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Original article

The ground-nesting bee *Anthophora plumipes* as a model species for assessing effects of soil-mediated pesticide exposure

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Abstract – Though many wild bee species nest in the ground, little is known of their potential exposure to pesticide residues in soil, or the effects of such exposure. Here, we introduce *Anthophora plumipes* as a potential model ground-nesting solitary bee species for controlled exposure to pesticides through soil. Bees from a naturally occurring population were allowed to nest in loam blocks containing varying concentrations of the neonicotinoid imidacloprid. Measured residues of imidacloprid in brood provisions and in bee bodies remained at $<0.01\%$ of the concentration in surrounding soil, suggesting limited migration of contaminants from soil to brood. Furthermore, imidacloprid contamination had no marked effect on the number, survival, body size or rate of parasitism of offspring at the tested concentrations (≤ 10 mg/kg). This species native to Eurasia and North Africa may be a suitable model for further research on the ecotoxicology of ground-nesting solitary bee species.

Ground-nesting bee / Solitary bee / Plant protection product / Risk assessment / Neonicotinoid / Kleptoparasitism

1. INTRODUCTION

Modern agriculture is largely dependent on the use of agrochemicals (fertilizers, fungicides, herbicides and insecticides) to combat pest species and maximize yield (Godfray et al. 2010). There is a trade-off between using these compounds effectively against pests while avoiding excessive harm to beneficial organisms, such as pollinating insects and the natural enemies of those pests. Wild bees provide substantive pollination services to crops and wild plants (Garibaldi et al. 2013). Yet there is mounting evidence that wild bee communities are negatively affected by agricultural practices relating to intensification of land use, including use of pesticides (Rundlöf et al. 2015; Woodcock et al. 2016; Dicks et al. 2021), with bee species diversity decreasing in areas where agricultural land use is intense (Bommarco et al. 2012; Bartomeus et al. 2013; Ollerton et al. 2014; Powney et al. 2019).

In order to promote safer use of pesticides, environmental risk assessments are performed on agrochemical compounds, including assessing the risks of exposure and harm to bees and

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other beneficial arthropods, often requiring strategies for mitigation of risks before a substance is licensed for use (Siviter et al. 2023). However, risk assessment traditionally focuses on ecotoxicological testing of just one bee species, the honey bee, *Apis mellifera* (Godfray et al. 2014; Pisa et al. 2014; Woodcock et al. 2016). Because of the varied physiology, life histories and ecology of bee species, there have been repeated calls for a more thorough risk assessment of additional bee species (Franklin and Raine 2019; Dietzsch and Jütte 2020; Topping et al. 2020; Willis Chan and Rondeau 2024), especially with regard to possible exposure routes and associated exposure risks that are not applicable to the honey bee (Boyle et al. 2019). Indeed, the impact of pesticides on non-*Apis* bees receives ever-increasing interest from the scientific community, although the honey bee still dominates as a model species in most studies (Siviter et al. 2021; Dirilgen et al. 2023). Other bee species that have more recently been used in ecotoxicological studies include bumble bees (*Bombus* spp.) and above-ground cavity-nesting solitary bees such as mason and leafcutter bees (Family Megachilidae) (Dirilgen et al. 2023). Most recently, acute pesticide exposure of multiple novel ground-nesting bee species, including members of the genera *Andrena*, *Colletes* and *Halictus*, has been conducted to assess intraspecific sensitivity (Jütte et al. 2023; Dewaele et al. 2024).

Exposure to agrochemicals can be dietary (ingestion of contaminated pollen/nectar) or through physical contact (e.g. through nesting material, plant material and dust). For bee species that nest in the ground (i.e. fossorial), a largely overlooked potential agrochemical exposure route is through soil contact (Sanchez-Bayo and Goka 2014; Franklin and Raine 2019). Pesticide residues in soils are widespread, with 80% of sampled European topsoils containing at least one compound (Silva et al. 2019). Rondeau et al. (2022) found one or more pesticide residue in soil sampled from bumble bee (*Bombus* spp.) queen hibernation sites, showing that some bee species may come into close contact with these residues.

The majority of all bee species are ground-nesting (Michener 2007; Danforth et al. 2019) spending their larval, pupal, teneral and hibernating stages in brood chambers built in soil, and females additionally spend their adult life constructing and provisioning nests and therefore handling or being in contact with soil (Harmon-Threatt 2020). Yet, almost nothing is known about the potential hazards of exposure to soil-bound pesticides for ground-nesting bee species. This knowledge gap is partly due to the lack of suitable experimental model ground-nesting bee species that can be readily reared and manipulated in large numbers. Proposed model organisms include the alkali bee (*Nomia meladeri* Cockerell 1906), commercially managed but restricted to alkaline soils in western USA (Boyle et al. 2019), and the hoary squash bee (*Eucera* (*Peponapis*) *pruinosa* Say 1837), on which Willis Chan et al. (2019) conducted the first-ever risk assessment of a ground-nesting wild bee species to insecticide exposure through soil. This species was also the subject of experimental exposure scenarios in greenhouses, which revealed that soil-drench treatment with various insecticides and fungicides led to decreased nesting activity and offspring survival, although residues were present in both the forage (nectar and pollen) and in the soil, so that the effects of food-borne exposure *versus* soil exposure could not be disentangled (Willis Chan and Raine 2020; see also Rondeau and Raine 2024b). Bumble bee queens have been experimentally exposed to contaminated soil during hibernation, with no effect on survival but with varying effects on colony founding success (Linguadoca et al. 2024; Rondeau and Raine 2024c). Other experiments attempting to assess the risk to solitary bees from soil exposure have instead relied on cavity-nesting species that use mud for nest construction (e.g. *Osmia* spp.) as a proxy for ground-nesting wild bee species, which may not capture the full extent of soil contact of ground-nesters (Jütte et al. 2017; Anderson and Harmon-Threatt 2019). So far, no European ground-nesting solitary bee has been investigated with respect to pesticide exposure through soil.

