Full length article

Exposure to sublethal levels of insecticide-fungicide mixtures affect reproductive success and population growth rates in the solitary bee *Osmia cornuta*

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ABSTRACT

In agricultural environments, bees are routinely exposed to combinations of pesticides. For the most part, exposure to these pesticide mixtures does not result in acute lethal effects, but we know very little about potential sublethal effects and their consequences on reproductive success and population dynamics. In this study, we orally exposed newly emerged females of the solitary bee *Osmia cornuta* to environmentally-relevant levels of acetamiprid (a cyano-substituted neonicotinoid insecticide) singly and in combination with tebuconazole (a sterol-biosynthesis inhibitor (SBI) fungicide). The amount of feeding solution consumed during the exposure phase was lowest in bees exposed to the pesticide mixture. Following exposure, females were individually marked and released into oilseed rape field cages to monitor their nesting performance and assess their reproductive success. The nesting performance and reproductive success of bees exposed to the fungicide or the insecticide alone were similar to those of control bees and resulted in a 1.3–1.7 net population increases. By contrast, bees exposed to the pesticide mixture showed lower establishment, shortened nesting period, and reduced fecundity. Together, these effects led to a 0.5–0.6 population decrease. Female establishment and shortened nesting period were the main population bottlenecks. We found no effects of the pesticide mixture on nest provisioning rate, offspring body weight or sex ratio. Our study shows how sublethal pesticide exposure may affect several components of bee reproductive success and, ultimately, population growth. Our results calls for a rethinking of pollinator risk assessment schemes, which should target not only single compounds but also combinations of compounds likely to co-occur in agricultural environments.

1. Introduction

The intensive use of plant protection products is one of the main components of mainstream agriculture, and global pesticide sales have increased by nearly 50 percent over the last two decades (FAO, 2022). Although applied to control specific pests and diseases, pesticides may also have undesired effects on non-target organisms, and pesticide exposure is recognized as one of the main drivers of widespread bee population declines (Goulson et al., 2015; Nicholson et al., 2023; Van Der Sluijs et al., 2013; Woodcock et al., 2016), with consequences on pollination services (Raven et al., 2021; Stanley et al., 2015). To protect

pollinators, pesticide regulation prohibits or severely restricts insecticide applications during the flowering period of entomophilous crops (EFSA, 2012, 2013; Simon-Delso et al., 2015; USEPA, 2014). Consequently, insecticide levels in crop and wild flowers in agricultural areas are, for the most part, relatively low (Botías et al., 2016; Heller et al., 2020; Main et al., 2020). In general, these levels are not expected to have lethal effects on bees (Azpiazu et al., 2023a; Heller et al., 2020; Siviter et al., 2021a; Zioga et al., 2020), but it is unclear to what extent they may elicit sublethal effects. Sublethal effects do not cause the immediate death of the organism, but may nonetheless affect reproductive success and compromise population persistence (Richardson et al., 2024;

Rondeau and Raine, 2024; Tosi et al., 2022). In addition, pollinators in agricultural environments are not exposed to single pesticides but to combinations of compounds (Knapp et al., 2023; Sanchez-Bayo and Goka, 2014). Multiple exposure may result from bees foraging on crops treated with different chemicals, either applied sequentially or together in a tank mix, and from bees foraging on various flower species contaminated with different compounds (Botías et al., 2017; David et al., 2016). Multi-exposure is of great concern because certain pesticide mixtures produce synergistic effects (e.g., Biddinger et al., 2013; Gill et al., 2012; Siviter et al., 2021b; Thompson et al., 2014). In particular, sterol-biosynthesis-inhibiting (SBI) fungicides, with low or no toxicity to bees (Artz and Pitts-Singer, 2015; Ladurner et al., 2005, 2008), enhance the toxicity of various insecticides, including pyrethroids, phenylpyrazoles, butenolides and neonicotinoids (Iverson et al., 2019; Pilling and Jepson, 1993; Raimets et al., 2018; Rondeau and Raine, 2024; Sgolastra et al., 2017). This synergistic effect occurs because the fungicide inhibits the P450 enzyme involved in the detoxification of these insecticides (Berenbaum and Johnson, 2015). Co-occurrence of SBI fungicides and insecticides is frequent in flowers and bee matrices in agroecosystems (Knapp et al., 2023; Rondeau et al., 2022; Zioga et al., 2020). Residues of systemic insecticides applied shortly before bloom to control sap-feeding pests appear in the pollen and nectar of crop flowers in combination with fungicides applied during bloom to control fungal diseases.

In this study, we tested the potential effects of oral exposure to field-realistic levels of acetamiprid, a cyano-substituted neonicotinoid, alone and in combination with tebuconazole, an SBI fungicide, on the nesting performance and reproductive success of the solitary bee *Osmia cornuta* in semi-field conditions. Compared to other insecticides, acetamiprid has a relatively low toxicity to bees (Buszewski et al., 2019; EFSA, 2016; Iwasa et al., 2004; Schuhmann et al., 2022). For this reason, it was not included in the EU ban on neonicotinoids (EFSA, 2022; EU, 2018/113). Studies testing acetamiprid toxicity on bees report lethal and sublethal effects at relatively high concentrations (Biddinger et al., 2013; Camp et al., 2020; Ferreira et al., 2022; Mokkapati et al., 2021; O'Reilly and Stanley, 2023; Shi et al., 2019, 2020) but no or only minor effects at lower, field-realistic, concentrations (Azpiazu et al., 2019; Chandler et al., 2020; Schuhmann and Scheiner, 2023; Schuhmann et al., 2024). Both acetamiprid and tebuconazole are commonly applied to entomophilous crops (EFSA, 2014, 2022), including oilseed rape (Wen et al., 2021) and fruit trees (González-Núñez et al., 2022; Heller et al., 2020; Li et al., 2015). Co-occurrence of acetamiprid and tebuconazole has been documented in flowers and bee-collected pollen, as well as on the body of bees (Botías et al., 2017; David et al., 2016; Knapp et al., 2023). *Osmia cornuta* is commonly exposed to pesticides because it shows a strong preference to forage on fruit tree flowers (Jaumejoan et al., 2023) and because, along with other *Osmia* species in different parts of the world, it is increasingly being used as a managed orchard pollinator (Bosch and Kemp, 2002).

