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

Article (Accepted Version)

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

Cristina Botías[1,2,*], Julia C. Jones[2,3], Tobias Pamminger[2,4], Ignasi Bartomeus[5], William O.H. Hughes[2] and Dave Goulson[2]

1. Instituto Regional de Investigación y Desarrollo Agroalimentario y Forestal de Castilla La Mancha (IRIAF), CIAPA de Marchamalo, Guadalajara, Spain

2. School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK

3. School of Biology and Environmental Science, University College Dublin, Dublin, Ireland

4. BASF SE, APD/EE - Li 425, Speyerer Strasse 2, 67117 Limburgerhof, Germany

5. Estación Biológica de Doñana (EBD-CSIC). Dpto. Ecología Integrativa, ES-41092, Sevilla, Spain

***** Corresponding author: cristinabotias@gmail.com

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 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).

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 &

156 Jepson, 1993; Sgolastra et al., 2017). EBI-fungicides inhibit the cytochrome P450
157 enzymatic detoxification mechanism in insects which is necessary for oxidative
158 metabolism of a variety of xenobiotics (Brattsten, Berger, & Dungan, 1994). Metabolic
159 detoxification mediated by cytochrome P450s contributes significantly to bee tolerance
160 to some insecticides (Beadle et al., 2019; Hayward et al., 2019; Johnson, Wen, Schuler,
161 & Berenbaum, 2006), and cytochrome P450 activity has been shown to be an important
162 determinant of neonicotinoid sensitivity in bumblebees (Manjon et al., 2018). The
163 inhibition of this detoxification mechanism may lead to insecticide residues being
164 metabolised more slowly, although the effect is dose dependent, and the extent of
165 synergism in field-realistic conditions is unclear (Thompson, Fryday, Harkin, & Milner,
166 2014). Therefore, the toxic effects of pesticides encountered by bees in the field are
167 difficult to predict from single-compound laboratory studies. Exposure to EBI-fungicides
168 can also reduce the anti-feeding response of bees to pyrethroids, and consequently
169 increase the toxicity of these insecticides by raising the levels of exposure (Thompson &
170 Wilkins, 2003). Moreover, exposure to neonicotinoids and to EBI-fungicides has been
171 shown to alter the feeding behavior of bees (Azpiazu et al., 2019; Elston, Thompson, &
172 Walters, 2013; Kessler et al., 2015). Therefore, the repellency or attractiveness of the food
173 contaminated with different pesticide should also be assessed to better understand the
174 extent of exposure to certain mixtures.

175 Bees are also impacted by a diversity of pathogens, including one suspected to
176 seriously affect their health, the microsporidium *Nosema ceranae* (Graystock, Yates,
177 Darvill, Goulson, & Hughes, 2013a; Higes et al., 2008), which infects gut epithelia of
178 adult bees. This obligate intracellular eukaryotic parasite was initially detected in the
179 Asian honeybee *Apis cerana* (Fries, Feng, Silva, Susan, & Pieniazek, 1996), but is now
180 globally distributed in *A. mellifera* (Klee et al., 2007), and has been more recently

181 detected in several species of solitary bees and bumblebees (Li et al., 2012; Plischuk et
182 al., 2009; Ravoet et al., 2014). *N. ceranae* has been suggested to be the causative agent
183 of an emergent infectious disease in bumblebees because detrimental effects in survival,
184 behavior and colony growth have been reported for *Bombus terrestris* with spores of this
185 microsporidium (Graystock et al., 2013a; Rotheray, Osborne, & Goulson, 2017).
186 However, the results of a recent study question the classification of *N. ceranae* as an
187 emerging infectious agent for bumblebees as a new host (Gisder et al., 2020). The
188 detrimental effects detected in *N. ceranae* PCR-positive bumblebees may instead be due
189 to the specific energetic and corresponding physiological costs that immune responses
190 entail (Ardia, Gantz, Schneider, & Strebel, 2012). In addition, exposure to neonicotinoid
191 insecticides and some fungicides has been reported to increase the levels and impact of
192 *N. ceranae* in honeybees (Alaux et al., 2010; Pettis et al., 2013; Pettis, vanEngelsdorp,
193 Johnson, & Dively, 2012), possibly due to a reduced immunocompetence in bees
194 challenged by some types of pesticides (Di Prisco et al., 2013; Garrido et al., 2013).
195 Understanding how environmental stressors affect immune responses in bees is crucial
196 for developing more informed strategies for mitigating the impacts of pesticides on the
197 health of pollinators (Pamminger, Botías, Goulson, & Hughes, 2018). In bumblebees, as
198 in other insects, the immune responses to parasites have been identified, and consist of
199 both cellular and humoral responses (Barribeau et al., 2015). Phagocytosis, encapsulation
200 and melanization are related to cellular immunity (Osta, Christophides, Vlachou, &
201 Kafatos, 2004), while humoral immunity involves antimicrobial peptide (AMPs)
202 synthesis (Evans, 2006). Four antimicrobial peptides which provide a broad-spectrum of
203 activity against microorganisms, *abaecin*, *apidaecin*, *defensin* and *hymenoptaecin*, have
204 been identified in *B. terrestris* during infection by trypanosomatid parasites (Barribeau et
205 al., 2015; Brunner, Schmid-Hempel, & Barribeau, 2013; Riddell, Sumner, Adams, &

206 Mallon, 2011), and during *N. ceranae* infection in honeybees (Antúnez et al., 2009;
207 Chaimanee et al., 2013). The synthesis of these AMPs is under the control of two
208 important signaling pathways, the Toll and the Imd pathways (Evans et al., 2006;
209 Lemaitre & Hoffmann, 2007). Another molecule with a potential role in defense against
210 gut-infecting parasites (Schlüns, Sadd, Schmid-Hempel, & Crozier, 2010) such as the
211 microsporidium *N. ceranae*, in bumblebees, is hemomucin, a surface glycoprotein
212 involved in inducing an immune response and found to be highly expressed in the midgut
213 (Theopold et al., 1996).

214 Here we conducted an experiment on bumblebees (*B. terrestris*) to study the
215 combined effects of chronic exposure to field-relevant doses of three abiotic stressors, the
216 neonicotinoid thiamethoxam, the pyrethroid cypermethrin, and the EBI-fungicide
217 tebuconazole, as well as the biotic stressor, *N. ceranae*. Bumblebee colonies were pulse
218 exposed to the stressors in controlled laboratory conditions for two weeks and then
219 subsequently placed in the field for a further seven weeks (depuration period under
220 environmentally relevant conditions). We compared the effect of simultaneous exposure
221 to the four stressors and to different ternary combinations of them, by evaluating the
222 impact of such stressors during the pulsed exposure and the second potential demographic
223 impact due to failure to fully eliminate the toxicants and the pathogen during the
224 depuration period. In addition, one group was exposed solely to the microsporidium *N.*
225 *ceranae* in order to discern if pesticide exposure alters the prevalence and gene expression
226 profile related to the immune response of inoculated bees. Thus, after the two-week
227 pulsed exposure period ended, we evaluated the prevalence of *N. ceranae* in bumblebee
228 colonies. Next, to further explore this effect we measured gene expression in four AMPs
229 (*abaecin, apidaecin, defensin* and *hymenoptaecin*), a signal molecule of the Toll (*pelle*),
230 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 ± s.d. = 15.8 ± 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, &

256 Smagghe, 2010), based on amplification of the 18S rDNA gene alone, has been shown to
257 be unsuitable to classify tripanosomatids infecting bees without sequencing (Bartolomé
258 et al., 2018; Ravoet et al., 2015). Colonies were then randomly assigned to one of seven
259 treatments (see below for details on the treatments; 10 colonies/treatment). There were
260 no significant differences between treatments in the worker population or weight of
261 colonies at the start of the experiment (ANOVA; worker population: $F_{6,63} = 0.563$, $P =$
262 0.758; mean weight = 119 ± 7.85 g, $F_{6,63} = 0.242$, $P = 0.961$).

