




## RESEARCH ARTICLE

# Chronic exposure to the pesticide flupyradifurone can lead to premature onset of foraging in honeybees *Apis mellifera*

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## Abstract

1. Honeybees *Apis mellifera* and other pollinating insects suffer from pesticides in agricultural landscapes. Flupyradifurone is the active ingredient of a novel pesticide by the name of 'Sivanto', introduced by Bayer AG (Crop Science Division, Monheim am Rhein, Germany). It is recommended against sucking insects and marketed as 'harmless' to honeybees. Flupyradifurone binds to nicotinic acetylcholine receptors like neonicotinoids, but it has a different mode of action. So far, little is known on how sublethal flupyradifurone doses affect honeybees.
2. We chronically applied a sublethal and field-realistic concentration of flupyradifurone to test for long-term effects on flight behaviour using radio-frequency identification. We examined haematoxylin/eosin-stained brains of flupyradifurone-treated bees to investigate possible changes in brain morphology and brain damage.
3. A field-realistic flupyradifurone dose of approximately 1.0 µg/bee/day significantly increased mortality. Pesticide-treated bees initiated foraging earlier than control bees. No morphological damage in the brain was observed.
4. *Synthesis and applications.* The early onset of foraging induced by a chronic application of flupyradifurone could be disadvantageous for honeybee colonies, reducing the period of in-hive tasks and life expectancy of individuals. Radio-frequency identification technology is a valuable tool for studying pesticide effects on lifetime foraging behaviour of insects.

## KEYWORDS

flight behaviour, flupyradifurone, foraging, histology, honeybee, insecticide, mortality, radio-frequency identification

## 1 | INTRODUCTION

Animal pollination is worth 235–577 billion USD per year (IPBES, 2016) and can enhance the quality of fruits (Klatt et al., 2014). In addition, honeybees and other insects foraging on flowers play a major role in ecology, because they are important pollinators of trees

and wild flowers, thus contributing to biodiversity (Biesmeijer et al., 2006; Devillers, 2002). The decline in pollinating insects (Goulson, Nicholls, Botías, & Rotheray, 2015) is therefore a matter of concern not only for agriculture but also for nature conservation. Pesticides are considered to be a major cause for insect decline, although other causes include parasites and pests, climate change and intensified

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agriculture (Goulson et al., 2015). The three main neonicotinoids imidacloprid, thiamethoxam and clothianidin, which affect insect behaviour through modulating the nervous system, were recently banned from use in the field in Europe because of concerns for honeybees and wild bees (European Commission, 2018a, 2018b, 2018c). However, alternative substances have been launched.

Flupyradifurone (4-[(2,2-difluoroethyl)amino]-2(5H)-furanone) is a new pesticide which was launched under the name of 'Sivanto' by Bayer AG (Bayer AG, Crop Science Division). It belongs to the company's new chemical class of butenolides and is highly effective against sucking insects, particularly whiteflies and aphids (Nauen et al., 2015). Similar to the neonicotinoids, flupyradifurone binds to nicotinic acetylcholine receptors in the insect brain and acts as a reversible agonist (Nauen et al., 2015). Activation of acetylcholine receptors leads to depolarization and eventually blocking of the synaptic transmission of cholinergic synapses (Casida, 2018).

As the oral LD<sub>50</sub> of flupyradifurone in honeybees is specified to be 1.2 µg ai/bee ('active ingredient') (Nauen et al., 2015), the primary way of intake is ingestion through nectar and pollen. Mortality studies often vary with respect to the lethal concentration of pesticides. The different sensitivities of honeybees for one and the same pesticide in different studies can often be traced back to different genetic backgrounds and different age groups. (Laurino, Manino, Patetta, & Porporato, 2013; Rinkevich et al., 2015). We therefore determined a sublethal concentration of flupyradifurone under our experimental conditions and subsequently applied this dose of flupyradifurone in our behavioural experiments.

So far, little has been known about sublethal effects of flupyradifurone on honeybees. Flupyradifurone can impair learning and memory in the honeybee (Hesselbach & Scheiner, 2018; Tan, Wang, Dong, Li, & Nieh, 2017), but studies on chronic exposure of honeybees in the field are lacking.

