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Sublethal effects of clothianidin and *Nosema spp*. on the longevity and foraging activity of free flying honey bees

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Abstract
Neonicotinoids alone or in combination with pathogens are considered to be involved in the worldwide weakening of honey bees. We here present a new approach for testing sublethal and/or synergistic effects in free flying colonies. In our experiment individually marked honey bees were kept in free flying mini-hives and chronically exposed to sublethal doses of the neonicotinoid clothianidin. Additional groups of bees were challenged with *Nosema* infections or with combinations of the pesticide and pathogens. Longevity and flight activity of the differentially treated bees were monitored for a period of 18 days. In contrast to previous laboratory studies, no effect of the neonicotinoid treatment on mortality or flight activity could be observed. Although the lifespan of *Nosema* infected bees were significantly reduced compared to non-infected bees a combination of pesticide and pathogen did not reveal any synergistic effect. Our results indicate that individual bees are less impaired by neonicotinoids if kept within the social environment of the colony. The effect of such a "social buffering" should be considered in future risk assessments.

Keywords Neonicotinoid clothianidin · Honey bees · *Nosema* · Sublethal effects · Field realistic · Colony level

Introduction

The global use of neonicotinoid insecticides has been considered a crucial driver for the decline of insect biodiversity in many parts of the world (Gallai et al. 2009; Potts et al. 2010; Scholer and Krischik 2014; Stankus 2014). Neonicotinoids mainly act as specific agonists by binding to acetylcholine receptors (AChR) leading to depolarization and blocking of the synaptic transmission at the postsynaptic membrane of cholinergic synapses. Therefore, they are highly effective in disrupting central nervous system function by overstimulation (Matsuda et al. 2001). In particular bees as the most important pollinator of many agricultural crops (Cresswell 2011; Staveley et al. 2014) have a high risk to come into contact with these neonicotinoids. Due to the systemic property of the neonicotinoids they are often used for seed coating in order to protect the growing plant against herbivores (Elbert et al. 2008). This might result in trace residues of these compounds in pollen/ nectar (Pohorecka et al. 2012) or guttation fluid (Reetz et al. 2011) and therefore, beneficial insects might be exposed to sublethal concentrations. Seed coating is also the preferred application of those neonicotinoid compounds that exhibit an extraordinary high toxicity to bees like imidacloprid, thiametoxam and clothianidin (Iwasa et al. 2004). This high toxicity to bees has been demonstrated in spring 2008 at the Upper Rhine-Valley. Here, clothianidin treated corn was sowed with pneumatic drilling machines. The abrasion of the contaminated seed was released into the environment and deposited on surrounding blossoms of orchards and oilseed rape. As a result, 12,000 honey bee hives were heavily damaged (Würfel 2008).

Besides such obvious impacts through acute poisoning, bees might also come into contact with sublethal concentrations of these neonicotinoids. Traces of the active substances can be translocated into pollen and nectar of the flowering plants (Van der Sluijs et al. 2013) or into guttation drops (Girolami et al. 2009; Reetz et al. 2011). Bees might therefore be exposed over longer time periods to sublethal concentrations of neonicotinoids either by foraging in treated crops or later on by consumption of contaminated food storage within the nest which might lead to loss of individual bees (Lu et al. 2014). For individual bees

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it has been impressively shown that even such traces of certain neonicotinoids can impair life span (Girolami et al. 2009), memory and orientation (Schneider et al. 2012), foraging efficacy (Henry et al. 2012; Matsumoto 2013; Karahan et al. 2015), reproductive output (Dussaubat et al. 2016) and immune status (Di Prisco et al. 2013). Additionally, neonicotinoids are supposed to have synergistic effects in combination with honey bee pathogens like honey bee viruses and the intracellular gut parasite *Nosema spp*. (Doublet et al. 2015). Of particular interest in this context is *Nosema ceranae* which is originally a parasite of the Asian honey bee *Apis cerana* and has only recently become invasive in the new host *Apis mellifera* where it is obviously replacing *Nosema apis* in many parts of the world (Paxton 2010; Fries 2010). There are contradictory statements concerning the impact of *Nosema* infections on colony damages (Chen et al. 2008; Forsgren and Fries 2010; Gisder et al. 2010; Higes et al. 2013), however several reports confirmed synergistic interactions between *Nosema* infections and neonicotinoids (Alaux et al. 2010; Vidau et al. 2011; Pettis et al. 2012; Doublet et al. 2015).

Consequently, neonicotinoids have been frequently made responsible for periodically high losses of honey bee colonies in Europe and Northern America (Bryden et al. 2013; Lu et al. 2014). Although the absolute number of global honey bee colonies is not decreasing (Moritz and Erler 2016) the chronic exposure to sublethal concentrations of neonicotinoids together with synergistic interactions are considered a main factor for the weakening of honey bee colonies worldwide (Pettis et al. 2013; Goulson et al. 2015; Sánchez-Bayo et al. 2016).

