Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze e Tecnologie Agrarie, Ambientali e Alimentari

Ciclo XXXV

Settore Concorsuale: 07/D1

Settore Scientifico Disciplinare: AGR/11

Advancing of risk assessment of pesticides on insect pollinators:

Beyond honey bees (*Apis mellifera* L.)

Presentata da: Cátia Ariana Henriques Martins

Coordinatore Dottorato

Prof. Massimilliano Petracci

Supervisore

Prof. Fabio Sgolastra

Co-supervisore

Prof. Giovanni Burgio

Esame finale anno 2023

“Há uma primavera em cada vida:
é preciso cantá-la assim florida”

- *Florbela Espanca*

Abstract

Pesticides are to date the most common strategy used by farmers to control pests and pathogens in agriculture. Because of detrimental effects, both on environment and human health related to agrochemical use, before approval pesticides are first tested within a tiered risk assessment scheme to assure that they do not pose an unacceptable risk to non-target organisms. However, this scheme is currently under revision by the European Food Safety Authority (EFSA), because it failed to account for several effects, particular on the pollinator communities, that emerged after the registration of neonicotinoids.

The western honey bee, *Apis mellifera* L., is currently the model specie for pesticide risk assessment on pollinators with the assumption that the worst-case scenarios for this species are sufficiently conservative to protect other insect pollinators. However, recent studies have showed that wild species may be more sensitive to plant protection products, due to differences in biology and life cycles. Therefore, there is the need to extend the risk assessment within a more ecological approach, in order to ensure that there are no irreversible effects on non-target organisms and in the environment.

Additionally, multi-pesticide exposure is a common scenario for pollinators present in agricultural environments, by simultaneous pesticide treatments in tank mixtures or by sequential applications in the same field. Thus, while foraging for flower sources, pollinators may be exposed to several chemicals through different routes (e.g. ingestion or contact), with the contaminated nectar/pollen or surfaces (i.e. plant materials, water and soil).

My dissertation aims to expand the risk assessment to other insect pollinators (including wild and managed pollinators), in order to cover some of the gaps of the current schemes. In this thesis, it is presented three experiments that cover the early stages of a solitary bee (chapter 1), the development of molecular tools for early detection of sub-lethal effects (chapter 2) and the development of protocols to access lethal and sub-lethal effects on other pollinator taxa (Diptera; chapter 3).

In the chapter 1 it was conducted an experiment to determine the effects of two pesticides (insecticide imidacloprid and fungicide tebuconazole) on *Osmia bicornis* L. larvae in order to assess whether and how pesticides can affect the larval development. Our experiment was based on recent findings that nectar-pollen provisions of solitary bees host an abundant microbial community (Steffan et al., 2019), essential for the correct developing of bee larvae (Dharampal et al. 2019). Our aim was to access the interactions pesticide-bee, in order to answer the following questions: (a) do pesticides affect the provision's microflora and therefore bee development and adult longevity?; (b) the pesticides could affect larval development directly, for example by causing early egg or larval mortality; and (c) do the mixture potentiate these effects?. Our results, show an effect of the imidacloprid on the larval development. However, we did not observe a direct effect of the fungicide, neither alone or in combination with imidacloprid.

In chapter 2, we tested the same pesticides (imidacloprid and tebuconazole) under laboratory conditions, with females of the solitary bee, *O. bicornis*, however through other exposure scenario (ingestion of contaminated syrup solution). For each treatment, we aimed to assess, at two different time points, the following endpoints: syrup consumption, survival, and four biomarkers (acetylcholinesterase -AchE-, carboxylesterase -CaE-, glutathione S-transferase -GST-, and alkaline phosphatase -ALP-), later used in the integrated biological response index (IBRv2), to assess the full toxicological status. The neonicotinoid significantly reduced syrup consumption, survival, and the neurological activity of the enzymes. The co-exposure of neonicotinoid-fungicide

did not increase the toxicity at the tested concentrations. Overall, AChE proved to be an efficient biomarker for the detection of early effects for both pesticides.

In chapter 3 the aim was to start cover risk assessment gaps by studying the sensitivity of adult females of three dipteran species, two hoverflies, *Sphaerophoria rueppellii* and *Eristalinus aeneus* (Syrphidae), and a parasitoid fly *Exorista larvarum* (Tachinidae), to a neonicotinoid insecticide (imidacloprid) through an acute contact exposure. The species sensitivity distribution (SSD) approach was used to compare the calculated median lethal doses (LD_{50}s) of the three dipteran species with those of other pollinator species obtained from literature (bees and beetles). The testing protocol for dipterans developed under this study allowed to obtain information on an ecological endpoint, the fecundity, which is impracticable to obtain for bees under laboratory conditions. This information was used to assess, for each species, the potential trade-off between fecundity and survival through the sub-lethal sensitivity index (SSI). *S. rueppelli* resulted to be the most sensitive species, both for lethal and sub-lethal effects, even when the doses were normalized with body weight. However, the highest value of SSI was observed in *E. larvarum*. All three species revealed to be less sensitive to imidacloprid than the surrogate species, honey bee.

The results across the three chapters show that the methods developed for the different studied species are valid and may be integrated in the future environmental risk assessment of pesticides.

Acknowledgements

First of all, I would like to thank my supervisor Prof. Fabio Sgolastra for his constructive advices and the availability for brainstorming during these 3 years. I would also like to thank Prof. Claudio Ratti, Prof. Giovanni Burgio, Prof. Maria Luisa Dindo and Prof. Jordi Bosch, for their scientific contributions and knowledge that helped me so many times. The present work would have never been realised without the collaboration of my colleagues Celeste Azpiazu, Martina Parrilli, Santolo Francanti and Serena Magagnoli, my team mates that helped me through the most challenging days, and many times lift my mood up to keep me going. Thank you, Alberto Lanzoni for your collaboration and providing me the aphids. Thanks to the team of Prof. Ratti, especially Chiara, Jin and Nicola that helped me so many times, through the long hours in the lab. A special thanks to the group of University of Siena, Prof. Ilaria Caliani, Prof. Silvia Casini, Prof. Antonella D'Agostino and Agata di Noi, for all your inputs and collaboration, that made possible a part of my PhD project. Thanks to all the grad students, for their contributions and will to learn more.

Thank you to Prof. Emilia Silva, Prof. Maria Cerejeira and Prof. Elisabeth Duarte, from University of Lisbon, who did not contribute to this work, but encouraged my desire to pursue a PhD (thank you for your enthusiasm and for believing in me).

Thank you to my family and all friends that even physically distant always gave me the strength to keep going, especially during the covid pandemic that kept us so far apart. Thank you for the long video calls, laughs and warm hugs after so many months without see each other. Thank you, Armin for fighting side-by-side with me this journey and for your unconditional support and love.

I am surrounded by wonderful people. Thank you!

Table of Contents

General introduction

The importance of pollinators

Pollination is the transfer of pollen grains from the anther (male sex organ) to the stigma (female sex organ) in flowering plants. Plants have evolved by developing mechanisms that prevent self-fertilization allowing the maintenance of genetic variability, thus relying on external biotic (e.g. animals) or abiotic (e.g. wind) factors to promote plant fertilization, seed production and dispersal, and reproduction (Frankie and Thorp, 2009). As for pollination performed by animals, insects are the most efficient and dominant pollination vectors while they visit the flowers for resources (Garibaldi et al., 2013; Rader et al., 2016). Entomophilous plants have established plant–insect mutualism networks providing, in exchanged for their pollination services, primary rewards that attract and keep insects returning to flowers. These rewards include nutrition (e.g. nectar and pollen), lipid secretions, resins, place to mate and materials for nesting (Frankie and Thorp, 2009).

Undoubtedly, pollination is a key ecosystem service for preservation of plant biodiversity and provision of crop pollination services worldwide, with approximately 75 % of crops relying on pollinators (Klein et al., 2007), and around 90 % of wild flowering plants depending at least to some extent on animal pollination (Potts et al., 2016). In terms of global food production, pollinator-dependent crops account for an estimated economic value of $235 billion-$577 billion, with a contribute of approximately 35 % on the global crop production volume (Lautenbach et al., 2012; Potts et al., 2016). However, the real value is to date still not fully defined. In addition, many of these crops also contribute to healthy human diets since many species encompass fruits, vegetables, seeds, nuts and oil crops, supplying important micronutrients, vitamins and minerals (Klein et al., 2018).

Crop yield and seed/fruit quality are strictly related with the abundance and diversity of pollinators, since different species vary in their functional traits, greater richness will consequently lead to foraging complementarity or even into synergies (Blüthgen and Klein, 2011; Brittain et al., 2013). Insect pollinator group comprises the four largest orders of insects: Coleoptera (beetles), Diptera (flies), Lepidoptera (butterflies and moths), and Hymenoptera (bees, ants, and wasps). Among them, the western honey bee (*Apis mellifera*) is the most widespread managed pollinator in the world, with an estimated of 81 million hives distributed globally, producing around 1.6 million tonnes of honey annually (Potts et al., 2016). However, apart from other few managed pollinators available commercially [Bees: *Bombus* spp., *Osmia* spp., *Megachile rotundata* F., *Nosmia melendri* Cockerell (Sgolastra et al., 2019) Flies: *Eristalinus aeneus* Scopoli, *Eristalis tenax* L., *Lucilia sericata* (Sánchez et al., 2022), *Eupeodes corollae* and *Sphaerophoria rueppellii* (Pekas et al., 2020)], majority of species are wild, with more than 20,000 – 30,000 described species only when considering bees (superfamily Apoidea; Ascher and Pickering, 2017). Both wild and managed pollinators play important roles, however a high diversity of wild pollinators is critical to pollination even when managed bees are present in high numbers (Garibaldi et al., 2013), and wild bees alone contribute substantially to crop production value up to $963 per crop ha^{-1} per species (Kleijn et al., 2015). In fact, several studies have shown that honey bees are not always the most effective pollinators on a per flower basis [e.g. almonds, blueberries, coffee, tomatoes, cherries (reviewed in Klein et al., 2007), pumpkin (Hoehn et al., 2008), apple (Mallinger and Gratton, 2015)]. Thus, the adoption of strategies in agricultural landscapes that promote biodiversity are extremely crucial (Garibaldi et al., 2014; Winfree, 2013). Moreover, increasing the diversity of pollinator species that may contribute to other ecological functions, such as pest

control, can increase ecosystem complexity and consequently its resilience against biotic and abiotic stressors, such as invasive species or climate change (Aizen et al., 2020; Burkle et al., 2017).

Such a wide group of insect pollinators presents very distinctive life history traits and strategies. Pollinators may have different food preferences, while some are considered as specialists, when they present constancy in the visits towards one plant species, others may be generalists, visiting many plant species. However, in a broader spatial scale, pollinators may switch from specialist to generalist as a respond to their surrounding environment and the availability of floral and nesting resources (Willcox et al., 2017). In fact, these complex mutualisms may be influenced by different factors, such as the spatial scale and life history features of the pollinator species, that will directly impact plant population dynamics, and consequently pollinator abundance (Cussans et al., 2010; Jha and Dick, 2010). Other aspects related with other ecological traits, such as differences in the level of sociality, nesting period and materials, and migratory status will ultimately influence their behaviour in the landscape and their capacity to respond to external environmental stressors (Aguirre-Gutiérrez et al., 2016; Schmolke et al., 2021).

Pollinator decline: trend, causes and consequences

During the past decades, global demand for pollination services has increase together with the pressure for high crop productivity, however, there is growing evidence that the populations of pollinators, both wild and managed, have been facing severe declines in diversity and abundance (Dicks et al., 2021; Koh et al., 2016; Potts et al., 2010). These declines were particularly noted in North America and Europe, initially by beekeepers describing annual colony losses of the managed honey bee, as twice as high from the historical registered before the 2000's (Aston, 2010; Gray et al., 2020; Kulhanek et al., 2017; Topolska et al., 2010). Although in other parts of the world, such as Africa, South America and Asia, the numbers of honey bee colonies have been actually increasing, between 1961 and 2007 (Hristov et al., 2020). Despite this, the demand for pollination services is increasing much more rapidly (>300%) worldwide (Aizen and Harder, 2009), leading to potential risk the food safety.

Moreover, there are growing evidences that declines in wild species may be even more pronounced than overall trends in managed species (Ollerton et al., 2014; Powney et al., 2019), however, to date wild pollinator populations and communities are still poorly documented. Among bees, the best documented are *Bombus* sp., from which several studies have been reported severe declines in the past years (Carvell et al., 2011; Goulson et al., 2008; Grixti et al., 2009; Williams and Osborne, 2009). Apart from them, information for other bee species is mostly lacking (Biesmeijer et al., 2006; Rasmont et al., 2005).

In an attempt to assess global pollinator decline, a recent study by Zattara and Aizen (2021), performed a long-term analysis based on worldwide records from the Global Biodiversity Information Facility (GBIF), with available data from 1900's to 2018. The authors found a noticeable decline starting near the end of the 20th century of Anthophila families (Figure I).

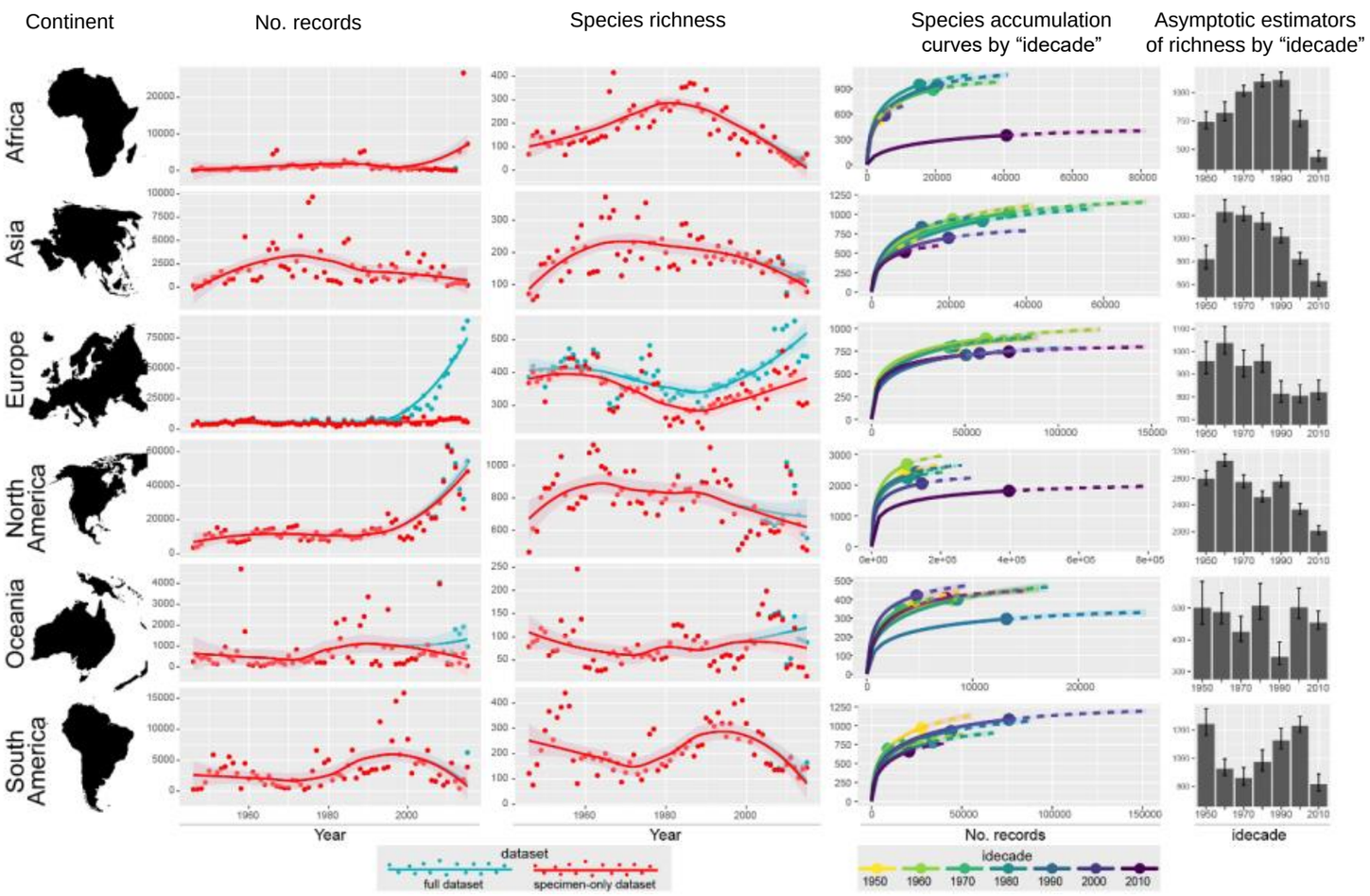


Figure I: Trends shown in GBIF records for each continent. The left two rows of plots show number of yearly bee records and species in GBIF (blue: full dataset; red: specimens-only dataset); the right two rows show Chao’s interpolation/extrapolation curves based on the specimens-only dataset grouped every ten years (idecades) for the period 1946-2015 and bar plots of the asymptotic estimates of richness by idecade for the same period (error bars mark upper and lower 95% confidence intervals). Source: Zattara and Aizen, 2021

Efforts have also been made for butterflies, through coordinated initiatives and international monitoring programs, especially at European level (European Butterfly Monitoring Scheme – eBMS[1], SAFEGUARD[1], SPRING[3], EU PoMS[4]). With the exception of butterflies, information for other groups of pollinators, such as flies or beetles, are fragmentary because of the lack of coordinated monitoring programmes. However, recent studies have unveiled that the trend of decline is also a reality for hoverflies (Barendregt et al., 2022; Biesmeijer et al., 2006; Hallmann et al., 2017, 2021; Powney et al., 2019). There are several functional traits and ecological differences between bees and hoverflies, however analysis revealed that specialists (diet and habitat) and species without migratory status tend to decline, whereas mobile generalists tend to thrive (Biesmeijer et al., 2006).

The observed trends have been mostly linked with changes in land management (that influence the abundance of floral resources and the availability of nesting places; Senapathi et al., 2017), climate change (Nicholson and Egan, 2020), pesticide use, spread of pests, pathogens and alien

1. https://butterfly-monitoring.net/
2. https://www.safeguard.biozentrum.uni-wuerzburg.de/Project/About.aspx
3. https://butterfly-monitoring.net/spring
4. https://joint-research-centre.ec.europa.eu/jrc-news/proposal-eu-pollinator-monitoring-scheme-eu-poms-2021-01-18_en

species (Brittain et al., 2010; Goulson et al., 2015; Vanbergen, 2013). All these stressors have long-term impacts in the pollinator populations, and interactions between the different sources of pressure are likely contributing to the observed declines. Therefore, for understanding the health status of pollinators it is necessary to acknowledge that such declines may be a response to the combined effects of malnutrition, diseases and pesticides (Vanbergen, 2013). In a recent study, López-Uribe and colleagues (López-Uribe et al., 2020) have proposed a holistic assessment of pollinator's health through the characterization of impacts at individual level: growth, survival, reproduction and host–microbial interactions; social immunity and nest microbiota; and population-level aspects, such as genetic diversity. Ultimately, the development of such methodologies would help to quantify the resilience of different pollinator species to the environmental conditions, and allow for the assessment of multiple stressors.

Consequently, pollinator declines have impacts on the food security and in the world agricultural economic output. Gallai et al. (2009) estimated the insect pollination economic value of the 100 most important crops, as a measure of the world's agricultural production. Considering only food production for human consumption, the losses would be 9.5 % in a scenario of total pollinator loss. The most affected crops would be stimulant crops, with vulnerability ratios of 39 %, followed by nuts (31 %), fruits (23.1 %), oilseed crops (16.3 %) and vegetables (12 %). In addition, a scenario of plant–pollinator networks collapse would also jeopardize wild plants, causing potential extinction of some species, in particular the ones that require more specialized pollination (Potts et al., 2010).

Pesticides and pollinators

The intensification of agricultural has relied closely on the increase use of agrochemicals. Schreinemachers and Tipraqsa (2012) showed that by 1 % increase in crop output per hectare 1.8 % of pesticide use per hectare would increase, however the growth in intensity of pesticide use reduces as the countries reach a higher level of economic development. In the past decades, pesticide use has received considerable attention being pointed as one of the main causes for pollinator declines, as studies have demonstrated widespread exposure and its adverse effects (Mitchell et al., 2017; Zioga et al., 2020). Pesticides can be uptake by the pollinators through direct consumption of nectar and/or pollen of contaminated flowering crops (Dively and Kamel, 2012; Heller et al., 2020; Stoner and Eitzer, 2012), or from wildflowers growing near agricultural areas (Botías et al., 2016; David et al., 2016). In addition, they can be exposed by contact with treated plant surfaces, or directly by the pesticides, when foraging during applications on field (spray or seed dressing treatments with systemic insecticides) (Thompson, 2012). In addition, pesticide residues in soil are an important route of exposure for many terrestrial invertebrates and ground nesting species (Gunstone et al., 2021; Main et al., 2020; Sgolastra et al., 2019). Other less explored route of exposure is through the feeding of honeydew excretions from hemipteran insects such as aphids, mealybugs, whiteflies, and psyllids that can also impair pollinators (in particular hoverflies; Calvo-Agudo et al., 2019).

The intensive use of systemic insecticides, such as neonicotinoids, has particularly raised growing concern among the scientific community, with studies showing the negative effects on honey bees and wild pollinators (Sánchez-Bayo et al., 2016; Woodcock et al., 2016). Neonicotinoids target the nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001), and due to their systemic nature and high persistence in the environment, pollinators are chronically exposed to residual amounts of these compounds. At first, these pesticide levels were considered safe but the impacts towards the exposed pollinators are well documented, as they can cause sub-lethal effects such

as the reduction of cognitive abilities, foraging performance, nesting success, colony development and ultimately survival (Decourtye and Devillers, 2010; Gill and Raine, 2014; Rundlöf et al., 2015; Tosi et al., 2017; Zhu et al., 2017). These findings led the European Commission to ban the outdoor use of the three most common neonicotinoids, imidacloprid, thiamethoxam and clothianidin, in 2018. Meanwhile, these neonicotinoids are still widely used in other parts of the world (Goulson, 2020), and in Europe novel pesticides or other less toxic neonicotinoids remain approved, although it is still expected that they will cause lethal or sub-lethal effects (Azpiazu et al., 2021; Capela et al., 2022).

Despite the well-established effects of neonicotinoids on non-target organisms, to date we still do not know the full extension of the effects to multi-compounds exposure, underestimating the complete risk of pesticides on pollinators. In fact, the interaction between several pesticides may result in additive or even synergistic toxic effects. Several studies have shown that some fungicides (in particular sterol biosynthesis inhibitors) can potentiate the toxicity of insecticides by reducing the detoxification capacity (Biddinger et al., 2013; Iverson et al., 2019; Iwasa et al., 2004; Raimets et al., 2018; Sgolastra et al., 2017). Fungicides are commonly applied during bloom due to the general concept that they are mostly harmless to insect pollinators, however even when pollinators are exposed to them alone, fungicides may have sub-lethal and lethal effects (Belsky and Joshi, 2020; Bernauer et al., 2015; Campbell et al., 2016; Zhu et al., 2014). In addition, fungicides may have an indirect impact by disrupting beneficial fungi (Yoder et al., 2013) and benefit pathogen prevalence (Degrandi-Hoffman et al., 2015; Krichilsky et al., 2021). Besides, fungicides may impact the microbes present in the pollen provisions (Yoder et al., 2011), that play an important role by altering their nutritional value (Steffan et al., 2019). Herbicides can also affect pollinators directly (Weidenmüller et al., 2022) or indirectly, by eliminate wild flowering plants that could serve as potential sources of food for insect pollinators (Bretagnolle and Gaba, 2015).

Pesticide regulations and the environmental risk assessment for insect pollinators in Europe

The European commission traditionally approved or authorized the use of pesticides based only on available survival data of honey bees. Now-a-days, recommendations take into consideration the detection of “unacceptable” acute or chronic effects (short and long-term assessment) on colony survival and development, taking into account effects on honey bee larvae and honey bee behaviour (Regulation (EC) No. 1107/2009). Therefore, pesticides have to undergo a risk assessment process to assure they do not pose an unacceptable risk to honey bees. Specific data requirement for their authorization and indication of the standard protocols to applied are set by Regulation (EC) No. 283/2013 and Regulation (EC) No. 284/2013. Risk assessment procedure follows a tiered approach, from cost-effective laboratory assays (first tier), based on acute exposure and LD_{50} calculation, to more environmentally-relevant semi-field and field tests (tiers 2 and 3), in order to evaluate their safety and efficacy. This scheme developed by the European Plant Protection Organization (EPPO) is currently under revision by the European Food Safety Authority (EFSA) because failed to account for several pesticide properties emerged after the registration of neonicotinoids (Sgolastra et al., 2020). In addition, current pesticide application guidelines provide less protection to wild pollinators, that have different physiologies, behaviours, and phonologies from the surrogate species (Sponsler et al., 2019; Uhl and Brühl, 2019).

Following a request from the European Commission in 2011, EFSA was asked to review the current environmental risk assessment (ERA) scheme for bees (EPPO, 2010) and deliver a scientific opinion on the science behind the development of a risk assessment of Plant Protection

Products on bees. In this scientific opinion published in 2012 (EFSA, 2012), EFSA highlighted several shortcomings in the EPPO schemes and the need to improve laboratory, semi-field and field tests. Specifically, EFSA recommended to: a) include other possible ways of exposure, such as dust generated during the sowing of coated seed and guttation drops seeped out by vegetation; b) detect sub-lethal effects due to chronic exposure to low doses of pesticides; c) improve the field studies in order to detect effects on bee mortality with high statistical power; d) include other bees besides honey bees. Some of these recommendations were integrated in the EFSA Bee Guidance Document (EFSA, 2013) published in 2013 but this guidance was never implemented or approved by the EU Member States and the EU Commission. To date, EFSA is revisioning the EFSA Bee Guidance Document whose publication is foreseen in 2023. Because the improvement of ERA is a continuous process, EFSA is already working to move forward with several initiatives. MUST-B is an EFSA project ('EU efforts towards a holistic and integrated risk assessment approach of multiple stressors in bees'), which aimed to move the current ERA for honey bees, based on single exposure/single stressor approach, to a more systems-based ERA (EFSA, 2021).

By 2030, EFSA aims to further advance the ERA methodology to better protect insect pollinators (including wild and managed pollinators), their diversity, ecological functions and the ecosystem services they provide, including pollination (IPol-ERA). The main innovation on this framework concerns the broadening of the environmental risk assessment to other insect pollinators, other than bees, and to assess the ecological consequences of pesticide effects (EFSA, 2022). This is also in line with the EU pollinator initiative which aims to protect pollinator diversity to guarantee the optimal pollination service for crops and wild plants.

Motivation and general research aim

The western honey bee, *Apis mellifera*, is considered the model specie for pesticide risk assessment on pollinators with the assumption that the worst-case scenarios for the honey bee are sufficiently conservative to protect other pollinator species. However, recent evidence has showed other species may be more sensitive to plant protection products, due to differences in biology and life cycles (Arena and Sgolastra, 2014). Therefore, there is the need to extend the risk assessment to other pollinator species and to develop standard protocols for the new test species. In addition, pollinators may be routinely exposed to several pesticides in the agricultural area (Tosi et al., 2018), however a recent systematic review has highlighted that the toxicity levels of binary pesticide combinations are only available for a very small fraction of possible binary combinations (<0.0005 %; Tosi et al., 2022), leaving uncovered many other pesticide interactions, that may even potentially present synergetic effects. Probably linked with the lack of standard toxicological protocols until a few years ago (OECD 237, OECD 239), ecotoxicological studies on honey bee larvae are also rare (Benuszak et al., 2017) and very rare for other bee species (Lehmann and Camp, 2021). As for studies performed on non-bee pollinators, the information is virtually negligible, and covering mainly hoverflies (Basley et al., 2018; Jansen et al., 2011; Moens et al., 2011).

The aim of this thesis is to cover some of these gaps and improve the current environmental risk assessment by assessing the impacts of two pesticides, alone and in combination, on larvae (**chapter 1**) and adults (**chapter 2**) of the solitary bee *Osmia bicornis* L. In the **chapter 3**, I developed and applied a new testing protocol to assess the acute contact exposure on three dipteran pollinator species.

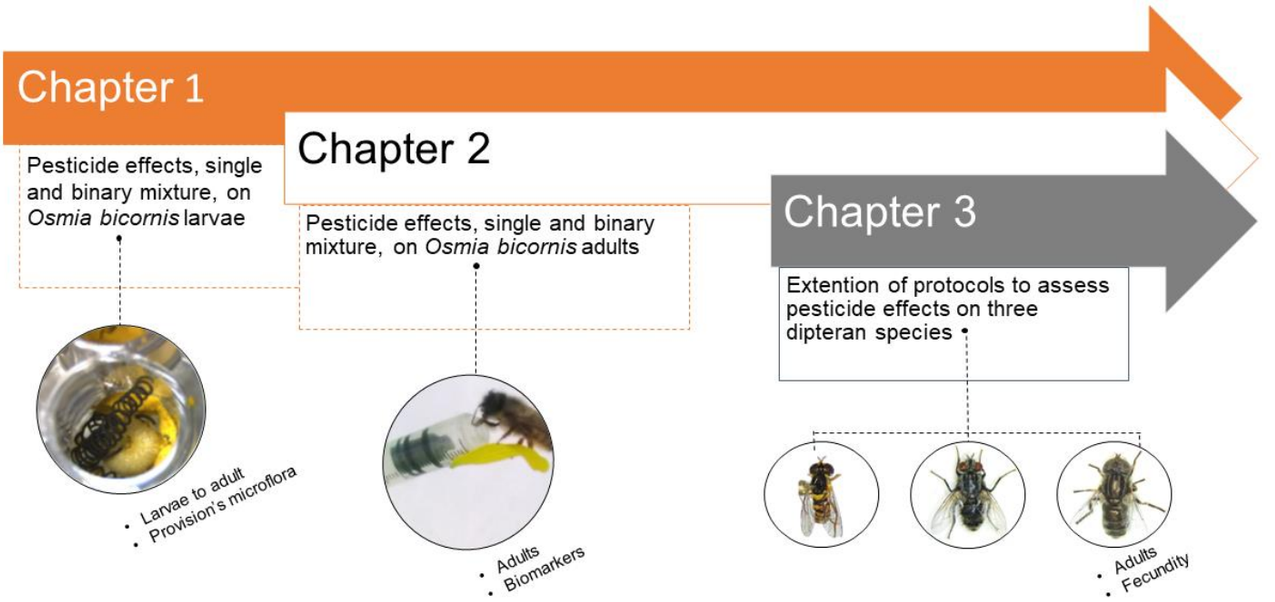


Figure II: Conceptual framework of the thesis. Each chapter focuses on the development of new methodologies for improvement of the risk assessment of pesticides on pollinators.

In all three chapters, I used, as case study, the neonicotinoid insecticide Confidor® whose active ingredient, imidacloprid, has been, from 1999 through at least 2018, the most widely used insecticide in the world, and, although currently banned in the European Union, it remains one of the top selling insecticides in the world (Sparks and Nauen, 2015). In the first two chapters, the experiments were conducted by evaluating the effects of imidacloprid and integrating the effects of the triazole fungicide Folicur®, whose active ingredient is tebuconazole.

References

Aguirre-Gutiérrez, J., Kissling, W. D., Carvalheiro, L. G., WallisDeVries, M. F., Franzén, M., and Biesmeijer, J. C. (2016). Functional traits help to explain half-century long shifts in pollinator distributions. *Scientific Reports*, *6*(1), 24451. https://doi.org/10.1038/srep24451

Aizen, M. A., Arbetman, M. P., Chacoff, N. P., Chalcoff, V. R., Feinsinger, P., Garibaldi, L. A., Harder, L. D., Morales, C. L., Sáez, A., and Vanbergen, A. J. (2020). Chapter Two - Invasive bees and their impact on agriculture. In D. A. Bohan and A. J. Vanbergen (Eds.), *Advances in Ecological Research* (Vol. 63, pp. 49–92). Academic Press. https://doi.org/https://doi.org/10.1016/bs.aecr.2020.08.001

Aizen, M. A., and Harder, L. D. (2009). The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Current Biology*, *19*(11), 915–918. https://doi.org/https://doi.org/10.1016/j.cub.2009.03.071

Arena, M., and Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology*, *23*(3), 324–334. https://doi.org/10.1007/s10646-014-1190-1

Ascher, J. S., and Pickering, J. (2017). Discover Life bee species and world checklist (Hymenoptera: Apoidea: Anthophila). *Availabe from: Http://Www.Discoverlife.Org./Mp/20q*.

Aston, D. (2010). Honey bee winter loss survey for England, 2007-8. *Journal of Apicultural Research*.

Azpiazu, C., Bosch, J., Bortolotti, L., Medrzycki, P., Teper, D., Molowny-Horas, R., and Sgolastra, F. (2021). Toxicity of the insecticide sulfoxaflor alone and in combination with the fungicide fluxapyroxad in three bee species. *Scientific Reports*, *11*(1), 6821. https://doi.org/10.1038/s41598-021-86036-1

Barendregt, A., Zeegers, T., van Steenis, W., and Jongejans, E. (2022). Forest hoverfly community collapse: Abundance and species richness drop over four decades. *Insect Conservation and Diversity*, *15*(5), 510–521. https://doi.org/https://doi.org/10.1111/icad.12577

Basley, K., Davenport, B., Vogiatzis, K., and Goulson, D. (2018). Effects of chronic exposure to thiamethoxam on larvae of the hoverfly Eristalis tenax (Diptera, Syrphidae). *PeerJ*, *2018*(1), 1–15. https://doi.org/10.7717/peerj.4258

Belsky, J., and Joshi, N. K. (2020). Effects of Fungicide and Herbicide Chemical Exposure on Apis and Non-Apis Bees in Agricultural Landscape. *Frontiers in Environmental Science*, *8*. https://doi.org/10.3389/fenvs.2020.00081

Benuszak, J., Laurent, M., and Chauzat, M.-P. (2017). The exposure of honey bees (Apis mellifera; Hymenoptera: Apidae) to pesticides: Room for improvement in research. *Science of The Total Environment*, *587–588*, 423–438. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.02.062

Bernauer, O. M., Gaines-Day, H. R., and Steffan, S. A. (2015). Colonies of Bumble Bees (Bombus impatiens) Produce Fewer Workers, Less Bee Biomass, and Have Smaller Mother Queens Following Fungicide Exposure. *Insects*, *6*(2), 478–488. https://doi.org/10.3390/insects6020478

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. A., Rajotte, E. G., Joshi, N. K., and Vaughn, M. (2013). Comparative Toxicities and Synergism of Apple Orchard Pesticides to Apis mellifera (L.) and Osmia cornifrons (Radoszkowski). *PLOS ONE*, *8*(9), e72587. https://doi.org/10.1371/journal.pone.0072587

Blüthgen, N., and Klein, A.-M. (2011). Functional complementarity and specialisation: The role of biodiversity in plant–pollinator interactions. *Basic and Applied Ecology*, *12*(4), 282–291. https://doi.org/https://doi.org/10.1016/j.baae.2010.11.001

Botías, C., David, A., Hill, E. M., and Goulson, D. (2016). Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. *Science of The Total Environment*, *566–567*, 269–278. https://doi.org/https://doi.org/10.1016/j.scitotenv.2016.05.065

Bretagnolle, V., and Gaba, S. (2015). Weeds for bees? A review. *Agronomy for Sustainable Development*, *35*(3), 891–909. https://doi.org/10.1007/s13593-015-0302-5

Brittain, C. A., Vighi, M., Bommarco, R., Settele, J., and Potts, S. G. (2010). Impacts of a pesticide on pollinator species richness at different spatial scales. *Basic and Applied Ecology*, *11*(2), 106–115. https://doi.org/10.1016/j.baae.2009.11.007

Brittain, C., Williams, N., Kremen, C., and Klein, A. M. (2013). Synergistic effects of non-Apis bees and honey bees for pollination services. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1754). https://doi.org/10.1098/rspb.2012.2767

Burkle, L. A., Delphia, C. M., and O'Neill, K. M. (2017). A dual role for farmlands: food security and pollinator conservation. *Journal of Ecology*, *105*(4), 890–899. https://doi.org/10.1111/1365-2745.12784

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A. P., Potts, S. G., Kleukers, R., Thomas, C. D., Settele, J., and Kunin, W. E. (2006). Parallel Declines in Pollinators and Insect-Pollinated Plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354. https://doi.org/10.1126/science.1127863

Calvo-Agudo, M., González-Cabrera, J., Picó, Y., Calatayud-Vernich, P., Urbaneja, A., Dicke, M., and Tena, A. (2019). Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. *Proceedings of the National Academy of Sciences*, *116*(34), 16817–16822. https://doi.org/10.1073/pnas.1904298116

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., and Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function in vitro but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/https://doi.org/10.1016/j.jinsphys.2015.12.003

Capela, N., Sarmento, A., Simões, S., Azevedo-Pereira, H. M. V. S., and Sousa, J. P. (2022). Sub-lethal doses of sulfoxaflor impair honey bee homing ability. *Science of The Total Environment*, *837*, 155710. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.155710

Carvell, C., Osborne, J. L., Bourke, A. F. G., Freeman, S. N., Pywell, R. F., and Heard, M. S. (2011). Bumble bee species' responses to a targeted conservation measure depend on landscape context and habitat quality. *Ecological Applications*, *21*(5), 1760–1771. https://doi.org/https://doi.org/10.1890/10-0677.1

Cussans, J., Goulson, D., Sanderson, R., Goffe, L., Darvill, B., and Osborne, J. L. (2010). Two Bee-Pollinated Plant Species Show Higher Seed Production when Grown in Gardens Compared to Arable Farmland. *PLOS ONE*, *5*(7), e11753-. https://doi.org/10.1371/journal.pone.0011753

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., and Goulson, D. (2016). Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International*, *88*, 169–178. https://doi.org/https://doi.org/10.1016/j.envint.2015.12.011

Decourtye, A., and Devillers, J. (2010). Ecotoxicity of neonicotinoid insecticides to bees. *Insect Nicotinic Acetylcholine Receptors*, 85–95. https://doi.org/10.1007/978-1-4419-6445-8_8

Degrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., and Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

Dicks, L. v, Breeze, T. D., Ngo, H. T., Senapathi, D., An, J., Aizen, M. A., Basu, P., Buchori, D., Galetto, L., Garibaldi, L. A., Gemmill-Herren, B., Howlett, B. G., Imperatriz-Fonseca, V. L., Johnson, S. D., Kovács-Hostyánszki, A., Kwon, Y. J., Lattorff, H. M. G., Lungharwo, T., Seymour, C. L., et al., Potts, S. G. (2021). A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nature Ecology & Evolution*, *5*(10), 1453–1461. https://doi.org/10.1038/s41559-021-01534-9

Dively, G. P., and Kamel, A. (2012). Insecticide Residues in Pollen and Nectar of a Cucurbit Crop and Their Potential Exposure to Pollinators. *Journal of Agricultural and Food Chemistry*, *60*(18), 4449–4456. https://doi.org/10.1021/jf205393x

EFSA (2012). Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (Apis mellifera, Bombus spp. and solitary bees). *EFSA Journal*, *10*(5), 2668. https://doi-org.ezproxy.unibo.it/10.2903/j.efsa.2012.2668

EFSA (2013). Guidance on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). *EFSA Journal*, *11*(7), 3295. https://doi.org/https://doi.org/10.2903/j.efsa.2013.3295

EFSA (2021). More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Bennekou, S. H., Koutsoumanis, K., Machera, K., Naegeli, H., Nielsen, S. S., Schlatter, J., Schrenk, D., Silano, V., Turck, D., Younes, M., Arnold, G., Dorne, J.-L., et al., Rortais, A. A systems-based approach to the environmental risk assessment of multiple stressors in honey bees. *EFSA Journal*, *19*(5), e06607. https://doi.org/https://doi.org/10.2903/j.efsa.2021.6607

EFSA (2022). Auteri, D., Devos, Y., Fabrega, J., Pagani, S., Rortais, A., de Seze, G., Heppner, C., and Hugas, M. Theme (concept) paper – Advancing the Environmental Risk Assessment of Chemicals to Better Protect Insect Pollinators (IPol-ERA). *EFSA Supporting Publications*, *19*(5), E200505E. https://doi.org/https://doi.org/10.2903/sp.efsa.2022.e200505

EPPO (2010). Alix, A., Chauzat, M. P., Duchard, S., Lewis, G., Maus, C., Miles, M. J., Pilling, E. D., Thompson, H. M., and Wallner, K. Environmental risk assessment scheme for plant protection products-Chapter 10: Honeybees–Proposed scheme. *Julius-Kühn-Archiv*, *423*, 27.

