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Pesticide residues in nectar and pollen of melon crops: Risk to pollinators and effects of a specific pesticide mixture on *Bombus terrestris* (Hymenoptera: Apidae) micro-colonies☆

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ABSTRACT

Residues detected in pollen collected by honey bees are often used to estimate pesticide exposure in ecotoxicological studies. However, for a more accurate assessment of pesticides effect on foraging pollinators, residues found directly on flowers are a more realistic exposure approximation. We conducted a multi-residue analysis of pesticides on pollen and nectar of melon flowers collected from five fields. The cumulative chronic oral exposure Risk Index (RI) was calculated for *Apis mellifera, Bombus terrestris* and *Osmia bicornis* to multiple pesticides. However, this index could underestimate the risk since sublethal or synergistic effects are not considered. Therefore, a mixture containing three of the most frequently detected pesticides in our study was tested for synergistic impact on *B. terrestris* micro-colonies through a chronic oral toxicity test. According to the result, pollen and nectar samples contained numerous pesticide residues, including nine insecticides, nine fungicides, and one herbicide. Eleven of those were not applied by farmers during the crop season, revealing that melon agroecosystems may be pesticide contaminated environments. The primary contributor to the chronic RI was imidacloprid and *O. bircornis* is at greatest risk for lethality resulting from chronic oral exposure at these sites. In the bumblebee micro-colony bioassay, dietary exposure to acetamiprid, chlorpyrifos and oxamyl at residue level concentration, showed no effects on worker mortality, drone production or drone size and no synergies were detected when pesticide mixtures were evaluated. In conclusion, our findings have significant implications for improving pesticide risk assessment schemes to guarantee pollinator conservation. In particular, bee pesticide risk assessment should not be limited to acute exposure effects to isolated active ingredients in honey bees. Instead, risk assessments should consider the long-term pesticide exposure effects in both pollen and nectar on a range of bees that reflect the diversity of natural ecosystems and the synergistic potential among pesticide formulations.

1. Introduction

Agricultural intensification involves landscape uniformity and simplification, enlarged field sizes and increased inputs of fertilizers and pesticides, all of which have the potential to alter the functioning of ecosystems which may lead to the reduction or even extinction of many wild plant and animal species (Geiger et al., 2010). At present, there is a global decline in bee species richness which may seriously compromise the reproduction of many wild flowering plants and the yield of about 85% of our cultivated crops, affecting food production stability

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(Fontaine et al., 2005; Garibaldi et al., 2011; Klein et al., 2007; Zattara and Aizen, 2021). Among the factors involved, there is growing concern about the impact of pesticides, which are often identified as one of the main causes of bee decline (Goulson et al., 2015; Zattara and Aizen, 2021).

One primary route of pesticide exposure to bees is the consumption of contaminated pollen and nectar both from crop flowers (Bonmatin et al., 2005; Dively and Kamel, 2012; Stoner and Eitzer, 2012) and nearby vegetation (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017). Most laboratory studies attempting to test field realistic concentrations of pesticides based exposure levels on residues detected in pollen collected from honey bees returning to their hive or in beebread (i.e., the pollen stored in comb cells within the hive) as a proxy for the field pesticide exposure (Laycock et al., 2012; Zhu et al., 2017) of adult bees. Although these residues can be realistic for in-hive bees (i.e., nurse and larval bees), which consume beebread, they are not the optimal estimation for foraging honey bees and for solitary bee species. According to the Kyriakopoulou et al. (2017) meta-analysis, residues detected in flower resources directly collected from plants should be used in pesticide risk assessment as the worst-case scenario because they are usually higher than those found in the pollen collected from honey bees. Thus, the use of the residues from honey bee-collected pollen may underestimate pesticide exposure for three reasons. Firstly, pollen collected by honey bees is mixed with nectar and glandular secretions, or derived from untreated and treated plants, and therefore, in both cases, pesticide residues are diluted (Bonmatin et al., 2015; Rolke et al., 2016). Secondly, bees exposed to lethal or sublethal doses might not be able to return to the hive (Fischer et al., 2014; Henry et al., 2012; Stanley et al., 2016). Thirdly, pesticide levels in beebread can decrease over time due to pesticide degradation.

Data on pesticide residues in flowers are available for several crops (Bonmatin et al., 2005; Botías et al., 2015; David et al., 2015; Dively and Kamel, 2012; Heller et al., 2020; Stoner and Eitzer, 2012; Zioga et al., 2020) and wildflowers (Botías et al., 2015; David et al., 2015). However, most of these studies has examined a single pesticide or few active ingredients or chemical groups (e.g., neonicotinoids) overlooking the risk from multi-pesticide exposures, which is, on the contrary, a frequent scenario when bees forage in agricultural landscapes. In our study, we aimed to assess pesticide risk for bees in the melon (*Cucumis melo* L.) agroecosystem, managed according to the current pesticide regulations in Spain. This pollinator-dependent crop is frequently sprayed with insecticides mainly to control aphids and whiteflies and with fungicides during bloom to prevent powdery mildew and other fungal diseases (Duncan and Ewing, 2015; Khetereli et al., 2016).

Exposure to multiple pesticides may pose a risk to the wide variety of bee species visiting melon flowers (Azpiazu et al., 2020; Rodrigo Gómez et al., 2016; Tschoeke et al., 2015; Winfree et al., 2007). It is well established that different bee species have different sensitivity to the various classes of pesticides (Arena and Sgolastra, 2014; Azpiazu et al., 2021; Rundlöf et al., 2015; Sgolastra et al., 2017a; Woodcock et al., 2017). For these reasons, in addition to honey bees (*Apis mellifera* L.), European Food Safety Authority (EFSA) has recommended including bumblebees (*Bombus terrestris* L.) and solitary bees (*Osmia cornuta* Latreille and/or *Osmia bicornis* L.) in risk assessment schemes (EFSA, 2013).