However, most experiments that confirmed these results have been exclusively performed with individual bees in cage experiments under artificial conditions (Lundin et al. 2015). The few published field studies indicate that the damages of neonicotinoids to honey bees at the colony level are significantly lower than calculated and expected from the results on individual bees (Cutler and Scott-Dupree 2007; Pilling et al. 2013; Pohorecka et al. 2013; Cutler et al. 2014; Rundlöf et al. 2015). Due to this discrepancy between the individual and colony level more field studies with a chronic application of the pesticides have been required in order to establish a realistic risk assessment for honey bee colonies that forage in treated crops (EFSA 2012; Blacquière et al. 2012; Lundin et al. 2015).

General problems for field studies with full sized colonies are the standardization of the colonies and the measurement of weak pesticide effects within the colony. Honey bees can buffer against stressors such as reducing brood production or overcompensating for a particular task allocation. As a superorganism with division of labor and specialization they can afford to overcompensate in response to a particular stress, however only on a group level. Therefore, measuring brood and population dynamics to assess colony health may simply not have enough resolution to detect the harmful effects of stressors such as chronic exposition to.

We here present a novel approach to combine advantages of laboratory testing in terms of monitoring individual bees over their entire life span with field realistic conditions of free flying honey bee colonies, where treated bees are able to perform age dependent social tasks.

We used newly hatched and individually marked worker bees that were infected or non-infected with *Nosema* spores and put them into small colonies that were chronically fed with either a clothianidin contaminated syrup or a control syrup. With this comprehensive approach we could analyze both, sublethal and synergistic effects of a neonicotinoid and a pathogen on bees. As vitality parameter we used the longevity and the foraging behavior of individual bees. Such approaches are even more important since the ban of three neonicotinoids by the European Union (EFSA 2013). A final decision whether these pesticides will be available for the agricultural production in future should be taken on the basis of robust field data.

Materials and methods

Experimental hive setup

All hive experiments were performed in a styrofoam mating nuc system ("Kieler mating nuc", KMN) in July and August of the year 2013. Each KMN colony was equipped with four top bars and a strip of a beeswax foundation attached to it (Fig. 1). Every nuc was filled with approximately 800 bees originated from brood frames of two full sized colonies that have been treated against Varroosis and have been proven to be free of *Nosema* spores (Fries et al. 2013).

Fig. 1 Kieler Mating nuc (KMN), equipped with four top bars and stripes of wax foundation and a food container in the back. Outside measurements W 21.5 cm × L 26.0 cm × H 17.0 cm

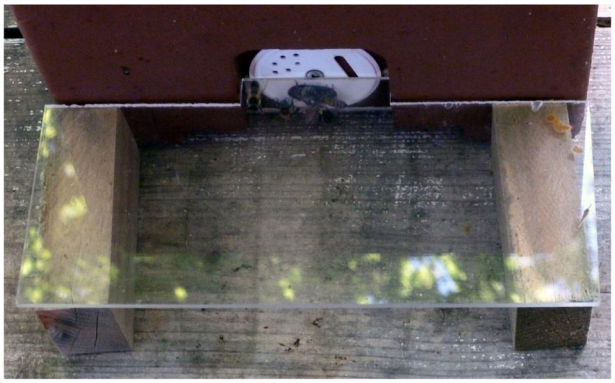

Fig. 2 Hive entrance with a lucent tunnel device for the observation of flight behavior

Subsequently, freshly hatched sister queens were introduced to the KMN´s. After one night in a dark and chilled room the KMN colonies were established at a protected apiary of the institute for mating. After a period of five weeks, 12 successfully mated KMN colonies with all stages of brood and freshly built wax combs were used for the following experiments.

In front of the hive entrance we installed a special tunnel of lucent plastic material. Thus, the bees had to walk a distance of about 10 cm to enter or leave the hive and marked foraging bees could therefore easily be recorded (Fig. 2).

Experimental field site and weather conditions

The KMN hives were set up at the Apicultural State Institute in Stuttgart-Hohenheim (48°42'31.8"N 9°12'38.2"E). Within the closer range of approximately 250 m, no other honey bee colonies were present. In the wider range (>250 m), other experimental hives as well as observation hives were placed. Main natural food source from local flora mainly was nectar and honeydew from *Tilia spp*.

The average temperature within the observation period was 22.5 °C with a precipitation of 101.6 L/m². Overall, good weather conditions prevailed to perform the experiment (DWD 2013).

