nature ecology & evolution

ARTICLES

DOI: 10.1038/s41559-017-0260-1

Pesticide reduces bumblebee colony initiation and increases probability of population extinction

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Pollinators are in global decline and agricultural pesticides are a potential driver of this. Recent studies have suggested that pesticides may significantly impact bumblebee colonies—an important and declining group of pollinators. Here, we show that colony-founding queens, a critical yet vulnerable stage of the bumblebee lifecycle, are less likely to initiate a colony after exposure to thiamethoxam, a neonicotinoid insecticide. *Bombus terrestris* queens were exposed to field-relevant levels of thiamethoxam and two natural stressors: the parasite *Crithidia bombi* and varying hibernation durations. Exposure to thiamethoxam caused a 26% reduction in the proportion of queens that laid eggs, and advanced the timing of colony initiation, although we did not detect impacts of any experimental treatment on the ability of queens to produce adult offspring during the 14-week experimental period. As expected from previous studies, the hibernation duration also had an impact on egg laying, but there was no significant interaction with insecticide treatment. Modelling the impacts of a 26% reduction in colony founding on population dynamics dramatically increased the likelihood of population extinction. This shows that neonicotinoids can affect this critical stage in the bumblebee lifecycle and may have significant impacts on population dynamics.

Bees play a vital role as pollinators in both agricultural and natural systems[1–4]. However, there is increasing concern about the state of wild bee populations. Nearly 10% of European bee species are currently considered threatened[5] and bumblebees are declining on a global scale[5–9]. The cause of these declines is thought to be a combination of factors, particularly habitat loss[10], parasites and diseases[11–13], invasive species[14], and climate change[15,16]. Pesticide use is also considered a major threat to wild bees[17–20], and both laboratory[21–26], semi-field[27–33] and field studies[34,35,36] have found negative impacts of pesticides on bumblebee behaviour, reproduction and colony success. However, information on the impacts of pesticides on the key life history stages of wild bees is still lacking. Bumblebees, like solitary bees, have an annual lifecycle whereby reproductive females (queens) initiate a colony in the spring[37]. Bumblebee queens are functionally solitary at this stage and do not have a colony to buffer them from environmental stress. Success depends entirely on the queen's survival and ability to initiate a colony and, as such, this represents a critical but vulnerable period in the lifecycle. Although bumblebee queens are likely to be exposed to a range of pesticides throughout their lifecycle, particularly when foraging in the early spring on flowering crops such as OSR, to date there has been no research on the impacts of pesticides on founding queens and their ability to initiate a colony. Rundlöf et al.[34] found that neonicotinoid treatment of OSR crops resulted in a lack of brood-cell building in solitary bees, but the mechanism remained unexplored. Negative impacts of neonicotinoids on the reproduction of the honeybee *Apis mellifera* queen have also been found[38,39], but honeybee colonies are perennial and the way in which this relates to the annual cycle in bumblebees remains unknown. However, given these results, it is vital that we understand the potential impacts of pesticides on bumblebee queens[40,26] and the resultant implications for wild populations.

We examined the impact of thiamethoxam (a neonicotinoid insecticide) exposure on colony-founding bumblebee (*Bombus terrestris*) queens. Neonicotinoids are the most widely used class of pesticide in the world[41] and thiamethoxam is one of three neonicotinoids currently under a European Union usage moratorium for flowering, bee-attractive crops. Neonicotinoids have been implicated in the decline of wild bees[20], butterflies[42] and other taxa[43]. A range of regulations on the use of neonicotinoids have also recently come into force in North America. Therefore, research on the risks to beneficial insects associated with exposure to these compounds has important global policy implications.

In addition to the potential threat from pesticide exposure, bumblebee queens are faced with a range of environmental stressors that can reduce their survival and fitness. Before initiating a colony in the spring, queens must first survive hibernation over winter, during which time they can lose up to 80% of their fat reserves[44], which may make them vulnerable to additional stress. Little is known about the overwintering survival of bumblebee queens in the wild, but studies in the laboratory have shown that a range of factors, such as pre-hibernation weight[45,46], hibernation duration[46] and the genotype of the queen and her mate[47,48], can be important. Furthermore, exposure to parasites and pesticides can also impact hibernation survival[40] and parasites have been shown to affect the post-hibernation success of queens. For example, *Crithidia bombi*, a prevalent trypanosome parasite of bumblebees, has a context-dependent impact on its queen host[49]. Under laboratory conditions, parasitized queens lost up to 11% more mass during hibernation and had up to a 40% reduction in fitness compared with uninfected queens[49].

In natural environments, bumblebee queens face not only potential pesticide impacts, but also other simultaneous environmental stressors. To reflect this, we investigated the effects of thiamethoxam exposure on *B. terrestris* queens and tested for interactions with two natural environmental stressors: infection with the parasite *C. bombi* and variation in hibernation duration. To extrapolate our results to field populations, we used a Bayesian framework to assess their implications for population sustainability.

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Table 1 | Species of bumblebee queen observed foraging in and around OSR fields during two visits in April 2014

Species	Number of queens observed per hour of searching	
	Foraging on OSR	Foraging on other flowers[a]
Bombus terrestris	3.6	0.4
Bombus lapidarius	9.1	1.5
Bombus lucorum complex	0.4	0.0
Bombus hortorum	1.5	0.5
Bombus pratorum	0.0	0.2
Bombus hypnorum	0.2	0.2
Bombus pascuorum	0.4	0.4

Numbers indicate the average number of each species observed per hour of searching (5.5 h in total). [a]The other flowers were *Lamium album*, *Glechoma hederacea* and *Veronica chamaedrys*.

