Field-level clothianidin exposure affects bumblebees but generally not their pathogens

Dimitry Wintermantel1,2,3, Barbara Locke1, Georg K.S. Andersson4,5, Emilia Semberg1, Eva Forsgren1, Julia Osterman1,6,7, Thorsten Rahbek Pedersen8, Riccardo Bommarco1, Henrik G. Smith4,5, Maj Rundlöf4,9 & Joachim R. de Miranda1

Neonicotinoids are implicated in bee declines and laboratory studies imply that they impair the bee immune system, thereby precipitating a rise in pathogen levels. To establish whether such synergisms reduce bee performance in real-world agricultural landscapes, we analysed the microbial composition of the bumblebee (Bombus terrestris) samples from our recent landscape study on the impacts of field-level clothianidin exposure. We related clothianidin exposure and microbial composition to both individual- and colony-level performance parameters, to better understand the direct and indirect mechanistic effects of neonicotinoid exposure on bumblebees. We show that exposure to clothianidin from seed-coated oilseed rape reduces bumblebee size and numbers, particularly of reproductives. However, exposure does not affect the levels of non-pathogenic bacteria or viruses, nor induce rises in the levels or virulence of intracellular parasites. We conclude that field exposure to the neonicotinoid clothianidin affects bumblebee performance but generally not their pathogenic or beneficial microbiota.
Bees are essential pollinators and their recent declines may have adverse effects on both natural plant biodiversity and the production of crops that depend on insect pollination. Global declines of bees have been attributed to habitat destruction, pesticide use, pathogens, climate change, or some combination of these factors. The conversion of often flower-rich natural or semi-natural habitat to arable land is thought to play a major role in the long-term decreases in bee diversity through habitat loss and fragmentation. Moreover, chronic exposure to agricultural pesticides, particularly the neurotoxic neonicotinoids, has recently been implicated in bee declines (reviewed in refs. 2, 3).

Neonicotinoids are used worldwide to control insect pests of economically important crops. They are taken up systemically to all parts of the plant, including pollen and nectar—the major foods for bees. Artificial feeding experiments with neonicotinoid exposure levels that are comparable to residue concentrations found in pollen and nectar of crops and wild flowers showed a variety of sublethal effects on bee reproduction,6,7,8 homing success,8,9 foraging behaviour,9–11, crop pollination12 and immune function,13, all of which may ultimately cause colony failure.14 Field studies15–17 and UK-wide surveys18,19 confirmed adverse effects of neonicotinoid seed dressings in oilseed rape on bees, but results varied with spatial location17 and bee species16 and some studies found no effects20–22.

To understand the sources of this variation, it is important to identify the mechanisms by which neonicotinoids impact bee performance under field conditions. Mortality in social bees can be masked by colony compensation mechanisms, such as the trade-off between worker production and more energy-costly males,15 or between colony size and individual body size of all bee castes.23 Regional differences in the effects of field-level neonicotinoid exposure on wild and managed bees have been partly ascribed to regional differences in parasite levels,17, all of which may ultimately cause colony failure.14 Field studies15–17 and UK-wide surveys18,19 confirmed adverse effects of neonicotinoid seed dressings in oilseed rape on bees, but results varied with spatial location17 and bee species16 and some studies found no effects20–22.

| Table 1 Bee numbers in relation to clothianidin seed treatment |
|---------------------------------------------------------------|
| **Response** | **Model** | **Predictor** | **Estimate (number)** | **Estimate (%)**<sup>a</sup> | **P-value**<sup>b</sup> |
| Bees (adults + cocoons) | LMM<sup>c</sup> | Treatment | −234.4 | −40.8 | 11.05 | <0.001 |
| Adult workers | LMM<sup>c</sup> | Treatment | −24.4 | −12.5 | 0.04 | 0.526 |
| Queens (adults + cocoons) | GLMM<sup>d</sup> | Treatment | −71.1 | −73.5 | 17.52 | <0.001 |
| Adult males | GLMM<sup>d</sup> | Treatment | −32.8 | −65.7 | 17.63 | <0.001 |

