A combination of the frequent fungicides boscalid and dimoxystrobin with the neonicotinoid acetamiprid in field-realistic concentrations does not affect sucrose responsiveness and learning behavior of honeybees

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ABSTRACT

The increasing loss of pollinators over the last decades has become more and more evident. Intensive use of plant protection products is one key factor contributing to this decline. Especially the mixture of different plant protection products can pose an increased risk for pollinators as synergistic effects may occur. In this study we investigated the effect of the fungicide Cantus® Gold (boscalid/dimoxystrobin), the neonicotinoid insecticide Mospilan® (acetamiprid) and their mixture on honeybees. Since both plant protection products are frequently applied sequentially to the same plants (e.g. oilseed rape), their combination is a realistic scenario for honeybees. We investigated the mortality, the sucrose responsiveness and the differential olfactory learning performance of honeybees under controlled conditions in the laboratory to reduce environmental noise. Intact sucrose responsiveness and learning performance are of pivotal importance for the survival of individual honeybees as well as for the functioning of the entire colony. Treatment with two sublethal and field relevant concentrations of each plant protection product did not lead to any significant effects on these behaviors but affected the mortality rate. However, our study cannot exclude possible negative sublethal effects of these substances in higher concentrations. In addition, the honeybee seems to be quite robust when it comes to effects of plant protection products, while wild bees might be more sensitive.

1. Introduction

Recent years have shown a clear decline in pollinators worldwide, which might ultimately lead to the loss of many plants as the pollination by wild animals is crucial for the persistence of many wild flowers and cultivated plants (Klein et al., 2007). More than three quarters of European crops depend on animal pollinators (Williams, 1994). The reasons that can lead to such a decline are manifold as pollinators are exposed to several external risks during their life such as climate change, habitat fragmentation or pathogens (Potts et al., 2010). One of the most important factors in insect decline, however, is the use of plant protection products (PPPs[1]) in intensive agriculture (Sánchez-Bayo and Wyckhuys, 2019). The honeybee (*Apis mellifera*) displays an excellent model organism for studying effects of PPPs on insect pollinators as there is a variety of behavioral paradigms and the bees can be easily bred

for research (Scheiner et al., 2013). However, the honeybee generally appears to be quite robust when it comes to effects of chemical pollutants, not least because of their sociality, which can increase their resilience towards pesticides. Wild bees, particularly solitary ones, might be much more sensitive than honeybees when they get into contact with PPPs. The effects of PPPs on different wild bees may also differ from each other (Willow et al., 2019; Wood et al., 2020).

Over the last decades, more and more PPPs have come to the market. Meanwhile, over 1000 PPPs are on offer in Europe alone. These PPPs include insecticides, fungicides, herbicides and a few other substances (El-Nahhal, 2020). Neonicotinoids are described as one of the most effective group of insecticides. However, increased evidence of negative impacts on pollinators led to a recent ban of almost all neonicotinoids in the European Union (European Commission, 2022). Acetamiprid belongs to the cyanoamidine group of neonicotinoids which is considered

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less toxic, when applied on its own (Schuhmann et al., 2022). It is the last neonicotinoid approved by the European Union (European Commission, 2022). Insecticides have been investigated intensively for possible negative effects on pollinators (Köhler and Triebskorn, 2013) and some fungicides have also been studied for side effects (e.g. Artz and Pitts-Singer, 2015; DeGrandi-Hoffman et al., 2015; Simon-Delso et al., 2018), although there are fewer studies on fungicides than on insecticides (Köhler and Triebskorn, 2013). In this study, we investigated possible effects of the frequent fungicides boscalid and dimoxystrobin (in the mixture Cantus® Gold) (Rosenkranz et al., 2020). In addition, we tested effects of acetamiprid (in Mospilan®) and of the mixture of these PPPs on honeybee behavior. The fungicide Cantus® Gold and the neonicotinoid Mospilan® can be applied sequentially to the same flowers, e.g. oilseed rape. It is therefore a highly realistic scenario for honeybees to be exposed to these two plant protection productions in the same time windows. Since oilseed rape is a favorite plant of honeybees (Stanley et al., 2013), they can consume relatively large amounts of PPPs sprayed on this plant during their daily foraging trips.

In the field, PPPs are frequently applied in combination or sequentially with a variety of active substances (Thompson et al., 2014). The resulting PPP mixtures can lead to synergistic effects of the different substances, i.e. effects that are more harmful than those of the sum effects of the different PPPs (Cedergreen, 2014; Folt et al., 1999; Piggott et al., 2015). Especially mixtures of sterol biosynthesis inhibiting (SBI[2]) fungicides and neonicotinoids or pyrethroids lead to synergistic effects, since the detoxification enzyme P450 can be inhibited by the fungicides which prevents the degradation of the insecticides. Intriguingly, not every mixture of the mentioned PPP groups leads to synergistic effects. This suggests that detoxification with the P450 enzymes plays different roles for different PPPs (Johnson et al., 2006; Raimets et al., 2018; Thompson et al., 2014). The background of synergistic effects is still unknown for some mixtures. In addition to disturbances of metabolic enzyme activity, possible causes of synergistic effects may be modifications of excretion or uptake rate and transport to the target site (Cedergreen, 2014). Such effects have not only been demonstrated in the honeybee (*A. mellifera*) (e.g. Vandame and Belzunces, 1998), but also in other beneficial insects such as *Osmia bicornis* (e.g. Sgolastra et al., 2017, 2018), *Bombus terrestris* (Raimets et al., 2018) and *Aphelinus abdominalis* (Willow et al., 2019).

We challenged the hypothesis that a combination of a frequent non-SBI fungicide and a neonicotinoid which is still used in the EU do not lead to synergistic effects on mortality, sucrose responsiveness and learning performance of honeybees. Since the formulated products which are applied to the fields contain other substances in addition to the active ingredients that could change their effects (Cox and Surgan, 2006), the formulations Cantus® Gold and Mospilan® were used.

Mospilan® contains the neonicotinoid acetamiprid (200 g/kg) as active ingredient (a.i.[3]), while Cantus® Gold comprises the fungicides boscalid and dimoxystrobin in equal parts (200 g/l respectively) (BASF SE, 2021; FMC Agricultural Solutions, 2021). Risk assessment toxicity tests are usually conducted to quantify mortality rates. However, sublethal effects on the behavior of the bees can also lead to severe consequences, which might ultimately result in the death of individuals or entire colonies. We tested the effects on the responsiveness to sucrose and the olfactory learning performance of honeybees in addition to mortality. For this, we used an established protocol which allows us to test PPP action on individual honeybees under controlled conditions and to compare our data with existing literature on the action of other fungicides and neonicotinoids. Both sucrose responsiveness and learning performance play an essential role in the effective persistence of a honeybee hive (Menzel, 1993; von Frisch, 1965) and allow us to estimate the degree of possible negative impacts on honeybee behavior and physiology.

2. Material and methods

2.1. Bees

Same age honeybee workers (*A. mellifera carnica*) were randomly collected from a hive maintained in the departmental apiary of the University of Würzburg. The hives were kept outdoor according to normal beekeeping standards. Bees were transferred into small cages (7.8 ×5.0 ×8.2 cm) where they were treated with the respective feeding solution for one week, which was some days longer than the established protocol for honeybees (Medrzycki et al., 2013) to simulate the exclusive foraging behavior of a bee for one week. The back of the cages was made of untreated wood and the side walls were made of plexiglass. For easy opening of the cages, a sliding metal grid was attached at the front. In the wooden lid of the cages there were two holes for the feeding tubes. The cages were maintained in an incubator (30 °C, 50 % humidity, constant darkness) for the duration of the treatment.

