

Uptake of Neonicotinoid Insecticides by Water-Foraging Honey Bees (Hymenoptera: Apidae) Through Guttation Fluid of Winter Oilseed Rape

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Abstract

The water-foraging activity of honey bees (*Apis mellifera* L.) on guttation fluid of seed-coated crops, such as winter oilseed rape (WOR; *Brassica napus* L.), has not yet been evaluated. We analyzed the uptake of active substances (a.s.) in guttation fluid by evaluating residues of honey-sac contents. In autumn, insecticide residues of up to 130 µg a.s. per liter were released in WOR guttation fluid; this concentration is noticeably lower than levels reported in guttation fluid of seed-coated maize. Until winter dormancy, the concentrations declined to <30 µg a.s. per liter. In spring, residues were linked to prewintered plants and declined steadily until flowering. The maximum release of residues in guttation fluid of seed-coated WOR occurs on the first leaves in autumn when the colonies' water demand decreases. For the first time, proof for the uptake of guttation fluid from seed-coated WOR by honey bees was provided by measuring residues in individual honey-sac contents. In total, 38 out of 204 samples (19%) showed residues of thiamethoxam at concentrations ranging from 0.3 to 0.95 µg per liter while the corresponding concentrations in guttation fluid of WOR varied between 3.6 to 12.9 µg thiamethoxam per liter. The amounts of thiamethoxam we found in the honey sacs of water-foraging honey bees were therefore below the thresholds in nectar and pollen that are considered to have negative effects on honey bees after chronic exposure.

Key words: guttation, neonicotinoid, seed coating, winter oilseed rape, honey sac

Seed coating with systemic insecticides, such as neonicotinoids, has become a common protection practice to protect many agricultural and horticultural crops against plant-feeding insects. High water solubility and xylem mobility of the active substances (a.s.) represent essential physicochemical prerequisites for their distribution within the plant tissue, and therefore for the efficacy of seed treatment. Neonicotinoids (i.e., acetamiprid, clothianidin, dinotofuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam) act on the insect's central nervous system as strong agonists of the postsynaptic nicotinic acetylcholine receptors (Tomizawa and Casida 2003). These active agents are used worldwide every year in several agricultural and horticultural crops, including high-yield crops such as cotton, maize, potato, and rape, to control a widespread range of insect pests (Weichel and Nauen 2003; VanTimmeren et al. 2011, 2012; Wallingford 2012; Vernon et al. 2013; Van Rozen et al. 2013).

However, the compounds clothianidin, imidacloprid, and thiamethoxam are also known for side effects (acute and sublethal) on nontarget beneficial organisms. In 2008, damage to honey bees (*Apis mellifera* L.) occurred in ~12,000 hives located in the Rhine Valley, Germany, and was caused by the drift of insecticidal particles from neonicotinoid-treated seeds of maize (*Zea mays* L.; Forster 2009; Pistorius et al. 2009; Rosenkranz and Wallner 2009). Comparable incidences were also reported in Italy (Greatti et al. 2003, 2006; Bortolotti et al. 2009; Marzaro et al. 2011). As a consequence, the seed coating with neonicotinoids in maize and cereals was suspended in 2009 in Germany by the Federal Office of Consumer Protection and Food Safety (BVL). In addition to these acute poisoning incidences, neonicotinoids are considered to have sublethal effects on honey bees and other pollinators (Sandrock et al. 2013, 2014; Goulson 2015). Even the application of sublethal

doses of clothianidin, imidacloprid, or thiamethoxam caused severe problems in the learning and orientation ability of individual honey bee foragers (Schneider et al. 2012; Henry et al. 2012; Fischer et al. 2014). Neonicotinoids together with pathogens and environmental factors have therefore been suspected to contribute to colony collapse disorder, in which honey bee hives are largely abandoned by the adult bees (Oldroyd 2007; VanEngelsdorp et al. 2009; Alaux et al. 2010; Paxton 2010; Vidau et al. 2011; Pettis et al. 2012). Chronic exposure to sublethal concentrations of clothianidin, thiamethoxam (Sandrock et al. 2013; Rundlöf et al. 2015), and imidacloprid (Whitehorn et al. 2012) leads to reduced reproductive capacity in bumble bees and solitary bees. Due to their high toxicity to insects and the large-area application in seed coating, neonicotinoids are perceived to be a contributor to colony and pollinator decline.

In spring 2013, the controversial debate on the use of neonicotinoids led to a risk assessment of the three active agents clothianidin, imidacloprid, and thiamethoxam, which are extremely toxic to honey bees (acute oral LD₅₀ of 3.7 ng/bee for clothianidin, 3.7 ng/bee for imidacloprid, and 5 ng/bee for thiamethoxam). In this process, the European Food Safety Authority (EFSA) revealed a data gap for the exposure of honey bees to active substances in drifted dust, nectar, and pollen as well as guttation fluid (European Food Safety Authority 2013a, b; European Food Safety Authority Panel 2013). This resulted in a temporary ban of the three neonicotinoids as seed coating by the EU Regulation No. 485/2013 issued on 24 May 2013 by the European Commission. Several agricultural and horticultural crops are affected, including maize and winter oilseed rape (WOR; *Brassica napus* L.).

