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Short-term lab assessments and microcolonies are insufficient for the risk assessment of insecticides for bees

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HIGHLIGHTS

- Risk assessment methods were compared for the effect of neonicotinoids on bumblebees.
- Microcolony results contradicted results of gyne-producing queenright colonies.
- Thiacloprid negatively impacted colonies in the field and the laboratory.
- Acetamiprid only showed a trend for reduced number of reproductives in the field.
- We emphasize the need for field experiments and assessment of gyne production.

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ABSTRACT

Risk assessment studies addressing effects of agrochemicals on bumblebees frequently use microcolonies. These are queenless colonies consisting of workers only in which typically one worker will lay unfertilized male-destined eggs. In the first tier of risk assessment for bees, short-term laboratory experiments (e.g. microcolonies) are used, the results of which will determine whether higher tier (semi-) field experiments are needed. To evaluate the suitability of microcolonies for risk assessment, a direct comparison between different assessment methods for the neonicotinoid pesticides acetamiprid and thiacloprid was made: microcolonies and queenright colonies under short-term laboratory conditions, queenright colonies under long-term laboratory conditions, and queenright colonies under field conditions. Here, we demonstrate that results from microcolonies contradict results from queenright colonies. While thiacloprid negatively impacted gyne production in queenright colonies, it had a positive effect on microcolony size. By contrast, thiacloprid had no significant effect on fitness parameters of queenright colonies under short-term laboratory conditions when mostly workers are produced. These results thus highlight both the need for long term assessments, allowing evaluation of gyne production, and the risk of reaching erroneous conclusions when using microcolonies. The negative effect of thiacloprid on colony fitness was confirmed under field conditions, where thiacloprid affected the production of reproductives, colony weight gain, worker weight, and foraging behaviour. For acetamiprid, a negative trend on colony fitness could only be shown in a field setup. Therefore, field-realistic setups, which allow colonies to forage freely, are most appropriate to assess sublethal effects of pesticides affecting behaviour and learning.

1. Introduction

Wild and domesticated bees provide pollination services to crops and wild plants, and their importance for human food security is invaluable (IPBES, 2019). Over the past decades, there has

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been mounting evidence of global pollinator declines (e.g. Biesmeijer et al., 2006; Potts et al., 2010; Goulson et al., 2015; Jacobson et al., 2018). There are multiple potential factors driving these declines, such as habitat loss and fragmentation, the use of agrochemicals, climate change, pathogens and invasive alien species. Neonicotinoids have been identified as a major threat to bees due to their toxicity, frequency and concentration in which they are detected in honey bee hives (Sanchez-Bayo and Goka, 2016) and their persistence in the environment (Bonmatin et al., 2015). Recently, multiple studies have demonstrated the role of neonicotinoids imidacloprid, thiamethoxam, and clothianidin in current bee declines (e.g. Rundlöf et al., 2015; Williams et al., 2015; Woodcock et al., 2016; Sanchez-Bayo and Goka, 2016; Tsvetkov et al., 2017).

Neonicotinoids are a group of systemic insecticides, which means that they are translocated throughout the entire plant via the phloem, ending up in the flowers, pollen, and nectar (Bonmatin et al., 2015) where bees and other non-target pollinating insects can be exposed to them (Sanchez-Bayo and Goka, 2016). In a comparative study of the acute toxicity of different topically applied neonicotinoids in honey bees, neonicotinoids were ranked according to their toxicity; imidacloprid was placed highest with an LD_{50} of 18 ng/bee and acetamiprid and thiacloprid were placed lowest with an LD_{50} of 7000 ng/bee and 15,000 ng/bee, respectively (Iwasa et al., 2004). The lower toxicity of *N*-cyanoamidines, such as thiacloprid and acetamiprid (Jeschke et al., 2011), compared to nitroguanidines, such as imidacloprid, thiamethoxam, and clothianidin (Jeschke et al., 2011), is explained by differences in the detoxification process in bees (Suchail et al., 2004; Brunet et al., 2005) because bees produce enzymes that can metabolise *N*-cyanoamidines but cannot effectively metabolise nitroguanidines (Feyereisen, 2018). Chronic exposure to field-realistic levels of neonicotinoids has been shown to affect learning and memory in bees for both groups of neonicotinoids (Decourtye et al., 2004; Williamson and Wright, 2013; Stanley et al., 2015; Tison et al., 2016). This can reduce their fitness through different pathways related to food collection, such as a reduced ability to navigate through complex environments (Stanley et al., 2015).

Pesticide risk assessment is complex and even more complex in social insect species, such as honey bees and bumblebees, because the end point is not the survival or reproduction of a single individual but of the colony as a whole (Blacquière et al., 2012). In 2013, the European Food Safety Authority (EFSA) published a guidance document with a tiered risk assessment scheme for both solitary and social bees (EFSA European Food Safety Authority, 2013) with a first tier assessment to evaluate toxicity levels by acute and chronic exposure of individuals and a higher tier assessment (semi-field or field) that needs to be carried out when risks are unacceptable in the first tier assessment. However, there is still little consensus on how to best evaluate toxicity on a colony-level. Studies are often difficult to compare because of the use of different chronic exposure periods, ranging from a couple of days to several weeks; exposure during different colony phases, ranging from colony foundation to the production of reproductives; the use of different parameters to evaluate effects on colony fitness, etc. Examples of direct colony fitness parameters or proxies for fitness include learning and memory (e.g. Decourtye et al., 2004; Stanley et al., 2015), foraging behaviour (e.g. Mommaerts et al., 2010; Gill and Raine, 2014), hygienic behaviour (e.g. Tsvetkov et al., 2017), colony weight (e.g. Whitehorn et al., 2012; Stanley and Raine, 2017), queenlessness (e.g. Tsvetkov et al., 2017), immune function parameters (Czerwinski and Sadd, 2017), queen weight or size (e.g. Wu-Smart and Spivak, 2018), queen reproductive physiology (e.g. Williams et al., 2015), queen colony founding success (e.g. Baron et al., 2017; Wu-Smart and Spivak, 2018), production of adults (workers, males, and queens) (e.g. Whitehorn et al., 2012; Stanley and Raine, 2017), production of reproductives (males and queens) (e.g. Siviter et al., 2018), egg laying rate (e.g. Siviter et al., 2020), and (stored) sperm quality (e.g. Williams et al., 2015; Straub et al., 2016). Furthermore, different model species are used (mostly the honey bee *Apis mellifera* and the bumblebee *Bombus terrestris*) while results from one species are usually not directly extrapolatable to others, e.g. due to differences in life cycle and physiology (Estoup et al., 1995; Stoner, 2016).

