# Synergistic interactions between a variety of insecticides and an EBI fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.)

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## Abstract

In recent years, concern has been raised over declines of farmland bees, including honey bees and bumble bees, in Europe and North America. Pesticides have been proposed as a potential cause of this decline. Bees could be exposed to variety of agrochemicals at the same time, which may cause synergistically detrimental impacts, but these 'cocktail effects' are incompletely understood. We therefore investigated the toxicity of the fungicide imazalil in mixture with one of four commonly used insecticides in three major chemical classes: fipronil (phenylpyrazoid); cypermethrin (pyrethroid); thiamethoxam; and imidacloprid (both neonicotinoids). EBI fungicides like imazalil can inhibit P450 detoxification systems in insects and therefore fungicide-insecticide co-occurrence might produce synergistic toxicity in bees. We assessed the impact of dietary fungicide-insecticide mixtures on the mortality and feeding rates of laboratory bumble bees (*Bombus terrestris* L.).

Regarding mortality, imazalil synergised the toxicity of fipronil, cypermethrin and thiamethoxam, but not imidacloprid. We found no synergistic effects on feeding rates. Our findings suggest that P450-based detoxification processes are differentially important in mitigating the toxicity of certain insecticides, even those of the same chemical class. Additionally, our results indicate that risk assessment schemes should in future address the potential for cocktail effects to amplify the toxicity of farmland agrochemicals to wild bees.

**Keywords:** Bumble bees, EBI fungicide, insecticides, synergy

## 1 INTRODUCTION

Recently, concern has been raised over pollinator declines in Europe and North America[1]. In some regions, beekeepers have experienced severe losses among colonies of managed honey bees (*Apis mellifera* L.)[2] and among some wild bees[3] there is evidence for extinctions[4] and range contractions[5]. Bee declines are of concern because of the valuable pollinator services that they provide to crops and wildflowers[6, 7]. The declines probably have various anthropogenic causes, including the use of pesticides in intensively cultivated farmland[8].

In farmland, pollinators may be exposed to several pesticides during their lifetime because numerous pesticide residues are present in bee forage plants[9] and in various hive matrices of managed honey bees[10]. For example, Mullin *et al.*[11] found 118 different pesticides and their metabolites among the various matrices (e.g. stored honey and bee bread) of honey bee hives. Contemporary intensive agriculture involves protecting crop plants with a variety of pesticides, including fungicides and insecticides, and bees will almost certainly encounter these residues in mixture when they forage in agrochemically treated bee-attractive crops[12, 13].

The existence of disproportionate, or non-additive, toxicity of pesticides in mixture is known as a 'cocktail effect', 'synergistic interaction'[14], or 'potentiation'[15]. Our focal example arises from the capacity of certain fungicides, which typically have low toxicity to insects, to greatly increase the toxicity of an insecticide by inhibiting the insect's capacity to metabolically degrade the insecticide. Specifically, the widely used group of fungicides known as ergosterol biosynthesis inhibitors (EBIs) are well known to increase toxicity to honey bees of pyrethroid insecticide in mixture[16]. However, while mixture effects have been tested widely in honey bees[17, 18], the susceptibility of wild bees to these synergistic interactions has not been fully explored. We therefore investigated the potential for an EBI fungicide, imazalil, to synergise (or, more strictly, potentiate) the toxicity to bumble bees of environmentally relevant insecticides from a varied range of chemical families, namely the neonicotinoids (thiamethoxam and imidacloprid), pyrethroids (cypermethrin) and phenylpyrazoles (fipronil).

The four focal insecticides that we studied all target the insect nervous system. The neonicotinoids block the ligand-gated ion channels of the nicotinic acetylcholine receptors. In bees, dietary exposure to neonicotinoids can impair a wide range of behavioural and life history-related characteristics[19] including homing behaviour[20], colony performance[21] and foraging activity[22]. The pyrethroid cypermethrin affects insect sodium channels[23] and has been demonstrated to affect longevity[24] and respiratory patterns[25] in bees. The phenylpyrazole fipronil blocks GABA-gated chloride channels in the insect central nervous system and can affect longevity in bees[26].

