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# Romance in peril: A common pesticide impairs mating behaviours and male fertility of solitary bees (*Osmia bicornis*)

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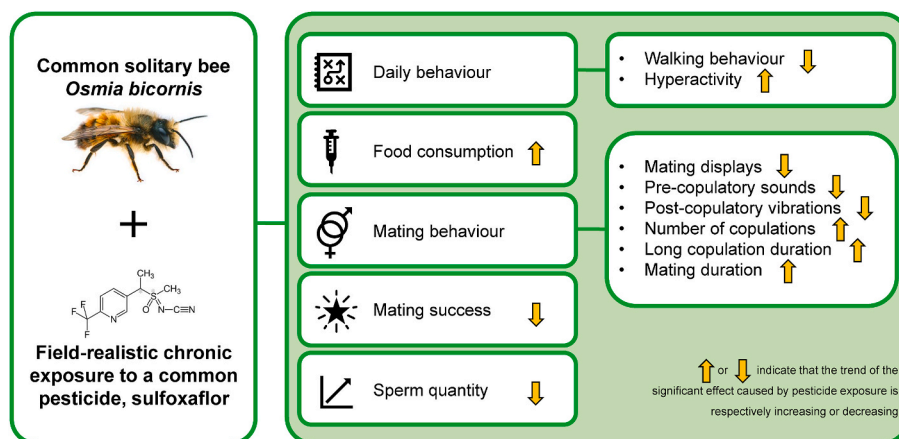
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## HIGHLIGHTS

- Field-realistic levels of common pesticide (sulfoxaflor) widely impaired bee health.
- Pesticide exposure increased food consumption (+27 %), mating duration (+166 %).
- Pesticide exposure reduced courtship behaviours (up to −36 %), mating success (−43 %).
- Pesticide exposure altered bee physiology, reducing sperm quantity (−25 %).
- Field-realistic pesticide levels can alter fertility, reproduction of pollinators.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Mating behaviour and fertility are strong selective forces, driving the reproductive trends of animals. Mating disorders may therefore contribute to the recent decline in insect and pollinators health worldwide. While the impact of pesticides on pollinators is widely considered as a driving factor for reducing pollinators health, their effect on mating behaviour and male fertility remains widely overlooked. Here, we assessed the effects of field-realistic exposure to a common pesticide used as a neonicotinoid substitute worldwide, sulfoxaflor, on the behaviour and male physiology of the solitary bee, *Osmia bicornis*. We measured a variety of parameters focusing on behaviours occurring before, and during mating, as well as sperm quantity. For the first time, we demonstrate that short-term chronic, field-realistic exposure to a common pesticide reduced pre-copulatory display (−36 %) and sounds (−27 %), increased the number of copulations (+110 %) and the mating duration (+166 %), while finally reducing sperm quantity (−25 %) and mating success (−43 %). Our research raises considerable concern on the impact of field-realistic, low sublethal pesticide levels on the fertility and reproductive success of

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pollinators. Assessing the impact of pesticides on fitness parameters and implementing more sustainable agricultural solutions would allow mitigating the ongoing threat of pesticide pollution on wild insect populations and the broader environment.

## 1. Introduction

Wild bees, including the red mason bee (*Osmia bicornis* L.), provide essential pollination service for both crops and wild plants sustaining food production and biodiversity while serving as bioindicator of environmental health (Monzón et al., 2004; Garibaldi et al., 2013; Winfree et al., 2008). However, their abundance and diversity have globally been drastically declining over the past decades, putting biodiversity and the production of pollination-dependent crops at considerable risk (López-Urbe et al., 2020; Garibaldi et al., 2013). While the reasons for these declines and losses are most certainly multifaceted (Potts et al., 2016; Goulson et al., 2015), considerable concern arises from the ubiquitous use of agrochemicals that are frequently found contaminating pollen and nectar of flowering crops and wild plants (Botías et al., 2015; Zioga et al., 2020). Mounting evidence suggests that environmental pesticide pollution can negatively affect insect biomass and diversity (Hallmann et al., 2017; Woodcock et al., 2016), including bees (Potts et al., 2010; Ollerton et al., 2014).

Pesticides can cause both lethal and sublethal negative effects on bees, individually or in combination with other stressors (Tosi et al., 2022). These effects include impairments in learning and memory (Decourtye et al., 2003), decision time (Ludicke and Nieh, 2020), feeding behaviour (Azpiazu et al., 2022), ovary development (Sgolastra et al., 2018), colony functionality (Crall et al., 2018), nesting behaviour (Artz and Pitts-Singer, 2015), immune response (Brandt et al., 2020), motor functions and phototaxis (Tosi and Nieh, 2017), respiratory rhythm (Hatjina et al., 2013), thermoregulation (Tosi et al., 2016; Tong et al., 2019; Azpiazu et al., 2019), orientation and navigation (Fischer et al., 2014; Jin et al., 2015), and flight abilities (Tosi and Nieh, 2017; Tong et al., 2019). However, our understanding of how pesticide exposure may affect insect mating, fertility and reproductive physiology remains scarce, in particular for solitary bees.

Considering that reproductive traits underpin individual fitness and ultimately population dynamics (Moretti et al., 2021), it is rather surprising that these traits remain largely overlooked, in particular for bee ecotoxicology. Pesticide exposure can lead to detrimental effects on the reproductive physiology and fertility (i.e., the capacity to produce viable sperm in males, and viable offspring in females) of honey bees (Straub et al., 2016; Williams et al., 2015), and bumble bees (Laycock et al., 2012; Linguadoca et al., 2021; Straub et al., 2022a). Laboratory and semi-field research have assessed the impact of sublethal pesticide exposure on *Osmia* females, including alterations of ovary development (Sgolastra et al., 2018), fecundity and offspring production (Centrella et al., 2020; Knauer et al., 2022), sex ratio (Sandrock et al., 2014; Stuligross and Williams, 2020; Albacete et al., 2024), and overall population growth rate (Stuligross and Williams, 2021; Albacete et al., 2024). The neonicotinoid insecticides, thiamethoxam and clothianidin, have been shown to impair male acceptance and mating success (Sandrock et al., 2014), and male fertility (Strobl et al., 2021) in *Osmia* spp; likely translating to considerable negative effects at the population level (Woodcock et al., 2016; Stuligross and Williams, 2020). There is finally concerningly limited knowledge on male reproduction parameters such as mating behaviour and fertility, especially in wild pollinators.

Male bees play a key role in sexual reproduction and can significantly influence population dynamics and offspring sex ratios (Stuligross and Williams, 2021). *Osmia* spp. is a protandrous genus, wherein males upon emergence feed on pollen and nectar while locating and waiting for newly emerged females at their mating sites (Seidelmann, 1999). During successful mating, the ejaculated semen coagulates in the vagina to form a mating plug (Seidelmann, 2015), facilitating the sperm to reach the

spermatheca, where the sperm can be preserved and stored until required (Conrad et al., 2010). Immediately after mating, females begin to establish their nests within cavities, where approximately 30 separated brood chambers are built and provisioned with pollen and small amounts of nectar (Raw, 1972; Batra, 1984). If the female has not been successfully inseminated by the males, only unfertilized eggs would be laid on the brood chambers, thus producing only males and leading to a bias in the offspring sex ratio (Stuligross and Williams, 2020). A successfully inseminated female will follow their natural laying process finally producing both females and males (Woodcock et al., 2016; Stuligross and Williams, 2020). In light of the limited data, additional studies assessing the potential inadvertent effects of pesticides on the male fertility and behaviour of bees are urgently required, in particular for agrochemicals beyond neonicotinoids.

