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The direct and indirect effects of environmental toxicants on the health of bumblebees and their microbiomes

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Bumblebees (*Bombus* spp.) are important and widespread insect pollinators, but the act of foraging on flowers can expose them to harmful pesticides and chemicals such as oxidizers and heavy metals. How these compounds directly influence bee survival and indirectly affect bee health via the gut microbiome is largely unknown. As toxicants in floral nectar and pollen take many forms, we explored the genomes of bee-associated microbes for their potential to detoxify cadmium, copper, selenate, the neonicotinoid pesticide imidacloprid, and hydrogen peroxide—which have all been identified in floral nectar and pollen. We then exposed *Bombus impatiens* workers to varying concentrations of these chemicals via their diet and assayed direct effects on bee survival. Using field-realistic doses, we further explored the indirect effects on bee microbiomes. We found multiple putative genes in core gut microbes that may aid in detoxifying harmful chemicals. We also found that while the chemicals are largely toxic at levels within and above field-realistic concentrations, the field-realistic concentrations—except for imidacloprid—altered the composition of the bee microbiome, potentially causing gut dysbiosis. Overall, our study shows that chemicals found in floral nectar and pollen can cause bee mortality, and likely have indirect, deleterious effects on bee health via their influence on the bee microbiome.

1. Introduction

Despite the high value pollinators have in agriculture and wild ecosystems, many populations are declining across Europe and North America [1,2]. Habitat change, disease, and chemical exposure are all thought to play a role in bee declines [3,4]. The primary chemicals responsible are pesticides, which bees come into contact with when foraging on treated crops [5]. However, when foraging on flowers bees do not only get exposed to pesticides, floral pollen and nectar may also contain environmental toxicants such as natural oxidizers (hydrogen peroxide), and heavy metals sequestered by the plants when growing in contaminated soils [6–9]. These environmental toxicants accumulate in bees and their hive materials, often at greater concentrations than in the flowers they were collected from, prompting an urgent need to obtain a complete understanding of their influence on bee health at field-realistic concentrations [10,11].

Several environmental toxicants are directly lethal to bees at high concentrations; honeybees (*Apis* spp.), bumblebees (*Bombus* spp.), and mason bees (*Osmia* spp.), all show rapid death after exposure in laboratory experiments [12–14]. There is evidence that in addition to lethality, exposure to high doses may cause indirect damage to bee health via disrupting their core microbiome [15]. A healthy gut microbiome is considered to be a crucial factor in bee health [16] and may positively influence bee tolerance to toxicants via toxicant metabolism [17], immune system stimulation [18], and protection against pathogens [19]. Likewise, there is apparent variation in the microbiomes of *Bombus* spp.

[20], with some guts harbouring non-core bacteria such as taxa within the order Enterobacterales [21] including the pathogen *Serratia* [22]. Increasing evidence finds that a number of stressors can break down a normal healthy microbiome and in doing so prevents their beneficial influence on the host. In particular, stressors such as inconsistent forage availability [23], antibiotics [24], infection [25], and pesticides [26], all disrupt the microbiome, potentially leading to dysbiosis and reduced host health. It could therefore be that the quality, quantity, and toxicity of a bee's diet may shape their microbiome and if these lead to microbial dysbiosis the cascading effects could make bees increasingly vulnerable to further nutritional stress and disease [27,28].

The effects of environmental toxicants and other xenobiotics on animal microbiomes is emerging as an integral part of modern ecotoxicology [29], where the microbiome potentially protects its host from metal(loid) toxicity. For example, host-associated bacteria have been shown to detoxify chromium and lead [30], copper [31], arsenic [32], and selenate [33]. In bees, the presence of a microbiome reduces selenate-induced mortality [34], and bee-associated microbes can remove cadmium from their environment [15]. The mechanisms for microbe-mediated protection against toxicants in bees remains unknown, but as the majority of bee symbionts reside in the hindgut [35] and most metal absorption in insects likely occurs in the midgut [36], the hindgut bacteria may be stimulating the bees' own inherent protective mechanisms. Also, the midgut of insects has a peritrophic matrix, which has been shown to directly protect against pesticides [37] and metals [38]. Bacterial genomes often encode metal(loid) transporters and detoxification pathways for stress responses, and symbionts simultaneously interact with environmental exposures and their host. This may result in bacterial detoxification genes also benefiting the host, possibly by preventing metal-induced damage to the hindgut, so by annotating pathways in symbiont genomes, we may begin to understand the mechanisms behind microbial toxicant protection.

Given the importance of environmental toxicants and the microbiome in host health, we investigated interactions between multiple chemical poisons, the bumblebee *B. impatiens*, and its microbes. First, we searched the genomes of bee-associated microbes for evidence they could play a role in the metabolism/detoxification of common chemicals (selenate, copper, cadmium, imidacloprid, and hydrogen peroxide). Second, we tested the direct lethality of these toxicants to bumblebees. Third, we determined if exposure to natural concentrations of selenate, copper, cadmium, imidacloprid, or hydrogen peroxide altered the composition of a healthy bee microbiome.