263 The seven treatments were applied using a stepwise removal approach, consisting
264 of exposure to three abiotic and one biotic stressor applied simultaneously or in different
265 ternary combinations. These treatments were applied in order to determine the relative
266 importance of each stressor or combination of stressors in affecting bumblebees at the
267 individual and colony levels (Table 1). In comparison with factorial approaches, the
268 stepwise removal approach allowed us to empirically test complex combinations of up to
269 four stressors with a limited number of treatments, and thus, to simplify the otherwise
270 unwieldy number of potential interactions by using experimental treatments that reflect
271 combinations of stressors that are likely to occur in real-world agricultural landscapes
272 (Côté, Darling, & Brown, 2016). The abiotic stressors were the pesticides thiamethoxam
273 (neonicotinoid insecticide), cypermethrin (pyrethroid insecticide), and tebuconazole
274 (demethylation inhibitor fungicide). These pesticides were selected based on their
275 extensive use in UK arable crops including oilseed rape, wheat and spring barley (FERA,
276 2017), consequent prevalence on crop and field margin flowers, and their regular
277 occurrence in bee food stores worldwide (Sánchez-Bayo & Goka, 2014). The biotic
278 stressor was the microsporidian pathogen *N. ceranae* (Table 1).

279 The colonies were kept in laboratory conditions (25°C, 50–60% relative humidity)
280 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

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

319 Pollen was spiked according to treatment by spreading the corresponding volume
320 of 0.01 mg/ml solutions of each pesticide over it with a micropipette to obtain the
321 concentrations required for the experiment: 5 ppb of thiamethoxam (Botías et al., 2015),
322 10 ppb of cypermethrin (Mullin et al., 2010) and/or 36 ppb of tebuconazole (David et al.,
323 2016; Mullin et al., 2010). Once spiked, the pollen with the pesticide solutions was
324 homogenized by means of a mortar and pestle. Sugar solution was spiked according to
325 treatment with 1.5 ppb of thiamethoxam (Botías et al., 2015), 10 ppb of cypermethrin
326 (Mukherjee, 2009) and/or 36 ppb of tebuconazole (Büchler & Volkmann, 2003). The
327 thiamethoxam concentration used was that detected previously in pollen and nectar
328 collected from flowers (Botías et al., 2015). The cypermethrin and tebuconazole
329 concentrations used were those detected previously in bee food stores (beebread and
330 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 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

356 was touched with a droplet of the spore solution at the tip of a micropipette. After parasite
357 inoculation on Day 5 colonies were monitored daily for worker mortality until they were
358 placed in the field on Day 15, with very low mortality observed during this period (<3
359 workers in all treatments). *Nosema* spores were not detected in the dead bees collected.
360
361 **2.3. DNA and RNA extraction, pathogen screening and gene expression analysis.**
362 Ten days post-inoculation, five marked bees per colony were flash-frozen in liquid
363 nitrogen and stored at – 80°C for subsequent parasite and gene expression analysis. The
364 midgut is the site of infection by *N. ceranae* and also the main site of exposure to orally
365 administered chemicals, so the midguts of these bees were individually dissected and used
366 for the pathogen screening and gene expression analyses. Once dissected, midguts were
367 immediately homogenized in 600 µL of buffer RTL Plus within a Pathogen Lysis Tube
368 (Cat No./ID: 19092, Qiagen), grinding first with a microtube pestle and then with a Tissue
369 Lyser LT (5 min at 50 Hz, Qiagen). Isolation of DNA and RNA from bumblebee guts
370 was performed using the AllPrep DNA/RNA Mini kit (Qiagen, UK) according to the
371 AllPrep DNA/RNA protocol. Genomic DNA was removed from RNA isolated using the
372 RNA-free DNase set (Qiagen) during the RNA extraction. DNA was stored at -20°C until
373 pathogen screening was performed, and RNA was stored at -80°C until gene expression
374 analysis started.
375 The presence of common bumblebee parasites (*Nosema bombi*, *Nosema ceranae*,
376 Trypanosomatida, *Apicystis bombi*), which are regarded as a threat to bumblebee health
377 (Graystock et al., 2013b; Meeus, Brown, De Graaf, & Smagghe, 2011), was evaluated via
378 PCR by analysing the 5 workers/colony that had been collected 10 days post-inoculation
379 with *N. ceranae* spores (N = 50 bees per treatment group). A pool of the 5 DNA extracts
380 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 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 3 qPCR replicates at 1:10, 1:100, 1:1000 and 1:5000 concentrations were generated to

406 test the amplification efficiency of each primer set. The efficiency of all the primer sets
407 used in our experiment were between 93.3 – 100.2%. Each sample was tested with the
408 reference genes *AK* and *PLA2* (Horňáková, Matoušková, Kindl, Valterová, & Pichová,
409 2010) and all seven immunity related genes and 2 detoxification related genes. Three
410 technical replicates were run per reaction. Reactions for qPCR were performed on an ABI
411 OneStep™ RT-PCR instrument using the following program: 95°C for 5 m, followed by
412 40 cycles of a 30 s at 94°C denaturation, 30 s at 59°C annealing and 30 s at 72°C extension
413 steps. Results were analysed using the OneStep™ analysis software. The amplification
414 results from the different genes were expressed as the threshold cycle (C_T) value, which
415 represents the number of cycles needed to generate a fluorescent signal greater than a
416 predefined threshold. Fluorescence was measured in the elongation step and negative
417 controls (without cDNA) were included in each reaction run. Fold change in expression
418 was calculated relative to expression levels in the control samples and using the geometric
419 mean of reference genes *AK* and *PLA2* as the endogenous control value with the $2^{(\Delta-\Delta CT)}$
420 approximation method (Livak & Schmittgen, 2001).
421
422 **2.4. Bumblebee monitoring under field conditions**
423 Monitoring under field conditions was timed to correspond to wild colony development
424 in the region where the study was performed (East Sussex, South East England, UK), with
425 colonies being placed out in the field on Day 15 after the colonies arrived (Day 13 post-
426 exposure). The field site was situated in an orchard at Stanmer Organics (Brighton, East
427 Sussex, UK), which has been Soil Association certified organic for the past 10 years and
428 is located within Stanmer Park Local Natural Reserve (about 5,000 ha of open and
429 wooded land), that covers a larger area than the typical foraging range of *B. terrestris*
430 (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 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

456 experiment, the values of number of workers, males, brood cells and queen cells recorded
457 upon collection from the field (on week 8) were the ones used for the statistical analyses.
458 Residuals of all models were inspected to ensure model assumptions were met.

