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Impacts of chronic sublethal exposure to clothianidin on winter honeybees

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Abstract A wide application of systemic pesticides and detection of their residues in bee-collected pollen and nectar at sublethal concentrations led to the emergence of concerns about bees' chronic exposure and possible sublethal effects on insect pollinators. Therefore, special attention was given to reducing unintentional intoxications under field conditions. The sensitivity of winter bees throughout their long lifespan to residual exposure of pesticides is not well known, since most previous studies only looked at the effects on summer bees. Here, we performed various laboratory bioassays to assess the effects of clothianidin on the survival and behavior of winter bees. Oral lethal and sublethal doses were administered throughout 12-day. The obtained LD_{50} values at 48, 72, 96 h and 10 days were 26.9, 18.0, 15.1 and 9.5 ng/bee, respectively. Concentrations <20 µg/kg were found to be sublethal. Oral exposure to sublethal doses was carried out for 12-day and, the behavioral functions were tested on the respective 13th day. Although slight reductions in the responses at the concentrations 10 and 15 µg/kg were observed, all tested sublethal concentrations had showed non-significant effects on the sucrose responsiveness, habitation of the proboscis extension reflex and olfactory learning performance. Nevertheless, chronic exposure to 15 µg/kg affected the specificity of the early long-term memory (24 h). Since the tested concentrations were in the range of field-relevant concentrations, our results strongly suggest that related-effects on winter and summer bees' sensitivity should also be studied under realistic conditions.

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Introduction

In the past two decades, systemic insecticides, e.g. neonicotinoids, have substituted many of the traditional insecticides, e.g. organophosphorus and pyrethroids. As they can be applied to soil or directly to seeds at low concentrations and along with their systemic properties, they provide the plants with prolonged protection from the root and foliar pests. Nowadays, seeds of various crops such as canola and maize are treated primarily with systemic insecticides, e.g. neonicotinoids, intending such treatment to decrease the environmental contamination and exposure to pesticides in humans and non-target organisms, e.g. bees, compared with foliar applications.

However, their diffusion through the xylem in growing plants leads to contaminate nectar, pollen (Schmuck et al. 2001; Cutler and Scott-Dupree 2007; Krupke et al. 2012; Rundlöf et al. 2015), which are collected by honeybee foragers and transported to the hive. Moreover, some studies recently reported that residues of neonicotinoids were detected in many water resources such as surface water (Samson-Robert et al. 2014; Sánchez-Bayo and Hyne 2014) and guttation water (Girolami et al. 2009; Joachimsmeier et al. 2012), which could be considered as another potential oral exposure route for bees.

Therefore, the tradeoff between insecticides controlling the wide variety of agricultural pests without any threat to forager bees and/or the whole colony, which inadvertently come into contact with pesticides, is a vital issue.

The concerns about chronic exposure and possible sublethal effects of pesticides on foraging bees and their

consequence on the whole colony should be taken into account to reduce unintentional intoxications under field conditions (Desneux et al. 2007; Decourtye et al. 2013). Furthermore, the combination between chronic and sublethal effects of pesticides and other natural stressors, such as prevalent parasites and diseases, should be also considered during the risk assessment process (Sánchez-Bayo et al. 2016).

Neonicotinoids including imidacloprid, clothianidin and thiamethoxam are acetylcholine mimics and act as agonists of nicotinic acetylcholine receptors (nAChRs), which in turn persistently activate the cholinergic receptors, leading to hyper-excitation and death (Jeschke and Nauen 2008). In the honeybee brain, α-bungarotoxin-sensitive and -insensitive nAChRs have been described (Gauthier et al. 2006). These receptors play an important role in tactile and olfactory learning as well as memory formation, especially long-term memory formation in the honeybee (Cano Lozano et al. 1996, 2001; Dacher et al. 2005; Thany and Gauthier 2005; Gauthier et al. 2006) which are essential functions for foraging behavior and cognitive functions.

The scientific literature already has great deal of information about the effects of imidacloprid (e.g. Maus et al. 2003; Halm et al. 2006; Gill et al. 2012) and its side-effects especially on the learning and memory performance of honeybees (e.g. Decourtye et al. 2003, 2004; Ramirez-Romero et al. 2005; Han et al. 2010a; Williamson and Wright 2013). Nevertheless, chronic rather than acute exposure to pesticides is reported to have an effect on feeding activity of the *Apis* and non-*Apis* bees (Han et al. 2010b; Fauser-Misslin et al. 2014; Ceuppens et al. 2015).

On the other hand, only a few studies assessed clothianidin-related effects on honeybees. Although clothianidin and imidacloprid are members of the chloronicotinyl family, clothianidin is a different chemical entity and its mode of action on bees is not well known.

A previous laboratory study on acute oral and contact toxicity showed a higher toxicity of clothianidin than imidacloprid for different genotypes of honeybees, where the oral LD_{50} value at 48 h ranged from 1.1 to 6.3 ng/bee for clothianidin and from 28.8 to 242.5 ng/bee for imidacloprid (Laurino et al. 2013). The 10 day-chronic toxicity of clothianidin on the honey bee was determined to be more than 10 µg/L (EFSA 2013). Schneider et al. (2012) reported that clothianidin elicited detrimental sublethal effects on the homing performance after acute oral exposure at the dose of 0.5 ng/bee compared to 1.5 ng/bee for imidacloprid.

