



Does winter oilseed rape grown from clothianidin-coated seeds affect experimental populations of mason bees and bumblebees? A semi-field and field study

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Abstract

Impacts of neonicotinoid-containing pesticides on pollinators have been heavily debated in recent years. While bees in the field get rarely exposed to lethal concentrations of neonicotinoids applied as seed coating, sublethal levels found in pollen and nectar may affect bee population development. We assessed a realistic and a worst-case scenario of clothianidin exposure to mason bees (*Osmia bicornis*) and bumblebees (*Bombus terrestris*) by conducting a small-scale field and semi-field experiment at sites planted with winter oilseed rape. Flight activity, mortality and population development (brood, colony strength and weight) of bumblebees and number of mason bee brood cells were assessed at three locations. We also analysed clothianidin residues in bee-collected pollen and nectar. We detected clothianidin at low concentrations in nectar and pollen; residues in pollen were higher than in nectar but did not exceed a maximum field concentration of 2.7 µg/kg for bumblebees and 4.7 µg/kg for mason bees. Exposure did not result in significant negative impacts on bumblebee colony development and potential reproductive success of mason bees in either semi-field or field setup. During exposure bumblebees in semi-field treatment tunnels were flying less actively than in control tunnels. Bumblebee colonies in treatment tunnels weighed more than control colonies at the end of the experiment. Our findings suggest that field-realistic exposure to oilseed rape grown from clothianidin-treated seeds (10 g clothianidin/kg seeds) poses little risks to mason bees, bumblebees and their population development. However, an impairment of flower visitation under a worst-case scenario may have implications for pollination services and crop production.

Keywords *Osmia bicornis* · *Bombus terrestris* · *Brassica napus* · Neonicotinoids · Sublethal effects · Field-realistic exposure

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1 Introduction

Pollinating insects, in particular commercially used bee species like honeybees, bumblebees and mason bees, are of high economical importance with e.g. honeybees being responsible for about 90% of commercial pollination (Allsopp et al. 2008). Even in wind-pollinated crop species like oilseed rape (OSR) their presence can increase seed pod weight and pod set (Garratt et al. 2014). Among the biotic and abiotic drivers that interfere with pollinators in the environment, agrochemicals used for crop protection have long been discussed as a major factor to disrupt pollination processes and bee health. There are different exposure routes to be considered when evaluating the risk of a pesticide to bees. Foraging bees may be exposed via direct contact with a sprayed or drifted pesticide (Koch and

Weisser 1997; Nuyttens et al. 2013). Even if a pesticide is not directly applied to the plant (and flower) surface, bees may be orally exposed to residues of systemic pesticides via pollen, nectar, guttation droplets or other contaminated water sources (Blacquière et al. 2012; Girolami et al. 2009; Samson-Robert et al. 2014). Amongst the systemic pesticides, neonicotinoids are the most widely used insecticides in the world (Jeschke et al. 2011). They are applied not only as a foliar spray but also as a seed coating agent in OSR and various other crops. Different agricultural application practices may result in different intensities of exposure to pollinators.

A growing body of studies has investigated the effects of neonicotinoids on honeybees (e.g. Krupke et al. 2012; Pilling et al. 2013). However, extrapolation of such study results to other bee species may not always be reasonable due to differences in life history (social-solitary, body size etc.) and foraging preferences (flower constancy, seasonality of forage etc.), which result in distinct exposure profiles for each species (Thompson and Hunt 1999; van der Valk and Koomen 2013). It is essential to investigate the risk of a pesticide to the particular bee species of interest. Besides laboratory studies that give a valuable insight into mechanisms and dose-response relationships, extrapolation of results to population level in the field often overestimates field-realistic dosage factors (Carreck and Ratnieks 2014). Semi-field (*worst case scenario*) and field experiments are essential to evaluate potential hazards of pesticides to pollinating insects in a “real-world” setting. Some recently published studies have reported negative effects of neonicotinoid seed treatment on population development and pollination services of bumblebees (Rundlöf et al. 2015; Stanley et al. 2015; Whitehorn et al. 2012; Woodcock et al. 2017) and solitary bees (Rundlöf et al. 2015; Sandrock et al. 2014; Woodcock et al. 2017). In contrast, other field studies were not able to reveal any biologically significant effects of neonicotinoids on *Apis* (Cutler et al. 2014; Pilling et al. 2013; Rundlöf et al. 2015) and non-*Apis* pollinators (Cutler and Scott-Dupree 2014; Peters et al. 2016; Sterk et al. 2016; Thompson et al. 2013). Differences in study results are likely due to the crop variety (summer vs. winter OSR) and local environmental conditions, which may affect residue concentrations in pollen and nectar (Dively and Kamel 2012), and therefore, the level of exposure. Equivocal results may also be due to low replication and a resulting low statistical power in some studies (Cresswell 2011).

In this study, we investigated the exposure risk for non-*Apis* bees that were exposed to OSR grown from clothianidin coated seeds in northern Germany. Clothianidin has been widely used as a seed treatment in OSR in Europe until its restriction in 2013 and its ultimate ban for outdoor use in 2018 (European Commission 2018). In order to

assess its sublethal and lethal effects on bees in the field, we recorded residues in collected pollen and nectar as well as flight activity, population development and reproductive potential of two non-*Apis* bee species, the solitary red mason bee *Osmia bicornis* and the social buff-tailed bumblebee *Bombus terrestris* in both a (*worst-case*) semi-field setting and a field setup.

2 Material and methods

2.1 Study sites

The semi-field study was conducted at a location in Lower Saxony (NI). In addition to the semi-field study, a field study was established at three locations: the semi-field location, one further location in Lower Saxony (NI 2) and a location in Brandenburg (BR). All three locations were situated in the north-eastern part of Germany (Fig. S1). At each location one treatment and one control site were arranged in a block design to minimise variability between sites within a location (Table 1). Treatment and control sites were situated on average a minimum of $917 \text{ m} \pm 667 \text{ m}$ standard error (SE) apart from each other. Each site was planted with winter OSR (*Brassica napus* L., hereafter WOSR) between August and September 2012. While control sites were planted with organically produced WOSR seeds (variety VISBY® or V140 OL®) that were not treated with insecticides, treatment sites received seeds (variety VISBY® or HAMMER®) with an ELADO FS 480® plus TMTD seed coating containing 10 g clothianidin, 2 g beta-cyfluthrin and 4 g thiram/kg seeds. Seed coating at location NI 2 contained in addition DMM (5 g dimetomorph/kg seeds). Seed treatment was applied in the form of a water-based suspension. Sowing quantities averaged $59 \text{ seeds/m}^2 \pm 7.2 \text{ SE}$ (Table 1). Control sites were farmed in compliance with organic farming standards. Treatment sites were managed under a conventional farming scheme with the intention to ensure a high WOSR yield using additional pesticides before bees were exposed (Table S2). However, additional products did not contain highly bee toxic active substances like clothianidin, imidacloprid or thiamethoxam.