Moreover, several studies have shown that some fungicides (e.g., triazole) can increase the toxicity of insecticides on social and solitary bees by reducing their detoxification capacity (Gill et al., 2012; Iwasa et al., 2004; Sgolastra et al., 2018, 2017a; Zhu et al., 2017). Therefore, mixtures containing more than one pesticide may have synergistic toxicity effects (e.g., on mortality, ovary maturation and reproduction; Carnesecchi et al., 2019; Gill et al., 2012; Sgolastra et al., 2018, 2017a). However, in the European Union (EU), for example, there are almost 500 active substances approved for use in plant protection product (EC, 2022) and testing all possible combinations of active ingredients and formulations is impractical due to the high cost. The solution could be to identify and then assess the most likely combinations of pesticide residues in a real field scenario.

Traditionally, pesticide toxicity to bees has been evaluated with acute oral or contact tests in the laboratory, in most cases, with a lethal endpoint only (Sgolastra et al., 2020). These tests neither consider the wide range of sublethal effects caused by pesticides (Decourtye et al., 2005; Sgolastra et al., 2018) nor the effects of long-term exposure to sublethal concentrations (Azpiazu et al., 2019; Gill and Raine, 2014) due to their persistence in the environment (Botías et al., 2015; Goulson, 2013; Silva et al., 2019). Therefore, to protect bee biodiversity, we should rethink the current procedures for risk evaluation of pesticides, including different bee species, multi-pesticide exposure, and chronic exposure tests at field concentrations.

In line with a more holistic approach in the environmental risk assessment, in this work we aim to: 1) identify and quantify the pesticides residues in pollen and nectar of five melon open-fields in Central Spain, 2) assess in each field the potential cumulative chronic risk of multiple pesticide exposure in different bee species, 3) identify the predominant pesticide mixtures (co-occurrence), and 4) test one of them on *B. terrestris* micro-colonies through a chronic oral toxicity test.

2. Materials and methods

2.1. Pesticide residues in pollen and nectar of melon flowers

Melon flowers sampling and nectar and pollen collection: Melon flowers were collected in July 2017 in five commercial fields on the basin of the Madrilenian Tajo River, an area for melon cultivation in the southeast of Madrid, Spain (Fig. 1). The insecticide and fungicide treatments performed during the 2017 crop cycle on the different fields are given in supplementary Table S1. In each field, three areas, separated by at least 100 m, were randomly selected and about 400 melon flowers (male and hermaphrodite) per area were collected. Nectar was extracted from 30 to 70 flowers using 5 μl microcapillaries (Blaubrand® intraMARK) to obtain 50 μl per sample (n = 3 per field). Collected nectar and the remaining flowers (about 250) for later pollen collection were transported to the laboratory in a portable refrigerator. Nectar was stored at −80 °C and flowers were dried in an incubator at 37 °C for 24 h to facilitate the removal of the pollen from the anthers according to Botías et al. (2015). Hereafter, anthers were collected and 0.1 g of pollen per sample (n = 3 per field) was extracted using a 150 μm pore size sieve [melon pollen grain Ø = 50–100 μm (PalDat, 2017)].

Multi-residue analysis of pesticides: multi-residue analyses were carried out externally at the Chemical Microbiological Laboratory of Seville (www.lqmsa.com), an analytical testing company officially accredited by ENAC (Spanish Entity for Accreditation to analyze pesticide residues). Pollen and nectar pesticide residue extractions were performed using a modified version of the QuEChERS methodology (David et al., 2015), which is particularly sensitive and satisfy quality standards (EU Directive 96/23/EC and Commission Decision, 2002/657/EC). High performance liquid chromatography with quantification and confirmation by triple-quadrupole mass spectrometer detector (HPLC-QQQ) and gas chromatography coupled to triple-quadrupole mass spectrometer detector (GC-QQQ) were used to analyze more than 200 compounds (Table S2). The recoveries were over the detection limit (LOD) of 3 ng/g for each analyte. More details of analytical techniques are provided in Supplementary Information.

2.2. Cumulative chronic risk index for multiple pesticide exposure

Using the maximum concentration of pesticide residues detected in the pollen and nectar in every melon field (mg of active ingredients/Kg of product) (Table S3), we estimated the cumulative chronic Risk Index (RI) of multiple pesticides simultaneously to bees, including honey bee (*A. mellifera*), bumblebees (*B. terrestris*) and mason bees (*O. bicornis*). The RI is calculated by adding the Toxic Unit (TU) of each compound found

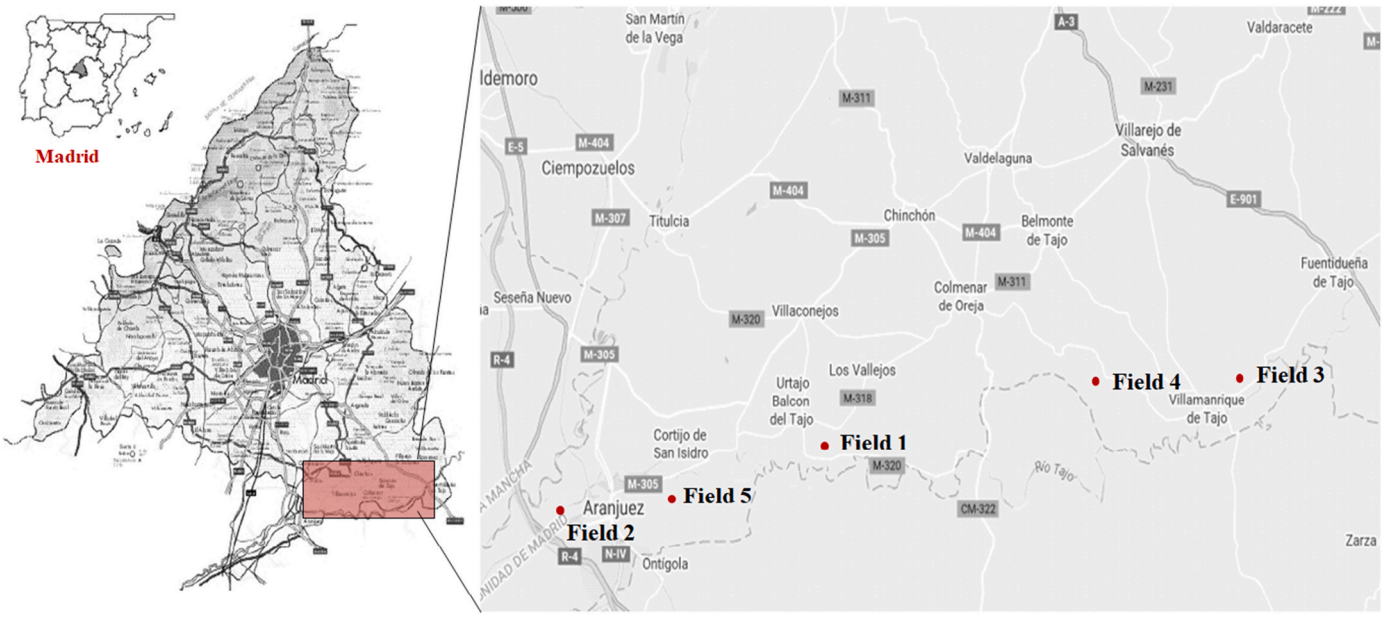

Fig. 1. Location of the five conventional melon fields in the study area, southeast of Madrid, Spain.

