 91) and 211 bees from colony 103 (controls = 104, comb Y & G = 107). Bees were individually examined for the presence of *N. ceranae* spores and spore levels were quantified using a haemocytometer (Cantwell 1970).

Our results revealed a higher proportion of bees reared from contaminated combs Y & G were infected with *Nosema ceranae* spores, compared to bees reared from relatively uncontaminated control comb for both colonies ($p<0.0001$)(Fig. 1). *Nosema ceranae* spore inoculants had no effect on the proportion of infected bees. However, infected bees sampled from colony 103 (added *Nosema*) had higher mean spore counts for both control and treatment combs, compared to infected bees sampled from colony 295. Infected bees from treatment comb Y & G had spore counts

averaging 5,059,000 and 142,000 in colonies 103 and 295, respectively. To compare, infected control bees had spore levels averaging 1,925,000 and 67,000 in colonies 103 and 295, respectively. Similarities in the proportion of infected bees among treatments between the two experimental colonies suggest that, while spores may be readily available in the hive or environment, individual susceptibility to spore infection is an important factor in driving epidemiological consequences of *N*. *ceranae* infection. Additionally, *N. ceranae* spores were detected in a greater proportion of comb Y & G bees at 2 weeks old than in control bees and infectivity continued to increase over time for comb Y & G bees. Two percent of control bees were infected with spores at week 2 compared to 20% infection in comb Y & G bees. Although *Nosema* spore infection is typically detected in older forager bees, these results suggest that bees exposed to pesticide residues in brood comb during larval development are more susceptible to *Nosema ceranae* infection and at a younger age.

Comparisons of *Nosema* infection between bees reared from comb Y and G revealed higher mean spore counts (overall and by week) in bees reared from comb G had than compared to bees from comb Y (Fig. 1). Although the quantity and identification of the mixed pesticide residues contained in comb Y and G are known, we cannot definitively pinpoint causative active ingredients. Nonetheless, interaction effects between pesticide exposure and *Nosema ceranae* infection need to be investigated further, especially when considering the level of pesticide contamination found in brood comb (Mullin 2010).

REFERENCES

Alaux C., Brunet, J., Dussaubat, C., Mondet, F.,Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P., Le Conte, Y. 2009. Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Envirnomental microbiology. 12, 774-782.

Antúnez K., Martín-Hernández R., Prieto L., Meana A., Zunino P., Higes M. 2009. Immune suppression o=in the honey bee (*Apis mellifera)* following infection by *Nosema ceranae* (Microsporidia). Environmental Microbiology. 11, 2284-2290.

Bogdanov S, Kilchenmann V, Imdorf A. 1996. Acaricide residues in beeswax and honey. In Mizrahi A, Lensky Y, eds, *Bee Products*. Plenum Press, NY. pp. 239-247.

Bortolotti L., Montanari R., Marcelino J., Medrzycki P., Maini S., and Porrini C. 2003. Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. Bulletin of Insectology 56 (1): 63-67.

Cantwell G.E (1970). Standard methods for counting *Nosema* spores. Am. Bee. J. 110, 222-223.

Chen Y., Evans J.D. Smith B.I., Pettis J.S. 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97, 186-188.

Desneux N., Decourtye A., and Delpuech J. M. 2007. The sublethal effects of pesticides on beneficial arthropods. Ann. Rev. Entomol. 52: 81-106.

Haarmann T., Spivak M., Weaver D., Weaver B., and Glenn T. 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. J. Econ. Entomol. 95: 28-35.

Haynes K.F. 1988. Sublethal effects of neurotoxic insecticides on insect behavior. Ann. Rev. Entomol. 33: 149-68.

Higes M., Martín R., Meana A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 93-95.

Higes M., Martín-Hernández R., Botías C., Bailón E.G., González-porto A.V., Barrios L., Nozal M.J.D., García-Palencia P., Meana A. 2008. How natural infection by *Nosema ceranae* causes honey bee colony collapse. Environmental Microbiology 10: 2659-2669.

Ladas A. 1972. The influence of some internal and external factors upon the insecticide resistance of honeybee. Apidologie. 3: 55-78.

Mayack C. and Naug D. 2009. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100, 185-188.

Mullin CA, Frazier M, Frazier JL, Ashcraft S., Simonds R, van Engelsdorp D, Pettis JS. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLOS one* 5: e9754

Naug D. and Gibbs A. 2009. Behavioral changes mediated by hunger in honeybees infected with *Nosema ceranae.* Apidologie. 40, 595-599.

Taylor K.S., Waller G.D., and Crowder L.A. 1987. Impairment of a classical conditioned response of the honey bee (*Apis mellifera* L.) by sublethal doses of synthetic pyrethroid insecticides. Apidologie 18 (3): 243-252.

van Engelsdorp D., Evans J., Saegerman C., Mullin C., Haubruge E., Nguyen B.K., Frazier M., Frazier J., Cox-Foster D., Chen Y., Underwood R., Tarpy D.R., Pettis J.S. 2009. Colony collapse disorder: a descriptive study. *PLosONE* 4: e6481.

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Fig. 1. Proportion of *N. ceranae* infected bees (x-axis) and mean spore level within infected bees (y-axis) reared from control & treatment combs at week 2, 3, 4 post adult emergence.

