



Sublethal fungicide-insecticide co-exposure affects nest recognition and parental investment in a solitary bee[☆]

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ABSTRACT

Fungicides may interact synergistically with insecticides. However, our understanding of the impacts of sublethal insecticide-fungicide combinations on solitary bees is mostly restricted to laboratory studies, providing no information about potential consequences on behavior and reproductive success. We analyzed the effects of a fungicide application, alone and in combination with sublethal levels of an insecticide, on the nesting behavior and reproductive output of the solitary bee *Osmia cornuta*. We released individually-marked females into oilseed rape field cages, and subsequently sprayed the plants with four treatments: control (water), fungicide (tebuconazole), insecticide (acetamiprid at a sublethal concentration), and mixture (fungicide + insecticide). We recorded nesting activity before and after the sprays and assessed post-spray individual reproductive success. Bees of the single pesticide treatments were unaffected by the sprays and did not differ from control bees in any of the parameters measured. The longevity of bees of the mixture treatment was unaffected. However, these bees showed reduced foraging activity, shorter in-nest pollen-nectar deposition times, and increased difficulty recognizing their nesting cavity, leading to a decrease in provisioning rate, parental investment, and offspring production. Our study demonstrates that co-exposure to a fungicide with otherwise harmless levels of an insecticide caused behavioral effects with consequences on reproductive success. Because longevity was unaffected, these effects would not have been easily detected in a chronic laboratory test. Our results have important implications for bee risk assessment, which should account for exposure to multiple compounds and address behavioral effects and reproductive output under semi-field and/or field conditions.

1. Introduction

Global agriculture is highly dependent on the use of pesticides. However, notwithstanding their role in pest and disease control, pesticides may have a wide range of undesired effects, including negative impacts on populations of non-target organisms, such as natural enemies and pollinators. Pesticides in general, and insecticides in particular, are considered one of the main drivers of wild and managed bee population declines (Goulson et al., 2015; Nicholson et al., 2024; Woodcock et al., 2016).

Understandably, most studies assessing the impact of pesticides on

bees focus on insecticides, while other plant protection products, such as fungicides, herbicides, and acaricides, have received comparatively less attention (Colin and Belzunces, 1992; Cullen et al., 2019; Iwasaki and Hogendoorn, 2021; Johnson et al., 2013). Owing to their low toxicity to bees (Ladurner et al., 2005), fungicides are not considered a threat to bee populations and fungicide sprays are allowed during bloom. In years with favorable conditions for the proliferation of fungal diseases, crops may be sprayed several times throughout the blooming period (Rondeau and Raine, 2022). As a result, fungicide residues are frequently found at high concentrations in both crop and wild flowers, and in bee-collected pollen and nectar (Bernal et al., 2010; Chauzat et al., 2006; David et al.,

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2016; Knapp et al., 2023; Sanchez-Bayo and Goka, 2014).

Although fungicides are labelled and marketed as non-harmful to bees, pollination studies with mason bees (*Osmia* spp.) in commercial orchards in the USA have reported important changes in foraging and nesting behavior following fungicide sprays during full bloom (Artz and Pitts-Singer, 2015; Ladurner et al., 2005, 2008). Females returning from foraging trips in recently-sprayed cherry orchards, were unable to recognize their nests and spent long periods checking various nesting cavities. This anomalous behavior entailed a temporary (3–4 days) or permanent arrest of nesting activity, with consequent declines in cell provisioning and reproductive success (Ladurner et al., 2005). Following these field observations, contact and oral toxicity of the fungicides involved in these sprays were tested under laboratory conditions. However, with one exception, toxicity at the field-recommended application rates was found to be very low (Ladurner et al., 2005). In subsequent semi-field studies, nesting females in cages planted with wildflowers were exposed to fungicides, by themselves and in tank mixtures including foliar fertilizers and surfactants similar to those observed to cause behavioral effects in commercial orchards (Artz and Pitts-Singer, 2015; Ladurner et al., 2008). However, these sprays produced no or only minor effects on nesting performance. In sum, semi-field experiments failed to induce the kind of behavioral effects observed in commercial orchards.

