RESEARCH ARTICLE

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Low toxicity crop fungicide (fenbuconazole) impacts reproductive male quality signals leading to a reduction of mating success in a wild solitary bee

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Abstract

1. Recent reports on bee health suggest that sublethal doses of pesticides have negative effects on wild bee reproduction and ultimately on their population growth.

2. Females of the solitary horned mason bee *Osmia cornuta*, evaluate thoracic vibrations and odours of males to assess male quality. When certain criteria are met, the female accepts the male and copulates. However, these signals were found to be modified by sublethal doses of pesticides in other hymenopterans. Here, we tested whether sublethal doses of a commonly used fungicide (Fenbuconazole) impact male quality signals and mating success in *O. cornuta*.

3. Males exposed to fenbuconazole exhibited reduced thoracic vibrations and an altered cuticular hydrocarbon profile compared to the control bees. Moreover, males exposed to the fungicide were less successful in mating than control males.

4. *Synthesis and applications*. Our results indicate that a low toxicity fungicide can negatively affect male reproductive success by altering behavioural and chemical cues. This could explain the decreasing pollinator populations in a pesticide-polluted environment. This study highlights the need for a more comprehensive approach, including behaviour and chemical cues, when testing new pesticides and a more cautionary approach to the pesticides already used on crops.

KEYWORDS
bee reproduction, biotremology, crop environment, cuticular hydrocarbons, female choice, fungicide, mating success, *Osmia cornuta*

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1 | INTRODUCTION

The contamination of agricultural landscapes with pesticides is a major driver of the global decline in pollinators (Potts et al., 2010; Raven & Wagner, 2021; Sánchez-Bayo & Wyckhuys, 2019). Impacts of pesticides on pollinators and more specifically on bees is of great concern due to their essential role in the reproduction of both wild and crop plants (Aizen et al., 2009; Dainese et al., 2018; Giannini et al., 2015; Klein et al., 2007). Recent studies, based on field, semi-field and laboratory experiments, on the impact of pesticides on wild bees (Baron, Jansen, et al., 2017; Klaus et al., 2021; Rundlöf et al., 2015; Woodcock et al., 2017) have reported negative effects of pesticides on wild bee reproduction and consequently on their population growth (Powney et al., 2019; Willis Chan & Raine, 2021).

The detrimental effect of pesticides on the reproduction of wild bees, including trap-nesting and underground nesting solitary bee species (e.g. *Megachile rotundata*, *Osmia lignaria*, *Osmia bicornis* and *Eucera pruinosa*), has been highlighted recently. According to these studies, wild bees are exposed to agrochemicals through different routes of exposure with direct and indirect effects on their reproductive potential (Anderson & Harmon-Threatt, 2019; Sandrock et al., 2014; Stuligross & Williams, 2020; Willis Chan et al., 2019; Willis Chan & Raine, 2021). The available information on reproductive success reveals that trap-nesting bees, genus *Osmia*, produced significantly fewer offspring in neonicotinoid-exposed environments (Rundlöf et al., 2015; Woodcock et al., 2017). The impact of sublethal doses of neonicotinoid on offspring production of wild solitary bees is high, ranging from a reduction of nearly 40%–90% (Sandrock et al., 2014; Stuligross & Williams, 2020; Willis Chan & Raine, 2021) to areas where apparently they were completely prevented from reproducing (Rundlöf et al., 2015).

According to Baron, Raine, and Brown (2017) and Sgolastra et al. (2018), field-realistic doses of the neonicotinoid thiamethoxam and a combination of clothianidin and propiconazole have an effect on ovary development, suggesting a reduced ability of females to lay eggs in several wild bee species. A direct reduction on egg laying of bumblebee queens was additionally reported from the sublethal effects of imidacloprid and by the new class of pesticide, sulfoxaflor (Laycock & Cresswell, 2013; Siviter et al., 2018, 2020). Furthermore, pesticides were found to affect the reproductive cells of male bees. Honeybee drones, for example, lose the viability of their spermatic cells when they are exposed to neonicotinoids (clothianidin and thiamethoxan) as well as amitraz and miticides at an immature developmental stage (Fisher II & Rangel, 2018; Straub et al., 2016). Moreover, clothianidin and thiamethoxam have adverse effects on the sperm viability of solitary mason bees (Strobl, Albrecht, et al., 2021; Strobl, Bruckner, et al., 2021) suggesting different toxicity levels in the reproductive cells of solitary male bees of the genus *Osmia*.

These results highlight that bees of both sexes exposed to agrochemical stressors might experience a reduction of their optimum fitness, suggesting, at least in part, that wild bee decline in agricultural areas may be a consequence of their reduced fertility due to pesticide exposure. In addition to cryptically impaired reproductive cells contributing to bee population decline through a post-copulatory issue, pesticides might influence reproductive impairment in earlier steps of sexual reproduction, that is, during pre-copulatory display. Knowledge about such a potential effect of pesticides on wild solitary bees is lacking, despite accumulating evidence on wasps (Tappert et al., 2017; Wang et al., 2018), on beetles (Müller et al., 2017), on moths (Lalouette et al., 2016) and on spiders (Tietjen, 2006).

Previous studies on chemical cues in a model species, the wasp *Nasonia vitripennis*, an important natural enemy of insect pests, found that couples that were exposed topically to sublethal doses of imidacloprid were less likely to perform courtship behaviour, leading to a reduction of 80% in mating rate (Tappert et al., 2017). Some of these negative effects were found to be a consequence of pesticide impact on the composition of fatty acids and terpenoid lipids, both components of cuticular hydrocarbons and pheromones in insects (Blomquist et al., 2018). According to Tappert et al. (2017), females of *N. vitripennis* topically exposed to imidacloprid were unable to locate male sex pheromones, culminating in a significant reduction of copulations. Furthermore, topical exposure of another pyrethroid on females of the wasp *Trichogramma chilonis* has been suggested to affect female reproductive pheromones and reduces the response of males to odour perception (Wang et al., 2018). Pyrethroid applied in the food supply of the mustard leaf beetle *Phaedon cochleariae* altered beetles' cuticular hydrocarbon composition, leading to a reduction of 35% in female reproductive success (Müller et al., 2017). Sublethal effects of pesticides were also observed to lower the capacity to vibrate thoracic muscles in bumblebees (Switzer & Combes, 2016; Whitehorn et al., 2017), a mechanism used by mason bee males in behavioural courtship displays.