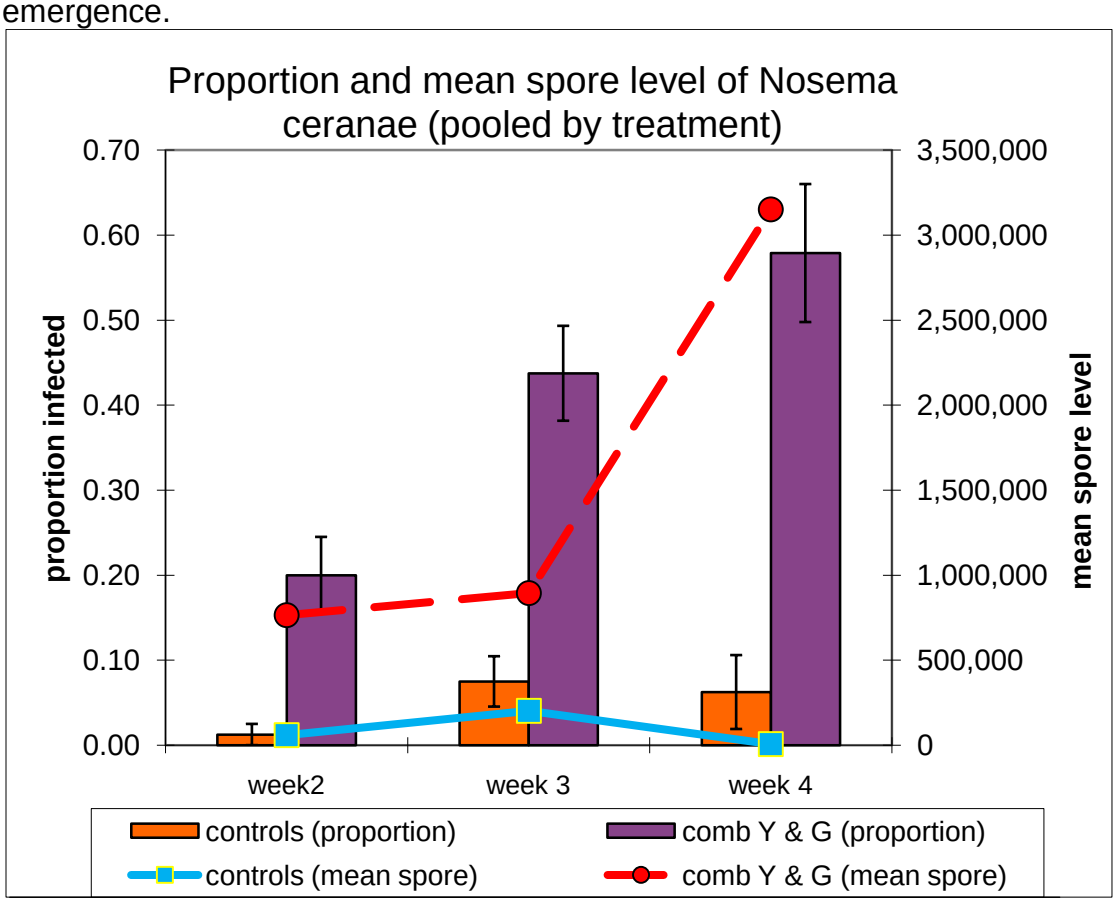

Table 1. Characteristics of pesticide residues detected in experimental treatment combs Y & G

	Active ingredient	Purpose of use	Chemical family	Toxicity	LD50-bee (µg/g)	ppb/bee	Detection (ng/g)	LOD (ng/g)
Comb Y	2,4 Dimethylphenyl formamide (DMPF)	Metabolite of INSECT (amitraz)	Amidine				142	4
	Chlorpyrifos	INSECT	OP	High	0.11	858	8.5	1
	Coumaphos	INSECT	OP	Mod	4.6	36114	7230	1
	Coumaphos oxon	Metabolite of INSECT (coumaphos)	OP				231	5
	Endosulfan I	INSECT	OC	Mod	7.0	54600	2.1	1
	Endosulfan II	INSECT	OC	Mod	7.0	54600	1.6	1
	Esfenvalerate	INSECT	PYR	High	0.41	3198	12.3	1
	Fluvalinate	INSECT	PYR	High	0.88	6864	6800	1
	Phosalone	INSECT	OP	Mod	4.5	35100	31.7	1
	THPI	Metabolite of FUNG (captan)	Thiophthalimide				98.7	50
Comb G	2,4 Dimethylphenyl formamide (DMPF)	Metabolite of INSECT (amitraz)	Amidine				147	4
	Coumaphos	INSECT	OP	Mod	4.6	36114	281	1
	Coumaphos oxon	Metabolite of INSECT (coumaphos)	OP				10.2	5
	Chlorothalonil	FUNG	Chloronitrile				65.7	1
	Fluvalinate	INSECT	PYR	High	0.88	6864	11280	1
	Permethrin total	INSECT	PYR	High	0.11-0.24	226-1248	103	10
	Pyrethrins	INSECT	PYR	High	0.02	172	229	50

Active ingredient, purpose of use (FUNG, fungicide; INSECT, insect/acaricide), chemical family (OC, organochlorine; OP, organophosphate; PYR, pyrethroid), toxicity to honey bees (High, highly toxic LD_{50}<2µg/bee; Mod, moderately toxic LD_{50}= 2-11 µg/bee), LD_{50} values for honey bees, *ppb/bee (converted LD_{50}), detections (ng/g), LOD (limit of detection)

* ppb/bee (based on LD/LC50-bee and average fresh weight per bee (128 mg) (Pollinator protection: a bee & pesticide handbook. 1990. C.A Johansen, D.F. Mayer)