A previous laboratory study showed that exposure to neonicotinoid-SBI fungicide mixtures, but not to the two compounds singly, prompted slow ovary development and reduced adult life span in *Osmia* (Sgolastra et al., 2018). Slow ovary maturation results in delayed nesting activity and, together with shortened life span, contributes to reduce the effective nesting period, which is strongly correlated to realized fecundity in *Osmia* (Bosch and Vicens, 2006; Sgolastra et al., 2016). Shortened life span may be indicative of accelerated aging, which in *Osmia* is associated with the production of smaller offspring and greater investment in males, the sex of smaller size (Bosch and Vicens, 2005; Seidelmann et al., 2010; Sugiura and Maeta, 1989; Torchio and Tepedino, 1980). Another laboratory study has demonstrated that exposure to neonicotinoid–SBI fungicide mixtures results in reduced thermoregulation capability (Azpiazu et al., 2019), a key factor limiting foraging activity in *Osmia* (Stone and Willmer, 1989; Vicens and Bosch, 2000). A semi-field study analyzed the effects of a tank mixture application of the neonicotinoid thiacloprid and the SBI fungicide prochloraz, on the nesting activity of

Osmia bicornis (Alkassab et al., 2020). Bees exposed to the application decreased their flight activity at the nest entrance and showed a reduction in offspring production of over 40 % compared to control bees. However, the tank mixture was applied during full bloom (something not allowed for insecticides) and at the maximum recommended application rates. Therefore, it remains to be established whether field-relevant pesticide exposure would yield similar results.

The first objective of our study is to measure the effects of field realistic concentrations of a neonicotinoid insecticide, singly and in combination with an SBI fungicide, on the reproductive success of a solitary bee. We have two hypotheses associated with this objective: a) bees exposed to the pesticide mixture will start nesting later, and have a shorter nesting period and reduced fecundity; b) bees exposed to the pesticide mixture will produce smaller offspring and a male-biased progeny sex ratio. Our second objective is to measure the potential effects of the expected reduction in reproductive success on population growth rate.

2. Materials and methods

2.1. Study organism

Osmia cornuta is a spring-flying solitary bee occurring in most of central and southern Europe and parts of northern Africa and western Asia (Müller, 2022). As in other *Osmia* species, males emerge a few days ahead of females and mate with newly-emerged females close to their natal nest or on flowers. Upon emergence, females fly to nearby flowers and feed on nectar (Torchio et al., 1987). Following this first meal, females undergo a pre-nesting period of 3–6 days during which they complete ovary maturation (Sgolastra et al., 2016), and then start searching for a nesting cavity. Individual females nest for 2–3 weeks, during which time they build one or more nests consisting of linear series of cells delimited by mud partitions, with each cell containing one pollen–nectar provision and one egg (Bosch et al., 2008). Upon nest completion, the nesting female seals the nest entrance with a mud cap and starts provisioning a new nest. Nesting females spend the night in their nesting cavity.

2.2. Bee population and rearing methods

The study was conducted in March-April 2022. At the end of March, wintered male and female cocoons from a population reared at our laboratory were incubated at 20–22 °C to elicit emergence. To avoid potential effects of body size on nest establishment and other variables related to reproductive success (Bosch and Vicens, 2006), we selected females of similar size (cocoon length range, 1.2–1.4 cm). Upon emergence, females were transferred to a screen holding cage (40 × 30 × 30 cm) to allow them to deposit the meconium (gut metabolic waste products). Females emerging on a given day were kept in the holding cage overnight and, the following morning, were evenly distributed among six pesticide exposure treatments (Table 1). We were concerned that mixture treatments could have a strong effect on female establishment, which would have resulted in a small sample size on which to measure reproductive success. For this reason, we used somewhat larger initial sample sizes in the two Mixture treatments.

2.3. Pesticide concentrations and preparation of test solutions

We worked with formulated products (Folicur® 25 EW, 25 % tebuconazole [Bayer CropScience]; and Carnadine®, 20 % acetamiprid [Nufarm Spain, S.A]). We tested two field-realistic concentrations of acetamiprid: 0.009 and 0.068 mg·L^{-1} (Table 1). These concentrations correspond to levels measured following pre-bloom spray applications in the nectar of oilseed rape (Wen et al., 2021) and apple flowers (Heller et al., 2020), respectively. To obtain these concentrations, we prepared a primary stock solution with a concentration of 5000 mg·L^{-1} by

Table 1
Pesticide exposure treatments and samples sizes (number of females exposed). All females were allowed to feed *ad libitum* for 4 h on a sugar solution (33 % w: w) with or without pesticides.