Sulfoxaflor is a sulfoximine insecticide, acting as an agonist of the nicotinic acetylcholine receptors (nAChR) (Sparks et al., 2013). By disrupting the cholinergic signaling on insect's central nervous system, even low doses can cause uncontrolled nerve impulses and muscle tremors that can affect behaviour and locomotion (Boff et al., 2021; Watson et al., 2021; Sparks et al., 2013). Sulfoxaflor has globally been promoted as a substitute of the neonicotinoid insecticides since their use restriction (Wang et al., 2017; Watson et al., 2021). While sulfoximines and neonicotinoids have both been extensively commercialized for broad pest control and share a similar mode of action (i.e., IRAC group 4), sulfoxaflor was reported as more selective and less toxic on sap-feeding pest insects (Sparks et al., 2013; James et al., 2023) and non-target organisms (Maloney et al., 2020; Liu et al., 2021; Azpiazu et al., 2021). Recent evidence however suggests that sulfoxaflor may be more toxic than certain neonicotinoids, such as acetamiprid and thiacloprid (Azpiazu et al., 2021). Given that sulfoxaflor can cause adverse effects on honey bees (e.g., reduced survival and disrupted metabolism; Cheng et al., 2023), bumble bees (e.g., reduced reproductive success, egg-laying rate, and colony growth; Siviter et al., 2018; Siviter et al., 2020; Tamburini et al., 2021), and solitary bees (e.g., reduced survival, flight and foraging performances, feeding behaviour; Azpiazu et al., 2022; Boff et al., 2021), the European Commission has restricted its usage to greenhouses. Nonetheless, pivotal crop-producing countries - such as the USA, Australia, and South Africa - have only implemented minor restrictions, if any (OJEU, 2022; Cortevea, U.S., 2022; US EPA, 2019; Cortevea Australia, 2022; Cortevea South Africa, 2022). Despite rising concerns of the adverse effects of sulfoxaflor on bees, studies investigating the potential inadvertent negative effects of pesticides on *Osmia bicornis* males, and more specifically on their mating behaviour and fertility are lacking.

Here, we aim to fill these knowledge gaps by investigating the effects of field-realistic sulfoxaflor exposure on lethal (i.e., survival) and sublethal reproductive parameters (i.e., food consumption, behavioural activity, mating behaviour, mating success, and sperm quantity) of a common solitary bee species, *O. bicornis*.

## 2. Material and methods

### 2.1. Bee rearing

We used the red mason bee, *O. bicornis* L. (Hymenoptera: Megachilidae) as a model species because this solitary bee species a) is one of the most abundant in Central Europe (Westrich, 1989); b) can be reared through established laboratory methods, allowing scientifically-sound experimental studies (Sgolastra et al., 2016); c) have a well-known biology (Westrich, 1989; Seidelmann, 2014) with monandrous females

(i.e., typically only mate once) and polygynous males (i.e., they can mate with several females).

The experiment was conducted during seven weeks, covering May and part of July 2023. A total of 369 cocoons (215 males and 154 females) were used. Of these, 297 (143 males and 154 females) were used to assess pesticide effects on survival, food consumption, behavioural activity, and mating behaviour, and 72 were used to assess pesticide sublethal effects on the sperm quantity.

*Osmia bicornis* cocoons were maintained at  $4 \pm 1$  °C in a refrigerator (Seidelmann and Rolke, 2019). Cocoons were sexed using the reference measures of *O. bicornis* (males: 8–10 mm; females: 10–12 mm; Gruber et al., 2011). To facilitate emergence, sex sorted cocoons were transferred daily to a rearing room maintained at  $22 \pm 2$  °C,  $50 \pm 20$  % relative humidity, and indirect natural light (Seidelmann and Rolke, 2019; 2023; Sgolastra et al., 2017). Each cocoon was placed in an individual falcon tube (50 mL) perforated with small holes to allow air circulation. Upon emergence, the date was recorded, sex was verified, and all individuals were visually assessed for the presence of ectoparasites or abnormalities, e.g., clinical disease symptoms, or morphological deformations (Bosch, 1992; Seidelmann, 2006). Males were then starved for 24 h within their falcon tube, where the meconium was expelled (Henriques-Martins et al., 2023) and then weighed to obtain the bee body mass (mg) which was used as a proxy for individual size. Each bee was then transferred to an individual cage that consisted of transparent plastic ice-cream cups (width: 7.5 cm, height: 5.5 cm, volume: 150 cc) adapted for solitary bee rearing using perforated lids allowing air circulation (Henriques-Martins et al., 2023). Each cage was provided with a sucrose solution feeder consisting of a 1 mL calibrated syringe (Ber-ject®, accuracy: 0.01 mL) inserted laterally and slightly tilted to facilitate consumption. Bees were fed *ad libitum* with sucrose solution (33 % weight by weight) prepared following standard protocols (Tosi et al., 2017; Sgolastra et al., 2018). The sucrose solutions were freshly prepared prior to the initiation of the three-day exposure period.

## 2.2. Pesticide exposure

*O. bicornis* males were chronically exposed to either a control or pesticide treatment throughout the rearing period of three days via oral exposure ( $n = 215$ ). The sucrose solution (33 %, w/w) was thus either pure (control) or spiked with 20 ppb of the active substance sulfoxaflor (CAS 946578-00-3; 99 % purity, Sigma-Aldrich, UK). Distilled water and white granulated sugar (Landi AG®, Dotzingen, Switzerland) were used for both control and pesticide treatments. We followed standard procedures to avoid cross contamination, including the appropriate handling of cages and laboratory instruments between treatment groups (Mędrzycki et al., 2013). A sulfoxaflor concentration of 20 ppb aligns with realistic field exposures, given reports of up to 40 ppb in cotton nectar (*Gossypium* spp.) collected by bees 1–5 days post-spraying (Adriaanse et al., 2023; US EPA, 2016, 2019). Residues of 22 ppb were similarly found in *Hibiscus rosa-sinensis* nectar after foliar application (Zhou et al., 2023), and 20 ppb in *Salvia splendens* nectar after combined soil and foliar treatments (Zhou et al., 2022).

Bee males were exposed for three days because males naturally emerge a few days before females, making pesticide exposure likely during this period as they feed before mating (Seidelmann, 1999). Furthermore, bees can realistically be exposed to single and multiple pesticides for three days (Tosi et al., 2018).

## 2.3. Survival assessment

Bee survival was registered for all male individuals reared for the mating behaviour assessment ( $n = 143$ ). Bee survival was recorded daily during the three-day exposure period of each male individual. A bee was considered dead when the individual was immobile, showed no spontaneous movements of abdomen and antennae, and did not react upon stimulation.

