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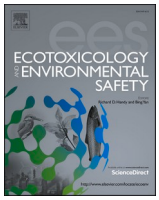

Description and validation of an improved method to feed solitary bees (*Osmia* spp.) known amounts of pesticides

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ABSTRACT

Pesticide exposure is an important driver of bee declines. Laboratory toxicity tests provide baseline information on the potential effects of pesticides on bees, but current risk assessment schemes rely on one species, the highly social honey bee, *Apis mellifera*, and there is uncertainty regarding the extent to which this species is a suitable surrogate for other pollinators. For this reason, *Osmia cornuta* and *Osmia bicornis* have been proposed as model solitary bee species in the EU risk assessment scheme. The use of solitary bees in risk assessment requires the development of new methodologies adjusted to the biology of these species. For example, oral dosing methods used with honey bees cannot be readily applied to solitary bees due to differences in feeding behaviour and social interactions. In this study, we describe the "petal method", a laboratory feeding method, and validate its use in acute and chronic exposure oral tests with *Osmia* spp. We conducted five experiments in which we compared the performance of several artificial flowers combining visual and olfactory cues against the petal method, or in which variations of the petal method were confronted. We then use the results of these experiments to optimize the feeding arenas and propose standardized methods for both acute and chronic exposure tests. The petal method provides high levels of feeding success, thus reducing the number of bees needed. It works with a wide variety of petal species and with both female and male *Osmia* spp., thus ensuring reproducibility across studies. To validate the use of the petal method in ecotoxicology tests, we assess the toxicity of a standard reference insecticide, dimethoate, in *O. cornuta* adults and determine LD50 values for this species. The petal method should facilitate the inclusion of solitary bees in risk assessment schemes therefore increasing the protection coverage of pesticide regulation.

1. Introduction

Pollinators in agricultural environments, are exposed to various pesticides, including insecticides, fungicides and herbicides (Azpiazu et al., 2023; Botías et al., 2015), and this exposure is considered an important driver of worldwide bee declines (Goulson et al., 2015; IPBES, 2016; Janousek et al., 2023). Insecticides are clearly the pesticide group most toxic to bees, but their toxicity may vary greatly among insecticide families (Sanchez-Bayo and Goka, 2014), and may be synergistically enhanced by the combined effects of certain fungicides and herbicides (Almasri et al., 2020; Azpiazu et al., 2021; Carnesecchi et al., 2019; Sgolastra et al., 2018; Tosi et al., 2022). In this context, basic toxicity laboratory tests provide a first level of information on the potential effects of pesticides and pesticide mixtures on bees. Bee pesticide risk assessment schemes (EC, 2002; EFSA, 2013; USEPA, 2014), currently rely on a single species, the western honey bee, *Apis mellifera,* a highly social species. However, the vast majority (ca. 90%) of the bee species worldwide are either solitary or cleptoparasitic on solitary bees

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(Danforth et al., 2019). Further, there are uncertainties regarding how differences in life history traits between social and solitary bee species may result in different potential risks (Schmolke et al., 2021). First, solitary bees have different routes and levels of pesticide exposure compared to honey bees (Sgolastra et al., 2019). Second, different bee species have different levels of sensitivity to various groups of pesticides (Arena and Sgolastra, 2014; Arthurs et al., 2007; Azpiazu et al., 2021; Sgolastra et al., 2017; Uhl et al., 2019). For these reasons, the European Food Safety Authority (EFSA) proposes the incorporation of mason bees, *Osmia cornuta* and/or *O. bicornis,* as surrogates of solitary bee species in pesticide risk assessment schemes (EFSA, 2013).

The use of mason bees as model species in ecotoxicology studies and risk assessment schemes calls for the development of appropriate protocols accounting for the biology and behaviour of *Osmia* spp. Topical toxicity tests with adult honey bees, which are based on the direct application of a drop of test solution on the thorax (EFSA, 2013; OECD, 1998a), can be easily applied to *Osmia*. However, oral toxicity tests with honey bees rely on group feeding and food exchange between individuals (trophallaxis) (OECD, 1998b). Because solitary bees do not perform trophallaxis, this method cannot be used in solitary bee tests. Ladurner et al. (2003) tried to feed females of two solitary bee species, *Osmia lignaria* and *Megachile rotundata*, with individual feeding methods available at the time (film canister and glass vial methods; Johansen et al., 1984; Van der Steen et al., 1996). However, most bees were unable to locate the feeders and did not consume the feeding solution even after exposure times of more than one hour, resulting in very low feeding success (mean: 14.7%; range 0–60%). A subsequent study with the same two species tested artificial flowers with and without the addition of a floral scent (Ladurner et al., 2005a), but feeding success then was even lower (mean: 0.75%; range 0–3%). These low feeding rates prompted Ladurner and collaborators to build a feeder inserted into a natural flower ("flower method") with a drastic increase in feeding success (mean: 84.7%; range 70–100%) (Ladurner et al., 2005a, 2003). The flower method was later used to assess oral toxicity of five fungicides and one insecticide in *O. lignaria* (Ladurner et al., 2005b). Feeding success was again high (97.7% of the bees consumed the entire test solution within one hour), even at the highest pesticide doses tested. Clearly, the addition of a flower to the feeder enhanced the capacity of *Osmia* to quickly locate and consume the test solution.

Because of its effectiveness, the flower method greatly reduces the number of feeding arenas and number of bees required to reach desired sample sizes in ecotoxicology studies (Ladurner et al., 2003). However, the method requires a flower for each tested bee, and the preparation of the feeding arenas is time-consuming. In this study, we propose a simplification of the flower method based on the use of a single petal (henceforth "petal method") and describe its application in both acute and chronic exposure experiments. We then provide results of five experiments in which the petal method is confronted with other individual feeding methods, or in which variations of the petal method are compared. We used both males and females of *O. cornuta* and *O. bicornis*. Finally, to validate the petal method for use in ecotoxicological studies, we assess the acute oral effects on *O. cornuta* females to dimethoate, a toxic reference insecticide.