2. Materials and methods

To identify the genomic basis for toxicant tolerance, we annotated publicly available genomes of bee symbionts with the RAST Server (Rapid Annotations using Subsystems Technology) [39] using whole-genome sequence data obtained from the National Center for Biotechnology Information (NCBI). Based on known 'core' microbes and opportunistic microbes commonly found within the microbiomes of bumblebees and those shared with honeybees [40], we annotated genomes from strains of the following species: *Bifidobacterium bombi*, *Bifidobacterium commune*, *Bombella intestini*, *Bombiscardovia coagulans*, Candidatus *Schmidhempelia bombi*, *Commensalibacter intestini*, *Gilliamella apicola*, *Lactobacillus bombicola*, *Serratia marcescens*, and *Snodgrassella alvi* (see electronic supplementary material, file SF1 for strain IDs and accession numbers). We also used CheckM to assay genome quality and removed genomes that were less than 90% complete and/or more than 10% contaminated from further analysis. We searched the following RAST subsystems: 'cobalt-zinc-cadmium resistance', 'copper homeostasis', 'copper homeostasis copper tolerance', 'copper transport system', 'oxidative stress tolerance', 'selenate/selenite uptake', and 'selenocysteine metabolism'.

We purchased 10 commercial *Bombus impatiens* colonies from Koppert Biological Systems, Inc. (Howell, MI) that contained a mated queen, approximately 200 workers, pollen, and a proprietary sugar solution. We immediately replaced the proprietary sugar solution with 60% sucrose and provided colonies with pollen patties ad libitum. To allow the colonies to develop, we kept them under constant darkness at 29°C for two weeks before starting the experiment. We collected 60 adult workers from each of three colonies ($N = 180$ bees for each compound) and sorted them by colony into cohorts of five bees in 475 ml polypropylene containers (WebstaurantStore, Lancaster, PA). Based on published ranges (electronic supplementary material, table ST1), we exposed bees to the following treatments: 10 mg l^{-1}, 1.0 mg l^{-1}, 0.1 mg l^{-1}, 0.01 mg l^{-1}, 0.001 mg l^{-1}, and 0 mg l^{-1} spiked into 60% sucrose for sodium selenate, cadmium chloride, and imidacloprid, 100 mg l^{-1}, 10 mg l^{-1}, 1.0 mg l^{-1}, 0.1 mg l^{-1}, 0.01 mg l^{-1}, and 0 mg l^{-1} copper chloride spiked into 60% sucrose, and 1.0 M, 0.1 M, 0.01 M, 0.001 M, 0.0001 M, and 0 M hydrogen peroxide spiked into 60% sucrose. We allowed bees to feed ad libitum for 14 days and recorded mortality daily. To analyse survivorship, we used the R packages 'drc,' [41] to calculate log-logistic functions for model selection, and 'survival' [42] to calculate statistical significance and hazard models, and 'survminer' to visualize survival curves [43].

We purchased three additional bumblebee colonies from Koppert Biological Systems, Inc. and reared the bees as above. We isolated 60 mature workers from each colony ($N = 180$) in 60 ml polypropylene containers (WebstaurantStore, Lancaster, PA). We exposed bees to chemical treatments by chronically feeding 30 bees 60% sucrose spiked with either 0.25 mg l^{-1} cadmium chloride, 0.5 mg l^{-1} sodium selenate, 25 mg l^{-1} copper chloride, 0.001 mg l^{-1} imidacloprid, 0.025 mol l^{-1} hydrogen peroxide, or 60% sucrose as a control ($N = 30$ per treatment), based on concentrations within the dose–response assay and published ranges (electronic supplementary material, table ST1). We allowed the bees to feed on either treatment ad libitum for 4 days, then flash froze and stored the bees at −80°C.

We used a DNA extraction protocol based on Engel *et al.* [44], Pennington *et al.* [45], and Rothman *et al.* [46]. We first surface sterilized individual bees using 0.1% sodium hypochlorite followed by three rinses with water. We then sterilely dissected the whole gut out of each bee and transferred the gut into DNeasy Blood and Tissue Kit lysis plates (Qiagen, Valencia, CA) containing 100 µl of 0.1 mm glass beads, one 3.4 mm steel-chrome bead, and 180 µl of buffer ATL, then homogenized with a Qiagen Tissuelyser at 30 Hz for 6 min. We followed the remainder of the kit protocol after homogenization. We also included four blanks to control for reagent contamination, which we prepared and sequenced in the same way as samples.

We prepared 16S rRNA gene libraries for paired-end Illumina MiSeq sequencing for each bee ($N = 134$) using the protocol from McFrederick and Rehan [47], Pennington *et al.* [48], and Rothman *et al.* [23]. We incorporated the 16S rRNA gene primer sequence, unique barcode sequence, and Illumina adapter sequence with PCRs as in [49]. We ran one round of PCR to ligate barcodes, then cleaned these PCRs with a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA) and used the cleaned amplicons as the template for another PCR to complete the Illumina adapter sequence [49]. We normalized the libraries with a SequalPrep Normalization kit (ThermoFisher Scientific,

Waltham, MA), pooled 5 µl of each normalized library and performed a final clean-up with a single-column PureLink PCR Purification Kit, then sequenced the libraries using a V3 Reagent Kit at 2 × 300 cycles on an Illumina MiSeq Sequencer in the UC Riverside Genomics Core Facility. Raw sequencing data are available on the NCBI Sequence Read Archive under accession numbers SRR6788889 – SRR6789022, and microbiome data of selenate versus control treatments were previously published in Rothman *et al*. [34].