## 2.4. Food consumption assessment

Food consumption was calculated for all reared bees which survived the three-day exposure period ( $n = 117$ ). Food consumption (i.e., ingestion of sucrose solution by each bee) was recorded at the end of the three-day exposure period, and calculated based on the mass of food consumed by each bee, corrected by the evaporation rate. Evaporation rate was measured using three cages with a feeder but without bees that were placed under the same experimental conditions. Daily evaporation rate was calculated for each cage by subtracting the daily syringe weight to the weight of the day before. To accurately assess food consumption, evaporation rate subtracted from the food consumption of each bee. Based on the sulfoxaflor concentration tested and daily food consumption, we defined the dose of sulfoxaflor ingested by each bee both daily (ng/bee/day) and over three days (ng/bee).

## 2.5. Daily behaviour assessment

The *O. bicornis* males ( $n = 117$ ) which survived the three-day exposure period were considered for the daily behaviour assessment analysis. We monitored the behaviour of each bee for 10 s once a day for three consecutive days ( $n = 351$  observations).

We adapted and refined the standardized honey bee behavioural assessment proposed by Tosi and Nieh (2019) as well as the official ecotoxicological guidelines (OECD, 2017) for application to solitary bees. The newly developed ethogram (Table 1) was designed to enhance the standardization, repeatability, and accuracy of bee behavioural assessments. Using this ethogram, we monitored eight behavioural categories (Table 1). All behaviours defined in the ethogram could be observed in the assessed experimental bees. For simplicity and statistical purposes, we classified behaviours as either “normal” or “abnormal” (Tosi and Nieh, 2019). Daily behaviours were classified into these two groups based on their correlation and biological relevance, using targeted statistical analysis (see section 2.8). The abnormal behaviour group included hyperactive, thanatosis, weak, and moribund behaviours, while the normal behaviour group included still, walking, feeding, and flying. A key challenge in this approach lies in defining whether a behaviour is normal or abnormal. For instance, while thanatosis is a natural behaviour in *Osmia* spp., it typically occurs in response to stress. Therefore, we categorized it as an abnormal behaviour.

**Table 1**

**Ethogram of *Osmia* bees.** The behaviours of *O. bicornis* (Hymenoptera: Megachilidae; maintained in cages) males were recorded daily for three consecutive days.

Behaviour	Description
<i>Still</i>	The bee stands on its legs, without exhibiting any active behaviour (i.e., walking, flying, or feeding).
<i>Walking</i>	The bee walks actively exhibiting coordinated movements.
<i>Feeding</i>	The bee approaches the feeder and unfolds its proboscis, inserting it in the syringe and actively feeds by sucking the sucrose solution.
<i>Flying</i>	The bee performs a coordinated flight using its wings, with all legs off the ground. The flight may consist of horizontally/vertically moving from one point to another or being steady.
<i>Thanatosis</i>	The bee lays on the ground with no active movement of its body, while typically laying on its back. The bee looks dead but exhibits regular ventilatory cycles. Upon stimulation, the bee reacts and then moves actively exhibiting coordinated movements.
<i>Hyperactive</i>	The bee manifests signs of excitement through chaotic and rapid expression of multiple behaviours, i.e., walking, flight attempts. Overall, the bee manifests poor coordination.
<i>Weak</i>	The bee may be actively moving or laying on one side. The bee movements are characterized by poor coordination and slowness.
<i>Moribund</i>	The bee lays on the ground with no active movement of its body, typically laying on one side or on its back. The bee exhibits regular ventilatory cycles and may move the antennae. Upon stimulation, the bee does not actively react.

## 2.6. Mating behaviour assessment

A total of 116 males and 114 females were tested for the mating behaviour assessment. *O. bicornis* mating behaviour was tested 96 h after male emergence (i.e., one day starvation and three days exposure) when *O. bicornis* males are considered sexually mature and known to mate (Seidelmann, 2015; Strobl et al., 2019). *Osmia* females are known to mate immediately after emergence (Felicioli et al., 2023), thus the age of the females tested was within 0–3 days. Females older than 24 h were fed with sucrose solution (33 %, w/w) and maintained at the same rearing conditions as male individuals until their use in the mating test.

Prior to the mating test, the females were individually placed inside a transparent mating arena (45 × 45 × 45 cm<sup>3</sup>) and then randomly paired with a male from either the control or pesticide treatment. The cage with a single male was gently placed in the middle of the mating arena to let the bee spontaneously exit to reduce observer biases. Once both male and female were in the arena, the following fourteen variables were recorded: mating latency, male acceptance, courtship duration, occurrence of copulatory display and sounds, number of copulations, long copulation duration, inter-copula looping (ICL) duration, post-copulatory phase duration, occurrence of post-copulatory display and vibrations, total mating duration, and mating success (Felicioli et al., 2023; Pilakouta and Baillet, 2022; Seidelmann and Rolke, 2019; Table S1).

Following the protocol defined by Amin et al. (2012), bee couples that did not start mating within 30 min were considered rejected, and the mating trial was thus aborted. Considering that mating is determined by the females (i.e., female choice; Seidelmann, 2014), rejected males were granted a second opportunity by being placed in a new arena with a naive virgin female. To ensure independence of observations, mating success of rejected males was assigned using only the second measure. All mating behaviours were recorded exclusively for accepted males, ensuring independent observations. To remove potential pheromones emitted by bees during the mating trial (Yin et al., 2013; Ayasse et al., 2001), the mating arena was thoroughly cleaned using water before being reused.

## 2.7. Sperm quantity assessment

After three days of exposure, control and pesticide-treated males were used for the sperm quantity assessment. A subset of 70 males ( $n_{\text{control}} = 33$ ,  $n_{\text{pesticide}} = 37$ ; July 2023) was reared following the harmonized rearing protocol and then were anesthetized by placing the individuals on ice (Brandt et al., 2020). They were pinned to a wax plate for the dissection of the entire reproductive genitalia (Strobl et al., 2019). The removed genitalia were placed in 150 µL of 0.9 % (w/w) NaCl solution, gently crushed to form a sperm stock solution, and then frozen. Before being assessed, the sperm stock solution was thawed and slightly vortexed (i.e., to homogenize the concentration of cells). Then, a 50 µL of this solution were diluted with 50 µL of 0.9 % (w/w) NaCl (1:1 dilution). Sperm quantity was measured using a Neubauer-improved chamber (Marienfeld®, 0.1 mm depth), and a light microscope at 400x magnification. The final sperm quantity was calculated by applying the following formula:

$$\text{Number of sperm cells } (n) = \frac{\text{Total volume in which semen was suspended } (\mu\text{L})}{\text{Volume counted in Neubauer chamber } (\mu\text{L})} \times \text{Average number of counted sperm } (n)$$

## 2.8. Statistical analyses

A linear model (LM) was applied to confirm that bee body mass did not differ significantly across treatments for both May- and July-

emerged bees. We used a Log-Rank test to assess the impact of pesticide treatment on the survival of May-born bees. To evaluate the effect of pesticide exposure on cumulative and daily food consumption (log-transformed to achieve normality), an LM was used, with treatment as a fixed factor (control or pesticide) and bee body mass as a covariate.