2.2 Study species

We studied two non-*Apis* bee species, which are used for commercial pollination purposes in Germany, *O. bicornis* and *B. terrestris*. Their ecology and life history contrasts each other. While *O. bicornis* females live solitarily and found and provision their own nest after having hatched and mated with a male, *B. terrestris* is a primitively eusocial species forming colonies with a reproducing

Table 1 Study locations, their geographic coordinates and setup information for the semi-field (SF) and field (FL) experiment

Location	Setup	Treatment	Locality	Geographic coordinates	Variety	Min. distance (m)	Sowing date	Acreage (ha) ^a	WOSR	Sowing quantity (seed/m ²)
NI	SF	C	Wolfenbüttel	52.165472, 10.578750	Visby	250	07.09.12	0.004	60	
		T	Wolfenbüttel	52.167361, 10.581639	Visby	250	30.08.12	0.004	70	
	FL	C	Wolfenbüttel	52.165472, 10.578750	Visby	250	07.09.12	1.5	60	
		T	Wolfenbüttel	52.167361, 10.581639	Visby	250	30.08.12	4.0	70	
NI 2	FL	C	Groß Twülpstedt	52.345940, 10.902682	V 140 OL	250	01.09.12	3.5	88	
		T	Groß Twülpstedt	52.345021, 10.895539	Visby	250	20.08.12	2.0	50	
BR	FL	C	Putlitz	53.246831, 12.057997	Visby	2250	22.08.12	26.0	50	
		T	Putlitz	53.265725, 12.022375	Hammer	2250	24.08.12	16.6	38	

NI = Lower Saxony, BR = Brandenburg, C = control sites, T = sites with clothianidin seed-coated winter oilseed rape (WOSR). Geographic coordinates displayed in [decimal degrees N, decimal degrees E], Min. distance = minimum distance between treatment and control field sites. Sowing date displayed in the format DD.MM.YY

^aFor setup SF given as acreage per tunnel

queen and a few 100 non-reproductive female individuals (workers).

Cocoons of male and female *O. bicornis* individuals were ordered from a commercial supplier (WAB-Mauerbienenzucht, Konstanz, Germany) and kept at 4 °C until the start of the experiment. Then, they were placed at control and treatment sites and exposed to local environmental conditions.

Bumblebee colonies were obtained from a commercial supplier (Biobest, Westerlo, Belgium) three to five days before the start of the experiment. They were delivered in a plastic breeding box surrounded by a cardboard box and kept at room temperature and ambient humidity in a dark room. Commercial feeding solution (Biogluc®, Biobest) and pollen was provided *ad libitum* until the day of placement. On the day of placement (Day 0), access to sugar solution and pollen was stopped and colonies were exposed to local experimental conditions. However, colonies still contained some stored pollen and nectar. On the day of placement, colonies were randomly assigned to treatment and control conditions. Colony strength (number of worker bees) was comparable between the 12 treatment and the 8 control colonies in tunnels [linear mixed model (LMM), $t_{18} = 0.85$, $p = 0.41$]. Colony strength was slightly lower (-7 ± 2.1 SE worker bees) in the 14 treatment than in the 14 control colonies at field sites (LMM, $t_{24} = -3.38477$, $p = 0.0024$); this difference in the number of worker bees was included as a covariate in subsequent statistical analysis. Initial colony weight of treatment

colonies resembled weight of control colonies at field sites (LMM, $t_{24} = 0.06$, $p = 0.95$). Colonies started with one queen and on average 32 ± 1.2 SE worker bees in the semi-field tunnels and 34 ± 2.6 SE worker bees at field sites.

2.3 Experimental setup

2.3.1 Semi-field

At the NI field location, four tunnels were placed at the control site and six tunnels at the treatment site. Each tunnel measured 10 m × 4 m and was covered with a net of 1.2 mm mesh width. Within each tunnel, two green gauze stripes (10 m × 0.5 m and 4 m × 0.5 m) acted as a ground cover for walking and collection of dead bees leaving a total area of flowering WOSR of 30 m² (Fig. 1a). Three squares (1 m × 1 m) were established in the flowering plant stand for survey of flight activity. One mason bee trap nest assembled from wooden boards with a total of 234 nesting tubes and a hatching container containing 75 female and 100 male cocoons was placed at the tunnel entrance (Fig. 1b). Each trap nest was protected from rain by a sheet of plastic roofing. Each tunnel was also equipped with two bumblebee colonies on a stand which were wrapped in protective roofing (Fig. 1d). In addition to the two focal bee species in this study, each tunnel also housed one small honeybee colony with 3281 workers on average (Fig. 1a). Bumblebee colonies were pre-assessed (i.e.

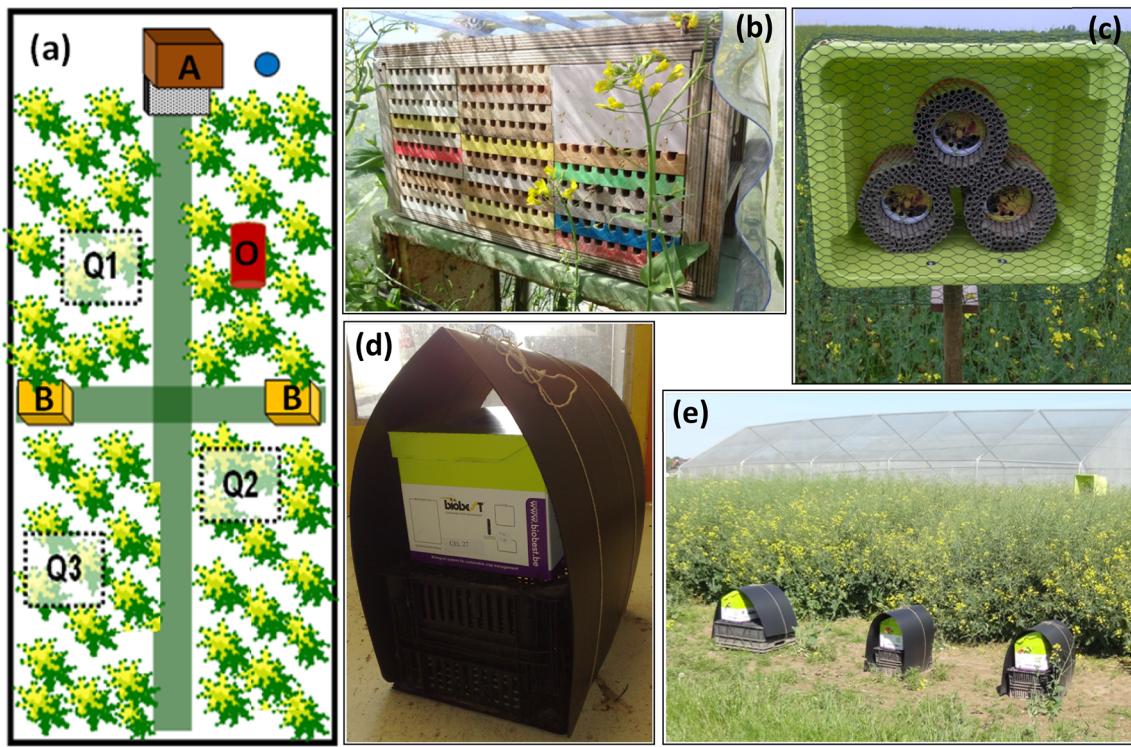


Fig. 1 **a** Schematic semi-field setup. Each tunnel measured 4 m × 10 m and was equipped with ground gauze (green crossed band) and a water and loam source (blue circle). **A** = honeybee colony, **B** = bumblebee colony, **O** = solitary bee trap nest,

Q = observation square for survey of flight activity. **b** *O. bicornis* trap nest in semi-field tunnels. **c** *O. bicornis* trap nest in the field setup. **d** *B. terrestris* colony with weather shield and stand. **e** *B. terrestris* colonies in the field setup (colour figure online)

Table 2 Start and end dates of exposure and assessment periods of flight activity displayed in the format DD.MM. and the corresponding days since first exposure (in brackets) for each study location