For bumblebees, microcolonies – which are colonies consisting of only workers where one or several workers will start egg-laying to produce males – are considered to be reliable models for trends in queenright colonies and are often used in studies evaluating dietary or pesticide effects (Tasei and Aupinel, 2008; Klingel et al., 2019). They are frequently used because of their cost-effectiveness, allowing larger numbers of replicates (Klingel et al., 2019), and their easy standardisation (Cabrera et al., 2016). The EFSA recommends the use of microcolonies for first tier risk assessment of agrochemicals (EFSA European Food Safety Authority, 2013). A recent review by Klinger et al. (2019) concluded that "microcolonies are a useful tool for studies of bumblebee biology and for assessing the effects of stressors on these bees" but they also pointed out that care must be taken to standardise protocols. Indeed, the numerous studies using microcolonies for risk assessment of pesticides show a remarkable variation in their experimental design, which underlines the need for standardisation in microcolony methodology. Variation is found in many methodological aspects, such as the number of workers used per microcolony, varying from three (e.g. Elston et al., 2013) to five (e.g. Besard et al., 2010; Barbosa et al., 2015), as well as the age of the workers and their relatedness (e.g. non-sister callow workers in Besard et al. (2010) and adult sister workers in Dance et al. (2017)), the timing of the end point (from 14 days in Laycock et al. (2012) to 11 weeks in Besard et al. (2010) and Barbosa et al. (2015)), and the evaluated parameters. The assumption that microcolonies are adequate indicators of trends in queenright colonies has rarely been tested (but see Tasei and Aupinel, 2008 and Mommaerts et al., 2010) and Cabrera et al. (2016) acknowledge that bumblebee microcolonies are not suited for higher tier pesticide risk assessment because they do not produce workers or new queens, which are key endpoints for higher tier regulatory risk assessment.

Laboratory studies are a useful tool for first tier pesticide risk assessment because they allow for an accurate evaluation of colony fitness parameters using controlled concentrations of agrochemicals under standardised conditions. However, the main shortcoming of laboratory studies is the fact that they do not reflect the complex environment with multiple stressors that bees face in the wild, such as pathogens, other agrochemicals or food scarcity that may act synergistically (Goulson et al., 2015; Sgolastra et al., 2017;Tosi et al., 2017). To approach the conditions that bees encounter in the wild, higher tier risk assessment is usually carried out in semi-field (tier II, typically in tunnels with surrogate crops) or field (tier III, typically in the proximity of agricultural crops) settings (Cabrera et al., 2016). The higher complexity in semi-field or field conditions may lead to differences in outcome when compared to laboratory conditions, especially when testing pesticides like neonicotinoids that affect learning, memory, and/or motor skills (Decourtye et al., 2004; Stanley et al., 2015; Tison et al., 2016; Whitehorn et al., 2017).

The aim of this study was to directly compare different risk assessment methods in bumblebees and to evaluate the adequacy of short-term laboratory methods, such as microcolonies. The effect of the neonicotinoids thiacloprid and acetamiprid were evaluated by comparing four different methods. Firstly, the effect of these insecticides was evaluated using microcolonies that were exposed

to the insecticides for nine weeks (short-term laboratory experiment). Secondly, queenright colonies were evaluated over the same exposure time, starting at the time of colony foundation (short-term laboratory experiment). Thirdly, a laboratory experiment in which gyne production of queenright colonies was assessed during a longer term assessment until gyne production ceased (long-term laboratory experiment). Lastly, effects of these insecticides were evaluated on colonies exposed in the field over a period of four weeks, where production of reproductives, flight activity, and colony weight gain were assessed. We hypothesized that results of queenright colonies in the laboratory may not be directly extrapolatable to outcomes under field conditions.

2. Materials and methods

2.1. Pesticide treatments

Two commercial products, Gazelle® (Certis Europe, active ingredient acetamiprid) and Calypso® (Bayer, active ingredient thiacloprid), were fed to bumblebee colonies in the sugar water (50° Brix), while sugar water only was used as a control treatment. The concentration at which both insecticides were offered was based on published concentrations found in nectar following crop treatment with commercial products containing either acetamiprid or thiacloprid (Pohorecka et al., 2012; Ellis et al., 2017; Table S1). These concentrations were measured several weeks after spraying and should therefore not be considered as peak concentrations found in nectar. Hence, the exposure to these doses is field-realistic, albeit conservative, as we did not add the insecticides to the pollen fed to the colonies. In the field, the systemic compounds can be expected to be concurrently expressed in both nectar and in pollen. Acetamiprid was added in a 7.6 ppb concentration and thiacloprid was added in a 561 ppb concentration (calculated based on the amounts of the active substances listed on the products' labels, Table S1). Both commercial products were dissolved in water and then added to the sugar water to obtain the correct concentrations (Table S1).