Frankie, G. W., and Thorp, R. W. (2009). Chapter 206 - Pollination and Pollinators. In V. H. Resh & R. T. Cardé (Eds.), *Encyclopedia of Insects (Second Edition)* (pp. 813–819). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-374144-8.00215-0

Gallai, N., Salles, J.-M., Settele, J., and Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*(3), 810–821. https://doi.org/https://doi.org/10.1016/j.ecolecon.2008.06.014

Garibaldi, L. A., Carvalheiro, L. G., Leonhardt, S. D., Aizen, M. A., Blaauw, B. R., Isaacs, R., Kuhlmann, M., Kleijn, D., Klein, A. M., Kremen, C., Morandin, L., Scheper, J., and Winfree, R. (2014). From research to action: enhancing crop yield through wild pollinators. *Frontiers in Ecology and the Environment*, *12*(8), 439–447. https://doi.org/https://doi.org/10.1890/130330

Garibaldi, L. A., Steffan-Dewenter, I., Winfree, R., Aizen, M. A., Bommarco, R., Cunningham, S. A., Kremen, C., Carvalheiro, L. G., Harder, L. D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N. P., Dudenhöffer, J. H., Freitas, B. M., Ghazoul, J., Greenleaf, S., et al., Klein, A. M. (2013). Wild Pollinators Enhance Fruit Set of Crops Regardless of Honey Bee Abundance. *Science*, *339*(6127), 1608–1611. https://doi.org/10.1126/science.1230200

Gill, R. J., and Raine, N. E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology*, *28*(6), 1459–1471. https://doi.org/https://doi.org/10.1111/1365-2435.12292

Goulson, D. (2020). Pesticides, Corporate Irresponsibility, and the Fate of Our Planet. *One Earth*, *2*(4), 302–305. https://doi.org/https://doi.org/10.1016/j.oneear.2020.03.004

Goulson, D., Lye, G., and Darvill, B. (2008). The decline and conservation of bumblebees. *Annual Review of Entomology*, *53*, 191–208. https://doi.org/10.1146/annurev.ento.53.103106.093454

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E. L. (2015). Bee declines driven by combined Stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229). https://doi.org/10.1126/science.1255957

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., Amaro da Costa, C., Dahle, B., Danihlík, J., Dražić, M. M., Evans, G., Fedoriak, M., Forsythe, I., Gajda, A., de Graaf, D. C., Gregorc, A., et al., Brodschneider, R. (2020). Honey bee colony winter loss rates for 35 countries participating in the COLOSS survey for winter 2018–2019, and the effects of a new queen on the risk of colony winter loss. *Journal of Apicultural Research*, *59*(5), 744–751. https://doi.org/10.1080/00218839.2020.1797272

Grixti, J. C., Wong, L. T., Cameron, S. A., and Favret, C. (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation*, *142*(1), 75–84. https://doi.org/https://doi.org/10.1016/j.biocon.2008.09.027

Gunstone, T., Cornelisse, T., Klein, K., Dubey, A., and Donley, N. (2021). Pesticides and Soil Invertebrates: A Hazard Assessment. *Frontiers in Environmental Science*, *9*. https://doi.org/10.3389/fenvs.2021.643847

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., and de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE*, *12*(10), e0185809. https://doi.org/10.1371/journal.pone.0185809

Hallmann, C. A., Ssymank, A., Sorg, M., de Kroon, H., and Jongejans, E. (2021). Insect biomass decline scaled to species diversity: General patterns derived from a hoverfly community. *Proceedings of the National Academy of Sciences*, *118*(2), e2002554117. https://doi.org/10.1073/pnas.2002554117

Heller, S., Joshi, N. K., Chen, J., Rajotte, E. G., Mullin, C., and Biddinger, D. J. (2020). Pollinator exposure to systemic insecticides and fungicides applied in the previous fall and pre-bloom period in apple orchards. *Environmental Pollution*, *265*, 114589. https://doi.org/https://doi.org/10.1016/j.envpol.2020.114589

Hoehn, P., Tscharntke, T., Tylianakis, J. M., and Steffan-Dewenter, I. (2008). Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1648), 2283–2291. https://doi.org/10.1098/rspb.2008.0405

Hristov, P., Shumkova, R., Palova, N., and Neov, B. (2020). Factors associated with honey bee colony losses: A mini-review. *Veterinary Sciences*, *7*(4), 166. https://doi.org/10.3390/vetsci7040166

Iverson, A., Hale, C., Richardson, L., Miller, O., and McArt, S. (2019). Synergistic effects of three sterol biosynthesis inhibiting fungicides on the toxicity of a pyrethroid and neonicotinoid insecticide to bumble bees. *Apidologie*, *50*(5), 733–744. https://doi-org.ezproxy.unibo.it/10.1007/s13592-019-00681-0

Iwasa, T., Motoyama, N., Ambrose, J. T., and Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. *Crop Protection*, *23*(5), 371–378. https://doi.org/https://doi.org/10.1016/j.cropro.2003.08.018

Jansen, J. P., Defrance, T., and Warnier, A. M. (2011). Side effects of flonicamide and pymetrozine on five aphid natural enemy species. *BioControl*, *56*(5), 759–770. https://doi.org/10.1007/s10526-011-9342-1

Jha, S., and Dick, C. W. (2010). Native bees mediate long-distance pollen dispersal in a shade coffee landscape mosaic. *Proceedings of the National Academy of Sciences*, *107*(31), 13760–13764. https://doi.org/10.1073/pnas.1002490107

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., Klein, A.-M., Kremen, C., M'Gonigle, L. K., Rader, R., Ricketts, T. H., Williams, N. M., Lee Adamson, N., Ascher, J. S., Báldi, A., Batáry, P., Benjamin, F., Biesmeijer, J. C., Blitzer, E. J., et al., Potts, S. G. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nature Communications*, *6*(1), 7414. https://doi.org/10.1038/ncomms8414

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Klein, A. M., Boreux, V., Fornoff, F., Mupepele, A. C., and Pufal, G. (2018). Relevance of wild and managed bees for human well-being. *Current Opinion in Insect Science*, 26, 82-88. https://doi.org/10.1016/j.cois.2018.02.011

Koh, I., Lonsdorf, E. v, Williams, N. M., Brittain, C., Isaacs, R., Gibbs, J., and Ricketts, T. H. (2016). Modeling the status, trends, and impacts of wild bee abundance in the United States. *Proceedings of the National Academy of Sciences*, *113*(1), 140–145. https://doi.org/10.1073/pnas.1517685113

Krichilsky, E., Centrella, M., Eitzer, B., Danforth, B., Poveda, K., and Grab, H. (2021). Landscape Composition and Fungicide Exposure Influence Host–Pathogen Dynamics in a Solitary Bee. *Environmental Entomology*, *50*(1), 107–116. https://doi.org/10.1093/ee/nvaa138

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D. M., Sagili, R. R., Pettis, J. S., Ellis, J. D., Wilson, M. E., Wilkes, J. T., Tarpy, D. R., Rose, R., Lee, K., Rangel, J., and vanEngelsdorp, D. (2017). A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research*, *56*(4), 328–340. https://doi.org/10.1080/00218839.2017.1344496

Lautenbach, S., Seppelt, R., Liebscher, J., and Dormann, C. F. (2012). Spatial and Temporal Trends of Global Pollination Benefit. *PLOS ONE*, *7*(4), e35954-. https://doi.org/10.1371/journal.pone.0035954

Lehmann, D. M., and Camp, A. A. (2021). A systematic scoping review of the methodological approaches and effects of pesticide exposure on solitary bees. *Plos One*, *16*(5), e0251197. https://doi-org.ezproxy.unibo.it/10.1371/journal.pone.0251197

López-Uribe, M. M., Ricigliano, V. A., and Simone-Finstrom, M. (2020). Defining pollinator health: a holistic approach based on ecological, genetic, and physiological factors. *Annu. Rev. Anim. Biosci*, 8:269–94. https://doi.org/10.1146/annurev-animal-020518- 115045

Main, A. R., Webb, E. B., Goyne, K. W., and Mengel, D. (2020). Reduced species richness of native bees in field margins associated with neonicotinoid concentrations in non-target soils. *Agriculture, Ecosystems & Environment*, *287*, 106693. https://doi.org/https://doi.org/10.1016/j.agee.2019.106693

Mallinger, R. E., and Gratton, C. (2015). Species richness of wild bees, but not the use of managed honeybees, increases fruit set of a pollinator-dependent crop. *Journal of Applied Ecology*, *52*(2), 323–330. https://doi.org/https://doi.org/10.1111/1365-2664.12377

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M., and Sattelle, D. B. (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*, *22*(11), 573–580. https://doi.org/https://doi.org/10.1016/S0165-6147(00)01820-4

Mitchell, E. A. D., Mulhauser, B., Mulot, M., Mutabazi, A., Glauser, G., and Aebi, A. (2017). A worldwide survey of neonicotinoids in honey. *Science*, *358*(6359), 109–111. https://doi.org/10.1126/science.aan3684

Moens, J., de Clercq, P., and Tirry, L. (2011). Side effects of pesticides on the larvae of the hoverfly Episyrphus balteatus in the laboratory. *Phytoparasitica*, *39*(1), 1–9. https://doi.org/10.1007/s12600-010-0127-3

Nicholson, C. C., and Egan, P. A. (2020). Natural hazard threats to pollinators and pollination. *Global Change Biology*, *26*(2), 380–391. https://doi.org/https://doi.org/10.1111/gcb.14840

Ollerton, J., Erenler, H., Edwards, M., and Crockett, R. (2014). Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science*, *346*(6215), 1360–1362. https://doi.org/10.1126/science.1257259

Pekas, A., de Craecker, I., Boonen, S., Wäckers, F. L., and Moerkens, R. (2020). One stone; two birds: concurrent pest control and pollination services provided by aphidophagous hoverflies. *Biological Control*, *149*, 104328. https://doi.org/https://doi.org/10.1016/j.biocontrol.2020.104328

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/https://doi.org/10.1016/j.tree.2010.01.007

Potts, S. G., Fonseca, V. I., Ngo, H. T., Biesmeijer, J. C., Breeze, T. D., Dicks, L., Garibaldi, L. A., Hill, R., Settele, J., and Vanbergen, A. J. (2016). *Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production*.

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C., Breeze, T. D., Dicks, L. v, Garibaldi, L. A., Hill, R., Settele, J., and Vanbergen, A. J. (2016). Safeguarding pollinators and their values to human well-being. *Nature*, *540*(7632), 220–229. https://doi.org/10.1038/nature20588

Powney, G. D., Carvell, C., Edwards, M., Morris, R. K. A., Roy, H. E., Woodcock, B. A., and Isaac, N. J. B. (2019). Widespread losses of pollinating insects in Britain. *Nature Communications*, *10*(1), 1018. https://doi.org/10.1038/s41467-019-08974-9

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K. S., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., et al., Woyciechowski, M. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(1), 146–151. https://doi.org/10.1073/pnas.1517092112

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., and Cresswell, J. E. (2018). Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). *Pest Management Science*, *74*(3), 541–546. https://doi-org.ezproxy.unibo.it/10.1002/ps.4756

Rasmont, P., Pauly, A., Terzo, M., Patiny, S., Michez, D., Iserbyt, S., Barbier, Y., and Haubruge, E. (2005). The survey of wild bees (Hymenoptera, Apoidea) in Belgium and France. *Food and Agriculture Organisation, Rome*, *18*.

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., and Yourstone, J. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, *521*(7550), 77–80. https://doi-org.ezproxy.unibo.it/10.1038/nature14420

Sánchez, M., Belliure, B., Montserrat, M., Gil, J., and Velásquez, Y. (2022). Pollination by the hoverfly Eristalinus aeneus (Diptera: Syrphidae) in two hybrid seed crops: celery and fennel (Apiaceae). *The Journal of Agricultural Science*, *160*(3–4), 194–206. https://doi.org/DOI: 10.1017/S0021859622000314

Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., and Desneux, N. (2016). Are bee diseases linked to pesticides? — A brief review. *Environment International*, *89–90*, 7–11. https://doi.org/https://doi.org/10.1016/j.envint.2016.01.009

Schmolke, A., Galic, N., Feken, M., Thompson, H., Sgolastra, F., Pitts-Singer, T., Elston, C., Pamminger, T., and Hinarejos, S. (2021). Assessment of the Vulnerability to Pesticide Exposures Across Bee Species. *Environmental Toxicology and Chemistry*, *40*(9), 2640–2651. https://doi.org/10.1002/etc.5150

Schreinemachers, P., and Tipraqsa, P. (2012). Agricultural pesticides and land use intensification in high, middle and low income countries. *Food Policy*, *37*(6), 616–626. https://doi.org/https://doi.org/10.1016/j.foodpol.2012.06.003

Senapathi, D., Goddard, M. A., Kunin, W. E., and Baldock, K. C. R. (2017). Landscape impacts on pollinator communities in temperate systems: evidence and knowledge gaps. *Functional Ecology*, *31*(1), 26–37. https://doi.org/https://doi.org/10.1111/1365-2435.12809

Sgolastra, F., Hinarejos, S., Pitts-Singer, T. L., Boyle, N. K., Joseph, T., Luckmann, J., Raine, N. E., Singh, R., Williams, N. M., and Bosch, J. (2019). Pesticide Exposure Assessment Paradigm for Solitary Bees. *Environmental Entomology*, *48*(1), 22–35. https://doi.org/10.1093/ee/nvy105

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., and Porrini, C. (2020). Bees and pesticide regulation : Lessons from the neonicotinoid experience. *Biological Conservation*, *241*(June 2019), 108356. https://doi.org/10.1016/j.biocon.2019.108356

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M. T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., and Bosch, J. (2017). Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Management Science*, *73*(6), 1236–1243. https://doi.org/https://doi.org/10.1002/ps.4449

Sparks, T. C., and Nauen, R. (2015). IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology*, *121*, 122–128. https://doi.org/https://doi.org/10.1016/j.pestbp.2014.11.014

Sponsler, D. B., Grozinger, C. M., Hitaj, C., Rundlöf, M., Botías, C., Code, A., Lonsdorf, E. v, Melathopoulos, A. P., Smith, D. J., Suryanarayanan, S., Thogmartin, W. E., Williams, N. M., Zhang, M., and Douglas, M. R. (2019). Pesticides and pollinators: A socioecological synthesis. *Science of The Total Environment*, *662*, 1012–1027. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.01.016

Steffan, S. A., Dharampal, P. S., Danforth, B. N., Gaines-Day, H. R., Takizawa, Y., and Chikaraishi, Y. (2019). Omnivory in Bees: Elevated Trophic Positions among All Major Bee Families. *The American Naturalist*, *194*(3), 414–421. https://doi.org/10.1086/704281

Stoner, K. A., and Eitzer, B. D. (2012). Movement of Soil-Applied Imidacloprid and Thiamethoxam into Nectar and Pollen of Squash (Cucurbita pepo). *PLOS ONE*, *7*(6), e39114. https://doi.org/10.1371/journal.pone.0039114

Thompson, H. M. (2012). Interaction between pesticides and other factors in effects on bees. *EFSA Supporting Publications*, *9*(9), 340E.

Topolska, G., Gajda, A., Pohorecka, K., Bober, A., Kasprzak, S., Skubida, M., and Semkiw, P. (2010). Winter colony losses in Poland. *Journal of Apicultural Research*, *49*(1), 126–128.

Tosi, S., Burgio, G., and Nieh, J. C. (2017). A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. *Scientific Reports*, *7*(1), 1201. https://doi.org/10.1038/s41598-017-01361-8

Tosi, S., Costa, C., Vesco, U., Quaglia, G., and Guido, G. (2018). A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Science of The Total Environment*, *615*, 208–218. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.09.226

Tosi, S., Sfeir, C., Carnesecchi, E., vanEngelsdorp, D., and Chauzat, M.-P. (2022). Lethal, sublethal, and combined effects of pesticides on bees: A meta-analysis and new risk assessment tools. *Science of The Total Environment*, *844*, 156857. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.156857

Uhl, P., and Brühl, C. A. (2019). The Impact of Pesticides on Flower-Visiting Insects: A Review with Regard to European Risk Assessment. *Environmental Toxicology and Chemistry*, *38*(11), 2355–2370. https://doi.org/10.1002/etc.4572

Vanbergen, A. J., and Initiative, the I. P. (2013). Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment*, *11*(5), 251–259. https://doi.org/https://doi.org/10.1890/120126

Weidenmüller, A., Meltzer, A., Neupert, S., Schwarz, A., and Kleineidam, C. (2022). Glyphosate impairs collective thermoregulation in bumblebees. *Science*, *376*(6597), 1122–1126. https://doi.org/10.1126/science.abf7482

Willcox, B. K., Aizen, M. A., Cunningham, S. A., Mayfield, M. M., and Rader, R. (2017). Deconstructing pollinator community effectiveness. *Current Opinion in Insect Science*, *21*, 98–104. https://doi.org/https://doi.org/10.1016/j.cois.2017.05.012

Williams, P. H., and Osborne, J. L. (2009). Bumblebee vulnerability and conservation world-wide. *Apidologie*, *40*(3), 367–387. https://doi.org/10.1051/apido/2009025

Winfree, R. (2013). Global change, biodiversity, and ecosystem services: What can we learn from studies of pollination? *Basic and Applied Ecology*, *14*(6), 453–460. https://doi.org/https://doi.org/10.1016/j.baae.2013.07.004

Woodcock, B. A., Isaac, N. J. B., Bullock, J. M., Roy, D. B., Garthwaite, D. G., Crowe, A., and Pywell, R. F. (2016). Impacts of neonicotinoid use on long-term population changes in wild bees in England. *Nature Communications*, *7*(1), 12459. https://doi.org/10.1038/ncomms12459

Yoder, J. A., Hedges, B. Z., Heydinger, D. J., Sammataro, D., and DeGrandi-Hoffman, G. (2011). *Differences among fungicides targeting beneficial fungi associated with honey bee colonies* (pp. 181–192). CRC Press.

Yoder, J. A., Jajack, A. J., Rosselot, A. E., Smith, T. J., Yerke, M. C., and Sammataro, D. (2013). Fungicide Contamination Reduces Beneficial Fungi in Bee Bread Based on an Area-Wide Field Study in Honey Bee, Apis mellifera, Colonies. *Journal of Toxicology and Environmental Health, Part A*, *76*(10), 587–600. https://doi.org/10.1080/15287394.2013.798846

Zattara, E. E., and Aizen, M. A. (2021). Worldwide occurrence records suggest a global decline in bee species richness. *One Earth*, *4*(1), 114–123. https://doi.org/https://doi.org/10.1016/j.oneear.2020.12.005

Zhu, W., Schmehl, D. R., Mullin, C. A., and Frazier, J. L. (2014). Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae. *PLOS ONE*, *9*(1), e77547-. https://doi.org/10.1371/journal.pone.0077547

Zhu, Y. C., Yao, J., Adamczyk, J., and Luttrell, R. (2017). Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (Apis mellifera). *PLOS ONE*, *12*(6), e0178421. https://doi.org/10.1371/journal.pone.0178421

Zioga, E., Kelly, R., White, B., and Stout, J. C. (2020). Plant protection product residues in plant pollen and nectar: A review of current knowledge. *Environmental Research*, *189*, 109873. https://doi.org/https://doi.org/10.1016/j.envres.2020.109873

Chapter 1

Effects of a fungicide and an insecticide, alone and in combination, on larval development and adult longevity of a solitary bee

Cátia Ariana Henriques Martins, Celeste Azpiazu, Claudio Ratti, Jordi Bosch, Piotr Medrzycki, Serena Magagnoli, Fabio Sgolastra

Manuscript in preparation

Chapter 1- Graphical abstract

ABSTRACT

Bees have long been considered strictly herbivorous since they rely on pollen and nectar to grow and survive during the larval and adult stage. However, recent studies have shown that bee's pollen provisions are host to abundant microbial communities, which feed on the pollen before and/or while it is consumed by bee larvae. In the process, microbes convert pollen into a complex of plant and microbial components, that are later ingested by bee larvae. However, it is likely that the microbiota associated to pollen provision may be affected by environmental conditions, including pesticides. We studied the chronic effects on male *Osmia bicornis* individuals through all their development phases, after the contamination of their provisions with a neonicotinoid insecticide Confidor® (imidacloprid) and the fungicide Folicur® SE (tebuconazole), both alone or in combination. We assessed several endpoints related to bee health, i.e., time of larval development, survival, food/body conversion rate, and adult body size and longevity. Our results, show an effect of the imidacloprid by slowing the process of larval development. However, we did not observe a direct effect of the fungicide, neither when administrated in combination with the neonicotinoid insecticide. Microbiome results may confirm the mechanisms of action through the high or low diversity of pathogens and/or beneficial symbionts.

Keywords: *Osmia bicornis*, larval development, fungicide, neonicotinoids, provision's microflora

1.1. Introduction

Bees allow the maintenance and well-functioning of terrestrial environments by providing essential ecosystem services (Fründ et al., 2013; Winfree et al., 2018). This diverse group of insects, comprises more than 20,000 species, that together with other insect pollinators are responsible for the pollination of approximately 75 % and 90 % of cultivated crops and wild flowering plants, respectively (Klein et al., 2007; Potts et al., 2016). However, the decline in pollinator populations has been observed alongside with the increasing demand for optimal pollination services in agricultural systems (Aizen and Harder, 2009), particularly relevant on large scale productions of orchard crops (Grab et al., 2019; Reilly et al., 2020). As modern agriculture highly relies on agrochemical applications for pest and pathogen protection (Schreinemachers and Tipraqsa, 2012; Sharma et al., 2019), pesticides may be found through the year, or even for several years, as residues (including their metabolites) in several environmental matrices such as in soil, water, pollen and nectar (Bonmatin et al., 2015; Kopit et al., 2022). Therefore, while bees forage to feed and collect resources for their offspring, they can encounter several residues of pesticides both in crop and off-crop areas (Xavier et al., 2020; Zioga et al., 2020). The toxicity of most of these compounds to pollinators has been widely demonstrated in laboratory conditions (Biddinger et al., 2013; Sanchez-Bayo and Goka, 2014). However, until now what we know is mainly based on the effects of pesticides on the western honey bee, *Apis mellifera* L. (Tosi et al., 2022). Moreover, most studies have only been focused on the pesticide effects on bee adults, leaving practically uncover the effects on other stages, such as larvae (Benuszak et al., 2017). Besides, the honey bee is a highly social insect, however most bees are solitary (ca. 70 % in temperate ecosystems; Sgolastra et al., 2019), meaning that the exposure and known effects on honey bee may not be directly extrapolated to solitary bees (Schmolke et al., 2021). In fact, the necessity to expand the knowledge to other species urges as increasing body of knowledge shows that the impact of pesticides on bees strongly depends on their life history traits (Arena and Sgolastra, 2014; Brittain and Potts, 2011; Kopit and Pitts-Singer, 2018; Stoner, 2016; Thompson, 2016).

Solitary bees have different routes and level of exposure that differ from honey bees in particular during larval stages, as larval food of solitary bees usually consists in a large amount of unprocessed pollen mixed with nectar, in contrast to the honey bee larvae that consume royal jelly, a specific glandular secretion. This may indicate a higher vulnerability for the solitary bee larvae since pollen has usually higher concentrations of pesticides, comparing to the honey bee processed food (Böhme et al., 2018). The presence of agrochemicals in the solitary bee's food source and nesting environments may induce lethal or fitness-reducing effects on larva (Anderson and Harmon-Threatt, 2019; Huntzinger et al., 2008; Kopit et al., 2022; Sgolastra et al., 2015; Tesoriero et al., 2003). Apart from a direct toxicological effect on larva an alternative or complementary hypothesis involves an alteration of the bee-microbe symbioses. A recent study, from Steffan et al. (2019), revealed that microbial communities play an important role to larval development of solitary bees, by degrading the pollen proteins to rend them more easily assimilated, and protecting the pollen provision from pathogens. Therefore, these bee-microbe symbioses are ultimately linked with larval growth and development, and adult fitness (Dharampal et al., 2019, 2020; Lozo et al., 2015; Philipp et al., 2016). However, pesticides, and in particular fungicides are likely to disrupt the microbial community dynamics, compromising bee health (Krichilsky et al., 2021; Steffan et al., 2017).

In this study, we exposed *Osmia bicornis* L. male larvae via ingestion of pollen-nectar provisions contaminated with one neonicotinoid insecticide (imidacloprid) and one fungicide (tebuconazole), separately and in combination. Mixtures of pesticides are usually not addressed in pesticide risk assessments despite the fact that bees are usually exposed to various combinations of chemical products. This is particular important because studies have been showing that some groups of pesticides may present synergistic effects on pollinators (e.g. neonicotinoids and triazole fungicides. Biddinger et al., 2013; Iverson et al., 2019; Iwasa et al., 2004; Raimets et al., 2018; Sgolastra et al., 2017). Yet, these studies were primarily focused on adult bees.

Our aim was to establish whether field-realistic concentrations of these agrochemicals have negative effects on larval development, survival and on subsequent adult longevity. We had three main hypothesis through which pesticides could mechanistically affect bees: (1) pesticides, in particular the neonicotinoid insecticide, could affect larval development directly, by causing early egg or larval mortality, or reducing their capacity to convert food in bee body with negative consequences on adult longevity; (2) pesticides would have an effect on the microbiome's provision, in particular the impact of fungicide residues on the microbiota communities, and its consequences on bee development; and (3) if the mixture could potentiate the effects.

1.2. Materials and methods

1.2.1. Bee rearing and collection

Osmia bicornis is a cavity-nesting solitary bee, with univoltine life cycle which emerges at mid-April and has its flight period until the end of June in Central Europe (Steffan-Dewenter and Schiele, 2008). Nests of *O. bicornis* are arranged in sequential brood cells, separated by mud partitions. The females collect pollen and nectar, from different flower sources (polylectic), and provisioned them as a mixture before laying the eggs. The larva hatches in its individual cell and starts to consume the provision before spinning a cocoon and pupating. Bees overwinter as adult and emerge in the following spring (Bosch and Kemp, 2002).

Cocoons were supplied by Pollinature Srl. and shipped to the laboratory of Agricultural Entomology (DISTAL), at the University of Bologna, where they were kept at wintering temperature of 3-4 °C, until April 2020. A total of 334 females, 534 males, and 500 nests (grass reeds) were set up and distributed in two different sites, with favorable conditions for the nesting activities. Cocoons were left to emerged in semi-natural areas (no expected pesticide application and abundant flower sources), located on the outskirts of Bologna.

Nesting activity was checked weekly for presence of eggs, and nests were collected when plugged and taken to the laboratory on mid-May 2020. All nests were kept at room temperature during collection. Eggs or young larvae and their pollen provisions were removed from the nests with the help of forceps, that were disinfected with alcohol (70º) between samples. Old larvae (older than the second instar stage) and eggs or larvae that looked damaged were discarded. In this study, only males were collected, their Identification was performed based on the cell position and size of the provision (males have smaller provisions and are usually located in the external part of the nest; Bosch et al., 2008). Upon removal from the nests, eggs/larvae were separated from the provision using a small brush. This procedure was done in order to perform the contamination of the pollen provision.

1.2.2. Pesticides

Stock solutions were prepared by diluting 100 µl of Confidor ® (imidacloprid, 20% w/v) in 50 ml of distilled water, and 616 µL Folicur ®SE (tebuconazole, 43.1% w/v) in 3.9 ml of distilled water. These solutions were sequentially diluted in distilled water for posterior pollen preparation in order to reach the desired concentrations (10 ppb for imidacloprid and 9 ppm for tebuconazole; see Table A1.1).

For imidacloprid we used the median value of residue concentrations detected on pollen and nectar from literature (Byrne et al., 2014; Chauzat et al., 2006, 2009, 2011; Dively and Kamel, 2012; Lentola et al., 2017; Mullin et al., 2010; Ostiguy et al., 2019; Pohorecka et al., 2012; Stoner and Eitzer, 2012). For tebuconazole, the value near 90th percentile of Residue per Unit Dose (RUD), referred to an application rate of 1 kg a.s./ha derived from foliar spray application (EFSA, 2013), was applied to the maximum field application rate expressed in the commercial formulate label, during blooming. This was done in order to estimate the concentration of product that arrives to pollen and nectar.

1.2.3. Larval exposure

O. bicornis provisions were kept as individual units for the natural control (CTRL), or were manually mixed (ratio of provision:water of 5:1 w/w), to obtain a uniform source for each treatment. Artificially mixed provisions were separated and prepared accordingly with the treatment: distilled water (Water), imidacloprid (INS), tebuconazole (FUNG), and the mixture between the two pesticides (MIX). All larvae from the artificially manipulated treatment groups received a new pollen provision with approximately 150 mg (fresh weight, P_i), prepared with a modified 2.5-mL sterile syringe. Provisions and egg/larvae were placed in sterile 48-well ELISA plates, where they were kept at 24.7 ± 3˚C and 40-50 % relative humidity, in dark conditions until the end of larval development.

1.2.4. Pollen-nectar provision collection

Upon the preparation of the manipulated provisions, 100 provisions, each of 200 mg of fresh weight, were separated for molecular analyses. Fifty samples were attributed for time 1 (T1), provisions collected 24h after contamination, and other 50 samples for time 2 (T2), provisions collected 15 days after contamination. Samples of T2 were kept in the climate chamber under the same conditions as the larvae, in order to standardize the degradation of pesticides and microbial development. Samples were then transferred to a − 80 °C freezer for storage until DNA extraction. In addition, 2 replicates per treatment and time (200 mg fresh weight) were collected at time 0 (T0), soon after the application, and T2 for liquid chromatography (LC–MS/MS) and gas chromatography (GC–MS/MS) with tandem mass spectrometry. Analysis at T0 were performed in order to detected the presence of possible undesired residues and confirm the nominal doses. Analysis at T2 assess the degradation of the pesticides during two-week period.

1.2.5. Larvae monitoring

Larvae mortality and provision consumption was checked daily. Larvae that died within 24h after exposure to the contaminated provisions, were discarded as we considered dead due to manipulation. Larval development started to count from the point that the fifth instar stage was reach (identify by the presence of feces). We continued daily controls after all provision was consumed by the larva, in order to assess the time of spinning the cocoon. When the cocoon was completely formed, the remaining pollen provision was weighed, if present, as well the feces.

Before overwintering, cocoons were weighed (with adult bees inside, P_w) accurately with an analytical balance. At this point, the temperature was gradually reduced to 12 ˚C, in a period of 2 weeks, until reach the overwinter temperature of 3-4 ˚C, and kept under dark conditions.

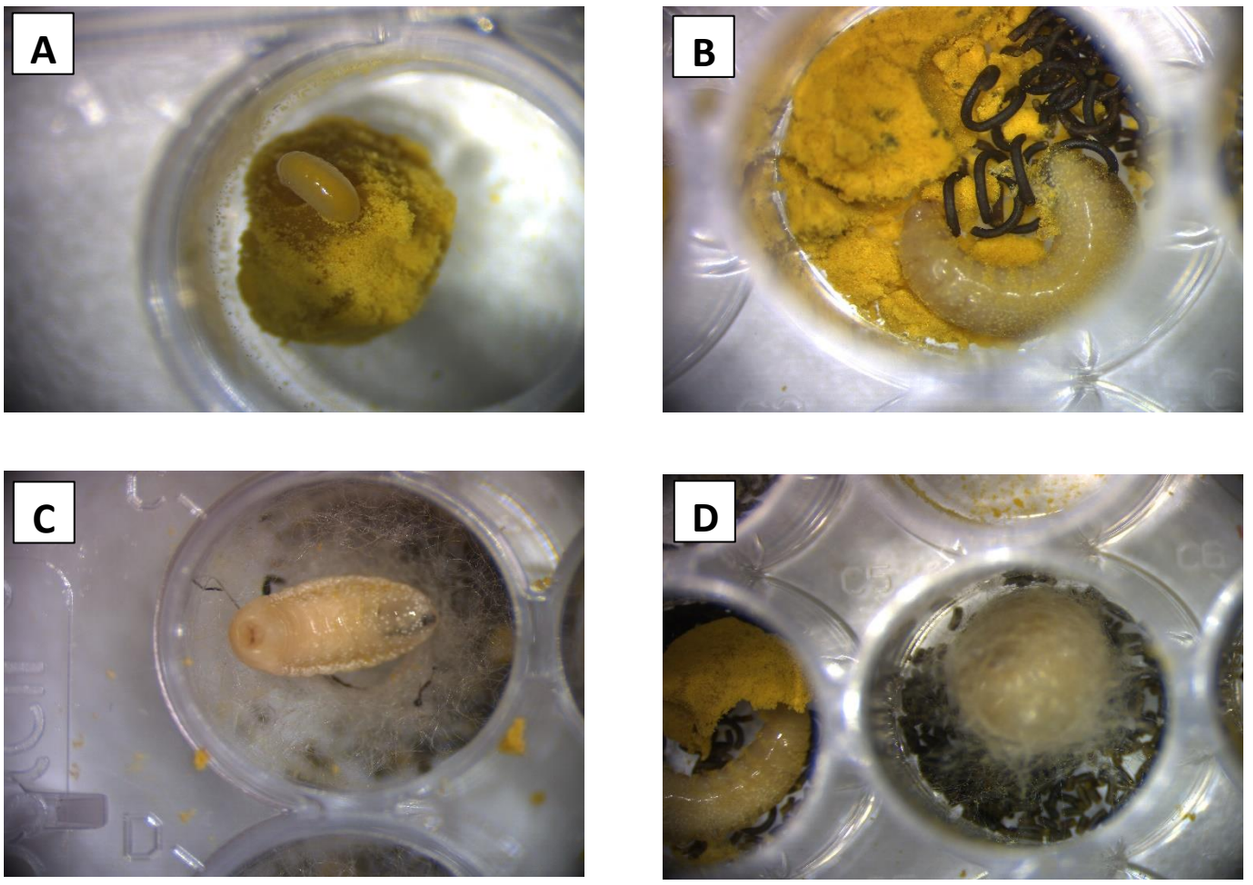


Figure 1.1: Larval development monitoring. **(A)** Early stages of larvae at T0; **(B)** Fifth instar larvae; **(C)** Initiation of spinning of the cocoon; **(D)** Completion of cocoon spin.

