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

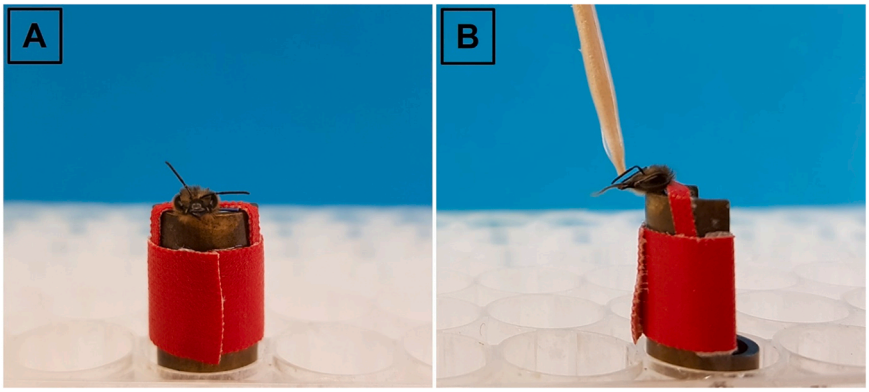

Fig. 1. Method for conditioning the bees in the laboratory. (A) The bee is harnessed in a holder and fixed with textile tape between head and thorax and over the abdomen. The antennae and mouthparts can still be moved freely. (B) The antennae of the bee are stimulated with a sucrose solution. The bee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