We chose imazalil to represent the EBI fungicides. Imazalil is environmentally relevant because its residues can occur in combination with imidacloprid in fruit orchards[27] and it is water soluble, which facilitates dose preparation. Due to their low toxicity to insects in pure exposures, EBI fungicides are not considered harmful to farmland bees provided that the 'good practice' label rates and prescriptions are followed[28]. However, the EBI fungicides can detrimentally affect bees' tolerance for other pesticides because of effects on metabolic detoxification pathways. A certain degree of insecticide tolerance in bees is possible due to metabolic detoxification of the active ingredients by enzymes of the cytochrome P450 system[17]. Impairment of the P450 system by EBI fungicides can result in the increase of insecticide toxicity for bees[29]. Therefore, the principal aim of our present study was to establish the involvement of metabolic detoxification in bumble bee-pesticide interactions by testing whether imazalil synergises various insecticides representing some of the major chemical families that are widely used in farmland crop protection.

# 2 MATERIAL AND METHODS

## 2.1 Bee provenance and husbandry

Bumble bees (*Bombus terrestris* L. ssp *audax*) were purchased as boxed queen-right colonies from commercial suppliers (Koppert Biological Systems, Berkel en Rodenrijs, Netherlands and BioBest, Westerlo, Belgium). For each of five separate experiments, adult workers were collected from a single colony under red light and individually allocated to a wooden cage (0.07 x 0.05 x 0.04 m) whose two largest faces were covered by ventilating mesh. Each cage was supplied with a small *ad libitum* syrup feeder. During experiments, the bees were kept in a semi-controlled environment (24±1°C, ~47% relative humidity and 12:12 h dim light:darkness). During experimental exposures, the caged bumble bees were fed on dose-appropriate syrup *ad libitum* and their feeders were weighed before and at the end of the experiment (after 48 h of exposure) in order to measure syrup consumption. We recorded mortality at 24 h and 48 h of exposure. Bees were considered dead when they did not move their legs or antennae and did not respond to stimulation.

## 2.2 Exposure to agrochemicals

In order to test for synergistic interactions between the fungicide and a single insecticide, each experiment comprised four treatments: (1) undosed controls; (2) fungicide alone; (3) insecticide alone; and (4) fungicide-insecticide mixture. At the University of Exeter laboratory, we conducted four separate experiments (one per focal insecticide) in which we delivered sublethal dietary doses of the four agrochemical treatments in feeder syrup (Attraker, Biological Systems, Berkel en Rodenrijs, Netherlands). At the Estonian University of Life Sciences laboratory, Tartu, we repeated the experiment conducted at Exeter (12 bees per treatment) on imidacloprid using both a larger number of replicates (i.e. 20 per treatment) and also the local procedures for dose preparation in order to validate the result previously obtained at Exeter. Except for the imidacloprid experiment at Exeter, each treatment was replicated in at least 20 bumble bee individuals in every experiment.

For each agrochemical, we used experimental doses (see below) that aimed to produce approximately 20% mortality in exposures to single dietary substances. The purpose of this level of dosing was both to demonstrate that the fungicide and insecticide were physiologically active in the exposed bees and also to provide enough capacity for the dietary mixture to reveal a statistically detectable synergistic interaction between the test substances, if it should exist. Specifically, if the two test substances each separately cause 20% mortality in treatment groups of 20 bees (i.e. 4 deaths per treatment), then their mixture is expected to cause 36% mortality (i.e. approximately 7 deaths) if they act independently (see Eq. 1 below) and a statistically significant non-independence (synergy) is detected when mortality exceeds 65% (13 deaths) in the mixture (see statistical testing below).

Before incorporation into diets, the active substances were dissolved initially in small volumes of acetone, which was subsequently adjusted so that syrup in each treatment group contained 1% of acetone including the undosed control diet according to the method described by Thompson *et al.*[24] The dietary concentrations of the active substances in the feeder syrups were as follows: imazalil (Sigma Aldrich), 300 mg $L^{-1}$; fipronil (Sigma Aldrich), 20 µg $L^{-1}$; thiamethoxam (Sigma Aldrich), 13 µg $L^{-1}$; imidacloprid (Sigma Aldrich), 500 µg $L^{-1}$; cypermethrin (Sigma Aldrich), 7 mg $L^{-1}$. The doses were established based on data from the literature and pilot experiments. The relatively high ratio of fungicide:insecticide concentrations in our diets facilitates the manifestation of synergistic interactions.[16]