2.2. Laboratory conditions: B. terrestris microcolonies and queenright colonies

We collected a total of 300 callow *B. terrestris* workers from different commercially reared colonies (Biobest Group NV, Belgium) to produce 60 microcolonies in total, each consisting of five callow workers. As callows were collected from hundreds of different colonies in the commercial rearing, few workers within a microcolony were sisters but all were still closely related. The workers were randomly placed together in a transparent plastic nest box (15 × 15 × 10 cm) and microcolonies were randomly assigned to the three treatments and placed in a climate room on carts in such a manner that all treatments were represented equally over all carts. Each treatment was replicated 20 times. After a nine-week-period, all microcolonies were frozen (Figure S1) and all emerged males, pupae, larvae, and eggs were counted. All adult (male) offspring were weighed together to obtain an average male weight per colony. The main microcolony fitness parameter is considered to be the total colony size, i.e. all larvae, pupae and adult males, as this reflects the total reproductive output of the microcolony after nine weeks. Eggs were not included in this parameter because the viability of the eggs was unknown.

A total of 75 four-month-hibernated *B. terrestris* queens were obtained from the mass rearing of Biobest Group NV (Belgium) and each queen was placed in a transparent plastic box (15 × 15 × 10cm) and then randomly assigned to one of the three treatments (N = 25 queens per treatment). They were placed on the same carts as the microcolonies so that each treatment was represented equally over each cart for both the microcolonies and the queenright colonies. Twenty colonies of each treatment were frozen after a nine-week-period and all offspring, pupae, larvae, and eggs were counted (N = 20 for queenright colonies in the growth phase, Figure S1). These queenright colonies were thus frozen after the same time period as the microcolonies. In order to directly compare the results obtained with queenright colonies frozen at nine weeks to the microcolonies we mainly focussed on the total colony size at nine weeks of age. An average worker weight was also determined per colony. There were five queenright colonies per treatment (15 in total) that were selected before nine weeks to be transferred into a larger nest box (28.5 × 22 × 13.5 cm) to allow for queen (and male) production to be assessed (N = 5 for queenright colonies in the reproductive-producing phase, Figure S1). These colonies were selected based on the criterium of being the first five within each treatment to reach a colony size of 60 workers. Potential new queen production was assessed by allowing queen production over a three-week-period since the first new queens had emerged, as the average time for queen production of a colony under these conditions is four weeks (Van Oystaeyen, unpublished data). By freezing colonies after these three weeks we will have captured the far majority of queens, either born or in the pupal stage (our data confirmed that few queen larvae were left after this time). By summing all queen developmental stages (adult queens, queen pupae and queen larvae) we have an accurate estimation of the total potential queen production of each colony. This method ensures that we correctly assess total queen production. Male production, on the other hand, was still ongoing at this time, which means that we do not have a final total male production. Colony size should not be considered as a main fitness parameter for these five colonies per treatment because they were frozen after different time periods, depending on when they started queen production. Therefore, the main parameter to evaluate fitness of these queenright colonies was queen production, which is also one of the main suggested end points for higher tier assessment (Cabrera et al., 2016). All males, queens, and workers produced in these colonies were weighed to obtain an average worker, male and queen weight per colony.

All colonies were kept at 27 ± 2 °C and 60 ± 10% relative humidity under continuous darkness (lights were exclusively switched on during short periods for feeding and hive assessments). All hives were fed ad libitum with pesticide-free sterilized honey bee-collected pollen and with 50° Brix sugar water (Biogluc®, Belgosuc, Belgium) provided by Biobest Group NV, Belgium. For all treatments, sugar water - with or without the insecticide - was refreshed once after 30 days for all (micro)colonies that were to be frozen after nine weeks. It was refreshed twice more, after 60 days and after 90 days, for those colonies that were kept for the production of new queens.

2.3. Field conditions: foraging queenright colonies

A total of 21 eight-week-old-colonies were obtained from the mass rearing of Biobest Group NV (Belgium) and placed in a 6150 m^2 field with rows of different flowering plants attractive to bumblebees (belonging to the following families: Boraginaceae, Malvaceae, Asteraceae, Brassicaceae and Papaveraceae) at the Biobest site in a rural area (51°07′49.5″N 4°53′22.7″E, Westerlo, Belgium). The experiment was carried out in April–May 2019 and there had been no pesticides used in the field in at least two years prior to the experiment. In order to minimize variation between colonies, we selected them from the same production batch and with similar sizes (i.e. around 70 workers and equal amounts of brood). No males or new queens were present in the colonies at the

start of the experiment. All colonies were weighed beforehand and they were assigned to the three treatments in such a manner that the average weight did not significantly differ between treatments (ANOVA, $P = 0.4$): thiacloprid 138.5 ± 11.3 g, control 148.7 ± 17.6 g and acetamiprid 139.7 ± 15.9 g. Each treatment was replicated seven times and consisted of 7.6 ppb acetamiprid in 60° Brix sugar water, 561 ppb thiacloprid in 60° Brix sugar water or 60° Brix sugar water without any insecticide added to it. A bottle with 2 L of this sugar water was placed underneath the colonies as soon as they were placed outside. Colonies were thus only exposed to the insecticides after placing them in the field and we can consider this as a highly conservative level of exposure for two reasons: 1) the colony is not exposed during the early growth phase and 2) because outside, workers will also collect nectar and pollen from the flowering field where no pesticides had been used, which can be expected to have a dilution-effect. Colonies were placed in the field with the nest entrance oriented to the southeast and following a rational design whereby treatments alternated within four longitudinal rows, while assuring a minimal distance of 5 m between colonies.

Flight activity of each colony was followed over a three-week-period. Number of workers flying in and out of each colony was counted in 5-min-rounds, repeated three times per counting day, totalling 15 min of observations per colony per counting day. Counts were carried out on nine days during the experiment. Number of workers returning with or without pollen loads were noted. We do not know whether workers returning without pollen were carrying nectar.

After a four-week-period in the field, colonies were collected at night to ensure that all individuals were inside the colony at time of collection. Colonies were subsequently frozen at −20 °C and all individuals, larvae and pupae were counted, new queens were weighed individually and the colony was weighed as a whole to obtain a final weight and calculate the weight change (final weight - initial weight). A relatively short period of four weeks was chosen for the field experiment to minimize the chance that new queens may have had left the colony. Queens were not observed to fly out during the four-week-experiment. The main parameter for fitness assessment was chosen as the total number of reproductives (i.e. the sum of males and gynes) because male and gyne production were both still ongoing at the time of colony freezing. Therefore, we do not have a total potential queen production for all colonies, as opposed to the laboratory experiment that was specifically set up to evaluate total queen production.