<sup>a</sup>Effect sizes in % were calculated in reference to the control group
<sup>b</sup>P-values were calculated by likelihood ratio tests with 1 degree of freedom and P < 0.05 is highlighted in bold
<sup>c</sup>Linear mixed-effects models (LMM; with normal error distribution)
<sup>d</sup>Generalized linear mixed-effects models (GLMM; with negative binomial error distribution and log link)
Because, the control colonies tended to be further along in their development than the exposed colonies, we were able to obtain male pupal samples from 28 of 32 colonies at untreated fields, but from only 16 of 32 colonies at clothianidin-treated fields. Similarly, we were able to obtain samples of at least 7 worker pupae more often from clothianidin-exposed (18) than control colonies (4). Samples of both male and worker pupae could be obtained from four clothianidin-exposed colonies that were in the transition from worker to male production, while two exposed colonies had neither worker nor male pupae. Generally, the male pupae were at an earlier developmental stage (LRT, \( P < 0.001 \)) and had a larger body mass than the worker pupae (LRT, \( P < 0.001 \)). Differences between castes are shown in reference to worker pupae that were in the transition from worker to male production, which in contrast to \( S. alvi \), was less frequently detected in colonies at clothianidin-treated fields than in control colonies (\( P = 0.037 \)). 

**Microorganism prevalence.** The two principal symbiotic gut bacteria of adult bees (\( G. apicola \) and \( S. alvi \)) were detected at all fields. In each treatment group, \( G. apicola \) was detected in adult worker bumblebee samples from 91% of the colonies. \( S. alvi \) tended to be more prevalent in colonies at clothianidin-treated fields than in colonies at control fields (LRT, \( P = 0.057 \), Fig. 3, Supplementary Table 1). The most prevalent pathogen was \( C. bombi \), which in contrast to \( S. alvi \), was less frequently detected in colonies at clothianidin-treated fields than in control colonies (\( P = 0.037 \)). Apicystis bombi was the only other pathogen that was detected in a majority of the colonies, but its prevalence did not differ between treatments (LRT, \( P = 0.40 \)). Nosema bombyx, Sacbrood virus (SBV), Slow bee paralysis virus (SBPV) and Acute bee paralysis virus (ABPV) were sporadically detected. Deformed wing virus type A (DWV-A), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), Lake Sinai virus types 1 and 2 (LSV-1, LSV-2), Apis mellifera filamentous virus (AmFV) and the microsporidians

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**Table 2 Bee size in relation to clothianidin seed treatment, caste and developmental stage**

| Sample          | Model       | Response | Predictor       | Estimate | \( \chi^2 \) | P-value\(^a\) | N Fields | N Colonies | N Bees |
|-----------------|-------------|----------|-----------------|----------|-------------|--------------|-----------|------------|--------|
| All pupae       | CLMM\(^b\)  | Stage    | Caste\(^d\)    | −1.32 mg | 34.09       | <0.001       | 16        | 62         | 678    |
| Male pupae      | LMM\(^c\)   | Body mass| Caste\(^d\)    | 45.0 mg  | 18.52       | <0.001       | 16        | 47         | 456    |
|                 | CLMM\(^b\)  | Stage    | Treatment      | −0.02    | 0.00        | 0.959        | 16        | 47         | 456    |
|                 | LMM\(^c\)   | Body mass| Treatment      | −85.1 mg | 20.40       | <0.001       | 16        | 47         | 456    |
|                 | LMM\(^c\)   | Intertegular distance | Treatment | −21.9 mg | 1.76        | 0.184        | 16        | 64         | 633    |
| Adult workers   | LMM\(^c\)   | Body mass| Treatment      | −8.0 mg  | 17.10       | <0.001       | 16        | 64         | 633    |

\(^a\)P-values were calculated by likelihood ratio tests with 1 degree of freedom and \( P < 0.05 \) is highlighted in bold

\(^b\)CLMM = Cumulative link mixed model (with logistic error distribution and logit link)

\(^c\)LMM = Linear mixed-effects model (with normal error distribution)

\(^d\)Differences between castes are shown in reference to worker pupae.
**Fig. 2** Size of pupae and adult bumblebees. **a** Body mass of male pupae in relation to treatment (control or clothianidin seed coating) and pupal developmental stage (1–6). Error bars represent 95% profile confidence intervals of linear mixed-effects model estimates at the earliest developmental stage (1). Circles indicate raw data on measured body mass (per bee). **b** Histograms of the intertegular distance and the body mass of adult worker bumblebees from colonies (32 per treatment) placed in oilseed rape fields (8 per treatment) sown from clothianidin-treated (dark grey; \( n = 320 \) bees) or insecticide-free seeds (white; \( n = 313 \)). Overlaps between the two treatment groups are shown in light grey and values are expressed in absolute terms and percentages of the total number of measured bees.

