



# The novel pesticide flupyradifurone (Sivanto) affects honeybee motor abilities

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## Abstract

Honeybees and other pollinators are threatened by changing landscapes and pesticides resulting from intensified agriculture. In 2018 the European Union prohibited the outdoor use of three neonicotinoid insecticides due to concerns about pollinators. A new pesticide by the name of “Sivanto” was recently released by Bayer AG. Its active ingredient flupyradifurone binds to the nicotinic acetylcholine receptor (AChR) in the honeybee brain, similar to neonicotinoids. Nevertheless, flupyradifurone is assumed to be harmless for honeybees and can even be applied on flowering crops. So far, only little has been known about sublethal effects of flupyradifurone on honeybees. Intact motor functions are decisive for numerous behaviors including foraging and dancing. We therefore selected a motor assay to investigate in how far sublethal doses of this pesticide affect behavior in young summer and long-lived winter honeybees. Our results demonstrate that flupyradifurone (830  $\mu\text{mol/l}$ ) can evoke motor disabilities and disturb normal motor behavior after a single oral administration (1.2  $\mu\text{g/bee}$ ). These effects are stronger in long-lived winter bees than in young summer bees. After offering an equal amount of pesticide (1.0–1.75  $\mu\text{g}$ ) continuously over 24 h with food the observed effects are slighter. For comparisons we repeated our experiments with the neonicotinoid imidacloprid. Intriguingly, the alterations in behavior induced by this pesticide (4 ng/bee) were different and longer-lasting compared to flupyradifurone, even though both substances bind to nicotinic acetylcholine receptors.

**Keywords** Honeybee · Motor ability · Acetylcholine receptor · Insecticide · Flupyradifurone · Imidacloprid

## Introduction

Honeybees, bumblebees and solitary bees play an essential role in crop pollination and a large amount of global crop production depends on insect pollinators (Klein et al. 2007). Bee pollination is also a key factor for fruit quality, as was recently shown for strawberries (Klatt et al. 2014). Nevertheless, the demand for pollination grows faster than the global stock of domesticated bees (Aizen and Harder 2009)

and wild bees like bumblebees have declined considerably (Goulson et al. 2015). This development is due to different factors including habitat loss and fragmentation, pathogens, climate change and agrochemicals (Goulson et al. 2015; Potts et al. 2010).

Honeybees are exposed to a large number of different pesticides especially when foraging on common blooming crops. As a variety of pesticides can be found in pollen, honey and wax, foraging bees and bees inside the hive have contact with these substances and can incorporate them (Mitchell et al. 2017; Mullin et al. 2010; Tosi et al. 2018). Sanchez-Bayo and Goka (2014) state that oral ingestion of contaminated pollen and honey is the main route of exposure to honeybees and bumblebees for systemic insecticides, including neonicotinoids, whereas hydrophobic insecticides and fungicides have a higher toxicity via contact exposure.

Recently, the European Union prohibited the outside use of three neonicotinoid insecticides (clothianidin, imidacloprid and thiamethoxam) due to concerns about adverse effects on insect pollinators (European Commission 2018a, 2018b, 2018c).

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“Sivanto” is a new pesticide which was recently launched by Bayer AG (Bayer AG, Crop Science Division, Monheim am Rhein, Germany). Its active ingredient flupyradifurone ( $C_{12}H_{11}ClF_2N_2O_2$ ) belongs to Bayer’s own new chemical class of butenolides (Nauen et al. 2015). In the US and other countries worldwide flupyradifurone has been on the market for several years now and it has recently been introduced to the first European country, the Netherlands. Generally, it is effective against sucking pests, especially whiteflies and aphids and it can even be applied on flowering crops (Nauen et al. 2015).

Flupyradifurone acts as an agonist on insect nicotinic acetylcholine receptors (AChR), similar to neonicotinoids, but the structure-activity relationship is different due to a different side of action (Jeschke et al. 2015) and flupyradifurone interacts in an additional way with tyrosine residues of the nicotinic AChR (Beck et al. 2015). Nicotinic AChR mediate fast cholinergic synaptic transmission and are important for diverse cognitive processes and mediating mechanosensory antennal input. Acetylcholine (ACh) is a major excitatory neurotransmitter in the insect nervous system (Breer and Sattelle 1987; Lee and O’Dowd 1999).

So far, there have been few studies about sublethal effects of flupyradifurone on honeybees. It was shown that flupyradifurone can reduce taste and learning abilities in the Western honeybee *Apis mellifera* (Hesselbach and Scheiner 2018) and in the Eastern honeybee *Apis cerana* (Tan et al. 2017). On the other hand, Campbell et al. (2016) could not show any adverse effects on colony strength after applying flupyradifurone in the maximum label rate on buckwheat fields.

A sublethal dose of a pesticide is “a dose or a concentration defined as inducing no statistically significant mortality in the experimental population” whereas a sublethal effect of pesticides is defined as “an effect (physiological or behavioral) on individuals that survive an exposure to a pesticide” with the possibilities that the “pesticide dose/concentration can be sublethal or lethal” (Desneux et al. 2007). In this review the authors also describe the variable sublethal effects pesticides have on honeybees. They may interfere with different behavioral aspects such as feeding behavior, learning processes but also mobility.

Motor activity is crucial for numerous tasks a honeybee has to perform, including foraging, homing and waggle-dancing, which is necessary to communicate the location of a food source. Those tasks are essential for the fitness of the whole colony. These tasks are shown to be negatively influenced by neonicotinoid pesticides (Eiri and Nieh 2016; Henry et al. 2012; Tison et al. 2016). Neonicotinoid pesticides can have different adverse effects on motor abilities in honeybees. Alkassab and Kirchner (2018) showed that the neonicotinoid clothianidin influences the distance a

honeybee moved after single treatment, depending on the time after a single administration. Williamson et al. (2014) treated honeybees with different neonicotinoids over the time course of 24 h and found that the bees spent more time on their backs. Application of some of the pesticides additionally affected walking, sitting, grooming and flying behavior.

The aim of this study is to examine the effects of flupyradifurone on motor abilities and motor behavior in short-lived summer bees and in long-lived winter bees. As we observed stronger effects in long-lived winter bees, we decided to repeat the experiment for comparisons with the neonicotinoid pesticide imidacloprid in those bees. We thus could evaluate the differences and similarities between those two compounds with regard to motor abilities in honeybees.

## Material and methods

### Bees

All experiments were carried out with honeybees (*Apis mellifera carnica*) from queen-right colonies maintained at the departmental apiary of Würzburg University. Colonies are regularly treated against *Varroa destructor* with a sufficient time interval to experiments.