Ground-nesting bees have, however, been successfully raised in field and laboratory setting to study their behaviour or to investigate their suitability as pollinators (Leonard and Harmon-Threatt 2019). Indeed, various species of anthophorid bees have been suggested as suitable for domestication as pollinators (Thalmann and Dorn 1990; Batra 1994; Louadi 2008; Graham et al. 2015; Adhikari and Miyanaga 2016b). Management has been attempted using portable blocks of loam as nesting material to encourage nesting activity, increase population size and permit translocation of brood to new localities. Thalmann and Dorn (1990) and Thalmann (1991) undertook studies on *A. plumipes* domestication for greenhouse pollination by optimizing nest box design as well as soil substrate and moisture levels. Batra (1994) described establishing new aggregations of the exotic *Anthophora villosula* Smith 1854 (then called *A. pilipes villosula*), a sister species to *A. plumipes*, using 15–20-cm-thick 'portable adobe blocks', while Graham et al. (2015) established new aggregations of *Anthophora abrupta* by subdividing an existing nest aggregation and encouraging nesting of emerging bees in 'hardened clay blocks' at a new location. Adhikari et al. (2016a) successfully reared *A. plumipes* (probably *A. villosula*) in a greenhouse using 20-cm-deep 'portable clay blocks'.

Building on this historical method development, we used the hairy-footed flower bee *Anthophora plumipes* (Pallas 1772, syn. *A. acervorum* Linnaeus 1758, *A. pilipes* Fabricius 1775), a ground-nesting wild bee species, for experimentally testing the off-target impacts of insecticide via soil exposure. Using a simple loam-block design as a trap nest, we induced the nesting of native *A. plumipes* in soil-filled nest units installed next to an existing nesting aggregation. To test the effects of pesticide exposure via soil, we used the neonicotinoid pesticide imidacloprid (IMI) as a test substance. With a contact median lethal dose (LD_{50}) of 0.025 and 0.22 µg/bee for honeybees and *Bombus terrestris* respectively, IMI is considered highly toxic to bees; it is widely known to cause lethal and sub-lethal effects on bees upon contact or ingestion (Lundin et al. 2015; Dirilgen et al. 2023) and to be persistent in soil (EFSA 2008). Residues of IMI in the soil matrix have been shown to have behavioural and physiological effects on multiple soil-living invertebrate taxa that can be lethal (reviewed in Pisa et al. 2014). Despite recent bans on its outdoor use in the European Union, the neonicotinoid IMI is still a widely used insecticide worldwide (Casida 2018).

To validate the methodology presented herein, we investigated if IMI mixed into the soil nesting substrate led to decreased *A. plumipes* nesting activity by assessing the number of brood cells constructed and the number of fully provisioned brood cells per trap nest. We assessed the survival of brood from early instar to eclosion and the incidence of infestation by parasites. The body size of eclosed brood was measured, as variation in body mass between IMI treatments could indicate either a direct cost of insecticide exposure itself (energetic investment in detoxification or other disruptions to development), or decreased maternal investment during provisioning (smaller provision sizes). The sex ratio of eclosed brood was assessed to investigate if there are sex-specific lethal effects of insecticide exposure during development. Finally, to determine the potential exposure to and translocation of IMI from soil to brood, we measured the concentration of IMI in soil substrate, brood cell provisions and bee bodies.

2. MATERIAL AND METHODS

2.1. Study species

The hairy-footed flower bee, *A. plumipes*, is a polylectic, univoltine, spring-flying bee native to central and southern Eurasia and North Africa (Westrich 2019). Nests of *A. plumipes* can be found in silt- and clay-rich soils, or even in soft sandstone, oriented horizontally or vertically in soil bluffs or in man-made structures such as old clay-rich walls (Loonstra 2009; Westrich 2019; Orr and Koch 2023). The nest consists of a short passage with clusters of brood cells (Fig. 1) at 2–20 cm depth, in which one egg

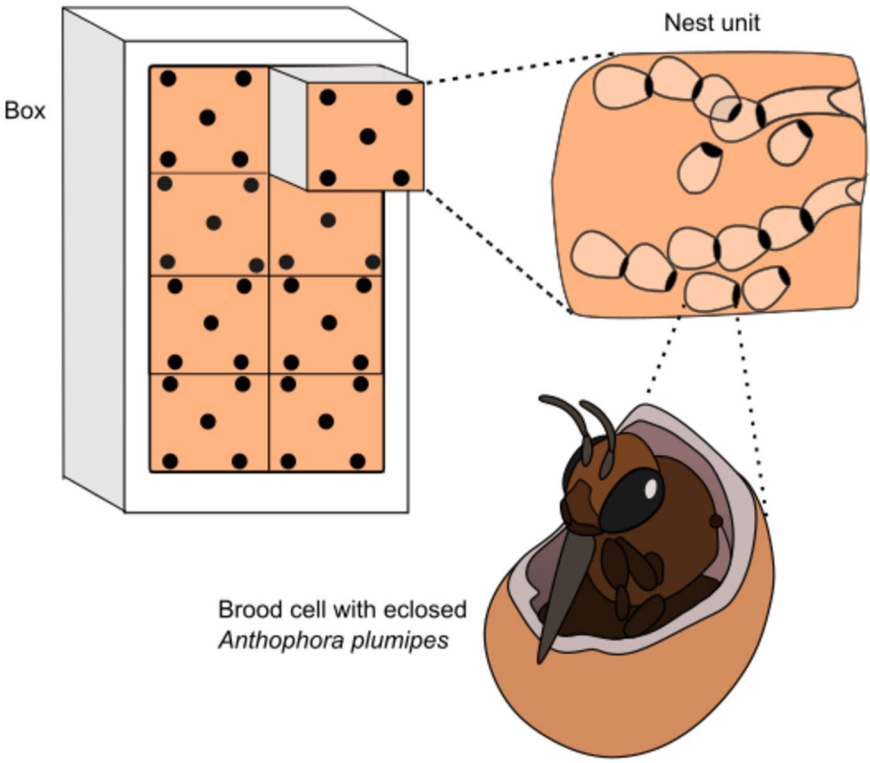

Fig. 1 Schematic illustration of the nest design with a box of eight removable 1 kg nest units (left), an example of brood cell distribution inside a section of such a nest unit (right top) and an opened brood cell with an ecolsed *A. plumipes* female (bottom right)

is placed per cell on a comparatively viscous pollen-nectar liquid provision (Loonstra 2009). The inside of the brood cell is lined with a layer of waxy secretion from the mother's Dufour's gland (described for *Anthophora abrupta* Norden et al. 1980). The larva consumes the brood provisions and parts of the cell lining, remains in the prepupal stage until August, metamorphoses and hibernates as an imago (Loonstra 2009). Known parasites of *A. plumipes* include the kleptoparasitic cuckoo bee *Melecta albifrons* Forster 1771 and the chalcid wasp *Monodontomerus obscurus* Westwood 1833 (Thalmann and Dorn 1990; Grissell 2007; Westrich 2019). In recent years, *Sitaris muralis* Forster 1771, a kleptoparasitic meloid beetle of anthophorid bees, has expanded across central Europe and is increasingly common in anthophorid nests within our study region (Lückmann 2016).