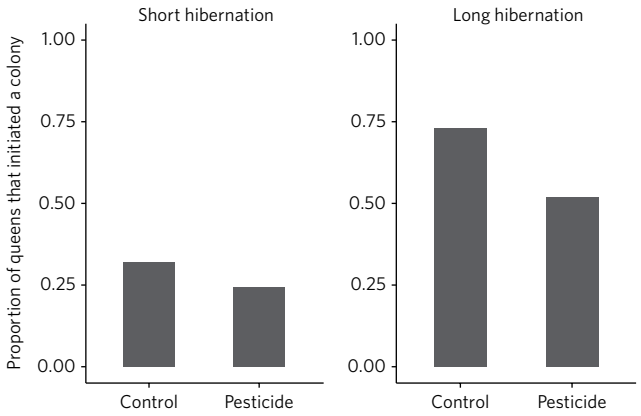

Fig. 1 | Effect of hibernation length and pesticide exposure on colony initiation. Proportion of *B. terrestris* queens that had undergone a short (6 week) or long (12 week) hibernation and been exposed to either the pesticide thiamethoxam or a control and then initiated a colony (by laying eggs) within 10 weeks of emergence from hibernation. The total sample sizes for each treatment group were as follows: short hibernation and control, $n=50$; short hibernation and pesticide, $n=49$; long hibernation and control, $n=48$; and long hibernation and pesticide, $n=50$ (includes queens that survived the entire experiment only).

Results

Are bumblebee queens exposed to neonicotinoids in the field? Bumblebee queens forage on oilseed rape (OSR; *Brassica napus*) treated with neonicotinoids. Transect walks in Oxfordshire, United Kingdom, identified seven species of queen in the vicinity of winter OSR crops, and six of these were actively foraging on OSR flowers (Table 1). *B. terrestris* and *Bombus lapidarius* were the species most commonly observed foraging directly on the crop. To establish whether neonicotinoids have an impact on queens during this colony-founding period, we conducted a laboratory trial, which exposed *B. terrestris* queens to a range of potential stressors, including dietary thiamethoxam.

Impacts of multiple stressors on *B. terrestris* queens. Mated *B. terrestris* queens were experimentally exposed to *C. bombi* or a control, hibernated for one of two durations and treated with the neonicotinoid thiamethoxam or a control in a fully crossed design. Survival and colony initiation (egg laying) were then monitored.

Colony initiation. Colony initiation was reduced by 26% in queens exposed to thiamethoxam (2.4 ppb for two weeks) during the colony-founding period (Fig. 1). Hibernation duration was also an important predictor of colony initiation, with fewer queens laying eggs after a 6 week hibernation (28% of all 231 queens in the experiment) compared with a 12 week hibernation (62% of all 231 queens in the experiment). Both pesticide treatment (estimate: −0.628, 95% confidence interval (CI): −1.240 to −0.017) and hibernation treatment (estimate: −1.514, 95% CI: −2.131 to −0.898) were included in the final composite generalized linear model for egg laying (Table 2). Neither parasite treatment nor any of the interactions were included in the final model as they did not improve model fit.

Pesticide-treated queens laid eggs earlier in the experiment than untreated queens. As pesticide treatment violated the assumption of proportional hazards in initial models, episode splitting was used to estimate two separate hazard ratios: during pesticide treatment (P1) and after treatment (P2)[46]. For P1, more pesticide-treated queens laid eggs (estimate: 1.400, 95% CI: 0.275 to 2.525), whereas for P2 the reverse effect was seen (estimate: −0.573, 95% CI: −1.034 to −0.112) (Fig. 2).

At the population level, no experimental factors predicted the presence or absence of adult workers. However, when only egg-laying queens were considered, pesticide was an important factor (estimate: 1.214, 95% CI: 0.320 to 2.107) and, unsurprisingly, due to the timing of egg laying (see above) a higher proportion of egg-laying queens in the pesticide treatment group had adult offspring by the end of the experiment.

The proportions of queens in the pesticide treatment groups, hibernation treatment groups and both groups combined that laid eggs and reared adult offspring are shown in Supplementary Table 1.

Syrup consumption. There is no evidence that pesticide exposure affected the amount of syrup consumed by queens (estimate: 0.024, 95% CI: −0.927 to 0.974), suggesting that thiamethoxam does not inhibit or promote feeding behaviour. However, queens that had hibernated for longer consumed more syrup post-hibernation (mean ± s.e. daily syrup consumptions: 0.805 ± 0.031 ml (long hibernation) and 0.527 ± 0.036 (short hibernation); estimate: −0.278, 95% CI: −0.373 to −0.183). Consequently, the average daily amount of active ingredient consumed by pesticide-treated queens in the long hibernation group was 1.977 ng day^{-1}, compared with 1.405 ng day^{-1} in the short hibernation group. Again, there were no effects of parasite treatment or any of the interactions on syrup consumption and, therefore, these factors were not included in the final model. The average daily syrup consumption for queens in each treatment group is shown in Supplementary Table 2.

Survival and weight loss. The only factor that predicted survival during hibernation was the initial weight of the queen (estimate: −7.195, 95% CI: −12.159 to −2.231), with heavier queens being more likely to survive. Post-hibernation queen survival was not predicted by any experimental treatments.

Queens lost more of their body weight after a long hibernation (mean weight loss ± s.e.: 17.2% ± 0.50) compared with a short hibernation (11.8% ± 0.45; estimate: −5.379, 95% CI: −6.700 to −4.059). Parasite exposure also caused an increase in weight loss, although it had a much smaller effect (mean weight loss ± s.e.: 15.19% ± 0.55) compared with controls (13.86% ± 0.53; estimate: 1.323, 95% CI: 0.006 to 2.641).