In this context, WOR became the focus of particular attention because of the large cultivation area and its attractiveness to nontarget insects due to the availability of pollen and nectar. WOR has nectar production of 0.6 mg per blossom per day and a mean sugar content of 44 to 59%, and produces 90 to 174 kg pollen per hectare (Maurizio and Graf 1980); these resources make WOR a highly attractive crop for honey bees and other pollinators. Unfortunately, WOR is generally very susceptible to pest and fungal infestation, which urges an intensive plant protection management beginning with coating the seeds with neonicotinoids. It is already known that traces of the neonicotinoid compounds from these seed coatings can be found in nectar and pollen of WOR (Pohorecka et al. 2012). An additional risk for the contamination of nontarget insects outside the flowering period is the uptake of guttation drops in seed-coated crops.

Guttation is a physiological process occurring in several plants for keeping up the internal water flow as an alternative process to transpiration, which is inhibited by an increasing humidity of the air. Thus, the exudation of surplus xylem fluid as water vapour by stomata (transpiration) is replaced by guttation (exudation in the form of water drops), which occurs at the leaf tips or along the leaf blades. Guttation fluid of nontreated plants presents no risk for honey bees and other nontarget organisms. Previous studies on seed-coated maize and triticale revealed that residues of systemic insecticides can be released in guttation fluid after they were applied as seed coatings or granules (Girolami et al. 2009; Joachimsmeier et al. 2010a, 2012a; Reetz et al. 2011; Tapparo et al. 2011; Pistorius et al. 2012). The highest concentration of active compounds in guttation fluid can be expected at the beginning of the growth phase of the crop. This applies to maize in Central Europe in early summer (May; Reetz et al. 2011) when the liquid demand of honey bee colonies is rather high; for WOR, however, this growth phase takes place during autumn (September), which coincides with the final preparation of honey bee colonies for overwintering.

There is a lack of detailed information on 1) the amount of guttation in WOR, 2) the concentration of neonicotinoid residues in guttation fluid of seed-coated WOR, and 3) the use of guttation fluid as a water source by water-foraging honeybees. We investigated these issues in a small-patterned landscape in southern Germany and in an intensive agricultural region in northern Germany. To assess practice-related data, commonly available seed coatings were used in WOR.

Materials and Methods

Experimental Sites, Treatments of WOR and Honey Bees

The experiments were performed 2009 to 2011 in southern Germany (Hohenheim, Baden-Wuerttemberg), and in northern Germany (Roggendorf, Mecklenburg-Western Pomerania). Both study sites provided agricultural areas planted with WOR. Seed coating with neonicotinoids and WOR sowing were performed according to the recommendations of the Agricultural Extension Service of the respective region (Table 1). These recommendations vary due to climatic differences and characteristics of the respective soils, but represented common agricultural practice before the ban of the three neonicotinoids. Table 1 also provides an overview of the different experiments during the investigation period.

The main difference between the two study sites was the intensity of the WOR cultivation: Hohenheim presented a more structured landscape, which provided alternative water foraging areas for honey bees, whereas the honey bees in Roggendorf were forced to forage water exclusively in WOR.

Hohenheim (HH; Southern Germany)

Experiments were carried out in the Heidfeldhof experimental area of the University of Hohenheim during the period 2009 to 2011. The site is situated at 400 m above sea level. The daily temperature and precipitation were recorded (Table 2). The prevalent type of soil is brown earth with partial silt; theoretical plate number 55 to 60. This location is characterized by a small-patterned landscape, a high biodiversity, and a wide range of water sources (permanent creeks and ponds; temporary puddles, water in ruts, WOR guttation fluid, dew, and rainfall).

Honey Bee Colonies

Six honey bee colonies belonging to the Apicultural State Institute were installed on a grassy road close to the field of freshly sown, seed-coated WOR, with the hive entrances pointing directly to the WOR area. WOR and the weeds and herbs in the tramline were the only plants growing within a distance of 300 m around the hives. The colonies were approximately of the same size (15,000 to 17,000 bees) and were headed by queens of the local breeding line. Hohenheim two-storey standard hives with 10 Zander frames per storey were used.

Winter Oilseed Rape

WOR was sown as standard with 50 seeds per square meter. For field trials, seeds with commonly available seed coatings were used to obtain data reflecting current agronomic practice. In 2009, the registered products Cruiser OSR (Syngenta Agro GmbH, Maintal, Germany) according to the German registration (4.2 g thiamethoxam/kg seed) and Elado (Bayer CropScience AG, Monheim, Germany) + TMTD + 98% Satec (SATEC HandelsGES. mbH, Elmshorn, Germany) (10.0 g clothianidin/kg seed) were used as seed coating in WOR; in 2010, Elado + TMTD 98% Satec + DMM (SATEC HandelsGES. mbH, Elmshorn, Germany) was used.

Table 1. Detailed information on the experiments in seed-coated winter oilseed rape performed at Hohenheim (HH; southern Germany) and Roggendorf (RD; northern Germany) during the period 2009 to 2011

Location	Year	Field size (ha)	Bee hives (<i>n</i>)	Trials	Samples (<i>n</i>)	Seed coating in WOR	Variety of WOR	Expected dose of active substance			Metabolites	
								(g a.s./kg seed)	(μ g a.s./grain ^a)	(g a.s./ha ^b)		
HH	2009	~0.2	6	GUT RES	22	CruiserOSR (German registration)	Hammer	4.2	18.5	9.25	Thiamethoxam	Clothianidin
RD	2011	42.5	16	GUT		CruiserOSR	Pioneer PR46W26	3.6	15.8	7.90	Thiamethoxam	Clothianidin
				RES WCH	11 232	(Polish registration)						
HH	2009	2 × ~3.0	6	GUT		Elado + TMTD 98% Satec	Hammer	10.0	44.0	22.00	Clothianidin	TZNG, TZMU
HH	2010	2 × ~2.3	6	RES GUT	23; 22	Elado + TMTD 98% Satec + DMM	Dimension	10.0	44.0	22.00	Clothianidin	TZNG, TZMU
				RES WCH	21; 15 204							

WOR, winter oilseed rape; GUT, observation of occurrence of guttation in WOR; RES, residual analysis of guttation fluid of WOR; WCH, observations of water-collecting honey bees on guttation in WOR and residual analysis of honey-sac contents.

