Synergistic effects of pathogen and pesticide exposure on honey bee (*Apis mellifera)* survival and immunity

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Abstract

Declines in native insect pollinator populations and substantial losses in managed honey bees have been reported on a global scale and become a widespread concern because of the importance of these insects for human food production and ecosystem stability. Several potential factors have been studied as possible causes of declining pollinator health, such as parasites and pathogens, exposure to agricultural pesticides, habitat loss and/or climate change. More recently, a combination of these factors rather than a single cause have been blamed for observed pollinator losses, but field studies of such interactions are challenging, especially in the presence of confounding environmental stressors. We therefore examined the impact of single and combined stressors on the honey bee (*Apis mellifera)* in a generally healthy Australian population. We exposed workers during their larval development and drones until they reached sexual maturity to the neonicotinoid pesticide Thiamethoxam, at concentrations more than 20 times lower than previously reported for field conditions, the microsporidian gut pathogen *Nosema apis* or both stressors at the same time. We found that simultaneous exposure significantly reduced bee health. We observed a substantial increase in mortality and a reduction of immunocompetence in workers exposed to both the pathogen and the pesticide. We conclude that the exposure of generally healthy bees to multiple environmental stressors results in synergistic effects where the effects are expected to negatively impact performance and could be sufficient to trigger colony collapse. We found that the vast majority of males did not survive to sexual maturity after exposure to very low levels of Thiamethoxam. This would not only reduce the reproductive success of individual colonies, but can also impact gene flow and genetic diversity at the population level, which are both known as key components of honey bee health.

1. Introduction

Pollination services of insects are of central importance for human food production and ecosystem stability (Breeze et al., 2011; Ollerton et al., 2011; Potts et al., 2010). Non-managed and native pollinators are especially important for flower pollination and increased fruit set (Garibaldi et al., 2013) but substantial declines in both wild and managed insect pollinator populations have been reported over recent years (Kosior et al., 2007; Nieto et al., 2014; Watanabe, 1994). The European honey bee (*Apis mellifera)* is a key insect pollinator of global significance (Breeze et al., 2011) and substantial losses in managed stock have been reported over recent years, especially in Europe and North America (Aizen and Harder, 2009; Godfray et al., 2014; Goulson et al., 2015; Potts et al., 2010). Because of their economic importance for managed pollination of agricultural crops, a substantial number of studies have been conducted to investigate the impact of environmental stressors on honey bee performance to quantify their effects on bee health. These include studies of various pathogens (Goulson et al., 2015), pesticide exposure (Budge et al., 2015; Calatayud-Vernich et al., 2016; Godfray et al., 2014; Samson-Robert et al., 2017; van der Sluijs et al., 2013; Woodcock et al., 2017), habitat loss, malnutrition and climate change (Goulson et al., 2015), which have all been proposed as possible contributors of declining pollinator health. No single factor investigated so far fully explains the losses observed in the field, implying that combinations of factors are responsible for observed declines (Bryden et al., 2013). There has been a substantial increase in publications investigating the effects of neonicotinoids on honey bee health and behaviour, as summarized in two recent reviews (Godfray et al., 2015; Pisa et al., 2017). More recent work specifically tested for possible synergistic, additive or antagonistic effects of pesticides and other stressors, in particular pathogens, on honey bee health, although most of these studies

were conducted under laboratory conditions (Alaux et al., 2010; Blanken et al., 2015; Di Prisco et al., 2013; Doublet et al., 2015; Pettis et al., 2012; Retschnig et al., 2014b). A key challenge for conducting such studies in the field is the difficulty in controlling for non-experimental stressors that are typically present (Poquet et al., 2016). But such limitations can be overcome when studying generally healthy bees.

We examined a population of generally healthy honey bees from the southern part of Western Australia (WA). The geographic isolation of this region, combined with strict quarantine regulations, has resulted in an environment largely free of a number of virulent honey bee pathogens, such as *Varroa* mites and their associated viruses, the small hive beetle, European foulbrood, and the microsporidium *Nosema ceranae (Roberts et al., 2015)*. Furthermore, Western Australia harbours large populations of native bees and non-managed honey bees, which currently provide the majority of crop pollination (Koh et al., 2016); commercially managed honey bees are primarily used for honey production resulting in minimal exposures to agricultural landscapes. Losses of honey bees, reported from other areas of the world, have never been observed in Western Australia, which therefore provides opportunities to study the effects and interactions of individual environmental stressors on otherwise healthy bee stock.

To test for the effects of two environmental stressors on individual honey bee health, either solely or in combination, we used the microsporidian pathogen *Nosema apis* and the neonicotinoid insecticide Thiamethoxam, which have both been linked to honey bee losses (Goulson et al., 2015; Henry et al., 2012; Henry et al., 2015). *N. apis* is a globally widespread fungal pathogen (Selman and Corradi, 2011) that infects and replicates in the midgut cells of infected bees (Fries, 1988). Our earlier work in Australian bees confirmed that the pathogen has low virulence in workers, but infections reduce survival of older bees (Lach et al., 2015; Milbrath et al., 2015), shift flight

activities towards younger bees (Lach et al., 2015) and reduce the length of foraging trips (Dosselli et al., 2016). In honey bee males, infections reduce longevity (Peng et al., 2015) and spores can be found in ejaculates of older males (Peng et al., 2015). Males respond to *Nosema* infections by a systemic upregulation of immune proteins in their seminal fluid (Grassl et al., 2016), which can efficiently kill *Nosema* spores (Peng et al., 2016). Nevertheless, surviving *Nosema* spores transferred within ejaculates to queens during mating can trigger novel infections (Roberts et al., 2015). The pathogen can impact colony performance, as previous research has shown that chronic infections can reduce a colony's ability to regulate hive temperature (Wang and Mofller, 1970) or even kill the entire colony (Fries, 1993).