2.2. Preparation of soil blocks as nest units

The design and preparation of the nest units for *A. plumipes* were modified from Thalmann and Dorn (1990). Soil was collected from a nearby garden area where pesticide had not been used for > 30 years. The soil was characterized as a silt loam (Supplementary information S1).

Dry soil was gradually mixed with water applied through a spray bottle (100 mL/kg of dry substrate). As containers for the soil, we used oblong cartons with a thin inner plastic coating (9 × 9 × 20 cm or 11 × 7 × 20 cm). Each carton was incrementally filled with 1 kg of soil–water mixture and lightly compressed to a depth of 15 cm. Thereafter, indentations (ca. 1 cm diameter and 4 cm deep) were made into

the soil every 3–5 cm along the outer perimeter of each block to encourage nesting. The soil substrate was intentionally softer than previously described (Thalmann and Dorn 1990) to facilitate removal of intact brood cells. The outermost layer of soil was sprayed with water to make it harden upon drying, preventing it from easily breaking during the field season. The basal corners of the cartons were cut to prevent excess moisture from collecting in the innermost layer of soil, and the soil-filled cartons were then left to dry for 6 days at room temperature. These 1 kg soil-filled cartons are from here on referred to as nest units. We placed up to 8 such nest units into a larger polystyrene box (inner measurements 18 × 40 × 20 cm) for easier transport and placement in the field (Fig. 2).

2.3. Field-realistic concentration of IMI

In the first experiment, a field-realistic concentration of 0.11 mg/kg IMI was selected, within the range of environmental concentrations in soil (EFSA 2008). A stock solution of 33.3 mg/L was created by mixing 10 mg pure IMI (PESTANAL analytic standard, Purity: ≥ 98.0%, CAS: 138261–41-3) in 300 mL distilled water on a magnetic stirrer for 6 h. The ensuing mix was diluted in water 1.1 mg/L and mixed with dry soil substrate 1:10 w/w (100 mL liquid to 1 kg dry soil substrate). For control treatment units, soil was mixed with distilled water lacking IMI using separate tools. Thirty nest units per treatment were prepared. Nest units were placed in 4 polystyrene boxes (Fig. 1, Fig. S2), always alternating control and treatment units within the same box in order to minimize effects based on location within the box. The boxes were placed in the Halle University's botanical gardens in Halle (Saale), Germany (51°48′90.1″N 11°95′90.1″E) on 30th of March 2021, directly in front of a large nesting aggregation of *A. plumipes* in a loam and stone wall, facing east, and protected from rain and direct sunlight by a roof. During their flight season, *A. plumipes* females colonized nest units and provisioned brood cells therein. In mid-July 2021, ca. 80 days after setting boxes out in the field and after the end of the *A. plumipes* nesting season, the boxes containing nest units were dismantled and moved to a protected outdoor location. In December the same year, nest units were excavated to count the number of brood cells produced and assess their contents.

2.4. Dose–response experiment with IMI

Using the same methodology as used in 2.3 (above), soil-filled nest units were prepared with a series of increasing IMI concentrations (0, 0.62, 1.25, 2.5, 5.0, 10.0 mg/kg, $N = 5$ nest units/concentration). For context, the median lethal IMI concentration (LC_{50}) for earthworms is 10.7 mg/kg and the 'no observable effects' concentration for springtails (*Folsomia candida*) is 1.25 mg/kg (EFSA 2008). A stock solution of 100 mg/L IMI mixed in distilled water was prepared, and subsequent concentrations were created via 2-factor serial dilution in distilled water. For each concentration and for the control treatment (distilled water only), 5 kg of dry soil substrate was mixed with 500 mL of treatment solution. From each 5 kg batch of soil, five 1 kg carton nesting units were prepared as described above. After drying, the treatments were mixed such that each of four polystyrene boxes contained nest units from all concentrations placed randomly within the box. Polystyrene boxes were then deployed at the same field site as described above. The nesting material was set out on 30th of March 2022 until mid-July 2022, when nesting activity had ceased. Nest boxes were subsequently dismantled and brood cells excavated from the 1 kg nest units in January of the following year to count the number of brood cells produced and assess their contents.

2.5. Assessment of IMI residues

We sampled soil, brood cell provisions and adult bees to assess the actual exposure to IMI and the longevity of IMI in these matrices. In the field-realistic experiment, soil samples for

