Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species

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ABSTRACT

BACKGROUND: Neonicotinoid insecticides have been identified as an important factor contributing to bee diversity declines. Nonetheless, uncertainties remain about their impact under field conditions. Most studies have been conducted on *Apis mellifera* and tested single compounds. However, in agricultural environments, bees are often exposed to multiple pesticides. We explore synergistic mortality between a neonicotinoid (clothianidin) and an ergosterol-biosynthesis-inhibitor fungicide (propiconazole) in three bee species (*A. mellifera*, *Bombus terrestris*, *Osmia bicornis*) following oral exposure in the laboratory.

RESULTS: We developed a new approach based on the binomial proportion test to analyze synergistic interactions. We estimated uptake of clothianidin per foraging bout in honey bees foraging on seed-coated rapeseed fields. We found significant synergistic mortality in all three bee species exposed to non-lethal doses of propiconazole and their respective LD_{10} of clothianidin. Significant synergism was only found in the first assessment times in *A. mellifera* (4 and 24 h) and *B. terrestris* (4 h), but persisted throughout the experiment (96 h) in *O. bicornis*. *Osmia bicornis* was also the most sensitive species to clothianidin.

CONCLUSION: Our results underscore the importance to test pesticide combinations likely to occur in agricultural environments, and to include several bee species in environmental risk assessment schemes.

KEY WORDS: *Apis mellifera*, *Bombus terrestris*, *Osmia bicornis*, clothianidin, propiconazole, field-realistic dose, synergism, binomial proportion test

1. INTRODUCTION

Bees and other flower-visiting animals provide pollination services for 87.5% of the angiosperms worldwide, thus playing an essential role in plant population dynamics and conservation of plant diversity.[1] At the same time, bees pollinate many crops, thus contributing decisively to human food supply.[2] Traditionally, managed honey bees, *Apis mellifera* L., have been credited with the largest share of these pollination services. However, an increasing number of studies are emphasizing the importance of bee diversity for crop pollination.[3,4] These studies have been accompanied by reports of wild bee declines, both in terms of abundance and species richness.[5-8] In addition, in several European countries and in North America, honey bee populations have also declined in the last decades.[9-11] The causes of this negative trend are complex and diverse[12] but neonicotinoid insecticides have often been signaled as one of the main factors contributing to bee declines.[13-15] As a result, the number of studies investigating the effects of neonicotinoids on bees has increased dramatically in the last years.[16] Nonetheless, uncertainties still remain about the magnitude of the impact of neonicotinoids on bees in field conditions.[14,17,18] Some of these uncertainties are related to the fact that most studies have tested single compounds, when, in agricultural environments, bees are often simultaneously exposed to a variety of compounds.[19,20] Multi-exposure scenarios occur when bees forage on a crop treated with different pesticides, either applied sequentially or in a tank mix, and when bees forage on various plants contaminated with different pesticides. Neonicotinoids have been found not only in pollen and nectar of various crops,[21,22] but also of wildflowers growing near treated crops.[23,24] Exposure to multiple compounds may result in synergistic toxic effects, as shown in some laboratory[25,26] and field studies.[27]

Here, we assess potential synergistic interactions between a neonicotinoid (clothianidin) and an ergosterol biosynthesis inhibitor (EBI) fungicide (propiconazole) on three bee species following oral exposure in the laboratory. In agricultural environments, bees are likely to be exposed to neonicotinoids and EBI fungicides in combination because these two groups of compounds are commonly applied to various crops such as oilseed rape, sunflower, fruit trees, maize and cereals.[28,29] The three species tested include two social and one solitary bee, the European honey bee (*A. mellifera*), the terrestrial bumblebee (*Bombus terrestris* (L.)), and the red mason bee (*Osmia bicornis* (L.)). In addition to sociality, these three species show important differences in other life history traits, including body size and pollen/nectar provisioning behavior, and therefore are likely to be exposed to different levels of pesticides. The comparative approach is important also because different bee species have different levels of sensitivity to various families of compounds.[30] For these reasons, all three species have been recently included in the European Food Safety Authority (EFSA) guidance document for the risk assessment of plant protection products on bees.[31]

To achieve our goal, we obtained dose-response curves to clothianidin for each bee species. Then, we assessed the effects of clothianidin LD_{10} alone and in combination with a non-lethal dose of propiconazole. In agreement with some studies finding synergism between various fungicides and insecticides,[26,32-34] we expected to find a synergistic effect. In addition, and in the face of differences among bee species in sensitivity to various compounds,[30] we also expected a different response by the three species tested. To our knowledge, this is the first time that synergistic mortality effects between insecticides and fungicides are tested through oral exposure in non-*Apis* bees.

2. EXPERIMENTAL METHODS

2.1 Bees and test conditions

Healthy, queen-right honey bee colonies (*A. mellifera ligustica*) were managed at the CREA-API (Council for Agricultural Research and Economics – Honey Bee and Silkworm Research Unit), Bologna, Italy, following standard beekeeping procedures. In the months prior to the experiments, these colonies received a single chemical treatment consisting of a liquid application of oxalic acid aimed to control *Varroa destructor* Anderson and Trueman infestation. In July 2015, we placed a funnel trap in front of the hives to collect forager bees[35]. Funnel traps are appropriate for this purpose because they discriminate between foragers and in-hive bees, as well as between bees that are exiting and entering the hive. We chose to work with forager bees, instead of in-hive bees, because they are more likely to be directly exposed to contaminated nectar. Following anesthetization with 60% CO_2 in synthetic air for ~ 30 minutes, groups of 10 bees were transferred to cardboard cages (9.5x6.5x5 cm) (Fig. S1, Supporting Information). Contrary to the use of pure CO_2,[36] this methodology does not to affect honey bee mortality. Following a starvation period of ~1 hour, 100 µL of the test solution were provided to each group of bees using a common feeder, assuming that, through trophallaxis, all individuals would ingest similar doses (10 µL).[37,38] Feeders were visually inspected at the end of the exposure phase. In all cases the test solution had been completely consumed. The cages were maintained in an incubator in complete darkness at 25±2°C and 50-70% relative humidity for the duration of the test. To minimize potential "incubator-microclimate" differences, the position of the various cages within the incubator was rotated daily.

Bumblebee colonies (*B. terrestris*) were purchased from BioPlanet s.c.a. (Cesena, Italy). Colonies contained 60-80 workers, brood in all stages of development and a laying queen. In September 2015, adult workers from four colonies were collected randomly under red light and individually transferred to Nicot cages (7.1 x 2.0 cm) (Fig. S1, Supporting Information).[39] Since large variation in body size exists among bumblebee workers, very small (approximately <0.13 g) and very large (>0.36 g) individuals were excluded. To avoid potential differences in sensitivity among different bee categories, newly emerged bees, recognizable by their grayish pubescence, were also excluded.[39] Prior to pesticide exposure bees were starved for 4 hours during which time they were maintained in a temperature cabinet at 26±2° C and 50-70% of relative humidity in continuous darkness. Since *Bombus* spp. do not perform trophallaxis, we could not use bulk feeders. Therefore, we used an individual feeding method whereby the test solution was offered through a 1 mL syringe inserted into the Nicot cage.[39] Each individual was provided with 10 µL of test solution for an exposure period of 4 hours. Feeders were visually inspected after the exposure phase and only bees that consumed 100% of the test solution were used in the statistical analyses. Following the exposure phase, bees were maintained individually in the Nicot cages and fed *ad libitum* through a 5 mL syringe filled with sucrose syrup. To avoid confinement side effects, the Nicot cages of each treatment were placed side by side on a tray, so that workers could perceive their mutual presence.