## 2.2 Statistical analyses

We tested statistically for synergistic interactions between the fungicide and a single insecticide with a modified binomial proportion test for additivity (BPA)[38]. The BPA test uses the 'Bliss independence criterion'[30], whose basis is that:

$$p_{AB}^{exp} = p_A + p_B - p_A \cdot p_B \qquad \text{Eq. 1}$$

where $p_A$ and $p_B$ denote the probabilities of mortality due to dietary substances A and B and $p_{AB}^{exp}$ denotes the expected probability of mortality due to a dietary mixture of A and B if they act independently. If $p_{AB}^{obs}$ denotes the observed proportion of bees that die by consuming the dietary mixture of A and B, then null hypothesis of an absence of interaction is:

$$H_0 \equiv D = \left(p_{AB}^{obs} - p_{AB}^{exp}\right) = 0 \qquad \text{Eq. 2}$$

An expression that evaluates the sampling distribution of *D* under $H_0$ as a *z*-score has been produced by Sgolastra *et al.*[31], which enabled us to obtain *p*-values by approximation to a standard normal distribution. For each insecticide, BPA tests were performed separately for mortality at 24 h and 48 h. For each focal insecticide, variation among treatments in feeding rate was analysed with one-way Analysis of Variance (ANOVA) and Tukey *post-hoc* tests. In analysing feeding rates at 48 h, only data from bumblebees alive at 24 hours were used.

## 3 RESULTS

No mortality was observed in any of the control exposures (undosed syrup). When mortality was the response variable, we detected synergistic interactions between imazalil and fipronil (24 h: BPA test, P < 0.005; 48 h, n.s.), thiamethoxam (24 h, 48 h: BPA test, P < 0.005) and cypermethrin (24 h, 48 h: P < 0.001) (Figs 1 & 2). Dietary exposure to imidacloprid alone (500 µg $L^{-1}$) caused little mortality and we did not detect positive synergistic interactions between imazalil and imidacloprid in the experiment at Tartu (Figs 1 & 2). Dietary imidacloprid reduced the mortality rate due to dietary imazalil in the Exeter experiment (24 h: BPA test, P < 0.005; 48 h: P < 0.001, Supplemental Information).

Feeding rates varied among the dietary treatments (one-way Analysis of Variance, fipronil: $F_{3,87}$ = 17.1, P < 0.001; thiamethoxam: $F_{3,60}$ = 15.6, P < 0.001; imidacloprid: $F_{3,73}$ = 5.2, P < 0.01; cypermethrin: $F_{3,64}$ =25.3, P < 0.001) and generally dietary agrochemicals reduced syrup consumption (Tukey *post-hoc* tests, P≤0.05; Fig 3), but no interactions were observed between insecticides and the fungicide.

## 3 DISCUSSION

### 4.1 Synergistic effects – physiological implications

Our present study revealed that dietary exposure to the fungicide imazalil increased the toxicity to bumble bees of three out of the four insecticides that we tested, which indicates that it has the capacity to cause a positive synergistic interaction, or cocktail effect, in these insects. Our findings are consistent with several previous studies of the effects on honey bees of fungicides in mixture. In honey bees, prochloraz synergises both pyrethroid[32] and pyrazole[29] insecticides, and thiamethoxam (a neonicotinoid insecticide) is synergised by both tebuconazole[16] and boscalid[13]. Fungicides that synergise the toxicity of insecticides in honey bees act by inhibiting detoxification systems, such as the P450 enzyme complex[33]. Taken together with previous work, our results suggest that the P450s could play an important role in both honey bees and bumble bees in the detoxification of a chemically varied group of active ingredients from three chemical families, namely the phenylpyrazoles (i.e. fipronil), the pyrethroids (cypermethrin) and the neonicotinoids (thiamethoxam). These findings have a straightforward adaptive explanation because the season-long activity of social bees makes them forage-generalists who must subsist on nectar and pollen from a wide variety of plant species, each of whose blooming period is shorter than the lifespan of the colony. Many plants protect their pollen against consumption by non-pollinating flower visitors with secondary chemicals[34], which vary in constitution among plant lineages. Social bees therefore have evolved to cope with a broad spectrum of plant secondary chemicals in their diet including metabolic detoxification by active enzymes (e.g. P450 systems) in the digestive tract. These considerations suggest that social bees, including bumble bees, are pre-adapted for tolerating dietary insecticides that are artificial analogues of naturally occurring plant toxins[35], such as the nicotine- and pyrethrum-based toxicants used in the present study. It also implies that oligolectic solitary bees could be more susceptible to insecticides than their social counterparts.

