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RESEARCH ARTICLE

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Multiple stressors interact to impair the performance of bumblebee *Bombus terrestris* colonies

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Abstract

1. Bumblebees are constantly exposed to a wide range of biotic and abiotic stresses which they must defend themselves against to survive. Pathogens and pesticides represent important stressors that influence bumblebee health, both when acting alone or in combination. To better understand bumblebee health, we need to investigate how these factors interact, yet experimental studies to date generally focus on only one or two stressors.
2. The aim of this study is to evaluate how combined effects of four important stressors (the gut parasite *Nosema ceranae*, the neonicotinoid insecticide thiamethoxam, the pyrethroid insecticide cypermethrin and the EBI fungicide tebuconazole) interact to affect bumblebees at the individual and colony levels.
3. We established seven treatment groups of colonies that we pulse exposed to different combinations of these stressors for 2 weeks under laboratory conditions. Colonies were subsequently placed in the field for 7 weeks to evaluate the effect of treatments on the prevalence of *N. ceranae* in inoculated bumblebees, expression levels of immunity and detoxification-related genes, food collection, weight gain, worker and male numbers, and production of worker brood and reproductives.
4. Exposure to pesticide mixtures reduced food collection by bumblebees. All immunity-related genes were upregulated in the bumblebees inoculated with *N. ceranae* when they had not been exposed to pesticide mixtures, and bumblebees exposed to the fungicide and the pyrethroid were less likely to have *N. ceranae*. Combined exposure to the three-pesticide mixture and *N. ceranae* reduced bumblebee colony growth, and all treatments had detrimental effects on brood production. The groups exposed to the neonicotinoid insecticide produced 40%–76% fewer queens than control colonies.
5. Our findings show that exposure to combinations of stressors that bumblebees frequently come into contact with have detrimental effects on colony health and performance and could therefore have an impact at the population level. These results also have significant implications for current practices and policies for pesticide risk assessment and use as the combinations tested here are frequently applied simultaneously in the field. Understanding the interactions between different stressors will be crucial for improving our ability to manage bee populations and for ensuring pollination services into the future.

KEYWORDS

Bombus terrestris, bumblebee health, colony performance, environmental stressors, *Nosema ceranae*, pesticide mixtures

1 | INTRODUCTION

Emerging evidence of widespread pollinator declines at local and regional scales (Nieto et al., 2015; Ollerton et al., 2014; Powney et al., 2019) raises concerns about reduced agricultural productivity and sustainability in natural ecosystems (Biesmeijer, 2006; Gill et al., 2015). Although the effects of environmental stressors on organisms are usually tested individually (Vouk et al., 1987), bees are often simultaneously exposed to a wide variety of biotic and abiotic stressors in nature (González-Varo et al., 2013). Protecting bee health is therefore a complex multifactorial issue that requires considering scenarios that include multiple stressors. Pathogens and pesticides have often been highlighted as key drivers of population declines in both wild and managed species (Goulson et al., 2015).

In agricultural environments, bees are frequently exposed to combinations of chemicals (David et al., 2016; Mullin et al., 2010; Tosi et al., 2018), some of which may produce additive, antagonistic or synergistic effects (Biddinger et al., 2013; Johnson et al., 2013; Sgolastra et al., 2017, 2018; Spurgeon et al., 2016; Zaragoza-Trello et al., 2020; Zhu et al., 2014). For instance, chronic exposure to a combination of field-relevant concentrations of two insecticides (neonicotinoid and pyrethroid) impaired natural foraging behaviour in *Bombus terrestris* colonies, and increased worker mortality leading to significant reductions in brood development and colony success (Gill et al., 2012). Moreover, the toxicity of neonicotinoids and pyrethroids in bees can increase when they are exposed to ergosterol-biosynthesis-inhibiting (EBI) fungicides simultaneously (Colin & Belzunces, 1992; Iwasa et al., 2004; Pilling et al., 1995; Pilling & Jepson, 1993; Sgolastra et al., 2017). EBI fungicide inhibit the cytochrome P450 enzymatic detoxification mechanism in insects which is necessary for oxidative metabolism of a variety of xenobiotics (Brattsten et al., 1994). Metabolic detoxification mediated by cytochrome P450s contributes significantly to bee tolerance to some insecticides (Beadle et al., 2019; Hayward et al., 2019; Johnson et al., 2006), and cytochrome P450 activity has been shown to be an important determinant of neonicotinoid sensitivity in bumblebees (Manjon et al., 2018). The inhibition of this detoxification mechanism may lead to insecticide residues being metabolized more slowly, although the effect is dose dependent, and the extent of synergism in field-realistic conditions is unclear (Thompson et al., 2014). Therefore, the toxic effects of pesticides encountered by bees in the field are difficult to predict from single-compound laboratory studies. Exposure to EBI fungicide can also reduce the anti-feeding response of bees to pyrethroids, and consequently increase the toxicity of these insecticides by raising the levels of exposure (Thompson & Wilkins, 2003). Moreover, exposure to neonicotinoids and to EBI fungicide has been shown to alter the feeding behaviour of bees (Azpiazu et al., 2019; Elston et al., 2013; Kessler et al., 2015). Therefore, the repellency or attractiveness of the food contaminated with different pesticide should also be assessed to better understand the extent of exposure to certain mixtures.

Bees are also impacted by a diversity of pathogens, including one suspected to seriously affect their health, the microsporidium *Nosema ceranae* (Graystock, Yates, Darvill, et al., 2013; Higes, Martín-Hernández, Botías, et al., 2008), which infects gut epithelia of adult bees. This obligate intracellular eukaryotic parasite was initially detected in the Asian honeybee *Apis cerana* (Fries et al., 1996), but is now globally distributed in *A. mellifera* (Klee et al., 2007), and has been more recently detected in several species of solitary bees and bumblebees (Li et al., 2012; Plischuk et al., 2009; Ravoet et al., 2014). *Nosema ceranae* has been suggested to be the causative agent of an emergent infectious disease in bumblebees because detrimental effects in survival, behaviour and colony growth have been reported for *B. terrestris* with spores of this microsporidium (Graystock, Yates, Darvill, et al., 2013; Rotheray et al., 2017). However, the results of a recent study question the classification of *N. ceranae* as an emerging infectious agent for bumblebees as a new host (Gisder et al., 2020). The detrimental effects detected in *N. ceranae* PCR-positive bumblebees may instead be due to the specific energetic and corresponding physiological costs that immune responses entail (Ardia et al., 2012). In addition, exposure to neonicotinoid insecticides and some fungicides has been reported to increase the levels and impact of *N. ceranae* in honeybees (Alaux et al., 2010; Pettis et al., 2012, 2013), possibly due to a reduced immunocompetence in bees challenged by some types of pesticides (Di Prisco et al., 2013; Garrido et al., 2013). Understanding how environmental stressors affect immune responses in bees is crucial for developing more informed strategies for mitigating the impacts of pesticides on the health of pollinators (Pamminger et al., 2018). In bumblebees, as in other insects, the immune responses to parasites have been identified, and consist of both cellular and humoral responses (Barribeau et al., 2015). Phagocytosis, encapsulation and melanization are related to cellular immunity (Osta et al., 2004), while humoral immunity involves antimicrobial peptide (AMPs) synthesis (Evans, 2006). Four antimicrobial peptides which provide a broad-spectrum of activity against microorganisms, *abaecin*, *apidaecin*, *defensin* and *hymenoptaecin*, have been identified in *B. terrestris* during infection by trypanosomatid parasites (Barribeau et al., 2015; Brunner et al., 2013; Riddell et al., 2011), and during *N. ceranae* infection in honeybees (Antúnez et al., 2009; Chaimanee et al., 2013). The synthesis of these AMPs is under the control of two important signalling pathways, the Toll and the Imd pathways (Evans et al., 2006; Lemaitre & Hoffmann, 2007). Another

molecule with a potential role in defence against gut-infecting parasites (Schlüns et al., 2010) such as the microsporidium *N. ceranae*, in bumblebees, is hemomucin, a surface glycoprotein involved in inducing an immune response and found to be highly expressed in the midgut (Theopold et al., 1996).

Here we conducted an experiment on bumblebees *B. terrestris* to study the combined effects of chronic exposure to field-relevant doses of three abiotic stressors, the neonicotinoid thiamethoxam, the pyrethroid cypermethrin and the EBI fungicide tebuconazole, as well as the biotic stressor, *N. ceranae*. Bumblebee colonies were pulse exposed to the stressors in controlled laboratory conditions for 2 weeks and then subsequently placed in the field for a further 7 weeks (depuration period under environmentally relevant conditions). We compared the effect of simultaneous exposure to the four stressors and to different ternary combinations of them, by evaluating the impact of such stressors during the pulsed exposure and the second potential demographic impact due to failure to fully eliminate the toxicants and the pathogen during the depuration period. In addition, one group was exposed solely to the microsporidium *N. ceranae* in order to discern if pesticide exposure alters the prevalence and gene expression profile related to the immune response of inoculated bees. Thus, after the 2-week pulsed exposure period ended, we evaluated the prevalence of *N. ceranae* in bumblebee colonies. Next, to further explore this effect we measured gene expression in four AMPs (*abaecin*, *apidaecin*, *defensin* and *hymenoptaecin*), a signal molecule of the Toll (*pelle*), and Imd immune pathways (*relish*), and an immune recognition/effector gene (*hemomucin*) in order to evaluate the effect of *N. ceranae* inoculation on the gene expression related to the immune response of bumblebees, and investigate the possible interference of pesticides on this effect (Aufauvre et al., 2014; Di Prisco et al., 2013). We also studied the expression of two genes related to cytochrome P450-mediated detoxification involved in the defence against insecticides in bumblebees and other insects (Colgan et al., 2019; Huang et al., 2015; Jing et al., 2018) to test if exposure to the EBI fungicide tebuconazole alters the cytochrome P450 enzymatic detoxification mechanisms in bumblebees, presumably exacerbating the effect of insecticide (Azpiazu et al., 2019; Berenbaum & Johnson, 2015; Iwasa et al., 2004; Johnson et al., 2013; Sgolastra et al., 2017). Collection of pollen and nectar with and without pesticides was also evaluated during the 2 weeks under laboratory-controlled conditions in order to assess if exposure to mixtures of pesticides had an effect on the feeding behaviour of bumblebees. Finally, colony growth and the production of workers and sexuals were measured during, and at the end of the depuration period respectively, to determine which combinations of stressors are more harmful to bumblebee colonies.

2 | MATERIALS AND METHODS

We obtained 70 early-stage bumblebee colonies of *B. terrestris audax* (Biobest, Westerlo, Belgium) on 21 May 2014, each consisting of a queen, brood and 10–21 workers (mean ± *SD* = 15.8 ± 3.16 workers/colony). We confirmed that all colonies were free of the most prevalent parasites (*Nosema bombi*, *N. ceranae*, Trypanosomatida and *Apicystis bombi*) at the start of the experiment by microscopic examination of faeces collected from the colonies and PCR of the guts of 20% of the workers present in each colony (Graystock, Yates, Evison, et al., 2013; Martín-Hernández et al., 2007). Tripanosomatids could not be identified to the species level because the method used for detection (Meeus et al., 2010), based on amplification of the 18S rDNA gene alone, has been shown to be unsuitable to classify tripanosomatids infecting bees without sequencing (Bartolomé et al., 2018; Ravoet et al., 2015). Colonies were then randomly assigned to one of seven treatments (see below for details on the treatments; 10 colonies/treatment). There were no significant differences between treatments in the worker population or weight of colonies at the start of the experiment (ANOVA; worker population: $F_{6,63} = 0.563$, $p = 0.758$; mean weight = 119 ± 7.85 g, $F_{6,63} = 0.242$, $p = 0.961$).