In field studies, clothianidin was detected in pollen from treated maize and in wild flowers growing near treated fields at levels of 3.9 and 9.4 µg/kg (Krupke et al. 2012). Cutler and Scott-Dupree (2007) reported that the maximum detected residue from clothianidin seed-treated canola were 3.0 and 3.7 µg/kg in pollen and nectar, respectively. In another large scale field study, the detected clothianidin residues in pollen of seed-treated canola (0.5–2 µg/kg) had no adverse effects on the honeybee colony's health, development and overwintering success (Cutler et al. 2014). Currently, assessments of clothianidin accumulation in soil and bee-relevant matrices showed no increase over time in fields receiving multiple applications of clothianidin. Relatively low residues in soil of 5.7–7.0 µg/kg, corn pollen 1.8 µg/kg and canola nectar 0.6 µg/kg were detected (Xu et al. 2015).

These concentrations were considered well below those reportedly required to elicit adverse effects. Nevertheless, the scoring of such chronic exposure of these highly toxic insecticides related to foraging behavior and learning and memory abilities requires a better understanding of the neonicotinoids' mode of action and their metabolites. However, these insecticides might have different interactions with different sub-types of nAChR in bees (Blacquière et al. 2012).

Although winter bees have longer survival capacities than summer bees, their sensitivity to exposure to lethal and sublethal doses of pesticides could be different. Most studies consider the effects on summer bees, whereas only few studies have looked at winter bees. Recently, Rondeau et al. (2014) reported a possible delayed and time-cumulative toxicity of imidacloprid in some arthropods using a toxicokinetic-toxicodynamic model. They suggested that prolonged exposure of winter bees throughout their lifespan (150 days) to contaminated honey with imidacloprid at 0.25 µg/kg would prove lethal to a large proportion of bees nearing the end of their life.

However, a previous study showed summer bees to be less sensitive to lethal but more sensitive to sublethal doses of imidacloprid than winter bees (Decourtye et al. 2003). Therefore, we conducted various laboratory bioassays to assess the effects of clothianidin on winter honeybees, testing acute and chronic oral exposures to different concentrations.

Materials and methods

Drug

Clothianidin was obtained in dry powder (99 % purity) from Bayer Crop Science, Germany. Due to the difficulty in dissolving the crystals in pure water, a stock solution was pre-dissolved in acetone to obtain a concentration of 200 mg/L. Then the stock solution was mixed with distilled water, thereby gaining a solution of 1 mg/L. The dilution series were done to obtain concentrations in a 2 M sucrose solution of 1, 5, 10, 15, 20, 50, 100 and 200 µg a.i./kg

syrup. The control group was fed a 2 M sucrose solution in addition to 0.1 % acetone in the acute and chronic experiment and 0.01 % acetone in the subsequent behavioral experiments. Fresh solutions were prepared weekly from frozen aliquots of the stock solution.

Bees

Winter workers of the honeybee *Apis mellifera* were collected from the same healthy colony maintained at the Ruhr University, Germany. The colony comprised about 9000 workers and a fertile 1-year-old queen. Bees were collected using vacuum and placed on ice using a plastic bag, then they were caged in groups of 20 individuals and maintained in darkness under controlled conditions (60 ± 2 % relative humidity, temperature of 29 ± 1 °C). The metal boxes (8.5 × 4.2 × 6.5 cm^3) had ventilation holes in the bottom, and two holes in the top to allow inserting feeding syringes. The sucrose solution and water were provided ad libitum with fresh solutions supplied daily.

Exposure protocols

Acute and chronic oral exposure

Preliminary experiments were carried out to determine sublethal doses. Three boxes (20 bees per box) per treatment were used for the oral exposure, where the sucrose solution contained either clothianidin (1, 10, 20, 50, 100 and 200 µg/kg) or only the solvent (control). Throughout the 12-day exposure period, the mortality was recorded daily in the control and treated groups. Then the effect of treatments on the survival time of bees was tested. On the basis of our results and the field-relevant concentrations, the sublethal concentrations (1–15 µg/kg) were used in the subsequent behavioral experiments (see Table 2). The cumulatively consumed amount of clothianidin per bee per treatment was estimated based on the consumed amount of sucrose syrup in the 12-day experimental protocol. The consumed amount per box was recorded daily, by weighing the feeding syringe before and after the bees had fed. Then, the average consumption per bee per day was calculated. All procedures, i.e. changing the feeding syringe and counting deed bees, were conducted under red light.

Sublethal oral exposure

Prior to the learning and memory experiments, five boxes (20 bees per box) per treatment were used to apply a 12-day oral exposure to clothianidin (1, 5, 10 and 15 µg/kg) by adding it to a 2 M sucrose solution and feeding it to winter workers ad libitum for 12 days. For an additional 4 days thereafter, bees of all treatments were fed 2 M uncontaminated sucrose solutions also after harnessing and training.