Treatment	Acronym	Compounds	Concentration [mg·L^{-1} of a.i.]	N females exposed	N females released per cage
Control	CTRL	–	0	41	10–11
Fungicide	TEB	Tebuconazole	150.0	40	9–11
Insecticide-1	ACT1	Acetamiprid	0.009	42	10–12
Insecticide-2	ACT2	Acetamiprid	0.068	41	10–11
Mixture-1	MIX1	Tebuconazole + Acetamiprid	150 + 0.009	60	15
Mixture-2	MIX2	Tebuconazole + Acetamiprid	150 + 0.068	64	15–17

dissolving Carnadine in distilled water. Then, the primary stock solution was diluted until we reached final stock solution concentrations of 0.45 and 3.40 mg·L^{-1}. These solutions were then diluted with a sucrose solution (33 % w: w; henceforth syrup) to achieve the final concentrations of 0.009 (insecticide-1; henceforth ACT1) and 0.068 mg·L^{-1} (insecticide-2; henceforth ACT2). We tested one tebuconazole concentration, 150 mg·L^{-1} (henceforth TEB) (Table 1). This concentration corresponds to the recommended field application rate of Folicur® 25 EW (250 g·L^{-1} of a.i.) (0.6 L product·ha^{-1} in a water volume of 1000 L·ha^{-1}; MAPA, 2024), and therefore represents a worst-case scenario in which bees are exposed during or immediately after a bloom spray. Levels of tebuconazole measured in nectar and pollen range from 0.05 to 22.7 mg·L^{-1} (Rondeau and Raine, 2022; USEPA, 2021), but concentrations as high as 1,568 mg·L^{-1} have been found in other bee matrices (bee bread, Bokšová et al., 2021). Other fungicides have also been reported at high concentrations in various studies (e. g., 350 mg·L^{-1} of prothioconazole in bee pollen, Roszko et al., 2016; 532.81 mg·L^{-1} of cyprodinil in flowers, Gierer et al., 2024; and 98.90 mg·L^{-1} of chlorothalonil in pollen loads, Mullin et al., 2010). To obtain the field application concentration, we prepared a stock solution with a tebuconazole concentration of 7498.88 mg·L^{-1} by dissolving Folicur® 25 EW in distilled water. The stock solution was then diluted with syrup (33 % w:w) to reach the desired concentration. The fungicide concentration was combined with the two insecticide concentrations to obtain two mixture treatments (MIX1 and MIX2) (Table 1). In addition, a group of control bees (CTRL) were exposed to syrup without pesticides (Table 1).

2.4. Pesticide exposure

As mentioned, information on the concentration of acetamiprid in the nectar of crop flowers was obtained from the literature. On the other hand, the amount of nectar consumed by a nesting *Osmia* female is hard to measure, especially because part of the nectar ingested is regurgitated onto the larval provision. However, during the pre-nesting phase, females collect nectar only for their own consumption. We took advantage of this phase of the life cycle and simulated the first nectar meal of newly-emerged females by offering them syrup laced with the test compounds at the desired concentrations. Meconium-free females were individually housed in transparent plastic containers (diameter: 11 cm; height: 7 cm) capped with a pin-perforated lid and a lateral hole through which a feeder made with a 1 ml syringe was inserted. Each feeder contained 300 μl of syrup (33 % w/w sucrose-water solution) with or without pesticides. A flower petal (*Euryops* spp., Asteraceae) was attached to the tip of the syringe to ensure that bees would locate the feeder promptly (see Azpiazu et al., 2023b for details). Bees were left to feed *ad libitum* for 4 h. Previous studies showed that extending this exposure phase up to 8 h did not result in increased syrup consumption (Sgolastra et al., 2018). The amount of solution ingested by each bee was assessed by checking the level of syrup in the calibrated syringe (accuracy: 0.01 ml). Three additional containers without bees were used to measure and account for potential evaporation. Final pesticide concentrations in the sucrose solution were not measured. Bees were maintained at 20 ± 2 °C and 50–70 % RH under indirect natural light throughout the exposure phase. Samples sizes are provided in Table 1.

2.5. Individual marking and release in field cages

After the exposure phase, females that had fed at least 15 μl of syrup were temporarily cooled at 4 °C and individually marked on the thorax with numbered tags (Fig. 1). Marked females were introduced in paper straws, which were then inserted into nesting materials in four field cages (length x width x height = 7 × 7 × 3 m) in an oilseed rape (*Brassica napus*) field (Fig. 1). The field had been sown with untreated seed and no pesticides were applied to the emerging crop. In each cage, a nesting station, consisting of a wooden box with the front side open and held 1.5 m above the ground, was installed facing SE. Each nesting station had six solid wood blocks (16 x 8.5 x 8.5 cm). Each nesting block had 25 drilled holes to accommodate a paraffin-coated paper straw (15 cm long, 8 mm inside diameter), so that nests could be easily extracted out of the nesting block. To ensure mud availability we dug a 20-cm deep trench next to each station and kept it moist throughout the study. The number of females of each treatment released per cage are reported in Table 1. Along with the marked females, newly-emerged unmarked males, were introduced in the cages at a rate of 1.7 per female (natural sex ratio; Bosch and Vicens, 2002). Four days after release, when mating activity had ceased, males were netted and removed from the cages.

2.6. Establishment and nesting period

Nesting activity was monitored daily through visual observations during the diel peak of activity (12:00–15:00). The cavity in which each female was nesting was noted throughout the nesting period. To measure nest provisioning rate, each day at dusk, when females were no longer active, paper straws containing active nests were removed with a pair of forceps, and nest progression (evidenced by placing the straw against a flashlight) was marked and dated. Females were considered dead when they were no longer seen at the nesting site or foraging. Dead females were often found in the vicinity of the nesting stations. For each female we obtained the following information: a) establishment (whether or not the female started a nest and laid at least one egg); b) pre-nesting period (days between release and the onset of nesting activity); c) nesting period (days between the onset and the end of nesting activity).