As a second stressor, we used the neonicotinoid Thiamethoxam. Neonicotinoids are among the most widespread agricultural insecticide used to protect crops from insect pests (Goulson, 2013; Woodcock et al., 2017). Neonicotinoids are readily absorbed by plants and kill pest insects such as aphids, leafhoppers, and whiteflies at very low doses, but seem to have low toxicity to vertebrates (Motohiro and John, 2005). They are typically administered by coating seeds with the pesticide prior to sowing. However, their continuous systemic presence in the growing plant results in pesticide residues in nectar and pollen (Rortais et al., 2017), to which pollinating insects are exposed. Systemic pesticides are known to be more toxic when ingested compared to surface contact and honey bees and their brood experience higher levels of toxicity if they consume contaminated pollen and nectar (Bonmatin et al., 2015; Pisa et al., 2015). A number of studies have confirmed that such exposure levels can trigger a range of effects such as an increase in queen supersedure (Sandrock et al., 2014), decreased nutritional stores (Mogren and Lundgren, 2016), suppression of the immune system (Aufauvre et al., 2014; Brandt et al., 2016; Di Prisco et al., 2013; Williams et al., 2015; Wood and Goulson, 2017), reduction in visual perception (Fischer et al.,

2014; Tison et al., 2016) or impairment of the bees' capacity for learning and memory (Belzunces et al., 2012; Blacquière et al., 2012; Decourtye et al., 2004a; Decourtye et al., 2004b; Han et al., 2010; Henry et al., 2012; Palmer et al., 2013; Papach et al., 2017; Piiroinen and Goulson, 2016; Williamson and Wright, 2013; Yang et al., 2012). More recently, increased mortality in honey bees exposed to pesticides and a second stressor have been reported (Alaux et al., 2010; Di Prisco et al., 2013; Doublet et al., 2015; Goulson et al., 2015; Papach et al., 2017). Consequently, neonicotinoid pesticides are prime suspects for sublethal effects that negatively impact honey bees.

Here, we quantified the effects of exposure to sublethal levels of a pathogen and a pesticide on males and workers, either solely or in combination. We compared the performance of stressed individuals with control bees and found that combined exposure significantly increased mortality and suppressed immunocompetence of workers. We provide field-based evidence for synergistic effects of pathogens and pesticides on honey bee worker health. When we exposed males to the same concentration of Thiamethoxam the majority of males did not survive to sexual maturity.

2. Materials and Methods

2.1 Field relevant exposure levels of Thiamethoxam

A number of previous studies have quantified the effects of neonicotinoid pesticides on bee health. However, these studies were criticised for using pesticide exposure levels that were deemed too high and therefore not field realistic (Australian Pesticides and Veterinary Medicines Authority, 2014; EFSA, 2012; Fairbrother et al., 2014; Godfray et al., 2014; Pisa et al., 2015). We therefore

149 began our study by conducting a field-based experiment to quantify the level of the neonicotinoid
150 Thiamethoxam contamination in bee bread produced by workers that were foraging on canola crops
151 in Western Australia. We assumed that local pesticide levels in pollen must be sublethal, given the
152 absence of large-scale honey bee losses, even for colonies used for crop pollination. We quantified
153 pesticide concentrations in bee bread, which is a mixture of pollen and honey stored by bees in the
154 hive and used to feed developing brood. We placed eight colonies next to flowering canola in Bindi
155 Bindi, Western Australia (30.56° S, 116.34° E) and Three Springs (29.32° S, 115.43° E) in 2013. At
156 both locations, no pesticide applications were made while our colonies were present and we
157 identified a Thiamethoxam seed-treated canola planting, as well as a field with untreated plants. The
158 distance of bee hives exposed to treated and untreated fields was 2.9 km in Three Springs and 1 km
159 in Bindi Bindi. Although foraging ranges of honey bees can be several kilometres (Beekman and
160 Ratnieks, 2000), they have been found to forage in close proximity to their hives if nectar and
161 pollen sources are provided close to the hive and from a dominant plant in bloom such as canola
162 (Sabbahi et al., 2005). Foraging ranges of honey bees in agricultural areas are therefore
163 substantially smaller and range between 600-800 m (Visscher and Seeley, 1982).

164 We placed two colonies into each crop field at each location, resulting in a total of eight
165 colonies or four per treatment group. The colonies were exposed to flowering canola over a period
166 of 4 weeks, after which we sampled bee bread from each colony and stored it at -20°C. To quantify
167 the concentration of Thiamethoxam in bee bread from the four locations, we used methods
168 previously described (Chen et al., 2013). Bee bread is known to contain neonicotinoid contaminants
169 ranging from 1 to 100 ng/g in pollen collected from colonies exposed to seed-treated canola
170 (Bonmatin et al., 2015; Mitchell et al., 2017). Because these pesticide concentrations in bee bread
171 are often below levels of quantitation (LOQ) they can be difficult to detect. To overcome this

problem we used the QuEChERS protocol to increase Thiamethoxam concentrations 80 times in samples prior to LC-QQQ-MS quantification, similar to Chen et al. (2013). We transferred 2 g of bee bread per sample into a polypropylene centrifuge tube (50 ml) and added 8 ml acetonitrile (ACN), 10 ml water and 2 ceramic homogenisers. After vortexing each sample for 2 min, we added the QuEChERS salt kit purchased from Agilent Technologies (Santa Clara, CA, USA) containing 4 g of anhydrous $MgSO_4$ and 1 g of sodium chloride. The solution was mixed for 1 min and centrifuged at 4,000 x g for 5 min. We transferred the acetonitrile fraction (8 ml) to a 15 ml dSPE polypropylene tube containing 150 mg of $MgSO_4$ and 25 mg of primary secondary amine (PSA). After mixing and vortexing the samples for 1 min, we centrifuged them at 4,000 x g for 1 min. Finally, 4 ml of the supernatant were dried under nitrogen and resuspended in 50 µl of H_2O, which was transferred into a glass auto sampler vial for analysis.