459 The differences in the collection of uncontaminated vs. pesticides-treated pollen
460 and sugar solution were analysed using generalized linear models (nlme package)
461 (Pinheiro & Bates, 2000). The differential collection of pollen or sugar solution divided
462 by the number of individuals per colony was modeled as a function of the treatment group
463 and the time when food consumption was measured. The temporal correlation between
464 errors was included in order to account for the repeated measures performed in each
465 colony and colony was included as a random effect. In addition, the total collection of
466 pollen and sugar solution measured after two weeks under laboratory conditions was
467 analysed using linear regression (LM; Gaussian error distribution), with the total
468 collection of pollen and sugar solution per group entered as fixed effects. Multiple
469 pairwise comparisons for total pollen and sugar solution collected were performed using
470 Tukey´s post-hoc tests (using the R package multcomp; Westfall, Tobias, Rom,
471 Wolfinger, & Hochberg, 1999). The relative percentage of pollen or sugar solution
472 collected per group from the uncontaminated feeder in relation to the pesticide feeder was
473 calculated by using the following formula: (grams of pollen or sugar solution collected
474 from the uncontaminated feeder in the 10 colonies of the group * 100) / grams of pollen
475 or sugar solution collected from both feeders in the 10 colonies of the group. The overall
476 percentage of food collected per group compared to controls was calculated using the
477 formula: 100 – [(total amount of pollen or sugar solution collected by the ten colonies of
478 the group * 100) / Total grams of pollen or sugar solution collected by the ten colonies of
479 control group].

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

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

497 Colony weight gain over time was analysed using a linear mixed effect model
498 (LMM; Gaussian error distribution). Weight gain data were log-transformed prior to
499 analysis to achieve normal error distribution. Treatment, week, and week^2 (to account for
500 the curved relationship of weight over time) were entered as fixed effects and the number
501 of workers present at Week 0 as a covariate. The interaction between week and treatment
502 was included in the model and colony was entered as a random effect. Maximum colony
503 weight, which was achieved at week 5 in all treatment groups, was also compared among
504 groups as a fitness proxy by using linear regression (LM; Gaussian error distribution),

505 followed by Tukey´s post-hoc tests (Westfall et al., 1999). The effect of treatment on the
506 total numbers of adult workers, adult males, worker and male brood cells (capped and
507 uncapped; worker and male cells cannot be distinguished), and queen cells (capped and
508 uncapped) was assessed using generalized linear-mixed-effects models (GLM; Poisson
509 error distribution), while the effect of treatment on the weight and thorax width of adult
510 workers and males were analysed using linear mixed-effects models (LMM; normal error
511 distribution). All models had treatment as a fixed factor and the LMMs included colony
512 as a random factor. Multiple pairwise comparisons for number of workers, males, brood
513 cells and queen cells produced were performed by using Tukey´s post-hoc tests (Westfall
514 et al., 1999). To evaluate the relative contribution of each stressor in affecting bumblebee
515 colony fitness, we assessed the mean effect size of each individual stressor on the
516 production of brood cells, new queens and males. To do this, we calculated the mean
517 effect size of each of the treatments containing a particular stressor, minus the effect size
518 of the treatment without it (e.g. average contribution of N to the effects on colony fitness:
519 mean [(NPFM – PFM); (NPF – PFM); (NPM – PFM); (NFM – PFM); (NPFM – M);
520 (NPF – M); (NPM – M); (NFM – M)].

521

522 **3. Results**

523 **3.1. Food collection**

524 Bumblebees collected 27, 16, 24, 24 and 19% more pollen respectively in colonies of
525 groups NPFM, NPF, PFM, NPM and NFM from the 'uncontaminated feeder' than from
526 the 'pesticides feeder' (GLM; $F_{6, 342} = 17.17$, $P < 0.0001$; Figure 1A; Table S2), and
527 reduced their overall pollen collection by 46, 21, 50, 57, and 38% respectively in colonies
528 of groups NPFM, NPF, PFM, NPM, and NFM compared to controls (LM; $F_{6,63} = 30.90$,
529 $P < 0.0001$; 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).

554 At the end of the experiment, after colonies had been foraging freely in the field
555 for seven weeks, *N. ceranae* was detected in 80, 60, 40, 80, 60% of the colonies in
556 treatments NPFM, PFM, NPM, NFM and M respectively (Figure 2B), and not detected
557 in the colonies of treatment NPF and controls. *Apicystis bombi* was detected in 60, 20, 40
558 and 20% of the colonies of treatments PFM, NFM, M and controls respectively.
559 Trypanosomatids were detected in all colonies of groups PFM, NPM, NFM, M and
560 Control, and in 80% of the colonies of groups NPFM and NPM (Figure 2B). The
561 prevalence of *N. ceranae*, *A. bombi* and trypanosomatids at the end of the study was not
562 affected by treatment (Table S8).

563 **3.3. Gene expression**

564 For the treatment groups examined (PFM, NPM, NFM, M), the genes analysed were
565 overall differentially expressed in treated groups compared to the control group ($F_{4,45}$ =
566 38.25, P = 0.002). *Hemomucin* was upregulated in treatments PFM, NPM, NFM and M;
567 *defensin* was upregulated in treatments PFM, NPM and M; *CYP4G15* was upregulated in
568 treatments NPM and M; and *pelle*, *relish*, *abaecin*, *hymenoptaecin*, *apidaecin* were
569 upregulated in treatment M with respect to control colonies (< 0.05 in all cases; Figure 3;
570 Table S9). In addition, multiple pairwise comparisons showed upregulation of *CPR* in
571 treatment NPM with respect to PFM (Tukey and Kramer (Nemenyi) test; P = 0.037), and
572 that *CYP4G15* was downregulated in treatment NFM with respect to M (P = 0.042), and
573 marginally downregulated with respect to NPM (P = 0.075).

574 **3.4. Fitness parameters**

575 The initial growth phase was followed in all colonies by a natural decline associated with
576 the production of new reproductives (Figure 4A). As indicated by a negative interaction
577 term, colonies of treatment NPFM gained less weight over the course of the experiment
578 compared to the control colonies (Table 2). Colonies of groups NPF and PFM also tended

579 to grow less over time, but only colonies of group NPM were significantly bigger than
580 controls when the maximum size was achieved in all groups (week 5) (LM followed by
581 Tukey´s post-hoc tests, $P = 0.046$; Table S10). Compared to control colonies, the number
582 of workers was lower in treatments PFM (GLM; estimate ± s.e. = - 0.18 ± 0.09, $z = - 2.04$,
583 $P = 0.04$) and NPM (GLM; estimate ± s.e. = - 0.18 ± 0.09, $z = - 2.04$, $P = 0.04$; Figure
584 4B), and the number of males was significantly reduced in colonies of treatment NPM
585 (GLM; estimate ± s.e. = - 0.45 ± 0.13, $z = - 3.47$, $P = 0.001$; Figure 4C), although effect
586 sizes were small in both cases (Tables 3, S11, S12 and S13). Male numbers in NPM
587 treatment were also smaller than in NPFM and PFM treatments (Tukey´s post hoc test;
588 both Ps < 0.001; Table S13). Treatments did not have an effect on the worker and male
589 weight (LMM, $P = 0.99$ and $P = 0.89$, respectively), or on the size (thorax width) of
590 workers and males (LMM, $P = 0.89$ and $P = 0.47$, respectively; Table S14). All treatments
591 had an influence on the number of brood (workers and males) cells produced (GLM, all
592 *P*s < 0.05; Table 3 and Table S11; Figure 4D), and this effect was stronger when the
593 neonicotinoid was present (Tables S15 and S17). Moreover, colonies of groups exposed
594 to the neonicotinoid (NPFM, NPF, NPM and NFM) produced fewer queen cells than
595 control colonies (C), the group with the pyrethroid and the EBI-fungicide (PFM), and the
596 group only inoculated with *N. ceranae* (M) (Tukey´s post-hoc tests; all $P \leq 0.01$; Tables
597 3, S16 and S17; Figure 4E).

