Acaricide, Fungicide and Drug Interactions in Honey Bees (*Apis mellifera*)

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Acaricide, Fungicide and Drug Interactions in Honey Bees (Apis mellifera)

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Abstract

Background: Chemical analysis shows that honey bees (Apis mellifera) and hive products contain many pesticides derived from various sources. The most abundant pesticides are acaricides applied by beekeepers to control Varroa destructor. Beekeepers also apply antimicrobial drugs to control bacterial and microsporidial diseases. Fungicides may enter the hive when applied to nearby flowering crops. Acaricides, antimicrobial drugs and fungicides are not highly toxic to bees alone, but in combination there is potential for heightened toxicity due to interactive effects.

Methodology/Principal Findings: Laboratory bioassays based on mortality rates in adult worker bees demonstrated interactive effects among acaricides, as well as between acaricides and antimicrobial drugs and between acaricides and fungicides. Toxicity of the acaricide tau-fluvalinate increased in combination with other acaricides and most other compounds tested (15 of 17) while amitraz toxicity was mostly unchanged (1 of 15). The sterol biosynthesis inhibiting (SBI) fungicide prochloraz elevated the toxicity of the acaricides tau-fluvalinate, coumaphos and fenpyroximate, likely through inhibition of detoxicative cytochrome P450 monooxygenase activity. Four other SBI fungicides increased the toxicity of tau-fluvalinate in a dose-dependent manner, although possible evidence of P450 induction was observed at the lowest fungicide doses. Non-transitive interactions between some acaricides were observed. Sublethal amitraz pre-treatment increased the toxicity of the three P450-detoxified acaricides, but amitraz toxicity was not changed by sublethal treatment with the same three acaricides. A two-fold change in the toxicity of tau-fluvalinate was observed between years, suggesting a possible change in the genetic composition of the bees tested.

Conclusions/Significance: Interactions with acaricides in honey bees are similar to drug interactions in other animals in that if P450-mediated detoxication appears to play an important role. Evidence of non-transitivity, year-to-year variation and induction of detoxication enzymes indicates that pesticide interactions in bees may be as complex as drug interactions in mammals.

Introduction

Chemical analysis of honey bees (Apis mellifera) and hive products show that most managed bee colonies in North America and Europe are repositories of a suite of chemical contaminants, including an assortment of insecticides, acaricides, herbicides and fungicides [1–3]. While a number of the residues detected were insecticides, many of which are highly toxic to bees, compounds of low acute toxicity were detected most frequently and at the highest concentrations in both bees and hive products. Some of the most ubiquitous contaminants of bees and bee products, coumaphos and tau-fluvalinate [3], are abundant in the hive environment because both are deliberately introduced as therapeutic acaricides to control the ectoparasitic mite, Varroa destructor. Varroa is the most serious pest of managed honey bee colonies in Europe and North America and clearly plays a role in the recent colony losses associated with colony collapse disorder [4–6]. Varroa weakens colonies in two ways: directly, by consuming the hemolymph of adult and pupal bees and, indirectly, by vectoring honey bee viruses and causing immunosuppression in parasitized bees [7].

In the face of the serious challenges presented by Varroa, beekeeping has become dependent on management techniques to control mite infestations, with apicultural acaricides playing a major role [4]. However, finding chemical control agents that selectively kill an arthropod pest of an arthropod host poses a unique pharmacological challenge. Synthetic pesticides that have been used as acaricides include the pyrethroid tau-fluvalinate (Apistan and Mavrik), the organophosphate coumaphos (Chek-Mite+, Perizin and Asuntol 50), the formamidine amitraz (Apivar and Taktik) and the pyrazole fenpyroximate (Hivastan and FujiMite). Natural products are also used for Varroa control, including the monoterpenoid thymol (ApilifeVar and ApiGuard).
and the organic acids, oxalic acid (Oxivar) and formic acid (MiteAway Quick Strips). There is little doubt that bees can benefit from reduced Varroa populations through the effective use of acaricides in combination with other management techniques [4,6,7]. In a chemical survey of honey bee colonies suffering from colony collapse disorder, the healthiest colonies were found to have higher concentrations of one acaricide, coumaphos [8].

The effectiveness of tau-fluvalinate [9] and coumaphos [10] has waned as Varroa populations have developed resistance to these acaricides. However, tau-fluvalinate and coumaphos remain common contaminants in the hive environment, partially as a result of continued application by beekeepers, and partially due to their lipophilic properties which lead to accumulation and persistence in beeswax [1,11]. Both coumaphos and tau-fluvalinate survive the wax recycling process and are present in newly manufactured wax foundation [3,12]. While amitraz itself does not accumulate in bee colonies [13], the amitraz metabolite 2,4-dimethyl formamide (DMF) has been detected in both bees and wax [3]. Oxalic acid is a natural product that can be found in honey and as an allelochemical in plants [14], though not at concentrations used for Varroa control. While thymol and other monoterpenoids may be naturally present in floral sources at low concentration, the high concentrations needed for acaridal activity may noticeably contaminate honey and wax [12,13]. With the wide range of acaricides currently in use and the continued presence of lipophilic acaricides in beeswax, it is quite likely that bees will be exposed to multiple acaricides simultaneously.