2.7. Nest analysis

At the end of the nesting period (late April), nest-blocks were collected and kept in a storage area at ambient temperatures to allow bees to develop. In September, when all offspring had reached the adult stage, paper straws containing nests were dissected and their contents analyzed. Each cocoon was dated, weighed and assigned a sex based on its size and position within the nest (Bosch and Vicens, 2002). When in doubt, we cut a small opening at the tip of the cocoon to verify the sex of the individual. Then, bees were wintered at 4 °C from 1 October until 15 March. After wintering, cocoons were incubated at 20–22 °C, and their emergence was monitored. From these nest analyses, we derived the following information for each female: a) fecundity (number of cells produced); b) female production (number of females produced); c) nest provisioning rate (number of cells built divided by the nesting period);
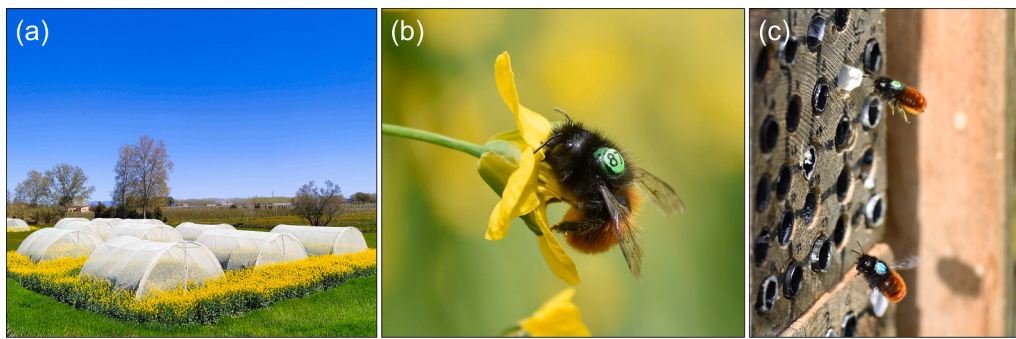
Fig. 1. Oilseed rape field cages into which individually marked females *O. cornuta* were released (a); female collecting pollen and nectar (b); females returning to their nests after a foraging trip (c).

d) mean offspring weight (separately for males and females); e) progeny sex ratio (males/males + females); f) percent offspring mortality; and g) number of female offspring emerging the next spring.

For each treatment, the female population growth rate (λ) was calculated as $\lambda = P$ (establishment) * N (♀ produced) * P (spring emergence), where P (establishment) is the establishment probability (0 for not established or 1 for established), N (♀ produced) is the number of female offspring produced, and P (spring emergence) is the proportion of female offspring emerging the following spring.

2.8. Statistical analysis

The effects of pesticide exposure on test solution consumption (amount of feeding solution ingested during the exposure phase) were analyzed by fitting a generalized linear model (GLM) with gamma error distribution. Insecticide concentration, fungicide concentration, and their interaction were the explanatory variables.

To analyze the effects of pesticide exposure on nesting performance and reproductive success, we fitted mixed-effects models, with insecticide concentration, fungicide concentration and their interaction as fixed effects, and cage as a random effect. Female establishment (binary variable) was analyzed with a binomial generalized linear mixed-effects model (GLMM). Nesting period (days), fecundity, female production, and female population growth rate were analyzed with GLMMs with Poisson error distribution. Pre-nesting period, nest provisioning rate and male and female offspring weight were analyzed using linear mixed-effects models (LMM) with normal error distribution. Progeny sex ratio (proportion of males) and offspring mortality were analyzed with a binomial GLMM.

The selection of model error distributions and link functions was based on residual plots and AIC, and we graphically validated the requirements of distribution and variance homogeneity for all models (Zuur et al., 2009). We calculated P values of fixed effects using likelihood ratio tests. All pairwise comparisons were done with the *emmeans* package and adjusted for multiple comparisons with Tukey's p-value adjustment method (Lenth et al., 2019). All analyses were conducted in R (R Core Team, 2020), packages "*nlme*" (Pinheiro et al., 2014) and "*lme4*" (Bates et al., 2014).

3. Results

3.1. Syrup consumption during the exposure phase

Syrup consumption during the 4-hour exposure phase was not affected by insecticide exposure (GLM, $\chi^2 = 4.4$, df = 2, p = 0.113), but decreased with fungicide exposure (GLM, $\chi^2 = 23.5$, df = 1, p < 0.001). The interaction between fungicide and insecticide was significant (GLM, $\chi^2 = 9.0$, df = 2, p = 0.011), with bees of the two mixture treatments showing a tendency to consume less syrup (Fig. 2). As a result, bees of the ACT treatments ingested higher levels of acetamiprid than bees of
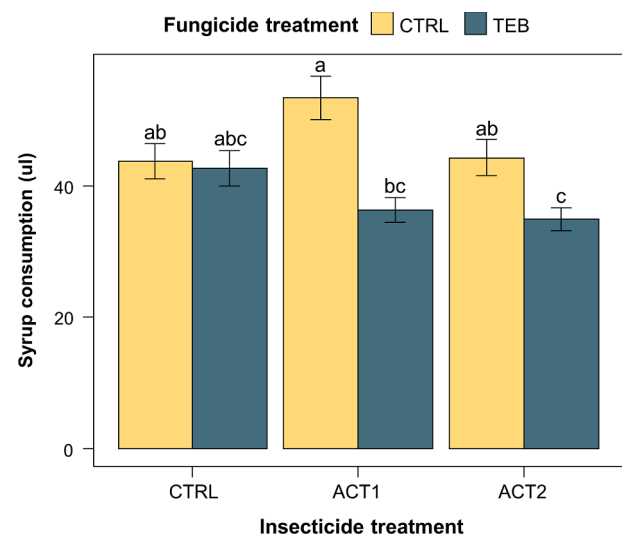

Fig. 2. Model-estimated means $\pm$ SE of test solution ingested during the 4 h-exposure phase in *O. cornuta* females orally exposed to six pesticide treatments: control (CTRL), fungicide (TEB), insecticide-1 (ACT1), insecticide-2 (ACT2), mixture-1 (TEB+ACT1), and mixture-2 (TEB+ACT2). Different letters denote significant differences (Tukey HSD test, p < 0.05; Supplementary Table 2).

the MIX treatments (mean $\pm$ SE/bee; ACT1: 0.5 $\pm$ 0.02 ng; MIX1: 0.3 $\pm$ 0.02 ng; ACT2: 3.0 $\pm$ 0.17 ng; MIX2: 2.4 $\pm$ 0.12 ng) (Supplementary Fig. S1). No bees died and no sublethal behavioral effects were observed during the exposure phase.