**Fig. 3** Microorganism prevalence. Percentage of bumblebee colonies (32 per treatment) infected with microorganisms in relation to treatment (white, control; grey, clothianidin seed coating) in oilseed rape fields (8 per treatment). For S. alvi, A. bombi, C. bombi and SBV generalized mixed-effects model estimates and their 95% confidence intervals are shown. For all other microbiota controlling for non-independence of colonies placed by the same field was not feasible. Therefore, the actual proportions of infected colonies per treatment and 95% confidence intervals calculated by two-sided binomial tests are illustrated. \( P \)-values were calculated based on likelihood ratio tests for model estimates and two-sided tests of equal proportions for measured proportions. NS not significant (\( P > 0.05 \)), \(* P < 0.05\).
Clothianidin exposure affected how *G. apicola* abundance in adult workers co-varied with the body mass of adult workers \((P = 0.006)\) and the number of adult males \((P = 0.027, \text{ Supplementary Data 2})\). In the clothianidin-exposed colonies, increase in *G. apicola* abundance was associated with an increase in worker body mass \((\text{LRT}, P < 0.001; \pm 47 \text{ mg between mean-max } \log_{10} \text{ G. apicola abundance, } n = 32 \text{ colonies})\) and in the number of adult males \((\text{LRT}, P = 0.001; \pm 28.2 \text{ bees between mean-max } \log_{10} \text{ G. apicola abundance})\), whereas no such co-variation was found for the control colonies \((\text{LRT, worker body mass: } P = 0.97; \text{ number of adult males: } P = 0.92)\).

No other interactions between treatment and microorganism abundance could be identified, although there was a weak indication that the relationship between the number of adult workers and *A. bombi* abundance differed between treatments \((\text{LRT, } P = 0.057; \text{ Supplementary Data 2})\).

Independently of treatment, the number of adult males declined with *S. alvi* abundance in adult workers \((\text{LRT, } P = 0.022; \pm 6.9 \text{ bees between mean-maximum } \log_{10} \text{ S. alvi abundance, } n = 64 \text{ colonies})\). We observed no other treatment-independent co-variation between microorganism abundance and bee performance parameters except for a non-significant tendency of the number of worker/male cocoons to decline with the abundance of *A. bombi* in adult worker bees \((\text{LRT, } P = 0.054)\).

**Discussion**

In this study, we confirm the previously reported negative effects of field exposure to clothianidin seed-treated oilseed rape on bumblebees (*B. terrestris*) at the colony level\(^{16}\) and show that this is also reflected at the individual bumblebee level, by a reduction in body size. In addition, we provide further evidence for reduced production of reproducitves\(^{5, 16, 17}\) in colonies next to clothianidin-treated fields compared to colonies next to control fields and find that these adverse effects are unrelated to differences in microbiome composition. The shift from the production of workers to reproducitves, which typically occurs after a colony growth phase, seemed to be delayed in colonies at clothianidin-treated fields. Colonies at treated fields had about 70% fewer queens and adult males compared to colonies at control fields, although the number of adult worker bees was comparable between clothianidin-exposed and control colonies. Although, the number of workers may have been affected by the proportion of bumblebees that were not in their nest when these were removed, clothianidin-exposed colonies exhibited also a several-times lower ratio of males to workers among the examined pupae than the control colonies. This reduced, or delayed, production of reproducitves may be due to colony compensation mechanisms for worker losses\(^{15}\), impaired ovary development\(^{32, 47}\) and/or under-nourishment due to reduced pollen foraging or insufficient brood care\(^{9-11}\). When the colonies were terminated, the developmental stage of the worker brood was clearly more advanced than that of the male brood, suggesting that the colonies were switching from worker production to male/queen production. This suggests that the differential between exposed and control colonies in the preponderance of worker or male brood may represent a delay in, rather than an abandonment of, the production of reproducitves.