To quantify Thiamethoxam concentrations in these enriched samples, we used an Agilent 1100 Series chromatograph coupled to a model 6430A triple quadrupole mass spectrometer (Agilent Technologies), with a JetStream electrospray source in positive ionization mode, using the same transition ions, as described in Takino (2006). Acetonitrile (ACN), methanol (LC-MS Chromosolv, ≥ 99.9%), and HPLC water were obtained from Fluka (Sigma–Aldrich,). Thiamethoxam and the isotope labelled ISTD Thiamethoxam-d3 (99.8 %) were purchased from Sigma-Aldrich with purity of 99.7%. The initial stock standard solutions were prepared in acetonitrile at a concentration of 100 µg/ml and then stored in amber glass vials at -20 °C until use. The calibration standards and working standards were prepared by dilution with HPLC water on the day of analysis. Chromatographic separation was performed on a Poroshell 120 EC-C18 2.7 µm, 3 × 100 mm column (Agilent Technologies). The mobile phase consisting of: (A) water, and (B) methanol, both containing 10 mM of ammonium acetate, was used at a flow rate of 0.4 ml / min.

During each LC-MS run, we used 35-min multi-linear methanol gradients that increased from 20% to 50% during the first 10 min of the run, from 50% to 70% for the next 3.5 min, from 70% to 71% B for the next 6.5 minutes, and from 71% to 100% for 9 min followed by 100% for the final 6 minutes. Injection volume of the extract sample was 2 µl. Capillary voltage was set at 3.5 kV and the electrospray source sheath gas flow and temperature were 5 L/min and 300 °C, respectively. Drying gas was operated at a flow of 11 L/min and a temperature of 250 °C. The nebulizer pressure was kept constant at 45 psi. The mass spectrometer was operated in the MS/MS mode, using multiple reaction monitoring (MRM). Compounds of interest were identified by their retention times and relative intensities of qualifier ions in the positive ionization mode.

2.2 Honeybee breeding

All animal material used for the second experiment originated from Western Australian honey bee breeding stock with no previous history of agricultural crop pollination or chemical treatments against disease; the latter being prohibited under current local beekeeping regulations. None of the colonies initially used to quantify field realistic exposure levels to Thiamethoxam were used for the second experiment. To quantify the effects of *N. apis* infections and Thiamethoxam exposure on honey bees, we used eight colonies with unrelated queens maintained at an apiary at the University of Western Australia between March and May 2015. Prior to experiments, we confirmed that the colonies were in generally good health as indicated by the presence of an egg laying queen, worker brood, honey and pollen storage and the absence of signs of disease. Colony sizes were standardised at the start of the experiment by providing each hive with seven frames with developing brood, one empty frame ready for oviposition and eight frames of empty wax foundation for colony growth. We added pollen traps at the entrances of each colony to force bees

to consume the pollen patties provided. We prepared pollen feeds for four pesticide treated colonies by mixing 250 g irradiated red gum pollen, 50 ml of 150% (w/v) sucrose solution and 2.6 pg/g Thiamethoxam. The remaining four colonies were used as a control and received pollen patties prepared as described above but without the pesticide. We provided each colony with a single pollen patty per week over 5 weeks and placed them between the bottom and top box, which we separated using a riser to provide sufficient space for the patties and bees to feed as previously described (Somerville, 2005). The time span provided a field relevant exposure time because it is comparable to the flowering period of canola in Western Australia. The setup also ensured that bees bred from these colonies developed under controlled conditions, either in the presence or in absence of the pesticide.

2.3 Collection, purification and inoculation of Nosema apis *spores*

Sampling of microsporidian spores for subsequent infection of workers and males was done according to a previously developed protocol (Peng et al., 2015; Peng et al., 2014). In the absence of *N. ceranae* in Western Australia (Roberts et al., 2015), spore samples used for inoculations contained only *N. apis*. We collected 20 foraging workers from the entrances of five non-experimental hives with known *N. apis* infections. The midguts of 100 workers were dissected and pooled in an Eppendorf tube along with 1 ml of DDI water and a 3 mm tungsten bead (Qiagen, Australia). The sample was homogenized for 30 s in a mixer mill (Retsch MM301) at 25 Hz, and 0.5 ml was layered onto 1.5 ml of 100% Percoll (Sigma-Aldrich) in a 2 ml Eppendorf tube. The sample was centrifuged at 18,000 x g for 60 min at 4 °C. After removing the supernatant, 1.5 ml of DDI water was added before vortexing and centrifuging the sample 3 times at 20,700 x g for 5 min. The pellet was resuspended in DDI water and spore concentration was determined using a Neubauer

haemocytometer, adjusted to 1 x 10^9 spores/ml and frozen at -80 °C prior to further experiments. To infect bees, we suspended thawed *N. apis* spores in 150% (w/v) sucrose solution to a final concentration of 10,000 spores/µl and hand fed newly hatched individual bees with a pipet using either 1 µl of 150% (w/v) sucrose solution as a control or 1 µl sucrose solution with 10,000 spores, a dosage that reliably produces infections in all bees inoculated but does not result in any significant increases in bee mortality (Fries, 1988; Fries et al., 2013; Peng et al., 2015).