Population modelling. To extrapolate our results to field populations, the experimental procedure described above would, ideally, be carried out on populations in the field. This is practically unfeasible. To overcome this problem and to demonstrate how estimates can

Table 2 | Summary of the models used in the analysis of data on the impact of the three treatments on *B. terrestris* queens

	Model types	Fixed factors	Random factors	R packages used	Final/composite model			95% CI	
					Parameter	Estimate	s.e.	Lower	Upper
Presence or absence of egg laying	GLM; GLMM	Hibernation; pesticide; parasite; thorax	Qcolony; Mcolony	lme4	Intercept	5.969	5.200	−4.171	16.110
					Hibernation	−1.514	0.316	−2.131	−0.898
					Pesticide	−0.628	0.313	−1.240	−0.017
					Thorax	−0.990	0.582	−2.124	0.144
Timing of egg laying[a,b]	Cox regression	Hibernation; P1; P2; parasite; thorax	Qcolony; Mcolony	survival	Hibernation	−1.044	0.233	−1.499	−0.590
					P1	1.400	0.577	0.275	2.525
					P2	−0.573	0.236	−1.034	−0.112
					Infection	0.096	0.228	−0.349	0.540
					Thorax	−0.553	0.378	−1.291	0.185
Presence or absence of adult offspring[c]	GLM; GLMM	Hibernation; pesticide; parasite; thorax	Qcolony; Mcolony	lme4	Intercept	−0.750	0.360	−1.453	−0.048
					Pesticide	1.214	0.458	0.320	2.107
					Hibernation	0.963	0.495	−0.003	1.928
Weight lost during hibernation[d]	lm; lme	Hibernation; parasite; thorax	Qcolony; Mcolony	nlme	Intercept	17.895	3.284	11.491	24.298
					Hibernation	−5.379	0.677	−6.700	−4.059
					Parasite	1.323	0.676	0.006	2.641
					Thorax	−0.658	1.204	−3.006	1.691
Hibernation survival	GLM; GLMM	Hibernation;parasite; thorax; pre-weight	Qcolony; Mcolony	lme4	Intercept	2.355	1.597	−0.759	5.470
					Pre-weight	−7.195	2.546	−12.159	−2.231
					Parasite	0.447	0.497	−0.522	1.415
					Hibernation	0.778	2.786	−4.655	6.211
					Pre-weight × hibernation	−6.945	5.606	−17.877	3.987
Post-hibernation survival[a]	Cox regression	Hibernation; pesticide; parasite; thorax	Qcolony; Mcolony	lme4	Null model				
Syrup consumption	lm; lme	Hibernation; pesticide; parasite	Qcolony; Mcolony	nlme	Intercept	0.802	0.037	0.729	0.874
					Hibernation	−0.278	0.049	−0.373	−0.183
					Pesticide	0.024	0.488	−0.927	0.974

Details of the models used are included, along with the model selection process (see Analysis in Methods for full details), the specific R packages used, and the parameters and estimates from the final or composite models. The importance and reliability of each parameter in the final models was assessed by checking the CIs. Those that did not cross zero were considered reliable and important to the model. [a]For the Cox regression models, random factors were included as frailty terms[60]. Model selection was undertaken as described for the mixed models. [b]The timing of egg laying was analysed using Cox regression with proportional hazards. Examination of the residuals showed that the pesticide factor did not meet the assumption of proportional hazards. To deal with this, the interaction between pesticide and time was considered, and separate hazard functions were calculated for P1 (the first 17 days) and P2 (from 17 days until the end)[60]. These two interaction terms were included instead of pesticide in the model selection process. [c]The analysis included egg layers only. [d]For analyses during hibernation, the fixed factor 'parasite' indicates the exposure of queens to the parasite or a control (as the infection status was unknown at this stage). All other analyses including 'parasite' used data on the infection status (whether the queen was successfully infected or not). GLM, generalized linear model; GLMM, generalized linear mixed model.

be obtained for the effect of pesticide in the field, we integrated our results with existing data using a Bayesian framework to assess their implications for population sustainability.

For a bumblebee population to survive in the natural environment, each year a colony should produce, on average, at least one daughter colony in the absence of density dependence. Making an analogy with metapopulation theory, we call the long-term average number of new colonies produced by an existing colony the 'colony capacity'[50]. This number depends on the chances of a new queen mating, surviving hibernation, finding a nest site and initiating a new colony, combined with the number of queens produced by the colony. The existing data on all these factors are limited and therefore we cannot know the value of the colony capacity with certainty. We used a Bayesian framework (see Methods) to integrate the published data on hibernation survival, colony initiation and new queen production, as well as the associated data uncertainty, to map the dependence of these data on the probability of queens successfully mating and finding nest sites in the wild (p_{nm})—a parameter for which no empirical data are currently available. This allowed us to capture the existing information on bumblebee biology and integrate it with our results while quantifying the certainty we have about the value of the colony capacity. We estimated the probability that colony capacity takes a certain value (Fig. 3a) and how this value would change taking into account the effect of thiamethoxam on colony initiation (Fig. 3b).

For bumblebee populations to persist, the colony capacity must be at least 1 in natural environments. The data we used for the number of new queens produced by a colony came from one study and one year[51]. Population persistence requires the colony capacity to be at least 1 when suitably averaged over both good and bad years. Even though we lack information about the extent of annual variability in colony capacity, the data we used came from a favourable location (Swiss meadows) and from a year that was favourable for bumblebee reproduction (see Methods). As the Swiss and broader European *B. terrestris* population is extant, the colony capacity in the year from which these data were collected

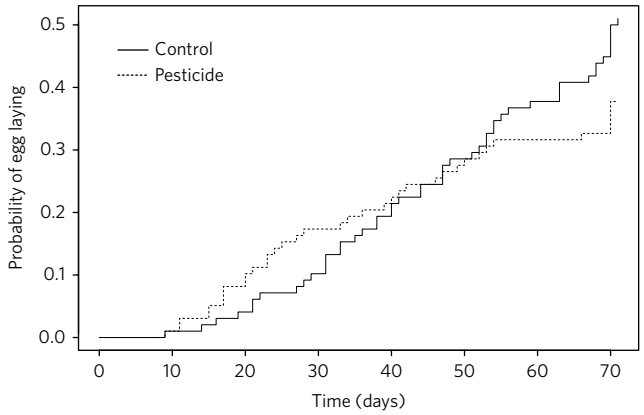

Fig. 2 | Effect of pesticide exposure on egg laying. Event history curve showing the cumulative probability of egg laying (from the end of hibernation (0 days) until the first egg was laid) by *B. terrestris* queens exposed to either the pesticide thiamethoxam or a control. Total sample sizes for each treatment group were: control, $n = 98$; and pesticide $n = 99$ (includes queens that survived the entire experiment only).