For summer experiments in July 2017 and July 2018 we utilized age-controlled honeybees. Newly emerged bees were marked in the course of 24 h after emergence and immediately placed into two alternating outdoor hives. Six to eight days later, the subjects (age six to eight days) were caught individually with glass vials and taken into the laboratory, where experiments were directly conducted. For the experiments with long-lived winter honeybees taking place from November 2017 until January 2018, we caught individuals from the bottom of one hive and kept them in a cage up to 1 h until experiments started.

### Determining sublethal doses

In a previous study we demonstrated that the concentrations we used for flupyradifurone are sublethal (Hesselbach and Scheiner 2018). In this experiment we showed that 5 µl per bee of the experimental flupyradifurone concentrations (830, 83 µmol/l) did not lead to a significantly increased mortality within 72 h after a single administration in age-controlled summer bees.

To identify sublethal doses of imidacloprid under our experimental conditions we repeated the experiment with this substance. In brief, winter bees received 5 µl of 50% sugar solution or this solution containing the pesticide individually (see “Administration of pesticides” below).

**Table 1** Applied concentrations, number of bees per treatment group, estimated consumption of 50% sugar solution and estimated consumption of pesticide feeding the different solutions ad libitum for 24 h

Pesticide	Season	Concentration (mol/l)	Number of bees	Estimated consumed volume per bee (μl)	Estimated consumed pesticide per bee (μg)
Flupyradifurone	Winter	83 μmol/l	18	73.0	1.75
Flupyradifurone	Winter	8.3 μmol/l	18	51.5	0.12
Flupyradifurone	Winter	Control	18	60.5	–
Flupyradifurone	Summer	83 μmol/l	15	43.6	1.04
Flupyradifurone	Summer	8.3 μmol/l	15	24.8	0.06
Flupyradifurone	Summer	Control	15	44.0	–
Imidacloprid	Winter	0.31 μmol/l	16	56.9	0.0046
Imidacloprid	Winter	0.031 μmol/l	16	64.6	0.0005
Imidacloprid	Winter	Control	16	61.5	–

This is a volume a bee consumes easily within a few seconds. We tested ten bees per treatment group and repeated the experiment twice. We tested concentrations comparable to Suchail et al. (2001). The imidacloprid concentrations we used were 62 μmol/l (equals 80 ng/bee), 6.2 μmol/l (equals 8 ng/bee), 3.1 μmol/l (equals 4 ng/bee) and 0.31 μmol/l (equals 0.4 ng/bee). Then we released the individuals into cages, fed them with 50 % sugar solution from a prepared 5 ml Eppendorf tube ad libitum and held them in an incubator (temperature 28 °C, relative humidity 60%). We checked for dead animals after 72 h.

### Administration of pesticides

We first determined the effect of the acute ingestion of the substances. The experiment was conducted in the morning and/or in the afternoon. For each recording, one bee per tested concentration was treated. After flupyradifurone (Molecular Weight: 288.679 g/mol) and imidacloprid (Molecular Weight: 255.662 g/mol) were diluted in deionized water, tap water and household sugar were added to prepare stock solutions (8.3 mmol/l for flupyradifurone; 0.31 mmol/l for imidacloprid). Those were kept at –20 °C. Experimental solutions were prepared at least every fourth day by adding 50% sucrose solution (1:10 ratios until experimental solutions achieved), made from household sugar. Experimental solutions were stored at 4 °C.

Individuals were anaesthetized on ice until they showed first signs of immobility. They were harnessed in holders and fixed with one strip of textile tape between head and thorax and another strip over the abdomen. This way they could freely move their antennae and mouth parts. After 20 min bees were fed individually with 5 μl of a 50% sugar solution containing the different pesticides using a pipette. No signs of regurgitation were observed in any treatment group throughout the entire experimental procedure.

For flupyradifurone, the highest concentration we applied was 830 μmol/l, which equals 1.2 μg/bee active

ingredient (a.i.). We further used a tenfold lower concentration (83 μmol/l). A 50% sucrose solution served as control.

For imidacloprid, the highest concentration we used was 3.1 μmol/l (equals 4 ng/bee) and 0.31 μmol/l (equals 0.4 ng/bee). A 50% sucrose solution served as control.

We next evaluated the effect of feeding the pesticides for 24 h. This study was done comparable to Williamson et al. (2014). As previous results showed that our caged bees consume 40–60 μl of a 50% sucrose solution per bee per day on average, we used tenfold lower concentrations in this experiment. This way the amount of pesticide exposure over 24 h was comparable to the single administration and the calculated dose of flupyradifurone ingested per bee was 1.75 μg/bee at maximum.

We placed 20 to 26 bees per concentration in a small cage (internal diameter 3.0 × 5.5 × 9.5 cm). With a 5 ml Eppendorf tube we fed a 50% sucrose solution or this solution containing the pesticide in different concentrations (s. Tab. 1) ad libitum. After 24 h we measured the residual volume and calculated the average exposure volume per bee (Table 1). Evaporation was not considered. All of the bees were recorded within 2.5 h and each time we recorded one bee per treatment group.

For all experiments we used flupyradifurone, Pestanal® analytical standard (CAS-Number: 951659-40-8, purity ≥98%), and imidacloprid, Pestanal® analytical standard (CAS-Number: 138261-41-3; purity ≥98%), both by Sigma Aldrich (Steinheim, Germany). For numbers of bees per experiment and treatment group see Tables 1 and 2.

### Observations in petri dishes

Petri dishes of 15 mm depth and 90 mm diameter with eight holes on their sides for ventilation served as observation arenas. The bottom of the dish was prepared with a paper sheet which was renewed for every bee to avoid pheromone traces. In summer, the lighted experimental room had a

**Table 2** Numbers of bees per treatment group in the single administration experiment

	Control	83 µmol/l	830 µmol/l
Flupyradifurone summer	19	19	19
Flupyradifurone winter	17	17	16
	Control	0.31 µmol/l	3.1 µmol/l
Imidacloprid summer	17	16	16

**Table 3** Behavioral categories of behavior in Petri-dish assay

Standing	Bee is remaining still and not carrying out any other behavior
Walking	Bee is walking around on the ground, the side or the top of the Petri dish, sometimes showing single grooming movements or brief attempts to fly
Grooming	Bee is standing still or slowly walking about and grooming its abdomen, legs, antennae or head
Circles	Bee is walking around in small circles with a diameter smaller than half the Petri dish or around her own axis
Upside down	Bee is lying on its back and fast moving its legs
Complete immobility/coma	Bee is lying on its side and not moving, but not dead

temperature of 25–26 °C and a relative humidity of 60%. In winter, it had a temperature of 20–22 °C and a relative humidity of 50%.