in the same field. The TU of an individual compound is the ratio between the exposure level via ingestion of pollen and nectar and its referent toxicity value for bees (i.e., LD50 – Lethal Dose 50) (Barmaz et al., 2010).

The RI for each field and bee species was calculated with the following formula:

$$RI = \sum_{i=1}^{n} \frac{[(RNi * NC) + (RPi * PC)]/1000}{LD50i} * AF$$

The RNi and RPi are the residues of the compound *i* expressed in mg/Kg found in the nectar and pollen, respectively. The NC and PC are the daily nectar and pollen consumption (expressed in mg/bee/day) by each bee species (Table S4). The LD50i is the acute oral lethal dose of the compound *i* expressed in μg/bee (Table S5). The AF is an assessment factor that converts acute toxicity in chronic toxicity, namely it converts LD50 in LDD50 (lethal daily dose) and we have assigned a value of 10 as recommend by Alix and Lewis (2010). When the LD50 values were not available for bumblebees and mason bees we applied a safety factor of 10 to the value for honey bees (EFSA, 2013). A RI higher than 1 indicates that bees in that field may consume a quantity of pesticide residues potentially lethal for them.

2.3. Occurrence and co-occurrence analysis

To identify the most commonly active ingredients in the nectar and pollen samples collected from the five melon fields, we performed a co-occurrence analysis between the active ingredients found using a probabilistic model for pair-wise patterns and the *co-occur* package in R (Prado et al., 2019). Occurrence was calculated using the number of positive samples out of the total (n = 15), and co-occurrence considering the number of fields in which both residues were detected (n = 5). One of the most likely combinations of three different compounds was selected for testing its effects on *B. terrestris* micro-colonies considering the impact of the mixtures and the single compounds.

2.4. Chronic bioassay with B. terrestris micro-colonies

Commercial bumblebee colonies were purchased from Agrobio S.L. (La Venta del Viso, Almería, Spain). From these original colonies, we created 64 queenless micro-colonies in three consecutive days. In each micro-colony, five newly emerged workers (silver hairs in color and crumpled and soft wings) from the same queen-right colony were placed in a circular plastic box (diameter 12 cm, height 9.5 cm) with a mesh in the lid to allow ventilation. Bumblebee workers develop their ovaries when the founding queen is absent (Amsalem et al., 2009). In the micro-colonies, one or several workers became dominant, developed her ovaries (Blacquière et al., 2012), and started to lay eggs during the first week and continued to the end of the study. Because workers were not inseminated, the brood resulted in a haploid male progeny (drones). Additionally, the workers continued to perform brood care activities, including feeding the larvae, building and heating cells. We used initially eight micro-colonies per treatment and excluded those with no egg laying during the first week (determined by the absence of wax-covered egg cups) as well as two micro-colonies where all the individuals died because the syrup spilled out from the feeder. Sample sizes in each treatment are shown in Fig. 4. Queenless micro-colonies were maintained in a walk-in chamber at 28–30 °C temperature, 50–60% relative humidity (RH), and continuous darkness throughout 11 weeks. This period was chosen according to previous studies (Barbosa et al., 2015; Mommaerts et al., 2010) and also coincides with the average duration of melon flowering (Lázaro et al., 2012). The position of micro-colonies was rotated weekly within the chamber to minimize the potential 'chamber–microclimate' differences.

After four days of chamber acclimatization the micro-colonies were randomly assigned to one of the following treatments: control (CONT), acetamiprid (A), chlorpyrifos (C), oxamyl (O) and the mixtures (A + C, A + O, C + O, A + C + O). Adults were exposed via pollen and nectar in laboratory with the same concentrations detected in the melon flowers in the fields sampled (Table 1), in an attempt to mimic field-realistic conditions. Before the treated food was offered, dead bees were replaced with workers from the same original queenright colony in accord with Laycock et al. (2012), but dead workers after pesticide exposure were not replaced.

Commercial syrup (Api 65®: 1.21 g/ml, fructose/glucose/saccharose solution; Agrobio S.L., Spain) and organic honey bee pollen (Bona Mel®, Alicante, Spain) treated with the pesticide concentrations selected were offered *ad libitum* until the end of the experiment. Stock pesticide solutions were prepared by mixing 500 mg of Epik® (acetamiprid, 20% w/w; Sipcam Inagra S.A.), 500 mg of Chas® (chlorpyrifos, 5% w/w, FMC Agricultural Solutions, S.A.U) and 100 μl of Afromyl® (oxamyl, 10% w/v, Industrias Afrasa S.A.) with 50 ml of distilled water.

Table 1
Pesticides detected in the pollen and nectar of melon flowers collected in five commercial melon fields in Madrid (Central Spain).

