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Sublethal neonicotinoid exposure attenuates the effects of electromagnetic fields on honey bee flight and learning

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ABSTRACT

Many environmental stressors are currently implicated in the decline of flying insects in general, and important pollinator species such as the honey bee. Recent studies have shown that extremely low frequency electromagnetic fields (ELF EMFs) affect many aspects of insect behaviour and cognition which raises the potential that ELF EMFs could interact with other environmental stressors such as neonicotinoid insecticides to have even greater impact on the decline of flying insects. Here we analysed the effects of individual and combined exposure of the honey bee to 50 Hz EMFs and sublethal exposure to clothianidin, in a tethered flight assay and an olfactory appetitive learning assay.

Clothianidin was significantly toxic to bees and exposure to field-realistic levels (2.00 ng/bee) led to 25% mortality. Exposure to ELF EMFs alone led to a significant increase in wingbeat frequency at levels above 100 μT. Prior exposure to clothianidin attenuated the effects of EMFs on wingbeat frequency. Exposure to EMFs alone reduced learning of a proboscis extension reflex (PER). Prior exposure to low doses of clothianidin attenuated the effects of EMFs on PER.

These results indicate no evidence of synergy between clothianidin and ELF EMFs as environmental stressors but suggest the potential for EMFs to affect the same susceptible fraction of the bee population that have been affected by clothianidin. Results lay the foundation to further explore the interactions of ELF EMFs with other environmental stressors and consider the key factors that may make bees susceptible to ELF EMFs.

Introduction

There is an inextricable link between environmental change from anthropogenic activities and adverse effects on ecosystems such as biodiversity loss (Butchart et al., 2010). Many human-derived activities have had unintended effects on insect populations, such as artificial light pollution affecting insect distributions and predator prey interactions (Longcore and Rich, 2004). Some activities have been linked to unintended effects on beneficial insect species such as bees, including the use of systemic insecticides (Goulson, 2013; Rundlöf et al., 2015), habitat fragmentation (Goverde et al., 2002), land-use/agricultural change (Ollerton et al., 2014), climate change (Kerr et al., 2015; Goulson et al., 2015a), environmental pollutants (Lusebrink et al., 2015) and even the anthropogenic spread of pollinator parasites (Goulson and Hughes, 2015b; Wilfert et al., 2016). The current consensus is that this cocktail of human-derived environmental stressors/pollutants in combination is leading to declines in honey bees and other pollinators (Potts et al., 2010; Ollerton et al., 2014; Goulson et al., 2015a).

Neonicotinoids are systemic insecticides (Goulson, 2013; Bonmatin et al., 2015) that have a broad range of targets, and are used on a variety of crops (EFSA, 2013; Goulson, 2013) to control various sucking/biting pests (Elbert et al., 1998). Their use increased dramatically in the early 2000's, with imidacloprid, clothianidin and thiamethoxam becoming the most commonly used neonicotinoids, largely due to their high toxicity (Goulson, 2013). The use of neonicotinoids is now known to be associated with increased honey bee mortality and risk of colony loss (Samson-Robert et al., 2017). Bees are most commonly exposed to neonicotinoids below lethal doses, and the

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sublethal effects of these insecticides are a major concern in assessing their environmental safety. Sublethal exposure to neonicotinoids has been shown to cause significant cognitive effects in bees, including reduced olfactory learning (Decourtye et al., 2004a,b; Han et al., 2010; Williamson and Wright, 2013) and learning performance associated with foraging activities (Decourtye et al., 2004b; Han et al., 2010). The effects of neonicotinoids have also been linked to reduced motor function in bees, leading to various behavioural alterations, including knockdown, poor coordination, increased locomotory activity and tremors (Lambin et al., 2001; Suchail et al., 2001).

Extremely low frequency electromagnetic fields (ELF EMFs) are pervasive in the environment, and anthropogenic ELF EMF pollution has increased greatly in recent years (Belpomme et al., 2018). Pellacani and Costa (2018) describe biological effects that ELF EMF pollution causes, including neurological effects that underpin behavioural processes in a variety of organisms. There is clear evidence that bees are affected by ELF EMFs in the range emitted by high-voltage transmission lines (HVTLs) (Shepherd et al., 2018, 2019), and hives kept under powerlines show evidence of biological stress (Greenberg et al, 1981). Powerline strips (i.e. the areas of land under powerlines) are thought to be important refuges for insect pollinators with beneficial host plants for pollinators, leading to relatively good species richness, including rare species of bees (Russell et al., 2005; Wojcik and Buchmann, 2012; Hill and Bartomeus, 2016) as well as other important pollinators such as butterflies (Berg et al., 2016). However, acute exposure to ELF EMFs at levels that can be encountered regularly in the environment around man-made sources such as high voltage transmission lines (HVTLs), has been shown to reduce olfactory learning, feeding and flight performance (Shepherd et al., 2018) in honey bees. The potential biological and environmental effects of ELF EMFs are poorly understood and we know nothing about how they interact with other stressors in the environment, such as neonicotinoid insecticides. HVTLs are built on flat land, and in general, away from residential areas, making farmland highly suitable for their construction. Consequently, in the natural environment it is likely that insects that are exposed to ELF EMFs will have also been exposed to agrochemicals, including potentially neonicotinoid insecticides, and that this exposure will occur during foraging activities.

Here, we demonstrate how individual and combined exposure to ELF EMFs and sublethal concentrations of clothianidin impact flight behaviour and learning in the honey bee, *Apis mellifera*. Our results can be considered in future risk assessment of environmental stressors on pollinators, which should include analysis beyond isolated risks of agrochemicals alone on non-target species.

Materials and methods

Electromagnetic coils

EMFs were generated using custom-made 25 cm (inner diameter) paired wire coils (Fig. 1A), fixed 14 cm apart (as described in detail in Shepherd et al., 2018). Briefly, coils were powered with 240 V AC through Variacs (RS Pro, RS Components, UK) to generate homogenous 50 Hz sinusoidal EMFs at the centre of the coils ranging from 10 μTto 10,000 μT.