1.2.6. Adult monitoring

The period of overwinter occurred from October 2020 to April 2021. In mid-April 2021, each individual was weighted and transferred from the ELISA plates to individual plastic cups (volume: 50 cc). Cocoons were incubated at 22-23 ˚C, 40-55% relative humidity and dark conditions, to promote the emergence. Upon emergence, the empty cocoons were collected and weighted (P_{cocoon}). Adult males were kept under these conditions until their death. Survival without feeding was used as a measure of remaining energy reserves (Bosch and Kemp, 2000). Daily controls were performed to assess the time of emergence and the adult longevity. Upon dead, bees were immediately frozen at – 20 °C, to later measure the head width (used as proxy of body size; Bosch and Vicens, 2002) of each individual bee under a stereomicroscope with a micrometer.

1.2.7. Molecular analyses

The DNA from each of the 100 pollen provisions, each sample with approximately 100 mg, was extracted using the Dneasy® Plant Mini Kit (Qiagen Group), following the manufacture's protocol. Firstly, in order to lyse the provisions, samples were bead beat each with 400 µl of lysis buffer and 10 ZR Bashing Beads of 0.1 mm, at 30 Hz for 6 min in the Tissue Lyser. The final elution volume was 100 µl, in nuclease free water. We used the resulting DNA extractions to prepare separate metabarcoding amplicon libraries for bacteria (the 16S rRNA gene [16S]) and fungi (the internal transcribed spacer [ITS]). We used previously described primers both for 16S (McFrederick and Rehan, 2016) and ITS (Smith and Peay, 2014) to design new primers. Primer

sequences used in this study are reported in Table A1.2. PCRs were conducted with 2 µl of template DNA in each reaction. Mixtures for PCR were always prepared separately for 16S and ITS, using the two indexed primers (1 µl each) for each sample and an appropriate quantity of nuclease free H_2O (15.3 µl), 0.5 µl of dNTPs (deoxyribonucleotide triphosphate), 5 µl of 5xGoTaq® buffer and 0.2 µl GoTaq® DNA Polymerase (Promega). PCR conditions were adjusted accordingly, samples were initially denatured at 94°C for 1 min and then amplified with 30 cycles of 94°C for 10 s, 54°C for 10 s, and 72°C for 40 s. For final extension, the program ended with a step of 72°C for 3 min and 4°C for 1 min. The PCR success was checked through gel electrophoresis in a 1% agarose gel.

A standard chloroform purification was performed to clean the PCR products. In brief, a mixture of chloroform:isoamyl alcohol (24:1) was added to the PCR product dissolved in nuclease free H_2O. Following centrifugation for 15 min, the upper phase was recovered and extracted for DNA precipitation. The samples were finally resuspended on nuclease free H_2O. Presence of the purified amplicon was again controlled through a gel electrophoresis (1% agarose). Samples will from this point be sent to the sequencing center, for library preparation trough a second PCR (index PCR, information needed for sample discrimination), followed by pooling and normalisation of the amplicons.

1.3. Statistical analysis

Statistical analyses were carried out with R software v4.2.1 (R Team, 2013). Pesticide effects were assessed by comparing differences between treatment groups for (1) larval development time: time from fifth larval instar to cocoon initiation, and cocoon spinning duration; (2) food/body conversion rate and body size; (3) emergence time; and (4) survival. All individuals confirmed to be females after adulthood were discarded from the statistical analysis. Analyses were first performed between the two controls (CTRL and Water). If no differences were found, hereafter, analysis were performed only between the Water control and treated groups. Assumptions of normality were verified using Shapiro-Wilk normality test and homogeneity of variance using Levene's test. Following tests for the endpoints: larval development time, food/body weight conversion rate and emergence time, were chosen accordingly. Food/body weight conversion rate was calculated by: $Food/body\ rate\ (\%) = \frac{P_w - P_{cocoon}}{P_i} \times 100$, where P_w is the individuals pre-wintering weight, P_i is the initial provisions weight, and P_{cocoon} is the weight of the cocoon.

To examine the impacts of the pesticides on the overall survival, Kaplan–Meier survival analysis was performed using the *survfit* function from the '*survival*' package, with no censored data. Analyses were performed considering the time that larvae reached the 5th instar stage to the adult death. Additionally, survival analysis was divided in two different time points: pre-emergence (period from the 5th larval stage to emergence) and post-emergence (adult longevity). The log-rank test was used to test for differences in survival between treatments with a Bonferroni correction for multiple comparisons. All results are reported with a 95 % confidence interval.

Microbiome analyses to bee's provision has not been performed to date, since we are waiting for the NGS (Next-Generation Sequencing) outcome from the sequencing centre, which is performed on Illumina MiSeq (2 x 300 bp). Therefore, the results will not be presented for this part of the experiment. The raw reads will be processed using QIIME2 (Caporaso et al., 2010) and R for downstream analyses.

1.4. Results

1.4.1. Larval development

Larval development time, from the fifth instar until the begging of cocoon spinning, significantly differed between CTRL and Water (Mann Whitney U: *U*= 75.5, p = 0.031) and among treatments (Kruskal-Wallis: χ^2 = 8.585, df = 3, p = 0.035). From the pesticide treatments, both INS and the MIX, differed significantly from the control (Figure 1.2 A).

Because most slow-developing bees from treatment INS and MIX died before starting to spin the cocoon, when considering the development period including the formation and conclusion of the cocoon (larval development from fifth instar + spinning duration), no significant differences were found (Kruskal-Wallis: χ^2= 1.0737, df = 3, p = 0.78).

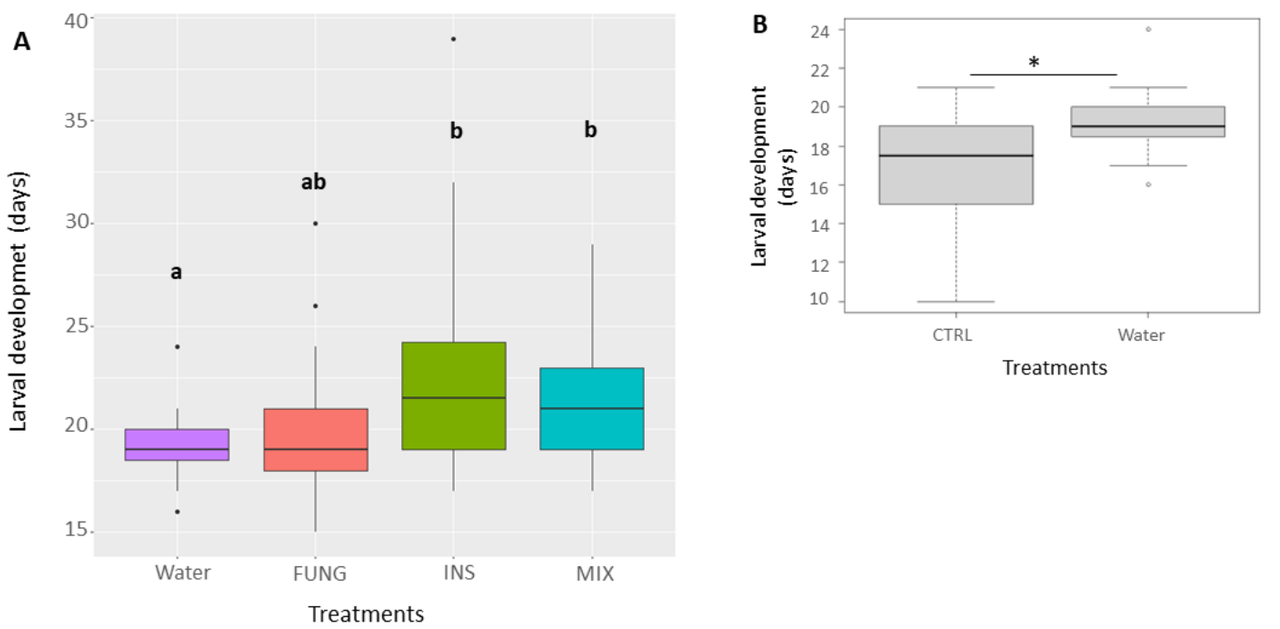


Figure 1.2: Larval development of *Osmia bicornis* males (between the fifth instar to the initiation of cocoon). **(A)** Differences are represented among treatments by different letters (Dunn's test; p< 0.05). Pesticides include: FUNG= fungicide tebuconazole, INS= insecticide imidacloprid, MIX= tebuconazole plus imidacloprid. **(B)** Differences between the two controls (CTRL =non-manipulated control and Water= manipulates control) are represented with asterisk (Mann Whitney U Test; *p< 0.05).
See: https://drive.google.com/drive/folders/123gSTUqikyKFDLhFBR_mSYplwr0wkDcC?usp=share_link

1.4.2. Food/body conversion rate and body size

At the end of larval development, only few bees did not consume completely the provision. Bees that left their provision, were the ones with observed early mortality. Regarding the food/body conversion rate there was significant differences between the non-manipulated control (CTRL) and Water (Mann Whitney U: *U*= 266.5, p = 0.00074). However, we did not identify significant differences between Water and the other treatments (Kruskal-Wallis: χ^2= 0.38, df = 3, p = 0.945; Figure 1.3).

Head size, used as a proxy for the body size of the adults was significantly different between the two controls (*T*-test: *t*(18)= 2.33, p =0.031), and between the treatments with imidacloprid and the Water (ANOVA: F= 8.574, df=3, p = 0.00014). Both parameters, conversion of provision into body mass and the body size, are positively correlated (Spearman's correlation: R = 0.62, p = 2.2×10^{-6}).

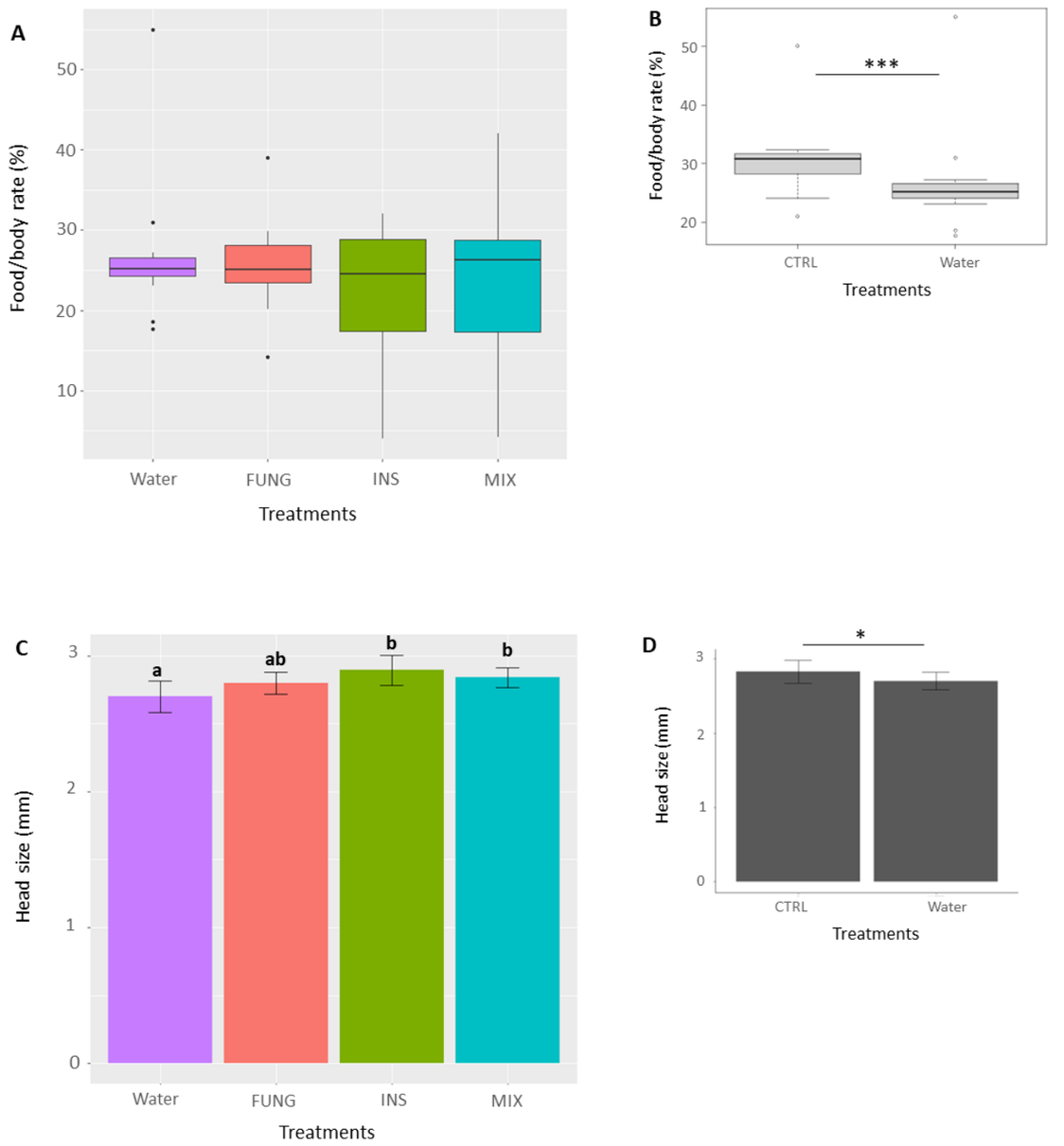


Figure 1.3: (A) Food/body conversion rate, shows the transformation of weight of the pollen-nectar provisions into body weight. **(B)** Differences are represented between the two controls with asterisk (Mann Whitney U Test; ***p< 0.001). **(C)** Mean head size (SD) across the different treatments (bars with different letters indicate significant differences. Tukey HSD; p<0.05), and **(D)** between controls (significant differences indicate with asterisks. *T*-test; p<0.05). Treatment groups include: CTRL= non-manipulated control, Water= manipulated control, FUNG= fungicide tebuconazole, INS= insecticide imidacloprid, MIX= tebuconazole plus imidacloprid.

1.4.3. Emergence time

Bees started to emerge after the second day of incubation. Differences on the emergence time were not observed within the treatment groups (Kruskal-Wallis: χ^2 = 0.516, df = 3, p = 0.93. Average ± SE: Water: 2.9 days ± 0.47; FUNG: 2.8 days ± 0.50; INS: 2.3 days ± 0.49; MIX: 1.94 days ± 0.52).

1.4.4. Survival

Larval exposure to the two pesticides and their mixture had no effect on the overall survival of *O. bicornis*. Cumulative survival curves did not differ significantly between the different treatments (Log-Rank test: χ^2=2.9, df= 3, p = 0.41, Figure 1.4 and Table 1). There were no differences in the first stages of the bees (pre-emergence; Log-Rank test: χ^2=0.5, df= 3 p = 0.92). Mortality on Water group was 10 %, during larval development. However, for survival analysis considering only the

adult period (post-emergence), significant differences between the bees were detected between the treatment group INS and the other groups (Log-Rank test: χ^2=12.2, df= 3, p = 0.0067).

Table 1: *O. bicornis* mortality during larval stage (counted until start of spinning the cocoon) and winter mortality (deaths confirmed after period of emergence). Median survival, and confidence interval (95% CI), is the overall time that individuals from each treatment group lived, from the 5th instar larval stage to the time of the dead as adults (counting the post-emergence period).

Treatment	Sample size	Larval mortality (%)	Winter mortality (%)	Median survival [95% CI] (days)
Water	20	10	15	332 [330; 334]
FUNG	18	16.6	11.1	332 [328; 334]
INS	19	36.8	5.2	332 [72; 335]
MIX	17	29.4	17.7	330 [28; 333]

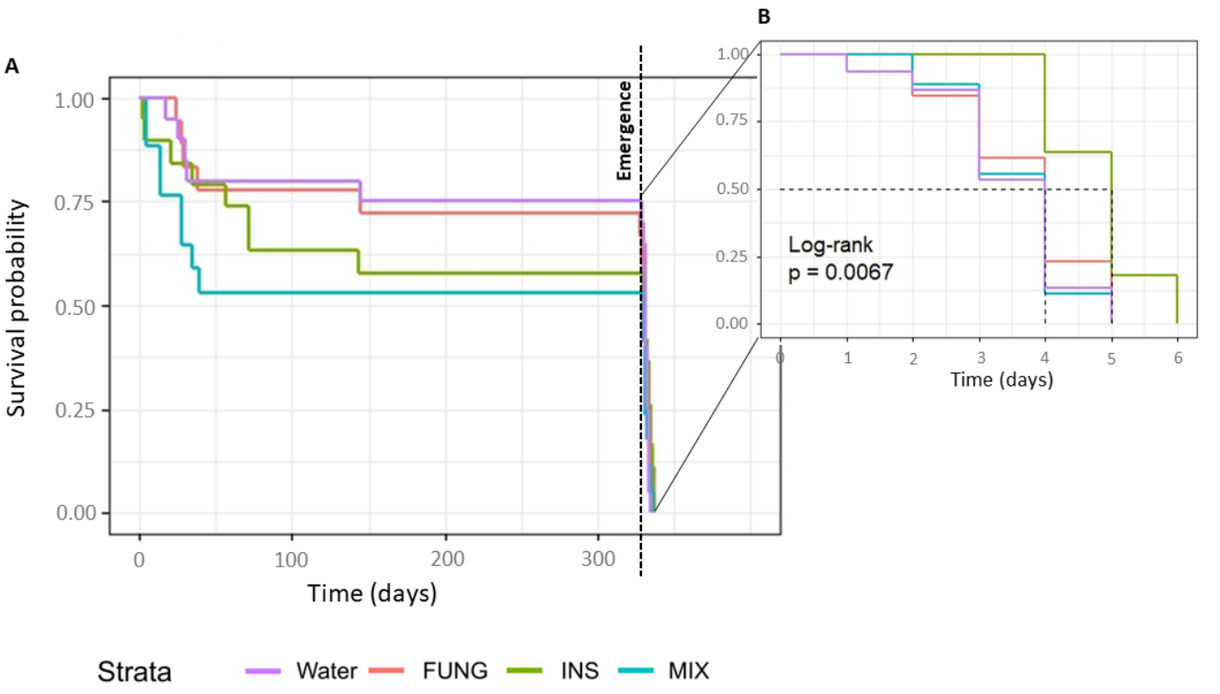


Figure 1.4: Survival curves from the **(A)** larvae to adulthood of *Osmia bicornis* males, after consumption of treated larval provisions. **(B)** Survival curves for the adults, considering only the post-emergence period (Kaplan–Meier analysis; p<0.05).

1.5. Discussion

The widespread use of pesticides results in the contamination of flowering plants through which different pollinators can be exposed during different development stages (Kopit and Pitts-Singer, 2018; Sanchez-Bayo and Goka, 2014). Pollinators, may therefore be exposed to such compounds through several routes, depending on their life cycles and life history traits (Sgolastra et al., 2019). Solitary bees in particular, may be more vulnerable to this exposure during the early stages, once females provide to their offspring unprocessed pollen and nectar as the main food. Thus, larvae ingest large quantities of contaminated food compared to their body size (Schmolke et al., 2021). However, we still have few understandings on how pesticides impact the early stages of solitary bees, because majority of the available information focuses on the effects on adults (Lehmann and Camp, 2021).

In this study, we performed a year-long assessment of *O. bicornis* larvae until adult emergence, after feeding in contaminated provisions with the two tested pesticides (imidacloprid and tebuconazole), alone or in combination. Based on previous studies showing direct effects on development and survival in honey bees (Tavares et al., 2017) and bumble bees (Gill et al., 2012) exposed to pesticides during larval stage, we expected higher mortality rate during larval development and lower capacity of *Osmia* to convert food in bee body, which will cause shorter adult life-span. Our results shown an effect of imidacloprid on the larval development in males of *O. bicornis*, by significantly slowing their development and increasing the mortality before bees were able to start their cocoons, even though this latter result was not statistically significant. Our findings support the results of previous studies performed in solitary bees, where exposure to systemic insecticides delayed larval development and increased larvae/pupa mortality (Abbott et al., 2008; Mokkapati et al., 2021; Phan et al., 2022; Preprint). Some studies have also unveiled the adverse effects of combination of pesticides in honey bee larvae (Tadei et al., 2019; Tomé et al., 2020; Wu et al., 2011; Zhu et al., 2014). However, contrary to our expectations, in our study the combination of imidacloprid with the SBI fungicide did not potentiate the toxicity, because effects were similar to the INS treatment.

Despite the observed effects on larval stage caused by the exposure to imidacloprid (INS) and fungicide-insecticide mixture (MIX), survival rate was not significantly different among treatments. Although only 58 and 53 % of bees exposed to INS and MIX, respectively, were able to emerge, compared to 75 % of the Water control. Surprisingly, when considering only the adult longevity, bees from INS treatment survived longer compared to all the other groups (1 day longer). This may be related to the lack of effects on the food conversion rate to body weight by the pesticides. Therefore, bees that were able to survive during development in the groups treated with imidacloprid may have had a higher adaptability to stressful conditions. This response was also observed on *Megachile rotundata* males, that responded to increasing concentrations of imidacloprid with a significant increase in adult longevity (Anderson and Harmon-Threatt, 2019).

The survival rate in the control groups (CTRL= 71 %; Water= 75 %) in our experiment respects the minimum value for the validation of the test according to standard protocols for honey bees, OECD guideline No. 239 (OECD, 2016), in which a minimum of 70 % emergence is required in the control groups. Despite this, we observed differences between the two controls (CTRL and Water) in some of the endpoints, i.e., larval development, food/body conversation rate and ultimately body size. These differences may be expected due to the higher variability in the weight of the provisions in the CTRL, while this factor was uniformized in the Water control, where all

bees received 150 mg of provision. To increase the level of standardization of the protocol, Eeraerts et al. (2020) recommends the method of the “new pollen”, where new provisions are made with uncontaminated honey bee pollen mixed with 50 % sugar water. However, this method may compromise the larval bee development (Kopit et al., 2022), by removing the beneficial symbionts, and potentially disrupting the microbe-derived services (Dharampal et al., 2019), as solitary bees have highly diverse and fluctuating bacterial communities in respect to social bees (Voulgari-Kokota et al., 2019). Another critical issue in the standardization of a test protocol for solitary bee larvae is the uniform distribution of the pesticide inside the provision. In this point, the analysis of pesticide residues showed similar values across the provision samples thus confirming the nominal concentrations and the uniform distribution of the pesticides.

It is already known that the host-associated symbiotic communities can affect the host immunity (Dai et al., 2018; O’Neal et al., 2018; Zhang et al., 2022) and this is also valid for the microbes that harbor in the provisions of solitary bees. At the same time the host interacts with the microbes modifying their composition (Kueneman et al., 2023). Pesticides can alter this equilibrium by affecting the host, the symbiotic communities or both. Thus, the second aim of our study was to assess the impact of pesticides in the provision’s microbiome. The analysis of microbiome in treated and untreated pollen provision is ongoing but, based on the minor effects observed in bee health, we can hypothesize a low alteration of the microbes due to pesticide exposure. Alternatively, *Osmia* individuals may present a certain plasticity and adaptation to lower microbial communities, if the analysis will show the beneficial fungi and bacteria were disrupt by the treatments. A recent study found that developing *Osmia cornifrons* larvae exert a selective pressure on the pollen borne microbiome through the time they feed, modifying and narrowing the pollen bacterial community, that is initially more diverse (Kueneman et al., 2023). However, although bee health in our experiment was not affected by the treatments, we cannot exclude indirect effects as shown by Tadei et al. (2020), who reported that bees contaminated during larval stages developed later cytotoxic effects on the midgut as adults, allowing the proliferation of pathogens. The presence of fungicides was also linked with the chalkbrood disease *in O. cornifrons* (Krichilsky et al., 2021). Results on the impact of imidacloprid in the host-microbes symbiotic are controversial and likely species-specific. A previous study on honey bees has shown long-term impact on microbial gut community leading to dysbiosis after chronic exposure to imidacloprid (Alberoni et al., 2021). Another study performed on bumble bees, however shown that despite the lethal effects on imidacloprid, this compound did not have any effect on the gut microbiome (Rothman et al., 2020). These evidences highlight the specificity of the functional of bee-microbial of different species and how this interaction can be complex.

References

Abbott, V. A., Nadeau, J. L., Higo, H. A., and Winston, M. L. (2008). Lethal and Sublethal Effects of Imidacloprid on *Osmia lignaria* and Clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae). *Journal of Economic Entomology*, 101(3), 784–796. https://doi.org/10.1093/jee/101.3.784

Aizen, M. A., and Harder, L. D. (2009). The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Current Biology*, 19(11), 915–918. https://doi.org/https://doi.org/10.1016/j.cub.2009.03.071

Alberoni, D., Favaro, R., Baffoni, L., Angeli, S., and di Gioia, D. (2021). Neonicotinoids in the agroecosystem: In-field long-term assessment on honeybee colony strength and microbiome. *Science of The Total Environment*, 762, 144116. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.144116

Anderson, N. L., and Harmon-Threatt, A. N. (2019). Chronic contact with realistic soil concentrations of imidacloprid affects the mass, immature development speed, and adult longevity of solitary bees. *Scientific Reports*, 9(1), 3724. https://doi.org/10.1038/s41598-019-40031-9

Arena, M., and Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology*, 23(3), 324–334. https://doi.org/10.1007/s10646-014-1190-1

Benuszak, J., Laurent, M., and Chauzat, M.-P. (2017). The exposure of honey bees *(Apis mellifera*; Hymenoptera: Apidae) to pesticides: Room for improvement in research*. Science of The Total Environment*, 587–588, 423–438. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.02.062

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. A., Rajotte, E. G., Joshi, N. K., and Vaughn, M. (2013). Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLOS ONE*, 8(9), e72587. https://doi.org/10.1371/journal.pone.0072587

Böhme, F., Bischoff, G., Zebitz, C. P. W., Rosenkranz, P., and Wallner, K. (2018). From field to food—will pesticide-contaminated pollen diet lead to a contamination of royal jelly? *Apidologie*, 49(1), 112–119. https://doi.org/10.1007/s13592-017-0533-3

Bonmatin, J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E. A. D., Noome, D. A., Simon-Delso, N., and Tapparo, A. (2015). Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research*, 22(1), 35–67. https://doi.org/10.1007/s11356-014-3332-7

Bosch, J., and Kemp, W. (2002). How to Manage the Blue Orchard Bee as an Orchard Pollinator. In *Sustainable Agriculture Network/National Agricultural Library*

Bosch, J., and Kemp, W. P. (2000). Development and Emergence of the Orchard Pollinator *Osmia lignaria* (Hymenoptera: Megachilidae). *Environmental Entomology*, 29(1), 8–13. https://doi.org/10.1603/0046-225x-29.1.8

Bosch, J., Sgolastra, F., and Kemp, W. P. (2008). Life cycle ecophysiology of *Osmia* mason bees used as crop pollinators. *Bee Pollination in Agricultural Ecosystems*, 83–104.

Bosch, J., and Vicens, N. (2002). Body size as an estimator of production costs in a solitary bee. *Ecological Entomology*, 27(2), 129–137.

Brittain, C., and Potts, S. G. (2011). The potential impacts of insecticides on the life-history traits of bees and the consequences for pollination. *Basic and Applied Ecology*, 12(4), 321–331. https://doi.org/https://doi.org/10.1016/j.baae.2010.12.004

Byrne, F. J., Visscher, P. K., Leimkuehler, B., Fischer, D., Grafton-Cardwell, E. E., and Morse, J. G. (2014). Determination of exposure levels of honey bees foraging on flowers of mature citrus trees previously treated with imidacloprid. *Pest Management Science*, 70(3), 470–482. https://doi.org/https://doi.org/10.1002/ps.3596

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., et al., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Chauzat, M. P., Martel, A. C., Cougoule, N., Porta, P., Lachaize, J., Zeggane, S., Aubert, M., Carpentier, P., and Faucon, J. P. (2011). An assessment of honeybee colony matrices, *Apis mellifera* (Hymenoptera: Apidae) to monitor pesticide presence in continental France. *Environmental Toxicology and Chemistry*, 30(1), 103–111. https://doi.org/10.1002/etc.361

Chauzat, M.-P., Carpentier, P., Martel, A.-C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M., and Faucon, J.-P. (2009). Influence of Pesticide Residues on Honey Bee (Hymenoptera: Apidae) Colony Health in France. *Environmental Entomology*, 38(3), 514–523. https://doi.org/10.1603/022.038.0302

Chauzat, M.-P., Faucon, J.-P., Martel, A.-C., Lachaize, J., Cougoule, N., and Aubert, M. (2006). A Survey of Pesticide Residues in Pollen Loads Collected by Honey Bees in France. *Journal of Economic Entomology*, 99(2), 253–262. https://doi.org/10.1093/jee/99.2.253

Dai, P., Yan, Z., Ma, S., Yang, Y., Wang, Q., Hou, C., Wu, Y., Liu, Y., and Diao, Q. (2018). The Herbicide Glyphosate Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in Vitro. *Journal of Agricultural and Food Chemistry*, 66(29), 7786–7793. https://doi.org/10.1021/acs.jafc.8b02212

Dharampal, P. S., Carlson, C., Currie, C. R., and Steffan, S. A. (2019). Pollen-borne microbes shape bee fitness. *Proceedings of the Royal Society B: Biological Sciences*, 286(1904). https://doi.org/10.1098/rspb.2018.2894

Dharampal, P. S., Hetherington, M. C., and Steffan, S. A. (2020). Microbes make the meal: oligolectic bees require microbes within their host pollen to thrive. *Ecological Entomology*, August. https://doi.org/10.1111/een.12926

Dively, G. P., and Kamel, A. (2012). Insecticide Residues in Pollen and Nectar of a Cucurbit Crop and Their Potential Exposure to Pollinators. *Journal of Agricultural and Food Chemistry*, 60(18), 4449–4456. https://doi.org/10.1021/jf205393x

Eeraerts, M., Pisman, M., Vanderhaegen, R., Meeus, I., and Smagghe, G. (2020). Recommendations for standardized oral toxicity test protocols for larvae of solitary bees, *Osmia* spp. *Apidologie*, 51(1), 48–60. https://doi.org/10.1007/s13592-019-00704-w

EFSA (2013). Guidance on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA Journal*, 11(7), 3295. https://doi.org/https://doi.org/10.2903/j.efsa.2013.3295

Fründ, J., Dormann, C. F., Holzschuh, A., and Tscharntke, T. (2013). Bee diversity effects on pollination depend on functional complementarity and niche shifts. *Ecology*, 94(9), 2042–2054. https://doi.org/https://doi.org/10.1890/12-1620.1

Gill, R. J., Ramos-Rodriguez, O., and Raine, N. E. (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature*, 491(7422), 105–108. https://doi.org/10.1038/nature11585

Grab, H., Branstetter, M. G., Amon, N., Urban-Mead, K. R., Park, M. G., Gibbs, J., Blitzer, E. J., Poveda, K., Loeb, G., and Danforth, B. N. (2019). Agriculturally dominated landscapes reduce bee phylogenetic diversity and pollination services. *Science,* 363(6424), 282–284. https://doi.org/10.1126/science.aat6016

Huntzinger, C. I., James, R. R., Bosch, J., and Kemp, W. P. (2008). Laboratory Bioassays to Evaluate Fungicides for Chalkbrood Control in Larvae of the Alfalfa Leafcutting Bee (Hymenoptera: Megachilidae). *Journal of Economic Entomology*, 101(3), 660–667. https://doi.org/10.1093/jee/101.3.660

Iverson, A., Hale, C., Richardson, L., Miller, O., and McArt, S. (2019). Synergistic effects of three sterol biosynthesis inhibiting fungicides on the toxicity of a pyrethroid and neonicotinoid insecticide to bumble bees. *Apidologie*, 50(5), 733–744.

Iwasa, T., Motoyama, N., Ambrose, J. T., and Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera. Crop Protection*, 23(5), 371–378. https://doi.org/https://doi.org/10.1016/j.cropro.2003.08.018

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, 274(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Kopit, A. M., Klinger, E., Cox-Foster, D. L., Ramirez, R. A., and Pitts-Singer, T. L. (2022). Effects of Provision Type and Pesticide Exposure on the Larval Development of *Osmia lignaria* (Hymenoptera: Megachilidae). *Environmental Entomology*, 51(1), 240–251. https://doi.org/10.1093/ee/nvab119

Kopit, A. M., and Pitts-Singer, T. L. (2018). Routes of Pesticide Exposure in Solitary, Cavity-Nesting Bees. *Environmental Entomology*, 47(3), 499–510. https://doi.org/10.1093/ee/nvy034

Krichilsky, E., Centrella, M., Eitzer, B., Danforth, B., Poveda, K., and Grab, H. (2021). Landscape Composition and Fungicide Exposure Influence Host–Pathogen Dynamics in a Solitary Bee. *Environmental Entomology*, 50(1), 107–116. https://doi.org/10.1093/ee/nvaa138

Kueneman, J. G., Gillung, J., van Dyke, M. T., Fordyce, R. F., and Danforth, B. N. (2023). Solitary bee larvae modify bacterial diversity of pollen provisions in the stem-nesting bee*, Osmia cornifrons* (Megachilidae). *Frontiers in Microbiology*, 13. https://doi.org/10.3389/fmicb.2022.1057626

Lehmann, D. M., and Camp, A. A. (2021). A systematic scoping review of the methodological approaches and effects of pesticide exposure on solitary bees. *PLOS One*, 16(5), e0251197.

Lentola, A., David, A., Abdul-Sada, A., Tapparo, A., Goulson, D., and Hill, E. M. (2017). Ornamental plants on sale to the public are a significant source of pesticide residues with implications for the health of pollinating insects. *Environmental Pollution*, 228, 297–304. https://doi.org/https://doi.org/10.1016/j.envpol.2017.03.084

Lozo, J., Berić, T., Terzić-Vidojević, A., Stanković, S., Fira, D., and Stanisavljević, L. (2015). Microbiota associated with pollen, bee bread, larvae and adults of solitary bee *Osmia cornuta* (Hymenoptera: Megachilidae). *Bulletin of Entomological Research*, 105(4), 470–476. https://doi.org/10.1017/S0007485315000292

McFrederick, Q. S., and Rehan, S. M. (2016). Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee. *Molecular Ecology*, 25(10), 2302–2311. https://doi.org/10.1111/mec.13608

Mokkapati, J. S., Bednarska, A. J., and Laskowski, R. (2021). The development of the solitary bee *Osmia bicornis* is affected by some insecticide agrochemicals at environmentally relevant concentrations. *Science of The Total Environment*, 775, 145588. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.145588

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., van Engelsdorp, D., and Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE*, 5(3), e9754. https://doi.org/10.1371/journal.pone.0009754

O'Neal, S. T., Anderson, T. D., and Wu-Smart, J. Y. (2018). Interactions between pesticides and pathogen susceptibility in honey bees. *Current Opinion in Insect Science*, 26, 57–62. https://doi.org/https://doi.org/10.1016/j.cois.2018.01.006

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M., and Sheppard, W. S. (2019). Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study. *Insects*, 10(1), 13. https://doi.org/10.3390/insects10010013

Phan, N. T., Joshi, N. K., Rajotte, E. G., Zhu, F., Peter, K. A., López-Uribe, M. M., and Biddinger, D. J. (2022). Systemic pesticides in a solitary bee pollen food store affect larval development and increase pupal mortality. *BioRxiv*, 2022.05.12.491686. https://doi.org/10.1101/2022.05.12.491686

Philipp, E., K, K. W., Quinn, M., E, A. K., Michael, B. S., Angus, C. J., Scott, C. R., Jacques, D., R, de M. J., Vincent, D., Olivier, E., D, E. J., Laurent, F., L, F. M., Fredrik, G., A, G. J., Laurent, G., Juliette, H., Hauke, K., et al., Benjamin, D. (2016). The Bee Microbiome: Impact on Bee Health and Model for Evolution and Ecology of Host-Microbe Interactions. *MBio*, 7(2), e02164-15. https://doi.org/10.1128/mBio.02164-15

Pohorecka, K., Skubida, P., Miszczak, A., Semkiw, P., Sikorski, P., Zagibajło, K., Teper, D., Kołtowski, Z., Skubida, M., Zdańska, D., and Bober, A. (2012). Pozostałości Insektycydów Neonikotynoidowych w Nektarze i Pyłku Zbieranym Przez Pszczoły z Upraw Rzepaku i Ich Wpływ na Rodziny Pszczele. *Journal of Apicultural Science*, 56(2), 115–134. https://doi.org/10.2478/v10289-012-0029-3

Potts, S. G., Fonseca, V. I., Ngo, H. T., Biesmeijer, J. C., Breeze, T. D., Dicks, L., Garibaldi, L. A., Hill, R., Settele, J., and Vanbergen, A. J. (2016). Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. *IPBES*

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., and Cresswell, J. E. (2018). Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). *Pest Management Science*, 74(3), 541–546.