2. Materials and methods

2.1. Description of the petal method

Below, we provide a brief description of the petal method. A more detailed account can be found in Supporting Information.

Cocoons containing wintered *Osmia* adults are taken from the wintering facility and exposed to 20–23 °C to enhance emergence. Newly-emerged bees are transferred to a holding cage (50 × 50 × 50 cm) and subjected to a 24 h-starvation period, during which they deposit the meconium. Then, bees are individually transferred to a feeding cage provided with a feeder containing the test compound dissolved in a feeding solution (33–50% w/w sucrose/water) (Fig. 1). We devised two types of feeders for studies of acute (type 1) and chronic (type 2) exposures, respectively. In the type 1 feeder, the test solution (10–20 μl) is pipetted into a tiny plastic receptacle inserted into a holding base, and a petal is inserted next to the receptacle (Fig. 1a). After an exposure phase of 1 h, the receptacle is checked and feeding success (consumption of the entire solution) is scored. In the type 2 feeder, the test solution is placed in a calibrated syringe with a petal attached to its tip. The syringe is inserted through the side of the holding cage (Fig. 1b). Solution consumption can be measured periodically by checking the level of test solution remaining in the calibrated syringe. To account for potential evaporation of the solution, an additional number of cages without bees are also monitored. Only bees that feed within the first 24 h of exposure are maintained for the duration of the chronic test.

Experimental conditions should be adjusted to the biology of *Osmia* spp. Although the timing of adult emergence can be modified somewhat through appropriate management of wintering temperatures, tests should be conducted in coincidence with the activity period within the population area of origin. Tests with *O. cornuta* and *O. bicornis* are best conducted at 22 ± 2 °C and 60 ± 10% relative humidity. Light is a critical factor in promoting feeding success. The best results are obtained when bees are exposed to indirect sunlight, but feeding rates drop on cloudy days. High rates of feeding success can also be achieved under artificial light (see experiment 6 below and Supporting Information for further details). Unless otherwise stated, all experiments described hereafter were conducted at-22 °C under indirect natural light.

2.2. Experiment 1: Comparison of the type 1 petal feeder and various types of artificial flowers

We designed this experiment to test the effectiveness of the petal method against various artificial flower models and to determine if the feeding success with artificial flower models could be enhanced by adding an olfactory attractant.

We exposed *O. bicornis* females to a feeding solution using type 1 feeders with a petal of *Bidens ferulifolia* (Asteraceae) (Fig. 1a) and four types of artificial flower models (Fig. 2a). The simplest artificial flower model had no petals, it had only a feeding receptacle inserted into a base of foam (Fig. 2a). The other flower models had five paper rectangles (1.3 × 0.5 cm) simulating petals arranged radially from the feeding receptacle (Fig. 2a). We tested three types of paper: white printing paper, white glossy photography paper and yellow-UV paper (printing paper painted with yellow UV-reflecting paint, Sparwar 3104®). To establish whether the addition of an olfactory cue increased feeding success, the four artificial flower models were tested with and without a drop of linalool deposited on the foam base next to the feeding receptacle. Linalool is a common component of the scent of many flowers and is often used to mimic floral fragrance in laboratory experiments (Decourtye et al., 2004; Sandoz et al., 2001).

The feeding solution was 10 μl of sucrose solution (50% w/v), and sample sizes were 30 females per treatment. We used an *O. bicornis* population reared at the University of Belgrade, Serbia. Cocoons were sent to CREAF in February 2014, and placed in a wintering cabinet at 4 °C. In April, cocoons were incubated and handled as described above. After a one-hour exposure in the feeding cages, we examined the feeding receptacles. Only bees that had consumed 100% of the feeding solution were scored as successful feeders.

We used a generalized linear model (GLM) to analyse feeding success (binary variable: consumption of 100% of the feeding solution or no consumption, < 100%) of the artificial flower models by fitting a binomial error distribution and using a logit link function. We included flower model type (feeding receptacle, white paper, photo paper and yellow-UV paper), scent (with and without linalool) and their interaction as fixed factors. Replicates were individual bees isolated in separate containers. We tested the significance of the main effects with the likelihood ratio test ($p < 0.05$). Post-hoc pairwise comparisons were