We observed that colonies at clothianidin-treated fields had not only fewer but also smaller bees. Lighter male pupae and smaller worker bees suggest that clothianidin exposure may directly or indirectly interfere with the development of individual bees. The size of bumblebees as adults is strongly influenced by the amount of food provisioned at the larval stage\(^{48}\), suggesting that pupae in clothianidin-exposed colonies may have been undernourished as larvae, as a result of impaired pollen foraging success or deficient brood care by the adult bees\(^{9-11}\). Such reduced brood care may

**Microorganism abundance.** Quantitative analyses of microorganism abundance were restricted to the positive samples of the four most prevalent microorganisms (*G. apicola, C. bombi, S. alvi, A. bombi*), in order to test the effects of clothianidin exposure on microorganism abundance independently of any effects on prevalence. Datasets from low-prevalence microorganisms were not analysed, as these were either too small (if non-detections are excluded) or too distorted by excess zero values (if non-detections were included) for meaningful analysis. Clothianidin exposure did not affect the abundance of any of these microorganisms in the infected bumblebee colonies \((\text{LRT, } P \geq 0.1, \text{ Supplementary Table 2; Fig. 4})\). Neither did the abundance of any of these microorganisms co-vary with the abundance of any of the other microorganisms \((\text{Supplementary Data 1})\).

**Interaction between clothianidin exposure and microorganisms.** Here, we assessed the interactive effect of clothianidin exposure and the abundance of the four most frequently detected microorganisms on the body size and the numbers of bees (of different castes) as well as on the previously in Rundlöf et al.\(^{16}\) reported colony weight and number of worker/male cocoons.

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**Fig. 4** Microorganism abundance. The \(\log_{10}\) DNA copy numbers per bee and colony of the most frequently detected microorganisms (*Apicystis bombi* \((n = 40 \text{ colonies in } N = 15 \text{ fields})\), *Crithidia bombi* \((n = 53, N = 16)\), *Giillemella apicola* \((n = 58, N = 16)\), *Snodgrassella alvi* \((n = 50, N = 16)\)) in relation to treatment (white, control; grey, clothianidin seed coating) in oilseed rape fields \((8 \text{ per treatment})\). The error bars represent 95% profile confidence intervals of linear mixed-effects model estimates. Circles indicate measured values \((\text{per colony})\). NS not significant \((P > 0.05)\). P-values were calculated by likelihood ratio tests on linear mixed-effects models.
reflect a quantitative shift by the colony towards foraging to compensate for a perceived deficit in foraging success, either through insufficient food intake or neuronal changes. *Bombus terrestris* and honeybee workers can be attracted to neonicotinoid-contaminated food, even though its consumption can reduce the bees' overall food intake. However, this effect was not observed for clothianidin. As smaller bumblebees are less efficient foragers, decreased worker size may exacerbate undernourishment, although our previous results showed that clothianidin exposure did not affect the number of food storage cells. Larger body size is also thought to be advantageous for the mating ability of males, therefore the drastic reduction in body mass of male pupae may have implications for their future reproductive success.

The adverse effects on body size and the production of reproductive are likely not due to increased pathogen susceptibility. Clothianidin-exposed and control colonies showed only little difference in the prevalence or the abundance of symbiotic and pathogenic microbiota. Of the eight RNA viruses tested, only three were detected in the adult bee samples, and furthermore only in a minority of colonies, while only one (ABPV) was detected in pupal samples. Of the six DNA pathogens tested, only the three bumblebee-specific parasites (*A. bombi*, *C. bombi*, *N. bombi*) were detected. The prevalences of these pathogens were generally comparable to other studies on commercially reared bumblebees, although *C. bombi* was detected more frequently in our study. This parasite showed a higher prevalence in the control than in the clothianidin-exposed group, even though previous laboratory-based research could not detect an effect of neonicotinoid exposure on *C. bombi*. This may be due to reduced *C. bombi* proliferation, which then leads to lower transmission or detection rates of the parasite. Clothianidin exposure may directly reduce *C. bombi* proliferation or indirectly by inducing changes in bumblebee size demographics, nourishment and foraging activity that also affect *C. bombi* proliferation. Beta-proteobacteria in the bee gut have previously been suggested to protect bumblebees against *C. bombi* infection and in our study *S. alvi* tended to be, contrary to *C. bombi*, more prevalent in colonies at clothianidin-treated fields. However, we did not find a relationship between the abundances of *S. alvi* and *C. bombi*. As those bees that failed to return to their nests or were removed by their nest mates after dying could not be sampled, it is conceivable that a decreased survival rate or homing success of *C. bombi* infected bees that lacked *S. alvi* or were additionally immune-challenged by the neonicotinoid caused a lower detection rate of *C. bombi* and masked a negative relation between *C. bombi* and *S. alvi*.