Reilly, J. R., Artz, D. R., Biddinger, D., Bobiwash, K., Boyle, N. K., Brittain, C., Brokaw, J., Campbell, J. W., Daniels, J., Elle, E., Ellis, J. D., Fleischer, S. J., Gibbs, J., Gillespie, R. L., Gundersen, K. B., Gut, L., Hoffman, G., Joshi, N., Lundin, O., et al., Winfree, R. (2020). Crop production in the USA is frequently limited by a lack of pollinators*. Proceedings of the Royal Society B: Biological Sciences*, 287(1931), 20200922. https://doi.org/10.1098/rspb.2020.0922

Rothman, J., Russell, K., Leger, L., McFrederick, Q., and Graystock, P. (2020). The direct and indirect effects of environmental toxicants on the health of bumble bees and their microbiomes. *Proceedings of the Royal Society B*, 44(951), 287(1937), 20200980. https://doi.org/10.1101/2020.04.24.060228

Sanchez-Bayo, F., and Goka, K. (2014). Pesticide residues and bees–a risk assessment. *PLOS One*, 9(4), e94482.

Schmolke, A., Galic, N., Feken, M., Thompson, H., Sgolastra, F., Pitts-Singer, T., Elston, C., Pamminger, T., and Hinarejos, S. (2021). Assessment of the Vulnerability to Pesticide Exposures Across Bee Species. *Environmental Toxicology and Chemistry*, 40(9), 2640–2651. https://doi.org/10.1002/etc.5150

Schreinemachers, P., and Tipraqsa, P. (2012). Agricultural pesticides and land use intensification in high, middle and low income countries. *Food Policy*, 37(6), 616–626. https://doi.org/https://doi.org/10.1016/j.foodpol.2012.06.003

Sgolastra, F., Hinarejos, S., Pitts-Singer, T. L., Boyle, N. K., Joseph, T., Luckmann, J., Raine, N. E., Singh, R., Williams, N. M., and Bosch, J. (2019). Pesticide Exposure Assessment Paradigm for Solitary Bees. *Environmental Entomology*, 48(1), 22–35. https://doi.org/10.1093/ee/nvy105

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M. T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., and Bosch, J. (2017). Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Management Science,* 73(6), 1236–1243. https://doi.org/https://doi.org/10.1002/ps.4449

Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., and Burgio, G. (2015). Toxicity of spirotetramat on solitary bee larvae, *Osmia cornuta* (Hymenoptera: Megachilidae), in laboratory conditions. *Journal of Apicultural Science*, 59(2), 73–83. https://doi.org/10.1515/JAS-2015-0024

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., and Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, 1(11), 1446. https://doi.org/10.1007/s42452-019-1485-1

Smith, D. P., and Peay, K. G. (2014). Sequence Depth, Not PCR Replication, Improves Ecological Inference from Next Generation DNA Sequencing. *PLOS ONE*, 9(2), e90234-. https://doi.org/10.1371/journal.pone.0090234

Steffan, S. A., Dharampal, P. S., Danforth, B. N., Gaines-Day, H. R., Takizawa, Y., and Chikaraishi, Y. (2019). Omnivory in Bees: Elevated Trophic Positions among All Major Bee Families. *The American Naturalist*, 194(3), 414–421. https://doi.org/10.1086/704281

Steffan, S. A., Dharampal, P. S., Diaz-Garcia, L., Currie, C. R., Zalapa, J., and Hittinger, C. T. (2017). Empirical, Metagenomic, and Computational Techniques Illuminate the Mechanisms by which Fungicides Compromise Bee Health. *JoVE*, 128, e54631. https://doi.org/doi:10.3791/54631

Steffan-Dewenter, I., and Schiele, S. (2008). DO RESOURCES OR NATURAL ENEMIES DRIVE BEE POPULATION DYNAMICS IN FRAGMENTED HABITATS. *Ecology*, 89(5), 1375–1387. https://doi.org/https://doi.org/10.1890/06-1323.1

Stoner, K. A. (2016). Current Pesticide Risk Assessment Protocols Do Not Adequately Address Differences between Honey Bees (*Apis mellifera*) and Bumble Bees (*Bombus* spp.). *Frontiers in Environmental Science*, 4. https://doi.org/10.3389/fenvs.2016.00079

Stoner, K. A., and Eitzer, B. D. (2012). Movement of Soil-Applied Imidacloprid and Thiamethoxam into Nectar and Pollen of Squash (*Cucurbita pepo*). *PLOS ONE*, 7(6), e39114. https://doi.org/10.1371/journal.pone.0039114

Tadei, R., Domingues, C.E.C., Malaquias, J.B., Camilo, E.V., Malaspina, O., and Silva-Zacarin, E. C. M. (2019). Late efect of larval co-exposure to the insecticide clothianidin and fungicide pyraclostrobin in Africanized *Apis mellifera. Scientific Reports, 9:3277.* https://doi.org/10.1038/s41598-019-39383-z

Tadei, R., Menezes-Oliveira, V. B., and Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, 267, 115622. https://doi.org/https://doi.org/10.1016/j.envpol.2020.115622

Tavares, D. A., Dussaubat, C., Kretzschmar, A., Carvalho, S. M., Silva-Zacarin, E. C. M., Malaspina, O., Bérail, G., Brunet, J. L., and Belzunces, L. P. (2017). Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages. *Environmental Pollution*, 229, 386–393. https://doi.org/10.1016/j.envpol.2017.05.092

Team, R. C. (2013). R: A language and environment for statistical computing.

Tesoriero, D., Maccagnani, B., Santi, F., and Celli, G. (2003). Toxicity of three pesticides on larval instars of Osmia cornuta: preliminary results. *Bulletin of Insectology*, 56(1), 169–171.

Thompson, H. (2016). Extrapolation of acute toxicity across bee species. *Integrated Environmental Assessment and Management*, 12(4), 622–626. https://doi.org/https://doi.org/10.1002/ieam.1737

Tomé, H. V. v, Schmehl, D. R., Wedde, A. E., Godoy, R. S. M., Ravaiano, S. v, Guedes, R. N. C., Martins, G. F., and Ellis, J. D. (2020). Frequently encountered pesticides can cause multiple disorders in developing worker honey bees. *Environmental Pollution*, 256, 113420. https://doi.org/https://doi.org/10.1016/j.envpol.2019.113420

Tosi, S., Sfeir, C., Carnesecchi, E., vanEngelsdorp, D., and Chauzat, M.-P. (2022). Lethal, sublethal, and combined effects of pesticides on bees: A meta-analysis and new risk assessment tools. *Science of The Total Environment*, 844, 156857. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.156857

Voulgari-Kokota, A., McFrederick, Q. S., Steffan-Dewenter, I., and Keller, A. (2019). Drivers, Diversity, and Functions of the Solitary-Bee Microbiota. *Trends in Microbiology*, 27(12), 1034–1044. https://doi.org/10.1016/j.tim.2019.07.011

Winfree, R., Reilly, J. R., Bartomeus, I., Cariveau, D. P., Williams, N. M., and Gibbs, J. (2018). Species turnover promotes the importance of bee diversity for crop pollination at regional scales. *Science*, 359(6377), 791–793. https://doi.org/10.1126/science.aao2117

Wu, J. Y., Anelli, C. M., and Sheppard, W. S. (2011). Sub-Lethal Effects of Pesticide Residues in Brood Comb on Worker Honey Bee (*Apis mellifera*) Development and Longevity. *PLOS ONE*, 6(2), e14720-. https://doi.org/10.1371/journal.pone.0014720

Xavier, K. v, KC, A. N., and Vallad, G. E. (2020). Fungicide Application Timing Essential for the Management of Leaf Spot and Fruit Rot on Pomegranate (*Punica granatum* L.) in Florida. *Plant Disease*, 104(6), 1629–1637. https://doi.org/10.1094/PDIS-10-19-2224-RE

Zhang Q, Wang Q, Zhai Y, Zheng H, Wang X (2022). Impacts of Imidacloprid and Flupyradifurone Insecticides on the Gut Microbiota of *Bombus terrestris. Agriculture*, 12(3):389. https://doi.org/10.3390/agriculture12030389

Zhu, W., Schmehl, D. R., Mullin, C. A., and Frazier, J. L. (2014). Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae. *PLOS ONE*, 9(1), e77547-. https://doi.org/10.1371/journal.pone.0077547

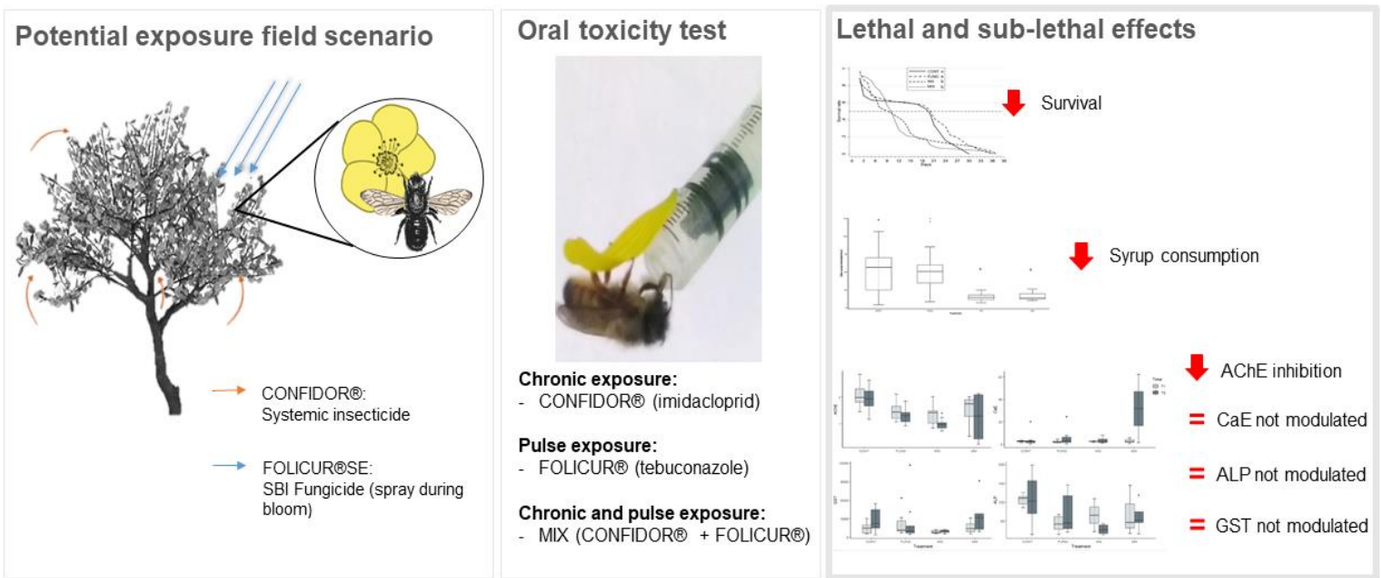
Zioga, E., Kelly, R., White, B., and Stout, J. C. (2020). Plant protection product residues in plant pollen and nectar: A review of current knowledge. *Environmental Research*, 189, 109873. https://doi.org/https://doi.org/10.1016/j.envres.2020.109873

Chapter 2

Biochemical responses, feeding and survival in the solitary bee *Osmia bicornis* following exposure to an insecticide and a fungicide alone and in combination

Cátia Ariana Henriques Martins, Ilaria Caliani, Antonella D'Agostino, Agata Di Noi, Silvia Casini, Martina Parrilli, Celeste Azpiazu, Jordi Bosch, Fabio Sgolastra

Published in *Environmental Science and Pollution Research* (2022), pp. 1-14



Chapter 2- Graphical abstract

ABSTRACT

In agricultural ecosystems, bees are exposed to combinations of pesticides that may have been applied at different times. For example, bees visiting a flowering crop may be chronically exposed to low concentrations of systemic insecticides applied before bloom and then to a pulse of fungicide, considered safe for bees, applied during bloom. In this study, we simulate this scenario under laboratory conditions with females of the solitary bee, *Osmia bicornis* L. We studied the effects of chronic exposure to the neonicotinoid insecticide, Confidor® (imidacloprid) at a realistic concentration, and of a pulse (1 day) exposure of the fungicide Folicur® SE (tebuconazole) at field application rate. Syrup consumption, survival, and four biomarkers: acetylcholinesterase (AChE), carboxylesterase (CaE), glutathione S-transferase (GST), and alkaline phosphatase (ALP) were evaluated at two different time points. An integrated biological response (IBRv2) index was elaborated with the biomarker results. The fungicide pulse had no impact on survival but temporarily reduced syrup consumption and increased the IBRv2 index, indicating potential molecular alterations. The neonicotinoid significantly reduced syrup consumption, survival, and the neurological activity of the enzymes. The co-exposure neonicotinoid-fungicide did not increase toxicity at the tested concentrations. AChE proved to be an efficient biomarker for the detection of early effects for both the insecticide and the fungicide. Our results highlight the importance of assessing individual and sub-individual endpoints to better understand pesticide effects on bees.

Keywords: Biomarkers, IBRv2 index, Imidacloprid, Pesticides, Sub-lethal effects, Tebuconazole

2.1. Introduction

Pesticide use associated with agricultural intensification, is considered one of the main drivers of pollinator declines (Goulson et al., 2015). Although most studies focus on single products or active ingredients, pollinators are usually exposed to combinations of products (Woodcock et al., 2016; Heather et al., 2019). Multi-pesticide exposure may occur due to the application of tank mixtures, but also when different products are applied sequentially. For example, residues of systemic insecticides from treatments performed before bloom (e.g., as seed coating) may appear in the flowers and get mixed with fungicides applied during bloom. Due to their low toxicity for bees, many entomophilous crops are sprayed with fungicides at least once during bloom (Xavier et al., 2020; Almasri et al., 2021). Under this scenario, pollinators experience chronic exposure to residual concentrations of systemic insecticides and acute exposure to high concentrations of fungicides. The levels of insecticides applied before bloom appearing in the pollen and nectar of crop flowers are typically low (Zioga et al., 2020). Some studies have reported that such concentrations pose no lethal risk to bees (Faucon et al., 2005; Maus et al., 2003; Nguyen et al., 2009), but may cause sub-lethal effects. On the other hand, fungicides are not supposed to directly harm insects, but sub-lethal effects, including genotoxicity (Caliani et al., 2021a) and alterations of the feeding behaviour (Zhu et al., 2017a), have been found in *Apis mellifera*. In addition, several studies have demonstrated that some insecticide-fungicide combinations induce synergistic toxicity effects in bees (Johnson et al., 2013; Mengoni Goñalons and Farina, 2018; Pilling et al., 1995; Thompson and Wilkins, 2003; Thompson et al., 2014; Wang et al., 2020 a,b). In particular, sterol biosynthesis inhibiting (SBI) fungicides have been shown to interact with neonicotinoids (Biddinger et al., 2013; Iwasa et al., 2004; Iverson et al., 2019; Raimets et al., 2018; Sgolastra et al., 2017). This interaction occurs because SBI fungicides modify the metabolic detoxification processes in bees by inhibiting cytochrome P450-monooxygenase (Berenbaum and Johnson 2015; Carnesecchi et al. 2019).

Sub-lethal effects are not easy to detect over the course of customary toxicological tests in the laboratory. Syrup consumption is an easy-to-measure fitness endpoint that may provide insights on pesticide-induced changes at the individual level. At the sub-individual level, the detoxification energy costs related with enzymatic activity, may have repercussions on fitness-related traits (Castañeda et al., 2009). For this reason, biomarkers, which can provide signals of early-stage alterations at lower biological levels, represent an important tool to evaluate sub-lethal effects (Caliani et al., 2021a). Different biochemical and cellular biomarkers have been developed and applied in honey bees to assess ecotoxicological health status and the sub-lethal effects of different pollutant compounds such as pesticides, heavy metals and PAHs (Badiou-Bénéteau et al., 2012; Caliani et al., 2021b; Carvalho et al., 2013; Han et al., 2019; Zhu et al., 2017b). Acetylcholinesterase (AChE) and carboxylesterases (CaE) have been widely used as biomarkers to assess the effects of different insecticide classes, such as organophosphates and carbamates, since they mechanistically interact with the nervous tissues of organisms (Sanchez-Hernandez, 2011). Other important biomarkers, such as glutathione-S-transferase (GST) and alkaline phosphatase (ALP), are involved in the biotransformation and detoxification of pollutants, and were first appointed as good candidates to monitor the defences of the honey bee by a neonicotinoid insecticide (Badiou-Bénéteau et al., 2012). In the last years, the search for biomarkers indicative of sub-lethal effects to various organisms has become a priority in ecotoxicological research (López-Uribe et al., 2020; Tlili and Mouneyrac, 2021); however, most studies on bees have only targeted the western honey bee, *A. mellifera*, and studies on solitary bees are mostly lacking (Mokkapati et al., 2022). A research effort on this topic is fundamental

because solitary bees are more sensitive than honey bees to certain pesticides (Arena and Sgolastra 2014; Azpiazu et al., 2021; Sgolastra et al., 2017) and have different routes and levels of exposure (Sgolastra et al., 2019). In fact, the European Food Safety Authority pointed out the necessity to include Osmia spp. as representative species of solitary bees in pesticide risk assessment (EFSA, 2013). *Osmia bicornis* is common European solitary bee that is managed for crop pollination in some areas (Sedivy and Dorn, 2014), and therefore is often exposed to pesticides.

In this study, we conducted a laboratory experiment in which we combined a chronical exposure to a field-realistic concentration of an insecticide (Confidor®, imidacloprid) with a single exposure to a fungicide (Folicur® SE, tebuconazole) in females of the solitary bee *O. bicornis*. Despite the ban on the use of neonicotinoids (imidacloprid, thiamethoxam and clothianidin) in the European Union, their presence in the environment is still reported due to the high persistence of neonicotinoids (Botías et al., 2016; Wintermantel et al., 2020), and therefore they may still pose a threat to pollinators. In addition, neonicotinoids are still widely used in non-EU countries (Goulson, 2020). We thus simulated a scenario in which bees foraging on flowers with residual concentrations of a systemic insecticide are exposed to a high fungicide dose applied during bloom. Our study has important implications for pesticide risk assessment: first, current risk assessment schemes are mostly based on single compounds (Rortais et al., 2017), even in the face of increasing evidence that pollinators are exposed to mixtures of pesticides (Sgolastra et al., 2020); second, current risk assessment schemes mostly overlook sub-lethal effects such as behavioural and physiological responses that may affect bee health even when no effects on survival are detected (Azpiazu et al., 2019; Cresswell, 2011; Sandrock et al., 2014); third, pesticide risk assessment has traditionally relied on a single species, the western honey bee, although pesticide effects may be species-dependent (Schmolke et al., 2021), and extrapolation from honey bees to wild bees may not adequately reflect realistic scenarios due to colony resilience in honey bees (Rundlöf et al., 2015).

In this study we measured syrup consumption and survival at the individual level, as well as a set of biomarkers covering various biological responses, including neurotoxicity (AChE and CaEs) and metabolic activity (GST and ALP). We also propose the development of an Integrated Biological Response (IBRv2) index (Sanchez et al., 2013) providing a measure of the overall response of the target organism to the exposure of pesticides in *O. bicornis*. This index is based on the biomarker deviation from the reference site, allowing the identification of how each selected biomarker contributes to the final toxicological status (Arrighetti et al., 2019). To our knowledge, IBRv2 indexes have not been developed for insect pollinators except for honey bees (Caliani et al., 2021 a,b), but they are widely used to investigate the effects of different contaminants on other groups of organisms. Our goal was to assess whether the chronic exposure to the insecticide, the fungicide pulse, and the insecticide-fungicide combination elicited some biomarker responses that could be related to syrup consumption and survival.

2.2. Materials and methods

2.2.1. Pesticides

We used commercially available formulates, Confidor® (imidacloprid 20% w/v) and Folicur® SE (tebuconazole 4.35% w/v), rather than active ingredients. The two pesticides were chosen because they are extensively used for pest and disease control in bee-pollinated crops such as fruits, nuts and vegetables. Many studies have documented co-occurrence of the two active

ingredients in nectar and pollen samples (Chauzat et al., 2006, 2009, 2011; David et al., 2015, 2016; ; Lentola et al., 2017; Mullin et al., 2010; Ostiguy et al., 2019; Pohorecka et al., 2012).

Stock solutions of each pesticide were prepared by dissolving the products in distilled water at nominal concentrations of 50 µg L^{-1} of Confidor®, and 1850 mg L^{-1} of Folicur® SE. The stock solutions were then diluted in a feeding solution (sugar and distilled water at 47.5% w/v; henceforth syrup) to achieve the desired concentrations of 5 µg L^{-1} and 185 mg L^{-1} of imidacloprid and tebuconazole, respectively. The final concentration of the syrup given to bees was 38% w/v (Azpiazu et al., 2019). The concentration of imidacloprid was within the range of residues found in nectar collected from flowers of different crops, either grown from imidacloprid-coated seed or treated via soil or spray applications (citrus: 0.8 – 6.82 ng mL^{-1} [Byrne et al., 2014]; apples: 2 – 70 ppb [Heller et al., 2020]; cucurbits: 3.8 - 7.3 ng g^{-1} and 6.7 - 16 ng g^{-1} [Dively and Kamel, 2012], 5 – 14 ppb [Stoner and Eitzer, 2012]; sunflower: 0.0019 (±0.001) mg kg^{-1} [Schmuck et al., 2001]; ornamental plants: < 1.2 - 5.7 ng g^{-1} [Lentola et al., 2017]). For tebuconazole, we worked with the potential concentration immediately after spray application, calculated as the maximum field application rate of its commercial formulation (6.45 L ha^{-1}) in orchards.

2.2.2. *Osmia bicornis* and test conditions

Bees were supplied by Pollinature Srl. Cocoons were shipped to the Department of Agricultural and Food Sciences, University of Bologna, Italy, and kept at wintering temperatures of 3-4 °C and 55 ± 10% relative humidity. In May 2021, large cocoons, expected to be females, were incubated at 22-23 °C until emergence. We worked with newly emerged females (<24h old). Over a period of 5 days, emerging females were distributed randomly and equally among the four exposure treatments (see below). Upon emergence, females were transferred to a Plexiglas flight cage (50 x 50 x 50 cm) for meconium deposition and 24 h starvation. 240 bees (60 bees per treatment) were then transferred to individual cages (transparent plastic cups; volume: 150 cc), with perforated lids to allow air circulation. Each cup was provided with a syrup feeder consisting of a 1 mL calibrated syringe (BEROJECT® III, accuracy: 0.02 mL) inserted laterally and slightly inclined. A petal of *Euryops* (Asteracea) was attached to the tip of the syringe to enhance prompt location of the feeder by the bee (Azpiazu et al., 2019; Sgolastra et al., 2018). From emergence until death, bees were maintained at 21-24 °C and 50-55% relative humidity under natural light, avoiding direct sunlight to reduce pesticide degradation.

2.2.3. Exposure conditions

After 24 h of starvation, bees were divided into 4 groups: control (CTRL), insecticide Confidor® (INS), fungicide Folicur® SE (FUNG) and the two pesticides (MIX). Bees of the CTRL treatment were fed regular syrup throughout the experiment. Bees of the FUNG treatment were also fed regular syrup throughout the experiment except on day 3 when they were offered syrup with fungicide. Bees of the INS treatment were fed syrup with insecticide throughout the experiment. Bees of the MIX treatment were also fed syrup with insecticide throughout the experiment, except on day 3 when they were fed syrup with insecticide and fungicide. In the treatment groups FUNG and MIX, the solution with fungicide was only offered for a period of 24 h to simulate a pulse exposure. This exposure scenario represents a compromise between a worst-case scenario, which does not account for fungicide degradation during the 24 hours, and a best-case scenario in which the fungicide is completely degraded in one day. Tebuconazole is known to be a stable compound under hydrolytic and photolytic conditions (EFSA, 2014; Lewis et al., 2006). In all cases, bees were fed ad libitum throughout the experiment. To avoid fungal proliferation, feeding solutions were freshly prepared every 3 days.

2.2.4. Syrup consumption and survival

Syrup consumption and survival were monitored daily until all bees died. To account for potential evaporation, syrup levels were measured in eight cages without bees. After dead, the head width of each bee was measured under a stereomicroscope with a micrometer as a proxy of body size (Bosch and Vicens, 2002).

2.2.5. Collection of tissue samples

Ten bees per treatment were collected for biomarker assessment at two different time points: T1 (on the fourth day of exposure, that is 24 h after the fungicide pulse) and T2 (on the sixth day of exposure, that is 72 h after the fungicide pulse). Bees were anesthetized in ice (4 °C) for 30 min and then the midgut and the head were removed and immediately frozen at - 80 °C.

2.2.6. Biomarker analysis

For each specimen, the head and midgut were processed separately to obtain the extracts on which to perform the enzymatic tests. Nervous tissue extracts from the head were used to evaluate AChE and CaE, and midgut extracts were used to measure GST and ALP activities. Tissues were weighted, and extraction medium was added proportionally to the weight of the tissue at a ratio of 10% (w/v). The buffer contained 40 mM Na phosphate buffer (pH 7.4), a mixture of protease inhibitors enzymes and 1% Triton X-100. The samples were homogenized by a tissue lyser (Qiagen) at 20 F for three periods of 30 s at 30 s intervals. The homogenates were centrifuged at 4 °C for 20 min at 13,000g and 15,000g for head and gut samples, respectively. The resulting supernatants were frozen at - 80 °C and used for the analyses.

AChE

The AChE activity was assayed in the head extracts according to Ellman et al. (1961) with modification from Caliani et al. (2021b). The reaction mixture was prepared in a 3 mL cuvette and contained 0.1 M sodium phosphate buffer (pH 7.4), 10 mM DTNB, 41.5 mM acetylthiocholine and 5 µL head extract. The activity was monitored continuously with a spectrophotometer (Agilent CARY UV60) for 5 min at 410 nm (25 °C) and expressed in μmol^{-1} g $tissue^{-1}$ min.

CaE

The CaE activity was measured in the head extracts and quantified at 538 nm according to Caliani et al. (2021a). A mixture containing 100 mM sodium phosphate buffer (pH 7.4) and 0.1 mL head extract was prepared and incubated at 25 °C for 5 min. The reaction was started by adding 0.4 mM α-NA as a substrate. After 3 min, the reaction was stopped adding 1.5 % SDS and 0.4 mg/L Fast Garnet GBC. The products of the reaction were quantified spectrophotometrically (Agilent CARY UV60) at 538 nm (25 °C) and the enzyme activity was expressed as nmol α-NA min^{-1} mg^{-1} protein ($\varepsilon = 23.59 \times 103$ mM^{-1} cm^{-1}).

GST

The GST activity was measured in the midgut samples following the method of Habig et al. (1974), modified. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.4), 8 mM GSH (reduced glutathione), 8 mM CDNB and 30 µL extract. The conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) was recorded spectrophotometrically (Agilent CARY UV60) at 340 nm (25 °C) and expressed as nmol CDNB conjugate formed min-1 mg-1 protein ($\varepsilon = 9.6 \times 103$ mM-1 m^{-1}).

ALP

The ALP activity was assayed in the midgut samples following the formation of p-nitrophenol, a product of the hydrolysis of the substrate (PNPP) due to the enzyme's activity, according to Bounias et al. (1996), modified. The reaction mixture consisted of 100 mM Tris-HCl buffer (pH 8.5), 100 mM MgCl2, 100 mM p-NPP as the substrate and 25 µL gut extract. The reaction was monitored continuously for 5 min at 405 nm (25 °C) at the spectrophotometer (Agilent CARY UV60) and the activity was expressed as nmol p-nNPP min^{-1} mg^{-1} protein (ε = 18.81 × 103 mM^{-1} cm^{-1} cm^{-1}).

2.2.7. Protein concentrations

Protein concentrations were measured according to the method of Bradford (1976) by BioRad Protein Assay (BioRad), using bovine serum albumin (BSA) as standard.

2.3. Statistical analysis

Statistical analysis was carried out with STATA (StataCorp, 2015) and data visualization with R software (R Core Team, 2013). Only bees that consumed at least 10 µL on the first 2 days of exposure were included in the analyses. Individuals collected for biomarker analysis were not included in the syrup consumption and survival analyses.

To avoid confounding effects of reduced syrup consumption due to ageing, we used daily syrup consumption data only up to the median survival date of each treatment. Differences in daily consumption among treatments were analysed at three different times (the first 2 days of exposure or "pre-pulse", the day of fungicide exposure or "pulse", and between the day after fungicide exposure until the median survival date "post-pulse"). We used the Kruskall-Wallis (KW) non-parametric test to detect differences among treatments in daily syrup consumption at each time. Dunn's test (with Benjamini–Hochberg correction) was performed for pairwise multiple-comparison. A Mann-Whitney U Test for paired samples was used to test for differences among pre-pulse and post-pulse periods and treatments. Bees that died before the post-pulse period, were excluded from this analysis.

Survival functions $S(t)$ were estimated using a Kaplan-Maier estimator with no censoring. Accordingly, $S(t)$ was estimated as $1 - F_n(t)$, where $F_n(t)$ is the empirical cumulative distribution function.

The comparison of survival rates between CTRL and the other treatments was performed using the Fleming–Harrington test, belonging to the weighted log-rank test $G^{\rho,\lambda}$ class (Fleming and Harrington, 2011). We used $G^{1,1}$ to detect differences between treatments especially in the intermediate section of the survival curves.

We performed a Kernel regression (KR) to detect a possible relationship between body size (measured as head width) and syrup consumption, and Cox regression model to detect the potential effect of body size on survival time.

Biomarker data were first analysed by comparing the median of the two collection times (T1 and T2) for each biomarker and treatment. KW non-parametric test and Dunn's test were conducted. Spearman's rank correlation coefficient was used to explore the relationship between pairs of biomarkers. Lastly, Integrated Biological Response (IBRv2) index (Sanchez et al., 2013) was used to quantify in a single value the overall degree of response to each treatment, in which higher IBRv2 values represent a higher stress level. Results are reported with a significance level of 5%.

2.4. Results

2.4.1. Syrup consumption

Significant differences in syrup consumption were found between treatments with and without the insecticide (Dunn's test; p≤ 0.0001; Fig. 1 and Table A2.1); overall, bees from INS and MIX consumed approximately 74% less syrup than bees of the CTRL and FUNG treatments. The fungicide pulse (FUNG) caused a decrease in feeding rate, which returned to control levels over the post-pulse period. Overall, syrup consumption significantly (p<0.0001) decreased from the pre-pulse to the post-pulse assessments in all treatments (see Table A2.2 for Mann-Whitney U Test results). Kernel regression analysis indicates that body size had no effect on daily syrup consumption (Table A2.3).

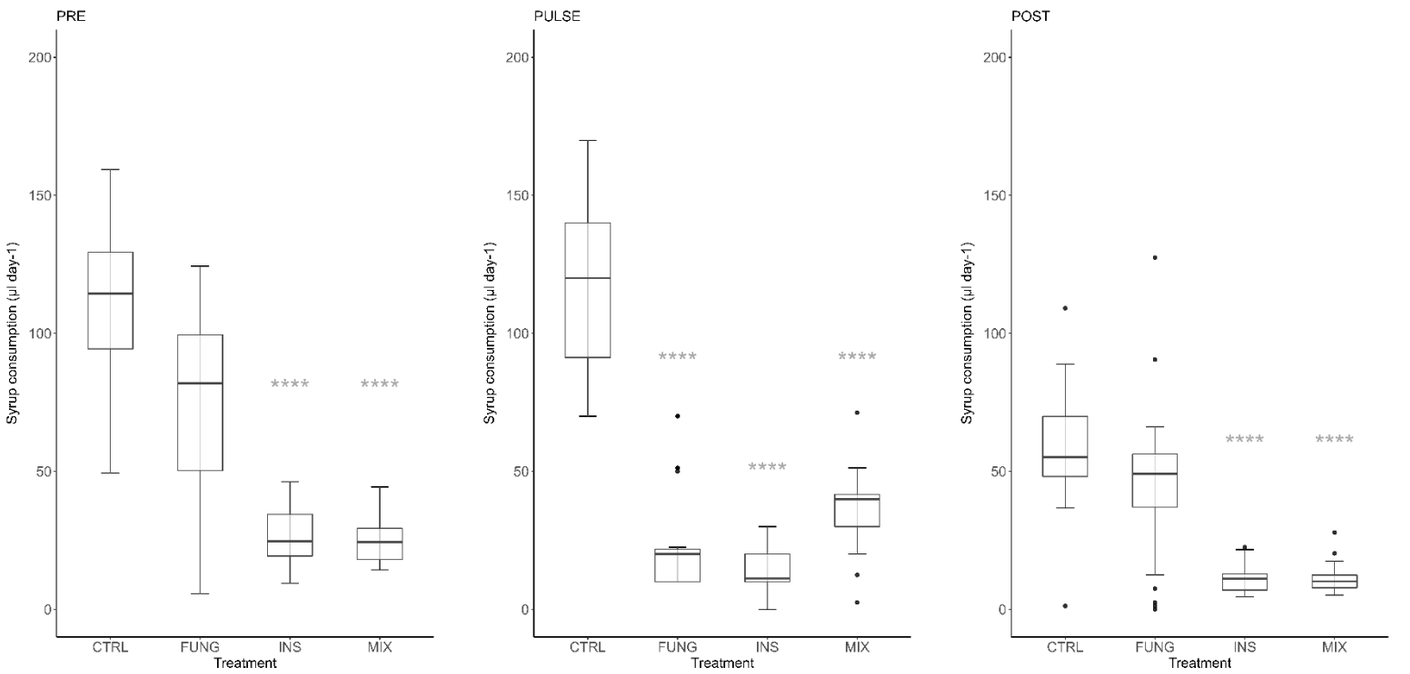


Figure 2.5: Daily syrup consumption (µl day^{-1}) up to the date of 50% mortality within each treatment. CTRL: control (n=25), FUNG: tebuconazole (n=26), INS: imidacloprid (n=26), MIX: tebuconazole + imidacloprid (n=24). PRE: first 2 days of exposure; PULSE: day 3; POST: after the 3rd day up to the date of 50% mortality within each treatment. Boxplots with asterisks are significantly different from the control (Dunn's Pairwise Comparison, ****p < 0.0001).

2.4.2. Survival analysis

Exposure to INS and MIX had an effect on survival of *O. bicornis* females. Survival significantly differed among treatments (p < 0.01) in the intermediate part of the distribution curves (Fig. 2 and see Table A2.4 for results of Fleming–Harrington tests). Median survival time dropped from approximately 21 days for CTRL and FUNG bees to 11 days for INS and MIX bees. Body size had no effect on survival (see Table A2.5 for Cox model results).