The seven treatments were applied using a stepwise removal approach, consisting of exposure to three abiotic and one biotic stressor applied simultaneously or in different ternary combinations. These treatments were applied in order to determine the relative importance of each stressor or combination of stressors in affecting bumblebees at the individual and colony levels (Table 1). In comparison with factorial approaches, the stepwise removal approach allowed us to empirically test complex combinations of up to four stressors with a limited number of treatments, and thus, to simplify the otherwise unwieldy number of potential interactions by using experimental treatments that reflect combinations of stressors that are likely to occur in real-world agricultural landscapes (Côté et al., 2016). The abiotic stressors were the pesticides thiamethoxam (neonicotinoid insecticide), cypermethrin (pyrethroid insecticide) and tebuconazole (demethylation inhibitor fungicide). These pesticides were selected based on their extensive use

TABLE 1 Summary of the seven treatments applied to *Bombus terrestris* colonies in the study (10 colonies/treatment), involving three abiotic pesticide stressors (the neonicotinoid insecticide thiamethoxam, the pyrethroid insecticide cypermethrin and the fungicide tebuconazole) and one biotic stressor (the microsporidian pathogen *Nosema ceranae*)

Group	Neonicotinoid (N)	Pyrethroid (P)	EBI fungicide (F)	*N. ceranae* (M)
NPFM	√	√	√	√
NPF	√	√	√	
PFM		√	√	√
NPM	√	√		√
NFM	√		√	√
M				√
Control				

in the UK arable crops including oilseed rape, wheat and spring barley (FERA, 2017), consequent prevalence on crop and field margin flowers, and their regular occurrence in bee food stores worldwide (Sánchez-Bayo & Goka, 2014). The biotic stressor was the microsporidian pathogen *N. ceranae* (Table 1).

The colonies were kept in laboratory conditions (25°C, 50%–60% relative humidity) in complete darkness for the first 2 weeks of the experiment. They received 5 g of fresh pollen in each of two feeders and 50 g of sugar solution in each of two feeders every 2–3 days, with any remaining pollen and sugar solution being weighed and discarded after replacement. The pollen food provided was a honeybee-collected polyfloral pollen blend, purchased from Biobest (Belgium) through Agralan Ltd (Swindon, UK), that was sterilized to exclude honeybee pathogen spill-over effects by the application of gamma irradiation with a cobalt-60 source at dose rates between 25 and 45 kGy (Graystock et al., 2016; Higes, Martín-Hernández, Garrido-Bailón, García-Palencia, 2008; Singh et al., 2010). Gamma irradiation has also been shown to be an effective tool to degrade pesticide residues in different matrices (Dessouki et al., 1999; Pargi & Bhatt, 2018). The sugar solution provided was 50% inverted sugar syrup (w/v with Ambrosia syrup, E H Thorne Ltd, and distilled water). Pesticide exposure started on Day 2 after arrival, when the colonies had acclimatized to the laboratory conditions. *Nosema ceranae* inoculation was performed on Day 5 (see below for details), such that bees had already been exposed to pesticide mixtures for 3 days at the time of inoculation. The 2-week pesticide exposure duration was based on the blooming period of flowering crops, when exposure of bumblebees to pesticide mixtures applied to the crops is more likely (Botías et al., 2017). Therefore, on Day 15, samples were collected for analysis of gene expression and colonies were then placed in the field where the workers could forage under natural conditions for a further 7 weeks (Figure S1). The weight of the colonies was measured once per week throughout the experiment. By the end of 9 weeks, the colonies were reaching the natural ends of their lives (indicated by production of reproductives, and decreases in number of workers and colony weight; Goulson, 2010).

2.1 | Pesticide exposure

Colonies were exposed to pesticides via the pollen and sugar solution provided according to their treatment group (Table 1), with pesticide-spiked food provided in the relevant treatments in one of the two pollen feeders and one of the two sugar solution feeders ('pesticide feeder'), and pesticide-free food provided in the other feeders ('uncontaminated feeder') in order to simulate exposure in field conditions in which bees may forage on flowers with and without residues, and also to detect a possible inhibitory feeding effect of contaminated food. Stock solutions were prepared in acetone of 1 mg/ml thiamethoxam (TMX) ($C_8H_{10}ClN_5O_3S$ powder grade: PESTANAL®, analytical standard; brand: Fluka), cypermethrin (CYPER) ($C_{22}H_{19}Cl_2NO_3$ powder; grade: PESTANAL®, analytical standard; brand: Fluka) and tebuconazole (TEB) ($C_{16}H_{22}ClN_3O$ powder; grade: PESTANAL®, analytical standard; brand: Fluka). A sample of each was subsequently diluted with distilled water to obtain the required concentrations for the experiment (0.01 mg/ml).

Pollen was spiked according to treatment by spreading the corresponding volume of 0.01 mg/ml solutions of each pesticide over it with a micropipette to obtain the concentrations required for the experiment: 5 p.p.b. of thiamethoxam (Botías et al., 2015), 10 p.p.b. of cypermethrin (Mullin et al., 2010) and/or 36 p.p.b. of tebuconazole (David et al., 2016; Mullin et al., 2010). Once spiked, the pollen with the pesticide solutions was homogenized by means of a mortar and pestle. Sugar solution was spiked according to treatment with 1.5 p.p.b. of thiamethoxam (Botías et al., 2015), 10 p.p.b. of cypermethrin (Mukherjee, 2009) and/or 36 p.p.b. of tebuconazole (Büchler & Volkmann, 2003). The thiamethoxam concentration used was that detected previously in pollen and nectar collected from flowers (Botías et al., 2015). The cypermethrin and tebuconazole concentrations used were those detected previously in bee food stores (beebread and honey), doubled to reflect the fact that beebread and honey are mixtures of pollen and nectar from different plants that may or may not contain pesticide residues. Pesticide-free food was spiked with the same concentration of acetone as applied to pesticide-contaminated food (5 μl/g). Since we were interested in measuring the potential feeding inhibition effect of the treatments applied, the location of the 'pesticide feeder' and the 'uncontaminated feeder' within the nests were interchanged every time the food was renewed in case the bumblebees preferred collecting food from a particular side of the nest.

2.2 | *Nosema ceranae* inoculation

On Day 5 of the experiment, when the colonies had been exposed to pesticide mixtures for 3 days, all bees within each colony were individually marked on the thorax and fed 4 μl of a single meal of either 30% sugar water (controls and the pathogen-free treatment NPF) or 30% sugar water containing *c*. 120,000 freshly prepared *N. ceranae* spores (other treatments, Table 1; viability 98.9% based on 0.4% Trypan blue staining; Higes, Martín-Hernández, Garrido-Bailón, et al., 2008) using a micropipette. The *N. ceranae* spores were obtained by homogenizing abdomens of adult honeybees from a naturally infected honeybee colony and purifying the homogenate by centrifugation in 95% Percoll (Sigma-Aldrich). Identity of the parasite was confirmed by PCR (Martín-Hernández et al., 2012). The dose administered is typical of that used in honeybee studies (Alaux et al., 2010; Doublet et al., 2014; Higes et al., 2007) and dosages of fewer than 100,000 spores have been found to infect bumblebees (Fürst et al., 2014; Graystock, Yates, Darvill, et al., 2013). Bees were starved for 4 hr before the inoculation of spores, and then immobilized by placing them in a cooler bag with ice blocks for approximately 10–15 min for ease of handling. Recovering bees ingested the inoculum when their proboscis was touched with a droplet of the spore solution at the tip of a micropipette. After parasite inoculation on Day 5, colonies were monitored daily for worker mortality until they were placed in the field on Day

15, with very low mortality observed during this period (<3 workers in all treatments). *Nosema* spores were not detected in the dead bees collected.

2.3 | DNA and RNA extraction, pathogen screening and gene expression analysis

Ten days post-inoculation, five marked bees per colony were flash-frozen in liquid nitrogen and stored at −80°C for subsequent parasite and gene expression analysis. The midgut is the site of infection by *N. ceranae* and also the main site of exposure to orally administered chemicals, so the midguts of these bees were individually dissected and used for the pathogen screening and gene expression analyses. Once dissected, midguts were immediately homogenized in 600 μl of buffer RTL Plus within a Pathogen Lysis Tube (Cat No./ID: 19092, Qiagen), grinding first with a microtube pestle and then with a Tissue Lyser LT (5 min at 50 Hz, Qiagen). Isolation of DNA and RNA from bumblebee guts was performed using the AllPrep DNA/RNA Mini kit (Qiagen) according to the AllPrep DNA/RNA protocol. Genomic DNA was removed from RNA isolated using the RNA-free DNase set (Qiagen) during the RNA extraction. DNA was stored at −20°C until pathogen screening was performed, and RNA was stored at −80°C until gene expression analysis started.

The presence of common bumblebee parasites (*N. bombi*, *N. ceranae*, Trypanosomatida, *A. bombi*), which are regarded as a threat to bumblebee health (Graystock, Yates, Evison, et al., 2013; Meeus et al., 2011), was evaluated via PCR by analysing the five workers/colony that had been collected 10 days post-inoculation with *N. ceranae* spores ($N = 50$ bees per treatment group). A pool of the five DNA extracts per colony was analysed using PCR protocols and parasite-specific primers following the methods of Graystock, Yates, Evison, et al. (2013), except for *N. ceranae* detection protocols where we followed methods described by Martín-Hernández et al. (2007). In the case of the *N. ceranae*-positive DNA pooled extracts, PCR analysis was performed in individual DNA templates in order to estimate the percentage of bees per colony with this microsporidian parasite. At the end of the experiment, a further subset of five bees per colony were examined for pathogen presence as described above.

For the gene expression analysis, we used the RNA of one bumblebee per colony (10 bumblebees per treatment). Since we were interested in the influence of pesticide treatments on the immune response, and the possible interference of the EBI fungicide tebuconazole in the detoxification mechanisms, we only analysed bumblebees from treatments PFM (CYPER + TEB + *N. ceranae*), NPM (TMX + CYPER + *N. ceranae*), NFM (TMX + TEB + *N. ceranae*), M (*N. ceranae*) and control, for the gene expression assay. All the specimens from groups inoculated with *N. ceranae* (PFM, NPM, NFM and M) used for gene expression were confirmed to be positive for this microsporidium by PCR.

The concentration and purity of RNA was determined on a Qubit fluorometer using a Qubit RNA HS Assay kit together with a Qubit® dsDNA HS Assay Kit (Qiagen), and 300 ng of total RNA was used for reverse transcription using the Phuson RT-PCR kit (Thermo Scientific). A set of seven immunity-related target genes, namely *abaecin*, *apidaecin*, *defensin*, *hymenoptaecin*, *hemomucin*, *relish* and *pelle*, were analysed using primers described previously (Brunner et al., 2013; Schlüns et al., 2010). In addition, the expression of two detoxification-related genes (*CPR* and *CYP4G15*) was evaluated by using two sets of primers, which were designed using Primer3 and published sequences (see Table S1 for primer specifications and putative gene function). Standard curves with three qPCR replicates at 1:10, 1:100, 1:1,000 and 1:5,000 concentrations were generated to test the amplification efficiency of each primer set. The efficiency of all the primer sets used in our experiment were between 93.3% and 100.2%. Each sample was tested with the reference genes *AK* and *PLA2* (Horňáková et al., 2010) and all seven immunity-related genes and two detoxification-related genes. Three technical replicates were run per reaction. Reactions for qPCR were performed on an ABI OneStep™ RT-PCR instrument using the following program: 95°C for 5 m, followed by 40 cycles of a 30 s at 94°C denaturation, 30 s at 59°C annealing and 30 s at 72°C extension steps. Results were analysed using the OneStep™ analysis software. The amplification results from the different genes were expressed as the threshold cycle (C_T) value, which represents the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. Fluorescence was measured in the elongation step and negative controls (without cDNA) were included in each reaction run. Fold change in expression was calculated relative to expression levels in the control samples and using the geometric mean of reference genes *AK* and *PLA2* as the endogenous control value with the $2^{(\Delta-\Delta CT)}$ approximation method (Livak & Schmittgen, 2001).