We used QIME2-2018.6 [50] to process the 16S rRNA gene sequence libraries. First, we trimmed low-quality ends off reads with QIIME2 and used DADA2 [51] to bin sequences into exact sequence variants (ESVs; 16S rRNA gene sequences that are 100% matches). We assigned ESV taxonomy using the q2-feature-classifier and SILVA database [52]. We also conducted BLASTn searches against the NCBI 16S microbial database (July 2018). We filtered out ESVs from the resulting table that corresponded to reagent contaminants as identified in blanks or were assigned as chloroplast or mitochondria. We then generated an ESV table (electronic supplementary material, File SF2) and UniFrac distance matrices. We visualized the UniFrac distances through Principal Coordinates Analysis (PCoA) with the R package 'ggplot2' [53], analysed the alpha diversity of our samples through the Shannon Diversity Index and the Kruskal–Wallis test. Lastly, we tested our beta diversity data for statistical significance in R v3.5.1 with the packages 'vegan' [54] and 'DESeq2' [55]. Data and representative code can be found on Data Dryad (doi:10.7280/D14T2K) and a preprint of this study was posted to the bioRxiv [56].

3. Results

(a) Genomic bases of chemical resistance

Through our RAST annotations of core microbial genomes, we identified several putative homologous genes that suggest that microbial genera commonly associated with bumblebees and honeybees could reduce the negative effects of these toxic compounds to bee health. Several bee symbionts and other bacteria identified by our next-generation sequencing study had homologs to some or all of the following genes in their genomes (electronic supplementary material, figure S1). For selenium ion resistance, we found genes corresponding to the selenium ion transporters DedA [57], TsgA [58], and putative selenium ion and sulfate importer CysA [59]. For cadmium ion resistance, we found the genes CzcABC, which encode the components of a cation transporter [60], its response regulator CzcD [61], and a cadmium-responsive transcriptional regulator, CadR [62]. We identified the following genes involved in copper resistance: a copper-translocating ATPase [63], two copper-binding multicopper oxidases [64,65] (SufI and CueO, respectively), the likely copper-binding proteins ScsABCD and CutEF [66], components of a copper-sequestering protein complex CopCD [67], and a copper-responsive transcriptional regulator, CueR [68]. Lastly, we searched for genes involved in oxidative stress response and found genes encoding paraquat-inducible superoxide dismutase (SOD) PqiAB, Mn- and Fe-SODs [69], the SOD response regulon SoxS [70], a LysR-family peroxide-inducible transcriptional regulator [71], ferroxidase, a ferric uptake regulation protein (FUR) [72], the zinc/copper uptake regulation protein Zur, which may protect against oxidative stress [73], the antioxidant gene NnrS [74], an Fnr-like transcriptional regulator [75], catalase/peroxidase [76], and alkyl hyperoxide reductase C (AhpC) [77].

As *S. alvi* and *G. apicola* genomes are known to vary between strains [78,79], and there are several genomes for each taxon publicly available, we compared the above-mentioned detoxification/tolerance genes between strains within these species and across isolates from *Apis* and *Bombus* spp. (51 strains of *S. alvi* [33 strains isolated from *Apis* spp. and 18 strains from *Bombus* spp.] and 65 strains of *G. apicola* [37 strains isolated from *Apis* spp. and 28 strains isolated from *Bombus* spp.]). We found that *G. apicola* had notable variation across genes involved in responding to oxidative stress (specifically NnrS, SoxS, Fnr, and catalase), copper tolerance (the copper-translocating ATPase and SufI), cadmium tolerance (CadR), and overall selenate tolerance. The variation in SufI, CadR, TsgA, and CysA appeared to be mainly driven by the bee genus that *G. apicola* was isolated from. There was less overall variation in detoxification/tolerance genes across *S. alvi* strains: we found strain variation in copper (CueR, CueO, and the copper-translocating ATPase) and cadmium tolerance (CzcA and CadR), while there was no genetic variation in oxidative stress response or selenate tolerance. Again, the bee genus each strain was derived from added to the apparent variability, with *Apis* isolates having fewer CueR and CzcA genes present, and Bombus isolates having fewer CadR and no CueO genes present (figure 1).

(b) Direct toxicity of each compound on bumblebee survival

We found that the concentration ranges of cadmium, copper, selenate, imidacloprid, and hydrogen peroxide went from no deaths to complete mortality. Over 7 days of continuous exposure, survival in the various concentrations differed significantly (Cox proportional hazard test log rank $p < 0.001$ for each compound, figure 2 and electronic supplementary material, figure S2). We note that the lowest concentrations did not affect survival, and that concentrations above 1 mg l^{-1} cadmium, 100 mg l^{-1} copper, 1 mg l^{-1} selenate, 0.1 mg l^{-1} imidacloprid, and 1.0 M hydrogen peroxide significantly reduced bee survival, which indicated a dose-dependent response (electronic supplementary material, table ST2). We also calculated the LC_{50} after 7 days continuous exposure for each toxicant: cadmium: 0.83 mg l^{-1}, copper: 66.55 mg l^{-1}, imidacloprid: 0.22 mg l^{-1}, selenate: 0.75 mg l^{-1}, and hydrogen peroxide: 0.39 mol l^{-1} (electronic supplementary material, figure S3). We note that while we exposed bees to treatments for 14 days, the data from copper and cadmium did not fit the proportional hazards assumptions due to high mortality in controls after 11 and 9 days, respectively. Survival results of these compounds up to 7 days fit the assumptions, so all five treatments were tested over a total of 7 days. Additionally, we tested selenate, imidacloprid, and hydrogen peroxide for 14 days and report the 14-day LC_{50} as follows: selenate: 0.09 mg l^{-1}, imidacloprid: 0.11 mg l^{-1}, and hydrogen peroxide: 0.27 mol l^{-1} (electronic supplementary material, figures S3 and S4).