The females of red mason bees evaluate male quality using at least two distinct sexual signals: thoracic vibration and cuticular hydrocarbons (Conrad et al., 2017). In this mating system, females will mate when male quality requirements are met (Conrad et al., 2010). Thus, mating success in mason bees relies on sexual signals that were shown to be potentially modulated by environmental stressors such as sublethal doses of pesticide: thoracic vibration (Switzer & Combes, 2016; Whitehorn et al., 2017) and cuticular volatiles (Müller et al., 2017; Tappert et al., 2017; Wang et al., 2018).

Beside the sublethal effects of insecticides, fungicides are commonly used to control diseases in crops and some are suggested to be safe for flower visitors such as bee pollinators (Heller et al., 2020; Tamburini et al., 2021). The active compound fenbuconazole, for example, which is applied via spraying to control powdery mildew, black rot and yellow and brown rusts on several crops, was found to have a low toxicity to mason bees and honeybees in mortality tests (European Food Safety Authority (EFSA), 2010, Biddinger et al., 2013). Moreover, sublethal effects of fenbuconazole in the formulation of Indar™ 5EW were found to have only a short-term effect over flower visitation rate of a mason bee in poor quality environments (Boff et al., 2020). Despite evidence of the direct and/or indirect impact of fungicides (i.e. chlorothalonil) on bee health (McArt et al., 2017), potential antagonistic effects of fungicides may

be unnoticed because of their different modes of action (Belsky & Joshi, 2020), which could potentially mask their impairment of wild bee population health.

Based on the reports of sublethal effects of several pesticides on the vibrational skills of bees (Switzer & Combes, 2016, Whitehorn et al., 2017) and their role on chemical communication (Lalouette et al., 2016; Müller et al., 2017; Tappert et al., 2017; Wang et al., 2018), we hypothesize that sublethal effects can affect mason bee reproduction by direct impairment of pre-copulatory displays and female choice. To test these hypotheses, we exposed males of the horned mason bee, *Osmia cornuta* (Latreille, 1805), to Indar™ 5EW with fenbuconazole as an active ingredient and conducted analyses on sexual signals such as thoracic vibrations and the composition of cuticular hydrocarbons. Moreover, we performed mating experiments with exposed and unexposed males in the presence of fungicide-free virgin females and compare their copulation success.

We explored four different pathways to evaluate the effect of the fungicide on mating of the horned mason bee:

(i) Choice experiments between fungicide-exposed and control males
(ii) Choice experiments among fungicide-exposed and among control males
(iii) Measurement of signal vibrations of pre-copulatory behaviour
(iv) Analysis of cuticular hydrocarbon composition (CHC)

2 | MATERIALS AND METHODS

2.1 | Study species

The mason bee *Osmia cornuta* (Hymenoptera: Megachilidae) is a cavity-nesting solitary bee with a geographic distribution within Europe and the northern parts of Africa and Asia (Ungricht et al., 2008). In Europe, *O. cornuta* adult bees emerge from overwintering in early spring and are important pollinators for several wild plant species and crops such as almond, pear and apple (Bosch & Blas, 1994; Bosch & Kemp, 2002). Because of its importance for crop pollination, *O. cornuta* is considered a key species in pesticide exposure assessments (Sgolastra et al., 2019). For the purpose to mitigate the effects of genetic variability, *O. cornuta* bees were obtained as pupae or pharate adults in cocoons derived from a unique locality in Germany.

2.2 | Mating behaviour of *Osmia*

Males of horned mason bee *O. cornuta* perform pre-copulatory behaviours to persuade females to mate. These include male thoracic vibrations and rubbing their antennae over the female eyes and antennae, during which odours on the body surface are thought to be transmitted. These quality signals (vibrations and odours) may trigger a female to accept mating. In the case of rejection, the female pushes the male off her back and waits for another male.

2.3 | Fungicide

The commercial systemic fungicide in water emulsion Indar™ 5EW (50g/L fenbuconazole), Dow Agro-Sciences, was used in the experiments. Indar™ 5EW belongs to the triazole fungicides family. It targets an enzyme (C14-demethylase) involved in the biosynthesis of ergosterols, causing a toxic accumulation of methylated sterols in fungal tissues (see also Supporting Information 1). According to the label of Indar™ 5EW, the fungicide aims to control leaf spots, apple scab, pear scab and apple powdery mildew. In crop fields, bees can be exposed to Indar™ 5EW through direct contact via spraying during the flowering and fruiting seasons. The field-realistic concentration was prepared according to the information provided by the producer of Indar™ 5EW for spraying in the field: 1.4 L of Indar™ 5EW (50g of fenbuconazole per litre) diluted in 1,000L of deionized water (0.07g of fenbuconazole per litre). The solution for use in the laboratory was obtained by diluting 70 µl of Indar™ 5EW in 50 ml of deionized water. This dilution produced 70ng of fenbuconazole per microlitre of deionized water.

2.4 | Assay 1 – Choice experiments between fungicide-exposed and control males

Newly emerged bees (females and males) were placed individually in Petri dishes (6 cm diameter, 3 cm height) and fed ad libitum with 25% (w/v) sucrose solution. Since ageing causes changes in the CHC of females, affecting male mating behaviour towards females (Seidelmann & Rolke, 2019), we used newly (<24 h after emergence) emerged females. The males were individually labelled using numbered and coloured Opalith tags (round convex shape of 3mm) and they were randomly assigned to a treatment. Control bees received 1 µl of deionized water on the right side of their thorax. In the same way, T50 males were exposed to 50% of the field-realistic concentration (35 ng µl.bee^{-1}) and T100 males were exposed to field-realistic concentrations of Indar™ 5 EW (70 ng µl.bee^{-1}), known to be non-lethal to mason bees (Boff et al., 2020). This approach simulates the worst case acute topical exposure scenario of spraying with the use of tank mix strength fungicide and exposure onto the cuticle of bees. This is effectively like the bees being active on the crop during the application of the fungicide and therefore being hit directly by the droplets of spray before it hits the crop. Directly after application, five control, five T50 and five T100 males were placed together in a flight cage (30×30×32cm) with one virgin female free of pesticide (Supporting Information 1, Figure S1). The use of five individuals of each group, rather than one of each group, was chosen, because increasing the number of individuals in an arena of a fixed size was found to enhance the possibility of encounters between potential mating partners in a reasonable time. The interactions were observed in a mating arena covered with solid steel on the bottom part

and a steel net on top and on two of the sides. Two opposite sides were covered with a transparent plastic to facilitate the observation of mating behaviours by two observers (SB and MB) simultaneously. In this scenario, we documented: (a) male mating success, based on the proportion of successful male individual copulations per treatment per trial (= proportion of successful copulations), (b) the order and the identity of males that performed copulation attempts, (c) the time latency (i.e. time of the first mating attempt) after a female was introduced into the cage, (d) the total number of copulation attempts of males and (e) the time a given male spent mounting on the back of a given female performing pre-copulatory display.