2.4 | Bumblebee monitoring under field conditions

Monitoring under field conditions was timed to correspond to wild colony development in the region where the study was performed (East Sussex, South East England, UK), with colonies being placed out in the field on Day 15 after the colonies arrived (Day 13 post-exposure). The field site was situated in an orchard at Stanmer Organics (Brighton, East Sussex, UK), which has been Soil Association-certified organic for the past 10 years and is located within Stanmer Park Local Natural Reserve (about 5,000 ha of open and wooded land), that covers a larger area than the typical foraging range of *B. terrestris* (Osborne et al., 2008). Colonies were randomly and evenly distributed across the orchard, leaving at least 5 m of separation between them. All colonies were placed in shaded positions (Figure S1). At the time of placement in the field, none of the flowering crops (mainly oilseed rape) within 2 km were in bloom. Although the doors on the nest-boxes were designed to ensure none of the queens were able to leave the colony, the numbers of newly emerged queens were unequal to the number of uncapped queen cells in their corresponding nests, so we assumed that some of the queens had managed to escape and therefore did not include the number of queens in our analyses of colony performance. The fresh

weight of all colonies was recorded at the start of the experiment and weekly thereafter. The nests were housed in a plastic box, which was in turn placed within a cardboard box. Since it is not possible to remove the nest material from the inner plastic box without causing severe disturbance, we weighed the inner box and all biological material within (bees, wax, brood, honey pots, etc.). In general, colony mortality over the course of the experiment was very low, with no colonies dying during the first 8 weeks. In the last week, five colonies died (one from group NPFM, one from group NPF, two from group PFM and one from group M). At the end of the experiment, all colonies were freeze-killed and then dissected. The number of empty and capped queen cells, males, workers and empty and capped worker/male pupal cells (brood cells) were counted. Also, 10 workers and 10 males were randomly collected from each colony (or all the bees available in colonies with <10 individuals), individually weighed and their thorax width was measured using digital callipers. A sample of five workers per colony was collected and processed for pathogen analysis, using methods described above.

2.5 | Statistical analysis

All data were analysed using the statistical software R, version 3.5.1 (R Core Team, 2018). For the five colonies that collapsed in the field 1 week before the end of the experiment, the values of number of workers, males, brood cells and queen cells recorded upon collection from the field (in week 8) were the ones used for the statistical analyses. Residuals of all models were inspected to ensure model assumptions were met.

The differences in the collection of uncontaminated versus pesticides-treated pollen and sugar solution were analysed using generalized linear models (NLME package; Pinheiro & Bates, 2000). The differential collection of pollen or sugar solution divided by the number of individuals per colony was modelled as a function of the treatment group and the time when food consumption was measured. The temporal correlation between errors was included in order to account for the repeated measures performed in each colony and colony was included as a random effect. In addition, the total collection of pollen and sugar solution measured after 2 weeks under laboratory conditions was analysed using linear regression (LM; Gaussian error distribution), with the total collection of pollen and sugar solution per group entered as fixed effects. Multiple pairwise comparisons for total pollen and sugar solution collected were performed using Tukey's post-hoc tests (using the R package MULTCOMP; Westfall et al., 1999). The relative percentage of pollen or sugar solution collected per group from the uncontaminated feeder in relation to the pesticide feeder was calculated by using the following formula: (grams of pollen or sugar solution collected from the uncontaminated feeder in the 10 colonies of the group × 100)/grams of pollen or sugar solution collected from both feeders in the 10 colonies of the group. The overall percentage of food collected per group compared to controls was calculated using the formula: 100 − [(total amount of pollen or sugar solution collected by the 10 colonies of the group × 100)/Total grams of pollen or sugar solution collected by the 10 colonies of control group].

The number of bees positive for *N. ceranae* 10 days post-inoculation was compared among the treatment groups that were inoculated with this microsporidium using a GLM (binomial error distribution). A similar model was used to examine the possible influence of treatments on the detection of pathogens in the colonies at the end of the study (modelled as the presence/absence of the pathogens in each colony).

Fold change in gene expression data was log-transformed to account for a right skew, and comparisons of all genes among the experimental groups were performed using model based analysis of multivariate data with a negative binomial error distribution (MVABUND package; Wang et al., 2012). Multivariate linear models allowed us to fit the responses of all genes simultaneously (Wang et al., 2012; Warton et al., 2012). The *summary.manylm* function in mvabund was used to test the effects of treatments on gene expression using the Lawley-Hotelling trace statistic, and we corrected for multiple tests using a stepdown resampling procedure. For multiple pairwise comparisons, differences in the fold change in gene expression between treatments were examined using a Tukey and Kramer (Nemenyi) test with Tukey-Dist approximation for independent samples (Wang et al., 2012).

Colony weight gain over time was analysed using a linear mixed effects model (LMM; Gaussian error distribution). Weight gain data were log-transformed prior to analysis to achieve normal error distribution. Treatment, week and week^2 (to account for the curved relationship of weight over time) were entered as fixed effects and the number of workers present at week 0 as a covariate. The interaction between week and treatment was included in the model and colony was entered as a random effect. Maximum colony weight, which was achieved at Week 5 in all treatment groups, was also compared among groups as a fitness proxy by using linear regression (LM; Gaussian error distribution), followed by Tukey's post-hoc tests (Westfall et al., 1999). The effect of treatment on the total numbers of adult workers, adult males, worker and male brood cells (capped and uncapped; worker and male cells cannot be distinguished), and queen cells (capped and uncapped) was assessed using generalized linear mixed effects models (GLM; Poisson error distribution), while the effect of treatment on the weight and thorax width of adult workers and males were analysed using (LMM; normal error distribution). All models had treatment as a fixed factor and the LMMs included colony as a random factor. Multiple pairwise comparisons for number of workers, males, brood cells and queen cells produced were performed by using Tukey's post-hoc tests (Westfall et al., 1999). To evaluate the relative contribution of each stressor in affecting bumblebee colony fitness, we assessed the mean effect size of each individual stressor on the production of brood cells, new queens and males. To do this, we calculated the mean effect size of each of the treatments containing a particular stressor, minus the effect size of the treatment without it (e.g. average contribution of N to the effects on colony fitness: mean [(NPFM − PFM); (NPF − PFM); (NPM − PFM); (NFM − PFM); (NPFM − M); (NPF − M); (NPM − M); (NFM − M)].

3 | RESULTS

3.1 | Food collection

Bumblebees collected 27%, 16%, 24%, 24% and 19% more pollen respectively in colonies of groups NPFM, NPF, PFM, NPM and NFM from the 'uncontaminated feeder' than from the 'pesticides feeder' (GLM; $F_{6,342} = 17.17$, $p < 0.0001$; Figure 1a; Table S2), and reduced their overall pollen collection by 46%, 21%, 50%, 57% and 38% respectively in colonies of groups NPFM, NPF, PFM, NPM and NFM compared to controls (LM; $F_{6,63} = 30.90$, $p < 0.0001$; Figure 1b; Table S3). There was a synergistic effect of pesticide mixtures and *N. ceranae* on total pollen collection, with both the colonies that were only inoculated with *N. ceranae* (M) and those exposed to the three-pesticide mixture (NPF), collecting significantly more pollen than colonies exposed to the full combination (NPFM; Tukey´s post-hoc test; both *p*s < 0.001; Table S4). Bumblebees collected 9%, 6%, 5%, 7% and 4% more sugar solution respectively in colonies of groups NPFM, NPF, PFM, NPM and NFM from the 'uncontaminated feeder' than from the 'pesticides feeder' (GLM; $F_{6,342} = 6.001$, <0.0001; Figure 1c; Table S2), and the overall sugar solution consumption was reduced by 21%, 9%, 13%, 11% and 14% respectively in colonies of groups NPFM, PFM, NPM, NFM and M, compared to controls (LM; $F_{6,63} = 3.92$; $p = 0.002$; Figure 1d; Tables S3 and S5). The total amount of pesticides collected per bee (estimated using the number of bees present in the colony at the beginning of the study) during 2 weeks in lab conditions are detailed in Table S6.

3.2 | Pathogen presence

Nosema ceranae was detected in 46%, 38%, 64%, 60% and 66% of the bees analysed from treatments NPFM, PFM, NPM, NFM and M respectively 10 days after spore inoculation (Figure 2a). Since we were interested in evaluating if pesticide mixtures had an effect on *N. ceranae* prevalence, we compared *Nosema*-inoculated groups that had been exposed to pesticide mixtures (NPFM, PFM, NPM, NFM) with the *Nosema*-inoculated group that was not exposed to pesticides (M). Workers from treatment NPF (TMX + CYPER + TEB) and the controls were confirmed to be free of this microsporidium by PCR. The percentage of bees with *N. ceranae* was significantly lower in treatments NPFM (TMX + CYPER + TEB + *N. ceranae*) and PFM (CYPER + TEB + *N. ceranae*) compared to treatment

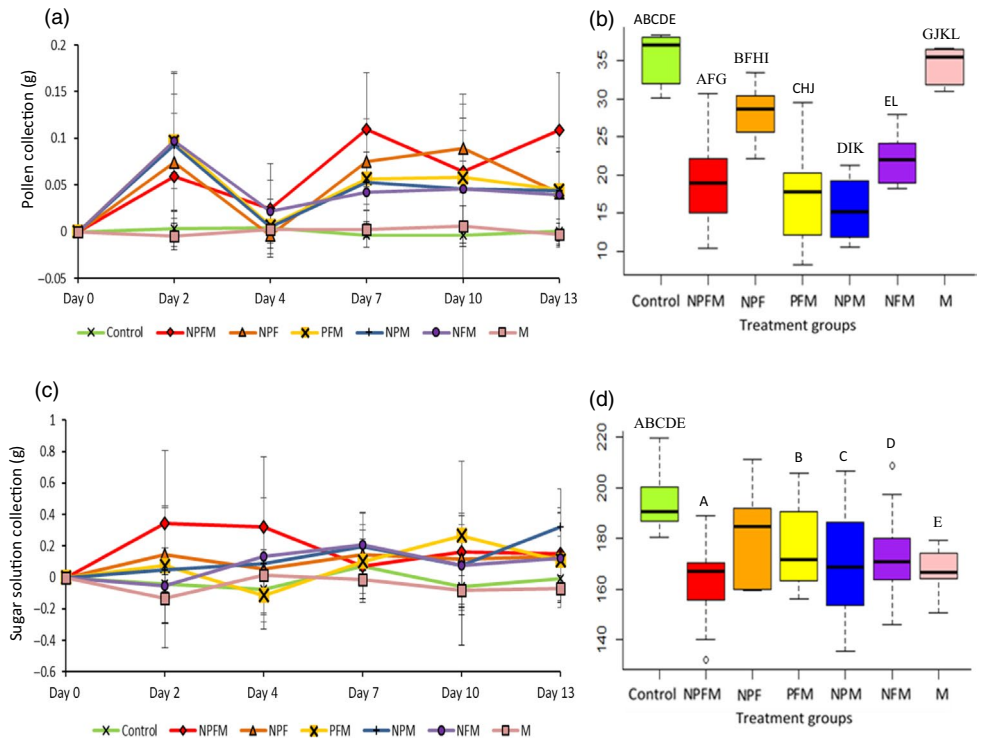

FIGURE 1 Food collection of *Bombus terrestris* bumblebee colonies exposed to combinations of pesticides and the *Nosema ceranae* parasite. (a) Differential collection of uncontaminated versus pesticide-treated pollen (g) during 13 days in laboratory conditions. (b) Total collection of pollen (g) per treatment group measured after 13 days in laboratory conditions. Graph shows median percentage (bar), interquartile ranges (boxes) and 95% CI (whiskers). (c) Differential collection of uncontaminated versus pesticide-treated sugar solution (g) during 13 days in laboratory conditions. (d) Total collection of sugar solution (g) per treatment group measured after 13 days in laboratory conditions. Graph shows median percentage (bar), interquartile ranges (boxes) and 95% CI (whiskers). NPFM = TMX + CYPER + TEB + *N. ceranae*; NPF = TMX + CYPER + TEB; PFM = TEB + CYPER + *N. ceranae*; NPM = TMX + CYPER + *N. ceranae*; NFM = TMX + TEB + *N. ceranae*; M = *N. ceranae*; Control = untreated. The M and Control treatment groups did not receive any pesticide in the 'pesticide-treated' feeder. Boxplots with similar letters are significantly different ($p < 0.05$, LMs with Gaussian error distribution)