2.4. Data analysis

All data were analysed in R 3.5.1 (R Core Team, 2019) using generalized mixed models (GLMM: 'glmer'-function; package: lme4) (Bates et al., 2015), linear and generalized models (GLM: 'glm'-function; package: base,R Core Team, 2019) as well as Kruskal-Wallis tests ('kruskal.test'-function; package: base,R Core Team, 2019). Model fit was assessed by visual inspection of the homogeneity of residuals and fit to the assumed distribution using the 'simulateResiduals'-function (package: DHARMa, Hartig, 2020). In addition, we tested over/underdispersion ('testDispersion'-function; package: DHARMa) for generalized models, as well as zero-inflation ('testZeroInflation'-function; package DHARMa) for generalized and linear models. Model simplification was based on p-values of GLM and GLMM models obtained from type III Wald chi square tests ('Anova'-function; package: car) (Fox and Weisberg, 2019). Multiple comparisons with Holm-adjustment of p-values using the 'contrast'-function were carried out when treatment effects were significant or when there was a trend ($P < 0.1$) (package: emmeans, Searle et al., 1980) for GLMM and GLM models. Dunn's tests ('dunn.test'-function; package: dunn.test, Dinno, 2014) were carried out following Kruskal-Wallis tests. Data of microcolonies and queenright colonies under laboratory and field conditions were analysed separately.

Microcolony development after a period of nine weeks under laboratory conditions was assessed on colony level and modelled using GLMs with treatment as a fixed effect. The number of predicted males (i.e. sum of males and male pupae) was modelled with a GLM with negative binomial distribution to handle overdispersion. Total colony size (i.e. all larval stages, pupae and adults summed) and average male weight were modelled with gaussian GLMs. As dead larvae were commonly observed in microcolonies, the proportion of dead larvae out of all larvae produced was analysed with a Kruskal-Wallis test because assumptions of a binomial GLM were violated.

The development of queenright colonies was assessed on colony level after a period of nine weeks under laboratory conditions for all colonies and modelled using GLMs with treatment as a fixed effect. GLMs with gaussian distribution were used for the number of predicted workers (i.e. sum of workers and worker pupae), total colony size and average worker and male weight. For the proportion of males to females we used a Kruskal-Wallis test because model assumptions for GLM models with binomial distribution were not met. In contrast to microcolonies, dead larvae were only rarely observed in queenright colonies and therefore this parameter was not analysed for queenright colonies.

For the subset of the colonies that were given the time to produce new queens, the total number of reproductives produced (i.e. a sum of adult males and queens) as well as total colony size were analysed using Kruskal-Wallis tests because model assumptions of GLMs could not be met. The total number of females (i.e. sum of adult queens and workers), the predicted number of queens (i.e. sum of queens and queen pupae) and average gyne weight were analysed using a gaussian GLM. The average weight of workers and the average weight of males were analysed using Kruskal-Wallis tests, as assumptions of gaussian GLMs were violated.

Flight activity (total number of workers flying in and out of the colonies during an observation round) and pollen income (number of workers returning with pollen) were analysed for queenright colonies under field conditions by generalized linear mixed models (GLMM) with a poisson distribution in which colony and counting day were included as random factors. The effect of treatment over time was analysed by using treatment in interaction with counting day as fixed effects. Potential non-linear effects of counting day were assessed by plotting raw data of flight activity and pollen income vs. counting day ('xyplot'-function; package: lattice; Sarkar, 2008) and a quadratic term of counting day included into both models.

The development of colonies under field conditions was analysed for the increase in colony weight after a period of four weeks using a Kruskal-Wallis test. The relationship between colony weight gain and total colony size was analysed by a GLM with gaussian distribution and a post hoc for the significant interaction effect was carried out by the function joint_tests (emmeans package, Searle et al., 1980). The number of reproductives, the potential number of queens (i.e. sum of queens emerged, queen pupae and queen larvae), total colony size and the relationship between males and potential queens were all analysed using GLMs with negative binomial distribution to account for overdispersion. The average weight of queens and the average weight of males were modelled with gaussian GLMs, whereas a Kruskal-Wallis test was used for the average weight of workers.

3. Results and discussion

3.1. Laboratory: microcolonies and queenright colonies

Most remarkably, our results show that thiacloprid had a significant positive effect on the total colony size (30% increase) of microcolonies compared to both the control and the acetamiprid treatment (control - thiacloprid: $z = -2.7$, $P = 0.01$, Fig. 1, Table S2), while we found no such effect in queenright colonies after a short assessment period (equal to the assessment period of the microcolonies). Moreover, an opposite result to the microcolony result was found in queenright colonies after a longer assessment period, as thiacloprid significantly reduced colony fitness through a reduced gyne production (see further). In the short-term assessment of queenright colonies, we found a non-significant negative trend of thiacloprid treatment on parameters related to colony development, such as colony size (control - thiacloprid: $z = 2.1$, $P = 0.1$, Fig. 1, Table S3). By contrast, assessment of queenright colonies over a longer time period, allowing gynes to be produced, revealed a highly significant treatment effect on the number of potential new queens ($X^2 = 40.4$, $P < 0.001$). Here, thiacloprid had a significant negative effect on the number of potential new queens when compared to the control (control - thiacloprid: $z = 5.6$, $P < 0.001$) and the acetamiprid treatment (acetamiprid - thiacloprid: $z = 5.2$, $P < 0.001$, Fig. 1 Table S4), reducing the number of potential queens by 30% compared to the control treatment, even