Tested honeybees were cold-anesthetized and individually fixed in plastic tubes using sticky tape on the backside of the thorax. The antennae and mouthparts were left free.

Experimental procedures

Sucrose responsiveness

To evaluate the sucrose responsiveness of the honeybees using the proboscis extension reflex (PER), individuals with equal motivation to sugar were selected. Two hours after being fed a 2 M sucrose solution, the honeybees' antennae were stimulated at 3-min intervals with sucrose solutions of ascending concentrations of 0.03, 0.1, 0.3, 1, 3, 10 and 30 % (w/v) (Aliouane et al. 2009). Over 3 days, the test was repeated daily until $N \geq 25$ tested bees were achieved for each treatment. The bees responding to sucrose stimulation by a proboscis extension were recorded as the percentage of the total tested bees per treatment.

Olfactory learning assays

To assess cognitive functions in winter workers of honeybees after exposure to clothianidin, the PER assays were used.

Harnessed honeybees were fasted for 2 h then trained for six trials with 3-min inter-trial intervals, where both the conditioned stimulus (CS) and the unconditioned stimulus (US) were paired. Bees responding to the CS in the first trial were excluded from the experiment. A sucrose solution (2 M) was used as US, and geraniol odor (98 %, Sigma-Aldrich, St. Louis, MO, USA) was used as CS+. Also, for (CS−) a different odor of peppermint extract was used (viva GmbH, Köln, Germany). Daily drops (4 µL) of each oil were diffused on a piece of filter paper and dispensed using a glass Pasteur pipettes. During each trial, a controlled solenoid valve was used to direct a 6 s pulse of airflow into the pipette which in turn was guided toward the bee's antennae. To avoid any possible mechanosensory stimulation occurring from a change in airflow, another glass Pasteur pipette delivered a constant flow of raw air from a gas cylinder to the bee's antennae after the 6 s CS pulse. This constant airflow was adjusted to 90–120 mL/min throughout each experiment. Both odor and non-odor pipettes were passed through a heated plate (35–37 °C). Twenty harnessed bees were equally distributed on a turntable. A small vacuum was positioned above the tested bee to remove the odor from the experiment's sphere. During training trials, after three seconds of the onset of the olfactory stimulus (CS+), the antennae were touched with

a drop of sugar water (2 M) using microcapillary tubes for 3 s to stimulate proboscis extension. Then, the bee was allowed to feed on a sucrose solution for 3 s.

A positive response of PER during the first 3 s of the CS pulse was recorded as a '1' and otherwise as a '0' event. Groups of approximately twelve bees per treatment were performed daily until $N \geq 35$ tested bees were achieved for each treatment.

The PER response rate was calculated as the percentage of honeybees responding. Mid-term (MTM) and early long-term (e-LTM) memory tests (see Hammer and Menzel 1995) were carried out by testing the responses of bees after 1 and 24 h to CS+/CS−.

Bees that did not respond to the sugar stimulation during the training phase were excluded from the experiment.

Habituation of the proboscis extension

Lambin et al. (2001) defined the habituation criterion as three consecutive sucrose stimulations without proboscis extension.

Bees fasted for 2 h were stimulated repeatedly with a 50 % sucrose solution applied to one antenna at 1-min intervals (see Fig. 1). Then, the sucrose solution was applied to the other antenna to set aside the probability of motor weariness. Individuals not responding to the 50 % sucrose solution and to the restoration test of the reflex were discarded. All experiments were performed at room temperature.

Statistical analysis

The Cox proportional hazard model (Cox 1972) was used to compare the hazard ratio between the control and treated groups, where treatments were entered as covariates. In this model, the hazard function represents the instantaneous death rate at time (t), given that the bees are still alive. It is based on the baseline hazard function (h_0) at the time (t) and the values of the treatments (d) as covariates. The assumption of the Cox model depends on the proportional hazards, implying that two bees with particular values for the time-independent covariates have a constant impact on the hazard over time. Thus, in the Cox model the individual hazard functions are relative to the hazard function under treatment (d) at the time (t) and are modeled as:

$$h_d(t) = h_0(t) \cdot \exp[\beta_d + \gamma_d X_d(t)]$$

where $h_0(t)$ is unspecified baseline hazard function of time; β_d is the regression coefficient representing the effect of the treatment; γ_d is the interaction between treatment (d) and death rate of the bees; $X_d(t)$ is time-dependent death rate of the bees under treatment (d).

Furthermore, a Probit analysis was performed to estimate the values of LD_{50} at different time points, i.e. 48, 72, 96 h and 10-day, depending on amount of clothianidin daily ingested per bee per treatment.

For the PER paradigm, the response of each bee to the stimulus during trials was scored as a binary response (0/1). Comparisons of responses at each trial between the control and treated groups were conducted with the Fisher's exact test. Mean values for the probability of response, and standard deviation of the means are presented for each treatment. The discrimination index (DI) was calculated for each bee (McCabe et al. 2007) to compare the specificity of olfactory learning and memory, where the DI of the responded bee is either +1 (response only to the CS+), 0 (responses to both odors) or −1 (response only to the CS−). Kruskal–Wallis test was used to examine the difference between the control and treatments.