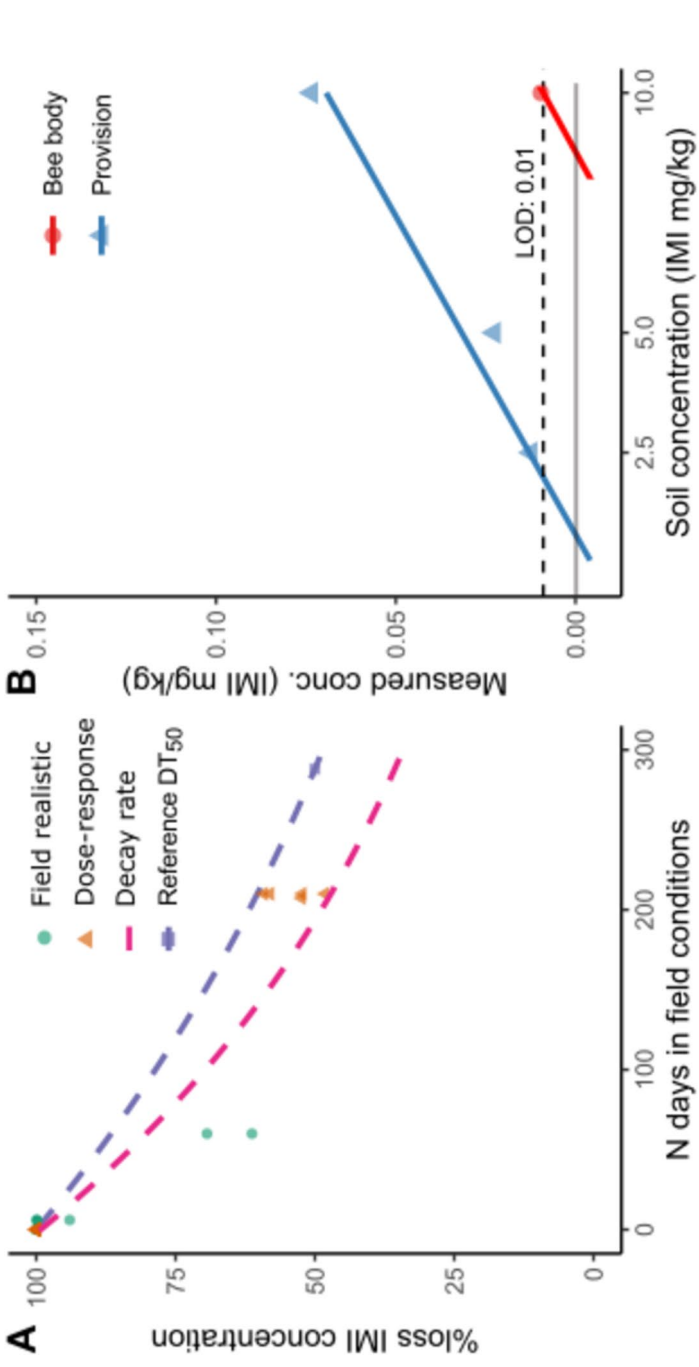

Fig. 2 **A** The percentage loss of IMI in samples taken from the nest units used in the field compared to the measured concentration at nest establishment. Dashed lines represent decay rate as calculated from the measured samples (purple dashed line), together with a reference value from EFSA (blue dashed line). **B** Measured concentration of IMI in adult bee bodies and brood cell provisions. Trend lines are included to show the estimated concentration below the limit of detection (LOD, dashed line)

residue analysis (see Supplementary information S3) were taken from freshly prepared substrate, and subsequently at day 60 after nest preparation ($N = 2$ samples per time point). In the dose–response experiment, freshly prepared substrate and substrate at 210 days after nest preparation were sampled ($N = 1$ sample per time point and concentration). Samples of live adult imagoes and unconsumed pollen provisions were collected each year at the time of nest excavation. All samples were stored at − 20 °C until residue analysis (Supplementary information S2). Based on the results from the residue analysis, the decay rate and corresponding half-life, or decay time (DT_{50}) was calculated (Supplementary information S3).

2.6. Assessment of nesting and brood parameters

Nest units were removed from their carton containers and carefully broken apart to reveal the individual brood cells within. Nest-provisioning females moisten the cell walls during nest construction, making them harder than the surrounding soil matrix when dry, and allowing brood cells to be excavated intact. Due to the cluster-like distribution of brood cells sometimes filling up large parts of a nest unit, reliably associating any given brood cell with a specific nest entrance was not possible (Fig. 1). Additionally, usurpation of nests between females is common, thus calculating brood cells per nest would not accurately reflect the effort of one female (Černá et al. 2013). Instead, the number of brood cells per nest unit (i.e. per ca. 1 kg of soil) was counted.

The relative depth of brood cells in the nest units (0–5, 6–10 or 11–15 cm from the soil surface) was recorded in a subset of nest units. In the field-realistic experiment, three nest units from the control treatment had broken apart so that a significant part of the soil was missing. With these excluded, the final sample size in the field-realistic experiment was $N = 27$ and $N = 30$ nesting units per control and treatment respectively. In the dose–response experiment, all 30 nest units (5 per IMI concentration) were intact.

Brood cells were stored at 4 °C or temporarily on ice while handling so as to not disturb hibernating imagoes. Each brood cell was examined by carefully scraping a small hole in its side to visually determine its contents. The following parameters were noted: total number of brood cells per nest unit, the number of empty brood cells (no or minor provision), the number of parasitized brood cells (and parasite identity, when possible) and the survival status of the brood in the remaining cells. The survival status at different developmental stages was scored in three categories: 1 = early instar mortality (pollen provision intact, partly consumed or moldy), 2 = late instar mortality (dead larva/prepupa/pupa, pollen provision fully consumed), 3 = eclosed imago (alive or dead as imago). Imagoes were briefly observed at room temperature for movement and those that did not respond to prodding were recorded as dead.

As few adult offspring were dead at the time of nest excavation and inspection of brood cells in January ($N = 21$ in the field-realistic experiment and $N = 6$ in the dose–response experiment across all treatments), all other bees that survived until eclosion were placed in the same category. In brood cells containing a provision mass largely consumed by fungus, early instar mortality was assumed even if no egg/larva could be directly observed. Imagoes were sexed based on the colour of the clypeus (pale: male; dark; female) or leg morphology, then returned to 4 °C until the following spring. In the dose–response experiment, imagoes were additionally removed from their brood cells and weighed to the nearest mg before being returned to their brood cells (or to opaque Petri dishes for offspring from broken brood cells) and held at 4 °C.

Additional methods for housing *A. plumipes* adults in the laboratory and orally administering known doses of pesticide to test adult bee sensitivity are described in the supplementary information S4, S5.

2.7. Statistical analysis

All statistical analyses were performed in R version 4.0.2 (R Core Team 2019).

The impact of pesticide treatment (with/without IMI) on the total number of brood cells per nest unit was assessed using a negative binomial generalized linear mixed model (NBGLMM) to avoid overdispersion of model residuals. The box in which the nesting unit was located was included as a random effect variable in both field-realistic and dose–response experiments. Model residuals were tested for normality using a Shapiro-Wilks test and found to comply with assumptions. In the field-realistic experiment, variation in the position in the nest unit (0–5, 6–10 or 11–15 cm depth) was assessed with an ANOVA, with treatment as a predictor variable.