598

599 **4. Discussion**

600 The results show that exposure to common environmental stressors interact to
601 affect bumblebees at the individual and the colony levels. One of the sub-lethal effects of
602 exposure to pesticide mixtures was an antifeedant effect, with all bees collecting both less
603 pollen and less sugar solution from the pesticide-contaminated feeders than

604 uncontaminated feeders. This was regardless of the pesticide contaminant, indicating that
605 at least two of the pesticides applied have an inhibitory effect on food consumption.
606 Furthermore, pesticide mixtures acted synergistically with *N. ceranae* to reduce total
607 pollen collection. Bumblebee colonies with low pollen consumption have previously been
608 shown to gain less weight over time even when nectar availability is high (Rotheray et
609 al., 2017), and this was true here for colonies of treatment NPFM (TMX + CYPER + TEB
610 + *N. ceranae*). The reduced intake of pesticide-contaminated food might be due to
611 avoidance or a secondary antifeedant response following ingestion (Thompson, Wilkins,
612 Harkin, Milner, & Walters, 2015). Thiamethoxam, other neonicotinoids such as
613 imidacloprid and clothianidin, and pyrethroids have previously been found to exert a
614 repellent or antifeedant effect on bees (Cresswell et al., 2012; Dance, Botias, & Goulson,
615 2017; Elston et al., 2013; Laycock, Cotterell, O'Shea-Wheller, & Cresswell, 2014; Rieth
616 & Levin, 1988; Thompson et al., 2015; Zhu, Yao, Adamczyk, & Luttrell, 2017). There is
617 some evidence that EBI-fungicides such as tebuconazole can reduce this effect
618 (Thompson & Wilkins, 2003), and studies of neonicotinoid-fungicide mixtures have
619 found that pesticide-contaminated food affects the post-consumption behavior of
620 bumblebees rather than being an olfactory repellent (Jiang et al., 2018). Therefore, the
621 effects of exposure to pesticide mixtures are complex and difficult to infer from single
622 compound tests, as confirmed by our results.

623 With respect to susceptibility to *N. ceranae* infection, the prevalence of *N. ceranae*
624 was lower when a combination of the fungicide and the pyrethroid were applied in the
625 diet. Although previous research showed that exposure to fungicides increased *N. ceranae*
626 levels in bees (Glavinic et al., 2019; Pettis et al., 2013) the suppressive effect of
627 tebuconazole on the infection of a fungal endoparasite in *Daphnia* has also been reported
628 before (Cuco, Abrantes, Gonçalves, Wolinska, & Castro, 2017). Tebuconazole, as with

629 all the other EBI-fungicides, obtains its fungicidal activity through disrupting
630 biosynthesis of ergosterol, the dominant lipid in fungal cell membranes (Köller &
631 Scheinpflug, 1987). Therefore, this azole fungicide may have led to a reduction in
632 ergosterol necessary for *N. ceranae* membrane functioning, thus inhibiting fungal growth
633 and possibly causing spore death (Dijksterhuis, Van Doorn, Samson, & Postma, 2011).
634 Why the effect of the fungicide should depend on the presence of the pyrethroid is
635 unclear, but previous research has found interactions between acute sub-lethal exposure
636 to cypermethrin and pathogen infections in honeybees (Bendahou, Bounias, & Fleche,
637 1997), and no significant impact on the susceptibility of *B. terrestris* workers to the gut
638 parasite *C. bombi* upon exposure to the pyrethroid lambda-cyhalothrin (Baron, Raine, &
639 Brown, 2014). While the potential suppression of infection by tebuconazole and/or
640 cypermethrin may seem positive for the host, the possible ecological consequences of this
641 antagonist interaction between pollution and disease are still poorly understood and may
642 have costs in terms of host-pathogen population dynamics (Cuco et al., 2017).

643 By exploring the possible interaction between pesticide exposure and *N. ceranae*
644 inoculation at the gene expression level, we found that all genes related to the immune
645 response were upregulated in the group that was only inoculated with *N. ceranae*
646 (Treatment M), indicating that the bumblebee immune system responded against this
647 microsporidium. This upregulation was reduced in groups that were inoculated with *N.*
648 *ceranae* spores and exposed to pesticide mixtures. This result suggests that pesticide
649 mixtures may interfere with the transcription of some genes encoding defense
650 mechanisms to pathogen challenge. Variation between genes in the strength of the effect
651 indicates that pesticides may not affect the transcription of all AMPs to the same extent,
652 or that different types of pesticide may alter the expression of specific immunity-related
653 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

678 gene could indicate the involvement of this enzyme in the formation of ROS upon *N.*
679 *ceranae* infection.

680 At the colony level, combined exposure to the three pesticides mixture and *N.*
681 *ceranae* (NPFM) reduced bumblebee colony growth over the course of the study.
682 Colonies that only received the three pesticides (NPF) or pyrethroid + fungicide + *N.*
683 *ceranae* (PFM) also grew marginally less than control colonies over time, suggesting that
684 the combination of a pyrethroid + EBI-fungicide produced detrimental effects on colony
685 growth. Previous research indicates that EBI-fungicides synergize pyrethroid toxicity in
686 honeybees (Colin & Belzunces, 1992; Pilling & Jepson, 1993; Thompson & Wilkins,
687 2003) and bumblebees (Raimets et al., 2018), and our study provides evidence that the
688 enhanced toxicity of this pesticide combination may translate into detrimental effects at
689 the bumblebee colony level (Whitehorn, O´Connor, Wackers, & Goulson, 2012).
690 Furthermore, colonies exposed to mixtures containing neonicotinoid + pyrethroid + *N.*
691 *ceranae* (NPM) were smaller at the time of maximum growth in all treatment groups
692 (week 5), indicating that this combination may also impair colony success, as only the
693 largest bumblebee colonies succeed in producing queens (Müller & Schmid-Hempel,
694 1992). Indeed, colonies of group NPM produced the lowest number of queens (mean ±
695 s.d. = 2.9 ± 3.65) in the present study. Our findings are of great concern given the high
696 prevalence of *N. ceranae* detected in wild and commercial bumblebee colonies in some
697 regions (Arbulo et al., 2015; Graystock et al., 2013a; Rotheray et al., 2017), and the
698 widespread use of neonicotinoid and pyrethroid insecticides (Botías et al., 2015; FERA,
699 2017).