Overhead transmission lines operate at different voltages in different countries up to a maximum of 1150 kV. In the UK, the largest power lines operate at 400 kV using Larger L6 pylons with ratings up to 4 kA per circuit and a minimum ground clearance of 7.6 m. In theory, this produces EMFs up to 100 μT at ground level directly beneath the conductor (WHO, 2007), depending on current loading and ground clearance. Shepherd et al. (2018) modelled the magnitude of the ELF EMFs around Larger L6 Pylon conductors using a current rating of 3.4 kA and found the field at 1 cm from the conductor to be 3000 μT, while at 10 cm to be 2700 μT, and at 1 m below the lowest conductor to be 1200 μT. For a 400 kV T-Pylon the EMFs are higher (Shepherd et al. 2018). For reference, the average magnetic field 1 m above ground under a Pylon is 5–10 μT, and the maximum theoretical level is 100 μT (ENA 2015, WHO 2007). We therefore chose a range of EMFs that reflected those found in the environment of 0, 20, 100 and 1000μT. Fields were measured using a magnetometer (Model GM2, Alphalab Inc., USA) to ensure they matched the modelled equivalents (Fig. 1B). For control treatments no current was passed through the coils.

Bee collection and harnessing

Honey bees, *Apis mellifera*, were kept in the University of Southampton campus apiary (50° 56′ 10″N, 1° 23′ 39″W) in summer 2016. Returning forager bees from 3 hives were collected and transported into the Insectary in the Institute for Life Sciences for analysis.

Clothianidin exposure and toxicity

COLOSS standard methods for toxicology research (Medrzycki et al., 2013) were used in designing toxicity assays. Analytical standard Clothianidin (PESTANAL - 33589; Sigma-Aldrich, UK) was used for all experiments diluted in a syrup (sucrose solution 50% w:v). Clothianidin was dissolved in acetone, and diluted to treatment concentrations with distilled water. For control treatment, acetone diluted as clothianidin treatments was added to the same sucrose solution. All solutions had a final concentration of 0.000125% (v:v) acetone and 50% (w:v) sucrose.

To establish the toxicity of clothianidin to bees a dose-response mortality curve and LD_{50} were determined. For clothianidin treatment, bees were held individually in Eppendorf tubes and fed with different doses of clothianidin in 10 μl of sucrose solution. Subsequently bees were grouped for further experiments. For toxicity experiments bees were fed 1 of 6 different doses of clothianidin (0.025, 0.50, 1.00, 2.00, 4.00 and 10.00 ng/bee). Immediately after exposure bees were transferred to plastic containers with feeders filled with a 50% (w:v) sucrose solution, from which they could feed freely. 15 bees were added to the same container (>10 bee OECD minimum), which in LD_{50} analyses formed a single cohort. For each of the 6 doses of clothianidin there were 9 cohorts (135 bees) for each treatment (3 × 15 bee cohorts per hive) (See Supplementary Table 1). Containers were kept at 29 ± 1 °C and assessed 24 hrs after exposure. Twenty-four hours after clothianidin treatment, bee mortality in each container was assessed using *COLOSS* recommendations for determination of dead bees (Medrzycki, et al., 2013). The number of dead bees in each container was totalled, and then analysed to determine the LD_{50}.

For clothianidin exposure in PER and flight experiments bees were fed 0.00, 0.25, and 2.00 ng treatments of clothianidin after collection. EFSA (2013) calculated a worst-case scenario through clothianidin residues in oilseed rape foraging of 4.3–13.7 ng per day. We therefore chose a high clothianidin treatment that is a conservative estimate for daily clothianidin exposure (and relatable to the 2.00 ng treatment used in LD_{50} analysis), and a low clothianidin treatment to test for sublethal effects that could occur for highly common clothianidin residues in the environment. Bees were placed in the same style free feeding containers as the toxicity assay. Flight experiments were carried out 24 h after clothianidin exposure. In total 369 bees were included in flight analyses (Supplementary Table 2). For PER experiments a total of 1051 bees were included (Supplementary Table 3). After clothianidin treatment bees were immobilized on ice and secured in custom-cut 1 ml pipette tips with adhesive tape (Tesa® SE, Germany). Bees were fed with a 40% (w/v) sucrose solution to satiation and maintained overnight at 29 ± 1 °C in a plastic perforated container with wet tissue paper. PER assays were conducted 17 h after clothianidin exposure.

Proboscis extension reflex

For combined clothianidin and ELF EMF effects on PER the protocol from Shepherd et al (2018) was followed. An experimental arena (W × D × H = 60 cm × 45 cm × 55 cm) and custom odour delivery