(c) Amplicon sequencing alpha diversity and library statistics

We obtained 743 529 quality-filtered 16S rRNA gene sequences with a mean of 5467 reads per sample ($N = 136$) that clustered into 113 ESVs (sequences that are 100% identical). We determined that our samples had a representative coverage of bacterial diversity at a sequencing depth of 2182 reads through

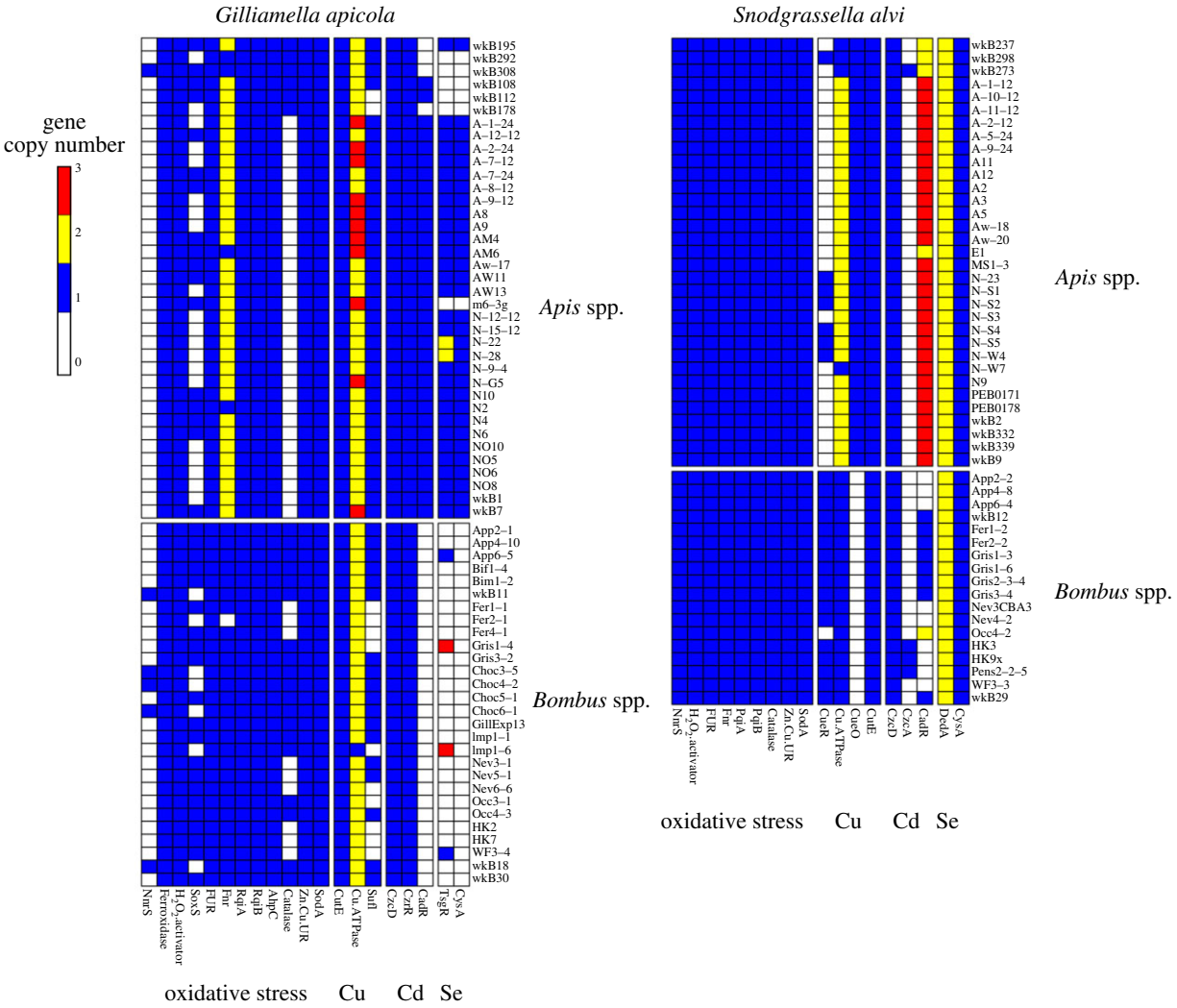

Figure 1. Illustration of the toxicant tolerance genes found in strains of *Snodgrassella alvi* and *Gilliamella apicola* as annotated by RAST and split by the bee genera they were isolate from. Coloured cells represent the copy number of each gene, row names indicate bacterial strain, and column names denote gene abbreviation or name. Nonstandard gene abbreviations are as follows: 'H_2O_2.activator' is a peroxide-inducible genes activator, 'FUR' is a ferric uptake regulation protein, 'Fnr' is a fumarate and nitrate reduction regulatory protein, 'Zn.Cu.UR' is a zinc/copper uptake regulation protein, and 'Cu.ATPase' is a copper-translocating ATPase. (Online version in colour.)

rarefaction analysis (electronic supplementary material, figure S5). Overall, alpha diversity was significantly different due to treatment (Shannon's $H = 24.21$, $p < 0.001$), although pairwise Kruskal–Wallis tests indicated that only selenate treatments had higher diversity as compared to controls (Benjamini-Hochberg corrected $p_{adj} < 0.05$, electronic supplementary material, table ST3).