2.2. Food supply

Food was provided via prepared 5 ml Eppendorf centrifuge tubes. The amount of food per cage was adapted to the number of individuals, so that the bees could feed ad libitum. Each day, the tubes were removed and replaced by new ones to guarantee a controlled and fresh food supply. The control bees received a 30 % sugar water solution (based on sucrose). Therefore, the feeding solutions of the treatment groups were also based on 30 % sugar water.

2.3. Plant protection products (PPPs)

To test for possible synergistic effects of PPPs on different behaviors of honeybees, the fungicide Cantus® Gold (suspension concentrate, active ingredients: boscalid 200 g/l and dimoxystrobin 200 g/l) (BASF SE, Ludwigshafen, Germany) and the insecticide Mospilan® (water soluble granules, active ingredient: acetamiprid 200 g/kg) (Nisso Chemical Europe GmbH, Düsseldorf, Germany) were investigated. Both are applied on oilseed rape fields (BASF SE, 2021; FMC Agricultural Solutions, 2021). For all behavioral experiments, four treatments consisting of a (1) control treatment, (2) a fungicide treatment, (3) an insecticide treatment and (4) a mix treatment of the insecticide and the fungicide were always tested together. To determine suitable concentrations for the experiments, studies were performed to calculate the LD50[4] value (see Supplementary Information, Fig. S1). Two sublethal doses (low dose/high dose) were chosen for the following experiments, that were both well below the LD50 value and which were based on PPP residuals. The active ingredients of Cantus® Gold (boscalid and dimoxystrobin) have been found in a quantity of 5 µg/kg (Luken and von der Ohe, 2018). For the active ingredient of Mospilan® (acetamiprid) residue levels of 72.5 µg/kg were reported (El-Nahhal, 2020). Taking into account these residue levels, the realistic daily honey consumption rate per bee (Rortais et al., 2005) and the daily consumption rate of feeding solution of caged bees (Hesselbach and Scheiner, 2019), both solutions for the behavioral tests can be considered as field relevant. For the fungicide, the calculated concentration 10 µg/l was used as the low dose. A bee ingested 0.0008 µg of both active ingredients per day. The high dose was 100 µg/l, which is why the uptake of active ingredient per bee per day increased to 0.008 µg. The low concentration of the insecticide corresponded to 200 µg/l. The intake of active ingredient per bee per day was 0.012 µg. The high dose was 2000 µg/l and corresponded to an intake of active ingredient per bee per day of 0.12 µg (for overview see Table 1). These concentrations were all below the recommended field doses (BASF SE, 2021; FMC Agricultural Solutions, 2021).

The feeding solutions were prepared with sugar water. First, a stock solution was prepared, which was then diluted accordingly until the concentrations of the feeding solutions were reached. The feeding solutions were renewed every two days. In the meantime, they were stored

Table 1

The concentrations of the active ingredients (a.i.) of the plant protection products (PPPs) used for mortality studies and behavioral experiments. The mixture always contained both PPPs in the indicated concentrations. The sample size of the experiments was as follows: Mortality: Control low: 100, Control high: 100, Cantus® Gold low: 100, Cantus® Gold high: 100, Mospilan® low: 100, Mospilan® high: 100, Mixture low: 100, Mixture high: 100. Sucrose responsiveness and learning: Control low: 44, Control high: 52, Cantus® Gold low: 41, Cantus® Gold high: 43, Mospilan® low: 46, Mospilan® high: 48, Mixture low: 39, Mixture high: 45. Reversal learning: Control low: 27, Control high: 21, Cantus® Gold low: 28, Cantus® Gold high: 19, Mospilan® low: 18, Mospilan® high: 23, Mixture low: 16, Mixture high: 19.

		Cantus® Gold	Mospilan®	Mixture
low	concentration	10 µg/l	200 µg/l	10 µg/l + 200 µg/l
	a.i./bee/day	0.0008 µg	0.012 µg	0.0008 µg + 0.012 µg
high	concentration	100 µg/l	2000 µg/l	100 µg/l + 2000 µg/l
	a.i./bee/day	0.008 µg	0.12 µg	0.008 µg + 0.12 µg

at 6 °C.

2.4. Mortality

For the determination of the toxicity of the PPPs used for the behavioral tests, between 20 and 50 honeybees were transferred to cages and maintained in an incubator (low concentration: five cages per treatment with 20 bees, high concentration: two cages per treatment with 50 bees). In each experiment, control group and treatment groups had the same number of bees at the start of the experiment. There was one cage per treatment group. Dead individuals were removed and counted daily when the food was changed.

2.5. Sucrose responsiveness

PER[5] (proboscis extension response) experiments were performed to quantify the responsiveness of honeybees to increasing concentrations of sucrose (Scheiner et al., 2013).

After one week of exposure to PPPs, the bees were individually anaesthetized on ice. Then they were harnessed in holders and fixed with one strip of textile tape between head and thorax and one strip over the abdomen, so that they could still move their antennae and mouth parts freely (Fig. 1) (see also Hesselbach and Scheiner, 2018). The test started two hours after the last bee had been harnessed.

First, it was controlled that each bee could move its proboscis freely. Afterwards, water and a series of sucrose concentrations were presented to the antennae of the honeybees in ascending concentrations (water, 0.1 %, 0.3 %, 1 %, 3 %, 10 %, 30 %) (Fig. 1). After each stimulation, it was recorded whether the bee had shown a PER or not. The intertrial interval was two minutes to avoid intrinsic sensitization (Scheiner et al., 2013). Finally, the response to 50 % sugar water was tested, because no response to 50 % sucrose was a criterion for exclusion from the subsequent learning experiments as 50 % sucrose was used as reward during conditioning.

2.6. Differential olfactory conditioning

The effect of the PPPs on the olfactory learning performance of honeybees was tested by classical differential conditioning followed by reversal learning which represents a complex cognitive task (Komischke et al., 2002). Bees not responding to the highest sucrose concentration (50 %) were not used for the learning experiment. Differential learning experiments were performed with two different odors as conditioned stimuli (1-nonanol (74278 1-nonanol, Sigma Aldrich, Steinheim, Germany) and eugenol (E51791 eugenol, Sigma Aldrich, Steinheim, Germany)). The odors were presented to the antennae of the honeybees via a syringe. The syringe contained a piece of filter paper soaked with 5 µl of the respective odor. During training, one odor (conditioned stimulus (appetitive): CS+[6]) was rewarded with 50 % sugar water (unconditioned stimulus (appetitive): US+[7]), while the other one (conditioned stimulus (aversive): CS-[8]) was punished with quinine (60 mM) (unconditioned stimulus (aversive): US-[9]).

Before training started, the spontaneous reactions to the two odors were tested, as only bees that did not show a spontaneous response to either odor could be used for the learning experiment (Hesselbach and Scheiner, 2018). During the whole learning experiment, the released odors were extracted by a fume hood to avoid contamination. For this reason, the bees were left in front of the fume hood for further 15 s after the end of the odor application, until the complete absence of the odor was ensured.

