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Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees

Dara A. Stanley¹, Michael P.D. Garratt², Jennifer B. Wickens², Victoria J. Wickens², Simon G. Potts², and Nigel E. Raine^{1,3}

¹School of Biological Sciences, Royal Holloway University of London, Egham, TW20 0EX, UK

²Centre for Agri-Environmental Research, School of Agriculture, Policy and Development, University of Reading, Reading, RG6 6AR, UK

³School of Environmental Sciences, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

Abstract

Recent concern over global pollinator declines has led to considerable research on the effects of pesticides on bees¹⁻⁵. Although pesticides are typically not encountered at lethal levels in the field, there is growing evidence indicating that exposure to field-realistic levels can have sub-lethal effects on bees affecting their foraging behaviour^{1,6,7}, homing ability^{8,9} and reproductive success^{2,5}. Bees are essential for the pollination of a wide variety of crops and the majority of wild flowering plants¹⁰⁻¹², but until now research on pesticide impacts has been limited to direct effects on bees themselves and not on the pollination services they provide. Here we show the first evidence that pesticide exposure can reduce the pollination services bumblebees deliver to apples, a crop of global economic importance. Colonies exposed to a neonicotinoid pesticide provided lower visitation rates to apple trees and collected pollen less often. Most importantly these pesticide exposed colonies produced apples containing fewer seeds demonstrating a reduced delivery of pollination services. Our results also suggest reduced pollination service delivery is not due to pesticide-induced changes in individual bee behaviour but most likely due to impacts at the colony level. These findings show that pesticide exposure can impair the ability of bees to provide pollination services, with important implications for both the sustained delivery of stable crop yields and the function of natural ecosystems.

Biotic pollination is required by a large proportion of crops worldwide¹, disproportionately including those with economically high values and nutritional content¹³. The contribution of pollination services to global agriculture has been steadily increasing and was estimated at \$361 billion in 2009¹⁴. In addition, animal-vectored pollination is required by an estimated 87.5% of all angiosperms to reproduce¹¹, making this process fundamental to the functioning of natural ecosystems. Therefore any threats to the delivery of pollination

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darastanley@gmail.com

Author contributions

DAS & NER conceived the project, DAS, NER & MPDG designed the research, DAS, JBW & VJW carried out the research, DAS, NER, MPDG & SGP contributed equipment for the research, DAS analysed the data, all authors were involved in writing the manuscript.

services could have serious consequences for both food security and wider ecosystem function. Neonicotinoid pesticides, the most widely used group of insecticides worldwide¹⁵, are implicated in the global declines of bee pollinators^{3,16}. Although previous work has shown that bumblebee foraging activity, colony growth and reproduction can be altered by sub-lethal exposure to neonicotinoid pesticides^{1,2,5-7}, all research on pesticide impacts has focussed on bees as the service providers, but has not assessed the pollination service itself. Therefore it is unknown whether pesticide exposure actually results in changes to the delivery of pollination services to crops and wild plants (for a discussion of potential mechanisms see¹⁷). This information is essential to assess the severity of pesticide effects on ecosystem services, and to inform actions to mitigate negative impacts.

Apples are an important global crop, with 75 million tonnes harvested from 95 countries in 2012 and an estimated export value of \$71 billion (Food and Agriculture Organisation statistics: <http://faostat3.fao.org>). Apple crops benefit from insect pollination with seed number, fruit set, fruit size and shape all improved with increased pollination services¹⁸. Bumblebees are major pollinators of apples¹⁹ and many other crops across the world¹², and are exposed to low levels of pesticides when foraging in agricultural areas. Here, we investigated how exposure to low, field-realistic levels of a widely used neonicotinoid insecticide (thiamethoxam) could affect the ability of bumblebees to pollinate apple trees. We pre-exposed colonies to 2.4ppb thiamethoxam, 10ppb thiamethoxam or control solutions (containing no pesticide: rationale for selecting pesticide concentrations and relevance of results are outlined in methods and supplementary materials) in their nectar source (artificial sugar water) for a period of 13 days (8 colonies per treatment, i.e. 24 colonies in total). Subsequently, colonies were brought to the field and allowed access to virgin apple trees of a dessert (Scrumptious) variety, along with trees of a polleniser (Everest) variety, in pollinator exclusion cages in which we observed both individual and colony level behaviour. At the end of the season, apples from tested trees were collected to assess pollination service delivery in terms of fruit and seed set.