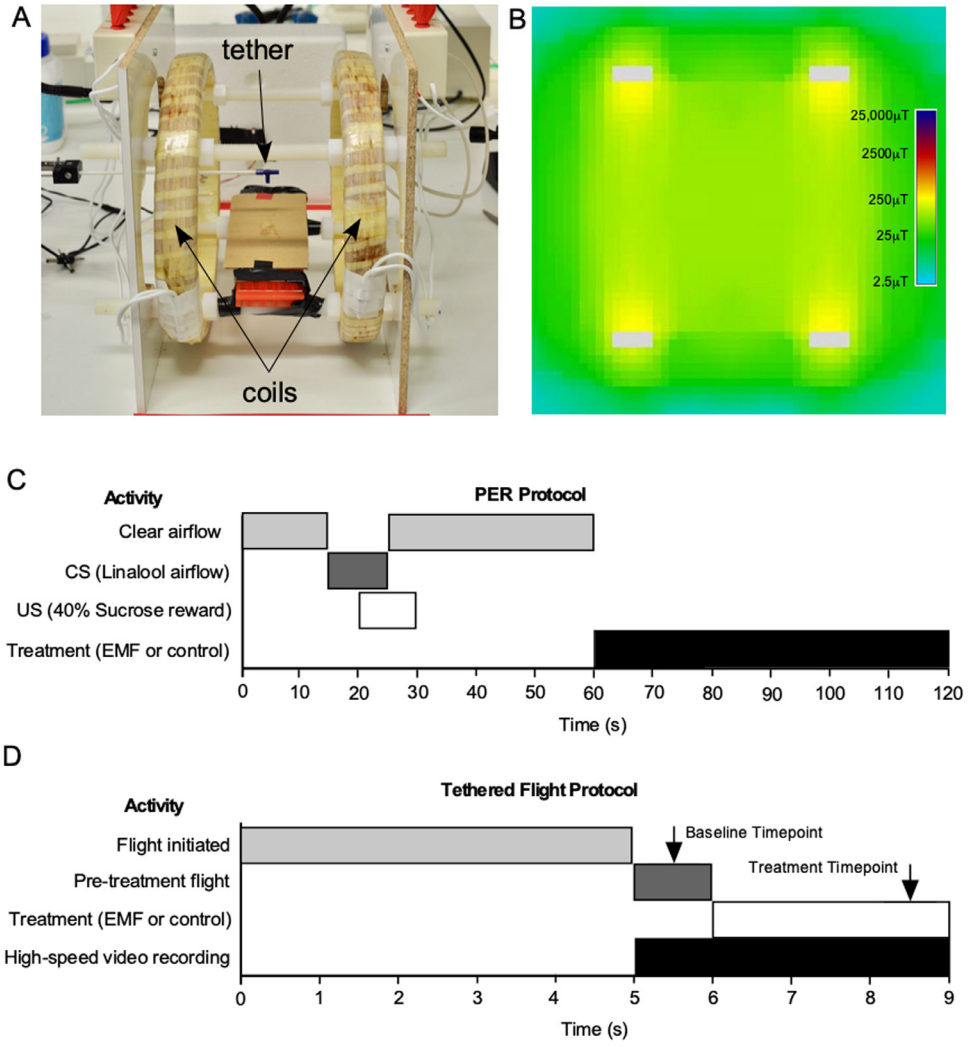


Fig. 1. The electromagnetic field (EMF) coils and experimental paradigms. **A.** Photograph of the coils showing the mount (tether) to which bees were attached for flight studies. B. Model of the EMF between the coils with 100 μT at the centre. **C.** Protocol for the proboscis extension reflex (PER) assessment during 50Hz EMF exposure. Five seconds after CS onset a 40% w/v sucrose solution reward (US) was given. Bees fed on the sucrose solution for 10 s (pairing of CS and US, 5 s), given 30s clear airflow and then removed from the arena. **D.** Flight trial for tethered flight assay. Flight was initiated for 5 s. 1000 fps high-speed video recording (4 s) captured pre-treatment period (1 s) and a treatment (Control or EMF) period (3 s). Baseline wingbeat frequency was recorded 0.5 s before initiation of the treatment and wingbeat frequency recorded 2.5 s after initiation of the treatment.

system were used. The conditioned stimulus (CS) used was 8 μl of 97% linalool (Sigma-Aldrich, UK) pipetted onto filter paper and used in the odour delivery system.

Bees were tested for gustatory responsiveness with a 40% sucrose solution. Those that failed were excluded along with overnight mortalities. For conditioning experiments bees were placed individually in the arena and allowed to adapt to surroundings for 15 s before being presented with the CS for 10 s (Fig. 1C). 5 s after the CS onset a 40% sucrose solution reward (unconditioned stimulus, US) was applied to an antenna. The bee was allowed to feed on the sucrose solution for 10 s (pairing CS and US for 5 s), given 30s clear airflow and then removed from the arena (Fig. 1C). 5 conditioning trials were completed for each bee in the assay with an inter-trial interval of 10 min.

If a bee did not extend its proboscis during the assay 'no response' was recorded. If a bee only extended its proboscis after the sugar reward (US) application began, then a 'gustatory response' was recorded. Bees that failed gustatory responsiveness were excluded from analysis. PER was recorded if a bee extended its proboscis during the CS application, but before US presentation. Bees that exhibited PER in response to linalool in the first conditioning trial were excluded from analysis, as learning cannot be recorded in bees that already respond to the CS. In total, 1051 bees were collected and 890 (84.7%) survived overnight treatments. Of these, 120 (11.4%) were excluded from trials for failing gustatory responsiveness and 83 (7.8%) for a pre-learned response to the CS. A total of 687 bees was therefore included in assessment of learning acquisition.

To analyse the combined effects of ELF EMFs and clothianidin on PER, bees that had been exposed to clothianidin treatments (0.00 ng/bee, 0.25 ng/bee, 2.00 ng/bee) were then exposed to EMFs for 1 min immediately following conditioning in each of the 5 conditioning trials in the PER assay. Bees were exposed to 3 different 50Hz EMF levels (20, 100, and 1000μT) or control exposures (bees were evenly grouped based on clothianidin survival). The number of bees (n) assigned to each *Clothianidin*EMF* combination treatment ranged from 78 to 100 (Supplementary Table 3).

Flight

Tethered flight assays were carried out as previously described in Shepherd et al. (2018). Briefly, hair on the dorsal side of the thorax was removed to provide an attachment point. Bees were attached to a tether using UV activated glue (Bug-Bond™, Veniard Ltd., Croydon, UK). The tether allowed bees to be placed on, and raised from, a platform in the centre of the coils. Raising bees vertically from the platform initiated flight. A high-speed video camera (MotionScope 1000S, Redlake Imaging, CA, USA) was used to record flight at 1000 fps. Following 5 s of consistent flight an EMF was applied and the video camera triggered to capture flight 1 s prior to EMF exposure and 3 s after exposure (Fig. 1D). Bees (369 in total, min n = 30 for each treatment, max n = 35, from 3 hives) were exposed to 4 different EMF strengths (0, 100, 1000, and 7000 μT). High-speed video was analysed to determine wingbeat frequencies of bees 0.5 s before EMF exposure (pre-treatment)

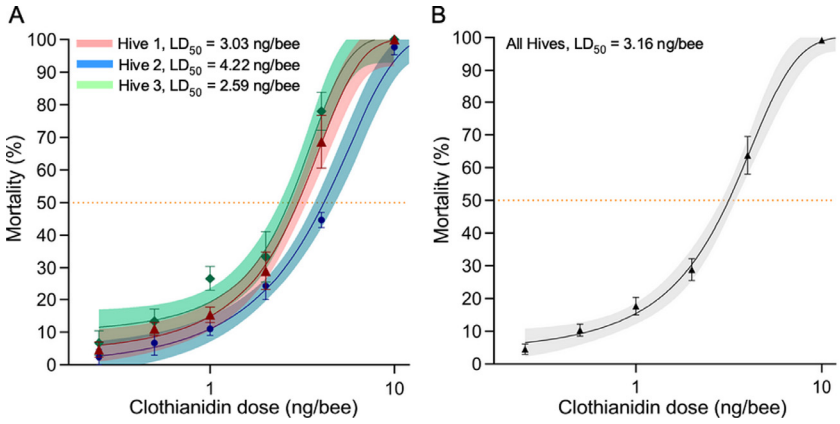


Fig. 2. Toxicity of clothianidin to honey bees. Dose-response curves for the effect of clothianidin on bee mortality for each of three individual hives and all hives combined. Log dose is plotted. Dotted lines show 95% confidence intervals. Data points and error bars show mean ± SEM. A. Mortality in three different hives and B. All hives combined. Hive 2 was the most resilient to clothianidin exposure while Hive 3 was the most susceptible.

and 2.5 s after the EMF onset (treatment). To determine the effect of acute EMF exposure on flight, the change in wingbeat frequency from pre-treatment to treatment was calculated for each bee, for both control and exposed bees.