We examined the effect of pesticide exposure on each daily behaviour separately (i.e., whether the bee exhibited the behaviour or not; Table 1) and on daily behaviours categorized as either normal or abnormal. The correlation between daily behaviour variables was analyzed using a phi test. Behaviours with a correlation coefficient equal to or greater than 0.3 were grouped together, whereas those with a negative correlation equal to or lower than −0.3 were placed in separate groups (Table S2). Behaviours that did not show significant correlations were grouped according to biological criteria established by previous studies (Tosi et al., 2021; Ludicke and Nieh, 2020). To examine the effect of pesticide exposure on each daily behaviour (i.e., whether the bee exhibited the behaviour or not, Table 1) and normal/abnormal behaviours, we implemented a generalized linear mixed-effects model (GLMM) with a binomial error distribution and logit link. Pesticide exposure was set as a fixed factor, while bee identity and the day of observation (days 1–3) were included as random factors to account for repeated observations. For the behaviour “hyperactive,” however, the GLMM did not converge when day was included as a random factor, so only bee identity was retained in the final model.

Each mating behaviour (Table S1) — mating choice, pre-copulatory display, pre-copulatory sound, mating success, post-copulatory display, and post-copulatory vibrations — was modeled as a binary outcome (whether the bee displayed the behaviour or not) using generalized linear models (GLMs) with a binomial error distribution and logit link, with pesticide exposure as the fixed factor and male body mass as covariate. The number of copulations per mating was modeled with a GLM using a Poisson error distribution and log link, with pesticide exposure as the fixed factor and the male weight as covariate. Mating duration, mating latency, courtship duration, time between copulations, ICL duration, post-copulatory phase duration, and long copulation duration (Table S1) are time measures (in minutes). These variables were log-transformed for normality and analyzed using an LM with pesticide exposure as the fixed factor.

To identify associations between the mating behaviours we calculated Pearson associations between each pair of mating behaviour variables. We identified significant correlations between mating behaviours ( $p < 0.05$ ; Table S3). The correlations were considered positive when the coefficient was equal or greater than 0.3 and negative when it was equal to or lower than −0.3, (Table S3).

The impact of pesticide exposure on sperm quantity in July-reared bees was evaluated using a GLM, with pesticide exposure as a fixed factor and bee body mass as a covariate. Given the overdispersion in the data, we specified a quasi-poisson error distribution and log link. Overall, model characteristics are summarized in the Supplementary information (Dataset 6). Model conditional means and their 95 % confidence intervals were calculated for all statistically significant variables, with the results reported in the Supplementary information (Table S4).

Data analysis was conducted in R Studio 3.4.4 (R Core Team, 2023).

Data management and visualization were carried out using the packages *readxl* (Wickham and Bryan, 2019), *tidyverse* (Wickham et al., 2019), *ggplot2* (Wickham, 2011), and *lubridate* (Grolemund and Wickham, 2011). Survival analysis was performed with the following packages:

survival (Therneau and Lumley, 2013), *ggsurvfit* (Sjoberg et al., 2024), *tidycmprsk* (Sjoberg and Fei, 2024), and *condSURV* (Meira-Machado and Sestelo, 2016). GLMM were implemented using the *lme4* package (Bates et al., 2015). Numerical continuous variables and their log transformations were tested for normality both visually and with the Shapiro–Wilk test. Model residuals were visually inspected with *plot* (model) for LMs and GLMs and with the *DHARMA* package (Hartig, 2022) for GLMMs, allowing us to determine the appropriate error distributions to meet model assumptions and ensure optimal data fitting. Model conditional means were estimated using the package *ggeffects* (Lüdtke, 2018).

### 3. Results

There was no significant difference in the body mass (recorded before exposure) across treatment groups (LM:  $\chi^2 = 0.0003$ ,  $F = 1.76$ ,  $df = 1$ ,  $p > 0.05$ ). On average, male bee body mass was  $47.1 \pm 1.1$  mg (mean  $\pm$  SE).

#### 3.1. Survival assessment

No significant effect of pesticide exposure on bee survival was observed (Log-Rank tests:  $\chi^2 = 0.7$ ,  $df = 1$ ,  $p = 0.4$ ,  $n_{\text{control}} = 72$ ,  $n_{\text{pesticide}} = 71$ ), demonstrating that the tested concentration was sublethal. At the end of the three-day exposure period, 80.3 % and 83.3 % of the males survived in control and pesticide groups, respectively.

#### 3.2. Food consumption assessment

There was a significant positive effect of pesticide on daily food consumption, as compared to control (+27 %, LM:  $F = 6.78$ ,  $df = 1$ ,  $p < 0.05$ ;  $n_{\text{control}} = 57$ ,  $n_{\text{pesticide}} = 60$ , Fig. S1; Table S2). The cumulative food consumption over three days was  $0.257 \pm 0.015$  g/bee and  $0.328 \pm 0.019$  g/bee (mean  $\pm$  SE) for respectively control and pesticide-exposed bees. This led to a daily and cumulative sulfoxaflor intake dose of respectively  $2.19 \pm 0.13$  and  $6.56 \pm 0.39$  ng/bee (mean  $\pm$  SE). Increasing body mass significantly increased food consumption (LM:  $F = 5.1$ ,  $df = 1$ ,  $p < 0.05$ ; Fig. S1).

#### 3.3. Daily behaviour assessment

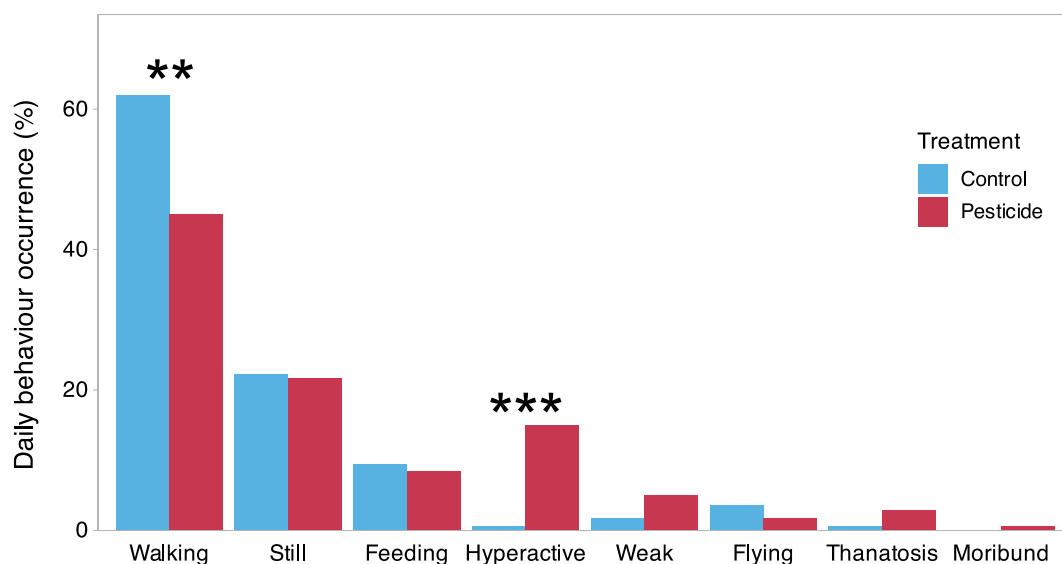
Pesticide exposure altered the behavioural activity of *O. bicornis* males ( $n = 117$ ) before mating. Specifically, there was a significant negative effect of pesticide on bee ‘walking’ (–31 % in pesticide-exposed bees as compared to control, GLMM:  $\chi^2 = 9.09$ ,  $df = 1$ ,  $p < 0.01$ ; Table S2) and a significant increased effect on ‘hyperactive’ behaviour (+2500 %, GLMM:  $\chi^2 = 30.85$ ,  $df = 1$ ,  $p < 0.001$ ; Table S2), as compared to control ( $n_{\text{daily behaviours, control}} = 171$ ,  $n_{\text{daily behaviours, pesticide}} = 180$ ; Fig. 1). There were no significant effects of pesticide exposure on ‘still’, ‘feeding’, ‘flying’, ‘moribund’, ‘thanatosis’, and ‘weak’ behaviours (Fig. 1; GLMM:  $\chi^2$ 's  $< 2.75$ ,  $df$ 's = 1,  $p$ 's  $> 0.05$ ). The frequency of abnormal behaviours was significantly greater in pesticide-exposed bees, as compared to control (+840 %; Fig. S2; GLMM:  $\chi^2 = 17.75$ ,  $df = 1$ ,  $p < 0.001$ ).