(d) Beta diversity and differential abundance of bacterial taxa between treatments

Regardless of treatment, we found that the gut communities of our samples were composed of bacteria of the genera *Gilliamella*, *Snodgrassella*, *Lactobacillus*, *Bifidobacterium*, *Bombiscardovia*, *Commensalibacter*, and *Serratia*, while other bacteria accounted for less than 1% of the relative abundance. To visualize the bumblebee gut microbiota, we generated a stacked bar plot representing bacteria present in greater than 1% relative abundance in each sample (figure 3 and electronic supplementary material, figure S6) and beta diversity through PCoA (figure 3), with only copper clearly clustering separately from control. We analysed the Generalized UniFrac distance matrix of our samples with Adonis PERMANOVA using colony and treatment as covariates and found that overall, there was a significant effect of treatment ($F = 4.57$, $R^2 = 0.14$, $p < 0.001$), colony ($F = 6.71$, $R^2 = 0.08$, $p < 0.001$), and interaction of these factors (treatment × colony, $F = 1.63$, $R^2 = 0.10$, $p < 0.001$). As we had multiple separate treatments, we analysed the pairwise interactions between each treatment versus control and found that each treatment except imidacloprid significantly changed the beta diversity of the bees' microbiomes (Benjamini-Hochberg corrected for each treatment $p_{adj} < 0.02$; imidacloprid: $p_{adj} = 0.96$, electronic supplementary material, table ST3).

We used 'DESeq2' to identify differences in proportional abundances of ESVs in our treatments versus controls. Several ESVs significantly differed in proportional abundance versus controls ($p_{adj} = < 0.05$, figure 4, electronic supplementary material, table ST4): in cadmium treatments: one *Commensalibacter* ESV was lower; copper treatments: two *Serratia* ESVs were higher, four *Gilliamella* ESVs (two higher and two lower), two *Bombiscardovia* ESVs were higher, one *Commensalibacter* ESV was higher, two *Lactobacillus* ESVs were higher, and two *Snodgrassella* ESVs were lower; hydrogen peroxide: one *Commensalibacter* ESV was higher; selenate treatments: two *Commensalibacter* ESVs were higher, and two *Lactobacillus*, two *Snodgrassella*, and two *Gilliamella*

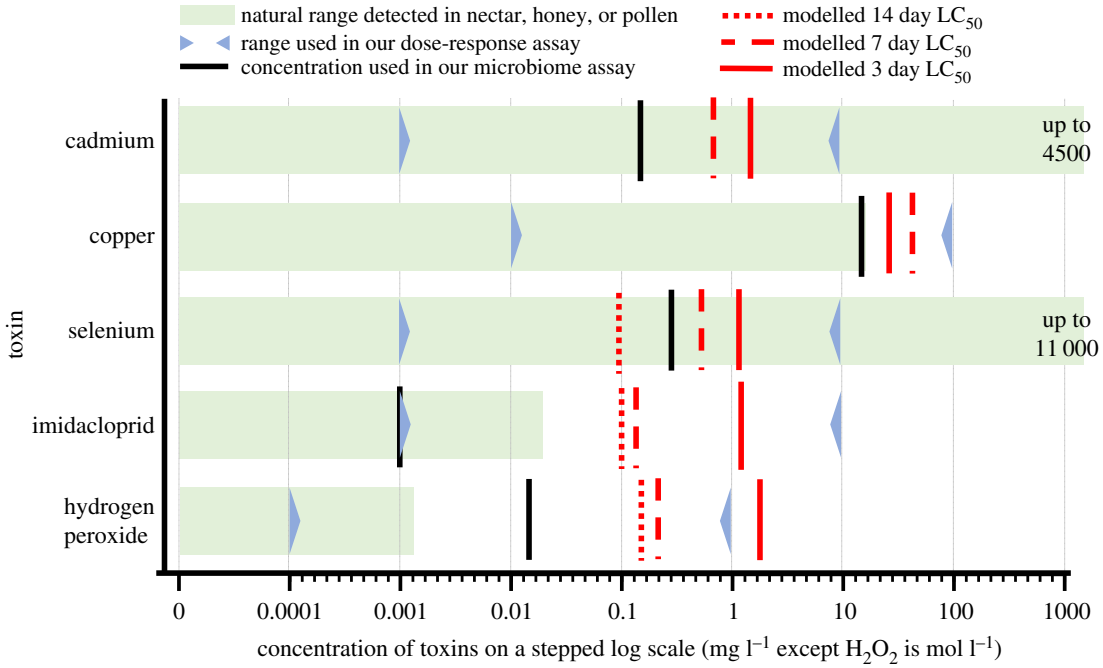

Figure 2. Natural ranges reported in nectar, honey, or pollen for each cadmium, copper, imidacloprid, hydrogen peroxide, and selenate along with the concentration ranges used in our dose–response and microbiome assays. Also annotated are the Cox proportional hazard modelled LC_{50} values for each toxicant after 3 days, 7 days, and 14 days of continuous exposure. Note in the microbiome assay, the concentration of H_2O_2 used was 10 times greater than the natural range. (Online version in colour.)

