LETTER

doi:10.1038/nature11585

Combined pesticide exposure severely affects individual- and colony-level traits in bees

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Reported widespread declines of wild and managed insect pollinators have serious consequences for global ecosystem services and agricultural production[1–3]. Bees contribute approximately 80% of insect pollination, so it is important to understand and mitigate the causes of current declines in bee populations [4–6]. Recent studies have implicated the role of pesticides in these declines, as exposure to these chemicals has been associated with changes in bee behaviour[7–11] and reductions in colony queen production[12]. However, the key link between changes in individual behaviour and the consequent impact at the colony level has not been shown. Social bee colonies depend on the collective performance of many individual workers. Thus, although field-level pesticide concentrations can have subtle or sublethal effects at the individual level[8], it is not known whether bee societies can buffer such effects or whether it results in a severe cumulative effect at the colony level. Furthermore, widespread agricultural intensification means that bees are exposed to numerous pesticides when foraging[13–15], yet the possible combinatorial effects of pesticide exposure have rarely been investigated[16,17]. Here we show that chronic exposure of bumblebees to two pesticides (neonicotinoid and pyrethroid) at concentrations that could approximate field-level exposure impairs natural foraging behaviour and increases worker mortality leading to significant reductions in brood development and colony success. We found that worker foraging performance, particularly pollen collecting efficiency, was significantly reduced with observed knock-on effects for forager recruitment, worker losses and overall worker productivity. Moreover, we provide evidence that combinatorial exposure to pesticides increases the propensity of colonies to fail.

The majority of studies to date have focused on pesticide exposure in honeybees, but bumblebees are also crucial pollinators and have smaller colonies, making them ideally suited to investigate effects at both the individual (worker) and colony level. This study mimicked a realistic scenario in which 40 early-stage bumblebee (*Bombus terrestris*) colonies received long-term (4-week) exposure to two widely used pesticides frequently encountered when foraging on flowering crops, the neonicotinoid imidacloprid and the pyrethroid λ-cyhalothrin. Imidacloprid is a systemic pesticide found in all plant tissues, including the pollen and nectar consumed by bees (oral exposure[18–20]). λ-cyhalothrin is sprayed directly on to crops, including their flowers, to which bees will be topically exposed (details in Supplementary Information). Foraging bees are thus simultaneously exposed to both chemicals in the field, making them excellent candidates to investigate the potential for combinatorial effects of pesticide exposure. Using a split block design (see Methods), we monitored colonies exposed to each pesticide independently and in combination (ten control colonies, ten exposed to imidacloprid (I), ten exposed to λ-cyhalothrin (LC) and ten exposed to I and LC (mix = M)). Imidacloprid (dissolved in 40% sucrose solution) was provided at a concentration (10 p.p.b. (parts per billion (10^9)) within the range found in crop nectar and pollen in the field[9,21]. λ-cyhalothrin was administered following label guidance for field-spray application (see Supplementary Information). Bees were able to forage in the field, providing a realistic and demanding behavioural setting, and the foraging behaviour of individual workers was recorded using radio frequency identification (RFID) tagging technology[10,11,22] (Supplementary Figs 1 and 2). Colonies were motivated to forage because we provided them with no pollen and limited amounts of sucrose solution.

During colony development, the production of workers (and their survival) is vital to colony success because workers provide the labour (for example, brood care and foraging) for the colony. Total worker production at the end of the experiment was significantly lower in imidacloprid-treated colonies (reduced by 27% in I and 9% in M colonies) compared to control colonies (mean ($\pm$ s.e.m.) workers per colony, I = 19.7 $\pm$ 3.0, M = 24.4 $\pm$ 3.2 versus control = 27.0 $\pm$ 4.0; linear mixed effects model (LMER), I, $Z = -3.71$, $P < 0.001$; M, $Z = -2.62$, $P = 0.009$; Fig. 1a). Two of the forty colonies, both M colonies, did not survive the experiment (they 'failed' after 3 and 8 days; see Supplementary Information), a colony failure rate significantly higher than other treatments (Fisher's Exact test: mid-P correction = 0.029). These two colonies were excluded from statistical analyses to provide a conservative assessment of worker production in M colonies (when included in analysis = 20.0 $\pm$ 3.9 workers). During the experiment, 223 (21% of total) workers were found dead inside nest boxes. On average, 36 $\pm$ 7.3% and 39 $\pm$ 7.5% of workers from LC and M colonies, respectively, died in the nest box; a figure four times higher than control (9 $\pm$ 3.4%) colonies (LMER, LC, $t = 4.31$, $P < 0.001$; M, $t = 4.23$, $P < 0.001$; Fig. 1b). Moreover, 43% of the workers found dead in LC and M colonies lived fewer than 4 days after eclosion—an apparent waste of resources required for future colony growth given that such young members are unlikely to have contributed any work (for example, foraging) to offset the resources invested to produce them. Queen loss occurred in 14 colonies, although loss rate did not differ significantly among treatments (control = 4; I = 5; LC = 2; M = 3; Fisher's exact test: mid-P-correction = 0.40) and we accounted for queen loss in our analyses (see Supplementary Information).