While foraging, honeybees are exposed to various environmental stressors including pesticides, which can impact flight performance (Gill, Ramos-Rodriguez, & Raine, 2012). Factors allowing us to estimate foraging success include foraging activity, time spent outside the hive and the capability to return to the colony. One method to automatically investigate flight behaviour is the RFID ('radio-frequency identification') technology (Scheiner et al., 2013). This system is based on radio waves of a certain frequency which are emitted from an antenna. These radio waves activate a nearby tag which is fixed to the thorax of a bee. The time of tag activation is registered continually (Nunes-Silva et al., 2019). Using two scanners at the entrance of the hive, it can be determined whether individual honeybees enter the hive or leave it. The RFID method was first used in honeybees by Streit, Bock, Pirk, and Tautz (2003). RFID was already used to test the influence of the neonicotinoids imidacloprid, clothianidin (Schneider, Tautz, Grünwald, & Fuchs, 2012) and thiamethoxam (Henry et al., 2012) and the phenylpyrazole fipronil (Decourtye et al., 2011) on honeybee foraging and homing. In those studies, however, the pesticides were only administered once. Thus, the studies did not take into account the storage of contaminated pollen and nectar and the subsequent chronic exposure of bees. In contrast, Thompson, Coulson,

Ruddle, Wilkins, and Harkin (2016) applied thiamethoxam-coated oilseed rapeseed, leading to detectable residues of the pesticide in pollen (1.0 µg/kg) and nectar (1.8 µg/kg). Tracking honeybees foraging in the contaminated fields, the authors failed to detect an effect of the pesticide on flight activity of the honeybees. To the best of our knowledge, there are no further studies on the effect of chronic pesticide exposure on honeybee foraging behaviour using RFID. Studies on bumblebees *Bombus terrestris*, in contrast, are abundant. Gill et al. (2012) showed that chronic exposition of bumblebee colonies to imidacloprid leads to significantly prolonged foraging flights and reduced pollen collection. Stanley, Russell, Morrison, Rogers, and Raine (2016) and Feltham, Park, and Goulson (2014) report similar results. This leads to the conclusion that the foraging efficacy of bumblebees is negatively influenced by the tested pesticides.

For these reasons, one aim of this study was to ask how an early and chronic exposure to a sublethal dose of flupyradifurone affects foraging behaviour in free-flying honeybees. Using separate cages inside the hive, we exposed a subgroup of honeybees to the pesticide, while not affecting the other bees of the colony.

Our results display effects of the pesticide on foraging activity. For the neonicotinoids imidacloprid (Gill et al., 2012) or thiamethoxam (Henry et al., 2012), which similarly affect foraging behaviour or homing success in bumblebees or honeybees, clear effects on brain structure of honeybees have been shown: Oliveira, Roat, Carvalho, and Malaspina (2014) treated newly emerged bees with different concentrations of thiamethoxam. Using haematoxylin/eosin staining, they found Kenyon cells with a pycnotic profile in the mushroom bodies, which indicates cell death through apoptosis. Similar alterations indicating cell death were observed after imidacloprid treatment in the antennal lobes and in the optic lobes (Almeida Rossi, Roat, Tavares, Cintra-Socolowski, & Malaspina, 2013). The affected brain regions have an important function in the processing of sensory information and multimodal integration (Galizia, Eisenhardt, & Giurfa, 2012), which could explain their effects on foraging behaviour.

To ask whether flupyradifurone induces similar damage in bee brain as the neonicotinoids imidacloprid, thiamethoxam and other pesticides (Almeida Rossi et al., 2013; Oliveira et al., 2014), we further analysed the brain structure using light microscopy after chronic treatment of honeybees.

## 2 | MATERIALS AND METHODS

### 2.1 | Chronic exposure—Mortality

Experiments were conducted with newly emerged bees in May/June 2018 and with *diutinus* (winter) honeybees in January/February 2018. Bees were gained from queen-right colonies located at the University of Würzburg which were treated regularly against *Varroa* mites. Essays for testing mortality were compiled according to OECD guidelines (OECD, 2017) and comparable to Suchail, Guez, and Belzunces (2001) and Alkassab and Kirchner (2016) with slight differences in the

number of bees per cage and the experimental duration. As age can affect the outcome of mortality studies (Rinkevich et al., 2015), the experiments were replicated four times for each age group.

*Diutinus* bees were collected from the bottom of one hive for all replicates. To obtain newly emerged bees, a frame with sealed brood (near emergence) was collected from two different hives and maintained in an incubator (darkness, 32°C, 70% relative humidity, RH) until emergence. Bees were anaesthetized on crushed ice within one to five minutes. For each experiment, 30 animals were grouped in a cage (internal dimensions: 8 cm x 5 cm x 5 cm). The cages were maintained in an incubator (darkness, 28°C, 60% RH) for 10 days. Bees were fed using a 5-ml Eppendorf tube. The control group received 50% sugar water. The treatment groups received this solution containing different concentrations of flupyradifurone. LD<sub>50</sub> values for pesticides often vary within the range of one 10-fold or more (Nauen, Ebbinghaus-Kintscher, & Schmuck, 2001; Rinkevich et al., 2015). Therefore, we decided to test 1.2 µg per bee per day, as this is a known acute oral LD<sub>50</sub> (Nauen et al., 2015). We furthermore tested a 10-fold higher and a 10-fold lower concentration. Preliminary experiments showed that *diutinus* bees consumed more food, so that we calculated 80 µl of food per bee per day for *diutinus* bees, while the newly emerged bees received 30 µl per bee per day. The resulting concentrations we applied in winter were  $5.2 \times 10^{-6}$  mol/L,  $5.2 \times 10^{-5}$  mol/L and  $5.2 \times 10^{-4}$  mol/L. In summer, the concentrations were  $1.4 \times 10^{-5}$  mol/L,  $1.4 \times 10^{-4}$  mol/L and  $1.4 \times 10^{-3}$  mol/L.