A possible explanation for the discrepancy between observations in commercial orchards and in those semi-field experiments is that bees in the commercial orchards were not only exposed to the fungicides sprayed during bloom, but also to insecticide residues. Insecticide residues at low (non-lethal) concentrations are commonly found in pollen and nectar of crop flowers (Azpiazu et al., 2023; Kyriakopoulou et al., 2017; Smodiš Škerl et al., 2009), and may originate from pre-bloom sprays with systemic insecticides (Heller et al., 2020), from neonicotinoid seed treatments (Goulson, 2013), and from drift during applications in neighboring fields (Krupke et al., 2012). *Osmia* females may also be exposed to low levels of insecticides when they collect mud to build their nests (Silva et al., 2019; Suzuki et al., 2023). Although in all these scenarios insecticide exposure is expected to occur at levels substantially below lethal thresholds for bees (Raine and Rundlöf, 2023), co-exposure with fungicides may synergistically enhance insecticide toxicity (Carneseccchi et al., 2019; Iwasa et al., 2004). Synergistic effects occur when fungicides inhibit or compete for cytochrome P450s enzymes, that are involved in insecticide detoxification (Berenbaum and Johnson, 2015; Schuhmann et al., 2022). A number of laboratory studies report sublethal effects of low levels of insecticides mixed with fungicides (Biddinger et al., 2013; Raimets et al., 2018; Sgolastra et al., 2017, 2018; Thompson et al., 2014), but the extent to which this kind of exposure may affect bee behavior and reproductive success in field conditions is mostly unknown.

In this study we measured the effects of fungicide applications, alone and in combination with low sublethal levels of insecticide, on the foraging and nesting behavior of the solitary bee *Osmia cornuta* (Latreille) (Megachilidae). We used tebuconazole (a sterol-biosynthesis-inhibiting (SBI) fungicide) and acetamiprid, a cyano-substituted neonicotinoid that shows low toxicity to bees (EFSA, 2016; Iwasa et al., 2004) and is often advertised as “safe for pollinators”. The two compounds are widely used on a range of entomophilous crops, including fruit trees, and have been found together in flowers and bee-collected pollen, as well as on the body of bees (Botías et al., 2017; David et al., 2016; Mullin et al., 2010). The objectives of our study are: (1) to test whether exposure to sublethal sprays of fungicides and insecticides, alone and in combination, alters nest recognition and nesting behavior in *O. cornuta* females under semi-field conditions; and (2) to assess the potential effects of these sprays on female longevity and progeny production.

2. Materials and methods

2.1. Study organism

Osmia cornuta is a spring-flying solitary bee occurring in most of central and southern Europe. Individual females live for approximately 20 days (Bosch and Vicens, 2006), during which time they build one or more nests in pre-existing cavities. They use mud to build their nests and deposit a pollen-nectar provision in each individual cell (Bosch, 1994). In field conditions, females build and provision an average of 0.5–1 cells per day (Bosch and Vicens, 2006). Upon returning from foraging trips, females use visual landmarks to locate their cavity (Fauria and Campan, 1998) and olfactory cues to recognize it (Guédot et al., 2006). *Osmia* females mark their nest entrance with an abdominal secretion (Guédot et al., 2006). If the nest marking is removed, females are not able to recognize their nest, inspect other nesting cavities, and show erratic behavior (Guédot et al., 2006).

2.2. Field cages and nesting stations

The study was conducted in March–April 2021 at the IRTA-Mas Badia Experimental Station (Girona, NE Spain). We set up twelve field cages (length x width x height = 7 × 7 × 3 m) in an oilseed rape (*Brassica napus* L.; Brassicaceae) field. The field had been sown with untreated seed and no pesticides were applied to the emerging crop. In each cage, we installed a nesting station consisting of a wooden box with the front side open and held 1.5 m above the ground facing SE. Each nesting station had three solid wood blocks (16 × 8.5 × 8.5 cm). Each 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. Cages were randomly assigned to one of four treatments: a) control (water spray); b) fungicide (formulated fungicide at the recommended field dose); c) insecticide (formulated insecticide at a low sublethal concentration); d) mixture (fungicide + insecticide). Each treatment was replicated three times in three different cages.

2.3. Bee population and rearing methods

When sufficient bloom was available, wintered cocoons from a native population reared at CREAF were incubated at 20–22 °C to elicit emergence. Upon emergence, females were temporarily cooled at 4 °C for 10 min and individually marked on the thorax with numbered tags. We released 20 marked unmated females and 34 unmarked males in each cage (1.7 male/female natural sex ratio; Bosch and Vicens, 2002). All bees of all treatments within each replicate were released in the cages on the same day. Four days after release, when mating activity had seemingly ceased, males were netted and removed from the cages.

2.4. Pesticide application

Pesticides were applied approximately one week after bee release, when most females had established in the nesting units and were building and provisioning their first nest. At 6:00–7:00 a.m., when bees were still inactive, we sprayed the plants using hand-held sprayers. Applications were conducted under dry weather conditions with no wind and nesting stations were covered with a plastic bag during the sprays. Sprays were conducted on the 2nd, 8th, and 13th of April (one cage of each treatment per day).