In addition to the acaricides, beekeepers may also apply antimicrobial drugs to control bacterial and microsporidial pathogens. Fumagillin (Fumadil-B) is fed in sucrose syrup to control infection by the microsporidian gut pathogen Nosema apis and Nosema ceranae. Oxytetracycline (TerramycinTM), and tylosin (TylanTM) are applied in powered sugar or syrup to control American foulbrood (Paenibacillus larvae) and other bacterial infections. To prevent harvested honey from contamination many prists, and acaricides are subject to a withholding period during which these therapeutics cannot be applied. As such, beekeepers are left with a relatively narrow window during which antimicrobial or acaricide applications are possible, potentially leading to a situation where multiple treatments are applied simultaneously.

In addition to compounds applied by the beekeeper, bees may also be exposed to plant protection products applied to flowers and flowering crops. Fungicides are the most abundant and common of the plant protection products found in bees and bee products because fungicides can be applied during bloom when bees are present [3,8,16]. While fungicides generally appear safe for adult bees [17], these compounds may, in certain situations, produce harmful effects [10]. For instance chlorothalonil (Bravo), the most commonly detected fungicide in bees and bee products [3], was found in “entombed pollen” in colonies suffering from colony collapse disorder [19]. Larval and pupal mortality has been reported in bees exposed to the fungicides pyraclostrobin and boscalid, which together constitute Pristine [16]. There are also documented interactions between the sterol biosynthesis inhibiting (SBI) fungicides and pyrethroid insecticides in honey bees [20–22]. For example, prochloraz is a SBI fungicide that functions through the inhibition of fungal cytochrome P450-monoxygenase (P450) mediated synthesis of ergosterols. Prochloraz has been shown to inhibit detoxicative P450 activity in honey bees as well, particularly in relation to detoxication of pyrethroid pesticides [20,21,23,24]. Pristine and a variety of SBI fungicides are used during bloom on almond orchards, when bees are present, and have been detected in pollen samples [3,16].

With the potential for managed honey bees to experience simultaneous exposure to acaricides, antimicrobials and fungicides it is important to consider the potential for harmful interactions between these compounds. Any interactions observed could provide an insight into honey bee physiology and will shed light on bees’ mechanisms of tolerance for both natural and synthetic xenobiotics. The present study aims to test for interactions between these most abundant contaminants of the hive environment using pair-wise lethal dose bioassays in which a sublethal pre-treatment with one acaricide, fungicide or antimicrobial is followed by a series of lethal doses of an acaricide. Mortality counts were then used to fit log-probit regression lines and determine lethal dose values (LD50). Acaricides were also combined with model enzyme inhibitors to characterize the classes of detoxicative enzymes that may be the basis for interactive effects.

Bliss [25], recognized three principal types of interactive effects that pesticide or drug combinations may elicit [26]. If no interaction occurs, and a combination is found to be only as deadly as its most toxic constituent, then the components of a mixture are understood to act independently. Such “independent joint action” is the null hypothesis for the experiments presented here and is characterized by acaricide toxicity that is unchanged by prior exposure to any other compound.

Interactive effects between compounds are observed when the toxicity of a drug or pesticide combination is either more or less toxic than expected based on the toxicity of the most toxic constituent. In the context of these experiments an interactive effect is defined as a change in the toxicity of an acaricide following sublethal pre-treatment with a fungicide, antimicrobial drug or another acaricide. An agonistic interaction is defined by the elevated toxicity of a drug or pesticide combination, while an antagonistic interaction is characterized by decreased toxicity. Interpreting the biological basis of interactive effects between compounds requires that the mode of action of the drugs or pesticides are known. Additive agonistic interactions are most likely to occur when different compounds work through the same mode of action. Synergistic agonistic interactions probably occur when the compounds work through different modes of action. The mode of action for each compound is listed in Figure 1 and 2 for each acaricide, fungicide and antimicrobial compound [27–34].

Materials and Methods

Bees were first treated with a sublethal dose of either an acaricide, a fungicide, an antimicrobial drug, or an enzyme inhibitor. Bees were then treated with a range of lethal doses of an acaricide to estimate the dose-response curve. Sublethal pretreatments included: six acaricides (tau-fluvalinate, coumaphos, fenpyroximate, amitraz, thymol and oxalic acid); eight fungicides of which three were non-SBI (pyraclostrobin, boscalid, chlorothalonil) and five were SBI fungicides (prochloraz, propiconazole, fenbuconazole, metconazole and myclobutanil); three antimicrobials (oxytetracycline, tylosin and fumagillin); and three enzyme inhibitors (diethyl maleate, S,S,S-trifluorophosphorothioate, and piperonyl butoxide). Lethal doses of five acaricides (i.e. all except oxalic acid) were applied as a second treatment. We tested 25 of the 6×6 possible acaricide-antimicrobial combinations, 24 of the 6×5 fungicide-acaricide combinations, 15 of the 6×3 antimicrobial-acaricide combinations and 9 of the 6×3 enzyme inhibitor-acaricide combinations. The acaricide oxalic acid was never used as a lethal agent in pairwise combinations because it was not
possible to deliver a lethal dose of this compound in the standardized volume of solvent. Enzyme inhibitors were not tested with tau-fluvalinate or coumaphos as these combinations have been tested previously [22,35]. The combination of pyraclostrobin and coumaphos was tested, but insufficient data were collected to allow for a meaningful comparison. The fungicides propiconazole, fenbuconazole, metconazole and myclobutanil were tested in combination with a single acaricide, tau-fluvalinate.