The solutions were kept at 4°C for a maximum of three days. Stock solutions were prepared at room temperature by solving flupyradifurone powder (flupyradifurone PESTANAL®, analytical standard by Sigma Aldrich) in deionized water and adding household sugar. Stocks were stored at -20°C. Food was exchanged daily, and dead bees were counted and removed at the same time (±2 hr). The individual consumption of the solution was determined by pipetting the remaining amount of sugar water. The mean individual consumption of the pesticide per day was calculated (Table S1).

## 2.2 | Radio-frequency identification RFID

The RFID experiments were conducted in July/August 2018 and September/October 2018 with the same queen-right host colony, which differed from the colonies used in the mortality experiments. Providing the same host colony in both replicates has the advantage that the biological background is the same in both replicates. On the other hand, replicate experiments had to be conducted one after another. The colony was maintained in a six-frame mini plus hive. Two specifically designed scanners (MAJA Bundle Bee Identification System: iID 2000 ISO 15693 optimized, Micro-Sensys GmbH) were positioned in front of the hive entrance to track the bees leaving or returning to the hive. These two scanners were connected through a narrow tunnel, which only allowed the sequential passage of bees.

Newly emerged honeybees were obtained in the same way as for the mortality experiment from two or three brood frames. They were

equally distributed into the subsequent treatment groups and then anaesthetized on crushed ice until they showed first signs of immobility. A transponder (mic3-TAG 16k, Micro-Sensys GmbH) was glued on the thorax of each bee with superglue (UHU® Sekundenkleber blitzschnell Pipette), and each bee was colour-marked on the abdomen consistent with the following treatment. Prior to gluing, each transponder was scanned by a USB-Pen and ID numbers were allocated to the subsequent treatment groups.

For each experiment, 100 bees per treatment group were placed in two cages (8 cm x 5 cm x 5 cm). These cages were placed on top of the frames in a second super on top of the original hive. The cages were equipped with a second grid in order to avoid trophallaxis with the hive bees. Feeding 30 µl food per bee per day was conducted via two 5-ml Eppendorf tubes. Control bees received 50% sugar solution and treated bees received this solution containing flupyradifurone ( $1.4 \times 10^{-5}$  mol/L), because this concentration did not lead to increased mortality (see Section 3). Stock solutions were prepared and stored the same way as for the mortality study, and food was refilled every second day or third day. After 7 days, the bees were released into the hive and tracked for 40 days. As we used a small colony consisting of approximately 2,000 individuals, we decided to insert only 100 bees per treatment group to ensure their acceptance.

## 2.3 | Data acquisition

Using the RFID data, we developed a custom python code (Anaconda Navigator Spyder 3.3.3) to calculate selected aspects of foraging behaviour. Bees which only left the hive once and bees which never left the hive were excluded from analysis. Based on this specification, we gained data of 60 flupyradifurone-treated bees in replicate one and of 40 flupyradifurone-treated bees in replicate 2. Of the control group, we could analyse 61 bees in the first replicate experiment and 45 control bees in the second replicate.

Data were excluded when the time interval between the first and the second scan was larger than 300 s. This way, we have excluded entering or leaving bees where one of the scanners most likely did not work. For this reason, we furthermore only used data when pairings of readings of the first and second scanners matched. Based on our own observations, a foraging trip was defined as a bee being outside the hive for more than 180 s. This includes bees on a short mission as defined by Susanto et al. (2018). Onset of foraging was defined as follows: when a bee left the hive for two trips on one day, this day was counted as day of initiation of foraging. Alternatively, when a bee left the hive for one trip on one day and for one or more trips on a subsequent day, the first day was counted as the day of onset of foraging. Thus, we included the days of orientation flights, which always precede the foraging flights (Winston, 1987). If there were several days between the first and the second trips, the day of the second trip was regarded as the onset of foraging. In addition, we measured the number and duration of foraging trips per bee per day. We divided the total number of trips by the days a bee was foraging in order to obtain the number of trips per active days. Furthermore,

the total duration of trips was divided by the number of trips to gain the total duration per trip.