The fungicide Folicur (tebuconazole 25 %, Bayer CropScience) was applied at the field-recommended dose for oilseed rape (0.25 kg a.i./ha). Considering that each cage covers an approximate area of 50 m² and that recommended application rate for oilseed rape is 400 L/ha, we applied a solution containing 5 g of Folicur dissolved in 2 L of tap water (625 ppm a.i. solution) in each cage. As for the insecticide, our goal was to attain field-relevant exposure levels. Acetamiprid concentrations of 0.009 mg/kg have been measured in the nectar of oilseed rape (Wen et al., 2021).

To achieve this concentration, we used the residue unit dose (RUD) approach proposed by EFSA (2013) for nectar (0.87 mg a.i. residue/kg). The RUD is an estimate of the concentration of pesticide expected in nectar for a reference application rate of 1 kg a.i./ha. To use the same tank volume as in the fungicide treatment, we dissolved 0.25 mL of Carnadine (acetamiprid 20%, Nufarm Spain) in 2 L of water per cage (25 ppm a.i. solution), which corresponds to a field application dose of 0.01 kg a.i./ha. The mixture treatment was prepared by mixing the same amounts of fungicide and insecticide used in the previous treatments in 2 L of water per cage. Control cages were sprayed with 2 L of water.

2.5. Nesting performance

To determine the number of released females that established successfully at the nesting units, as well as their longevity and nest provisioning rate, nesting materials were inspected daily at night, when females were no longer active. Each night, we counted the number of females roosting inside the nesting cavities and the progression of each active nest was marked and dated on the outside of the paper straw. Bee identity was established the following morning as bees set out for their first foraging activity. Females were considered dead when they were no longer seen at the nesting site or foraging.

Nesting behavior was assessed on the day before the application (day -1), and then on the day of the application (day 0) and the day after (day +1) (Fig. 1), when levels of exposure are expected to be highest (Gierer et al., 2024). To obtain behavioral data, we filmed bee activity using camcorders placed in front (at 30 cm) of the nesting stations, coupled with direct observations (one observer per cage). Recordings took place during two 45-min intervals at midday and at 4:00 p.m., simultaneously in all treatments, coinciding with the diel peak of nesting activity.

A total of 54 h of video recordings including 4253 foraging trips were analyzed using BORIS software (Friard and Gamba, 2016). We identified several behaviors related to foraging and nesting performance and measured the time each bee spent performing each behavior. For each nesting female, we quantified the following behaviors: a) bee activity (whether the female was entering/leaving the nest at least once during the recording period); b) pollen-nectar foraging trip duration; c) mud foraging trip duration; d) number of pollen-nectar foraging trips; e) number of mud foraging trips; f) in-nest pollen-nectar time (time spent inside the nest depositing pollen-nectar loads); g) in-nest mud time (time spent inside the nest depositing mud loads); h) nest recognition errors (the number of nesting cavities inspected by a female returning from a foraging trip before finding her own).

2.6. Nest contents analysis

Seventeen days after release, when only a small number of senile bees were still alive but no longer provisioning, nesting materials were collected and kept in a storage area at ambient temperature to allow larvae 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 and assigned a sex based on its size and position within the nest (Bosch and Vicens, 2002). Cocoons were wintered at 4 °C from 1 October until 15 March. Then, cocoons were incubated at 20 °C and emergence was assessed daily. For each female, we characterized the nesting performance after the spray application as follows: a) fecundity (number of cells produced); b) nest provisioning rate (number of cells built per day); c) parental investment (total progeny weight produced); d) mean offspring weight (separately for males and females); e) progeny sex ratio (males/males + females); f) percent offspring mortality.

2.7. Data analysis

To analyze variation in nesting behavior through time and across treatments, we fitted mixed-effects models with pesticide treatment, assessment day and their interaction as fixed effects, and replicate as random effect. Bee activity was analyzed with a binomial generalized linear mixed-effects model (GLMM) with nesting activity as a binary response ("active": entered or left the nest at least once; "inactive": was not observed entering or leaving the nest during the recording period). For the number of pollen-nectar and mud trips, we fit zero-inflated GLMMs with a negative binomial error distribution (to account for overdispersion) and a log-link function. The time spent foraging for pollen and nectar (pollen-nectar trip time) or mud (mud trip time), along with the time spent inside the nest depositing pollen-nectar loads (in-nest pollen-nectar time) or mud loads (in-nest mud time), was analyzed using linear mixed-effects models (LMM) with the response variables log-transformed to meet normality and homogeneity of variance assumptions. To analyze nest recognition errors, we employed hurdle models to address zero-inflation in the data. Initially, we assessed the probability of erratic behavior (bees making at least one mistake) as a binary response variable using a binomial GLMM with a logit link function. Then, for females that exhibited this kind of behavior, we analyzed the number of nest recognition errors by fitting a GLMM with a Poisson error distribution and an identity link function. For models that included multiple observations of a single female, we also included bee identity (nested within replicate) as random effects.