Setup	Organism	Parameter	BR	NI	NI 2
Semi-field	<i>O. bicornis</i>	Start of exposure		10.05. (0)	
		End of placement		01.07. (52) ^a	
		Flight activity		14.05. to 10.06. (4–31)	
	<i>B. terrestris</i>	Start of exposure		17.05. (0)	
		End of exposure		11.06. (25)	
		Flight activity		18.05. to 10.06. (1–24)	
Field	<i>O. bicornis</i>	Start of exposure	15.05. (0)	17.05. (0)	16.05. (0)
		End of exposure	06.06. (22)	12.06. (26)	07.06. (22)
		Flight activity	Not recorded	Not recorded	Not recorded
	<i>B. terrestris</i>	Start of exposure	17.05. (0)	17.05. (0)	16.05. (0)
		End of exposure	08.06. (22)	11.06. (25)	12.06. (27)
		Flight activity	Not recorded	Not recorded	Not recorded

BR = Brandenburg, NI = Lower Saxony

^aFemale mason bees were allowed to further forage until the end of WOSR flowering (around 15.06.) before trap nest units were sealed off and pollen samples were taken on 18.06.; however, trap nests were removed from the tunnels later (end of placement on 01.07.) due to operational difficulties

workers were counted, the condition of the queen was assessed, a photo and the weight of the each colony was taken), and mason bee cocoons and bumblebee colonies were then exposed to experimental conditions on 10th and

17th May 2013 respectively (Day 0, Table 2) when WOSR was in full flower (BBCH 65). Mason bees were placed in the tunnels a week before placement of bumblebees so that the females would have sufficient time to hatch (females

hatch later than males, depending on weather conditions and time of the year, Dietzsch et al. 2015). This was to ensure that both mason bees and bumblebees would be actively foraging as soon as WOSR was in full flower. They were kept exposed for 25–36 days (Table 2). However, mason bees were allowed to forage and use nest tubes approximately as long as bumblebees were exposed before trap nest units were sealed off (footnote Table 2).

Mason bee trap nests were sampled for pollen and left in the semi-field tunnels for approximately two more weeks after the exposure period before transported to the Julius Kühn Institut (JKI) in Braunschweig, Germany, and frozen at –20 °C. Cocoons and brood cells were counted in November 2013. *B. terrestris* colonies were moved to the JKI in Braunschweig for assessment and sampling immediately after the exposure period. Depending on initial colony size, peak in colony development and therefore onset of offspring production occurs at different points in time even when developmental patterns are similar (Dietzsch et al. 2018). In order to monitor the peak in colony development, production of reproductive offspring and to ensure the same level of maturity at freezing, colonies were kept in the laboratory for another seven to nine weeks before they were frozen and stored at –20 °C in the semi-field.

2.3.2 Field

Each control and treatment field site was equipped with one mason bee trap nest and four (site NI 2 and BR) to six (site NI) bumblebee colonies, respectively. While bee trap nests were set up approximately 10 m from the field edge within the WOSR stand, bumblebee colonies were established with a distance of approximately 1.5 m from each other, adjacent to the WOSR field and at least 15 m apart from any bee trap nest (Fig. 1e). Both, trap nests and colonies were placed in a south-facing direction and at opposing sides of control and treatment sites to allow the furthest possible distance between treatment and control bees. Trap nests were protected with chicken wire from predators; they contained three trap nest units (Fig. 1c) each enclosing a hatching container with 33 female and 33 male mason bee cocoons and 100 cardboard tubes for egg laying (WAB-Mauerbienenzucht, Konstanz). Bumblebee colonies were protected with a plastic sheet from weather impacts and placed on a stand like in the semi-field setup (Fig. 1d). *B. terrestris* colonies were pre-assessed, and cocoons and bumblebee colonies were then exposed to experimental conditions between 15th and 17th May 2013 (Day 0, Table 2) when WOSR was (almost) in full flower (BBCH 63–65). They were kept exposed for 22–27 days (Table 2) until the end of flowering.

After the exposure period, one *O. bicornis* trap nest unit of each trap nest was frozen and stored at –20 °C. Pollen

was extracted from these trap nest units in the laboratory at a later stage. The remaining two units of each trap nest were moved to the JKI in Braunschweig in order to assess reproductive success. After pupation, mason bee larvae were kept at 4 °C and 78% relative humidity in a climatic chamber until cocoons were counted in November 2013. As in the semi-field study, *B. terrestris* colonies were moved to the JKI in Braunschweig immediately after the exposure period where they were assessed, sampled and kept for further seven to nine weeks in the laboratory in order to monitor the peak in colony development, production of reproductive offspring and to ensure the same level of maturity at freezing. They were stored at –20 °C.

2.4 Data collection

2.4.1 Population parameters

Population parameters of interest in the field experiment included population strength, growth, reproduction and bee mortality; in addition, the semi-field experiment also provided the assessment of flight activity.

To assess reproductive success of *O. bicornis*, nest tubes were opened either by lifting the wooden plates (semi-field experiment) or by cutting the cardboard tubes with a pair of fine scissors (field experiment). Then the number of nest tubes that showed building activity of at least one cell (“occupied tubes”) and the number of brood cells per nest tube were counted. Sampling was conducted during (field study) and after (semi-field and field study) the exposure period (Table 3).

In order to measure population strength, reproductive success and bee mortality of *B. terrestris* colonies in the semi-field and field experiment, each colony box had to be opened in the laboratory. Colony strength, measured as the number of worker bees, and reproductive success, measured as the number of brood cells (i.e. egg, larvae and pupae cells), were assessed by taking a picture of each colony at each assessment day under red-light conditions and counting the number of bee individuals and brood cells on the images afterwards. Dead bumblebee individuals inside the colonies were counted immediately when boxes were opened (for assessment dates see Table 3). Population growth of *B. terrestris* colonies measured as colony weight was assessed by weighing each closed plastic box without its card board case (scale precision ± 2 g) in the laboratory.

Flight activity of mason bees and bumblebees in the semi-field experiment was assessed by counting the number of individuals in each of three 1 m × 1 m squares in each tunnel as a snapshot once a day during the exposure period. In order to complement activity patterns with weather data, hourly temperatures (in °C), cloud cover during observation periods (% cover) and daily

Table 3 (a) Assessment dates of mason bee population parameters (number of occupied tubes, number of brood cells) with corresponding days since first exposure; (b) assessment dates of bumblebee colonies with corresponding days since first exposure

(a) Location	Nest unit	Semi-field			Field					
		Date	Day	Pollen sample	Date	Day	Pollen sample			
NI	1	18.06.	39	✓	24.05.	7	✗			
	2	Not applicable			12.06.	26	✓			
	3				12.06.	26				
NI 2	1	Not carried out			31.05.	15	✗			
	2				08.06.	23	✓			
	3				08.06.	23				
BR	1	Not carried out			23.05.	8	✓			
	2				06.06.	22	✓			
	3				06.06.	22				
(b) Location	Semi-field				Field					
	Date	Day	Pop	Nectar	Pollen	Date	Day	Pop	Nectar	Pollen
NI	15.05.	– 2	✓			15.05.	– 2	✓		
	24.05.	7		✓	✗	24.05.	7		✓	✗
	29.05.	12	✓	✓	✓	29./30.05.	12/13		✓	✓
	06.06.	20		✗	✗	06.06.	20		✓	✓
	13.06.	27	✓			13.06.	27	✓		
	15.07.	59	✓			15.07.	59	✓		
	31.07.–	75–87	✓			31.07.–	75–87	✓		
		12.08.					12.08.			
NI 2	Not carried out					15.05.	– 1	✓		
						23.05.	7		✗	✓
						31.05.	15		✗	✗
						07.06.	22		✓	✓
						13.06.	28	✓		
						15.07.	60	✓		
						31.07.–	76–88	✓		
							12.08.			
BR	Not carried out					15.05.	– 2	✓	a	a
						13.06.	27	✓		
						15.07.	59	✓		
						07.–08.08.	82–83	✓		