Daily counts of newly eclosed bees showed that worker production in I colonies did not become significantly lower than control colonies until the end of week 2, and for M colonies until the end of week 4 (Fig. 1c; see Supplementary Information and Supplementary Table 1). Daily counts of dead bees also revealed that worker mortality in LC colonies did not become significantly higher than that in control colonies until the end of week 3, but worker mortality in M colonies became significantly higher than that in control colonies as early as the end of week 1. The delayed effect of imidacloprid exposure on worker productivity in I and M colonies coincides with the time taken by workers to develop from egg to adult (approximately 22 days), suggesting that the observed effect is a result of imidacloprid on brood development. Indeed, the total number of larvae and pupae combined that were found in colonies at the end of the experiment ('brood number') was significantly lower in I and M colonies compared to control colonies (LMER, I, $Z = -6.23$, $P < 0.001$; M, $Z = -5.60$, $P < 0.001$). Overall, this represented a 22% reduction in brood production in I colonies and a 7% reduction in M colonies (mean ($\pm$ s.e.m.) brood number, I = 36 $\pm$ 8.0, M = 43 $\pm$ 11.7 (including failed colonies:

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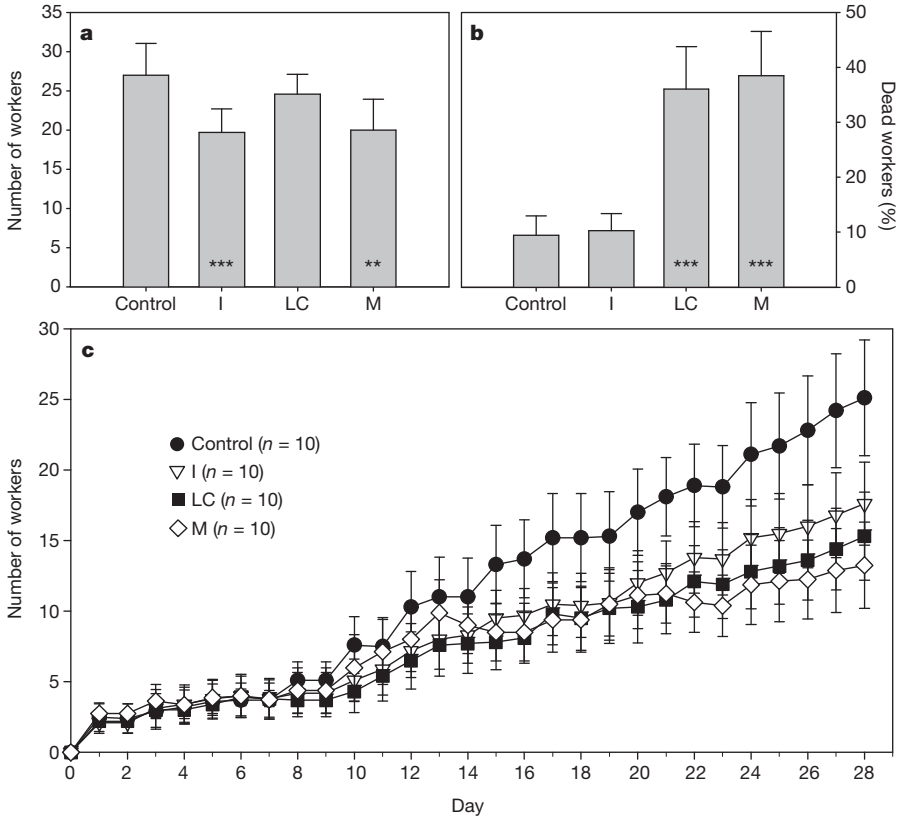

Figure 1 | Worker production and mortality. **a**, Mean (± s.e.m.) number of workers per colony that eclosed by the end of the experiment. **b**, Mean percentage of workers per colony found dead inside the nest box by the end of the experiment. **c**, Colony growth shown by daily counts of the cumulative number of workers eclosed minus the cumulative number of workers found dead (mean (± s.e.m.) per colony). Data shown on the *x* axis indicate the number of days since the start of the experiment (day 1 = 24 h after the start of experimentation). M treatment includes the two collapsed colonies. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (comparison with control).