To analyze the effects of pesticide applications on the post-treatment nesting performance and reproductive success, we fitted mixed-effects

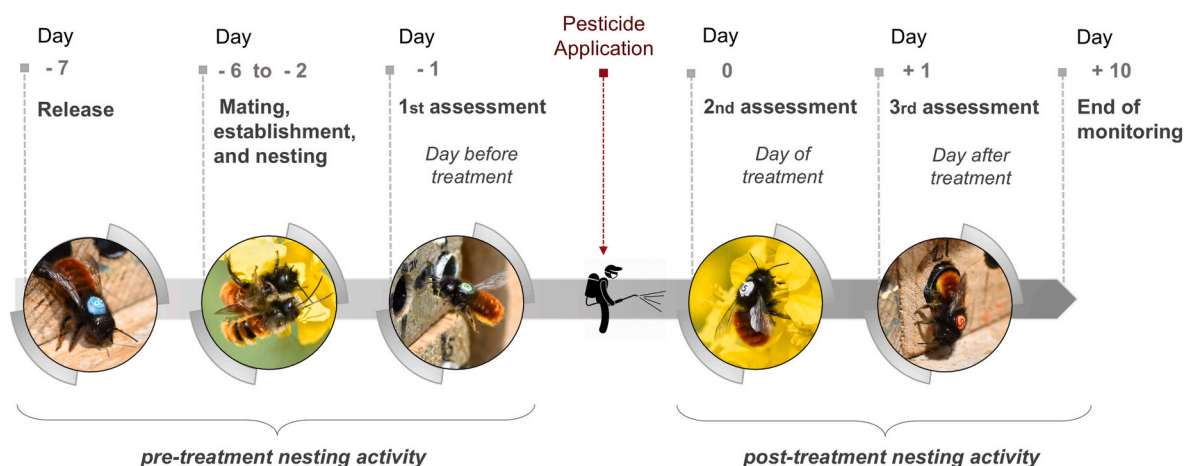


Fig. 1. Timeline of the experiment.

models with pesticide treatment as a fixed factor and replicate as random effect. Post-treatment longevity was analyzed with GLMM using a Poisson error distribution and a square-root link function. Fecundity was analyzed with a GLMM with a negative binomial error distribution (to account for overdispersion) and a log-link function. Nest provisioning rate and parental investment were analyzed by fitting GLMM with a Gaussian error distribution. Male and female offspring weights were analyzed using linear mixed-effects models (LMM) with a normal error distribution. Progeny sex ratio and offspring mortality were analyzed using binomial GLMMs with a logit link function.

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 (package “DHARMA”; Hartig, 2022). 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 “lme4” (Bates et al., 2014) and “glmmTMB” (Brooks et al., 2023).

3. Results

3.1. Nesting behavior

Be activity (proportion of bees actively nesting during the recordings), was influenced by treatment (GLMM, $\chi^2 = 12.6$; $df = 3$; $p = 0.006$) and the interaction between treatment and assessment day (GLMM, $\chi^2 = 12.8$; $df = 6$; $p = 0.046$; Fig. 2; Table A1). Bee activity remained similarly high across time in Control, Fungicide, and Insecticide cages (Fig. 2), but decreased significantly after the spray (to 71% on day 0 and to 55% on day +1) in Mixture cages (Fig. 2).

We recorded 1706 pollen-nectar trips and 2547 mud trips. The number of pollen-nectar or mud trips per female were not related to the interaction between treatment and assessment day (pollen-nectar: GLMM, $\chi^2 = 5.4$; $df = 6$; $p = 0.50$; mud: GLMM, $\chi^2 = 5.3$; $df = 6$; $p = 0.51$) (Tables A1 and A2). Pollen-nectar foraging trips lasted 3–4 min, and mud-collecting trips 2–3 min (Table A2). Both pollen-nectar and mud trip duration increased over time, but were not affected by treatment (pollen-nectar: LMM, $F_{3,132} = 0.08$; $p = 0.97$; mud: LMM, $F_{3,128} =$

0.68; $p = 0.59$) or the interaction between treatment and assessment day (pollen-nectar: LMM, $F_{6,1559} = 1.7$; $p = 0.13$; mud: LMM, $F_{6,2406} = 2.0$; $p = 0.06$). In-nest pollen-nectar time was significantly influenced by the interaction between treatment and assessment day (LMM, $F_{6,1540} = 5.0$; $p < 0.0001$; Fig. 3, Table A1). In general, in-nest pollen-nectar time lasted 3–4 min, but dropped to ~2.5 min 24 h after the application (day +1) in the mixture treatment (Fig. 3, Table A2). Mud load deposition lasted 1–2 min and decreased over time, but was not affected by treatment (LMM, $F_{3,128} = 3.6$; $p = 0.08$) or by the interaction between treatment and assessment day (LMM, $F_{6,2477} = 1.8$; $p = 0.09$).