700 All treatments led to a small reduction in worker and male brood production. Adult
701 worker numbers were slightly lower in the groups in which bumblebees had been exposed
702 to the pyrethroid + *N. ceranae* (treatments PFM and NPM), and male numbers were fewer

703 when the pyrethroid and the neonicotinoid were applied in combination (NPM). Our
704 findings are in agreement with previous research showing that exposure of bumblebees
705 to neonicotinoids alone and in combination with pyrethroids impair brood cell and male
706 production (Fauser-Misslin, Sadd, Neumann, & Sandrock, 2014; Gill et al., 2012;
707 Rundlöf et al., 2015; Wintermantel et al., 2018). This may have detrimental consequences
708 for colony fitness and development as the rate of colony growth depends on the number
709 of adult workers (Whitehorn et al., 2012). However, it is worth noting that the number
710 of workers, even in the control colonies (mean = 27.7 workers), was very low compared
711 to what is reported for wild *B. terrestris* nests (ca. 300-400 workers; Duchateau &
712 Velthuis, 1988). Our experimental colonies may have performed poorly compared to
713 natural ground-nesting colonies due to differences in the incubation conditions in surface
714 *vs*. fossorial situations (Vogt, 1986a, 1986b). Furthermore, brood production was not only
715 affected by exposure to pesticide mixtures, but also by *N. ceranae* inoculation (treatment
716 M). The changes in gene expression related to immune and detoxification responses in
717 the *N. ceranae*-challenged bumblebees may involve fitness costs that led to the
718 detrimental effects observed at the individual and colony levels (Graystock et al., 2013a;
719 Rotheray et al., 2017). More significantly, exposure to the neonicotinoid thiamethoxam
720 led to a large reduction in queen cell production, with colonies of treatments NFM, NPF,
721 NPM and NPFM producing 40%, 60%, 76% and 72% fewer queen cells than control
722 colonies. These findings are consistent with previous studies that reported a decrease in
723 queen production when bumblebee colonies were exposed to neonicotinoids (Fauser-
724 Misslin et al., 2014; Rundlöf et al., 2015; Whitehorn et al., 2012; Wintermantel et al.,
725 2018; Woodcock et al., 2017). The fitness of a bumblebee colony is determined by its
726 production of new queens and males (Goulson, 2010), so the fact that colonies exposed
727 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.

Acknowledgements: The authors thank A. Herrera-Bachiller, E. Nordén, E.L. Rotheray, E. Nicholls, R. Fowler, T.J. Wood, C. Hynes and M. Stenning for assistance in the field and the lab, and for comments. We thank Stanmer Park managers for permission to use their facilities for our fieldwork. This study was supported by Defra (Research Project PS2372; awarded to DG). Graphical abstract by Amparo Hidalgo Galiana (Ig:ahg_ilustracion)

Author contributions: CB and DG conceived the project and designed the experiment; CB performed the semi-field experiment; CB, JJ and TP carried out the laboratory molecular work with input from WOHH; CB and IB analysed the data; CB 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: 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).

References

Alaux, C., Brunet, J.-L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., … Le Conte, Y. (2010). Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology*, *12*(3), 774–82. doi: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), 2284–90. doi:10.1111/j.1462-2920.2009.01953.x

Arbulo, N., Antúnez, K., Salvarrey, S., Santos, E., Branchiccela, B., Martín-Hernández, R., … 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. doi: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. doi: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. doi: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. doi: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. doi:10.1111/1365-2664.12205

Barribeau, S. M., Sadd, B. M., du Plessis, L., Brown, M. J., Buechel, S. D., Cappelle, K., … Schmid-Hempel, P. (2015). A depauperate immune repertoire precedes evolution of sociality in bees. *Genome Biology*, *16*, 83. doi: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*, *111*(9), 3496–3501. doi:10.1073/pnas.1318628111

Bartolomé, C., Buendía, M., Benito, M., De la Rúa, P., Ornosa, C., Martín-Hernández, R., … 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. doi:10.1016/j.jip.2018.03.015

Beadle, K., Singh, K. S., Troczka, B. J., Randall, E., Zaworra, M., Zimmer, C. T., … Bass, C. (2019). Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*. *PLoS Genetics*, *15*(2), 1–19. doi: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. doi:10.1016/j.cois.2015.03.005

Biddinger, D. J., Robertson, J. L., Mullin, C., Frazier, J., Ashcraft, S. a, Rajotte, E. G., … Vaughn, M. (2013). Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PloS One*, *8*(9), e72587. doi: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. doi: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. doi: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. doi:10.1021/acs.est.5b03459

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.

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. doi: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. doi: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), 260–5. doi:10.1016/j.vetpar.2012.12.004

Colgan, T. J., Fletcher, I. K., Arce, A. N., Gill, R. J., Rodrigues, A. R., Stolle, E., … Wurm, Y. (2019). Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees. *Molecular Ecology*, *28*(8), 1964–1974. doi: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. doi: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. doi:10.1098/rspb.2015.2592

Cresswell, J. E., Page, C. J., Uygun, M. B., Holmbergh, M., Li, Y., Wheeler, J. G., … Tyler, C. R. (2012). Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology*, *115*(6), 365–371. doi: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. doi: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. doi: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. doi: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. doi:10.1007/s10582-999-0071-y

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., … 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*, *110*(46), 18466–18471. doi: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. doi: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), 969–83. doi:10.1111/1462-2920.12426

Duchateau MJ and Velthuis HHW. 1988. Development and Reproductive Strategies in *Bombus terrestris* Colonies. Behaviour 107: 186–207.

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. doi:10.1007/s13592-013-0206-9

Evans, J D, Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., … Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, *15*(5), 645–56. doi:10.1111/j.1365-2583.2006.00682.x

Evans, Jay D. (2006). Beepath: An ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of Invertebrate Pathology*, *93*(2), 135–139.

886 doi:10.1016/j.jip.2006.04.004

887 Fauser-Misslin, A., Sadd, B. M., Neumann, P., & Sandrock, C. (2014). Influence of
888 combined pesticide and parasite exposure on bumblebee colony traits in the
889 laboratory. *Journal of Applied Ecology*, *51*(2), 450–459. doi:10.1111/1365-
890 2664.12188

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

895 Fries, I., Feng, F., Silva, A., Susan, B., & Pieniazek, N. J. (1996). *Nosema ceranae* n . sp . (
896 Microspora , Nosematidae), Morphological and Molecular Characterization of a
897 Microsporidian Parasite of the Asian Honey bee *Apis cerana* (Hymenoptera ,
898 Apidae), *365*, 356–365. doi:10.1016/S0932-4739(96)80059-9

899 Fürst, M. A., McMahon, D. P., Osborne, J. L., Paxton, R. J., & Brown, M. J. F. (2014).
900 Disease associations between honeybees and bumblebees as a threat to wild
901 pollinators. *Nature*, *506*(7488), 364–6. doi:10.1038/nature12977

902 Garrido, P. M., Antúnez, K., Martín, M., Porrini, M. P., Zunino, P., & Eguaras, M. J.
903 (2013). Immune-related gene expression in nurse honey bees (*Apis mellifera*)
904 exposed to synthetic acaricides. *Journal of Insect Physiology*, *59*(1), 113–9.
905 doi:10.1016/j.jinsphys.2012.10.019

906 Gill, R. J., Baldock, K. C. R., Brown, M. J. F., Cresswell, J. E., Dicks, L. V., Fountain, M. T.,
907 … Potts, S. G. (2015). *Protecting an Ecosystem Service: Approaches to*
908 *Understanding and Mitigating Threats to Wild Insect Pollinators*. *Advances in*
909 *Ecological Research* (1st ed., Vol. 54). Elsevier Ltd.
910 doi:10.1016/bs.aecr.2015.10.007

911 Gill, R. J., Ramos-Rodriguez, O., & Raine, N. E. (2012). Combined pesticide exposure
912 severely affects individual- and colony-level traits in bees. *Nature*, *491*(7422),
913 105–8. doi:10.1038/nature11585

914 Gisder, S., Horchler, L., Pieper, F., Schüler, V., Sima, P., & Genersch, E. (2020). Rapid
915 gastrointestinal passage may protect *Bombus terrestris* from becoming a true host
916 for *Nosema ceranae*. *Applied and Environmental Microbiology*,
917 10.1128/AEM.00629-20. doi:10.1128/MCB.00386-19

918 Glavinic, U., Tesovnik, T., Stevanovic, J., Zorc, M., Cizelj, I., Stanimirovic, Z., & Narat, M.
919 (2019). Response of adult honey bees treated in larval stage with prochloraz to
920 infection with *Nosema ceranae*. *PeerJ*, *7*, e6325. doi:10.7717/peerj.6325

921 González-Varo, J. P., Biesmeijer, J. C., Bommarco, R., Potts, S. G., Schweiger, O., Smith,
922 H. G., … Vilà, M. (2013). Combined effects of global change pressures on animal-
923 mediated pollination. Trends in Ecology & Evolution, 28(9), 524–30.
924 doi:10.1016/j.tree.2013.05.008

925 Goulson, D. (2010). *Bumblebees: behaviour, ecology, and conservation (2nd edn).*
926 *Oxford University Press, New York*.