Our present investigation found no evidence for a synergistic interaction during dietary exposure to a mixture of a known P450 inhibitor, imazalil, and imidacloprid in bumble bees. Similarly, previous research that exposed honey bees to imidacloprid using oral doses found little synergistic interaction with EBI fungicides[16]. Contact applications of active ingredients to the thorax of honey bees also found very weak synergistic effects of piperonyl butoxide (PBO, another P450 inhibitor) on imidacloprid, even though PBO strongly synergised the toxicity of two other neonicotinoids, acetamiprid and thiacloprid[36]. Based on these results, we tentatively propose two hypotheses. First, it is conceivable that separate detoxification systems deal with imidacloprid and the other toxicants and that one hallmark of the proposed imidacloprid-specific enzyme system is insensitivity to inhibition by imazalil and PBO. However, it is unclear what detoxification enzyme could be both specific to imidacloprid and also selectively immune to interference from imazalil and PBO. Second, it is possible that imazalil suppresses the metabolic activation of imidacloprid by a P450 enzyme system. Imidacloprid has toxic metabolites, 5-hydroxyimidacloprid and olefin, that are implicated in causing mortality[37]. Disruption of metabolic activation may also explain why the synergistic effects of imazalil on fipronil that were evident at 24 h had disappeared by 48 h; specifically, inhibition of P450 oxidative enzymes may reduce the production of fipronil's highly toxic sulfone metabolite[38]. Consequently, we postulate that complex mixture effects can arise when both detoxification and metabolic activation of an insecticide are inhibited by a second active substance, such as a fungicide.

In contrast to the effects on mortality that we observed in our experiment, no synergism was detected in regard to feeding rate, although the separate exposures to the fungicide and insecticides decreased it. These results provide further confirmation of differential sensitivity to pesticides among various endpoints like mortality and feeding rate[39]. Despite the reductions in feeding rates caused by dietary agrochemicals, it is unlikely that any of the individuals in our experiments died from starvation within the 48 h exposure, because dosed bumble bees can live for 35 days while feeding at less than half the rate of undosed controls[40].

We observed differences among our separate experiments in the levels of mortality caused by exposure to dietary imazalil. We expect that these differences originated in either intrinsic or environmental variation in the bumble bee colonies used, because our experiments were conducted in different times of year and for each experiment new bumble bee colonies were purchased. However, while the differences indicates that the severity of mixture effects can be expected to vary among real-world instances, it is unlikely that the existence of synergistic interactions (i.e. our main conclusion) can itself be governed by environmental influences or genetic variation among bees.

**4.2 Synergistic effects – environmental relevance**

Our results indicate that exposures to environmentally relevant mixtures of pesticides could be potentially harmful to wild bees even when the impacts of separate exposures to the mixture's single components are negligible. Specifically, our experiments confirm that cocktail effects arising from agrochemical pesticides are physiologically possible in bumble bees, but we recognize that further research is needed to establish their potency when bees are exposed to residues at environmentally realistic levels, which are likely to be lower than those we studied here. Thus, further empirical testing of pesticide mixtures is warranted and should be taken into account in regulations that govern the use of fungicides and insecticides in farmland.

**4.4 Summary**

Our present study revealed that certain insecticide-fungicide mixtures (except imidacloprid-imazalil) positively synergised the effect of the insecticide in bumble bees when assessed by levels of mortality, but not when assessed by variation in feeding rates. The efficacy of imazalil (an EBI fungicide) to synergise the toxicity of chemically varied insecticides suggests that P450 systems are involved in broad-spectrum detoxification in bumble bees. As previously found, imidacloprid alone was weakly synergised and the physiological basis of this differentiation is a target for future research. Our evidence that cocktail effects can arise in bumble bees should extend concern over the potential impacts of agrochemical mixtures to include wild bee species in farmland.

## ACKNOWLEDGEMENTS

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## FIGURE LEGENDS

Figure 1. Bumblebees' mortality (proportion dying) after 24 hours (with 95% confidence interval) in different treatment groups at different experiments. Different letters behind the numerical values at the same experiment indicate statistically significantly different groups ($p \leq 0.05$; pairwise Fisher exact tests followed by Bonferroni-Holm correction for multiple testing). The p-values (denoted $P_{synergistic\ effect}$) show the statistical significance of synergistic effect (one-tailed binomial proportion test).