Compound	Pesticide class[b]	Concentration (ppb) in melon flowers (mean ± S. E.)[c]		N of fields sprayed[e]/N of fields in which pesticides were detected	Days between application and sample collection	Occurrence (% samples)
		pollen	nectar			
Acetamiprid (A)	neonicotinoid-I	**482.93 ± 215.85**	**6.41 ± 3.53**	**5/5**	2–11	100
Imidacloprid	neonicotinoid-I	369.36 ± 186.31	15.34 ± 7.62	-[f]/4	45–71	66.70
Oxamyl (O)	carbamate- I	**<3**[d]	**0**	**-/5**	-	46.70
Metalaxyl-m	acylalanines- F	<3[d]	0	-/5	-	46.70
Chlorpyrifos (C)	organophosphate- I	**3.97 ± 0.93**	**1.45 ± 1.45**	**-/3**	-	40.00
Abamectin	avermectins- I	32.67 ± 12.83	0	2/2	2–5	40.00
Azoxystrobin	methoxyacrylates- F	5.92 ± 2.92	0	**-/3**	-	36.70
Myclobutanil	triazole- F	0	5.58 ± 0.70	4/2	2–15	26.70
Boscalid	pyridine-carboxamide- F	266.38 ± 152.77	0	2/2	5	26.70
Flonicamid	flonicamid- I	27.10 ± 6.08	0	1/1	18	20.00
Atrazine[a]	triazine- H	5.10 ± 0.74	0	-/1	-	20.00
Quinomethionate[a]	quinoxaline- F	52.50 ± 19.62	0	-/1	-	16.70
Chlorantraniliprol	diamides- I	5.65 ± 2.65	0	-/2	-	13.30
Difenoconazole	triazole- F	3.80 ± 0.70	0	-/2	-	13.30
Kresoxim-methyl	oximino-acetates- F	29.60 ± 15.20	0	2/1	5	13.30
Chlorothalonil	chloronitriles- F	25.85 ± 5.75	0	-/1	-	13.30
Thiacloprid	neonicotinoid- I	<3[d]	0	-/1	-	13.30
Alpha-cypermethrin	pyrethroids - I	153	0	-/1	-	6.70
Quinoxyfen	aryloxyquinoline- F	<3[d]	0	3/1	2–11	6.70
Triadimenol	triazole- F	0	0	1/0	5	0

More details about pesticide treatments are provided in the Supplementary Information. In bold, pesticides selected to the chronic bioassay with *Bombus terrestris* micro-colonies.

[a] Unauthorized pesticides in the EU (MAPA, 2017).

[b] Based on Insecticide (IRAC, 2022), Fungicide (FRAC, 2022) and Herbicide resistance action committees (HRAC, 2022). I = insecticide; F = fungicide; H = herbicide.

[c] Analyzed by high performance liquid chromatography with quantification and confirmation by triple-quadrupole mass spectrometer detector (HPLC-QQQ) and gas chromatography with triple-quadrupole mass spectrometer detector (GC-QQQ).

[d] Under the Limit of quantification (LOQ).

[e] The hyphen indicates that pesticides were not sprayed by farmers.

[f] Imidacloprid was only applied as seed coating in the nursery.

These solutions were diluted in the syrup or in the distilled water used for the pollen preparation to reach the desired concentrations (Table 1).

Pollen balls (i.e., honey bee pollen mixed with distilled water; mean mass: 6.32 g, SE = 0.07 g) were renewed every 3–4 days, and the syrup was replaced once a week (Fauser-Misslin et al., 2014) throughout the 11 weeks. Forty millilitres of the commercial syrup were offered to the bumblebees in bird drinking troughs (capacity 70 ml, diameter 4 cm, height 8.5 cm). Each time food was replaced, fresh pesticide solution was prepared to be diluted in the syrup or distilled water for pollen preparation.

To know the amount of pollen and syrup bees were in contact with, we used syrup and pollen collection instead of pollen and syrup consumption following Dance et al. (2017), because some syrup was stored in the wax honey pots and some pollen was used as provision brood. Accordingly, pollen was weighed every 3–4 days and the volume of syrup was measured weekly. Six identical plastic boxes to those used for micro-colonies were kept with full syrup feeders and pollen but without bumblebees to measure the amount evaporated.

Twice per week, worker mortality was evaluated as well as the production of drones (i.e., males coming from unfertilized eggs). To identify a possible sublethal effect on body size, we measure the thorax width (inter-tegulae span) as a proxy of the body size (Kapustjanskij et al., 2007), in three of the last drones emerged in every micro-colony as the most unfavorable case, because that is when the workers had been feeding on the pesticides for a longer period. These male offspring were removed from the micro-colonies and kept in the freezer (−20 °C) until measurements were done using a stereomicroscope (S6E Leica®) at 20x magnification and an ocular micrometer (precision ± 0.01 mm). At the end of the assay (week 11), brood production was evaluated by dissecting the micro-colonies and counting the number of egg cups, larvae and pupae.

2.5. Statistics

We used one-way analysis of variance (ANOVA, $P < 0.05$) with statistical software package SPSS Statistics® (IBM Corp. Released, 2013) to analyze the effect of treatments on total pollen and syrup collected, on brood and male production and on their thorax width. The linearly independent pairwise comparisons of estimated marginal means were separated using the Fisher's Least Significant Difference (LSD) test. The nonparametric tests of Kruskal-Wallis ($P < 0.05$) were used to establish differences on mortality, because data violated the premises of the ANOVA after the transformation [ln (x + 1)] of the dependent variable. The number of males that emerged per week was analyzed using a Linear Mixed Model (LMM). The model considered treatment as a fixed factor, week as a fixed factor repeated within subjects, and their interaction. The means were compared using Fisher's LSD test ($p < 0.05$).

3. Results

3.1. Pesticides residues found in melon flowers

Multi-residue analyses of pesticides in melon flowers collected from five commercial melon fields identified a total of 19 active ingredients (nine insecticides, nine fungicides and one herbicide; Table 1) in the pollen and nectar. Most of them (i.e., 11) had not been applied by the farmers during the current crop season as documented in their compulsory field notebook (Table S1). Oxamyl, chlorpyrifos and metalaxyl-m were the most frequently detected pesticides (>40%; Table 1) that were not applied by the farmers (Table S1). Acetamiprid and imidacloprid were found in 100% and 66.7% of the samples, respectively, and at high residue level in both pollen and nectar. Acetamiprid was applied from 2 to 11 days before sample collection and imidacloprid was applied by seedling treatment in the nursery before transplanting to the field instead of foliar treatment. Pesticides (i.e., abamectin, boscalid, flonicamid, kresoxim-methyl and *alpha*-cypermethrin) with high residue concentration (i.e., >25 ppb) were also applied before sample collection (except *alpha*-cypermethrin). No residues of the fungicide triadimenol, applied in one field, were detected (Tables 1 and S1). Pollen contained more pesticides and higher levels of pesticides than nectar (<3 to 482.93 ± 215.85 ppb in pollen vs 1.45 ± 1.45 to 15.34 ± 7.62 ppb in nectar). Only three insecticides were detected in both pollen and nectar (acetamiprid, imidacloprid, chlorpyrifos). Myclobutanil, a fungicide, was only found in nectar.