End of foraging was defined as the last time a bee was detected. If the bee was detected within the last three days of the experiment (e.g. days 44–47), data were censored in further statistical analysis regarding the end of foraging. We like to outline here that the end of foraging does not equal the day of death, because we found many live bees at the end of the experiment whose last day of foraging had been much earlier.

## 2.4 | Statistics

Statistical analyses were conducted using SPSS Statistics 23 (IBM) and GraphPad Prism 7.0 (GraphPad). Graphs were produced using the same programs.

The Kaplan–Meier method with log-rank test was applied for comparing the mortality rates (Dechaume Moncharmont, Decourtye, Hennequet-Hantier, Pons, & Pham-Delègue, 2003) and for comparing onset and end of foraging between the two treatment groups (Fox, 2006; Woyciechowski & Moroń, 2009). As data were not distributed normally (Kolmogorov–Smirnov Test), trips per active day and duration per trip between the different treatment groups were compared using Mann–Whitney *U* tests. We applied repeated-measures analysis of variance (ANOVA) for comparing duration per trip per day for the first 20 days of foraging between the different treatment groups. Here, we only used data of bees whose last day of foraging was after the 20th day in order to make sure that all bees included were still alive during the test phase. As Mauchly's test revealed no sphericity of data, we applied Greenhouse–Geisser correction to adjust the degrees of freedom for the averaged tests of significance. All tests were two-tailed.

## 2.5 | Morphological analysis of honeybee brains

To show possible alterations in honeybee brains, 20 newly emerged bees were collected per treatment group. Bees were maintained and fed in the same way as for the mortality study. The control group received 50% sugar water. The treatment group received this solution containing flupyradifurone ( $1.4 \times 10^{-4}$  mol/L). Food was replaced every second or third day. We chose this treatment regime, because preliminary data had not shown any morphological brain alterations after treating bees for one, five or ten days in lower concentrations ( $1.4 \times 10^{-5}$  mol/L,  $1.4 \times 10^{-6}$  mol/L). Furthermore, this was a concentration which increased mortality in our experiments. Acute administration of this concentration leads to behavioural abnormalities (Hesselbach & Scheiner, 2018).

On day ten, bees were removed from the cages and quickly killed at  $-20^{\circ}\text{C}$ . Brains were dissected in bee Ringer solution (Table S3) under a Zeiss stereomicroscope (Stemi 508; Zeiss).

The brains were fixed in formaldehyde 4%. The organs were dehydrated in a series of ethanol solutions (Table S3) at room temperature. Subsequently, they were embedded in paraffin after treatment

with isopropanol and n-butyl acetate for two times. 5- $\mu\text{m}$  sections were cut after deparaffining (Roti<sup>®</sup>-Histol [Carl Roth<sup>®</sup> Germany]) for two times, using a series of ethanol (Table S3). Sections were stained with haematoxylin and eosin (H/E) according to Gill II (Mulisch & Welsch, 2015) and embedded using Roti<sup>®</sup>-Histokitt II (Carl Roth<sup>®</sup> Germany).

Ten brains of each group were examined under a light microscope (Zeiss Axioskop 40) and photomicrographs were taken using a digital camera (Zeiss AxioCam 105 colour, 5MP) which was connected to the microscope. We used Zeiss AxioVision software for taking the images. Because some pesticides were shown to affect certain areas of the honeybee brain (Almeida Rossi et al., 2013; Jacob, Soares, Nocelli, & Malaspina, 2015; Oliveira et al., 2014), we focused on the optic lobes and the mushroom body calyces. To verify that we analysed identical regions in the brain of different subjects, we chose slices for analysis in which the central complex and the lateral and medial calyces were visible.