After exposure to the fungicide (Supporting Information 1, Table S1) a total of 68 males ($n_{control}$ = 22, n_{T50} = 21 males and n_{T100} = 25 males) were observed three times a day for a period of 1 hr each trial, with 1 hr intervals between the trials. In each trial, a new virgin female was used. A total of 28 females was used in this assay leading to a total of 28 hs of observations. To simulate field scenarios in croplands, where males can copulate with several females and where they can be exposed to pesticides multiple times, 39 males were used for more than 1 day ($n_{reused\_control\male}$ = 13, $n_{reused\_T50\male}$ = 12 and $n_{reused\_T100\male}$ = 14). Male reuse ranged from 2 to 5 days (mean ± SD = 1.86 ± 1.09 days), but males were always exposed to the same treatment (water or the fungicide). The number of males used for more than 1 day did not differ between the treatment groups (GLM, Tukey HSD post hoc test; T100 vs. control, z = 0.182, p = 0.855; T50 vs. control, z = 0.695, p = 0.487; and T100 vs. T50, z = 0.825, p = 0.409). Moreover, additive chronic exposure (males exposed to a treatment more than once) had no significant effect upon mating (GLMM; χ^2 = 0.771, df = 1, p = 0.379). After the experiment, we measured the body size of each bee (see Supporting Information 1) and used this information to evaluate the potential effect of male size on performance.

Observations were carried out at room temperature at three different time points of the day from 9 am to 2 pm with provision of artificial and natural sunlight, from two windows with the arena sheltered from direct sunlight.

2.5 | Assay 2 – Choice experiments among fungicide-exposed and among control males

Fungicide exposure was performed as in Assay 1. Individuals were provided ad libitum with a 25% (w/w) sugar solution of API-Invert (72.7%; Südzucker AG). Mating pairs were established by introducing one virgin female to the mating cage (30 × 30 × 30 cm) with either five control males or five males exposed to the 100% field-realistic concentration of the fungicide (T100). Once a mating pair was established, the pair was removed from the arena following the protocol established by Conrad et al. (2010) and subsequent matings were recorded while signal vibrations were measured (see below). Mating pairs were not reused in any further observations. Observations were carried out on 13 control bees and 14 treated bees in a climate chamber with artificial light, at a constant temperature of 21°C

and humidity ranging from 56% to 72%. Although we used a climate chamber, the time at which the experiment was conducted and humidity were measured for each mating attempt and used in our statistical analyses as predictors.

2.6 | Assay 3 – Signal vibrations of pre-copulatory behaviour

To test the effect of the fungicide on the vibrational signal (burst length and modulation frequency) of males, we recorded the thoracic vibrations produced by males during the pre-copulatory phase. Males exposed to 100% field-realistic concentration of the fungicide (T100) and control bees were recorded with a laser vibrometer (Polytec PDV-100, Waldbronn) connected to a computer using a 32-bit sound card and Soundforge Pro 10.0 software (SonicFoundry) at a sampling rate of 96 kHz. The data were analysed using Spike 2 (Cambridge Electronic Design). All males were marked with a white spot on the dorsal side of their thorax to enhance the reflection of the laser beam. While evaluating the acquired data, we compared the average pulse duration (burst length) and frequency modulation ranges. For a standardized measure, the analysed frequency was acquired by a power spectrum (with 16,384 FFT and a Hanning window) during the 10 s right before a copulation or rejection event occurred. For burst length, we measured the length of at least 10 clearly visible pulses (within 10 s before the copulation/rejection event) and averaged the results per individual. The modulation range was acquired by measuring the maximum height of the main frequency modulations within the 10 s window prior to the copulation/rejection event. We analysed 27 recordings—13 from control and 14 from fungicide-exposed males.

2.7 | Analysis of cuticular hydrocarbons

Males used in Assay 1 were freeze-fixed and stored individually at −20°C. Cuticular hydrocarbons of each male were extracted with n-hexane for 10 min. The extracts ($n_{control}$ = 24, n_{T50} = 10 and n_{T100} = 17) were analysed using gas chromatography coupled with mass spectrometry (6890 GC coupled to a 5975C inert XL MSD from Agilent Technologies®). The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 μl at 300°C) was equipped with a DB-5 Fused Silica capillary column (30 m × 0.25 mm ID, df = 0.25 μm, J&W Scientific). Helium served as a carrier gas at a constant flow of 1 ml per minute. The following temperature program was used: initial temperature of 60°C, temperature increase by 5°C per minute up to 300°C and isotherm at 300°C for 10 min. The electron ionization mass spectra (EIMS) were acquired at an ionization voltage of 70 eV (source temperature: 230°C). For recording and analysing chromatograms and mass spectra, we used the HP Enhanced ChemStation G1701AA Version A.03.00 software. Once all peaks were quantified, we eliminated all compounds representing <0.1% of the total abundance in all groups from the dataset. In addition, we characterized

each hydrocarbon using retention indices and diagnostic ions. We used dimethyl disulphide derivations to determine double bond positions. Ethical approval was not required in the study.