The absence of interactive effects between clothianidin exposure and the abundance of intracellular parasites suggests that clothianidin did not affect the virulence of the parasites or the tolerance of the host to parasite infection. Gilliamella apicola was the only microorganism whose relationship with the bumblebee performance parameters depended on clothianidin exposure. In clothianidin-exposed colonies, the body mass of adult worker bees and the number of adult males increased with the abundance of *G. apicola* in adult workers, whereas no co-variation was observed in the control group. Gilliamella spp. promote weight gain in bees through the decomposition of otherwise indigestible or toxic carbohydrates, which may be one possible explanation as to why adult worker bees of the two treatment groups did not significantly differ in body mass, but did differ in intertropical distance. In contrast, to *G. apicola*, *S. alvi* abundance in adult workers showed a negative co-variation with the number of adult males independently of treatment. We are aware that caution has to be applied when interpreting marginally significant effects (such as the effects of microorganisms in interaction or independently of treatment on the number of adult males or the treatment effect on *C. bombi* prevalence), since the probability of a false-positive finding increases with the number of parameters tested and can be much greater than the probability of falsely rejecting the null hypothesis evaluated by P-values.

The exposure of bumblebees to clothianidin from seed-coated oilseed rape during one flowering season, which was confirmed by residue analysis of bumblebee-collected nectar, did not affect the levels of symbiotic gut bacteria or viruses, nor did it induce rises in the levels or virulence of intracellular parasites. This suggests that the bumblebees’ combined individual, adaptive and social immune defences, since the probability of a false-positive finding increases with the number of parameters tested and can be much greater than the probability of falsely rejecting the null hypothesis evaluated by P-values. The exposure of bumblebees to clothianidin from seed-coated oilseed rape during one flowering season, which was confirmed by residue analysis of bumblebee-collected nectar, did not affect the levels of symbiotic gut bacteria or viruses, nor did it induce rises in the levels or virulence of intracellular parasites. This suggests that the bumblebees’ combined individual, adaptive and social immune defences, since the probability of a false-positive finding increases with the number of parameters tested and can be much greater than the probability of falsely rejecting the null hypothesis evaluated by P-values. The exposure of bumblebees to clothianidin from seed-coated oilseed rape during one flowering season, which was confirmed by residue analysis of bumblebee-collected nectar, did not affect the levels of symbiotic gut bacteria or viruses, nor did it induce rises in the levels or virulence of intracellular parasites. This suggests that the bumblebees’ combined individual, adaptive and social immune defences, since the probability of a false-positive finding increases with the number of parameters tested and can be much greater than the probability of falsely rejecting the null hypothesis evaluated by P-values.
queens and males suggest that neonicotinoids may deleteriously influence bumblebee population sizes\textsuperscript{19}, which may further be exacerbated by a neonicotinoid-induced reduction in colony initiation after hibernation\textsuperscript{32}. Long-term studies at the population level are needed to investigate whether bumblebee colonies can recover from temporarily reduced brood production during periods when they are no longer exposed to neonicotinoids, as shown under laboratory conditions\textsuperscript{19}. 

