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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.

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3. We established seven treatment groups of colonies that we pulse exposed to different combinations of these stressors for two weeks under laboratory conditions. Colonies were subsequently placed in the field for seven 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-pesticides 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*, colony performance, environmental stressors, pesticide mixtures, *Nosema ceranae*, bumblebee health

## Resumen

1. Los abejorros están constantemente expuestos a una amplia gama de agentes estresantes bióticos y abióticos de los que deben defenderse para sobrevivir. Los patógenos y los pesticidas son importantes factores estresantes que influyen en la salud de los abejorros, tanto cuando actúan solos como en combinación. Para tener un mejor conocimiento sobre la salud de los abejorros, debemos investigar cómo interactúan estos factores estresantes, pero los estudios experimentales hasta la fecha generalmente se centran en estudiar solo uno o dos factores.

2. El objetivo de nuestro estudio es evaluar cómo los efectos combinados de cuatro importantes factores estresantes (el parásito intestinal *Nosema ceranae*, el insecticida neonicotinoide tiametoxam, el insecticida piretroide cipermetrina y el fungicida EBI tebuconazol) interactúan para afectar a los abejorros a nivel individual y de colonia.

3. Establecimos siete grupos de tratamiento de colonias de abejorros que expusimos a diferentes combinaciones de estos factores estresantes durante dos semanas en condiciones de laboratorio, y posteriormente se colocaron en el campo durante siete semanas, para evaluar el efecto de los tratamientos sobre la prevalencia de *N. ceranae* en abejorros inoculados, los niveles de expresión de genes relacionados con la inmunidad y la desintoxicación, la recolección de alimentos, el aumento de peso, el número de obreras y machos, y la producción de cría de obreras, machos y reinas.

4. La exposición a mezclas de pesticidas redujo la recolección de alimentos por parte de los abejorros. Todos los genes relacionados con la inmunidad se sobre-expresaron en los abejorros inoculados con *N. ceranae* cuando no habían estado expuestos a mezclas de pesticidas, y los abejorros expuestos al fungicida y al piretroide presentaron menos probabilidades de tener *N. ceranae*. La exposición combinada a la mezcla de tres pesticidas y *N. ceranae* redujo el crecimiento de la colonia de abejorros y todos los

tratamientos tuvieron efectos perjudiciales en la producción de crías. Los grupos expuestos al insecticida neonicotinoide produjeron entre un 40 y un 76% menos de reinas que las colonias control.

5. Nuestros hallazgos muestran que la exposición a combinaciones de factores estresantes con los que los abejorros entran frecuentemente en contacto tiene efectos perjudiciales sobre la salud y el rendimiento de la colonia y, por lo tanto, podría tener un impacto a nivel poblacional. Estos resultados también tienen importantes implicaciones para las prácticas y políticas actuales de evaluación de riesgos y uso de plaguicidas, ya que las combinaciones probadas aquí se aplican con frecuencia simultáneamente en el campo. Comprender las interacciones entre los diferentes factores de estrés es fundamental para mejorar nuestra capacidad de gestión de las poblaciones de abejas y así garantizar los servicios de polinización en el futuro.

## **Introduction**

Emerging evidence of widespread pollinator declines at local and regional scales (Nieto et al., 2015; Ollerton, Erenler, Edwards, & Crockett, 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, Butler, Upton, Parke, & Asher, 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, Nicholls, Botías, & Rotheray, 2015).