2.8 | Statistical analysis

Assay 1: To explore the reproductive investment of males, we tested whether the number of copulation attempts predicted successful copulations regardless of treatment, using a GLMM with binomial error structure. Copulation success was used as a response variable and the number of copulation attempts as a fixed factor. Male and female identity were included as random factors to account for pseudoreplication. To investigate the effects of control and fungicide treatments on the proportion of successful male individuals copulating per treatment per trial, we used a GLMM with a binomial error structure. In addition to treatment (control, T50 and T100), the number of mating attempts and male body size were included in the model as fixed factors and the identity of males and females used in our experiments were incorporated as random factors to account for pseudoreplication due to the multiple use of a given male (despite its non-effect over mating, see above). Further Tukey HSD post hoc tests were used for pairwise comparisons between the treatments (control vs. T50 vs. T100). Furthermore, we used GLMMs with negative binomial error structure to investigate the effects of control and fungicide treatments on untreated males having preferred access to females compared to treated males; the time of the first male mating attempt; number of mating attempts; and mounting time (MT). Treatment (control, T50 and T100) and male body size were used as fixed factors and the identity of males and females used in our experiments were incorporated as random factor to account for pseudoreplication. Furthermore, Tukey HSD post hoc tests were used for pairwise comparisons between the treatments (control vs T50 vs. T100).

Assay 2: We used a GLM with binomial error structure to test for the effects of treatment on male copulation success. Treatment (control and T100) and relative humidity were used as fixed factors.

Assay 3: We used a linear model (LM) to investigate the effect of the fungicide on burst length and the modulation frequency. Treatment (control and T100), time and relative humidity were used as fixed factors.

We performed a non-metric multidimensional scaling analysis (NMDS) with Bray–Curtis distances with respect to treatment group and their cuticular hydrocarbons. Lastly, we verified the effects of treatment by multivariate analysis of variance. To discriminate the cuticular compounds that were most important for the separation of the controls and the males exposed to the fungicide, we analysed the CHC profile data using a random forest with the package RANDOMFOREST in R (Liaw & Wiener, 2002), with number of trees adjusted to 10,000. We used the mean decrease of accuracy to interpret substance importance (Cutler et al., 2007).

In order to investigate the effect of treatment on the relative abundance of cuticular hydrocarbons, we ran a robust LM using the R package MASS (Venables & Ripley, 2002) with subsequent post hoc tests with Bonferroni correction using the R package MULTCOMP (Hothorn et al., 2008). To visualize the relative abundance of each hydrocarbon in each treatment group, we generated heat maps of the log transformed mean values of compounds with the package GGPLOT2 in R (Wickham, 2009). The same analyses were done for the subset of males that have successfully mated.

All model (GLMMs, GLMs and LM) assumptions were visually checked and conformed to expectations (e.g. normality of the distribution of residuals, homogeneity of variances, linearity).

Mixed model analyses were performed using the R package LME4 v.1.0-6 (Bates et al., 2015). Tukey HSD post hoc tests were performed using the R package MULTCOMP (Hothorn et al., 2008).

3 | RESULTS

3.1 | Assay 1 – Choice experiments between fungicide-exposed and control males

Regardless of treatment, the number of mating attempts was a strong determinant of copulation success (GLMM; $\chi^2 = 9.209$, $df = 1$, $p = 0.002$; Figure 1). The observed number of successful copulations was significantly lower in the fungicide-treated bees compared to the control bees (GLMM; Tukey HSD post hoc tests; $p < 0.05$, see below). Control males performed a total of 16 successful copulation events, while males treated with the 50% field-realistic concentration (T50) and males treated with the 100% field-realistic concentration (T100) copulated in five and six events, respectively (GLMM; Tukey HSD post hoc test; control vs. T50, $z = 2.02$, $p = 0.043$; control vs. T100, $z = 1.973$, $p = 0.048$; and T50 vs. T100, $z = 0.135$, $p = 0.893$; see Figure 2, Table 1). Experimental treatment did not affect the likelihood of a male accessing a female first (control males were the first
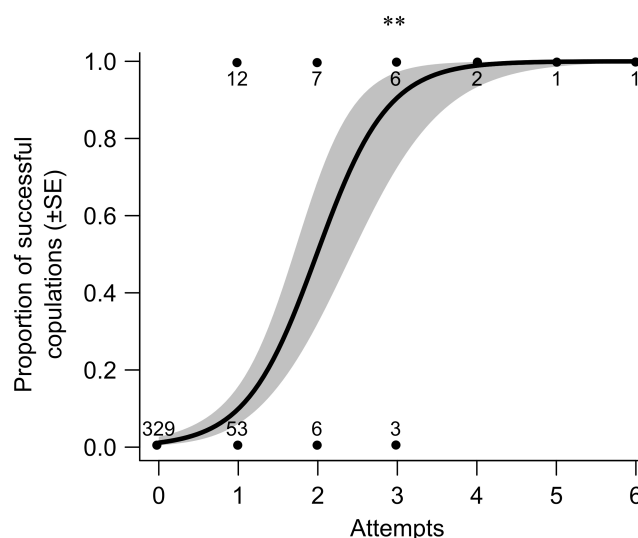

FIGURE 1 The proportion of successful copulations increased with the number of mating attempts. Level of significance: **$p < 0.01$. The plotted line represents the predicted relationship, and the shaded area indicates the standard error
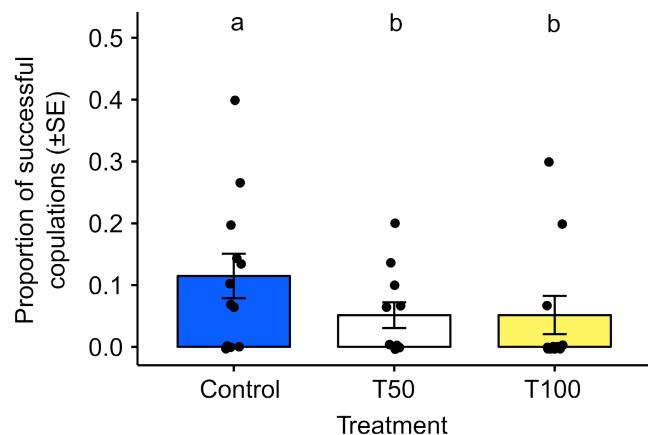
FIGURE 2 Proportion of successful copulations between the treatments. Different letters above the bars indicate significant differences (GLMM; Tukey HSD test, $p < 0.05$). T50 (males exposed to 50% field-realistic concentration) and T100 (males exposed to 100% field-realistic concentration of fenbuconazole). Means $\pm SE$ are shown