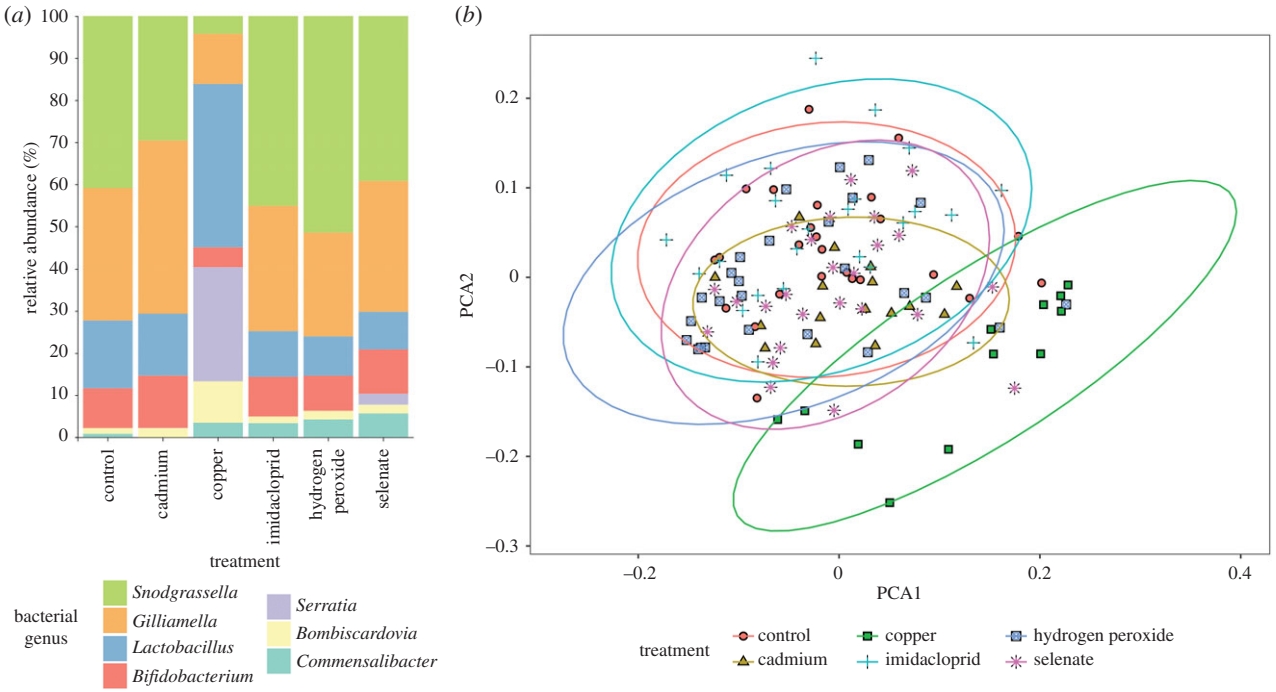

Figure 3. (*a*) Stacked bar plot showing the relative abundance of bacterial genera present in bumblebee microbiomes following 4 days of exposure to the various chemicals. Rare genera (less than 1% relative abundance) were removed for clarity. (*b*) PCoA plot of the Generalized UniFrac distance matrix of bumblebee microbiome samples following a 4-day chemical exposure. Overall, treatment ($F = 4.57$, $R^2 = 0.14$, $p < 0.001$) significantly affected the microbiomes of our samples, and *post-hoc* testing showed each treatment except imidacloprid significantly altered the bees' microbiomes (BH corrected $p_{adj} < 0.02$; imidacloprid: $p_{adj} = 0.96$). Ellipses represent 95% confidence intervals around the centroid for each treatment and colour/shape corresponds to treatment. (Online version in colour.)

ESVs were all lower; lastly, we did not find any differentially abundant ESVs in our imidacloprid-treated bees.

4. Discussion

A range of environmental toxicants negatively influenced bee health indirectly and directly by perturbing their microbiome composition and reducing their survival respectively. Field-realistic doses of cadmium, selenate, and copper impacted the bumblebee microbiome—potentially having an indirect negative effect on bumblebee health. Furthermore, there are individual ESVs of symbiotic or pathogenic bacteria that are tolerant or susceptible to most of these chemicals. Previous studies have examined whether the microbiome is affected by our assayed poisons in several non-bee species [80–84]