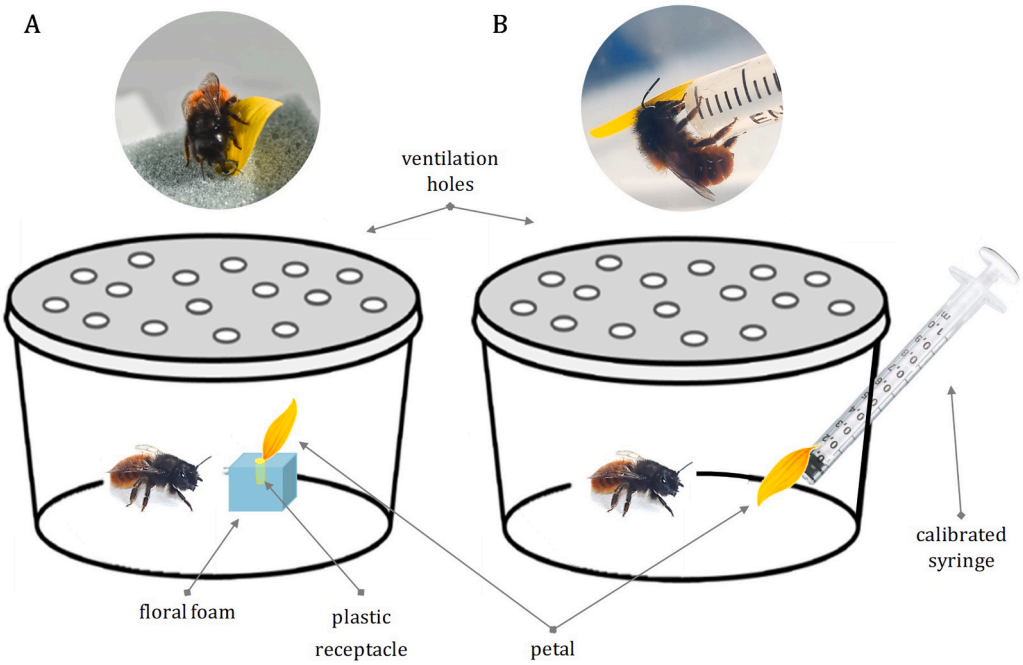

Fig. 1. Cages with type 1 (A) and type 2 (B) petal feeders for acute and chronic exposure experiments, respectively.

conducted with Fisher's LSD test ($p < 0.05$). Then, the feeding success of the best artificial feeder was compared against the petal method with Pearson's chi-squared test, testing the null hypothesis that bees showed no preference for feeder type (expected proportion = 0.5).

2.3. Experiment 2: Comparison of type 1 feeders with petals from different plant species and between male and female bees

The aim of this experiment was to establish whether feeding success was affected by petal origin and whether it differed between *O. cornuta* males and females. We also tested whether feeding success would increase with increasing exposure time.

In March 2021, we worked with an *O. cornuta* population reared at CREAF. Female and male bees were individually exposed for two hours to a feeding solution (20 μl of sucrose solution, 33% w/w) in type 1 feeding arenas (Fig. 1a). We used the petals of three yellow composite plants: *Bidens ferulifolia*, *Euryops chrysanthemoides* and *Osteospermum ecklonis* (Fig. 2b). Sample sizes were 28–30 bees per treatment and sex. Feeding solution consumption was checked at 15, 30, 60 and 120 min following the introduction of the bees in the arena.

We analysed feeding success (binary variable) with a generalized linear mixed model (GLMM), including repeated measures (time), by fitting a binomial error distribution and a logit link function. We used petal origin, bee sex, time and their interaction as fixed factors. Bee identity was added as a random factor to control for repeated measures on the same bee. We tested the significance of the main effects with the likelihood ratio test ($p < 0.05$) and pairwise comparisons were conducted with Fisher's LSD test ($p < 0.05$).

2.4. Experiment 3: Comparison of type 1 feeders with petals from different plant species

In this experiment, we again tested the effects of petal origin and time on feeding success, but with *O. bicornis* females only.

We used an *O. bicornis* population reared at CREAF for this test. In May 2021, newly emerged females were handled as in the previous experiment. We used petals from two Asteraceae species: *Euryops chrysanthemoides* and *Tagetes erecta* (Fig. 2c). Sample sizes were 32–33 bees per treatment. Feeding consumption was checked at 15, 30 and 60 min

As in the previous experiment, we used a GLMM with a binomial error distribution and a logit link function to analyze feeding success (binary variable). We used petal origin (*E. chrysanthemoides* and *T. erecta*), time (15, 30, 60 min) and their interaction as fixed factors. Time was considered a repeated measure. Bee identity was added as a random factor to control for repeated measures on the same bee. We tested the significance of the main effects using the likelihood ratio test ($p < 0.05$) and Fisher's LSD test ($p < 0.05$) as post-hoc analysis.

2.5. Experiment 4: Comparison of type 2 feeders with and without petal in Nicot cages

In this experiment, we compared the effectiveness of type 2 feeders (intended for chronic exposure tests) with and without petals. Bees were held in Nicot cages, which are commonly used in toxicology experiments with bumblebees (Azpiazu et al., 2021; Linguadoca et al., 2022; OECD, 2017a; Sgolastra et al., 2017; Siviter et al., 2022).

We used *O. bicornis* females from a population reared at CREAF. In May 2021, emerging females were handled as in the above experiments. Bees were then individually placed in the Nicot cages (Nicot® queen breeding systems; 7.1 × 2.0 cm) with an inserted 1-mL calibrated syringe containing the feeding solution (Fig. 2d). To facilitate syrup flow, the syringe was slightly slanted, with its tip 5–10 mm from the bottom of the cage. A petal of *E. chrysanthemoides* was attached to the tip of the syringes of the petal treatment. Samples sizes were 30 bees per treatment divided into 3 groups (10 bees per group) corresponding to 3 incubation times one week apart from each other. Feeding success in this test of continued exposure was defined as the % of bees that consumed at least 10 μl, and was measured at 4 and 24 h after the introduction of the bees in the cages. After 24 h, we checked cages daily to measure syrup levels (daily syrup consumption) and bee longevity until all bees died. We controlled for potential changes in syrup levels due to evaporation, using three extra Nicot cages that did not contain any bees.

We analysed feeding success of three groups of bees of each treatment with a general linear model (GLM), with feeder type, time (repeated measures within subjects) and their interaction as fixed factors. Means were separated using Fisher's LSD test ($p < 0.05$). Daily syrup consumption and longevity were not analysed because, in both