M = 39 ± 9.6) versus control = 46 ± 9.7). Despite this, there was no significant difference in the mass of the wax nest structure (see Supplementary Information for details) across treatments at the end of the experiment (LMER, I, $t = -1.12$, $P = 0.27$; M, $t = -1.22$, $P = 0.23$; Supplementary Fig. 3) indicating that I and M colonies attempted to raise similar brood numbers but that a lower proportion of larvae and pupae survived to eclosion.

Although imidacloprid could be directly affecting brood (physiological) development, it could also indirectly affect the brood by causing changes to colony behaviour and/or structure: for example, changes to foraging behaviour leading to food limitation[23,24]. We tested this hypothesis by studying worker foraging performance using RFID technology to automatically record the exact time workers left or entered each colony (Supplementary Figs 1 and 2). Overall, we collected data from 259 recognized foragers from 32 colonies (*n* colonies: control = 7; I = 10; LC = 8; M = 7) making 8,751 foraging bouts (median (interquartile range) per worker = 23 (10–44); for criteria used to classify foragers and foraging bouts see Methods). We examined whether pesticide treatment affected foraging activity and forager recruitment. We found that foragers from M colonies performed fewer foraging bouts compared to control colonies (LMER, $t = -2.55$, $P = 0.011$; Fig. 2a), and that there were significantly more foragers in both I and M colonies compared to control colonies over the 4 weeks (LMER, I, $Z = 4.20$, $P < 0.001$; M, $Z = 3.49$, $P < 0.001$; Fig. 2a). The higher number of foragers in I and M colonies (compared to control) is unlikely to be due to either pesticide causing a significant repellent or anti-feedant effect (this corroborates the lack of published evidence for pyrethroid repellency in bumblebees despite reports of pyrethroids being repellent to honeybees[25]). This is because workers did not have to visit the feeder, as they could forage for nectar outside, yet we found no difference among treatments in the amount of sucrose collected from feeders (LMER, $t \leq 1.63$, $P \geq 0.11$; Supplementary Fig. 6).

Given that I and M colonies recruited higher numbers of workers to forage compared to control colonies, we evaluated whether this was a response to reduced individual foraging efficiency by monitoring pollen foraging performance and observing the size of pollen loads (load size scored as: small = 1, medium = 2, large = 3; see Methods) brought back by foragers ($n = 20$ h of observation per colony). Crucially, imidacloprid-exposed foragers returned with significantly smaller pollen loads per foraging bout compared to control colonies (LMER, I, $t = -3.31$, $P = 0.0011$; M, $t = -3.38$, $P < 0.001$; Fig. 2b). Imidacloprid-exposed foragers collected pollen successfully in a significantly lower percentage of their foraging bouts (mean (± s.e.m.), I = 59 ± 7.3%, M = 55 ± 8.6% versus control = 82 ± 5.8%; LMER, I, $t = -3.16$, $P = 0.0018$; M, $t = -3.05$, $P = 0.0026$; Supplementary Fig. 4) and we also found that the average duration of successful foraging bouts (during which pollen was collected) was significantly longer for imidacloprid-exposed foragers than for control foragers (LMER, I, $t = 2.10$, $P = 0.037$; M, $t = 2.87$, $P = 0.005$; Fig. 2c). Together, these data show that imidacloprid-exposed workers were significantly less efficient at collecting pollen in the field.