Osmia bicornis individuals were obtained from a population reared in Poland since 2000. The parental population was released in a pesticide free area of the Kazimierz Landscape Park and the progeny was reared outdoors. In early October 2014, adults within their cocoons were wintered at 3 °C at CREA-API. In May 2015, female cocoons were incubated at 24 °C until

emergence and then transferred to a Plexiglas flight cage (50 x 50 x 50 cm) to allow them to deposit the meconium. Approximately 24 hours after emergence, these unmated, meconium-free females were individually housed in test cages made of cardboard ice cream cups (width: 7.5 cm; height: 5.5 cm) with a perforated (for aeration), transparent plastic lid (Fig. S1, Supporting Information). These cages were kept in the laboratory at 22±2 °C and 50-70% of relative humidity under natural light. Unlike honey bees and bumblebees, mason bees are only active for a short period of time in the spring. For this reason, *Osmia* ecotoxicology tests are conducted at lower temperatures,[40-43] than *Apis* and *Bombus* tests.[38,39] As with bumblebees, *Osmia* do not perform trophallaxis. To feed bees individually, we used the "petal method",[40] a modification of the "flower method".[41] The test solution (10 µL) was pipetted into a tiny plastic ampoule (internal diameter 2 mm, external diameter 3 mm, height 5 mm) attached to a natural petal inserted into a foam holder (diameter: 1 cm; height: 1 cm). We used several Asteraceae (*Bidens* and *Coleostephus*) as petal sources. As with bumblebees, only bees that consumed 100% of the test solution were used in the statistical analyses. After 1 hour of exposure bees were placed in groups of 3-5 individuals in cages similar to those used in the feeding test provided with an artificial feeder (a 2.5 mL syringe). A flower petal was attached to the tip of this feeder to enhance prompt location. To avoid bees stacking up, a wire mesh in the form of a small bridge was introduced in each cage.[40]

Sample sizes were approximately 30 individuals per bee species and dose in all tests. In an attempt to minimize stress from manipulation, test bees were not weighed. Instead, 30 individuals of each species were randomly selected and weighed to obtain an average fresh body weight per species.

2.2 Test solutions

We used active ingredients in all test solutions. Propiconazole (purity 98.4%) was purchased from Sigma-Aldrich. To obtain the stock solution we diluted 133 mg of propiconazole in 1 mL of acetone and then conducted subsequent dilutions until we reached a final concentration of 35 mg/mL. This solution was added to the feeding solution (500 g of sucrose in 1 L of purified distilled water – 33% w:w) at the ratio of 20 µL/mL to obtain the desired concentration (7 µg of propiconazole in 10 µL of solution, the per-capita volume provided to the bees) (see 2.3 Experimental design).

Clothianidin (purity 99%) was purchased from Dr Ehrenstorfer Gmbh. The stock solution with a concentration of 1.05 mg of clothianidin/mL of acetone was used to prepare the test solutions. To obtain a range of appropriate concentrations based on the desired exposure level, the stock solution was first diluted in acetone until we reached the final nominal concentrations ranging from 2 to 160 mg/L (actual concentrations: 2.7 to 176.8 mg/L). These solutions were added to the feeding solution (500 g of sucrose in 1 L of purified distilled water) at the ratio of 10 µL/mL (see 2.3 Experimental design).

The final concentration of acetone in the feeding solution was adjusted to 3% (v:v) in all treatments (solvent control, propiconazole, clothianidin and the combination fungicide-neonicotinoid). To reach this level, pure acetone was added as needed. Following the exposure phase, bees were fed *ad libitum* with a sucrose solution (33% w:w).

2.3 Experimental design

To obtain clothianidin dose-response curves, we exposed bees to 6 doses (5 in *O. bicornis*) of clothianidin in a geometric series. We used a factor of 2 ranging from 0.25 to 8 ng/bee (nominal doses) in *A. mellifera*; a factor of 2 ranging from 0.5 to 16 ng/bee in *B. terrestris*; and a factor of 3 ranging from 0.2 to 16 ng/bee in *O. bicornis*. A negative control (only water) and a solvent control (3% v:v) were also included in the tests. Following preliminary trials with several doses of propiconazole based on previous studies reporting oral LD_{50}s for *A. mellifera* and *O. lignaria*,[43] we established 7 µg/bee as a non-lethal dose for all three species.

Then, in the synergism experiment, bees were exposed to four treatments: solvent control (3% v:v), fungicide (7 µg/bee), neonicotinoid (LD_{10} of each species), and the combination fungicide-neonicotinoid. Post-test chemical analysis of the doses of neonicotinoid applied to the feeding solution confirmed that they were within the range of 95% CL of the respective LD_{10} of each species.

2.4 Statistical analysis

Mortality was assessed 4 hours after the end of the exposure phase, and then checked every 24 hours for 4 days. LD_{10} and LD_{50} values and their 95% confidence limits for each assessment time were determined using Probit analysis (PoloPlus, LeOra Software). To be conservative, the lowest LD_{10} values for each species were used in the synergism experiment. For each species, Log-rank Kaplan-Meier (K-M) survival analyses were carried out to compare survival in water and solvent controls. In the synergism experiment, K-M survival analyses with pairwise multi comparison procedures (Holm-Sidak method) were conducted to compare survival among the four treatments. Survival analyses were conducted with SigmaPlot 12.3.

Synergistic interactions are customarily tested with χ^2 and GLM procedures.[44] However, the high proportion of zeroes in the mortality assessments prevented the use of these approaches in our study. Instead, we used a modified binomial proportion test, which we propose as a new means to test for synergistic interactions. The rationale and description, as well as the scripts of this new procedure are provided in the Appendix S2 and S3, Supporting Information. First, we calculated the expected mortality proportion of the combination treatment as $P_{PCExp}=P_P + (1-P_P)\, P_C$, where P_P and P_C are the observed mortality proportion in the propiconazole and clothianidin treatments, respectively. Then, we tested whether the difference between the observed and the expected mortality from the clothianidin-propiconazole combination could arise by chance alone (null hypothesis), or whether this difference was significantly larger than zero, indicating the existence of synergism between the two substances (alternative hypothesis). Binomial proportion confidence intervals (see Supporting Information) enable us to build a hypothesis test for the difference between two proportions based on the Wald confidence interval. Numerical simulations (Fig. S2, Supporting Information) show that this test performs very satisfactorily for the range of values of our study. We used a significance level of 0.05 throughout the study. To avoid type I error rate inflation (i.e. rejecting the null hypothesis when it is true) arising from multiple comparisons, we implemented the Holm correction. These analyses were conducted with the p.adjust function of the in-built "stats" package of the R software.[45]

In the *A. mellifera* experiment, bees were grouped in cages (10 individuals per cage) during the exposure and post-exposure phases. To check for a potential cage effects, we used rank-transformed, repeated-measures ANOVA analyses for each species separately, with cage as the

between-subjects factor and time (4, 24, 48, 72 and 96 hours) as the within-subjects factor. This test may be regarded as a non-parametric equivalent of repeated-measures ANOVA.[46] We found no differences among cages for any of the treatments (Table S1, Supporting Information).