After individual treatment of summer bees, the animals from the holder were directly taken into the Petri dish. Bees remained in their dish for the entire 3 h of the experimental procedure. After feeding in the cages, individual winter bees were caught with forceps and placed in the Petri dish immediately before the experiment started.

After 15 min of adaption, every individual was recorded on camera (SONY Digital HD Video Camera Recorder HDR-CX405 with 50 fps) or mobile phone (Samsung Galaxy A3 with 30 fps) for 10 min. To detect the progression of motor changes after acute ingestion, we recorded behavior at 15, 60 and 180 min after feeding. After administering the solution for 24 h we recorded each bee one time for 10 min.

Preliminary observations showed that the behavior of honeybees under these conditions fell into one of the following six exclusive categories: Standing, walking, grooming, walking in circles, lying upside down or complete immobility (Table 3, online resource Movies S1, S2).

Further, we recorded the number of times a bee was falling on its back. We calculated the number of fallings per time walking or walking circles, because bees only showed this behavior when walking about. Attempts to fly were not recorded separately, because we were unable to detect events shorter than 1 s, which these attempts usually were. We used BORIS (Behavioral Observation Research Interactive Software 4.1.3. (Friard et al. 2016)) to record the time spent engaged in each behavioral state and the number of fallings. The observations were all carried out by the same person.

## Statistics

Statistical analyses were conducted using SPSS Statistics 23, (IBM, Armonk, NY USA) and GraphPad Prism 7.0 (GraphPad, Las Jolla, CA USA). Graphs were made using GraphPad Prism 7.0.

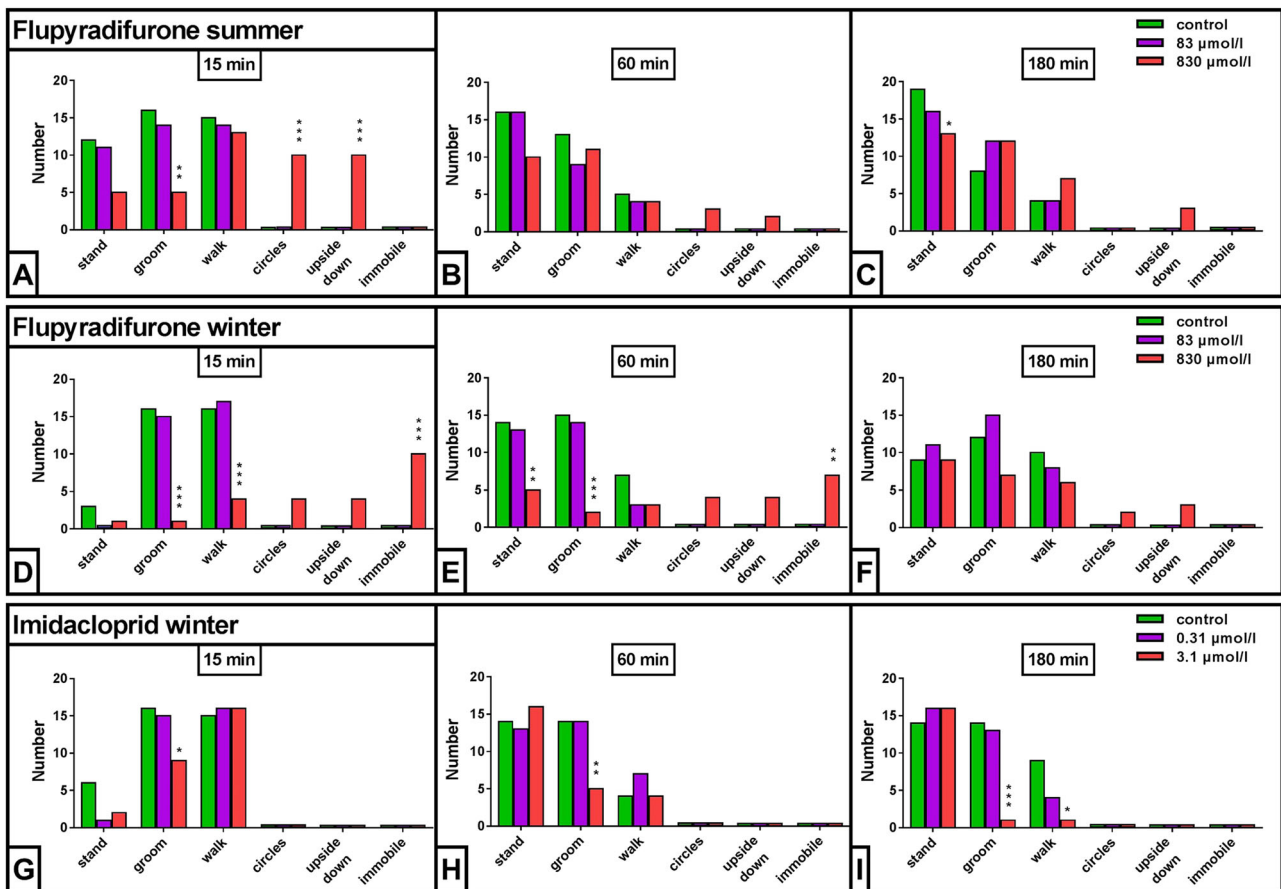
The mortality rates of the differently treated groups and the number of bees showing different behaviors were compared using Pearson Chi-Square Test. Fisher's Exact Tests with Bonferroni correction ( $p < 0.025$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*)) were applied for pairwise comparisons.

The time spent in each behavioral state as well as the counts of falling per time walking about or walking in circles were tested for normal distribution with a Kolmogorov-Smirnov Test. As they were not distributed normally, we applied non-parametric analysis of variance (Kruskal–Wallis  $H$  Test) to determine the effect of flupyradifurone and imidacloprid on motor behavior between the different treatment groups. Dunns' post hoc-tests were applied for pairwise comparisons.