A consequence of recruiting a greater number of workers to forage is that it increases the proportion of colony workforce going outside to undertake a potentially hazardous task[22]. Indeed, our RFID data show the number of foragers per colony was significantly correlated with the number of workers leaving the colony and getting 'lost' outside (that is, workers that did not return: Spearman's Rank, $\rho = 0.801$, $P < 0.001$; Supplementary Fig. 5). Consequently, we found that on average the percentage of workers getting lost in I and M colonies was 50% and 55% higher than control colonies (I = 30 ± 3.1%, M = 31 ± 5.3% versus control = 20 ± 2.9%; LMER, I, $t = 2.83$, $P = 0.008$; M, $t = 2.26$,

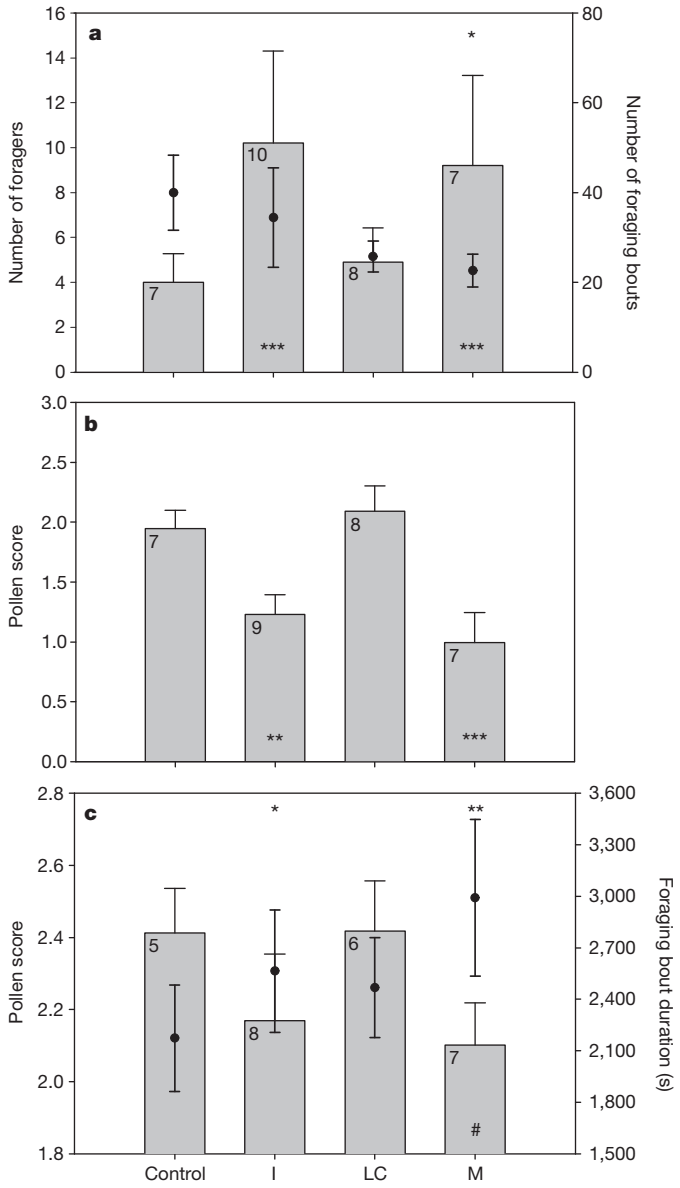

Figure 2 | Foraging performance. **a**, Mean (± s.e.m.) number of foragers per colony (column), and foraging bouts per worker per colony (filled circles: $n = 259$ foragers). **b**, Mean pollen score per worker per colony for all observed foraging bouts ($n = 228$ foragers). **c**, Mean pollen score per successful (pollen) foraging bout for each worker per colony (column), and mean duration of successful foraging bouts per worker per colony (filled-circles) ($n = 147$ foragers). *n* colonies shown in top left corner of columns. Significant differences from control treatment for column data are shown at the bases of columns, and for filled-circle data are shown above columns (**a** and **c**). $\#P \leq 0.1$, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (comparison with control).

$P = 0.03$). Furthermore, when considering worker mortality and losses combined over the 4 weeks (mean (± s.e.m.): I = 41 ± 4.2%, LC = 51 ± 6.8%, M = 69 ± 7.1% versus control = 30 ± 5.0%, LMER, I, $t = 1.79$, $P = 0.08$; LC, $t = 3.25$, $P = 0.0026$; M, $t = 5.24$, $P < 0.001$; Table 1 and Fig. 3), we found that colonies treated with both pesticides (M) suffered most severely. Moreover, M colonies had significantly higher overall worker losses than either I colonies (LMER, $t = -3.69$, $P < 0.001$) or LC colonies (LMER, $t = -2.31$, $P = 0.027$).