2.4 Worker breeding

To generate an age-matched cohort of worker bees, we restricted queens in each colony to three frames for 3-6 days. We removed frames containing capped worker brood from hives after 20 days and placed them in an incubator at 32 °C, 60% humidity. We collected 100 newly eclosed workers per colony and inoculated 50 bees with 1 µl of 150 % sucrose solution containing 10,000 *N. apis* spores and 50 individuals with sucrose solution as a control. To perform inoculations, we starved bees for 2 hours before randomly allocating them to one of the two treatments. Each bee was hand fed by offering the 1 µl inoculum in a pipette tip. After dosing, workers were held in separate cages by treatment (*N. apis* or control) and pesticide exposure (Thiamethoxam or control) and were placed into surrogate colonies. We provided workers with 200% sucrose solution (w/v) *ad libitum* and retrieved them after 15 days, corresponding to an age when workers engage in foraging activities and are therefore likely to become infected (Dosselli et al., 2016; Lach et al., 2015). We quantified worker mortality per cage by counting the number of surviving workers and randomly selected 10 infected and 10 uninfected workers per colony to measure encapsulation response as described below.

264 *2.5 Male breeding*

265 Previous research revealed that honey bee males are particularly susceptible to environmental stress
266 (Sturup et al., 2013) and we therefore decided to quantify effects of pathogen and pesticide
267 exposure on males as well as female workers. We bred an age-matched cohort of males in each of
268 our eight experimental colonies by restricting queens to one frame of male and two frames of
269 worker comb for 3-6 days. Male brood was removed from the hives after 23 days and placed in an
270 incubator at 32 °C, 60% humidity. We collected up to 180 newly eclosing drones per colony and
271 inoculated half with 1 µl of 150% sucrose solution containing 10,000 *N. apis* spores with a pipette
272 tip, and half with 1 µl of 150% sucrose solution as a control. After treatment, males were placed in
273 small cages of 30 each, separated by infection treatment (*N. apis* or control) and pesticide exposure
274 (Thiamethoxam or control) and returned to their maternal colonies to allow them to sexually
275 mature. When we retrieved the cages 15-18 days later to quantify encapsulation response, sperm
276 viability and sperm number, we found that a large number of males had not survived, especially
277 those exposed to pesticide; we therefore used survival data per cage to test for treatment effects.
278

279 *2.6 Measuring immune response*

280 To evaluate immunocompetence, we quantified encapsulation response, a cellular response
281 commonly used to measure insect immunity. The process involves haemocyte recognition and
282 attachment to a foreign particle. Haemocytes melanise and eventually form a capsule around the
283 object. Encapsulation response correlates with pathogen resistance in bumblebees (Doums and
284 Schmid-Hempel, 2000) and honey bees (Evans et al., 2006; Strand, 2008) and has been used to
285 compare innate immune responses in bees and ants (Baer et al., 2006; Baer et al., 2005; Baer and
286 Schmid-Hempel, 2003). We randomly selected 10 infected and 10 uninfected surviving workers per

287 colony. Each bee was anaesthetised with CO_2 and placed into equipment normally used for
288 artificially inseminating honey bee queens (Ruttner and Drescher, 1976). Two steel hooks were
289 used to pull apart the terga and expose the inter-segmental membrane between the third and fourth
290 tergites. A small hole was pierced into the membrane using a sterilized injection needle. We then
291 implanted a 1 mm long piece of nylon, sterilized in 70% ethanol, into the bees' haemocoel. We
292 allowed bees to recover and placed them in cages separated by treatment and colony, held in an
293 incubator at 32°C, 60% humidity and with sucrose solution *ad libitum*. All bees were killed after 24
294 hours and stored at -20°C. Nylon implants were retrieved by dissection, embedded on a microscope
295 slide with Eukitt (Sigma Aldrich) and protected with a cover slip. We photographed implants using
296 a Canon EOS D 60 digital camera connected to a Leica 9.5 dissecting microscope. Photographs
297 were analysed with ImageJ (http://rsb.info.nih.gov/ij/download.html) to quantify grey values of
298 implants and backgrounds. For statistical analyses, we calculated encapsulation response as the
299 difference between the grey value of the implant minus the background.
300
301 *2.7 Data analysis*
302 All statistical analyses were performed using SPSS version 21 for Macintosh. To compare
303 pesticide concentrations between canola fields in Experiment 1 we used a Generalised Linear Model
304 (GLM) with location (Bindi Bindi and Three Springs) and seed treatment (Thiamethoxam versus
305 control) as independent factors. To compare survival in both sexes and encapsulation responses in
306 workers in Experiment 2, we used GLMs with gamma distributions and log Link functions.
307 Pesticide exposure and pathogen infection were used as independent factors, and colony was nested
308 within pesticide treatment. To test for significant effects of co-exposure to both stressors, we
309 inspected the pathogen x pesticide interaction terms and kept them in all models, independently of

whether they were statistically significant or not. Male mortality data were x + 1 transformed prior to statistical analysis due the presence of a number of zeros in this dataset.