^a Calculated on the thousand grain weight of 4.4 g.

^b Calculated on the seed rate of 50 seed per square meter.

Table 2. Temperature (°C; min., max.) and mean precipitation (mm) per month during the experiments on guttation of winter oilseed rape at the experimental sites in Hohenheim (HH; southern Germany) and Roggendorf (RD; northern Germany)

Hohenheim (HH; southern Germany)				
Month	Year	Ø Temp (°C)		Ø Precipitation (mm)
		Min.	Max.	
Aug.	2009	13	27	2
Sept.	2009	10	22	1
Oct.	2009	5	14	2
Nov.	2009	3	11	2
Dec.	2009	-2	4	2
Jan.	2010	-5	0	1
Feb.	2010	-2	5	1
Mar.	2010	1	10	1
April	2010	4	16	0
May	2010	8	16	2
June	2010	11	24	2
July	2010	14	28	3
Aug.	2010	12	23	3
Sept.	2010	7	19	2
Oct.	2010	3	14	1
Nov.	2010	2	9	2
Dec.	2010	-5	1	2
Jan.	2011	-2	4	1
Feb.	2011	-1	7	0
Mar.	2011	1	12	1
April	2011	5	20	1
May	2011	7	22	1
Roggendorf (RD; northern Germany)				
Aug.	2011	14	21	4
Sept.	2011	11	19	2

Roggendorf (RD; Northern Germany)

Experiments were carried out in autumn 2011 (21–30 September). The field site is situated at 48 m above sea level. The daily temperature and the daily precipitation were recorded, beginning with the

sowing until the end of the experiments (Table 2). The prevalent type of soil is brown earth; theoretical plate number 48 to 52. This location is characterized by intensive agriculture with reduced water sources (two permanent ponds at a distance of ~1 km; temporary WOR guttation fluid, dew, and one rainfall).

Honey Bee Colonies

Sixteen hives belonging to an apiary of a local beekeeper were used for experiments, and were positioned centrally in the field of 42.5 hectares of seed-coated WOR. Placing the bee hives in the center of a WOR field does not conform to the common beekeeping practice but displays worst case conditions for water foragers.

Depending on the weather conditions, groups of four hives each were moved onto the field of WOR for intervals of 2 or 3 d. The colonies were headed by young queens of the local breeding line, and were comparable in size. The colonies were kept in Frankenbeute two-storey standard hives with nine Langstroth frames per storey. The feeding of the colonies had been finished before starting the experiments. Nevertheless, in order to increase the honey bees' water demand and for stimulating the water-foraging activity, some sugar paste feed (Apifonda; Südzucker, Mannheim, Germany) was offered inside the hives.

Winter Oilseed Rape

Seeds are sown as standard with 50 seeds per square meter in a field of 42.5 hectares. WOR seed was treated with the registered product CruiserOSR according to the Polish registration (3.6 g thiamethoxam/kg seed). Thus, the maximum authorized amount of active substance per seed was lower than during the trials in 2009–2010 carried out in HH.

Sampling Methods

Guttation

The observations on the occurrence of guttation in WOR as well as the sampling of the guttation fluid were done between 6:30 a.m. and 10:00 a.m. In case of rainfall, the sampling was postponed. The occurrence of guttation and the growth stages of the plants were documented using the BBCH Monograph (Meier 2001). In case of

guttation, single drops were randomly sampled from at least 20 to 30 plants (pooled sample) and transferred drop by drop by means of glass Pasteur pipettes (VWR International GmbH, Darmstadt, Germany) with rubber bulbs (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) into sealable glass vials (1.5 ml; Chromatographie-Zubehör Trott, Krißtel, Germany). Laboratory investigations required an amount of 3 ml guttation fluid. All samples of guttation (HH: 2009: $n=67$; 2010: $n=36$; RD: 2011: $n=10$) were stored in the freezer at -20°C until residue analysis.

Water-Foraging Honey Bees

Targeted observations on water-foraging honey bees in the field are nearly impossible due to the wide foraging range. Therefore, returning foraging honey bees were sampled in autumn 2010 (HH) and 2011 (RD) from hives which were exposed to germinating seed-coated WOR (Table 1). After observing the flight activity in front of the entrance (5 min), the hive entrances were closed. Subsequently, samples of returning foragers could be collected by using a vacuum cleaner (RosyTec GmbH, Kirchhain, Germany). Inside the vacuum cleaner, honey bees were frozen with dry ice and stored in the freezer at -20°C . Honey-sac contents were analyzed using a centrifugation method (Reetz and Wallner 2014). In autumn 2010 (20–24 September, 11 October; HH), water-foraging bees were sampled on 6 d and 232 individual honey sacs were analyzed for residues. In autumn 2011 (2–30 September; RD), water-foraging bees were sampled on 10 d and 204 individual honey sacs were analyzed for residues.