927 Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by
928 combined stress from parasites, pesticides, and lack of flowers. *Science*,

347(6229), 1255957. doi:10.1126/science.1255957

Graystock, P., Jones, J. C., Pamminger, T., Parkinson, J. F., Norman, V., Blane, E. J., … Hughes, W. O. H. (2016). Hygienic food to reduce pathogen risk to bumblebees. *Journal of Invertebrate Pathology*, *136*, 68–73. doi:10.1016/j.jip.2016.03.007

Graystock, Peter, Yates, K., Darvill, B., Goulson, D., & Hughes, W. O. H. (2013a). Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. *Journal of Invertebrate Pathology*, *114*(2), 114–9. doi:10.1016/j.jip.2013.06.005

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

Hayward, A., Beadle, K., Singh, K. S., Exeler, N., Zaworra, M., Almanza, M. T., … 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. doi: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. doi: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), 211–7. doi:10.1016/j.jip.2006.11.001

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V, Barrios, L., … Meana, A. (2008). How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environmental Microbiology*, *10*(10), 2659–69. doi: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), 1374–9. doi: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), 76–8. doi: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. doi:10.1016/j.ab.2009.09.019

Huang, Y., Lu, X.-P., Wang, L.-L., Wei, D., Feng, Z.-J., Zhang, Q., … Wang, J.-J. (2015). Functional characterization of NADPH-cytochrome P450 reductase from *Bactrocera dorsalis*: Possible involvement in susceptibility to malathion. *Scientific Reports*, *5*, 18394. doi: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. doi: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. doi: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. doi: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. doi: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. doi:10.1093/jee/99.4.1046

Kessler, S. C., Tiedeken, E. J., Simcock, K. L., Derveau, S., Mitchell, J., Softley, S., … Wright, G. a. (2015). Bees prefer foods containing neonicotinoid pesticides. *Nature*. doi:10.1038/nature14414

Klee, J., Besana, A. M., Genersch, E., Gisder, S., Nanetti, A., Tam, D. Q., … 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. doi: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*, 153–8. doi: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. doi: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. doi: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. doi: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.

doi:10.1006/meth.2001.1262

Manjon, C., Troczka, B. J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., … Nauen, R. (2018). Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides. *Current Biology*, *28*(7), 1–7. doi: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), 2127–38. doi: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), 6331–8. doi:10.1128/AEM.00270-07

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), 107–15. doi:10.1111/j.1365-2672.2009.04635.x

Meeus, Ivan, 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. doi:10.1111/j.1523-1739.2011.01707.x

Mukherjee, I. (2009). Determination of pesticide residues in honey samples. *Bulletin of Environmental Contamination and Toxicology*, *83*(6), 818–21. doi: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. doi: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. doi:10.1371/journal.pone.0009754

Nieto, A., Roberts, S. P. M., Kemp, J., Rasmont, P., Kuhlmann, M., Criado, M. G., … Rúa, P. De. (2015). *European Red List of Bees*. Luxembourg: Publication Office of the European Union. Retrieved from http://cmsdata.iucn.org/downloads/erl_of_bees_low_res_for_web.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), 406–15. doi: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. doi: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. doi: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. doi: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), 153–8. doi: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, Edward D., & Jepson, P. C. (1993). Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pesticide Science*, *39*(4), 293–297. doi:10.1002/ps.2780390407

Pinheiro, J., & Bates, D. (2000). Mixed effects models in S and S-Plus. *New York: Springer-Verlag.*

Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A., … 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. doi: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 2019 10:1*, *10*(1), 1018. doi:10.1038/s41467-019-08974-9

R Core Team. (2018). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

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. doi:10.1002/ps.4756

Ravoet, J., Schwarz, R. S., Descamps, T., Yañez, O., Tozkar, C. O., Martin-Hernandez, R., … de Graaf, D. C. (2015). Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *Journal of Invertebrate Pathology*, *130*, 21–7. doi:10.1016/j.jip.2015.06.007

Ravoet, J., Smet, L. De, 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. doi: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), 529–40. doi: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. doi: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. doi:10.1080/00218839.2017.1307712

Rundlöf, M., Anderson, G. K. S., Bommarco, R., Fries, I., Hederstrom, V., Herbertsoon, L., … Smith, H. G. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, *521*, 77–80. doi:10.1038/nature14420

Sánchez-Bayo, F., & Goka, K. (2014). Pesticide residues and bees - a risk assessment. *PloS One*, *9*(4), e94482. doi: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), 705–9. doi:10.1016/j.dci.2010.02.002

Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D., … Mediche, S. (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., … 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. doi:10.1002/ps.4449

Singh, R., Levitt, A. L., Rajotte, E. G., Holmes, E. C., Ostiguy, N., Vanengelsdorp, D., … 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. doi:10.1371/journal.pone.0014357

Spurgeon, D., Hesketh, H., Lahive, E., Svendsen, C., Baas, J., Robinson, 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).

Theopold, U., Samakovlis, C., Erdjument-bromage, H., Dillon, N., Axelsson, B., Schmidt, O., … 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. doi:10.1007/s13592-014-0273-6

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. doi:10.1002/ps.3868

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

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. doi: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. doi:10.1086/physzool.59.1.30156090

Vogt, F. D. (1986). Thermoregulation in Bumblebee Colonies. II. Behavioral and Demographic Variation throughout the Colony Cycle. Physiological Zoology, 59(1), 60–68. doi: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. Chichester : Wiley.*

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. doi: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. doi: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. Cary, NC: 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. doi:10.1002/ps.1616

Wintermantel, D., Locke, B., Andersson, G. K. S., Semberg, E., Forsgren, E., Osterman, J., … de Miranda, J. R. (2018). Field-level clothianidin exposure affects bumblebees but generally not their pathogens. *Nature Communications*, *9*(1), 5446. doi:10.1038/s41467-018-07914-3

Woodcock, B. A., Bullock, J. M., Shore, R. F., Heard, M. S., Pereira, M. G., Redhead, J., … Pywell, R. F. (2017). Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. *Science*, *356*(6345), 1393–1395. doi:10.1126/science.aaa1190

Zaragoza-Trello, C., Vilá, M., Botías, C., Bartomeus, I. (2020). Interactions among global change pressures act in a non-additive way on bumblebee individuals and colonies. Functional Ecology, in press.

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. doi: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. doi: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. doi:10.1371/journal.pone.0178421

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).