3. Results

3.1 Determining field relevant exposure levels of Thiamethoxam

We identified Thiamethoxam in all bee bread samples evaluated during our first experiment, irrespectively of whether they were collected from colonies placed at seed-treated or control fields (Figure 1). Pesticide concentrations were more than three times higher ($p < 0.001$, see Table 1 for statistical details) in colonies exposed to seed-treated canola plantings (55.196 ± 17.816 pg/g (mean ± sem)) compared to colonies placed at untreated fields (17.035 ± 4.291 pg/g, (mean ± sem)). Thiamethoxam concentrations were also higher in samples from Three Springs (79.068 ± 26.664 pg/g (mean ± sem)) compared those from Bindi Bindi (31.323 ± 7.333 pg/g (mean ± sem)), although the difference between locations was not statistically significant ($p = 0.708$, Table 1). Because our primary aim was to expose bees to sublethal levels of the pesticide during our second experiment using a completely different set of colonies, we applied a highly conservative approach to set up exposure levels for our main experiment and used an exposure level of 2.6 pg/g of Thiamethoxam. This concentration was marginally lower than the 95% confidence interval of Thiamethoxam contaminations measured in colonies exposed to plantings that were not seed-treated and was more than 21-times lower than those found in bee bread from seed-treated canola. Our exposure dose was therefore statistically lower than any pesticide contamination we measured in bee bread collected from colonies exposed to Australian agricultural environments.

333

334 *3.2 Effects of Thiamethoxam exposure during development on workers*

335 A total of 800 workers (100 workers per colony) were available for the inoculation
336 with *N. apis*. Co-exposure to Thiamethoxam and *N. apis* substantially increased worker mortality
337 16-18 days after the inoculation procedure as indicated by a significant pathogen x pesticide
338 interaction term in the GLM analysis (GLM: Wald Chi square 5.413, $p = 0.020$, Figure 2, see Table
339 2 for statistical details). When we compared encapsulation responses among the 144 surviving
340 workers (18 ± 0.378 individuals per colony) we also found a significant Thiamethoxam x *N. apis*
341 interaction (GLM: Wald Chi-Square 4.367, $p = 0.037$, see Table 3 for statistical details); showing
342 that encapsulation response in workers was lowest in individuals co-exposed to the pathogen and
343 pesticide at the same time (Figure 3).

344

345 *3.3 Effects of continuous Thiamethoxam exposure on males*

346

347 A total of 1248 males (156 ± 17.49 (mean ± sem) individuals per colony) were available for the
348 inoculation treatments. At 15 - 18 days after treatment, the majority of males had not survived in
349 their maternal colonies (Figure 4). Mortality was significantly higher in males that originated from
350 colonies fed with Thiamethoxam contaminated pollen patties compared to males from control
351 colonies (GLM: Wald Chi square 113.28, $p < 0.001$, see Table 4 for statistical details). Mortality
352 was also higher for *N. apis* infected males than for uninfected males (GLM: Wald Chi square 7.89,
353 $p = 0.005$) but the pathogen x pesticide interaction term was not significant (GLM: Wald Chi square
354 1.737, $p = 0.188$ n.s.), implying that *Nosema* infections had no additional effects. However, because
355 male mortality was high and was driven by pesticide exposure, any potential effects of co-occurring

356 *N. apis* infections would have been difficult to detect in our data set (Figure 4). As a result of the
357 low survival of Thiamethoxam-exposed males (no male survivors in two of four Thiamethoxam-
358 treated colonies), the remaining sample sizes were too small to analyse other life history traits such
359 as sperm number, sperm viability or encapsulation response.
360
361
362 **4. Discussion**
363 We conducted a field-based study of honey bees from a population where major losses or declines
364 are absent in wild and managed stock. The bees were exposed to two different environmental
365 stressors, a pathogen and a neonicotinoid pesticide. Our experimental setup exposed honey bees to a
366 pesticide concentration significantly lower than levels we initially detected during our first
367 experiment in the field. Our design for the second experiment therefore recreated a situation where a
368 cohort of workers and males was raised with pesticide-contaminated pollen and a exposure of some
369 of these bees to a pathogen during adult life.
370 Overall, we found strong effects of these stressors on bee health. We confirmed the presence
371 of synergistic effects of both environmental stressors on worker bee health and mortality was high
372 in males exposed to very low levels of Thiamethoxam.
373
374 *4.1 Effects on workers*
375 We confirm significant synergistic effects of *N. apis* infection and pesticide exposure in honey bees;
376 exposure to both stressors at the same time resulted in a significant increase in mortality and
377 immune suppression. Our findings are in line with earlier reports that infection with *N. ceranae* or

exposure to Thiamethoxam negatively impact the honey bee immune system (Antunez et al., 2009) (Brandt et al., 2016; Brandt et al., 2017; Sánchez-Bayo et al., 2016).

Because we transferred workers to surrogate colonies after eclosion and inoculation with *N. apis*, these test individuals experienced no further exposure to contaminated bee bread. We therefore conclude that the effects of reduced survival and immunity must result, at least partially, from pesticide exposure during worker development. Although not quantified, we found no indication of substantial mortality occurring in workers during their larval and pupal stage, which may have been indicated by patchy brood or failure to eclose. Similar results were found by Papach *et al*. (2017), who reported impaired learning and memory of workers that were exposed to Thiamethoxam only during larval development. This implies that sublethal pesticide exposure during larval and/or pupal phase can have long term consequencesbecasue it can impact life history traits stages later in life, and becomes significant when the bees become exposed to further environmental stress such as a pathogen infection. Co-exposure to Thiamethoxam and *N. apis* killed over 70% of workers, which was substantially higher than mortalities observed in the remaining treatments, as well as in previous experiments with comparable experimental setups. Synergistic effects of pesticides and pathogens on worker mortality have also been reported in other studies (Alaux et al., 2010; Pettis et al., 2012; Retschnig et al., 2014a; Vidau et al., 2011). Worker losses of this magnitude are expected to negatively impact colony performance, although additional research is required to test whether these effects are sufficient to trigger colony collapses, especially when they continue to occur over multiple cohorts.