## Results

### Survival after imidacloprid treatment

We first determined sublethal doses of imidacloprid in two replicate experiments, comparable to Hesselbach and Scheiner (2018). We counted the dead animals in each cage 72 h after initial feeding of imidacloprid. None of the bees in the control groups or in the lower concentration groups had died (3.1 and 0.31 µmol/l). In the group with the next



**Fig. 1** Number of bees per treatment group showing different behaviors at 15, 60 and 180 min after a single administration of flupyradifurone in summer bees (a–c), winter bees (d–f) and imidacloprid in winter bees (g–i). Significant differences between the treatment groups

and the control are shown with asterisks. Note the lower significance level due to Bonferroni correction (Fisher's Exact Test with Bonferroni correction,  $p < 0.025$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*)

higher concentration ( $6.2 \mu\text{mol/l}$ ), in the first trial, three out of ten and in the second trial, one out of ten bees had died, which was not significantly different from the control ( $p = 0.211$  and  $p = 1.0$ ). In the highest treatment group ( $62 \mu\text{mol/l}$ ) in the first trial, eight out of ten, and in the second trial, all ten bees had died, which was significantly different from the control ( $p < 0.01$  and  $p < 0.001$ ). We used the two lower concentrations for the following experiments, because they differed by one power of ten, comparable to the flupyradifurone concentrations.

### Number of bees showing different behaviors after single administration of pesticides

Flupyradifurone treatment resulted in a number of unusual behaviors. Fifteen minutes after a single administration of flupyradifurone ( $830 \mu\text{mol/l}$ ) summer bees started to walk in circles (“circles”) and some bees lay on their back (“upside down”) (Fig. 1a–f, red bars). More precisely, after 15 min, significantly more summer bees ( $830 \mu\text{mol/l}$ ) were walking in circles ( $p < 0.0005$ , Fig. 1a) and lying upside down ( $p <$

$0.0005$ , Fig. 1a) compared to the control. Winter bees additionally displayed a state of immobility (Fig. 1d–f, red bars).

Though some bees still exhibited these behaviors after 60 min (Fig. 1b, e) and 180 min (Fig. 1c, f), there was no longer a significant difference between the treatment groups and the control after these intervals. In winter bees, flupyradifurone-induced immobility lasted from 15 min ( $p < 0.0005$ , Fig. 1d) till 60 min ( $p < 0.005$ , Fig. 1e) after receiving flupyradifurone ( $830 \mu\text{mol/l}$ ) compared to the control. Though walking in circles and lying upside down was noted after this treatment in winter bees, there was no significant difference compared to the control. Neither in summer bees nor in winter bees were these differences found between the lower dose of flupyradifurone ( $83 \mu\text{mol/l}$ ) and the control (Fig. 1a–f).

Other behaviors were also slightly impaired by flupyradifurone ( $830 \mu\text{mol/l}$ ). The number of bees standing was significantly reduced after the higher dose of flupyradifurone ( $830 \mu\text{mol/l}$ ) compared to the control at 180 min in summer bees ( $p < 0.025$ , Fig. 1c) and after 60 min in winter



bees ( $p < 0.025$ , Fig. 1e). There was no such difference at the other time points or between the lower dose of flupyradifurone (83  $\mu\text{mol/l}$ ) and the control (Fig. 1a–f).

In addition to the unusual behaviors displayed by flupyradifurone, this substance (830  $\mu\text{mol/l}$ ) significantly reduced grooming behavior 15 min after application both in summer bees ( $p < 0.005$ , Fig. 1a) and in winter bees ( $p < 0.0005$ , Fig. 1d) compared to the control. In winter bees, reduced grooming behavior was still observable after 60 min ( $p < 0.0005$ , Fig. 1e). At the other time points and between the lower concentration of flupyradifurone (83  $\mu\text{mol/l}$ ) and the control were no significant differences (Fig. 1a–f).

In summer bees walking behavior was unaffected by flupyradifurone at any time after one-time administration (Fig. 1a–c). In winter bees significantly fewer flupyradifurone treated animals (830  $\mu\text{mol/l}$ ) showed walking at 15 min ( $p < 0.0005$ , Fig. 1d) compared to the control. This was not observable at the other time points and between lower concentration of flupyradifurone and the control (Fig. 1d–f).

For imidacloprid, which also binds to nicotinic AChRs, similar to flupyradifurone, neither walking in circles, lying upside down nor immobility were observed after one-time treatment. The most striking effect of imidacloprid was the reduced number of bees showing grooming at 15 min ( $p < 0.025$ , Fig. 1g), 60 min ( $p < 0.005$ , Fig. 1h) and 180 min ( $p < 0.0005$ , Fig. 1i) after administering the pesticide (3.1  $\mu\text{mol/l}$ ), while this substance had no effect on the number of bees standing. At 180 min after treatment, significantly fewer bees showed walking ( $p < 0.025$ , Fig. 1i) compared to the control, which was not observed at 15 or 60 min after treatment. These differences were not seen after administering the lower dose of imidacloprid (0.31  $\mu\text{mol/l}$ , Fig. 1g–i).

### Duration of behaviors displayed after single administration of pesticides

As walking in circles, lying upside down and immobility were exclusively noted after flupyradifurone treatment (830  $\mu\text{mol/l}$ ) the durations of these behaviors were excluded from the following analysis.

Summer bees treated with flupyradifurone (830  $\mu\text{mol/l}$ ), spent significantly less time standing at 60 min after treatment compared to the lower dose of flupyradifurone (83  $\mu\text{mol/l}$ ,  $p < 0.01$ , Fig. 2a) but not compared to the control ( $p = 0.191$ ). At 180 min, standing was significantly reduced compared to the control ( $p < 0.05$ , Fig. 2a). The same difference was observed in winter bees treated with flupyradifurone (830  $\mu\text{mol/l}$ ) at 60 min ( $p < 0.01$ , Fig. 2d).

Furthermore, grooming was shown during shorter phases in flupyradifurone-treated summer bees (830  $\mu\text{mol/l}$ ) compared to the control after 15 min ( $p < 0.01$ , Fig. 2b). This

effect was gone after 60 and 180 min. Flupyradifurone-treated winter bees (830  $\mu\text{mol/l}$ ) showed shorter durations of grooming after 15 min ( $p < 0.001$ ) and 60 min ( $p < 0.01$ , Fig. 2e). After 180 min, there was less time spent grooming compared to the lower dose of flupyradifurone (83  $\mu\text{mol/l}$ ,  $p < .05$ , Fig. 2e) but not compared to the control ( $p = 0.773$ , Fig. 2e). These differences were not seen between the lower dose of flupyradifurone (83  $\mu\text{mol/l}$ ) and the control neither in summer bees, nor in winter bees (Fig. 2b, e).

The duration of walking was unaffected by flupyradifurone in summer bees (Fig. 2c). After administering flupyradifurone (830  $\mu\text{mol/l}$ ) once, winter bees showed shorter durations of walking at 15 min ( $p < 0.001$ , Fig. 2f) compared to the control. There was no such difference after 60 and 180 min and between the lower dose of flupyradifurone (83  $\mu\text{mol/l}$ ) and the control (Fig. 2f).