When whole colonies were given access to apple trees we found an effect of insecticide treatment on visitation rates to apple flowers ($F_{2,86}=3.1$, $p=0.05$); colonies exposed to 10ppb pesticide provided lower visitation rates to apple flowers than controls (Fig. 1a; Extended Data Table 1). We also found an impact of treatment on the number of foraging trips from which bees returned carrying pollen ($\chi^2=9.65$, $df=2$, $p=0.008$), with fewer bees from colonies exposed to 10ppb pesticide returning with pollen than workers from control colonies (Fig. 1b). Apple abortion rate was affected by treatment ($\chi^2=5.94$, $df=2$, $p=0.05$) with trees pollinated by 2.4ppb pesticide exposed colonies aborting more fruit than controls (Fig. 2a), although overall levels of fruit set did not differ ($\chi^2=4.1$, $df=2$, $p=0.13$) and there was no difference in the proportion of trees that produced fruit among treatments ($\chi^2=1.2$, $df=2$, $p=0.55$). However, we found a significant effect of treatment on the number of seeds produced per apple, an indicator of fruit quality, ($\chi^2=8.27$, $df=2$, $p=0.02$); flowers pollinated by colonies exposed to 10ppb pesticide produced significantly fewer seeds than those pollinated by 2.4ppb colonies (Fig. 2b). These results show that colonies exposed to pesticide can deliver reduced pollination services to apple crops.

These colony level impacts could be explained by several mechanisms, including individual behavioural changes. Individual bees exposed to 10ppb pesticide spent longer foraging ($F_{2,57}=3.72$, $p=0.03$; Fig. 3a), visited more Scrumptious flowers ($\chi^2=12.79$, $df=2$, $p=0.002$) and switched more frequently between varieties during each trip ($\chi^2=11.32$, $df=2$, $p=0.003$; Fig. 3b; Extended Data Table 2), which suggests a modification of their floral preferences⁹. Neonicotinoids target neurotransmitter receptors in insects and, as well as causing neuronal inactivation²⁰, some have been shown to be partial neuronal agonists²¹; therefore increases in individual foraging activity may be explained by acute increases in neuronal activity causing hormesis (a biphasic response in which low levels of an otherwise toxic compound can result in stimulation of a biological process²²). However, we found no effect of treatment on whether flowers visited by these individual bees produced apples ($\chi^2=0.88$, $df=2$, $p=0.64$), showed higher rates of fruit abortion ($\chi^2=0.42$, $df=2$, $p=0.81$) or different levels of seed set ($\chi^2=0.11$, $df=2$, $p=0.95$). This suggests that bees exposed to pesticide must somehow be behaving differently on flowers, in a way that was not readily observable in our experiment (e.g. changes in stigma contact²³), such that increased visit frequency did not result in better pollination service delivery at the individual level.

Our results suggest that impacts on pollination service delivery are not due to individual behavioural modification, but instead are most likely due to changes in colony activity levels as evidenced by reduced floral visitation rates and pollen collection. Bees collecting pollen may be more effective pollinators as they can deposit more pollen on plant stigmas²⁴; therefore if pesticide-exposed colonies are collecting less pollen they are also likely to be depositing less on stigmas than bees from control colonies. While individual bees exposed to pesticides visited more flowers, overall pesticide exposed colonies provided lower visitation rates and collected less pollen, thus explaining why reduced pollination services were delivered. Gill & Raine⁷ found that control (untreated) bees improved their pollen foraging performance over time whereas imidacloprid treated bees became less successful foragers; foragers in our colony level experiment may have carried out multiple trips and become more experienced foragers, potentially explaining why we find impacts on pollen collection here but not in the individual level experiment. Interestingly, for almost all parameters measured in this study we found significant effects on both individual behaviour and colony level function following 10 ppb thiamethoxam exposure, but not at the 2.4 ppb level. This suggests that there are dose dependent effects that lie between these two exposure levels. Both these exposure levels are highly relevant as they are within the range measured in the field, but further work is necessary to elucidate the lowest level at which these effects become significant (for further discussion of rationale for exposure and relevance of results, see methods and supplementary information).