Apart from increased worker mortality, we found an additional synergistic effect in surviving workers; the encapsulation response was substantially lower in individuals exposed to both stressors compared to bees exposed to a single or no stressor. We conclude that neonicotinoid

401 exposure reduces the immune response capabilities of the affected bees. A reduced encapsulation
402 response is known to correlate with other key responses and life history traits such as resistance to
403 viral infections (Trudeau et al., 2001; Washburn et al., 1996), pathogen resistance (Doums and
404 Schmid-Hempel, 2000), colony size (Baer and Schmid-Hempel, 2003), foraging activity (Doums
405 and Schmid-Hempel, 2000; König and Schmid-Hempel, 1995) and the amount of sperm stored
406 (Baer et al., 2006). A reduction in individual encapsulation response, therefore, may impact colony
407 performance. It would be interesting to unravel the proximate factors responsible for these long-
408 term effects and lag times of sublethal pesticide exposure during development, especially because
409 previous studies reported delayed increases in mortality in response to pesticide exposure during
410 larval development (Oliveira et al., 2014; Rondeau et al., 2014; Van den Brink et al., 2016). This
411 may be a result of irreversible binding of the pesticide to insect nicotinic acetylcholine receptors
412 (nAChR), resulting in continuous neuronal activity (Matsuda et al., 2001; Motohiro Tomizawa and
413 John, 2003) and accumulation of the pesticide on neuronal synapses until it reaches a critical
414 threshold and results in the death of the animal (Pisa et al., 2017). Two previous studies also
415 confirmed immunosuppressive effects of neonicotinoids in honey bees (Brandt et al., 2016; Di
416 Prisco et al., 2013), which resulted from up-regulation of an inhibitor of a member of the gene
417 family NF-jB within the TOLL pathway (Evans et al., 2006). More work is required to confirm the
418 physiological suppression of individual immune pathways in response to pesticide exposure.
419
420 *4.2 Effects on males*
421 Exposure of honey bee males to Thiamethoxam, at concentrations significantly lower than those
422 measured in the field, resulted in high mortality whether or not the bees were infected with *N. apis*.
423 Similar to results for workers, we found no indication of increased mortality in males during

development. Our findings were similar to a recently published study reporting reduction in male survival (but not workers) after exposure to the two neonicotinoid pesticides, Thiamethoxam and Clothianidin, during larval development (Straub et al., 2016). However, the levels of Thiamethoxam used to contaminate pollen feeds were more than 1,700 times higher than the dosages used in our study and Straub et al (2016) also discontinued pesticide exposure of adult bees . Survival rates of males were very low at 15-18 days of age and post treatment and were comparable to those we observed in our study. The absence of improvement in survivorship, despite the low exposure, reiterates the potency of Thiamethoxam as an insecticide. Pesticide exposure impacting the production of reproductives is also known for bumble bees (Rundlöf et al., 2015), suggesting that effects on sexual offspring is not honey bee specific.

Although we did not quantify male or worker mortality during development, we found no indication of increased larval or pupal mortality during the dual stressor experiment; all brood was fully laid up on the frames with no apparent indication of developmental or eclosing failure such as missing or patchy brood. The observed lethal effects of Thiamethoxam became evident during the adult stage, similar to a recent study investigating the effects of co-exposure to neonicotinoid pesticides and bacterial infections (Papach et al., 2017). Although our experimental setup did not allow continuous quantification of individual survival over time, the majority of pesticide-exposed males died prior to reaching sexual maturity (Ruttner, 1966; Tofilski and Kopel, 1996). The observed mortality levels are expected to have substantial consequences because they reduce both the reproductive success and fitness of colonies affected and ultimately impact bee populations by reductions in gene flow and genetic diversity, two key components with known relevance to colony health (Amiri et al., 2017; Baer and Schmid-Hempel, 2001; Mattila and Seeley, 2007; Tarpy et al., 2013; Whitehorn et al., 2011).

Our data show that honey bee males are especially vulnerable to pesticide exposure; mortality of drones was 100% in some of the Thiamethoxam-exposed colonies. Susceptibility of male social insects to environmental stress has been reported previously (Baer et al., 2005; Gerloff et al., 2003; Vainio et al., 2004), and was hypothesised to result from reduced genetic diversity in haploid males (O'Donnell and Beshers, 2004) or lower investment of males into somatic life in response to selection for high fecundity (Rolff, 2002; Schmid-Hempel, 2005). However, the high mortality rates in males also could have resulted from our experimental design. We returned inoculated males to their maternal colonies where they were continually exposed to the pesticide in Thiamethoxam-treated colonies.

Because we found no obvious signs of male mortality during the developmental stages, we confirmed that the lethal effects of Thiamethoxam exposure become only expressed in adult life of workers and males, whether animals continue to be exposed to the pesticide (males) or not (workers). Consequently, quantifying effects of pesticide exposure on bee life history traits requires long term monitoring because they may only be observable after a time lag and later in the life cycle (Thorbek et al., 2017).