#### 3.4. Mating behaviour and sperm quantity assessment

In total, 116 males ( $n_{\text{control}} = 57$ ,  $n_{\text{pesticide}} = 59$ ) and 114 females were reared for the mating behaviour tests. Of the 116 males, only 90 were accepted by the female, and thus retained for the mating test ( $n_{\text{control}} = 46$ ,  $n_{\text{pesticide}} = 44$ ). Seventy-six males ( $n_{\text{control}} = 42$ ,  $n_{\text{pesticide}} = 34$ ) were accepted at the first attempt, while fourteen ( $n_{\text{control}} = 4$ ,  $n_{\text{pesticide}} = 10$ ) needed a second opportunity to be accepted by the female.

There was no significant effect of pesticide, as compared to control, on male acceptance (%), courtship duration (min), ICL duration (min), or post-copulatory phase duration (min) (Table 2; Figs. S2b–e). Pesticide exposure significantly altered nine mating behaviours, as compared to the control treatment (Table 2; Fig. 2a–h, S2a). We provided detailed summary statistics highlighting the pesticide effects on the fourteen measured mating and physiological variables in Table 2.

There was a significant negative effect of exposure on mating latency (–45 %; Table 2; Fig. S3a). Nonetheless, male acceptance rate was not significantly affected by the pesticide treatment (78 % overall, Table 2; Fig. S3b). Courtship duration was not significantly affected by the pesticide treatment (2min 18sec  $\pm$  46sec overall; Table 2; Fig. S3c). During courtship, sulfoxaflor led to a significant negative effect on the occurrence of pre-copulatory display (Table 2; Fig. 2a), where this specific mating behaviour was observed in 93.5 % of the controls and

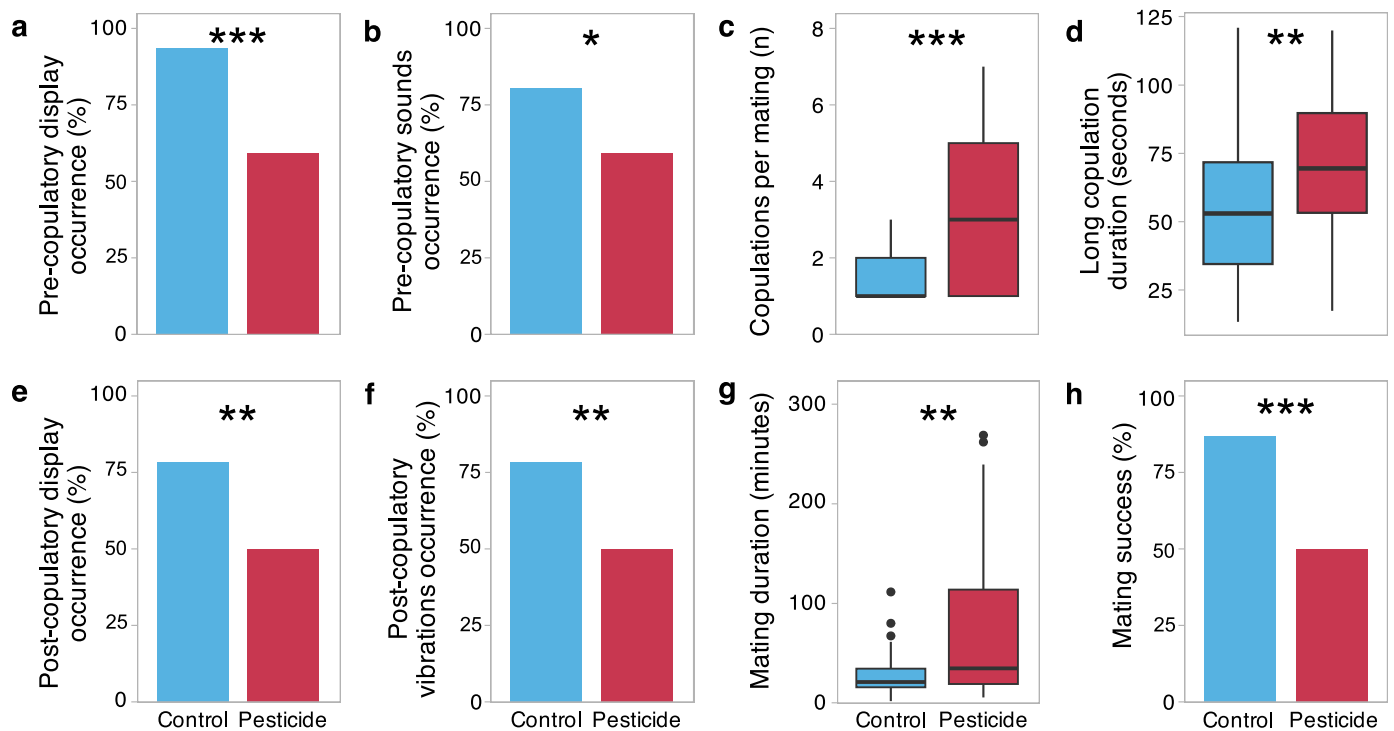


**Fig. 1.** Sublethal effects of field-realistic chronic pesticide exposure on solitary bee behaviours. The behaviours of 117 *Osmia bicornis* males were monitored during the three-day of chronic exposure to 33 % (w/w) sucrose solution (control) or 20 ppb of sulfoxaflor (pesticide) dissolved in 33 % (w/w) sucrose solution. The barplot shows how frequently each behaviour (see Table 1) was observed within the treatment groups. Significant differences (GLMM,  $n_{\text{control}} = 171$ ;  $n_{\text{pesticide}} = 180$ ) are indicated by asterisks (\*\* and \*\*\* respectively correspond to  $p < 0.01$  and  $p < 0.001$ ).

Table 2

**Summary of the effects caused by sublethal, field-realistic pesticide exposure on solitary bee (*Osmia bicornis*) reproduction.** We report average and Standard Error (SE), as well as significant differences among groups (indicated by asterisks: \*, \*\*, and \*\*\* respectively correspond to  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ ). Model estimated means of each statistically significant variable are reported in the [Supplementary Table S2](#).