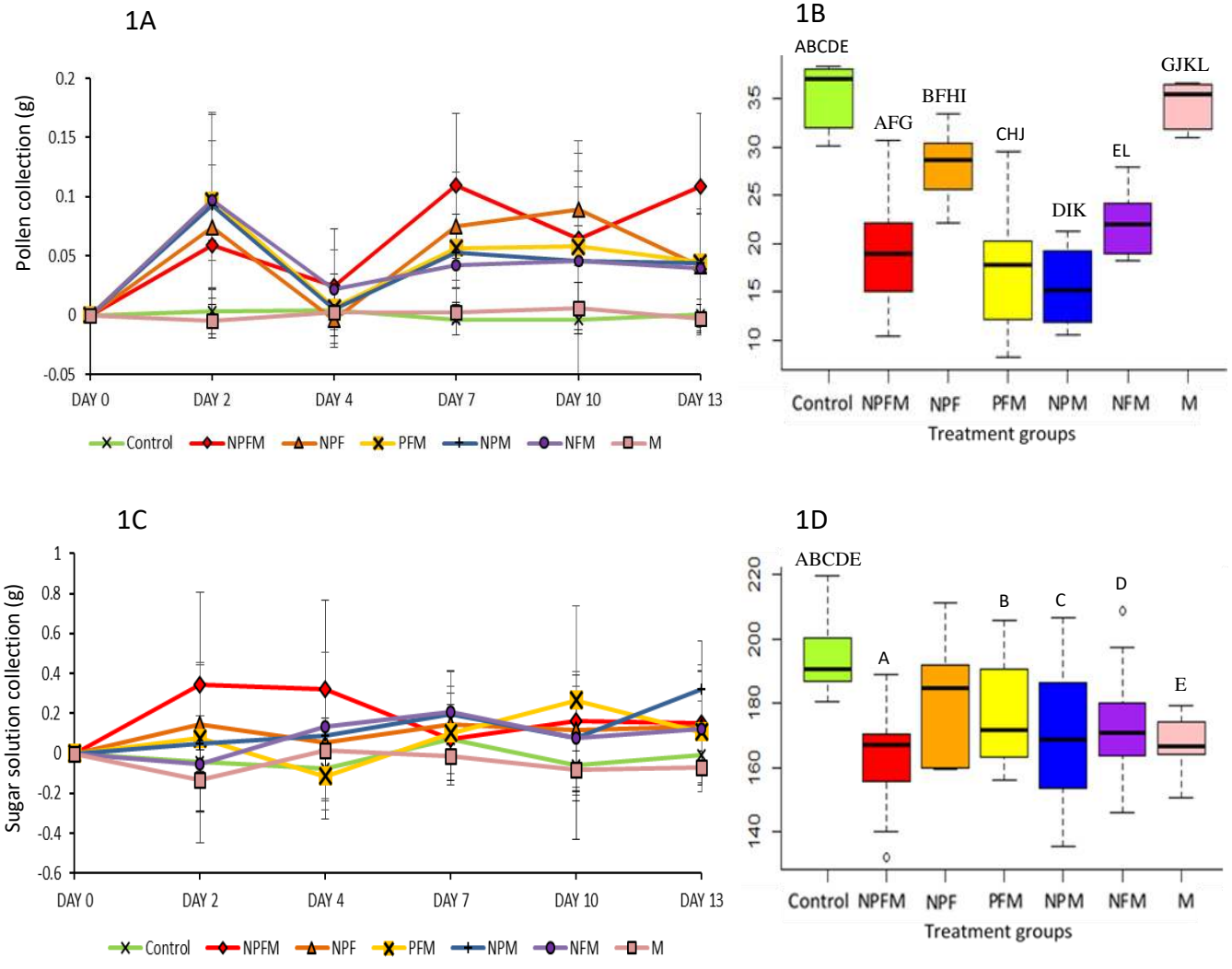

Figure 2. Pathogens detected in the bumblebee colonies. A: Percentage of *Bombus terrestris* in which the pathogen *Nosema ceranae* was detected 10 days after inoculation with a controlled dose of *N. ceranae* in sugar solution (treatments NPFM, PFM, NPM, NFM and M) or pathogen-free solution (controls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

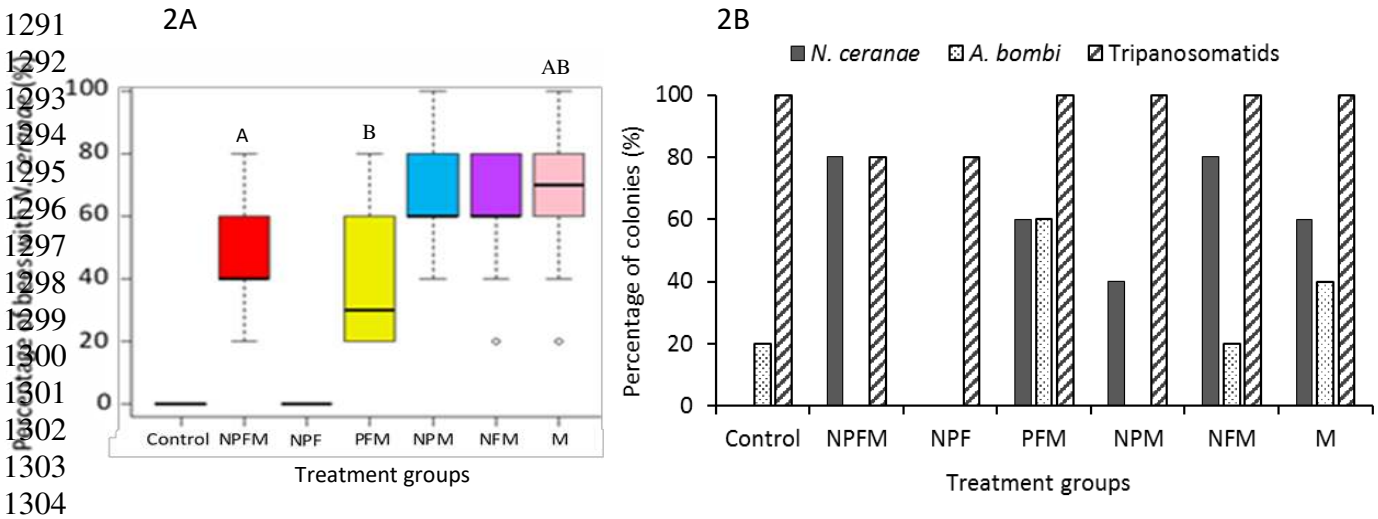

Figure 3. Boxplots showing the effect of treatments on the expression of genes related to the immune (*hemomucin*, *pelle*, *relish*, *abaecin*, *defensin*, *hymenoptaecin*, *apidaecin*) and detoxification (*CPR* and *CYP4G15*) response of bumblebees (*B. terrestris*) after 13 days of exposure to pesticide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