The probability that a female returning from a foraging trip inspected other nesting cavities was significantly affected by treatment (GLMM, $\chi^2 = 8.5$; $df = 3$; $p = 0.037$) and by the interaction between treatment and assessment day (GLMM, $\chi^2 = 23.0$; $df = 6$; $p = 0.001$; Fig. 4a, Table A1). The proportion of bees making nest recognition errors remained similarly low over time in Control, Fungicide, and Insecticide cages, but increased to 38% on day 0 and to 56% on day +1 in Mixture cages (Fig. 4a–Table A2). Among bees that had problems recognizing their nest, the number of errors per bee was also significantly influenced by treatment (GLMM, $\chi^2 = 13.9$; $df = 3$; $p = 0.003$) and by the interaction between treatment and assessment day (GLMM, $\chi^2 = 19.8$; $df = 6$; $p = 0.003$; Fig. 4b–Table A1). The number of nest recognition errors per bee remained consistent around 1–3 in Control, Fungicide, and Insecticide cages, but increased up to 6 errors per bee after the application (day 0) in Mixture cages (Fig. 4b–Table A2).

3.2. Nesting performance and reproductive success

Post-spray longevity was similar across treatments (GLMM, $\chi^2 = 4.5$; $df = 3$; $p = 0.214$), indicating that the sprays had no lethal effects. However, nest provisioning rate (GLMM, $\chi^2 = 15.5$; $df = 3$; $p = 0.001$), fecundity (GLMM, $\chi^2 = 9.8$; $df = 3$; $p = 0.021$), and parental investment (GLMM, $\chi^2 = 9.5$; $df = 3$; $p = 0.024$) were influenced by treatment. These variables were lower in Mixture than in Control cages (Table 1). Nest provisioning rates dropped the day after application and resumed levels comparable to those of control cages thereafter (Fig. B1).

Body weights of male and female offspring were similar across treatments (males: LMM, $F_{3,108} = 0.1$; $p = 0.974$; females: LMM, $F_{3,28} =$

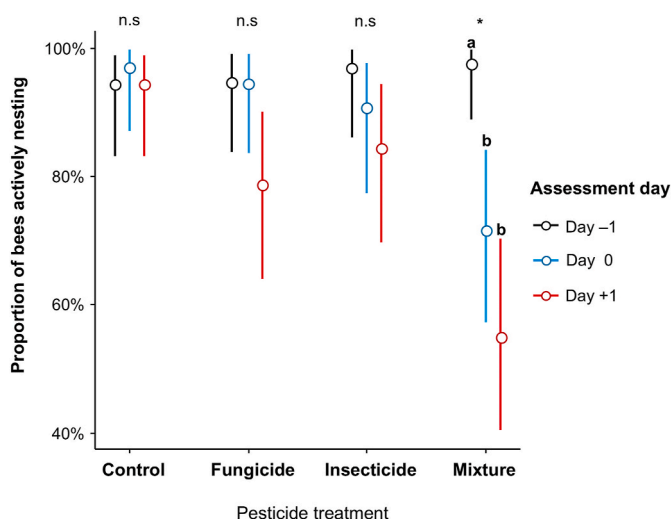


Fig. 2. Model-estimated means and 95% confidence intervals for the proportion of females actively nesting in field cages sprayed with four treatments: Control (N = 35), Fungicide (N = 37), Insecticide (N = 33), and Mixture (N = 42). Observations were conducted on the day before the application (day -1), shortly after the application (day 0) and the day after the application (day +1). Different letters denote significant differences between assessment days within a treatment (Tukey HSD test, $p < 0.05$).

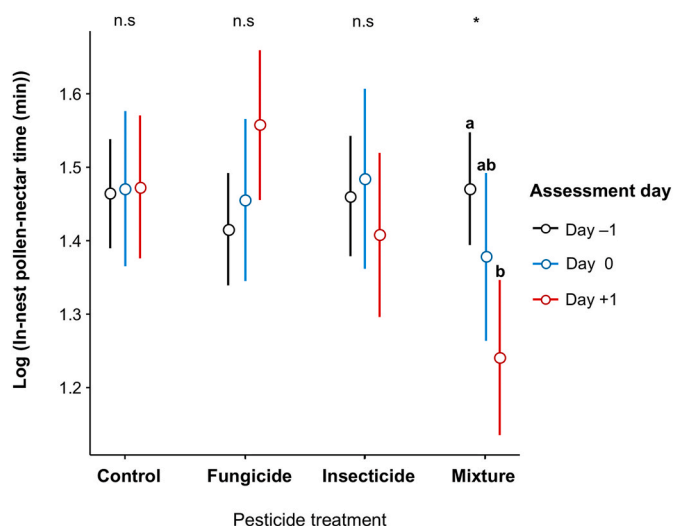


Fig. 3. Model-estimated means and 95% confidence intervals for log-transformed in-nest pollen-nectar time (time spent inside the nest depositing pollen-nectar loads) in females nesting in field cages sprayed with four treatments: Control (N = 35), Fungicide (N = 37), Insecticide (N = 33), and Mixture (N = 42). Observations were conducted on the day before the application (day -1), shortly after the application (day 0) and the day after the application (day +1). Different letters denote significant differences between assessment days within a treatment (Tukey HSD test, $p < 0.05$).