During the differential learning experiment, the CS+ and the CS- were presented five times each, in an alternating order. Each time, the odor was presented for eight seconds to the antennae. For the first three seconds the odor was presented alone, while for the following five seconds the odor was presented in combination with the reward or the punishment. If the bee showed a PER in the three-second time window in which only the CS+ was applied, the response was considered positive, i.e. the bee had learned the association. In each trial, it was recorded whether the bee had shown a conditioned PER to the CS+ or CS- alone. After the odor application, the US+ or US- were presented for another two seconds, so that the bee could drink the solution.

To investigate a more complex form of learning, the experiment was continued with reversal learning trials (Hadar and Menzel, 2010). The stimuli were switched, so that the former CS+ became the CS-, and vice versa. The rest of the experimental setup remained the same. Since only bees that had previously learned should be evaluated during reversal
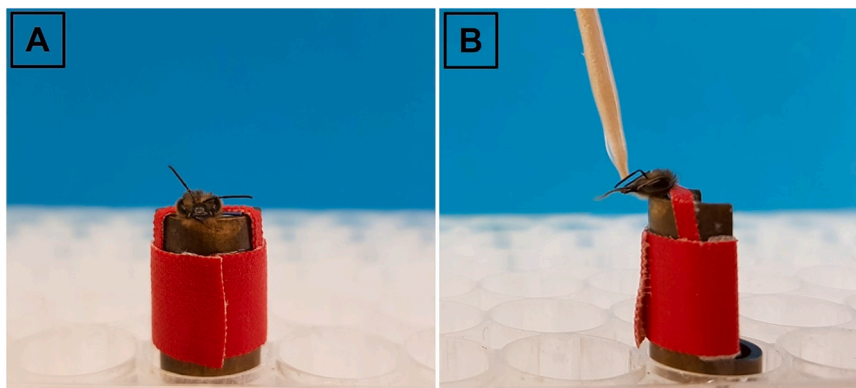

Fig. 1. Method for conditioning the bees in the laboratory. (A) The bee is harnessed in a holder and fixed with textile tape between head and thorax and over the abdomen. The antennae and mouthparts can still be moved freely. (B) The antennae of the bee are stimulated with a sucrose solution. The bee is showing a proboscis extension response (PER) in response to the stimulation.

learning, a test was performed between the learning and reversal learning trials (Fig. 2) (For details on test trial see Supplementary Information, Fig. S2).

2.7. Statistics

Kaplan Meier curves with Log-rank tests were used for comparing the mortality rates as there was no replicate effect. For pairwise comparisons Bonferroni-Dunn method was used (GraphPad Prism® version 7.03 for Windows, GraphPad Software, La Jolla, CA USA).

To compare the PER performance in the sucrose responsiveness tests of the different treatment groups a generalized linear model (GLM[10]) was applied with sucrose concentration as within-subject factor and treatment as between-subject factor (Šidák test for pairwise comparisons). Only bees displaying the PER to 50 % sucrose which did not show any spontaneous response to either odor were analyzed (SPSS® Statistics 26 (Version 26, IBM®, Armonk, NY USA), GraphPad Prism® version 7.03 for Windows, GraphPad Software, La Jolla, CA USA)).

Learning performance and reversal learning performance are shown by learning and reversal learning curves. For the learning trials all conditioned bees were included in the GLM, while for the reversal learning performance only the bees that had learned before (i.e. honeybees that showed a response to the CS+ and no response to the CS- during the test (see Supplementary Information)) were analyzed. The learning or reversal learning trials were used as within-subject factor while the treatment was used as between-subject factor. During the learning trials, there were almost no responses to the CS-. Therefore, we did not perform a GLM (SPSS® Statistics and GraphPad Prism®).

3. Results

3.1. Mortality

The concentrations of 10 µg/l Cantus® Gold and 200 µg/l Mospilan® or their mixture did not increase the mortality of the bees (mortality rate: control: 3 %, Cantus® Gold: 2 %, Mospilan®: 2 %, mix: 1 %) (Log-rank test with Bonferroni correction; $p_{Control\ vs\ Cantus®\ Gold} = 1.000$, $p_{Control\ vs\ Mospilan®} = 1.000$, $p_{Control\ vs\ Mix} = 0.952$). The tenfold higher concentrations of 100 µg/l Cantus® Gold and 2000 µg/l Mospilan® also did not increase the mortality, but the mixture of both led to a higher mortality rate compared to the control group (mortality rate: control: 4 %, Cantus® Gold: 8 %, Mospilan®: 9 %, mix: 15 %) (Log-rank test with Bonferroni correction; $p_{Control\ vs\ Cantus®\ Gold} = 0.749$, $p_{Control\ vs\ Mospilan®} = 0.509$, $p_{Control\ vs\ Mix} = 0.028(*)$) (Fig. 3 A and B).

3.2. Responsiveness to sucrose

For testing responsiveness to sucrose, we used two sublethal concentrations of Cantus® Gold (low: 10 µg/l and high: 100 µg/l) and Mospilan® (low: 200 µg/l and high: 2000 µg/l). The proportion of bees showing PERs increased with increasing sucrose concentration in all groups (proportion PER after low treatment: control: 84 %, Cantus® Gold: 85 %, Mospilan®: 87 %, mix: 90 %; proportion PER after high treatment: control: 92 %, Cantus® Gold: 84 %, Mospilan®: 92 %, mix: 82 %) (GLM: effect of trial; $p_{low\ dose} < 0.001$; $p_{high\ dose} < 0.001$). Response of trained bees to sucrose was unaffected by treatment with PPPs (GLM: treatment effect on sucrose responsiveness; $p_{low\ dose} = 0.505$; $p_{high\ dose} = 0.355$). Bees treated with the different concentrations of Cantus® Gold and Mospilan® did not differ from control bees in their responses to increasing sucrose concentrations (Fig. 4 A and B).
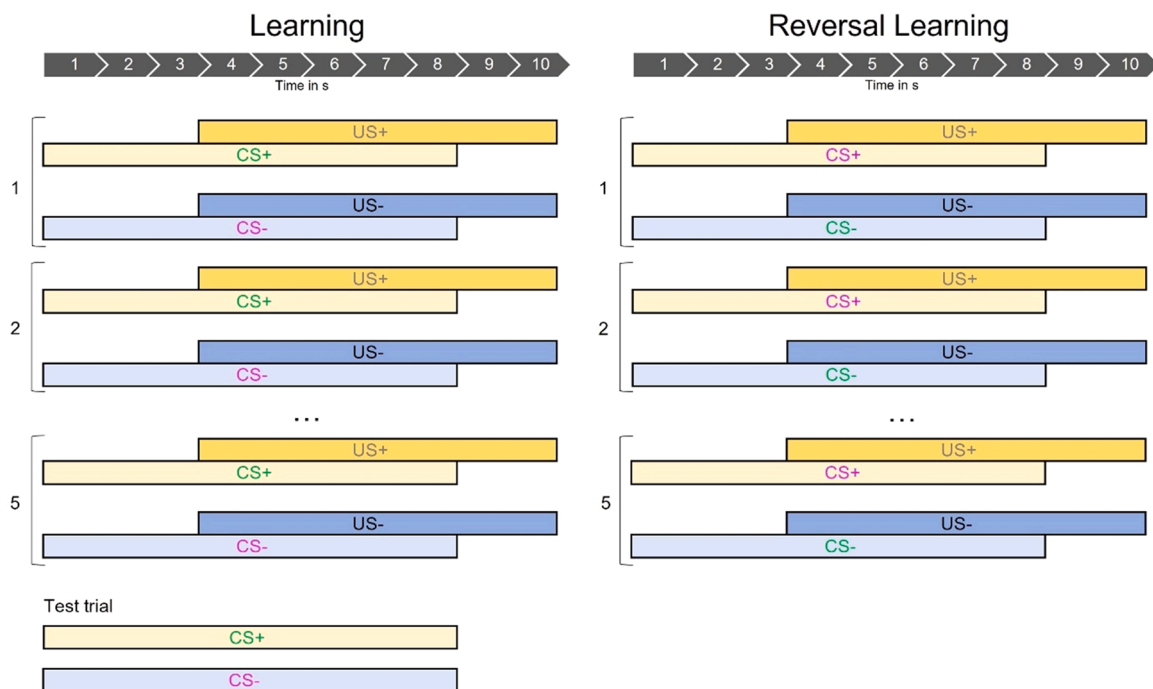

