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

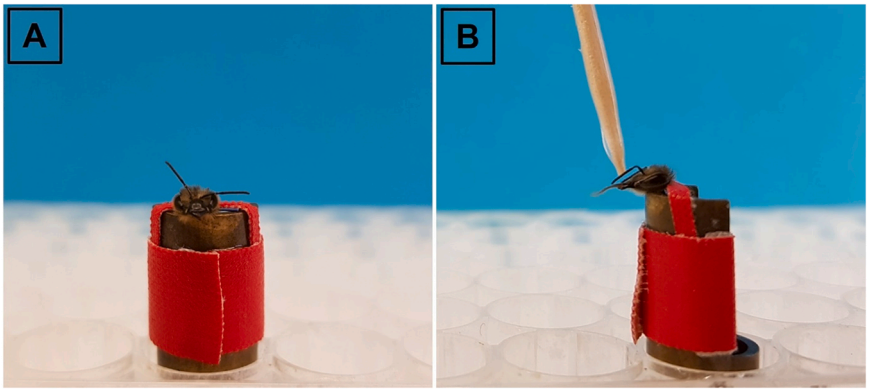

Fig. 1. Method for conditioning the bees in the laboratory. (A) The bee is harnessed in a holder and fixed with textile tape between head and thorax and over the abdomen. The antennae and mouthparts can still be moved freely. (B) The antennae of the bee are stimulated with a sucrose solution. The bee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

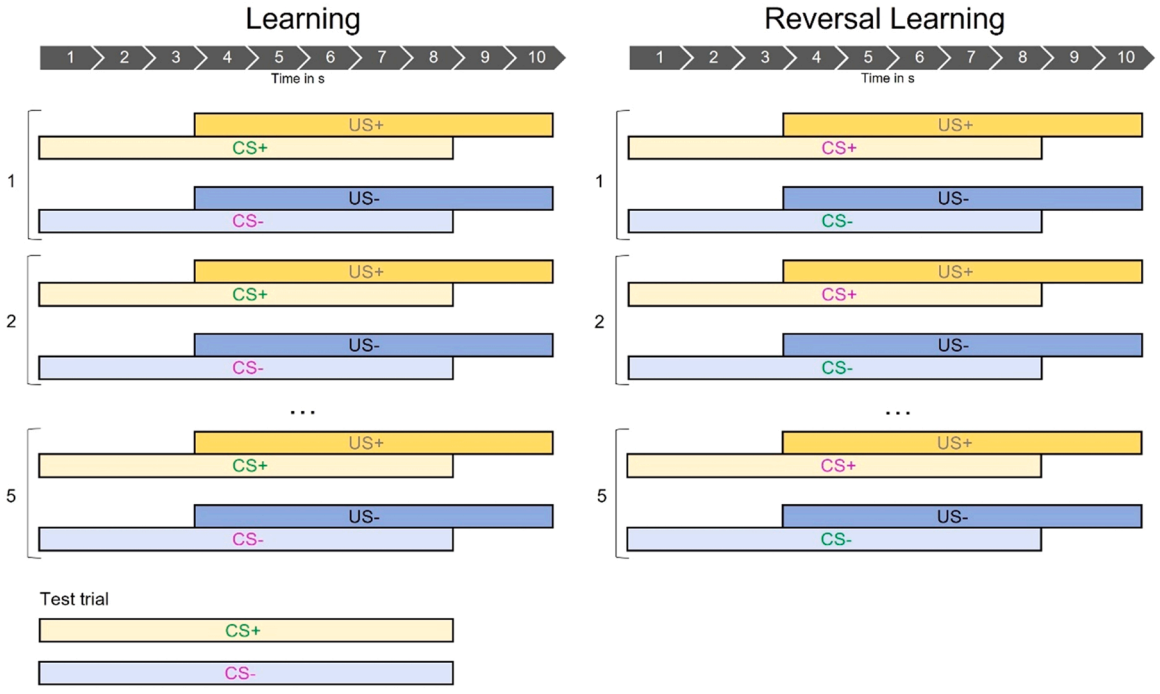

Fig. 2. Procedure of the learning (left) and reversal learning (right) paradigm. First, the odor (CS+/CS-) was presented for three seconds. During the following five seconds the odor was presented in combination with the reward or the punishment (US+/US-). When the bee showed a proboscis extension response (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.)