Residues of Neonicotinoid Insecticides in Guttation Fluid of Seed-Coated WOR and Honey-Sac Contents

The chemical analysis was performed using different HPLC-MS systems equipped with electrospray ionization. The ion sources were operated in the positive mode. All reagents and solvents were HPLC or MS grade. Individual MS acquisition parameters and the retention times for the analytes are given as supporting information (please see [Supp Material](#); [Supp Table 1](#) [online only]).

Guttation

Analysis of guttation fluid required no specific preparation; samples were injected directly into the HPLC-MS systems.

Honey-Sac Contents

Samples of 2010

The cooled samples of honey-sac contents were fortified with ice water (60 μl) and internal reference standard (imidacloprid- d_4 , 40 ng). After the addition of acetonitrile (150 μl), the samples were mixed on an automated shaker (30 min). The reduced final extract ($\sim 30 \mu\text{l}$) was injected into the HPLC-HR/MS system (Table S1, HPLC-MS system No. 2). Compounds were identified and quantified by HPLCHR-MS (LTQ-Orbitrapspectrometer, Thermo Fisher Scientific GmbH, Bremen, Germany). Retention times and exact masses were consistent with the reference standards (Sigma-Aldrich Produktions GmbH, Steinheim, Germany). Separation was performed on a Surveyor-LC HPLC system (Thermo Fisher Scientific GmbH, Bremen, Germany) using an RP-Nucleodur Gravity column (1.8 μm , 3 by 50 mm; Macherey-Nagel GmbH + Co. KG, Düren, Germany). The spectrometer was operated in positive mode (mass range, 80–400, high-accuracy mass measurements within 2 ppm deviation using internal lock mass; m/z 391.284290; bis-(2-ethylhexyl) phthalate). Compounds were monitored at their exact masses, imidacloprid (m/z of 256.059–256.060; Rt 8.43 min), imidacloprid- d_4 (m/z of 260.084–260.085; Rt 8.43 min), thiacloprid (m/z of 253.0305–253.0315;

Rt 9.25 min), clothianidin (m/z of 250.0150–250.0170; Rt 8.55 min), and thiamethoxam (m/z of 292.026–292.028; Rt 7.84 min). External calibration was linear, ranging from 1 to 5,000 ng/ml.

Samples of 2011

The cooled samples of honey-sac contents were diluted with acetonitrile (100 μl) and well mixed by vortex shaker (~ 1 min). A saline solution (100 g/liter NaCl, 100 μl) was added, and then the samples were well mixed again and centrifuged (2 min, 500 rpm) for phase separation. The upper phase of each sample (50 μl) was transferred into a glass vial, diluted with dH_2O (50 μl), and 5 μl of this solution was injected into the HPLC-MS/MS system (Table S1; HPLC-MS system No. 3), which consisted of a Accela UHPLC-System coupled to a LTQ Velos mass spectrometer (both Thermo Fisher Scientific Inc, San Jose, CA) equipped with a Heated Electrospray Ionization source (HESI). The HESI parameters in positive polarity were as follows: 350°C , ion spray voltage +3500 V, capillary temperature 300°C , sheath gas 50, auxiliary gas 20, sweep gas flow rate 1, S-Lens RF level 60. Chromatography was carried out on a Triart C18 column (3 μm ; 100 by 3.0 mm; YMC Europe GmbH, Dinslaken, Germany) at a flow rate of 0.4 ml/min. The column was maintained at 40°C . For separation of analytes following linear gradient program was used (min/% B): 0/15; 1/15; 16/98; 18/98; 19/15; 22/15. Analytes were quantified via an SRM (Selected Reaction Monitoring) approach using a collision-induced dissociation (CID) mechanism. The signals of the most abundant fragment ions in the CID spectrum were used for quantification. Thiamethoxam and clothianidin standards were obtained from the manufacturer Dr. Ehrenstorfer GmbH (Augsburg, Germany), TZNG and TZMU from Bayer CropScience GmbH (Monheim, Germany). Individual pesticide stock solutions (1,000 mg/liter) were prepared in acetonitrile and stored in amber screw-capped glass vials in the dark at 4°C . External standard solutions used for calibration were prepared from the stock solutions by dilution with acetonitrile (50%).

Results

Occurrence of Guttation in WOR

Guttation in WOR occurs regularly beginning with the growth of the first leaves (BBCH 10) until the winter dormancy (BBCH 16/17). During the winter (period of vernalization), guttation could be observed in only a few cases with very small volumes. In spring, WOR guttation continued up to flowering (BBCH 51) and through the end of flowering, but less regularly than before vernalization. Thus, further sampling was not practical due to the small volume of fluid, even though guttation could be observed sporadically until harvest. Guttation in WOR occurred concurrently with the guttation of surrounding plants, e.g., monocotyledons.