Fig. 2. Procedure of the learning (left) and reversal learning (right) paradigm. First, the odor (CS+/CS-) was presented for three seconds. During the following five seconds the odor was presented in combination with the reward or the punishment (US+/US-). When the bee showed a proboscis extension response (PER), the US+ /US- was presented for another two seconds, while the CS+ /CS- was removed. Learning and reversal learning consisted of 5 trials. CS+ and CS- were always shown in alteration. The green color represents one odor, while the magenta color represents the other odor. The odors in their function as CS+ and CS- were switched between the learning and the reversal learning paradigm. After the five learning trials a test was performed. The CS+ and CS- were shown without any reward our punishment for eight seconds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
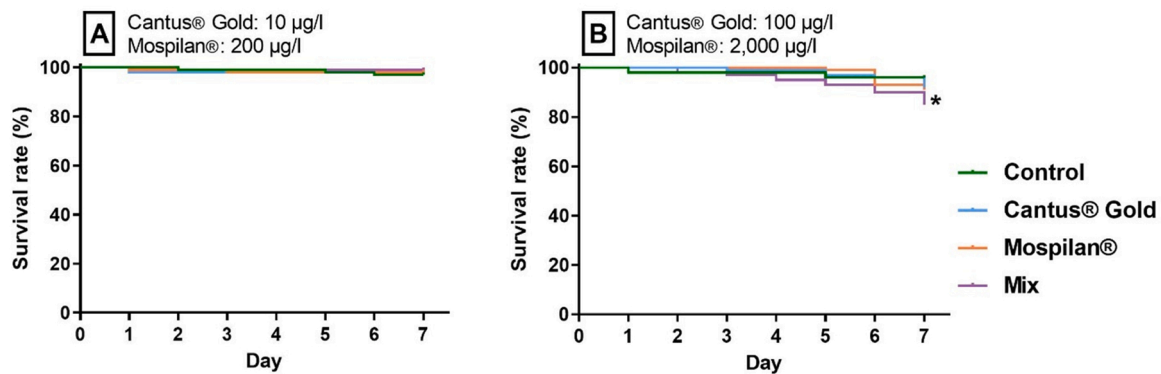
Fig. 3. Survival curves. (A) Kaplan Meier survival curves of the honeybees that were treated with the low sublethal concentrations of Cantus® Gold (blue; n = 100; 10 µg/l), Mospilan® (orange; n = 100; 200 µg/l) or the mixture of both (purple; n = 100). The control bees received a sucrose solution (green; n = 100). There was no treatment effect on the survival rate of the different groups (mortality rate: control: 3 %, Cantus® Gold: 2 %, Mospilan®: 2 %, mix: 1 %) (Log-rank test with Bonferroni correction; $p_{Control\ vs\ Cantus®\ Gold} = 1.000$, $p_{Control\ vs\ Mospilan®} = 1.000$, $p_{Control\ vs\ Mix} = 0.952$). (B) Kaplan Meier survival curves of the honeybees that were treated with the high sublethal concentrations of Cantus® Gold (blue; n = 100; 100 µg/l), Mospilan® (orange; n = 100; 2000 µg/l) or the mixture of both (purple; n = 100). The control bees received a sucrose solution (green; n = 100). There was a treatment effect as significantly more mix animal died compared to control bees (mortality rate: control: 4 %, Cantus® Gold: 8 %, Mospilan®: 9 %, mix: 15 %) (Log-rank test with Bonferroni correction; $p_{Control\ vs\ Cantus®\ Gold} = 0.749$, $p_{Control\ vs\ Mospilan®} = 0.509$, $p_{Control\ vs\ Mix} = 0.028$ (*)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
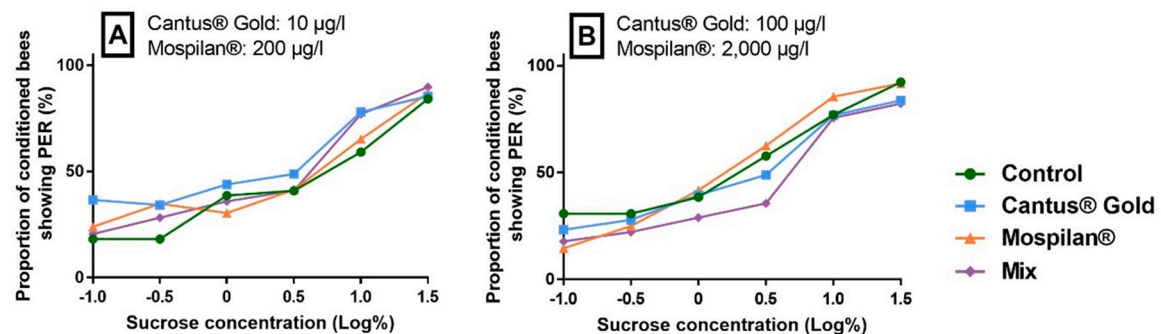

Fig. 4. PER curves. (A) Proportion of conditioned honeybees showing a proboscis extension response (PER) to increasing sucrose concentrations after oral treatment with a control solution (green circle; n = 44) or a low sublethal concentration of Cantus® Gold (blue square; n = 41; 10 µg/l), Mospilan® (orange triangle; n = 46; 200 µg/l) or the mixture of both (purple rhombus; n = 39). There were no treatment effects on sucrose responses (proportion PER after low treatment: control: 84 %, Cantus® Gold: 85 %, Mospilan®: 87 %, mix: 90 %) (GLM: treatment effect on sucrose responsiveness, $p_{low\ dose} = 0.505$). (B) Proportion of conditioned honeybees showing a PER to increasing sucrose concentrations after oral treatment with a control solution (green circle; n = 52) or with a high sublethal concentration of Cantus® Gold (blue square; n = 43; 100 µg/l), Mospilan® (orange triangle; n = 48; 2000 µg/l) or the mixture of both (purple rhombus; n = 45). There was no treatment effect on sucrose responses (proportion PER after high treatment: control: 92 %, Cantus® Gold: 84 %, Mospilan®: 92 %, mix: 82 %) (GLM: treatment effect on sucrose responsiveness, $p_{high\ dose} = 0.355$).