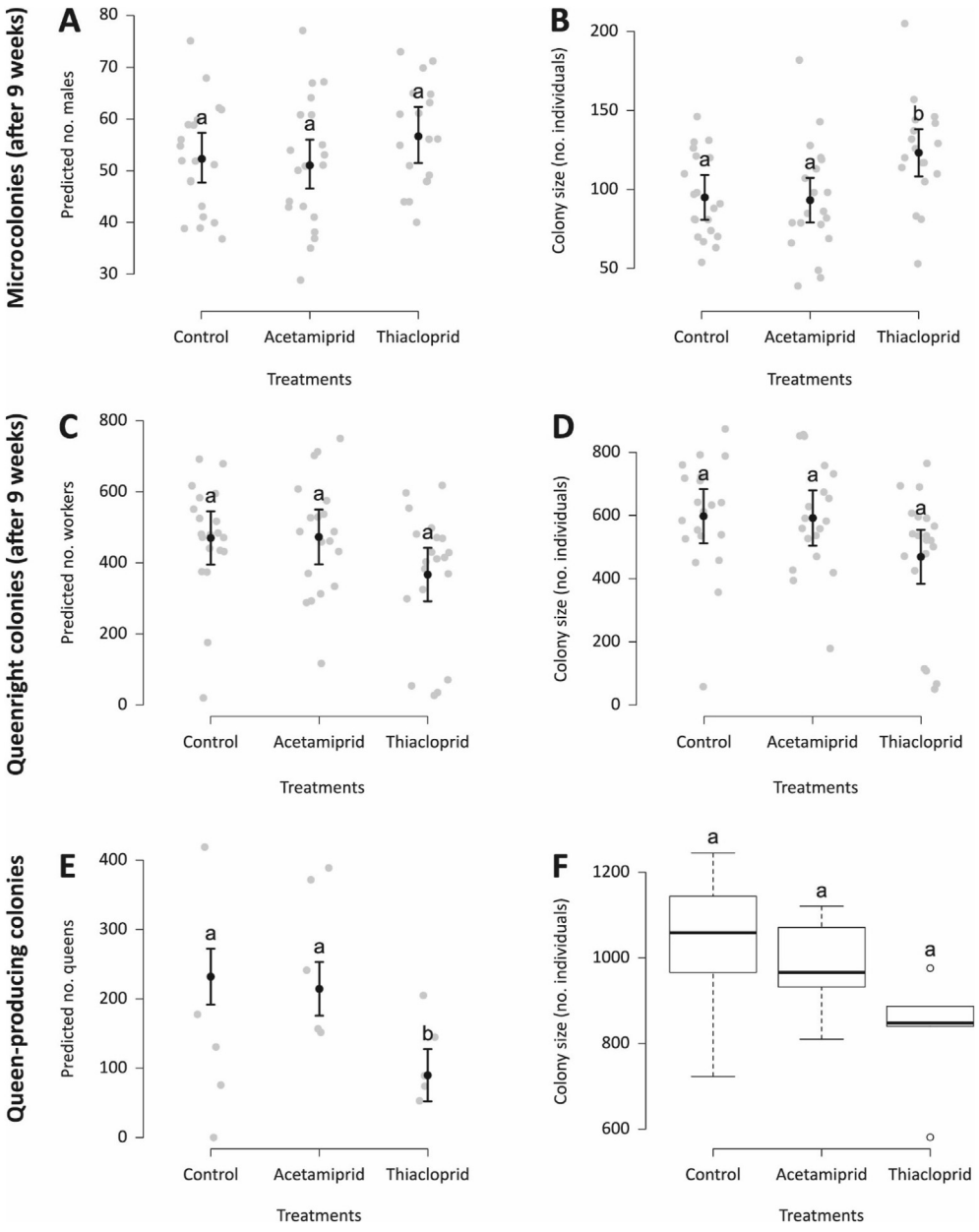

Fig. 1. Comparison of different risk assessment methods in the laboratory: microcolonies and short-term and long-term queenright colonies. Treatment effects are shown for (A) predicted number of males and (B) total colony size of microcolonies, (C) predicted number of workers and (D) total colony size of queenright colonies, as well as (E) predicted number of queens and (F) total colony size of queen-producing colonies. Dot plots show mean values obtained from model estimates (black dots) and bars show ± 95% confidence intervals. Box plots are used to visualize data analysed with Kruskal-Wallis tests and show median (horizontal line), 75% quartiles (box) as well as quartiles + 1.5 × quartile range (whiskers). Significant ($p < 0.05$) treatment effects are shown with letters.

though one out of the five control colonies did not produce any gynes. All other colonies produced gynes.

Together, these results demonstrate that the use of microcolonies, which are commonly used and recommended for the risk assessment of agrochemicals in bumblebees (EFSA European Food Safety Authority, 2013), lead to outcomes which differ substantially from queenright colonies, even under the exact same climatic and nutritional conditions and with an equal insecticide exposure (time and concentrations). Furthermore, results indicate that a long-term assessment of queenright colonies in which gyne production is the end point is needed to detect negative effects of sublethal pesticides such as thiacloprid. Microcolonies do not produce female offspring and can therefore lead to erroneous conclusions when pesticides act on the reproductive physiology of queens.