Clothianidin treatment

As a metabolite of thiametoxam, clothianidin is a nitro-substituted neonicotinoid of high toxicity to honey bees (Iwasa et al. 2004). The oral LD_{50} was calculated to be 37 µg/kg (37 ppb) or 3.7 ng/bee, respectively with a NOEL of 20 µg/kg (20 ppb) (Würfel 2008).

For the application of clothianidin (Clo) we used the dry compound (99% purity, Dr. Ehrenstorfer GmbH), which was sonicated in pure water for a stock solution. The amount of stock solution was calculated for a final concentration of 15 µg/kg (or 15 ppb, which was considered to be below an acute toxic concentration; Alkassab and Kirchner 2016) and diluted in sucrose syrup (Apiinvert, Südzucker GmbH). The same amount of pure water without clothianidin was used for the control treatment.

Treatment groups

Ten of the 12 established KMN colonies were split randomly into two groups of five KMN each. One group received sugar syrup free of any pesticide (Table 1) while the other group was chronically fed with 1.12 kg sugar syrup/18 days/KMN containing clothianidin in a concentration of 15 µg/kg (see 2.3), corresponding to a total amount of 16.8 µg clothianidin/18 days/KMN (Table 1). The remaining two KMN colonies served as a reserve for potential queen loss. Therefore, bees of each treatment group were allocated to five mini-hives (= replicates).

The effects of clothianidin and/or *Nosema* infection were analyzed in individually marked bees. For this purpose, brood combs from two full sized donor colonies were put into an incubator for 24 h. Then the freshly hatched bees were mixed and prepared for the experiment. Six groups of 70 freshly hatched bees each were individually labelled with a colored and numbered opalith plate on their thorax. In addition to the individual label per bee we marked the abdomen with a hive specific color (Fig. 3) in order to determine drifting bees that enter "wrong" colonies. Three groups of differently treated bees were added to each KMN colony.

Infection with *Nosema apis* and *Nosema ceranae*

Before the introduction into the KMN the hatched and marked bees were put into a stainless steel cage and fed with sucrose solution (n = 35 bees per cage). We used three reversed caps of Eppendorf cups as feeding dish, which were put into each cage and filled with a total amount of 650 µL sucrose solution per cage, corresponding to 18.6 µL solution/bee. Depending on the treatment group, the sucrose solution contained spores of *N. apis*, *N. ceranae* or no spores as a control.

The *Nosema* spores were extracted from the midgut of artificially infected bees, which were previously reared in cages at our institute. Differentiation between *N. ceranae* and *N. apis* species were confirmed via qPCR (Fries et al. 2013). Only freshly extracted spore suspensions were used and purified twice via centrifugation and then diluted in sucrose syrup. The spore count of the solution was performed with a Thoma counting device to approximately 488,000 spores/650 µL per cage or, on average, 14,000 spores per bee. We waited until the bees consumed all of the food which usually was the case after 24 h.

Table 1 Setup and color codes of the six different experimental bee groups — 70 of each experimental group split across five mini-hives (KMN), each hosting initially 210 marked bees. Bees from five colonies formed one experimental group of 350 bees

	Treatment group	Colour code on the thorax	No. of KMN	No. of bees per group per KMN	No. of marked bees per KMN	No. of bees per group in total
Fed with sugar syrup only	***Control (C)***	yellow	5	70	210	350
	N.ceranae (N.cer)	green		70		350
	N.apis (N.apis)	red		70		350
Fed with *Clothianidin* sugar syrup solution	***Clothianidin (Clo)***	light yellow	5	70	210	350
	N.ceranae+Clothianidin (N.cer+Clo)	light green		70		350
	N.apis+Clothianidin (N.apis+Clo)	light red		70		350
Total amount			**10**			**2100**

Subsequently the bees were fed for another 24 h with pure sucrose solution (without spores) in order to provide enough time that the spores have passed the proventriculus which minimize the risk of cross infections between the different treatment groups.

Analysis of *Nosema* infection

After the observation period (see 2.6) ten bees per group and colony were inspected for *Nosema* infection, respectively. Single bees were crushed with 500 mL of pure water each in Bioreba extraction bags. Spores then were counted according to the "Standard methods for *Nosema* research using a light microscope and a Thoma counting chamber (Fries et al. 2013).

Mortality and flight activity

After the artificial *Nosema* infection all marked bees were introduced into the KMN colonies according to Table 1. The experiment started 24 h after the introduction for a period of 18 days. The observation included a daily mortality check, for which all combs including the inside of the hive were photographed for the later on counting of the marked bees on a computer screen. The pictures were taken outside the foraging activity, early in the morning (Fig. 4). The overall recovery rate is also shown in Table 2.

