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Review on imidacloprid diffusion route and a case study: from apple orchard to the honey bee colony matrices

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Abstract

Honey bees play a pivotal role in natural and rural ecosystems by providing human and animal food sources through pollination services. However, in cultivated areas, they can be exposed to the chemicals utilized for crop protection. Neonicotinoid insecticides can adversely affect honey bee colonies impairing their survival, immunity and biological activities at lethal and sublethal doses. For this reason, neonicotinoids, together with other stress factors, like pathogens (e.g. viruses and *Varroa* mites), climate change and food shortage, are considered one of the causes of worldwide colony losses. Nevertheless, the natural way of entry and diffusion of these pesticides in field colonies is not completely clear. Here, we wanted to fill this gap by studying the diffusion route of imidacloprid and its metabolites by analysing different matrices collected from honey bee colonies used for pollination of apple orchards, in the framework of applied Integrated Pest Management strategies. Pollen, honey bees, honey, royal jelly, bee wax and bee bread were sampled from 6 honey bee colonies placed in two different apple orchards before blooming, exposed to chemicals application and removed from the site after that. Samples were analysed using a liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) in order to detect imidacloprid, olefin imidacloprid and 5-hydroxy imidacloprid. The results demonstrate that the primary way of entrance of imidacloprid was the pollen transported by foragers, while the main accumulation matrices were bee bread, honey and wax. These findings allow us to hypothesize that the accumulation of this insecticide, especially in bee bread, the main larval food, could potentially impact negatively on honey bee wellbeing at the adult stage. Moreover, our data could implement the honey bee colony simulator.

Key words: pesticides, honey bee, toxicodynamic, residues, bee bread.

Introduction

During their regular foraging activity, honey bees (*Apis mellifera* L.) perform an essential ecosystem service (i.e. pollination) for about 90% of wild flowering plant species and 75% of the world's most common crops (Garibaldi *et al*., 2011; Ollerton *et al*., 2011). Thus, the evident decline of honey bees and heavy colony losses reported in different countries of Europe and North America (Mutinelli *et al*., 2010; vanEngelsdorp and Meixner, 2010; Dainat *et al*., 2012; Brodschneider *et al*., 2018; Gray *et al*., 2019), poses a risk to the fundamental service these insects provide. Honey bees are the primary pollinators in agricultural systems contributing to improving the quantity and quality of agricultural production (Hristov *et al*., 2020; Khalifa *et al*., 2021). However, they are easily exposed to pesticides used for plant protection (Schmuck *et al*., 2001; Porrini and Bortolotti, 2003; Stadler *et al*., 2003; Dively and Kamel, 2012; Tsvetkov *et al*., 2017; Douglas *et al*., 2020) through direct contact with the treated and non-target contaminated plants (Koch and Weißer, 1997; David *et al*., 2016; Gradish *et al*., 2019; Ward *et al*., 2020; Main *et al*., 2021), pesticides polluted waters (Samson-Robert *et al*., 2014; Giroux, 2019) and soils (Silva *et al*., 2019), or through the contact with contaminated dust particles or tank mixture cloud (Sanchez-Bayo and Goka, 2016; Sanchez-Bayo *et al.*, 2016; Rortais *et al*., 2017; Krahner *et al*., 2021) during their flight.

Neonicotinoids are widely used insecticides (Jeschke *et al*., 2011; Simon-Delso *et al*., 2015; Craddock *et al*., 2019) applied against a broad spectrum of sucking and certain chewing pests (Liu and Casida, 1993; Matsuda *et al*., 2001; Tomizawa and Casida, 2003; Thany, 2010). However, also beneficial insects and pollinators, such as bumblebees and honey bees, can be harmed by these systemic insecticides, potentially present in the nectar or pollen of contaminated plants (Rortais *et al*., 2005; Girolami *et al*., 2009; 2023; Cresswell, 2011; Simon-Delso *et al*., 2015; Jiang *et al*., 2018). Since the 1990s one of the most used neonicotinoids has been imidacloprid (Zhang *et al.*, 2011; Godfray *et al*., 2014; 2015), which can be applied by foliar spray application, soil application, irrigation, or seed treatment (Elbert *et al*., 1991; Chmiel *et al.*, 2020). Imidacloprid and its metabolites may be found in hive food stores (Mullin *et al*., 2010; Wu *et al*., 2011) and it is presumably picked up by honey bee foragers when gathering resources such as nectar and pollen, transported to the hive and stored (Cresswell, 2011; Schneider *et al*., 2012). Once in the hive, contaminated food is shared among the colony components and ingested by larvae and adults (Nixon and Ribbands, 1952; Farina, 1996).