3.3. Olfactory learning

The learning experiments were performed following the same sublethal oral treatments as the experiments investigating sucrose responsiveness (Cantus® Gold; low: 10 µg/l, high: 100 µg/l; Mospilan®; low: 200 µg/l, high: 2000 µg/l). After oral treatment, bees in all groups learned to respond to the CS+ during acquisition (proportion of responses to CS+ after low treatment: control: 66 %, Cantus® Gold: 63 %, Mospilan®: 48 %, mix: 49 %; proportion of responses to CS+ after high treatment: control: 46 %, Cantus® Gold: 44 %, Mospilan®: 52 %, mix: 38 %) (GLM: effect of trial; CS+: $p_{low\ dose} < 0.001$; $p_{high\ dose} < 0.001$). Only a maximum of 2 % of the bees showed a response to the CS-. Treatment with the fungicide, the insecticide, or the combination of both did not affect learning performance (GLM: treatment effect on learning; CS+: $p_{low\ dose} = 0.165$; $p_{high\ dose} = 0.612$). (Fig. 5 A and Fig. 6 A). During the reversal learning phase, responses to the new CS+ became more frequent (proportion of responses to CS+ after low treatment: control: 28 %, Cantus® Gold: 27 %, Mospilan®: 18%, mix: 21 %; proportion of responses to CS+ after high treatment: control: 13 %, Cantus® Gold: 21 %, Mospilan®: 24 %, mix: 18 %), while the responses to the former CS+ , which now represented the punished CS-, became

less frequent (proportion of responses to CS- after low treatment: control: 21 %, Cantus® Gold: 38 %, Mospilan®: 18 %, mix: 16 %; proportion of responses to CS- after high treatment: control: 21 %, Cantus® Gold: 11 %, Mospilan®: 28 %, mix: 18 %) (GLM: effect of trial; CS+: $p_{low\ dose} < 0.001$; $p_{high\ dose} < 0.001$; CS-: $p_{low\ dose} < 0.001$; $p_{high\ dose} < 0.001$). There was no treatment effect on the reversal learning performance independent of the concentration used (GLM: treatment effect on reversal learning; CS+: $p_{low\ dose} = 0.500$; $p_{high\ dose} = 0.748$; CS-: $p_{low\ dose} = 0.197$; $p_{high\ dose} = 0.484$) (Fig. 5B and Fig. 6B).

4. Discussion

4.1. Effects of PPP mixtures

The results of our study show that the responsiveness to sugar water and the learning behavior of honeybees were not affected by the combination of Cantus® Gold and Mospilan®. However, the mortality studies showed a synergistic effect, as the mix group (100 µg/l Cantus® Gold + 2000 µg/l Mospilan®) differed significantly from the other groups.

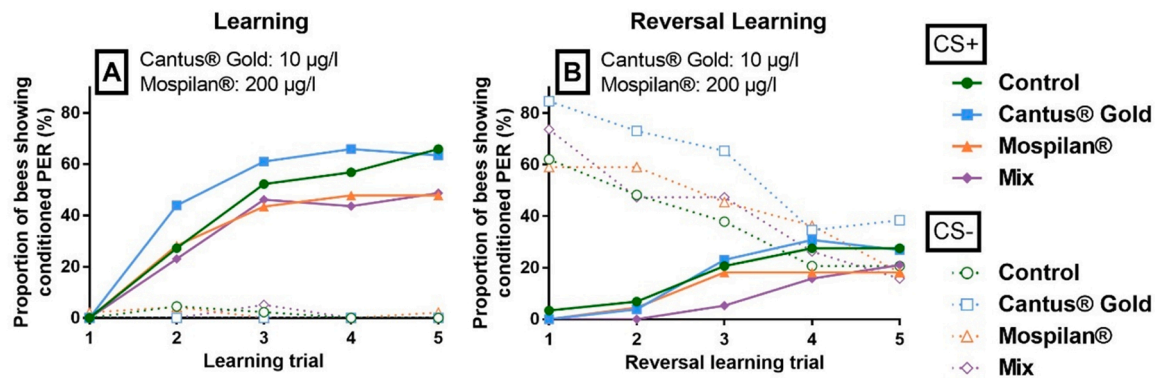Different studies on PPP mixtures already showed similar synergistic

Fig. 5. Learning and reversal learning curves of bees treated with a low field realistic dose of PPPs. (A) Learning curves of honeybees treated with a control solution (green circle; n = 44) or with a low sublethal dose of Cantus® Gold (blue square; n = 41; 10 µg/l), Mospilan® (orange triangle; n = 46; 200 µg/l) or the mixture of both (purple rhombus; n = 39). The proportion of bees showing a conditioned proboscis extension response (PER) is shown for each group. The honeybees learned well to respond to the CS+ (solid lines) (GLM: effect of trial, CS+: $p_{low\ dose} < 0.001$) and not to react to the CS- (dotted lines) (proportion of responses to CS+ after low treatment: control: 66 %, Cantus® Gold: 63 %, Mospilan®: 48 %, mix: 49 %; proportion of responses to CS- after low treatment: control: 0%, Cantus® Gold: 0 %, Mospilan®: 2 %, mix: 0 %). Treatment with the different PPPs had no significant effect on learning performance (GLM: treatment effect on learning, CS+: $p_{low\ dose} = 0.165$). (B) Reversal learning curves of honeybees treated with a control solution (green circle; n = 27) or with a low sublethal dose of Cantus® Gold (blue square; n = 28; 10 µg/l), Mospilan® (orange triangle; n = 18; 200 µg/l) or the mixture of both (purple rhombus; n = 16). The proportion of bees showing a conditioned proboscis extension response (PER) is shown for each group. The proportion of responses to the new CS+ increased slowly with progressive trial (solid lines), while the responses to the former CS+ decreased (dotted lines) (proportion of responses to CS+ after low treatment: control: 28%, Cantus® Gold: 27 %, Mospilan®: 18 %, mix: 21 %; proportion of responses to CS- after low treatment: control: 21 %, Cantus® Gold: 38 %, Mospilan®: 18 %, mix: 16 %) (GLM: effect of trial, CS+: $p_{low\ dose} < 0.001$, CS-: $p_{low\ dose} < 0.001$). There was no effect of treatment on the reversal learning performance (GLM: treatment effect on reversal learning, CS+: $p_{low\ dose} = 0.500$, CS-: $p_{low\ dose} = 0.197$).
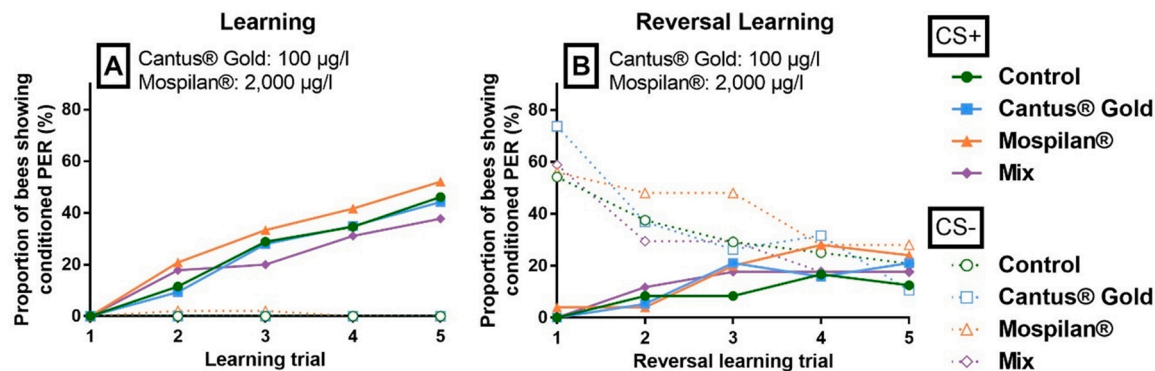