The mechanisms behind the different outcomes of microcolonies and queenright colonies were not examined in this study, but a few possible underlying mechanisms that may lead to differences between both types of colonies are suggested hereafter. Firstly, there are important differences between the two female castes used to found these two types of colonies (workers for microcolonies and queens for queenright colonies), both morphologically and physiologically. The most pronounced morphological caste-difference is body size (Alford, 1975). Evidence for differences in susceptibility to pesticides according to body size within species seems to be lacking but an inverse relationship between body size and pesticide susceptibility has been found between bee species (Devillers et al., 2003). If such an inverse relationship were to exist within species, one could expect that workers are more susceptible to pesticides than queens. Our results do not support this as they, by contrast, indicate a higher susceptibility of queens, in particular in the case of thiacloprid. Similarly, Mobley and Gegear (2018) found a higher susceptibility of reproductives, both queens and males, to chronic sublethal doses of clothianidin than workers in the bumblebee *B. impatiens*. They found that neonicotinoid consumption induced changes to several genes associated with reproduction. Hence, physiological differences linked to reproductive status and sexual versus asexual reproduction are presumably important in explaining the observed differences. A second potential explanation for differences between microcolonies and queenright colonies is that sperm needed to fertilise female-destined eggs can be affected by pesticides. This is a plausible scenario because the viability of sperm cells stored inside honey bee queens' spermathecae has been shown to be severely affected by the neonicotinoid imidacloprid, even at the lowest tested concentration of 0.02 ppm (Chaimanee et al., 2016). In a study by Williams et al. (2015) this effect on spermatozoa was confirmed for the other two banned neonicotinoids, thiamethoxam and clothianidin, and they additionally observed ovariole hyperplasia, suggesting that neonicotinoids can affect both the queen's reproductive anatomy and physiology. A third possibility is that there may be differences in susceptibility to pesticides during larval development between males, i.e. the offspring in the microcolonies, and females, i.e. the offspring in queenright colonies. Indeed, a recent study demonstrated that honey bee males are more susceptible to neonicotinoids than workers during their development (Friedli et al., 2020). Here, male larval mortality in microcolonies was frequently observed, while (worker) larval mortality in queenright colonies was rarely observed. This observation could point to a higher susceptibility of males to environmental stress during development. However, at the same time, a lower proportional larval mortality was found in the thiacloprid-treated microcolonies compared to the control and acetamiprid treatment ($P = 0.01$ and $P = 0.004$, respectively; Figure S2, Table S2), which is a direct explanation for the positive effect of thiacloprid on microcolony size. The finding that thiacloprid increases male larval survival is difficult to explain and it is in stark contrast with the theory of high male susceptibility postulated by Friedli et al. (2020). Both the use of different study organisms (honey bees versus bumblebees) and the type of parameters that were assessed (parameters for developmental instability versus mortality) may explain this discordance. Further research would be needed to shed light on the mechanistic explanation for the observed positive effect of thiacloprid on male larval survival in the microcolonies.

Acetamiprid did not have any significant effects on the measured fitness parameters in the tested concentrations for both the microcolonies and queenright colonies and neither of the two insecticide treatments affected male weight in microcolonies or worker weight in short-term queenright colonies (Table S2 and S3). Similar to the microcolonies and queenright colonies evaluated at nine weeks, no significant effects of acetamiprid could be demonstrated for any of the measured fitness parameters in gyne-producing queenright colonies. Neither insecticide treatment had a significant effect on male, queen or worker weight (Table S4).

The clear negative effect of thiacloprid on gyne production after a long-term evaluation of queenright colonies and the lack of such a clear significant effect in queenright colonies after a shorter evaluation period, emphasize the importance of long-term risk assessment studies. The production of gynes has been put forward as one of the main endpoints in risk assessment of agrochemicals (Cabrera et al., 2016). In queen-producing colonies, there were no significant effects of thiacloprid on male production or total colony size (Table S4), for which needs to be pointed out that our setup was intended to evaluate new queen production. Male production was still ongoing at the time of colony termination and colony size cannot be directly compared between colonies and treatments because colonies were frozen at different time points when gyne production ceased. Male production is often included as an endpoint in pesticide risk assessment (e.g. Stanley and Raine, 2017) but we argue that the assessment of male production in species where workers can produce them asexually is more ambiguous. Some authors correct for the fact that males are haploid in the calculation of the colony's reproductive output by multiplying the number of males by 0.5 and summing this up with the number of produced queens (e.g. Ellis et al., 2017).

There are several reasons for considering gyne production as the main indicator of bumblebee colony fitness. Firstly, gyne production is limited more by both external and internal factors than male and worker production. Externally, food provisions play a key role in gyne production because queen larvae consume larger amounts of pollen and are fed more frequently than male or worker larvae (Plowright and Jay, 1977; Ribeiro et al., 1999). A limitation of food income, caused by negative effects of pesticides on foraging activity, can therefore directly affect gyne production. Internal or physiological factors limiting gyne production are related to the longer developmental time of queens compared to males and workers (Ribeiro et al., 1999; Cnaani et al., 2002) and the haplodiploid sex determination system (Crozier, 1971) because only mother queens can fertilise eggs and produce gynes. Therefore, gyne production is limited by the presence of a healthy egg-laying mother queen, implicating that pesticides that reduce mother queen longevity (Sandrock et al., 2014; Scholer and Krischik, 2014;Tsevtkov et al., 2017) or the quality of stored sperm (Williams et al., 2015) may affect new gyne production. However, this is different for male production because males can also arise from non-fertilised worker-laid eggs (Owen and Plowright, 1982). Secondly, queens have the longest solitary phase of all individuals within a bumblebee colony, during which they are more vulnerable to stressors than during the social phase (Straub et al., 2015). Thirdly, most bumblebee species are monandrous (Röseler, 1973;

Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000), while males can fertilise multiple females (Röseler, 1973; Tasei et al., 1998). Therefore, in theory, fewer males than queens are needed but sex ratios are typically male biased (Owen and Plowright, 1982; Bourke, 1997; Beekman and Van Stratum, 1998), indicating that males are often produced in surplus. Finally, an evident reason for queen production as the main risk assessment parameter is that mated queens eventually need to found new colonies. As a consequence, even small reductions in queen production at the colony level could have profound consequences at the population level.