We have shown that imidacloprid exposure at concentrations that can be found in the pollen and nectar of flowering crops causes impairment to pollen foraging efficiency, leading to increased colony demand for food as shown by increased worker recruitment to forage. However, imidacloprid-treated colonies (I and M) were still unable to collect as much pollen as control colonies. Such pollen constraints, coupled with a higher number of workers undertaking foraging rather than brood care, seemed to affect brood development, resulting in reduced worker production that can only exacerbate the problem of having an impaired colony workforce. These findings show a mechanistic explanation to link recently reported effects on individual worker behaviour[10,11,26–29] and colony queen production[12] as a result of neonicotinoid exposure. Moreover, exposure to a second pesticide λ-cyhalothrin (pyrethroid) applied at label-guideline concentration for crop use caused additional worker mortality in this study highlighting another potential risk. Bee colonies typically encounter several classes of pesticides when foraging in the field[13–15], potentially exposing them to a range of combinatorial effects. Indeed, M colonies in our study were consistently negatively affected in all our measures of worker behaviour, suffered the highest overall worker losses (worker mortality and forager losses), which were twice as great as for control colonies, and two colonies did in fact fail (Table 1).

Table 1 | Summary of observed pesticide effects for each treatment group (I, LC or M) in comparison to the control group

Effect level	Effect type	I	LC	M
Effects on individual behaviour	Number of foragers	+	ND	+
	Foraging bout frequency	ND	ND	−
	Amount of pollen collected	−	ND	−
	Duration of pollen foraging bouts	+	ND	+
Effects at colony level	Worker production	−	ND	−
	Brood number	−	ND	−
	Nest structure mass	ND	ND	ND
	Worker mortality	ND	+	+
	Worker loss	+	−	+
	Worker mortality & loss	ND	+	+
	Colony failure (*n* failed/*n* survived)	0/10	0/10	2/8

Significant decrease (−), significant increase (+) and no detected effect (ND) at the 5% significance level.

Pesticide-label-guidance concentrations and application rates are approved on the basis of ecotoxicological tests using single pesticides and set at a level for field use deemed 'sublethal' (below a dose lethal to 50% of animals tested (LD_{50})). However, the risk of exposure to multiple pesticides, or of the same pesticide being applied to different (adjacent) crops, is currently not considered when evaluating the safety of pesticides for bees. Given the serious impacts on M colonies it is concerning that pesticide products containing mixtures of neonicotinoids and pyrethroids are in current use[18]. At present there are also no guidelines for testing chronic or sublethal effects of pesticides on

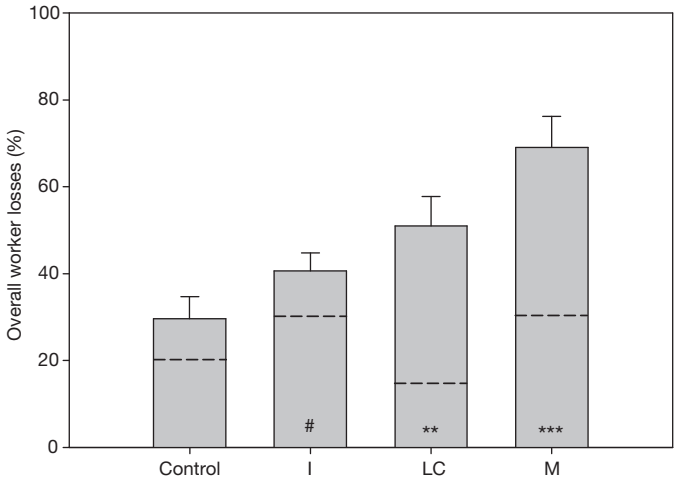

Figure 3 | Overall worker losses. Mean (± s.e.m.) overall percentage of workers lost per colony, including workers lost outside (below the dashed line) and worker mortality (dead workers found in nest box; above the dashed line), during the 4-week experiment. $n = 40$ colonies. $\#P \leq 0.1$, $**P \leq 0.01$, $***P \leq 0.001$ (comparison with control).

bees[30], and considering that we did not detect significant effects until 2 to 4 weeks into our study, the current European and Mediterranean Plant Protection Organisation (EPPO) and Organization for Economic Co-operation and Development (OECD) guideline of a maximum exposure of 96 h (for testing acute effects of pesticides on honeybees) appears to be insufficient. Our results emphasize the importance of recent recommendations by the European Food Safety Authority (EFSA) Panel on Plant Protection Products and their Residues (http://www.efsa.europa.eu/en/efsajournal/pub/2668.htm) proposing the need for longer term toxicity testing on both adult bees and larvae, new protocols to detect cumulative toxicity effects and separate risk assessment schemes for different bee species. Our findings have clear implications for the conservation of insect pollinators in areas of agricultural intensification, particularly social bees with their complex social organization and dependence on a critical threshold of workers performing efficiently to ensure colony success.