Honey is the primary energy source for adult honey bees, while bee bread is the primary source of proteins, amino acids, fat and micronutrients. Bee bread is a mixture of pollen, honey and honey bee secretion and must

be consumed in large quantities by larvae of more than three days old and by nurse bees to produce royal jelly (Haydak, 1943; 1970; Malone *et al*., 2002).

Realistically, maximum exposure to imidacloprid is expected among adult honey bees that consume the most significant amounts of contaminated pollen and honey. Large amounts of pollen are consumed by nurse bees, and to a lesser extent by larvae, whereas large amounts of nectar are consumed by wax-producing bees, brood attending bees, winter bees, and foragers (Babendreier *et al*., 2004). While collecting pollen or nectar, foragers get their bodies covered by pollen (Parker, 1981; Thorp, 2000), and topical exposure of foragers to contaminated pollen is possible (Crenna *et al*., 2020). Moreover, larvae and adults inside the hive are in contact with wax, thus a topical exposure of larvae and honey bees to contaminated wax should also be taken into consideration (Wilmart *et al*., 2021). Several studies have concluded that bees exposed to neonicotinoid pesticides, and in particular to imidacloprid, show different negative effects on their life, health and reproduction (Anderson and Harmon-Threatt, 2019; Morfin *et al*., 2019; Inouri-Iskounen *et al*., 2020; Pereira *et al*., 2020; Power *et al*., 2020). A single most important cause of bee colony losses was indicated as related only to pests and pathogens (Ratnieks and Carreck, 2010) but it was hypothesized that neonicotinoids may decrease honey bee resistance to diseases and parasite attacks (Maini *et al*., 2010). Later on, it has been shown that exposure to neonicotinoids, namely imidacloprid and clothianidin, leads to immunosuppression in honey bees, promoting viral infections. This effect was found at very low concentrations, well below those that honey bees are likely to encounter in the field (Di Prisco *et al*., 2013; Brandt *et al*., 2017). Moreover, the products of imidacloprid environmental degradation and metabolization in the honey bee body are also toxic (Goulson and Kleijn, 2013). For instance, a primary metabolic byproduct (5-OH imidacloprid) significantly impairs short-term and longer-term olfactory learning (Williamson and Wright, 2013).

A study by Gooley and Gooley (2020) has outlined that administration of field-realistic dosage of imidacloprid for 48 hours, significantly disrupted honey bees' non-flight metabolic rates resulting in less available energy for foraging and performing hive duties which in the end could negatively affect colony health.

As mentioned above, adult honey bees can get into contact with agrochemical residues present in the environment, while larvae can be exposed by prolonged close contact with combs contaminated with pesticide residues or by ingestion of royal jelly produced by exposed nurse bees. The colony can consequently suffer higher brood mortality and reduced adult lifespan (Wu *et al*., 2011). Studies on the sublethal effects showed during adulthood after sublethal exposure during larval stages are not copious, however some publications provide evidence of this occurrence. It was shown that larvae of honey bees exposed to very low doses of imidacloprid (0.04 ng/larvae) significantly reduced subsequent adult learning by 58%-63% compared to control bees (Bortolotti *et al.*, 2003; Yang *et al*., 2012; Matsumoto, 2013). Considering the detrimental effect of imidacloprid on honey bee health, in 2013 the European Community restricted its use to some bee attractive crops such as maize, oilseed rape and sunflowers, after a risk assessment carried out in 2012 by the European Food Safety Authority (EFSA, 2013). A new evaluation was carried out by EFSA in 2018, resulting in restriction of imidacloprid use to greenhouse productions and expiration of its approval on December 1st 2020 (EFSA, 2018). Despite these restrictions residues of imidacloprid and its metabolites can be found in honey, pollen and bee bread (Kasiotis *et al*., 2023).

Moreover, even if the use of imidacloprid in agriculture has been severely limited in Europe, the study of the effects of this chemical on honey bees and in particular of its dislocation within the bee nests and the evaluation of its sublethal effects can greatly contribute to the understanding of similar problems related to other chemicals (Tosi *et al.*, 2021).

Even though the adverse effects of imidacloprid on honey bees are well known and now are evident, the natural way of entrance and diffusion of this chemical in the honey bee colony is still not completely clear. To fill this gap, in this study, we analysed the diffusion route of imidacloprid and its metabolites in the honey bee colonies exposed to this pesticide. We monitored the content of imidacloprid, olefin imidacloprid and 5-hydroxy imidacloprid in honey bee matrices reared in the apple orchards, where foliar application was common in the context of Integrated Pest Management strategy. Data obtained in this study could be used to update mathematical models such as ApisRAM which will be used in the new approach for the risk assessment of honey bees (Duan *et al*., 2022).