**Methods**

**Study sites.** In 2013, a total of 16 fields (field size = 8.9 ± 1.4 ha (mean ± s.e.m)) in southern Sweden, intended for the production of spring-sown oilseed rape (Brassica napus L.), were selected by the absence of other oilseed rape fields within a 2 km radius and paired based on geographical proximity and the land use in the surrounding landscapes (r = 2 km; for details see Rundlöf et al\textsuperscript{17}). For each field pair, one field was randomly assigned to be sown with clothianidin-treated oilseed rape seeds (25 mL Elado (Bayer; 400 g L\textsuperscript{−1} clothianidin = 180 g L\textsuperscript{−1} b-cyfluthrin) per kg seed and the fungicide thiram) while the paired field was sown with insecticide-free oilseed rape treated only with thiram. Farmers at both treated and untreated fields used non-neonicotinoid insecticide sprays but were instructed not to use other neonicotinoids for pest control (see Rundlöf et al\textsuperscript{17}). However, one control field was accidently sprayed with 0.3 L ha\textsuperscript{−1} Biscaya, which contains the neonicotinoid thiacloprid as active ingredient. Thiacloprid has a considerably lower acute toxicity to bees than clothianidin\textsuperscript{2}. Residue analyses of bee-collected pollen and nectar revealed that the overwhelming insecticide exposure, and greatest difference between control and treated fields, was from clothianidin, with minor traces of the spray insecticides distributed equitably between control and treated fields\textsuperscript{16}.

**Bumblebee colonies.** Six commercially reared Bombus terrestris colonies (Naturall, Koppert Biological Systems) were placed in triplets in two wooden, ventilated houses placed in shaded areas along the field edge in each of the 16 fields between June 14 and 28, 2013, at the onset of oilseed rape flowering in each field (for details see Rundlöf et al\textsuperscript{16}). Field allocation was randomized and there was no difference in weights between colonies at treated (73 ± 19 g (mean ± s.e.m), n = 32 colonies) and control (73 ± 18 g, n = 32) fields at placement\textsuperscript{17}. The colonies were –10 weeks old at the time of placement, containing roughly 50 workers, one queen, and both pupae and larvae. All 12 bumblebee colonies at a field pair were freeze-killed simultaneously at –20 °C between July 7th and August 5th 2013, at first sighting of new queens in any one of those 12 colonies, one pair after closing nests with check valves for >24 h and the rest at night when most bees were assumed to be in the nest. Since oilseed rape flower phenology influenced placement time and the switch to queen production determined termination time, the duration of field placement varied between 23 and 38 days for different sets of colonies.

**Bee performance parameters.** The two outer bumblebee colonies in each housing unit (total of four from each field) had been assessed previously for the number and weight of queen cocoons, the number and weight of worker/male cocoons, the number of pollen and nectar cells, and the weight of the nest structure\textsuperscript{18}. These samples were stored frozen at –20 °C. In this study, we determined the following additional parameters for these same colonies: the total number of bees (adults and pupae), the total number of queens (adults and pupae), the number of adult workers and the number of adult males, as well as the caste, weight and developmental stage of individual pupal cocoons, and the body mass and interterritorial distance of individual adult workers. We were unable to categorize ~0.7% of the adult bees, which were not used in analyses. Intertegular distance is the distance between the insertion points of the wings\textsuperscript{19} and a standard measure of adult body size in bees\textsuperscript{22}. Intertegular distance was measured using a digital caliper, with the individual body mass of adults and pupae was measured using a balance with 0.1 mg resolution. Only intact bees were analysed. The developmental stage of individual pupae was rated into 6 categories based on eye colour (white = 1, pink = 2, brown = 3), body colour (white = 1–3, brown = 4, black = 5) and the presence of wings (6).

**Pathogens and beneficial bacteria.** The colonies that were assessed for bee performance parameters were also examined for the presence and abundance of the most common and important pathogens and beneficial microbes, including the RNA viruses Deformed wing virus type A (DWV-A), Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Sacbrood virus (SBV), Slow bee paralysis virus (SBPV), Chronic bee paralysis virus (CBPV), Lake Sinai virus types 1 and 2 (LSVr & LSV2), the DNA virus Apis mellifera filiformis virus (AmFV), the microsporidian gut parasites Nosema apis, N. ceranae and N. bombi, and two other common internal parasites (Apis cerana bombi, Crabidius bombi) and the non-pathogenic gut bacteria Gilliamella apicola and Sphingobacterium alvei (Supplementary Table 3).