Residues of Neonicotinoid Insecticides in Guttation Fluid of Seed-Coated WOR

Due to the favorable climate in southern Germany, WOR seeds sown in late August germinate in September. The first guttation fluid occurs at the cotyledons, which can be contaminated with clothianidin in concentrations of 70 to 130 μg a.s. per liter (BBCH 10; Fig. 1B). After the growth of the first leaves, the amount of guttation fluid increases. The level of active substances released in the guttation fluid of seed-coated WOR is highest during autumn (up to 130 μg /liter clothianidin at BBCH 10; Fig. 1B, C). Until the winter dormancy, WOR continues its growth up to BBCH 16/17. This leads to an accumulation of active substance within the plant cells, but simultaneously to a dilution of the

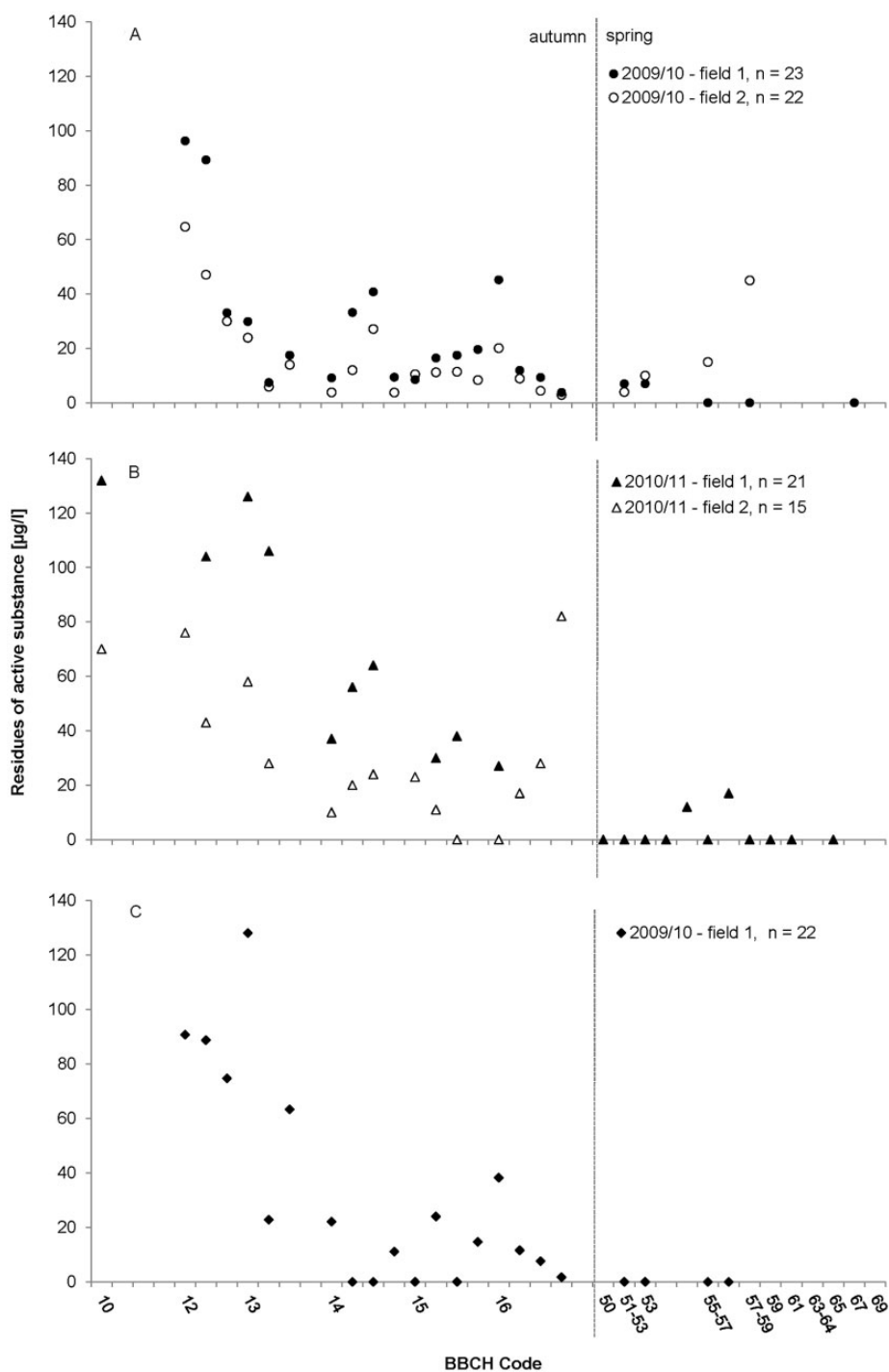


Fig. 1. Concentrations of active substances ($\mu\text{g}/\text{liter}$) in guttation fluid of seed-coated winter oilseed rape cultivated in Hohenheim (HH; southern Germany). Samples on guttation fluid were collected during several growth stages (BBCH Codes). (A) In 2009–2010, variety ‘Hammer’ was treated with 10 g clothianidin/kg seed. (B) In 2010–2011, variety ‘Dimension’ was treated with 10 g clothianidin/kg seed. (C) In 2009–2010, variety ‘Hammer’ was treated with 4.2 g thiamethoxam/kg seed (German registration; clothianidin [metabolite of thiamethoxam]). For all compounds LOQ = 0.5 $\mu\text{g}/\text{liter}$.

active substance in the xylem fluid, and also in the guttation fluid. Prewinter guttation of seed-coated WOR had $<30 \mu\text{g}$ clothianidin per liter (Fig. 1). The release of residues in postwintered guttation fluid (March, April) is linked to the level of concentrations measured before winter dormancy of WOR. In spring, further growth of the plants (until flowering) leads to a further decrease in released concentrations of active substances in the WOR guttation fluid.

In the intensive agriculture region of northern Germany, samples of guttation from seed-coated WOR (3.6 g thiamethoxam/kg seed) could only be collected 10 times during the earliest growth stages (BBCH 11/12 to 13/14) due to the experimental duration of 10 d. These guttation fluids had $\leq 19 \mu\text{g}$ a.s. per liter (Σ thiamethoxam and clothianidin; LOQ = 0.3 $\mu\text{g}/\text{liter}$; Table 3).