to attempt mating in 24 cases, T50 in 15 cases and T100 in 26 cases, GLMM; $\chi^2 = 0.678$, $df = 2$, $p = 0.712$, Supporting Information 2, Figure S2 a) or the time a male needed to start the first copulatory attempt (GLMM; $\chi^2 = 2.047$, $df = 2$, $p = 0.359$). Pesticide-treated males reached females and performed copulatory attempts as quickly as control males ((mean $\pm SD$); Time$_{control}$ = 173 $\pm$ 555 s, Time$_{T50}$ = 101 $\pm$ 395 s and Time$_{T100}$ = 276 $\pm$ 727 s, Supporting Information 2, Figure S2b). In addition, treatment did not affect the number of mating attempts ($n_{control}$ = 55, n_{T50} = 35 and n_{T100} = 47; GLMM; $\chi^2 = 0.584$, $df = 2$, $p = 0.746$, Supporting Information 2, Figure S2c) and the time that males spent performing pre-copulatory behaviours ((mean $\pm SD$); MT$_{control}$ = 68 $\pm$ 262 s, MT$_{T50}$ = 29 $\pm$ 200 s and MT$_{T100}$ = 54 $\pm$ 354 s; GLMM; $\chi^2 = 0.673$, $df = 2$, $p = 0.713$, Supporting Information 2, Figure S2d). See Table 1 for a complete presentation of the effect of the fungicide on mating behaviour explored in Assay 1.

3.2 | Assay 2 – Choice experiments among fungicide-exposed and among control males

In the experiment where fungicide-exposed males (T100) and control males were kept separately with virgin females, the proportion of successful copulations in control males (20%) was significantly higher compared to males exposed to 100% field-realistic concentration of the fungicide (7%) (GLM; $\chi^2 = 5.479$, $df = 1$, $p = 0.019$, Table 1; Supporting Information 2, Figure S3). Relative humidity had an effect in the proportion of successful copulations (GLM; $\chi^2 = 6.434$, $df = 1$, $p = 0.011$).

3.3 | Assay 3 – Signal vibrations of pre-copulatory behaviour

Among the pairs formed with a male on top of a female, we did not find a significant difference in male vibrational burst length

TABLE 1 Summary of statistical models. Sexual signals and copulation success of *Osmia cornuta* exposed to fenbuconazole-based fungicide

Assays	Study variable	Treatment			Statistical test	Significances		
		C	T50	T100		C versus T50	C versus T100	T50 versus T100
Assay 1	Copulation success	16	5	6	GLMM (Tukey HSD test)	z = 2.02, p = 0.043	z = 1.973, p = 0.048	z = 0.135, p = 0.893
	Likelihood of a male to access female	24	15	26	GLMM (Tukey HSD test)	z = 0.408, p = 0.912	z = 0.410, p = 0.911	z = 0.823, p = 0.689
	Time first copulatory attempt (sec) Mean (±SD)	173 (±555)	101 (±395)	276 (±727)	GLMM (Tukey HSD test)	z = 0.805, p = 0.421	z = 0.246, p = 0.805	z = 1.098, p = 0.272
	Attempts	55	35	47	GLMM (Tukey HSD test)	z = 0.738, p = 0.741	z = 0.178, p = 0.983	z = 0.572, p = 0.835
	Time spent on pre-copulatory behaviours (sec)	68 (±262)	29 (±200)	54 (±354)	GLMM (Tukey HSD test)	z = 0.727, p = 0.748	z = 0.026, p = 1	z = 0.707, p = 0.760
	CHC				Pillai test (PT)	PT = 0.605, p < 0.001	PT = 0.837, p < 0.001	PT = 0.618, p < 0.001
Assay 2	Copulation events	5		2	GLM	χ^2 = 5.479, df = 1, p = 0.019		
Assay 3	Average burst length (sec) (±SD)	0.36 (±0.17)		0.38 (±0.17)	LM	t = 1.452, p = 0.160 F = 2.1, df = 1, p = 0.16		
	Modulation frequency (Hz)	336.67 (range)		164.25 (range)	LM	t = 2.092, p = 0.047 F = 4.4, df = 1, p = 0.04		

(BL) between control males (mean $\pm$ SD) ($BL_{control} = 0.359 \pm 0.174$ s) and males treated with 100% field-realistic concentration of the fungicide ((mean $\pm$ SD); $BL_{T100} = 0.384 \pm 0.173$ s; LM; $t = 1.452$, $p = 0.16$). However, the modulation frequency (MF) of vibrations was significantly higher in the control group ((mean $\pm$ SD); $MF_{control} = 289.5 \pm 85.8$ Hz) compared to bees that were exposed to fungicide ((mean $\pm$ SD); $MF_{T100} = 266.6 \pm 53.73$ Hz; LM, $t = 2.092$, $p = 0.047$, Table 1, Figure 3). Time and relative humidity also had an effect over modulation (LM, $F = 7.248$, $df = 1$, $p = 0.01$; LM, $F = 4.377$, $df = 1$, $p = 0.037$ respectively).

3.4 | Chemical profile of male bees

We identified a total of 47 cuticular hydrocarbons on the cuticle of *O. cornuta* males. The NMDS (Figure 4) recovered 85% of the data variance and displayed three different groups based on CHC's ($Pillai_{2,4,96} = 0.843$, $p < 0.001$). With pairwise comparisons between the treatment groups, we found that the CHC profiles identified on control males differed significantly between T50 and T100 males ($Pillai_{1,2,31} = 0.605$, $p < 0.001$; $Pillai_{1,2,38} = 0.837$, $p < 0.001$ respectively). The CHC profiles of T50 and T100 were also different from each other ($Pillai_{1,2,24} = 0.618$, $p < 0.001$, Figure 4).

Overall, the relative abundance of alkenes (61%) was higher compared to alkanes (25%) and alkadienes (13%). The percentage of each cuticular hydrocarbon group remained similar among treatments, that is, control males: alkenes (56%), alkanes (28%) and alkadienes (14%); T50 males: alkenes (55%), alkanes (30%) and alkadienes (14%); T100 males: alkenes (56%), alkanes (28%) and alkadienes (16%). Random forest discrimination analysis indicated that the compounds 7C24en (7-tetracosene), 5C25en (5-pentacosene), 7C28en (7-octacosene), 5C24en (5-tetracosene) and 9C24en (9-tetracosene)
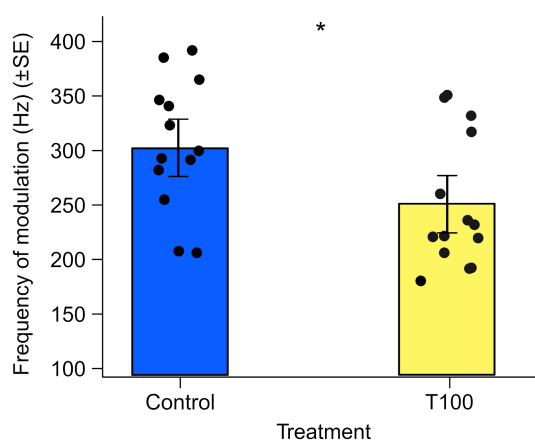
were the top five cuticular hydrocarbons responsible for differentiation between control, T50 and T100 male groups (Supporting Information 2, Figure S4).