3. RESULTS

Mean ± SE body size was 88.2 ±1.7, 261.8±11.4, 124.1±2.5 mg in *A. mellifera*, *B. terrestris* and *O. bicornis*, respectively. The lowest clothianidin LD_{10} and LD_{50} values were obtained at 24 h in *A. mellifera* and *B. terrestris*. Instead, *O. bicornis* showed a delayed response to this compound, and the lowest LD_{10} and values of LD_{50} values were obtained at 72 h (Table S2 and S3 in Supporting Information). Based on these values, *O. bicornis* was the most sensitive species, both in terms of ng of clothianidin per bee and per g of bee body weight (Table 1). There were no significant differences in survival between the water and solvent controls at 96 h (Log-rank, df=1, χ^2=0.84, p=0.36; χ^2=0.88, p=0.35; χ^2=3.62, p=0.06; in *Osmia bicornis*, *Bombus terrestris* and *A. mellifera,* respectively).

Post-test chemical analyses showed that the actual clothianidin doses used in the synergism experiment for *A. mellifera*, *B. terrestris* and *O. bicornis* were 0.79, 1.81 and 0.63 ng/bee, respectively, and thus very close to their respective LD_{10} (0.86, 1.87, 0.66 ng/bee).

In *A. mellifera*, the Log-rank statistic showed significant differences between cumulative survival curves of bees exposed to the different treatments (Fig. 1, Log-rank χ^2=11.69, df=3, p=0.009). The high mortality in the control treatment can be attributed to the fact that we worked

with (aged) forager bees. Pairwise analyses at the end of the experiment (96 hours) showed close-to-significant results in the control *vs* combination and the propiconazole *vs* combination comparisons (Table 2). Synergistic interaction, indicating that the clothianidin-propiconazole combination was significantly more toxic than the sum of the toxicity of the two compounds separately, were found only in the first two assessment times (4 and 24 hours) (Fig. 1).

We also found significant differences between cumulative survival curves in *B. terrestris* (Fig. 2, Log-rank χ^2=41.24, df=3, p<0.001). In the pairwise comparisons, the clothianidin-propiconazole combination was more toxic than the control and the propiconazole treatments, but differences with the clothianidin treatment were not significant (Table 2). In addition, the clothianidin treatment was more toxic than the control and the propiconazole treatments (Table 2). A synergistic effect between clothianidin and propiconazole was statistically significant 4 hours after exposure, but not in subsequent assessment times (Fig. 2).

Significant differences between cumulative survival curves were also found in *O. bicornis* (Fig. 3, Log-rank χ^2=37.71, df=3, p<0.001). The pairwise comparisons yielded higher mortality in the propiconazole-clothianidin combination than in the control and the two single compound treatments (Table 2). At 96 hours, mortality in the clothianidin-propiconazole combination reached 50%, compared to 6.0, 3.0 and 12.5% in the control, propiconazole and clothianidin treatments, respectively. The clothianidin-propiconazole combination produced a clear synergistic effect at all assessment times (Fig. 3).

4. DISCUSSION AND CONCLUSIONS

The aim of this study was to assess potential synergistic mortality interaction between a neonicotinoid insecticide and an EBI fungicide likely to co-occur in agricultural environments on three bee species with highly contrasting life history traits. A key question is whether the doses administered in our experiment can be considered to be field-realistic. The amount of clothianidin ingested over a day by a honey bee foraging in a rape field planted with clothianidin-coated seed has been estimated by EFSA at 4.27-13.65 ng/bee/day.[28] The LD_{10} doses administered in our synergism experiment (0.63-1.81 ng/bee) is 0.046-0.42 times this estimate. The acute exposure used in our study, however, is more directly comparable to the amount of clothianidin ingested in a single foraging bout. Following the same rationale as in the EFSA calculations of daily clothianidin intake[28], we estimated clothianidin intake per foraging bout by a honey bee foraging in an oilseed rape field planted with clothianidin-coated seed. Our calculations are based on information on sugar consumption per unit time during flight (8-12 mg / h),[47] foraging bout duration (30-80 min),[48] and the proportion of this time spent flying (80%)[49] (see Appendix S6, Supporting Information for details). The resulting clothianidin intake per foraging bout is 0.11-1.36 ng. The doses administered to *O. bicornis* (0.63 ng) and *A. mellifera* (0.79 ng) in our synergism experiment fall well within this range and therefore can be considered to be field-realistic. On the other hand, the dose administered to *B. terrestris* (1.81 ng) is higher, in agreement with the larger body size in this species.

In *A. mellifera* and *O. bicornis*, mortality following oral administration of LD_{10} doses of clothianidin was not significantly different from control mortality. In *B. terrestris*, on the other hand, mortality was significantly higher in the LD_{10} treatment than in the control. This unexpected result may be explained by differences in sensitivity between the colonies used to calculate the clothianidin dose-response curve and those used in the synergism experiment.

Mortality in bees of the dose-response experiment exposed to 2.75 ng/bee was 23%, compared to 31% in bees of the synergism experiment exposed to 1.81 ng/bee (the estimated LD_{10}). Inter-colony variability in pesticide sensitivity is well documented in honey bees.[50,51] As for the propiconazole dose administered (7 µg/bee), it did not induce significant mortality in any of the three bee species. This dose corresponds to 0.125 and 0.2 times the propiconazole oral LD_{50} obtained in a previous study for *A. mellifera* and *Osmia lignaria* Say, respectively,[43] and falls within the range (from 0.0224 to 22.4 µg/bee) tested by Thompson *et al.*[34] yielding no oral toxic effects in *A. mellifera*. Even if our test doses did not produce significant mortality (with the exception of clothianidin LD_{10} in *B. terrestris*), sub-lethal effects cannot be ruled out. Previous studies have shown sub-lethal effects on bees exposed to doses of neonicotinoids similar to those in our study.[52,53] A study on *Osmia cornuta* (Latreille) found alteration of the navigation behavior under laboratory conditions in bees orally exposed to a 0.76 ng/bee dose of clothianidin (similar to our LD_{10}: 0.63 ng/bee).[54] A greenhouse study with *O. lignaria* and *Megachile rotundata* (Fabricius) showed disruption of nest recognition in females exposed to field doses of fungicides (iprodione and a mixture of pyraclostrobin + boscalid).[55]