Figure 2. Bumblebees' mortality (proportion dying) after 48 hours (with 95% confidence interval) in different treatment groups at different experiments. Different letters behind the numerical values at the same experiment indicate statistically significantly different groups ($p \leq 0.05$; pairwise Fisher exact tests followed by Bonferroni-Holm correction for multiple testing). The p-values (denoted $P_{synergistic\ effect}$) show the statistical significance of synergistic effect (one-tailed binomial proportion test).

Figure 3. Average 48-hours feeding rate (mg per 48 h per $bee^{-1}$) in different treatment groups at different experiments. Different letters behind the numerical values at the same experiment indicate statistically significantly different groups ($p \leq 0.05$; Tukey post-hoc test). Numbers of bumblebees with 48-hours feeding rate in each group are shown under the group name.

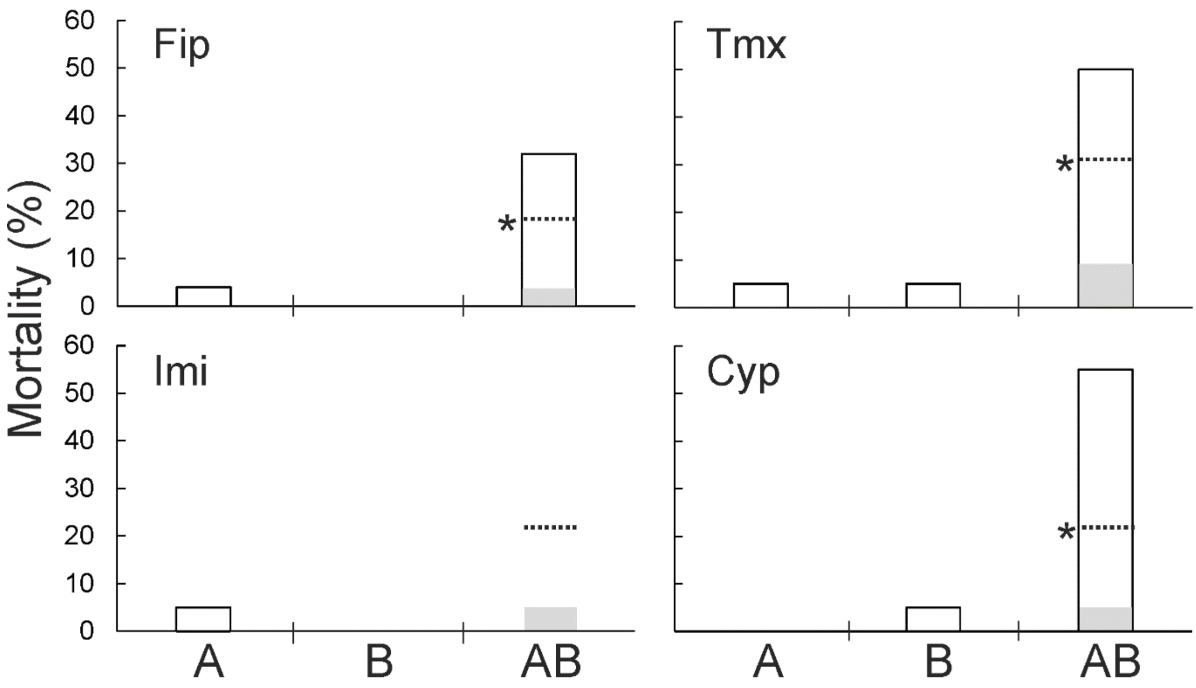



Figure 1. Mortality (*y*-axis: proportion dying, %) after 24 hours in three exposure treatments: A = dietary imazalil; B = insecticide (Fip = fipronil; Tmx = thiamethoxam; Imi = imidacloprid; and Cyp = cypermethrin); and AB = imazalil-insecticide mixture. In the AB column, the grey fill indicates the expected mortality if the components of the dietary mixture act independently ($H_0$) and the dashed horizontal line indicates the upper 95% confidence interval on the sampling distribution under $H_0$. An asterisk indicates that the mixture has produced a statistically significant synergistic effect (one-tailed binomial proportion test). A column is blank (has no bar) if no mortality occurred.