(a) Nest unit = number of trap nest unit sampled (there was only one unit in the semi-field setup), Pollen = ✓ indicates pollen sampled from nest tubes at this date, ✗ indicates that the amount of pollen was too small for chemical analysis. Empty fields indicate that no pollen sampling was conducted but brood cells were counted. Dates are in the format DD.MM. BR = Brandenburg, NI = Lower Saxony; (b) Pop = ✓ indicates assessment of population parameters (number of workers, weight, number of brood cells), Nectar = ✓ indicates nectar was sampled from nest, Pollen = ✓ indicates pollen was sampled from nest; ✗ indicates the amount of nectar or pollen was too small for chemical analysis. Empty fields indicate that no assessment, nectar or pollen sampling was conducted

Dates are in the format DD.MM. NI = Lower Saxony, BR = Brandenburg

^aNo nectar or pollen sampling at this location

precipitation (in mm) was recorded for the entire period of exposure.

2.4.2 Residue analysis

Clothianidin residues were assessed in pollen of mason bee brood cells and in pollen and nectar sampled from

bumblebee colonies. Mason bee pollen was extracted from nest tubes of trap nests, which were frozen after collection and cut open for preparation (see Table 3 for dates of sampling). Eggs or larvae were removed from the pollen cluster, pollen was carefully separated from cell wall divisions, and if necessary and/or possible pollen from multiple cells of the same trap nest unit was pooled to

obtain an optimal sample weight. Bumblebee pollen and nectar was sampled from the colony before and during exposure (Table 3). Samples were pooled for each location and treatment in the semi-field and field setup respectively. All samples were stored in Eppendorf tubes at -20°C .

We aimed for an optimal sample weight of 1 g nectar and pollen respectively. However, in many cases optimal sample weight could not be obtained so that smaller sample weights had to be accepted. Any sample smaller than 0.2 g was discarded and not chemically analyzed to assure reliable and precise results. Limit of Quantification (LOQ) was 0.63 $\mu\text{g}/\text{kg}$ for an exact sample weight of 1.0 g nectar/pollen, and limit of detection (LOD) was 0.20 $\mu\text{g}/\text{kg}$. Clothianidin concentrations were analyzed via liquid chromatography-mass spectrometer-system with acetamiprid D3 as a surrogate following the procedure described in Pistorius et al. (2015).

2.5 Data analysis

Due to the low number of replication for the parameters *Number of occupied tubes* and *Number of brood cells* of the

mason bee data set, data were not normally distributed. All other data sets were normally distributed. We used non-parametric Wilcoxon–Mann–Whitney tests (semi-field data) and Wilcoxon–Pratt Signed Rank tests (field data) for the parameters *Number of occupied tubes* and *Number of brood cells*. Linear mixed models (LMM) and generalized linear mixed models (GLMM) were used for all other data (Supplement 3). Data were analyzed using the packages *coin* and *Rfit* for non-parametric data, package *nlme* for parametric data and *lsmeans* for *post-hoc* testing within the statistical software *R* (R Core Team 2014). Multiple comparisons were adjusted using the multivariate *t* distribution (Bretz et al. 2010). From full models, which in general included all assessed parameters, optimal models were selected using AIC criteria and procedures described in Zuur et al. (2009) (Table S3). *Treatment* as the main factor of interest was default in all models. We used different variance structures for different strata where necessary.

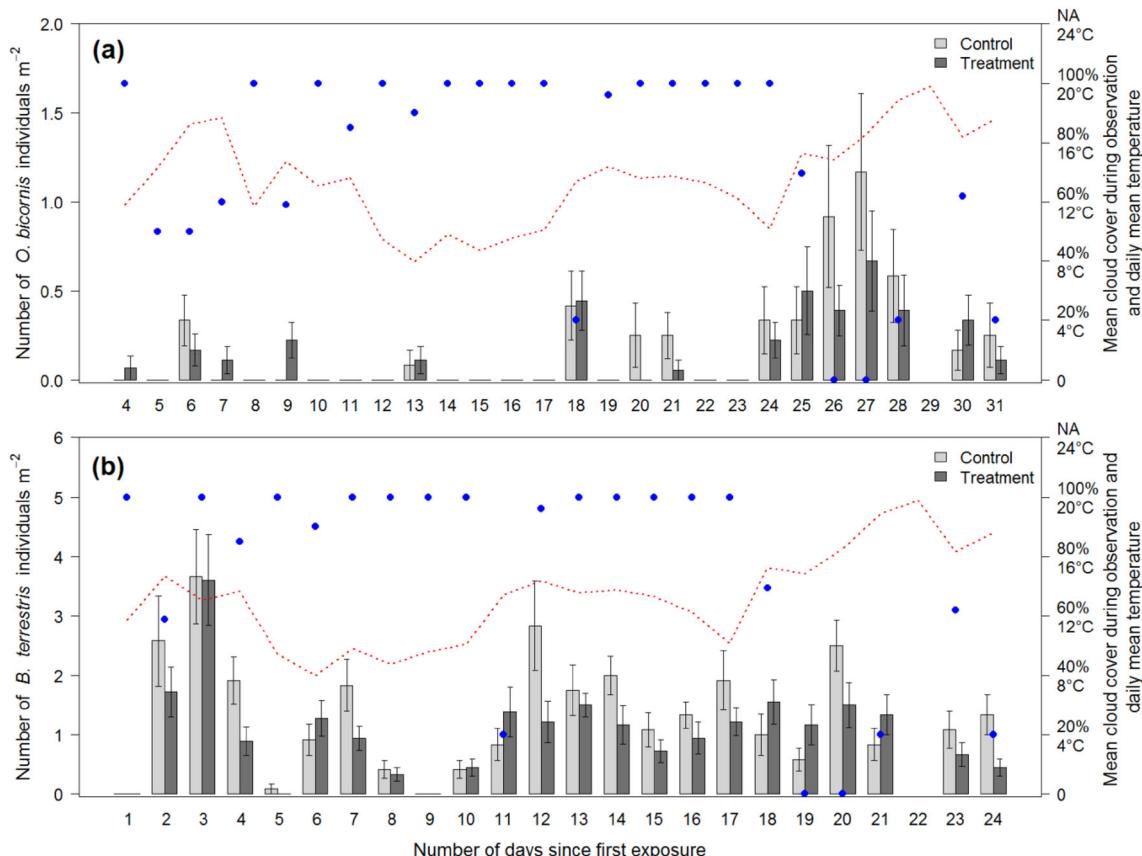


Fig. 2 Mean number \pm standard error of **a** *O. bicornis* and **b** *B. terrestris* individuals counted daily over the entire exposure period in each of the $1 \times 1 \text{ m}^2$ flight squares in semi-field tunnels. The red line

and blue dots depict mean daily temperature and mean cloud cover during flight square observation periods, respectively (colour figure online)

Table 4 Statistical test results for the main factors in (a) the semi-field and (b) the field setup with reference to figures (Fig.) that show data summaries