For the number of fallings per time walking or per time walking circles we did not find a significant difference between the different treatment groups and the control neither in summer bees nor in winter bees after administering flupyradifurone.

In contrast to flupyradifurone, the neonicotinoid imidacloprid (3.1  $\mu\text{mol/l}$ ), increased the duration of standing significantly compared to the control at 60 min ( $p < 0.01$ ) and at 180 min ( $p < 0.001$ ) after treatment (Fig. 2g).

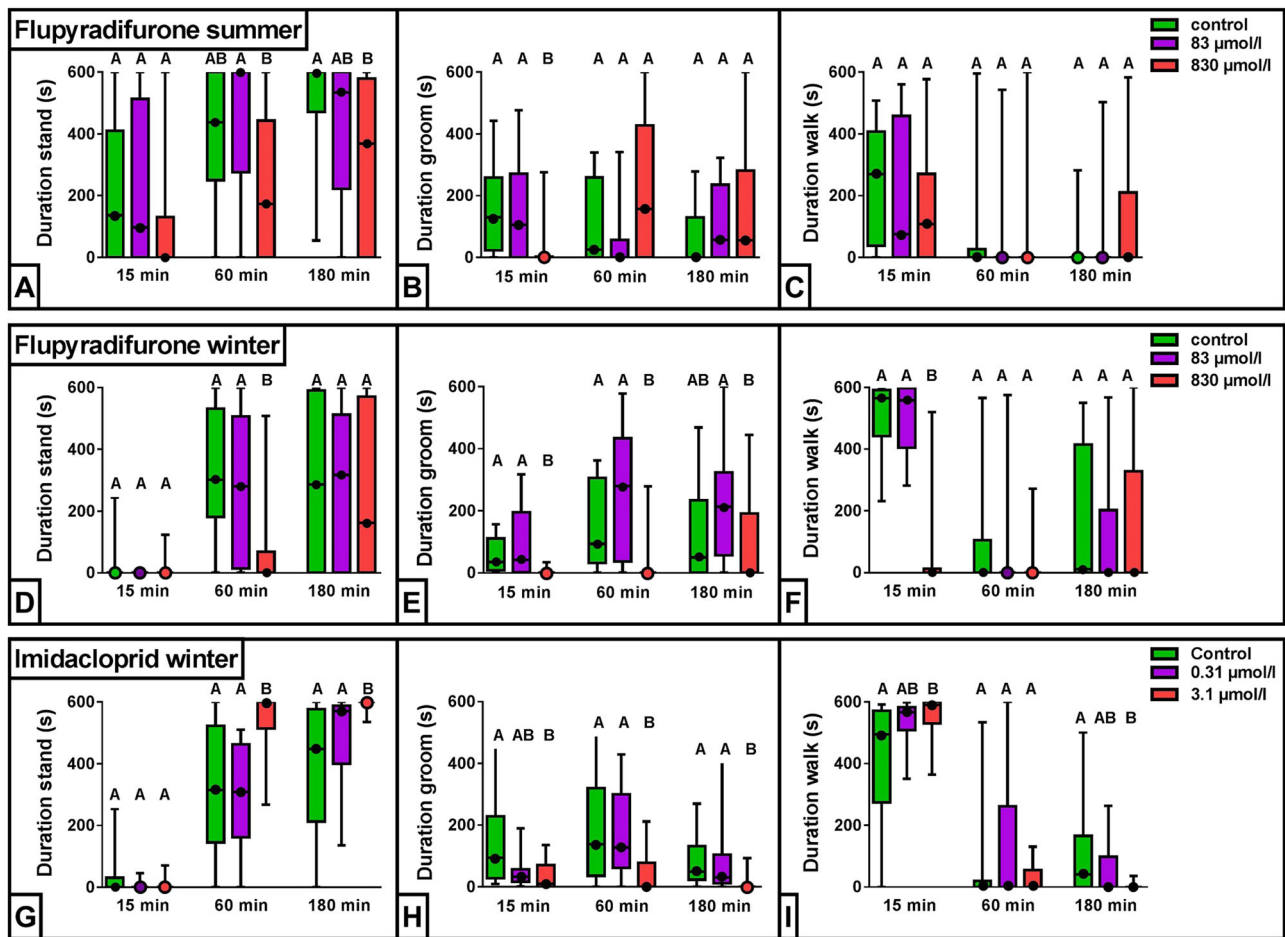
The time spent grooming was significantly reduced at 15 min ( $p < 0.01$ ), 60 min ( $p < 0.01$ ) and 180 min ( $p < 0.001$ , Fig. 2h) compared to the control. This was similar to the effects of flupyradifurone in winter bees and consistent with fewer bees showing grooming at all time points.

Bees treated with imidacloprid (3.1  $\mu\text{mol/l}$ ) displayed a significantly longer walking time at 15 min after administration ( $p < 0.05$ ), but decreased at 180 min ( $p < 0.05$ , Fig. 2i). Between the lower dose of imidacloprid (0.31  $\mu\text{mol/l}$ ) and the control there were no differences (Fig. 2i).

There was a significant difference in the count of falling per time walking after 15 min between the two treatment groups ( $p < 0.05$ ) but not compared to the control group. After 60 and 180 min there was no such difference.

### Providing pesticides for 24 h

After treating the bees for 24 h no bee in either treatment group died. During the assay walking in circles, lying upside down or immobility were not observed after flupyradifurone or imidacloprid were provided for 24 h neither in summer nor in winter bees, although these unusual behaviors occurred after a single administration of flupyradifurone. Furthermore, in flupyradifurone-treated summer bees there was no significant difference between the treatment groups regarding the duration showing the different behaviors (Fig. 3a–c). There was also no significant difference in the count of falling per time walking (Fig. 4a).



**Fig. 2** Duration of periods when bees were showing different behaviors 15, 60 and 180 min after a single administration of flupyradifurone in summer bees (a–c), winter bees (d–f) and imidacloprid in winter bees (g–i). Graphs show medians (dots are colored at median =

0 for better overview), interquartile range and 10–90 percentile. Different letters indicate the significant difference between different treatment groups (Kruskal Wallis  $H$  Test,  $p < 0.05$ )

For winter bees treated with flupyradifurone, there was no significant difference regarding the time spent standing and walking compared to the control (Fig. 3d, f). For grooming, we found a significant difference, but only between the lower and the higher doses of flupyradifurone ( $p < 0.05$ ) and not between the control and flupyradifurone ( $8.3 \mu\text{mol/l}$ ;  $p = 1.0$ ) or flupyradifurone ( $83 \mu\text{mol/l}$ ;  $p = 0.145$ ; Fig. 3e). Furthermore, the occurrence of falling per time walking was significantly increased in flupyradifurone-treated bees ( $83 \mu\text{mol/l}$ ) compared to the control ( $p < 0.001$ ; Fig. 4b).