A 36% reduction in the number of seeds produced in apples pollinated by colonies exposed to 10ppb pesticide in comparison to control colonies has important agronomic implications for crop production. The number of seeds in apples is closely linked to fruit crop quality in most, but not all, varieties^{18,25} and the enhancement of fruit quality, particularly the proportion of Class 1 fruit, underpins the economic value of UK orchards²⁶: growers must typically thin out their apple crops making the quality of each fruit very important. Therefore impacts on seed set and fruit quality have direct implications for apple production value, and as seed set and fruit set are positively linked in many varieties reduced seed set

can have direct negative implications for fruit set and total crop yield^{26,27}. As certain apple varieties in the UK currently experience pollination deficits^{19,26}, mitigating the impacts of pesticides on bumblebee pollinators could improve pollination service delivery. Apple crops are visited by a wide variety of pollinator groups, and neonicotinoid pesticides differentially affect insect taxa^{4,28}. Apart from bumblebees, one of the other main pollinator groups that visit apple flowers are solitary bees¹⁹, and it has been suggested that pesticide sensitivity of solitary bees is likely to be higher than for larger, social species like bumblebees^{4,5,17,29}. Therefore, apple pollination in a field setting could be more vulnerable to pesticide exposure than measured here.

Bumblebees are essential pollinators of many important crops other than apples, including field beans, berries, tomatoes and oilseed rape¹⁷. If exposure to pesticides alters pollination services to apple crops, it is likely that these other bee pollinated crops would also be affected. Most importantly, the majority of wild plant species benefit from insect pollination services¹¹. Therefore reduced pollination by pesticide-affected colonies, as evidenced by reduced seed set, also has significant implications for pollination in wild systems. Many wild plant species are both self-incompatible and pollen limited³⁰, so any reduction in the delivery of pollination services could have substantial impacts on wild plant communities and therefore wider ecosystem function.

Concerns over global bee declines are strongly driven by the need for the essential pollination services they provide to both crops and wild plants. The use of neonicotinoid pesticides presents a potential threat to bee health and, although the evidence base reporting sub-lethal (behavioural) effects of pesticides on bees is mounting³, we have shown for the first time that there is also an important impact of pesticide exposure on the pollination services bees provide. This information provides a new perspective when trying to fully understand the trade-offs involved when using insecticides, showing both the potential benefits and the true costs of pest control options need to be considered.

Methods

Pesticide preparation

A stock pesticide solution was made by dissolving 100mg thiamethoxam (PESTANAL, Analytical Standard, Sigma Aldrich) in 100ml Acetone (1mg/ml). Aliquots of stock solution were added to 40% sucrose to create treatment solutions of 10µg/L (10ppb) and 2.4µg/L (2.4ppb) thiamethoxam. These concentrations were chosen as field-realistic; the lower concentration (2.4ppb) was based on thiamethoxam concentrations found in nectar pots of bumblebee colonies foraging in agricultural areas in the UK³¹ and in pollen collected by honeybees³², and the higher concentration (10ppb) is within the range measured in pollen and nectar and of a variety of treated crops^{33,34,35} and contaminated wild flowers^{35,36,37}, and has been used in previous studies examining impacts of another neonicotinoid (imidacloprid) on bee behaviour^{38,39}. A control solution was also made by repeating the process outlined above but using an aliquot of 10ppb acetone only (i.e. no pesticide).