Moreover, the differences of trials to habituation among treatments were tested using the One-way ANOVA and the Fisher least significant differences tests. All statistical analyses were performed using SPSS.22 software (SPSS, Chicago, IL, USA). The level of significance in all tests was set to $p \leq 0.05$.

Results

Determination of sub-lethal concentrations of clothianidin

The obtained acute LD_{50} values for 48, 72 and 96 h and after a 10-day chronic oral exposure show a high level of

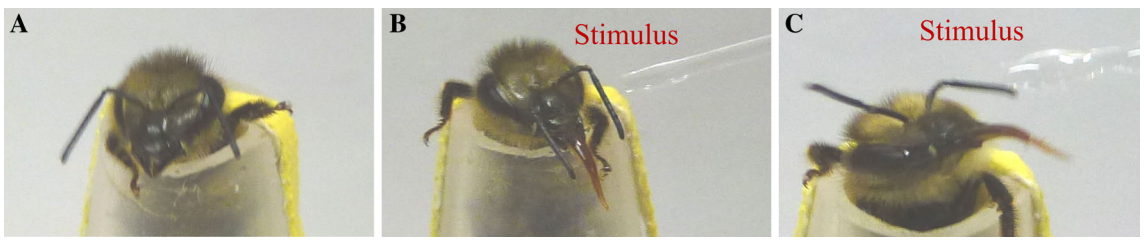


Fig. 1 Antennal posture through habituation process. **a** At rest period, the bee's antennae are moving randomly in all directions. **b** During the initial phase, stimulation of one antenna with a sugar solution leads to both antennae stretching forward. **c** After repetitive stimulation, only the stimulated antenna is curved at the back and toward the stimulation, whereas the other non-stimulated antenna is stretched in the other direction

toxicity of clothianidin to winter honeybees (Table 1). Moreover, our results demonstrate a decreasing trend in LD_{50} values with exposure time, where the cumulated dose ingested by the bee after 10 days is three time lower than the dose required to produce the same effect after the 48 h toxicity test. The daily ingested amount per bee is approximately 60 mg of contaminated sugar solution. Therefore, daily ingested doses of clothianidin range from 0.06 to 12 ng a.i./day for the range of applied concentrations.

Based on our results, concentrations lower than 20 µg/kg are found to be sublethal, as all treatments significantly affect the survival of winter bees throughout the 12 days experimental period except for 10 and 1 µg/kg (Fig. 2). Also, the p-values and hazard ratio for significant differences between treatments are shown in Table 2. Estimated hazard ratio values indicate that a living bee in a treatment group at a certain time point has an appraised probability of having died by the next time point compared to a bee in the control group.

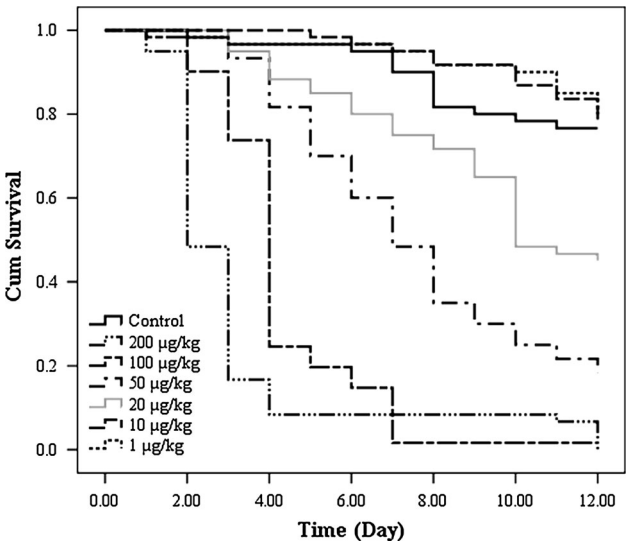


Fig. 2 Survival rate of caged bees after 12 days due to feeding a contaminated sugar solution with different concentrations of clothianidin

Sucrose responsiveness

At the orally applied concentrations, no significant effects are observed on response rates of winter bees to different concentrations of sucrose solutions (Fig. 3a–d). However, exposed bees to clothianidin show partial and non-significant reductions of sucrose responsiveness to 0.3 and 1 % at 15 µg/kg (Fisher's exact test, $p_{0.3\ \%} = 0.08$ and $p_{1\ \%} = 0.18$) and to 1 % at 10 µg/kg (Fisher's exact test, $p = 0.07$).

Olfactory learning and memory

Bees in treatments (10 and 15 µg/kg) exhibit a poorer non-significant probability of responding during the third and fourth trials (Fisher's exact test, $p_{T2} = 0.37$, $p_{T3} = 0.36$), then reach a lower asymptote on the fifth and sixth trials (Fig. 4a–d). A non-significant lower memory performance (lower response to CS+) at 1 and 24 h of the treated bees with 10 and 15 µg/kg compared to control is observed (Fig. 5a).