Materials and methods

Six colonies of *Apis mellifera carnica* Pollmann, homogeneous in population and with sister queens, were installed in two different apple orchards (3 colonies/orchard) in the agricultural landscape of the Trentino (Northern Italy) (supplemental material S1) during the season 2012. The fields were both managed following the Integrated Pest Management strategies and were selected considering the intensity of imidacloprid use in terms of the application rate per hectare: Low Impact Field (LIF: 450 ml/ha) and High Impact Field (HIF: 600 ml/ha) (supplemental material S2). Additional information is detailed in the supplemental material S3.

Colonies were installed in early April, just before the apple blooming and were left in place for one month and a half until being moved (June) to a natural area with no cultivated fields and zero pesticide exposure.

Pollen, honey bees, honey, royal jelly, wax and bee bread were sampled from each colony.

Pollen loads were collected using frontal pollen traps fitted to the entrance of the hive in two moments: before (April, 28th), and five days after (May, 8th in LIF and May, 11th in HIF) the spray application of imidacloprid. Pollen traps were removed after 48 hours and the collected pollen was stored at −20 °C until analysis. Pollen loads were destined for both multi-residual and palynological analyses. Pollen loads collected by colonies in

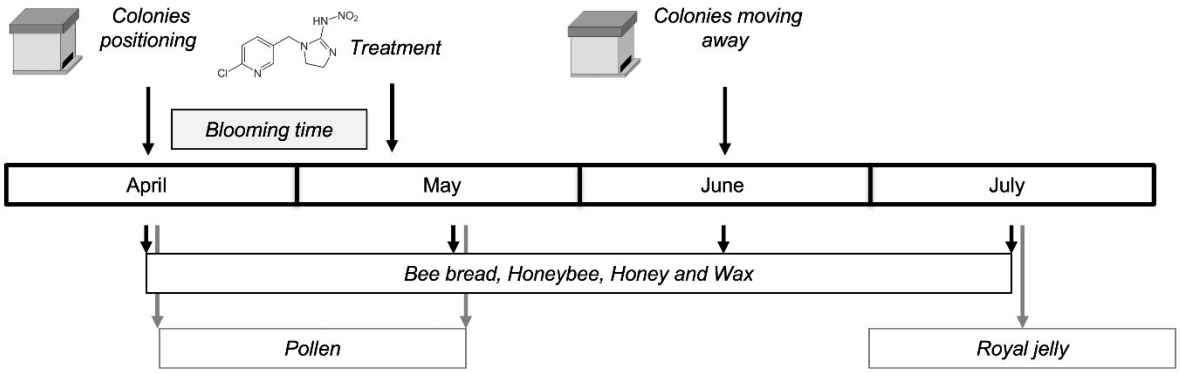

Figure 1. Experimental design. The diagram reports the field activities (above the time line) and the matrices collected (below the time line) for the entire experiment.

each data and site were pooled so that we analysed in total four pollen samples. Honey bees, honey and wax were sampled directly from the combs every month from April to July. Royal jelly samples were collected at the end of July by creating an orphan colony from each original hive used in the field trials using 5 combs with mostly capped brood covered by their workers bees. Royal cells (42 royal cells each nucleus containing 24 h-old larvae, taken from the mother colony using the normal grafting technique, were offered to the orphan nucleus, which raised a number of these larvae to obtain queens and after 72 hours from the insertion of the cells, larvae were removed and royal jelly collected. Royal jelly collected in nucleus originated by LIF colonies and that collected in HIF colonies was pooled separately to obtained two royal jelly samples.

All samples were immediately stored in dry ice to avoid degradation of active ingredients and, after reaching the laboratory, they were kept frozen at −20 °C until analysis. The workflow is summarized in figure 1.

Pollen wax and honey bees were ground using a mill in liquid nitrogen, while honey and royal jelly did not require preliminary treatment.

An aliquot of two grams of each sample was extracted according to the method QuEChERS EN 15652. The extracts were analysed using a liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS). Imidacloprid, olefin imidacloprid and 5-hydroxy imidacloprid standards were provided by Sigma Aldrich and LGC Standards. Analyses were performed using UHPLC-MS/MS model TSQ Quantum Access Max (Thermo Fisher Scientific), recording two specific transitions for each pesticide, the column used was a C18 column 2.7 µm particle size 100 × 3 mm, and the mobile phase was a gradient of 4 mM ammonium formate 0.1% formic acid in water/ 4 mM ammonium formate 0.1% formic acid in methanol. Analyses were also performed in GC-MS/MS model TSQ Quantum XLS (Thermo Fisher Scientific), with a Rxi 5 ms column.