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In agricultural environments, bees are frequently exposed to combinations of chemicals (David et al., 2016; Mullin et al., 2010; Tosi, Costa, Vesco, Quaglia, & Guido, 2018), some of which may produce additive, antagonistic or synergistic effects (Biddinger et al., 2013; Johnson, Dahlgren, Siegfried, & Ellis, 2013; Sgolastra et al., 2017, 2018; Spurgeon et al., 2016; Zhu, Schmehl, Mullin, & Frazier, 2014; Zaragoza-Trello, Vilá, Botías & Bartomeus, 2020). 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, Ramos-Rodriguez, & Raine, 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, Motoyama, Ambrose, & Roe, 2004; Pilling, Bromley-Challenor, Walker, & Jepson, 1995; Pilling & Jepson, 1993; Sgolastra et al., 2017). EBI-fungicides inhibit the cytochrome P450 enzymatic detoxification mechanism in insects which is necessary for oxidative metabolism of a variety of xenobiotics (Brattsten, Berger, & Dungan, 1994). Metabolic detoxification mediated by cytochrome P450s contributes significantly to bee tolerance to some insecticides (Beadle et al., 2019; Hayward et al., 2019; Johnson, Wen, Schuler, & Berenbaum, 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 metabolised more slowly, although the effect is dose dependent, and the extent of synergism in field-realistic conditions is unclear (Thompson, Fryday, Harkin, & Milner, 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-fungicides

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-fungicides has been shown to alter the feeding behavior of bees (Azpiazu et al., 2019; Elston, Thompson, & Walters, 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, Goulson, & Hughes, 2013a; Higes 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, Feng, Silva, Susan, & Pieniazek, 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). *N. ceranae* has been suggested to be the causative agent of an emergent infectious disease in bumblebees because detrimental effects in survival, behavior and colony growth have been reported for *Bombus terrestris* with spores of this microsporidium (Graystock et al., 2013a; Rotheray, Osborne, & Goulson, 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, Gantz, Schneider, & Strebel, 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., 2013; Pettis, vanEngelsdorp,

Johnson, & Dively, 2012), 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, Botías, Goulson, & Hughes, 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, Christophides, Vlachou, & Kafatos, 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, Schmid-Hempel, & Barribeau, 2013; Riddell, Sumner, Adams, & Mallon, 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 signaling pathways, the Toll and the Imd pathways (Evans et al., 2006; Lemaitre & Hoffmann, 2007). Another molecule with a potential role in defense against gut-infecting parasites (Schlüns, Sadd, Schmid-Hempel, & Crozier, 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 two weeks and then subsequently placed in the field for a further seven 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 two-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 defense 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 two weeks under laboratory-controlled conditions in order to assess if exposure to mixtures of pesticides had an effect on the feeding behavior of



bumblebees. Finally, colony growth and the production of workers and sexuals was 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 21st May 2014, each consisting of a queen, brood and 10-21 workers (mean  $\pm$  s.d. =  $15.8 \pm 3.16$  workers/colony). We confirmed that all colonies were free of the most prevalent parasites (*Nosema bombi*, *Nosema 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 et al., 2013b; Martín-Hernández et al., 2007). Tripanosomatids could not be identified to the species level because the method used for detection (Meeus, de Graaf, Jans, & Smaghe, 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 =  $119 \pm 7.85$  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, Darling, & Brown, 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 in 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 two 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 25–45 kGy (Graystock et al., 2016; Higes, Martín-Hernández, Garrido-Bailón, García-Palencia, & Meana, 2008; Singh et al., 2010). Gamma irradiation has also been shown to be an effective tool to degrade pesticide residues in different matrices (Dessouki, Aly, & Sokker, 1999; Pargi & Bhatt, 2018). The sugar solution provided was 50% inverted sugar syrup (w/v with Ambrosia syrup, E H Thorne Ltd, Market Rasen, UK, and distilled water). Pesticide exposure started on Day 2 after arrival, when the colonies had acclimatised to the laboratory conditions.