Fig. 3 Individually labelled honeybees with a group specific colored and numbered opalith plate on the thorax and a hive specific color on the upper side of the abdomen. An amount of 35 bees were put into a stainless steel cage (outside measurements: W 8.5 cm × L 4.5 cm × H 6.5 cm) for mass feeding with either spores of *N. apis*, *N. ceranae* or no spores at all for control

The flight activity of marked bees of all 10 colonies was analyzed by counting leaving and returning bees at the entrance over a period of 60 min per colony and day. Due to the weather conditions flight activity could be recorded at 10 days during the 18 day observation period.

Both, mortality and flight activity were analyzed using individual bees of the 6 treatment groups whereby each treatment group was distributed over five mini-hives.

Fig. 4 Picture of a brood comb from the KMN colonies for the daily mortality assessment

Residue analysis

Before start of the experiment, a sample of the feeding syrup mixed with clothianidin was collected. Pooled samples of pollen (bee bread) and stored food of the control and clothianidin colonies were collected at the end of the observation period (day 18) out of in-hive storage cells. These samples were analyzed using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive Solid Phase Extraction (SPE) - QuEChERS-method; German version EN 15662:2009 in certified labs (feeding syrup and food: eurofins Dr. Specht Labs Hamburg, LOQ 3 µg/kg; pollen: LUFA Speyer, LOQ 0.3 µg/kg).

Statistical analysis

We evaluated the mortality data with a Kaplan-Meier-Survival analysis. Survivorship between control and treatment(s) was compared pairwise and tested for significance with Log-Rank Tests (Cox-Mantel) followed by a Bonferroni correction. Workers which were collected at the end of the experiment were considered censored, equal to those observed but not collected on the last day of the experiment.

Flight activity data were checked with a Shapiro-Wilk test, refusing normal distribution ($p < 0.05$). Therefore, a Kruskal-Wallis-H-Test was performed on the six experimental groups for bees returning to the mini-hives. In case of significant differences, groups then were further tested pairwise using a Mann-Whitney-U-Test with Bonferroni correction ($p = 0.003$).

The different *Nosema* spore counts per group did also not fulfill normal distribution (Shapiro-Wilk test, $p < 0.05$). Therefore a Kruskal-Wallis-H-Test was performed and in case of significant differences, groups then were further tested pairwise using a Mann-Whitney-U-Test with Bonferroni correction ($p = 0.003$). All tests were performed with WinSTAT (R. Fitch Software, Bad Krozingen).

Results

Recovery rate of introduced bees

The recovery rate was calculated by the number of bees that could be rediscovered 24 h after the introduction of 70 particularly treated worker bees per mini-hive. The high recovery rates in all groups ranging from 90.3 to 96.3% (Table 2) indicate that the prior treatment (feeding of clothianidin and *Nosema* spores) did not have an acute negative impact.

Residue analysis

Samples of feeding syrup, pooled pollen (bee bread) and food from combs of the control and clothianidin colonies were collected at the end of the observation period (day 18) from in-hive storage cells. The intended clothianidin concentration in the feeding syrup could be verified by laboratory analysis. Additionally, we found measurable residues between ~2 and 6 µg/kg in stored food and pollen of the clothianidin treated KMN. We could also confirm that the untreated controls were free of clothianidin residues (Table 3).

Mortality of worker bees

The Kaplan-Meyer analysis of the differentially treated bees revealed highly significant differences between the six groups (Log-Rank $p < 0.001$) (Fig. 5). A pairwise post hoc analysis with Bonferroni correction of all treatments showed that only the two groups treated with *N. ceranae* ("*N. ceranae*" and "*N. ceranae* + Clo") had a significant higher mortality when compared to the control ($p < 0.003$) (Fig. 5). Neither the "*N. apis*" groups nor the clothianidin group had a significant higher mortality compared to the control. Within the untreated control group we analyzed colony-specific effects and did not find significant

Table 2 Recovery rates of all treatment groups. "Recovered bees" represent the number of all bees that were identified 24 h after the introduction into the respective mini-hive

	Introduced bees	Recovered bees[a]	Recovery rate [%]
Control	70	66.0 ± 3.5	94.3
N. ceranae	70	65.8 ± 2.3	94.0
N. apis	70	65.8 ± 2.6	94.0
Clothianidin	70	63.2 ± 3.5	90.3
N. ceranae + Clo	70	65.0 ± 4.8	92.9
N. apis + Clo	70	67.4 ± 1.9	96.3

[a]mean of all grouped bees in $n = 10$ KMN colonies at day one of the experiment

Table 3 Residue analysis of control and clothianidin treated feeding syrup prior to observation period

	Control	clothianidin
Stock solution	-	15 mg/kg
Feeding syrup	0 µg/kg	15 µg/kg
Stored food	0 µg/kg	6 µg/kg
Stored pollen	< 0.3 µg/kg	1.79 µg/kg