**Chemicals**

Technical grade chemicals were used for all trials, with the exception of the antimicrobial drugs. The acaricides coumaphos, fenpyroximate and tau-fluvalinate were obtained from Chem Services Inc. (West Chester, PA), thymol from Aldrich Chemical Co. (Milwaukee, WI) and oxalic acid from Fisher Scientific (Rochester, NY). The model enzyme inhibitors piperonyl butoxide (PBO) and diethyl maleate (DEM) were obtained from Acros Organics (Morristown, NJ) and S,S,S-tributylphosphorotrithioate (DEF) from Chem Services. The fungicides boscalid, pyraclostrobin, prochloraz, fenbuconazole, metconazole, myclobutanil and propiconazole were also purchased from Chem Services, and chlorothalonil from Fluka Analytical (St. Louis, MO). All chemicals were serially diluted in HPLC-grade acetone for topical application.
Insects

Fourteen honey bee colonies maintained at the University of Nebraska – Lincoln East Campus provided bees for bioassays conducted from April – September 2009 and 2010. Colonies were requeened in 2010 with naturally mated Italian queens (C. F. Koehne & Sons, Inc., Glenn, CA). Bacterial brood diseases were prevented with Terramycin (oxytetracycline) treatments in March of both years. Nosema spp. infection was controlled using Fumagilin-B (fumagillin) fed in March 2009. Apigard and oxalic acid were the only acaricides used in the apiary to control Varroa populations during the four years prior to conducting this study.

Frames of late-stage brood were collected from these colonies and placed in a dark, humid incubator (Darwin Chambers Co., St. Louis, MO, model H024) at 34°C. Newly emerged adults were brushed from frames daily into screened wooden cages (21 × 14 × 12 cm) and weighed as a group to estimate the number of bees. Each cohort of 200–800 bees was provisioned with 1:1 (w/w) granulated sugar dissolved in water and held for 3–4 days in the incubator.

Pre-treatment with Sublethal Doses of Acaricide, Fungicide, Antimicrobial Drug or Inhibitor

Each treatment series was conducted using 3–4 day-old adult worker bees divided into 8–13 subgroups of 20 bees each. Bees were narcotized with carbon dioxide and each subgroup was placed in a separate wax-coated paper cup (177 cm³; Solo S306, Highland Park, IL) covered with cotton cheesecloth and secured with two rubber bands. A maximum sublethal pre-treatment of each acaricide, fungicide, enzyme inhibitor, or solvent control, was then applied to bees in all subgroups. Model enzyme inhibitor pre-treatment for acaricides was determined in the current experiment. Each acaricide pre-treatments applied to each bee were: 1 µg tau-fluvalinate, 3 µg coumaphos, 1 µg fenpyroximate, 1 µg amitraz, 10 µg thymol and 100 µg oxalic acid. Sublethal fungicide pre-treatment doses for each bee were: 10 µg chlorothalonil, 10 µg prochloraz, 10 µg pyraclostrobin, 20 µg boscalid, and 10 µg pyraclostrobin together with 20 µg boscalid to replicate the ratio of active ingredients in Pristine. All doses were delivered topically in 1 µl of acetone and applied to the thoracic notum using a 50 µl syringe fitted in a repeating dispenser (Hamilton PB-600, Reno, NV). A control pre-treatment consisting of 1 µl of pure acetone was also included.

Bioassays testing the potential for interactive effects between tau-fluvalinate and three different doses of the SBI fungicides, prochloraz, fenbuconazole, metconazole, myclobutanil, prochloraz and propiconazole, plus PBO, were performed in 2010. To standardize for the different molecular weights of PBO and the SBI fungicides bees were pre-treated at eqimolar dose levels: 0.1, 1 or 10 nanomoles fungicide per bee (corresponding to approximately 0.03, 0.5 and 3 µg per bee). Acetone was also applied as a pre-treatment control.

Bees receiving formulated antimicrobial drug pre-treatments were fed Duramycin-10 (40% oxytetracycline, Durvet, Blue Springs, MO, USA), Tylan (100% tylosin, Elanco, Greenfield, IN, USA) and Fumagilin-B (2.1% fumagillin, Medivet, Mandeville, LA, USA), dissolved in 50% sucrose water and fed to bees at a concentration of 1 mg/ml oxytetracycline active ingredient (a.i.), 1 mg/ml tylosin (a.i.) and 0.5 mg/ml fumagillin (a.i.). Drug feeding began 24 h prior to acaricide treatment and continued until mortality was scored. The sublethal concentrations used are similar to label recommendations for application of these drugs to whole colonies. The drugs in 50% sucrose water, and a sucrose water control, were fed to large groups of bees for the first 24 h of exposure through 20 ml glass scintillation vials covered with cotton cheesecloth, which were weighed before and after feeding to determine drug consumption. After acaricide treatment drugs in 50% sucrose water were provided in punctured 1.5 ml microcentrifuge tubes.