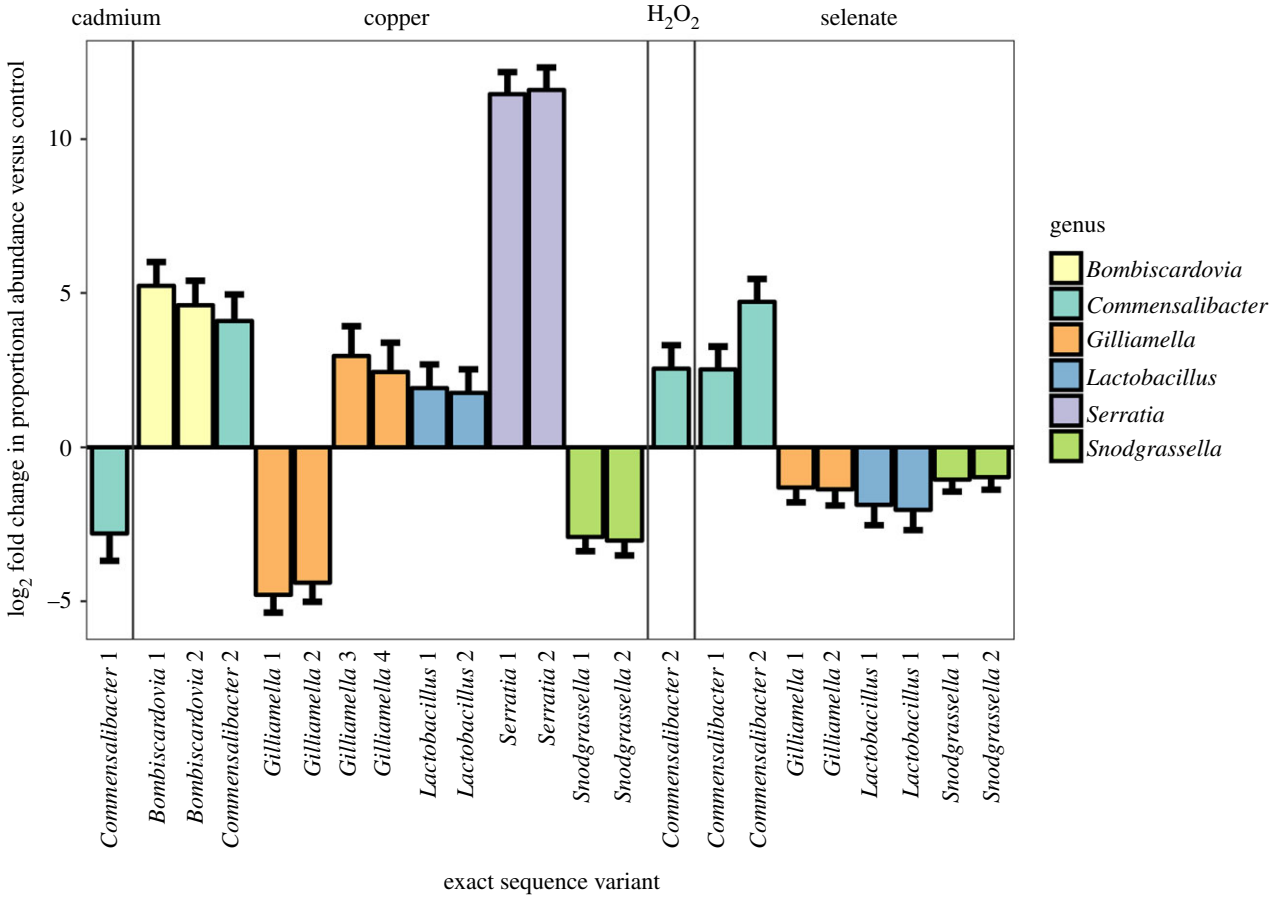

Figure 4. $\log_2$fold change of the differentially abundant ESVs in the bumblebee microbiomes following exposure to either cadmium, copper, hydrogen peroxide, or selenate treatments versus controls, coloured by genus. Each treatment had at least one significantly different ESV except imidacloprid (BH corrected $p_{adj} < 0.05$). Error bars denote the standard error of the $\log_2$fold change. (Online version in colour.)

and in honeybees and bumblebees [15,34,85]. We extend this work by screening a broad panel of toxicants on bees and their symbionts and further show that members of the bee microbiota vary in their tolerance to the chemicals. Additionally, we identify that the field-realistic concentrations of cadmium and selenate can cause mortality in the common eastern bumblebee, *Bombus impatiens*.

(a) Toxicants generally, but sometimes subtly, affect the bumblebee microbiome

The bumblebee gut microbiome exhibited a variety of responses to the toxicant challenges. Copper led to a striking compositional change of the opportunistic pathogen *Serratia*, which suggests a departure from the normal, presumably healthy gut community, potentially resulting in gut dysbiosis [24,86]. Selenate exposure altered the composition of non-core bacteria, while core symbiont ESVs were less proportionally abundant, further supporting our hypothesis of dysbiosis resulting from toxicant exposure [87]. Despite less extreme effects on survival, copper exposure had the most dramatic effect on the bees' overall microbial diversity and changed the proportional abundance of 13 individual ESVs: most taxa were compositionally enriched. Conversely, two *G. apicola* ESVs and two *S. alvi* ESVs decreased in compositional abundance. The effect on *G. apicola* is especially interesting, as two other *G. apicola* ESVs significantly increased in proportion, suggesting there is genomic variation in copper tolerance within this taxon, similar to other genomic differences within bee symbionts [78]. As a caveat, due to the compositional nature of amplicon-based microbiome sequencing, we are unable to definitively conclude the effects of treatments on any individual ESV.

Genomic analyses suggest putative mechanisms by which the bumblebee gut microbiome may be affected by copper and selenate. Each core symbiont varies in its complement of putative selenium ion resistance genes, with *Bifidobacterium bombi*, *Bombiscardovia coagulans*, *L. bombicola*, and *S. alvi* [79,88] all possessing homologous genes to the selenate transporter DedA [57], while *G. apicola* does not, with the effects of selenate exposure being overall relatively minor possibly due to the presence of selenate tolerance genes. All annotated strains of *S. alvi* putatively possess the sulfate/selenium ion transporter CysA, while only *Apis*-derived strains of *G. apicola* possess this gene but not those isolated from *Bombus* spp. Likewise, there was a slight variation in the presence and copy number of the selenium ion transporter TsgA [58] in the genomes of *Apis* isolates of *G. apicola*, while TsgA was much rarer in the genomes of *Bombus*-derived strains. Similar to selenium tolerance genes, between-strain variation likely exists in copper tolerance in *S. alvi* and *G. apicola*: by homology to other bacterial taxa, strains of *S. alvi* contain varying numbers of the genes CueO [65], CueR [68], and a copper-translocating ATPase, while *G. apicola* strains varied in SufI and copper-translocating ATPase genes; however, within *Bombus* isolates, there was almost no variation in these genes and they did not possess CueO. *Snodgrassella* isolated from *Apis* spp. accounted for most of the variation between strains. The strain variation in homologous *G. apicola* copper-translocating

genes may underlie the differential abundance of *G. apicola* strains under copper challenge, although our 16S rRNA gene data do not allow us to test this hypothesis. Both *Apis*- and *Bombus*-derived strains varied in their SufI and copper-translocating ATPase genes. Lastly, we note that the potential pathogen *Serratia marcescens*—which had a dramatic compositional increase during copper treatments—has numerous putative genomic bases for copper tolerance, although again we are unable to verify the genes present due to the limitations of 16S rRNA gene sequencing.