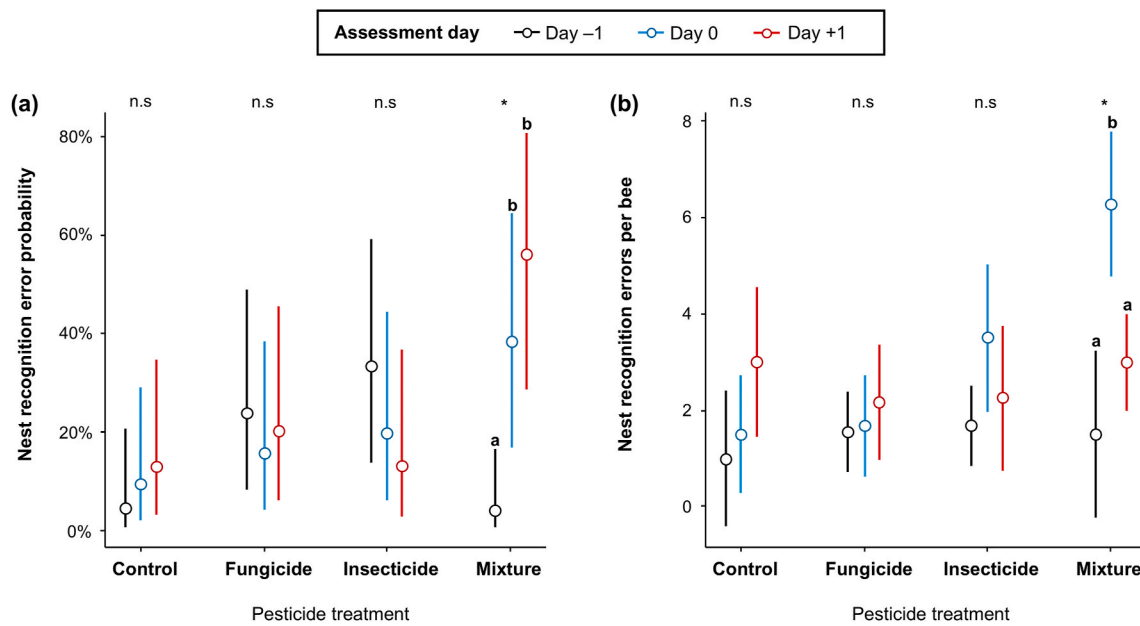


Fig. 4. Model-estimated means and 95% confidence intervals for **a)** nest recognition error probability (proportion of females that made at least one nest recognition error), and **b)** number of nest recognition errors in females nesting in field cages sprayed with four treatments: Control (N = 35), Fungicide (N = 37), Insecticide (N = 33), and Mixture (N = 42). Observations were conducted on the day before the application (day -1), shortly after the application (day 0) and the day after the application (day +1). Different letters denote significant differences between assessment days within a treatment (Tukey HSD test, p < 0.05).

Table 1

Model-estimated means and standard errors of parameters related to post-treatment nesting performance and reproductive success in *Osmia cornuta* females nesting in field cages sprayed with four pesticide treatments: Control, Fungicide, Insecticide, and Mixture (Fungicide + Insecticide). Values within a row followed by different letters are significantly different (Tukey HSD test, p < 0.05).

Parameter	Control	Fungicide	Insecticide	Mixture
Nesting females	35	37	33	42
Longevity ^a	5.77 ± 0.41 a	5.14 ± 0.37 a	5.64 ± 0.41 a	4.79 ± 0.34 a
Fecundity ^b	3.54 ± 0.39 a	2.42 ± 0.31 ab	2.66 ± 0.34 ab	2.18 ± 0.28 b
Nest provisioning rate ^c	0.58 ± 0.05 a	0.44 ± 0.05 ab	0.42 ± 0.06 ab	0.35 ± 0.05 b
Parental investment ^d	434.95 ± 40.56 a	307.02 ± 40.56 ab	323.27 ± 42.42 ab	276.51 ± 37.94 b
♂ Offspring weight ^d	127.55 ± 2.58 a	128.89 ± 2.75 a	127.61 ± 2.80 a	128.87 ± 3.03 a
♀ Offspring weight ^d	178.54 ± 8.24 a	160.03 ± 14.07 a	152.19 ± 7.94 a	179.91 ± 7.42 a
Progeny sex ratio ^e	0.90 ± 0.04 a	0.93 ± 0.03 a	0.84 ± 0.05 ab	0.75 ± 0.07 b
% Offspring mortality ^f	18.35 ± 5.67 a	22.18 ± 6.90 a	25.58 ± 7.76 a	14.96 ± 5.16 a

^a In days.
^b N of eggs laid.
^c Cells built per day.
^d In mg of progeny produced.
^e ♂♂/(♂♂+♀♀).
^f Offspring not emerging in spring.