Acaricide Dose-response Determination

Groups of pre-treated bees were allowed to recover for 1 h to minimize mortality due to extended carbon dioxide anaesthetization. Each subgroup of approximately 20 bees, of the 8–13 dose-groups in the bioassay, was then anaesthetized again and treated topically with either an acetone solvent control or one of a range of 7–12 acaricide doses, including doses eliciting 0% and 100%
Statistical Analyses

Lines were fitted to dose-mortality data on a log-probit scale for each pre-treatment-acaricide combination using glm in the R statistical package [36]. From these lines the lethal dose 50% (LD50) values and accompanying 95% confidence intervals were calculated using Fieller’s method, with correction for heterogeneity where appropriate [37]. Interactive effects between acaricides and the various pre-treatments were determined with pairwise tests comparing the dose-response lines for bees receiving pre-treatments with bees receiving a control pre-treatment using a test analogous to ANCOVA [26]. The full model, which includes the dose of acaricide as a covariate, pre-treatment as a categorical factor and the interaction between acaricide dose and pre-treatment, was compared with two simplified models (Figure 3). A process of model simplification was undertaken in which the explanatory power of model terms was assessed by reference to the likelihood ratio [36]. The first simplified model lacks the interaction term and tests the interaction between pre-treatment and acaricide dose, essentially testing for differences in the slope of the two dose-response lines (also known as a test of the “hypothesis of parallelism”) [26]. A significant change in slope, as determined by a significant “dose by pre-treatment” interaction, may indicate competitive inhibition between the pre-treatment and acaricide [26]. The second simplified model lacks the pre-treatment factor entirely and was used to test the effect of pre-treatment on acaricide toxicity (also known as the “hypothesis of equality”) [26]. A significant pre-treatment effect is evidence of an agonistic or antagonistic interaction between the two treatments.

Statistical significance for the model comparisons was determined by comparing likelihood ratios, corrected for heterogeneity, against an F-distribution [26,36]. P-values for the 73 pairwise comparisons were corrected using the Holm-Bonferroni procedure (Figures 1 and 2). The 15 pairwise comparisons between tau-fluvalinate and SBI fungicides were separately corrected for multiple comparisons. A sample R script showing the analysis is available (Methods S1).

Results

The relative toxicity of topical application of the five acaricides to bees receiving the control pre-treatment, from most to least toxic, was amitraz>fenpyroximate>tau-fluvalinate>coumaphos>thymol in 2009 (Figure 1 and Table S1). In 2010, the pattern of toxicity was similar, but fenpyroximate was found to be the most toxic acaricide and amitraz the second most toxic (Figure 2 and Table S2). Tau-fluvalinate, using identical methods, was more toxic to bees in 2010 than in 2009 (based on non-overlap of 95% confidence intervals) (Figures 1 and 2). Fenpyroximate also appeared to be more toxic to bees in 2010, though bees in 2010 were not subjected to acetone pretreatment.

The different acaricides varied greatly in their propensity for interaction with other compounds. Tau-fluvalinate interacted with most other compounds tested, including all 5 acaricides, 8 of 9 fungicides or a fungicide combination, and 2 of 3 antimicrobial compounds. Fewer interactions were observed with coumaphos, fenpyroximate and thymol. Coumaphos interacted with 3 of 5 acaricides, 1 of 4 fungicides and none of the 3 antimicrobials. Fenpyroximate interacted with 3 of 5 acaricides, 2 of 3 fungicides and 2 of 3 antimicrobials. Thymol interacted with 3 of 5 acaricides, 2 of 5 fungicides and none of the 3 antimicrobials. Amitraz did not interact with any fungicides or antimicrobials, but did demonstrate an antagonistic interaction with a single acaricide, oxalic acid.

Approximately half of the acaricide-acyaricide (15 of 25) and acaricide-fungicide (13 of 28) combinations tested showed evidence of interactions, nearly all of which were agonistic and resulted in increased acaricide toxicity (Figures 1 and 4). Four interactions were detected among the 15 antimicrobial-acaricide combinations tested, two of which were antagonistic interactions with fenpyroximate (Figure 2). Bees fed oxytetracycline demonstrated no change in tau-fluvalinate toxicity at the LD50 level, but a significant increase in the slope of the fitted dose-response line was observed. Bees fed antimicrobial drugs as a pre-treatment consumed 5.44±0.67 μl sugar water/bee/day, regardless of drug content (ANOVA, p>0.05, N = 27).

Piperonyl butoxide, a model P450 inhibitor, and DEF, a model carboxylesterase inhibitor, both increased the toxicity of fenpyroximate, but not amitraz or thymol (Figure 1).
indicated with a superscript letter a = significant pre-treatment effect, sterol biosynthesis inhibiting (SBI) fungicide at three dose levels. Significant differences compared to the control treatment are indicated with a superscript letter a = significant pre-treatment effect, b = significant pre-treatment*acaricide dose effect (Table S3).

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There was no single pre-treatment that interacted with all acaricides, although oxalic acid pre-treatment caused an interactive effect with all acaricides except coumaphos. Three compounds did not interact with any acaricide tested: the antimicrobial drug tylosin, the enzyme inhibitor DEM, and the fongicide boscalid.

The most potent agonistic interaction observed in the first year of the study was between prochloraz and tau-fluvalinate (Figure 1). To determine if similar interactions occur between tau-fluvalinate and other more widely used SBI fungicides, four additional fungicides in this class (propiconazole, fenbuconazole, metconazole, and myclobutanil) were tested in combination with tau-fluvalinate (Figure 4 and Table S3) in the second year of the study.