Tethered flight assays were conducted 24 hrs after clothianidin treatment (0.00 ng/bee, 0.25 ng/bee, 2.00 ng/bee), the same time point at which mortality was measured in the LD_{50} analysis and under exactly the same conditions (free feeding cage at 29°C). The number of bees (n) assigned to each *Clothianidin*EMF* combination treatment ranged from 30 to 35 (Supplementary Table 2).

Statistical analysis

Data were analysed using SPSS (v.24, IBM SPSS Inc.) and Graphpad Prism (v.8, Graph Pad Software Inc.). Where appropriate, homogeneity of variance and normality assumptions were tested. For all models assessing the impacts of treatments on binomial PER data (as well as mortality and gustatory responsiveness in the PER assay), binomial error structure and logit link function were used. 'Hive of origin' was considered as a random effect (Piiroinen and Goulson, 2016) in all mixed models.

LD_{50} analyses, which determines the lethal dose that kills 50% of tested bees, were conducted following standard methods (Medrzycki, et al., 2013). Regression analyses were used to plot mortality proportions in a dose-response curve with 95% confidence limits, where clothianidin doses were logarithmically transformed and mortality proportions were logit transformed. LD_{50} values and other median lethal doses were interpolated from the curve.

In the PER assays, it is possible that differential clothianidin treatments could affect the number of bees excluded from the PER assay by either changing mortality levels, or by affecting gustatory responsiveness. To determine the effect of clothianidin treatment (0.0, 0.25, or 2.00 ng/bee) on the proportion of mortality, and the proportion of gustatory responsiveness in surviving bees, was assessed using generalized mixed effect models (GLMMs). A GLMM was also used to analyse the effects of combined clothianidin and EMF treatments on learning acquisition (PER) in trials 2–5. Trial 1 was not included as bees cannot exhibit conditioned PER in trial 1, and to improve model fit, including 'trial number' as a repeated measure interactive factor. Where appropriate, pairwise contrasts with Fisher's least significant difference protocol (Fisher's LSD) were used in *post-hoc* analyses for GLMM's. A one-way Analysis of Variance (ANOVA) was used to determine the initial effect of clothianidin treatment on honey bee wingbeat frequency with Bonferroni protocol for *post-hoc* analyses. A linear GLMM with clothianidin and EMF treatments as interaction factors, was used to assess the combined effects of clothianidin and EMFs on the change in wingbeat frequency observed in the flight assay.

Results

Clothianidin toxicity

To determine the toxicity of clothianidin in bees we calculated dose-response curves for six clothianidin doses (0.25, 0.50, 1.00, 2.00, 4.00 and 10.00 ng/bee) and 24 h mortality were plotted for each individual hive (Fig. 2A), and all hives combined (Fig. 2B). Some hives appeared more susceptible to clothianidin exposure than others although there was considerable overlap of LD_{50} curves. The 24 h LD_{50} for Hive 1 was 3.03 ng/bee which was mid-range compared to the other two hives (Fig. 2A). Hive 2 was the most resilient to clothianidin exposure (Fig. 2A) with the highest 24 h LD_{50} at 4.22 ng/bee. In contrast, Hive 3 was the most susceptible to clothianidin exposure, with the lowest 24 h LD_{50} at 2.59 ng/bee. For all hives combined the 24 h LD_{50} for clothianidin was 3.16 ng/bee (95% CI: 2.88–3.44 ng/bee) (Fig. 2B). On average a dose as low as 0.97 ng/bee (95% CI: 0.73–1.24 ng/bee) was sufficient to cause 15% mortality, and doses greater than or equal to 5.89 ng/bee (95% CI: 5.11–6.91 ng/bee) caused at least 85% mortality. For doses relevant to the 24 h timescale used in the flight assay, 0.25 ng/bee caused 6.5% mortality (95% CI: 2.2–10.9%) and 2.00 ng/bee caused 30.4% mortality (95% CI: 25.6–35.3%).

Effects of clothianidin alone on flight

The effect of exposure to clothianidin alone on wingbeat frequency was assessed in the tethered flight assay (Fig. 3A). Across all hives with no clothianidin exposure, the mean wingbeat frequency was 116 ± 3 Hz, whereas bees exposed to 0.25 ng clothianidin had a mean wingbeat frequency of 111 ± 3 Hz, while bees exposed to 2.00 ng had a higher mean wingbeat frequency of 126 ± 3 Hz. Clothianidin had a statistically significant effect on wingbeat frequency (One-way ANOVA, $F_{2,366} = 7.68$, $P = 0.0005$), as bees that survived 2.00 ng exposure had significantly higher increased wingbeat frequencies than both 0.25 ng (Bonferroni: $P < 0.001$) and 0.00 ng (Bonferroni: $P = 0.039$) exposures. There was no significant difference between the wingbeat frequencies of bees following 0.00 ng and 0.25 ng treatments (Bonferroni: $P = 0.54$).

