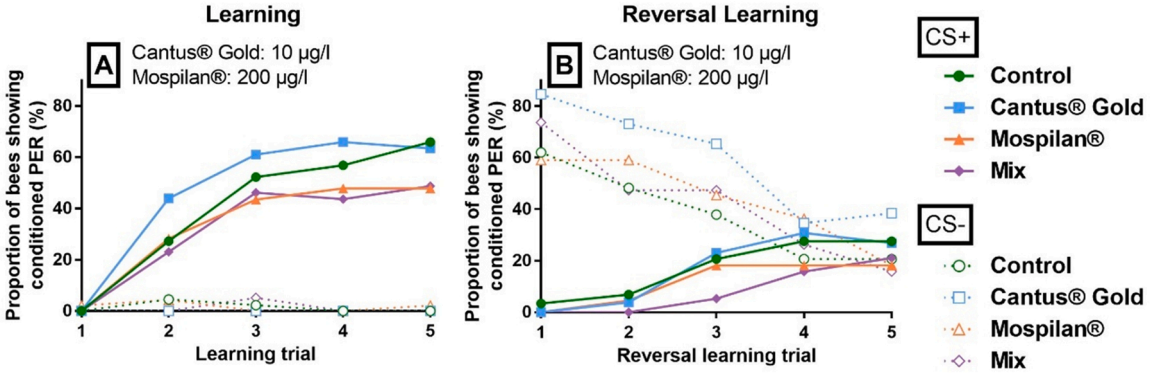

Fig. 5. Learning and reversal learning curves of bees treated with a low field realistic dose of PPPs. (A) Learning curves of honeybees treated with a control solution (green circle; n = 44) or with a low sublethal dose of Cantus® Gold (blue square; n = 41; 10 µg/l), Mospilan® (orange triangle; n = 46; 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-: $p_{low\ dose} = 0.197$).

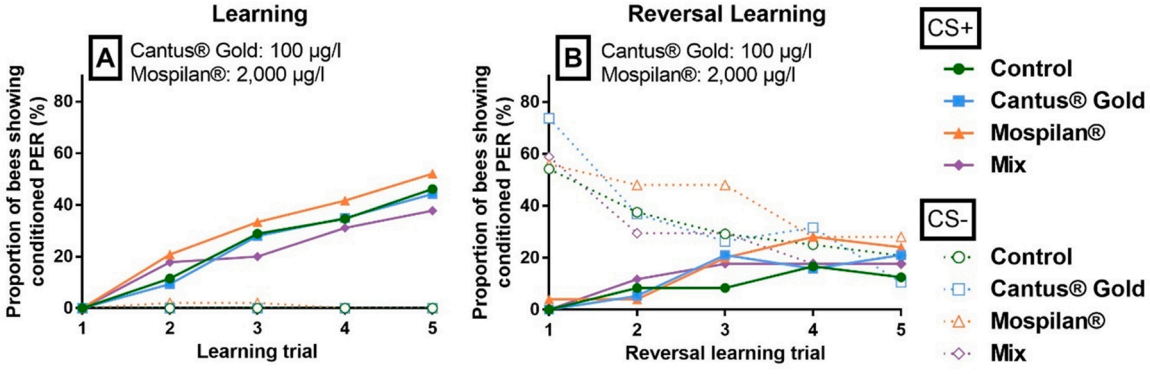

Fig. 6. Learning and reversal learning curves of bees treated with a high field realistic dose of PPPs. (A) Learning curves of the honeybees that were treated with a control solution (green circle; n = 52) or with a high sublethal dose of Cantus® Gold (blue square; n = 43; 100 µg/l), Mospilan® (orange 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