3.2. Queenright colonies in the field

To enable a comparison between field experiments and (short-term and long-term) laboratory experiments, a field experiment in which colonies were exposed to the neonicotinoids for four weeks was carried out to assess foraging activity and colony development parameters. During this relatively short but realistic insecticide exposure, there were significant effects of treatment on overall flight activity (X^2 = 14.594, P = 0.001) and pollen income (X^2 = 7.252, P = 0.03), with only thiacloprid significantly reducing both parameters compared to the control (control - thiacloprid: flight activity: z = 3.8, P = 0.001; pollen income: z = 1.4, P = 0.02, Fig. 2, and Table S5). Thiacloprid decreased total foraging activity by 30% and number of pollen load-carrying foragers by 42% compared to the control treatment. A similar negative effect on foraging activity and/or pollen income was already demonstrated for the three banned neonicotinoids imidacloprid (Mommaerts et al., 2010; Gill and Raine, 2014; Feltham et al., 2014), thiamethoxam (Henry et al., 2012; Tosi et al., 2017b), and clothianidin (Schneider et al., 2012; Scholer and Krischik, 2014) but our study is the first to demonstrate a comparable effect of the "low-risk" neonicotinoid thiacloprid on foraging activity in queenright bumblebee colonies. Exposure to thiacloprid can have adverse effects on memory retrieval during navigation (Fischer et al., 2014; Tison et al., 2017), as well as impair foraging behaviour in honey bees (Tison et al., 2016). Therefore, impaired learning and memory retrieval are plausible mechanisms explaining the observed effect on foraging activity in this study. The acetamiprid treatment did not affect flight activity or pollen income compared to the control treatment (Table S5).

A reduced pollen income is a logical consequence of lower foraging activity, which will inevitably hamper colony development, because pollen is essential for brood development and egg maturation by the mother queen (Alford, 1975; Mommaerts et al., 2010). Concordant to the expectations of a reduced colony development associated with a reduced foraging activity, a significantly lower colony weight gain was found over the four-week-period in the thiacloprid treatment compared to both the control and acetamiprid treatment (control - thiacloprid: z = 2.2, P = 0.03, Fig. 3B and Table S6). The average weight gain of thiacloprid-treated colonies was 25% below that of control colonies. By contrast, thiacloprid did not significantly reduce the total colony size of queenright colonies in the field (Table S6). Interestingly, there was a positive relationship between colony size and colony weight gain for the acetamiprid and the control treatment, while this was not the case for the thiacloprid treatment (interaction between treatment and colony size: $\chi 2$ = 10.3, P = 0.006; acetamiprid: F ratio = 16.6, P < 0.001; control: F ratio = 35.1, P < 0.001; thiacloprid: F ratio = 0.2, P = 0.6; Fig. S3). These findings thus emphasize the importance of evaluating multiple parameters, such as foraging behaviour, actual colony size, number of reproductives, next to colony weight gain, in higher tier risk assessment (Mommaerts et al., 2010; Cabrera et al., 2016). Presumably other factors, such as number of gynes or amount of food provisions (honey or pollen pots), influence the weight of colonies to a large extent. For colonies in the field, a total of all reproductives was additionally evaluated (i.e. the sum of all males and gynes). Our results demonstrate that thiacloprid significantly decreased the number of reproductives compared to the control (control - thiacloprid: z = 4, P < 0.001; Fig. 3A, Table S6). Therefore, our results correspond to the findings of Ellis et al. (2017) who tested bumblebee colonies in a field with an equal thiacloprid concentration in nectar to the concentration applied in this study, showing that colonies close to thiacloprid-treated fields had a reduced longevity, weight, and number of produced reproductives.

Interestingly, there was also a negative trend in the number of reproductives produced in acetamiprid-treated colonies compared to the control (control - acetamiprid: z = 1.7, P = 0.09; Fig. 3, Table S6). Even though there was no statistical significance at the 0.05 level, this is the first indication of a potential negative effect of acetamiprid on colony development under realistic conditions. These findings contest the assumption that this substance poses a low risk to bees (EFSA European Food Safety Authority, 2013). Further research would be needed to evaluate the effect of acetamiprid in higher, yet field-realistic, concentrations because our application dose can be considered highly conservative. In addition,

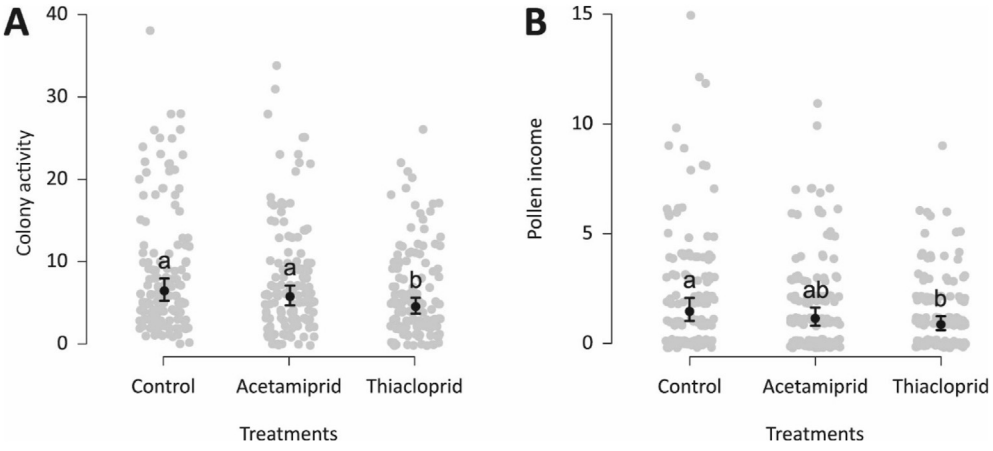

Fig. 2. Effect of 4-week-neonicotinoid exposure on foraging activity of queenright colonies in the field. Treatment effects are shown for (A) colony activity defined as the total number of workers leaving and entering the colony and for (b) pollen income defined as the number of workers returning to the colony with pollen. Dot plots show mean values obtained from model estimates (black dots) and bars show ± 95% confidence intervals. Significant (p < 0.05) treatment effects are shown with letters.