To compare the memory specificity of the treated bees during the tests for MTM and LTM, the discrimination index was calculated. The DI was greater than zero for all treatments which indicate that bees could distinguish between (CS+) and (CS−). Nevertheless, the treatments of 10 and 15 µg/kg slightly affected the specificity of memory at 1 h, but only the treatment of 15 µg/kg significantly reduced the specificity of e-LTM (Kruskal–Wallis test, $p = 0.04$; Fig. 5b), where the treated bees showed responses to both CS+ and CS− (Fig. 5c).

Table 1 Estimated lethal doses ($LD_{50} \pm SE$) values of clothianidin for *A. mellifera* (ng/bee)

Acute oral toxicity	Clothianidin ng/bee
LD_{50}	
48 h	26.9 ± 4.9
72 h	18.0 ± 4.4
96 h	15.1 ± 3.6
Chronic oral toxicity	9.5 ± 2.9
10-day	

Habituation of the proboscis extension

There is no significant effect of the treatments on the PER suppression compared to the control. However, we observed a slight decrease in the PER habituation of winter bees after long-term exposure to 15 µg/kg of clothianidin (one-way ANOVA, $p = 0.07$; Fig. 6).

Discussion

To our knowledge, no studies have been carried out on the toxicity of clothianidin to winter bees. In the present study, the clothianidin oral LD_{50} value after 48 h (26.9 ng/bee) obtained in winter bees *Apis mellifera carnica* were 3–6-folds higher than previous reported values (Laurino et al. 2013; EFSA 2013). However, Decourtye and Devillers (2010) reported that oral LD_{50} values of imidacloprid, as a

Table 2 Cox proportional hazard model fitted to the survival data after exposure to range of concentrations of clothianidin versus control

Treatments (µg/kg)	Regression coefficient (B)	Standard error $SE_{(B)}$	Hazard ratio (HR)	p-values*
200	3.16	0.30	23.62	<0.001
100	2.73	0.31	15.37	<0.001
50	1.73	0.31	5.63	<0.001
20	1.03	0.32	2.80	0.001
10	−0.15	0.39	0.86	0.70
1	−0.21	0.39	0.81	0.59

* Given p values is depending on the Wald's test for the significance of the estimated coefficient in Cox model

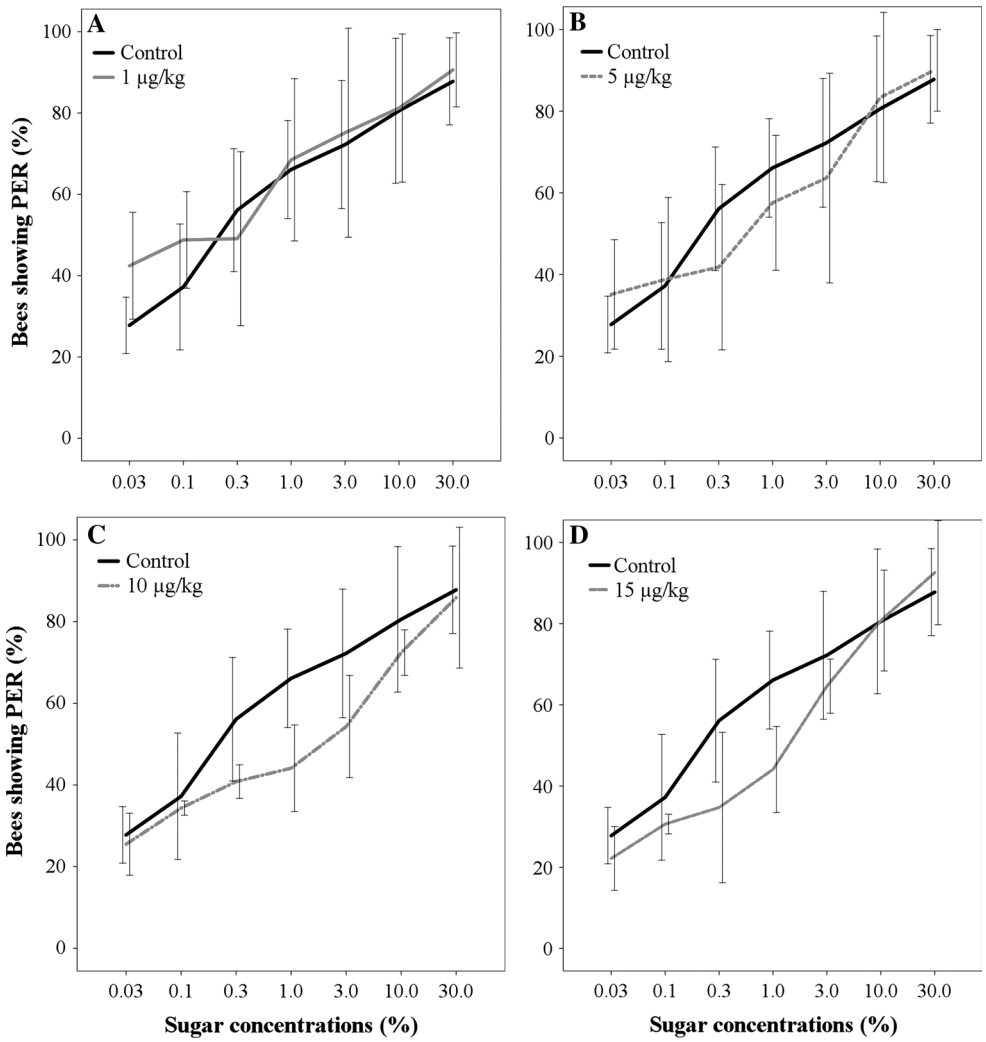


Fig. 3 a–d Sucrose responsiveness of winter honeybees after oral exposure to sublethal concentrations of clothianidin compared to control bees. The response of each treatment's bees to antennal stimulation with sucrose solutions of ascending concentrations is expressed as the percentage of the PER. The *bars* represent mean ± SD. Tested bees 32, 31, 26, 32 and 26 for control, 1, 5, 10 and 15 µg/kg, respectively