Test no	Study species	Response variable	Predictive variable	Comparison	N/df	Test statistic	p	Fig.
(a) Semi-field setup								
1	Osmia	Flight activity	Cloud cover	Medium vs. low	751	- 3.48	< 0.001	2a
2	Osmia	Flight activity	Cloud cover	High vs. low	751	- 6.19	< 0.001	2a
3	Osmia	Flight activity	Treatment	Treatment vs. control	751	- 1.70	0.090	2a
4	Osmia	Occupied tubes	Treatment	Treatment vs. control	10	- 1.08	0.350	3
5	Osmia	Number of cells	Treatment	Treatment vs. control	10	- 1.08	0.348	3
6	Bombus	Flight activity	Cloud cover	High vs. low	660	- 0.20	0.84	2b
7	Bombus	Flight activity	Temperature	Rising	660	0.395	0.69	2b
8	Bombus	Flight activity	Treatment	Treatment vs. control	660	- 2.95	0.003	2b
9	Bombus	Dead workers/colony	Treatment	Treatment vs. control	56	- 0.94	0.35	4
10	Bombus	Dead workers/gauze	Treatment	Treatment vs. control	207	1.71	0.09	
11	Bombus	No. of workers	Treatment	Treatment vs. control	60	1.07	0.29	5
12	Bombus	No. of workers	Treatment × week	Treatment vs. control	20	1.92	0.13	5
				Week 11–13				
13	Bombus	No. of brood cells	Treatment	Treatment vs. control	60	1.96	0.0504	5
14	Bombus	Colony weight	Treatment	Treatment vs. control	18	1.76	0.09	6
15	Bombus	Colony weight	Treatment × week	Treatment vs. control	18	2.71	0.010	6
				Week 12				
16	Bombus	Old queen absent	Treatment	Treatment vs. control	NA	0.21	1	
(b) Field setup								
1	Osmia	Occupied tubes	Treatment	Treatment vs. control	12	1.78	0.09	3
2	Osmia	Number of cells	Treatment	Treatment vs. control	6	1.60	0.25 ^a	3
3	Bombus	Dead workers/colony	Treatment	Treatment vs. control	20	- 0.17	0.86	4
4	Bombus	No. of workers	Treatment	Treatment vs. control	79	0.52	0.60	5
5	Bombus	No. of brood cells	Treatment	Treatment vs. control	77	1.54	0.13	5
6	Bombus	Colony weight	Treatment	Treatment vs. control	23	0.51	0.61	6
7	Bombus	Old queen absent	Treatment	Treatment vs. control	1	0	1	

Test results are referred to in the text by test number (Test No). Test statistic depends on data distribution (Table S3) and is either t, Z, F or χ^2 . Numbers in bold indicate significant difference between treatment and control setup. Osmia = *O. bicornis*, Bombus = *B. terrestris*

^aDue to the low number of replicates in each group, a significant difference at an α level of 0.05 between groups is not detectable with this non-parametric test. The reported p value is the lowest possible value

3 Results

3.1 *Osmia bicornis*

3.1.1 Semi-field

Flight activity of *O. bicornis* was generally low (Fig. 2a), at least in comparison to flight activity of bumblebees (Fig. 2b) and was mainly influenced by weather conditions. Medium and high cloud cover reduced flight and foraging of mason bee individuals observed in the 1 m × 1 m squares (Table 4a, Test 1 and 2). Observed mason bee flight activity did not significantly differ between control and treatment tunnels (Table 4a, Test 3).

The low flight activity was mirrored by the relatively low number of occupied nest tubes and constructed brood cells in semi-field tunnels (Fig. 3). The number of occupied tubes (Table 4a, Test 4) and brood cells (Table 4a, Test 5) did not significantly differ between control and treatment tunnels.

Only two adequate pollen samples were retrieved from semi-field nest tubes due to the relatively low nesting activity of bees in tunnels. Clothianidin residues were detected in low concentrations in the treatment pollen sample (Table 5). There was no clothianidin detected in control pollen.

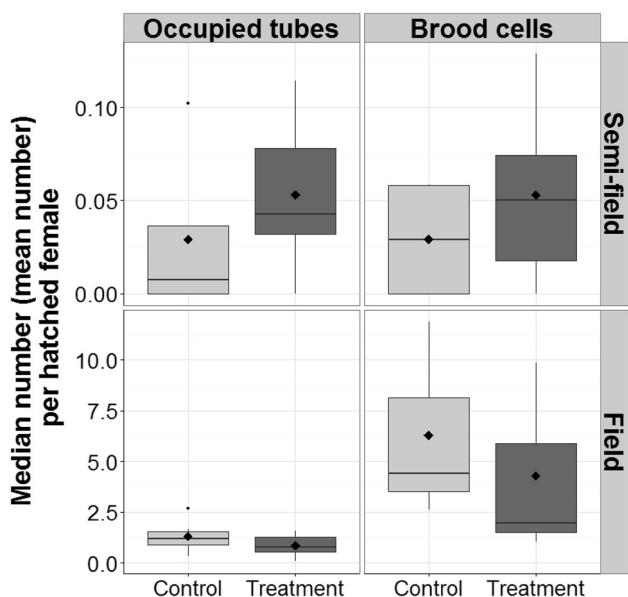


Fig. 3 Median number, interquartile ranges (IQR) $\pm 1.5 \times$ IQR whiskers of occupied nest tubes and brood cells per hatched female in trap nest units in semi-field tunnels and in the field setup. Trap nest units were equipped with 234 nesting tubes, 75 female as well as 100 male *O. bicornis* cocoons in the tunnels and with 100 nesting tubes, 33 female and 33 male *O. bicornis* cocoons in the field setup. Large bold black points indicate the mean number of the parameter. Small points represent outliers

3.1.2 Field

The number of occupied nest tubes and constructed brood cells per hatched female at the field sites was an order of magnitude larger than in the semi-field tunnels (Fig. 3). Although mason bees built slightly fewer cells in treatment than in control sites (Fig. 3), neither the number of occupied tubes (Table 4b, Test 1) nor the number of brood cells (Table 4b, Test 2) significantly differed between treatment and control sites.

Clothianidin residues were detected in low concentrations in seven of the 17 pollen samples collected from treatment sites (Table 5). Two of the 13 samples from control sites also showed clothianidin residues but at four times lower maximum concentrations than at treatment sites.

3.2 *Bombus terrestris*

3.2.1 Semi-field

Flight activity of *B. terrestris* in the semi-field tunnels was very variable between days as well as between tunnels across the same day (high SE, Fig. 2b). Bumblebee flight was not significantly influenced by weather conditions (Fig. 2b, Test 6 and 7); however, more bumblebees were

observed flower-visiting in control tunnels than in treatment tunnels (Fig. 2b, Table 4a Test 8).

Over the course of the study, bumblebee colonies grew bigger, and more dead bees accumulated inside the colonies (Fig. 4). Colonies in treatment tunnels did not reveal significantly more dead bees than colonies in control tunnels (Table 4a, Test 9). There was a high variation within the same treatment (*Tunnel* was an important random factor for the optimal statistical model, cf. Table S3). Hardly any dead bumblebee individuals were collected from the gauze in each tent during daily assessments ($\text{max}_{\text{control}} = 2$ individuals, $\text{max}_{\text{treatment}} = 6$ individuals). Mortality rates accessed via gauze counts increased slightly during the second half of exposure but were not significantly higher in treatment tunnels than in control tunnels (Table 4a, Test 10).