3.2. Nesting and reproductive success

3.2.1. Female establishment and nesting activity

Female establishment (% of females that initiated nesting activities and laid at least one egg) was affected by insecticide exposure (GLMM, $\chi^2 = 10.3$; df = 2; p = 0.006), fungicide exposure (GLMM, $\chi^2 = 10.2$; df = 1; p = 0.001), and their interaction (GLMM, $\chi^2 = 6.1$; df = 2; p = 0.047). Establishment was 85 % in CTRL bees, and did not significantly decrease in bees of the TEB, ACT or MIX1 treatments. However, establishment dropped to 40 % in bees of the MIX2 treatment (Fig. 3A; Table 2).

The effects of fungicide and insecticide exposure on pre-nesting period (time to start nesting activities following release) failed significance (Fig. 3B, Table 2, Supplementary Table S1). Nesting period was affected by insecticide exposure (GLMM, $\chi^2 = 13.8$; df = 2; p = 0.001), fungicide exposure (GLMM, $\chi^2 = 5.9$; df = 1; p = 0.015), and their interaction (GLMM, $\chi^2 = 12.8$; df = 2; p = 0.002). Bees of the MIX1 treatment, but not of the MIX2, had a significantly shorter nesting period than CTRL, TEB and ACT bees (Fig. 3C, Table 2).
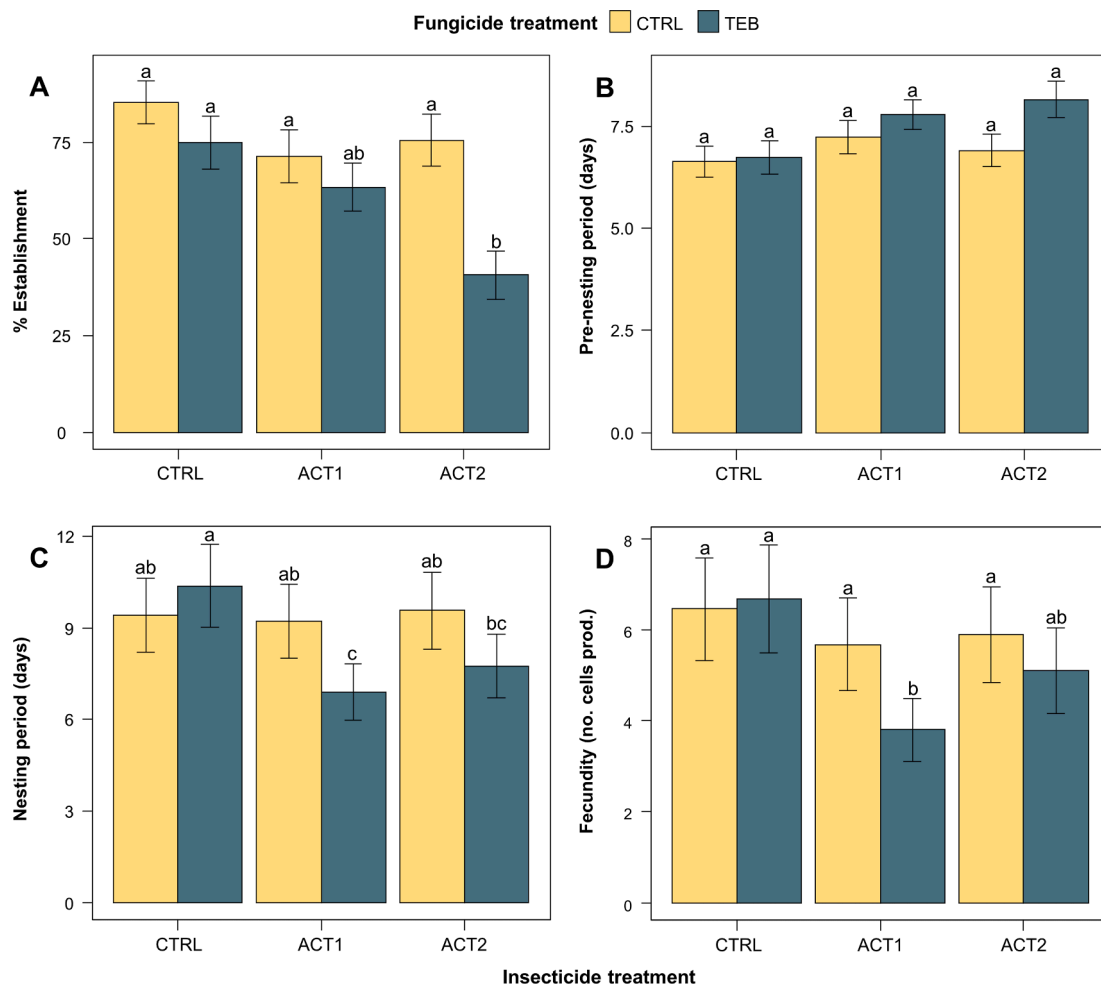
Fig. 3. Model-estimated means ± SE of percent nest establishment (**A**), pre-nesting period (**B**), nesting period (**C**) and fecundity (**D**) in *O. cornuta* females orally exposed to six pesticide treatments (control (CTRL), fungicide (TEB), insecticide-1 (ACT1), insecticide-2 (ACT2), mixture-1 (TEB+ACT1), and mixture-2 (TEB+ACT2)) and subsequently released in field cages. Different letters denote significant differences (Tukey HSD test, p < 0.05; Supplementary Table 2).

Table 2

Model-estimated means ± SE of parameters related to nesting activity and reproductive success in *O. cornuta* females released in field cages following exposure to sublethal concentrations of a fungicide (TEB), an insecticide (ACT), and mixtures of the two compounds. Values within a row followed by different letters are significantly different (Tukey HSD test, p < 0.05; Supplementary Table 2).