3.2. Cumulative chronic risk index of multiple pesticides to pollinators

The cumulative chronic risk index (RI) of exposure to multiple pesticide to pollinators was calculated in every field for honey bees (i.e., *A. mellifera*), bumblebees (i.e., *B. terrestris*) and mason bees (i.e., *O. bicornis*) (Fig. 2). Mason bees were the most threatened pollinators by pesticides, regardless of the field, followed by honey bees and bumblebees. Three fields (1, 2 and 4) exhibited RI values higher than 1 corresponding to those where the residue concentrations were higher (Table 1 and Table S3). The contribution of each compound to the RI indexes is shown in Fig. 2. For honey bees, imidacloprid was the main contributor to RI scores, accounting for 99.9% in field 1 and 4. For bumblebees, a RI > 1 was only detected in field 4 and, again, imidacloprid was the responsible active ingredient (99.8% of the time). In mason bees several compounds contributed more equally to the cumulative RI scores. The main contributors were: imidacloprid (63.7% in field 1; 17.6% in field 2; 99.9% in field 3), abamectin (27.3% in field 1; 81.2% in field 2) and *alpha*-cypermethrin (8.8% in field 1) (Fig. 2).

3.3. Effects on B. terrestris micro-colonies

Co-occurrence network and pesticide mixture selected: The most likely pesticide combination in the study area, according to the co-occurrence network of pesticide residues (Fig. 3), was acetamiprid + metalaxyl-m + oxamyl (co-occurrence in 5 fields), followed by the former three pesticides + imidacloprid (co-occurrence in 4 fields) and by the last four pesticides + chlorpyrifos + azoxystrobin (co-occurrence in 3 fields). Considering these results, we selected three insecticides with different modes of action and with a co-occurrence higher than 60% (co-occurrence in 3 fields): the neonicotinoid acetamiprid (A), the carbamate oxamyl (O) and the organophosphate chlorpyrifos (C). Commercial formulations of these pesticides registered for pest control in melon in Spain (MAPA, 2017) were used and tested at the concentration levels found in the nectar and pollen (Table 1).

Worker mortality: Over the 11 weeks of the experiment, worker mortality was not significantly different among treatments (Kruskal-Wallis: $\chi^2 = 6.96$, df = 7, P = 0.43) and the average was 4.6% ± 1.3 (CONT = 0; A = 6.7% ± 6.7; C = 5.0% ± 5.0; O = 7.5% ± 3.7; A + C = 8.6% ± 4.0; A + O = 2.9% ± 2.9; C + O = 0%; A + C + O = 5.0% ± 3.3).

Pollen and syrup collected: No significant differences were found in the total amounts of pollen and syrup collected during the bioassay period (ANOVA, pollen: $F_{7, 49} = 0.84$, P = 0.56; syrup: $F_{7, 49} = 0.54$, P = 0.1; Fig. 4a and b, respectively).

Brood and male production: The number of egg cups in the first week, brood production and total male number after 11 weeks of exposure to the three pesticides, and their combinations were not significantly different among treatments (ANOVA number of egg cells: $F_{7,49} = 2.16$, P = 2.16; brood production: $F_{7,49} = 0.25$, P = 0.97; males: $F_{7,49} = 0.67$, P = 0.70; Fig. 4c, d and f, respectively). The thorax width (i.e., intertegulae span) of the males emerged during the last week was also not different among treatments (ANOVA, $F_{7,163} = 2.04$, P = 0.06; Fig. 4e). All males started to emerge between 29 and 32 days (week 6 of the experiment) after pesticide exposure (except for two males from the CONT microcolony that emerged earlier, after 24–28 days). The highest number of males emerged in week 6 and week 9 (LMM: $F_{6,109.6} = 88.79$, P < 0.001; Fig. 4h), but no significant differences were found between treatments (LMM: $F_{7,274.26} = 0.53$, P = 0.81; Fig. 4g), nor for the treatment-week interaction (LMM: $F_{42,109.6} = 1.04$, P = 0.432).

4. Discussion

Multiple pesticide residues in pollen and nectar of melon crop flowers and their potential risks in three bee species have been assessed in this study to go one step further to previous studies, which focused on

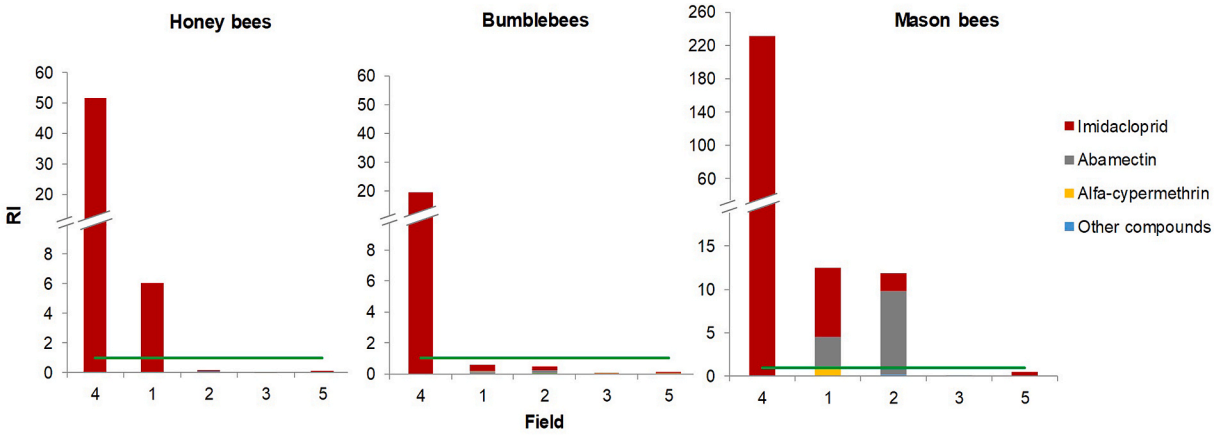

Fig. 2. Cumulative chronic Risk Index (RI) for honey bees (*A. mellifera*), bumblebees (*B. terrestris*) and mason bees (*O. bicornis*) in each melon field. Residue data from pollen and nectar, daily pollen and nectar consumption for each bee specie, oral LD50 of each compound and an assessment factor (AF = 10) that converts acute toxicity in chronic toxicity were used in the calculations. Greenline indicates RI = 1 representing the limit of the potentially lethal risk.