Table 3. Concentrations of active substance (a.s.) thiamethoxam and its metabolite clothianidin (LOQ = 0.3 µg/liter) in guttation fluid of seed-coated winter oilseed rape (variety 'Pioneer PR46W26', coated with 3.6 g thiamethoxam/kg seed; Polish registration) cultivated in 2011 in Roggendorf (RD; northern Germany)

BBCH code	Active substances (a.s.) (µg a.s./liter)		
	Thiamethoxam	Clothianidin	Σ
11–12	12.02	6.47	18.49
	12.94	5.93	18.87
	6.99	3.06	10.05
12	3.46	0.54	4.00
	3.25	0.70	3.95
12–13	4.51	0.67	5.18
	4.12	0.75	4.87
13	4.25	0.49	4.74
	3.97	0.46	4.43
13–14	7.64	0.91	8.55

Residues of Neonicotinoid Insecticides in Honey-Sac Contents of Honey Bees

Hohenheim

A total of 232 honey-sac contents from water-foraging honey bees that gathered in the small-patterned landscape were analyzed for clothianidin residues (LOQ = 3 µg/liter) according to the seed coating with Elado + TMTD 98% Satec + DMM. Residues of the active agents used in the seed coating could not be detected in any of these samples (Table 4).

Roggendorf (RD)

A total of 204 samples showing probability of coincidence between flight activity of honey bees and occurrence of guttation were analyzed for residues of neonicotinoids (Table 4). In 141 samples (69%), no residues of neonicotinoids were detected in the honey-sac contents, whereas 38 samples (19%) were contaminated with thiamethoxam at 0.3 to 0.95 µg per liter (LOQ = 0.3 µg/liter). Additionally, 24 samples (12%) had thiamethoxam at concentrations below LOQ, and one sample (0.5%) was contaminated below LOD. Clothianidin could be detected in just one sample at a concentration below LOQ (0.13 µg/liter < LOQ = 0.3 µg/liter). TZMU, one of its metabolites, was detected at a concentration below LOD (0.08 µg/liter < LOD = 0.1 µg/liter). TZNG, a second metabolite of clothianidin, could not be detected in any of the samples (LOQ = 3.0 µg/liter).

Discussion

In this study, we show for the first time that guttation fluid from seed-coated WOR contained measurable contamination residues from neonicotinoids, and that the contaminated guttation fluid was taken up by honey bees. This confirms that the translocation of systemic active substances is not only limited to pollen and nectar. Earlier studies had already shown that guttation fluid is excreted by WOR and that this guttation fluid of seed-coated plants presents an additional exposure route of active substances (Girolami et al. 2009; Joachimsmeier et al. 2010a, 2012a; Marzaro et al. 2011; Reetz et al. 2011; Tapparo et al. 2011; Pistorius et al. 2012).

We observed that guttation commonly occurred in field-grown WOR, frequently during the cultivation period and concurrently with the guttation of surrounding plants. However, during the winter dormancy (period of vernalization), the guttation process was

Table 4. Residual analysis by HPLC-MS systems of guttation fluid of seed-coated winter oilseed rape and honey-sac contents of returning foraging honey bees exposed to this seed-coated winter oilseed rape cultivated in Hohenheim (HH, 2010; southern Germany) and Roggendorf (RD, 2011; northern Germany)

Year	Location	Sample	Active substances (g a.s./kg seed)	Metabolites	LOQ	Samples (n)	Residues (µg a.s./liter) (n) (%)			Residues > LOQ (µg a.s./liter)			
							n.n.	< LOD	LOD > x < LOQ	> LOQ	Min.	Max.	Σ
2010	HH	WCH	10.0	Clothianidin	~3.0 ^a	232	232	—	—	—	—	—	—
2010	HH	GUT	10.0	Clothianidin	3.0	36	—	—	11 (31)	10.00	132.00	46.00	30.00
2011	RD	WCH	3.6	Thiamethoxam	0.3	204	141 (69)	1 (0.5)	24 (12)	0.30	0.95	0.39	0.37
				Clothianidin	0.3	204	203	—	1	—	—	—	—
				TZMU	0.3	204	203	1	—	—	—	—	—
				TZNG	3.0	204	204	—	—	—	—	—	—
2011	RD	GUT	3.6	Thiamethoxam	0.3	10	—	—	—	3.25	12.94	6.31	4.38
				Clothianidin	0.3	10	—	—	—	0.54	6.47	2.00	0.72

LOD, limit of detection (µg a.s./liter); LOQ, limit of quantitation (µg a.s./liter); GUT, guttation fluid of winter oilseed rape; WCH, honey-sac contents of water-collecting honey bees.
^a Dependent on volume of honey-sac content (cal. on 30 µl).

nearly inhibited. In spring, the amount of guttation fluid increased again for a few weeks before WOR increased stem elongation. The frequency and intensity of guttation decreased until flowering and kept decreasing until the maturation of the WOR, which might have been a result of the vernalization (Simming-Annefeld 1976). As a natural process, occurrence of guttation fluid indicates high environmental water availability and is correlated to soil temperature and moisture (Hughes and Brimblecombe 1994). However, due to the multitude of guttation influencing factors (e.g., plant species, growth stage, type of soil, soil moisture and water availability for roots, temperature, and humidity), threshold values for guttation in WOR have not been determined (Joachimsmeier et al. 2012b). Earliest guttation events already occur with the development of the cotyledons (BBCH 10). Under field conditions, up to 1.3 ml guttation fluid per plant can be released by young WOR plants during the night until the morning hours (K.W. and J.E.R., unpublished data). Based on the assumption that 80% of the seeds germinate (50 seeds per square meter), young WOR plants might deliver up to 520 liter of guttation fluid per hectare.