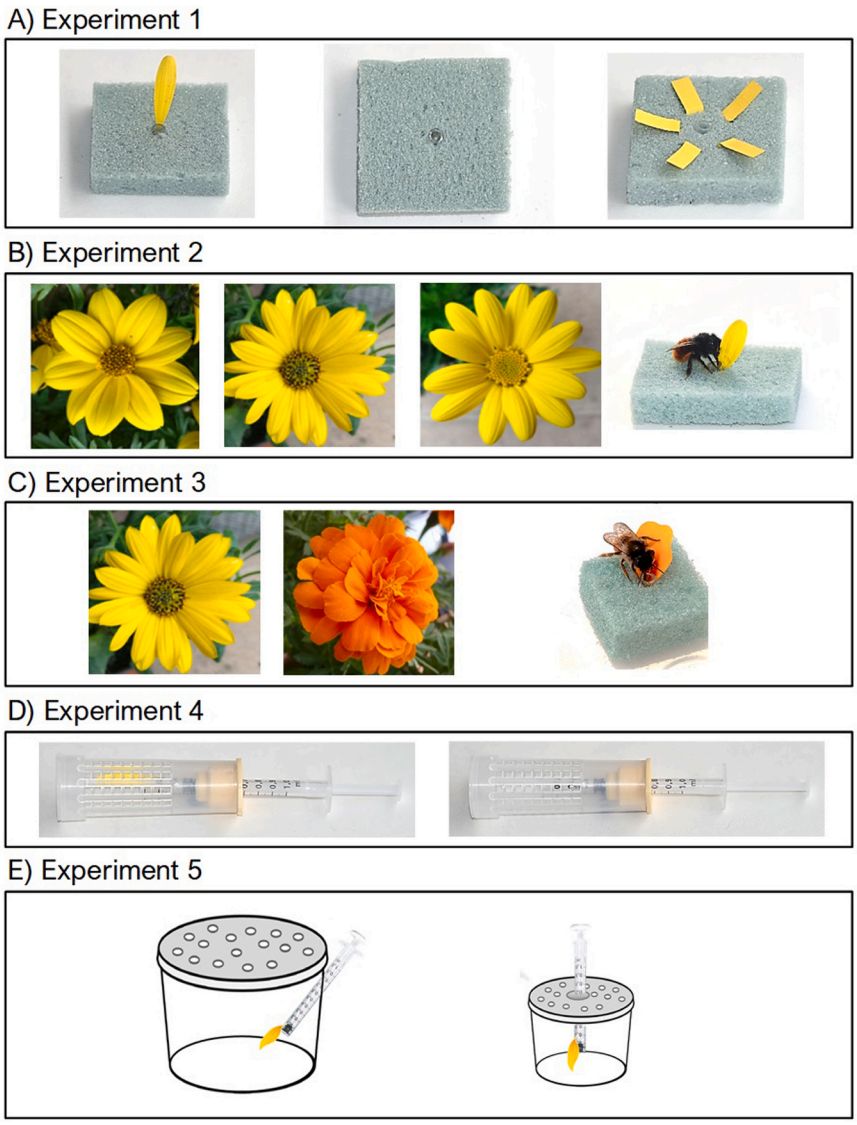

Fig. 2. A) Experiment 1: From left to right: type 1 petal feeder, feeder without petals, and yellow-UV paper flower feeder. B) Experiment 2: Flowers of the three plant species used as petal sources; from left to right: Bidens ferulifolia, Euryops chrysanthemoides and Osteospermum ecklonis. C) Experiment 3: Flowers of the two plant species used as petal sources; from left to right: E. chrysanthemoides and Tagetes erecta. D) Experiment 4: Nicot cage with type 2 feeders, with and without petal. E) Experiment 5: Feeding cages of two sizes with type 2 petal feeders.

types of cages, syrup spilled from the syringe and many bees were soaked with syrup (see below).

2.6. Experiment 5: Effect of cage size

To address the syrup-spill problems that occurred with the Nicot cages, we worked with two cage sizes (i.e., small and large cages) and tested the potential effects of cage size on feeding success, syrup consumption and longevity in bees exposed to type 2 petal feeders.

In this experiment, we used *O. bicornis* females from a population supplied by Pollinature SRL. In May 2022, we incubated cocoons containing adult bees as in the above experiments. One day after emergence, females were individually housed in plastic ice-cream cup containers (cages) of two sizes (Fig. 2e). Large cages (150 cc) measured 5.5–8 cm (diameter) x 7 cm (height), and small cages (50 cc) 3.5 cm (diameter) x 5.5 cm (height). Both types of cages had a transparent plastic lid. The feeder was a calibrated syringe with a petal of *E. chrysanthemoides* inserted so that the tip of the feeder was ca. 10 mm above the bottom of the cage (Fig. 2e). Sample sizes were 15 bees per treatment. We calculated feeding success (defined as the % of bees that consumed at least 10 μl) at 24 h and 48 h. After 48 h, daily syrup consumption and longevity were measured as in the previous experiment, and three additional cages of each size without bees were monitored to account for potential evaporation of the syrup.

Feeding success (binary variable: consumption or no consumption of at least 10 μl of the feeding solution) was analysed using a GLMM with a binomial error distribution and a logit link function. We used cage size (small vs. large cage), time (24 and 48 h) and their interaction as fixed factors. Time was considered a repeated measure. Bee identity was added as a random factor to control for repeated measures on the same bee. We used Kaplan-Meier (K-M) survival curves with pairwise comparison procedures (Log-Rank Test, $p < 0.05$) to compare survival between treatments.

2.7. Experiment 6: Acute oral effects of a reference insecticide on O. cornuta females

The objective of this experiment was to validate the use of the petal feeder as a method to assess the oral toxicity of pesticides in *Osmia* adults. We tested *O. cornuta* adults to acute exposure of dimethoate, an organophosphate insecticide often used as a toxic standard in honey bee and bumblebee toxicity tests (OECD, 2017a, 1998b).

We used bees from an *O. cornuta* population reared at CREAF. In March 2014, newly-emerged females were individually transferred to feeding cages with type 1 petal (*B. ferulifolia*) feeders (Fig. 1a). Bees were acutely exposed to six treatments, including a control (negative control)

and five insecticide doses. To prepare the test solutions, we dissolved a dimethoate commercial formulation (400 g/l emulsifiable formulation from C. Q. Massó) in a sucrose-water solution (50% w/v). In this acute exposure test, each bee was offered 10 μl of test solution. Test solutions were prepared on the day the tests started. An analytical verification of toxicant concentration (Eurofins Agroambiental, S.A) showed that the actual test doses were 0.1855, 0.2698, 0.5589, 1.152 and 2.589 μg dimethoate/bee.