Individual pairwise comparison through relative quantification of cuticular hydrocarbons displayed on heat maps via robust LMs between control and treated bees (T50 and T100) indicated significant differences in the relative representation of 21 CHC with most of the differences found in alkenes ($n = 19$ compounds) followed by alkadienes ($n = 2$ compounds). For better visualization, heat maps represent log transformed data (Figure 5).

The cuticular hydrocarbons on the surface of control males observed mating were significantly different from those on fungicide-exposed males that performed mating ($Pillai_{2,4,14} = 1.326$, $p = 0.002$, NMDS; Supporting Information 2, Figure S5). Moreover, there were significant differences in relative amounts of CHC between mated control and mated treatment (T50 and T100) males for 22 out of 47 cuticular hydrocarbons. For better visualization, heat maps represent log transformed data (Supporting Information 2, Figure S6).

4 | DISCUSSION

The fungicide Indar™ 5EW with the principle active compound fenbuconazole is commonly applied to crops and it has been characterized as a low toxicity pesticide due to low honeybee and mason bee mortality in survival tests (European Food Safety Authority (EFSA), 2010, Biddinger et al., 2013). However, to our knowledge, no studies have so far investigated the sublethal effects of fenbuconazole on the mating of solitary bees, as well as presenting empirical results suggesting that wild solitary bee population decline may result from the impact of

FIGURE 3 Frequency of modulation (measured in hertz) of *Osmia cornuta* males. The untreated control group (blue) showed a significantly higher modulation frequency compared to the T100 group (males exposed to 100% field-realistic concentration = yellow). Dots correspond to every measured bee. Means $\pm$ SE are shown; *$p < 0.05$ indicates significant difference
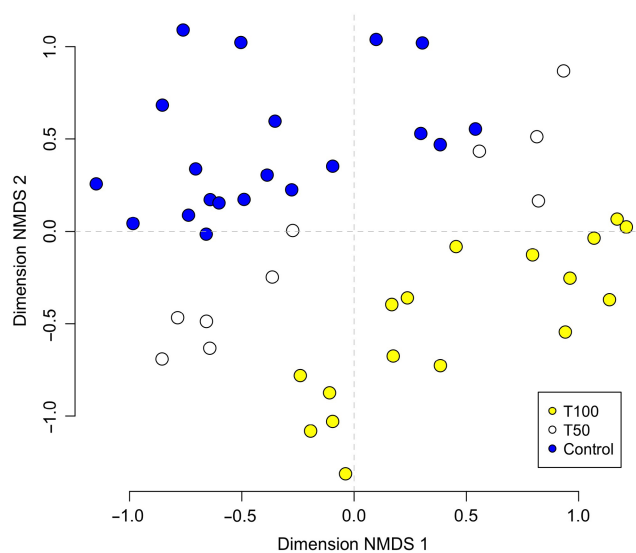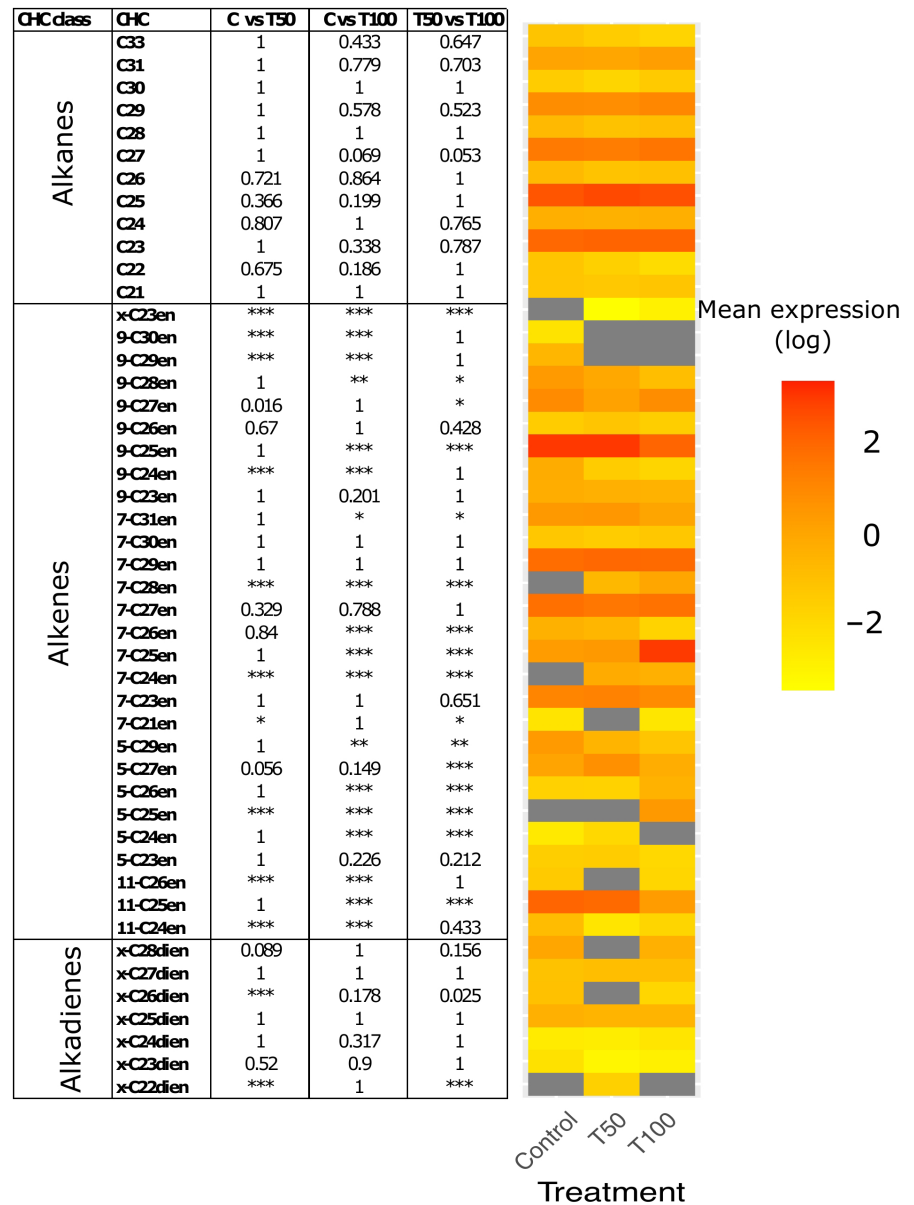