## 3 | RESULTS

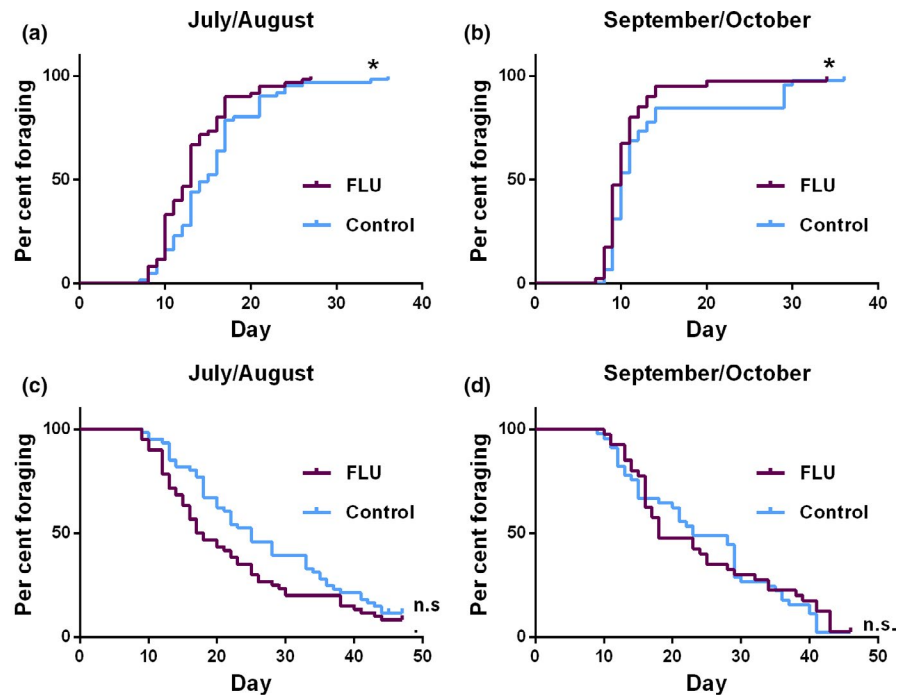
### 3.1 | Mortality

During a ten-day period, chronic toxicity was evaluated. In all replicates, the mortality of control groups was below 15%, as is recommended by OECD guidelines (OECD, 2017). The highest concentration applied ( $1.4 \times 10^{-3}$  mol/L in newly emerged bees and  $5.2 \times 10^{-4}$  mol/L in *diutinus* bees) led to a significantly increased mortality in all replicates with all bees dying within three days ( $p < .001$  in all replicates). The 10-fold lower concentration led to a significant difference in mortality between the treated bees and control bees in summer in three out of four replicates and in winter in two out of four replicates. Receiving this concentration between three and nine out of 30 bees died within ten days in summer and in winter. For the lowest concentration ( $1.4 \times 10^{-5}$  mol/L in newly emerged bees and  $5.2 \times 10^{-6}$  mol/L in *diutinus* bees), we found no difference towards the control in summer in all replicates. In winter, there was no significant difference in three out of four replicates. In the one replicate being significantly different, the mortality of the treated bees was significantly lower than that of the control. For all results, also see Table S2 and Figures S1 and S2. These results led to the assumption that a concentration of  $1.4 \times 10^{-5}$  mol/L was sublethal for newly emerged bees, and we therefore used this concentration for RFID experiments.

### 3.2 | Onset and end of foraging

Flupyradifurone-treated bees ( $1.4 \times 10^{-5}$  mol/L) started foraging significantly earlier in both replicates than control bees ( $p < .05$ ). The median times of foraging onset were day 15 (July/August) and day 10 (September/October) in the control group and day 13 (July/August) and day 10 (September/October) in the flupyradifurone-treated group (Figure 1).

**FIGURE 1** (a, b) Onset of foraging in July/August (a) and in September/October (b): In both experiments, pesticide-treated bees started foraging significantly earlier than control bees ( $p < .05$  (\*)). (c, d) End of foraging in July/August (c) and in September/October (d). In both experiments, the end of foraging did not differ between treatment groups ( $p = .064$ ; (n.s., c) and  $p = .975$  (n.s., d)). The y-axis represents the Kaplan–Meier estimates of the cumulative proportion of foraging honeybees for the flupyradifurone-treated bees ( $1.4 \times 10^{-5}$  mol/L (FLU)) and the control



The end of foraging did not differ between the two treatment groups in either replicates ( $p_{\text{July/August}} = .064$  and  $p_{\text{September/October}} = .735$ ), although the median days at the end of foraging varied strongly. The median end of foraging was day 25 (July/August) or day 23 (September/October) in the control group and day 17 (July/August) or day 18 (September/October) in the flupyradifurone-treated group (Figure 1).

### 3.3 | Number of trips and trip duration

The two replicates differed in the effects of flupyradifurone on the foraging trip frequency and the foraging duration. In replicate one, there were no differences in the frequency of foraging trips ( $p = .800$ ) or in the durations of foraging trips ( $p = .274$ ; Figure 2a,b) between flupyradifurone-treated bees and controls. In the second replicate, however, the flupyradifurone-treated bees foraged significantly more frequently ( $p < .01$ ) and for longer periods ( $p < .001$ ; Figure 2c,d).

In addition, we found a day effect on the duration per trip per day in both replicates (July/August:  $p < .05$ ,  $F_{(4,245)} = 2.7$ ; September/October:  $p < .05$ ,  $F_{(4,192)} = 3.2$ ).

However, we did not find an effect of treatment on the duration per trip per day during the first 20 days of foraging (Figure 3a,b; July/August:  $p = .74$ ,  $F_{(1,67)} = 0.1$ ; September/October:  $p = .98$ ,  $F_{(1,46)} = 0.0$ ).