Combined effects of clothianidin and ELF EMFs on flight

24 h after clothianidin treatment, tethered flight assays were conducted in which bees were also exposed to 50 Hz EMF (0 μT control, 100 μT, 1000 μT and 7000 μT). With no clothianidin exposure (0.00 ng/bee), and no EMF exposure, the wingbeat frequency increased by 2.4 ± 0.9 Hz, during the time period of the assay (Fig. 3B). With increasing EMF exposure, wingbeat frequency increased by 3.0 ± 0.8 Hz at 100 μT, by 5.6 ± 0.9 Hz at 1000 μT, and by 6.3 ± 1.1 Hz at 7000 μT. Following exposure to 0.25 ng clothianidin the increase in frequency

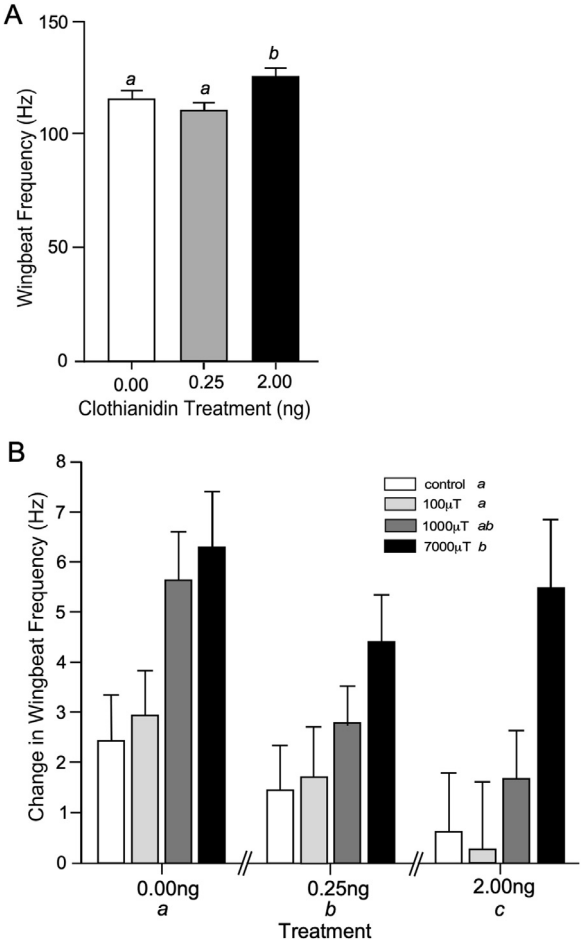


Fig. 3. Prior exposure to clothianidin reduced the effects of ELF EMFs on wingbeat frequency. **A.** Effect of 0.00 ng, 0.25 ng, and 2.00 ng/bee clothianidin exposure on wingbeat frequency 24 h after clothianidin exposure. **B.** Change in wingbeat frequency for all 12 combination treatments of clothianidin (0.00, 0.25 and 2.00 ng/bee) and EMFs (0 μT, 20 μT, 100 μT and 1000 μT). EMF exposure led to increased wingbeat frequency. Prior treatment with clothianidin reduced the effects of EMFs on wingbeat frequency. For all figures mean ± SEM are plotted. Different letters for both treatments and exposure correspond to significant differences ($\alpha = 0.05$).

caused by EMF was reduced to 1.7 ± 1.0 Hz at 100 μT, 2.8 ± 0.7 Hz at 1000 μT and 4.4 ± 0.9 Hz at 7000 μT. Following 2.00 ng clothianidin treatment the increases in wingbeat frequency found prior to clothianidin exposure were reduced even further to 0.6 ± 1.1 Hz in control bees, 0.3 ± 1.3 Hz in bees exposed to 100 μT, 1.7 ± 0.9 in bees exposed to 1000 μT and 5.5 ± 1.3 Hz in bees exposed to 7000 μT.

There was no two-way interaction effect of EMF exposure and clothianidin treatment on the change observed in mean wingbeat frequency (GLMM, $F_{6,357} = 0.59$, $P = 0.74$). EMF exposure significantly increased wingbeat frequency across all clothianidin treatments (GLMM, $F_{3,357} = 9.837$, $P < 0.0001$) with 7000 μT significantly increasing the mean wingbeat frequency from both control (Fisher's LSD: $P < 0.0001$) and 100 μT exposure (Fisher's LSD: $P < 0.0001$).

Clothianidin exposure reduced wingbeat frequency changes across all treatments, including controls (GLMM, $F_{3,357} = 6.127$, $P = 0.002$). In bees exposed to 2.00 ng clothianidin alone the mean wingbeat frequency increased by 1.8 ± 0.5 Hz, which was significantly less than the mean increase of 4.3 ± 0.5 Hz following 0.00 ng control (Fisher's LSD: $P = 0.002$). Bees treated with 0.25 ng clothianidin also exhibited a mean wingbeat frequency of 2.6 ± 0.5 Hz that was significantly less than control (Fisher's LSD: $P = 0.031$). There were no differences between 0.25 ng and 2.00 ng clothianidin treatment effects on a change in wingbeat frequency (Fisher's LSD: $P = 0.35$).

Thus, in control bees not exposed to clothianidin, 50Hz EMFs caused an increase in wingbeat frequency, with greater field strengths causing greater changes in wingbeat frequency. Prior exposure to clothianidin, reduced the effect of EMF on wingbeat frequency so that only exposure to 7000 μT caused a significant change in frequency.

Effects of clothianidin alone on bees excluded from PER (mortality and gustatory responses)

In preparation for the PER assay, bees given sucrose treatment exhibited 10.4% mortality, a 0.25 ng/bee clothianidin dose caused 14.7% mortality, and a 2.00 ng/bee dose caused 30.7% mortality (Fig. 4A). Increased doses of clothianidin significantly increased mortality (GLMM: $F_{2,1048} = 22.33$, $P < 0.001$). A dose of 2.00 ng/bee significantly increased mortality compared to both control (Fisher's LSD : $P = 0.001$), and 0.25ng/bee treatments (Fisher's LSD: $P < 0.001$). The 0.25 ng/bee dose did not significantly increase mortality from control (Fisher's LSD: $P = 0.054$).

Hive 3 (the most susceptible hive for 24 h clothianidin LD_{50} at 2.59 ng/bee) exhibited higher mortality levels prior to PER assays with 14.2% mortality for sucrose treatment, 19.8% mortality for a 0.25 ng/bee dose, and 53.8% mortality for a 2.00 ng/bee dose (Fig. 4B). Hive 1 (the 2nd most susceptible hive for 24 h clothianidin LD_{50} at 3.03 ng/bee) was intermediate in terms of susceptibility to clothianidin exposure prior to PER assays, with 6.2% mortality for sucrose treatment, 15.7% mortality for a 0.25 ng/bee dose, and 24.4% mortality for a 2.00 ng/bee dose. Hive 2 (the least susceptible hive for 24 h clothianidin LD_{50} at 4.22 ng/bee) was the most resilient to clothianidin exposure prior to the PER assay with 7.0% mortality for sucrose, 7.8% mortality for a 0.25 ng/bee dose, and 12.9% mortality for a 2.00 ng/bee dose.