Residue analysis of guttation fluid of seed-coated WOR revealed that the systemic properties of neonicotinoids lead to measurable residues in guttation fluid. In autumn, up to 130 µg/liter clothianidin was released by the first leaves of WOR (BBCH 10 to 16/17) cultivated in southern Germany. The residues in guttation fluid of seed-coated WOR were noticeably lower than the highest residues measured in guttation fluid of seed-coated maize (up to 8,000 µg/liter clothianidin; Reetz et al. 2011). In WOR, the maximum concentrations were merely limited to a few weeks during autumn. In spring, the release of guttation fluid and residues contained therein started at lower levels (<30 µg/liter clothianidin). Guttation in WOR continued to occur until flowering but with decreasing neonicotinoid residues. Higher values of residues (up to mg a.s./liter) measured in WOR guttation fluid were reported from Central Germany (Lower Saxony; Joachimsmeier et al. 2010b). A noteworthy lower level of residues (20 µg/liter Σ thiamethoxam and clothianidin) was measured in guttation fluid of seed-coated WOR cultivated in northern Germany in 2011. These findings were not consistent with our results from southern Germany during the period 2009 to 2011. This leads to the conclusion that local factors influencing guttation (e.g., weather conditions, microclimate in the plant stand, physicochemical properties of active substances, variety-specific translocation of active substances in the plant tissue) might be responsible for the differences in residue levels in guttation fluid of seed-coated WOR cultivated in different years and different sites, e.g., soils. The low residues in WOR guttation fluid of northern Germany might be less associated with the lower amount of active substance per seed (expected dose: CruiserOSR according to Polish registration with 15.8 µg thiamethoxam per grain in comparison to the German registration with 18.5 µg a.s. per grain) than with faster processes of leaching into the soil due to the high soil moisture resulting from the heavy precipitations in northern Germany during the summer months of 2011.

The collection by honey bees of guttation fluid from seed-coated WOR in combination with the residue analysis of honey-sac contents from those bees displays the third aspect of these experiments. The methodology of analyzing the bees' honey-sac loads delivered precise information about the active uptake of residues through guttation fluid of seed-coated WOR. Samples were first collected from honey bee colonies located in the small-patterned landscape at the Hohenheim study site. This site was characterized by a high diversity of alternative water sources, such as permanent (stream, ponds) and temporary (puddles, water in ruts, guttation fluid, dew,

raindrops) sources. The WOR guttation fluid found there was contaminated with residues of the seed coating (up to 130 µg/liter clothianidin in BBCH 10), and there was an intake of water into the hives at the same time (Reetz et al. 2012). Even though honey bees gathering guttation fluid from WOR could be observed in only a few cases, no residues of neonicotinoids could be measured in the honey-sac contents ($n = 232$). Therefore, the Northern study site was used which was characterized by a landscape with a more intensive cultivation area of WOR. Here, the honey bee colonies were positioned centrally in a field of 42.5 hectares planted with young seed-coated WOR. In this worst-case scenario, the diversity of water sources was reduced to guttation fluid and dew from the WOR plants. The nearest permanent water sources were documented to be $\sim 1,000$ m away. At this study site honey bees could regularly be observed gathering WOR guttation fluid. The uptake of contaminated water confirms current experiments revealing that honey bees as well as bumble bees do not avoid food which is contaminated by field-realistic concentrations of clothianidin, imidacloprid, or thiamethoxam (Kessler et al. 2015). These observations were also verified by the residual analyses of individual honey-sac contents ($n = 204$): In total, 19% of the samples ($n = 38$) were contaminated with thiamethoxam in concentrations ranging from 0.3 to 0.95 µg per liter (LOQ = 0.3 µg/liter), and 12% ($n = 24$) showed residues of thiamethoxam below LOQ; corresponding concentrations in guttation fluid of WOR varied between 3.25 to 12.94 µg thiamethoxam per liter. Residues of clothianidin and TZMU were detected in one sample of honey-sac content (each 0.5%) below LOQ and LOD, respectively (clothianidin: 0.13 µg/liter; TZMU 0.08 µg/liter); corresponding concentrations in guttation fluid of WOR varied between 0.54 to 6.47 µg clothianidin per liter. These results indicated that honey bees forage on guttation fluid of seed-coated plants in case of a reduced or absent variety of alternative water sources in the surrounding area, and supported the conclusion that in a landscape with alternate water sources, guttation fluid of seed-coated WOR does not represent an unacceptable risk to water-foraging honey bees.

Previous work determined that there is an overlap in time and space between the occurrence of guttation fluid and the activity of water-foraging honey bees (Reetz et al. 2012). The data presented here on honey-sac contents confirmed that the uptake of contaminated guttation fluid by honey bees occurs, at least under the particular conditions at the Northern study site without alternative water sources. Guttation in WOR occurs temporarily during the morning hours until the single drops at the leaf blades evaporate. In contrast, maize plants initially deliver guttation fluid as single drops at the leaf tips and blades, which later coalesce in the leaf sheaths. Due to this funnel function of the maize leaves, the fluid is present in the leaf sheaths during the day unlike in WOR. Increasing the distance to the treated fields (≥ 500 m; Visscher et al. 1996) might be one measure for minimizing the uptake of residues by guttation fluid into beehives in regions with fewer alternative water sources. In addition to guttation fluid, a variety of temporary water-delivering sources, like dew, raindrops, or nectar, are usually available to honey bees during the day and throughout the year.