*N. ceranae* inoculation was performed on Day 5 (see below for details), such that bees had already been exposed to pesticide mixtures for three days at the time of inoculation. The two-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, David, Hill, & Goulson, 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 seven weeks (Figure S1). The weight of the colonies was measured once per week throughout the experiment. By the end of nine 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 ppb of thiamethoxam (Botías et al., 2015), 10 ppb of cypermethrin (Mullin et al., 2010) and/or 36 ppb 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 ppb of thiamethoxam (Botías et al., 2015), 10 ppb of cypermethrin (Mukherjee, 2009) and/or 36 ppb 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 three days, all bees within each colony were individually marked on the thorax and

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fed 4 µl of a single meal of either 30% sugar water (controls and the pathogen-free treatment NPF) or 30% sugar water containing ca. 120,000 freshly prepared *N. ceranae* spores (other treatments, Table 1; viability 98.9% based on 0.4% Trypan blue staining) (Higes et al., 2008) using a micropipette. The *N. ceranae* spores were obtained by homogenising 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, Labarussias, de Miranda, Moritz, & Paxton, 2014; Higes, García-Palencia, Martín-Hernández, & Meana, 2007) and dosages of fewer than 100,000 spores have been found to infect bumblebees (Fürst, McMahon, Osborne, Paxton, & Brown, 2014; Graystock et al., 2013a). Bees were starved for 4 h 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, UK) 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 (*Nosema bombi*, *Nosema ceranae*, Trypanosomatida, *Apicystis bombi*), which are regarded as a threat to bumblebee health (Graystock et al., 2013b; Meeus, Brown, De Graaf, & Smagghe, 2011), was evaluated via PCR by analysing the 5 workers/colony that had been collected 10 days post-inoculation with *N. ceranae* spores (N = 50 bees per treatment group). A pool of the 5 DNA extracts per colony was analysed using PCR protocols and parasite-specific primers following the methods of Graystock et al. (2013b), 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 5 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 Phusion 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 3 qPCR replicates at 1:10, 1:100, 1:1000 and 1:5000 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 – 100.2%. Each sample was tested with the reference genes *AK* and *PLA2* (Horňáková, Matoušková, Kindl, Valterová, & Pichová, 2010) and all seven immunity related genes and 2 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 any queens were not 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



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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 eight 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, ten workers and ten males were randomly collected from each colony (or all the bees available in colonies with less than ten individuals), individually weighed and their thorax width was measured using digital calipers. A sample of 5 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 5 colonies that collapsed in the field one 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 (on 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 vs. 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 modeled 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 two weeks under laboratory conditions was

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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, Tobias, Rom, Wolfinger, & Hochberg, 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:  $(\text{grams of pollen or sugar solution collected from the uncontaminated feeder in the 10 colonies of the group} \times 100) / \text{grams of pollen or sugar solution collected from both feeders in the 10 colonies of the group}$ . The overall percentage of food collected per group compared to controls was calculated using the formula:  $100 - [(\text{total amount of pollen or sugar solution collected by the ten colonies of the group} \times 100) / \text{Total grams of pollen or sugar solution collected by the ten colonies of control group}]$ .

The number of bees positive for *N. ceranae* ten days post-inoculation was compared among the treatment groups that were inoculated with this microsporidium using a generalized linear model (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 (modeled as the presence/absence of the pathogens in each colony).

Fold-change in gene expression data were 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, Naumann, Wright, & Warton, 2012). Multivariate linear models allowed us to fit the responses of all genes simultaneously (Wang et al., 2012; Warton, Wright, & Wang, 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 effect model (LMM; Gaussian error distribution). Weight gain data were log-transformed prior to analysis to achieve normal error distribution. Treatment, week, and week<sup>2</sup> (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 linear mixed-effects models (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; Table 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 two 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 ten 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 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 seven weeks, *N. ceranae* was detected in 80, 60, 40, 80, 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  $\pm$  s.e. =  $-0.18 \pm 0.09$ ,  $z = -2.04$ ,  $P = 0.04$ ) and NPM (GLM; estimate  $\pm$  s.e. =  $-0.18 \pm 0.09$ ,  $z = -2.04$ ,  $P = 0.04$ ; Figure 4B), and the number of males was significantly reduced in colonies of treatment NPM (GLM; estimate  $\pm$  s.e. =  $-0.45 \pm 0.13$ ,  $z = -3.47$ ,  $P = 0.001$ ; Figure 4C), although effect sizes were small in both cases (Tables 3, S11, S12 and S13). Male numbers in NPM treatment were also smaller than in NPFM and PFM treatments (Tukey's post hoc test; both  $P$ 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