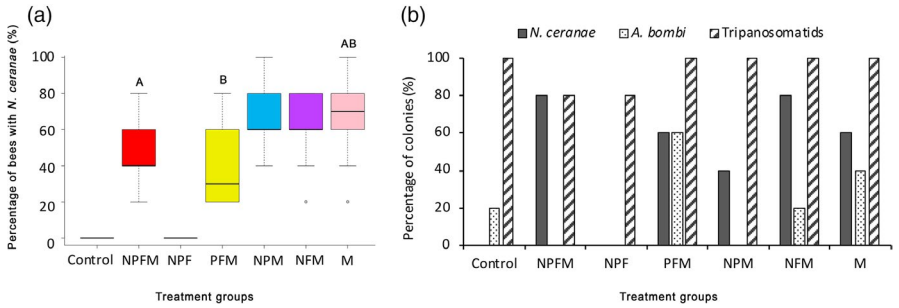

FIGURE 2 Pathogens detected in the bumblebee colonies. (a) Percentage of *Bombus terrestris* in which the pathogen *Nosema ceranae* was detected 10 days after inoculation with a controlled dose of *N. ceranae* in sugar solution (treatments NPFM, PFM, NPM, NFM and M) or pathogen-free solution (controls and treatment NPF). Bumblebee colonies in treatments NPFM, NPF, PFM, NPM and NFM had been exposed to treatment-specific combinations of pesticides. Graph shows median percentage (bar), interquartile ranges (boxes) and 95% CI (whiskers). Boxplots with similar letters are significantly different ($p < 0.05$; GLM with binomial error distribution). (b) Percentage of colonies where *N. ceranae*, *Apicystis bombi* and tripanosomatids were detected at the end of the study. The microsporidium *N. bombi* was not detected in any of the colonies of the study. NPFM = TMX + CYPER + TEB + *N. ceranae*; NPF = TMX + CYPER + TEB; PFM = TEB + CYPER + *N. ceranae*; NPM = TMX + CYPER + *N. ceranae*; NFM = TMX + TEB + *N. ceranae*; M = *N. ceranae*; Control = untreated

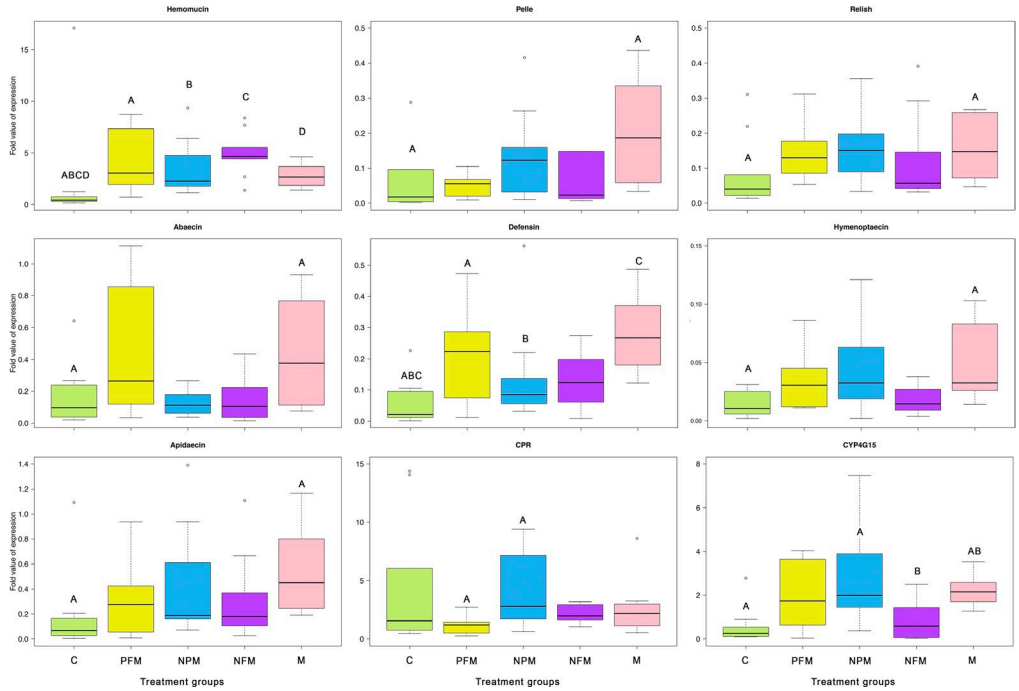

FIGURE 3 Boxplots showing the effect of treatments on the expression of genes related to the immune (*hemomucin*, *pelle*, *relish*, *abaecin*, *defensin*, *hymenoptaecin*, *apidaecin*) and detoxification (*CPR* and *CYP4G15*) response of bumblebees (*Bombus terrestris*) after 13 days of exposure to pesticide treatment and 10 days post-*Nosema ceranae* inoculation. Fold values of expression were calculated with ΔCt values (see main text) and are therefore on a scale defined by reference gene expression. Boxplots with similar letters are significantly different ($p < 0.05$; multivariate linear models, with binomial error distribution). Since we were interested in the influence of pesticide treatments on the immune response, and the possible interference of the EBI fungicide tebuconazole in the detoxification mechanisms, we only analysed bumblebees from groups PFM (TEB + CYPER + *N. ceranae*), NPM (TMX + CYPER + *N. ceranae*), NFM (TMX + TEB + *N. ceranae*), *M* (*N. ceranae*) and C (control), for the gene expression assay

M (*N. ceranae*; GLM; respectively: estimate = −0.82, *SE* = 0.29, $z = -2.83$, $p = 0.005$; estimate = −1.15, *SE* = 0.30, $z = -3.91$, $p < 0.001$; Table S7).

At the end of the experiment, after colonies had been foraging freely in the field for 7 weeks, *N. ceranae* was detected in 80%, 60%, 40%, 80% and 60% of the colonies in treatments NPFM, PFM, NPM, NFM and M respectively (Figure 2b), and not detected in the colonies of treatment NPF and controls. *Apicystis bombi* was detected in 60%, 20%, 40% and 20% of the colonies of treatments PFM, NFM, M and controls respectively. Trypanosomatids were detected in all colonies of groups PFM, NPM, NFM, M and Control, and in 80% of the colonies of groups NPFM and NPM (Figure 2b). The prevalence

of *N. ceranae*, *A. bombi* and trypanosomatids at the end of the study was not affected by treatment (Table S8).

3.3 | Gene expression

For the treatment groups examined (PFM, NPM, NFM, M), the genes analysed were overall differentially expressed in treated groups compared to the control group ($F_{4,45} = 38.25$, $p = 0.002$). *Hemomucin* was upregulated in treatments PFM, NPM, NFM and M; *defensin* was upregulated in treatments PFM, NPM and M; *CYP4G15* was upregulated in treatments NPM and M; and *pelle*, *relish*, *abaecin*, *hymenoptaecin*, *apidaecin* were upregulated in treatment M with respect to control colonies (<0.05 in all cases; Figure 3; Table S9). In addition, multiple pairwise comparisons showed upregulation of *CPR* in treatment NPM with respect to PFM (Tukey and Kramer [Nemenyi] test; $p = 0.037$), and that *CYP4G15* was downregulated in treatment NFM with respect to M ($p = 0.042$), and marginally downregulated with respect to NPM ($p = 0.075$).

3.4 | Fitness parameters

The initial growth phase was followed in all colonies by a natural decline associated with the production of new reproductives (Figure 4a). As indicated by a negative interaction term, colonies of treatment NPFM gained less weight over the course of the experiment compared to the control colonies (Table 2). Colonies of groups NPF and PFM also tended to grow less over time, but only colonies of group NPM were significantly bigger than controls when the maximum size was achieved in all groups (Week 5; LM followed by Tukey's post-hoc tests, $p = 0.046$; Table S10). Compared to control colonies, the number of workers was lower in treatments PFM (GLM; estimate ± *SE* = −0.18 ± 0.09, $z = -2.04$, $p = 0.04$) and NPM (GLM; estimate ± *SE* = −0.18 ± 0.09, $z = -2.04$, $p = 0.04$; Figure 4b), and the number of males was significantly reduced in colonies of treatment NPM (GLM; estimate ± *SE* = −0.45 ± 0.13, $z = -3.47$, $p = 0.001$; Figure 4c), although effect sizes were small in both cases (Table 3; Tables S11–S13). Male numbers in NPM treatment were also smaller than in NPFM and PFM treatments (Tukey's post hoc test; both

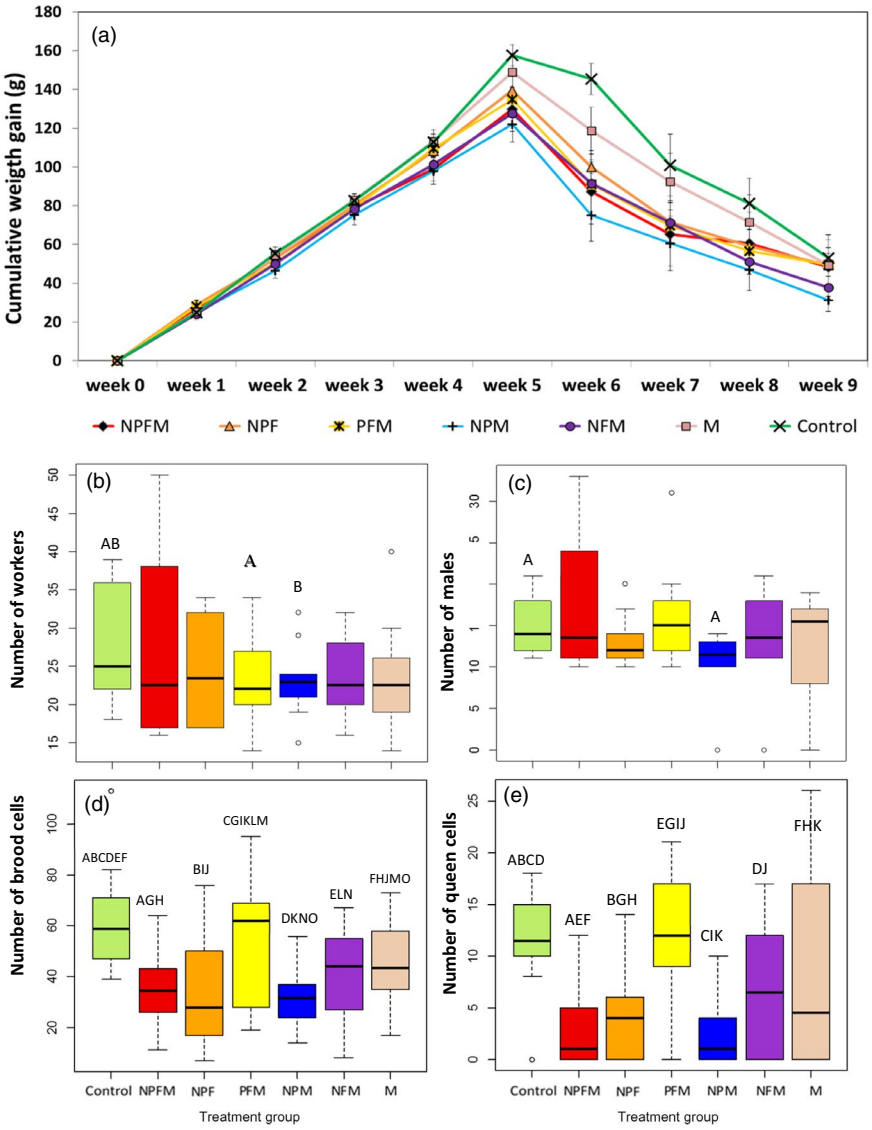

FIGURE 4 Fitness parameters measured in the Bombus terrestris colonies. (a) Mean colony weight observed for the control and treatment groups at weekly intervals. The change in weight over time was significantly smaller ($p = 0.009$) in the colonies that received the four stressors (NPFM) compared to control colonies. (b) Boxplots of the number of workers in each treatment group. (c) Boxplots of the number of males in each treatment group. (d) Boxplots of the number of brood (workers and males) cells produced in each treatment group. (e) Boxplots of the number of queen cells produced in each treatment group. Boxplots with similar letters are significantly different ($p < 0.05$; GLMs with Poisson error distribution followed by Tukey´s post-hoc tests). NPFM = TMX + CYPER + TEB + *N. ceranae*; NPF = TMX + CYPER + TEB; PFM = TEB + CYPER + *N. ceranae*; NPM = TMX + CYPER + *N. ceranae*; NFM = TMX + TEB + *N. ceranae*; *M* = *N. ceranae*; Control = Untreated