Initial sample sizes were 30 bees per treatment. During the exposure phase, cages with bees were kept in an incubator (22 ± 2 °C and 60 ± 10% relative humidity) under artificial light (two L 18 W/77 Fluora® fluorescent tubes placed 15 cm above the cages, Supporting Information). After 1 h of exposure, the feeding receptacles were checked, and bees that had not entirely consumed the test solution were discarded. As a result, the final number of bees per treatment ranged from 22 to 27.

Following the exposure phase, bees were housed in groups of 5–10 individuals in plastic cups similar to those used as feeding cages, and fed ad libitum with sucrose solution (50% w/v). This solution was provided in a feeder made with a 5-mL Eppendorf vial with the tip cut out and plugged with a cigarette filter. The feeder was inserted through the cup lid and a *B. ferulifolia* petal was attached to its feeding end. Bees confined together in a reduced space show a tendency to form clumps of individuals. To avoid this confinement effect, a small wire mesh structure was provided (Supporting Information). Mortality was determined at 4, 24, 48, 72 and 96 h after exposure.

We used a GLM with a binomial error distribution and a logit link function to establish whether feeding success (binary variable) was dependent on insecticide dose (fixed factor). We tested the significance of the main effects with the likelihood ratio test ($p < 0.05$). Post-hoc pairwise comparisons were conducted with Fisher's LSD test ($p < 0.05$). The LD50 values and their 95% confidence limits for each assessment time (4, 24, 48, 72 and 96 h) were determined using Probit Regression analysis.

3. Results

3.1. Experiment 1: Comparison of type 1 petal feeder and various types of artificial flowers

Both flower model and scent influenced feeding success in artificial flowers, and the interaction between these two factors was non-significant (Table 1). Feeding success increased from 0% to 17% in flower models without linalool to 23–27% in flower models with linalool (Fig. 3a). However, the effectiveness of the best artificial flower model (yellow UV-paper + linalool) (27%) was much lower than that of the petal feeder (76.6%) (Table 1, Fig. 3a).

3.2. Experiment 2: Comparison of type 1 feeders with petals from different plant species and between male and female bees

Neither petal origin nor sex affected feeding success (Table 1). Time, on the other hand, significantly affected feeding success (Table 1). At 15 min, feeding success was ca. 50% in all treatments, and then it increased over time to reach ca. 80% at 60 min (Fig. 3b). This percentage did not increase by exposing bees up to 120 min (Fig. 3b). None of the interactions were significant.

3.3. Experiment 3: Comparison of type 1 feeders with petals from different plant species

Feeding success was not affected by petal origin, but increased significantly over time (Table 1). The interaction was non-significant. At 60 min, feeding success reached levels > 80% (Fig. 3c), similar to those obtained with *O. cornuta* in Experiment 2.

Table 1
Effects of the different factors tested in each experiment and their interaction on bee feeding success. Experiment 1: Feeding success of *O. bicornis* females exposed to different models of artificial flowers with and without a scent (linalool) cue, and to a type 1 petal feeder. Experiment 2: Feeding success over time of *O. cornuta* females and males exposed to type 1 feeders with petals of three different plant species. Experiment 3: Feeding success over time of *O. bicornis* females exposed to type 1 feeders with petals of two different plant species. Experiment 4: Feeding success over time of *O. bicornis* females exposed to type 2 feeders with and without petals in Nicot cages. Experiment 5: Feeding success over time of *O. bicornis* females exposed to type 2 petal feeders in large (150 cc) and small (50 cc) cages.

Species	Feeder type[a]		% Feeding success			
Experiment 1		**Analysis**	**Variables**	**χ2**	**gl**	**P-value**
O. bicornis females	1	GLM	*a) Comparison among artificial flowers*			
			Flower model (F)	9.42	3	**0.024**
			Scent (S)	23.98	1	**< 0.001**
			F x S	4.67	3	0.198
		χ^2 test	*b) Comparison between best artificial flower and petal feeder*			
			Feeder	9.77	1	0.002
Experiment 2			**s**	**F**	**gl**	**P-value**
O. cornuta females and males	1	GLMM	Petal origin (P)	1.03	2	0.359
			Sex (S)	0.03	1	0.860
			Time (t)	16.79	3	**< 0.001**
			P x S	0.95	2	0.386
			P x t	0.36	6	0.907
			S x t	0.18	3	0.913
			P x S x t	0.54	6	0.780
Experiment 3				**F**	**gl**	**P-value**
O. bicornis females	1	GLMM	Petal origin (P)	0.74	1	0.390
			Time (t)	15.66	2	**< 0.001**
			P x t	0.26	2	0.771
Experiment 4				**F**	**gl**	**P-value**
O. bicornis females	2	GLM	Feeder (F)	21.05	1	**0.010**
			Time (t)	64.80	1	**0.001**
			F x t	20.00	1	**0.011**
Experiment 5				**χ2**	**gl**	**P-value**
O. bicornis females	2	GLMM	Cage size (S)	0.25	1	0.618
			Time (t)	3.65	1	0.237
			S x t	0.25	1	0.618

[a] Type 1 intended for acute exposure and type 2 for chronic exposure experiments.

3.4. Experiment 4: Comparison of type 2 feeders with and without petal in Nicot cages

Both feeder type and exposure time influenced feeding success, and their interaction was significant (Table 1). Feeding success increased with time, and bees with the petal feeder had higher feeding success, especially at 4 h, when it was more than two times greater than for bees with the feeder without a petal (Fig. 3d). Unfortunately, due to the small size of the cage, most bees in both treatments contacted the tip of the syringe during the post-exposure phase, and their bodies and the inner walls of the cage ended up covered with syrup. This problem prevented a reliable measurement of syrup consumption and bee longevity.