Our first expectation was that oral exposure to the clothianidin-propiconazole combination would produce a synergistic effect on bee mortality. This expectation was confirmed, since significant synergism was detected in the three species. The biochemical mechanism behind this synergism is probably related to the capacity of EBI fungicides to inhibit P450-mediated detoxification.[56] Different bee species, including *A. mellifera*, *Bombus huntii* and *Megachile rotundata* (in the same family as *Osmia*) have been found to share similar P450s detoxification genes, although the number of genes involved in the detoxification process varies from species to species.[56,57] The level of synergism between neonicotinoid insecticides an EBI fungicides has

been shown to be fungicide dose-dependent.[34] Thus, further studies would be necessary to assess variation in the magnitude of synergism with different doses of fungicide. However, Thompson *et al.*[34] found synergistic interaction between propiconazole and clothianidin in orally exposed *A. mellifera* at fungicide doses much lower (0.224 µg/bee) than our test dose (7 µg/bee).

Previous multi-species experiments have shown different sensitivities to various pesticides.[30] In our study *Osmia bicornis* was the most sensitive species to clothianidin (lowest LD_{50} in ng/g of bee body weight) followed by *B. terrestris* and *A. mellifera*. In a study in which *A. mellifera*, *Bombus impatiens* Cresson and *O. lignaria* were topically exposed to clothianidin, mason bees were again the most sensitive species followed by honey bees and bumblebees.[58] Our second expectation was that the three bee species would show a different response to the clothianidin-propiconazole combination. This expectation was also met, since synergism was apparent throughout the entire assessment period in *O. bicornis*, but only in the first assessment times in *B. terrestris* and in *A. mellifera*. A previous study tested combinations of neonicotinoid insecticides (imidacloprid and acetamiprid) and EBI fungicides (fenbuconazole) through contact exposure in *A. mellifera* and *Osmia cornifrons* (Radoszkowski).[33] This study also found synergistic mortality effects in both bee species but, as opposed to our study, synergism was stronger in *Apis* than in *Osmia*. Overall, the magnitude of synergism observed in our study (expressed as the ratio between observed and expected mortality proportions) ranged from 3.3 (in *A. mellifera* at 24 h) to 8.0 (in *O. bicornis* at 4 h). These values are in the same order of magnitude as values reported in other studies on nitro-substituted neonicotinoids (imidacloprid, thiamethoxam and clothianidin).[26,33,34] On the other hand, studies on cyano-substituted neonicotinoids (acetamiprid, thiacloprid) found much higher synergistic effects with EBI

fungicides.[26,33] These results are important, because cyano-substituted neonicotinoids are less toxic to bees than nitro-substituted neonicotinoids.[26,33]

Differences among bee species in detoxification mechanisms may explain the observed differences in sensitivity to pesticides and pesticide mixtures.[59] Ability to detoxify pesticides that, like neonicotinoids, are based on plant defensive chemicals is likely to be dependent on the evolutionary history of the species. In relation to their wider diet breadth, social species are expected to be pre-adapted to a wider range of plant defensive chemicals than solitary bees. [60] In addition, other behavioral and life history traits, such as body weight, foraging range, levels of pollen/nectar consumption, and exposure to other potentially contaminated materials (soil, leaves) are likely to result in different levels of exposure and different responses under field conditions. In general, eusocial bees are considered to be less vulnerable because effects at the individual level can be buffered by the rest of the colony ("superorganism resilience").[61] Conversely, in solitary bees, individual effects have direct repercussions on reproductive success.[62] A recent study found that foraging in oilseed rape fields planted with clothianidin dressed seeds, had negative effects on wild bee density, *O. bicornis* nesting, and *B. terrestris* colony growth, but no significant effects were observed on *A. mellifera* colony development.[63] *B. terrestris* colonies are much smaller than *A. mellifera* colonies and therefore are expected to be less resilient. Finally, temperature has been shown to have an effect on pesticide toxicity.[64] For this reason, and because we did not use the same temperature in all three species, a potential effect of temperature on our results cannot be ruled out. As mentioned, temperatures used in the experiments were adjusted to the thermal requirements of each species.

In this study we have developed a new approach based on a modified binomial proportion test to study synergistic interactions. Using this approach, we have shown that the combination of

sublethal doses of two agrochemical compounds routinely used on a variety of crops produces a synergistic effect, in terms of mortality, on three bee species with contrasting life history traits. Our results also show important differences among species. These findings have important consequences for environmental risk assessment. First, since bees are susceptible of being exposed to a wide range of pesticides both on crops and off-fields,[24,65] synergism between compounds likely to co-occur in agricultural environments should be assessed. This is particularly important for fungicides, which are routinely sprayed during bloom under the assumption they are safe for bees. Synergistic interaction between fungicides and insecticides could explain the dramatic changes in nest recognition and cell production rates observed in *O. lignaria* following fungicide applications in cherry orchards.[66] Second, our study underscores the need to include other model species besides the honey bee in risk assessment schemes. Given the observed differences among species in pesticide sensitivity and level of exposure[30,65] extrapolations should not be made between species with contrasting life histories.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1. Ollerton J, Winfree R, Tarrant S, How many flowering plants are pollinated by animals? *Oikos* **120**: 321–326 (2011).
2. Klein A-M, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T, Importance of pollinators in changing landscapes for world crops. *Proc Biol Sci* **274**: 303–313 (2007).
3. Garibaldi LA, Steffan-Dewenter I, Winfree R, Aizen MA, Bommarco R, Cunningham SA, Kremen C, Carvalheiro LG, Harder LD, Afik O, Bartomeus I, Benjamin F, Boreux V, Cariveau D, Chacoff NP, Dudenhöffer JH, Freitas BM, Ghazoul J, Greenleaf S, Hipólito J, Holzschuh A, Howlett B, Isaacs R, Javorek SK, Kennedy CM, Krewenka KM, Krishnan S, Mandelik Y, Mayfield MM, Motzke I, Munyuli T, Nault BA, Otieno M, Petersen J, Pisanty G, Potts SG, Rader R, Ricketts TH, Rundlöf M, Seymour CL, Schüepp C, Szentgyörgyi H, Taki H, Tscharntke T, Vergara CH, Viana BF, Wanger TC, Westphal C, Williams N, Klein AM, Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science* **339**: 1608–1611 (2013).
4. Mallinger RE, Gratton C, Species richness of wild bees, but not the use of managed honeybees, increases fruit set of a pollinator-dependent crop. *J Appl Ecol* **52**: 323-330 (2014).
5. Biesmeijer JC, Roberts SPM, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers AP, Potts SG, Kleukers R, Thomas CD, Settele J, Kunin WE, Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* **313**: 351–354 (2006).