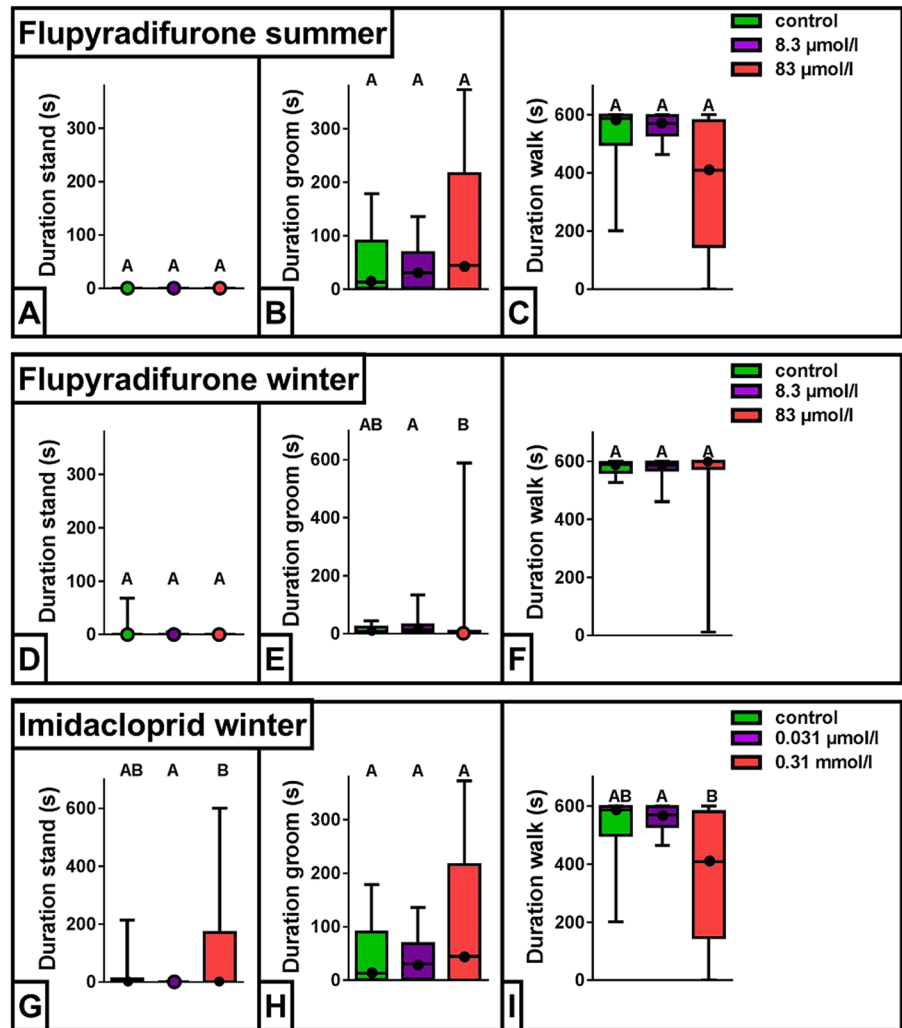
For imidacloprid-treated winter bees, there was a significant difference between the two applied concentrations with respect to the duration of standing ( $p < 0.05$ ) and walking ( $p < 0.05$ ), but the treatment groups were not significantly different from the control (Fig. 3g, i). There was no significant difference in the duration of grooming (Fig. 3h) and the counts of falling per walking when winter bees were treated with imidacloprid (Fig. 4c).

For all statistical results see online resource Tables S1–S3. The complete dataset is available under the following link: <https://figshare.com/s/818f15bb365a925670dc>.

## Discussion

The aim of this study was to examine the effects of two pesticides acting on the nicotinic AChR on honeybee motor behavior. We therefore compared the actions of the novel pesticide flupyradifurone with those of the neonicotinoid imidacloprid in short-lived summer honeybees and long-lived winter honeybees. Using video tracking we could observe in detail behavioral changes in honeybees at three different time points (15, 60, 180 min) after a single administration of the pesticide and after administering the pesticide for 24 h. To our knowledge, this is the first study in which a single and a permanent application of a pesticide were compared in the same study, simulating a single

**Fig. 3** Duration of periods showing different behaviors after administering flupyradifurone in summer bees (a–c), winter bees (d–f) and imidacloprid in winter bees (g–i) over a period of 24 h. Graphs show medians (dots are colored at median = 0 for better overview), interquartile range and 10–90 percentile. Different letters indicate a significant difference between the different treatment groups (Kruskal Wallis  $H$  Test,  $p < 0.05$ )



ingestion and a more naturally occurring prolonged ingestion of a substance.

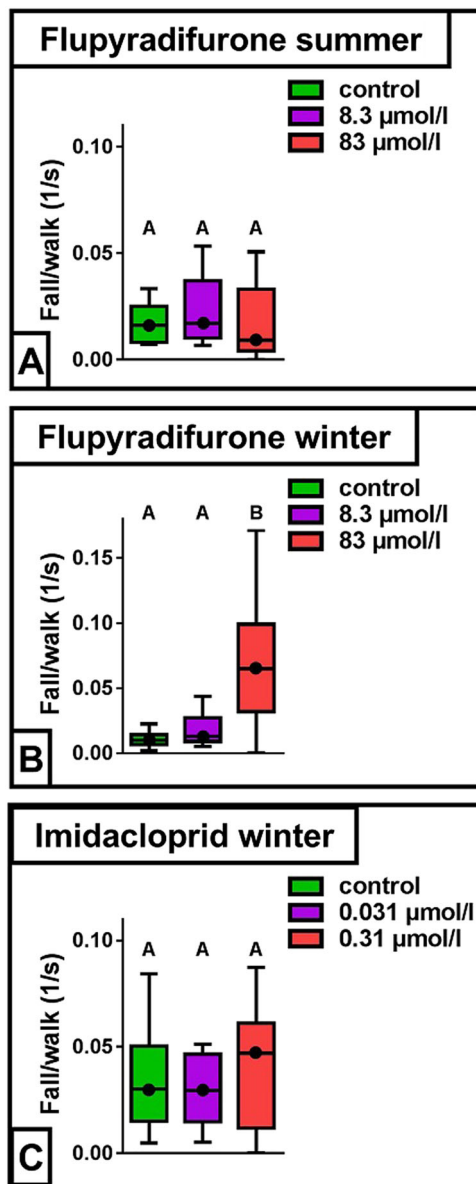
At 15, 60 and 180 min after administering the pesticides, the different phases of intoxications were seen. The effects changed in a time-dose-dependent manner. Interestingly, though having the same target receptor, flupyradifurone and imidacloprid led to different behavioral changes.