Fixed effect	Estimate	*SE*	*df*	*t* value	*p*
(Intercept)	2.332	0.247	541	9.446	<0.001
NPFM	0.394	0.234	62	1.681	0.098
NPF	0.319	0.234	62	1.360	0.179
PFM	0.276	0.235	62	1.175	0.244
NPM	0.119	0.234	62	0.508	0.614
NFM	0.061	0.234	62	0.263	0.793
M	0.010	0.234	62	0.043	0.966
Week	0.961	0.065	541	14.886	**<0.001**
Week^2	−0.091	0.006	541	−14.380	**<0.001**
No. workers at week 0	0.002	0.012	62	0.213	0.832
NPFM × week	−0.241	0.092	541	−2.624	**0.009**
NPF × week	−0.180	0.092	541	−1.963	0.050
PFM × week	−0.178	0.092	541	−1.932	0.054
NPM × week	−0.131	0.091	541	−1.433	0.152
NFM × week	−0.077	0.091	541	−0.848	0.397
M × week	0.000	0.092	541	0.004	0.997
NPFM × week^2	0.017	0.009	541	1.848	0.065
NPF × week^2	0.015	0.009	541	1.654	0.099
PFM × week^2	0.015	0.009	541	1.685	0.093
NPM × week^2	0.006	0.009	541	0.662	0.508
NFM × week^2	0.003	0.009	541	0.372	0.710
M × week^2	−0.003	0.009	541	−0.339	0.735

TABLE 2 Linear mixed effects model for colony weight. Effects that are statistically significant ($p < 0.05$) are highlighted in boldface. Parameter estimates are with reference to the control group. NPFM = TMX + CYPER + TEB + *N. ceranae*; NPF = TMX + CYPER + TEB; PFM = TEB + CYPER + *N. ceranae*; NPM = TMX + CYPER + *N. ceranae*; NFM = TMX + TEB + *N. ceranae*; M = *N. ceranae*; Control = Untreated

TABLE 3 Mean and standard deviation (*SD*) of the number, weight, thorax width (size) of workers and males, and the number of brood cells and queen cells present in the colonies at the end of the experiment

		Workers			Males				
		Number	Weight (g)	Size (mm)	Number	Weight (g)	Size (mm)	Number of brood cells	Number of queen cells
NPFM	Mean	26.6	0.09	4.05	17.1	0.17	5.52	34.9	3.2
TMX + CYPER + TEB + *Nosema*	*SD*	11.96	0.04	0.61	8.03	0.04	0.41	14.93	4.21
NPF	Mean	24.6	0.08	4.11	13.1	0.16	5.46	34.3	4.6
TMX + CYPER + TEB	*SD*	7.4	0.03	0.66	3.14	0.04	0.47	23.63	5.08
PFM	Mean	23.1	0.08	4.16	16.2	0.17	5.6	55.1	11.5
CYPER + TEB + *Nosema*	*SD*	5.67	0.04	0.57	6.16	0.05	0.5	25.04	7.28
NPM	Mean	23.2	0.08	4.1	9.6	0.17	5.43	31.4	2.7
TMX + CYPER + *Nosema*	*SD*	4.75	0.04	0.56	5.19	0.05	0.39	13.2	3.65
NFM	Mean	23.7	0.08	4.16	12.6	0.16	5.5	41.5	6.8
TMX + TEB + *Nosema*	*SD*	5.27	0.04	0.64	7.49	0.05	0.53	18.96	6.16
M	Mean	23.8	0.08	4.16	12	0.16	5.41	45.3	8.9
Nosema	*SD*	7.29	0.03	0.58	7.12	0.04	0.37	17.15	10.43
Control	Mean	27.7	0.08	4.25	15.1	0.17	5.44	63	11.4
	SD	7.51	0.03	0.7	3.57	0.04	0.48	22.15	5.06

*p*s < 0.001; Table S13). Treatments did not have an effect on the worker and male weight (LMM, $p = 0.99$ and $p = 0.89$, respectively), or on the size (thorax width) of workers and males (LMM, $p = 0.89$ and $p = 0.47$, respectively; Table S14). All treatments had an influence on the number of brood (workers and males) cells produced (GLM, all *p*s < 0.05; Table 3; Table S11; Figure 4d), and this effect was stronger

when the neonicotinoid was present (Tables S15 and S17). Moreover, colonies of groups exposed to the neonicotinoid (NPFM, NPF, NPM and NFM) produced fewer queen cells than control colonies (C), the group with the pyrethroid and the EBI fungicide (PFM), and the group only inoculated with *N. ceranae* (M; Tukey's post-hoc tests; all $p \leq 0.01$; Table 3; Tables S16 and S17; Figure 4e).

4 | DISCUSSION

The results show that exposure to common environmental stressors interact to affect bumblebees at the individual and the colony levels. One of the sub-lethal effects of exposure to pesticide mixtures was an antifeedant effect, with all bees collecting both less pollen and less sugar solution from the pesticide-contaminated feeders than uncontaminated feeders. This was regardless of the pesticide contaminant, indicating that at least two of the pesticides applied have an inhibitory effect on food consumption. Furthermore, pesticide mixtures acted synergistically with *N. ceranae* to reduce total pollen collection. Bumblebee colonies with low pollen consumption have previously been shown to gain less weight over time even when nectar availability is high (Rotheray et al., 2017), and this was true here for colonies of treatment NPFM (TMX + CYPER + TEB + *N. ceranae*). The reduced intake of pesticide-contaminated food might be due to avoidance or a secondary antifeedant response following ingestion (Thompson et al., 2015). Thiamethoxam, other neonicotinoids such as imidacloprid and clothianidin, and pyrethroids have previously been found to exert a repellent or antifeedant effect on bees (Cresswell et al., 2012; Dance et al., 2017; Elston et al., 2013; Laycock et al., 2014; Rieth & Levin, 1988; Thompson et al., 2015; Zhu et al., 2017). There is some evidence that EBI fungicide such as tebuconazole can reduce this effect (Thompson & Wilkins, 2003), and studies of neonicotinoid-fungicide mixtures have found that pesticide-contaminated food affects the post-consumption behaviour of bumblebees rather than being an olfactory repellent (Jiang et al., 2018). Therefore, the effects of exposure to pesticide mixtures are complex and difficult to infer from single compound tests, as confirmed by our results.

With respect to susceptibility to *N. ceranae* infection, the prevalence of *N. ceranae* was lower when a combination of the fungicide and the pyrethroid were applied in the diet. Although previous research showed that exposure to fungicides increased *N. ceranae* levels in bees (Glavinic et al., 2019; Pettis et al., 2013) the suppressive effect of tebuconazole on the infection of a fungal endoparasite in *Daphnia* has also been reported before (Cuco et al., 2017). Tebuconazole, as with all the other EBI fungicide, obtains its fungicidal activity through disrupting biosynthesis of ergosterol, the dominant lipid in fungal cell membranes (Köller & Scheinpflug, 1987). Therefore, this azole fungicide may have led to a reduction in ergosterol necessary for *N. ceranae* membrane functioning, thus inhibiting fungal growth and possibly causing spore death (Dijksterhuis et al., 2011). Why the effect of the fungicide should depend on the presence of the pyrethroid is unclear, but previous research has found interactions between acute sub-lethal exposure to cypermethrin and pathogen infections in honeybees (Bendahou et al., 1997), and no significant impact on the susceptibility of *B. terrestris* workers to the gut parasite *C. bombi* upon exposure to the pyrethroid lambda-cyhalothrin (Baron et al., 2014). While the potential suppression of infection by tebuconazole and/or cypermethrin may seem positive for the host, the possible ecological consequences of this antagonist interaction between pollution and disease are still poorly understood and may have costs in terms of host-pathogen population dynamics (Cuco et al., 2017).

By exploring the possible interaction between pesticide exposure and *N. ceranae* inoculation at the gene expression level, we found that all genes related to the immune response were upregulated in the group that was only inoculated with *N. ceranae* (Treatment M), indicating that the bumblebee immune system responded against this microsporidium. This upregulation was reduced in groups that were inoculated with *N. ceranae* spores and exposed to pesticide mixtures. This result suggests that pesticide mixtures may interfere with the transcription of some genes encoding defence mechanisms to pathogen challenge. Variation between genes in the strength of the effect indicates that pesticides may not affect the transcription of all AMPs to the same extent, or that different types of pesticide may alter the expression of specific immunity-related genes only, as reported by previous research (Di Prisco et al., 2013).

When gene expression related to detoxification mechanisms was investigated, we found that the *CPR* gene was upregulated in treatment NPM (CYPER + TMX + *N. ceranae*) compared to PFM (CYPER + TEB + *N. ceranae*). The *CYP4G15* gene was upregulated in the two treatments that did not receive the EBI fungicide, M (*N. ceranae*) and NPM (CYPER + TMX + *N. ceranae*), compared to the controls and treatment NFM (TMX + TEB + *N. ceranae*). This differential expression suggests that the EBI fungicide may have inhibited the expression of the genes related to the cytochrome P450-mediated detoxification (Berenbaum & Johnson, 2015; Johnson et al., 2013), but the cytochrome P450 monooxygenase activity should be studied to confirm this hypothesis. Although fungicides such as tebuconazole are designed to inhibit the fungal CYP51, a family of P450 enzymes involved in ergosterol biosynthesis (Lepesheva & Waterman, 2007), they have been shown to have non-selective inhibition of P450s (Zhang et al., 2002), which likely results in drug–drug interactions. It remains to be shown if the altered transcription of these putative CYP/P450 genes are specific detoxification-responses and whether the encoded enzymes are capable of metabolizing the pesticides applied, but there is evidence suggesting that they are involved in the defence against insecticides in bumblebees and other insects (Colgan et al., 2019; Huang et al., 2015; Jing et al., 2018). In treatment M, (*N. ceranae* only), upregulation of the *CYP4G15* gene may indicate a specific response to the parasite, as previously shown in *B. terrestris* cytochrome P450s which showed altered expression upon *C. bombi* inoculation (Barribeau et al., 2014). Bees respond to *N. ceranae* infection by producing reactive oxygen species (ROS) in the gut, and the involvement

of cytochrome P450 enzymes in the transformation of toxic metabolites into ROS in biological systems has been demonstrated before (He et al., 2017). Therefore, upregulation of the cytochrome P450 gene could indicate the involvement of this enzyme in the formation of ROS upon *N. ceranae* infection.

At the colony level, combined exposure to the three pesticides mixture and *N. ceranae* (NPFM) reduced bumblebee colony growth over the course of the study. Colonies that only received the three pesticides (NPF) or pyrethroid + fungicide + *N. ceranae* (PFM) also grew marginally less than control colonies over time, suggesting that the combination of a pyrethroid + EBI fungicide produced detrimental effects on colony growth. Previous research indicates that EBI fungicide synergize pyrethroid toxicity in honeybees (Colin & Belzunces, 1992; Pilling & Jepson, 1993; Thompson & Wilkins, 2003) and bumblebees (Raimets et al., 2018), and our study provides evidence that the enhanced toxicity of this pesticide combination may translate into detrimental effects at the bumblebee colony level (Whitehorn et al., 2012). Furthermore, colonies exposed to mixtures containing neonicotinoid + pyrethroid + *N. ceranae* (NPM) were smaller at the time of maximum growth in all treatment groups (week 5), indicating that this combination may also impair colony success, as only the largest bumblebee colonies succeed in producing queens (Müller & Schmid-Hempel, 1992). Indeed, colonies of group NPM produced the lowest number of queens (mean ± *SD* = 2.9 ± 3.65) in the present study. Our findings are of great concern given the high prevalence of *N. ceranae* detected in wild and commercial bumblebee colonies in some regions (Arbulo et al., 2015; Graystock, Yates, Darvill, et al., 2013; Rotheray et al., 2017), and the widespread use of neonicotinoid and pyrethroid insecticides (Botías et al., 2015; FERA, 2017).