*Ps* < 0.05; Table 3 and 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  $P \leq 0.01$ ; Tables 3, 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, Wilkins, Harkin, Milner, & Walters, 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, Botias, & Goulson, 2017; Elston et al., 2013; Laycock, Cotterell, O'Shea-Wheller, & Cresswell, 2014; Rieth & Levin, 1988; Thompson et al., 2015; Zhu, Yao, Adamczyk, & Luttrell, 2017). There is

some evidence that EBI-fungicides 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 behavior 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, Abrantes, Gonçalves, Wolinska, & Castro, 2017). Tebuconazole, as with all the other EBI-fungicides, 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, Van Doorn, Samson, & Postma, 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, Bounias, & Fleche, 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, Raine, & Brown, 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 defense 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 metabolising the pesticides applied, but there is evidence suggesting that they are involved in the defense 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, Sadd, du Plessis, & Schmid-Hempel, 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, up-regulation 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-fungicides 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, O'Connor, Wackers, & Goulson, 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  $\pm$  s.d. =  $2.9 \pm 3.65$ ) 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 et al., 2013a; 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, Sadd, Neumann, & Sandrock, 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 (ca. 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 vs. 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 et al., 2013a; 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-fungicides 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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**Data availability:** All data from this study are provided as a public figshare repository (<https://doi.org/10.6084/m9.figshare.11844711.v1>)

**Code availability:** All R codes are provided as a public figshare repository (<https://doi.org/10.6084/m9.figshare.11844711.v1>).

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Figure 1. Food collection of *Bombus terrestris* bumblebee colonies exposed to combinations of pesticides and the *Nosema ceranae* parasite. A) Differential collection of uncontaminated vs. 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 vs. 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).