To determine which plant species had been visited by foragers, a two-gram representative aliquot of each sample of pollen pellet was analysed in the Fondazione Edmund Mach laboratory (Trento, Italy). Each aliquot was split into batches or subsamples according to their colour. Then, one pollen pellet for each subsample was dispersed in 1 mL of distilled water. The palynological analysis was carried out on aliquots of 0.01 mL of this suspension spread on a microscopy slide. A minimum of 500 grains for each subsample were counted and identified according to literature (Barth *et al*., 2010). The identification of pollen types was done at a magnification of 400× or 1000× which evidenced the pollen morphology according to Persano and Ricciardelli d'Albore (1989), groups of 100 pollen grains were counted following 5 parallel lines through the slide (El-Labban, 2020). Pollen profile was obtained by summing all the data of each subsample and was expressed as the percentage of each pollen type.

For statistical analysis, normality of the data distribution was verified with the Shapiro-Wilk test and Kolmogorov-Smirnov test, while homogeneity of variance was tested with Levene's test.

Overall imidacloprid and metabolites concentration differences between honey bee matrices were analysed with One-Way ANOVA and LSD post hoc test, by considering one dependent variable (pesticide concentration) and one independent variable (honey bee matrices).

Two-Way ANOVA and LSD post hoc test with 95% CI of bootstrap with one dependent variable (pesticide concentration) and two independent variables (honey bee matrices and time) was used to analyse the imidacloprid and metabolites concentration differences between honey bee matrices and concerning the sampling time. The imidacloprid and metabolites concentration was expressed in ppb ± expanded uncertainty.

All statistical analyses were performed by using Prism v.5 for Mac OSX (GraphPad Software, San Diego, CA, USA), setting the significance level at 0.05.

Results

To evaluate the level of contamination of apple orchards, the content of imidacloprid and its metabolites in different honey bee matrices was analysed. For each treatment (LIF and HIF) a total of 102 samples were used for chemical analyses, particularly: 24 for honey, 24 for honey bee bodies, 24 for bee bread, 24 for wax, 4 for pollen load and 2 for royal jelly. The overall results have shown that in both fields (LIF and HIF) the level of imidacloprid was

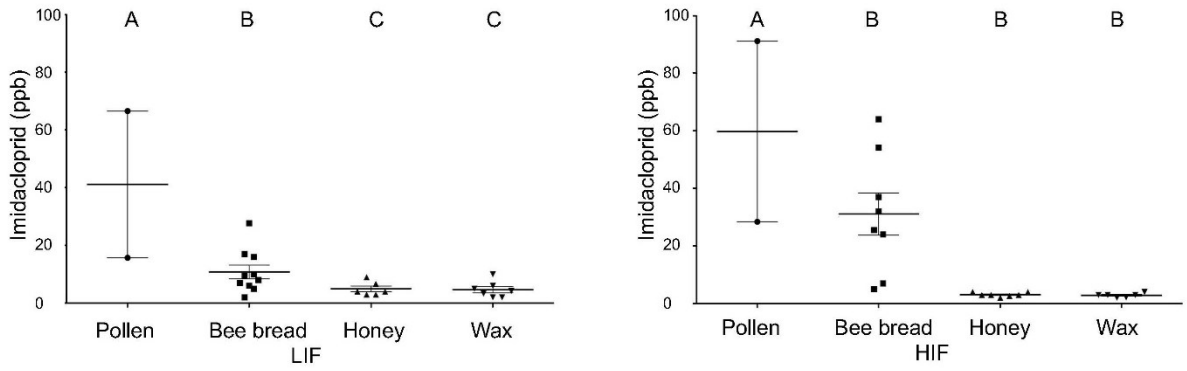

Figure 2. Average concentration of imidacloprid in honey bee matrices (bee bread, honey and wax) from LIF and HIF. All values are expressed in ppb ± standard error, and the differences have been statistically compared with One-Way ANOVA and LSD post hoc test. Different letters indicate statistically differences between groups.