After administering flupyradifurone (830 µmol/l) once, different motor disabilities could be observed. These effects seem to be stronger in long-lived winter bees, where a state of complete immobility or apparent “coma” was shown. The other motor disabilities, walking in circles, sometimes with an arched abdomen, and lying upside down with fast movements of the legs, could be observed in winter bees and summer bees. It is notable that the effects were mostly noticed at 15 and 60 min after administration and to a smaller degree at 180 min after administration. The time course of these alterations could indicate a fast diffusion of the substance in the bee and a fast metabolism of flupyradifurone in honeybees.

The stronger alterations in winter bees could indicate a slower metabolism and detoxification of these animals. A differential activity of detoxifying enzymes in worker honey bees was shown by Smirle (1993). He found a positive relationship between the intensity of brood rearing and the activities of detoxifying enzymes. His explanation for this was that young workers are responsible for the processing of food brought to the colony (Winston 1987) and that the detoxification of xenobiotics before feeding to the brood could be vital to colony survival. This would explain the lower effects on young, six-to-eight-day-old summer bees, currently most probably being nurse bees and therefore having higher detoxification capacities compared to old winter bees.

Our findings are similar to those found for the neonicotinoid pesticide clothianidin (Alkassab and Kirchner 2018). Here the authors report that the bees moved a greater distance and spent more time upside down after a single treatment with clothianidin ( $\geq 1$  ng/bee). As we did not measure the walking speed of bees, we cannot make an





**Fig. 4** Count of fallings per time walking after feeding flupyradifurone ad libitum in summer bees (a) and winter bees (b) and imidacloprid in winter bees (c) over a period of 24 h. Graphs show medians, interquartile range and 10–90 percentile. Different letters indicate a significant difference between the different treatment groups (Kruskal Wallis *H* Test,  $p < 0.05$ )

objective statement about the distance moved. Schneider et al. (2012) also observed bees lying upside down, showing paddling movements unable to return and bees walking with an arched abdomen after administration of doses  $\geq 1$  ng clothianidin. Similarly, acute consumption of the neonicotinoid thiametoxam altered locomotion and caused hyperactivity shortly after exposure and impaired motor functions over a longer period (Tosi and Nieh 2017).

Interestingly, the time spent grooming was reduced in summer bees as well as in winter bees after administering

flupyradifurone (830 µmol/l) once. There are several explanations conceivable for that. It is possible that the bee had an impaired olfactory perception and therefore does not sense a necessity for grooming. Another explanation is that its motor ability needed for grooming is reduced due to the pesticide. Alternatively, the bee could just be preoccupied with the striking behaviors and therefore not be grooming itself.

The effects of a single administration of imidacloprid (3.1 µmol/l) were often contrary to the effects of flupyradifurone. After the application of imidacloprid no motor disabilities could be observed. Furthermore, the effects seem to get stronger over the time course of 180 min with the bees getting less and less mobile. The findings for imidacloprid are consistent with a study by Lambin et al. (2001) where they found a significant reduction in movement and an increase in immobility phases 30 min post-treatment with imidacloprid in higher doses ( $\geq 5$  ng/bee). Similar observations were also made by Schneider et al. (2012). In addition, these effects fit well with previous studies. Beketov and Liess (2008) reported delayed toxic effects 4–12 days following exposure of freshwater arthropods to thiacloprid. Similarly, it was stated that neonicotinoids have a delayed and time-cumulative toxic effect on honeybees when administered for several days (Rondeau et al. 2014; Tennekes 2010).

On the other hand, administering flupyradifurone or imidacloprid in any concentration we used for 24 h did not lead to any observable motor disability except for the winter honeybees treated with flupyradifurone (83 µmol/l), which fell over significantly more often.

These findings are in contrast with the results of Williamson et al. (2014) who administered 3.7 ng/bee imidacloprid for 24 h. The authors showed that bees spent more time standing and upside down but less time walking, flying and grooming after imidacloprid treatment. Nonetheless, in a preliminary study in which we applied 8.0 ng imidacloprid per bee over the course of 24 h, we could see a trend in the same direction as Williamson et al. (2014) with bees tending to stand more and fall over more often. These findings indicate a different tolerance of bees towards pesticides which could be due to lower detoxification rates. This variation could be related to the different age, but also to colony strength as was described by Smirle (1993). Likewise, there are two studies comparing the effects of imidacloprid on mobility and clearance of ingested pesticide which come to different results. While both authors find no effect on locomotion, Cresswell et al. (2014) report a 100% clearance of ingested imidacloprid and reports no motor disabilities. Sánchez-Bayo et al. (2017), on the other hand, observed motor disabilities (restless, apathetic, trembling and falling over), reported higher mortality in treated bees and incomplete clearance of ingested pesticide.

The highest concentration we applied once (830  $\mu\text{mol/l}$  equals 1.2  $\mu\text{g/bee}$ ) is reported to be the acute oral  $\text{LD}_{50}$  for honeybees (EFSA 2015). In our experiment, this amount of pesticide was sublethal. A great variability in the  $\text{LD}_{50}$  dose has also been described for other insecticides. Nauen et al. (2001) found a twofold difference regarding the  $\text{LD}_{50}$ , depending on the location of the honeybees in Europe. Depending on the genetic background of honeybees even higher differences (16 to 33 fold difference) regarding the sensitivity for imidacloprid were described (Rinkevich et al. 2015). These authors also found age dependent effects for other pesticide categories. It is therefore plausible that our experimental bees have a lower sensitivity towards flupyradifurone. For this reason it would be interesting to repeat our experiments with differently aged bees and with bees from a different genetic background.