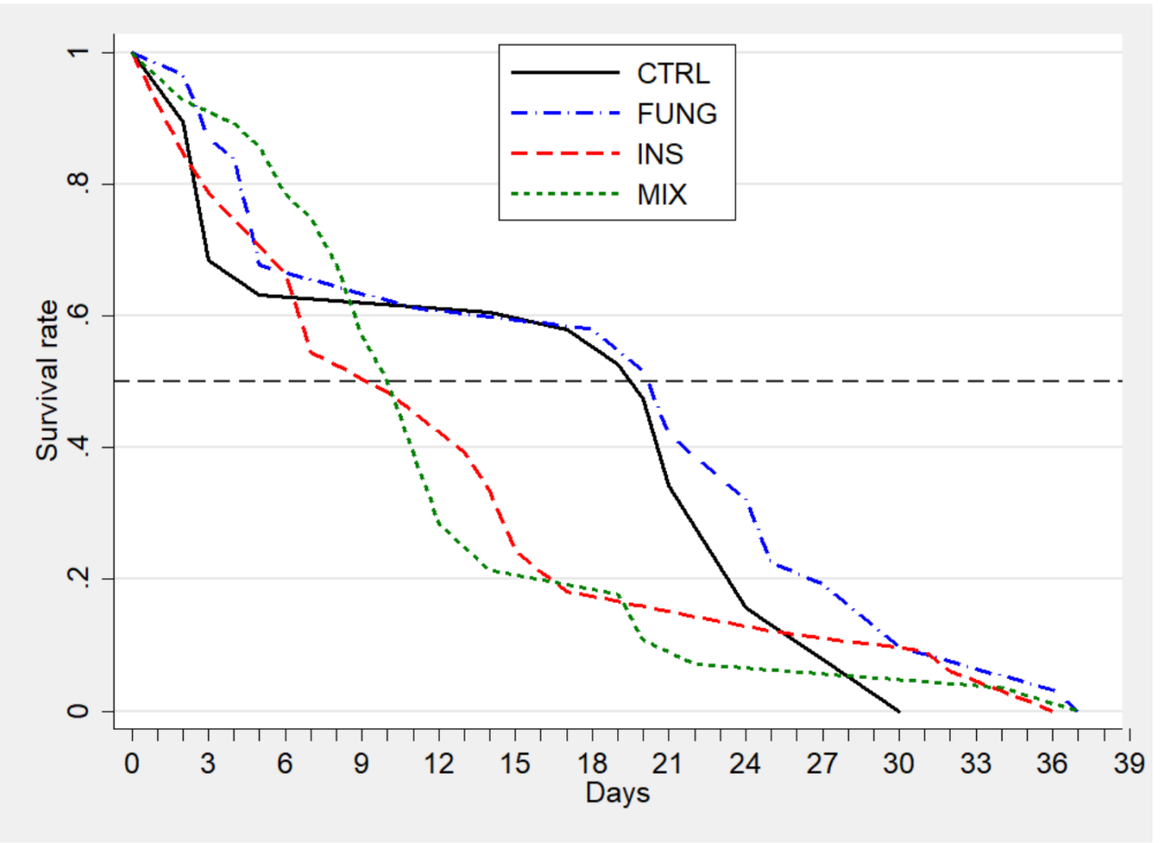


Figure 2.6: Survival curves of *Osmia bicornis* females orally exposed to various pesticide treatments. CTRL: control (n=38); FUNG: tebuconazole (n=31); INS: imidacloprid (n=33); MIX: tebuconazole + imidacloprid (n=27). The dashed line indicates 50% survival rate.

2.4.3. Biomarkers

Biomarkers of neurotoxicity (AChE and CaE) and metabolic activity (ALP and GST) were assessed on days 4 (T1) and 6 (T2), that is 24 h and 72 h after the fungicide pulse, respectively. The results of the four biomarkers at T1 and T2 are shown in Figure 3. The results of the descriptive statistics, the Kruskal-Wallis tests for the assessment of statistically significant differences among groups for each biomarker and syrup consumption at T1 and T2, and the Dunn's Pairwise Comparison with the control group are summarized in the supplementary material (Tables A2.6, A2.7 and A2.8). AChE activity was significantly inhibited in the INS treatment at both times compared to the control (Dunn's test; T1, $p<0.01$; T2, $p<0.001$); AChE was also significantly inhibited in FUNG treatment at T1 (Dunn's test; $p<0.05$). No significant differences were observed for CaE, GST and ALP activity, in none of the assessment times. Overall, we found a significant positive correlation between ALP and GST at T1 ($p<0.05$; $\rho= 0.666$) and at T2 ($p<0.001$; $\rho=0.806$) and a positive and significant correlation between syrup consumption and AChE activity at T1 ($p<0.05$; $\rho=0.4072$) and T2 ($p<0.01$; $\rho=0.4710$).

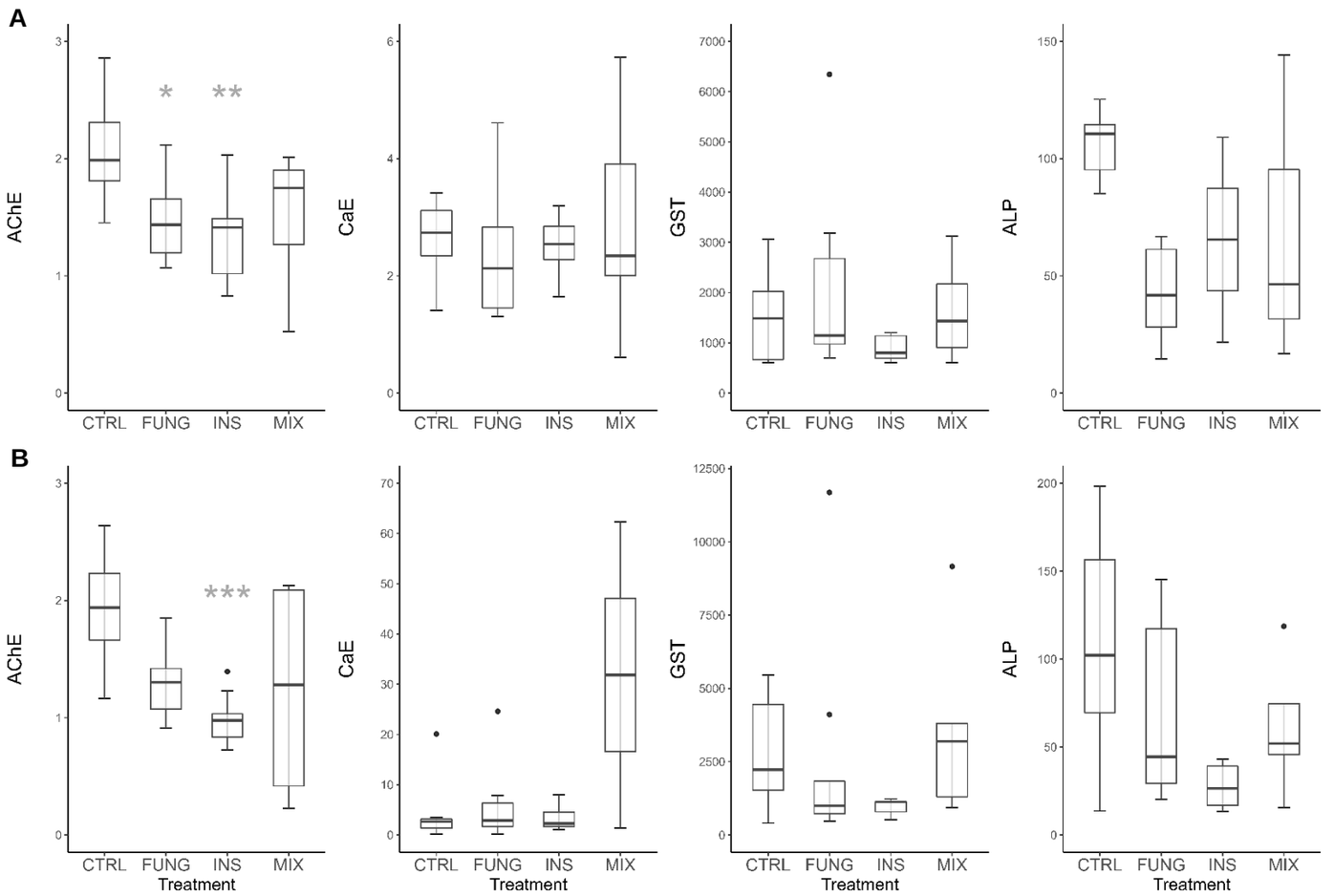
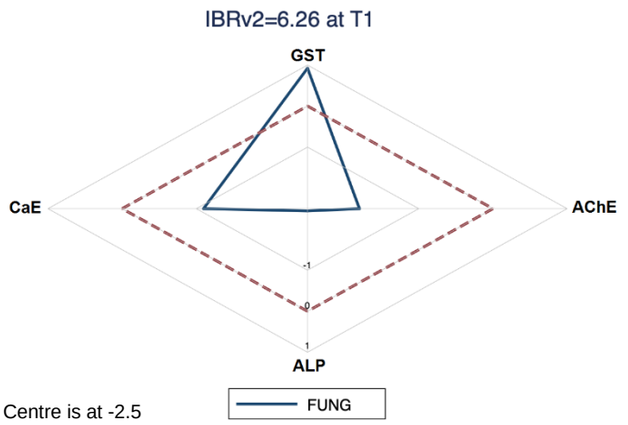


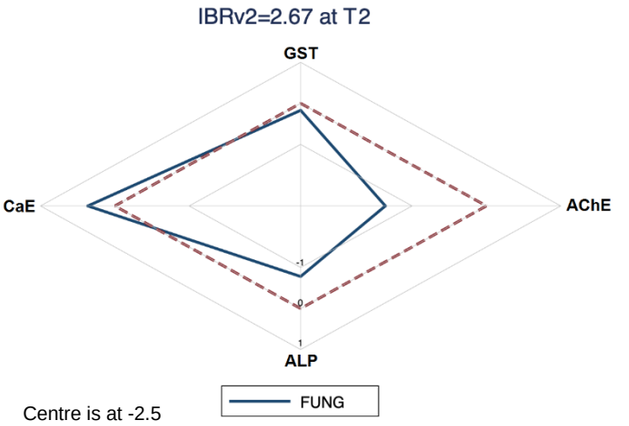
Figure 2.7: Activity of biomarkers AChE (μmol^{-1} g tissue^{-1} min), CaE (nmol min^{-1} mg^{-1} protein), GST (nmol min^{-1} mg^{-1} protein) and ALP (nmol min^{-1} mg^{-1} protein) in *Osmia bicornis* females orally exposed to various pesticide treatments. CTRL: control, FUNG: tebuconazole, INS: imidacloprid, MIX: tebuconazole + imidacloprid. Measurements were taken at T1 (day 4 of exposure; **A**) and T2 (day 6 of exposure; **B**). Boxplots with asterisks are significantly different from the control (Dunn's Pairwise Comparison, *p <0.05; **p<0.01, ***p<0.001).

2.4.4. IBRv2

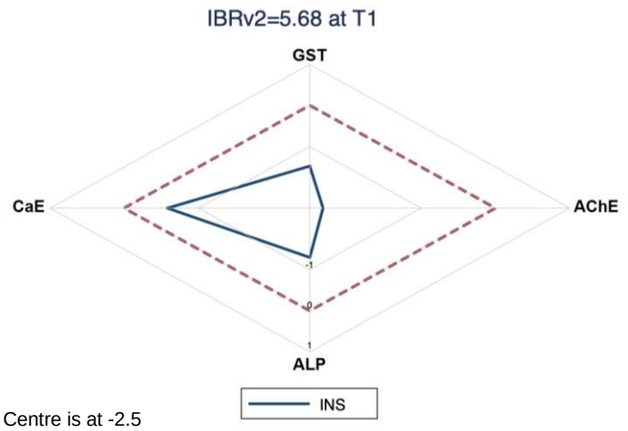
The results of the Integrated Biological Response (IBRv2) for each treatment are shown in Figure 4. In the FUNG treatment, the IBRv2 value declined from T1 (6.26) to T2 (2.67). The most discriminant factor for this treatment shifted from GST at T1 to CaE at T2. Bees exposed to INS and MIX showed increasing IBRv2 values from T1 to T2, with CaE as the predominant factor in all the star plots. The MIX treatment showed the lowest IBRv2 at T1 (4.09).



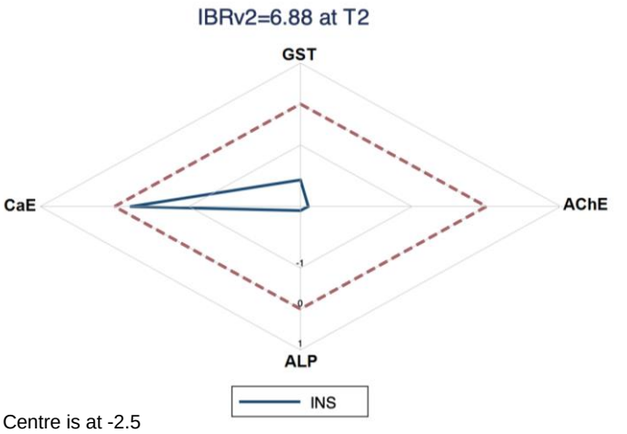
Centre is at -2.5



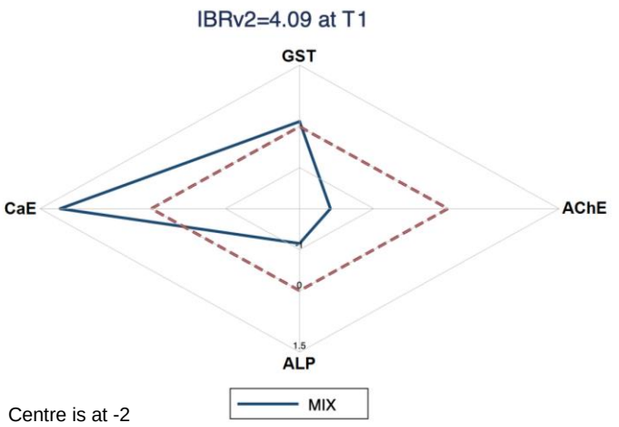
Centre is at -2.5



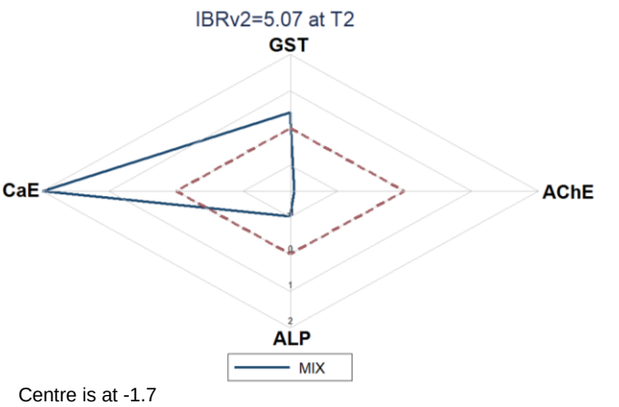
Centre is at -2.5



Centre is at -2.5



Centre is at -2



Centre is at -1.7

Figure 2.8: Star plots of the integrated biological response (IBRv2) in *Osmia bicornis* females orally exposed to three pesticide treatments. FUNG, tebuconazole; INS, imidacloprid; MIX, tebuconazole + imidacloprid. Measurements were taken at T1 (day 4 of exposure) and T2 (day 6 of exposure). The dashed line indicates the control values.

2.5. Discussion

In this study, we tested the effects of oral co-exposure to a neonicotinoid, imidacloprid, and a SBI fungicide, tebuconazole, on adult *O. bicornis* females. Our first objective was to establish whether exposure to a fungicide pulse could enhance the toxicity of low-level chronic exposure to the neonicotinoid. Our second objective was to identify neurotoxicity and metabolic activity biomarkers that could act as early warning signals of sub-lethal effects.

Our results clearly indicate a feeding reduction due to the continued feeding of imidacloprid, which agrees with the findings of other studies (Azpiazu et al., 2019; Zhu et al., 2017a). The fungicide pulse at the maximum field application concentration caused a temporary decrease in feeding rate but did not affect post-pulse syrup consumption. In addition, the fungicide pulse did not impact the feeding of bees of the MIX treatment. A study in which bumble bees were exposed to the combination imidacloprid-imazalil also failed to find synergistic effects on feeding rate (Raimets et al. 2018). The observed effect of imidacloprid on syrup consumption may be related to the reduction of bee mobility, leading bees to ingest less syrup (Medrzycki et al., 2003; Wu et al., 2017).

In addition, the median survival time of bees exposed to imidacloprid at 5 µg L^{-1} (alone and in combination with the fungicide) was significantly shorter (e.g. 10 days) than in bees from CRTL and FUNG treatments. In our study, bees were chronically exposed to a constant concentration of imidacloprid throughout their lifespan, an approach that does not account for pesticide degradation over time or “dilution effect” due to visitation to uncontaminated flowers. However, levels of imidacloprid higher than the concentration tested in our study have been found in the flowers of crop and non-crop plants potentially extending the period of pesticide exposure beyond the blooming of the target crop (Botías et al., 2016; Wintermantel et al., 2020). Interestingly, the toxicity of imidacloprid in our study was higher than in a previous study that used a 3-fold higher concentration of the same commercial product, Confidor®, on *O. bicornis* females (Azpiazu et al., 2019). Median mortality time in the control bees of the two studies was similar (19 and 20 days, respectively), but the median mortality time of the group treated with imidacloprid was 10 days in our study compared to 16 days in Azpiazu’s study (Azpiazu et al., 2019). These differences could be explained by the different diet offered to the bees. Our bees were provided with syrup only whereas those of Azpiazu et al. (2019) also had access to pollen. Several studies have shown that pollen feeding positively affects health and longevity in honey bees (Huang, 2012; Pasquale et al., 2013) and may mitigate the negative impact of pesticides (Castle et al., 2022).

In our study, the fungicide did not affect bee survival, even when combined with the insecticide. Some oral acute exposure studies have found a synergistic effect of the fungicide propiconazole on the toxicity of the neonicotinoid clothianidin (Sgolastra et al., 2018, 2017). In contrast, the tebuconazole pulse did not reduce the survival of *O. bicornis* females chronically exposed to imidacloprid in our study. These results are in line with other studies in which honey bees chronically exposed to imidacloprid-tetraconazole (Zhu et al., 2017b) and imidacloprid-difenoconazole (Pal et al., 2022) mixtures did not yield synergistic effects; similarly, no interactions between imidacloprid (15 µg L^{-1}) and myclobutanil were found following chronic oral exposure in *O. bicornis* (Azpiazu et al., 2019).

We assessed four selected biomarkers (AChE, CaE, GST and ALP) to determine the impact of the two pesticides at the neurological and metabolic levels. AChE, an important enzyme responsible for the hydrolyses of acetylcholine at the cholinergic synapses (Badiou-Bénéteau et

al., 2012), allows the control and modulation of neural transmission (Badiou et al., 2008). In our work, AChE was significantly inhibited by the tebuconazole (27% reduction, at T1) and by the imidacloprid (29% reduction, at T1 and 49% at T2), indicating a clear neurotoxic effect of the two pesticides and confirming AChE as an excellent biomarker for the assessment of sub-lethal effects in *O. bicornis*.

The levels of inhibition can be considered relevant in altering the proper functioning of the nervous system. AChE inhibition has been associated with exposure to some classes of insecticides, such as carbamates and organophosphates (Fulton and Key, 2001; Rabea et al., 2010). To date, AChE activity is also used for studying the neonicotinoids and their metabolites neurotoxic effects (Boily et al., 2013; Gyori et al., 2017; Samson-Robert et al., 2015; Shao et al., 2013). As observed by Badawy et al. (2015), neonicotinoids such as dinotefuran (nitro-substituted compound) and acetamiprid (cyano-substituted), enhance the inhibition of AChE activity in honey bees after exposure to different field relevant doses, even though AChE is not the target site of neonicotinoids. In our study, we also observed a neurotoxic effect positively correlated to alterations in the feeding behaviour of *O. bicornis*. Caliani et al. (2021b) found a neurotoxic effect of Amistar® Xtra (a.i. azoxystrobin), but no data were produced regarding syrup consumption. The inhibitory effect on the AChE activity by tebuconazole, related with immobility, has been also reported in aquatic organisms (Altenhofen et al., 2017; Lebrun et al., 2021). The use of fungicides may also be associated with sublethal effects disrupting the bee's overall fitness and behaviour (Artz and Pitts-Singer, 2015; Fisher II et al., 2021). As for the MIX group, in our study non-statistically significant alterations in this enzyme activity were observed. We can hypothesize the absence of a synergic effect of the two pesticides because we did not observe the highest inhibition in the MIX group. Yet, we cannot exclude an antagonistic or a predominant effect of one compound over the other.

CaE are phase-I detoxifying enzymes that mainly hydrolyse non-polar carboxyl esters (Badiou-Bénéteau et al., 2012; Barata et al., 2005; Stone et al., 2002). Besides, they also play a role in the defence mechanism, protecting AChE from the inactivation caused by organophosphates and carbamates. Several studies have also shown differential expression of CaEs after exposure to pesticides (Badiou-Bénéteau et al., 2012; Zhu et al., 2017a,b). In our study, CaE was not modulated by the fungicide or the insecticide. This result, together with the AChE inhibition, leads us to hypothesize that the AChE was the most affected enzyme.

The main role of the phase-II metabolizing GST isoenzymes is to catalyse the reaction with reduced glutathione (GSH) and conjugate xenobiotic compounds, facilitating their detoxification (Shi et al., 2012). The tendency for the decreased of GST activity, in particular in INS treatment at T1 and T2, could be indicative of an organism's attempt to respond to an oxidative stress condition. This result could be expected, since imidacloprid is known to induce metabolic disruptions and oxidative stress in honey bees and other animals (Nicodemo et al., 2014; Powner et al., 2016).

ALP is included in the final process of digestion and in the mechanism of active membrane transport (Cheung and Low, 1975; Srivastava and Saxena, 1967). Although ALP is not involved in detoxification processes, its activity can be modulated in reaction to chemical stress. In our study, the ALP activity was not statistically inhibited by any treatment or time, although we observed an overall decrease in its activity. Other studies showed a modulation of ALP in honey bees exposed to insecticides, such as fipronil, spinosad, imidacloprid, or following infection by Nosema (Carvalho et al., 2013; Dussaubat et al., 2012; Kairo et al., 2017; Paleolog et al., 2020).

An inhibition was also found by Caliani et al. (2021a), after honey bee exposure to fungicides and heavy metals. A previous study (Almasri et al., 2020) on honey bees, did not find ALP modulation after the administration of combinations of imidacloprid, glyphosate and difenoconazole. We also found a positive correlation between GST and ALP at both times. The positive correlation between these two enzymes could indicate that both are affected by imidacloprid and tebuconazole.

We used the IBRv2 index to integrate the responses of the selected biomarkers (AChE, CaE, GST and ALP). This approach facilitates the visualization of the spatial arrangement of different enzymatic responses and the possible effects of different contaminant compounds. At T1, the FUNG treatment showed the highest IBRv2 value, followed by the INS treatment and finally the MIX treatment. This result indicates that the fungicide alone induced a high oxidative stress, particularly expressed by GST activity, although no difference was found between treatments. Previous studies also found increased GST response after fungicide exposure (Han et al., 2014; Johansen et al., 2007). Since the fungicide was administered as a pulse, we expected an improvement of the organisms' health status at T2 that was confirmed by the IBRv2 lowest value. This result suggests that the bees are able to biotransform and detoxify when they are not chronically exposed to the fungicide. On the other hand, the IBRv2 value increased from T1 to T2 in the INS treatment, as expected given the continuous exposure to the pesticide. This treatment group is also the one that shows the highest IBRv2 value at T2. This could be due to the fact that the bees of FUNG group at T2 were not exposed anymore to the fungicide, and they were recovering from the fungicide pulse exposure, while bees of the INS treatment were in contact with the pesticide for a prolonged period of time. As with the INS treatment, the IBRv2 value of the MIX treatment was higher at T2 than at T1. The MIX IBRv2 value confirms the results of FUNG at T2, indicating recovery from the fungicide pulse. The obtained MIX value is probably due to the insecticide action only. These results confirm that biomarkers can be a useful tool in the framework of pesticide risk assessment as an early warning signal of pesticide side effects on bees in post-registration monitoring programs.

2.6. Conclusions

Our study demonstrates that exposure to the commercial insecticide Confidor® and fungicide Folicur® may impact the solitary bee *O. bicornis* at different levels of biological organization: from enzymatic responses to feeding rate and survival. Our results showed that: (i) chronic exposure to residual concentrations of imidacloprid affected feeding and survival of this solitary bee; (ii) an acute exposure to a fungicide, considered safe to use during bloom, had a temporary sub-lethal impact; (iii) contrary to our expectation, the pulse of fungicide did not exacerbate the effects of imidacloprid. As for the molecular tools, one of the four biomarkers tested, AChE, was inhibited by the fungicide and the insecticide, showing promise as an indicator of sub-lethal effects in *O. bicornis*. The IBRv2 index proved to be a powerful tool to describe the toxicological status of *O. bicornis*, highlighting a good ability of the bees to recover from the fungicide pulse, while a chronic exposure to INS caused increased sub-lethal effects. No effects of the binary mixture were observed. Overall, this study provides evidence for improving the current risk assessment procedures by including sub-lethal endpoints and other bee species in addition to *A. mellifera*.

Acknowledgements

We appreciate the assistance of R. Spinelli and K. Palpurtha during data collection.

References

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tehamitchian, S., Cousin, M., Brunet, J.L., and 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', *Ecotoxicology and Environmental Safety*, 203:111013. https://doi.org/10.1016/j.ecoenv.2020.111013

Almasri, H., Tavares, D.A., Tchamitchian,S., Pélissier, Sené, D., Cousin, M., Brunet, J-L., and Belzunces, L.P. (2021) 'Toxicological status changes the susceptibility of the honey bee *Apis mellifera* to a single fungicidal spray application', *Environmental Science and Pollution Research*, 28(31):42807–42820. https://doi.org/10.1007/s11356-021-13747-3

Altenhofen, S., Nabinger, D. D., Wiprich, M. T., Pereira, T. C. B., Bogo, M. R., and Bonan, C. D. (2017) 'Tebuconazole alters morphological, behavioral and neurochemical parameters in larvae and adult zebrafish (*Danio rerio*)', *Chemosphere*, 180, 483-490. https://doi.org/10.1016/j.chemosphere.2017.04.029

Arena, M. and Sgolastra, F. (2014) 'A meta-analysis comparing the sensitivity of bees to pesticides', *Ecotoxicology*, 23(3):324–334. https://doi.org/10.1007/s10646-014-1190-1

Arrighetti, F., Landro, S.M., Lambre, M.E., Penchaszadeh, P.E., and Teso, V. (2019) 'Multiple-Biomarker Approach in the Assessment of the Health Status of a Novel Sentinel Mussel *Brachidontes rodriguezii* in a Harbor Area', *Marine Pollution Bulletin* 140: 451–61. https://doi.org/10.1016/j.marpolbul.2019.02.002

Artz, D. R., and 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(8), e0135688. https://doi.org/10.1371/journal.pone.0135688

Azpiazu, C. Bosch, J., Viñuela, E., Medrzycki, P., Teper, D. and Sgolastra, F. (2019) 'Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in a solitary bee', *Scientific Reports*, 9(1):1–11. https://doi.org/10.1038/s41598-019-50255-4

Azpiazu, C., Bosch, J., Bortolotti, L., Medrzycki, P., Teper, D., Molowny-Horas, R. and Sgolastra, F. (2021) 'Toxicity of the insecticide sulfoxaflor alone and in combination with the fungicide fluxapyroxad in three bee species', *Scientific Reports*, 11(1):6821. https://doi.org/10.1038/s41598-021-86036-1

Badawy, M. E. I., Nasr, H. M. and Rabea, E. I. (2015) 'Toxicity and biochemical changes in the honey bee Apis mellifera exposed to four insecticides under laboratory conditions', *Apidologie*, 46(2):177–193. https://doi.org/10.1007/s13592-014-0315-0

Badiou-Bénéteau, A., Carvalho, S.M., Brunet, J-L., Carvalho, G.A., Buleté, A., Giroud, B. and Belzunces, L.P. (2012) 'Development of biomarkers of exposure to xenobiotics in the honey bee *Apis mellifera*: Application to the systemic insecticide thiamethoxam', *Ecotoxicology and Environmental Safety*, 82:22–31. https://doi.org/10.1016/j.ecoenv.2012.05.005

Badiou, A., Meled, M. and Belzunces, L. P. (2008) 'Honeybee Apis mellifera acetylcholinesterase—A biomarker to detect deltamethrin exposure', *Ecotoxicology and Environmental Safety*, 69(2):246–253. https://doi.org/10.1016/j.ecoenv.2006.11.020

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N. and Porte, C. (2005) 'Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basin (NE Spain)', *Aquatic Toxicology*, 74(1): 3-19. https://doi.org/10.1016/j.aquatox.2005.04.002

Berenbaum, M. R. and Johnson, R. M. (2015) 'Xenobiotic detoxification pathways in honey bees', *Current Opinion in Insect Science*, 10:51–58. https://doi.org/10.1016/j.cois.2015.03.005

Biddinger, D.J., Robertson, J.L., Mullin, C., Frazier, J., Ashcraft, S.A., Rajotte, E.G., Joshi, N.K., and Vaughn, M. (2013) 'Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski)', *PLOS ONE*. Public Library of Science, 8(9):e72587. https://doi.org/10.1371/journal.pone.0072587

Boily, M., Sarrasin, B., DeBlois, C., Aras, P., and Chagnon, M. (2013) 'Acetylcholinesterase in Honey Bees (*Apis Mellifera*) Exposed to Neonicotinoids, Atrazine and Glyphosate: Laboratory and Field Experiments', *Environmental Science and Pollution Research* 20 (8): 5603–14. https://doi.org/10.1007/s11356-013-1568-2

Bosch, J. and Vicens, N. (2002) 'Body size as an estimator of production costs in a solitary bee', *Ecological Entomology*, 27(2):129–137. https://doi.org/10.1046/j.1365-2311.2002.00406.x

Botías, C., David, A., Hill, E.M., Goulson, D. (2016) 'Contamination of wild plants near

neonicotinoid seed-treated crops, and implications for non-target insects', *Science of The Total Environment*, 566:269-278. https://doi.org/10.1016/j.scitotenv.2016.05.065

Bounias, M., Kruk, I., Nectoux, M., and Popeskovic, D. (1996) 'Toxicology of Cupric Salts on Honeybees. V. Gluconate and Sulfate Action on Gut Alkaline and Acid Phosphatases', *Ecotoxicology and Environmental Safety*, 35(1):67–76. https://doi.org/10.1006/eesa.1996.0082

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, 72(1):248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Byrne, F.J., Visscher, P.K., Leimkuehler, B., Fischer, D., Grafton-Cardwell, E.E., and Morse, J.G. (2014) 'Determination of exposure levels of honey bees foraging on flowers of mature citrus trees previously treated with imidacloprid', *Pest Management Science*. John Wiley & Sons, Ltd, 70(3):470–482. https://doi.org/10.1002/ps.3596

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Ammendola, A., Di Noi, A. Gori, A. and Casini, S. (2021a) 'Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of *Apis mellifera*', *Ecotoxicology and environmental safety*, 208:111486. https://doi.org/10.1016/j.ecoenv.2020.111486

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Giovanetti, L., Di Noi, A. and Casini, S. (2021b) 'First application of an Integrated Biological Response index to assess the ecotoxicological status of honeybees from rural and urban areas', *Environmental Science and Pollution Research*, p.1–11. https://doi.org/10.1007/s11356-021-14037-8

Carnesecchi, E., Svendsen, C., Lasagni, S., Grech, A., Quignot, N., Amzal, B., Toma, C., Tosi, S., Rortais, A., and Cortinas-Abrahantes, J. (2019) 'Investigating combined toxicity of binary mixtures in bees: Meta-analysis of laboratory tests, modelling, mechanistic basis and implications for risk assessment', *Environment international*, 133:105256. https://doi.org/10.1016/j.envint.2019.105256

Carvalho, S.M., Belzunces, L.P., Carvalho, G.A., Brunet, J-L. and Badiou-Beneteau, A. (2013) 'Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honeybee *Apis mellifera* to insecticides', *Environmental toxicology and chemistry*. Wiley Online Library, 32(9):2117–2124. https://doi.org/10.1002/etc.2288

Castañeda, L.E, Figueroa, C.C., Fuentes-Contreras, E., Niemeyer, H.M., and Nespolo, R.F. (2009) 'Energetic Costs of Detoxification Systems in Herbivores Feeding on Chemically Defended Host Plants: A Correlational Study in the Grain Aphid, *Sitobion avenae*',*Journal of Experimental Biology* 212 (8): 1185–90. https://doi.org/10.1242/jeb.020990

Castle, D., Alkassab, A.T., Bischoff, G., Steffan-Dewenter, I., and Pistorius, J. (2022) 'High Nutritional Status Promotes Vitality of Honey Bees and Mitigates Negative Effects of Pesticides', *Science of The Total Environment* 806: 151280. https://doi.org/10.1016/j.scitotenv.2021.151280

Chauzat, M-P., Faucon, J-P., Martel, A-C., Lachaize, J., Cougoule, N. and Aubert, M. (2006) 'A Survey of Pesticide Residues in Pollen Loads Collected by Honey Bees in France', *Journal of Economic Entomology*. Oxford Academic, 99(2):253–262. https://doi.org/10.1093/jee/99.2.253

Chauzat, M-P., Carpentier, P., Martel, A-C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M., and Faucon., J-P. (2009) 'Influence of Pesticide Residues on Honey Bee (Hymenoptera: Apidae) Colony Health in France', *Environmental Entomology*. Oxford University Press (OUP), 38(3):514–523. https://doi.org/10.1603/022.038.0302

Chauzat, M-P., Martel, A-C., Cougoule, N., Porta, P., Lachaize, J., Zeggane, S., Aubert, M., Carpentier, P. and Faucon, J-P. (2011) 'An assessment of honeybee colony matrices, Apis mellifera (Hymenoptera: Apidae) to monitor pesticide presence in continental France', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 30(1):103–111. https://doi.org/10.1002/etc.361

Cheung, W. W. K. and Low, K. W. (1975) 'Ultrastructural and functional differentiation of the midgut of the sugar cane beetle, Protaetia acuminata (F.)(Coleoptera: Cetoniidae)', *International Journal of Insect Morphology and Embryology*, 4(4):349–361. https://doi.org/10.1016/0020-7322(75)90023-9

StataCorp. (2015). Stata 14 Base Reference Manual. College Station, TX: Stata Press.

Cresswell, J. E. (2011) 'A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees', *Ecotoxicology*, 20(1):149–157. https://doi.org/10.1007/s10646-010-0566-0

David, A., Botías, C., Abdul-Sada, A., Goulson, D. and Hill, E.M. (2015) 'Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment', *Analytical and bioanalytical chemistry*. Springer, 407(26):8151–8162. https://doi.org/10.1007/s00216-015-8986-6

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E.L., Hill, E.M. and Goulson, D. (2016) 'Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops', *Environment international*, 88:169–178. https://doi.org/10.1016/j.envint.2015.12.011

Dively, G. P. and Kamel, A. (2012) 'Insecticide Residues in Pollen and Nectar of a Cucurbit Crop and Their Potential Exposure to Pollinators', *Journal of Agricultural and Food Chemistry*. American Chemical Society, 60(18):4449–4456. https://doi.org/10.1021/jf205393x

Dussaubat, C., Brunet, J-L., Higes,M., Colbourne, J.K., Lopez, J., Choi, J-H., Martin-Hernandez, R., Botias, C., Cousin, M. and McDonnell, C. (2012) 'Gut pathology and responses to the microsporidium Nosema ceranae in the honey bee Apis mellifera', *PloS one*, 7(5):e37017. https://doi.org/10.1371/journal.pone.0037017

EFSA (2013) 'Guidance on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees)', *EFSA Journal*, 11(7):3295. https://doi.org/10.2903/j.efsa.2013.3295

EFSA (2014) 'Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole' *EFSA Journal*, 2(1):3485. https://doi.org/10.2903/j.efsa.2014.3485

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) 'A new and rapid colorimetric determination of acetylcholinesterase activity', *Biochemical Pharmacology*, 7(2):88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Faucon, J.-P., Aurières, C., Drajnudel, P., Mathieu, L., Ribière, M., Martel, A.C., Zeggane, S., Chauzat, M.-P., and Aubert, M.F.A. (2005) 'Experimental Study on the Toxicity of Imidacloprid given in Syrup to Honey Bee (*Apis Mellifera*) Colonies', *Pest Management Science* 61 (2): 111–25. https://doi.org/10.1002/ps.957

Fleming, Thomas R, and David P Harrington. (2011). *Counting Processes and Survival Analysis*. John Wiley & Sons.