	Control group		Pesticide group		Effect change of pesticide treatment, as compared to control (%)	Test	$X^2$	F	df	p-value
	Average	SE	Average	SE						
Mating latency	3min 48sec	19sec	2min 7sec	29sec	-45	LM	NA	32.58	1	***
Male acceptance	80.7 %	NA	74.6 %	NA	-8	GLM	0.62	NA	1	$\geq 0.05$
Pre-copulatory display occurrence	93.5 %	NA	59.1 %	NA	-36	GLM	16.08	NA	1	***
Pre-copulatory sounds occurrence	80.4 %	NA	59.1 %	NA	-27	GLM	4.94	NA	1	*
Courtship duration	1min 36sec	34sec	3min 1sec	1min 27sec	+89	LM	NA	3.30	1	$\geq 0.05$
Number of copulations	1.63	0.11	3.45	0.30	+110	GLM	24.93	NA	1	***
ICL duration	9min 9sec	58sec	15min 1sec	2min 50sec	+64	LM	NA	2.05	1	$\geq 0.05$
Long copulation duration	55sec	5sec	1min 13sec	6sec	+33	LM	NA	5.39	1	*
Post-copulatory display occurrence	78.3 %	NA	50 %	NA	-36	GLM	8.40	NA	1	**
Post-copulatory vibrations occurrence	78.3 %	NA	50 %	NA	-36	GLM	7.98	NA	1	**
Post-copulatory phase duration	19min 34sec	2min 16sec	33min 28sec	6min 37sec	+71	LM	NA	0.77	1	$\geq 0.05$
Mating duration	29min 13sec	3min 13sec	75min 51sec	11min 25sec	+166	LM	NA	9.84	1	**
Sperm quantity	131732	11090	173664	16872	-25	GLM	6.70	NA	1	**
Mating success	87 %	NA	50 %	NA	-43	GLM	14.98	NA	1	***



**Fig. 2. Sublethal field-realistic pesticide exposure affects solitary bee (*Osmia bicornis*) mating behaviours.** Pesticide (sulfoxaflor) effects on solitary male bees (a–b) pre-copulatory, (c–d) copulatory, (e–f) post-copulatory, and (g–h) overall mating phases are shown. The (a–b, e–f) mating displays, sounds, and vibrations, (c) number of copulations per mating, (d) duration of the long copulation, (g) duration of the overall mating process, and (h) overall mating success were recorded. Each box plot (c, d, g) shows the 25th and 75th percentiles (respectively the upper and lower side of the box, representing the central 50 % of the data), the median (horizontal line within the box), the highest and lowest values (respectively the upper and lower whiskers), and the outliers (individual points above the whiskers). Significant differences among groups are indicated by asterisks (\*, \*\*, and \*\*\* correspond to  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively;  $n_{\text{control}} = 46$ ,  $n_{\text{pesticide}} = 44$ ).

only 59.1 % of the exposed bees. Further, a significant negative effect of sulfoxaflor exposure was observed on the occurrence of pre-copulatory sounds made by exposed bees when compared to controls (Table 2; Fig. 2b). Pre-copulatory sounds were observed in 80.4 % of the control mating's, whereas in only 59.1 % of the sulfoxaflor exposed males.

Sulfoxaflor exposure significantly increased the number of

copulations (Table 2; Fig. 2c). Control males attempted  $1.63 \pm 0.11$  copulations, whereas sulfoxaflor exposed bees revealed on average  $3.45 \pm 0.30$  (mean [n]  $\pm$  SE), resulting in an increase of copulation attempts by 110 %. In addition, sulfoxaflor did not significantly affect ICL duration (Table 2; Fig. S3d), resulting in an average ICL duration across both treatments of  $12\text{min } 38\text{sec} \pm 1\text{min } 53\text{sec}$ . However, sulfoxaflor had a

significant positive effect on the long copulation duration (Table 2; Fig. S3d), resulting in a 32 % increase between the exposed (1min 13sec  $\pm$  6sec) and the controls (55sec  $\pm$  5sec) (Fig. S3d).

Overall, there was a significant negative effect of sulfoxaflor on the mating success of male bees (Table 2; Fig. 2h), resulting in 43 % fewer success in the pesticide group (50 %) as compared to the control group (87 %). Consequently, sulfoxaflor exposure significantly reduced the occurrence of post-copulatory display and post-copulatory vibrations (Table 2; Fig. 2e and f). Post-copulatory display and vibrations were observed in 78.3 % of control matings, whereas exposed bees exhibited post-copulatory display and vibrations in 50 % of the mating's (Fig. 2d and e). Sulfoxaflor did not significantly affect post-copulatory phase duration (Table 2; Fig. S3e), resulting in an average duration of 26min 22sec  $\pm$  3min 30sec. However, sulfoxaflor exposure had a significant negative effect on the total mating duration (Table 2; Fig. 2g). Sulfoxaflor exposure increased the average mating duration by 166 %: the duration of mating was 29min 1sec  $\pm$  3min 13sec and 75min 51sec  $\pm$  11min 25sec, respectively in control and pesticide groups (Fig. 2g).

Male body mass had a significant positive effect on the number of copulations (GLM:  $\chi^2 = 4.75$ ,  $df = 1$ ,  $p < 0.05$ ), and subsequently on the total mating duration (LM:  $F = 4.50$ ,  $df = 1$ ,  $p < 0.05$ ). In contrast, there was no significant effect of male body mass on the remaining mating behaviours, including male acceptance, pre-copulatory display and sounds occurrences, post-copulatory display and vibrations occurrences (GLM:  $\chi^2$ 's  $< 0.88$ ,  $df$ 's = 1,  $p$ 's  $> 0.347$ ), as well as in courtship duration, ICL duration, and post-copulatory phase duration (LM:  $F$ 's  $< 1.84$ ,  $df$ 's = 1,  $p$ 's  $> 0.181$ ). The pesticide treatment caused a significant reduction of sperm quantity in male bees (-25 %;  $n_{\text{control}} = 33$ ,  $n_{\text{pesticide}} = 37$ ; Table 2; Fig. 3a). Irrespective of the treatment, bee body mass had a significant positive effect on sperm quantity (GLM:  $\chi^2 = 10.21$ ,  $df = 1$ ,  $p < 0.01$ ;  $n_{\text{control}} = 33$ ,  $n_{\text{pesticide}} = 37$ ; Fig. 3b): heavier bees had higher sperm counts.

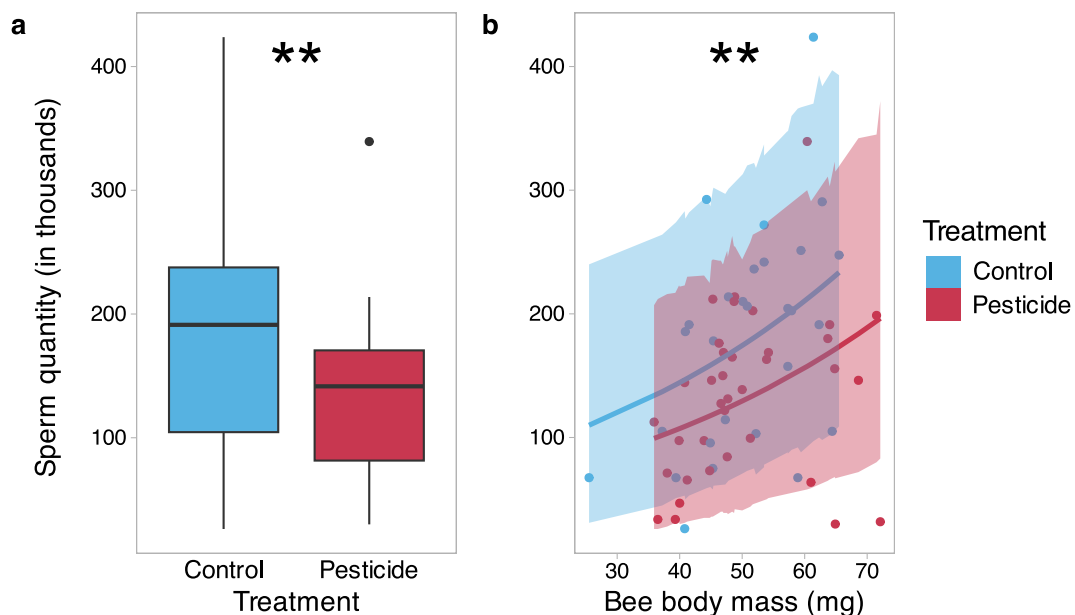
The data revealed significant correlations between specific mating behaviours ( $p < 0.05$ ; Table S3). The mating duration resulted positively correlated with the number of copulation (Pearson; 0.6), ICL duration (0.6), and Courtship duration (0.3). There was also a positive correlation between pre and post-copulatory display (0.3) and between ICL duration