Experimental setup

Twenty-four *Bombus terrestris audax* colonies were obtained from Biobest (Westerlo, Belgium) at the start of April 2014, each containing a queen and an average of 99 workers (range 57-133). Colonies were weighed on arrival to estimate the overall colony size, and each assigned sequentially to one of three treatment groups (2.4ppb thiamethoxam, 10ppb thiamethoxam and control) based on decreasing mass (but randomly assigned within block). Each day, three colonies (one from each treatment) were assigned to treatment groups, until after 7 days all colonies were receiving treated sucrose (16 colonies exposed to thiamethoxam and 8 to control solution). We chose this sequential exposure regime to mimic subsequent field testing and ensure all colonies had comparable durations of exposure to their treatment. Colonies were fed treated sucrose solution from a gravity feeder inserted at the base of the nest box. Feeders were initially refilled every 2-3 days, and then every 1-2 days when the colonies had grown significantly. Untreated, defrosted honeybee collected pollen was provided to colonies every 2-3 days. Colonies were exposed to treatments for an average of 13 days (range 12-15) prior to field testing. Before being moved to the field, colonies had access to a feeder containing sucrose (40%) in a laboratory flight arena for 48 hours to become accustomed to leaving the nest to forage. There was no difference in colony weights at the start (ANOVA: $F_{2,21}=0.091$, $p=0.91$) or end (ANOVA: $F_{2,21}=0.88$, $p=0.43$) of the experimental period, indicating no treatment effect on colony size.

Field testing

Cage experiments were carried out at Sonning Farm, University of Reading, UK. 100 apple trees of a commercial dessert apple (Scrumptious variety) were moved into holding pollinator exclusion cages in mid-March 2014 prior to flowering to prevent insect visitation. Field experiments began when trees were entering full flower in mid-April. Each day, one colony from each treatment was taken from the laboratory, placed individually in one of the three test cages and observed simultaneously (with one observer per cage) in a randomised block design (see below for details of observations). Each day a different treatment was assigned to each observer. Cages were $4.8 \times 2.1 \times 2.1$ m frames covered in polyethylene mesh (gauge size = 1.33mm, Fig. S1). Observations were carried out on 8 dry, bright days from 16–26 April 2014 spanning the peak flowering of apples (daily means: max temperature 16°C , rainfall 2.5mm). This flowering period limited the number of days on which testing could be carried out, and therefore the number of colonies that could be tested.

Individual level measurements—Each morning, three cages were populated with two virgin Scrumptious trees each from the holding cages (mean \pm SE = 130 ± 8.5 flowers per tree) as well as two polleniser trees (Everest variety, mean \pm SE = 305 ± 15 flowers per tree, Extended Data Fig. 1). The number of flowers of each variety was standardised across cages to ensure equal floral density, and 40 open and receptive Scrumptious flowers were marked with cable ties on each Scrumptious tree for subsequent estimation of pollination services (fewer flowers were marked on the last day of observations as there were no longer 40 full-bloom flowers – flower numbers on these days were noted). The nest boxes in each cage were then opened to allow a single worker to exit. This bee was observed for the duration of its foraging trip (until it attempted to return to the nest), or until 60 minutes had elapsed (Extended Data Fig. 2). The duration of the foraging trip, the number of flowers of each

apple variety visited, and the handling time for each flower visit was recorded using Etholog software (EthoLog: Behavioural observation transcription tool, University of Sao Paulo, Brazil, 2011). If the individual bee did not visit any flowers within the first 20 minutes, it was assumed not to be a forager and was captured, returned to the colony and another bee released. All bees that foraged were paint marked before they were returned to the colony to ensure the same individuals were not observed twice. This process was repeated until all cages had the same number of active foragers recorded (3–5 bees per colony each day). Individual level observations took place between 1000–1630hrs.

Colony level measurements—After individual level observations, the two focal Scrumptious trees in each cage were removed and replaced with two new virgin trees. Again we standardised the number of flowers of each variety across cages with 40 open and receptive flowers on each tree marked with cable ties. Colony boxes were opened to allow free entry and exit to all active bees for a period of 60 minutes. This time period was chosen to avoid over-pollination of test flowers based on pilot observations. Colony activity was monitored at the nest entrance using video cameras. After an initial 10-minute period to allow the bees to become accustomed to the setup, four 10-minute focal observations were carried out on separate patches of Scrumptious flowers in each cage to estimate visitation rates. At the end of the 60-minute period, the Scrumptious trees were immediately removed to prevent further visitation. Colony level observations were carried out between 1430–1830hrs.

Estimation of pollination services—At the end of both the individual and colony observation periods, all test trees were returned to holding cages in which they were not visited by any other insects until apples were harvested at the end of the season. An initial assessment of fruit set from marked flowers (indicating flowers open during cage tests) was made at the end of May for all test Scrumptious trees to assess how many flowers were proceeding to fruit set stage (and how many aborted: Fig. 2a). Marked apples were collected on 27 August, and a final assessment made of the proportion of marked flowers that had produced mature fruit (Extended Data Fig. 2). In the lab, seed number was counted per apple for all collected fruit (274 apples from 96 trees across both experiments). Details of all data analyses carried out are given in the supplementary information.