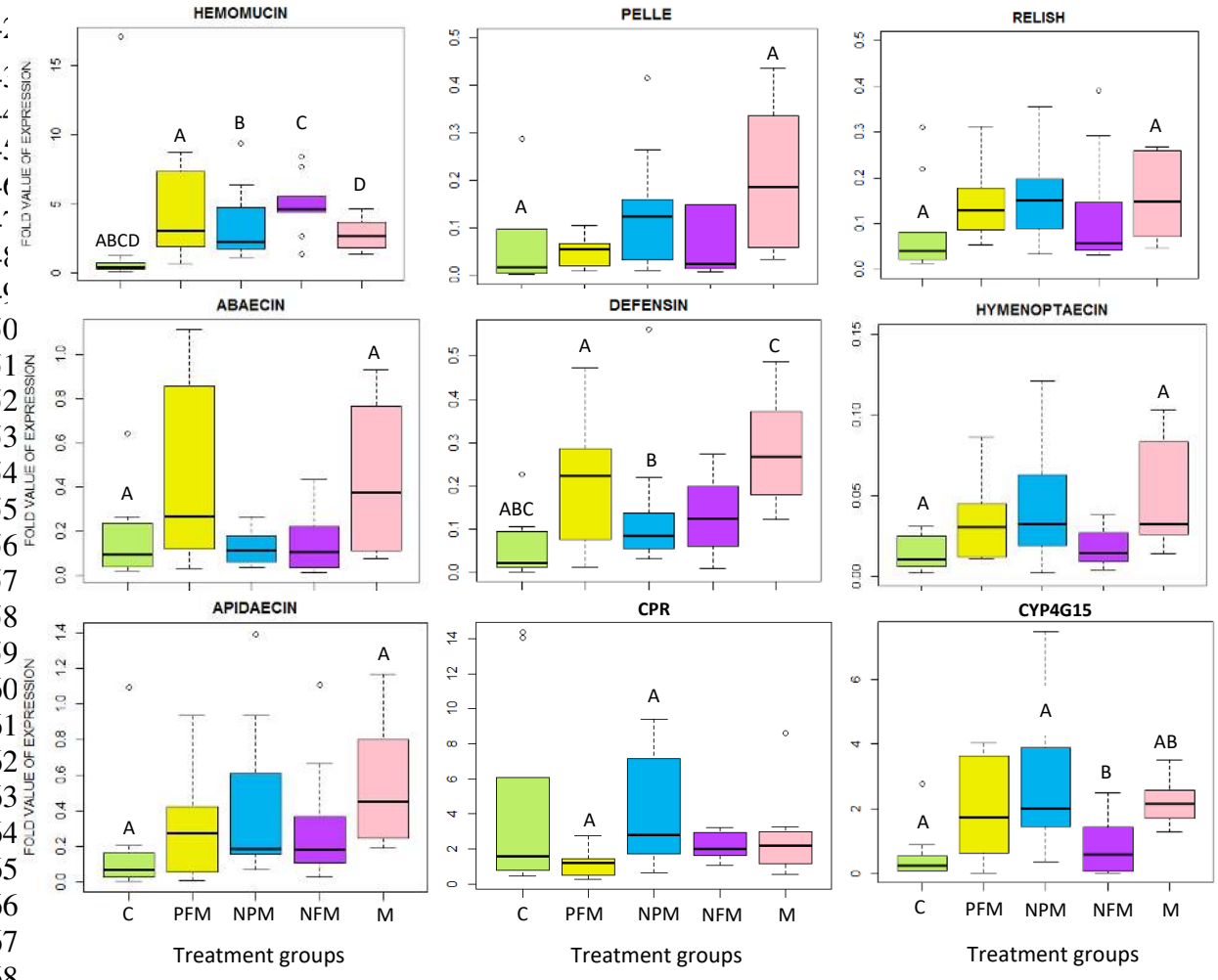

Figure 4. Fitness parameters measured in the *Bombus terrestris* colonies. A: Mean colony weight observed for the control and treatment groups at weekly intervals. The change in weight over time was significantly smaller ($P = 0.009$) in the colonies that received the four stressors (NPFM) compared to 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 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.

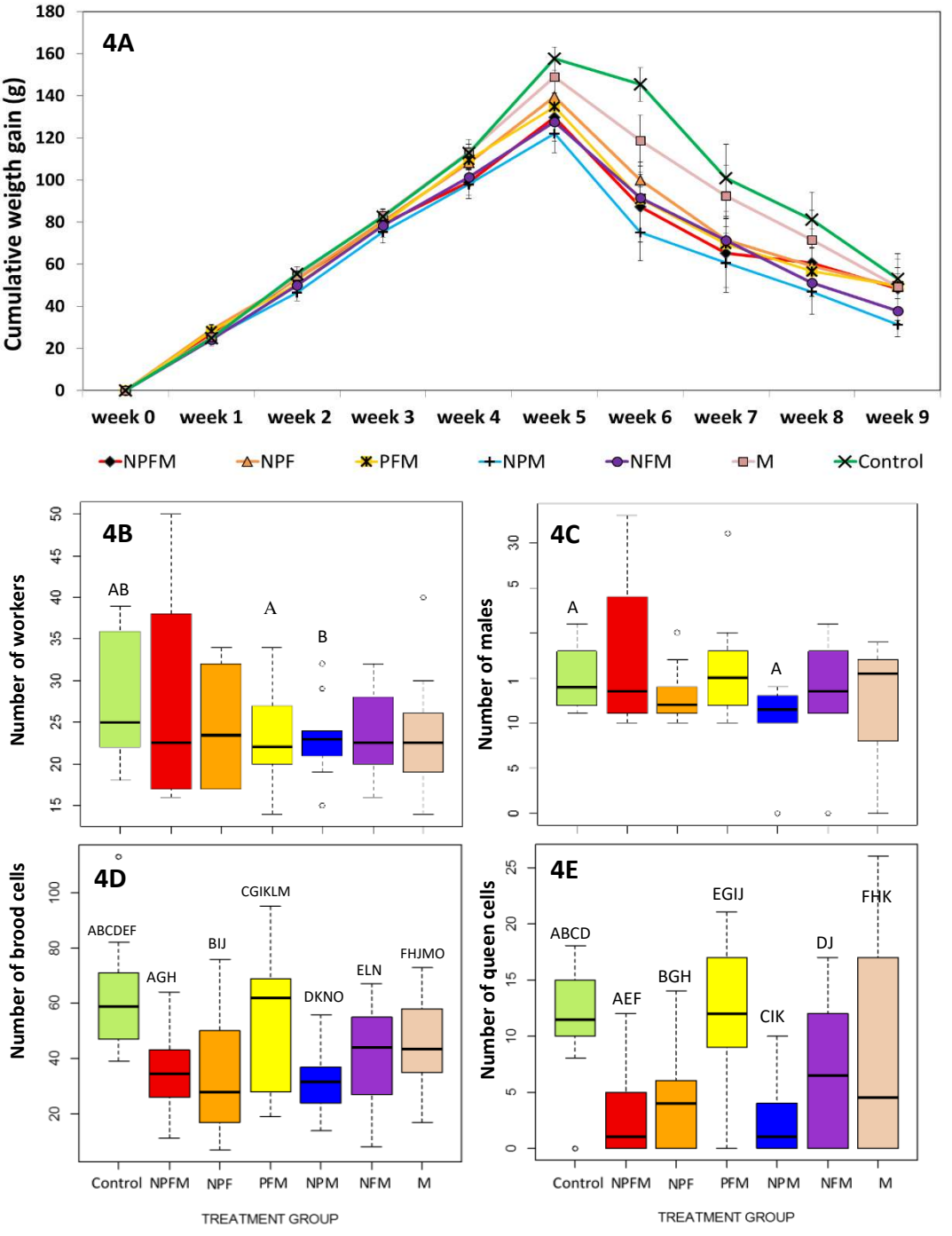

Table 1. Summary of the seven treatments applied to *B. terrestris* colonies in the study (10 colonies/treatment), involving three abiotic pesticide stressors (the neonicotinoid insecticide thiamethoxam, the pyrethroid insecticide cypermethrin and the fungicide tebuconazole) and one biotic stressor (the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

GROUP	NEONICOTINOID (N)	PYRETHROID (P)	DMI FUNGICIDE (F)	*N. ceranae* (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**
Week²	-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 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	Number of
		Number	Weight (g)	Size (mm)	Number	Weight (g)	Size (mm)	brood cells	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