Fig. 6. Learning and reversal learning curves of bees treated with a high field realistic dose of PPPs. (A) Learning curves of the honeybees that were treated with a control solution (green circle; n = 52) or with a high sublethal dose of Cantus® Gold (blue square; n = 43; 100 µg/l), Mospilan® (orange triangle; n = 48; 2000 µg/l) or the mixture of both (purple rhombus; n = 45). The proportion of bees showing a conditioned proboscis extension response (PER) is shown for each group. The honeybees learned well to respond to the CS+ (solid lines) (GLM: effect of trial, CS+: $p_{high\ dose} < 0.001$) and not to react to the CS- (dotted lines) (proportion of responses to CS+ after high treatment: control: 46 %, Cantus® Gold: 44 %, Mospilan®: 52 %, mix: 38 %; proportion of responses to CS- after high treatment: control: 0 %, Cantus® Gold: 0 %, Mospilan®: 0 %, mix: 0 %). Treatment with the different PPPs had no significant effect on learning performance (GLM: treatment effect on learning, CS+: $p_{high\ dose} = 0.612$). (B) Reversal learning curves of the honeybees treated with a control solution (green circle; n = 21) or with a high sublethal dose of Cantus® Gold (blue square; n = 19; 100 µg/l), Mospilan® (orange triangle; n = 23; 2000 µg/l) or the mixture of both (purple rhombus; n = 19). The proportion of bees showing a conditioned proboscis extension response (PER) is shown for each group. The proportion of responses to the new CS+ increased slowly with progressive trial (solid lines), while the responses to the former CS+ decreased (dotted lines) (proportion of responses to CS+ after high treatment: control: 13 %, Cantus® Gold: 21 %, Mospilan®: 24 %, mix: 18 %; proportion of responses to CS- after high treatment: control: 21 %, Cantus® Gold: 11 %, Mospilan®: 28 %, mix: 18 %) (GLM: effect of trial, CS+: $p_{high\ dose} < 0.001$, CS-: $p_{high\ dose} < 0.001$). There was no effect of treatment on the reversal learning performance (GLM: treatment effect on reversal learning, CS+: $p_{high\ dose} = 0.748$, CS-: $p_{high\ dose} = 0.484$).

effects. The combination of the SBI fungicide propiconazole and the neonicotinoid clothianidin led to synergistic effects on mortality in *A. mellifera* and different wild bee species (Sgolastra et al., 2017). The SBI fungicides triflumizole and propiconazole significantly increased the toxicity on honeybees when applied in combination with the neonicotinoids thiacloprid or acetamiprid (Iwasa et al., 2004; Manning et al., 2017). The toxicity on honeybees was also synergistically increased when, among others, the SBI fungicide tetraconazole and the neonicotinoid imidacloprid were applied in combination (Zhu et al., 2017). The SBI fungicide difenoconazole and the neonicotinoid imidacloprid

also reduced the survival rate of honeybees significantly (Almasri et al., 2020). Small synergistic effects were observed when SBI fungicides (myclobutanil, propiconazole, flusilazole, tebuconazole) were applied together with neonicotinoids (thiamethoxam, clothianidin, imidacloprid, thiacloprid) via oral or contact exposure to honeybees (Thompson et al., 2014). The combined application of different SBI fungicides with the pyrethroid lamda-cyhalothrin to the thorax of honeybees increased the toxicity as part of a synergistic effect (Pilling and Jepson, 1993).

Synergistic effects have also been discovered in other bee species, wild bees and other beneficial insects. The combined action of the SBI

fungicide propiconazole and the neonicotinoid acetamiprid on the Asian honeybee (*Apis cerana cerana*) led to a synergistic effect on survival (Han et al., 2019) and the SBI fungicide tebuconazole led to an increased mortality in the parasitoid wasp *A. abdominalis* when applied in combination with the neonicotinoid thiacloprid (Willow et al., 2019).

The SBI fungicide difenoconazole interacted synergistically with the pyrethroid bifenthrin on *B. impatiens*, while the SBI fungicide fenhexamid showed no synergistic effect in combination with the same pyrethroid (Iverson et al., 2019). The toxicity for *B. terrestris* was synergistically increased when the SBI fungicide imizalil was applied together with the pyrethroid cypermethrin or the neonicotinoid thiamethoxam. The combination of imizalil and imidacloprid did not show a synergistic effect (Raimets et al., 2018). These two results suggest that the occurrence of a synergistic effect cannot be made dependent on either the fungicide selected or the insecticide used but is always due to the exact combination of the two. Even PPPs from the same group can lead to different effects depending on the mixture partner with which they are combined (Iverson et al., 2019; Raimets et al., 2018).

While most of these studies used combinations of SBI fungicides and neonicotinoids or pyrethroids, we have tested the effect of the mixture of a non-SBI fungicide (Cantus® Gold) and a neonicotinoid (Mospilan®) and could also find a synergistic effect on mortality.

The occurrence of such synergistic effects might be explained by the disruption of the detoxification process (Cedergreen, 2014). The detoxification mechanism can be divided into three phases. First, the toxic substances are modified by enzymes so that they can no longer interact with lipophilic structures. P450 enzymes are crucially involved in this process. Then the substances are conjugated to increase their solubility. Finally, the substances are transported out of the cell (Berenbaum and Johnson, 2015). It has been shown that SBI fungicides can inhibit the detoxification enzyme P450 in bees (Johnson et al., 2006; Schmuck et al., 2003; Wilkinson et al., 1974). Such modifications of P450s have also been shown in other animals (e.g. Brattsten et al., 1994, Ronis et al., 1994). The described mechanism may promote synergistic effects as the detoxification process that is responsible for the degradation of toxins like insecticides is impaired, leading to an increase in adverse effects in bees (Gong and Diao, 2017; Iwasa et al., 2004; Schuhmann et al., 2022). As the fungicides boscalid and dimoxystrobin contained in Cantus® Gold are non-SBI fungicides, they have a different mode of action (Fungicide Resistance Action Commitee, 2021). However, synergistic effects on mortality were also shown with this fungicide in combination with the neonicotinoid Mospilan®. In addition to the modification of P450 enzymes, there are other possibilities that can explain synergistic effects. Other metabolic enzymes can be influenced, and modifications of excretion or uptake rate and transport to the target site are possible reasons (Cedergreen, 2014).

As our results show that synergistic effects of non-SBI fungicide-insecticide mixtures cannot be excluded, further investigations are needed for other combinations.

In addition to mortality, synergistic effects were also examined on the responsiveness to sugar water and the learning performance. The bees tested in the learning experiments had survived the one-week feeding period and were thus already more resilient than those bees that died during the treatment. Surprisingly, they did not show any behavioral abnormalities. While we did not find any sublethal effects on learning with our substances, synergistic effects of an herbicide-insecticide mixture on learning behavior of honeybees have already been demonstrated. The application of the mixture led to a poorer learning performance compared to that of the single application of the PPPs and the control bees (Mengoni Goñalons and Farina, 2018).