Colony strength (number of worker bees) increased during the course of the experimental period (Fig. 5; *week* was a significant factor, Supplement 3). Colonies contained less brood cells (eggs, larvae plus pupae) and were lighter compared to initial brood and weight assessments at the end of the exposure period but gained brood cells and weight during the post-exposure period (Figs. 5, 6; *week* was a significant factor, Table S3). At the end of the experiment, no further weight changes were detected and brood cell numbers dropped. While variability across tunnels was high on some assessment dates for both colony strength and the number of brood cells, either in control colonies or treatment colonies or both (Fig. 5), the variation was low for the measurement of colony weight (Fig. 6). Control colonies did not contain significantly more worker bees (Table 4a, Test 11 and 12) or a significantly higher number of brood cells (Table 4a, Test 13), nor did they generally weigh significantly more than treatment colonies (Table 4a, Test 14). However, treatment colonies were significantly heavier than control colonies at the end of the trial (Table 4a, Test 15; significant interaction, *post-hoc comparison week 12*).

Old queens were absent in two of eight control colonies (from week 4 onwards) and in two of 12 treatment colonies (from week 5 and 6 onwards), with no significant difference (Table 4a, Test 16). Two control colonies and one treatment colony were lacking brood of any stage (eggs, larvae or pupae) as of week 9.

Only three pollen and two nectar samples were retrieved from bumblebee colonies due to relatively small amounts of stored food in the colonies. There was no clothianidin detected in nectar samples (Table 5). However, two pollen samples from consecutive weeks retrieved from treatment tunnels contained relatively high clothianidin concentrations (Table 5).

Table 5 Concentrations of clothianidin in pollen and nectar (matrix) collected by *O. bicornis* and *B. terrestris* in control (C) and treatment (T) tunnels (SF) and at field sites (FL)

Organism	Setup	Treatment	Matrix	Week	N Loc	N total	N > LOD	N > LOQ	Max ^a (µg/kg)	Mean ± SE ^b (µg/kg)
<i>O. bicornis</i>	SF	C	Pollen	6	1	1	0	0	0	0
		T	Pollen	6	1	1	0	1	3.89	3.89
	FL	C	Pollen	2	1	1	0	0	0	0
				4	1	12	1	1	1.20	0.19 ± 0.13
	T		Pollen	2	0	0				
				4	3	17	0	7	4.70	1.32 ± 0.44
<i>B. terrestris</i>	SF	C	Nectar	1	1	1	0	0	0 ^c	0
				2	0	0				
		T	Pollen	1	0	0				
				2	0	0				
		T	Nectar	1	0	0				
				2	1	1	0	0	0	0
	FL	C	Pollen	1	1	1	1	0	7.00 ^d	7.00
				2	1	1	1	0	6.40 ^c	6.40
		T	Nectar	1	1	1	0	0	0 ^d	0
				2	1	2	0	0	0.18 ^c	0.18
		T	Pollen	1	1	1	0	0	0 ^c	0
				2	1	1	0	0	0 ^d	0
		T	Nectar	1	1	2	0	1	2.68 ^c	1.34 ± 1.34
				2	1	1	1	0	0.71	0.71
		T	Pollen	1	1	1	1	0	1.90 ^c	1.90
				2	1	1	0	0	0 ^c	0
				3	2	2	0	0	0 ^c	0

Mason bee pollen in tunnels was sampled from nest tubes supplied with pollen over the entire period of exposure (Week = 6). Mason bee pollen at field sites was sampled from nest tubes supplied with pollen over either the first two weeks (Week = 2) or the entire period of exposure (Week = 4). Bumblebee pollen and nectar was sampled from the nest once a week during exposure. Empty cells indicate a lack of samples due to scarcity of resources. N [Loc] = Number of locations with samples larger than pre-defined lowest weight of 0.2 g, N [total] = Total number of samples, N [> LOD] = Number of samples with a clothianidin concentration > LOD but ≤ LOQ, N [> LOQ] = Number of samples with a clothianidin concentration > LOQ, Max = Highest concentration detected, Mean ± SE = Mean (± standard error) clothianidin concentration

^aSamples with a concentration ≤ LOD listed as 0

^bIncludes samples with concentrations ≤ LOD as 0 and samples with concentrations > LOD but ≤ LOQ as 0.63 µg/kg (= LOQ)

^cSample weight was smaller than 1.0 g

^dSample weight was 0.19 g

3.2.2 Field

As in the semi-field tunnels, bumblebee colonies grew bigger in individuals over the course of the study, and more dead bees were monitored inside the colonies (Fig. 4). Colonies at treatment sites did not contain significantly more dead bees than colonies at control sites (Table 4b, Test 3).

Colony development in the field experiment showed similar overall patterns as in the semi-field setup. Colony strength grew over the entire experimental period until the end of the experiment (Fig. 5) and was mirrored by a steady gain in weight (Fig. 6). This is particularly interesting since the number of brood cells peaked at the end of the exposure period and rapidly decreased in the weeks after exposure (Fig. 5). Minimum weight of control and

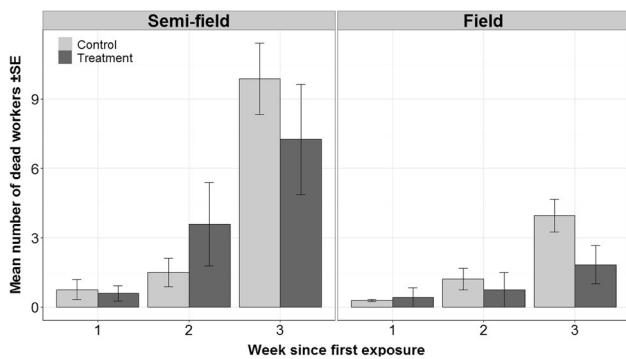


Fig. 4 Mean number \pm standard error of dead *B. terrestris* individuals inside each colony accumulatively counted during the exposure period in semi-field tunnels and in the field setup

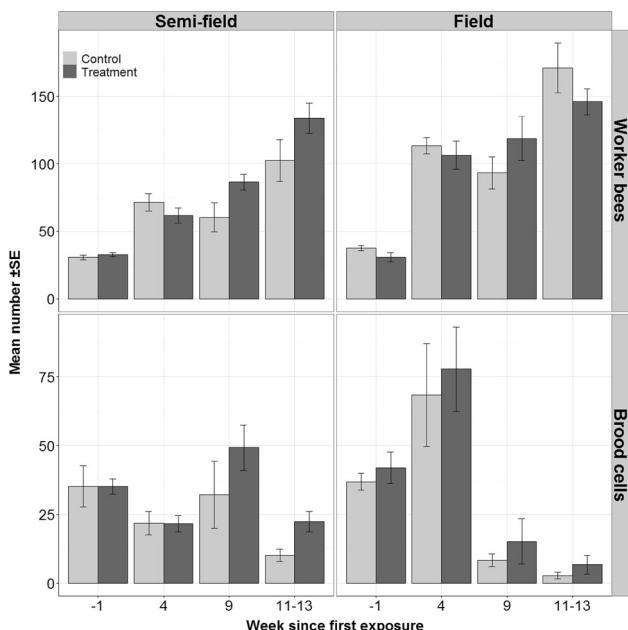


Fig. 5 Mean number of worker bees and brood cells \pm standard error found in *B. terrestris* colonies from control and treatment sites before and after exposure in semi-field tunnels and in the field setup. Numbers were obtained counting individuals and cells on photographic images taken during population assessments in the laboratory. Brood cell numbers only represent occupied brood cells i.e. empty cells of already hatched brood were not counted anymore

treatment colonies was 504 g and 472 g, respectively. Maximum weight of control and treatment colonies reached 970 g and 1036 g, respectively. Variability across and within locations was high for two of the three developmental parameters on some of the assessment dates (*location* was an important random factor for the optimal statistical model of the number of brood cells and colony weight, cf. Table S3). Colonies at control sites did not contain significantly more worker bees (Table 4b, Test 4) or a higher number of brood cells (Table 4b, Test 5) and