Of the bees that survived clothianidin treatment there was no effect of treatment on gustatory responsiveness (GLMM $F_{2,887} = 2.098$, $P = 0.123$) which was 88.7% for 0.00 ng exposure, 87.4% for 0.25 ng exposure, and 83.0% for 2.00 ng exposure.

Combined effects of clothianidin and ELF EMFs on PER

To determine the combined effects of ELF EMFs and clothianidin on learning, bees were exposed to twelve combination treatments: 1 of 3 clothianidin treatments (0.00 ng, 0.25 ng, 2.00 ng/bee) 17 h prior to the PER assay, followed by 1 of 4 EMF treatments (0 μT control, 20 μT, 100 μT and 1000 μT) for 1 min after every conditioning trial during the PER assay. A GLMM was used to determine the interactive effects of clothianidin and EMF treatment on learning acquisition. As trial number increased bees learned the CS in the PER assay (GLMM, $F_{3,2700} = 16.89$, $P < 0.0001$). Bees that were not exposed to clothianidin overnight (i.e. 0.00 ng/bee) and not exposed to EMFs during conditioning had the highest learning levels in the PER assay with 60% in only trial 2, and a peak of 87% learning (Fig. 5A). Bees that were exposed to increasing intensities of EMFs during conditioning trials had reduced learning levels, with 68–87 % control bees responding in trials 3–5, 55–77 % responding under a 20 μT EMF, 43–72 % responding under a 100 μT EMF and 42–68 % responding under a 1000 μT EMF.

Prior treatment with clothianidin, with no EMF exposure, reduced the proportions of learning compared to control. For example, with no EMF exposure during conditioning and no clothianidin treatment, bees reached levels of 75–87 % learning (across trials 3–5), but 0.25 ng treated bees exhibited PER at 71-78 %, while 2.00 ng treated bees exhibited PER at 68–72 % (Fig. 5A–C). 2.00 ng clothianidin exposure significantly reduced learning levels when compared to control bees (when no EMF exposure occurred) (GLMM, Fisher's LSD: $P = 0.006$).

Clothianidin treatment and EMF exposure had an interactive effect on the proportions of PER in the assay (GLMM, $F_{6,2700} = 5.196$, $P < 0.001$). As the dose of clothianidin increased the effect of EMFs

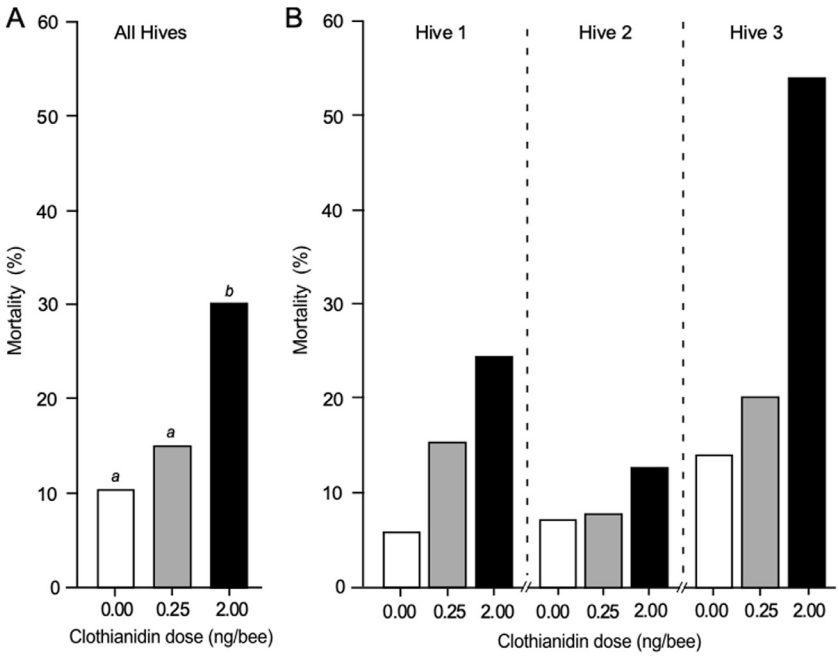


Fig. 4. Effects of clothianidin on mortality. The effects of 0.00 ng, 0.25 ng, and 2.00 ng/bee clothianidin exposure on bees from all three hives. **A.** Increasing doses of clothianidin led to significant increases in mortality. **B.** Variability in the effects of 0.00 ng, 0.25 ng, and 2.00 ng/bee clothianidin exposure on bees from three different hives. The exact percentage mortality is plotted. Binary mortality data were analysed in a generalized linear mixed model (GLMM) with binary logit link function. Mortality was greatest in Hive 3 and least in Hive 2. Different letters for both treatments and exposure correspond to significant differences ($\alpha = 0.05$).

on learning acquisition was reduced. With no clothianidin exposure (0.00 ng/bee treatment), all ELF EMF levels reduced the proportion of bees learning from 43 to 63% at 20 μT, 37–48% at 100 μT and 42–62% at 1000 μT, were significantly reduced from controls of 60–87% (Fisher's LSD: $P < 0.0001$) (Fig. 5A). With a clothianidin treatment of 0.25 ng/bee, however, only 100 μT and 1000 μT ELF EMF exposure reduced PER levels significantly (Fig. 5B) to 47-67% (Fisher's LSD: $P = 0.015$) and 48-67% (Fisher's LSD: $P = 0.001$) respectively from control levels (62–78% PER). In addition, after a 0.25 ng/bee treatment, learning levels were higher after 20 μT exposure by 60–74% compared with 100μT exposure (Fisher's LSD: $P = 0.018$) or 1000μT (Fisher's LSD: $P < 0.001$) exposure (Fig. 5B). With the highest clothianidin treatment (2.00 ng/bee) only EMFs of 1000 μT reduced learning acquisition significantly from controls (Fig. 5C), from 52–72% to 38–64% (Fisher's LSD: $P = 0.003$). After 2.00 ng/bee clothianidin treatment 1000 μT EMF exposure also significantly reduced learning levels by 55–77% learning levels after 20 μT EMF (Fisher's LSD: $P < 0.001$) and by 44–72% after 100 μT EMF (Fisher's LSD: $P = 0.011$) (Fig. 5C).

Other model effects that were tested regarding the 'trial number' interactions with 'EMF' or 'clothianidin' effects, were non-significant. There was no three-way interaction effect of *'clothianidin' x 'EMF' x 'trial'* (GLMM, $F_{18,2700} = 0.604$, $P = 0.90$), no two-way *'clothianidin' x 'trial'* (GLMM, $F_{6,2700} = 0.51$, $P = 0.80$) or *'EMF' x 'trial'* effect on learning (GLMM, $F_{9,2700} = 1.41$, $P = 0.18$).