Other properties have also been influenced by synergistic effects. Cardiotoxicity of honeybees was increased several times by the combination of the SBI fungicide prochloraz and the pyrethroid deltamethrin (Papaefthimiou and Theophilidis, 2001). Thermoregulation was synergistically affected by the combined application of prochloraz or difenoconazole and deltamethrin, as a joint hypothermia was observed

(Vandame and Belzunces, 1998). Honeybee larval mortality was synergistically affected by the non-SBI fungicide chlorothalonil and the pyrethroid fluvalinate (Zhu et al., 2014). The combination of propiconazole and the neonicotinoid clothianidin resulted in synergistic effects on ovary maturation and longevity in *O. bicornis* (Sgolastra et al., 2018). Deficits in motor abilities in the parasitoid wasp *A. abdominalis* were observed after a treatment with the SBI fungicide tebuconazole in combination with the neonicotinoid thiacloprid (Willow et al., 2019).

4.2. Effects of fungicides

Due to many fungal diseases, fungicides are among the most widely used PPPs worldwide. Their use leads to residues in pollen and nectar as well as in bee bread and other products (Schuhmann et al., 2022). Even though fungicides were not developed to control insect pests, their use can have negative effects on honeybees.

The fungicide Pristine® (a.i. boscalid and pyraclostrobin) negatively affected the cognition of honeybees as chronically treated bees showed a reduced learning performance (DesJardins et al., 2021). Pristine® also led to an earlier onset of foraging activity in honeybees, which may be associated with a shorter lifespan. In addition, the size of the hive was reduced by the fungicide (Fisher et al., 2021). The larval development of honeybees was impaired by the fungicides Captan® (a.i. captan), Rovral® (a.i. iprodion) and Ziram® (a.i. ziram), since the animals did not undergo a complete development to adult bees (Mussen et al., 2004). Boscalid and pyraclostrobin led to reduced ATP concentrations in honeybees treated with contaminated pollen (DeGrandi-Hoffman et al., 2015) and the fungicide difenoconazole negatively affected the survival of honeybees (Almasri et al., 2020).

Furthermore, wild bees and other non-target organisms can be affected by fungicides. It was shown that the fungicide Pristine® can lead to a disruption of nest recognition in *O. lignaria* and *M. rotundata* (Artz and Pitts-Singer, 2015) and the fungicide Captan® (a.i. captan) reduced the survival rate of *O. lignaria* (Ladurner et al., 2005). In bumblebees (*B. terrestris*) the fungicides diniconazole, fludioxonil, dithianon and difenoconazole interacted with the mitochondrial respiration leading to an uncoupling or inhibition (Syromyatnikov et al., 2017). The fungicide azoxystrobin altered the gut microbiome of the soil animal *Enchytraeus crypticus*. It also affected the mortality and reproduction of the animals (Zhang et al., 2019). The gut microbiome of mice was altered by the fungicide penconazole (Meng et al., 2019). In *Danio rerio* the sexual development was affected by the fungicide prochloraz (Kinnberg et al., 2007).

Our experiments showed no negative impact on honeybees when the fungicide Cantus® Gold was fed, but negative effects cannot be fully excluded. Due to the abundance of adverse fungicide effects but the imbalance between the number of insecticide and fungicide studies that has prevailed in recent years (Zubrod et al., 2019), more studies looking at the effects of fungicides on non-target organisms are desirable. In particular, factors such as feeding duration and concentration seem to be important factors determining the toxicity of the PPPs to bees. While some effects only become visible when the animals are fed with the corresponding solutions for two to three weeks due to cumulative potential, other effects appear earlier (Simon-Delso et al., 2018). However, factors such as feeding duration differ depending on the experimental design and the research question.

4.3. Effects of insecticides

In our study, the single application of the neonicotinoid Mospilan® had no effect on the mortality, the responses to sucrose and the learning performance. Studies investigating the effect of acetamiprid on the toxicity of the Eastern honeybee *A. cerana cerana* showed that the mortality rate of newly emerged bees is affected while adult bees show no effect, directly supporting our findings (Han et al., 2019). When testing the action of acetamiprid on honeybee sensory responses

minimal effects on water responsiveness were shown following oral application in another study (Aliouane et al., 2009). Lifespan and homing ability of honeybees were affected by acetamiprid treatment. Furthermore, foraging activity was disrupted, and fewer foraging flights were performed (Shi et al., 2020, 2019). Acetamiprid increased the sucrose sensitivity and had a negative effect on cognition (El Hassani et al., 2008; Thany et al., 2015). Other non-target organisms were also affected by acetamiprid. In *O. bicornis*, the energetic budget and the metabolism were modified by the neonicotinoid. In addition, the timing of cocoon formation was influenced during the development of the wild bee (Mokkapati et al., 2021a; b). Furthermore, acetamiprid can affect the nest growth and the development of *B. impatiens* microcolonies (Camp et al., 2020). In mammals like rats acetamiprid even led to adverse effects on the male reproductive system and the female immune system (Arıcan et al., 2020; Mondal et al., 2009).

Other neonicotinoids like thiamethoxam and imidacloprid led to a reduced sucrose responsiveness in honeybees (Jiang et al., 2018; Mengoni Goñalons and Farina, 2018). Clear effects on learning behavior were found when testing the neonicotinoids imidacloprid and thiamethoxam or an insecticide with the same mode of action (e.g., flupyradifurone). In these studies, learning performance was impaired and memory functions decreased (Hesselbach and Scheiner, 2018; Jiang et al., 2018; Mengoni Goñalons and Farina, 2018). In *O. cornuta*, fertility was negatively affected after a thiamethoxam treatment and navigational skills were impaired after treatment with clothianidin (Jin et al., 2015; Strobl et al., 2021). Furthermore, the sensitivity of *O. bicornis* and *B. terrestris* was impaired by clothianidin (Straub et al., 2021). Memory performance was impaired in male rats after clothianidin treatment (Kubo et al., 2022).

Neonicotinoids target the nicotinic acetylcholine receptors (nAChRs[11]) in the brain of insects and act as agonists. The transmission of nerve impulses is impaired via this pathway, since the blocking of the nAChRs results in a continuous Na^+-influx and K^+-efflux (Casida, 2018). The expression of nAChRs was detected in brain regions that are responsible for gustatory and olfactory stimulus processing (Dupuis et al., 2012). Therefore, these regions are potential target areas and sublethal effects of neonicotinoids are likely to occur there.

As described for the fungicides, the concentration of the PPPs and the treatment duration might explain the discrepancy between different studies. The different groups of neonicotinoids which differ from each other in their chemical structure could also lead to different outcomes. While acetamiprid belongs to the cyano-substituted neonicotinoids, the substances imidacloprid, thiamethoxam and clothianidin display the group of nitro-substituted neonicotinoids. Due to the varying degree of polarity and reactivity, they can lead to different effects. This is also true for mixtures of neonicotinoids with fungicides (Schuhmann et al., 2022).

4.4. Relevance for the environment

The PPPs Cantus® Gold and Mospilan® can both be applied to rapeseed fields (BASF SE, 2021; FMC Agricultural Solutions, 2021). The sublethal concentrations used for the behavioral tests were both well below the LD50 value (see Supplementary Information) and are in line with realistic residue levels of the respective active ingredients (see 2.3).