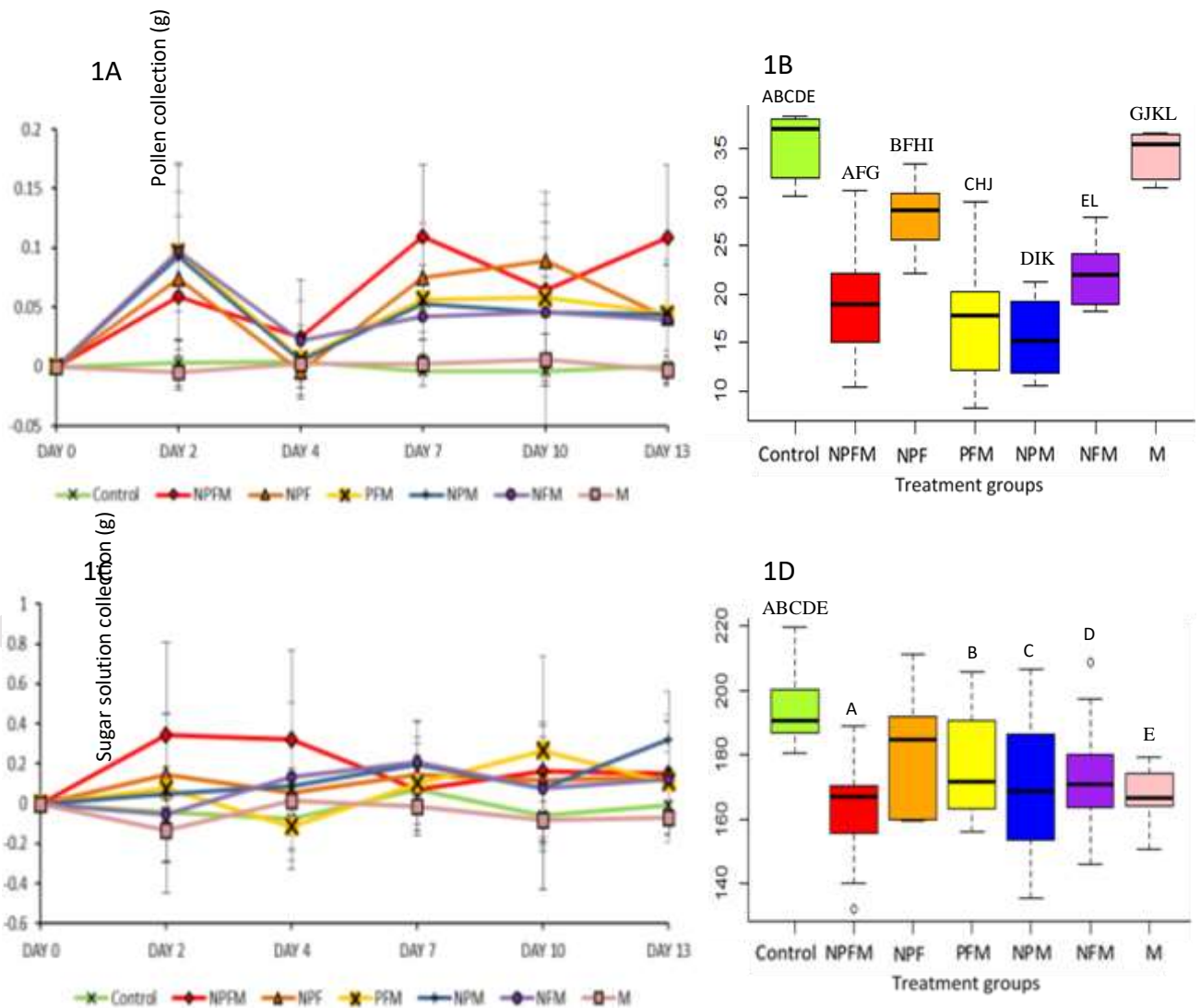




Figure 2. Pathogens detected in the bumblebee colonies. A: Percentage of *Bombus terrestris* in which the pathogen *Nosema ceranae* was detected 10 days after inoculation with a controlled dose of *N. ceranae* in sugar solution (treatments NPFM, PFM, NPM, NFM and M) or pathogen-free solution (controls 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*, *A. 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.