Antagonistic interactions were observed between tau-fluvalinate and 5 of 5 SBI fungicides when pre-treated with 0.1 nmol per bee. Tau-fluvalinate became more toxic (1.4 to 2.3-fold) when bees were pre-treated with any of the 5 fungicides or PBO at the 1 nmol dose, and much more toxic (12 to 74-fold) at the 10 nmol dose. An interaction between tau-fluvalinate and PBO (5.8-fold) was only observed at the 10 nmol dose level.

Discussion

For each major group of toxicants applied in combination with acaricides – model enzyme inhibitors, acaricides, fungicides, and antimicrobials – we first consider the physiological implications of our results, especially as they relate to the involvement of detoxification enzyme systems, as well as non-transitivity that was observed in some of the interactions. Finally, we address the environmental relevance of our findings for assessing risks of the toxicants to bee health.

Model Enzyme Inhibitor Interactions

The agonistic interactions observed between PBO and tau-fluvalinate [33], coumaphos [22] and fenpyroximate imply that P450 enzymes play a role in detoxifying these acaricides in honey bees. Heterologous expression of three honey bee P450 enzymes demonstrated that P450s are capable of metabolizing both tau-fluvalinate and coumaphos in vitro [39]. Agonistic interactions with DEF suggest that tau-fluvalinate, coumaphos and fenpyroximate are also detoxified through carboxylesterase activity. No interactions were observed between DEM and the acaricides. Therefore, either glutathione-S-transferase enzyme activity is not important for the detoxication of acaricides or DEM is not an effective inhibitor of the relevant enzymes in honey bees.

Thymol and amitraz did not interact with any of the inhibitors, which indicates that detoxication is not important for bee’s tolerance of these compounds. However, despite their unchanged toxicity in the presence of enzyme inhibitors, both thymol and amitraz have the potential to interact with detoxicative enzymes [40,41] and may interfere with detoxication of other xenobiotics.

Acaricide-acaricide Interactions

The cluster of interactions observed between tau-fluvalinate, coumaphos and fenpyroximate, all of which appear to be detoxified by P450s and carboxylesterases, suggest that interactions between these compounds may be occurring because these acaricides interact with detoxicative enzymes [22,39]. However, not every combination of these acaricides caused an interaction. Since acaricides were used both as treatments and as pre-treatments, we were able to investigate the temporal transitivity of the interactions. A combination is ‘transitive’ if the same effect occurs irrespective of the order of exposure and ‘non-transitive’ if an interaction is only observed when one of the pair of compounds is applied first. A non-transitive interaction was observed between tau-fluvalinate and fenpyroximate. While fenpyroximate pre-treatment increased the toxicity of tau-fluvalinate 8-fold, the opposite was not true. Tau-fluvalinate pre-treatment did not significantly change the toxicity of fenpyroximate. The non-transitivity exhibited between fenpyroximate and tau-fluvalinate may indicate that fenpyroximate can competitively inhibit the specific P450 isoforms involved in tau-fluvalinate detoxication [39] while tau-fluvalinate does not interact with the specific P450s that allow bees to tolerate fenpyroximate exposure.

The toxicity of thymol increased following pre-treatment with either tau-fluvalinate or coumaphos. Since it is not known how bees tolerate thymol, it is difficult to speculate about the basis for these interactions. Coumaphos toxicity was unchanged by thymol, but tau-fluvalinate toxicity was transitorily increased when thymol was applied as a pre-treatment. Monoterpenoids, like thymol, have been shown to inhibit P450 activity in human liver microsomes [40], so this agonistic interaction may be a result of thymol inhibition of the P450s on which tolerance of tau-fluvalinate depends.

Amitraz participated in three non-transitive acaricide-acaricide interactions. The toxicity of amitraz was unchanged when bees were pre-treated with tau-fluvalinate. However, amitraz pre-treatment increased the toxicity of tau-fluvalinate 5-fold. Interactions between formamidines and pyrethroids are known in other insects and may be due to synergism at the target site through cooperative binding [42], or through inhibition of pyrethroid detoxication, through inhibition of P450s or carboxylesterases [41].

Amitraz is bioactivated through a hydrolysis reaction to DMPF which is an octopamine receptor agonist [29,43]. Bees may tolerate amitraz as an acaricide because they are poor at amitraz bioactivation, possibly due to the paucity of genes that encode P450s and carboxylesterases in the honey bee genome [44]. Despite the apparent lack of amitraz bioactivation in bees, this compound may have some affinity for the P450 enzymes that are important for tau-fluvalinate and fenpyroximate detoxication.

In one of the few antagonistic interactions observed, oxalic acid pre-treatment reduced the toxicity of amitraz nearly four-fold.
However, this interaction may be an artifact of the methods used. Amitraz was applied to the same location on the thoracic notum as the oxalic acid pre-treatment, which may have caused degradation of amitraz, which is known to undergo hydrolytic breakdown under acidic conditions [45], while tau-fluvalinate and coumaphos do not [46]. All other acaricides showed some agonistic effects with oxalic acid, possibly due to increased cuticular penetration caused by the abrasive effect of oxalic acid crystals on the epicuticle or because of the elevated production of reactive oxygen species initiated by oxalic acid [47,48]. Future studies may seek to avoid the effects of local site interactions by using different application sites or different routes of administration.