Both imidacloprid and flupyradifurone bind to nicotinic AChR in the honeybee brain, though the agonistic efficacy of flupyradifurone is 0.56 relative to ACh, while imidacloprid exhibits a relative efficacy of 0.15 (Nauen et al. 2015). In the insect central nervous system, ACh is the most frequent neurotransmitter and thought to be the primary excitatory transmitter. Stainings for acetylcholinesterase (AChE), the enzyme hydrolyzing ACh, indicate the presence of ACh in various regions of the honeybee brain, including the antennal lobes, parts of the mushroom bodies and the dorsal lobes (Bicker 1999). Pressure injections of ACh or agonists on nAChR (nicotine and carbachol) into the prothoracic neuropil of locusts elicited excitation of a ventral cord interneuron which synapses with motor neurons of special wing steering muscle (Gauglitz and Pflüger 2001). The presence and distribution of nicotinic AChR in these regions of the honeybee has not been examined so far, but a homology between the ventral unpaired median neurons and the dorsal unpaired medial neurons has been suggested (Schröter et al. 2007). This leads to the presumption that the lack of motor coordination seen after application of flupyradifurone and imidacloprid could be mediated through these neurons. In addition, the somata of insect motor neurons are assumed to be sensitive to neuromodulators (Nation 2008). Therefore, it is also possible that nicotinic AChR located in motor neurons are a target of the pesticides. This could directly lead to the motor disabilities observed after the administration of the pesticides. As ACh is not the synaptic mediator at neuromuscular junctions in insects (Nation 2008), it is unlikely that the observed effects emerge directly from this region.

Although the main target of flupyradifurone is the nAChR, it cannot be excluded that other molecular targets are modified through this pesticide. It was shown for other systemic pesticides that they can alter immune responses in honeybees (James and Xu 2012) and that especially neonicotinoids lead to an enhanced production of reactive

oxygen species. To our knowledge, there are no such studies on flupyradifurone but further investigation in this direction would be useful.

*Drosophila melanogaster* is a model for drug discovery for Parkinson's disease (Whitworth et al. 2006). Coulom and Birman (2004) showed that chronic exposure to rotenone, a pesticide inhibiting the mitochondrial complex I, leads to symptoms of Parkinson's disease with major locomotor defects and dopaminergic neuron degeneration. Recently it was shown that Ach and nicotinic AChR agonists such as nicotine and the neonicotinoids imidacloprid and nitanpyram mediate dopamine release in *Drosophila* larval ventral nerve cords. Agonists evoke the release of dopamine, whereas antagonists, like  $\alpha$ -bungarotoxin, decrease the release (Pyakurel et al. 2018). The motor disabilities in honeybees we observed after the administration of flupyradifurone could also be compared to deficits seen in Parkinson's disease. Either the agonistic effect on nicotinic AChR or the additional modification of dopaminergic neurons could have led to this outcome. Although this is only a speculation, the honeybee might thus have the potential to become a future model for Parkinson's disease.

Residues of flupyradifurone in nectar and pollen vary strongly, depending on the crop (Glaberman and White 2014). In apples, the flupyradifurone residues after two foliar applications (205 g ai/ha) were found to be 1.5 mg ai/kg in nectar and 39 mg ai/kg in pollen. The overall values in nectar ranged from <0.001 mg ai/kg in watermelons to 21.83 mg ai/kg in cotton and those in pollen from 0.002 mg ai/kg in watermelons to 67.7 mg ai/kg in blueberries.

Similarly the amount of food a bee consumes per day is strongly related to its age or task (Rortais et al. 2005). The task of winter bees is to maintain a constant nest temperature (Winston 1987). This can cost varying amounts of energy, depending on outside weather conditions. On average, a winter bee is thought to consume 8.8 mg of sugar per day. A brood-attending nurse bee, in contrast, consumes up to 50 mg sugar and up to 12 mg pollen per day. For forager honeybees the amount of food consumed depends on their flight activity. It is reported that a foraging honeybee needs 8–12 mg sugar per hour of flight or rather up to 128 mg sugar per day (Rortais et al. 2005).

Because the amount of nectar consumed by bees depends mainly on the sugar content of the plants, the pesticide consumption varies according to crops. The concentration of sugar found in the nectar varies between 15 and 84% among crops (Knopper et al. 2016). For apple nectar the concentration of sugar is described to vary between 32 and 56% (Butler 1944).

Apple nectar and pollen are attractive to honeybees, but also to bumblebees and different solitary bees (Delaplane and Mayer 2000). Based on the consumption results by

Rortais et al. (2005), and on the assumption that the sugar concentration of apple nectar is 32%, we calculated the probable intake of flupyradifurone by the different worker honeybees. A winter bee would thus incorporate 0.041 µg flupyradifurone, a nurse bee would take 0.7 µg flupyradifurone in and a forager would consume 0.05 µg flupyradifurone per hour or up to 0.6 µg flupyradifurone per day. The amount of flupyradifurone leading to motor disabilities was 1.2 µg per bee of all ages, which is approximately 1.7 to 30 fold higher than the amount consumed with apple nectar and pollen. However, as higher residues of flupyradifurone were found in other crops, it cannot be excluded that bees are exposed to higher concentrations when foraging on nectar from other crops.

Consulting the results of Rortais et al. (2005) the European Food Safety Authority (2012) concluded the maximum intake of imidacloprid for a nectar foraging honeybee to have a broad range up to 1.037 ng per bee per day. This is 25.9% of the highest dose we administered once. Bees performing other tasks would consequently have a lower intake. As the next lower concentration we used was only 0.4 ng per bee, we cannot exclude finding adverse results with this intermediate, field realistic dose. In this context testing an intermediate concentration, using for example a progression factor of two, could have led to further, field-realistic results and should be considered in future experiments.

Depending on the location of their hive, the honeybees of one colony usually forage not only on one single crop but in a diverse landscape. Danner et al. (2017) showed that honeybees increased their foraging distance when facing a lower landscape diversity in order to maintain pollen amount and diversity. As a multitude of pesticides is found in pollen, honey and wax (Mitchell et al. 2017; Mullin et al. 2010; Tosi et al. 2018), foraging bees and consecutively bees inside the hive have contact with a mixtures of these substances and feed on them. Oral ingestion of contaminated pollen and honey is the main route of exposure to honeybees for systemic insecticides, including neonicotinoids (Sanchez-Bayo and Goka 2014). Furthermore, it should be considered that the application of flupyradifurone over multiple years could lead to residues in the soil, as was shown for neonicotinoid pesticides and herbicides (Krupke et al. 2012). This would lead to another possible route of pesticide intake for honeybees.

So far, only few studies on the effects of flupyradifurone on honeybees have been available. Our results demonstrate an urgent need for more studies investigating the effects of flupyradifurone in combination with other current pesticides on honeybees. Regarding the declining numbers of insects (Hallmann et al. 2017) and pollinators (Potts et al. 2010) worldwide, it is urgently necessary to study effects of flupyradifurone on wild pollinators.

## Data availability

All necessary data are available in the online supplementary information or under the following link: <https://figshare.com/s/818f15bb365a925670dc>.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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