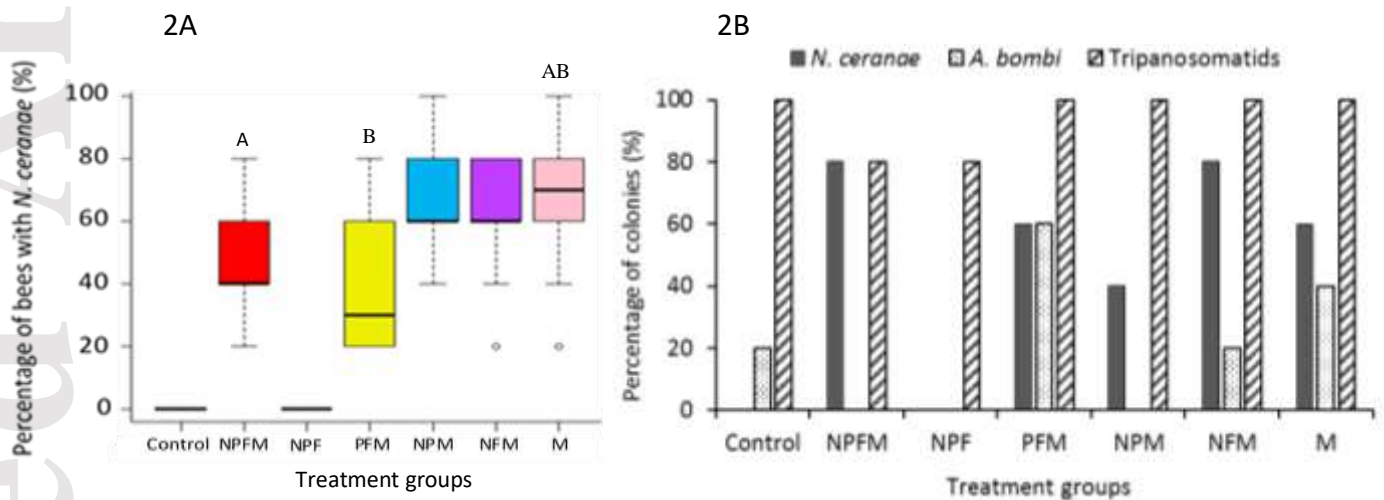


Figure 3. Boxplots showing the effect of treatments on the expression of genes related to the immune (*hemomucin*, *pelle*, *relish*, *abaecin*, *defensin*, *hymenoptaecin*, *apidaecin*) and detoxification (*CPR* and *CYP4G15*) response of bumblebees (*B. terrestris*) after 13 days of exposure to pesticide treatment and 10 days post-*N. ceranae* inoculation. Fold values of expression were calculated with  $\Delta C_t$  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.