Data analysis

Individual level—Measures of the number of flowers visited, numbers of switches between apple varieties, duration of total time in cage (from when the bee left the colony box until it returned/end of 60 minute period) and time taken to visit the first flower (latency) were recorded for all individual bees. For 68 of 93 bees observed (evenly distributed across cages and treatments) a number of additional response variables were also recorded including mean duration of the first 5 flower visits, number of inter flower intervals longer than 60 seconds, mean duration of flower visits, mean period of time between flower visits, length of time spent foraging (time between first and last flower visit) and total time spent on flowers (sum of durations for all individual flower visits). We tested for differences in these measures among treatments by constructing mixed effects models with pesticide treatment as a fixed effect. As several variables differed among days, including weather,

floral abundance and the identity of colonies used, day of testing was included as a random blocking factor in all models. Data were analysed in R version 3.1.0⁴⁰, using either linear mixed effects (LME) models with the lmer function in the nlme package for continuous data⁴¹, generalised mixed effects (GLMM) models with Poisson distribution used for response variables that were counts using the glmer function in the lme4 package⁴², or the glmmPQL function in the MASS package⁴³ when data were overdispersed. Models were validated by plotting standardized residuals versus fitted values, normal qq-plots and histograms of residuals, and continuous response variables were logarithmically transformed ($\log(X + 1)$) if necessary to improve residual fit. If treatment was significant, Tukey's post hoc tests were performed using the glht function in the multcomp package⁴⁴.

To assess differences in apple production on trees visited by pesticide exposed and control bees, we examined a number of variables including the number of fruits produced at the start of the season (May) and at the end (September), the change in proportion of apples forming from marked flowers per tree between the start and end measures (fruit abortion levels) and number of seeds per apple (measured in early September; Fig. 2a). Models were run as described previously with treatment as a fixed effect, although the tree on which fruits were produced, the number of bees released and date of testing were included as random effects. As a number of trees produced no fruit, seed set data were analysed in two steps. Firstly, we tested whether there was a treatment difference in the number of trees that produced any fruit. Secondly, we tested for treatment differences in seeds per apple (a measure that only included trees that had produced some fruit).

Colony level—We tested for differences in colony activity levels (the combined number of entries and exits by workers to the colony box) and the number of bees carrying pollen among treatments using GLMM models in the MASS package⁴³, with Poisson distribution for count data. Treatment differences in flower visitation rate to Scrumptious trees were tested using LME models⁴¹. Date of testing was used as a random effect in all models, and models were validated as described above. Fruit abortion and seed set variables were analysed as described for the individual level experiment, using tree and date of testing as random effects.

Extended Data



Extended Data Figure 1.

An example of the experimental setup at the Sonning Farm field site; experimental pollinator exclusion cages containing a bumblebee colony (located in the corner of the cage) and potted experimental apple trees. Photos: Dara Stanley



Extended Data Figure 2.

An experimental bumblebee (*Bombus terrestris*) worker visiting an apple flower (left), and an example of an apple produced from a marked (yellow cable tie) apple flower (Scrumptious variety: right). Photos: Dara Stanley & Louise Truslove

Extended Data Table 1

Results from the colony level experiment, including means and standard errors per treatment, and summaries of models run. Significant differences ($p < 0.05$) are highlighted in bold.

Colony level	Mean \pm SE	Model summary						
		control	2.4ppb	10ppb		df	p	
<i>Activity</i>								
No. of bee visits returning with pollen	7.13 \pm 4.28	3.57 \pm 3.41	1.5 \pm 1.13	$\chi^2=9.65$	2	0.008	glmmPQL	
<i>Fruit set</i>								
End no. of fruit	3.44 \pm 0.99	3 \pm 0.85	2.25 \pm 0.60	$\chi^2=4.1$	2	0.13	glmer	
Proportion of trees producing apples	0.69	0.69	0.81	$\chi^2=1.2$	2	0.55	glmer	

Extended Data Table 2

Results from the individual level experiment, including means and standard errors per treatment, and summaries of models run. Significance differences ($p < 0.05$) are highlighted in bold.