Our concentrations are significantly lower compared to other studies investigating the same active ingredients or PPPs (e.g. Shi et al., 2019). While these studies found effects of PPPs, no effects on behavior could be detected in our experiments. Since our concentrations were based on current residue values, it may well be that our residual values already reflect a reduced use of these PPPs in agriculture, at least in Germany. Under some conditions, the real intake amounts of the bees might, nevertheless, be significantly higher, since the PPPs might already degrade by the time the residue values are determined (Fantke and Juraske, 2013). However, the investigation of the tenfold higher dose also showed no effects on behavior. But as the concentrations used were below the recommended field doses (BASF SE, 2021; FMC Agricultural

Solutions, 2021) and also other factors in the field may always have an influence (Sánchez-Bayo and Wyckhuys, 2019), research into even higher concentrations continues to be necessary, particularly since the honeybee seems to be quite resilient with respect to PPPs. Furthermore, there are strong fluctuations in the residue values (see e.g. Rosenkranz et al., 2020) and the expected daily intake of a honeybee can also vary (Rortais et al., 2005). In recent years, beekeepers have repeatedly detected symptoms of poisoning in honeybee hives. This could be explained by various reasons. Residues of PPPs that are already restricted can be found in soil due to illegal application or emergency registrations, even if this happens rarely and in rather low concentrations (Rosenkranz et al., 2019). The number of active ingredients detected in bee products varies. In bee bread, around 20 substances have been found in individual samples (Rosenkranz et al., 2019). A large number of PPPs has also been detected in pollen, nectar and wax. Thus, bees may be exposed to a large amount of chemicals in various concentrations (Bokšová et al., 2021; Chauzat and Faucon, 2007; Daniele et al., 2018; El-Nahhal, 2020; Rosenkranz et al., 2019; Tong et al., 2018). Additionally, beebread consists of a variety of pollen species (Urcan et al., 2017) and based on this assumption, calculations for residue levels are made (Rosenkranz et al., 2019). However, honeybee hives in mixed landscapes contain a lower amount of certain pollen species compared to hives in fruit plantations or oilseed rape fields. Therefore, the amount and diversity of different PPPs strongly depends on the location of the hive (Rortais et al., 2017).

The lack of effects in our study can therefore be explained by the lower concentrations compared to other studies on the effects on honeybee behavior, in addition to methodological differences. Further, the exclusion of external factors from the environment in our laboratory study may have affected the outcome of our experiments. However, our approach provides a good basis for investigating the side effects of PPPs currently used in the EU and worldwide under field realistic conditions.

In the field, tank mixtures of fungicides and insecticides or sequential sprayings can result in PPP cocktails. In addition, the combination of seed and spray treatment can lead to mixtures. In seed treatments, different systemic agents that coat the seeds are transported into the plants and lead to residues in nectar and pollen. When other PPPs are sprayed to these plants, the pollinators are confronted with the combination (Thompson et al., 2014). Even if attention is paid that no harmful mixtures are applied in agriculture, such mixed PPP intakes can still occur due to the foraging behavior of the bees, because honeybees forage on different crops and over long distances (Steffan-Dewenter et al., 2002).

While some PPP mixtures containing neonicotinoids are already banned, e.g. in the European Union, parts of Canada or the United States, due to negative effects on non-target organisms, they can still be used in other countries (European Commission, 2022; La Ville de Montréal, 2015; New York State, 2023). PPPs containing neonicotinoids banned in the European Union, such as clothianidin, imidacloprid, thiacloprid or thiamethoxam can still be applied e.g. in Brazil and cause harmful effects on beneficial insects there (Agência Nacional de Vigilância Sanitária, 2022). Testing of higher concentrations, but also testing of PPPs and mixtures already banned in some parts of the world, is therefore still necessary.

4.5. Effects on wild bees

Moreover, besides honeybees, wild bees are particularly confronted with PPPs and their mixtures. Results from studies with honeybees can usually not be directly transferred to wild bees, since they can differ from honeybees in many aspects (Drossart and Gérard, 2020; Wood et al., 2020). First of all, there is a large diversity of wild bee species. The various species not only differ in their lifestyle, which ranges from eusocial to solitary, but also exhibit morphological differences. In addition, there are clear differences in the foraging behavior of honeybees and wild bees. Many wild bees are specialized on certain pollen

species and have a smaller foraging radius than honeybees. Moreover, due to their social lifestyle and the large number of workers inside the hive, honeybees can often compensate for PPP effects. This is not possible for solitary wild bees. In addition, nurse bees can filter larval food before feeding honeybee larvae, so fewer PPPs are ingested by them. Particularly at the individual level, honeybees and wild bees also exhibit different sensitivities to PPPs (Drossart and Gérard, 2020; Thompson, 1999; Wood et al., 2020). This was already shown by investigating the effect of a neonicotinoid to three different bee species. The solitary wild bee *O. bicornis* was most sensitive to the insecticide clothianidin, followed by the social wild bee *B. terrestris* and finally the honeybee *A. mellifera*. Synergistic effects were also most evident in *O. bicornis* (Sgolastra et al., 2017). Wild bees, such as the bumblebee, also have different activity patterns compared to honeybees. They show higher activity in the morning and in the evening while honeybees display their activity peak in the midday. In addition, wild bees often fly in unfavorable weather conditions. These different flight times can also lead to higher exposure to PPPs for wild bees, as regulations for PPP application are usually based on honeybee activity (Thompson, 1999).

Wild bees are indispensable pollinators of crops but especially of wild plants. At the same time, they suffer from a significant decline. Thus, it is important to focus research on stressors and their effects on pollinators not only on the honeybee but also on wild bees (Drossart and Gérard, 2020).

5. Conclusion

A correct evaluation of food sources is crucial for the foraging success of a honeybee, as the nectar concentration determines whether a food source should be exploited (Seeley, 1995; von Frisch, 1965). Furthermore, individual responsiveness to sucrose has a direct impact on the learning behavior (Scheiner et al., 2005, 2001). A good learning performance also maximizes the foraging success, as honeybees have to learn important features of the food source like the shape, the color or the odor and have to be able to orient themselves to the sun compass and to landmarks. Sharing this information with other foragers increases the foraging efficiency (Menzel, 1993). Accordingly, adverse effects of PPPs on learning behavior or sucrose responsiveness would have far-reaching consequences for the honeybee colony.

Our behavioral experiments revealed no negative effects of a field-realistic treatment with the fungicide Cantus® Gold, the insecticide Mospilan® or the mixture of both PPPs on sucrose responsiveness or learning performance of honeybees. Nevertheless, the mortality rate was synergistically affected.

Although our finding suggests that the PPPs tested do not have a sublethal effect on the honeybees in the field realistic concentrations, this does not mean that higher concentrations would not have negative side effects. But our experiments were based on a field-realistic situation in Europe and should be interpreted in this background.

Additionally, the interaction of PPPs needs to be addressed further, because we could only test one combination of fungicides and a neonicotinoid, but other combinations of different PPPs are also frequent. In fact, future studies should try to investigate a matrix of different PPPs and their interaction using more concentrations to estimate the real threat of PPP mixtures on honeybee behavioral performance and cognition.

Since honeybees differ from wild bees in many aspects, no direct comparisons can be made with wild bees. However, as wild bees are suffering from a significant decline, further studies with different wild bee species are inevitable. Also other non-target organisms should be examined in more detail since negative effects of PPPs can occur (Drossart and Gérard, 2020; Thompson, 1999; Willow et al., 2019; Wood et al., 2020).

CRediT authorship contribution statement

Antonia Schuhmann: Data acquisition, Visualization, Writing – original draft, Writing – review & editing. **Ricarda Scheiner:** Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data for the mortality studies, the PER tests and the learning experiments is available on Mendeley Data (doi: 10.17632/4r839t38sb.1).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114850.

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