Pooled food and pollen from storage combs of all control and clothianidin treated KMN colonies after 18 days of observation (LC-MS/MS, LOQ: 3 µg/kg for food, 0.3 µg/kg for pollen)

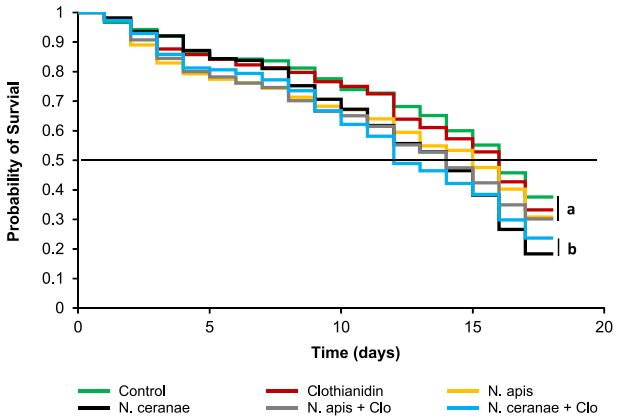

Fig. 5 All six groups were compared with a Kaplan-Meier-Survival analysis. A post-hoc Log-Rank test revealed highly significant differences between those groups (Log-Rank $p < 0.001$), therefore we tested groups pairwise. Different letters indicate statistically significantly higher mortality when compared to the control group ($p < 0.003$)

differences between the 5 mini-hives (Cox regression with pairwise comparison and Bonferroni correction). The results indicate that *N. ceranae* but not clothianidin represented the crucial factor for shortened life span.

Flight activity

Bees from the "*N. ceranae*" group revealed the highest, and bees from the "clothianidin" group the lowest flight activities (Fig. 6). However only slightly significant differences in the overall flight activity of the six treatment groups (=returning foragers) were found (Kruskal–Wallis-H-Test; $p = 0.04$), but no significant differences were confirmed with a pairwise comparison of the groups (Mann–Whitney-U-Test, $p > 0.003$).

Nosema spore counts and infection ratio

The average numbers of spores per bee from approximately $n = 50$ individuals per treatment group ranged from 925,500 (control) to 7,839,286 ("*N. ceranae* + Clo") after 18 days of incubation (Fig. 7). A Kruskal–Wallis H-Test

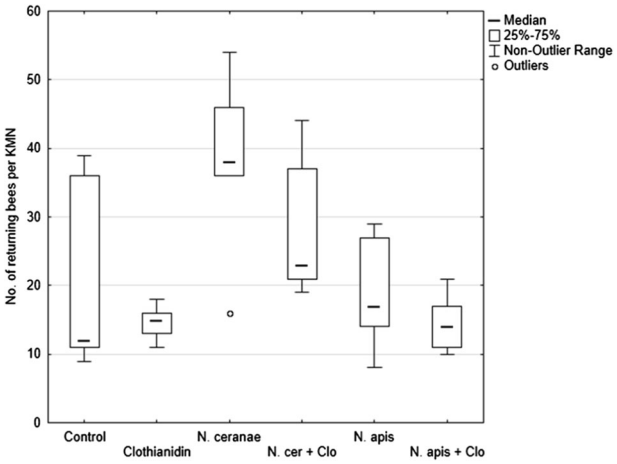

Fig. 6 Box-Whisker-Plot of incoming forager bees of all six treatment groups ($n = 5$ KMN) within the 18 days observation period. *N. ceranae* infected bees revealed the highest and the bees of the control group the lowest flight activities. However, no significant differences were found with a pairwise post-hoc comparison of all groups (Mann-Whitney-U-Test, $p > 0.003$, Bonferroni correction)

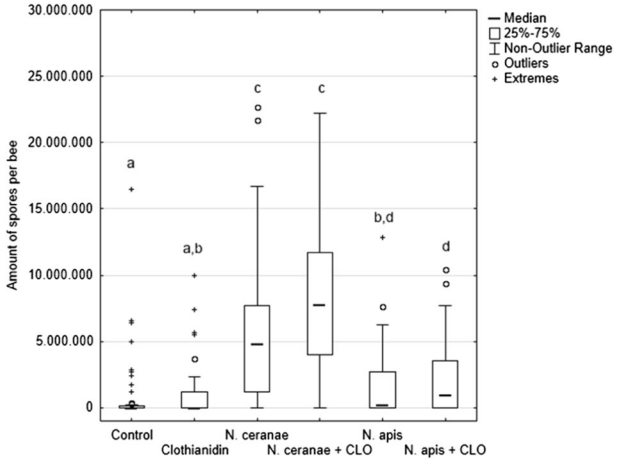

Fig. 7 Box-Whisker-Plot of the amount of spores per bee after 18 days of incubation. Columns with different letters indicate significant differences (Mann-Whitney-U-Test, post-hoc Bonferroni correction, $p < 0.003$)