All treatments led to a small reduction in worker and male brood production. Adult worker numbers were slightly lower in the groups in which bumblebees had been exposed to the pyrethroid + *N. ceranae* (treatments PFM and NPM), and male numbers were fewer when the pyrethroid and the neonicotinoid were applied in combination (NPM). Our findings are in agreement with previous research showing that exposure of bumblebees to neonicotinoids alone and in combination with pyrethroids impair brood cell and male production (Fauser-Misslin et al., 2014; Gill et al., 2012; Rundlöf et al., 2015; Wintermantel et al., 2018). This may have detrimental consequences for colony fitness and development as the rate of colony growth depends on the number of adult workers (Whitehorn et al., 2012). However, it is worth noting that the number of workers, even in the control colonies (mean = 27.7 workers), was very low compared to what is reported for wild *B. terrestris* nests (*c*. 300–400 workers; Duchateau & Velthuis, 1988). Our experimental colonies may have performed poorly compared to natural ground-nesting colonies due to differences in the incubation conditions in surface versus fossorial situations (Vogt, 1986a, 1986b). Furthermore, brood production was not only affected by exposure to pesticide mixtures, but also by *N. ceranae* inoculation (treatment M). The changes in gene expression related to immune and detoxification responses in the *N. ceranae*-challenged bumblebees may involve fitness costs that led to the detrimental effects observed at the individual and colony levels (Graystock, Yates, Darvill, et al., 2013; Rotheray et al., 2017). More significantly, exposure to the neonicotinoid thiamethoxam led to a large reduction in queen cell production, with colonies of treatments NFM, NPF, NPM and NPFM producing 40%, 60%, 76% and 72% fewer queen cells than control colonies. These findings are consistent with previous studies that reported a decrease in queen production when bumblebee colonies were exposed to neonicotinoids (Fauser-Misslin et al., 2014; Rundlöf et al., 2015; Whitehorn et al., 2012; Wintermantel et al., 2018; Woodcock et al., 2017). The fitness of a bumblebee colony is determined by its production of new queens and males (Goulson, 2010), so the fact that colonies exposed to field-relevant mixtures containing pyrethroids and neonicotinoids, and especially the latter, produced fewer males and queens, may lead to the most serious effects on bumblebees through detrimental consequences at the population level.

Our findings are worrying since the variety of chemical, physical and biological stressors associated with global change that represent potential environmental hazards to pollinators, such as bumblebees, has increased rapidly in recent years. According to our results, pesticide mixtures including neonicotinoids, pyrethroids and EBI fungicide may not be compatible with bumblebee conservation, so their simultaneous use in the field should be regulated to protect these essential pollinators. Studying the responses to field-relevant combinations of stressors may guide us to mitigate the detrimental consequences of multiple stressor interactions on pollinator health, and thus, on biodiversity conservation, ecosystem functioning and for the global agricultural sector and food production.

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AUTHORS' CONTRIBUTIONS

C.B. and D.G. conceived the project and designed the experiment; C.B. performed the semi-field experiment; C.B., J.C.J. and T.P. carried out the laboratory molecular work with input from W.O.H.H.; C.B. and I.B. analysed the data; C.B. led the writing of the manuscript, and all authors contributed to drafts and gave final approval for publication.

DECLARATION OF COMPETING INTERESTS

The authors declare the following competing interests: Tobias Pamminger is affiliated with BASF SE.

DATA AVAILABILITY STATEMENT

All data and code from this study are provided as a public figshare repository: https://doi.org/10.6084/m9.figsh are.11844 711.v1 (Botías et al 2020).

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REFERENCES

Alaux, C., Brunet, J.-L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., & Belzunces, L. P., & Le Conte, Y. (2010). Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology*, *12*(3), 774–782. https://doi.org/10.1111/j.1462-2920.2009.02123.x

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., & Higes, M. (2009). Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). *Environmental Microbiology*, *11*(9), 2284–2290. https://doi.org/10.1111/j.1462-2920.2009.01953.x

Arbulo, N., Antúnez, K., Salvarrey, S., Santos, E., Branchiccela, B., Martín-Hernández, R., Higes, M., & Invernizzi, C. (2015). High prevalence and infection levels of *Nosema ceranae* in bumblebees *Bombus atratus* and *Bombus bellicosus* from Uruguay. *Journal of Invertebrate Pathology*, *130*, 165–168. https://doi.org/10.1016/j.jip.2015.07.018

Ardia, D. R., Gantz, J. E., Schneider, B. C., & Strebel, S. (2012). Costs of immunity in insects: An induced immune response increases metabolic rate and decreases antimicrobial activity. *Functional Ecology*, *26*(3), 732–739. https://doi.org/10.1111/j.1365-2435.2012.01989.x

Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., & Blot, N. (2014). Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. *PLoS ONE*, *9*(3), e91686. https://doi.org/10.1371/journal.pone.0091686

Azpiazu, C., Bosch, J., Viñuela, E., Medrzycki, P., Teper, D., & Sgolastra, F. (2019). Chronic oral exposure to field- realistic pesticide combinations via pollen and nectar: Effects on feeding and thermal performance in a solitary bee. *Scientific Reports*, *9*(19), 13770. https://doi.org/10.1038/s41598-019-50255-4

Baron, G. L., Raine, N. E., & Brown, M. J. F. (2014). Impact of chronic exposure to a pyrethroid pesticide on bumblebees and interactions with a trypanosome parasite. *Journal of Applied Ecology*, *51*(2), 460–469. https://doi.org/10.1111/1365-2664.12205

Barribeau, S. M., Sadd, B. M., du Plessis, L., Brown, M. J. F., Buechel, S. D., Cappelle, K., Carolan, J. C., Christiaens, O., Colgan, T. J., Erler, S., Evans, J., Helbing, S., Karaus, E., Lattorff, H. M. G., Marxer, M., Meeus, I., Näpflin, K., Niu, J., Schmid-Hempel, R., … Schmid-Hempel, P. (2015). A depauperate immune repertoire precedes evolution of sociality in bees. *Genome Biology*, *16*, 83. https://doi.org/10.1186/s13059-015-0628-y

Barribeau, S. M., Sadd, B. M., du Plessis, L., & Schmid-Hempel, P. (2014). Gene expression differences underlying genotype-by-genotype specificity in a host–parasite system. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(9), 3496–3501. https://doi.org/10.1073/pnas.1318628111

Bartolomé, C., Buendía, M., Benito, M., De la Rúa, P., Ornosa, C., Martín-Hernández, R., Higes, M., & Maside, X. (2018). A new multiplex PCR protocol to detect mixed trypanosomatid infections in species of *Apis* and *Bombus*. *Journal of Invertebrate Pathology*, *154*, 37–41. https://doi.org/10.1016/j.jip.2018.03.015

Beadle, K., Singh, K. S., Troczka, B. J., Randall, E., Zaworra, M., Zimmer, C. T., Hayward, A., Reid, R., Kor, L., Kohler, M., Buer, B., Nelson, D. R., Williamson, M. S., Davies, T. G. E., Field, L. M., Nauen, R., & Bass, C. (2019). Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*. *PLoS Genetics*, *15*(2), 1–19. https://doi.org/10.1371/journal.pgen.1007903

Bendahou, N., Bounias, M., & Fleche, C. (1997). Acute toxicity of cypermethrin and fenitrothion on honeybees according to age, formulations and insecticide interaction. *Journal of Environmental Biology*, *18*(1), 55–65.

Berenbaum, M. R., & Johnson, R. M. (2015). Xenobiotic detoxification pathways in honey bees. *Current Opinion in Insect Science*, *10*, 51–58. https://doi.org/10.1016/j.cois.2015.03.005

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. A., Rajotte, E. G., Joshi, N. K., & Vaughn, M. (2013). Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLoS ONE*, *8*(9), e72587. https://doi.org/10.1371/journal.pone.0072587

Biesmeijer, J. C. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354. https://doi.org/10.1126/science.1127863

Botías, C., David, A., Hill, E. M., & Goulson, D. (2017). Quantifying exposure of wild bumblebees to mixtures of agrochemicals in agricultural and urban landscapes. *Environmental Pollution*, *222*, 73–82. https://doi.org/10.1016/j.envpol.2017.01.001

Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., & Goulson, D. (2015). Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. *Environmental Science and Technology*, *49*(21), 12731–12740. https://doi.org/10.1021/acs.est.5b03459

Botías, C., Jones, J. C., Pamminger, T., Bartomeus, I., Hughes, W. O. H., & Goulson, D. (2020). Data from: Multiple stressors interact to impair the performance of bumblebee (*Bombus terrestris*) colonies [Dataset]. *figshare*, https://doi.org/10.6084/m9.figshare.11844711.v1

Brattsten, L. B., Berger, D. A., & Dungan, L. B. (1994). In vitro inhibition of midgut microsomal P450s from Spodoptera eridania caterpillars by demethylation inhibitor fungicides and plant growth regulators. *Pesticide Biochemistry and Physiology*, *49*, 234–243. https://doi.org/10.1006/pest.1994.1025

Brunner, F. S., Schmid-Hempel, P., & Barribeau, S. M. (2013). Immune gene expression in *Bombus terrestris*: Signatures of infection despite strong variation among populations, colonies, and sister workers. *PLoS ONE*, *8*(7), e68181. https://doi.org/10.1371/journal.pone.0068181

Büchler, R., & Volkmann, B. (2003). Rückstände von carbendazim undanderen fungiziden im bienenhonig aufgrund der blütespritzungvon winterraps. *Gesunde Pflanzen*, *55*(8), 217–221. https://doi.org/10.1007/s10343-003-0011-x

Chaimanee, V., Pettis, J. S., Chen, Y., Evans, J. D., Khongphinitbunjong, K., & Chantawannakul, P. (2013). Susceptibility of four different honey bee species to *Nosema ceranae*. *Veterinary Parasitology*, *193*(1–3), 260–265. https://doi.org/10.1016/j.vetpar.2012.12.004

Colgan, T. J., Fletcher, I. K., Arce, A. N., Gill, R. J., Ramos Rodrigues, A., Stolle, E., Chittka, L., & Wurm, Y. (2019). Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees. *Molecular Ecology*, *28*(8), 1964–1974. https://doi.org/10.1111/mec.15047

Colin, M.-E., & Belzunces, L. P. (1992). Evidence of synergy between prochloraz and deltamethrin in *Apis mellifera* L.: A convenient biological approach. *Pesticide Science*, *36*(2), 115–119. https://doi.org/10.1002/ps.2780360206

Côté, I. M., Darling, E. S., & Brown, C. J. (2016). Interactions among ecosystem stressors and their importance in conservation. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20152592. https://doi.org/10.1098/rspb.2015.2592

Cresswell, J. E., Page, C. J., Uygun, M. B., Holmbergh, M., Li, Y., Wheeler, J. G., Laycock, I., Pook, C. J., de Ibarra, N. H., Smirnoff, N., & Tyler, C. R. (2012). Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology*, *115*(6), 365–371. https://doi.org/10.1016/j.zool.2012.05.003

Cuco, A. P., Abrantes, N., Gonçalves, F., Wolinska, J., & Castro, B. B. (2017). Interplay between fungicides and parasites: Tebuconazole,

but not copper, suppresses infection in a Daphnia-Metschnikowia experimental model. *PLoS ONE*, *12*(2), 1–16. https://doi.org/10.1371/journal.pone.0172589

Dance, C., Botias, C., & Goulson, D. (2017). The combined effects of a monotonous diet and exposure to thiamethoxam on the performance of bumblebee micro-colonies. *Ecotoxicology and Environmental Safety*, *139*, 194–201. https://doi.org/10.1016/j.ecoenv.2017.01.041

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., & Goulson, D. (2016). Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International*, *88*, 169–178. https://doi.org/10.1016/j.envint.2015.12.011