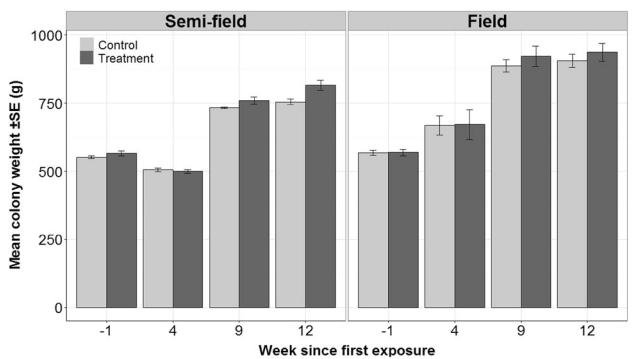


Fig. 6 Mean colony weight (in g) \pm standard error of *B. terrestris* colonies from control and treatment sites before and after exposure in semi-field tunnels and in the field setup

did not weigh significantly more than treatment colonies (Table 4b, Test 6).

Old queens were absent from nine of the 14 control colonies (three after week 4 and six after week 6) and from eight of the 14 treatment colonies (four after week 4 and four after week 6) before the end of the colony life cycle (control not significantly different from treatment, Table 4b, Test 7). Five control colonies and seven treatment colonies were lacking brood of any stage (eggs, larvae and pupae) as of week 9.

Residues were revealed in some of the four pollen and five nectar samples from treatment sites (Table 5). Mean and maximum concentrations found in pollen samples were lower than those found in the semi-field setup. While one of the five nectar samples from control sites contained traces of clothianidin, one of the four pollen samples revealed clothianidin residues similar to those found at treatment sites.

4 Discussion

4.1 *Osmia bicornis*

Mason bees are highly sensitive in their flight activity when it comes to unfavorable weather conditions. In contrast to bumblebees, which can partially regulate their body temperature by buzzing (Heinrich 1979), mason bees are entirely dependent on ambient conditions. Rain or dense clouds could disrupt their flight activity. Despite the weather dependency, we did not see any effect of the seed treatment on the flight activity of mason bees in tunnels.

Foraging resulted in provisioning of brood cells within nesting tubes. Female bees in the tunnels had a much more limited access to pollen resources than bees in the field due to the size of foraging area and the competition for resources with bumblebees and honeybees. Hence, tunnel bees produced much fewer cells than field bees. Nutritional

stress has been shown to synergistically reduce honeybee survival when experienced in combination with clothianidin exposure (Tosi et al. 2017). By stressing both control and treatment bees in tunnels we expected the food shortage to elevate any negative effects of clothianidin exposure on treatment bees. Neither the number of occupied tubes nor cell production changed in response to clothianidin treatment in tunnels or at field sites. This is in line with other semi-field and field studies in WOSR (Ruddle et al. 2018; Woodcock et al. 2017) where *O. bicornis* cell production was not directly affected by the exposure to neonicotinoid-treated WOSR. Residue concentrations in pollen collected in WOSR studies have been shown to be relatively low (cf. Peters et al. 2016). Higher concentrations of clothianidin in summer OSR trials (Rundlöf et al. 2015; but see Cutler and Scott-Dupree 2007) or combined concentrations of a neonicotinoid mixture (Woodcock et al. 2017) may be more potent and may correlate negatively with cell production.

Cell production may not translate to reproductive output if parasitism or environmental factors impair larval development and/or hatching of adult offspring. We did not investigate these parameters in our study. Previous laboratory and field experiments did not reveal any detrimental effects on larval development and overwintering when mason bees were exposed to clothianidin at field-realistic levels (Nicholls et al. 2017; Peters et al. 2016; but see Bailey and Greenwood 2018 cautioning against the latter).

Effects of pesticide exposure are usually correlated to pesticide concentrations. We found that the clothianidin concentration in mason bee pollen sampled in a treatment tunnel was in the same range as maximum residue concentrations in pollen from field sites; they did not exceed 4.7 µg/kg. This is considerably lower than maximum concentrations in field experiments reported elsewhere (Rundlöf et al. 2015; however, this study used summer OSR, i.e. on average a 2.5 times higher seeding density with a shorter period between seeding and flowering, hence a considerably lower dilution factor). They are a fraction of the chronic toxicity no observed effect concentration (NOEC) of 10 µg clothianidin/L liquid diet (for adult honeybees) which is used as a toxicity endpoint by the European Food Safety Authority (2013).

Mean residue values in tunnels were three-fold higher than in the field setup confirming that the use of tunnels as a worst-case exposure setting was appropriate. While mean residue concentrations in mason bee pollen in the field experiment mirrored those in bumblebee pollen and were in the range of concentrations found in other field studies on mason bees in WOSR (Peters et al. 2016; Woodcock et al. 2017), maximum concentrations were more than twice as high as residues in bumblebee pollen. Flight distances of mason bees are relatively small compared to

larger, social bee species (Greenleaf et al. 2007). They depend on food resources in close proximity. Individual bees are likely to use OSR as their main forage when placed close to it (Holzschuh et al. 2013; but see Peters et al. 2016). Hence few single samples taken from nests at treatment sites showed high clothianidin concentrations while an amalgamation of stored pollen may reveal low clothianidin concentrations comparable to our mean residue values (cf. Woodcock et al. 2017).

Clothianidin was not detected in control pollen in the semi-field experiment. However, one sixth of the samples from control field sites (i.e. site 2 in Lower Saxony) showed very low concentrations of clothianidin residues. These findings suggest that some bee individuals from nesting tubes at control sites were able to fly the distance to treated OSR fields, other crops treated with clothianidin or wildflowers in arable field margins (Botías et al. 2015). In future experiments distances between control sites and any other treated site should be larger than the minimum distance of 250 m in our study to minimise the risk of contamination.

4.2 *Bombus terrestris*

Bumblebees were more active than mason bees when confronted with unfavorable weather conditions. Neither higher cloud cover nor precipitation or lower temperatures significantly disrupted their flight activity. Bumblebees are adapted to forage in colder climates (Corbet et al. 1993), which makes them excellent pollinators particularly early in the year.

Bumblebees foraged significantly more often on flowers in control than in treatment tunnels. Visitation rate to single flowers may have increased due to resource scarcity; untreated OSR in tunnels produced fewer flowers and grew more patchily than treatment OSR (personal observation). Food shortage has been shown to change foraging behavior of bumblebee individuals (Landry et al. 2000) and is a plausible cause for the observed contrast since higher flower visitation rates in control tunnels did not translate to greater colony growth. Unfortunately we did not quantify flower density and cannot include it as a statistical covariate. Further, bumblebee individuals may have changed their foraging behavior in response to clothianidin residues in pollen. While exposure to neonicotinoids may result in decreased bumblebee foraging activity in the field (Gill and Raine 2014), Arce et al. (2017) and Stanley et al. (2016) showed that bumblebees from control colonies conducted as many foraging bouts as bees from treatment colonies. Bumblebees cannot taste clothianidin and are not repelled by it (Kessler et al. 2015). Low concentrations of neonicotinoids may even increase motor activity in bees (Decourtey and Devillers 2010).