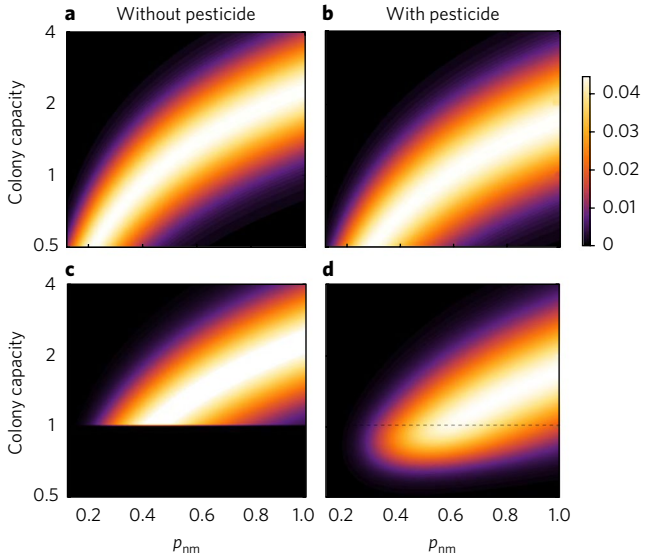

Fig. 3 | Effect of pesticide exposure on colony capacity. a–d, Heat maps showing the likelihood profiles for the colony capacity taking certain values without (**a**,**c**) or with thiamethoxam exposure (**b**,**d**). Each heat map represents the probability distribution (normalized likelihood) of the colony capacity (where lighter colours indicate a higher probability; see the scale to right of the graphs) as a function of p_{nm} and the colony capacity itself. The colony capacity is the multiplication of p_{nm} with the number of gynes produced[51], the probability of surviving hibernation[45] and the probability of being able to initiate a colony following survival. **a**, Probability that the colony capacity takes a certain value for different values of p_{nm} (using data from[45,52]). **b**, Likelihood profile of the colony capacity after exposure to thiamethoxam; the distribution is lower than in **a** due to the reduction in colony initiation (as found in our empirical results; see Supplementary Material for methodology). **c**, Likelihood profile of the colony capacity without exposure to thiamethoxam, conditioned to take a value of at least 1 to take account for the natural bumblebee population of *B. terrestris* being extant. The colony capacity must therefore be at least 1. The likelihoods for the colony capacities below 1 were therefore set to 0 and the profile was re-normalized. **d**, Likelihood profile for the colony capacity as in **c** after the effect of thiamethoxam exposure was taken into account. Because of the reduction in colony initiation caused by thiamethoxam exposure, colony capacities below 1 have positive probability. The total probability of a colony capacity below 1 is found by integrating all probabilities below the dashed line in **d**.

must, therefore, have been at least 1. Using our Bayesian framework, we added this information to our estimate of the probability of the colony capacity taking a certain value.

Figure 3c shows the probability heat map for colony capacity, accounting for the fact that colony capacity must exceed 1 in self-sustaining natural populations. At a minimum, the colony capacity must exceed 1 in good years. As noted above, the data we used represent a favourable year, and therefore we set the likelihood of colony capacities below 1 to 0 in Fig. 3c. After adding the impact of thiamethoxam exposure on colony initiation, values of colony capacity below 1 became likely (Fig. 3d). After adding up all the probabilities for colony capacities below 1, the probability of a colony capacity with a value below 1 was 28%.

If the colony capacity is less than 1, the population will eventually go extinct. We can therefore say that, based on these data, widespread thiamethoxam use would lead to eventual population extinction with a probability of at least 28%. This is likely to be a conservative estimate as it is based on the data taken in a favourable location in a year with good conditions and does not take the small probabilities of extinction for colony capacities exceeding 1 into account. Note that for intermediate ranges of p_{nm}, there is a substantially higher probability of neonicotinoid exposure leading to population extinction.

Discussion

We show for the first time that exposure to field-relevant levels of the neonicotinoid pesticide thiamethoxam significantly reduces successful colony founding in *B. terrestris* queens. Two weeks of insecticide exposure resulted in a shift in the timing of colony initiation, and ultimately a 26% reduction in the proportion of queens that laid eggs by the end of the experiment. These results add significantly to our understanding of the impacts of neonicotinoid on a key life-stage in an essential agricultural pollinator. Including this 26% reduction in models of population dynamics indicated a dramatically increased risk of population extinction after pesticide exposure.

Interestingly, despite its overall negative effect, thiamethoxam exposure caused an increase in the number of queens laying eggs early in the experiment (Fig. 2). However, by day 40, colony initiation by pesticide- and control-treated queens had levelled off and by the end of the experiment (day 70) a higher proportion of control queens had laid eggs. There is evidence that individuals from various taxa respond to natural enemies by shifting reproductive effort earlier; for example, the snail *Biomphalaria glabrata* increases oviposition soon after exposure to a trematode worm[53], while *Daphnia* species lay larger clutches earlier when exposed to a microsporidian parasite[54] and mature at an earlier instar after exposure to predatory fish cues[55]. Such plasticity in life-history traits is thought to be an adaptive response to threats to survival or reproduction. Moret and Schmid-Hempel[56] found that bumblebee colonies shift reproduction earlier in response to immune challenges of the workers, as well as harsh conditions (lower temperature). This resulted in an increase in the production of sexual offspring early on in these treatment groups, followed by a drop compared with controls later in the experiment. Although this effect was seen at the colony level rather than the individual level, it shows that social insects such as bumblebees exhibit life-history changes in response to physiological stress. Our study is the first to suggest that similar processes may occur in response to pesticide exposure. Pesticides cause metabolic changes in honeybees, including the regulation of genes associated with immune function and detoxification[57,58]. It therefore seems likely that the shift in the

timing of colony initiation observed in the current experiment was a response to physiological stress from pesticide exposure.