FIGURE 4 Non-metric multidimensional scaling (NMDS) of cuticular hydrocarbon profiles (quantity and quality) of control males (blue), males exposed to 50% field-realistic concentration (T50 group: white) and males exposed to 100% field-realistic concentration (T100 group: yellow)

FIGURE 5 Heat maps of the log transformed mean values of individual cuticular hydrocarbon compounds (CHC), as relative amount in %, for control males, T50 males and T100 males; yellow indicating low expression, red indicating a high expression and silver indicating no expression. The table on the left-hand side shows the individual compound and the respective *p*-values of the robust linear models computed for each pairwise comparison between the treatments. C = control, T50 = males treated with the 50% field-realistic dose and T100 = males treated with the 100% field-realistic dose. Level of significance: *0.05 > *p* > 0.01, **0.01 > *p* > 0.001, ****p* < 0.001

CHC class	CHC	C vs T50	C vs T100	T50 vs T100
Alkanes	C33	1	0.433	0.647
	C31	1	0.779	0.703
	C30	1	1	1
	C29	1	0.578	0.523
	C28	1	1	1
	C27	1	0.069	0.053
	C26	0.721	0.864	1
	C25	0.366	0.199	1
	C24	0.807	1	0.765
	C23	1	0.338	0.787
	C22	0.675	0.186	1
	C21	1	1	1
Alkenes	x-C23en	***	***	***
	9-C30en	***	***	1
	9-C29en	***	***	1
	9-C28en	1	**	*
	9-C27en	0.016	1	*
	9-C26en	0.67	1	0.428
	9-C25en	1	***	***
	9-C24en	***	***	1
	9-C23en	1	0.201	1
	7-C31en	1	*	*
	7-C30en	1	1	1
	7-C29en	1	1	1
	7-C28en	***	***	***
	7-C27en	0.329	0.788	1
	7-C26en	0.84	***	***
	7-C25en	1	***	***
	7-C24en	***	***	***
	7-C23en	1	1	0.651
	7-C21en	*	1	*
	5-C29en	1	**	**
	5-C27en	0.056	0.149	***
	5-C26en	1	***	***
	5-C25en	***	***	***
	5-C24en	1	***	***
	5-C23en	1	0.226	0.212
	11-C26en	***	***	1
	11-C25en	1	***	***
	11-C24en	***	***	0.433
Alkadienes	x-C28dien	0.089	1	0.156
	x-C27dien	1	1	1
	x-C26dien	***	0.178	0.025
	x-C25dien	1	1	1
	x-C24dien	1	0.317	1
	x-C23dien	0.52	0.9	1
	x-C22dien	***	1	***

Mean expression (log)

Treatment: Control, T50, T100

pesticide on pre-copulatory behaviour. We found that horned mason bee males with topical exposure to the fungicide showed reduced thoracic vibration patterns and altered cuticular hydrocarbon composition, two sexual signals known to be used by females to choose a mating partner. Moreover, the number of copulations was significantly greater in control males. Based on our results, we assume that females can recognize when male bees have been exposed to fungicides and might avoid copulating with them, probably due to altered male quality reproductive signals caused by the fungicide.

4.1 | Effects of pesticides on bee reproduction

Studies reporting significant decreases in solitary bee populations in croplands (Rundlöf et al., 2015; Willis Chan & Raine, 2021; Woodcock et al., 2017) have suggested the need to evaluate how sublethal doses of pesticide affect bee reproduction. The results of our experiments

indicate that pesticides may impact bee reproduction by altering male quality signals used by females to choose a mating partner. Our hypothesis, that the observed lower copulation rates of males are due to female choice, is supported by the finding that treated as well as control males were similarly engaged in approaching females, that is, there was no difference in accessing females or in the time spent on mating attempts, but actual copulations achieved differed between control and treated groups. We rejected the possibility that the reduced number of copulations of treated males is due to impairment of movement, since engagement (numbers of attempts, time between attempts and mounting) of treated males did not differ from that of control males. Our results rather point to differences in male quality signals, such as cuticular hydrocarbon composition and thoracic vibrations, which are affected by fungicide treatment and may explain the observed difference in copulation success.

Osmia cornuta females mate with a single male and males are similarly accessible to females by displaying scramble competition (Ayasse

et al., 2001). In our study, this condition did not change with pesticide exposure. Topical exposure of pyrethroid on the bed bug *Cimex lectularius* showed that pyrethroid-exposed males mated with both treated and untreated females; however, similar to our findings, treated males had decreased success in copulatory events compared to control males (Crawley et al., 2017). In the spider *Rabidosa rabida*, males that failed in copulation due to the effect of a pesticide (malathion) on the initiation of courtship ended by being eaten by the female, suggesting severe disruption of reproductive output (Tietjen, 2006). From our study, we still do not know for how long the effect of this fungicide on the alterations of vibrational signals and CHC remains on horned mason bee males. If pesticide exposure permanently changes male quality and females remain selective towards a pattern of sexual signals, the local population theoretically could decrease due to female mating avoidance. In the scenario with permanent signal changes, female sexual reproduction could rely on the presence of males dispersing from pesticide-free areas. It is unlike that local females of horned mason bees would engage in dispersion since they are mostly philopatric (Bosch et al., 2021). For *O. bicornis*, it has been shown that females prefer males from surrounding populations over males from distant locations (Conrad et al., 2010; Conrad & Ayasse, 2015). Alternatively, to balance a lack of potential mating partners, females could maintain their reproductive output by biasing their offspring production towards males. Due to the haplodiploid sex determination system of bees, production of males occurs independently of a mating partner from unfertilized eggs (Cook & Crozier, 1995). Interestingly, male-biased offspring sex ratios were reported in semi-field reproductive experiments where bees of *O. cornuta* and *O. lignaria* were exposed to pesticides (Sandrock et al., 2014; Stuligross & Williams, 2020). However, results from ground nesting squash bees showed no measurable change in offspring sex ratios in response to exposure to three insecticides tested (Willis Chan & Raine, 2021), suggesting that this effect might be species specific and may depend on different variables, including the route of pesticide exposure.