### 3.4 | Histology

Focusing on the mushroom body calyces and the optic lobes, we analysed ten brains of control bees and ten brains of flupyradifurone ( $1.4 \times 10^{-4}$  mol/L)-treated bees. We looked for Kenyon cells with a pycnotic profile, characterized by margination and condensation of

nuclear chromatin, and for Kenyon cells, which were greater in size, indicating cell swelling. These alterations may indicate the occurrence of cell death by apoptosis (Orchard & Nation, 2011). Similarly, we looked for pycnotic cells and cells with a swollen appearance in the area of the optic lobes (Almeida Rossi et al., 2013; Oliveira et al., 2014). We did not find any cell alterations in H/E stainings in the inner compact cells and non-compact cells of mushroom bodies or the lamina, medulla, outer or inner chiasmata of the optic lobes of flupyradifurone-treated bees (Figures S3 and S4).

## 4 | DISCUSSION

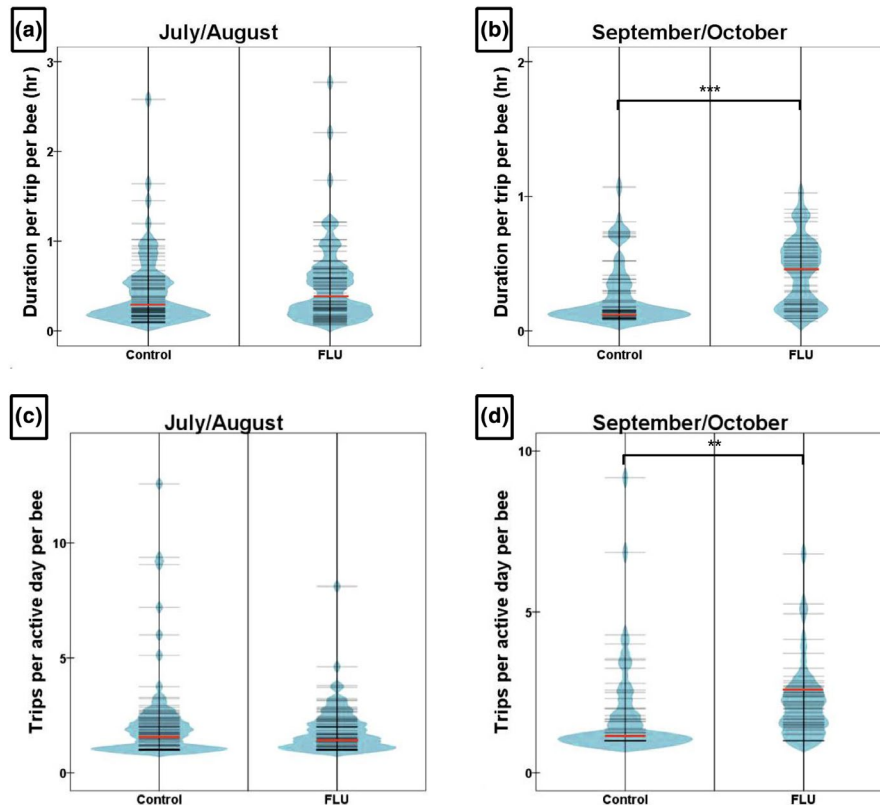
### 4.1 | Mortality, onset and end of foraging

The aim of the present study was to determine the effects of flupyradifurone on honeybee mortality, foraging activity and brain morphology. Our results reveal for the first time that a chronic dose of approximately 1 µg flupyradifurone per bee per day over the time course of ten days increases mortality significantly compared to a control group in newly emerged summer bees and long-lived *diutinus* bees. A 10-fold lower concentration ( $\sim 0.1$  µg per bee per day) led to a survival rate comparable to that of the control. It can therefore be assumed that this concentration fed chronically would only lead to sublethal effects according to the definition of Desneux, Decourtye, and Delpuech (2007).

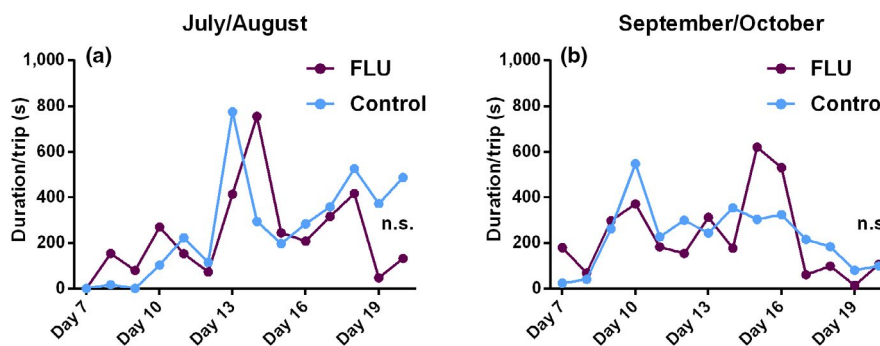
Overall, initiation of foraging in our set-ups was relatively early, which is typical of small hives (Sakagami, 1953). Importantly, bees which were chronically treated with a sublethal dose of flupyradifurone showed an earlier onset of foraging compared to the control group.