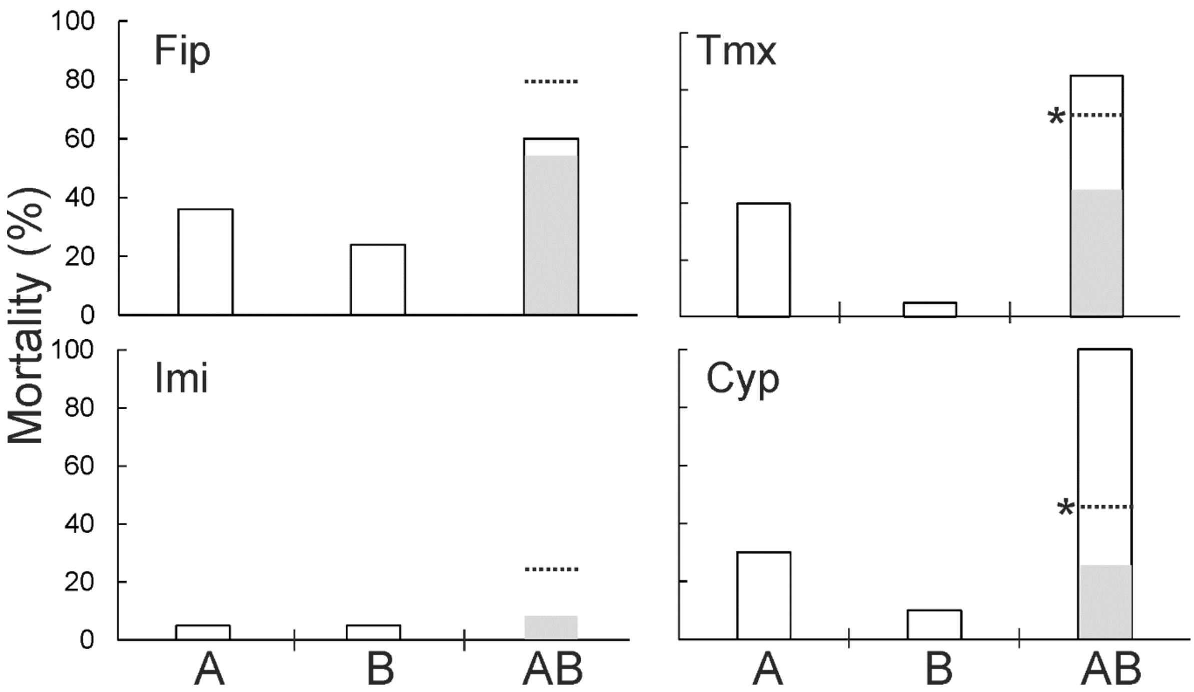



Figure 2. Mortality (*y*-axis: proportion dying, %) after 48 hours in three exposure treatments: A = dietary imazalil; B = insecticide (Fip = fipronil; Tmx = thiamethoxam; Imi = imidacloprid; and Cyp = cypermethrin); and AB = imazalil-insecticide mixture. In the AB column, the grey fill indicates the expected mortality if the components of the dietary mixture act independently ($H_0$) and the dashed horizontal line indicates the upper 95% confidence interval on the sampling distribution under $H_0$. An asterisk indicates that the mixture has produced a statistically significant synergistic effect (one-tailed binomial proportion test). A column is blank (has no bar) if no mortality occurred.