4.2 | Vibrations as a cue for male fitness

Modulation ranges differed significantly between males treated with fungicide and untreated males. This supports the hypothesis that vibrational signals of males are influenced by the pesticide, which could potentially hinder their mating success. We already know that male vibrations play a vital role in the mating process of *O. bicornis* (Conrad et al., 2017; Conrad & Ayasse, 2015) and strongly suspect a similar role in *O. cornuta* as the mating system of these two species is very similar. Modulation range seems to be an important parameter within the vibrational signal of males since it differs between males from different areas (Conrad & Ayasse, 2015) and can be influenced by environmental variables, such as humidity and temperature change (Conrad et al., 2017). Our study therefore provides evidence for the importance of using vibrational outputs as a component to measure bee health, as was proposed in a previous study (Conrad et al., 2017). Although there are various studies about

disrupting vibrational communication as a pest repellent instead of using pesticides (Eben et al., 2015; Eriksson et al., 2012; Krugner & Gordon, 2018), there are few studies about the influence of pesticides on these signals in bees (Switzer & Combes, 2016; Whitehorn et al., 2017). Even with different exposure routes, that is oral and topical, and different reported toxicities, pesticides likely cause changes in thoracic vibration patterns and are thus impacting the vibrational capacity of bees to some extent, potentially hampering their reproductive fitness.

4.3 | Cuticular hydrocarbons and female choice

Osmia cornuta males treated with Indar™ 5EW showed a different cuticular hydrocarbon composition compared to control males. For some beetles and wasps, it has previously been shown that individuals exposed to sublethal doses of pesticides (neonicotinoid) displayed changes in the cuticular hydrocarbon and pheromone composition, with a subsequent effect upon mating (Müller et al., 2017; Tappert et al., 2017; Wang et al., 2018). Our findings highlight a neglected major impact on the reproduction of wild *O. cornuta* at crop sites, by disruption of mating due to the critical alteration in chemical cues or signals. It is possible that the synthetic pathways of some of the key compounds that are used by females to assess male fitness were altered after fungicide exposure and that this leads to the observed changes in chemical profile. There is evidence of an indirect regulation of cuticular hydrocarbons through detoxification and metabolic resistance to pesticides in insects that are mediated by cytochrome P450 monooxygenases (Balabanidou et al., 2016; Zhang et al., 2019). P450 genes are modulators of biosynthetic production of hydrocarbons in insects (Chen et al., 2016) and subfamilies of these genes are associated with metabolic resistance to systemic pesticides in honeybees (Gong & Diao, 2017) and in *Osmia* bees (Beadle et al., 2019). Consequently, it has been suggested to inhibit detoxification processes when bees are exposed to a fungicide (Raimets et al., 2018).

Cuticular hydrocarbons as part of a few compounds or complex compounds arrangements have diverse and complex functions in insects, including sexual communication (Ayasse et al., 2001; Wyatt, 2014). We found significant differences in the relative composition of cuticular hydrocarbons such as alkenes and alkadienes between the three treatment groups; and the five most important components that differentiated groups all belong to the alkenes. The role of alkenes on mating systems has also been highlighted in the solitary bee species *Megachile rotundata* (Paulmier et al., 1999) and *O. bicornis* (Conrad et al., 2010). The table associated with our heat map shows that the expression of alkenes differed between control and T50 and between control and T100 males, in approximately in 40% and 65% of cases. For instance, a reduction of C25 alkenes with a double bond position at the 9th C atom in favour of C25 alkenes with a double bond position at the 7th C atom was recorded after treatment with the fungicide. These specific changes were still present among males that copulated after exposure to fungicide, suggesting that differences in mating success are not directly dependent

or uniquely dependent on these compounds. The specific blend of cuticular hydrocarbons to display cues for mating partner recognition has also been reported in other agriculturally important insects, such as the fruit-infesting flies *Drosophila suzukii* and hunting wasps (e.g. *Nasonia vitripennis*) (Mair & Ruther, 2019; Snellings et al., 2018). Thus, we present evidence of the impact of fungicide on cuticular hydrocarbons, but whether female choice is caused by changes in specific compounds or by the alteration in the whole CHC bouquet remains a challenge for future studies.

Our study helps to elucidate potential mechanisms that might be contributing to a reduction of mason bees in croplands. Nevertheless, it only partially covers the impact of the pesticides on the reproduction of this species. Further studies should consider investigating the perception of male quality when females of *O. cornuta* are exposed to pesticides and whether sublethal doses of other agrochemical classes impose similar impacts on mason bees.

5 | CONCLUSIONS

Our study shows the negative effects of a pesticide, fenbuconazole, on mating behaviour and copulation success of males of the solitary bee *O. cornuta*. The results provide evidence that can help to understand how pesticides might lead to reduced bee populations worldwide, especially in crop sites. Our study on mating behaviour and sexual signals sheds the light on a new path to explore the impact of environmental stressors on insect decline. Due to the importance of bees for pollination service provision, we stress the need for the inclusion of mating experiments on safety test programs of chemical products used in agriculture, to understand the effect of pesticides on the reproductive system of beneficial insects such as bees.

CONFLICT OF INTEREST
No conflict of interest has been declared by the authors.

AUTHORS' CONTRIBUTIONS
S.B., J.R. and D.L. designed the experiment; S.B. and M.B. performed Assays 1 and 4; T.C. performed analysis of bee vibration; S.B., M.W. and T.S. performed analysis on chemical compounds; S.B., A.F., J.R. and P.T. performed statistical analysis; D.L. provided funding; S.B. drafted the manuscript. All authors contributed to the final version of the manuscript and agreed with manuscript submission.

DATA AVAILABILITY STATEMENT
Data are available via Dryad Digital Repository https://doi.org/10.5061/dryad.kwh70rz5q (Boff et al., 2022).

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