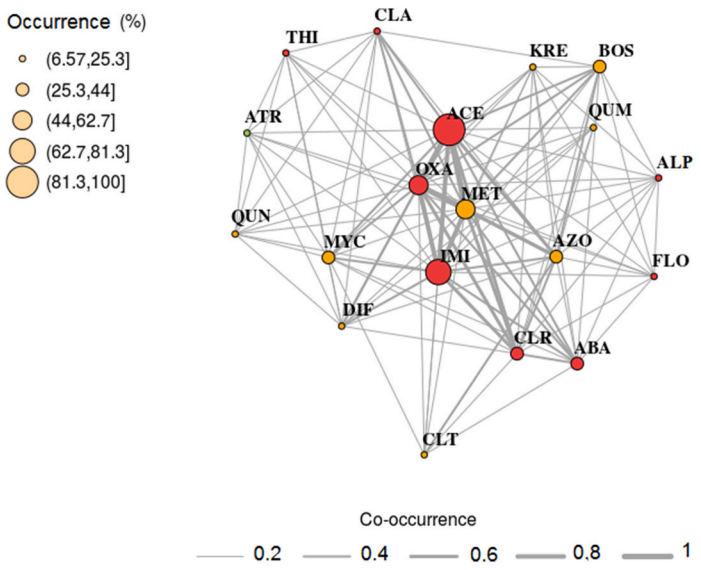

Fig. 3. Co-occurrence network of pesticide residues detected in pollen and nectar samples in five melon fields (n = 3 per field) of Madrid (Spain). Node sizes indicate the pesticide frequency in the samples. Links between pesticides represent the probability of co-occurrence in the fields. Probabilistic model for pair-wise patterns and the co-occur package in R was used. I = insecticides; H = herbicides; F = fungicides; ACE = acetamiprid; IMI = imidacloprid; OXA = oxamyl; MET = metalaxyl-m; CLR = chlorpyrifos; ABA = abamectin, AZO = azoxytrobin; MYC = myclobutanil; BOS = boscalid; FLO = flonicamid; ATR = atrazine; QUM = quimethionate; CLA = clorantraniliprol; DIF = difenoconazol; KRE = Kresoxim-methyl; CLT = chlorothalonil; THI = thiacloprid; ALP = *alpha*-cypermethrin; QUN = quinoxyfen.

analyzing residues of a few active ingredients or chemical groups (e.g., neonicotinoid insecticides or triazole fungicides) (Botías et al., 2015; David et al., 2015) or on hive products (e.g., corbicular pollen), due to the difficulty in obtaining samples of nectar and pollen directly from the flowers (Mullin et al., 2010; Porrini et al., 2016; Sanchez-Bayo and Goka, 2014; Tosi et al., 2018). Thus, pesticide residue compounds detected on melon flowers in an agroecosystem allowed us to identify the most likely combinations and test one of them and their single products in bumblebee micro-colonies under laboratory conditions.

Our results on pesticide residues showed that bees foraging in the melon fields of the study area are exposed to a high number of pesticides when collecting pollen and/or nectar (nine insecticides, nine fungicides and one herbicide), highlighting the fact that melon agroecosystems can be a very pesticide-contaminated environment for bees and other beneficial insects (i.e., predators and parasitoids). Surprisingly, 11 of them had not been applied by farmers during the current crop season.

Some pesticides that were detected at higher concentrations (i.e., acetamiprid, abamectin, boscalid, flonicamid and kresoxim-methyl) had been applied by the farmers close to the flower sampling date. Imidacloprid was applied to melon seeds in the nursery before transplanting to the field and *alpha*-cypermethrin was not applied in any of the studied fields, but both insecticides were found at high concentrations. Similarly, other pesticides not applied (e.g., oxamyl, metalaxyl-m and chlorpyrifos) were also detected, but a lower concentration. The presence of pesticides in the fields that were not applied by farmers during the crop cycle, may be due to external contamination, such as drift from nearby sprayed fields, contaminated soil (e.g., the persistent pollutant chlorpyrifos; Das et al., 2020; Leistra et al., 2006; Ngan et al., 2005; Wolters et al., 2003), or contaminated irrigation water (e.g., oxamyl and metalaxyl-m because of their high solubility; EPA, 2000; Struger et al., 2016).

Except for myclobutanil, all pesticides were detected in pollen while only four compounds (i.e., acetamiprid, imidacloprid, myclobutanil, and chlorpyrifos) were detected in the nectar. Notable, these pesticides were detected at lower concentrations in the nectar than pollen. This result, which is in line with previous studies (Botías et al., 2015; Dively and Kamel, 2012; Kyriakopoulou et al., 2017; Mullin et al., 2010; Stoner and Eitzer, 2012; Zioga et al., 2020), may be explained in part, by the higher exposure of the anthers during foliar pesticide applications compared to the nectaries.

By using the data from pesticide residues, we also estimated the cumulative chronic RIs in each field for three bee species. Imidacloprid was the main contributor to risk values in all bees assessed (i.e., *A. mellifera, B. terrestris* and *O. bicornis*). This compound was detected in four fields and was applied to melon seeds in the nursery before transplanting to the field. At present, in the EU, its use is only allowed in permanent greenhouses or on seeds whose resulting crops will be grown inside greenhouses during the entire life cycle (OJEU, 2018). However, these restrictions did not exist at the time our study was conducted (OJEU, 2013). The identification of the riskiest compounds may be useful for establishing better practices, for example, reducing the use of more dangerous pesticides (Sgolastra et al., 2017b). By comparing the RIs between bee species, *O. bicornis* were determined to have the greatest risk. Prior studies with neonicotinoid insecticides also observed that *Osmia* bees were more lethality sensitive than honey bees and bumblebees (Arena and Sgolastra, 2014; Biddinger et al., 2013; Sgolastra et al., 2017a). Moreover, the risk to *O. bicornis* was exacerbated because abamectin also contributed to the RI. These results highlight the need to incorporate other bee species (i.e., non-*Apis*) into pesticide risk assessments.