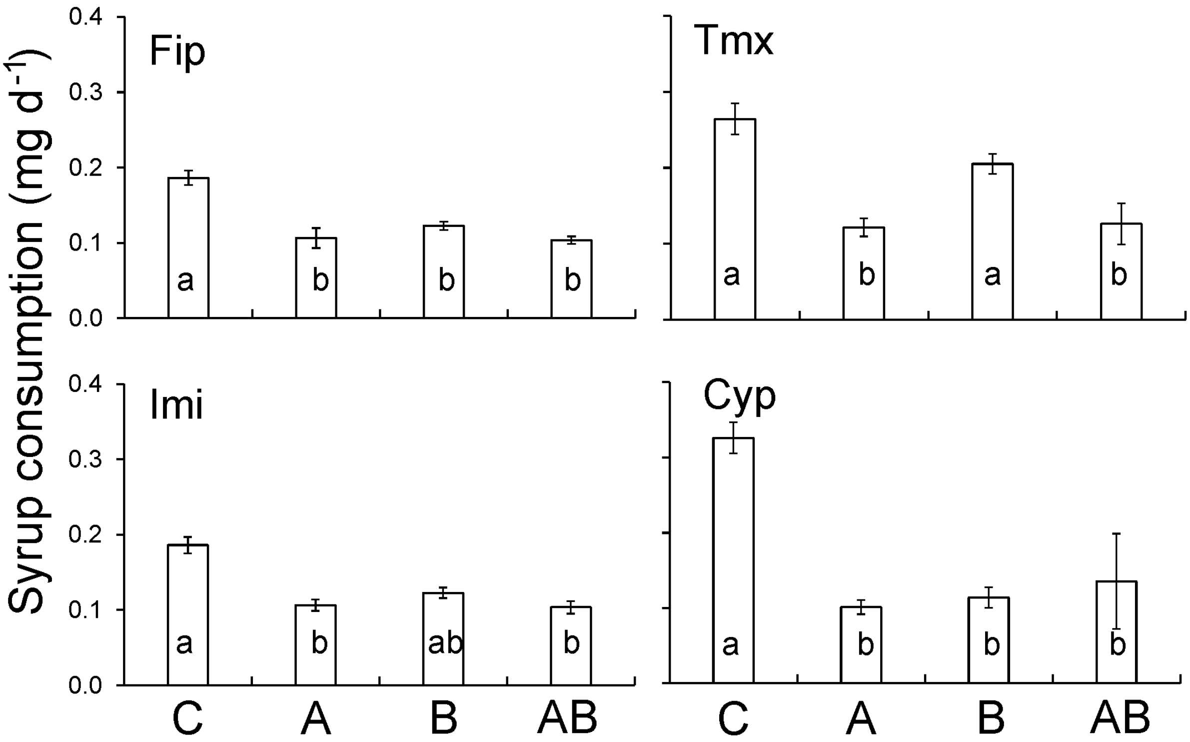
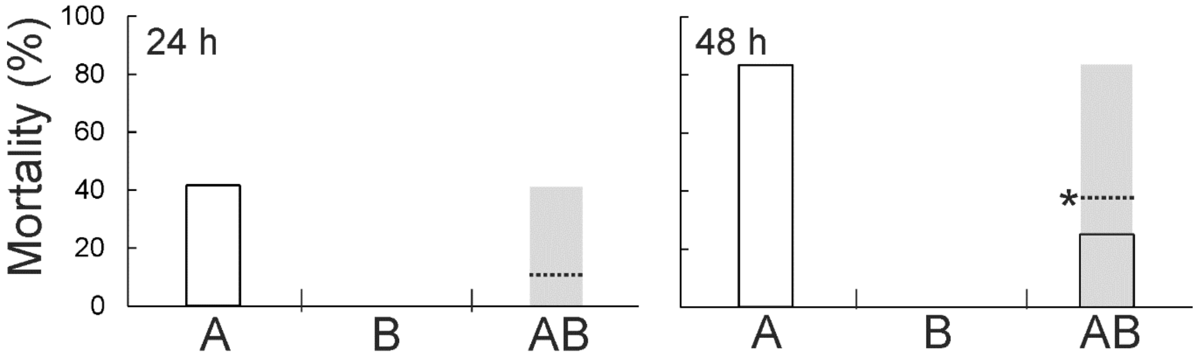



Figure 3. Variation in individual feeding rates (*y*-axis: mg syrup consumed per bee per day) during 48 hours of exposure among four dietary treatments: C = undosed controls; A = dietary imazalil; B = insecticide (Fip = fipronil; Tmx = thiamethoxam; Imi = imidacloprid; and Cyp = cypermethrin); and AB = imazalil-insecticide mixture. Among the histogram columns, different lower case letters indicate significant differences in mean feeding rate (Tukey test, $P < 0.05$). Error bars indicate 1 SE.

**SUPPLEMENTAL INFORMATION**




Supplemental Information Figure 1. Experiment conducted at the University of Exeter to investigate mixture effects between imazalil and imidacloprid. Mortality (*y*-axis: proportion dying, %) after 24 (left panel) and 48 hours in three exposure treatments: A = dietary imazalil; B = imidacloprid insecticide; and AB = imazalil-imidacloprid mixture. In the AB column, the grey fill indicates the expected mortality if the components of the dietary mixture act independently ($H_0$) and the dashed horizontal line indicates the lower 95% confidence interval on the sampling distribution under $H_0$. An asterisk indicates that the mixture has produced a statistically significant synergistic effect (one-tailed binomial proportion test). In this experiment, dietary imidacloprid in mixture reduced the mortality rate due to dietary imazalil. A column is blank (has no bar) if no mortality occurred.