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 species and have a smaller foraging radius than honeybees. Moreover, due to their social lifestyle and the large number of workers inside the hive, honeybees can often compensate for PPP effects. This is not possible for solitary wild bees. In addition, nurse bees can filter larval food before feeding honeybee larvae, so fewer PPPs are ingested by them. Particularly at the individual level, honeybees and wild bees also exhibit different sensitivities to PPPs (Drossart and Gérard, 2020; Thompson, 1999; Wood et al., 2020). This was already shown by investigating the effect of a neonicotinoid to three different bee species. The solitary wild bee *O. bicornis* was most sensitive to the insecticide clothianidin, followed by the social wild bee *B. terrestris* and finally the honeybee *A. mellifera*. Synergistic effects were also most evident in *O. bicornis* (Sgolastra et al., 2017). Wild bees, such as the bumblebee, also have different activity patterns compared to honeybees. They show higher activity in the morning and in the evening while honeybees display their activity peak in the midday. In addition, wild bees often fly in unfavorable weather conditions. These different flight times can also lead to higher exposure to PPPs for wild bees, as regulations for PPP application are usually based on honeybee activity (Thompson, 1999).

Wild bees are indispensable pollinators of crops but especially of wild plants. At the same time, they suffer from a significant decline. Thus, it is important to focus research on stressors and their effects on pollinators not only on the honeybee but also on wild bees (Drossart and Gérard, 2020).

5. Conclusion

A correct evaluation of food sources is crucial for the foraging success of a honeybee, as the nectar concentration determines whether a food source should be exploited (Seeley, 1995; von Frisch, 1965). Furthermore, individual responsiveness to sucrose has a direct impact on the learning behavior (Scheiner et al., 2005, 2001). A good learning performance also maximizes the foraging success, as honeybees have to learn important features of the food source like the shape, the color or the odor and have to be able to orient themselves to the sun compass and to landmarks. Sharing this information with other foragers increases the foraging efficiency (Menzel, 1993). Accordingly, adverse effects of PPPs on learning behavior or sucrose responsiveness would have far-reaching consequences for the honeybee colony.

Our behavioral experiments revealed no negative effects of a field-realistic treatment with the fungicide Cantus® Gold, the insecticide Mospilan® or the mixture of both PPPs on sucrose responsiveness or learning performance of honeybees. Nevertheless, the mortality rate was synergistically affected.

Although our finding suggests that the PPPs tested do not have a sublethal effect on the honeybees in the field realistic concentrations, this does not mean that higher concentrations would not have negative side effects. But our experiments were based on a field-realistic situation in Europe and should be interpreted in this background.

Additionally, the interaction of PPPs needs to be addressed further, because we could only test one combination of fungicides and a neonicotinoid, but other combinations of different PPPs are also frequent. In fact, future studies should try to investigate a matrix of different PPPs and their interaction using more concentrations to estimate the real threat of PPP mixtures on honeybee behavioral performance and cognition.

Since honeybees differ from wild bees in many aspects, no direct comparisons can be made with wild bees. However, as wild bees are suffering from a significant decline, further studies with different wild bee species are inevitable. Also other non-target organisms should be examined in more detail since negative effects of PPPs can occur (Drossart and Gérard, 2020; Thompson, 1999; Willow et al., 2019; Wood et al., 2020).

Ethical approval

Our protocols comply with standard welfare practice in our field. The experiment involved bees from an apiary dedicated to research.

Funding

This work was supported by a grant from the Bavarian State Ministry of the Environment and Consumer Protection (TGC01GCU-75253) to RS in the research network BayÖkotox (TP 2 Honig- und Wildbienen unter Stress) and by a grant of the German Federal Environmental Foundation to AS. This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

CRediT authorship contribution statement

Antonia Schuhmann: Data acquisition, Visualization, Writing – original draft, Writing – review & editing. **Ricarda Scheiner:** Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data for the mortality studies, the PER tests and the learning experiments is available on Mendeley Data (doi: 10.17632/4r839t38sb.1).

Acknowledgements

We would like to thank Anna Paulina Schmid, Sarah Manzer and Valerie Finke for their methodological support. We thank Dirk Ahrens for beekeeping. We would like to thank the Bavarian State Ministry of the Environment and Consumer Protection (research network BayÖkotox, TP 2 Honig- und Wildbienen unter Stress) and the German Federal Environmental Foundation for funding resources. This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114850.

References

Agência Nacional de Vigilância Sanitária, 2022. Monografias de agrotóxicos - Monografias autorizadas. https://www.gov.br/anvisa/pt-br/setorregulado/regularizacao/agrotoxicos/monografias/monografias-autorizadas-por-letra. (Accessed 11 February 2023).

Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M., Gauthier, M., 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environ. Toxicol. Chem. 28, 113–122. https://doi.org/10.1897/08-110.1.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.L., Belzunces, L.P., 2020. Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees. Ecotoxicol. Environ. Saf. 203. https://doi.org/10.1016/j.ecoenv.2020.111013.

Arıcan, E.Y., Gökçeoğlu Kayalı, D., Ulus Karaca, B., Boran, T., Öztürk, N., Okyar, A., Ercan, F., Özhan, G., 2020. Reproductive effects of subchronic exposure to acetamiprid in male rats. Sci. Rep. 10, 8985. https://doi.org/10.1038/s41598-020-65887-0.

Artz, D.R., Pitts-Singer, T.L., 2015. Effects of fungicide and adjuvant sprays on nesting behavior in two managed solitary bees, *Osmia lignaria* and *Megachile rotundata*. PLoS One 10. https://doi.org/10.1371/journal.pone.0135688.