and Courtship duration (0.3). Display occurrence resulted negatively correlated with mating duration and (-0.3) and the number of copulations (-0.3).

#### 4. Discussion

Our novel data reveal that sublethal field-realistic exposure to a commonly used pesticide negatively affects the mating behaviour, mating success, and male fertility of a key pollinator, *Osmia bicornis*. While pesticide exposure showed no effect on male survival, the pesticide caused numerous sublethal behavioural and physiological effects, such as increased hyperactivity, increased food consumption (+27 %), reduced courtship behaviours (display: 36 %, sounds: 27 %), increased mating duration (+166 %) while reducing mating success (-43 %) and sperm quantity (-25 %). These findings are likely explained by the pesticide altering the central nervous system of bees, thereby impairing their cognitive, sensory, and motor abilities as well as reproductive behaviour and physiology (Blacquière et al., 2012; Siviter and Muth, 2020; Tosi et al., 2022). The observed findings highlight that pesticides can reduce male fitness which may inevitably lead to drastic consequences at the population level. The data offer a plausible mechanistic explanation for the ongoing decline of wild bee populations and underscore the urgent need to find sustainable solutions in agriculture that safeguard pollinators and biodiversity.

As predicted, the tested concentrations revealed no significant lethal effects, confirming that field-realistic sulfoxaflor levels were sublethal, adding to previous results in honey bees and bumble bees (Cheng et al., 2023; Linguadoca et al., 2021). Similarly, other studies did not find differences for *Osmia* female survival rates. For example, Boff et al. (2021) exposed *O. bicornis* females to field-realistic concentrations of sulfoxaflor (10 ppb, and 50 ppb), obtaining 80 % of survival rate after 72 h exposure in both control and pesticide groups. Our data further support recent studies suggesting that *Osmia* spp. show greater sensitivity to compounds that target nicotinic acetylcholine receptors (nAChRs) receptors, compared to pesticides with a different mode of action (Arena and Sgolastra, 2014; Biddinger et al., 2013; Sgolastra et al., 2017; Uhl et al., 2018). Although lethal effects of field-realistic



**Fig. 3.** Sublethal effect of field-realistic pesticide exposure on solitary bee (*Osmia bicornis*) sperm quantity. The impact of the pesticide (sulfoxaflor) treatment on bee (a) quantity of sperm and (b) its relationship with body mass (mg) was assessed in control ( $n = 33$ ) and sulfoxaflor-treated ( $n = 37$ ) *O. bicornis* males. (a) Box limits indicate the range of the central 50 % of the data, with a central line marking the median value, whiskers extending to the lowest and highest values, and outliers indicated as dots. (b) Lines indicate the estimates marginal means by GLM (methods and results). Significant differences among treatments (GLM) are indicated by asterisks (\*\* corresponds to  $p < 0.01$ ).

concentrations of sulfoxaflor are rare, any decrease in fitness, beyond mere survival, will have profound implications for all species.

Our exposure scenario significantly increased hyperactivity and food consumption, as compared to control bees. Insecticides typically lead to overexcitation of the central nervous system of bees causing hyperactivity (Cresswell et al., 2014; Tong et al., 2019; Tosi and Nieh, 2019) which can lead to increasing their speed during walking (Tosi and Nieh, 2017) and flight (Tosi et al., 2017), as well as their respiration rates (Han et al., 2018). As locomotion and coordination are essential for flight ability and foraging success (Tosi et al., 2017), the behavioural impairments shown here may impact the quality of the pollination service provided by bees. The increased hyperactivity caused by pesticide-treated bees may have consequently increased bee energy – and thus food – consumption. Food consumption of *Osmia* females has been increased by sublethal sulfoxaflor exposure (Azpiazu et al., 2022, 2024), while other pesticides (i.e., clothianidin, glyphosate) did not cause significant effects (Strobl et al., 2020) and others (i.e., thiamethoxam, flupyradifurone) caused a decrease in food consumption in honey bees (Tosi et al., 2021; Tosi and Nieh, 2019; Tong et al., 2019). These effects can lead to wider behavioural consequences in the field (Straub et al., 2021; Mokkapati et al., 2021), potentially increasing the time bees spend on flowers consequently increasing the time needed to satisfy their nutritional requirements, and leading to metabolic disorders causing physiological disruptions, damaging, and weakening of bees.

Irrespective of the treatment groups, we reported an average daily sucrose solution (33 %, w/w) consumption in *O. bicornis* males of  $97.8 \pm 0.1$  mg per bee per day, during the three-day of exposure, meaning 32.3 mg of pure sucrose daily. Our results support previous results (Henriques-Martins et al., 2023) which reported an average daily sucrose solution (38 %, w/v) consumption of 118 mg per bee in male *O. bicornis*, which equals to 44.8 mg of pure sucrose daily. Similarly, Azpiazu et al. (2023) reported an average daily syrup (50 %, w/w) consumption of *O. bicornis* females around 90–100 mg per bee per day during the first three days of feeding, which means 45–50 mg of pure sucrose daily. Food consumption is typically maximum during the first days of life, and it decreases as they age (Azpiazu et al., 2023). Strobl et al. (2020) reported an average daily sucrose solution (50 %, w/w) consumption in *O. bicornis* females of 73 mg per bee, which means 36.5 mg of pure sucrose daily. These results are comparable to or exceed our findings, despite feeding bees a less concentrated sucrose solution (i.e., 33 % w/w). By contrast, Azpiazu et al. (2019, 2022) reported that *O. bicornis* females consumed around 50–60 mg of sucrose solution (33 %, w/w) per bee per day, which means 17–20 mg of pure sucrose daily. Taken together and considering the smaller body size of *O. bicornis* males, these results highlight that, as in other wild bee species, males consume more food than females. As in other wild bee species, a plausible explanation may be due to *O. bicornis* females have higher fat body reserves and a slower metabolism when compared to their male counterparts (Linguadoca et al., 2021). Our results concluded that male bee's body mass had a positive significant effect on the number of copulations performed. Heavier bees may have had greater energy reserves leading to additional copulations, especially when they did not achieve a long copulation at first.