Environmental Relevance of the Observed Acaricide-acaricide Interactions

High sublethal doses were deliberately chosen as pre-treatments to make the magnitude of any interactive effect between the pre-treatment and acaricide as large as possible. However, to determine the hazard posed by acaricide interactions in bee hives, the actual exposure bees receive when treated with formulated acaricides must be considered. Margins of safety for each acaricide formulation can be estimated by dividing the dose estimated to cause 10% mortality (LD10) by the estimated daily dose. The LD10 can be determined from the fitted dose response lines (Table S1). The daily dose can be estimated by first determining the amount of active ingredient present in each acaricide formulation, correcting this value by the amount of active ingredient available for uptake by the bees (e.g. 10% of the total acaricide content in strip formulations is taken up by bees [49,50]) and dividing this quantity by the number of days the acaricide formulation is present in the hive. The estimated daily dose calculated in this way can be validated using published pesticide residue data [3,51].

Based on these calculations, coumaphos and tau-fluvalinate formulations carry the largest margins of safety for honey bees (43- and 29-fold, respectively), while fenpyroximate, amitraz, thymol and oxalic acid formulations have narrower margins of safety (2.0 to 7.8-fold). A consequence of the great differences in margins of safety between acaricides is that the potent interactions observed in this study with tau-fluvalinate and coumaphos may be more likely to cause oxidative stress than the parent compound [2,3,57]. Five SBI fungicides demonstrated synergism with the pyrethroids alpha-cypermethrin and lambda-cyhalothrin in bees [23]. We found that a similar suite of SBI fungicides could interact synergistically with the pyrethroid tau-fluvalinate, likely through inhibition of P450s, when administered at 1 or 10 nmol of fungicide per bee. Unexpectedly, an antagonistic interaction was observed at the lowest fungicide doses (0.1 nmol or approximately 0.03 μg per bee). This antagonism suggests that, in addition to inhibiting bee P450 enzymes, the SBI fungicides may also induce P450 gene expression at low levels of exposure, including the genes encoding P450 isoforms that are involved in tau-fluvalinate detoxication [39]. Low doses of P450 inhibitors have been found to induce P450 gene expression in other insects [58] and SBI fungicides can induce P450 enzyme activity in mammals [59]. Quercetin, a pollen flavonol that is known to interact with bee P450s [60], caused a similar reduction in tau-fluvalinate toxicity in bees [61].

Pre-treatment of bees with the fungicide chlorothalonil increased the toxicity of both thymol and tau-fluvalinate. Chlorothalonil is a multi-site action fungicide in the chloronitrile family and is metabolized through P450 activity to 4-hydroxy-2,5,6-trichloroisophthalonitrile. This metabolite is both more toxic and more likely to cause oxidative stress than the parent compound [62,63]. Competitive P450 inhibition of tau-fluvalinate detoxication may account for this observed interaction as well. Chlorothalonil has previously been shown to have an interactive effect with alpha-cypermethrin and lambda-cyhalothrin, two pyrethroids, in bees [23].

Fungicide-acaricide Interactions

In 2009 experiments the SBI fungicide prochloraz produced an almost 2000-fold increase in the toxicity of tau-fluvalinate. Tau-fluvalinate, coumaphos and fenpyroximate — all acaricides for which P450-mediated detoxication was implicated through synergistic interactions with PBO — also showed a synergistic interaction with prochloraz. These results confirm previous reports of interactions between pyrethroid insecticides and prochloraz through P450s [20], and extend those findings to show that this fungicide can affect the metabolism of acaricides as well as field-applied pesticides.

Because of the potent interaction observed between prochloraz and tau-fluvalinate, additional P450-interacting SBI fungicides were tested that are extensively used in a variety of cropping systems. The SBI family of fungicides are known to be present in bee-collected pollen, wax and in bees themselves [2,3,57]. Five SBI fungicides demonstrated synergism with the pyrethroids alpha-cypermethrin and lambda-cyhalothrin in bees [23]. We found that a similar suite of SBI fungicides could interact synergistically with the pyrethroid tau-fluvalinate, likely through inhibition of P450s, when administered at 1 or 10 nmol of fungicide per bee. Unexpectedly, an antagonistic interaction was observed at the lowest fungicide doses (0.1 nmol or approximately 0.03 μg per bee). This antagonism suggests that, in addition to inhibiting bee P450 enzymes, the SBI fungicides may also induce P450 gene expression at low levels of exposure, including the genes encoding P450 isoforms that are involved in tau-fluvalinate detoxication [39]. Low doses of P450 inhibitors have been found to induce P450 gene expression in other insects [58] and SBI fungicides can induce P450 enzyme activity in mammals [59]. Quercetin, a pollen flavonol that is known to interact with bee P450s [60], caused a similar reduction in tau-fluvalinate toxicity in bees [61].