**Sample processing and homogenization.** For each colony, pooled samples of ten adult worker bumblebees, ten worker pupae and/or ten male pupae were prepared. Two bumblebee colonies (both from treated fields) did not contain any pupae and not all of the remaining colonies contained both worker and male pupae, so that the final sample set consisted of 64 adult samples, 22 worker pupae samples and 44 male pupae samples. Furthermore, five of the worker pupae samples contained only 7–10 individuals. The 10 bees in each pooled sample were placed in a polyethylene bag with an inner mesh (BioReba). The bees were finely ground with a pestle. One millilitre of nuclease-free water per bee was added and the slurry was mixed thoroughly until the suspension was homogenous. The homogenates were stored at –80 °C until nuclease-free DNA extractions.

**Nucleic acid extraction.** DNA was extracted from the adult bumblebee homogenates using the protocol for extracting DNA from Nosema spores\textsuperscript{33}, which is described in the Supporting Information. DNA from bacteria and DNA from the apomictic genus Apis mellifera was removed through strategic PCR amplification. One millilitre of primary bee homogenate was centrifuged for 5 min in a microtube at 13,000 rpm. The pellet was repeatedly frozen-thawed with liquid nitrogen and ground with a sterilized micro-pipe pestle until pulverized. The pulverized pellet was re-suspended in 400 µL Qiagen Plant tissues DNA buffer (including the Qiagen AlphaCard) eluted from Qiagen Plant RNAeasy protocol (including the Qiagen AlphaCard) and re-suspended in 400 µL Qiagen Plant tissues DNA buffer (including the Qiagen AlphaCard) and concentrated to 100 µL of DNA. A Nanodrop pipette was used to add one microlitre of DNA to 100 µL of 10× reaction buffer, followed by 5 min incubation on ice and centrifugation for 5 min at 14,000 rpm to remove the dye. DNA was purified from 500 µL of the supernatant by the Qiagen automated Qiaquick extraction robot, following the plant DNAeasy protocol and resuspended in 100 µL of 10× reaction buffer. This was used for reverse transcription and PCR amplification of the bumblebee internal reference gene Bt-RPL23, for correcting sample-specific differences in RNA quality\textsuperscript{66}, and N. ceranae and N. bombi DNA of the small subunit ribosomal RNA gene (Gilliamella apicola (based on the 16S ribosomal RNA gene). All sequences matched 100% their intended target.

**RT-qPCR and qPCR.** The DNA pathways were quantified using Reverse Transcription-quantitative PCR (RT-qPCR). Two technical assays were also included in the RNA assay: one for RNA250, a passive exogenous reference RNA of known concentration included in the RT-qPCR reaction mixture, for correcting sample-specific differences in assays performance\textsuperscript{67}, and one for the amplification of the bumblebee internal reference gene Bt-RPL23, for correcting sample-specific differences in RNA quality\textsuperscript{66}. The DNA pathways were quantified by qPCR. Novel qPCR assays were designed, experimentally optimized and confirmed through bidirectional Sanger sequencing of representative PCR products for Crithidia bombi (based on the GADH gene), Nosema ceranae and N. bombi (based on the small subunit ribosomal RNA gene, Sphingobacterium alvei (based on the 16S ribosomal RNA gene). All sequences matched 100% their intended target.

The PCR reactions were run in duplicate and conducted in 20 µL volumes containing 2 µL template, 0.2 µM (RNA) or 0.4 µM (DNA) of forward and reverse primers (Supplementary Table 3) and either the Bio-Rad EvaGreen qPCR master mix (DNA) or the Bio-Rad iScript One-Step RT-qPCR mix (RNA), both with SYBR Green detection chemistry. The reactions were incubated in 96-well optical qPCR plates in the Bio-Rad CFX connect thermocycler, using the following amplification cycling profiles for the RNA assays: 10 min at 50 °C for cDNA synthesis (RT-qPCR only; to inactivate the reverse transcriptase and activate the Taq polymerase) followed by 40 cycles of 10 s at 95 °C for denaturation and 10 s at 60 °C for primer annealing, extension, and data collection. The amplification cycles were followed by a melting curve analysis to determine the specificity of the amplification by holding the temperature for 10 s at 95 °C and then reading the fluorescence at 0.5 °C increments from 65 °C to 95 °C. Included on each reaction plate were positive and negative (template-free) assay controls. For each type of mutation (Supplementary Table 3) a 2-fold dilution series of a positive control of known concentration covering seven to eight orders of magnitude, for quantitative data conversion, establishing the reference melting curve profile of the amplicon and estimating the reaction performance statistics.