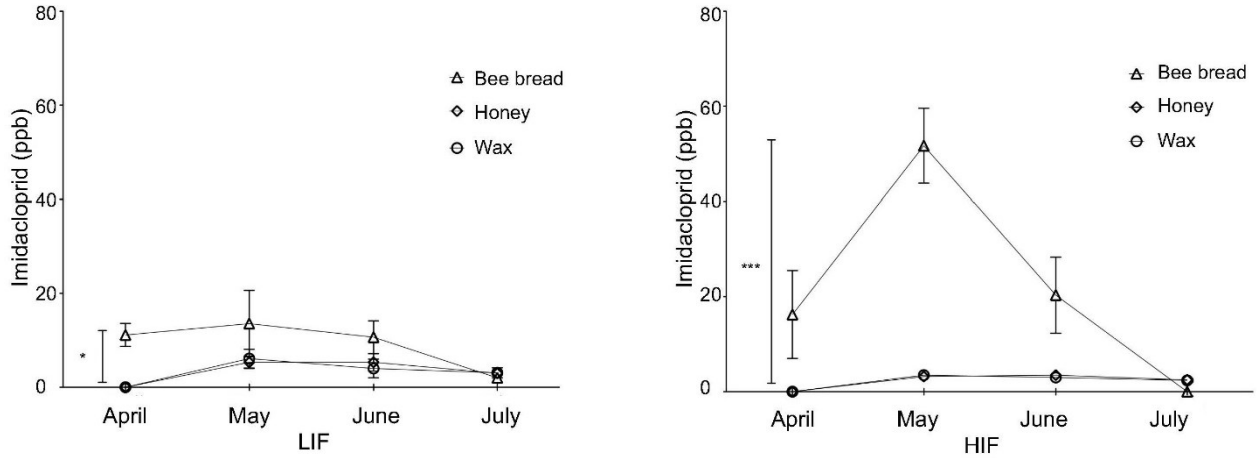

Figure 3. Monthly average concentration of imidacloprid in honey bee matrices (bee bread, honey and wax) in both LIF and HIF. Asterisks indicate the differences between bee bread and honey and wax, * = 0.01 to 0.05, *** = 0.0001 to 0.001. All values are expressed in ppb ± standard error, and differences have been statistically compared with Two-Way ANOVA and LSD post hoc test with 95% CI of bootstrap.

higher in pollen, followed by honey and wax (figure 2). There was no imidacloprid found in honey bee bodies nor in royal jelly in neither of the experimental fields for any replicate time (supplemental material S4A, S4F). In both LIF and HIF fields, there was a significant difference of the imidacloprid concentration between honey bee matrices. However, post hoc test analysis revealed that in LIF the concentration of imidacloprid in pollen was significantly higher than in the other matrices, while there was no difference between honey and wax. In the HIF, the concentration of imidacloprid in pollen was significantly higher than in other matrices. No metabolites were detected in any matrices from both experimental fields (supplemental material S4).

In both experimental fields, the trend of imidacloprid concentration in the bee bread was characterized by a significant increase in time, with a peak in May (13.57 ppb for LIF and 51.70 ppb for HIF), followed by a decrease that reached almost zero in July (figure 3). In both LIF and HIF field there was a significant main effect of time and matrices on imidacloprid concentration (LIF: Two-Way ANOVA with 95% CI of bootstrap, $F_{[3,\ 24]time}$ = 3.301, p = 0.037; $F_{[2,\ 24]matrix}$ = 7.706, p = 0.030; HIF: Two-Way ANOVA with 95% CI of bootstrap, $F_{[3,\ 24]time}$ = 11.818, p < 0.001; $F_{[2,\ 24]matrix}$ = 30.878, p < 0.001). Moreover, there is a significant main effect of the interaction between time and matrices on imidacloprid concentration in HIF and none in LIF (LIF: Two-Way ANOVA with 95% CI of bootstrap, $F_{[6,\ 24]time \times matrix}$ = 1.473, p = 0.229; HIF: Two-Way ANOVA with 95% CI of bootstrap, $F_{[6,\ 24]time \times matrix}$ = 9.919, p < 0.001). However, LSD post hoc test analysis revealed that, on average, only the concentration of imidacloprid from bee bread was significantly higher than the other matrices (LIF: Two-Way ANOVA with 95% CI of bootstrap, bee bread vs honey p = 0.002, bee bread vs wax p = 0.003; HIF: Two-Way ANOVA with 95% CI of bootstrap, bee bread vs honey p < 0.001, bee bread vs wax p < 0.001; LSD post hoc analysis) (figure 3).