Fisher II, A., DeGrandi-Hoffman, G., Smith, B.H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J.H. and Harrison, J.F. (2021) 'Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*)', *Environmental Pollution*, 269: 115964. https://doi.org/10.1016/j.envpol.2020.115964

Fulton, M. H. and Key, P. B. (2001) 'Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects', *Environmental Toxicology and Chemistry: An International Journal*. Wiley Online Library, 20(1):37–45. https://doi.org/10.1002/etc.5620200104

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E.L. (2015) 'Bee declines driven by combined Stress from parasites, pesticides, and lack of flowers', *Science*, American Association for the Advancement of Science, 347(6229). https://www.science.org/doi/10.1126/science.1255957

Goulson, D. (2020) 'Pesticides, corporate Irresponsibility, and the fate of our Planet', *One Earth*, 2(4), 302-305. https://doi.org/10.1016/j.oneear.2020.03.004

Gyori, J., Farkas, A., Stolyar, O., Székács, A., Mörtl, M., and Vehovszky, A. (2017) 'Inhibitory Effects of Four Neonicotinoid Active Ingredients on Acetylcholine Esterase Activity', *Acta Biologica Hungarica* 68 (4): 345–57. https://doi.org/10.1556/018.68.2017.4.1

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) 'Glutathione S-Transferases: THE FIRST ENZYMATIC STEP IN MERCAPTURIC ACID FORMATION', J*ournal of Biological Chemistry*, 249(22):7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

Han, W., Yang, Y., Gao, J., Zhao, D., Ren, C., Wang, S., Zhao, S. and Zhong, Y. (2019) 'Chronic toxicity and biochemical response of *Apis cerana cerana* (Hymenoptera: Apidae) exposed to acetamiprid and propiconazole alone or combined', *Ecotoxicology*, 28(4):399–411. https://doi.org/10.1007/s10646-019-02030-4

Han, Y., Zhu, L., Wang, J., Wang, J., Xie, H. and Zhang, S. (2014) 'Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin', *Ecotoxicology and Environmental Safety*, 107:214–219. https://doi.org/10.1016/j.ecoenv.2014.06.006

Heather, G., Branstetter, M.G., Amon, N., Urban-Mead, K.R., Park ,M.G., Gibbs, J., Blitzer, E.J., Poveda, K., Loeb, G. and Danforth, B.N. (2019) 'Agriculturally dominated landscapes reduce bee phylogenetic diversity and pollination services', *Science*. American Association for the

Advancement of Science, 363(6424):282–284. https://www.science.org/doi/10.1126/science.aat6016

Heller, S., Joshi, N.K., Chen, J., Rajotte, E.G., Mullin, C. and Biddinger, D.J. (2020) 'Pollinator exposure to systemic insecticides and fungicides applied in the previous fall and pre-bloom period in apple orchards', *Environmental Pollution*, 265:114589. https://doi.org/10.1016/j.envpol.2020.114589

Huang, Z. (2012) 'Pollen Nutrition Affects Honey Bee Stress Resistance', *Terrestrial Arthropod Reviews* 5 (2): 175–89. https://doi.org/10.1163/187498312X639568

Iverson, A., Hale, C., Richardson, L., Miller, O. and McArt, S. (2019) 'Synergistic effects of three sterol biosynthesis inhibiting fungicides on the toxicity of a pyrethroid and neonicotinoid insecticide to bumble bees', *Apidologie*, 50(5):733–744. https://doi.org/10.1007/s13592-019-00681-0

Iwasa, T., Motoyama, N., Ambrose, J.T. and Michael Roe, R. (2004) 'Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*', *Crop Protection*, 23(5):371–378. https://doi.org/10.1016/j.cropro.2003.08.018

Johansen, N. S., Moen, L. H. and Egaas, E. (2007) 'Sterol demethylation inhibitor fungicides as disruptors of insect development and inducers of glutathione S-transferase activities in *Mamestra brassicae*', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 145(3):473–483. https://doi.org/10.1016/j.cbpc.2007.02.004

Johnson, R.M., Dahlgren, L., Siegfried, B.D. and Ellis, M.D. (2013) 'Acaricide, Fungicide and Drug Interactions in Honey Bees (*Apis mellifera*)', *PLOS ONE*. Public Library of Science, 8(1):e54092. https://doi.org/10.1371/journal.pone.0054092

Kairo, G., Biron, D.G., Abdelkader, F.B., Bonnet, M., Tchamitchian, S., Cousin, M., Dussaubat, C., Benoit, B., Kretzschmar, A. and Belzunces, L.P. (2017) 'Nosema ceranae, Fipronil and their combination compromise honey bee reproduction via changes in male physiology', *Scientific Reports*, 7(1):1–14. https://doi.org/10.1038/s41598-017-08380-5

Lebrun, J. D., De Jesus, K., and Tournebize, J. (2021) 'Individual performances and biochemical pathways as altered by field-realistic exposures of current-use fungicides and their mixtures in a non-target species, *Gammarus fossarum*', *Chemosphere*, 277, 130277. https://doi.org/10.1016/j.chemosphere.2021.130277

Lentola, A., David, A., Abdul-Sada, A., Tapparo, A., Goulson, D. and Hill, E.M. (2017) 'Ornamental plants on sale to the public are a significant source of pesticide residues with implications for the health of pollinating insects', *Environmental Pollution*. Elsevier Ltd, 228:297–304. https://doi.org/10.1016/j.envpol.2017.03.084

Lewis, K., Tzilivakis, J., Green, A., and Warner D. (2006) 'Pesticide Properties DataBase (PPDB)', https://bit.ly/32dhDUj

López-Uribe, M.M., Ricigliano, V.A., and Simone-Finstrom, M. (2020) 'Defining Pollinator Health: A Holistic Approach Based on Ecological, Genetic, and Physiological Factors', *Annual Review of Animal Biosciences*, 8:269-294. https://doi.org/10.1146/annurev-animal-020518-115045

Maus, C., Curé, G., Schmuck, R., Porrini, C., and Bortolotti, L. (2003) 'Safety of Imidacloprid Seed Dressings to Honey Bees: A Comprehensive Overview and Compilation of the Current State of Knowledge', *Bulletin of Insectology* 56: 51–58.

Medrzycki, P., Montanari, R., Bortolotti, L., Sabatini, A.G., Maini, S., Porrini, C. (2003) 'Effects of imidacloprid administered in sublethal doses on honey bee behaviour. Laboratory tests', *Bulletin of Insectology*, 56:59–62

Mengoni Goñalons, C. and Farina, W. M. (2018) 'Impaired associative learning after chronic exposure to pesticides in young adult honey bees', *Journal of Experimental Biology*, 221(7):jeb176644. https://doi.org/10.1242/jeb.176644

Mokkapati, J. S., Bednarska, A. J. and Laskowski, R. (2022) 'Physiological and biochemical response of the solitary bee *Osmia bicornis* exposed to three insecticide-based agrochemicals', *Ecotoxicology and Environmental Safety*, 230:113095. https://doi.org/10.1016/j.ecoenv.2021.113095

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D. and Pettis, J.S. (2010) 'High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health', *PLoS ONE*. Edited by F. Marion-Poll. Public Library of Science, 5(3):e9754. https://doi.org/10.1371/journal.pone.0009754

Nguyen, B.K., Saegerman, C., Pirard, C., Mignon, J., Widart, J., Thirionet, B., Verheggen, F.J., Berkvens, D., De Pauw, E., and Haubruge, E. (2009) 'Does Imidacloprid Seed-Treated

Maize Have an Impact on Honey Bee Mortality? ' *Journal of Economic Entomology* 102 (2): 616–23. https://doi.org/10.1603/029.102.0220

Nicodemo, D., Maioli, M.A., Medeiros, H.C.D., Guelfi, M., Balieira, K.V.B., De Jong, D., and Mingatto, F.E. (2014) 'Fipronil and imidacloprid reduce honeybee mitochondrial activity', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 33(9):2070–2075. https://doi.org/10.1002/etc.2655

Ostiguy, N., Drummond, F.A., Aronstein, K., Eitzer, B., Ellis, J.D., Marla Spivak, M., and Sheppard, W.S. (2019) 'Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study', *Insects*. MDPI AG, 10(1):13. https://doi.org/10.3390/insects10010013

Pal, E., Almasri, H., Paris, L., Diogon, M., Pioz, M., Cousin, M., Sené, D., *et al*. (2022) 'Toxicity of the Pesticides Imidacloprid, Difenoconazole and Glyphosate Alone and in Binary and Ternary Mixtures to Winter Honey Bees: Effects on Survival and Antioxidative Defenses', *Toxics* . https://doi.org/10.3390/toxics10030104

Paleolog, J., Wilde, J., Siuda, M., Bąk, B., Wójcik, Ł. and Strachecka, A. (2020) 'Imidacloprid markedly affects hemolymph proteolysis, biomarkers, DNA global methylation, and the cuticle proteolytic layer in western honeybees', *Apidologie*, 51(4):620–630. https://doi.org/10.1007/s13592-020-00747-4

Pasquale, G.D., Salignon, M., Conte, Y.L., Belzunces, L.P., Decourtye, A., Kretzschmar, A., Suchail, S., Brunet, J-L. and Alaux, C. (2013) 'Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter?', *PLOS ONE*, 8(8):e72016. https://doi.org/10.1371/journal.pone.0072016

Pilling, E.D., Bromleychallenor, K.A.C., Walker, C.H. and Jepson, P.C. (1995) 'Mechanism of Synergism between the Pyrethroid Insecticide λ-Cyhalothrin and the Imidazole Fungicide Prochloraz, in the Honeybee (Apis mellifera L.)', *Pesticide biochemistry and physiology*, 51(1):1–11. https://doi.org/10.1006/pest.1995.1001

Pohorecka, K., Skubida, P., Miszczak, A., Semkiw, P., Sikorski, P., Zagibajło,K., Teper, D., *et al.* (2012) 'Pozostałości Insektycydów Neonikotynoidowych w Nektarze i Pyłku Zbieranym Przez Pszczoły z Upraw Rzepaku i Ich Wpływ na Rodziny Pszczele', *Journal of Apicultural Science*, 56(2):115–134. 10.2478/v10289-012-0029-3.

Powner, M.B., Salt, T.E., Hogg, C. and Jeffery, G. (2016) 'Improving Mitochondrial Function Protects Bumblebees from Neonicotinoid Pesticides', *PLOS ONE*. Public Library of Science, 11(11):1–11. https://doi.org/10.1371/journal.pone.0166531

Rabea, E. I., Nasr, H. M. and Badawy, M. E. I. (2010) 'Toxic effect and biochemical study of chlorfluazuron, oxymatrine, and spinosad on honey bees (*Apis mellifera*)', *Archives of Environmental Contamination and Toxicology*. Springer, 58(3):722–732. https://doi.org/10.1007/s00244-009-9403-y

Raimets, R., Karise, P., Mänd, M., Kaart, T., Ponting, S., Song, J. and Cresswell, J.E. (2018) 'Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.)', *Pest management science*, 74(3):541–546. https://doi.org/10.1002/ps.4756

Rortais, A., Arnold, G., Dorne, J-L., More, S.J., Sperandio, G., Streissl, F., Szentes, C.and Verdonck, F. (2017) 'Risk assessment of pesticides and other stressors in bees: Principles, data gaps and perspectives from the European Food Safety Authority', *Science of The Total Environment*, 587–588:524–537. https://doi.org/10.1016/j.scitotenv.2016.09.127

Rundlöf, M., Andersson, G.K.S., Bommarco, R., Fries, I., Hederström,V., Herbertsson, L., Jonsson, O., Klatt, B.K., Pedersen, T.R., and Yourstone, J. (2015) 'Seed coating with a neonicotinoid insecticide negatively affects wild bees', *Nature*. Nature Publishing Group, 521(7550):77–80. https://doi.org/10.1038/nature14420

Samson-Robert, O., Labrie, G., Mercier, P.L., Chagnon, M., Derome, N., and Fournier, V. (2015) 'Increased Acetylcholinesterase Expression in Bumble Bees During Neonicotinoid-Coated Corn Sowing', *SCIENTIFIC REPORTS* 5. https://doi.org/10.1038/srep12636

Sanchez-Hernandez, J.C. (2011) 'Pesticide Biomarkers in Terrestrial Invertebrates', *Pesticides in the Modern World-Pests Control and Pesticides Exposure and Toxicity Assessment.InTech*, 213–40.

Sanchez, W., Burgeot, T. and Porcher, J.-M. (2013) 'A novel "Integrated Biomarker Response" calculation based on reference deviation concept', *Environmental Science and Pollution Research*, 20(5):2721–2725. https://doi.org/10.1007/s11356-012-1359-1

Sandrock, C., Tanadini, L.G., Pettis, J.S., Biesmeijer, J.C., Potts, S.G. and Neumann, P. (2014) 'Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success', *Agricultural and Forest Entomology*, 16(2):119–128. https://doi.org/10.1111/afe.12041

Schmolke, A., Galic, N., Feken, M., Thompson, H., Sgolastra, F., Pitts-Singer, T., Elston, C., Pamminger, T., and Hinarejos, S. (2021) 'Assessment of the Vulnerability to Pesticide Exposures Across Bee Species', *Environmental Toxicology and Chemistry* 40 (9): 2640–51. https://doi.org/10.1002/etc.5150

Schmuck, R., Schöning, R., Stork, A. and Schramel, O. (2001) 'Risk posed to honeybees (*Apis mellifera* L, Hymenoptera) by an imidacloprid seed dressing of sunflowers', *Pest Management Science*. John Wiley & Sons, Ltd, 57(3):225–238. https://doi.org/10.1002/ps.270

Sedivy, C. and Dorn, S. (2014) 'Towards a sustainable management of bees of the subgenus *Osmia* (Megachilidae; *Osmia*) as fruit tree pollinators', *Apidologie*, 45(1):88–105. https://doi.org/10.1007/s13592-013-0231-8

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M.T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R. and Bosch, J. (2017) 'Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species', *Pest Management Science*. John Wiley & Sons, Ltd, 73(6):1236–1243. https://doi.org/10.1002/ps.4449

Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D. and Bosch, J. (2018) 'Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee', *Proceedings of the Royal Society B*, 285(1885):20180887. https://doi.org/10.1098/rspb.2018.0887

Sgolastra, F., Hinarejos, S., Pitts-Singer, T.L., Boyle, N.K., Joseph, T., Luckmann, J., Raine, N.E., Singh, R., Williams, N.M., and Bosch, J. (2019) 'Pesticide Exposure Assessment Paradigm for Solitary Bees', Environmental Entomology 48 (1): 22–35. https://doi.org/10.1093/ee/nvy105

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., Porrini, C., Simon-Delso, N., and Bosch, J. (2020). 'Bees and pesticide regulation: lessons from the neonicotinoid experience'. *Biological Conservation*, 241, 108356. https://doi.org/10.1016/j.biocon.2019.108356

Shao, X.S., Xia, S.S., Durkin, K.A., and Casida, J.E. (2013) 'Insect Nicotinic Receptor Interactions in Vivo with Neonicotinoid, Organophosphorus, and Methylcarbamate Insecticides and a Synergist', *Proceedings of the National Academy of Sciences of the United States of America,* 110 (43): 17273–77. https://doi.org/10.1073/pnas.1316369110

Shi, H., Pei, L., Gu, S., Zhu, S., Wang, Y., Zhang, Y. and Li, B. (2012) 'Glutathione S-transferase (GST) genes in the red flour beetle, Tribolium castaneum, and comparative analysis with five additional insects', *Genomics*. Elsevier Inc., 100(5):327–335. https://doi.org/10.1016/j.ygeno.2012.07.010

Srivastava, J. P. and Saxena, S. C. (1967) 'On the alkaline and acid phosphatase in the alimentary tract of *Periplaneta americana* L.(Blattaria: Blattidae)', *Applied Entomology and Zoology*, 2(2):85–92. https://doi.org/10.1303/aez.2.85

Stone, D., Jepson, P. and Laskowski, R. (2002) 'Trends in detoxification enzymes and heavy metal accumulation in ground beetles (Coleoptera: Carabidae) inhabiting a gradient of pollution', C*omparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 132(1): 105-112. https://doi.org/10.1016/S1532-0456(02)00052-2

Stoner, K. A. and Eitzer, B. D. (2012) 'Movement of Soil-Applied Imidacloprid and Thiamethoxam into Nectar and Pollen of Squash (Cucurbita pepo)', *PLOS ONE*. Public Library of Science, 7(6):e39114. https://doi.org/10.1371/journal.pone.0039114

Team, R Core. (2013) 'R: A Language and Environment for Statistical Computing.'Vienna, Austria.

Thompson, H. and Wilkins, S. (2003) 'Assessment of the synergy and repellency of pyrethroid/fungicide mixtures', *Bulletin of Insectology*, 56(1):131–134.

Thompson, H.M., Fryday, S.L., Harkin, S. and Milner, S. (2014) 'Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops', *Apidologie*, 45(5):545–553. https://doi.org/10.1007/s13592-014-0273-6

Tlili, S., and Mouneyrac, C. (2021) 'New Challenges of Marine Ecotoxicology in a Global Change Context', *Marine Pollution Bulletin* 166: 112242. https://doi.org/10.1016/j.marpolbul.2021.112242

Wang, Y., Zhu, Y. C. and Li, W. (2020a) 'Comparative examination on synergistic toxicities of chlorpyrifos, acephate, or tetraconazole mixed with pyrethroid insecticides to honey bees (Apis mellifera L.)', Environmental Science and Pollution Research, 27(7):6971–6980. https://doi.org/10.1007/s11356-019-07214-3

Wang, Y., Zhu, Y. C. and Li, W. (2020b) 'Interaction patterns and combined toxic effects of acetamiprid in combination with seven pesticides on honey bee (Apis mellifera L.)', *Ecotoxicology and environmental safety*, 190:110100. https://doi.org/10.1016/j.ecoenv.2019.110100

Wintermantel D., Odoux, J., Decourtye, A., Henry, M., Allier, F., Bretagnolle, V. (2020) 'Neonicotinoid-induced mortality risk for bees foraging on oilseed rape nectar persists despite EU moratorium', *Science of The Total Environment*, 704: 135400. https://www.sciencedirect.com/science/article/pii/S0048969719353938

Woodcock, B.A., Isaac, N.J.B., Bullock, J.M., Roy, D.B., Garthwaite, D.G., Crowe, A. and Pywell, R.F. (2016) 'Impacts of neonicotinoid use on long-term population changes in wild bees in England', *Nature* Communications, 7(1):12459. https://doi.org/10.1038/ncomms12459

Wu, Y. Y., Luo, Q. H., Hou, C. S., Wang, Q., Dai, P. L., Gao, J. and Diao, Q. Y. (2017) 'Sublethal effects of imidacloprid on targeting muscle and ribosomal protein related genes in the honey bee *Apis mellifera* L.', *Scientific Reports*. 7, 15943. https://doi.org/10.1038/s41598-017-16245-0

Xavier, K. V, KC, A. N. and Vallad, G. E. (2020) 'Fungicide Application Timing Essential for the Management of Leaf Spot and Fruit Rot on Pomegranate (Punica granatum L.) in Florida', *Plant Disease*. Scientific Societies, 104(6):1629–1637. https://doi.org/10.1094/PDIS-10-19-2224-RE

Zhu, Y.C., Yao, J., Adamczyk, J. and Luttrell, R. (2017a) 'Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (Apis mellifera)', *PLOS ONE*. Public Library of Science, 12(6):e0178421. https://doi.org/10.1371/journal.pone.0178421

Zhu, Y.C., Yao, J., Adamczyk, J. and Luttrell, R. (2017b) 'Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (Apis mellifera)', *PLOS ONE.* Public Library of Science, 12(5):1–16. https://doi.org/10.1371/journal.pone.0176837

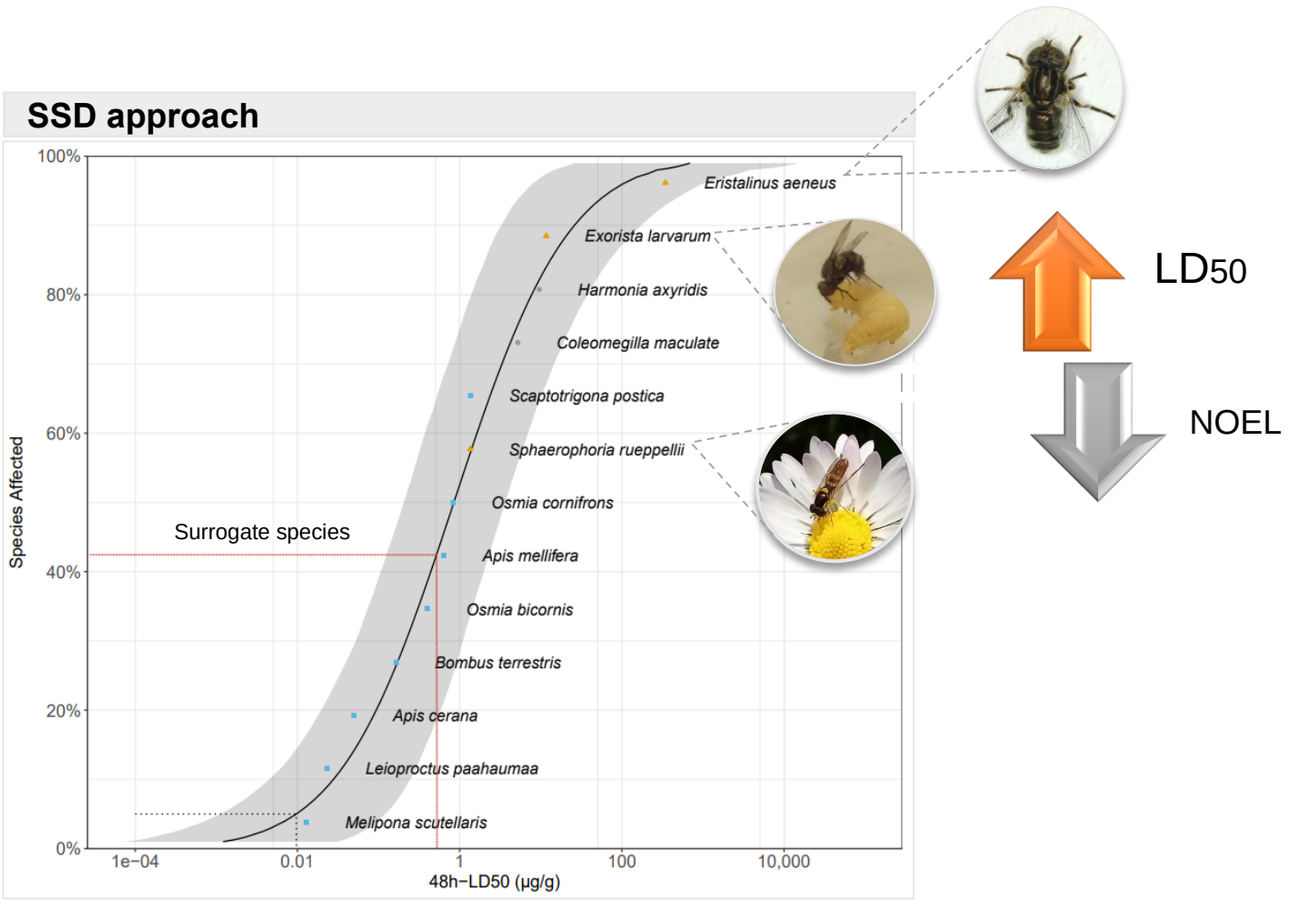
Zioga, E. Kelly, R., White, B., and Stout, J.C. (2020) 'Plant Protection Product Residues in Plant Pollen and Nectar: A Review of Current Knowledge', *Environmental Research* 189: 109873. https://doi.org/10.1016/j.envres.2020.109873

Chapter 3

Sensitivity of flower-visiting flies (Diptera) towards a neonicotinoid insecticide: A multiple species approach for environmental risk assessment

Cátia Ariana Henriques Martins, Celeste Azpiazu, Giovanni Burgio, Jordi Bosch, Maria Luisa Dindo, Santolo Francati, Daniele Sommaggio, Fabio Sgolastra

Manuscript in preparation



Chapter 3- Graphical abstract

ABSTRACT

Insect pollinators are a broadly diverse group essential for ecosystem functioning and crop pollination. However, especially in agricultural environments, pollinators are commonly exposed to pesticides, which can hamper their pollination services. Although pesticides are considered one of the main drivers of pollinator decline, information on pesticide sensitivity of non-bee pollinators is to date mostly lacking. Dipterans are a highly diverse group with over 160,000 species, including some important pollinators. In this study, we assessed the sensitivity of three dipteran species (*Sphaerophoria rueppellii*, *Eristalinus aeneus*, *Exorista larvarum*) to a neonicotinoid insecticide (Confidor®, imidacloprid) through an acute contact exposure. We calculated the median lethal dose (LD50) by adapting the standard protocol for honey bees and compared our results with those available in literature for other pollinator species through the species sensitivity distribution (SSD) approach. Additionally, we assessed the potential trade-off between fecundity and survival through the sub-lethal sensitivity index (SSI). Overall, dipterans were less sensitive to imidacloprid than most bee species, however, among them*, S. rueppelli* resulted the most sensitive. The SSI values indicated that fecundity is not a very sensitive endpoint for the species tested in our study.

Keywords: Pollinators, Diptera, Species Sensitivity Distribution, Fecundity

3.1. Introduction

Insect pollinators provide an important role in ecosystems, favouring plant reproduction and maintaining biodiversity (Klein et al., 2007). They compose a wide ecological group that includes bees, butterflies, flies, beetles, ants, moths and wasps. Thus, pollinator communities cover a broad variety of different life traits and strategies, which include specialist herbivores, predators, cleptoparasitic and parasitoids (Ollerton, 2017). Among them, flies (order Diptera) are one of the most diverse taxa on the planet and play an important role as pollinators (Larson et al., 2001), although they are often undervalued. Dipterans have a worldwide distribution and are common in both natural and managed habitats, comprising more than 160.000 species and 150 families, with at least 75 families as flower visitors (Courtney et al., 2017; Skevington and Dang, 2002; Ssymank et al., 2008). Flies can visit flowers for several reasons, primarily for pollen and nectar, but also for finding preys and hosts for their larvae, as well as for shelter, mating and oviposition sites (Woodcock et al., 2014). Recently, the diversity and abundance of non-bee pollinators, such as dipterans, have been of growing appreciation for their potential as alternative pollinators of honey bees in crops (Orford et al., 2015; Ssymank et al., 2008) and their use in integrated pest management (IPM), as agents for biological control (Dindo and Grenier, 2023; Dunn et al., 2020). In particular, hoverflies (Diptera: Syrphidae) have been acknowledged for their importance as pollinators (Doyle et al., 2020), disregarding other fly species from this role. However, non-hoverfly dipterans can represent the largest percentage of flower-visiting species (Galliott et al., 2017; Grass et al., 2016; Orford et al., 2015), evidencing the importance of extending the information available for other groups, that is to date limited.

Despite the high dependency of global food security on pollinator populations, in the last century, domestic and wild pollinators have been facing dramatic declines caused mostly by anthropogenic factors, such as intensification of agriculture, linked with the high reliance on pesticide applications (Biesmeijer. et al., 2006; Goulson et al., 2015; Zattara and Aizen, 2021). Hallmann et al. (2017) showed a substantial long-term decline in insect biomass in natural ecosystems in Germany, which included non-bee flower visitors (mainly hoverflies, see also Hallmann et al., 2021). Similar declines were also observed and reported in another recent study, with data from 1979–2021 on hoverflies' abundance and richness (Barendregt et al., 2022). The use of pesticides is considered a possible cause of decline, as insecticide residues were detected both in natural and semi-natural areas (Bernal et al., 2010; Botías et al., 2016; Wood and Goulson, 2017).

Typically, environmental risk assessment for pollinators is based on the calculation of the dose of a pesticide that is lethal to 50% of the population (LD_{50}) of the surrogate species, following acute contact and oral exposure (Arena and Sgolastra, 2014). This approach overlooks the potential sensitivity differences among species and disregards the occurrence of sub-lethal effects (Azpiazu et al., 2019; Biddinger et al., 2013; Heard et al., 2017; Robinson et al., 2017; Sgolastra et al., 2020). As pesticides can cause multiple adverse sub-lethal effects on pollinators (Tosi et al., 2022), it can be expected a trade-off between survival and other fitness components, such as their ability to reproduce (fecundity). This reproduction-survival trade-off occurs because the reproduction process may compete with the energetic demands of somatic maintenance (survival), which include stress resistance and immunity (Rodrigues and Flatt, 2016).

Currently, the ecotoxicological literature and the regulatory risk assessment framework for insect pollinators, in USA and Europe, is centred on the western honey bee *Apis mellifera* L. (EFSA, 2013; USEPA, 2014), at the expense of other pollinator species. To face these shortcomings, the European Food Safety Authority has published a scientific opinion on non-target arthropods (NTA)

risk assessment (EFSA, 2015) proposing to expand the current framework to other test species, following a list of the International Organisation for Biological and Integrated Control (IOBC) standard test methods (Candolfi, 2000). Nonetheless, non-bee pollinators are still underrepresented, and it remains unclear which test species would sufficiently cover the overall group (Uhl and Brühl, 2019). Since few attempts have been made to study the sensitivity to pesticides on other pollinator taxa, it is still uncertain to what extent pesticides impact non-bee species (Basley et al., 2018; Jansen et al., 2011; Moens et al., 2011; Nagloo et al., 2023). In this sense, the species sensitivity distributions (SSD) approach is an optimal method to compare pesticide sensitivity across species and to derive a protection exposure level for most or all species, within the same functional groups, when limited single-species toxicity data is available (Wheeler et al., 2002). This distribution statistically describes the variation among a set of species to a particular chemical or mixture, and attributes a 5^{th} percentile cut-off concentration/dose (HC5/HD5), that ensures a proper level of protection (Posthuma et al., 2001).

In the present study, we assessed the impact of a neonicotinoid insecticide (Confidor®, imidacloprid) in three different dipteran species and we compared their sensitivity with *A. mellifera* and other insect pollinators through a species sensitivity distribution curve (SSD). We conducted an acute contact toxicity test on adult females of a parasitoid fly, *Exorista larvarum* (Linnaeus) (Diptera: Tachinidae), and two hoverflies, *Sphaerophoria rueppellii* (Wiedemann) (Diptera: Syrphidae) and *Eristalinus aeneus* (Scopoli) (Diptera: Syrphidae). *E. larvarum* is a polyphagous gregarious larval parasitoid of several Lepidoptera species, e.g. the gypsy moth and cabbage moth, both identified as a pest in forest and agricultural environments (Benelli et al., 2018); and is a common visitor of Apiaceae flowering plants (Mellini and Coulibaly, 1991). *S. rueppellii* is an aphidophagous hoverfly (Amorós-Jiménez et al., 2012; 2014), commercially available due to their high efficacy as biological control of the aphid *Myzus persicae* (Sulzer) and important contributions in pollination, particularly used on sweet pepper production (Pekas et al., 2020). *E. aeneus* has saprophagous feeding habits during the larval stage and is an effective pollinator in the adult stage (Sánchez et al., 2022).

We have adapted the standardized acute contact test of honey bees (OECD no.214; OECD, 1998) to Diptera species to assess the LD_{50} and the effects on fecundity. In addition, we calculated for each species the sub-lethal sensitivity index (SSI) to assess their trade-off between survival and reproduction, following pesticide exposure. This index may predict population responses by integrating the life-histories strategies with the interspecific responses to a chemical, therefore providing an understanding of whether the species invest their energy in growth and reproduction or prioritize survival (van Straalen, 1994).

3.2. Materials and methods

3.2.1. Flies and test conditions

A colony of *E. larvarum* was maintained at the Department of Agricultural and Food Sciences (DISTAL - University of Bologna, Italy), following the standard rearing procedures described by Dindo et al. (1999). *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were used as a factitious host, reared at 30 ± 1˚C, 65 ± 5% relative humidity, and in complete darkness, as described by Dindo et al. (2007). For the trials, mature *E. larvarum* puparia were selected according to their weight, ranging from 35 to 55 mg. Newly-emerged individuals were kept in Plexiglas cages (40 x 30 x 30 cm) and left to mate for 3 days (72h), in a male:female proportion of 1:1. Individual mated females were later collected into Petri dishes and anaesthetized on ice blocks for 3 min at 4-5 °C,

in order to perform contact pesticide administration. Following anaesthetization, groups of 5 females were transferred to Plexiglas cages (20x20x20 cm), in accordance with their treatment group.

S. rueppellii were obtained from a population reared in Belgium (commercially available as Spharophoria-System, Biobest N.V.) and shipped as pupae to the laboratory of DISTAL in two periods (February and April 2021) in order to reach an adequate sample size. Upon arrival, pupae were immediately transferred to the climate chamber for incubation. We kept the pupae in standard mesh cages (60x40x40 cm) and left them to mate, after emergence, for 2 days (48h), in a male:female proportion of 1:1. Following the same procedure as described previously, females, after mating, where anaesthetized on ice blocks for 2 min at 4-5 °C, to proceed with the treatments. Each female was then transferred to individual cages (V=150 cc, with the lid perforated for air circulation) and maintained there until the end of the test.

E. aeneus pupae were obtained from a population reared in Spain (commercially available as Goldfly®, Polyfly S.L.) and shipped in two distinct periods (November 2021 and January 2022) to the laboratory of DISTAL. Pupae were incubated in the conditions of the climate chamber. Emerged individuals were kept in standard mesh cages (60x40x40 cm) for 5 days (120h), in a male:female proportion of 1:1. Mated females were then anaesthetized on ice blocks for 3 min at 4-5 °C, as described before, and transferred to individual cages (V=150 cc, with the lid perforated for air circulation), for all the duration of the test.

All flies were kept under light:dark conditions (16:8h) in a climate room at a temperature of 26 ± 1°C, ca. 70-80% relative humidity, during the emergence, mating and test duration. Upon emergence, all fly species were fed *ad libitum*, with honey bee pollen pellets from organic beekeeping (Bona Mel ®), sugar cubes and distillate water, provided through a humid cotton. Pollen was supplied to promote ovarian development (Woodcock et al., 2014). For *E. larvarum* water was provided by plastic drinking troughs of 50 mL capacity, whereas for the hoverflies, water was provided through a 1 mL Eppendorf, with the tip cut for insertion of cotton. Water was refilled when needed. Sample sizes were between 20-30 females per fly species and doses in all tests. A randomly selected group of mated females of each species was weighted to obtain an average fresh body weight.

3.2.2. Test solutions

We used commercially available formulate Confidor® (imidacloprid 20% w/v). Imidacloprid is a well-studied molecule due to its high acute risk for bees, which lead to be banned from use by the European Commission in 2018 (OJEU, 2018). Despite this, many Member States still provide emergency authorizations for its use and is still heavily used in many parts of the world (Goulson, 2020). To obtain the stock solutions, fresh treatment solutions were made with Confidor® in HPLC-grade acetone and serially diluted to appropriate concentration.

3.2.3. Experimental design

For all fly species, two control groups were included: negative control (untreated) and solvent control (pure acetone). In order to obtain imidacloprid dose-response curves, we exposed flies to six/seven doses in a geometric series. We used a factor of 2.5 from 40 to 1563 ng insect^{-1} (nominal doses) in *E. larvarum*; a factor of 2.5 from 0.41 to 16 ng insect^{-1} in *S. rueppellii;* and a factor of 3 ranging from 540 to 43,750 ng insect^{-1} in *E. aeneus*. Adult mated female flies were exposed to the test solutions by topical application (1 µL per female, applied to the dorsal side of the thorax, between the neck and wing base). Mortality was recorded daily (4, 24, 48, 72 and 96 h) and

compared with solvent control values. Given the fact that there are no official ecotoxicology tests for Diptera species, we did not establish a test validation criterion for the control mortality.

3.2.4. Sub-lethal effects: Fecundity

Fecundity, defined as the number of offspring produced by an individual insect (Awmack and Leather, 2002), has been assessed in our study starting from 24h after topical application of imidacloprid at different doses. To assess this parameter, we adjusted the protocol to each test species taking into account their specific biology and life cycle.

For *E. larvarum,* this parameter was assessed by counting the number of eggs laid on the host larvae*.* The mated females in groups of 5 were exposed for 1h to the last instar *G. mellonella* larvae (3 larvae per female = 15 larvae), following the methodology described by Dindo et al. (2019). After exposure, the overall number of eggs counted on the host larvae was divided by the number of females to determine fecundity, expressed as the average number of eggs laid per female in 1 h. We followed this methodology as Dindo et al. (1999) showing that *in vitro*-reared females lay most eggs from the 5th day following emergence and when they are in groups of 5 individuals.

As for *S. rueppellii,* eggs were counted daily until the end of the trial (96h). We added in each cage two sprouts of pea plant (*Pisum sativum* L.) infested with aphids (*M. persicae*), with one sprout less infested to leave free space for egg-laying. A colony of the aphids was maintained on the pea plant in a climate chamber at the laboratory of DISTAL (T= 20 ± 1˚C, 60-80% relative humidity and 16:8h light:dark). Sprout roots were wrapped in humid cotton (with distilled water) and aluminium foil, to keep the plant alive. Sprouts and aphids were substituted when needed. Eggs were counted daily and hoverfly larvae were removed, if detected. This last step was done to avoid predation of eggs since in aphidophagous species cannibalism can occur during the larval stage (Branquart et al., 1997). For this species, fecundity was expressed as the daily number of eggs per female.

Fecundity endpoint on *E. aeneus* was assessed daily for 7 days (168 h). To promote egg-laying, we provided a substrate of decaying soaked oat grains (proposed by Campoy et al., 2020; Gladis, 1994) for each individual female. The substrate was prepared with organic oat and distilled water (at the proportion of 200 g to 175 mL) and left for 24h at room temperature and completely dark. We provided ca. 9.3 g of substrate per female, which was changed every two days to avoid mould formation. Fecundity in this species was expressed as the daily number of eggs per female.

3.2.5. Ecotoxicological data from literature

Median lethal doses (LD_{50}) for imidacloprid were selected from literature and entered into a file including several variables: active substance pure or in formulation, pollinator species (Hymenoptera: bees and Coleoptera: beetles), treatment specifications (exposure and time-scale), value of the endpoint and references (see Supplementary Materials, Table A.3.1). Lepidoptera (butterflies) are not represented in this study, since no confrontable literature data was available (contact exposure on adults).