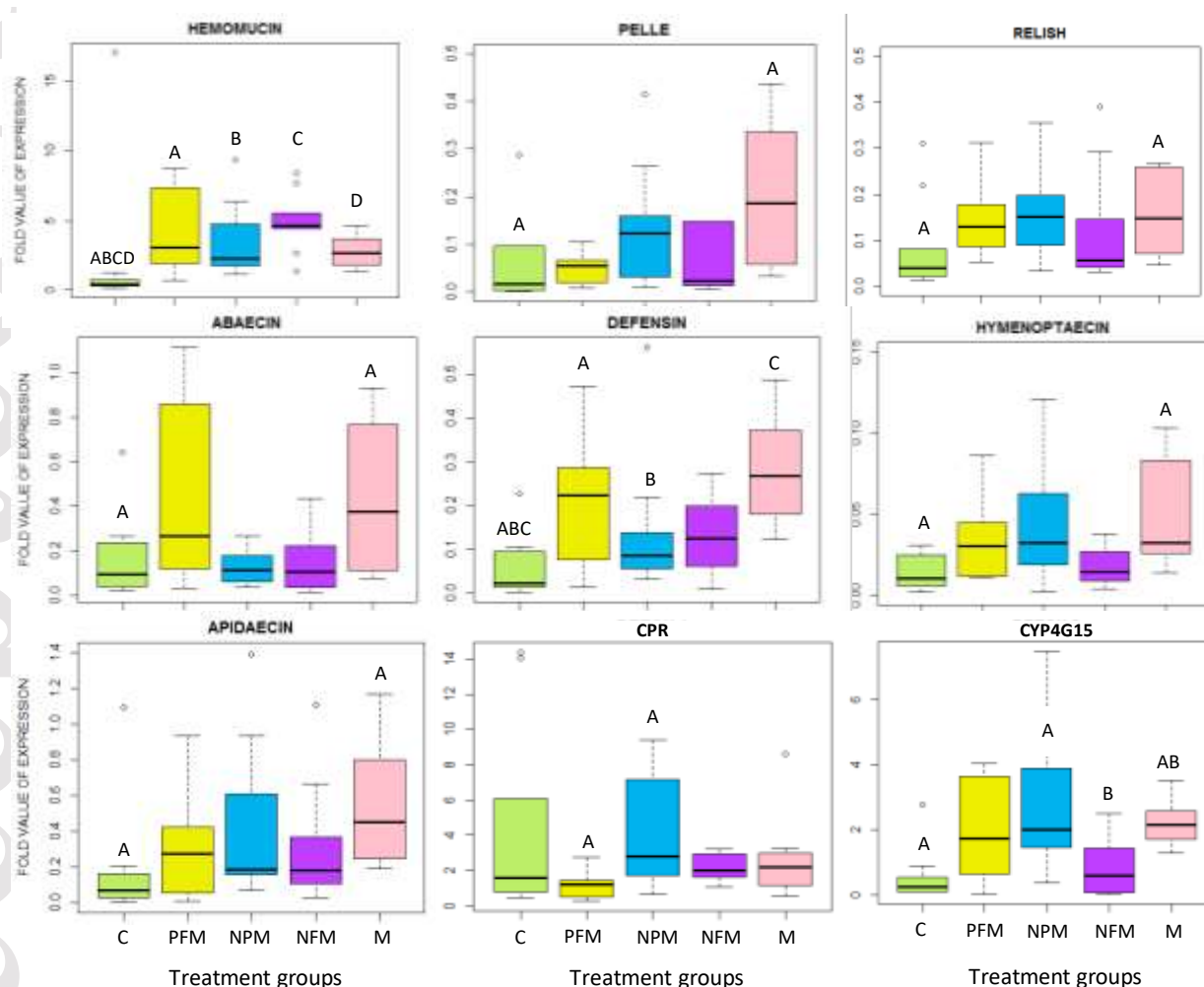


Figure 4. Fitness parameters measured in the *Bombus terrestris* colonies. A: Mean colony weight observed for the control and treatment groups at weekly intervals. The change in weight over time was significantly smaller ( $P = 0.009$ ) in the colonies that received the four stressors (NPFM) compared to control colonies. B: Boxplots of the number of workers and males in each treatment group. 2C: Boxplots of the number of brood (workers and males) cells and queen cells produced in each treatment group. Boxplots with similar letters are significantly different ( $P < 0.05$ ; GLMs with Posson 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.