6. Burkle LA, Marlin JC, Knight TM, Plant-Pollinator Interactions over 120 Years: Loss of Species, Co-Occurrence, and Function. *Science* **339**: 1611–1615 (2013).
7. Ollerton J, Erenler H, Edwards M, Crockett R, Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science* **346**: 1360–1362 (2014).
8. Nieto A, Roberts SPM, Kemp J, Rasmont P, Kuhlmann M, García Criado M, Biesmeijer JC, Bogusch P, Dathe HH, De la Rúa P, De Meulemeester T, Dehon M, Dewulf A, Ortiz-Sánchez FJ, Lhomme P, Pauly A, Potts SG, Praz C, Quaranta M, Radchenko VG, Scheuchl E, Smit J, Straka J, Terzo M, Tomozii B, Window J, Michez D, European Red List of bees. Luxembourg: Publication Office of the European Union (2014).
9. Potts S, Roberts S, Dean R, Marris G, Brown M, Jones R, Neumann P, Settele J, Declines of managed honey bees and beekeepers in Europe. *J Apic Res* **49**: 15-22 (2010).
10. Vanengelsdorp D, Meixner MD, A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invertebr Pathol* **103**: S80–S95 (2010).
11. Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K, Lengerich EJ, Pettis J, Skinner JA, Wilkes JT, Sagili R, vanEngelsdorp D, A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* **46**: 292-305 (2015).
12. Vanbergen AJ, Insect Pollinators Initiative, Threats to an ecosystem service: pressures on pollinators. *Front Ecol Environ* **11**: 251–259 (2013).
13. Maini S, Medrzycki P, Porrini C, The puzzle of honey bee losses: a brief review. *Bull Insectology* **63**: 153–160 (2010).
14. Godfray HCJ, Blacquière T, Field LM, Hails RS, Petrokofsky G, Potts SG, Raine NE,

Vanbergen AJ, McLean AR, A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc Biol Sci* **281**: 20140558 (2014).

15. Pisa LW, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Downs CA, Goulson D, Kreutzweiser DP, Krupke C, Liess M, McField M, Morrissey CA, Noome DA, Settele J, Simon-Delso N, Stark JD, Van der Sluijs JP, Van Dyck H, Wiemers M, Effects of neonicotinoids and fipronil on non-target invertebrates. *Environ Sci Pollut Res Int* **22**: 68–102 (2014).
16. Lundin O, Rundlöf M, Smith HG, Fries I, Bommarco R, Neonicotinoid Insecticides and Their Impacts on Bees: A Systematic Review of Research Approaches and Identification of Knowledge Gaps. *PLoS ONE* **10**: e0136928 (2015).
17. Carreck NL, Ratnieks FLW, The dose makes the poison: have "field realistic" rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? *J Apic Res* **53**: 607–614 (2014).
18. IPBES, Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. Potts SG, Imperatriz-Fonseca VL, Ngo HT, Biesmeijer JC, Breeze TD, Dicks LV, Garibaldi LA, Hill R, Settele J, Vanbergen AJ, Aizen MA, Cunningham SA, Eardley C, Freitas BM, Gallai N, Kevan PG, Kovács-Hostyánszki A, Kwapong PK, Li J, Li X, Martins DJ, Nates-Parra G, Pettis JS, Rader R, Viana BF (eds.). pp. 1–30 (2016).
19. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, VanEngelsdorp D, Pettis JS, High levels of miticides and agrochemicals in North American apiaries: implications for

honey bee health. *PLoS ONE* **5**: e9754 (2010).

20. Hladik ML, Vandever M, Smalling KL, Exposure of native bees foraging in an agricultural landscape to current-use pesticides. *Sci Total Environ* **542**: 469–477 (2016).
21. Bonmatin JM, Marchand PA, Charvet R, Moineau I, Bengsch ER, Colin ME, Quantification of imidacloprid uptake in maize crops. *J Agric Food Chem* **53**: 5336–41 (2005).
22. Pohorecka K, Skubida P, Miszczak A, Semkiw P, Sikorski P, Zagibajlo K, Teper D, Koltowski Z, Skubida M, Zdanska D, Bober A, Residues of Neonicotinoid Insecticides in Bee Collected Plant Materials from Oilseed Rape Crops and Their Effect on Bee Colonies. *J Apic Sci* **56**: 115–134. (2012).
23. Krupke CH, Hunt GJ, Eitzer BD, Andino G, Given K, Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS ONE* **7**: e29268 (2012).
24. Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill EM, Goulson D, Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. *Environ Sci Technol* **49**: 12731–12740 (2015).
25. Vandame R, Belzunces LP, Joint actions of deltamethrin and azole fungicides on honey bee thermoregulation. *Neurosci Lett* **251**: 57–60 (1998).
26. Iwasa T, Motoyama N, Ambrose JT, Roe RM, Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot* **23**: 371–378 (2004).
27. Gill RJ, Ramos-Rodriguez O, Raine NE, Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature* **491**: 105–108 (2012).
28. EFSA, Conclusion on the peer review of the pesticide risk assessment for bees for the

active substance clothianidin. *EFSA J* **11**(1):3066 (2013).

29. EFSA, Reasoned opinion on the review of the existing maximum residue levels (MRLs) for propiconazole according to Article 12 of Regulation (EC) No 396/2005. *EFSA J* **13**(1):3975 (2015).
30. Arena M, Sgolastra F, A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology* **23**: 324–334 (2014).
31. EFSA, EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA J* **11**(7):3295 (2013).
32. Pilling ED, Jepson PC, Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pestic Sci* **39**: 293–297 (1993).
33. Biddinger DJ, Robertson JL, Mullin C, Frazier J, Ashcraft S, Rajotte EG, Joshi NK, Vaughn M, Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLoS ONE* **8**: e72587 (2013).
34. Thompson HM, Fryday SL, Harkin S, Milner S, Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. *Apidologie* **45**: 545–553 (2014).
35. Medrzycki P, Funnel trap – a tool for selective collection of exiting forager bees for tests. *J Apic Res* **52**: 122–123 (2013).
36. Nicolas G, Sillans D, Immediate and latent effects of carbon dioxide on insects. *Ann Rev Entomol* **34**: 97-116 (1989).
37. Medrzycki P, Giffard H, Aupinel P, Belzunces LP, Chauzat M-P, Claßen C, Colin ME, Dupont T, Girolami V, Johnson R, Le Conte Y, Lückmann J, Marzaro M, Pistorius J, Porrini C, Schur A, Sgolastra F, Delso NS, van der Steen JJM, Wallner K, Alaux C,

Biron DG, Blot N, Bogo G, Brunet J-L, Delbac F, Diogon M, El Alaoui H, Provost B, Tosi S, Vidau C, Standard methods for toxicology research in *Apis mellifera*. *J Apic Res* **52**: 1–60 (2013).