revealed a highly significant difference between the six groups ($p < 0.003$). Bees from both *N. ceranae* groups had the highest amount of spores followed by the two *N. apis* groups. The originally uninfected control and clothianidin treated group also showed slight *Nosema* infections. All *Nosema* treated groups had significantly higher spore counts than the control group (U-Test, $p < 0.003$). No differences between clothianidin treated and non-treated groups could be observed, e.g. "clothianidin" vs. "control", "*N. ceranae* + Clo" vs. "*N. ceranae*" and "*N. apis* + Clo" vs. "*N. apis*" (Mann-Whitney-U-Test, $p > 0.003$).

A successful *Nosema spp.* infection of the respective groups could be validated with the ratio of infected bees (Fig. 8). All intentionally infected bees showed infection

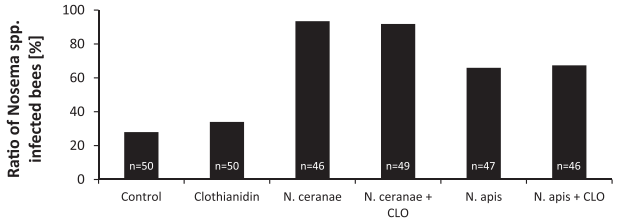

Fig. 8 Ratio of *Nosema spp*. infected bees per group after 18 days of incubation. Both groups originally not infected with *Nosema* spores (control, clothianidin) showed the least rate of infection. Both *N. ceranae* groups were above 90% and both *N. apis* groups above 66%

Table 4 All *Nosema spp*. infected bee samples were analyzed via qPCR for the ratio of both *Nosema* species

	Total bees (N)	Infected bees (N)	*N. apis* (%)	*N. ceranae* (%)
Control	50	11	0.0	100.0
Clothianidin	50	19	0.3	99.7
N. apis	46	27	46.2	53.8
N. apis + Clo	49	34	50.4	49.6
N. ceranae	47	42	0.0	100.0
N. ceranae + Clo	46	45	2.4	95.4

Bees from originally not infected groups were almost entirely infected with *N. ceranae*, so were both *N. ceranae* groups. The two *N. apis* groups showed an approximately 50/50 cross infection ratio with *N. ceranae*

rates from 66–93%, however 28–34% bees of the non-infected groups showed an infection too but with clearly lower numbers of spores per bee (Fig. 7).

All positive *Nosema* bee samples were analyzed with qPCR to differentiate from the species *N. apis* and *N. ceranae* to determine possible cross infections. Results are shown in Table 4. Bees from both *N. ceranae* groups had almost 0% cross infections, whereas bees from originally not infected groups were nearly entirely infected with *N. ceranae*. In contrast, both *N. apis* groups showed approximately 50/50 cross infection ratios with *N. ceranae*.

Discussion

With our new approach we could clearly show that the effects of a chronic exposure of sublethal concentrations of neonicotinoids on honey bees strongly depend on the experimental setup. Obviously, the way of application of the pesticide and the way how the bees are kept during the experiment has a huge impact on the toxicity of the pesticide at the colony level. In many studies, side effects of certain neonicotinoids on individual bees have been described when sublethal concentrations and/or dosages were applied. Among others, learning, memory, orientation and foraging behavior were negatively affected in individual worker bees (Henry et al. 2012; Van der Sluijs et al. 2013; Scholer and Krischik 2014; Fischer et al. 2014; Charreton et al. 2015; Karahan et al. 2015; Tosi et al. 2017) and moreover, the reproductive capacity of queens and drones was significantly reduced (Williams et al. 2015; Kairo et al. 2016; Chaimanee et al. 2016). Furthermore, synergistic effects in combination with honey bee diseases, mainly with viruses (Di Prisco et al. 2013) and *Nosema spp*. infections have been demonstrated (Vidau et al. 2011; Aufauvre et al. 2012; Pettis et al. 2012, 2013; Doublet et al. 2015). However, most of these experiments were performed with single bees that were kept and treated under laboratory conditions, often in cage tests. This was already criticized in a meta-analysis reviewing 268 primary research studies on neonicotionids and bees (Lundin et al. 2015) leading to the demand for more studies that measure effects on the colony level. In contrast to the large number of cage tests the few studies that measured effects on honey bee colony performance in the field could not confirm clear negative effects of neonicotinoids (Blacquière et al. 2012; Pilling et al. 2013; Rundlöf et al. 2015; Henry et al. 2015). A recent large study of Woodcock et al. (2017) in three European countries revealed negative effects on both, wild and managed bees but the effects were not consistent across countries. Another recent study confirms clear negative effects of neonicotinoids on the colony level (Tsvetkov et al. 2017), however after exposure of honey bee colonies to a large cocktail of more than 25 pesticides over a period of several months. To better understand the discrepancy among the various studies we here present an approach that combines the advantage of laboratory tests – i.e. the defined application of certain compound(s) and analysis of individual bees - with an experimental design where the bees could perform their natural task within the social environment of a bee colony.