Discussion

We have found that exposure to ELF EMFs alone reduced learning in an exposure-dependent manner. Similarly, exposure to clothianidin alone also led to a reduction in learning. When applied in combination, however, prior sublethal exposure to clothianidin reduced the effect of ELF EMFs on learning. Hives that were more susceptible to clothianidin in toxicity analyses also had higher mortality in PER assays. Clothianidin susceptibility may have led to some of the interactive effects of clothianidin and ELF EMFs on cognitive behaviour. Clothianidin and ELF EMFs alone each caused an increase in wingbeat frequency, however, clothianidin exposure reduced ELF EMF-induced changes in wingbeat frequency across all treatments.

Clothianidin and ELF EMF interactions with biological systems

The effects of sublethal levels of neonicotinoids on locomotion have been described in a variety of studies including locomotory deficits, but also increased locomotory activity (Lambin et al., 2001; Suchail et al., 2001; Williamson et al., 2014; Alkassab and Kirchner, 2018). The binding of neonicotinoids (including clothianidin) to nicotinic acetylcholine receptors (nAChRs), which have an essential role in fast excitatory synaptic transmission in the central nervous system, result in excitation, which may underlie the increase in locomotory activity and the increase in wingbeat frequency observed here (Matsuda et al., 2001; Jeschke and Nauen, 2008).

At low concentrations, the effects of neonicotinoids are excitatory, for example Tosi et al. (2017) found that a 1.37 ng/bee acute dose of thiamethoxam (another neonicotinoid of which clothianidin is a metabolite) had excitatory effects on honeybee flight. At higher levels neonicotinoids can lead to overstimulation, and death (Cabirol and Haase, 2019). As a result, it is possible that ELF EMFs, known to have excitatory effects, could have synergistic excitatory effects with neonicotinoids, as other stressors are known to have (Sgolastra et al., 2017). Our results showed, however, that there was no synergistic interactive effect of clothianidin and acute ELF EMF exposure on flight. Instead, where wingbeat frequency had increased from initial levels for all EMF exposures during the assay, the magnitude of this change was reduced following sublethal exposure to clothianidin. A possible underlying explanation for this effect is that for bees that survived clothianidin exposure during the flight assay, the effects of clothianidin at the neural level may have brought about the observed increased wingbeat frequency. As a result, bees treated with clothianidin in the flight assay had higher wingbeat frequencies before any exposure to EMFs and, consequentially, may have already been flying close to physiological limits under the experimental conditions before EMF effects on wingbeat frequency could be induced.

For cognitive behaviour the effects of neonicotinoids on neural systems may also provide a potential route for interactive effects of EMF and clothianidin. For example, clothianidin has been shown by Palmer et al. (2013) to cause a depolarization-block of firing in Kenyon cells, which have a critical function in honey bee learning and memory. This kind of neurobiological effect may underpin the reductions in learning caused by clothianidin found in this study, as well as nu-

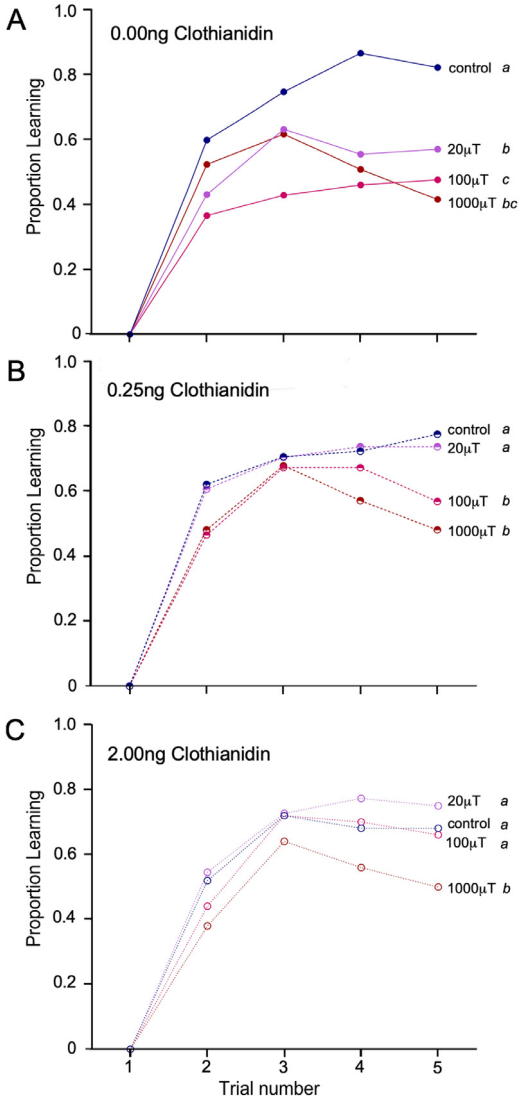


Fig. 5. Effects of clothianidin EMFs on PER. Proportions of bees showing PER following exposure to clothianidin and 50Hz EMFs. **A.** 0.00 ng/bee clothianidin exposure, **B.** 0.25 ng/bee clothianidin exposure, and **C.** 2.00 ng/bee clothianidin exposure. For all figures exact proportions are plotted. EMFs reduce learning in the PER assay. Prior clothianidin exposure reduced the effect of EMFs on learning. Different letters for both treatments and exposure correspond to significant differences ($\alpha = 0.05$).

merous other effects of neonicotinoids on learning and memory in bees (Decourtye et al., 2003, 2004a; Williamson and Wright, 2013; Stanley et al., 2015; Piiroinen and Goulson, 2016). There is also the possibility that through neural interactions clothianidin causes effects that may otherwise have been caused by ELF EMFs. The mode of action of clothianidin is through neuronal excitation in insects (Matsuda et al., 2001; Jeschke and Nauen, 2008), and ELF EMFs are known to have excitatory effects on cellular systems (Dimbylow, 1998; Jacobson et al., 2005). If reductions in learning and memory caused by ELF EMFs are related to their effects in cognitive neural systems, then pre-treatment of bees with clothianidin may reduce the ability of ELF EMFs to cause these same effects on cognitive behaviour.