3.5. Experiment 5: Effects of cage size

Feeding success was not affected by cage size or time (Table 1, Fig. 3e), and the interaction between cage size and time was non-significant (Table 1; Fig. 3e). Daily syrup consumption during the chronic exposure phase (48 h after introducing the bees into the arenas) followed completely different patterns in the two types of cages. Syrup consumption in the large cages was initially high and then decreased over time as bees aged (Fig. 4a). On the other hand, in the small cages, syrup consumption was very low at first, and then increased after days

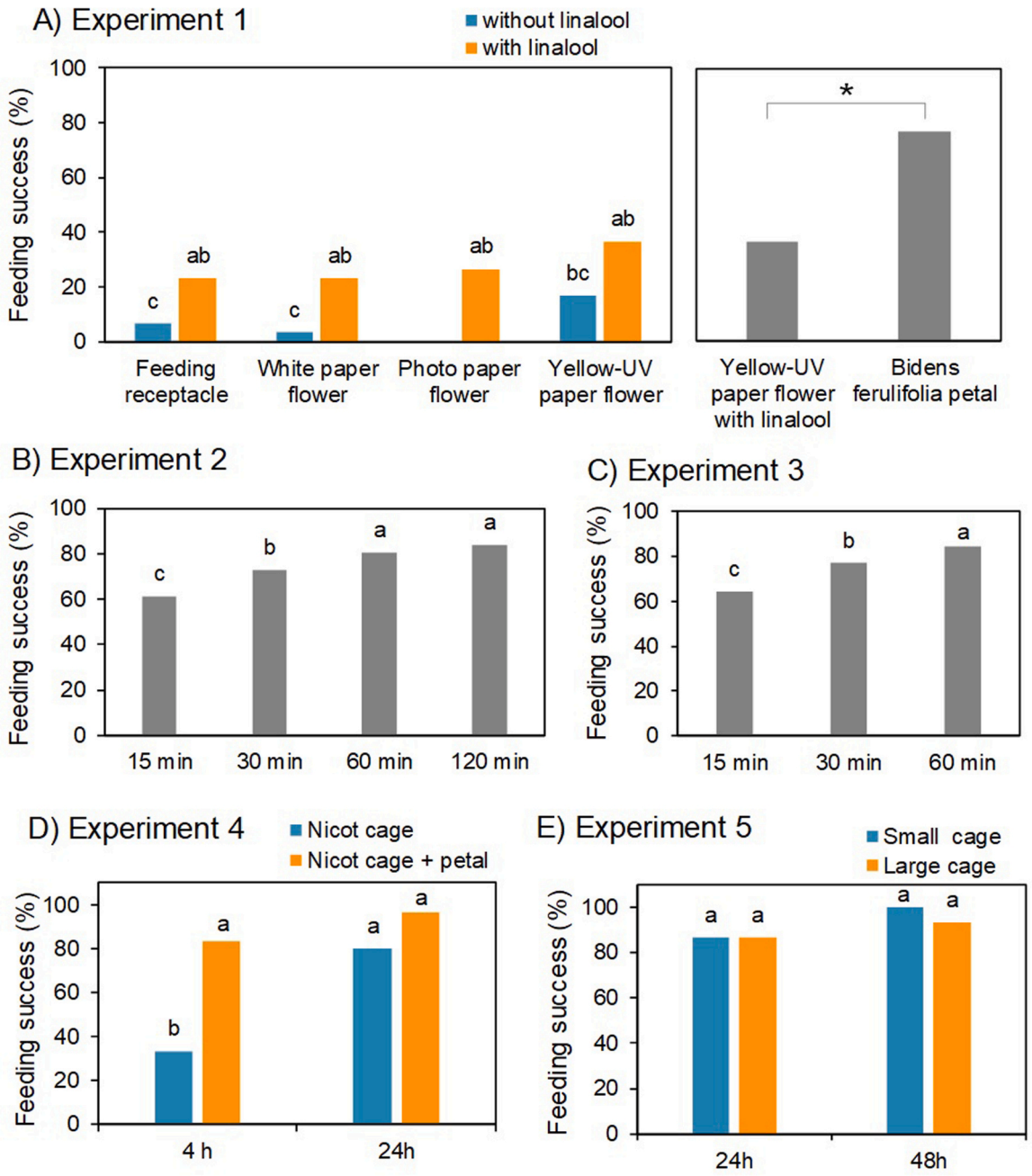

Fig. 3. Percent feeding success in *Osmia* spp. exposed to various types of feeders. A) Experiment 1: Feeding success of *O. bicornis* females exposed to different models of artificial flowers with and without a scent (linalool) cue, and to a type 1 petal (*Bidens ferulifolia*) feeder. B) Experiment 2: Feeding success over time of *O. cornuta* females and males acutely exposed to type 1 feeders with petals of three different plant species. C) Experiment 3: Feeding success over time of *O. bicornis* females exposed to type 1 feeders with petals of two different plant species. D) Experiment 4: Feeding success over time of *O. bicornis* females exposed to type 2 feeders with and without petals in Nicot cages. E) Experiment 5: Feeding success over time of *O. bicornis* females exposed to type 2 petal feeders in large and small cages. Values with the same letter are not significantly different (Fisher's LSD post hoc; $p < 0.05$) and * indicates significant differences between treatments ($p < 0.05$).

5–6 (Fig. 4c). However, this apparent increase in syrup consumption was confounded by the spill of syrup in some of the small cages. Cumulative survival curves differed significantly between cage sizes (Log-Rank Test: $\chi 2 = 12.12$, df $= 1$, $p = 0.002$; Fig. 4d), with much greater survival in the large (20.8 ± 1.9 days) than in the small cages (9.9 ± 1.8 days) (Fig. 4d).