Nevertheless, our cumulative chronic RI can underestimate the risk to pollinators for two reasons. Firstly, this index is focused on lethal effects, but sublethal effects can cause important consequences on bee health as well (Di Noi et al., 2021; Siviter et al., 2021). Secondly, the synergistic effects of pesticides are not factored into the RI, and our study shows that bees in melon agroecosystems are exposed to multiple pesticide combinations. Although data are available for laboratory-based toxicological studies of binary mixtures of chemicals (Carnesecchi et al., 2019; Lehmann and Camp, 2021), information on pesticide combinations with more than two compound is still lacking.

One of the most frequent pesticide co-occurrences (>60%) in the melon agroecosystem was comprised of acetamiprid (a neonicotinoid), oxamyl (a carbamate), and chlorpyrifos (an organophosphate). To our knowledge, no studies in bee ecotoxicology have focused on this particular combination. For this reason, we decided to test whether these compounds can synergistically interact on bees at the detected concentrations. In order to simulate as much as possible a realistic scenario, we chronically exposed *B. terrestris* micro-colonies to these three insecticides in both pollen and nectar simultaneously. Our results showed that the concentration levels detected in melon crops of Central Spain of acetamiprid, chlorpyrifos and oxamyl alone and in combination did not cause any adverse effects on worker mortality, the number of drones produced, and the size of drone developed.

The use of micro-colonies for this kind of study offers a good advantage compared to the use of individual bees because the social organization can be considered and therefore both effects on adults and

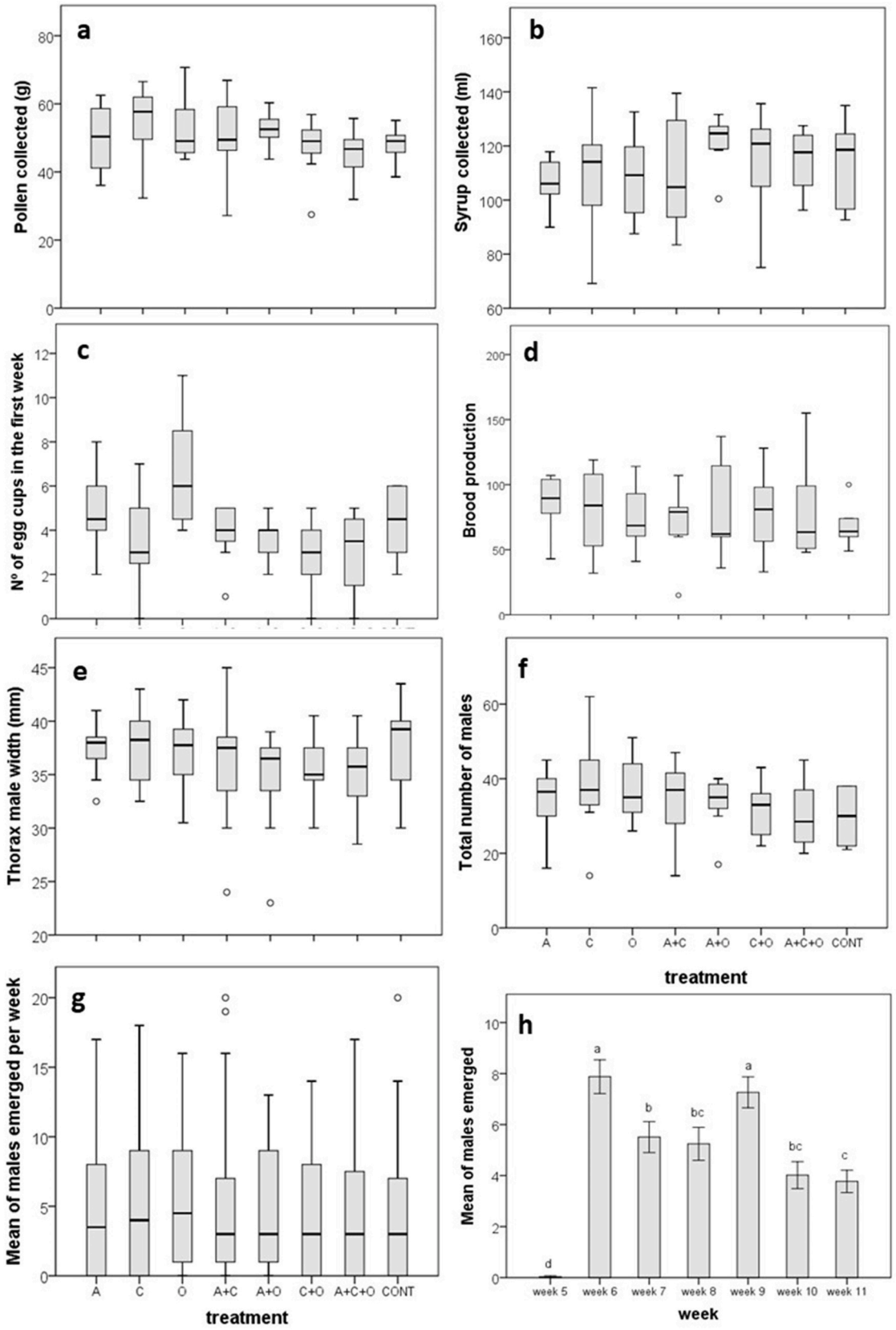

Fig. 4. *Bombus terrestris* micro-colonies: a) total pollen collected (g); b) total syrup collected (ml); c) number of egg cups during the first week; d) brood production; e) thorax male width (mm); f) total number of males produced in an 11-week period; g) mean of males emerged per week in every treatment (A: acetamiprid, n = 6; C: chlorphyrifos, n = 8; O: oxamyl, n = 8; A + C, n = 7; A + O, n = 7; C + O; n = 7; A + C + O, n = 8; CONT: control, n = 6); h) mean of males emerged per week. Boxplots indicate the lower, median and upper quartiles. Whiskers extending to the most extreme data point indicate that there is not more than 1.5 times the interquartile range from the edge of the box. Means with the same letter are not significantly different (Fisher's LSD post hoc; $p < 0.05$).