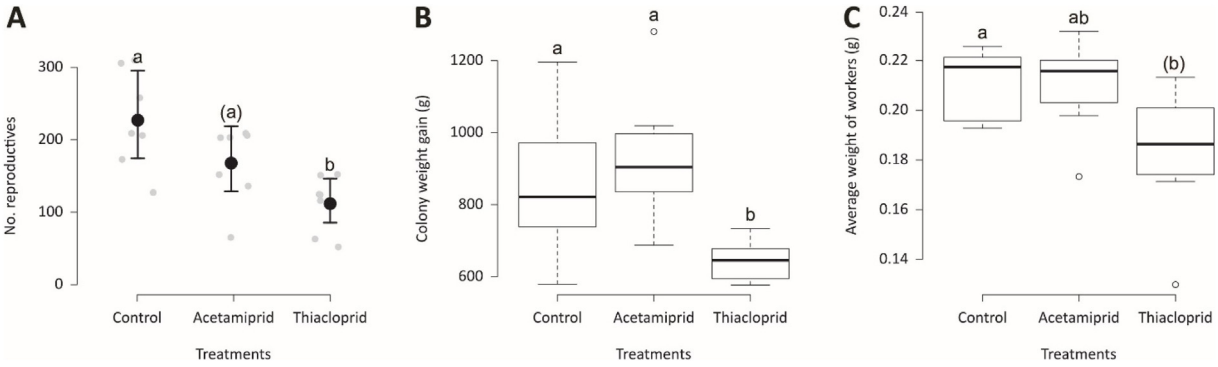

Fig. 3. Effect of 4-week-neonicotinoid exposure treatment on different parameters in the field. Treatment effects are shown for (A) average number of reproductives, (B) colony weight gain and (C) average worker weight. Dot plots show mean values obtained from model estimates (black dots) and bars show ±95% confidence intervals. Box plots are used to visualize data analysed with Kruskal-Wallis tests and show median (horizontal line), 75% quartiles (box) as well as quartiles + 1.5 × quartile range (whiskers). Significant ($p < 0.05$) treatment effects are shown with letters and trends ($0.05 \leq p < 0.1$) are shown with letters in brackets.

we need to stress that the concentrations tested for both insecticides differed and that the tested concentration of acetamiprid was ca. 80 times lower than that of thiacloprid. This limits a direct comparison between both tested insecticides.

All 21 colonies initiated queen production within the four-week-period in the field and in 5 out of 7 (71%) control colonies and in 3 out of 7 (43%) of both the acetamiprid- and thiacloprid-treated colonies queen production was still ongoing (queen larvae present) at the time the experiment was terminated. This may indicate that queen production was underestimated most in our control treatment. Furthermore, male production was also still ongoing at the time the experiment was stopped and a negative relationship between the number of males and gynes produced was found ($\chi^2 = 5.4$, $P = 0.02$, Figure S4), which justifies evaluating both sexes together.

In contrast with the laboratory study, a reduced worker weight for thiacloprid-treated colonies was found compared to the control (control - thiacloprid: $z = 2$, $P = 0.048$, Fig. 3C), while there were no significant effects on queen and male weight (Table S6). We hypothesize that thiacloprid affected worker weight in the field but not in the laboratory because flight activity and pollen income were significantly reduced in the field study. This had presumably led to a reduced pollen availability for larval feeding. Pollen are the main protein source needed for larval development (Alford, 1975) and larvae that are deprived of pollen will develop into adults with a smaller body size (Sutcliffe and Plowright, 1988; Pereboom et al., 2003).

In this study, commercial neonicotinoid products were used, as opposed to the sole active neonicotinoid ingredients. This reflects the situation that pollinators encounter in nature, as only commercial products are used for crop treatment. It also begs the question to what extent the observed negative effects on colonies are caused by certain adjuvants, the neonicotinoid itself or through synergistic interactions between the adjuvants and the active ingredient. Adjuvants are used to enhance the efficiency of the biological activity of the main active ingredient(s) and are often neglected in pesticide risk assessment, even though toxicity of these substances has been demonstrated (e.g. Surgan et al., 2010; Artz and Pitts-Singer, 2015). This is both due to a lack of transparency on the ingredients in commercial products and due to the fact that most pesticide risk assessments for bees is carried out without formulation ingredients (Mullin, 2015). Future research would need to aim at elucidating the role of adjuvants in commercial pesticides in bee declines.

4. Conclusions

The results of this study demonstrate the risk of reaching erroneous conclusions when microcolonies are used in first tier risk assessment of agrochemicals. Thiacloprid had a positive effect on microcolony size at nine weeks, while no significant effects of this treatment were found in queenright colonies at the same time point. When queenright colonies were evaluated over a longer period, thiacloprid negatively impacted gyne production. Therefore, our results also highlight the need for long-term laboratory evaluations in lower tier risk assessment in which gyne production should be considered as a main endpoint (Cabrera et al., 2016). The assessment of queenright colonies in the field confirmed the negative effect of thiacloprid found in the long-term laboratory assessment, while effects for acetamiprid were only found under field-realistic conditions, thereby further advocating for risk assessment of pesticides under field realistic conditions. This is especially important when the substances affect foraging behaviour (Mommaerts et al., 2010), which can be mediated through effects on learning, memory and/or motor skills (Decourtye et al., 2004; Stanley et al., 2015). For instance, the negative effects of thiacloprid on bumblebee colony fitness parameters in our field experiment are mediated through a reduced foraging activity and pollen income.

In conclusion, for first tier risk assessments (laboratory conditions), we advocate against the use of microcolonies but recommend the use of queenright colonies in which gyne production is included in the assessments. Furthermore, higher tier risk assessments (field-realistic conditions) remain indispensable to assess true risks for pollinators. Multiple parameters reflecting colony fitness should thereby be considered. For neonicotinoids and presumably other insecticides targeting the nicotinic acetylcholine receptor (e.g. sulfoximines; Sparks et al., 2013), the assessment of foraging behaviour and colony fitness parameters (e.g. production of new queens) remain the most important end points.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128518.

Author contributions

Annette Van Oystaeyen: Supervision, Conceptualization, Methodology, Formal Analysis, Writing. **Björn K. Klatt**: Formal Analysis, Visualization, Writing. **Clément Petit**: Investigation and Data Curation. **Nancy Lenaerts**: Investigation. **Felix Wäckers**: Supervision, Writing.

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