Using RFID technology, the same alteration has been shown for *Deformed Wing Virus*-infected honeybees (Lach, Kratz, & Baer, 2015).





**FIGURE 2** (a, b) Duration per trip per bee in July/August (a) and in September/October (b). In the first replicate, there was no significant difference in the duration per trip per bee ( $p = .274$ ; a). The duration per trip per bee differed significantly between the flupyradifurone-treated bees and the control in replicate two ( $p < .001$  (\*\*\*) b). Bean plots show the distribution of data with each black line representing data of one individual bee. Medians are shown by a red line. (c, d) Trips per active day per bee in July/August (c) and in September/October (d). Control bees and flupyradifurone-treated bees did not differ in the first replicate ( $1.4 \times 10^{-5}$  mol/L (FLU)) in the number of trips per active day per bee ( $p = .800$ ; c). In the second replicate, the treatment group differed from the control group in the number of trips per active day per bee ( $p < .01$  (\*\*); d). Bean plots show the distribution of the data with each black line representing data of one individual bee. Medians are shown with a red line



**FIGURE 3** Duration per trip per day over the first twenty days of foraging in July/August (a) and in September/October (b): We found a significant influence of day on the duration per trip per day in replicate one and two (July/August:  $p < .05$ ,  $F_{(4,245)} = 2.7$  (a); September/October:  $p < .05$ ,  $F_{(4,192)} = 3.2$  (b)), but no significant effect of treatment on the duration per trip per day during the first 20 days of foraging (July/August:  $p = .74$ ,  $F_{(1,67)} = 0.1$  (a); September/October:  $p = .98$ ;  $F_{(1,46)} = 0.0$  (b))

Similarly, accelerated maturation and premature foraging have been observed in *Varroa*-infected honeybees (Zanni, Değirmenci, Annoscia, Scheiner, & Nazzi, 2018) as well as in *Nosema apis*-inoculated bees (Woyciechowski & Moroń, 2009). Prado et al. (2019) found a significantly later onset of foraging after applying a pesticide mixture with pollen to newly emerged bees during their first days of adult

life. These different outcomes could result from the pesticide mixture they used, herbicides, fungicides and insecticides. The similar outcome of these different studies indicates that indirect effects, possibly due to chronic stress, are responsible for the earlier onset of foraging. Rueppell, Bachelier, Fondrk, and Page (2007) assume that mortality patterns in worker honeybees are mainly influenced by the

time point of transition to foraging, while the pre-foraging in-hive period is rather flexible in duration. Though we did not investigate this, the earlier onset of foraging could lead to bees performing in-hive tasks such as nursing for a shorter period of life and dying faster than control animals under hive conditions. Individual and colony factors determine the time of switching from in-hive tasks to foraging. On the individual level, foragers have lower levels of protein and stored lipids and haemocyte levels decline with age (Quigley, Amdam, & Rueppell, 2018). It was suggested that this change is related to altered hormonal states in foraging honeybees, as they have an increased level of juvenile hormone (JH). Goblirsch, Huang, and Spivak (2013) showed that *Nosema ceranae*-infected nurse-aged bees have higher JH titres in the haemolymph than control bees. These authors also observed more *Nosema ceranae*-infected bees at the hive entrance in earlier stages of their experiments, leading to the conclusion that the infection induces premature foraging along with higher JH titres. Furthermore, foraging honeybees have an elevated metabolic rate compared to in-hive workers, which leads to elevated release of free radicals, inducing oxidative stress (Quigley et al., 2018). This expresses itself in oxidative carbonylation. Seehuus, Krekling, and Amdam (2006) showed that oxidative carbonylation in the honeybee brain was strongly associated with social task, with bees in the foraging state expressing higher levels of oxidative carbonylation. Oxidative stress and hormonal state were not investigated in our study and should be taken into account in further studies.

Regarding the end of foraging, we did not find any statistical differences between treatment groups, though there was a trend in replicate one (July/August) towards an earlier end of foraging in flupyradifurone-treated bees. This supports the suggestion that transition to foraging determines life span of honeybees (Rueppell et al., 2007).

## 4.2 | Foraging activity and duration

Studies imply that imidacloprid-exposed bumblebees were less efficient at collecting pollen (Gill et al., 2012; Stanley et al., 2016). This effect was also reported for a certain fungicide-dominated pesticide mixture in honeybees (Prado et al., 2019). However, a single administration of the neonicotinoids imidacloprid or clothianidin to honeybees can lead to a reduction of foraging activity and to longer foraging flights, which corroborates the aforementioned thesis (Schneider et al., 2012). On the other hand, Thompson et al. (2016) did not find an altered foraging behaviour in honeybees foraging on thiamethoxam-treated oilseed rape.