The impacts of pesticides on reproduction have also been observed in bumblebee workers and queens[21,59,26] and solitary bees[34,60]. However, the mechanisms behind the reduced colony initiation in our study and the impacts on workers and solitary bees in previous studies remain unclear. If the metabolic cost of detoxification is high, this could lead to the reallocation of nutrients such as proteins, reducing nutrient availability for other biological processes (for example, ovary development). Studies of other taxa, such as the southern armyworm *Spodoptera eridania* have found that nicotine detoxification imposes a significant metabolic cost, leading to a reduction in growth[61]. It is known that honeybees and bumblebees can clear ingested pesticide rapidly[62]; however, the metabolic costs of the detoxification process in bees remain unknown.

We did not detect impacts of any of the experimental treatments on the ability of queens to produce adult offspring. However, when only egg-laying queens were considered, a higher proportion of thiamethoxam-treated queens had adult offspring by the end of the experiment. This is likely to have been due to a higher proportion of treated queens laying eggs early in the experiment, giving them more time to rear adult offspring. Given the temporal patterns in egg laying identified in our study, this pattern would most likely have reversed if the experiment had been continued beyond 14 weeks. Our results suggest that a pulse of thiamethoxam exposure after hibernation creates two populations of queens, with presumably weaker queens being effectively castrated, while stronger queens respond by bringing reproduction forward. Early initiation could have benefits, such as earlier access to foraging resources, a longer growth period and, thus, a higher reproductive output. However, it could also put colonies out of synchronisation with the broader population and the flowering times of key forage plants.

Our observations in the field indicate that bumblebee queens of multiple species forage on and around OSR crops that have been treated with a neonicotinoid seed treatment. Previous studies have shown that neonicotinoids are present in both the pollen and nectar of crops and forage plants in the surrounding area[63–65]. Bumblebee queens foraging in agricultural environments are therefore exposed to neonicotinoids while foraging in the early spring. The crops observed in this study had been treated with Modesto seed treatment, which contains clothianidin. In the laboratory trial, we tested the impacts of thiamethoxam (which is the most widely used neonicotinoid on OSR crops[66]). Neither thiamethoxam nor clothianidin are repellent to bumblebees[67], and we found no evidence for a reduction in feeding associated with thiamethoxam treatment in this study. The extent of pesticide exposure faced by bumblebee queens in the field, and how this impacts colony initiation under natural foraging conditions, requires further investigation.

A two-week exposure period was used in this experiment to represent the period of foraging immediately after hibernation and before colony initiation was likely to begin, and therefore specifically targeting queens rather than the developing brood[37]. However, wild foraging queens could be exposed to thiamethoxam and other pesticides for longer periods, and potentially well into the development of the colony. It would be useful to explore this further, as the impacts on colony initiation observed in this study may be further exacerbated if queens are exposed for longer periods or to a range of pesticides.

Using a Bayesian model for bumblebee population dynamics, we show that the impacts of pesticide exposure during colony founding carry a considerable risk of population extinction. Due to the lack of field data on mating success and the availability of nest sites (although given bumblebee sex ratios, at least mating success is likely to have a probability of 1; for example, refs [12,21]), we cannot give a precise prediction on whether or not populations will go extinct. However, our model shows that unless mating success and finding a nest site are assured, reductions in colony initiation resulting from thiamethoxam exposure are likely to lead to appreciable probabilities of population failure. Given the landscape of pesticide use, this could lead to local extinctions and drive a source–sink network in bumblebee populations. Detailed population genetic studies would be required to test for such a large-scale impact.

Queens that underwent a 12 week hibernation period were more likely to lay eggs compared with queens that underwent a 6 week hibernation period. This has been observed previously in laboratory studies[46,49]. Wild *B. terrestris* queens in the UK are likely to need to hibernate for up to 6–9 months through the winter[44] and this may reflect an adaptation allowing them to perform better under longer hibernation conditions. The relatively short hibernation periods used in the current study were chosen to maximize sample size for the pesticide phase of the experiment. Queens that undergo a longer, more stressful hibernation in the wild may therefore be even more susceptible to the effects of pesticide exposure. This hypothesis is supported by observations that queens exposed to neonicotinoids were more likely to die after longer hibernation periods of up to four months and lost weight more rapidly over this period[40]. None of the treatments or covariates measured in this study had an impact on the survival of queens after hibernation, although queens that were heavier before hibernation were more likely to survive until the end of hibernation, as was found in a number of previous studies[45,46]. Neonicotinoids were found to have an impact on queen survival in several previous studies[24,40,68]. However, in these cases, this effect was seen at much higher doses (20 ppb or higher in Scholer and Krischik[68]), during hibernation[40] or much later in the colony lifecycle[24]. Nevertheless, the current study was conducted under optimal laboratory conditions making our results conservative, and more stressful field conditions may increase impacts the impacts of pesticides.

Exposure to *C. bombi* resulted in greater weight loss during hibernation, as was the case in Brown, Schmid-Hempel and Schmid-Hempel[49]. However, there were no parasite impacts on the other traits measured. Brown, Schmid-Hempel and Schmid-Hempel[49] found an impact of *C. bombi* on colony founding and development, with fewer infected queens initiating a colony, and those which did producing fewer workers, males and gynes. However, the shorter hibernation period used in the current study is likely to explain this difference.