38. OECD. OECD guideline for testing of chemicals. Test No 213: Honey bees, acute oral toxicity test (1998).
39. Hanewald N, Roessink I., Mastitsky S, Amsel K, Bortolotti L, Colli M, Gladbach D, Haupt S, Jeker L, Kimmel S, Molitor C, Noel E, Schmitt H, Wilkins S, van der Steen J, Compilation of results of the ICPPR non-Apis working group with a special focus on the bumblebee acute oral and contact toxicity ring test 2014 ICPPR Non-Apis Working Group. 12th International Symposium of the ICP-PR. Hazards of pesticides to bees Ghent (Belgium), September 15-17, 2014. *Julius-Kuhn-Archiv* **450**: 194-296 (2015).
40. Hinarejos S, Domene X, Bosch J, Oral toxicity of dimethoate to adult *Osmia cornuta* using an improved laboratory feeding method for solitary bees. 12th International Symposium of the ICP-PR. Hazards of pesticides to bees Ghent (Belgium), September 15-17, 2014. *Julius-Kuhn-Archiv* **450**: 192 (2015).
41. Ladurner E, Bosch J, Maini S, Kemp WP, A method to feed individual bees (Hymenoptera : Apiformes) known amounts of pesticides. *Apidologie* **34**: 597–602 (2003).
42. Roessink I, Hanewald N, Schneider C, Medrzycki P, Bosch J, Hinarejos S, Gladbach D, Stanisavljević L, Krnjajić S, Schnurr A, Schmitt H, Jeker L, Noel E, Brühl C, van der Steen J. First results of a method proposal for a solitary bee (*Osmia* spp.) first tier acute contact laboratory test. SETAC Europe 25th Annual Meeting Abstract Book, Barcelona, 3-7 May 2015, pp. 314-315 (2015).

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

44. Pallmann P, Schaarschmidt F, Common pitfalls when testing additivity of treatment mixtures with chi-square analyses. *J Appl Entom* **140**: 135-141 (2016).

45. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/ (2016).

46. Zimmerman DW, Zumbo BD, Relative power of the Wilcoxon test, the Friedman test, and repeated measures ANOVA on ranks. *J Exp Educ* **62**: 75-86 (1993).

47. Balderrama NM, Almeida LO, Núñez JA, Metabolic rate during foraging in the honeybee. *J Comp Physiol B* **162**: 440–447 (1992).

48. Winston ML, The biology of the honey bee. Harvard University Press, Cambridge. (1987).

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

50. Smirle MJ, Winston ML, Intercolony variation in pesticide detoxification by the honey bee (Hymenoptera: Apidae). *J Econ Entomol* **80**: 5-8 (1987).

51. Laurino D, Manino A, Patetta A, Porporato M Toxicity of neonicotinoid insecticides on different honey bee genotypes. *Bull Insectology* **66**: 119-126 (2013).

52. Lambin M, Armengaud C, Raymond S, Gauthier M, Imidacloprid-induced facilitation imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Arch Insect Biochem Physiol* **48**:129–134 (2001).

53. Henry M, Béguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S, Decourtye A, A common pesticide decreases foraging success and survival in honey bees. *Science* **336**: 348–350 (2012).

54. Jin N, Klein S, Leimig F, Bischoff G, Menzel R, The neonicotinoid clothianidin interferes with navigation of the solitary bee *Osmia cornuta* in a laboratory test. *J Exp Biol* **218**: 2821–2825 (2015).

55. Artz DR, Pitts-Singer TL, Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, *Osmia lignaria* and *Megachile rotundata*. *PLoS ONE* **10**: e0135688 (2015).

56. Berenbaum MR, Johnson RM, Xenobiotic detoxification pathways in honey bees. *Curr Opin Insect Sci* **10**: 51–58 (2015).

57. Xu J, Strange JP, Welker DL, James RR, Detoxification and stress response genes expressed in a western North American bumble bee, *Bombus huntii* (Hymenoptera: Apidae). *BMC Genom* **14**: 874 (2013).

58. Scott-Dupree CD, Conroy L, Harris CR, Impact of currently used or potentially useful insecticides for canola agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae), *Megachile rotundata* (Hymentoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae). *J Econ Entomol* **102**: 177–182 (2009).

59. Cresswell JE, Robert F-XL, Florance H, Smirnoff N, Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*). *Pest Manag Sci* **70**: 332–337 (2014).

60. Cresswell JE, The impacts of agrochemical pesticides on bees in intensively cultivated farmland, in *Pollination Services to Agriculture: Sustaining and Enhancing a Key*

Ecosystem Service, ed. by Gemmill-Herren B, Routledge Taylor & Francis Group, London and New York, pp. 155-179 (2016).

61. Straub L, Williams GR, Pettis J, Fries I, Neumann P, Superorganism resilience: Eusociality and susceptibility of ecosystem service providing insects to stressors. *Curr Opin Insect Sci* **12**: 109–112 (2015).
62. Sandrock C, Tanadini LG, Pettis JS, Biesmeijer JC, Potts SG, Neumann P, Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success. *Agric For Entomol* **16**: 119-128 (2014).
63. Rundlöf M, Andersson GKS, Bommarco R, Fries I, Hederström V, Herbertsson L, Jonsson O, Klatt BK, Pedersen TR, Yourstone J, Smith HG, Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* **521**: 77-80 (2015).
64. Ladas A, The influence of some internal and external factors upon the insecticide resistance of honeybee [in German]. *Apidologie* **3**: 55-78 (1972).
65. David A, Botías C, Abdul-Sada A, Nicholls E, Rotheray EL, Hill EM, Goulson D, Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environ Int* **88**: 169–178. (2016).
66. Ladurner E, Bosch J, Kemp WP, Maini S, Foraging and nesting behavior of *Osmia lignaria* (Hymenoptera: Megachilidae) in the presence of fungicides: cage studies. *J Econ Entomol* **101**: 647–653 (2008).