member of the neonicotinoids family, could vary widely in the honey bee by a factor of twenty. An explanation for such difference might lie in various factors such as the detoxification capacity of bees from different colonies, the age of tested bees, the subspecies and the exposure duration (Decourtye and Devillers 2010; Rondeau et al. 2014). On the other hand, we suggest that winter bees in the current study ingested clothianidin gradually throughout the 24 h and could metabolize part of the taken amount, thereby delaying the accumulation of pesticide to a lethal level (see Suchail et al. 2004). Thus, our data confirmed previous studies, which demonstrated a high acute oral toxicity of neonicotinoids including clothianidin to honeybees compared to other groups of insecticides.

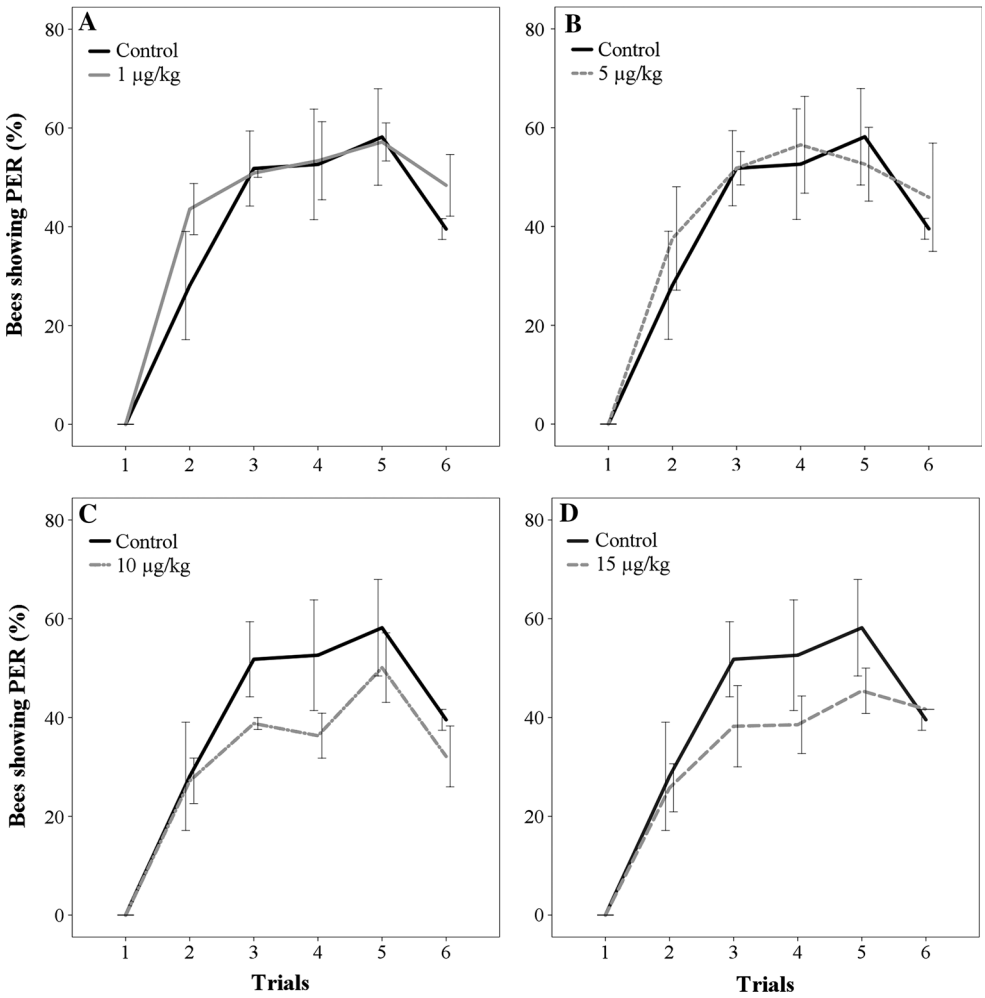
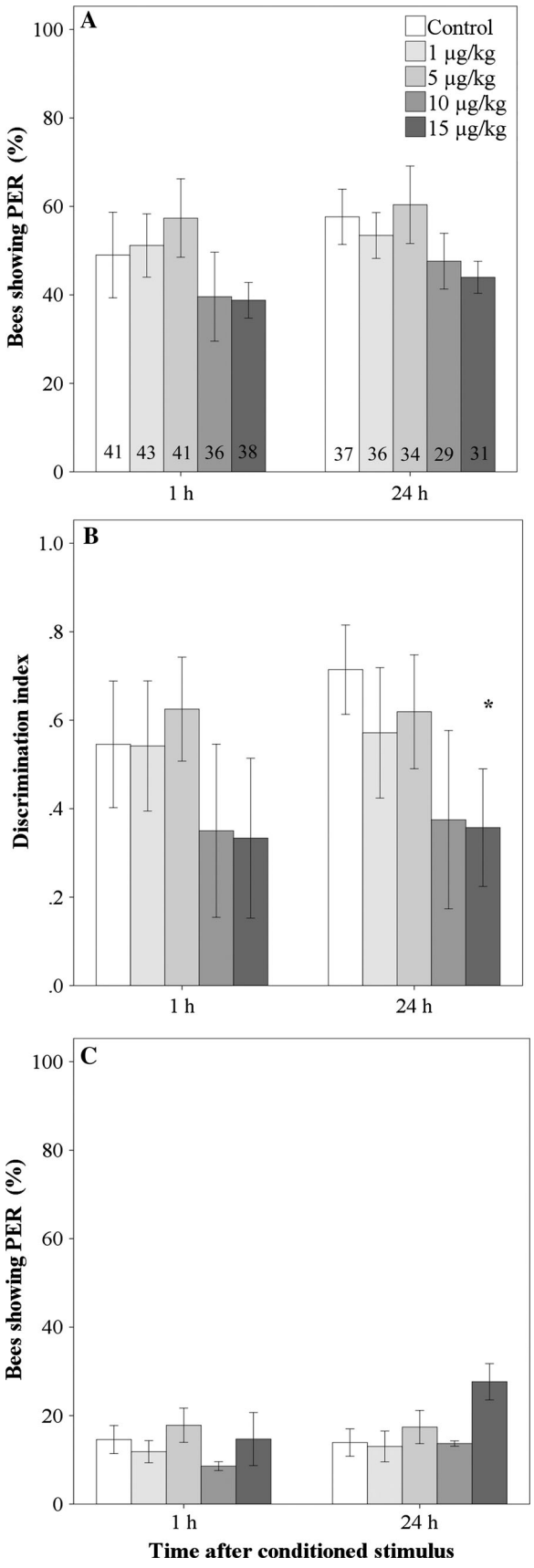