Independently of the proximate factors that caused the observed high mortality in males, we anticipate that males will make interesting study subjects for future research on effects and interactions of environmental stressors on bee health. Previous studies have shown that miticide and insecticide treatments of hives negatively impacts male fertility (Johnson et al., 2013; Kairo et al., 2017; Kairo et al., 2016) and Chaimanee *et al*. (2016) recently reported a significant reduction in sperm viability in drones exposed to the neonicotinoid, Imidacloprid, at doses as low as 0.02 ppm. Straub *et al*. (2016) showed similar sperm viability reductions in honey bee males exposed to Thiamethoxam at 4.5 ppb. If males are more sensitive to environmental stressors than female

workers, their performance could provide early indicators of colony deterioration. Colonies exposed to Thiamethoxam in the field were reported to compensate for worker losses by increasing worker brood production (Henry et al., 2015), potentially resulting in further decreases in drone production as the queen continues to invest in producing workers over drones. Collapsing male populations might not impact colony survival in the short run, if worker populations remain largely unaffected; however, longer-term, the unavailability of males may impact genetic diversity of colonies and reduce gene flow (Beaurepaire et al., 2014; Tarpy et al., 2013).

4.3 Thiamethoxam exposure under field conditions

Quantification of Thiamethoxam contamination in bee bread collected from honey bee colonies placed near flowering canola plantings confirmed that honey bees are exposed to the pesticide in quantifiable amounts in the field. The contamination levels were significantly higher in bee bread samples collected from colonies exposed to Thiamethoxam-treated canola fields but we also identified significant amounts of the pesticide in bee bread samples of colonies from control fields. There are two possible explanations for this finding. First, honey bees may have used larger foraging areas than we anticipated and foraged, albeit to a lesser extent, on more distant pesticide-treated crops. Alternatively, the untreated crops were grown on fields with a history of previous Thiamethoxam treatment, and pesticide residues of earlier applications remaining in the soil could be taken up by the growing plants. Neonicotinoids are known to be chemically stable and to persist over prolonged periods of time (Goulson, 2013; Qin et al., 2015), and a recent study confirmed residual background levels of neonicotinoid contamination even in crops grown under certified organic conditions (Mogren and Lundgren, 2016) or their presence in wildflowers growing near treated crops (Botías et al., 2015; Krupke et al., 2012). Honey bee colonies used for crop pollination

could therefore be exposed to pesticides from previous applications. Moreover, as we demonstrated in our experiments, low residual pesticide levels could be sufficiently high to negatively impact honey bee survival and health. It would have been interesting to determine pesticide concentrations in remaining bee bread and males/workers collected during the experimental treatments, but contamination levels were too low for reliable quantification by the available equipment, and we were not able to compare pesticide concentrations between treatments. Nevertheless, the potential risks of agricultural soils acting as long lasting pesticide sinks should be studied in more detail, especially where crop species are grown in rotation and bees are exposed to a variety or mixtures of pesticides, some of which might even be banned for use on pollinator-dependent crops.

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Tables

Table 1: Generalised Linear Model (GLM) of Thiamethoxam concentrations in bee bread samples collected from colonies exposed to seed-treated as well as untreated canola fields at two different locations (Bindi Bindi and Two Springs). Thiamethoxam concentrations were significantly higher in samples from seed-treated canola crops compared to untreated control fields (Figure 1).

Source	Type III		
	Wald Chi-Square	df	p-value
Intercept	393.383	1	< 0.001
Location	0.140	1	< 0.708
Thiamethoxam-treated fields	10.922	1	0.001

Table 2: GLM analysis of effects of *N. apis*-infection and/or Thiamethoxam exposure on honey bee worker survival using colony as a nested factor within Thiamethoxam treatment. A significant interaction term indicates that animals exposed to both stressors experienced substantially higher mortality compared to singly stressed workers or non-stressed control bees (Figure 2).

Source	Type III		
	Wald Chi-Square	df	p-value
Intercept	258.872	1	< 0.001
Thiamethoxam	0.079	1	0.779
N. apis	3.135	1	0.077
Colony (Thiamethoxam)	70.358	6	< 0.001
Thiamethoxam x *N. apis*	5.413	1	0.020

Table 3: GLM analysis of effects of Thiamethoxam exposure and *N. apis* infection on encapsulation response in worker honey bees. A significant *N. apis* x Thiamethoxam interaction term indicated that worker bees exposed to both stressors showed a substantially higher reduction in encapsulation response compared to workers that were exposed to the pesticide or *N. apis* infection solely (Figure 3).

Source	Type III		
	Wald Chi-Square	df	p-value
Intercept	1395.392	1	< 0.001
Thiamethoxam	4.595	1	0.032
N. apis	9.364	1	0.002
Colony (Thiamethoxam)	93.766	6	< 0.001
Thiametoxam * *N. apis*	4.367	1	0.037

Table 4: GLM analysis of significant effects of Thiamethoxam and *N. apis* exposure on honey bee male survival. Exposure to Thiamethoxam and infections with *N. apis* both reduced survival of honey bee males (Figure 4).

Source	Type III		
	Wald Chi-Square	df	p-value
Intercept	718.826	1	< 0.001
Thiamethoxam	113.282	1	< 0.001
N. apis	7.898	1	0.005
Colony (Thiamethoxam)	458.296	6	< 0.001
Thiamethoxam x *N. apis*	1.737	1	0.188

References

Aizen, M. A., Harder, L. D., 2009. The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. Current Biology. 19**,** 915-918.

Alaux, C., et al., 2010. Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Environmental Microbiology. 12**,** 774-782.

Amiri, E., et al., 2017. Queen quality and the impact of honey bee diseases on queen health: Potential for interactions between two major threats to colony health. Insects. 8**,** 48.

Antunez, K., et al., 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environ Microbiol. 11**,** 2284-90.

Aufauvre, J., et al., 2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. PLoS One. 9**,** e91686.

Australian Pesticides and Veterinary Medicines Authority, 2014. Overview Report: Neonicotinoids and the Health of Honey Bees in Australia. Australian Government. <https://archive.apvma.gov.au/news_media/docs/neonicotinoids_overview_report_february_2014.pdf>.