Dessouki, A. M., Aly, H. F., & Sokker, H. H. (1999). The use of gamma radiation for removal of pesticides from waste water. *Czechoslovak Journal of Physics*, *49*(1), 521–533. https://doi.org/10.1007/s10582-999-0071-y

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., & Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(46), 18466–18471. https://doi.org/10.1073/pnas.1314923110

Dijksterhuis, J., Van Doorn, T., Samson, R., & Postma, J. (2011). Effects of seven fungicides on non-target aquatic fungi. *Water, Air, and Soil Pollution*, *222*(1–4), 421–425. https://doi.org/10.1007/s11270-011-0836-3

Doublet, V., Labarussias, M., de Miranda, J. R., Moritz, R. F. A., & Paxton, R. J. (2014). Bees under stress: Sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental Microbiology*, *17*(4), 969–983. https://doi.org/10.1111/1462-2920.12426

Duchateau, M. J., & Velthuis, H. H. W. (1988). Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour*, *107*, 186–207. https://doi.org/10.1163/156853988X00340

Elston, C., Thompson, H. M., & Walters, K. F. A. (2013). Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie*, *44*(5), 563–574. https://doi.org/10.1007/s13592-013-0206-9

Evans, J. D. (2006). Beepath: An ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of Invertebrate Pathology*, *93*(2), 135–139. https://doi.org/10.1016/j.jip.2006.04.004

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., & Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, *15*(5), 645–656. https://doi.org/10.1111/j.1365-2583.2006.00682.x

Fauser-Misslin, A., Sadd, B. M., Neumann, P., & Sandrock, C. (2014). Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. *Journal of Applied Ecology*, *51*(2), 450–459. https://doi.org/10.1111/1365-2664.12188

FERA. (2017). Pesticide Usage Survey Report 271. The Food and Environment Research Agency. Arable Crops in the United Kingdom 2016. Garthwaite, D, Barker, I, Ridley, L Mace, A Parrish, G Macarthur, R Lu, Y. Land Use & Sustainability Team Fera Sand Hutton York YO41 1LZ.

Fries, I., Feng, F., Silva, A., Susan, B., & Pieniazek, N. J. (1996). *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). *European Journal of Protistology*, *32*(3), 356–365. https://doi.org/10.1016/S0932-4739(96)80059-9

Fürst, M. A., McMahon, D. P., Osborne, J. L., Paxton, R. J., & Brown, M. J. F. (2014). Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature*, *506*(7488), 364–366. https://doi.org/10.1038/nature12977

Garrido, P. M., Antúnez, K., Martín, M., Porrini, M. P., Zunino, P., & Eguaras, M. J. (2013). Immune-related gene expression in nurse honey bees (*Apis mellifera*) exposed to synthetic acaricides. *Journal of Insect Physiology*, *59*(1), 113–119. https://doi.org/10.1016/j.jinsphys.2012.10.019

Gill, R. J., Baldock, K. C. R., Brown, M. J. F., Cresswell, J. E., Dicks, L. V., Fountain, M. T., Garratt, M. P. D., Gough, L. A., Heard, M. S., Holland, J. M., Ollerton, J., Stone, G. N., Tang, C. Q., Vanbergen, A. J., Vogler, A. P., Woodward, G., Arce, A. N., Boatman, N. D., BrandHardy, R., & Potts, S. G. (2015). *Protecting an ecosystem service: Approaches to understanding and mitigating threats to wild insect pollinators. Advances in ecological research* (1st ed., Vol. 54). Elsevier Ltd. https://doi.org/10.1016/bs.aecr.2015.10.007

Gill, R. J., Ramos-Rodriguez, O., & Raine, N. E. (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature*, *491*(7422), 105–108. https://doi.org/10.1038/nature11585

Gisder, S., Horchler, L., Pieper, F., Schüler, V., Sima, P., & Genersch, E. (2020). Rapid gastrointestinal passage may protect *Bombus terrestris* from becoming a true host for *Nosema ceranae*. *Applied and Environmental Microbiology*, *86*. https://doi.org/10.1128/AEM.00629-20

Glavinic, U., Tesovnik, T., Stevanovic, J., Zorc, M., Cizelj, I., Stanimirovic, Z., & Narat, M. (2019). Response of adult honey bees treated in larval stage with prochloraz to infection with *Nosema ceranae*. *PeerJ*, *7*, e6325. https://doi.org/10.7717/peerj.6325

González-Varo, J. P., Biesmeijer, J. C., Bommarco, R., Potts, S. G., Schweiger, O., Smith, H. G., Steffan-Dewenter, I., Szentgyörgyi, H., Woyciechowski, M., & Vilà, M. (2013). Combined effects of global change pressures on animal-mediated pollination. *Trends in Ecology & Evolution*, *28*(9), 524–530. https://doi.org/10.1016/j.tree.2013.05.008

Goulson, D. (2010). *Bumblebees: Behaviour, ecology, and conservation* (2nd ed.). Oxford University Press.

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Graystock, P., Jones, J. C., Pamminger, T., Parkinson, J. F., Norman, V., Blane, E. J., Rothstein, L., Wäckers, F., Goulson, D., & Hughes, W. (2016). Hygienic food to reduce pathogen risk to bumblebees. *Journal of Invertebrate Pathology*, *136*, 68–73. https://doi.org/10.1016/j.jip.2016.03.007

Graystock, P., Yates, K., Darvill, B., Goulson, D., & Hughes, W. O. H. (2013). Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. *Journal of Invertebrate Pathology*, *114*(2), 114–119. https://doi.org/10.1016/j.jip.2013.06.005

Graystock, P., Yates, K., Evison, S. E. F., Darvill, B., Goulson, D., & Hughes, W. O. H. (2013). The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology*, *50*(5), 1207–1215. https://doi.org/10.1111/1365-2664.12134

Hayward, A., Beadle, K., Singh, K. S., Exeler, N., Zaworra, M., Almanza, M.-T., Nikolakis, A., Garside, C., Glaubitz, J., Bass, C., & Nauen, R. (2019). The leafcutter bee, *Megachile rotundata*, is more sensitive to N-cyanoamidine neonicotinoid and butenolide insecticides than other managed bees. *Nature Ecology and Evolution*, *3*(11), 1521–1524. https://doi.org/10.1038/s41559-019-1011-2

He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cellular Physiology and Biochemistry*, *44*(2), 532–553. https://doi.org/10.1159/000485089

Higes, M., García-Palencia, P., Martín-Hernández, R., & Meana, A. (2007). Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). *Journal of Invertebrate Pathology*, *94*(3), 211–217. https://doi.org/10.1016/j.jip.2006.11.001

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V., Barrios, L., del Nozal, M. J., Bernal, J. L., Jiménez, J. J., Palencia, P. G., & Meana, A. (2008). How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environmental Microbiology*, *10*(10), 2659–2669. https://doi.org/10.1111/j.1462-2920.2008.01687.x

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., Botías, C., García-Palencia, P., & Meana, A. (2008). Regurgitated pellets of *Merops apiaster* as fomites of infective *Nosema ceranae* (Microsporidia) spores. *Environmental Microbiology*, *10*(5), 1374–1379. https://doi.org/10.1111/j.1462-2920.2007.01548.x

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., García-Palencia, P., & Meana, A. (2008). Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees. *Journal of Invertebrate Pathology*, *97*(1), 76–78. https://doi.org/10.1016/j.jip.2007.06.002

Horňáková, D., Matoušková, P., Kindl, J., Valterová, I., & Pichová, I. (2010). Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. *Analytical Biochemistry*, *397*(1), 118–120. https://doi.org/10.1016/j.ab.2009.09.019

Huang, Y., Lu, X.-P., Wang, L.-L., Wei, D., Feng, Z.-J., Zhang, Q. I., Xiao, L.-F., Dou, W., & Wang, J.-J. (2015). Functional characterization of NADPH-cytochrome P450 reductase from *Bactrocera dorsalis*: Possible involvement in susceptibility to malathion. *Scientific Reports*, *5*, 18394. https://doi.org/10.1038/srep18394

Iwasa, T., Motoyama, N., Ambrose, J. T., & Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection*, *23*(5), 371–378. https://doi.org/10.1016/j.cropro.2003.08.018

Jiang, X., Wang, Z., He, Q., Liu, Q., Li, X., Yu, L., & Cao, H. (2018). The effect of neonicotinoid insecticide and fungicide on sugar responsiveness and orientation behavior of honey bee (*Apis mellifera*) in semi-field conditions. *Insects*, *9*(4), 130. https://doi.org/10.3390/insects9040130

Jing, T.-X., Tan, Y., Ding, B.-Y., Dou, W., Wei, D.-D., & Wang, J.-J. (2018). NADPH-cytochrome P450 reductase mediates the resistance of *Aphis* (Toxoptera) citricidus (Kirkaldy) to abamectin. *Frontiers in Physiology*, *9*, 986. https://doi.org/10.3389/fphys.2018.00986

Johnson, R. M., Dahlgren, L., Siegfried, B. D., & Ellis, M. D. (2013). Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). *PLoS ONE*, *8*(1), e54092. https://doi.org/10.1371/journal.pone.0054092

Johnson, R. M., Wen, Z., Schuler, M. A., & Berenbaum, M. R. (2006). Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. *Journal of Economic Entomology*, *99*(4), 1046–1050. https://doi.org/10.1093/jee/99.4.1046

Kessler, S. C., Tiedeken, E. J., Simcock, K. L., Derveau, S., Mitchell, J., Softley, S., Radcliffe, A., Stout, J. C., & Wright, G. A. (2015). Bees prefer foods containing neonicotinoid pesticides. *Nature*, *521*(7550), 74–76. https://doi.org/10.1038/nature14414

Klee, J., Besana, A. M., Genersch, E., Gisder, S., Nanetti, A., Tam, D. Q., Chinh, T. X., Puerta, F., Ruz, J. M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., & Paxton, R. J. (2007). Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology*, *96*(1), 1–10. https://doi.org/10.1016/j.jip.2007.02.014

Köller, W., & Scheinpflug, H. (1987). Fungal resistance to sterol biosynthesis inhibitors: A new challenge. *Plant Disease*, *71*(12), 1066–1074.

Laycock, I., Cotterell, K. C., O'Shea-Wheller, T. A., &Cresswell, J. E. (2014). Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees. *Ecotoxicology and Environmental Safety*, *100*, 153–158. https://doi.org/10.1016/j.ecoenv.2013.10.027

Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology*, *25*, 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615

Lepesheva, G. I., & Waterman, M. R. (2007). Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta – General Subjects*, *1770*(3), 467–477. https://doi.org/10.1016/j.bbagen.2006.07.018

Li, J., Chen, W., Wu, J., Peng, W., An, J., Schmid-Hempel, P., & Schmid-Hempel, R. (2012). Diversity of *Nosema* associated with bumblebees (*Bombus* spp.) from China. *International Journal for Parasitology*, *42*(1), 49–61. https://doi.org/10.1016/j.ijpara.2011.10.005

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Manjon, C., Troczka, B. J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K. S., Zimmer, C. T., Homem, R. A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M. S., Davies, T. G. E., Field, L. M., Bass, C., & Nauen, R. (2018). Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Current Biology*, *28*(7), 1–7. https://doi.org/10.1016/j.cub.2018.02.045

Martín-Hernández, R., Botías, C., Bailón, E. G., Martínez-Salvador, A., Prieto, L., Meana, A., & Higes, M. (2012). Microsporidia infecting *Apis mellifera*: Coexistence or competition. Is *Nosema ceranae* replacing *Nosema apis*? *Environmental Microbiology*, *14*(8), 2127–2138. https://doi.org/10.1111/j.1462-2920.2011.02645.x

Martín-Hernández, R., Meana, A., Prieto, L., Salvador, A. M., Garrido-Bailón, E., & Higes, M. (2007). Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Applied and Environmental Microbiology*, *73*(20), 6331–6338. https://doi.org/10.1128/AEM.00270-07

Meeus, I., Brown, M. J. F., De Graaf, D. C., & Smagghe, G. (2011). Effects of invasive parasites on bumble bee declines. *Conservation Biology*, *25*(4), 662–671. https://doi.org/10.1111/j.1523-1739.2011.01707.x

Meeus, I., de Graaf, D. C., Jans, K., & Smagghe, G. (2010). Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. *Journal of Applied Microbiology*, *109*(1), 107–115. https://doi.org/10.1111/j.1365-2672.2009.04635.x

Mukherjee, I. (2009). Determination of pesticide residues in honey samples. *Bulletin of Environmental Contamination and Toxicology*, *83*(6), 818–821. https://doi.org/10.1007/s00128-009-9772-y

Müller, C. B., & Schmid-Hempel, P. (1992). Correlates of reproductive success among field colonies of *Bombus lucorum*: The importance of growth and parasites. *Ecological Entomology*, *17*(4), 343–353. https://doi.org/10.1111/j.1365-2311.1992.tb01068.x

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. *PLoS ONE*, *5*(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Nieto, A., Roberts, S. P. M., Kemp, J., Rasmont, P., Kuhlmann, M., Criado, M. G., Biesmeijer, J. C., Bogusch, P., Dathe, H. H., & Rúa, P. D. (2015). *European red list of bees*. Publication Office of the European Union. Retrieved from https://ec.europa.eu/environment/nature/conservation/species/redlist/downloads/European_bees.pdf

Ollerton, J., Erenler, H., Edwards, M., & Crockett, R. (2014). Extintions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science*, *346*(6215), 1360–1362.