3.3. Statistical analysis

Median acute lethal dose values (contact 48h-LD_{50}) were calculated for the fly species by fitting a dose-response model to the data in R v4.2.1 (R Core Team, 2013). The values were determined by ED function of "*drc*" package (Ritz et al., 2015). Models were chosen using Akaike information criterion (AIC). Furthermore, it was ensured that appropriate models were used for tests with control mortality (no fixed lower limit). Mortality was corrected with Abbott's formula (Abbott,

1925), by using the untreated control as reference. Calculation of the LD_{50} values for *E. aeneus* was performed by diving the dose-effect curve into the two ascending parts, following a similar approach by Suchail et al. (2000), due to the non-monotonic response observed in this species. Weight-normalized LD_{50} values were further calculated by dividing LD_{50} values in ng/insect by the mean fresh weight of all species in the respective test. The species sensitivity distribution (SSD) was fitted to a log-normal dataset with the values of 48h-LD_{50}, through the package "*ssdtools*" (Thorley and Schwarz, 2018). We obtained from the curve the 5% (HD5, as the lower limit of the distribution) hazardous dose, and calculated its 95% confidence intervals (CI, 1000 interactions). Using the methodology of Arena and Sgolastra (2014), we further calculated the sensitivity ratio (R) between *A. mellifera* and the other pollinator species $\left(R = \frac{LD_{50}\ A.mellifera}{LD_{50}\ pollinator}\right)$.

Fecundity endpoint was assessed only on female flies that survived for a period of 24h after contact exposure. For both *S. rueppellii* and *E. aeneus*, eggs were counted daily. As for *E. larvarum*, we only assessed the number of eggs laid after 24h of the treatment. We used the Kruskall-Wallis non-parametric test to detect differences among treatments in the number of eggs laid per female. Dunn's test (with Benjamini–Hochberg correction) was performed for pairwise multiple-comparison with the solvent control values, after verifying that there were no significant differences between the two controls (untreated and solvent control). Moreover, a Chi-square (x^2) test was performed to detect the influence of the treatments on the number of females that laid eggs. All results are reported with a significance level of 5%.

The sub-lethal sensitivity index (SSI), which is the ratio between the median lethal dose (LD_{50}) and the no-effect level or dose for reproduction (NOEL), was calculated for each species (van Straalen, 1994). However, since NOEL is highly dependent on sample size and dose selection (Laskowski, 1995), the lower confidence bound of the Benchmark Dose (BMDL) has been used as denominator in the formula proposed by van Straalen (1994). In our study, the BMDLs were estimated through the US EPA's Benchmark Dose Software (BMDS Online version) (USEPA, 2023) using the data on the number of females that laid eggs for each treatment. A high SSI value (>1) indicates that reproduction is a very sensitive endpoint because it is inhibited at doses lower than the LD_{50}. On the contrary low SSI values (<1) indicate that the organism continues to invest its energy in reproduction till its death.

3.4. Results

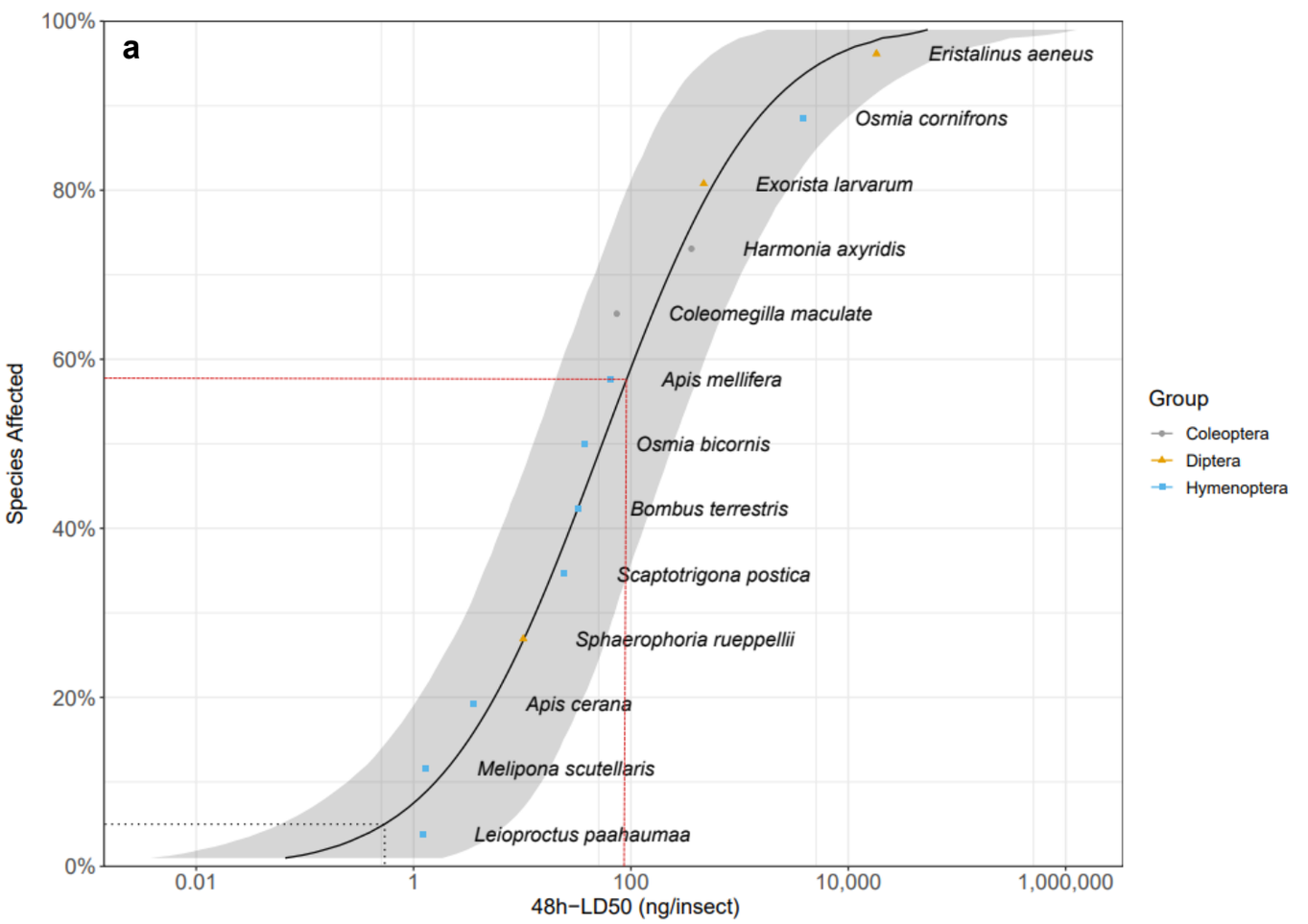
3.4.1. Species sensitivity distribution

The effect of imidacloprid was assessed on the three dipteran species estimating their respective LD_{50}, following topical exposure. Mortality control (untreated), used to correct the mortality percentages at 48h, was respectively, 0% for *S. rueppellii*; 17% for *E. larvarum*; 21% for *E. aeneus*. No significant differences in mortality rate were observed between untread and solvent control groups in the three tested species (*S. rueppellii*: $x^2 = 1.48$, p= 0.22; *E. larvarum*: $x^2 = 0.15$, p= 0.70; *E. aeneus*: $x^2 = 0.083$, p= 0.78). Imidacloprid dose-response curves varied substantially between the studied species and the LD_{50} values increased in the following order: *S. rueppellii* < *E. larvarum* < *E. aeneus* (Table 1). The same sensitivity ranking was maintained when the LD_{50} values were normalized with the fresh body weight of each species. For *E. aeneus* we were only able to calculate the LD_{50} for the first ascending part of its non-monotonic curve.

Table 3.1: LD_{50}s values and model parameters in ng/insect and in µg/g of insect body weight following acute contact exposure to Confidor® (a.i. imidacloprid) at 48h. *Values for *Eristalinus aeneus* were divided for the two ascending parts of the dose–effect curve.

Species	n	Slope	p-value	48h-LD_{50} (ng/insect)	95% CI	48h-LD_{50} (µg/g insect)	95% CI
Exorista larvarum	233	-0.765	0.0087	467.46	302.28 - 632.65	11.66	7.54 - 15.79
Sphaerophoria rueppellii	205	-1.668	0.012	10.23	7.81 - 12.65	1.35	0.83 - 1.86
*Eristalinus aeneus**	260	-1.062	0.036	18,176.20	8,005.6 - 28,346.9	344.77	151.85 - 537.69
		-9.692	NA	NA	NA	NA	NA

The HD5 (the 5% hazardous dose) was calculated fitting the 48h-LD_{50} of different pollinator species (Hymenoptera: bees; Diptera: flies; Coleoptera: beetles), resulting to be 0.615 ng/insect, 95% CI [0.087; 7.416], and 0.0105 µg/g insect, 95% CI [0.0016; 0.118] (Fig. 1). The SSD curves show that the sensitivity of *A. mellifera* is intermediate, resulting in ca. 59% (41% when normalized with body weight) of pollinator species uncovered by the honey bee. The calculated sensitivity ratio (R) showed that the 10-fold safety factor recommended by EFSA (2013) used to extrapolate the sensitivity of *A. mellifera* to other pollinator species, was protective for 75% of the tested species, including the three dipterans of our study. Therefore, the safety factor, with or without body weight normalization, would not be conservative for three bee species, *A. cerana*, *M. scutellaris* and *L. paahaumaa* (Supplementary Materials Table A.3.2).



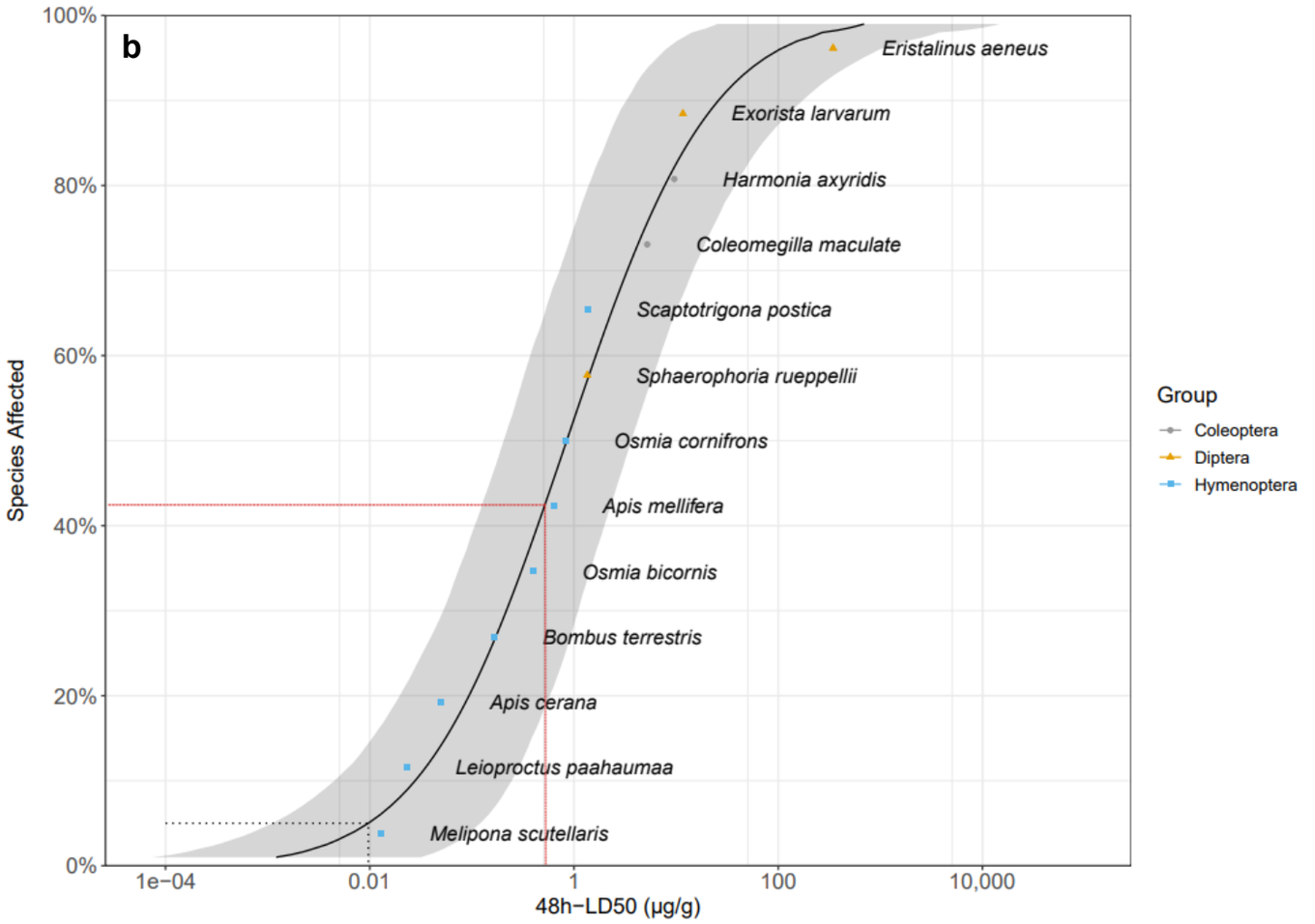


Figure 3.1: Species sensitivity distribution of imidacloprid calculated from multiple fly (Diptera), bee (Hymenoptera) and beetle (Coleoptera) species, **(a)** without and **(b)** with fresh body weight-normalization. Species names are aligned by sensitivity in ascendent order. Grey area shows the parametric 95% CI (1000 interactions). HDx are the hazardous dose for x% of the species. HD5 limit is represent by grey dashed lines and LD_{50} of *A. mellifera* is represent by red dashed lines. LD_{50} values for bee and beetle species are literature values (values for *A. mellifera, B. terrestris*, *O. bicornis* and *O. cornifrons* are represented as means of different studies).

3.4.2. Sub-lethal effects: Fecundity

Fecundity did not differ among the untreated and solvent control groups in all three dipteran species for this reason only the solvent control was included in the successive analysis. For *S. rueppellii* no difference was observed for the number of females that laid eggs (x^2 = 12.89, d.f.= 5, p= 0.62), however the number of daily eggs was significantly different between control and the highest tested dose (x^2= 12.65, d.f. = 5, p= 0.027. Figure 3.2a, Dunn's test: *p <0.05). In *E. larvarum* again no differences were observed on the number of females that laid eggs (x^2 = 22.15, d.f.= 5, p= 0.1), but the number of eggs differed between treatments and control (x^2= 30.61, d.f. = 5, p< 0.001. Figure 3.2b, Dunn's test: *p <0.05; **p<0.01, ***p<0.001), with exception of the lowest tested dose. As for *E. aeneus*, only between the control and the dose of 29,166 ng/insect, significant differences against control were detected for both the number of females that laid eggs (x^2= 14.74, d.f. = 6, p = 0.022) and the daily number of eggs (x^2= 16.43, d.f. = 6, p = 0.012; Figure 3.2c. Dunn's test: *p <0.05).

Comparison between LD_{50} and the fecundity are shown in Table 3.2. Our results showed a SSI below 1 in all dipteran species indicating that the reproduction was not inhibited even at concentrations higher than the LD50.

Table 3.2: Comparison of lethal and sub-lethal effects on the three dipteran species. n= sample size of females that survived > 24h after contact exposure; LD50= median lethal dose; BMDL= the lower confidence bound of the benchmark dose on fecundity effects; SSI= sublethal sensitivity index.

Species	**n**	**LD_{50} (ng/insect)**	**BMDL (ng/insect)**	**SSI**
Sphaerophoria rueppellii	174	10.23	12.99	0.78
Exorista larvarum	178	467.46	760.49	0.61
Eristalinus aeneus	209	18,176.20	41,378	0.43

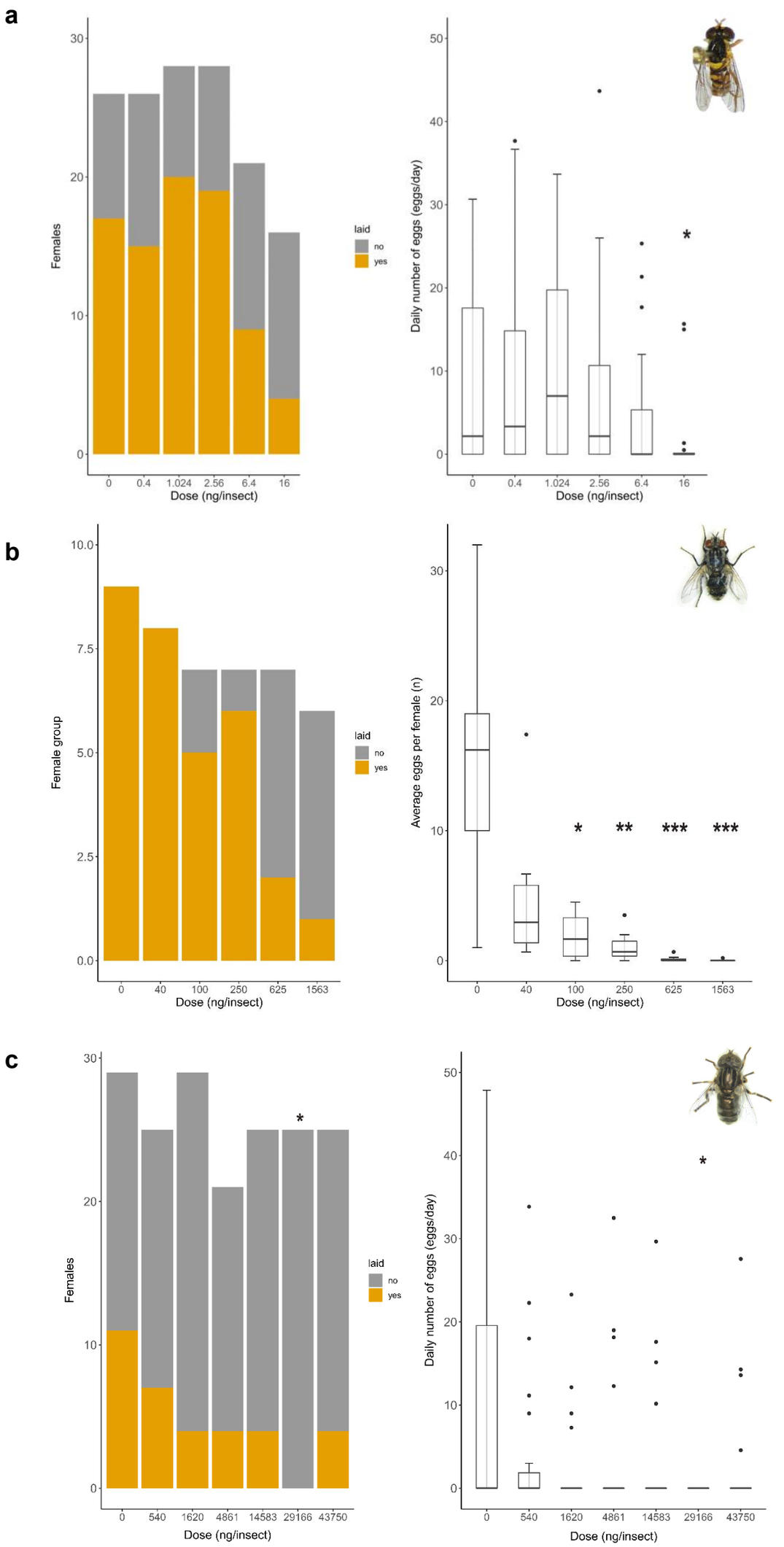


Figure 3.2: Fecundity comparison between control and treated females. **(a)** *Shaerophoria rueppellii*; **(b)** *Exorista larvarum;* **(c)** *Eristalinus aeneus.*

3.5. Discussion

Environmental risk assessment procedures for insect pollinators still rely almost exclusively on the surrogate species, *A. mellifera*, even though studies have already shown that this species is not always the most sensitive and the best representative species of all pollinators (Arena and Sgolastra, 2014). For these reasons, there have been already some attempts to include other bee species in the current risk assessment schemes (EFSA, 2013). In line with these initiatives, in the current study, we aimed to assess the impact of a common pesticide on another pollinator group, dipterans, about which little information on the response to pesticides (sensitivity) is known (Basley et al., 2018; Nagloo et al., 2023). To achieve this goal, we selected three pollinator species that cover different life history traits (the parasitoid *E. larvarum*, the predator *S. rueppellii* and the saprophagous *E. aeneus*), and adapted the existing standardized protocols for honey bees to them (OECD, 1998).

The acute contact LD_{50} values, obtained in the current study, showed that the sensitivity order of the three species, from the most sensitive (low LD_{50}, expressed in ng per insect) to the lowest (high LD_{50}), was: *S. rueppellii* < *E. larvarum* < *E. aeneus*. Following the species-sensitivity distribution (SSD) approach (Belanger et al., 2017), we extended our results by comparing them with literature values for other pollinator species acutely exposed to imidacloprid by contact (see Table S1). This approach, useful to provide a robust estimation of pesticide sensitivity of the pollinator community using a limited set of ecotoxicity data, allows us to observe and compare the sensitivity of different species and to predict the potentially affected species at certain concentrations. According to our results, the potential species affected below the LD_{50} of the honey bee are around 60%, including the hoverfly S. *rueppellii*, which is among the tested species the most sensitive to imidacloprid from dipterans. The high level of sensitivity of this species to imidacloprid has been also reported in a previous study (Calvo-Agudo et al. (2019), where *S. rueppellii* adults were fed with contaminated honeydew. This route of exposure is often overlooked in the current environmental risk assessment but may be very relevant for several pollinator species.

When introducing the 48 h-LD_{50} values corrected by the body weight in the SSD model, the protection derived by the honey bee LD_{50} increases with around 40% of potential affected species, which are all bees. Even tough body size can partially explain the interspecific sensitivity differences among bees (Arena and Sgolastra, 2014; Thompson, 2016), other biological traits can determine the intrinsic sensitivity of pollinators (e.g. hemolymph pH, lipid content, cuticle thickness), including their phylogenetic relationships (Pamminger, 2021).

Interestingly, especially when corrected with body weight, Coleoptera and Diptera species seem less sensitive to imidacloprid than bees (all bees species in the figure 1b, except *Scaptotrigona postica*, are in the lower part of the curve, thus potentially more affected). In this regard, toxicogenomic has strong potential to elucidate the molecular mechanisms evolved in different phylogenetic groups to respond to the different toxicants. *Sphaerophoria rueppellii*, the most sensitive non-bee species in our SSD analysis, has an increased number of genes in detoxification families, such as GSTs and CYP4 P450s, in comparison with bees (Bailey et al., 2022). In fact, as an adaptation to the exposure during their lifespans to more heterogenous environments, in terms of chemical stressors, hoverfly species may have evolved an expansion of their detoxification genes (Bailey et al., 2022; Doyle et al., 2022; Yuan et al., 2022). Bees also

depend on CYP9Q enzymes for xenobiotic detoxification, however, they are not universal in all species, which can explain their different intrinsic sensitives (Haas et al., 2022).

Across the tested dipteran species, the saprophagous hoverfly *E. aeneus* was the less sensitive. This result is in line with other studies that observed a relatively high tolerance of *Eristalis tenax* (Eristalinae) response to the neonicotinoids, imidacloprid and thiamethoxam (Basley et al., 2018; Nagloo et al., 2023). These results confirm that flies may be less sensitive to the neonicotinoid imidacloprid than bees, although other dipteran species should be tested using a standardized and comparable methodology. Moreover, it is important to notice that we have observed a non-monotonic response to the acute toxicity of *E. aeneus*, as at intermediate doses the toxicity dropped and started to increase again at higher doses. This unexpected dose response feature of imidacloprid was already reported in a study with *A. mellifera* (Suchail et al., 2000). Although its mechanistic explanation is unclear, this non-monotonic/biphasic dose-response could be much more common and general than expected (Agathokleous and Calabrese, 2020).

Specie Sensitive Distribution curves have been also used to derive a dose/concentration that would protect the 95th percentile of the species (HD5). In our study, the derived HD5 was approximately 100-fold lower than the 48h-LD_{50} of the surrogate species *A. mellifera*. This value is 10 times higher than the safety factor of 10 recommended by EFSA (2013) to estimate the sensitivity of untested bee species using honey bee data. Three (*A. cerana*, *M. scutellaris* and *L. paahaumaa*) of the 13 tested species in our dataset would show an LD_{50} lower than the *A. mellifera* LD_{50} even when corrected by a factor of 10. This outcome is in line with other studies showing the need to apply a safety factor higher than 10 to protect non-*Apis* bees (de Assis et al., 2022; Lourencetti et al., 2023).

An important aspect to take into consideration, when evaluating the effects of pesticides on insect pollinators, is not only their intrinsic sensitivity but also the population resilience. The latter may be considered as the capacity of the population to recover after disturbance, which is directly linked with its fecundity. In our study, we assessed the fecundity of three dipteran species, through the number of females that laid eggs and the number of eggs per batch; while for the *E. larvarum* we obtained 100% laying success in the females from the solvent control group, hoverflies reached only 65% and 38% for *S. rueppellii* and *E. aeneus*, respectively. Campoy et al. (2020), obtained a higher rate of *E. aeneus* females that laid at least one batch of eggs (around 64%), however, the differences may be explained by the time given to females to mate (in their study females were kept with males during all trial in contrast to the 5 days given in our study), or by the microbiologic composition of the fermented oat substrate used to induce oviposition (Campoy et al., 2022). In this species, the mortality in the water control group (at 48h) was also higher when compared to the other two dipteran species (21% vs 0% and 17% in *Shaerophoria rueppellii* and *Exorista larvarum*, respectively) indicating that the rearing or test conditions for *E. aenous* were probably not optimal. This issue highlights the importance to consider the rearing and maintenance conditions in developing standard laboratory tests for ecotoxicological studies with non-*Apis* pollinators.

Declines in fecundity can be considered early warning signals (EWSs) before population collapse. However different species can show different strategies or adaptive plasticity in reallocating resources from reproduction to maintain the survival of the individual. The SSI values calculated in our study show that fecundity is not a very sensitive endpoint (EWS) in the dipterans tested in

our study, as SSI values were lower than 1. In fact, the lethal and sub-lethal doses (e.g. BMDL) were relatively close indicating that these species continue to invest in fecundity even in presence of a stressor. However, in *E. larvarum* there was a significant reduction in the number of eggs laid per female already at the concentration 4 times lower than its LD_{50}.

In conclusion, our study provides important information on the sensitivity of three Diptera pollinator species in comparison with the surrogate species *A. mellifera* and presents a first step towards an advanced environmental risk assessment enlarged to non-bee pollinators. In addition, and on the contrary to bees, the possibility to measure the fecundity in some dipteran species under laboratory conditions, together with the calculation of the SSI, represents a great advantage to predict the impact of pesticides at population level. This crucial parameter for population dynamics can be in the future integrated in simulation models for their application in the environmental risk assessment (Topping et al. 2020).

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *J.Econ.Entomol*, *18*(2), 265–267.

Agathokleous, E., and Calabrese, E. J. (2020). Environmental toxicology and ecotoxicology: How clean is clean? Rethinking dose-response analysis. *Science of The Total Environment*, *746*, 138769. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.138769

Amorós-Jiménez, R., Pineda, A., Fereres, A., and Marcos-García, M. Á. (2012). Prey availability and abiotic requirements of immature stages of the aphid predator Sphaerophoria rueppellii. *Biological Control*, *63*(1), 17–24. https://doi.org/https://doi.org/10.1016/j.biocontrol.2012.06.001

Amorós-Jiménez, R., Pineda, A., Fereres, A., and Marcos-García, M. Á. (2014). Feeding preferences of the aphidophagous hoverfly Sphaerophoria rueppellii affect the performance of its offspring. *BioControl*, *59*(4), 427–435. https://doi.org/10.1007/s10526-014-9577-8

Arena, M., and Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology*, *23*(3), 324–334. https://doi.org/10.1007/s10646-014-1190-1

Awmack, C. S., and Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomology*, *47*(1), 817–844.

Azpiazu, C., Bosch, J., Viñuela, E., Medrzycki, P., Teper, D., and Sgolastra, F. (2019). Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in a solitary bee. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-50255-4

Bailey, E., Field, L., Rawlings, C., King, R., Mohareb, F., Pak, K.-H., Hughes, D., Williamson, M., Ganko, E., Buer, B., and Nauen, R. (2022). A near-chromosome level genome assembly of the European hoverfly*, Sphaerophoria rueppellii* (Diptera: Syrphidae), provides comparative insights into insecticide resistance-related gene family evolution. *BMC Genomics*, *23*(1), 198. https://doi.org/10.1186/s12864-022-08436-5

Barendregt, A., Zeegers, T., van Steenis, W., and Jongejans, E. (2022). Forest hoverfly community collapse: Abundance and species richness drop over four decades. *Insect Conservation and Diversity*, *15*(5), 510–521. https://doi.org/https://doi.org/10.1111/icad.12577

Basley, K., Davenport, B., Vogiatzis, K., and Goulson, D. (2018). Effects of chronic exposure to thiamethoxam on larvae of the hoverfly *Eristalis tenax* (Diptera, Syrphidae). *PeerJ*, *2018*(1), 1–15. https://doi.org/10.7717/peerj.4258

Belanger, S., Barron, M., Craig, P., Dyer, S., Galay-Burgos, M., Hamer, M., Marshall, S., Posthuma, L., Raimondo, S., and Whitehouse, P. (2017). Future needs and recommendations in the development of species sensitivity distributions: Estimating toxicity thresholds for aquatic ecological communities and assessing impacts of chemical exposures. *Integrated Environmental Assessment and Management*, *13*(4), 664–674. https://doi.org/https://doi.org/10.1002/ieam.1841

Benelli, M., Tóth, F., and Dindo, M. L. (2018). Low-temperature storage of *Exorista larvarum* puparia as a tool for assisting parasitoid production. *Entomologia Experimentalis et Applicata*, *166*(11–12), 914–924. https://doi.org/https://doi.org/10.1111/eea.12738

Bernal, J., Garrido-Bailón, E., del Nozal, M. J., González-Porto, A. v, Martín-Hernández, R., Diego, J. C., Jiménez, J. J., Bernal, J. L., and Higes, M. (2010). Overview of Pesticide Residues in Stored Pollen and Their Potential Effect on Bee Colony (*Apis mellifera*) Losses in Spain. *Journal of Economic Entomology*, *103*(6), 1964–1971. https://doi.org/10.1603/EC10235

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. A., Rajotte, E. G., Joshi, N. K., and Vaughn, M. (2013). Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLOS ONE*, *8*(9), e72587. https://doi.org/10.1371/journal.pone.0072587

Botías, C., David, A., Hill, E. M., and Goulson, D. (2016). Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. *Science of The Total Environment*, *566–567*, 269–278. https://doi.org/https://doi.org/10.1016/j.scitotenv.2016.05.065

Branquart, E., Hemptinne, J.-L., Bauffe, C., and Benfekih, L. (1997). Cannibalism in *Episyrphus balteatus* (Ditp: Syrphidae). *Entomophaga*, *42*(1), 145–152. https://doi.org/10.1007/BF02769892

Biesmeijer, J.C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A. P., Potts, S. G., Kleukers, R., Thomas, C. D., Settele, J., and Kunin, W. E. (2006). Parallel

Declines in Pollinators and Insect-Pollinated Plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354. https://doi.org/10.1126/science.1127863

Calvo-Agudo, M., González-Cabrera, J., Picó, Y., Calatayud-Vernich, P., Urbaneja, A., Dicke, M., and Tena, A. (2019). Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. *Proceedings of the National Academy of Sciences*, *116*(34), 16817–16822. https://doi.org/10.1073/pnas.1904298116

Campoy, A., Lutsyk, M., Pérez-Bañón, C., and Rojo, S. (2022). Age-stage two-sex life table analysis of *Eristalinus aeneus* (Diptera, Syrphidae) reared with two different larval media. *Bulletin of Entomological Research*, *112*(1), 13–20. https://doi.org/10.1017/S0007485321000328

Campoy, A., Sáez, L., Pérez-Bañón, C., and Rojo, S. (2020). Demography and population parameters of two species of eristaline flower flies (Diptera, Syrphidae, Eristalini). *Journal of Applied Entomology*, *144*(1–2), 133–143. https://doi.org/10.1111/jen.12717

Candolfi, M. P. (2000). *Guidelines to evaluate side-effects of plant protection products to non-target arthropods*. International Organization for Biological and Integrated Control of Noxious Animals and Plants.

Candolfi, M. P., Bakker, F., Cañez, V., Miles, M., Neumann, Ch., Pilling, E., Primiani, M., Romijn, K., Schmuck, R., Storck-Weyhermüller, S., Ufer, A., and Waltersdorfer, A. (1999). Sensitivity of non-target arthropods to plant protection products: Could *Typhlodromus pyri* and *Aphidius* spp. be used as indicator species? *Chemosphere*, *39*(8), 1357–1370. https://doi.org/https://doi.org/10.1016/S0045-6535(98)00489-5

Conceição de Assis, J., Tadei, R., Menezes-Oliveira, V. B., and Silva-Zacarin, E. C. M. (2022). Are native bees in Brazil at risk from the exposure to the neonicotinoid imidacloprid? *Environmental Research*, *212*, 113127. https://doi.org/https://doi.org/10.1016/j.envres.2022.113127

Courtney G.W., Pape T., Skevington J.H., Sinclair B.J., 2017. Biodiversity of Diptera. In Foottit R.G. & Adler P.H. (Eds): Insect Biodiversity: Science and Society, Volume I. John Wiley & Sons Ltd. pp.: 229-278

Dindo, M. L., Farneti, R., Scapolatempo, M., and Gardenghi, G. (1999). In Vitro Rearing of the Parasitoid *Exorista larvarum* (L.) (Diptera: Tachinidae) on Meat Homogenate-Based Diets. *Biological Control*, *16*(3), 258–266. https://doi.org/https://doi.org/10.1006/bcon.1999.0772

Dindo, M. L., Marchetti, E., and Baronio, P. (2007). In Vitro Rearing of the Parasitoid *Exorista larvarum* (Diptera: Tachinidae) from Eggs Laid Out of Host. *Journal of Economic Entomology*, *100*(1), 26–30. https://doi.org/10.1093/jee/100.1.26

Dindo, M. L., and Grenier, S. (2023). Chapter 4 - Production of dipteran parasitoids. In J. A. Morales-Ramos, M. G. Rojas, and D. I. Shapiro-Ilan (Eds.), *Mass Production of Beneficial Organisms (Second Edition)* (pp. 71–100). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-822106-8.00003-8

Dindo, M. L., Rezaei, M., and de Clercq, P. (2019). Improvements in the rearing of the tachinid parasitoid *Exorista larvarum* (Diptera: Tachinidae): Influence of adult food on female longevity and reproduction capacity. *Journal of Insect Science*, *19*(2). https://doi.org/10.1093/jisesa/iey122

Doyle, T., Hawkes, W. L. S., Massy, R., Powney, G. D., Menz, M. H. M., and Wotton, K. R. (2020). Pollination by hoverflies in the Anthropocene. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1927), 20200508. https://doi.org/10.1098/rspb.2020.0508

Doyle, T., Jimenez-Guri, E., Hawkes, W. L. S., Massy, R., Mantica, F., Permanyer, J., Cozzuto, L., Hermoso Pulido, T., Baril, T., Hayward, A., Irimia, M., Chapman, J. W., Bass, C., and Wotton, K. R. (2022). Genome-wide transcriptomic changes reveal the genetic pathways involved in insect migration. *Molecular Ecology*, *31*(16), 4332–4350. https://doi.org/https://doi.org/10.1111/mec.16588

Dunn, L., Lequerica, M., Reid, C. R., and Latty, T. (2020). Dual ecosystem services of syrphid flies (Diptera: Syrphidae): pollinators and biological control agents. *Pest Management Science*, *76*(6), 1973–1979. https://doi.org/https://doi.org/10.1002/ps.5807

EFSA (2013). Guidance on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus* spp. and solitary bees). *EFSA Journal*, *11*(7), 3295. https://doi.org/10.2903/j.efsa.2013.3295.

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2015. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for

non-target arthropods. *EFSA Journal*; 13(2):3996, 212 pp. https://doi.org/10.2903/j.efsa.2015.3996

European Commission (2018). Commission Implementing Regulation (EU) 2018/783 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance imidacloprid. O.J. 132, 31–34

Galliot J.-N., Brunel D., Bérard A., Chauveau A., Blanchetête A., Lanore L., Farruggia A., 2017. Investigating a flower-insect forager network in a mountain grassland community using pollen DNA barcoding. Journal of Insect Conservation, 21: 827-837.