Fig. 4 **a–d** Proportion of the PER to the conditioned stimulus (CS+) for winter bees exposed to clothianidin compared to control bees. The *bars* represent mean ± SD. Tested bees 41, 43, 41, 36 and 38 for control, 1, 5, 10 and 15 µg/kg, respectively

In the case of systemic insecticides like clothianidin, the effects of chronic cumulative exposure are not excluded, due to their present at low concentrations in nectar and pollen collected from treated plants (e.g. Cutler and Scott-Dupree 2007; Krupke et al. 2012). In most toxicity studies, the amount of pesticides required to become lethal decreased with exposure time (Dechaume-Moncharmont et al. 2003; Suchail et al. 2001). Furthermore, Rondeau et al. (2014) reported a delayed lethal effect after prolonged exposure over the honeybees' lifespan, especially in the case of long-lived winter bees.

According to our laboratory chronic exposure tests, clothianidin at 9.5 ng/bee exhibited a lethal effect on caged winter bees. This cumulated dose ingested by the bee after 10 days was three times lower than the dose needed to produce the same effect after a 48 h. These results indicate the sensitivity of winter bees to chronic poisoning, due to the physiological differences between summer and winter bees such as high hemolymph vitellogenin titers, well-developed hypopharyngeal glands, low titers of juvenile hormone and high amounts of adipose tissues (Winston 1987; Seehuus et al. 2006; Behrends and Scheiner 2010), which could play an important role in their response to the pesticide exposure. However, Decourtye et al. (2003) found summer bees to be less sensitive to lethal doses of imidacloprid, where the mortality of winter bees after chronic oral exposure to imidacloprid increased significantly at 48 µg/kg compared to 96 µg/kg for summer bees. On the other hand, clothianidin is considered as a super agonist of nAChR, whereas imidacloprid exhibited partial-agonist action (Brown et al. 2006). Hence, differences between acute and chronic intoxication could also vary depending on the variations in the structure and efficacy of neonicotinoids.

The sublethal effects of clothianidin on studied behavioral functions including sensory and cognitive functions indicate limited effects on winter bees. Our results showed that daily repeated oral exposure to clothianidin at 10 and 15 µg/kg induced only slight reductions of sucrose responsiveness. Similarly applied doses (0.1, 0.5 and 1 ng/bee) of thiamethoxam (a neonicotinoid precursor for clothianidin) had no significant effect on the sucrose



◀**Fig. 5** **a** Memory performance as a proportion of the PER after 1 and 24 h to the conditioned stimulus (CS+) for winter bees exposed to clothianidin compared to control bees. **b** Discrimination index of bees exposed to different concentrations of clothianidin after 1 and 24 h. **c** Response proportion to both (CS+) and (CS−) of bees exposed to different concentrations of clothianidin after 1 and 24 h compared to control bees. The *bars* represent mean ± SE. The sample size of each group is given in each column