For evaluating guttation fluid of seed-coated WOR as a water source for honey bees and possible effects on bees' health, the season-depending foraging activity needs to be regarded in view of the facility of a residue uptake by guttation. In this context, the following major aspects have to be considered: 1) Seasonality in occurrence of high amounts of guttation fluid and residues in WOR guttation fluid in autumn when the water demand of honey bee colonies decreases, except when feeding them with sugar paste for

overwintering. In autumn, the water content in honey bee colonies decreases due to the common practice of feeding honey bee colonies with sugar syrup. The syrup has to be thickened by evaporative processes of the honey bees. 2) Guttation of seed-coated WOR occurs at the same time as guttation of several mono- and dicotyledonous plants. 3) Residues of neonicotinoids measured in honey-sac contents are reduced by the factor of about 20 as compared to guttation fluid of seed-coated WOR. 4) Foraging honey bees are temporarily exposed to residues while carrying contaminated nectar or water in the honey sacs. Within the hive, nectar and water are distributed to several hive mates. 5) Due to the large number of unsealed larvae and the jelly they are fed with, there might be an increased demand for water in spring (average amount of water in jelly for drones is 62.9% and for workers is 66.8%; Nelson et al. 1924; Lindauer 1954).

Flowering WOR is an attractive crop for honey bees and other pollinators due to the daily nectar and pollen production (Maurizio and Grafl 1980). For this reason, many beekeepers move their colonies to fields of flowering WOR in spring. Residues of systemic neonicotinoids used as seed coating are present in nectar and pollen, with lower amounts in samples of WOR than in samples of spring rape (Pohorecka et al. 2012). During flowering of WOR, the release of systemic insecticides in pollen and nectar is not only of ecotoxicological importance for honey bees, but also for bumble bees and solitary bees, as well as for further flowering visiting insects, such as beetles, hoverflies, lacewings, butterflies, or spiders. The current evaluation of short-term effects of chronic exposure to sublethal concentrations of neonicotinoids in pollen on honey bees at colony level is based on the application of higher residue concentrations (2 ppb \triangleq $\mu\text{g a.s./liter}$; Sandrock et al. 2014) than detected in the honey-sac contents of the water-foraging honey bees in this experiment (up to 0.95 $\mu\text{g a.s./liter}$ \triangleq <1 ppb). Currently no information is available on effects that are caused by lower concentrations. Thus, the risk of negative effects on honey bees due to contaminated guttation fluid of seed-coated WOR seems to be relatively low compared to other contamination sources, e.g., pollen. However, other pollinators, such as bumble bees and solitary bees, might be more susceptible to neonicotinoids (Gill et al. 2012; Laycock et al. 2012; Whitehorn et al. 2012; Larson et al. 2013; Sandrock et al. 2013; Goulson 2015). This is confirmed by a recent field study conducted in Sweden where strong effects on bumble bees and solitary bees but not honey bees was thought to be caused by the exposure to large fields of spring rape treated with clothianidin (25 ml Elado/kg seed; maximum residues: honey bee-collected pollen 23 ng a.s./g and in honey bee-collected nectar 16 ng a.s./ml; Rundlöf et al. 2015). Even low risk to honey bee colonies due to thiamethoxam residues in pollen and nectar from seed-coated oilseed rape were detected by Pilling et al. (2013), whereas Cutler et al. (2014) estimate no adverse effects on honey bee colonies when exposed to canola grown from seed coated with clothianidin. Dively et al. (2015) found no strong effects at the colony level when honey bees were fed with pollen containing imidacloprid at concentrations relevant for seed-coatings (5 $\mu\text{g a.s./kg}$). During the past few years, the scientific focus has been shifted on side effects to other beneficial nontarget insects, on the pollinator decline, and loss of biodiversity in agriculture (Biesmeijer et al. 2006; Desneux et al. 2007; Klein et al. 2007; Byrne and Fitzpatrick 2009; Gallai et al. 2009; Carvalheiro et al. 2010; Mommaerts et al. 2010; Ollerton et al. 2011; Bommarco et al. 2012; Holzschuh et al. 2012; Laycock et al. 2012; Stoner and Eitzer 2012; Whitehorn et al. 2012; Easton and Goulson 2013; Laycock and Cresswell 2013; Larson et al. 2013; Sandrock et al. 2013; Cutler et al. 2014; Rundlöf et al. 2015). As individual and synergistic pollinating capacities are essential for a multitude of plants,

declines in biodiversity will cause ecological and economic interference in the prevalent cultural landscape, which needs to be prevented (Watanabe 1994; Ghazoul 2005; Klein et al. 2007; Gallai et al. 2009; Carvalheiro et al. 2010; Ollerton et al. 2011; Albrecht et al. 2012; Holzschuh et al. 2012; Rucker et al. 2012; Leonhardt et al. 2013; Stanley et al. 2013).

Current information about the translocation of systemic neonicotinoids in all plant tissues, and the release of neonicotinoid substances in pollen, nectar, and guttation fluid underlines the urgent need for a reassessment of the side effects on nontarget insects by the European Commission in order to accomplish the aims of integrated pest management.

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Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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