Gladis, T. (1994). Laborzucht einiger Eristalinen (Diptera, Syrphidae) und Möglichkeiten für ihren Einsatz in der Pflanzenzüchtung [Laboratory rearing of some eristalines (Diptera, Syrphidae) and the possibility of their use in plant cultures]. *Verhandlungen Der Westdeutscher Entomologentag*, *1993*, 139–152.

Goulson, D. (2020). Pesticides, Corporate Irresponsibility, and the Fate of Our Planet. *One Earth*, *2*(4), 302–305. https://doi.org/https://doi.org/10.1016/j.oneear.2020.03.004

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E. L. (2015). Bee declines driven by combined Stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229). https://doi.org/10.1126/science.1255957

Grass, I., Albrecht, J., Jauker, F., Diekötter, T., Warzecha, D., Wolters, V., and Farwig, N. (2016). Much more than bees—Wildflower plantings support highly diverse flower-visitor communities from complex to structurally simple agricultural landscapes. *Agriculture, Ecosystems & Environment*, *225*, 45–53. https://doi.org/https://doi.org/10.1016/j.agee.2016.04.001

Haas, J., Hayward, A., Buer, B., Maiwald, F., Nebelsiek, B., Glaubitz, J., Bass, C., and Nauen, R. (2022). Phylogenomic and functional characterization of an evolutionary conserved cytochrome P450-based insecticide detoxification mechanism in bees. *Proceedings of the National Academy of Sciences*, *119*(26), e2205850119. https://doi.org/10.1073/pnas.2205850119

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., and de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE*, *12*(10), e0185809. https://doi.org/10.1371/journal.pone.0185809

Hallmann, C. A., Ssymank, A., Sorg, M., de Kroon, H., and Jongejans, E. (2021). Insect biomass decline scaled to species diversity: General patterns derived from a hoverfly community. *Proceedings of the National Academy of Sciences*, *118*(2), e2002554117. https://doi.org/10.1073/pnas.2002554117

Heard, M. S., Baas, J., Dorne, J.- lou, Lahive, E., Robinson, A. G., Rortais, A., Spurgeon, D. J., Svendsen, C., and Hesketh, H. (2017). Comparative toxicity of pesticides and environmental contaminants in bees: Are honey bees a useful proxy for wild bee species? *Science of The Total Environment*, *578*, 357–365. https://doi.org/https://doi.org/10.1016/j.scitotenv.2016.10.180

Jansen, J. P., Defrance, T., and Warnier, A. M. (2011). Side effects of flonicamide and pymetrozine on five aphid natural enemy species. *BioControl*, *56*(5), 759–770. https://doi.org/10.1007/s10526-011-9342-1

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Larson, B. M. H., Kevan, P. G., and Inouye, D. W. (2001). Flies and flowers: taxonomic diversity of anthophiles and pollinators. *The Canadian Entomologist*, *133*(4), 439–465. https://doi.org/DOI: 10.4039/Ent133439-4

Lourencetti, A. P. S., Azevedo, P., Miotelo, L., Malaspina, O., and Nocelli, R. C. F. (2023). Surrogate species in pesticide risk assessments: Toxicological data of three stingless bees species. *Environmental Pollution*, *318*, 120842. https://doi.org/https://doi.org/10.1016/j.envpol.2022.120842

Mellini, E. and Coulibaly, A.K. (1991) Un decennio di sperimentazione sul sistema ospite – parassita *Galleria mellonella* L. – Pseudogonia rufifrons Wied.: sintesi dei risultati. Bollettino dell'Istituto di Entomologia "Guido Grandi" della Università degli Studi di Bologna, 45, 191–249.

Moens, J., de Clercq, P., and Tirry, L. (2011). Side effects of pesticides on the larvae of the hoverfly *Episyrphus balteatus* in the laboratory. *Phytoparasitica*, *39*(1), 1–9. https://doi.org/10.1007/s12600-010-0127-3

Nagloo, N., Rigosi, E., and O'Carroll, D. C. (2023). Acute and chronic toxicity of imidacloprid in the pollinator fly, *Eristalis tenax* L., assessed using a novel oral bioassay. *Ecotoxicology and Environmental Safety*, 114505, 251. https://doi.org/10.1016/j.ecoenv.2023.114505

OECD. (1998). Test No. 214: honeybees, acute contact toxicity test. *OECD Guidelines for the Testing of Chemicals*.

Ollerton, J. (2017). Pollinator diversity: distribution, ecological function, and conservation. *Annual Review of Ecology, Evolution and Systematics*, *48*.

Orford, K. A., Vaughan, I. P., and Memmott, J. (2015). The forgotten flies: The importance of non-syrphid Diptera as pollinators. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1805). https://doi.org/10.1098/rspb.2014.2934

Pamminger, T. (2021). Extrapolating Acute Contact Bee Sensitivity to Insecticides Based on Body Weight Using a Phylogenetically Informed Interspecies Scaling Framework. *Environmental Toxicology and Chemistry*, *40*(7), 2042–2050. https://doi.org/https://doi.org/10.1002/etc.5045

Pekas, A., de Craecker, I., Boonen, S., Wäckers, F. L., and Moerkens, R. (2020). One stone; two birds: concurrent pest control and pollination services provided by aphidophagous hoverflies. *Biological Control*, *149*, 104328. https://doi.org/https://doi.org/10.1016/j.biocontrol.2020.104328

Posthuma, L., Suter II, G. W., and Traas, T. P. (2001). *Species sensitivity distributions in ecotoxicology*. CRC press.

Ritz, C., Baty, F., Streibig, J. C., and Gerhard, D. (2015). No title. *Dose-Response Analysis Using R PloS One 10: E0146021*.

Robinson, A., Hesketh, H., Lahive, E., Horton, A. A., Svendsen, C., Rortais, A., Dorne, J. lou, Baas, J., Heard, M. S., and Spurgeon, D. J. (2017). Comparing bee species responses to chemical mixtures: Common response patterns? *PLoS One*, *12*(6), e0176289.

Rodrigues, M. A., and Flatt, T. (2016). Endocrine uncoupling of the trade-off between reproduction and somatic maintenance in eusocial insects. *Current Opinion in Insect Science*, *16*, 1–8. https://doi.org/https://doi.org/10.1016/j.cois.2016.04.013

Sánchez, M., Belliure, B., Montserrat, M., Gil, J., and Velásquez, Y. (2022). Pollination by the hoverfly *Eristalinus aeneus* (Diptera: Syrphidae) in two hybrid seed crops: celery and fennel (Apiaceae). *The Journal of Agricultural Science*, *160*(3–4), 194–206. https://doi.org/DOI: 10.1017/S0021859622000314

Sanchez-Bayo, F., and Goka, K. (2014). Pesticide residues and bees–a risk assessment. *PloS One*, *9*(4), e94482.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., and Porrini, C. (2020). Bees and pesticide regulation: Lessons from the neonicotinoid experience. *Biological Conservation*, *241*(June 2019), 108356. https://doi.org/10.1016/j.biocon.2019.108356

Skevington, J. H., and Dang, P. T. (2002). Exploring the diversity of flies (Diptera). *Biodiversity*, *3*(4), 3–27. https://doi.org/10.1080/14888386.2002.9712613

Ssymank, A., Kearns, C. A., Pape, T., and Thompson, F. C. (2008). Pollinating Flies (Diptera): A major contribution to plant diversity and agricultural production. *Biodiversity*, *9*(1–2), 86–89. https://doi.org/10.1080/14888386.2008.9712892

Suchail, S., Guez, D., and Belzunces, L. P. (2000). Characteristics of imidacloprid toxicity in two Apis mellifera subspecies. *Environmental Toxicology and Chemistry*, *19*(7), 1901–1905. https://doi.org/https://doi.org/10.1002/etc.5620190726

Team, R. C. (2013). *R: A language and environment for statistical computing*.

Thompson, H. (2016). Extrapolation of acute toxicity across bee species. *Integrated Environmental Assessment and Management*, *12*(4), 622–626. https://doi.org/https://doi.org/10.1002/ieam.1737

Thorley, J., and Schwarz, C. (2018). ssdtools: An R package to fit species sensitivity distributions. *Journal of Open Source Software*, *3*(31), 1082.

Topping C.J., Aldrich A., Berny P. (2020). Overhaul environmental risk assessment for pesticides. *Science*, 367, 360-363. DOI: 10.1126/science.aay1144

Tosi, S., Sfeir, C., Carnesecchi, E., vanEngelsdorp, D., and Chauzat, M.-P. (2022). Lethal, sublethal, and combined effects of pesticides on bees: A meta-analysis and new risk assessment tools. *Science of The Total Environment*, *844*, 156857. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.156857

Uhl, P., and Brühl, C. A. (2019). The Impact of Pesticides on Flower-Visiting Insects: A Review with Regard to European Risk Assessment. *Environmental Toxicology and Chemistry*, *38*(11), 2355–2370. https://doi.org/10.1002/etc.4572

(USEPA) Environmental Protection Agency, (PMRA) Health Canada Pest Management Regulatory Agency Health Canada, and (CDPR) California Department of Pesticide Regulation. 2014. Guidance for assessing pesticide risks to bees. https://www.epa.gov/sites/production/files/2014-06/documents/pollinator_risk_assessment_guidance_06_19_14.pdf

(USEPA) United States Environmental Protection Agency. 2023. BMDS Online (Model Library Version 2023.03.1) [Web App]. Available from https://bmdsonline.epa.gov/.

van Straalen, N. M. (1994). Biodiversity of ecotoxicological responses in animals. *Netherlands Journal of Zoology*, *44*(1), 112–129.

Vogt, H. (2000). Sensitivity of non-target arthropod species to plant protection products according to laboratory results of the IOBC WG Pesticides and Beneficial Organisms'. *IOBC WPRS BULLETIN*, *23*(9), 3–16.

Wheeler, J. R., Grist, E. P. M., Leung, K. M. Y., Morritt, D., and Crane, M. (2002). Species sensitivity distributions: data and model choice. *Marine Pollution Bulletin*, *45*(1), 192–202. https://doi.org/https://doi.org/10.1016/S0025-326X(01)00327-7

Wood, T. J., and Goulson, D. (2017). The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. *Environmental Science and Pollution Research*, *24*(21), 17285–17325. https://doi.org/10.1007/s11356-017-9240-x

Woodcock, T. S., Larson, B. M. H., Kevan, P. G., Inouye, D. W., and Lunau, K. (2014). Flies and flowers II: floral attractants and rewards. *Journal of Pollination Ecology*, *12*, 63–94.

Yuan, H., Gao, B., Wu, C., Zhang, L., Li, H., Xiao, Y., and Wu, K. (2022). Genome of the hoverfly *Eupeodes corollae* provides insights into the evolution of predation and pollination in insects. *BMC Biology*, *20*(1), 157. https://doi.org/10.1186/s12915-022-01356-6

Zattara E.E., Aizen M.A., (2021). Worldwide occurrence records suggest a global decline in bee species richness. One Earth, 4: 114-123.

Conclusions

Chapter 1

- Chronic larval exposure to residual concentrations of imidacloprid, alone or in combination with the fungicide tebuconazole, slower the larval development and caused early mortality, although the last was not statistically significant.
- Although the survival rate was not significantly different among treatments, only 58 and 53 % of bees exposed to INS and MIX, respectively, were able to emerge.
- The MIX treatment did not potentiate the toxic effect between the two pesticides.
- The observed differences between the two controls (non-manipulated and manipulated) for the endpoints of larval development and food/body conversion rate, may be expected due to the high variance of the weight of the provisions in the non-manipulated control. However, chemical residue analysis shows that the methods used for contamination of the pollen-nectar provisions is valid and can be suitable to develop a protocol to test pesticides on larvae of solitary bees

Chapter 2

- Chronic exposure to residual concentrations of imidacloprid affected feeding and survival of the solitary bee. The acute exposure to a fungicide, considered safe to use during bloom, had a temporary sub-lethal impact (feeding).
- The fungicide pulse increased the IBRv2 index, indicating potential molecular alterations, however, the individuals were able to recover when the contamination source was removed.
- The chronic exposure to the neonicotinoid, significantly reduced the neurological activity of the enzymes.
- In particular, AChE activity was inhibited by the fungicide and insecticide, showing to be a promising tool to be used as an indicator of sub-lethal effects in *O. bicornis*. Inhibition of AChE activity was positively correlated to alterations in the feeding behaviour, by causing an effect of immobility on the females.
- Contrary to our expectations the pulse of fungicide did not exacerbate the effects of imidacloprid.
- Our results highlight the importance of developing tools for potential use in pesticide post-registration monitoring programs and in the future framework of chemical risk assessment as early warning signals.

Chapter 3

- Acute contact LD_{50} values showed to be of high variability between the three studied species. Sensitivities were, from the most sensitive (low LD_{50}, expressed in ng per insect) to the lowest (high LD_{50}): *S. rueppellii* < *E. larvarum* < *E. aeneus*.
- Our Species Sensitivity Distribution (SSD) approach, revealed that the protection derived by the honey bee LD_{50} increases when body weight correction is performed, however still

40 % of the species are left uncovered (all of them bee species). The three Diptera species used in the study were sufficiently cover by the endpoint of honey bee.

- When combining the body weight correction with the safety factor of 10, proposed by EFSA, still the honey does not sufficiently cover all the bee species (leaving uncovered 3 bee species). Therefore, the SSD approach used in this study can be used to derive a conservative protection value (HD5).
- Sub-lethal Sensitivity Index (SSI) values, show that fecundity is a very sensitive endpoint in the tachinid fly *E. larvarum* compared to the other dipterans, as its SSI value was around ten times higher than the hoverflies of our study. This result indicates a trade-off between survival and reproduction in *E. larvarum*, while in hoverfly species, the lethal and sub-lethal doses were relatively close.
- The future risk assessment schemes should include more sensitive species than the honey bee.

APPENDIX 1

Supplementary materials - Chapter 1

Table A1.1: Determination of pesticide residues on the provisions by LC/MS-MS and GC/MS-MS. CTRL: control, Water: water control, FUNG: tebuconazole, INS: imidacloprid. MIX: tebuconazole + imidacloprid. T0: provision collected soon after the preparation and T2: provision collected 15 days of the preparation.

Treatment	Pesticide detected	Units	Value	IC (95%)
CTRL - T0	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.026	0.011
CTRL -T2	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.025	0.011
	Pendimethalin	mg/kg	0.01	0.004
Water -T0	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.024	0.011
Water -T2	multi-LC, multi-GC	mg/kg	<LOQ	-
FUNG - T0	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.024	0.011
	Tebuconazole	**mg/kg**	**9.056**	**2.08**
FUNG - T2	Captan	mg/kg	<LOQ	-
	Tebuconazole	**mg/kg**	**9.367**	**2.141**
INS - T0	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.015	0.007
	Imidacloprid	**mg/kg**	**0.007**	**0.003**
INS- T2	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.017	0.008
MIX - T0	Captan	mg/kg	<LOQ	-
	Folpet	mg/kg	0.021	0.009
	Imidacloprid	**mg/kg**	**0.01**	**0.004**
	Tebuconazole	**mg/kg**	**8.72**	**2.014**
MIX - T2	Folpet	mg/kg	0.013	0.006
	Imidacloprid	**mg/kg**	**0.006**	**0.003**
	Tebuconazole	**mg/kg**	**9.08**	**2.085**

The measurement uncertainty applied to the test results was calculated at a 95% confidence level (p) assuming a coverage factor (k) equal to 2.
LOQ values: Captan =0.100 mg/kg; Folpet =0.010 mg/kg; Pendimethalin = 0.010 mg/kg
In bold are the concentrations used in the experiment

Table A1.2: Primer sequences for preparation of amplicon library based on the 16S rRNA and ITS

Libraries	Primer name	Primer sequence (5'->3')
16S	V5-V6 799F-mod3_PG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCMGGATTAGATACCCKGG
	V5-V6 1115R_PG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGGGTTGCGCTCGTTG
ITS	ITS1F_PG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGTAA
	ITS2_PG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC

APPENDIX 2

Supplementary materials - Chapter 2

Table A2.1: Daily syrup consumption (µl day^{-1}) in *Osmia bicornis* females orally exposed to various pesticide treatments. CTRL: chronic exposure to regular syrup; FUNG: chronic exposure to regular syrup with a pulse of tebuconazole (185 mg L^{-1}) on day 3; INS: chronic exposure to syrup with imidacloprid (5 µg L^{-1}); MIX: chronic exposure to syrup with imidacloprid (5 µg L^{-1}) + a pulse of tebuconazole (185 mg L^{-1}) on day 3. Pre-pulse: first 2 days of exposure; Pulse: day 3; Post-pulse: after the 3rd up to the date of 50% mortality within each treatment; TOTAL: Daily syrup consumption from the beginning of exposure until the 50% mortality within each treatment.

		Pre-pulse		Pulse (day 3)		Post-pulse		TOTAL	
Treatment	**N**	**Mean (SD)**	**Median**	**Mean (SD)**	**Median**	**Mean (SD)**	**Median**	**Mean (SD)**	**Median**
CTRL	25	110 (30.6)	114.4	118 (27.3)	120	59.4 (21.1)	55.2	69.2 (16.2)	64.4
FUNG	26	77.3 (32.0)	81.9	25.6 (18.8)	20	45.4 (27.6)	49.1	52.2 (22.4)	53.0
INS	26	26.9 (9.7)	24.7	14.4 (9.79)	11.3	11.1 (4.9)	11.1	15.1 (4.1)	15.6
MIX	24	24.8 (8.3)	24.4	35.9 (14.3)	40	11 (5.15)	10.2	16.7 (4.8)	16.0

Table A2.2: Results of Mann-Whitney U Test (One-sided tests*) when syrup consumption is compared between pre- pulse and post pulse- time. *Note: Ho: median of pre-pulse -post-pulse assessment = 0 vs. Ha: median of pre-pulse -post-pulse assessment > 0

Treatment	p-value
CTRL	0.0000
FUNG	0.0003
INS	0.0000
MIX	0.0000

Table A2.3: Results of the Kernel regression analysis (dependent variable is daily syrup consumption).

	Average	SE	t	p-value
Body size	6.1768	6.64318	0.930	0.354

Table 2.4: Results of Fleming-Harrington test for equality of survivor functions (middle differences).

Treatment	Events observed	Events expected	Sum of ranks
CTRL	38	38.70	-1.4370414
FUNG	31	41.03	-1.9626036
INS	33	27.79	1.1195858
MIX	28	22.48	2.2800592
Total	130	130	0
$\chi^2(3) = 15.90$; Pr > $\chi^2 = 0.0012$			

Table A2.5: Results of Cox regression model.

	Haz. Ratio	SE	z	P>|z|	[95% Conf. Interval]
Body size	2.16989	2.474617	0.68	0.497	0.2321125 - 20.28509

Table A2.6: Activity of four biomarkers (AChE is expressed as μmol^{-1} g $tissue^{-1}$ min. CaE, GST and ALP are expressed as nmol min^{-1} mg^{-1} protein) in *Osmia bicornis* females orally exposed to various pesticide treatments. CTRL: chronic exposure to regular syrup; FUNG: chronic exposure to regular syrup with a pulse of tebuconazole (185 mg L^{-1}) on day 3; INS: chronic exposure to syrup with imidacloprid (5 µg L^{-1}); MIX: chronic exposure to syrup with imidacloprid (5 µg L^{-1}) + a pulse of tebuconazole (185 mg L^{-1}) on day 3. Measurements were taken at T1 (day 4 of exposure; A) and T2 (day 6 of exposure; B).

		AChE					CaE					GST					ALP				
Time	**Treatment**	**N**	**Min**	**Max**	**Mean (SD)**	**Median**	**N**	**Min**	**Max**	**Mean (SD)**	**Median**	**N**	**Min**	**Max**	**Mean (SD)**	**Median**	**N**	**Min**	**Max**	**Mean (SD)**	**Median**
A	**CTRL**	8	1.45	2.86	2.09 (0.48)	1.99	6	1.41	3.42	2.62 (0.73)	2.74	5	602.21	3058.69	1568.73 (1021.93)	1488.62	5	85.15	125.32	106.15 (15.94)	110.59
	FUNG	10	1.07	2.12	1.48 (0.35)	1.44	6	1.31	4.61	2.41 (1.26)	2.13	6	700.22	6344.31	2240.18 (2201.32)	1144.76	6	14.58	66.74	42.72 (21.40)	41.77
	INS	10	0.83	2.03	1.34 (0.42)	1.41	10	1.64	3.19	2.51 (0.48)	2.54	5	600.50	1201.85	886.56 (270.03)	800.14	2	21.71	109.15	65.43 (61.83)	65.43
	MIX	10	0.52	2.01	1.55 (0.48)	1.75	9	0.61	5.73	2.88 (1.50)	2.34	4	601.54	3119.65	1646.14 (1112.35)	1431.69	3	16.80	144.29	69.15 (66.73)	46.37
B	**CTRL**	10	1.17	2.64	1.94 (0.42)	1.94	9	0.17	20.1	4.06 (6.13)	2.66	7	405.02	5457.02	2865.88 (1924.03)	2230.08	7	13.64	198.33	109.47 (65.6)	102.21
	FUNG	10	0.91	1.85	1.33 (0.33)	1.30	8	0.21	24.59	5.91 (7.95)	2.86	8	466.39	11688.10	2593.15 (3855.46)	992.15	6	20.15	145.41	70.09 (56.77)	44.48
	INS	9	0.72	1.39	1 .00 (0.21)	0.98	9	1.06	8.02	3.23 (2.28)	2.27	5	521.50	1228.36	962.82 (297.54)	1129.54	5	13.25	43.12	27.74 (13.21)	26.43
	MIX	4	0.23	2.13	1.23 (1.02)	1.28	2	1.40	62.29	31.85 (43.06)	31.85	5	939.77	9160.21	3679.79 (3295.66)	3190.55	5	15.60	118.65	61.33 (38.37)	52.05

Table A2.7: Results of Kruskal-Wallis tests at T1 and T2 for the different tests: TSC (Mean daily syrup consumption; expressed as µl day−1); AChE (acetylcholinesterase; expressed as µmol^{-1} g tissue^{-1} min); CaE (carboxylesterase; expressed as nmol min^{-1} mg^{-1} protein); GST (glutathione-S-transferase; expressed as nmol min^{-1} mg^{-1} protein) and ALP (alkaline phosphatase; expressed as nmol min^{-1} mg^{-1} protein).

Biomarkers	p-value at TIME 1	p-values at TIME 2
TSC	0.0001	0.0001
AChE	0.0194	0.0023
CaE	0.8263	0.8770
GST	0.5519	0.1852
ALP	0.1138	0.0885

Table A2.8: Results of Dunn's Pairwise in comparison with the control group for TSC (Mean daily syrup consumption; expressed in µl day−1), AChE (acetylcholinesterase; expressed as µmol^{-1} g tissue^{-1} min), CaE (carboxylesterase; expressed as nmol min^{-1} mg^{-1} protein), GST (glutathione-S-transferase; expressed as nmol min^{-1} mg^{-1} protein) and ALP (alkaline phosphatase; expressed as nmol min^{-1} mg^{-1} protein).

Biomarkers	TIME1			TIME2		
	FUNG	INS	MIX	FUNG	INS	MIX
TSC	0.2927	0.0001	0.0012	0.2580	0.0000	0.0002
AChE	0.0403	0.0074	0.0956	0.0607	0.0005	0.1129
CaE	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
GST	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ALP	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

APPENDIX 3

Supplementary materials - Chapter 3

Figure A3.1: Boxplots of fresh weight for studied fly species.

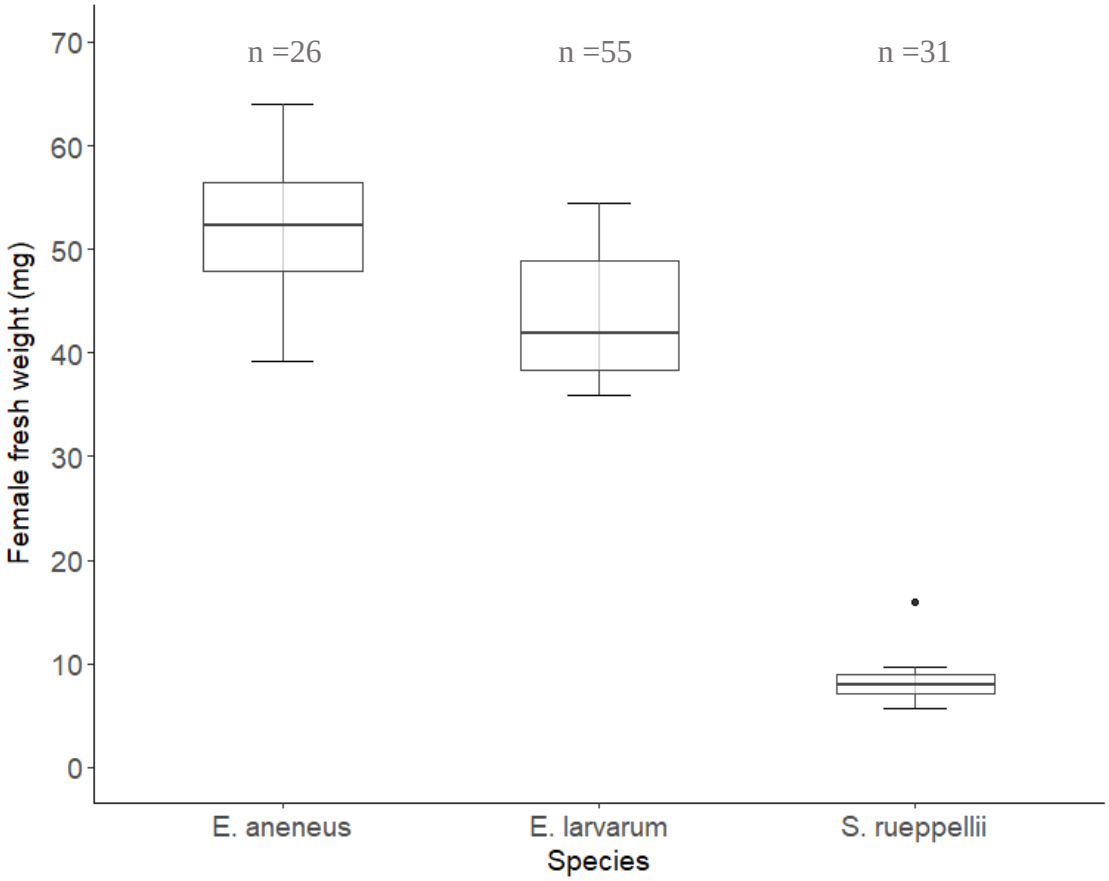


Table A3.1: Toxicity data used for fitting the Sensitivity Species Distribution (SSD). LD_{50} values: lethal dose of imidacloprid to 50% of the organisms after 48h. LD_{50} values are represent without and with weight standardization. Type shows the used product in the studies (a.i.: active ingredient, c.f.: commercial formulation). The reference column shows the respective studies. When no weight standardization was performed in the study, we indicate with letters the literature reference for the mean weight, used to transform the data.

Species	Family	Mean fresh weight (mg)	Contact LD_{50} (ng/insect)	Contact LD_{50} (µg/g insect)	Type	Reference
Flies (Diptera)						
Exorista larvarum	Tachinidae	40.1	467.5	11.7	c.f	This study
Sphaerophoria rueppellii	Syrphidae	7.9	10.2	1.35	c.f	This study
Eristalinus aeneus	Syrphidae	52.7	18176.2	344.8	c.f	This study
Bees (Hymenoptera)						
European *Apis mellifera*	Apidae	100a	6.7	0.07	a.i.	Suchail et al., 2000
European *Apis mellifera*	Apidae	100a	24.3	0.24	a.i.	Suchail et al., 2000
European *Apis mellifera*	Apidae	100a	81	0.81	a.i.	EFSA, 2015
European *Apis mellifera*	Apidae	100a	42	0.42	c.f.	EFSA, 2015
European *Apis mellifera*	Apidae	100a	245	2.45	a.i.	Uhl et al., 2019
European *Apis mellifera*	Apidae	112	234	2.09	a.i.	Tai et al., 2022
European *Apis mellifera*	Apidae	100a	150	1.5	c.f.	Biddinger et al., 2013
European *Apis mellifera*	Apidae	100a	60	0.6	a.i.	ECOTOX (U.S. Environment Protection Agency)
Apis cerana	Apidae	75b	3.6	0.05	a.i.	Yasuda et al., 2017
Bombus terrestris	Apidae	200c	14	0.07	a.i.	Bortolotti et al., 2002
Bombus terrestris	Apidae	200c	77	0.39	c.f.	Bortolotti *et al.,* 2002
Scaptotrigona postica	Apidae	18d	24.5	1.36	a.i.	Soares et al., 2015
Melipona scutellaris	Apidae	100e	1.29	0.013	a.i.	da Costa et al., 2015
Leioproctus paahaumaa	Colletidae	52.4	1.21	0.0231	a.i.	Tai et al., 2022
Osmia bicornis	Megachilidae	94.6f	30	0.33	a.i.	Uhl et al., 2019
Osmia bicornis	Megachilidae	94.6f	46	0.49	a.i.	Beadle et al., 2019
Osmia cornifrons	Megachilidae	131g	3820	29.16	c.f.	Biddinger et al., 2013
Osmia cornifrons	Megachilidae	100-110	-	0.023	a.i.	Phan et al., 2020
Beetles (Coleoptera)						
Harmonia axyridis	Coccinellidae	37.5g	360	9.60	c.f.	Youn et al., 2003
Coleomegilla maculate	Coccinellidae	14.2g	74	5.21	c.f.	Lucas et al., 2004

a. Mean weight from Suchail *et al*., 2004
b. Mean weight from Thompson, 2016
c. Mean weight from Hagen *et al*., 2011
d. Mean weight from Hartfelder and Engels, 1992
e. Attributed the same weight as honey bee *Apis mellifera* L. (Lourenço *et al*., 2012)
f. Mean weight from Uhl *et al*., 2019
g. Hätönen *et al*., 2022

Table A3.2: Species sensitivity ratio (R). Values in bold exceed the range of 10-fold safety factor from the endpoint of *A. mellifera*, recommended by EFSA Guidance document (2013).

Species	R (ng/insect)	R (µg/g insect)
Exorista larvarum	0.14	0.05
Sphaerophoria rueppellii	6.27	0.47
Eristalinus aeneus	0.00	0.00
Apis cerana	**17.81**	**12.69**
Bombus terrestris	1.95	3.84
Scaptotrigona postica	2.62	0.47
Melipona scutellaris	**49.71**	**49.20**
Leioproctus paahaumaa	**52.99**	**27.48**
Osmia bicornis	1.73	1.58
Osmia cornifrons	0.02	0.78
Harmonia axyridis	0.18	0.07
Coleomegilla maculate	0.87	0.12

References

Curated toxicity data were retrieved from the ECOTOXicology Knowledgebase, U.S. Environmental Protection Agency. http:/www.epa.gov/ecotox/ (1 November 2022)

Beadle, K., Singh, K. S., Troczka, B. J., Randall, E., Zaworra, M., Zimmer, C. T., Hayward, A., Reid, R., Kor, L., Kohler, M., Buer, B., Nelson, D. R., Williamson, M. S., Davies, T. G. E., Field, L. M., Nauen, R., and Bass, C. (2019). Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*. *PLOS Genetics*, *15*(2), e1007903-. https://doi.org/10.1371/journal.pgen.1007903

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. A., Rajotte, E. G., Joshi, N. K., and Vaughn, M. (2013). Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLOS ONE*, *8*(9), e72587. https://doi.org/10.1371/journal.pone.0072587

Bortolotti, L., Porrini, C., and Sbrenna, G. (2002). Effetti dell'imidacloprid nei confronti di *Bombus terrestris* (L.). *Informatore Fitopatologico*, 66.

da Costa, L. M., Grella, T. C., Barbosa, R. A., Malaspina, O., and Nocelli, R. C. F. (2015). Determination of acute lethal doses (LD50 and LC50) of imidacloprid for the native bee *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae). *Sociobiology*, *62*(4), 578–582. https://doi.org/10.13102/sociobiology.v62i4.792

EFSA (2013). Guidance on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus* spp. and solitary bees). *EFSA Journal*,11(7):3295. https://doi.org/10.2903/j.efsa.2013.3295

EFSA (2015). Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid considering all uses other than seed treatments and granules. *EFSA Journal* (Vol. 13, Issue 8). Wiley-Blackwell Publishing Ltd. https://doi.org/10.2903/j.efsa.2015.4211

Hagen, M., Wikelski, M., and Kissling, W. D. (2011). Space Use of Bumblebees (Bombus spp.) Revealed by Radio-Tracking. *PLOS ONE*, *6*(5), e19997. https://doi.org/10.1371/journal.pone.0019997

Hartfelder, K., and Engels, W. (1992). Allometric and multivariate analysis of sex and caste polymorphism in the neotropical stingless bee,Scaptotrigona postica. *Insectes Sociaux*, *39*(3), 251–266. https://doi.org/10.1007/BF01323946

Hätönen, M., Kantner, C., Lopez Losada, R., Ludwig, N., Benavent González, A., Riedhammer, C., Kunz, P., Panico, S. C., Laakkonen, E., Parramon Dolcet, L., Vangheel, M., Carlon, C., and Gutierrez Alonso, S. (2022). *European arthropods and their role in pollination: scientific report of their biodiversity, ecology and sensitivity to biocides.* https://doi.org/10.2823/147199

Kueh Tai, F., Pattemore, D. E., Jochym, M., Beggs, J. R., Northcott, G. L., and Mortensen, A. N. (2022). Honey bee toxicological responses do not accurately predict environmental risk of imidacloprid to a solitary ground-nesting bee species. *Science of the Total Environment*, *839*. https://doi.org/10.1016/j.scitotenv.2022.156398

Lourenço, C. T., Carvalho, S. M., Malaspina, O., and Nocelli, R. C. F. (2012). Oral Toxicity of Fipronil Insecticide Against the Stingless Bee *Melipona scutellaris* (Latreille, 1811). *Bulletin of Environmental Contamination and Toxicology*, *89*(4), 921–924. https://doi.org/10.1007/s00128-012-0773-x

Lucas, É., Giroux, S., Demougeot, S., Duchesne, R.-M., and Coderre, D. (2004). Compatibility of a natural enemy, *Coleomegilla maculata lengi* (Col., Coccinellidae) and four insecticides used against the Colorado potato beetle (Col., Chrysomelidae). *Journal of Applied Entomology*, *128*(3), 233–239. https://doi.org/https://doi.org/10.1111/j.1439-0418.2004.00843.x

Phan, N. T., Joshi, N. K., Rajotte, E. G., López-Uribe, M. M., Zhu, F., and Biddinger, D. J. (2020). A new ingestion bioassay protocol for assessing pesticide toxicity to the adult Japanese orchard bee (*Osmia cornifrons*). *Scientific Reports*, *10*(1), 9517. https://doi.org/10.1038/s41598-020-66118-2

Soares, H. M., Jacob, C. R. O., Carvalho, S. M., Nocelli, R. C. F., and Malaspina, O. (2015). Toxicity of Imidacloprid to the Stingless Bee *Scaptotrigona postica* Latreille, 1807 (Hymenoptera: Apidae). *Bulletin of Environmental Contamination and Toxicology*, *94*(6), 675–680. https://doi.org/10.1007/s00128-015-1488-6

Suchail, S., Debrauwer, L., and Belzunces, L. P. (2004). Metabolism of imidacloprid in *Apis mellifera*. *Pest Management Science*, *60*(3), 291–296. https://doi.org/https://doi.org/10.1002/ps.772

Suchail, S., Guez, D., and Belzunces, L. P. (2000). Characteristics of imidacloprid toxicity in two *Apis mellifera* subspecies. *Environmental Toxicology and Chemistry*, *19*(7), 1901–1905. https://doi.org/https://doi.org/10.1002/etc.5620190726

Thompson, H. (2016). Extrapolation of acute toxicity across bee species. *Integrated Environmental Assessment and Management*, *12*(4), 622–626. https://doi.org/https://doi.org/10.1002/ieam.1737

Uhl, P., Awanbor, O., Schulz, R. S., and Brühl, C. A. (2019). Is *Osmia bicornis* an adequate regulatory surrogate? Comparing its acute contact sensitivity to Apis mellifera. *PLOS ONE*, *14*(8), e0201081-. https://doi.org/10.1371/journal.pone.0201081

Yasuda, M., Sakamoto, Y., Goka, K., Nagamitsu, T., and Taki, H. (2017). Insecticide Susceptibility in Asian Honey Bees (Apis cerana (Hymenoptera: Apidae)) and Implications for Wild Honey Bees in Asia. *Journal of Economic Entomology*, *110*(2), 447–452. https://doi.org/10.1093/jee/tox032

Youn, Y. N., Seo, M. J., Shin, J. G., Jang, C., and Yu, Y. M. (2003). Toxicity of greenhouse pesticides to multicolored Asian lady beetles, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Biological Control*, *28*(2), 164–170. https://doi.org/https://doi.org/10.1016/S1049-9644(03)00098-7