In pollen loads, the imidacloprid concentration increased after the field treatment, passing from 15.70 ppb and 28.40 ppb to 66.50 and 91.10 in LIF and HIF, respectively (supplemental material S4E). However, the real effect of the field treatment was estimated by normalizing

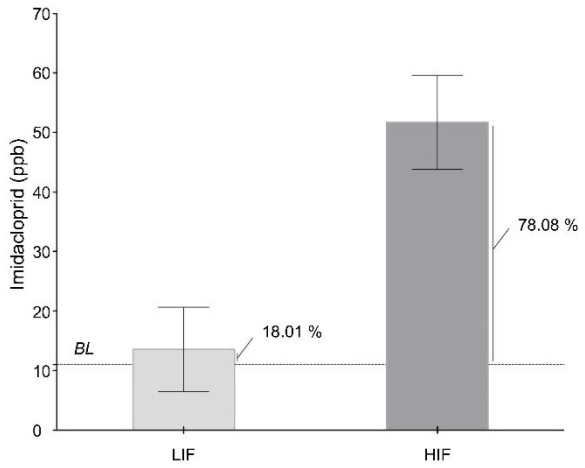

Figure 4. Average concentration of imidacloprid in bee bread analysed before and after the field treatment for both LIF and HIF. The percentage stands for the concentration increase of imidacloprid from the basal point of the experiment, calculated as the average of the pesticides detected before the field treatment (BL = background level). All values are expressed in ppb ± standard error.

the concentration of imidacloprid in the bee bread after the field treatment with the background level (BL) of imidacloprid already present before the field treatment. As shown in figure 4, in the bee bread from HIF, the imidacloprid concentration increased by 78.80%, while there was 18.01% in the pollen load from LIF.

Regarding palynological analysis of pollen loads before and after treatment in LIF and HIF see table 1.

Discussion and conclusion

Data obtained in this work underline that honey bees introduce imidacloprid in the hive through pollen collected on plants and how this is accumulated in the colony matrices, such as bee bread, honey, and wax. Results lead us to draw up an interpretative model of the entranceway and diffusion route of imidacloprid in honey bee colonies when they are used for pollination service in apple orchards (figure 5A-B). The insecticide is first sprayed in the field, outside the hive, when the apple trees are not in blooming and it potentially reaches the wildflowers already present in the surrounding area. Foragers collect the contaminated pollen and bring it inside the hive, where it is mainly processed and stored as bee bread (figure 5A-B). Imidacloprid can then persist for at least two months and a half in matrices. The presence of high quantities of imidacloprid in pollen loads confirm previous published data that found mixtures of neonicotinoids in pollen and nectar of wildflowers growing in arable field margins, at concentrations that are sometimes even higher than those found in the crop (Botías *et al*., 2015; Goulson *et al*., 2015; Ward *et al*., 2020; Main *et al*., 2021).

Considering that the estimated content of pollen in honey bee worker larvae could be approximate 5.4 mg (Babendreier *et al.*, 2004), in our experiments, a larva born at the end of April, just after the field treatment, could have been exposed to an imidacloprid dose of maximum of 0.09 ng (16.00 ppb in bee bread) and 0.14 ng (25.50 ppb in bee bread) for LIF and HIF, respectively. This dosage increases during the month of May with a maximum of 0.15 ng (27.7 ppb in bee bread) for LIF and 0.35 ng (64 ppb in bee bread) for HIF, then decreases during June with a maximum of 0.09 ng (17 ppb in bee bread) and 0.17 ng (32 ppb in bee bread) for LIF and HIF, respectively, and reaches zero in July. Therefore, although the concentration per larva is not enough to cause death, exposure to sub-lethal doses could still alter development, behaviour and longevity of these insects in the adult phase (Wu *et al*., 2011; Yang *et al*., 2012; Wu *et al*., 2017) especially considering the prolonged contact with contaminated wax and nectar. As a matter of fact, it has been proved (Wu *et al.*, 2014) that a dose and time-dependent increase of apoptosis of the brain cells occurs in honey bees exposed to 0.5-4.5 ng imidacloprid by triggering a caspase-dependent pathway of apoptosis and autophagy. It is already known that neonicotinoids, i.e. thiamethoxam at a dose of 4.5 ppb and clothianidin at a dose of 1.5 ppb, significantly reduce the reproductive capacity of honey bee drones. However, while no significant effects were observed for male teneral (newly emerged adult) body mass and sperm quantity, the data clearly

Table 1. Palynological analysis in pollen loads from honey bees collected before and after the field treatment in both LIF and HIF.