METHODS SUMMARY

Each colony contained a queen and ten or fewer workers at the start of the experiment, with no significant difference among treatments in worker number (Kruskal–Wallis: $H = 0.26$, $P = 0.97$). Colonies were housed in two-chambered nest boxes, with the rear chamber housing the nest and front chamber used for pesticide exposure (Supplementary Figs 1 and 6). Nest boxes were kept in the laboratory but connected via an outlet tube to the outside to allow natural foraging. Foraging activity of tagged workers was automatically recorded by RFID readers placed at the entrance to each nest box (Supplementary Fig. 2). The food chamber housed a feeder containing a specified volume (averaging 13ml) of control sucrose solution (control and LC) or 10 p.p.b. imidacloprid sucrose solution (I and M) provided every 2 to 3 days (Supplementary Table 2). The feeder was placed in a Petri dish lined with filter paper that was sprayed once at the start of each week with 0.69 ± 0.046 ml of control solution (control and I) or 37.5 p.p.m. (parts per million (10^6)) λ-cyhalothrin solution (LC and M). Workers walking across the filter paper to the feeder had contact exposure to λ-cyhalothrin (LC and M), and oral exposure to imidacloprid (I and M) when feeding. Colonies were not provided with pollen to motivate foraging behaviour. All workers were RFID tagged, with new workers tagged within 3 days of eclosion (Supplementary Fig. 2). We classified a foraging bout as a period of at least 5 minutes between a worker leaving and returning to a colony, and a forager as a worker that performed at least 4 foraging bouts. Pollen foraging was observed for 1 hour per colony per day (5 days per week) recording the presence and size of pollen loads collected (Supplementary Table 2). Colonies were frozen at the end of the experiment; the number of workers (and tag identifications) and brood was counted, and the mass of the nest structure was recorded.

Full Methods and any associated references are available in the online version of the paper.

Received 19 April; accepted 13 September 2012.
Published online 21 October 2012.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank M.J.F. Brown, M. Clook, J. Culverhouse, A. Dixon, M. Fürst, D. Garthwaite, A. Horsell, V. Jansen and I. Pedroso-Rovira for comments and technical assistance, and Syngenta Bioline Bees for supplying colonies. The study was supported by the Insect Pollinator Initiative (funded under the auspices of the Living with Environmental Change programme, Biotechnology and Biological Sciences Research Council (BBSRC), Wellcome Trust, Scottish Government, Department for Environment, Food and Rural Affairs (DEFRA) and Natural Environment Research Council (NERC): grant BB/I000178/1).

Author Contributions R.J.G., O.R.-R. and N.E.R. carried out the experiment; R.J.G. and N.E.R. designed the experiment and wrote the paper; N.E.R. conceived the project.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.J.G. (richard.gill@rhul.ac.uk) or N.E.R. (nigel.raine@rhul.ac.uk).

METHODS

Experimental setup. Each colony contained a queen and an average of four workers (range = 0–10) at the start of the experiment, reflecting the development stage of natural colonies when crops tend to flower in Europe[31,32], and when most pesticide treatments are applied (March to June)[33,34]. We used a split block design to account for variation in colony size, developmental stage and potential seasonal variation between replicates (20 colonies in July, and 20 colonies in September: see Supplementary Information). For each replicate, colonies were ranked according to the number of workers and pupae, with the 4 highest-ranked (largest) colonies assigned to block 1, the next 4 highest ranked to block 2, and so on. Each replicate consisted of 5 blocks ($n = 20$ colonies). Within each block the 4 treatments (control, I, LC and M) were randomly assigned among the 4 colonies. There was no significant difference among treatments in either the number of workers or pupae present at the start of the experiment (Supplementary Information). Colonies were provided a two-chambered nest box; the rear chamber housing the nest ('brood chamber') and front chamber used for pesticide exposure ('food chamber'; Supplementary Figs 1 and 6). Nest boxes were kept in the laboratory but connected to the outside environment through an outlet tube leading to an exit hole in the laboratory window, allowing natural foraging (for details see Supplementary Information and Supplementary Fig. 1). Between the outlet tube and nest box were three sections of transparent tubing allowing observation of bees as they left or entered nest boxes (Supplementary Fig. 2). Two RFID readers (Maja IV reader modules with optimized antenna for mic3 transponders: Microsensys GmbH) at the nest entrance allowed automatic monitoring of all tagged workers as they entered and left the colony with minimal disturbance to natural foraging patterns[22].