Parameter	CTRL	TEB	ACT1	ACT2	MIX1	MIX2
Released females	41	40	42	41	60	64
% Establishment [1]	85.4 ± 5.5 a	75.0 ± 6.8 a	71.4 ± 7.0 a	75.6 ± 6.7 a	63.3 ± 6.2 ab	40.6 ± 6.1**b**
Females nesting	35	30	30	31	38	26
Pre-nesting period [2]	6.6 ± 0.4 a	6.7 ± 0.4 a	7.2 ± 0.4 a	6.9 ± 0.4 a	7.8 ± 0.4 a	8.2 ± 0.4 a
Nesting period [2]	9.4 ± 1.2 **ab**	10.4 ± 1.4 a	9.2 ± 1.2 **ab**	9.6 ± 1.3 **ab**	6.9 ± 0.9**c**	7.7 ± 1.1 **bc**
Fecundity	6.5 ± 1.1 a	6.7 ± 1.2 a	5.7 ± 1.0 a	5.9 ± 1.1 a	3.8 ± 0.7**b**	5.1 ± 0.9 **ab**
Female production	2.7 ± 0.5 a	2.9 ± 0.6 a	2.5 ± 0.5 a	2.5 ± 0.5 a	1.5 ± 0.3**b**	2.0 ± 0.4 **ab**
Nest provisioning rate [3]	0.67 ± 0.05 a	0.62 ± 0.05 a	0.57 ± 0.05 a	0.64 ± 0.05 a	0.56 ± 0.05 a	0.68 ± 0.06 a
♂ Offspring weight [4]	124.0 ± 4.1 a	126.2 ± 4.5 a	118.5 ± 4.7 a	128.9 ± 4.3 a	126.9 ± 4.5 a	131.3 ± 4.9 a
♀ Offspring weight [4]	170.5 ± 4.9 a	178.8 ± 5.2 a	162.9 ± 5.4 a	177.3 ± 5.4 a	165.8 ± 5.3 a	174.0 ± 6.2 a
Progeny sex ratio [5]	0.59 ± 0.05 a	0.57 ± 0.05 a	0.55 ± 0.05 a	0.58 ± 0.05 a	0.60 ± 0.05 a	0.58 ± 0.06 a
% Offspring mortality [6]	22.9 ± 4.5 a	20.0 ± 4.5 a	22.5 ± 4.7 a	26.2 ± 4.8 a	24.0 ± 4.9 a	30.1 ± 5.3 a
♀ Pop. growth rate (λ) [7]	1.7 ± 0.4 a	1.6 ± 0.4 a	1.3 ± 0.3 a	1.4 ± 0.3 a	0.64 ± 0.16**b**	0.56 ± 0.14**b**

[1] of N released.

[2] in days.

[3] cells built per day.

[4] in mg.

[5] ♂♂/ (♂♂+♀♀).

[6] offspring not emerging in spring.

[7] λ = *P* (establishment) * N (♀ produced) * *P* (spring emergence).

3.2.2. Nesting performance and reproductive success

Nest provisioning rate (number of cells built per day) was unaffected by pesticide exposure (Table 2, Supplementary Table S1). On the other hand, fecundity (number of cells produced) was significantly influenced by insecticide exposure (GLMM, $\chi^2 = 21.2$; df = 2; p < 0.001), fungicide exposure (GLMM, $\chi^2 = 6.2$; df = 1; p = 0.013), and their interaction (GLMM, $\chi^2 = 9.0$; df = 2; p = 0.011). Fecundity dropped from ca. 6–7 eggs per female in the CTRL, TEB, and the two ACT treatments to ca. 4–5 in the two MIX treatments (Fig. 3D; Table 2). Female production was also affected by insecticide exposure (GLMM, $\chi^2 = 9.9$; df = 2; p = 0.007), but not by fungicide exposure (GLMM, $\chi^2 = 3.8$; df = 1; p = 0.052). However, the interaction between fungicide and insecticide exposure was significant (GLMM, $\chi^2 = 7.4$; df = 2; p = 0.025). Both fecundity and female production were significantly lower in bees of the MIX1 treatment, but not in bees of the MIX2 treatment (Table 2). Fecundity was strongly correlated to nesting period (fecundity: r = 0.9, p < 0.001).

Mean male and female offspring body weight were not affected by pesticide exposure (Table 2, Supplementary Table S1). Likewise, we found no effects of insecticide or fungicide exposure on offspring sex ratio or offspring mortality (Table 2, Supplementary Table S1).

3.2.3. Population growth

Female population growth rate was significantly affected by insecticide exposure (GLMM, $\chi^2 = 26.0$; df = 2; p < 0.001), fungicide exposure (GLMM, $\chi^2 = 19.3$; df = 1; p < 0.001), and their interaction (GLMM, $\chi^2 = 11.9$; df = 5; p = 0.002). Bees in the CTRL, TEB, and the two ACT treatments exhibited similar population growth rates (1.3–1.7; Table 2; Fig. 4). Bees exposed to the MIX treatments, on the other hand, experienced negative growth (0.56–0.64; Table 2; Fig. 4).