For the sublethal treatment we tried to simulate a field realistic worst case exposure (Pecenka and Lundgren 2015; Rundlöf et al. 2015; Tosi et al. 2017) while staying at the same time below the NOEL of 20 µg/kg clothianidin (Alkassab and Kirchner 2016; Würfel 2008). Therefore, we used sugar syrup spiked with clothianidin to a final concentration of 15 µg/kg for the chronic feeding of the test colonies. After each test colony received an amount of more than one kg of this contaminated syrup over a period of 18 days, the analysis of a pooled sample of stored food from all treated colonies confirmed a concentration of 6 µg/kg clothianidin suggesting an approximately 1:1 dilution of the fed syrup with the nectar collected by foraging. This dilution effect may explain why detrimental effects are rather absent in a full colony set-up when compared to lab-testing and may play a crucial role for the "buffering capacity" of a honey bee colony. The control colonies were free of

clothianidin residues. Due to our mass feeding approach we cannot exactly determine the pesticide consumption of each individually marked bee. However, since all bees had to use either the syrup or the stored food we can safely assume a chronic intoxication with clothianidin ranging from 6 µg/kg (food) to 15 µg/kg (syrup) over the experimental period of 18 days.

Effect of clothianidin on mortality and flight activity

The median life-span of the untreated control bees was somewhat lower than reported from large free flying colonies but laid in the range of other tests with small experimental units (Retschnig et al. 2015). A chronic feeding with clothianidin, however, did not have any effect on the life span of the bees within the treated colonies. This is in contradiction with experiments on the homing ability of foraging bees that have been treated with clothianidin or thiametoxam (Henry et al. 2012; Tosi et al. 2017). Though, in both studies the concentration of the applied pesticide was two to four times higher than in our experiment which does not correspond to field realistic conditions (Cresswell and Thompson 2012; Guez 2013) and is clearly higher than the concentrations recently measured in the nectar from clothianidin treated fields in Europe (Rundlöf et al. 2015; Henry et al. 2015; Rosenkranz et al. 2013). Furthermore, the bees in the studies of Henry et al. (2012) and Tosi et al. (2017) were fed in the laboratory over a period of several days prior to the homing experiments which might be an additional stress factor. A similar discrepancy between semi-artificial homing experiments and a long-term field study have been recently confirmed for thiacloprid, another commonly used neonicotinoid. While artificially treated bees revealed a clear reduced capacity in navigation and homing behavior (Fischer at al. 2014), a chronic exposure to high concentrations of thiacloprid over three years did not adversely affect the tested honey bee colonies (Siede et al. 2017). Several studies support our finding that sublethal and field realistic concentrations of neonicotinoids does not increase the bee mortality in free flying colonies (Schmuck et al. 2001; Faucon et al. 2005; Cutler and Scott-Dupree 2007; Pilling et al. 2013; Rundlöf et al. 2015; Woodcock et al. 2017).

It is noticeable that the low mortality of the clothianidin treated bees in our experiment was not a consequence of a reduced flight activity. There were no significant differences between bees from the control group compared to bees from the different treatment groups. This is in accordance with a recent field study (Henry et al. 2015) but again in disagreement with a former study of the same author (Henry et al. 2012). It is further noticeable that both groups infected with *N. ceranae* revealed the highest flight activity which is confirmed by the findings of Dussaubat et al. (2013).

There are several reasons why sublethal concentrations of neonicotinoids might act differently in cage tests, semi-artificial approaches or field tests with entire colonies. Obviously this is not only the consequence of the "buffering capacity" of a honey bee colony as a huge eusocial "superorganism" that is able to quickly compensate for the loss of a certain number of impaired individuals (Henry et al. 2015). Honey bees at the colony level seem to be less impaired and diversely affected than individual bees held under artificial conditions (Straub et al. 2015). This quality however, appears to be reserved to highly eusocial insects only (Ellis et al. 2017). Our results rather indicate that it even makes a difference whether individual bees are exposed to contaminated food within their social environment or whether they are isolated from their social entity for the application of the pesticide. So far it is unknown how social interaction on the colony level could alter the toxic effects for individuals. According to Sponsler and Johnson (2017), individual- and colony-level effects are linked in a complex and hardly understood way. In addition, the authors make very clear that even studies on the toxicity of pesticides on the colony level require individual-oriented approaches. Our experimental setup fulfills these requirements by applying a defined amount of pesticide and by analyzing individual bees within their social environment.