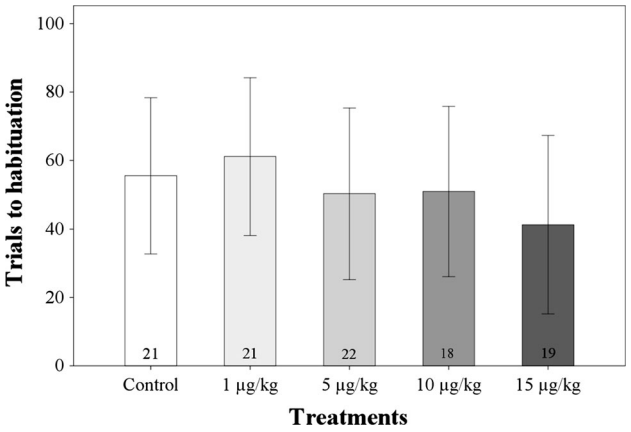


Fig. 6 Number of trials required to reach habituation in treated bees compared to control bees. The *bars* represent mean ± SD. The sample size of each group is given in each column

responsiveness in worker honeybees (El Hassani et al. 2008). Furthermore, a partial decrease of sucrose responsiveness was reported after repeated oral exposure with thiamethoxam at the dose of 1 ng/bee (Aliouane et al. 2009).

Winter bees chronically consume sublethal doses of clothianidin, but non-significant impacts on olfactory learning performance were observed in the current study. A previous study also showed that acute oral treatment of thiamethoxam at 1 ng/bee failed to cause any effect on honeybees' behavioral functions (El Hassani et al. 2008).

Otherwise, only the treatment of 15 µg/kg significantly affected the specificity of e-LTM but not MTM, where the treated bees showed responses to both CS+ and CS−. In light of these findings, treated bees showed a decrease in the ability to differentiate the olfactory stimuli during the test, suggesting possible effects on the consolidation phase at applied dose. Aliouane et al. (2009) showed that the different exposure routes had different subsequent effects. They did not observe a significant effect of thiamethoxam after chronic oral administration at 0.1 and 1 ng/bee on learning and memory performance. Otherwise, they reported that chronic topical application of thiamethoxam at 0.1 ng/bee induced adverse effects on memory performance 24 h after learning, whereas a significant impairment of learning performance with no effect on memory was observed at 1 ng/bee.

This effect indicates a high affinity of clothianidin to nicotinic alpha-bungarotoxin-sensitive nicotinic receptors which have been reported to be involved only in the formation of long-term memory (24 h) and not in medium-term memory (3 h) (Dacher et al. 2005; Gauthier et al. 2006). Our results are in agreement with another study suggesting differences in mechanisms of neonicotinoids toxicity (Brown et al. 2006).

Previous studies have demonstrated that imidacloprid modifies the habituation of the proboscis extension reflex by increasing the number of trials in bees younger than 7 days and reducing it in older bees (Guez et al. 2001, 2003). Another study reported facilitation of the PER-habituation after topical exposure to imidacloprid at 1.25 ng/bee (Lambin et al. 2001). We observed a slight and non-significant decrease in the PER-habituation of winter bees after long-term exposure to 15 µg/kg of clothianidin.

However, our observations showed that the associative and non-associative learning tasks of winter bees seem to be less sensitive after long-term exposure to a toxicant than those of summer bees. Decourtye et al. (2003) found that a significant learning impairment after 11 days of exposure to imidacloprid at 12 µg/kg in summer bees compared to 48 µg/kg in winter bee. Therefore, clothianidin-related effects on summer bees should be investigated.

In conclusion, our laboratory tests showed an increase in clothianidin toxicity with exposure time, which varied threefold from 48 h to 10 days (see Tennekes and Sánchez-Bayo 2013; Rondeau et al. 2014). Furthermore, limited sublethal effects on the tested behavioral functions of winter bees were observed, as only chronic exposure to clothianidin at 15 µg/kg affected the early long-term memory performance. Although many factors are different under field conditions compared to laboratory conditions, which affected the detoxification process in the honeybee, the laboratory results play an important role in assessing the exposure risk. Under field conditions and at the admitted application rate as seed dressing (max. 80 g/ha), the detected amounts of clothianidin in nectar and pollen from oilseeds plants were 3.0 and 3.7 µg/kg, respectively (Cutler and Scott-Dupree 2007). Also, the exposure to clothianidin at low concentrations (1–5 µg/kg), which could be considered similar to realistic residues found in nectar and/or pollen, had no recognizable effects under laboratory conditions on the behavior of winter bees. However, the interactions of different stress factors such as parasites, diseases and pesticides could increase the susceptibility of the bees (Sánchez-Bayo et al. 2016), especially during this essential and sensitive period of the honeybee life. Thus, we point out that the sublethal doses and their effects on the overwintering success of honeybees need to be investigated under realistic conditions, since prolonged exposure of winter bees throughout their lifespan (ca. 150 days) to contaminated honey is not excluded. On the other hand, possible different sensitivities of summer and winter bees to pesticides should be considered in further studies.

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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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