There are a variety of other mechanisms through which ELF EMFs and clothianidin may interact. Due to the range of neural effects of neonicotinoids caused by nAChR binding (Matsuda et al., 2001; Jeschke and Nauen, 2008; Jeschke et al., 2010), changes in sensory responses by neonicotinoids are likely. Jin et al. (2015) found that clothianidin exposure of 0.76 ng/bee reduced sensory responses of solitary bees (*Osmia cornuta*) to a visual environment.

While there is much to learn regarding magnetosensation, bees are well known to show magnetosensory abilities (Bazalova et al., 2016; Gegear et al., 2010; Kirschvink et al., 1997, 2001; Liang et al., 2016), and if magnetosensory abilities have a critical role in the effects of acute ELF EMF exposure in honey bees, as found here, then clothianidin-related effects on sensory perception could cause the reduction in EMF effects with increased clothianidin exposure found here. This could be important as if neonicotinoids can affect magnetosensation they would be likely to affect geomagnetic orientation by honeybees, which alongside the effects on learning and flight observed here, could contribute to the well-documented impact of neonicotinoids on foraging/homing flights and orientation e.g. longer foraging flights (Schneider et al., 2012), reduced proportions of successful homing flights (Matsumoto, 2013), and impacts on components of navigation, including causing longer homing flights (Fischer et al., 2014).

Other interaction mechanisms between ELF EMFs and clothianidin may also occur; for example, clothianidin has been shown to modify transcription of a range of critical genes related to stress, health and immunity (Christen et al., 2016) including heat shock responses (Esther et al., 2015) which ELF EMFs have been shown to activate (Wyszkowska et al., 2016), however, ELF EMF induced heat shock responses occur after much longer chronic exposures than were tested here. It seems unlikely that with the acute ELF EMF exposures applied in this study that a molecular interaction in stress pathways could occur.

Mortality

The overall median lethal dose found here was 3.16 ng/bee which is concerning given the high levels of clothianidin encountered in the environment. EFSA (2013) calculated a worst case scenario through clothianidin residues in oilseed rape foraging of 4.3–13.7 ng per day. While some studies have been critical (Carreck and Ratnieks, 2014) that a dose applied all at once, as was done here, is dramatically different from one applied over a week or even over the course of day, under the 'worst case scenario' described by the EFSA (2013) honeybees here would have to detoxify 1.4–4.3 times the LD_{50} a day, or 7.8–24.5 times the dose required to kill 10% of bees. In experiments where bees were allowed to freely feed clothianidin treatments at 25 ppb, the same concentration as the low clothianidin treatment used here (0.25 ng in 10 μl), they consumed the equivalent of 2.99 ng/bee/24 h (Williamson et al., 2014) resulting in 45% mortality. There does not appear to be a major difference here in mortality of a 2.99 ng dose (45%) received over 24 h *versus* to what was found here regarding mortality of a 3.16 ng dose (50%) received at once. Acetylcholinesterase, which would normally break down agonists of nAChRs, cannot break down neonicotinoids, making their binding irreversible (Matsuda et al., 2001). As a result, detoxification would have to be very efficient for bees not to be affected by clothianidin exposure, given the effects found here. Even under the circumstances where bees can detoxify neonicotinoids to survive, this process is known to modify gene expression, activate multiple stress pathways, and require intensive energetic investment (Esther et al., 2015; Gong and Diao, 2017). The LD_{50}'s tested here were in 10 μl of sucrose, and therefore the overall LD_{50} for the assay (3.16 ng/bee) was equivalent to 366 ppb. With clothianidin consistently found above 10,000 ppb in guttation fluid of treated plants (Girolami et al., 2009; Tapparo et al., 2011), bees from this assay would only need to consume 0.316 μl of guttation fluid of clothianidin treated crops to receive the median lethal dose, and for extremes such as 717,000 ppb (EFSA, 2013) would require only 0.0044 μl to receive the median lethal dose.

There were variations in clothianidin toxicity between hives. The median lethal dose for the strongest hive at 4.22 ng/bee was 63% higher than that of the weakest hive, 2.59 ng/bee. This kind of variation in the field is common. For example, different 24 h oral LD_{50}'s for clothianidin have been reported at different doses including 2.18 ng/bee

(Matsumoto, 2013), 3.79 ng/ bee (EFSA, 2013) and 2.8–3.7 ng/bee (Laurino et al., 2011). It is well understood that neonicotinoid toxicity is dependent on a variety of factors including underlying health, for example from substandard protein feeding (Wehling et al., 2009), infection (Alaux et al., 2010; Vidau et al., 2011), and from other factors such as age (Suchail et al., 2001; Guez et al., 2003). It is likely that underlying factors within each hive had a part to play in the variation in clothianidin toxicity that was observed.

The underlying health of hives may have had some part to play in the interactions found between clothianidin exposures and the responses of bees to ELF EMFs, particularly if ELF EMFs are more likely to affect bees that are unhealthy, aged or stressed. For example, in olfactory conditioning when higher clothianidin treatments were used (inducing higher levels of mortality in the test population), the impacts of ELF EMFs on learning performance were reduced. In this case unhealthy, aged, or stressed bees that may have been more susceptible to ELF EMF exposure may have already been eliminated from the assay through clothianidin induced mortality. Underlying health causing varying clothianidin effects could also cause variations in sub-lethal interactions of stressors. For example, unhealthy, aged, or stressed bees that may have been susceptible to ELF EMF-induced effects of learning may have already been limited in terms of performance by clothianidin exposure, thus reducing the effects of EMFs on learning in comparison to bees not treated with clothianidin. These findings could further be expanded to the flight assay, where clothianidin increased the pre-treatment wingbeat frequency of all bees. Increased mortality of bees in this assay from clothianidin exposure may have left healthier faster flying bees in the assay.

Conclusions

Ultimately ELF EMFs have been shown to impact honey bee learning, memory and flight, even when bees are treated with clothianidin, although the magnitude of the EMF effect is reduced. Clothianidin on the other hand had dramatic effects on honey bee behaviour and health, which are likely to cause large scale ecological damage. Clothianidin susceptibility may have a part to play in terms of this compound's interactions with ELF EMFs. If the impacts of ELF EMFs on important cognitive and locomotory behaviours in pollinators translate to field scenarios, then where the effects of neonicotinoids are reduced, ELF EMFs may become a greater factor as an environmental stressor of pollinators.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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