3.5; p = 0.090). Similarly, we found no effects of pesticide treatment on offspring mortality (GLMM, $\chi^2 = 6.1$; df = 3; p = 0.106). However, there were differences in progeny sex ratio among treatments (GLMM, $\chi^2 = 15.1$; df = 3; p = 0.002), with a higher proportion of females produced in Mixture cages (Table 1).

4. Discussion

Our study demonstrates that co-exposure to otherwise innocuous fungicide and insecticide applications may have sublethal effects on the nesting behavior of solitary bees, leading to reduced reproductive success. Our results provide an explanation for the sudden population declines observed in intensively managed commercial orchards following fungicide applications (Artz and Pitts-Singer, 2015; Ladurner et al., 2005, 2008), and raise concerns about the potential effects of sublethal pesticide combinations to which bees are routinely exposed in agricultural environments (Knapp et al., 2023; Nicholson et al., 2024; Ward et al., 2022).

The fungicide spray at the recommended field dose had no effect on nesting behavior or reproductive output. This result is congruent with the low toxicity of SBI fungicides on bees (Ladurner et al., 2005; Sgolastra et al., 2017, 2018; Thompson et al., 2014), and with commercial label specifications allowing their use during bloom. Importantly, neither the insecticide spray had any effect on nesting behavior, longevity, or reproductive success. We sprayed at a field application rate resulting in a concentration close to 0.009 ppm in nectar (RUD approach; EFSA, 2013), similar to concentrations measured in the nectar of crop flowers following pre-bloom acetamiprid sprays (melon: 0.006 ppm, Azpiazu et al., 2023; oilseed rape: 0.009 ppm, Wen et al., 2021; apple: 0.068 ppm, Heller et al., 2020). In a recent semi-field study, newly-emerged *O. cornuta* females allowed to feed once on syrup with 0.068 ppm of acetamiprid had establishment success, longevity, and fecundity similar to control females (Albacete et al., 2024). Overall, these results indicate that *Osmia* populations can survive in environments with certain background levels of insecticide. This conclusion is remarkable considering that *O. cornuta* is about 10 times more sensitive to acetamiprid (oral LD₅₀: 0.89 µg/bee; Barnadas, 2022) than honey bees (8.85 µg/bee; EFSA, 2016).

On the other hand, females nesting in cages sprayed with the same concentration of insecticide mixed with the fungicide experienced several behavioral changes, ultimately leading to declines in nest provisioning rate and offspring production. Mixture sprays did not affect the number and duration of pollen-nectar or mud trips. However, following the sprays, females in the mixture cages spent less time depositing

pollen-nectar loads inside the nest. This reduction could be indicative of lower amounts of pollen and nectar being collected per foraging trip. Exposure to field-relevant concentrations of a neonicotinoid insecticide affected pollen-collecting efficiency in bumblebees (Gill et al., 2012). In that study, foragers exposed to imidacloprid returned to the nest with smaller pollen loads than foragers from control colonies. Similarly, oral exposure to pollen-bound fungicide-insecticide mixtures was found to decrease the amount of pollen collected per foraging trip in honey bees (Prado et al., 2019). Depressed pollen collection may not only affect bee reproductive success but also reduce pollination services (Stanley et al., 2015).

Mixture sprays also caused an increase in the number of females that had difficulty locating and recognizing their nest. *Osmia* females use visual cues to locate their nesting cavity (Fauria and Campan, 1998), and olfactory cues to recognize it (Guédot et al., 2006). Females mark the entrance of their nests with an abdominal secretion and when these olfactory markings are experimentally removed, females are unable to recognize their nests, causing them to inspect other nesting cavities (Guédot et al., 2006). The inability of females in the mixture cages to recognize their nest following fungicide-insecticide sprays is thus congruent with a loss of olfactory capacity and/or olfactory memory. Laboratory studies using olfactory conditioning methods have found that exposure to the neonicotinoid imidacloprid reduces scent recognition and olfactory memory in honeybees (Williamson and Wright, 2013) and bumblebees (Muth et al., 2019). This loss of olfactory capability may be caused by disturbance of the mushroom bodies (Heisenberg, 1998; Rybak and Menzel, 2010), which are involved in olfactory learning and have been shown to be affected by neonicotinoid exposure (Palmer et al., 2013; Tomé et al., 2012).