Individual level	Mean \pm SE			Model summary			
	control	2.4ppb	10ppb		df	p	
<i>Behaviour</i>							
Mean duration of first 5 flower visits (secs)	12 \pm 1.3	18 \pm 2.7	16 \pm 2.3	F=1.97	2,58	0.15	lme
Total no. flower visited	83 \pm 15	97 \pm 14	125 \pm 22	$\chi^2=4.65$	2	0.1	glmmPQL
Total no. Scrumptious flowers visited	27.6 \pm 5.1	47.3 \pm 5.7	53.5 \pm 5.7	$\chi^2=12.79$	2	0.002	glmmPQL
Mean duration of flower visits (secs)	7 \pm 1	8 \pm 1.2	11 \pm 3.5	F=0.98	2,58	0.38	lme
Length of time spent foraging (time of last flower visit - time of first flower visit) (secs)	1157 \pm 231	1191 \pm 184	1856 \pm 217	F=3.72	2,57	0.03	lme
Duration of total time in cage (secs)	2041 \pm 239	2162 \pm 202	2383 \pm 204	F=1.338	2,84	0.27	lme
<i>Fruit set</i>							
End no. of fruit	1.38 \pm 0.52	2.06 \pm 0.80	3.13 \pm 1.17	$\chi^2=3.82$	2	0.15	glmer
Proportion of trees producing apples	0.44	0.5	0.56	$\chi^2=0.88$	2	0.64	glmer

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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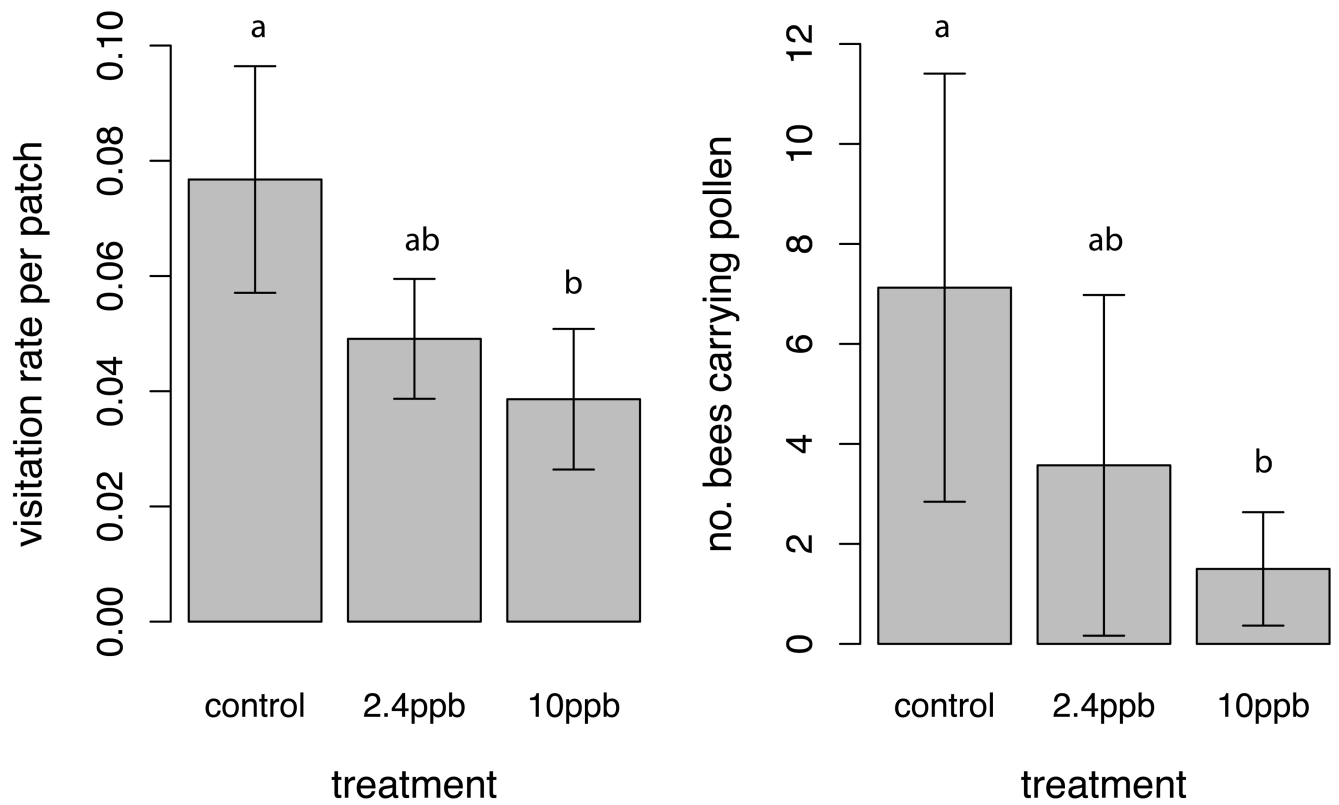