These results provide the first evidence that chronic exposure to thiamethoxam reduces colony initiation by bumblebee queens, with knock-on effects for population sustainability and extinction. In addition, there is considerable evidence that neonicotinoids can impair the development of bumblebee colonies and reduce the number of queens produced later in the lifecycle[22,24,28,29,34,35], which could additionally deplete populations. Further research is needed to explore the long-term impacts of the observed reduction in egg laying on colony success and population dynamics in the field. However, imminent policy decisions relating to the use of neonicotinoids should take into consideration the timing and mode of application of these compounds, the organisms and—crucially—lifecycle stages, which are likely to be impacted by exposure.

Methods

Are bumblebee queens exposed to neonicotinoids in the field? Two visits were made to two winter OSR (*B. napus*) fields (variety PR46W21) at Shiplake Farm, Oxfordshire, United Kingdom (51° 30' 15.2" N, 0° 54' 00.7" W) during early April 2014 when the crop was in flower. Crop seeds had been treated with Modesto seed treatment (clothianidin and β-cyfluthrin; Bayer Crop Science) and planted the previous year. Transects around the edge of the fields (distance around each field: 2 km and 0.94 km) and through the centre of the crop (0.3 km and 0.4 km respectively) were walked between 11:00 and 15:00 on days when weather conditions were suitable (sunny and dry with minimal wind). Transects were walked once per visit at a steady pace (total walking time per visit: 3 h) and all bumblebee species within 2 m of the transect were recorded, along with the caste

and activity of each bee. Queens of the *B. lucorum* complex (*B. lucorum*, *Bombus cryptarum* and *Bombus magnus*) cannot be reliably separated using morphological features alone[69], and so these were recorded as *B. lucorum* complex.

Impacts of multiple stressors on *B. terrestris* queens. *Colonies.* Fifteen *B. terrestris audax* colonies were obtained from Koppert. Colonies were kept in the laboratory in darkness at 22 °C and a red light was used for colony manipulation. Colonies were fed ad libitum with 50% Ambrosia (EH Thorne), an inverted sugar syrup solution (herein referred to as syrup) and frozen honeybee-collected pollen pellets (Koppert). On arrival, 10% of the workers from each colony were dissected and screened microscopically for the parasites *C. bombi* (Trypanosomatidae), *Nosema bombi* (Microsporidia) and *Apicystis bombi* (Neogregarinida) using a Nikon eclipse (50i) compound microscope at 400× magnification. No parasite infections were detected at this stage.

Mating. Males and gynes (reproductive females) were removed from colonies as callows (newly emerged bees) and kept communally in single-sex wooden boxes (24 cm × 14 cm × 10.5 cm) with nest mates of the same age and fed ad libitum with pollen and syrup.

Four days after eclosion, gynes were mated with unrelated males of at least four days of age. Mating took place in a 60 cm × 50 cm × 50 cm wooden framed arena with plastic mesh sides under natural light at a temperature of 22 °C. Up to 25 males from a single colony were placed into the arena and left to acclimatize for 10 min. Unrelated gynes from another single colony and age group were then added to the arena. Mating pairs were removed from the arena immediately and the time, date, male and female colony, and age were recorded. Once mating was complete, the male was removed and frozen at −20 °C. The mated queen was kept in an individual plastic box (13 cm × 11 cm × 6.8 cm) containing a small amount of tissue paper to remove excess moisture and immediately provided with 100 µl of inoculum (see below for inoculum preparation). When this full amount had been consumed, the queen was provided with ad libitum food (pollen and syrup) for between two and four days after mating (depending on how quickly the inoculum was consumed), at which point it was weighed and placed into hibernation (see below). Queens that did not consume the full amount of inoculum within four days were excluded from the experiment.

Gynes that did not mate on the first attempt were kept in their communal boxes as described above, and further mating attempts (up to five attempts per gyne) were made (with different groups of males), until mating took place. Males were also kept until mating had occurred, and mating attempts continued until males were two weeks of age, at which point they were frozen at −20 °C.

Preparation and delivery of *C. bombi* inoculum. *C. bombi* was obtained from naturally infected wild *B. terrestris* queens collected from Windsor Great Park, Surrey, United Kingdom (51° 25′ 05.9″ N, 0° 36′ 19.5″ W) during the spring of 2013. Queens were also screened for *N. bombi*, *S. bombi* and *A. bombi* and any queens co-infected with these parasites were removed. *Crithidia*-infected queens were placed in the laboratory in Perspex queen-rearing boxes (13.3 cm × 8 cm × 5.6 cm) with ad libitum syrup and pollen, and kept in a dark room at a constant temperature of 28 °C and 50% humidity (conditions suitable for colony initiation). Eleven naturally infected queens (and their colonies in six cases) were available at the start of the experiment; 10 µl of faeces was collected from each of these, combined and used to infect 20 stock worker bees collected from each of the experimental colonies. This ensured that a wide range of naturally occurring strains of *C. bombi* was available for the infection of experimental queens. All collected faeces was combined and diluted with 0.9% Ringer's solution to make 1 ml of solution. *C. bombi* cells were filtered using a modified protocol for purification[23] originally developed by Cole[70]. This process was repeated using wild-caught queens from the same population that were not infected with *C. bombi*, *A. bombi*, *N. bombi* or *S. bombi* to provide a control.

The stock bees were taken from the experimental colonies to account for any filtering of the parasite strains by workers before infection of the experimental queens[71]. Workers were removed from each colony and starved for a period of four hours. Each stock bee was then individually fed a 10 µl drop of inoculum (containing 10,000 *C. bombi* cells) and observed until all the liquid had been consumed. These stock bees were then kept communally in wooden boxes with their nest mates and fed pollen and syrup ad libitum. The same process was repeated using faeces from the uninfected wild queens to create a control stock.

To make the inoculum for the experimental queens, an equal volume of faeces (10 µl) was collected from each box of stock bees on each day that inoculation took place. This was combined and purified as described above. The resulting solution was diluted with syrup and 100 µl of this inoculum (containing at least 20,000 *C. bombi* cells) was provided in a feeding tube for each queen. The same process was repeated using the *C. bombi*-free faeces from the control stock bees.