4. Discussion

The aim of this study was to assess the effects of exposure to field-realistic levels of acetamiprid, a widely used neonicotinoid insecticide considered relatively safe for bees (Buszewski et al., 2019; EFSA, 2016; Iwasa et al., 2004; Schuhmann et al., 2022), alone and in combination with an SBI fungicide, on the reproductive success and population dynamics of a solitary bee. To do this, we simulated the first nectar meal of newly emerged females by offering them a feeding solution laced with the desired fungicide/insecticide concentrations, and then monitored their nesting activity and assessed their reproductive success. Importantly, bees exposed to either the fungicide or the two concentrations of the insecticide alone did not differ from control bees in any of the parameters related to reproductive success that we measured, and experienced a positive population growth that did not differ from the control. However, bees exposed to the pesticide mixtures were less likely to establish a nest and had a reduced nesting period, resulting in decreased offspring production and negative population growth. The two key bottlenecks determining this population decline were establishment success and nesting period. Our results demonstrate that co-exposure to sublethal concentrations of acetamiprid and tebuconazole reduces population growth in *Osmia* and raise concerns about the long-term persistence of solitary bee populations in intensively managed agricultural environments where these pesticides are frequently used.

Previous laboratory studies with honey bees, bumblebees, and *O. bicornis* have shown that exposure to neonicotinoids at high concentrations and/or under prolonged exposure, usually have an inhibitory effect on feeding (Azpiazu et al., 2019; Baron et al., 2017; Martins et al., 2023; Zhu et al., 2017). However, exposure to low concentrations stimulates feeding (Kessler et al., 2015; Sgolastra et al., 2018). SBI fungicides have also been shown to reduce feeding in both social and solitary bees (Jaffe et al., 2019; Martins et al., 2023; Rondeau and Raine, 2024). In our study, exposure to either the fungicide or the two concentrations of the insecticide did not adversely affect syrup consumption. However, when the two compounds were combined feeding rates were depressed. As a result of the depressed feeding rates in the mixture treatments, insecticide intake was actually lower in bees of the mixture treatments than in bees of the insecticide treatments.

Previous studies in which *Osmia* populations were released in enclosures consistently show that a fraction of females fail to establish (Ladurner et al., 2008; Schwarz et al., 2022; Sgolastra et al., 2016; Tepedino and Torchio, 1982). Establishment success is lower in small females (Bosch and Vicens, 2006; Tepedino and Torchio, 1982), and in females taking longer to emerge out of their cocoons (Sgolastra et al., 2016), suggesting a suboptimal physiological condition. In our study, exposure to acetamiprid or tebuconazole alone did not affected establishment success, which was high and similar to that of control bees. This result is consistent with previous studies conducted on bumblebee microcolonies, which showed no significant sublethal effects on bee behavior or colony development following oral exposure to field-relevant concentrations of acetamiprid (Camp et al., 2020;
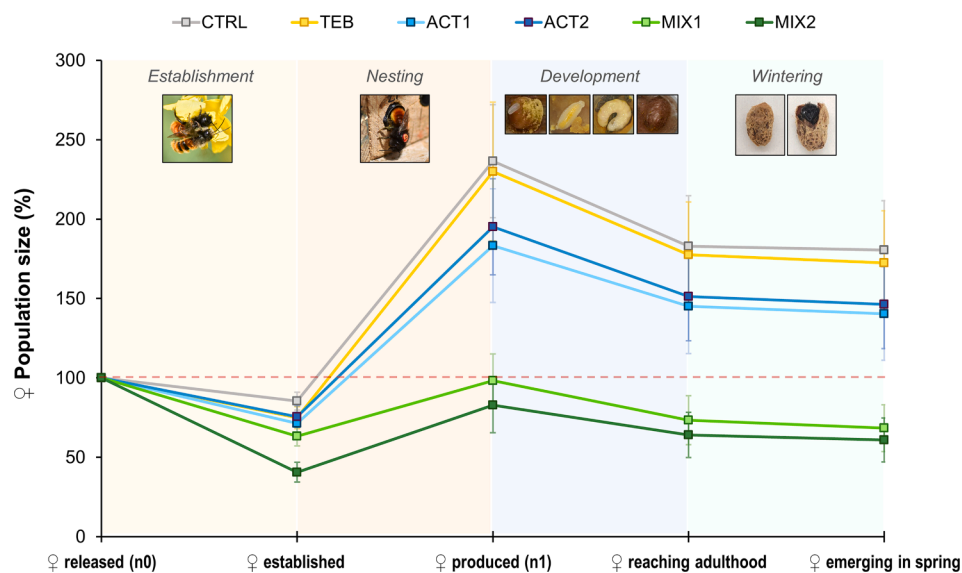

Fig. 4. Dynamics of *O. cornuta* female populations orally exposed to six pesticide treatments (control (CTRL), fungicide (TEB), insecticide-1 (ACT1), insecticide-2 (ACT2), mixture-1 (TEB+ACT1), and mixture-2 (TEB+ACT2)) and subsequently released in field cages. Squares with error bars represent arithmetic mean ± SE female population size from the initial release of the parental generation into the cages (n_0) until the emergence of the adults of the filial generation (n_1).

Schuhmann et al., 2024). It is also congruent with the commercial label specifications of the fungicide, which allow its use during flowering. Bees of our insecticide treatments ingested doses of acetamiprid ranging from 0.5 to 3 ng per bee (Supplementary Fig. S1), which are between 2,950 and 17,700 times lower than the acute oral LD50 for honey bees (8,850 ng per bee; EFSA, 2016). The ingested dose range of tebuconazole (2,500 to 11,500 ng per bee) is between 7–33 times lower than the estimated LD50 for honey bees (>83,050 ng per bee; USEPA, 2021). By contrast, establishment in the two mixture treatments decreased to ca. 60 % and 40 % (at the low and high concentrations, respectively). Importantly, because syrup consumption was lower in MIX bees, impaired establishment was reached at acetamiprid exposure levels as low as 0.3 to 2.4 ng per bee (between 3,687 and 29,500 times lower than the acute oral LD50 for honey bees). Many of the bees that failed to establish were seen repeatedly during the first days of monitoring, either feeding or resting on flowers. These observations demonstrate that these bees did not die immediately following insecticide exposure and had the opportunity to replenish energy reserves lost during wintering (Fliszkiewicz et al., 2012; Sgolastra et al., 2011) but nonetheless failed to establish a nest.