FIGURE CAPTIONS AND TABLE

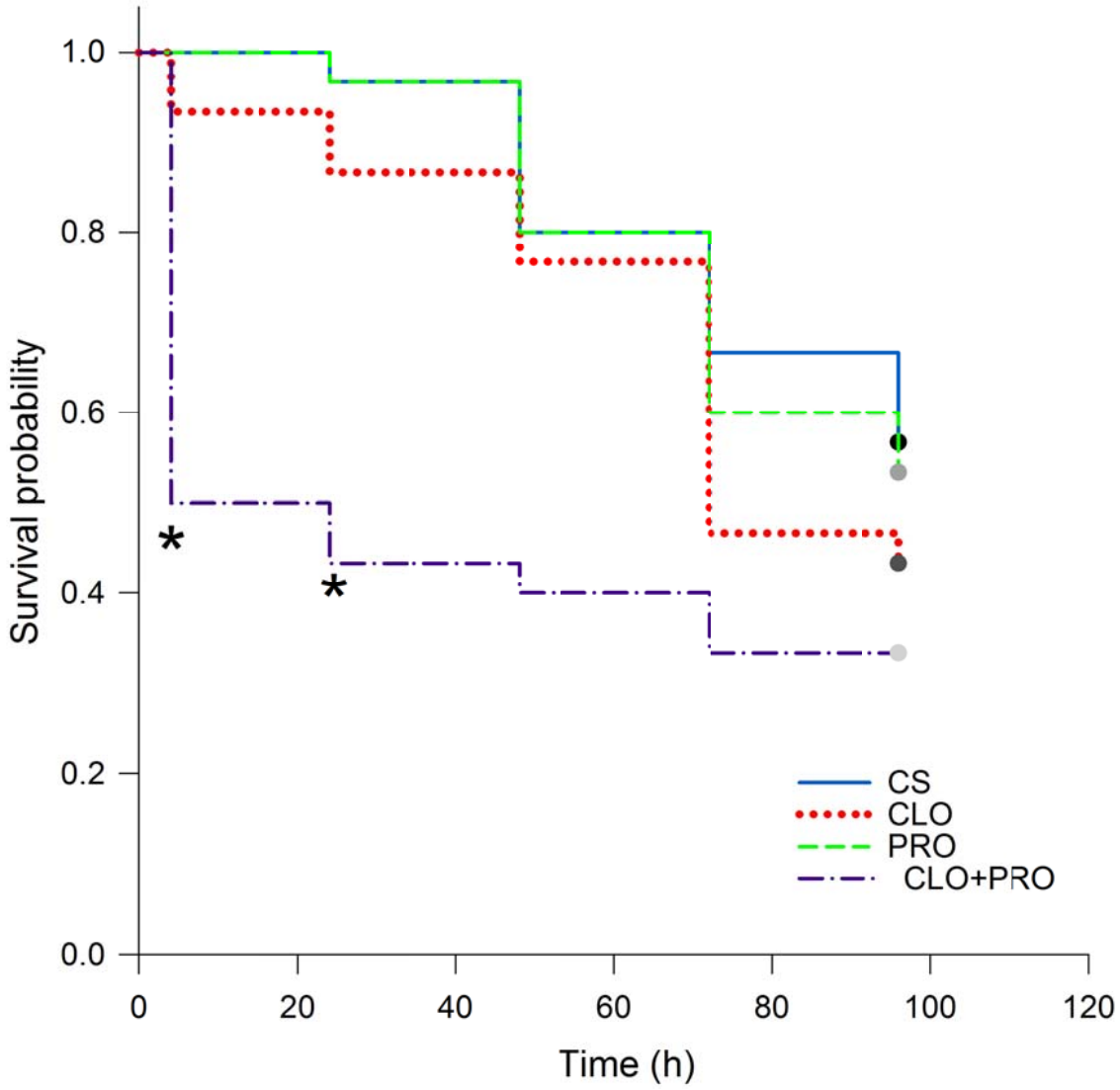

Figure 1. Cumulative proportion of surviving *Apis mellifera* foragers orally exposed to a control solution (CS - sugar water solution with 3% acetone), clothianidin (CLO - 0.79 ng/bee), propiconazole (PRO - 7 µg/bee), and clothianidin + propiconazole (CLO+PRO - 0.79 ng + 7 µg/bee). Statistically significant synergistic effects ($p<0.05$; one-tailed Binomial Proportion test

with Holm correction) at the various assessment times (4, 24, 48, 72, 96 h) are marked with an asterisk.

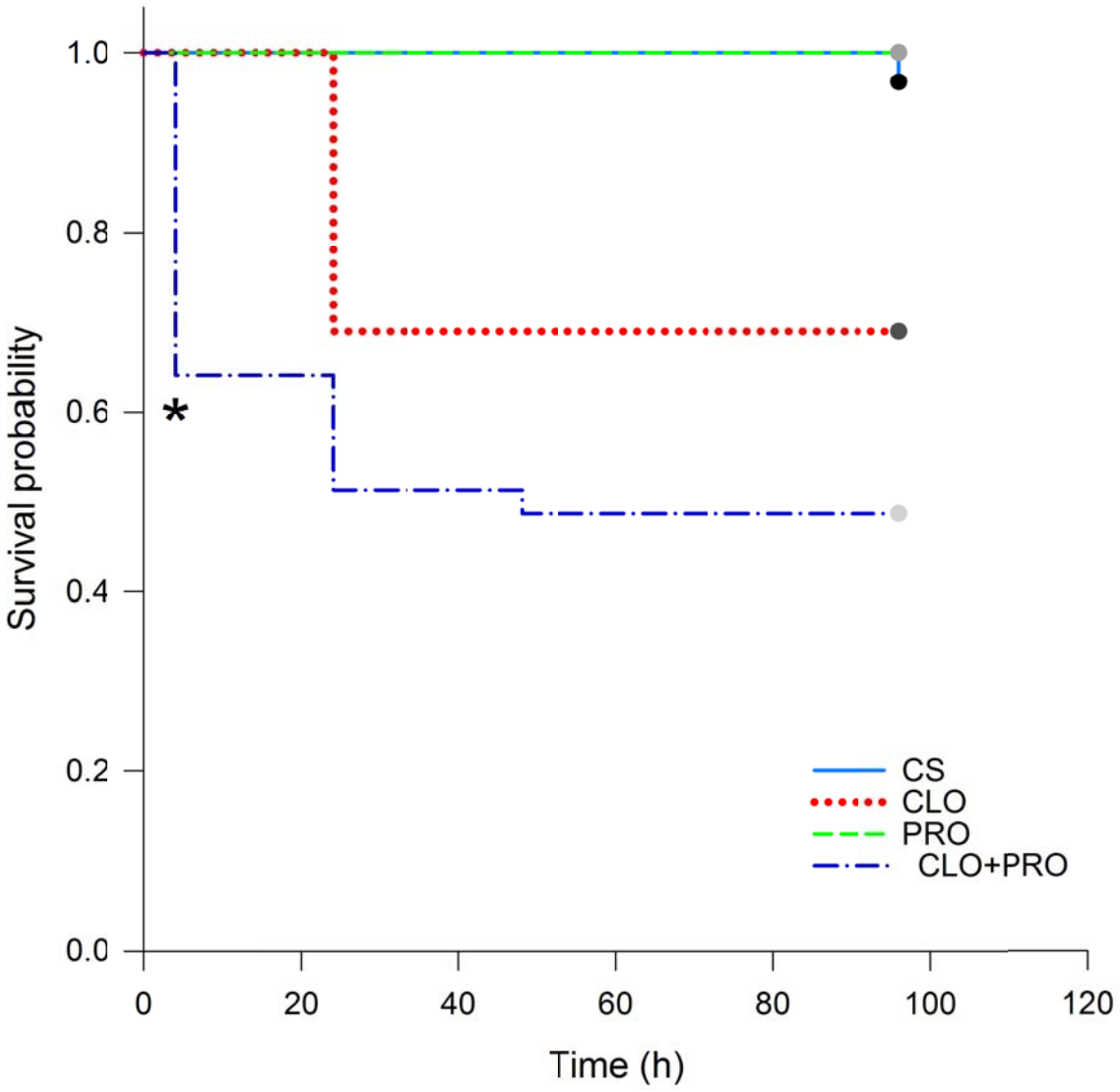

Figure 2. Cumulative proportion of surviving *Bombus terrestris* workers exposed to a control solution (CS - sugar water solution with 3% of acetone), clothianidin (CLO - 1.81 ng/bee), propiconazole (PRO - 7 µg/bee), and clothianidin + propiconazole (CLO+PRO - 1.81 ng + 7 µg/bee). Statistically significant synergistic effects ($p<0.05$; one-tailed Binomial Proportion test

with Holm correction) at the various assessment times (4, 24, 48, 72, 96 h) are marked with an asterisk.