Hibernation. Mated queens (only those that had consumed the full volume of inoculum) were weighed, placed into 50 ml tubes (Falcon) with damp sterilized sand and kept in a dark incubator at a constant temperature of 4 °C for either 6 weeks or 12 weeks. After this hibernation period, the queens were removed from the tubes and re-weighed. Surviving queens were then placed into Perspex queen-rearing boxes (13.3 cm × 8 cm × 5.6 cm) with ad libitum syrup and pollen and kept in a dark room at a constant temperature of 28 °C and 50% humidity.

Table 3 | Summary of queen numbers allocated to the eight treatment groups in the experiment

Hibernation	Pesticide	Parasite	*n*	Infection	*n*	% infected
Long	Pesticide	Parasite	31	Infected	20	64.5
		Control	27			
	Control	Parasite	29	Infected	18	62.1
		Control	27			
Short	Pesticide	Parasite	30	Infected	23	76.7
		Control	30			
	Control	Parasite	28	Infected	22	78.6
		Control	29			

Hibernation (long: 12 weeks; short: 6 weeks), pesticide (exposure to thiamethoxam or no exposure) and parasite (exposure to *C. bombi* or no exposure) were the three treatments. The infection status indicates the number and percentage for each parasite group that was successfully infected by the end of the experiment.

Pesticide exposure. A total of 319 mated queens were placed into hibernation. Of these, 20 died during hibernation and a further 68 were excluded from the final analysis. Exclusion was due to a lack of replication for their natal colony (as a result of nest mates being lost ($n=60$)), accidental infection with *C. bombi* ($n=6$) and accidental death ($n=2$). The remaining 231 queens (from eight colonies) were allocated to either the pesticide or control treatment. The distribution of queens across the eight treatment groups is shown in Table 3.

Three days after emergence from hibernation, queens in the pesticide treatment group were provided with syrup containing 2.4 ppb thiamethoxam, which is equivalent to that found in stored nectar in bumblebee colonies foraging in agricultural environments in the UK[72] and significantly below mean levels reported in stored pollen from bumblebee colonies foraging in agricultural environments in the UK[73]. As established in the field trial above, bumblebee queens forage on neonicotinoid-treated OSR crops and are therefore likely to be exposed to these pesticides as they establish a colony in the spring.

Analytical standard thiamethoxam (Pestanal; Sigma–Aldrich) was mixed with Acetone (Fluka; Sigma–Aldrich) to give a stock solution of 100 mg ml^{-1}. Aliquots of this stock were diluted with syrup to give a final concentration of 2.4 ppb thiamethoxam. Acetone alone was diluted in the same way to provide a solvent control. Solution was freshly made on each day of the experiment. Samples of treated syrup from two dates in the experiment were collected and analysed for thiamethoxam residues using liquid chromatography–mass spectrometry (Food and Environment Research Agency). Average residues were found to be 2.5 ± 0.085 µg kg^{-1}.

Queens were provided with the pesticide-treated syrup (or acetone-treated syrup in the case of the control group) for 14 days, and the amount consumed by the queen during this time was measured twice (once after 7 days, at which point the feeder was replenished with fresh treated syrup, and again after 14 days) using a 25 ml measuring cylinder to an accuracy of 0.25 ml. The average evaporation rate was measured by keeping feeders ($n=10$) in empty rearing boxes for one week and calculating the volume lost during this time. Syrup consumption data were then corrected for evaporation. Queens were provided with ad libitum untreated syrup for the remainder of the experiment.

Post-hibernation monitoring. After hibernation, all queens were provided with a pollen ball (ground pollen pellets mixed with syrup to form a soft dough, shaped into a cylinder of approximately 1 cm in height and diameter) in which to lay their eggs and as a source of food. Unused pollen balls (which contained no eggs or brood) were changed twice a week to provide a source of fresh pollen for the queens. Pollen balls containing brood were left in the box and an additional pollen ball or dish of loose pollen was provided twice a week.

Queens were monitored daily for mortality and egg laying. All bees that died during the experiment were frozen at −20 °C on the day of death. The first date of egg laying (colony initiation) was recorded, as was the date that the first adult worker eclosed. Queens that had not initiated a colony ten weeks after emergence from hibernation were frozen at −20 °C. Queens that had a brood were kept for an additional four weeks to monitor the development of the brood into adult workers.

Each queen was checked for the presence of *C. bombi* (by microscopic examination of a fresh faecal sample) three times during the experiment. The first check occurred 4 days after the end of hibernation, the second 11 days after hibernation and the third 30 days after hibernation.

Dissection. All dissections were performed using a Nikon microscope (SM2800) at a magnification of ×10 to ×30. At the end of the experiment, all queens were

dissected and checked microscopically for the presence of *C. bombi* (as described for the parasite screening above). Queens were also screened for *N. bombi* and *A. bombi* to verify the earlier colony-screening results. Neither of these parasites was found at this stage.

Analysis. Models were constructed for each analysis using some or all of the following factors: hibernation (short or long), pesticide (pesticide or control), parasite (exposed to the parasite or not exposed) and infection (infected or uninfected; this was assessed through the four parasite checks. If *C. bombi* was detected during any of these, the individual was considered to be infected). The following covariates were also considered: pre-weight (pre-hibernation weight), post-weight (post-hibernation weight), weight loss (proportion of weight lost during hibernation) and thorax (thorax width). The natal colony of the queen and of her mate (QColony and Mcolony) were considered as random factors in mixed models and compared with equivalent models without random factors. In the analysis of egg laying and colony development, all queens that died during the experiment were excluded, as they had not been present during the entire 10 (or 14) week observation period. The details of each analysis are summarized in Table 2.

All analyses were performed in R (version 3.1.1; ref. [74]) using the packages lme4[75] and survival[76].