In our study, only the flupyradifurone-treated bees of the second replicate in September/October showed significantly more and longer foraging flights per day than control bees, similar to pesticide-treated bumblebees (Gill et al., 2012). Availability of floral resources and thus the season as well as an elevated *Varroa* burden in late summer and autumn (Vidal-Naquet, 2015) might change pesticide toxicokinetics (Poquet, Vidau, & Alaux, 2016) could be possible explanations for the different outcomes of our two replicates. Repeated-measures ANOVA revealed no significant effect of treatment on the duration

per foraging trip in the first 20 days in both replicates. It therefore remains unclear whether the chronic exposure of honeybees to flupyradifurone for one week decreases foraging efficiency.

## 4.3 | Histological alterations

Kenyon cells of the mushroom bodies play an extraordinary role in learning and memory of honeybees (Gauthier, 2009). It was also suggested that Kenyon cells are sensitive to juvenile hormone, with this hormone regulating gene expression and possibly dendritic growth (Velarde, Robinson, & Fahrbach, 2009). Thus, Kenyon cells would also play a role in the nurse-forager transition of adult honeybees. It was shown that *Deformed Wing Virus*, a pathogen replicating in the mushroom bodies, the antennal lobes and the visual neuropils (Shah, Evans, & Pizzorno, 2009), can lead to premature foraging in honeybees (Lach et al., 2015). In this study, we show that flupyradifurone leads to premature foraging and previous studies revealed a negative influence of this pesticide on honeybee learning and memory (Hesselbach & Scheiner, 2018; Tan et al., 2017).

Thus, we searched for signs of structural alterations in honeybee brains, especially of the Kenyon cells in the mushroom bodies and in cells of the optic lobes, after treatment with flupyradifurone. In contrast to what has been shown for neonicotinoid pesticides (Almeida Rossi et al., 2013; Oliveira et al., 2014), we did not find any signs of apoptosis in Kenyon cells and in cells of the optic lobes of flupyradifurone-treated bees. Despite this, we cannot exclude ultrastructural alterations. It would further be interesting to investigate brains of foraging bees for those alterations.

## 4.4 | Honeybee exposure to flupyradifurone in the field

Flupyradifurone has a broad spectrum of target organisms and can be applied on a variety of plants (Nauen et al., 2015). The residues of flupyradifurone in nectar and pollen vary strongly between crops (Glaberman & White, 2014). In this study, the estimated environmental concentration (EEC) in apple nectar after two foliar applications was specified to be 0.44 µg/bee/day through nectar for adult worker honeybees. This is about four times higher than the average consumption rate per day in our foraging experiment. We thus consider our concentrations used as field-realistic. Nurse bees ingest a high amount of pollen to produce larval and queen food through their glands (Rortais, Arnold, Halm, & Touffet-Briens, 2005; Winston, 1987), and flupyradifurone residues in pollen often exceed those in nectar (Glaberman & White, 2014). We can thus not exclude an even higher consumption rate. Tosi and Nieh (2019) performed similar calculations with residues in oilseed rape and conclude that even an intake of up to 5.5 µg/bee/day in foragers and 2.4 µg/bee/day can be considered field-realistic, which is considerably higher than the concentrations we used. Hardly anything is known about combined effects of insecticides and fungicides, which are frequently co-applied

in the same crops. First experiments analysing these effects suggest synergistic effects and enhanced toxicity (Tosi & Nieh, 2019; Zhu, Yao, Adamczyk, & Luttrell, 2017). Interactions of flupyradifurone and further pesticides are therefore an important field for future studies.

In conclusion, we show effects of a chronic flupyradifurone application on the mortality of honeybees. To a lesser extent, flight behaviour was affected, leading to an earlier onset of foraging. The molecular pathways underlying the assumed faster development are unclear and deserve further investigation. The RFID technology has proved a valuable tool for investigating long-term effects of chronic pesticide exposure in field experiments.

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## AUTHORS' CONTRIBUTIONS

H.H. performed preliminary experiments and gained the data. M.A. and F.S. developed the software to analyse RFID data. H.H. and R.S. wrote the main manuscript text, and H.H. prepared all of the figures. All authors contributed critically to the drafts and gave final approval for publication.

## DATA AVAILABILITY STATEMENT

Data available in the online supplementary information or via the Figshare: <https://doi.org/10.6084/M9.FIGSHARE.7564790.V4> (Hesselbach, Seeger, Schilcher, Ankenbrand, & Scheiner, 2019).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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