**Data conversion and normalization.** The melting curves of individual reactions were evaluated visually in order to separate out non-specific amplifications, which differ in melting temperature profile from true target cDNA/DNA amplicons. Non-specific amplifications were deleted from the data set. All assays were run in duplicate, with the mean value of these two duplicates used in further calculations. Both duplicates had to yield a positive quantitative value and pass the melting curve analysis for the data to be included in the data set. The Cq-values (quantification cycle) of all confirmed amplifications were subsequently converted and estimated the Sρ-values (Starting Quantity) in copy numbers of each target DNA/RNA, using the corresponding calibration curves for the different assays. These data were
multiplied by the various dilution/conversion factors incurred throughout the extraction, cDNA synthesis and amplification procedures, to calculate the estimated error distribution and the other parameters were adjusted with the sample-specific data for the two technical assays: the exogenous (RNA250) and internal (Bt-RPL23) reference RNAs. The data for RNA250 was used to calculate the individual cDNA conversion efficiency for each sample, i.e. the ratio of the amount of RNA250 estimated by RT-qPCR (output) versus the known amount of RNA250 (input). Due to the reaction (input). As RNA was easily degraded there is a risk that differences between individual samples in RNA quality (i.e. degradation) can affect the results. The data for the RNA targets of interest were therefore normalized to the average value for Bt-RPL23 mRNA, thus correcting the data for sample-specific differences in RNA quality with respect to RT-qPCR performance.

Statistical analyses. The data consisted of a range of quantitative biological and pathological parameters, which were measured at colony level (bee numbers, microbiome composition) or at individual level (body mass and intertergular distance). The microbial data were analysed on both their binary (presence/absence) and quantitative (abundance) characters. As no samples were taken prior to exposure, the statistical analyses consisted largely of straight comparisons between colonies at clothianidin-treated fields and control fields. All analyses were done both including and excluding the field where Biscaya (containing the neonicotinoid thiacloprid) was sprayed, to determine whether this influenced the results. Except in two cases, excluding the field sprayed with Biscaya from the data analysis did not move the P-values from below to above the 0.05 threshold or vice versa (Supplementary Table 4).

The impact of treatment on the total numbers of bees (cocoons + adults) and the number of adult workers was assessed using linear mixed-effects models (LMM; with normal error distributions), while treatment effects on the total numbers of queens (cocoons + adults) and the number of adult males were analysed using generalized linear-mixed-effects models (GLMM) with negative binomial error distributions and log links. All models on bee numbers contained treatment as a fixed factor and field pair identity and field identity as random factors.

To test whether the developmental stage of male pupae differed from worker pupae, a cumulative link mixed model (CLMM; with logistic error distribution and cumulative link function) was used for CLMMs. All (C)LMMs for data on individual bees contained the fixed factor and microorganism abundance on bee performance, effect size of a log10 unit increase in microorganism abundance was shown relative to the estimated response value of a control colony with average microorganism log10 abundance. For microorganism prevalence, effect size was shown as a percentage change of infected colonies relative to the total number of colonies.

The power analysis suggested that 80% power was reached for effect sizes ranging from under 15% (number of adult workers by A. bombi x treatment) to over 25% (S. albi prevalence by treatment) (Supplementary Fig. 1).

Data availability. Supporting the findings of this study are available within the paper, its supplementary information files and/or Rundlöf et al. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
Conceptualization, J.R.d.M., M.R., R.B., H.G.S., T.R.P., J.O., D.W.; Methodology, J.R.d.M., D.W., E.F., E.S., M.R., G.K.S.A.; Investigation, D.W., E.S., G.K.S.A.; Formal analysis, D.W., M.R.; Data curation, J.R.d.M., M.R.; Writing, D.W., B.L.; Editing, All; Visualization, D.W.; Supervision J.R.d.M., M.R.; Project Administration, E.F., T.R.P., R.B., M.R., H.G.S.; Funding Acquisition, J.R.d.M., M.R., R.B., H.G.S., T.R.P.

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