Pre-treatment of bees with the fungicide chlorothalonil increased the toxicity of both thymol and tau-fluvalinate. Chlorothalonil is a multi-site action fungicide in the chloronitrile family and is metabolized through P450 activity to 4-hydroxy-2,5,6-trichloroisophthalonitrile. This metabolite is both more toxic and more likely to cause oxidative stress than the parent compound [62,63]. Competitive P450 inhibition of tau-fluvalinate detoxication may account for this observed interaction as well. Chlorothalonil has previously been shown to have an interactive effect with alpha-cypermethrin and lambda-cyhalothrin, two pyrethroids, in bees [23].

Pyraclostrobin and boscalid applied together in a blend like Pristine moderately increased the toxicity of tau-fluvalinate. When applied individually, however, only pyraclostrobin had an effect. Both boscalid and pyraclostrobin kill fungi through the inhibition of respiration by blocking mitochondrial electron transport at complexes II and III, respectively [64,65]. Pyraclostrobin also moderately increased the toxicity of fenpyroximate, which is itself an inhibitor of mitochondrial respiration at complex I [66]. Fenpyroximate, pyraclostrobin and boscalid in combination have been hypothesized to harm bees by starving them of energy through inhibition of oxidative phosphorylation. Alternatively, the interaction observed between fenpyroximate and pyraclostrobin may be the product of increased oxidative stress caused by their interference with mitochondrial electron transport [67]. Increased mortality related to oxidative stress may also explain the interaction observed between tau-fluvalinate and pyraclostrobin, as pyrethroids can also cause increased production of reactive oxygen species [68]. Other respiration-inhibiting fungicides including iprodione and vinclozlin [32] may also be of concern as these compounds have been
found at high concentration in chemical analyses of bees and bee products [3].

Environmental Relevance of Fungicide-acaricide Interactions

Consumption of contaminated pollen is the most likely route of exposure to fungicides [57]. Assuming a colony consumes an average of 20 kg of pollen per year to rear 150,000 bees or about 150 mg pollen consumed for each bee [69] the typical fungicide exposure a bee receives through pollen can be estimated. Assuming that most pollen is eaten by nurse bees during the first 10 days of adulthood [69], a nurse bee could be exposed to as much as 1.3 μg chlorothalonil per day by consuming 13 mg of pollen containing 99 ppm chlorothalonil [3]. While this dose is approximately one-eighth the dose of chlorothalonil applied in this study, we speculate that it may be sufficient to interact with tau-fluvalinate and thymol. The thymol-chlorothalonil interaction is of particular concern given the relatively low estimated margin of safety afforded by thymol formulations (Table 1).

Pollen has been found to contain as much as 1 ppm boscalid and 0.26 ppm pyraclostrobin [3]. Nurse bees consuming this pollen may take in as much as 0.015 μg and 0.003 μg per day of boscalid and pyraclostrobin, respectively, which is three orders of magnitude less than the doses of boscalid and pyraclostrobin that demonstrated agonistic interaction with acaricides in this study. Given the mild interactions observed between these fungicides and the acaricides, and the relatively low level of these fungicides found in pollen [3], it seems unlikely to us that adult bees would suffer acute mortality from Pristine™-acaricide combinations under field conditions.

The SBI fungicides are also known to enter the hive through contaminated pollen [57]. Mullin et al. [3] detected the SBI fungicide myclobutanil at levels as high as approximately 1 ppm in pollen. At this concentration a nurse bee would be expected to consume 0.013 μg of myclobutanil per day, which corresponds to about half the lowest dose of this fungicide administered. A single-dose exposure to myclobutanil at this level could counter intuitively protect the bee, to some extent, from tau-fluvalinate toxicity, possibly through induction of detoxicative enzymes. However, chronic dietary exposure at this level could lead to potent synergistic interactions, as we observed at higher fungicide doses, depending at the rate at which myclobutanil is metabolized or excreted.

### Table 1. Estimated margins of safety for six acaricides based on estimated daily dosages per bee and the estimated LD10.

| Formulated Product | Active Ingredient | g | Treatment Duration (Days) | Predicted Daily Exposure (μg/bee) | Reported Mean Exposure (μg/bee) | Max. (μg/bee) | LD10 (μg/bee) | Estimated Margin of Safety |
|--------------------|------------------|---|--------------------------|----------------------------------|--------------------------------|-------------|-------------|--------------------------|
| Apistan            | tau-fluvalinate  | 1.4| 56                       | 0.13                             | 0.04*                         | 0.70*       | 3.08        | 24                       |
| Checkmite+         | coumaphos       | 2.8| 45                       | 0.31                             | 0.09*                         | 3.20*       | 12.1        | 39                       |
| Hivistan           | fenpyroximate   | 0.7| 42                       | 0.40                             | –                              | –           | 2.19        | 5.5                      |
| Apivar             | amitraz         | 1.0| 42                       | 0.12                             | 0.15*                         | 9.04*       | 0.92        | 7.8                      |
| ApilifeVar         | thymol          | 49 | 32                       | 7.6                              | –                              | –           | 15.4        | 2.0                      |
| Apiguard           | thymol          | 25 | 28                       | 4.5                              | –                              | –           | 15.4        | 3.4                      |
| oxalic acid        |                  | 1.8| 1                        | 44                               | –                              | –           | 176.7c      | 4.0                      |

For reported concentrations of acaricides in bees: a = [3], and b = [51]. See the text for methods used to estimate daily exposure. Margins of safety are calculated by dividing the LD10 dose (Table S1 and c = [76]), by the predicted daily dose.