Our novel results demonstrate that sublethal, field-realistic pesticide exposure can turn mating into a long, risky, and unsuccessful process in solitary bees. The pesticide-exposed males that performed a successful mating required almost three times longer than control males. Typically, *Osmia* males hid underground to avoid predation mostly by birds and rodents (Kierat et al., 2017) and only expose themselves when in search of a female to mate (Raw, 1972, 1976; Krunic et al., 2005; Kierat et al., 2017). While assessing the potential impact of increased predation risk for *O. bicornis* during mating is valuable, quantitative population-level data remain unavailable. However, by extending all mating phases and increasing the number of copulations, the pesticide inevitably exposes the mating bees to an increased risk of predation. Further, our data

revealed that pesticide exposure can reduce male's pre-copulatory mating displays and sounds by respectively 36 % and 27 %. Considering that *O. bicornis* females prefer males performing longer pre-copulatory sounds (Conrad et al., 2010), the effect of pesticides on this mating display may considerably reduce the likelihood of the males being accepted by females. Our data supports previous studies in which *O. bicornis* males were exposed to sublethal concentrations of the fungicide fenbuconazole (Boff et al., 2022). Boff et al. (2022) demonstrated that pesticide exposure significantly reduced the pre-copulatory sounds frequency of modulation (Hz) in *O. bicornis*, with a subsequent impairment in their mating success. The observed negative effects of the pesticide on mating displays appears a plausible mechanistic explanation for impaired male acceptance and relative mating success. Indeed, sulfoxaflor exposure reduced the number of *O. bicornis* males which successfully mate. Given that such effects will inevitably reduce fitness, further negative effects on male and female reproductive physiology would further amplify the potential consequences of pesticide exposure at the individual fitness level.

We demonstrate for the first time that sulfoxaflor exposure can impair male bee fertility by reducing the sperm quantity. Our results extend previous results showing that neonicotinoids can impair mating success and fertility of male bees (Straub et al., 2016, 2022a), including solitary bees (Strobl et al., 2021; Sandrock et al., 2014). For instance, Sandrock et al. (2014) reported the pesticide exposure effects on *O. bicornis* fertility, resulting in a significant reduction in the offspring production, and a significantly biased on offspring sex ratio. Similarly, Strobl et al. (2021) demonstrated that field-realistic sublethal concentrations of a common neonicotinoid significantly reduced the sperm quantity as well as the sperm viability in *O. cornuta*. As sperm quantity is determined during the pupal stage when spermatogenesis occurs (Armbruster, 1913), potential reasons for the observed reduction in sperm may be the cytotoxic lethal effects on sperm cells induced by pesticides, or pesticide effects on completing sperm maturation inside the vesicula seminalis. Furthermore, previous studies revealed that sulfoxaflor increases oxidative stress that can induce apoptosis in honey bee sperm cells (Chakrabarti et al., 2020; Ibrahim et al., 2023). These findings would support our speculations as to why *O. bicornis* males had reduced sperm counts. Additional reasons may be that dead or dying sperm cells may undergo biological degradation or be reabsorbed by macrophages and phagocytic dendritic cells (Larsen et al., 2019; Chen and Zheng, 2021; McDonald, 2000; Ribas-Maynou et al., 2022). Lastly, sulfoxaflor may further influence the sperm developmental process inside the vesicula seminalis, previously studied in honey bees (Klein et al., 2021). Irrespective of the underlying mechanism, a reduction in sperm quantity will have down-stream negative consequences on the number of females a male can successfully inseminate. Given fewer inseminated females would yield reduced female offspring, our data provide an additional mechanistic explanation for recently observed declines in wild bee populations (e.g., Rundlöf et al., 2015; Woodcock et al., 2017). Future research should investigate a broader range of pesticide concentrations and their sublethal effects in field conditions, focusing on their impact on sperm development and viability.

We reveal that pesticides alter bee behaviours before, during, and after mating (see also Tosi et al., 2022 for a meta-analysis). This may translate to wider fitness impairments, including alterations on the ability of bees to forage, defend territories against competing males, and find females. Additional data are required to link the observed negative behavioural traits with potential down-stream negative consequences on behaviours displayed under field conditions (e.g., foraging efficiency, defending territory, finding females). Given that bees are exposed to pesticides for even longer periods (Tosi et al., 2018), future research should also include longer-term studies to better understand the chronic, sublethal impacts of pesticides on bee health and their reproductive capacities.

This study enhances understanding of *Osmia* mating behaviour, demonstrating that *O. bicornis* shares similar mating patterns, in terms of

both structure and timing, with *O. cornuta* (Felicioli et al., 1998, 2023). Consistent with previous research, we confirm a positive correlation between male body size and sperm quantity, as seen in *Melipona beecheii*, *O. cornuta*, and *Apis mellifera* (Pech-May et al., 2012; Schlüns et al., 2003; Strobl et al., 2019).

A key contribution of our work is the inclusion of male bees in ecotoxicological studies, addressing a critical gap in pollinator research (Koeniger, 2005). Our research underscores the urgency to include male bees in ecotoxicological assessments to enhance current risk evaluation frameworks. While most research on pesticide impacts focuses on females, particularly in solitary bees (Azpiazu et al., 2019, 2022, 2024; Stuligross and Williams, 2020, 2021), incorporating the neglected male sex in bee health assessments is essential, as impaired male health will inevitably have far-reaching negative consequences at the population-level.

## 5. Conclusion

We demonstrate that common, field-realistic pesticide exposure widely impairs solitary bee (*O. bicornis*) fitness, specifically revealing the subtle significant impacts caused on multiple male bee behaviours both before (hyperactivity) and during (alteration of courtship and copulation) mating. We finally demonstrate that sublethal exposure impairs fertility and mating success in this common bee species. In combination with real-world data on environmental contamination (Tosi et al., 2018; Traynor et al., 2021) and previous findings (Straub et al., 2022a, 2022b; Strobl et al., 2021; Stuligross and Williams, 2020), the available knowledge makes a strong case for how environmental pollution can negatively affect wild bees as well as potentially other wild animal populations (Goulson et al., 2015; MacInnis et al., 2023). The integration of fitness-relevant sublethal parameters in future risk assessments schemes (More et al., 2021; Tosi et al., 2022) appears crucial to better understand and mitigate the inadvertent negative effects of pesticides individually and in combination with other stressors. Given the ubiquitous use of pesticides, our results raise broader concerns over their impact on the reproduction and fitness of both popular and less-known pollinators and animals. More accurate assessments of pesticide exposure, toxicity, and risks are urgent to fully grasp their potential harm, support science-based regulatory decisions, and finally minimize inadvertent negative effects on our ecosystems.

## CRedit authorship contribution statement

**Luis Vélez-Trujillo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Luca Carisio:** Writing – review & editing, Validation, Methodology, Investigation. **Ewa Popiela:** Writing – review & editing, Methodology, Investigation. **Lars Straub:** Writing – review & editing, Methodology, Conceptualization. **Simone Tosi:** Writing – review & editing, Visualization, Resources, Project administration, Methodology, 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.chemosphere.2025.144335>.

## Data availability

All data are provided freely through the platform Open Science Framework (OSF).

[Survival, food consumption, daily behaviours, mating behaviour, sperm quantity, and model characteristics \(Original data\)](#) (OSF Storage)

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