We tested the number of empty (i.e. not provisioned) cells and the number of parasitized cells using two separate generalized linear mixed model (GLMM) with binomial error distribution and log-link function (i. empty vs. provisioned; ii. parasitized vs. not parasitized minus empty cells) separately for the field-realistic and dose–response data.

For those brood cells containing an *A. plumipes*, we tested the impact of IMI on brood survival to three different developmental stages (early instar mortality, late instar mortality, imago) using a cumulative link mixed model (CLMM, fitted with Laplace approximation) separately for the field-realistic and the dose–response experiment data. Nest unit identity nested in box was included as a random effect variable in all models. Log-odds of mortality at different developmental stages between treatments was predicted using the function 'ggpredict' from the 'ggeffects' package (Lüdecke 2018).

Potential differences in the primary sex ratio (i.e. the number of male and female eggs deposited by the parental generation) could not be assessed since sex could not be determined for non-eclosed brood. To evaluate whether there was a difference between the sexes in the likelihood of survival until eclosion with respect to IMI treatment, the sex ratio of eclosed adults (proportion females to males/nest unit) was tested against treatment in both experiments using LMMs. Nest box was included as a random effect, residuals were tested for normality using Shapiro-Wilks tests and *p*-values were adjusted with a Bonferroni correction in the dose–response experiment to account for the family-wise error rate.

To test whether exposure to soil-bound IMI affected the body mass of *A. plumipes* males and females in the dose–response experiment, we used a linear model with normal error distribution and with treatment (IMI soil concentration), sex and their interaction term as explanatory variables. A robust linear model (RLM) was used to account for non-normality of residual caused by outlier data points. A Kruskal–Wallis test followed by a pairwise Wilcoxon rank sum test with Benjamini and Hochberg *p*-value correction was used as a non-parametric alternative for testing the weight of the sexes separately against treatment.

The R package 'lme4' (Bates et al. 2015) was used for GLMs, GLMMs and LMMs, the R package 'ordinal' (Christensen 2023) was used for the CLMM, and the R package 'MASS' (Venables and Ripley 2002) was used for NB.GLMM and RLM. *p*-values of model estimates were calculated by likelihood ratio tests using the package 'lmtest' (Zeileis and Hothorn 2002) or by analysis of variance (ANOVA) in cases where sample sizes were low (< 10).

3. RESULTS

All nest blocks deployed at the field site were readily occupied by *A. plumipes* in both years (experiments). All indentations originally provided were excavated into nests, showing the suitability of the substrate and nest unit design. In many cases, additional entrance holes were dug after all pre-made indentations had been occupied. A total of 1666 brood cells (median: 27, range 69–13 per nest unit) and 1323 brood cells (median: 43, range 73–20) were constructed in the field-realistic and dose–response experiments, respectively.

3.1. Assessment of IMI residues

In the field-realistic experiment, the measured concentration of IMI in the soil at the start of the experiment diverged > 20% from the nominal concentration of 0.11 mg/kg. Thus, the measured concentration of 0.062 mg/kg is used here on.

In the field-realistic experiment, the IMI concentration decreased by an average of 5% SD ± 7% after 6 days and by 35 ± 6% after 60 days (Fig. 2A). In the dose–response experiment, the IMI concentration across treatments and soil layers decreased by an average of 46 ± 5% across 210 days (field deployment to brood cell excavation; Fig. 2A). The calculated half-life (DT_{50}) based on the data from both experiments was 194 days.

In the field-realistic experiment, brood cell provisions and brood samples contained no detectable IMI residues (limit of detection: 0.01 mg/kg). In the dose–response experiment, unconsumed brood cell provisions collected at nest excavation (210 days after placement in the field) had no measurable residues of IMI at the IMI treatment concentrations of 0.62 or at 1.25 mg/kg (< 0.01 mg/kg). At 2.5, 5 and 10 mg/kg IMI soil treatments, residues of a maximum of 0.013, 0.023 and 0.070 mg/kg were found, which corresponds to less than 1/100th of the concentration in the surrounding soil (Fig. 2B). In bee bodies, residues were only detectable in bees that had developed in the 10 mg/kg soil treatment. The mean IMI concentration in these adults was 0.01 ± 0.005 mg/kg, i.e. 14.3% of the residue concentration found in provisions (Fig. 2B).

3.2. Effects of IMI concentration on brood cell number and brood parameters

The number of brood cells per nest unit did not differ between uncontaminated soil and soil with a field-realistic concentration (0.06 mg/kg) of IMI (LRT: $p = 0.98$, Table I, Fig. 3A) in the field-realistic experiment, with a total of $N = 1666$ brood cells constructed across treatments. Brood cells were most often constructed at a depth of 6–10 cm within nest blocks compared to 1–5 cm or 11–15 cm depths (ANOVA: $F_{(2,149)} = 91.3$, $p < 0.001$), and this did not differ between treatments (control vs. IMI: ANOVA: $F_{(1, 149)} = 0.41$, $p = 0.52$, Supplementary information S7).

In the dose–response experiment, there were no observable differences in the total number of brood cells constructed between the control and across the five IMI concentrations (ANOVA: $p = 0.30$, Table I, Fig. 3B).

In the field-realistic experiment, 7.2% of brood cells were empty (i.e. without brood cell provisions or brood, $N = 103$ cells), and proportions did not differ between IMI treatments (LRT: $p = 0.29$). Parasitism affected 10.4% of brood cells across treatments ($N = 156$), with no impact of IMI treatment on the rate of parasitism (LRT: $p = 0.18$, Fig. 2C, Table I).

In the dose–response experiment, there were no effects of IMI concentration on either the rate of brood cell parasitism (14.2%, $N = 188$ parasitized cells, LRT: $p = 0.36$) or on empty cells (6.7%, $N = 88$, LRT: $p = 0.13$, Table I, Fig. 3D). Key parasites observed across both years were *M. albifrons* and *M. obscurus* as well as the first recorded appearance of the kleptoparasitic meloid beetle *S. muralis* at our study site (for the proportion of cells occupied by each parasitic species, see supplementary information S6).