Our first objective was to establish whether exposure to sublethal levels of the fungicide-insecticide combination affected reproductive output. We expected that bees exposed to the pesticide mixtures would start nesting later and have a reduced nesting period. In a previous laboratory study, Sgolastra et al. (2018) showed that a single syrup meal with a neonicotinoid-SBI fungicide mixture delayed ovary maturation (which signals the onset of nesting activities; Lee et al., 2015; Maeta, 1978; Wasielewski et al., 2011) and shortened longevity in *O. bicornis*. Our analysis failed to detected differences in female pre-nesting period. However, bees exposed to the pesticide mixture had shorter nesting periods compared to those exposed to either the fungicide or the insecticide alone. Because fecundity was strongly correlated to nesting period duration (see also Bosch and Vicens, 2006; Sgolastra et al., 2016), female offspring production was also reduced in bees exposed to the pesticide mixture.

We also expected that bees exposed to the pesticide mixture would produce smaller offspring and a male-biased progeny sex ratio. A shortened nesting period may be indicative of accelerated aging, which could be attributed to oxidative stress associated to pesticide exposure (Christen et al., 2016; Li-Byarlay and Cleare, 2020). In *Osmia*, aging causes females to reduce the size of larval provisions (resulting in smaller offspring body size) and to progressively shift the sex ratio of their progeny towards males, the sex requiring smaller provisions (Bosch and Vicens, 2005; Seidelmann et al., 2010; Sugiura and Maeta, 1989; Tepedino and Torchio, 1982). This shift in parental investment is explained by the increased amount of time aged females require to collect a pollen-nectar load (Bosch and Vicens, 2005). Male-biased sex ratios have been observed in *Osmia* females exposed to sublethal levels of pesticides (Sandrock et al., 2014; Stuligross and Williams, 2020), but sex ratio and offspring body size did not differ across pesticide treatments in our study. Importantly, bees in our study were exposed to pesticides only once during the pre-nesting period, whereas bees in Sandrock et al. (2014) and Stuligross and Williams (2020), experienced chronic exposure throughout the nesting period. Therefore, females in our study that overcame the single-exposure intoxication were able to initiate and maintain nesting activity at a normal pace, as indicated by the lack of differences in nest provisioning rates across treatments.

Our second objective was to establish whether co-exposure to the two compounds could affect bee population dynamics. Our results indicate that *O. cornuta* populations can withstand certain levels of insecticide or fungicide exposure, as evidenced by the positive population growth rates in the ACT and TEB treatments. However, exposure to both compounds, even at the lowest insecticide concentration, resulted in negative population growth rates. These findings agree with the documented relatively low population returns of managed *Osmia* populations in agricultural environments (Bosch and Kemp, 2002; and

references therein; Bosch et al., 2021; Monzón et al., 2004; Pitts-Singer et al., 2018). As mentioned, the main bottlenecks affecting population growth in our study, were female establishment success and nesting period duration. *Osmia* females have short life spans (ca. 20 days), low fecundity (0.5–1 egg per day) and produce male-biased progenies (1.7 ♂:♀) (this study; Bosch and Kemp, 2002; and references therein). Therefore, any reduction in the effective nesting period may critically compromise female population growth rates.

Our study demonstrates that exposure to a meal of syrup containing sublethal concentrations of an insecticide-fungicide mixture during the pre-nesting period has a negative impact on solitary bee establishment, fecundity, and population growth rates. Importantly, these effects were observed following a one-time exposure scenario involving a two-compound mixture. In agricultural environments, bees may experience repeated and/or chronic exposure (Sanchez-Bayo and Goka, 2014) to a wide array of chemicals (Knapp et al., 2023; Nicholson et al., 2023). Also importantly, none of these effects were observed when bees ingested the insecticide and the fungicide alone, suggesting that bee populations may be able to thrive in the presence of low levels of insecticides but not under certain pesticide combinations. The relatively low harmful effects of acetamiprid on *O. cornuta* align with previous studies on honey bees and bumblebees (Camp and Lehmann, 2021; Iwasa et al., 2004; Schuhmann and Scheiner, 2023). Future research should aim to characterize environmental pesticide loads and identify commonly-encountered pesticide combinations (Sanchez-Bayo and Goka, 2014; Zioga et al., 2020). Then, these pesticide combinations should be screened for their toxicity to bees and potential interactions should be identified, with special reference to fungicides, which are routinely sprayed during bloom under the assumption they are safe for bees (Rondeau and Raine, 2022). Information on the toxicity of pesticide combinations along with background pesticide levels would assist producers and farm advisor on spray decisions. Ultimately, this information should be used to develop integrated pest management programs that provide both effective pest control and pollinator protection (Biddinger and Rajotte, 2015; Topping et al., 2021). Our findings also call for a rethinking of pesticide risk assessment, which should target not only single products but also combinations of products likely to co-occur in agricultural environments (Carnesecchi et al., 2019; Sgolastra et al., 2020; Topping et al., 2020).

5. Author statement

Sergio Albacete: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Visualization. **Gonzalo Sancho**: Conceptualization, Methodology, Validation, Investigation, Writing – Review & Editing. **Celeste Azpiazu**: Conceptualization, Methodology, Validation, Investigation, Writing – Review & Editing. **Fabio Sgolastra**: Conceptualization, Methodology, Validation, Writing – Review & Editing. **Anselm Rodrigo**: Conceptualization, Methodology, Validation, Investigation, Writing – Review & Editing, Supervision, Project administration. **Jordi Bosch**: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Supervision, Funding acquisition.

CRediT authorship contribution statement

Sergio Albacete: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Gonzalo Sancho:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Celeste Azpiazu:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Fabio Sgolastra:** Writing – review & editing, Methodology, Conceptualization. **Anselm Rodrigo:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Jordi Bosch:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

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.envint.2024.108919.

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