Model selection. To select the optimal model for each analysis, Akaike information criterion (AIC)c values (AIC values corrected for small sample sizes) were compared for a set of candidate models. First, mixed models with one or both of the random factors Qcolony and Mcolony were compared with equivalent models with no random factors[77]. This was used to decide the random structure used in further model selection (one random factor, both random factors or no random factors). Candidate models were then constructed including biologically meaningful combinations of the fixed factors listed above. These were compared with the null model (no fixed factors) and full model (all fixed factors). Two random factors—queen colony and male colony—were included in the initial comparisons, but did not improve the fit of any of the models and so were not included. Two-way interactions between treatments were considered, but due to lack of coverage three-way interactions were not. Interactions between covariates and treatments were included if data visualisation indicated that this may be useful. The AICc values were used (these were chosen over AIC values due to the small sample sizes) and the optimal model (with the lowest AICc) was selected. When AICc values for different models were within two units of the lowest, model averaging was undertaken[78] (except in cases for which the null model was among these, in which case the null was assumed to be optimal). Final models were verified graphically for fit and to ensure all assumptions had been met[52,77]. Interpretation of the importance of factors within the final models was based on the size of the estimate (the larger the estimate, the greater the effect size of that factor) and 95% CIs (those that did not cross zero were considered reliable and important to the model). Model selection tables are available in the Supplementary Tables 2–8.

Modelling methods. The colony capacity is the average number of colonies produced over one season. It is calculated as the product of the average number of gynes produced per colony taking a certain value, the probability of surviving six months in hibernation and consequently initiating a colony and the probability of successfully mating and finding a new nest site—the p_{nm} variable. Although we do not know the value of any of the components of p_{nm} with absolute certainty, several studies have quantified the success of various stages of the lifecycle. Baer and Schmid-Hempel[51] studied the number of gynes produced by colonies under field conditions. They found that 18 colonies produced 155 gynes—an average of 8.61 gynes per colony. We assumed that the number of gynes produced follows a geometric distribution with mean m_g (the mean number of gynes). The probability of surviving hibernation (which we called p_h) and the probability of being able to initiate a colony following survival (which we called p_c) were studied by Beekman et al.[46]. They found that out of 45 queens, 23 survived a 6 month hibernation period, from which 11 (of 23) initiated a colony. We assumed that both the number of survivors and the number of colony-initiating queens is binomially distributed. Here, we use this information to calculate the likelihood of the colony capacity taking a certain value.

Following emergence from the natal colony, gynes need to mate, survive hibernation and then find a nest site. p_{nm} has not been studied quantitatively; therefore, no information on the value of this parameter exists. Figure 3a shows the probability of the colony capacity taking a certain value.

Next, we estimated the effect of pesticide exposure on the population. In this study, the effect of thiamethoxam on queen survival after three months of hibernation was tested experimentally using control queens and queens treated with pesticide. Assuming that the numbers surviving were binomially distributed, we established the likelihood of the multiplicative effect of pesticide exposure.

To do this, we conservatively assumed that the effect of thiamethoxam on colony initiation after hibernation is the same for six months as it is for three months (in reality it is likely to be higher) and inferred the likelihood of this effect. Subsequently, we normalized the likelihood before combining the results to establish the probability of the colony capacity after neonicotinoid exposure taking a certain value (Fig. 3b). For intermediate values of p_{nm}, it can be seen that the colony capacity is likely to be reduced below 1 (Fig. 3d), which means populations will become extinct.

As *B. terrestris* populations have persisted in natural environments, we know the colony capacity of an extant population has to be at least 1. The expected number of colonies produced per colony can vary over the years and the information we have is restricted to an observation in a single year. However, even though we have no comparable data on the number of gynes produced, additional studies indicate that the productivity of *B. terrestris* in the same location was around 50% lower in a previous year[51,79]. This indicates that the data used here to estimate the colony capacity represent a relatively good year. We can thus reason that if the number of daughter colonies falls below one in a good year, a population cannot persist and, therefore, the persistence of *B. terrestris* in the wild tells us that our estimate of the colony capacity based on a good year has to be at least 1. We therefore conditioned the result on the colony capacity exceeding 1 by setting the probability of colony capacities below 1 to 0 and renormalizing (see Fig. 3c). We then took the effect of pesticide into account and calculated the probability of the colony capacity taking a certain value under pesticide exposure (Fig. 3d). The total probability of the colony capacity estimated to be to less than 1 was 28%. As we made several restrictive assumptions to reach this result it is very likely to be a conservative estimate.

Full details of these probability calculations are provided in the Supplementary Information.

Data availability. The data that support the findings of this study are available in Supplementary Data 1. The Mathematica notebook used to generate Fig. 3 is available from V.A.A.J. on request.

Received: 14 October 2016; Accepted: 30 June 2017;
Published online: 14 August 2017

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Acknowledgements

The authors thank S. Baldwin, B. McCrea, O. Ramos-Rodriguez and D. Wells for assistance in the laboratory and L. Evans, M. Fürst, C. Jones, E. Leadbeater, F. Manfredini, K. Smith and D. Stanley for comments. We thank The Crown Estate for permission to collect wild bumblebees at Windsor Great Park and S. Doble for permission to survey fields at Shiplake Farm. This study was supported by the UK Insect Pollinators Initiative grants BB/I000178/1 (awarded to N.E.R.) and BB/1000151/1

(awarded to M.J.F.B. and V.A.A.J.), funded jointly by the Living with Environmental Change programme, Biotechnology and Biological Sciences Research Council, Wellcome Trust, Scottish Government, Department for Environment, Food and Rural Affairs and Natural Environment Research Council, and a Natural Environment Research Council studentship to G.L.B. N.E.R. is supported as the Rebanks Family Chair in Pollinator Conservation by The W. Garfield Weston Foundation.

Author contributions

G.L.B., M.J.F.B. and N.E.R. conceived the project and designed the experiment. G.L.B. carried out the experiment and statistical analyses. V.A.A.J. carried out the modelling. All authors contributed to writing the paper.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available for this paper at doi:10.1038/s41559-017-0260-1.

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