Figure 1.

Impacts of pesticide treatment on colony level behaviour. (a) Visitation rates provided by colonies to Scrumptious apple flowers (number of visits per flower per minute) and (b) number of foraging trips from which bees returned carrying pollen, from colonies exposed to different pesticide treatments. Means \pm SE are shown, and different letters indicate significant differences ($p < 0.05$).

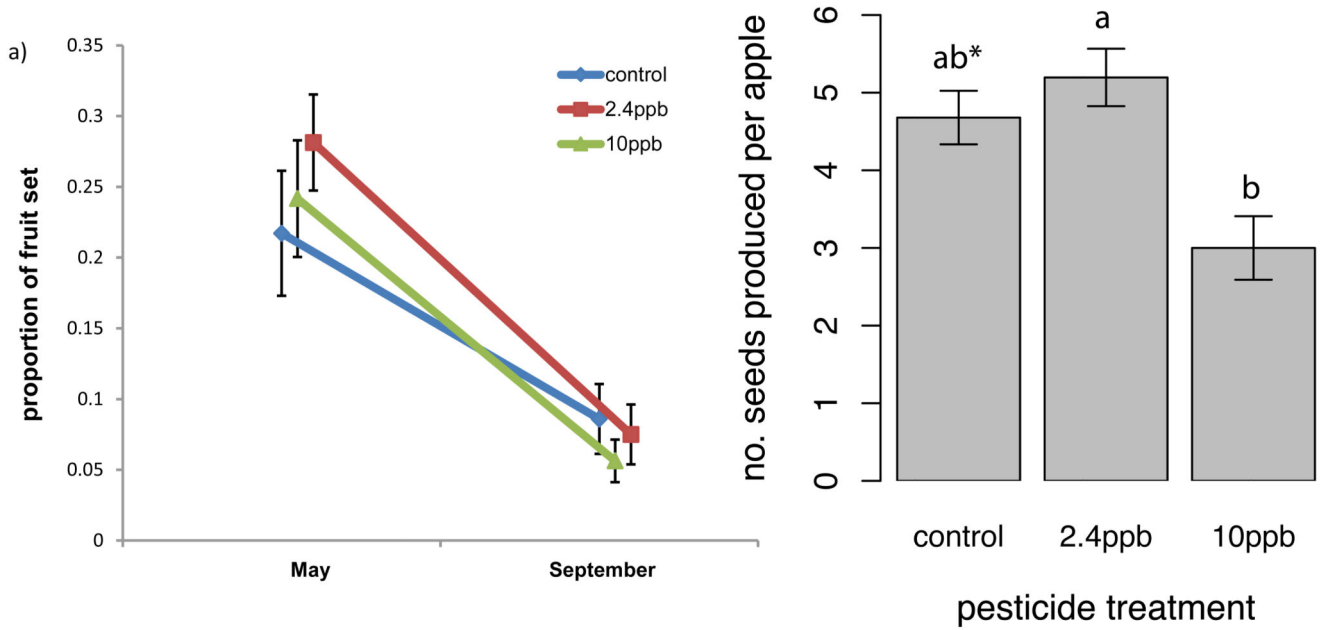


Figure 2.