Osmia females have short life spans (ca. 20 days on average) and low fecundity in field conditions (0.5–1 eggs per day) (Bosch and Vicens, 2006; Maeta, 1978; Seidelmann et al., 2010). In our study, exposure to the mixture treatment did not affect bee longevity, but prompted a decrease in nest provisioning rate and parental investment, significantly reducing offspring production. Female fecundity after the sprays was ca. 2 eggs in bees of the mixture cages, compared to ca. 3.5 eggs in bees of the control cages. Therefore, in bees with such short life spans and low fecundity, even transitory arrests in nesting activity may significantly affect cell production, compromising reproductive success and population dynamics. These results suggest that the sustainability of solitary bee populations could be at risk in agricultural environments with residual insecticide contamination and frequent fungicide sprays.

Alternatively, or in addition, to lower cell production, decreased nest provisioning rates could result in smaller pollen-nectar provision size. However, we did not find differences among treatments in offspring size (strongly correlated to provision size; Bosch and Vicens, 2002). In *O. cornuta*, offspring allocated small food provisions are more likely to die during development (Bosch, 2008) and wintering (Bosch and Kemp, 2004). In agreement to this, there were no differences between treatments in offspring mortality. This result also implies that the levels of pesticide residues in the provisions were not high enough to compromise immature development. By contrast, offspring sex ratio differed among treatments. Surprisingly, females of the mixture cages produced female-biased sex ratios compared to females of the other treatments. This result was unexpected because females are costlier to produce (require larger provisions) and studies in which *Osmia* females were exposed to insecticides have reported male-biased sex ratios (Sandrock et al., 2014; Stuligross and Williams, 2020). Our results can be explained by the occurrence of unmated females. Unmated *Osmia* females build and provision nests but lay only male (unfertilized) eggs (Bosch and Vicens, 2006; Maeta, 1978; Phillips and Klostermeyer, 1978). We revisited the results of our nest analyses to determine the number of females producing only male progeny (presumed to be unmated) and found that these were lower in mixture cages (24%) than in control (55%), fungicide (57%) and insecticide (38%) cages. We then re-analyzed sex ratio outcomes excluding presumably unmated females and

found no differences in progeny sex ratio among treatments (GLMM, $\chi^2 = 1.7$; $df = 3$; $p = 0.63$). The occurrence of unmated females in other *Osmia* studies with individually marked females under semi-field conditions is around 12–29% (Albacete et al., 2024; Frohlich and Tepedino, 1986; Tepedino and Torchio, 1982). In our study, the removal of males four days after release may have caused an unusually high occurrence of unmated females.

5. Conclusions

Synergistic effects between fungicide-insecticide mixtures have been documented in laboratory studies (Biddinger et al., 2013; Colin and Belzunces, 1992; Raimets et al., 2018; Sgolastra et al., 2017, 2018; Thompson et al., 2014). However, the extent to which these pesticide mixtures may affect bee populations in field conditions is mostly unknown. Our study reveals that exposure to a fungicide mixed with low field-realistic levels of an insecticide affects bee reproduction under semi-field conditions. Importantly, all observed effects were non-lethal. Longevity was not affected, but females exposed to the pesticide mixture reduced their foraging and nesting activity and experienced impaired nest recognition, leading to a reduction of reproductive output. The ecological consequences of sublethal pesticide effects are poorly understood, but our study demonstrates that behavioral effects may have ecologically relevant repercussions for bee population dynamics and pollination services.

Pesticide regulation is an essential component of bee protection. However, bee risk assessment has some important gaps, including the testing of single compounds, reliance on a single species with a unique level of colony-resilience, insufficient coverage of sublethal effects and insufficient understanding of populational consequences. Our study addresses these four gaps and underscores the need to develop robust methods to reliably detect sublethal effects under semi-field and field conditions. Our findings have also important implications for pesticide management in conventional and IPM agricultural systems. The screening of sublethal effects of different pesticide combinations at low, field realistic, concentrations, will allow us to predict the potential effects of fungicide sprays depending on background insecticide levels. This knowledge will assist fruit producers and farm advisors to manage pre-bloom insecticide applications and bloom fungicide sprays, thus mitigating pesticide impact on both wild and managed pollinators.

CRedit authorship contribution statement

Sergio Albacete: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Celeste Azpiazu:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Gonzalo Sancho:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Marta Barnadas:** Methodology, Investigation. **Georgina Alins:** Writing – review & editing, Resources. **Fabio Sgolastra:** Writing – review & editing, Validation, Methodology, Conceptualization. **Anselm Rodrigo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Jordi Bosch:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

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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.2024.125223>.

Data availability

Data will be made available on request.

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