larvae can be measured. Although reproductive fitness cannot be assessed as mating is not required and only males are produced, drone production has been identified as a crucial indicator of microcolony productivity as it encompasses potential impacts on various aspects such as fertility, growth and development (Belsky et al., 2020). The disadvantage is that the consumption of pesticides by adults and larvae cannot be directly measured, because pollen provisions are, to some extent, incorporated into the nest structure and the syrup is stored (Dance et al., 2017) weakening our ability to compare the dose we tested to the LD50 data. However, the amount of the active ingredient ingested may be estimated considering the syrup (adult: 400 mg/day; larva: 60 mg/day) and pollen (adult: 30 mg/day; larva: 40 mg/day) consumption of *B. terrestris* from literature (EFSA, 2013; Gradish et al., 2019), the calculated bee longevity of this study (adult: 77 days; larva: 14 days) and the concentrations tested (Table 1). Comparisons between the estimations of amounts of pesticide ingested (μg/bee) in our study after chronic exposure (acetamiprid: 1.31; chlorpyrifos: 0.05; oxamyl: 0.01) with the acute oral LD50s reported in adult bees (*B. terrestris*) for acetamiprid (22 μg/bee), chlorpyrifos (0.24 μg/bee) and oxamyl (0.38 μg/bee) (Sanchez-Bayo and Goka, 2014), revealed that our tested doses were 20–40 times lower. The difference is even higher (20–3500 times) in bee larvae because acute oral LD50's for acetamiprid is 5.56 μg/larva (Yang et al., 2020), for chlorpyrifos 0.6 μg/larva (Dai et al., 2017) and for oxamyl 7.15 μg/larva (Prezenská et al., 2019), while the amounts consumed in μg/larva in our experiment were 0.276, 0.03 and 0.02 μg/larva for acetamiprid, chlorpyrifos, and oxamyl, respectively. These differences help to explain why no lethal effects were observed in workers or larvae. However, caution must be exercised when making this comparison as the amount of adult pollen consumption remains unknown. We rely on data from EFSA reports and other relevant paper (Gradish et al., 2019) that are based on studies using microcolonies, but as previously stated, pollen consumption cannot be precisely measured. Therefore, further investigation is required to accurately determine the amount of adult pollen consumption, which will aid in gaining a complete understanding of the potential effects of pesticide exposure and its impact on bee health.

Besides, no effects on drone production were observed. Contrary to these findings, previous studies of *Bombus impatiens* microcolonies exposed to acetamiprid have shown varying impacts on parameters related to drone production, depending on the route of exposure (Weitekamp et al., 2022). A concentration of 1130 ppb via syrup was found to decrease the number of emerged drones (Camp et al., 2020b), while a lower concentration of 6.41 ppb in our study did not produce a significant effect. However, a similar concentration of 452 ppb via pollen, compared to the concentration of 482.93 ppb in our study, resulted in a significant reduction in drone weight (Camp et al., 2020a). The differences may be attributed to the fact that we used thorax width as a proxy for body size (Kapustjanskij et al., 2007), rather than directly weighing the bees. In addition, our study employed *B. terrestris* instead of *B. impatiens*. As previously stated, the impact of pesticides on bees can vary between species, even among species in the same genus (Baron et al., 2017).

Another of the most likely combination of pesticides in this study area (i.e., acetamiprid, imidacloprid, and mycrobutanil) showed important sublethal effects in the thermal performance and the activity of the solitary bee *O. bicornis*. However, these effects were mainly due to the action of a single compound, imidacloprid (Azpiazu et al., 2019). Nitro-substituted neonicotinoids, including imidacloprid, clothianidin, and thiamethoxam, are more toxic than cyano-substituted neonicotinoids, such as acetamiprid and thiacloprid (Manjon et al., 2018). Previous studies have shown that imidacloprid has a negative impact on bumblebees, affecting colony success, queen production, survival, and foraging behavior (Gill et al., 2012; Scholer and Krischik, 2014; Whitehorn et al., 2012). However, additional studies are necessary to fully assess the sublethal effects of acetamiprid on pollinators.

5. Conclusions

The high number of pesticides found in pollen and nectar of melon flowers suggests that bees in melon agroecosystems can be exposed to variety of pesticide mixtures throughout the extended blooming period of this crop (i.e., ~10 weeks). Many of them were found in melon flowers although they were not applied by farmers to the crop in the current season, probably due to pesticide accumulation in soils, contaminated irrigation water, or drift from the adjacent fields. This study provides valuable data on the most likely pesticide combinations in a specific crop agroecosystem, which can be useful when planning more realistic ecotoxicological studies that take into consideration the exposure to multiple pesticides, which is the most likely real-world scenario. The results of the cumulative chronic RI and a previous study (Azpiazu et al., 2019) emphasize the need to study different pesticides and incorporate non-*Apis* bees in risk assessment schemes. In addition, sublethal and synergistic effects should be considered under more accurate ecotoxicological protocols. Furthermore, if pollinators can visit other adjacent crops or wildflowers contaminated with pesticide residues, they may be exposed to an even greater number of compounds (Botías et al., 2015; Favaro et al., 2019; Graham et al., 2022; McArt et al., 2017), further increasing the risks to their health. Our findings underscore the importance of considering the broader spatio-temporal scope of pesticide exposure, and shifting from the current Environmental Risk Assessment (ERA) based on a single crop, single species, single use approach to a more holistic, systems-based ERA (Topping et al., 2020) that support pollinator health and overall ecosystem wellbeing.

Credit author statement

Celeste Azpiazu: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. Pilar Medina: Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition. Fabio Sgolastra: Conceptualization, Formal analysis, Writing – review & editing. Ana Moreno-Delafuente: Methodology, Investigation, Writing – review & editing. Elisa Viñuela: Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121451.

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