Soil treatment did not have any effect on *A. plumipes* brood mortality across developmental stages (LRT: $p = 0.302$, Table I, 1 = early instar mortality, 2 = late instar mortality, 3 = survival until eclosion) in the field-realistic experiment, and there were no differences in the likelihood of survival between stages and treatment ($p = 0.30$, log-odds ratio = 0.15 [95% CI − 0.14, 0.44], Table II, Fig. 3C). In the dose–response experiment, there was again no overall effect of IMI soil treatments on brood mortality (LRT: $p = 0.085$, Table I), but one treatment (5 mg/kg IMI) showed a significantly lower mortality (i.e. lower odds of death pre-eclosion) compared to the control treatment ($p = 0.044$, log-odds ratio = − 0.54 [95% CI − 1.1, − 0.01], Table II, Fig. 3D).

Table I Number of brood cells, brood cell parameters and adult bee parameters in relation to soil IMI treatment across both experiments, the field-realistic experiment (2021) and the dose–response experiment (2022)

Response	Model	Fixed effect	Chi-square	df	*p*-value	Statistic test	N nest unit; N box	N brood cells
Field-realistic experiment								
N brood cells	NBGLMM[1]	Treatment (0, 0.06 mg/kg IMI)	< 0.001	1	0.981	LRT	57 and 8	1666
Parasitized (y/n)	GLMM[2]		1.79	1	0.181	LRT	52 and 7	1499
Empty cell (y/n)	GLMM		1.261	1	0.261	LRT	52 and 7	1499
Brood mortality (1–3)	CLMM[3]		1.064	1	0.302	LRT	52 and 7	1236
Sex ratio (f/m)	LMM[4]		0.19	1	0.663	ANOVA	46 and 7	654
Dose–response experiment								
N brood cells	NBGLMM	Treatment (0, 0.6, 1.25, 2.5, 5.0, 10 mg/kg IMI)	6.218	5	0.286	LRT	30 and 4	1323
Parasitised (y/n)	GLMM		5.457	5	0.363	LRT	30 and 4	1323
Empty cell (y/n)	GLMM		8.531	5	0.129	LRT	30 and 4	1323
Brood mortality (1–3)	CLMM[3]		9.668	5	0.085	LRT	30 and 4	1047
Sex ratio (f/m)	LMM		7.404	5	0.192	ANOVA	29 and 4	539
Weight (mg)	RLM[5]	Treatment		5	0.019*	ANOVA	NA	411
		Sex		5	< 0.001*	ANOVA	NA	411

[1]Negative Binomial Generalized Linear Mixed Model

[2]Generalized Linear Mixed Model

[3]Cumulative Link Mixed Model

[4]Linear Mixed Model

[5]Robust Linear Model

Adult brood weight differed between the sexes (ANOVA: $p < 0.001$, Table II) and with IMI treatment (ANOVA: $p = 0.019$; Table II) in the dose–response experiment. The interaction term was dropped as its removal decreased the Akaike information criterion of the model. Across treatments, females weighed on average 13.7% more than males (females: 235 ± 22.6 mg; males: 203 ± 22.6 mg, Fig. 3E). When analyzing sexes separately, body weight did not differ across IMI treatments, neither in males (Kruskal–Wallis test: $x^2 = 9.37$, df = 5, $p = 0.09$) nor in females (Kruskal–Wallis test: $x^2 = 6.21$, df = 5, $p = 0.28$). Treatment did not have an effect on eclosed adult sex ratios in either of the experiments (Table II).