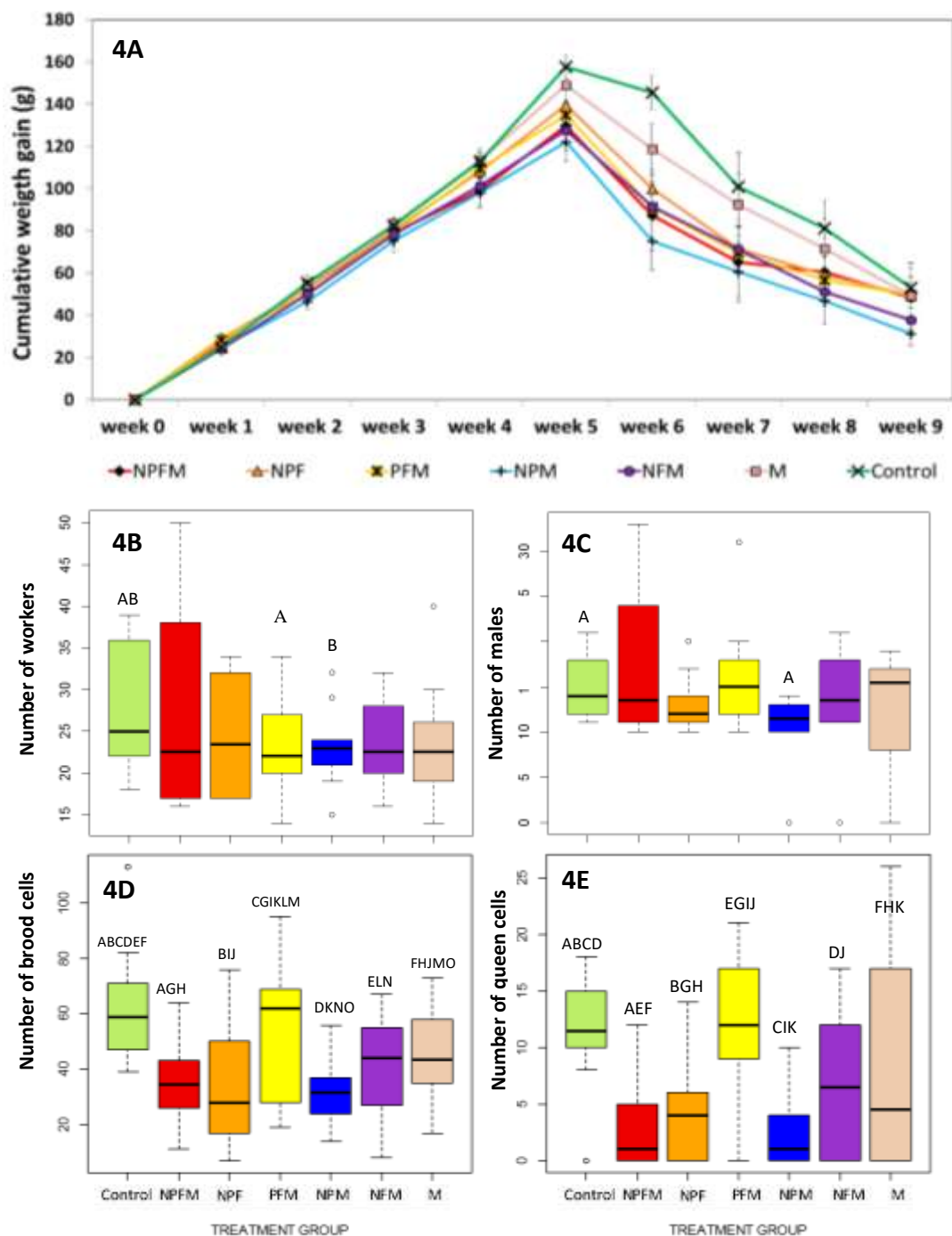


Table 1. Summary of the seven treatments applied to *B. terrestris* colonies in the study (10 colonies/treatment), involving three abiotic pesticide stressors (the neonicotinoid insecticide thiamethoxam, the pyrethroid insecticide cypermethrin and the fungicide tebuconazole) and one biotic stressor (the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

GROUP	NEONICOTINOID (N)	PYRETHROID (P)	DMI FUNGICIDE (F)	<i>N. ceranae</i> (M)
NPFM	✓	✓	✓	✓
NPF	✓	✓	✓	
PFM		✓	✓	✓
NPM	✓	✓		✓
NFM	✓		✓	✓
M				✓
Control				

Table 2. Linear mixed effect 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.

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 <sup>2</sup>	-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 <sup>2</sup>	0.017	0.009	541	1.848	0.065
NPF*week <sup>2</sup>	0.015	0.009	541	1.654	0.099
PFM*week <sup>2</sup>	0.015	0.009	541	1.685	0.093
NPM*week <sup>2</sup>	0.006	0.009	541	0.662	0.508
NFM*week <sup>2</sup>	0.003	0.009	541	0.372	0.710
M*week <sup>2</sup>	-0.003	0.009	541	-0.339	0.735

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 of brood cells	Number of queen cells
		Number	Weight (g)	Size (mm)	Number	Weight (g)	Size (mm)		
<b>NPFM</b>	mean	26.6	0.09	4.05	17.1	0.17	5.52	34.9	3.2
<b>TMX+CYP+TEB+Nosema</b>	sd	11.96	0.04	0.61	8.03	0.04	0.41	14.93	4.21
<b>NPF</b>	mean	24.6	0.08	4.11	13.1	0.16	5.46	34.3	4.6
<b>TMX+CYP+TEB</b>	sd	7.4	0.03	0.66	3.14	0.04	0.47	23.63	5.08
<b>PFM</b>	mean	23.1	0.08	4.16	16.2	0.17	5.6	55.1	11.5
<b>CYP+TEB+Nosema</b>	sd	5.67	0.04	0.57	6.16	0.05	0.5	25.04	7.28
<b>NPM</b>	mean	23.2	0.08	4.1	9.6	0.17	5.43	31.4	2.7
<b>TMX+CYP+Nosema</b>	sd	4.75	0.04	0.56	5.19	0.05	0.39	13.2	3.65
<b>NFM</b>	mean	23.7	0.08	4.16	12.6	0.16	5.5	41.5	6.8
<b>TMX+TEB+Nosema</b>	sd	5.27	0.04	0.64	7.49	0.05	0.53	18.96	6.16
<b>M</b>	mean	23.8	0.08	4.16	12	0.16	5.41	45.3	8.9
<b>Nosema</b>	sd	7.29	0.03	0.58	7.12	0.04	0.37	17.15	10.43
<b>CONTROL</b>	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