3.6. Experiment 6: Acute oral effects of a reference insecticide on *O. cornuta* females

As many as 84% of the bees tested consumed 100% of the test solution within one hour. No significant differences in feeding success were observed between the dimethoate doses and the control ($\chi^2 = 3.72$; gl $= 5$; $p = 0.6$), indicating that there were no repellence or attraction effects of the insecticide. During the first 4 h of exposure, 40% of the bees at the highest dose (2.6 μg a.i./bee) showed abnormal behaviour, including hyperactivity, impaired movement coordination, and proboscis extension. After 24 h, these symptoms were also observed in individuals exposed to lower doses. At 96 h, mortality was 4.5% in the control treatment, and ranged from 11.5% to 100% in the dimethoate treatments. The LD50s calculated at each assessment time (4, 24, 48, 72 and 96 h) are shown in Table 2.

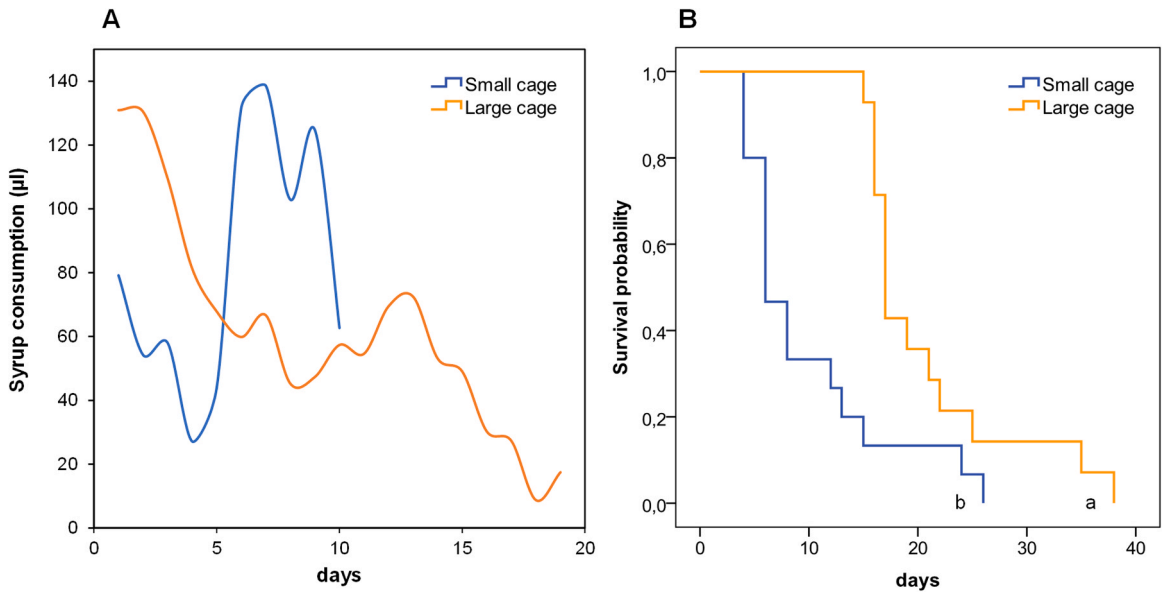

Fig. 4. Experiment 5. Daily mean syrup consumption (µl per bee) (A), and cumulative survival probability in *O. bicornis* exposed to type 2 petal feeders in small (50 cc) and large (150 cc) cages. Curves with different letters are significantly different (Log Rank test: $p < 0.05$).

Table 2
Oral toxicity of dimethoate in *Osmia cornuta* females: LD50 values and 95% confidence limits at 4, 24, 48, 72 and 96 h after exposure and statistical outputs of the probit dose-response models.

Exposure time	LD_{50} (µg a. i./bee)	95% confidence limits	Slope ± SE	t	*P*
4 h	1.057	0.829–1.413	2.53 ± 0.38	6.671	0.007
24 h	0.666	0.545–0.824	3.38 ± 0.47	7.223	0.005
48 h	0.803	0.617–0.980	5.203 ± 1.22	4.264	0.024
72 h	0.733	0.541–0.914	4.177 ± 0.912	4.579	0.019
96 h	0.755	0.546–0.942	4.485 ± 1.064	4.215	0.024

4. Discussion

In this study, we describe an individual feeding method (petal method) for laboratory toxicity studies with solitary bees, and demonstrate its effectiveness in acute and chronic oral-exposure tests. For both acute and chronic exposure, the petal method maintains levels of feeding success similar to those obtained with the flower method (Ladurner et al., 2003), while reducing the number of flowers required and shortening the time needed to prepare the feeding arenas.

Artificial syrup feeders (e.g., Eppendorf tube caps, plastic syringes, artificial flowers), are readily utilized by *A. mellifera* and *Bombus* spp. (Azpiazu et al., 2021; Cabezas and Farinós, 2022; Ladurner et al., 2005a; Mundy-Heisz et al., 2020; Sgolastra et al., 2017) and are used routinely in oral-exposure protocols (OECD, 2017a, 1998b). However, *Osmia* females in our study had difficulty recognizing artificial feeders, even with added color and scent cues. These results are consistent with previous studies in which *O. lignaria* and *Megachile rotundata* consistently performed worse than *A. mellifera* with artificial feeders (Ladurner et al., 2005a, 2003). Also with a study in which *O. cornifrons* individually exposed to artificial feeders took 12–24 h to feed (Phan et al., 2020). Other studies have used syringes with yellow squares of sponge-cloth as visual attractants for *O. bicornis* but feeding success was not reported (Mokkapati et al., 2022, 2021). A recent study with the solitary bee *Centris analis* used artificial pink cloth flowers to attract individuals to a syrup feeder (Tadei et al., 2022). Feeding success reached > 60% at 24 h and > 80% at 72 h. Surprisingly, this species performed better in the dark, as the bees exhibited high levels of agitation under light (Tadei et al., 2022). The different response between *Apis* and *Bombus* on the one hand, and *Osmia* and *Megachile* on the other hand, to artificial feeders may be attributed to differences in life history. Feeding on nectar and honey within the confinement of the hive is a common behaviour in both *Apis* and *Bombus*. In contrast, *Osmia* and *Megachile* store provisions of pollen mixed with nectar, and therefore never encounter nectar by itself in their nests. In these species, interaction with nectar occurs only in association with a flower. These behavioural differences between bee species illustrate the importance of accounting for the natural history of the model species when designing experimental protocols.