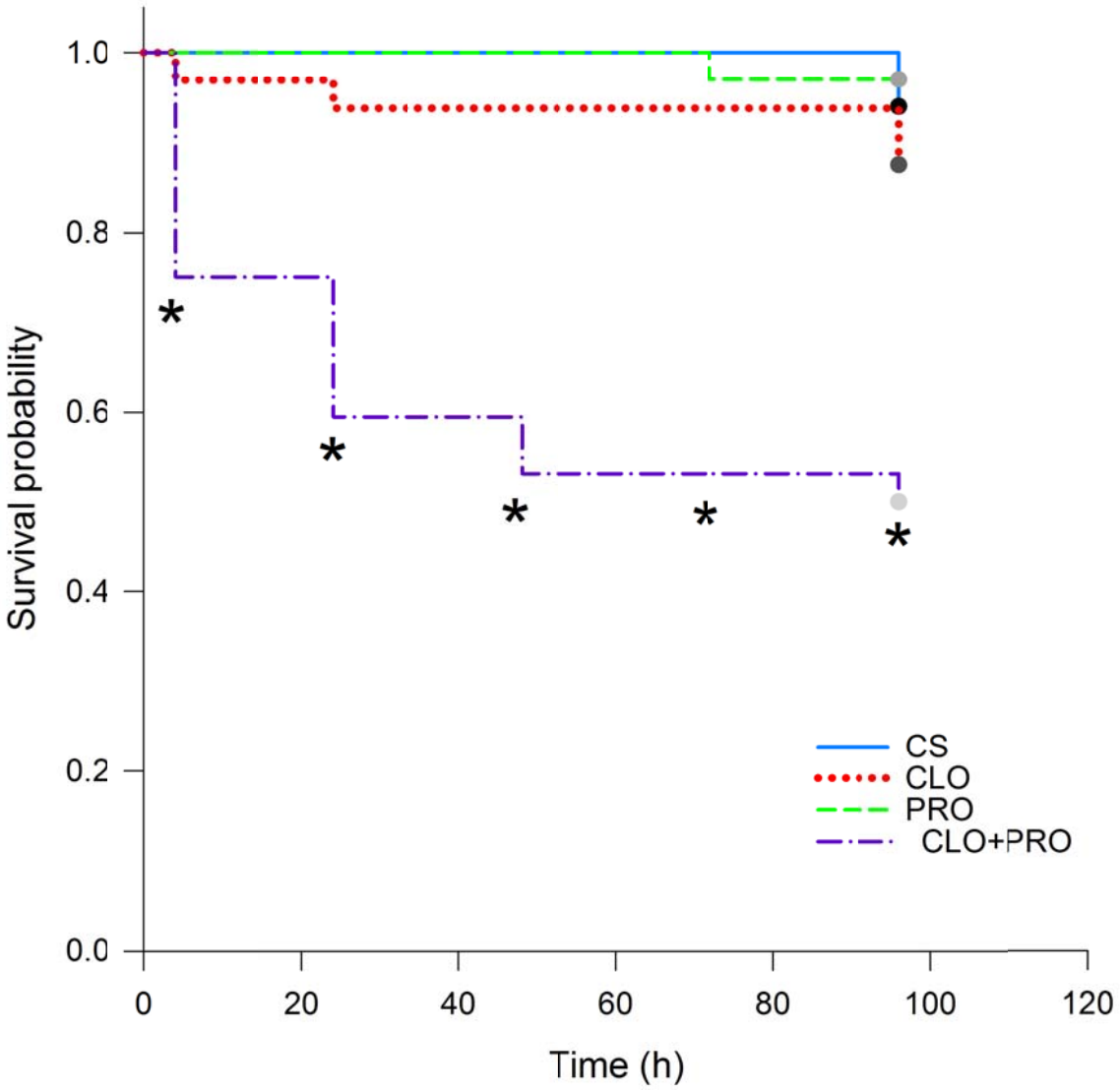

Figure 3. Cumulative proportion of surviving *Osmia bicornis* females exposed to a control solution (CS - sugar water solution with 3% acetone), clothianidin (CLO - 0.63 ng/bee), propiconazole (PRO - 7 µg/bee), and clothianidin + propiconazole (CLO+PRO - 0.63 ng + 7 µg/bee). Statistically significant synergistic effects ($p<0.05$; one-tailed Binomial Proportion test

with Holm correction) at the various assessment times (4, 24, 48, 72, 96 h) are marked with an asterisk.

Table 1. Lethal doses (LD) and 95% confidence limits (CL) expressed in ng/bee and in ng/g of bee body weight following acute oral exposure to clothianidin. Assessment times differ between species because clothianidin had a delayed effect on *O. bicornis*.

Species	**N**	**Assessment time**	**Slope±S.E.**	**LD_{50} (95% CL)**		**LD_{10} (95% CL)**		**% control mortality**	**% solvent mortality**
				ng/bee	ng/g	ng/bee	ng/g		
Apis mellifera	210	24 h	4.42±0.77	1.68 (1.28-2.04)	19.08 (14.50-23.11)	0.86 (0.50-1.16)	9.80 (5.61-13.20)	3.3	10
Bombus terrestris	212	24 h	5.76±0.98	3.12 (2.32-3.96)	11.90 (8.84-15.10)	1.87 (0.76-2.46)	7.13 (2.89-9.38)	2.9	0
Osmia bicornis	179	72 h	5.12±0.94	1.17 (0.93-1.45)	9.47 (7.52-11.70)	0.66 (0.43-0.85)	5.32 (3.44-6.82)	10	3.3

Table 2. Pairwise comparisons of the sensitivity of *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis* to clothianidin and propiconazole, alone and in combination, 96 hours after oral exposure (Holm-Sidak pairwise multi comparison test based on Log-rank Kaplan-Meier survival analyses).

Treatments	***Apis mellifera***		***Bombus terrestris***		***Osmia bicornis***	
	Statistic	P value	Statistic	P value	Statistic	P value
Control vs propiconazole	0.07	0.79	1.23	0.27	0.32	0.57
Control vs clothianidin	1.22	0.61	8.00	0.01	0.86	0.58
Control vs combination	6.91	0.05	18.48	<0.01	16.78	<0.01
Propiconazole vs clothianidin	0.72	0.64	13.09	<0.01	2.01	0.40
Propiconazole vs combination	6.04	0.07	25.73	<0.01	19.09	<0.01
Clothianidin vs combination	3.11	0.28	4.42	0.07	11.13	<0.01