Impacts of pesticide treatment on fruit and seed set. (a) The change in proportion of fruit set for trees pollinated by colonies exposed to different pesticide treatments measured early (May) and late (September), which represents fruit abortion level, and (b) number of seeds produced per apple pollinated by colonies exposed to different pesticide treatments. Means \pm SE are shown, and different letters indicate significant differences ($p < 0.05$, * indicates a difference of $p = 0.06$ between control and 10ppb).

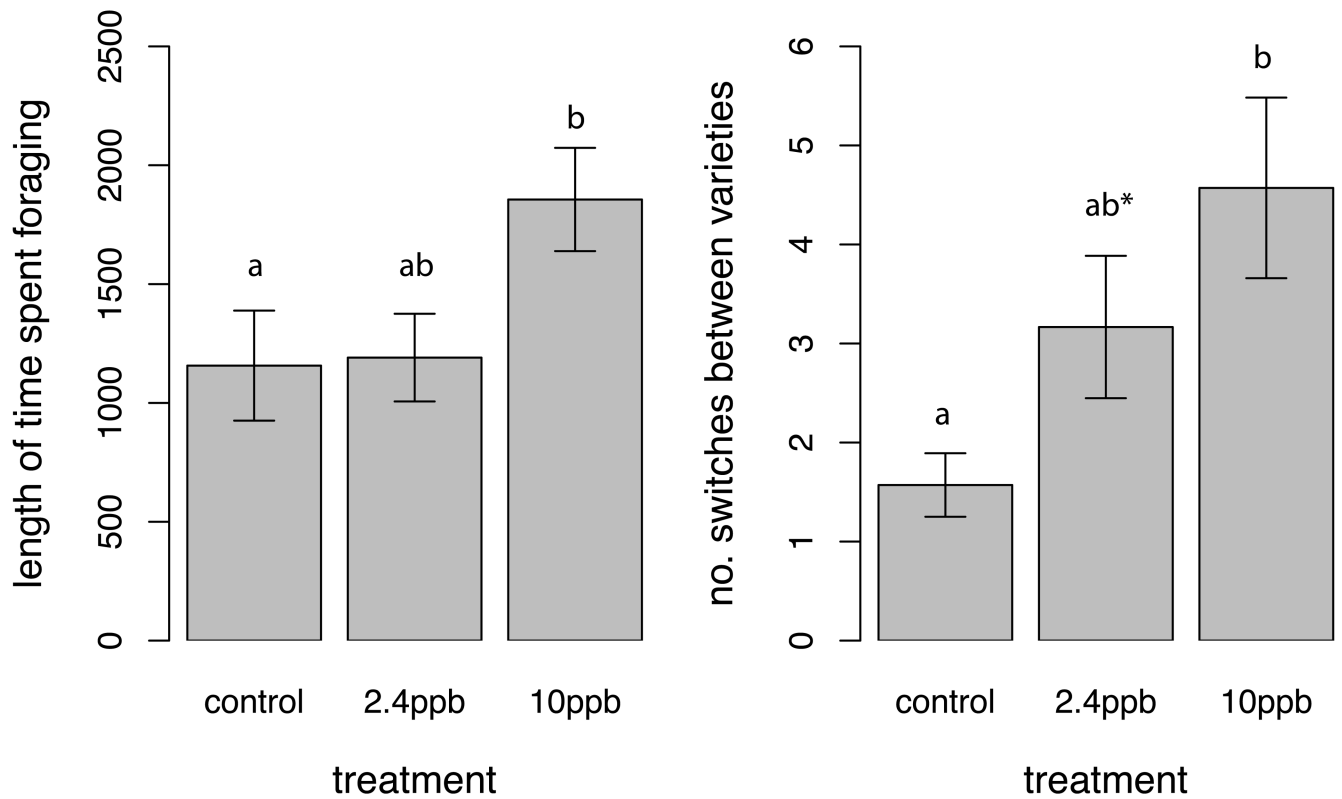


Figure 3.

Impacts of pesticide treatment on individual bee behaviour. (a) Time spent foraging per foraging trip (seconds; $n=68$ bees) and (b) number of switches between Scrumptious and Everest apple varieties ($n=93$ bees) of individual bees exposed to different pesticide treatments. Means \pm SE are shown, and different letters indicate significant differences ($p < 0.05$, * indicates a difference of $p=0.06$ between control and 2.4ppb).