Pesticide treatment. Bees were exposed to pesticide treatments in the food chamber using a gravity feeder placed on a Petri dish (90 mm diameter) lined with filter paper. The filter paper was sprayed with 0.69 ± 0.046 ml of either control solution (control and I) or 37.5 p.p.m. λ-cyhalothrin solution (LC and M); the maximum label-guidance concentration for spray application to oilseed rape in the United Kingdom. The gravity feeder contained either a control sucrose solution (control and LC) or 10 p.p.b. imidacloprid sucrose solution (I and M). This concentration falls within the range found in the pollen and nectar of flowering crops visited by bees[9,20,21,35–38] (for details on pesticide selection and application see Supplementary Information and Supplementary Box 1). During the experiment the sucrose treatment was applied every 2 days (3 days over weekends) between 13:00 and 14:00 (Supplementary Table 2). Before refilling feeders we measured the volume of any remaining solution to calculate what the bees had collected ($n = 12$ feeder replenishments per colony during the 28-day period). We provided 10 ml of sucrose treatment per application in week 1, with a 2-ml incremental increase in the volume of sucrose at the start of each subsequent week (week 2 = 12 ml, week 3 = 14 ml, week 4 = 16 ml) to reflect an increase in colony demand as they developed. The amount of sugar provided was less than each colony typically collects by nectar foraging[39], ensuring that workers were motivated to forage for nectar and pollen outside.

Spray treatments were applied once at the start of each experimental week (Supplementary Table 2) using a new piece of filter paper for each application. This follows label guidance for the maximum application of λ-cyhalothrin to crops that recommends at least 7 days between spraying events and a maximum of 4 applications within the flowering season.

Observations and measurements. To monitor colony condition and development, colonies were inspected every day to assess the number of newly eclosed (callow) workers, the number of dead workers (removed and frozen (–20 °C)), and queen condition. Three days before the start of the experiment faecal samples from each queen were checked for the presence of three parasites: the trypanosome *Crithidia bombi*, the microsporidian *Nosema bombi* and the neogregarine *Apicystis bombi*. This parasite assessment was repeated on the twenty-eighth experimental day using faecal samples from the queen (if present) and a subset of workers from each nest box (for details of parasite assessment see Supplementary Information).

To monitor foraging performance, all workers present at the start of the experiment (precise age unknown) were individually RFID tagged (for details see Supplementary Information), and during the experiment all newly produced workers were tagged within 3 days of eclosion (age known). Tagging stopped on the twenty-fourth day of the experiment because any workers emerging after this point were unlikely to become foragers[40]. In total, 854 workers were tagged, with each tag providing a unique (16-digit) code for unambiguous identification. We classified a foraging bout as a period of at least 5 minutes elapsing between a worker leaving and entering a colony. We also specified that workers must perform at least four foraging bouts to be considered a forager (for the rationale behind foraging rules see Supplementary Information).

Pollen foraging was observed in each colony for 1 hour per day (5 days a week) to record pollen foraging activity. Observation periods were always 2 h (at approximately 16:00) and 21 h (at approximately 10:00 the following day) after treatment application or renewal (Supplementary Table 2). We recorded the time that each tagged worker entered a colony (observing when it passed through the transparent tubes and under the RFID readers) using a stopwatch synchronised with the RFID (host) data logger. We scored the amount of pollen in the forager's corbiculae (pollen baskets) as small (score of 1), medium (score of 2) or large (score of 3) relative to the size of the worker.

Nest box entrances were closed after dark on the evening of the twenty-eighth experimental day. Each nest box, containing bees and brood, was placed in a freezer (–20 °C). Window exits remained open for 18 h with each outlet tube connected to an individual bottle trap to catch any returning foragers. All tagged workers were identified and recently eclosed (untagged) workers were assumed to have developed in the colony they were found in. Worker thorax width was measured using digital callipers. All pupae and larvae were dissected from each nest, counted and weighed to provide final measures of brood development, and the nest structure was also weighed.

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