	% before treatment		% after treatment	
	LIF	HIF	LIF	HIF
Malus/Pyrus	62.43	59.88	82.22	0.91
Fraxinus sp.	20.44	14.92	5.79	89.86
Compositae T-form	13.81	23.99	8.63	0.35
Prunus sp.	1.66	-	-	0.55
Papaveraceae	1.66	-	-	2.78
Ericaceae	-	1.21	-	-
Caprifoliaceae	-	-	3.03	-
Geraniaceae	-	-	-	3.43
Aesculus sp.	-	-	-	2.12
Apiaceae	-	-	0.33	-
Total	100.00	100.00	100.00	100.00

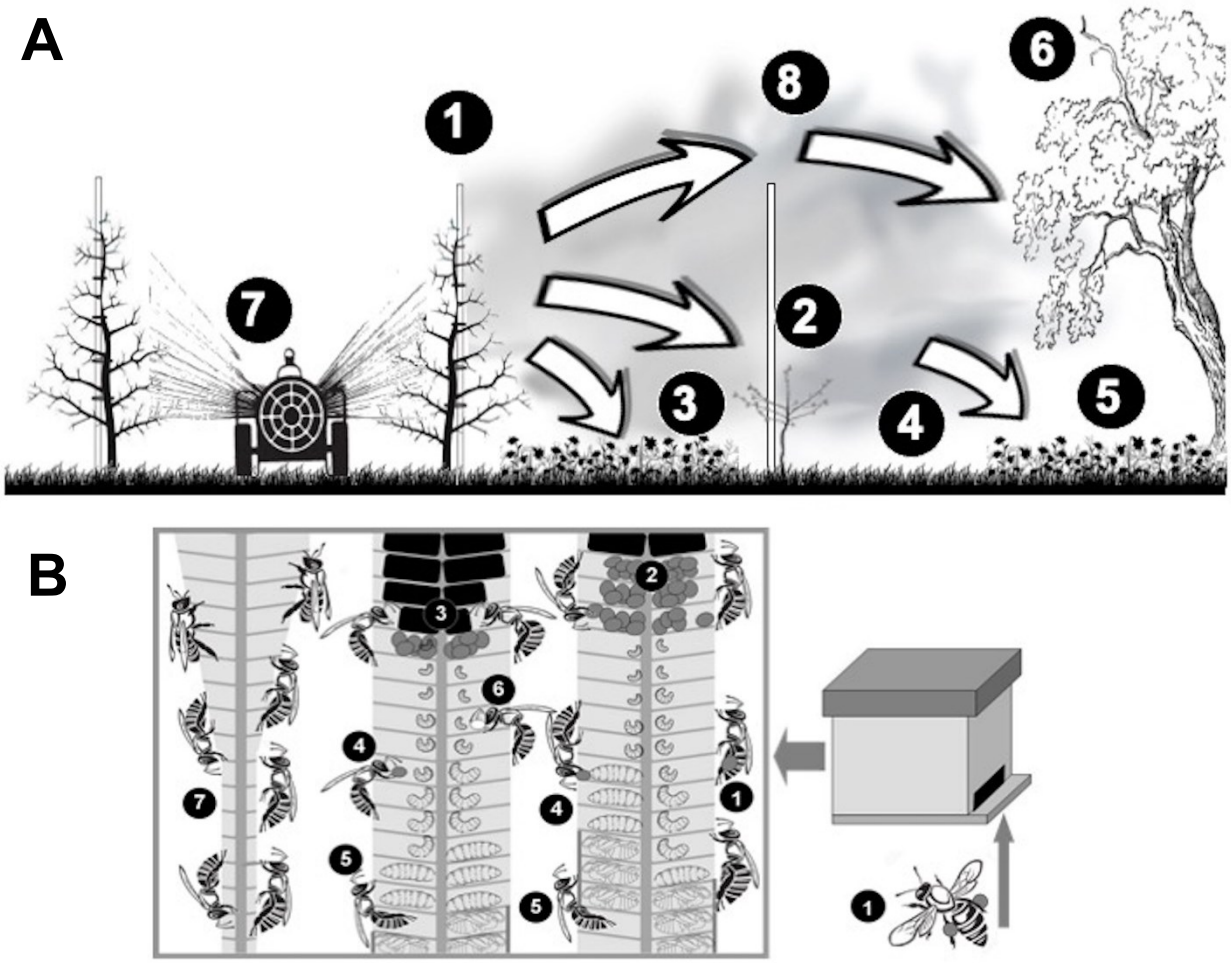

Figure 5. Interpretative model of a way of entry and diffusion of imidacloprid: (**A**) from the field - 1) apple trees in production with regularly ended flowering, 2) apple trees of new planting with late flowering, 3) inter-row with flowering herbs, 4) inter-row without flowering herbs, 5) spontaneous herbs contiguous to the orchard, 6) spontaneous trees or shrubs adjacent to the orchard, 7) application of crop protection products with atomizer, 8) drift effect; (**B**) to the honey bee colony - 1) the foragers bring contaminated nectar and pollen "grey oval" into the hive, 2) pollen is stored and transformed into bee bread, 3) the nectar is stored and transformed into honey, 4) mature larvae are fed with contaminated honey and bee bread, 5) bees raised with honey and contaminated bee bread are born, 6) bee bred with contaminated honey and bee bread secrete potential contaminated royal jelly "white oval", 7) bees bred with contaminated honey and bee bread secrete contaminated wax.

showed reduced drone lifespan, as well as reduced sperm viability (percentage living versus dead) and living sperm quantity (Straub *et al*., 2016). Based on the results we obtained, drone larvae born in May and June were fed with honey potentially contaminated with a maximum of 4 ppb of imidacloprid. Ciereszko *et al*. (2017) underlined the potential negative impact of also this chemical on the reproductive capacity of drones by reduction of sperm viability, motility and sperm mitochondrial potential. Impairment of queen's fertility as also been associated to imidacloprid exposure: queens in treated colonies exhibited reduced fecundity, likely due to imidacloprid acting directly on sensory and motor functions of the central nervous system that impacted egg-laying behaviour and locomotor activity (Wu-Smart and Spivak, 2016). Moreover, a study by Chaimanee *et al.* (2016) showed that sperm viability decreases by 50% also while stored in the spermatheca of queens exposed to sub-lethal doses of imidacloprid (0.02 ppm). Therefore, it is likely that also the drones sampled in our study suffer from possible alterations of the reproductive capacity, however more studies are needed to verify this hypothesis.