Both semi-field and field colonies revealed expected colony development patterns: while the number of worker bees and colony weight steadily increased and peaked at the end of the laboratory period, brood cell production peaked earlier and dropped to lower numbers compared to the beginning, before colonies were frozen, which indicates the natural end of a colony's life. The more colonies grew, the more worker bees died inside them, although at low numbers. Semi-field colonies were slightly delayed in their onset of increase, possibly due to food scarcity in tunnels compared to the field. Developmental parameters of control colonies resembled those of treatment colonies in the field setup. In a previous field study (Rundlöf et al. 2015) bumblebee colonies produced fewer brood cells and weighed less when exposed to treated summer OSR. In contrast, bumblebee colonies in clothianidin-treated WOSR fields revealed similar strength and (peak) weight like their control counterparts (Sterk et al. 2016; Woodcock et al. 2017; see Bailey and Greenwood 2018 for a critical review of the former). Exposure concentration is a key for effect size (Carreck and Ratnieks 2014): "field-realistic" doses have not always been appropriately estimated in laboratory and "controlled exposure" field trials and may not be representative for certain study locations (e.g. Gill et al. 2012).

While seed treatment did not significantly disrupt colony strength and brood production in tunnels, treatment colonies were heavier than control colonies at the end of the trial. This effect occurred long after the exposure period, when larvae fed on contaminated pollen during the exposure period were hatched, and active adult workers during exposure may still have been alive. If the weight difference was a (maybe behavioural) positive long-term effect of clothianidin exposure, our results contrast studies by Feltham et al. (2014) and Stanley et al. (2016), who revealed a decline in provisioning and pollen collection when adult bumblebees consumed neonicotinoid-contaminated nectar. They may indicate a hormetic effect (Dickel et al. 2018) that could be addressed in a future more extensively replicated study. To our knowledge, behavioral changes in adult bees induced by larval exposure to neonicotinoids have been studied for honeybees (Yang et al. 2012) and mason bees (Nicholls et al. 2017) but not for bumblebees. Treatment colonies also contained (non-significantly) more workers and brood cells in week 12. Variation in colony strength and brood was relatively high in comparison to variation in weight, indicating that bumblebees (like mason bees) show high phenotypic plasticity. Bumblebee colonies can be quite variable and this background noise may restrain the detection of any treatment effect. Due to the relatively low number of replicated locations in our study this variability may have obscured potentially present treatment effects (type II error) and hence lowered

statistical power. While our study design did not produce pseudo-replicated data as did other studies on clothianidin exposure (cf. Bailey and Greenwood 2018), a larger number of replicates would have decreased standard errors for some (e.g. number of brood cells) but not all of the parameters (e.g. colony weight). Replication of field sites is a key factor for reducing type II errors. However, finding the necessary number of site pairs in order to reach statistical power with adequate effect sizes poses a practical challenge (cf. Woodcock et al. 2016 referring to 68 replicate blocks for bee studies). Our study used a similar number of colonies/field sites like other published smaller-scale bumblebee studies on clothianidin exposure (e.g. Cutler and Scott-Dupree 2014; Arce et al. 2017; Scholer and Krischik 2014) but did not match the number of replicates of larger-scale studies (e.g. Rundlöf et al. 2015; Sterk et al. 2016). To cover plasticity and to increase the robustness of results we consider a larger number of replicate sites in future studies.

Sample sizes for residue analysis were small, and we detected no or very low concentrations of clothianidin in pollen and nectar from treatment sites. Maximum clothianidin values in pollen from treatment tunnels (7.0 µg/kg) were three-fold higher than in the field setup (2.7 µg/kg), confirming the use of tunnels as a worst-case scenario. Overall, maximum concentrations in bumblebee field samples were lower than in mason bee samples. Bumblebees are known to forage further away from the nest than solitary bees (Greenleaf et al. 2007; Osborne et al. 2008). Although they exhibit some flower constancy (Free 1970) and can benefit from a mass flowering crop like OSR, food provisions are often sourced from several plant species (Sterk et al. 2016; Woodcock et al. 2017). Mixing resources may dilute potentially higher concentrations of neonicotinoid residues in OSR nectar and pollen; on a colony level bumblebee individuals are likely to use OSR at different intensities which may decrease mean clothianidin concentration even further.

We also detected clothianidin residues in two control field samples. Due to relatively short distances between some control and treatment sites, bee foraging ranges may have overlapped between control and treatment sites so that control sites were not completely independent from treatment sites. The matrix of in-field crops and wild plant species along field edges surrounding our control sites may have potentially acted as a source of clothianidin-containing nectar and pollen as well. Contamination of bee-collected nectar and pollen was also found in other chronic exposure studies where control and treatment sites were further apart (e.g. Rundlöf et al. 2015, Woodcock et al. 2017). In our study, those clothianidin concentrations were substantially lower at control sites than at treatment sites. Negative impacts of clothianidin on population parameters

were absent in tunnels where control samples did not reveal any residues. However, one might still argue that the absence of any negative effect in the field may have been a result of a corrupted diet of control bees containing minimal levels of clothianidin. In general, control sites may not be true controls if field sites vary in multiple factors that interfere with the pesticide effect of interest. Such field site effects could be eliminated by controlled exposure experiments (e.g. Arce et al. 2017) where bees are fed with pre-defined, clothianidin-contaminated sucrose solution. However, controlled exposure studies do not always mimic local field realistic pesticide exposure (Carreck and Ratnieks 2014); for example agricultural practice varies considerably between geographic regions and can have a huge impact on exposure levels (cf. Rundlöf et al. 2015 vs. Woodcock et al. 2017). In order to get a general idea of field-realistic exposure levels, field studies like ours are essential in expanding our knowledge of actual residue levels found in bee-collected plant products.

5 Conclusions

Our study did not reveal significant negative impacts of exposure to WOSR grown from clothianidin-treated seeds (10 g active ingredient/kg seeds) on potential reproductive success of red mason bees and bumblebee colony development. This highlights that maximum concentrations of 4.7 and 7.0 ng clothianidin/g pollen found in mason bee and bumblebee pollen, respectively, represent little risk to the bees' population development. We confirmed that mean and maximum clothianidin residue levels detected in the central-north of Germany mirror the concentrations measured in WOSR (Botías et al. 2015; Cutler and Scott-Dupree 2007; Pohorecka et al. 2012; Thompson et al. 2013) and summer OSR studies (Cutler and Scott-Dupree 2007; Cutler et al. 2014; but see Rundlöf et al. 2015 for higher concentrations despite similarly high seeding densities as the former publications).

While pesticide-induced restrained population development and suppressed reproductive potential are considered as primary sublethal impacts of pesticides on bees, changes in foraging behavior represent another sublethal impact not currently considered in risk assessment (Stanley and Raine 2016). Results of our semi-field study show evidence that impacts on flight activity may be detectable (though possibly as a result of differences in flower density) but may not translate into impacts on reproduction. Although not threatening bee health directly, disrupted foraging behavior due to pesticide exposure can result in a reduction of pollination services and plant reproduction (Stanley et al. 2015) and may indirectly harm bees in the long term.

Although the European Commission has recently banned any outdoor use of clothianidin in the EU (European Commission 2018), further research on the impacts of neonicotinoids is still relevant for an informed risk assessment in the future. So far, the effects on bees are clearly dose-dependent, yet the dose at which they become negatively significant in the field is often unclear (Stanley et al. 2015). Our work highlights the benefit of combining different methods (semi-field and field) and bee taxa to elucidate the risk of pesticide exposure under realistic conditions on a regional scale that mirrors local conditions and agricultural practices.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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