Acaricide, Fungicide and Drug Interactions in Bees

Simultaneous exposure to tau-fluvalinate and oxytetracycline, which blocks multi-drug resistance transporters, has been shown in previous work to increase tau-fluvalinate toxicity [70]. In our testing, pretreating bees with oxytetracycline had no significant effect on the LD50 of tau-fluvalinate; however, the “pre-treatment by acaricide dose” effect was significant, indicating that the slope is significantly steeper for bees receiving oxytetracycline treatment (Table S1). This may be evidence of oxytetracycline effects on the drug transporter enzymes [70]. While amitraz and oxytetracycline have been shown to induce programmed cell death in the honey bee midgut [71], this combination had no effect on acute mortality.

An antagonistic interaction was observed between fenpyroximate and oxytetracycline. Oxytetracycline has been reported to have antioxidant properties [72], which may ameliorate the effects of fenpyroximate, a pesticide known to cause oxidative-stress in mammals [67].

Between-year Variability

Honey bees treated in 2009 were more tolerant of tau-fluvalinate than bees in both previous studies [22] and bees treated in 2010. A study conducted in 2008 using an identical protocol determined an LD50 for tau-fluvalinate of 6.75 μg per bee [22]. The LD50 for tau-fluvalinate in 2009 was determined to be 19.8 μg per bee, which is substantially higher than 9.2 μg and 9.0 μg per bee determined in 2010 for oral and topical control pre-treatments, respectively. This substantial difference in susceptibility to tau-fluvalinate and other acaricides between years occurred despite the fact that bees were kept in the same apiary, in the same woodenware, and were collected for bioassays over the same months of the year and, for the most part, were subjected to bioassays by the same workers using the same equipment and technical grade tau-fluvalinate purchased from the same source. The only obvious difference between years was the genetic stock as queens in all colonies were replaced at the beginning of the season in 2010. Elzen et al. [73], noted that European honey bees (A. mellifera ligustica) were significantly more tolerant of the pyrethroid cyfluthrin than African honey bees (A. mellifera scutellata) and suggested that this difference may be the product of inadvertent selection for pyrethroid tolerance resulting from the widespread use of tau-fluvalinate as an acaricide in managed European honey bee colonies. In the same manner, the observed difference in tau-fluvalinate toxicity between populations of European honey bees...
may be due to inadvertent selection for tolerance through queen breeders’ choice of acaricides.

Conclusions

Detoxification by P450s appears to be the basis for the tolerance bees show toward tau-fluvalinate, coumaphos and fenproximate, but not amitraz or thymol. Any SBI fungicide that inhibits P450-mediated detoxification has the potential to interact with tau-fluvalinate, and likely coumaphos and fenproximate as well. Given the large number of pesticides to which bees are potentially exposed, and the practical impossibility of testing every possible combination, examining pesticide interaction with P450s holds promise as a method to simplify pesticide interaction testing in a rational way. Testing for P450 interactions could serve as the first tool in a lab-based “toolbox” testing for potential pesticide interactions in bees.

While these laboratory bioassays point to potential problems associated with the various acaricide treatments, any management recommendations must be based on additional information gained from field experiments using whole colonies. Lethal-dose bioassays, by definition, require the use of doses that are high enough to cause acute mortality in bees – doses that are often much higher than bees are likely to encounter under field conditions. However, documentation of these interactions provides a foundation for future experiments using field-relevant doses and helps to focus the limited resources available for field experiments on those pesticide combinations with the greatest potential to cause harm. The routes of exposure to acaricides, fungicides and antimicrobials in beehives may be different from the topical and oral applications used in these bioassays. The actual exposure bees receive to formulated acaricides, sequestered acaricides in beecwax, and fungicide applications in agriculture need to be quantified to accurately assess the risk posed by interactions. Additionally, bees may experience sublethal effects that are, by definition, not quantifiable in lethal dose bioassays, but may have a substantial effect on colony health [74,75]. Until more is known about the potential for lethal-dose bioassays, but may have a substantial effect on colony health [74,75]. Until more is known about the potential for interaction between acaricides, fungicides and antimicrobials in the real world it would be prudent for beekeepers to avoid concurrent use of acaricides that are detoxified by P450s – tau-fluvalinate, coumaphos, and fenproximate – especially in settings where honey bees may be simultaneously exposed to the P450-inhibiting SBI fungicides.

Supporting Information

Table S1 Dose-response line parameters and pairwise comparisons for topical application of five acaricides following sublethal topical pre-treatment with another acaricide, fungicide or detoxicative enzyme inhibitor. (DOCX)

Table S2 Dose-response line parameters and pairwise comparisons for topical application of five acaricides following 24 h oral treatment with antimicrobial drugs. (DOCX)

Table S3 Dose-response line parameters and pairwise comparisons for tau-fluvalinate following treatment with sterol biosynthesis inhibiting fungicides. (DOCX)

Methods S1 Sample R script for lethal dose determination and pairwise comparison of two dose-response curves. (DOC)

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Author Contributions

Conceived and designed the experiments: RMJ LD BDS MDE. Performed the experiments: RMJ LD MDE. Analyzed the data: RMJ. Wrote the paper: RMJ LD BDS MDE.

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