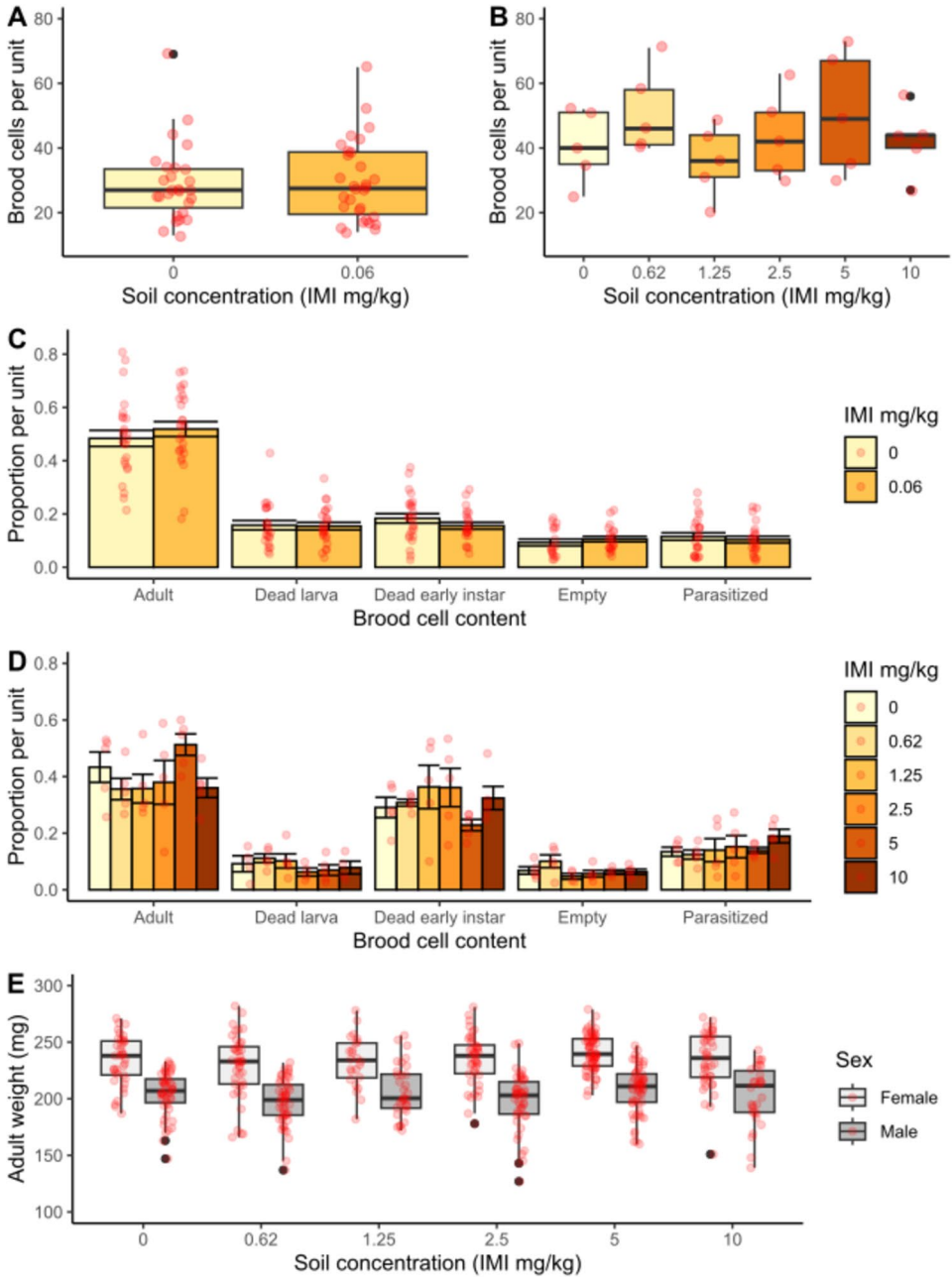

Fig. 3 **A** Boxplots showing number of brood cells per nest unit at different IMI soil concentrations in the field-realistic experiment and **B** the dose–response experiment. Boxes show median (line), interquartiles (box) and whiskers extend to 1.5 times the interquartile range. **C** The relative proportion in each nest unit of eclosed adult bees, brood dying during development (larvae or early instar), empty brood cells and parasitized brood cells (mean and standard errors) in the field-realistic and **D** dose–response experiment. **E** Boxplots of fresh weight of eclosed adults from brood cells constructed in different soil concentrations of IMI in the dose–response experiment. Dots represent individual data points

Table II Predicted log-odds ratios for brood mortality to different developmental stages (1 = early instar mortality, 2 = late instar mortality, 3 = survival until eclosion) for the ordinal mixed model. Individual treatments are compared to the control treatment in each year. A negative log-odds ratio means lower odds of death to any stage and positive means higher odds of death compared to the control treatment

Treatment (mg/kg IMI)	log-odds ratio	95% CI	*p*-value	N nest units; N boxes	N brood cells
Field-realistic experiment					
0	—	—		52 and 7	1237
0.062	0.15	−0.14, 0.44	0.3		
Dose–response experiment					
0	—	—		30 and 4	1236
0.62	−0.32	−0.85, 0.21	0.2		
1.25	0.34	−0.17, 0.86	0.2		
2.5	−0.29	−0.80, 0.22	0.3		
5	−0.54	−1.1, −0.01	0.044*		
10	−0.29	−0.79, 0.21	0.3		

4. DISCUSSION

Here, we demonstrate a novel methodology for exposing a ground-nesting bee, *A. plumipes*, including brood cell provisioning females and developing brood, to agrochemicals through their nesting substrate, and expand upon the merits and demerits of this species. Moreover, we found no negative impact of IMI soil contamination on the number of brood cells, brood development or parasitism of brood cells.

The decay rate and corresponding half-life of IMI in the soil (DT_{50}: 196 days) calculated here were consistent with previous reports on the half-life of IMI (geometric mean: 174 days, max: 288 days in soil; EFSA 2008). We thereby show that IMI was present in the soil during nest excavation by nesting females, and IMI remained in the soil matrix throughout the bees' development period and beyond. However, we found the IMI concentrations in the brood provisions to be much lower than in the soil matrix, and measurable only when soil concentrations were several orders of magnitude higher than are field-realistic (i.e. only at 2.5, 5 and 10 mg/kg IMI). If a completed brood cell provision is assumed to weigh 500 mg, it would correspond to an oral dose of 0.035 µg per larva at the highest concentration measured here (0.07 mg/kg IMI), which is considerably lower than the IMI LD_{50} of 4.17 µg of honey bee larvae (Dai et al. 2017). The waxy brood cell lining typical of anthophorid bees (Norden et al. 1980) may partly protect the brood from acutely harmful levels of IMI exposure through brood cell provisions. However, the waxy layer may also be consumed by the developing larva (Norden et al. 1980, personal communication), which could lead to oral exposure through this medium, especially if lipophilic compounds are applied. Such brood cell linings are common among ground-nesting bees, and may present some level of protection from leaching of pesticides, though the chemical properties of the linings may vary considerably (Cane 1981).

It should be noted that, in this assay, nest units were protected from rain and remained dry throughout the nesting season. Under conditions in which nests are exposed to rain, or in species that nest in other types of soil with a lower clay content and/or higher moisture level, the presence of even relatively low concentrations of water-soluble compounds such as IMI may pose a greater risk to bee brood, as contaminants may be mobilized in water (Rondeau and Raine 2024c). This has been demonstrated for nesting females of *Osmia lignaria* (Fortuin et al. 2021),

in which negative effects of contact exposure to contaminated soil (simulating exposure during mud collection for cell wall construction) were more pronounced with increasing moisture in the substrate. To study the effect of water and agrochemicals on our system, one could deploy horizontal nest units with controlled water application regimes, mimicking precipitation. Provisions of ground-nesting bees can absorb water from the surrounding soil, though this effect likely depends on the chemical properties of the cell lining (Cane and Love 2021). Whether this hygroscopic action is responsible for the transfer of contaminants to provisions, and if this would increase with higher moisture content, remains to be investigated. During nest construction, females of *A. plumipes* and other anthophorids also moisten the soil to facilitate digging (Batra 1994; personal communication). This behaviour may also mobilize contaminants and poses an additional risk, even when nests are constructed in dry localities.

We found no impact of IMI soil treatment on the number of excavated brood cells, the number of empty or parasitized cells, or the developmental stage reached by individual brood, except in one treatment: 5 mg/kg IMI, where survival was higher than in all other treatments including control treatment (Table II). Though replication was relatively low, this hormetic rather than linear response has been observed in other studies on neonicotinoid exposure (i.e. Anderson and Harmon-Threatt 2019).