Osborne, J. L., Martin, A. P., Carreck, N. L., Swain, J. L., Knight, M. E., Goulson, D., & Sanderson, R. A. (2008). Bumblebee flight distances in relation to the forage landscape. *The Journal of Animal Ecology*, *77*(2), 406–415. https://doi.org/10.1111/j.1365-2656.2007.01333.x

Osta, M. A., Christophides, G. K., Vlachou, D., & Kafatos, F. C. (2004). Innate immunity in the malaria vector *Anopheles gambiae*: Comparative and functional genomics. *Journal of Experimental Biology*, *207*(15), 2551–2563. https://doi.org/10.1242/jeb.01066

Pamminger, T., Botías, C., Goulson, D., & Hughes, W. O. H. (2018). A mechanistic framework to explain the immunosuppressive effects of neurotoxic pesticides on bees. *Functional Ecology*, *32*(8), 1921–1930. https://doi.org/10.1111/1365-2435.13119

Pargi, J., & Bhatt, H. G. (2018). Effect of gamma irradiation on degradation of pesticide residues in fruits and vegetables. *Trends in Biosciences*, *11*(28), 3519–3523.

Pettis, J. S., Lichtenberg, E. M., Andree, M., Stitzinger, J., Rose, R., & vanEngelsdorp, D. (2013). Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. *PLoS ONE*, *8*(7), e70182. https://doi.org/10.1371/journal.pone.0070182

Pettis, J. S., vanEngelsdorp, D., Johnson, J., & Dively, G. (2012). Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. *Die Naturwissenschaften*, *99*(2), 153–158. https://doi.org/10.1007/s00114-011-0881-1

Pilling, E. D., Bromley-Challenor, K. A. C., Walker, C. H., & Jepson, P. C. (1995). Mechanism of synergism between the pyrethroid insecticide alpha-cyhalothrin and the imidazole fungicide prochloraz in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology*, *51*, 1–11.

Pilling, E. D., & Jepson, P. C. (1993). Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pesticide Science*, *39*(4), 293–297. https://doi.org/10.1002/ps.2780390407

Pinheiro, J., & Bates, D. (2000). *Mixed effects models in S and S-Plus*, Statistics and Computing Book Series. Springer Nature Switzerland AG.

Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A., Abrahamovich, A. H., Lange, C., Higes, M. (2009). South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). *Environmental Microbiology Reports*, *1*(2), 131–135. https://doi.org/10.1111/j.1758-2229.2009.00018.x

Powney, G. D., Carvell, C., Edwards, M., Morris, R. K. A., Roy, H. E., Woodcock, B. A., & Isaac, N. J. B. (2019). Widespread losses of pollinating insects in Britain. *Nature Communications*, *10*(1), 1018. https://doi.org/10.1038/s41467-019-08974-9

R Core Team. (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., & Cresswell, J. E. (2018). Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). *Pest Management Science*, *74*(3), 541–546. https://doi.org/10.1002/ps.4756

Ravoet, J., Schwarz, R. S., Descamps, T., Yañez, O., Tozkar, C. O., Martin-Hernandez, R., Bartolomé, C., De Smet, L., Higes, M., Wenseleers, T., Schmid-Hempel, R., Neumann, P., Kadowaki, T., Evans, J. D., & de Graaf, D. C. (2015). Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *Journal of Invertebrate Pathology*, *130*, 21–27. https://doi.org/10.1016/j.jip.2015.06.007

Ravoet, J., Smet, L. D., Meeus, I., Smagghe, G., Wenseleers, T., & de Graaf, D. C. (2014). Widespread occurrence of honey bee pathogens in solitary bees. *Journal of Invertebrate Pathology*, *122*, 55–58. https://doi.org/10.1016/j.jip.2014.08.007

Riddell, C. E., Sumner, S., Adams, S., & Mallon, E. B. (2011). Pathways to immunity: Temporal dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut parasite. *Insect Molecular Biology*, *20*(4), 529–540. https://doi.org/10.1111/j.1365-2583.2011.01084.x

Rieth, J. P., & Levin, M. D. (1988). The repellent effect of two pyrethroid insecticides on the honey bee. *Physiological Entomology*, *13*(2), 213–218. https://doi.org/10.1111/j.1365-3032.1988.tb00925.x

Rotheray, E. L., Osborne, J. L., & Goulson, D. (2017). Quantifying the food requirements and effects of food stress on bumble bee colony development. *Journal of Apicultural Research*, *56*(3), 288–299. https://doi.org/10.1080/00218839.2017.1307712

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., Yourstone, J., & Smith, H. G. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, *521*, 77–80. https://doi.org/10.1038/nature14420

Sánchez-Bayo, F., & Goka, K. (2014). Pesticide residues and bees – A risk assessment. *PLoS ONE*, *9*(4), e94482. https://doi.org/10.1371/journal.pone.0094482

Schlüns, H., Sadd, B. M., Schmid-Hempel, P., & Crozier, R. H. (2010). Infection with the trypanosome *Crithidia bombi* and expression of immune-related genes in the bumblebee *Bombus terrestris*. *Developmental and Comparative Immunology*, *34*(7), 705–709. https://doi.org/10.1016/j.dci.2010.02.002

Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D., & Bosch, J. (2018). Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee. *Proceedings of the Royal Society B: Biological Sciences*, *285*, 20180887.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M. T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., & Bosch, J. (2017). Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Management Science*, *73*, 1236–1243. https://doi.org/10.1002/ps.4449

Singh, R., Levitt, A. L., Rajotte, E. G., Holmes, E. C., Ostiguy, N., vanEngelsdorp, D., Lipkin, W. I., dePamphilis, C. W., Toth, A. L., & Cox-Foster, D. L. (2010). RNA viruses in hymenopteran pollinators: Evidence of inter-Taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. *PLoS ONE*, *5*(12), e14357. https://doi.org/10.1371/journal.pone.0014357

Spurgeon, D., Hesketh, H., Lahive, E., Svendsen, C., Baas, J., Robinson, A., Horton, A., & Heard, M. (2016). *Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees* (Apis mellifera, Osmia bicornis *and* Bombus terrestris*)*. EFSA Supporting Publications, EN-1076 (February). https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2016.EN-1076

Theopold, U., Samakovlis, C., Erdjument-bromage, H., Dillon, N., Axelsson, B., Schmidt, O., Tempst, P., & Hultmark, D. (1996). Helix pomatia lectin, an inducer of *Drosophila immune* response, binds to hemomucin, a novel surface mucin*. *The Journal of Biological Chemistry*, *271*(22), 12708–12715.

Thompson, H. M., Fryday, S. L., Harkin, S., & Milner, S. (2014). Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. *Apidologie*, *45*(5), 545–553. https://doi.org/10.1007/s13592-014-0273-6

Thompson, H., & Wilkins, S. (2003). Assessment of the synergy and repellency of pyrethroid/fungicide mixtures. *Bulletin of Insectology*, *56*(1), 131–134.

Thompson, H. M., Wilkins, S., Harkin, S., Milner, S., & Walters, K. F. A. (2015). Neonicotinoids and bumblebees (*Bombus terrestris*): Effects on nectar consumption in individual workers. *Pest Management Science*, *71*(7), 946–950. https://doi.org/10.1002/ps.3868

Tosi, S., Costa, C., Vesco, U., Quaglia, G., & Guido, G. (2018). A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Science of the Total Environment*, *615*, 208–218. https://doi.org/10.1016/j.scitotenv.2017.09.226

Vogt, F. D. (1986a). Thermoregulation in bumblebee colonies. I. Thermoregulatory versus brood-maintenance behaviors during acute changes in ambient temperature. *Physiological Zoology*, *59*(1), 55–59. https://doi.org/10.1086/physzool.59.1.30156090

Vogt, F. D. (1986b). Thermoregulation in bumblebee colonies. II. Behavioral and demographic variation throughout the colony cycle. *Physiological Zoology*, *59*(1), 60–68. https://doi.org/10.1086/physzool.59.1.30156091

Vouk, V. B., Butler, G. C., Upton, A. C., Parke, D. V., & Asher, S. C. (1987). *Methods for assessing the effects of mixtures of chemicals. Prepared by Scientific Group on Methodologies for the Safety Evaluation of Chemicals*. Wiley. https://apps.who.int/iris/handle/10665/41771

Wang, Y., Naumann, U., Wright, S. T., & Warton, D. I. (2012). mvabund–an R package for model-based analysis of multivariate abundance

data. *Methods in Ecology and Evolution*, *3*(3), 471–474. https://doi.org/10.1111/j.2041-210X.2012.00190.x

Warton, D. I., Wright, S. T., & Wang, Y. (2012). Distance-based multivariate analyses confound location and dispersion effects. *Methods in Ecology and Evolution*, *3*(1), 89–101. https://doi.org/10.1111/j.2041-210X.2011.00127.x

Westfall, P. H., Tobias, R. D., Rom, D., Wolfinger, R. D., & Hochberg, Y. (1999). *Multiple comparisons and multiple tests using the SAS system*, (1st ed.). SAS Institute Inc.

Whitehorn, P. R., O'Connor, S., Wackers, F. L., & Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science*, *336*, 351–352. https://doi.org/10.1002/ps.1616

Wintermantel, D., Locke, B., Andersson, G. K. S., Semberg, E., Forsgren, E., Osterman, J., Rahbek Pedersen, T., Bommarco, R., Smith, H. G., Rundlöf, M., & de Miranda, J. R. (2018). Field-level clothianidin exposure affects bumblebees but generally not their pathogens. *Nature Communications*, *9*(1), 5446. https://doi.org/10.1038/s41467-018-07914-3

Woodcock, B. A., Bullock, J. M., Shore, R. F., Heard, M. S., Pereira, M. G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., & Pywell, R. F. (2017). Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. *Science*, *356*(6345), 1393–1395. https://doi.org/10.1126/science.aaa1190

Zaragoza-Trello, C., Vilà, M., Botías, C., & Bartomeus, I. (2020). Interactions among global change pressures act in a non-additive way on bumblebee individuals and colonies. *Functional Ecology*. https://doi.org/10.1111/1365-2435.13703

Zhang, W., Ramamoorthy, Y., Kilicarslan, T., Nolte, H., Tyndale, R. F., & Sellers, E. M. (2002). Inhibition of cytochromes P450 by antifungal imidazole derivatives. *Drug Metabolism and Disposition*, *30*(3), 314–318. https://doi.org/10.1124/dmd.30.3.314

Zhu, W., Schmehl, D. R., Mullin, C. A., & Frazier, J. L. (2014). Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. *PLoS ONE*, *9*(1), e77547. https://doi.org/10.1371/journal.pone.0077547

Zhu, Y. C., Yao, J., Adamczyk, J., & Luttrell, R. (2017). Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). *PLoS ONE*, *12*(6), 1–19. https://doi.org/10.1371/journal.pone.0178421

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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