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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

Abbreviations: PPP, plant protection product; SBI, sterol biosynthesis inhibiting; a.i., active ingredient; LD50, lethal dose at which 50 % of experimental animals die; PER, proboscis extension response; CS+, conditioned stimulus (appetitive); US+, unconditioned stimulus (appetitive); CS-, conditioned stimulus (aversive); US-, unconditioned stimulus (aversive); GLM, generalized linear model; nAChR, nicotinic acetylcholine receptor.

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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