The exposure regime deployed in our experiments was generally not harmful to developing brood. These results could be corroborated by laboratory experiments in which larvae are allowed to develop on provisions with varying concentrations of IMI, which would allow determination of the lower limit of harmful effects (see e.g. Eeraerts et al. 2019). Although we could confirm pesticide exposure through brood cell provisions and pesticide presence in or on bee bodies, brood body mass was not affected by IMI exposure in a conclusive way, as significant effects were present when sexes were analyzed together but only borderline significant in males when analyzed separately. This effect may be due to a limited sample size, but indicates that the physiological impact of the IMI concentrations reached in provisions was likely limited. The vitality and longevity of eclosed adults post-hibernation, an endpoint potentially of greater fitness relevance, would now be important to measure so as to assess the lifetime effects of pesticide exposure through soil on a bee's fitness.

The effects on *A. plumipes* female longevity or fecundity to prolonged exposure to the contaminated soil matrix during nest construction could not be assessed in our assays as individual nesting females were not tracked. Contrary to the results presented by Rondeau and Raine (2024a), nesting bees did not avoid nor favour contaminated soil, as the number of brood cells was comparable across treatments. Moreover, we did not see a difference in the number of empty (potentially abandoned) brood cells between treatments, suggesting that females also successfully completed brood cells at the same rate. To study the effects of soil contamination on female fitness, one could use a greenhouse or cage setup with nest units, an approach which has been demonstrated to work well for *A. plumipes* (Thalmann and Dorn 1990; Adhikari et al. 2016a). However, assessing female fecundity based on counting brood cells in a nest can be difficult as brood cells can be densely clustered and nest abandonment or usurpation is common in these species, i.e. the brood cells associated with one nest entrance may belong to several females (Černá et al. 2013).

We have illustrated the possibility to collect a significant number of *A. plumipes* eggs, larvae and adults using soil-filled containers as a type of 'trap nest' that encourages colonization by wild bees when placed in proximity to an existing nest aggregation. Nest units containing brood can be moved to new locations to enable experimental work on multiple sites. However, we found brood mortality pre-eclosion to be high and independent of IMI treatment across both experiments(37.7% and 48.5% in the field-realistic and dose–response experiment, respectively). In most cases, provisions were spoiled by fungus (early instar mortality), possibly leading to collapse of the egg or early instar larva as brood could not

always be located. We found late instar larvae and prepupae with symptoms similar to chalkbrood disease (i.e. mummification; Jensen et al. 2013), though we did not attempt to identify the causative agent. This varying but significant presence of fungus could be a hinderance when establishing new populations with the aim to produce large numbers of individuals for toxicological studies. Information on the mortality rate from fungal pathogens in the wild is understudied, and the rate of mortality due to such effects likely varies greatly between years and locations. As an example, in an experimental nesting bed for *N. melanderi*, a comparable rate of 35% was recorded (Batra 1970). Reducing the prevalence of molds may be achieved by sterilizing soil prior to use in nesting units (freeze-drying or autoclaving, see Leonard and Harmon-Threatt 2019), even though it is unknown whether the fungal/bacterial pathogens responsible for larval mortality reside in the soil or are brought into brood cells by nesting bees. In toxicology assessments of soil organisms, standardized soil is often used (Martikainen 1996). To further limit mortality due to parasitism, brood cells containing parasites should be removed yearly to prevent buildup of parasite populations. New aggregations should, when possible, be established away from native ones to protect from spillover of parasites.

Here, we demonstrate limited to no effect of soil-mediated exposure to high concentrations of IMI, a substance known to be highly toxic to adult bees, indicating that the acute risks to brood (i.e. mortality) through soil exposure alone are low in *A. plumipes*, at least under dry conditions. However, exposure to sub-lethal doses during development can have delayed behavioural and physiological effects (Stuligross and Williams 2021), and these warrant further attention. With our presented design, nest units could easily be translocated to more controlled environments such as greenhouses, and with standardized diets and parasites excluded, more rigorously controlled experiments could be conducted, e.g. see Rondeau and Raine (2024b).

In order to expand the basis of experimental ecotoxicological evidence, and ultimately to understand whether the risk from soil-mediated pesticide exposure is a potential driver of wild bee decline, *A. plumipes* holds great potential as a tool for the research community. However, particularities of this species' biology, such as its waxy brood cell lining and its preference for clay/loam-rich soils, prevent us from drawing any general conclusions on risks to ground-nesting species as a whole. Ground-nesting bees use a wide variety of soil structures and habitats. Anthophorid species are relatively rare in their preference for soils with high clay and loam content, whereas it is more common for ground-nesting species to use soils with a higher percentage of sand (Antoine and Forrest 2020). The movement and bioavailability of agrochemical molecules in the soil will also vary depending on the soil properties and moisture content, and this should be considered in each specific scenario where soil-mediated pesticide exposure is a risk, e.g. in contrast to sandy soils, clay soils may bind pesticides, reducing bioavailability and exposure of *A. plumipes* to those pesticides. Choice of soil substrate, nest architecture, presence or absence of brood cell lining as well as temperature and precipitation at the nesting site will all influence the magnitude of exposure to adults and to brood. It should be noted that only a subset of species may be at risk of exposure to pesticides by nesting in proximity to agricultural fields. Specific cases of close crop-bee relationships, in which ground-nesting bees nest directly in the field, are not common (Willis Chan et al. 2019; Tschanz et al. 2023), though the potential risk posed by pesticides has recently been judged to be high for ground-nesting bee species in North America (Willis Chan and Rondeau 2024). Risk from soil-mediated pesticide exposure thus poses a complex problem that is not solvable with experimental evidence from one model species, but will warrant a gradual expansion of knowledge with a focus on risk scenarios where bee-crop interaction is plausible. Continued methodological development and a variety of protocols for successful experimentation on ground-nesting bees are therefore essential for future ecological risk mitigation.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

S.H.: conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing—original draft. K. S.: methodology, writing—review and editing. G. S.: methodology, investigation, data curation, writing—original draft. R.C., G. L., G. C. G.: methodology, investigation, data curation. R. J. P.: conceptualization, funding acquisition, writing—review and editing, supervision, project administration.

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DATA AVAILABILITY

The raw data associated with this study can be found at https://doi.org/10.5281/zenodo.10839835.

DECLARATIONS

Conflict of interest The authors declare no competing interests.

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