Both *O. bicornis* and *O. cornuta* are generalist bees and have been reported to forage on a wide range of flowering plants (Haider et al., 2014; Jaumejoan et al., 2023; Kratschmer et al., 2020; Raw, 1974; Splitt et al., 2021; Tasei, 1973; Westrich, 1990). In this study, we focused on ornamental Asteraceae because these plants are easy to grow and produce many petals per plant. Our results show that petal origin did not influence the effectiveness of the method, with high rates of feeding success in both females and males. Linguadoca et al. (2022) obtained high levels of feeding success in *O. bicornis* using petals of *Brassica rapa* and *Diplotaxis tenuifolia* (Brassicaceae). Using entire corollas instead of petals, Ladurner et al. (2003) obtained similar results in *O. lignaria* with flowers of *Prunus avium*, *Malus domestica* (Rosaceae), *Convolvulus arvensis* (Convolvulaceae), and *Vinca minor* (Apocynaceae). Taken together, these results suggest that the effectiveness of the petal method is robust to a wide range of flower species.

Our research shows that the petal method can be used effectively in both acute and chronic exposure studies. In acute toxicity tests, the petal method reduces exposure variability between test individuals for two reasons. First, in contrast to group feeding methods, it ensures that the dose consumed is exactly the same for all bees. Second, the short time needed by bees to locate the feeder (<1 h) and consume the test solution homogenizes the timing of exposure across individuals. Our results show that feeding success does not increase over 77–84% by exposing bees for periods longer than one hour, so our method minimizes the time invested in the exposure phase. The effectiveness of the petal method is also high in chronic tests, with feeding success > 80% after 24 h. The use of a calibrated syringe allows for the measuring of the levels of syrup consumption, a fundamental endpoint in chronic studies. Importantly, however, our results show that small cages are not suitable for chronic

studies with *Osmia*. Bees in the Nicot® cages and in the small cages of experiment 5, contacted the tip of the syringe with their bodies, causing the syrup to spill, which hindered measurement of syrup consumption and affected bee longevity. Mortality at 10 days in these two cage types exceeded 15%, the validity threshold established for honey bee chronic test (OECD, 2017b). In contrast, no syrup spilling occurred in the large cages, with 0% mortality at 10 days. Importantly, the mean longevity recorded in our large cages (20 days) is within the range of longevity recorded for *Osmia* females nesting in greenhouses (16–30 days) and in the field (18–30 days) (Bosch, 1994; Bosch and Vicens, 2006; Frohlich and Tepedino, 1986; Maeta, 1978; Sgolastra et al., 2016; Sugiura and Maeta, 1989; Tepedino and Torchio, 1982).

Our sixth experiment demonstrates the suitability of the petal method in acute oral toxicity bioassays. The dimethoate oral LD50 for *O. cornuta* at 24 h after exposure (0.66 μg a.i./bee) is consistent with values found in the literature for a smaller *Osmia* species (*O.* lignaria: 0.25–0.27 μg a.i./bee) and for *A. mellifera* (0.15–0.31 μg a.i./bee) (Ladurner et al., 2005b). Mortality in our control group (4.5%, at 96 h) complied with the criteria established in toxicity protocols for honey bees ($\leq$ 10%; OECD, 1998), and feeding success in our experiment was high (84%), even at the highest dimethoate concentration. The petal method also has been validated in other ecotoxicological acute (Albacete et al., in press; Azpiazu et al., 2021; Linguadoca et al., 2022; Sgolastra et al., 2017) and chronic (Azpiazu et al., 2022, 2019) exposure studies with *Osmia* spp. with levels of feeding success ranging from 66% to 88% and from 71% to 77%, respectively.

5. Conclusions

The European Food Safety Authority (EFSA) has recommended including *Osmia* spp. in risk assessment schemes (EFSA, 2013) but standardized protocols for solitary bees are not yet available. Our research describes a feeding method for laboratory studies that results in high levels of feeding success for *Osmia* (thus reducing the number of bees required per test), and homogeneous dosage and timing of exposure (thus reducing variability across individuals). Additionally, our method ensures low percent mortality and realistic bee longevity in the control group. The validation of the petal method in ecotoxicological studies provides further evidence of its potential contribution towards improved risk assessment schemes and ultimately supports the protection of pollinator biodiversity and ecosystem pollination services.

CRediT authorship contribution statement

C. Azpiazu: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **S. Hinarejos:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing. **G. Sancho:** Methodology, Investigation, Writing – review & editing. **S. Albacete:** Methodology, Investigation, Writing – review & editing. **F. Sgolastra:** Investigation, Resources, Supervision, Conceptualization, Methodology, Writing – review & editing. **C.A.H. Martins:** Methodology, Investigation, Writing – review & editing. **X. Domene:** Methodology, Investigation, Writing – review & editing. **J. Benrezkallah:** Methodology, Investigation, Writing – review & editing. **A. Rodrigo:** Methodology, Supervision, Funding acquisition, Writing – review & editing. **X. Arnan:** Formal analysis, Writing – review & editing. **J. Bosch:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115398.

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