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