Nosema infection

The artificial infections with *N. ceranae* were highly successful which is confirmed by the average number of spores per bee ranging from 5.8 to 7.8 million spores for the "*N. ceranae*" and the "*N. ceranae* + Clo" group, respectively. These infection rates match the results of natural infected bees of similar age (Smart and Sheppard 2012). In contrast, the artificial infection with *N. apis* spores was less successful leading only to infection rates ranging from 1.8 to 2.1 million spores per bee for the "*N. apis*" and the "*N. apis* + Clo" group, respectively. Because we used the same amount of fresh spore material for both *Nosema* species, these differences indicate a slower growth of the *N. apis* infection (Natsopoulou et al. 2015). This is in accordance with studies showing a better growth of *N. ceranae* under higher temperature conditions (Martín-Hernández et al. 2009; Gisder et al. 2010) and consequently *N. ceranae* is meanwhile the predominant *Nosema* species in Southern Germany (Rosenkranz et al. 2013).

Because infected and non-infected bees were kept within the same colony, some cross infection was inevitable. However, due to the low spore load of the non-infected groups a pathogenic effect seems unlikely.

The bees infected with *N. ceranae* showed a significantly reduced lifespan. Also the bees infected with *N. apis* showed a similar but not significant tendency. However,

due to the above mentioned lower infection rates the interpretation of pathogenic effects in the *N. apis* groups must be taken with care. This is in agreement with many studies confirming a shorter lifespan in *Nosema* infected bees, primarily caused by an earlier start of foraging (reviewed in Higes et al. 2013). Accordingly, also in our experiments the two *Nosema* infected experimental groups revealed the highest flight activities.

Although *N. ceranae* had a clear negative impact on the infected bees we could not prove any synergistic or additive effects when *Nosema* infected bees were additionally exposed to chronic clothianidin feeding. This clothianidin feeding did neither shorten the lifespan nor change the flight activity compared to *Nosema* infected bees that received untreated syrup.

At least in terms of an increased mortality we clearly contradict the results of Alaux et al. (2010) and Vidau et al. (2011), who both showed synergistic effects with *N. ceranae* and a neonicotinoid pesticide. However, these studies were conducted in cage experiments under laboratory conditions where bees probably react more sensitive to *Nosema* infections. In addition, *Nosema* strains may vary in infectivity and virulence (Genersch 2010) and a number of experiments provide evidence that related to the genetic background of the honey bee host, the level of tolerance and resistance to *N. ceranae* can produce a different outcome (Dussaubat et al. 2013; Fontbonne et al. 2013; Huang et al. 2013; Huang et al. 2014). Similar findings of field studies performed in observation hives or full sized colonies assessing synergistic effects between neonicotinoids (thiacloprid, clothinaidin) and *N. ceranae* support and augment our conclusion (Goss 2014; Retschnig et al. 2015; Rolke et al. 2016). Yet, the results of this experiment cannot certainly exclude synergistic effects between neonicotinoids and parasites of other degrees. Further studies should therefore include a positive control and comprise different concentrations of pesticides and other pathogens like *Varroa* mites or bee viruses (Fries et al. 2011). For such applications, our test system represents a suitable approach.

Conclusion

Our study strongly indicates that in free flying honey bee colonies the effects of sublethal concentrations of neonicotinoids – alone or in combination with a pathogen – on bee mortality are substantial lower compared to *in vitro* experiments with caged bees. According to our results this "buffering effect" is not a simple replacement of dead worker bees by the huge amount of brood in a full sized colony but rather a lower susceptibility of the individual bee when the pesticide is applied within the well-balanced social community. The physiological mechanisms responsible for this lower susceptibility still need to be clarified.

We could also show that the KMN mini-hives used in our study are suitable for testing effects of pesticide and pathogens on the colony level. As we did not detect synergistic effects in the present approach, further studies have to prove how synergistic interactions are measurable under these colony conditions. The "colony" is the crucial endpoint for a final risk assessment, however typical colony-level performance parameters like population dynamics, honey yields and overwintering rates depend strongly on environmental factors and are difficult to record (Sponsler and Johnson 2017). As colony level effects are finally the result of the intoxication of individual bees, our approach offers the possibility to measure the impact of pesticide treatments on individual bees in consideration of the complex effects of "social buffering".

Our results cannot finally answer the question whether certain neonicotinoids should be excluded from the agricultural practice. The great number of studies dealing with the impact of neonicotinoids on honey bees came to varying results and therefore different recommendations concerning the future use of these pesticides. For regulatory authorities and political decision-makers a scientific-based risk assessment is therefore extremely difficult. A better regulation and standardization of the methods that are used for the study of neonicotinoids and honey bees would be an important first step.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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