Cadmium, imidacloprid, and hydrogen peroxide all had moderate (cadmium and hydrogen peroxide) to no (imidacloprid) effects on the microbiome. Cadmium changed the bumblebees' bacterial community but resulted in decreased proportional abundance of only one ESV of *Commensalibacter*. While neither *S. alvi* nor *G. apicola* were affected by cadmium treatments, there were notable differences between *Apis* and *Bombus*-derived isolates. For example, the presence of the putative cadmium-responsive regulator gene CadR was highly variable between both *G. apicola* (CadR is absent in *Bombus* isolates) and *S. alvi* (CadR was present more commonly and in greater copy number, in *Apis* isolates than *Bombus* isolates). Imidacloprid did not affect the gut microbiome in *B. impatiens*, which agrees with a previous experiment that showed imidacloprid did not affect the honeybee microbial community [85]. As imidacloprid targets acetylcholine receptors in insects [89], it is perhaps not surprising that the bumblebee gut microbiome is not affected by this insecticide. Hydrogen peroxide modestly changed the microbial community of *B. impatiens* at higher-than-natural concentrations and increased the proportional abundance of one ESV of *Commensalibacter*. As hydrogen peroxide is thought to have antimicrobial properties in flower nectar [90], bumblebee-associated microbes may be resistant due to routine peroxide exposure.

The ubiquity of hydrogen peroxide exposure in nature may explain why members of the core bee gut microbiome have combinations of putative genes to cope with oxidative stress. While *S. alvi* did not exhibit any strain variation in the presence of homologous genes known to underlie oxidative stress response, *G. apicola* did: our genomic analysis indicated a variable presence of SoxS, an Fnr regulator, and NnrS. Cadmium resistance is less clear, as *Commensalibacter intestini* has several cadmium resistance genes, but is still susceptible to the treatment *in vivo*, while core bumblebee symbionts' resistance pathways are more depauperate. As with other resistance pathways, *G. apicola* varied in genes predicted to underlie cadmium tolerance, while *S. alvi* exhibited substantial strain variation, notably in CadR between *Apis* and *Bombus* isolates. These results suggest that individual core bee microbiome members largely resist cadmium on a community-level scale, and we hypothesize that bacteria may be partitioning cadmium detoxification between each other, as has been shown in other metabolic processes [79]. We also note that in insects, most metal uptake occurs in the midgut [36], so the concentrations of the metals are likely lessened through the bee absorbing some metal before hindgut bacteria may encounter these toxicants.

(b) Mortality effects of each compound

By exposing bees to cadmium, copper, hydrogen peroxide, imidacloprid, or selenate, we show that each toxicant is lethal to bumblebees at varying concentrations—following the mantra that the dose makes the poison. For example, constant ingestion of selenate and cadmium at levels that bees may encounter on flowers grown in polluted soils are toxic even on the third day of chronic exposure [91,92]. Bees were more tolerant of copper, with lethal doses higher than the levels likely encountered when foraging on plants in contaminated areas [92]. In regard to non-metallic toxicants, the insecticide imidacloprid and hydrogen peroxide were both lethal to bees at doses above normal exposure, and we note that bees appeared to avoid the highest doses of hydrogen peroxide. While adult bees tolerated above-field-relevant doses of copper and imidacloprid, sublethal exposure to these chemicals is known to reduce brood production and larvae population, which may cause negative colony-level effects [14,93]. Lastly, bees seemed to tolerate natural levels of hydrogen peroxide, which is supported by studies showing high hydrogen peroxide levels in some flowers [94] and that bees can detoxify peroxide [95]. Our data suggest that exposure to these chemicals should be investigated further, and studies should focus on interactions between bees, gut microbes, parasites, and their environment, to understand more about the subtle and potentially synergistic effects of stressors on pollinator health.

5. Conclusion

Bees have been recognized for their use as bioindicators to monitor environmental pollution, and our work supports this claim by showing that bees are susceptible to many environmental toxicants. Our interdisciplinary study reports the direct effects of cadmium, copper, hydrogen peroxide, imidacloprid, and selenate exposure, and we conclude that direct effects are only part of the story. To fully appreciate the risks of exposure we must also consider the effects on the microbiome as indirect effects on bee health. Encouragingly, we have identified several putative genomic bases for microbial tolerance or susceptibility to each toxicant and found that there can be substantial strain variation in these genes in the bacteria *S. alvi* and *G. apicola*, especially between strains isolated from different bee genera. This variation suggests that the bee gut microbiome harbours diverse strains that may be resilient to various environmental challenges. As we have indicated, there is a wide diversity of putative stress response genes between bee symbiont strains, and culture-based toxicology assays should be conducted to characterize bacterial susceptibility to toxicants *in vitro*. We suggest that future studies investigate the multipartite interactions between host, symbiont, and the environment, and the potential for microbiomes and hosts to reciprocally protect each other from environmental insults.

Data accessibility. Raw data and representative code are available from the Dryad Digital Repository: https://doi.org/10.7280/D14T2K [96].

Authors' contributions. J.A.R., K.A.R., L.L., Q.S.M., and P.G. conceived the project; L.L. performed survival experiments; J.A.R., K.A.R., and P.G. performed microbiome exposure work; J.A.R. performed genome scans and analysed microbiome data; P.G. analysed survival data; J.A.R. wrote the first draft of the manuscript, and all authors helped make subsequent additions to the final version.

Competing interests. We declare we have no competing interests.

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