Regarding worker honey bees, energy and feeding requirements, and consequently frequency and amount of possible exposure to imidacloprid, are strictly connected to the "role" attended in the hive. For nurse bees, the total amount of pollen consumed within 10 days is 65 mg on average (Pain and Maugenet, 1966; Crailsheim *et al*., 1992), while wax-producing bees require 18 mg of sugar per day during the maximum periods of production, (Tokuda, 1955; Taranov, 1959; Hepburn, 1986). Brood attending bees need energy to maintain the brood temperature around 34 °C (Simpson, 1961; Heinrich, 1985). During the sampling period of our study, in temperate climates, temperatures average 15-20 °C, outside the hive and in such conditions, a brood attending bee will

consume between 50 mg (at 15 °C) and 34 mg (at 20 °C) of sugar per day (Free and Spencer-Booth, 1959; Simpson, 1961) and a total of 272-400 mg of sugar over the entire 8 day brood attendance period. During the three months winter period, in temperate regions a "winter" honey bee requires on average 8.8 mg of sugar per day (equivalent to 11 mg of honey) to maintain the nest at favourable temperatures (5-8 °C in the periphery and 15-20 °C in the centre) (Farrar, 1952; Johansson and Johansson, 1969; Winston, 1987). Foragers will consume 32-128.4 mg of sugar for the collection of pollen and 10.4-15.6 mg of sugar for nectar collection per day to perform their tasks (Rortais *et al*., 2005). Moreover, sublethal exposure to neonicotinoids can possibly impair honey bee colonies overwintering (Lu *et al*., 2014). Considering the results of our study, it becomes clear that the possibilities of exposure to imidacloprid, through pollen and honey ingestion, are likely to occur also in adult honey bees, however in different quantities connected to feeding habits and for long periods as suggested from the presence of residues in honey, pollen and wax detected until the middle of July. Unfortunately, it was not possible to verify this hypothesis as none of the honey bee samples collected and immediately refrigerated, showed the presence of imidacloprid contamination, possibly due to rapid degradation of the chemical in living honey bees (Schott *et al*., 2017).

Our data clearly have shown how the neonicotinoid imidacloprid can quickly enter into honey bee colonies through the pollen load and be accumulated in the bee bread, honey and wax being a potential source of sublethal intoxication, mainly for the more susceptible larva stage but also for the coming adults. We believe that this study could contribute to completing the intricate picture of the relation between neonicotinoids and honey bee colonies and could be taken into account for risk assessment programming in the use of imidacloprid in apple orchards pollinated by honey bee services. Our data may also be used to implement mathematical models such as Apis-RAM which will be used in the honey bees risk assessment (Duan *et al*., 2022).

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VM and PF have contributed equally to this work and share first authorship.

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