Baer, B., et al., 2006. Sperm storage induces an immunity cost in ants. Nature. 441**,** 872-875.

Baer, B., et al., 2005. Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection. Insectes Soc. 52**,** 298-303.

Baer, B., Schmid-Hempel, P., 2001. Unexpected consequences of polyandry for parasitism and fitness in the bumblebee, *Bombus terrestris*. Evolution. 55**,** 1639-43.

Baer, B., Schmid-Hempel, P., 2003. Effects of selective episodes in the field on life history traits in the bumblebee *Bombus terrestris*. Oikos. 101**,** 563-568.

Beaurepaire, A. L., et al., 2014. Extensive population admixture on drone congregation areas of the giant honeybee, Apis dorsata (Fabricius, 1793). Ecology and Evolution. 4**,** 4669-4677.

Beekman, M., Ratnieks, F. L. W., 2000. Long-range foraging by the honey-bee, *Apis mellifera* L. Functional Ecology. 14**,** 490-496.

Belzunces, L. P., et al., 2012. Neural effects of insecticides in the honey bee. Apidologie. 43**,** 348-370.

Blacquière, T., et al., 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology. 21**,** 973-992.

Blanken, L. J., et al., 2015. Interaction between *Varroa destructor* and imidacloprid reduces flight capacity of honeybees. Proceedings of the Royal Society B: Biological Sciences. 282**,** 20151738.

Bonmatin, J. M., et al., 2015. Environmental fate and exposure; neonicotinoids and fipronil. Environmental Science and Pollution Research. 22**,** 35-67.

Botías, C., et al., 2015. Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. Environmental Science & Technology. 49**,** 12731-12740.

Brandt, A., et al., 2016. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera L.*). Journal of Insect Physiology. 86**,** 40-47.

581 Brandt, A., et al., 2017. Immunosuppression in Honeybee Queens by the Neonicotinoids
582 Thiacloprid and Clothianidin. Scientific Reports. 7**,** 4673.
583 Breeze, T. D., et al., 2011. Pollination services in the UK: how important are honeybees?
584 Agriculture, Ecosystems & Environment. 142**,** 137-143.
585 Bryden, J., et al., 2013. Chronic sublethal stress causes bee colony failure. Ecology Letters. 16**,**
586 1463-1469.
587 Budge, G. E., et al., 2015. Evidence for pollinator cost and farming benefits of neonicotinoid seed
588 coatings on oilseed rape. Scientific Reports. 5**,** 12574.
589 Calatayud-Vernich, P., et al., 2016. Influence of pesticide use in fruit orchards during blooming on
590 honeybee mortality in 4 experimental apiaries. Science of The Total Environment. 541**,** 33-
591 41.
592 Chaimanee, V., et al., 2016. Sperm viability and gene expression in honey bee queens (*Apis*
593 *mellifera*) following exposure to the neonicotinoid insecticide imidacloprid and the
594 organophosphate acaricide coumaphos. Journal of Insect Physiology. 89**,** 1-8.
595 Chen, M., et al., 2013. Simultaneous determination of residues in pollen and high-fructose corn
596 syrup from eight neonicotinoid insecticides by liquid chromatography–tandem mass
597 spectrometry. Analytical and Bioanalytical Chemistry. 405**,** 9251-9264.
598 Decourtye, A., et al., 2004a. Imidacloprid impairs memory and brain metabolism in the honeybee
599 (*Apis mellifera* L.). Pesticide Biochemistry and Physiology. 78**,** 83-92.
600 Decourtye, A., et al., 2004b. Effects of imidacloprid and deltamethrin on associative learning in
601 honeybees under semi-field and laboratory conditions. Ecotoxicology and Environmental
602 Safety. 57**,** 410-419.
603 Di Prisco, G., et al., 2013. Neonicotinoid clothianidin adversely affects insect immunity and
604 promotes replication of a viral pathogen in honey bees. Proceedings of the National
605 Academy of Sciences, USA. 110**,** 18466-18471.
606 Dosselli, R., et al., 2016. Flight behaviour of honey bee (*Apis mellifera*) workers is altered by initial
607 infections of the fungal parasite *Nosema apis*. Scientific Reports. 6**,** 36649.
608 Doublet, V., et al., 2015. Bees under stress: sublethal doses of a neonicotinoid pesticide and
609 pathogens interact to elevate honey bee mortality across the life cycle. Environmental
610 Microbiology. 17**,** 969-983.
611 Doums, C., Schmid-Hempel, P., 2000. Immunocompetence in workers of a social insect, *Bombus*
612 *terrestris* L., in relation to foraging activity and parasitic infection. Canadian Journal of
613 Zoology. 78**,** 1060-1066.
614 EFSA, 2012. Statement on the findings in recent studies investigating sub-lethal effects in bees of
615 some neonicotinoids in consideration of the uses currently authorised in Europe. EFSA
616 Journal. 10**,** 2752-2779.
617 Evans, J. D., et al., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*.
618 Insect Molecular Biology. 15**,** 645-656.
619 Fairbrother, A., et al., 2014. Risks of neonicotinoid insecticides to honeybees. Environmental
620 Toxicology and Chemistry. 33**,** 719-731.
621 Fischer, J., et al., 2014. Neonicotinoids interfere with specific components of navigation in
622 honeybees. PLoS ONE. 9**,** e91364.
623 Fries, I., 1988. Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee.
624 Apidologie. 19**,** 319-328.
625 Fries, I., 1993. *Nosema apis* - A parasite in the honey bee colony. Bee World. 74**,** 5-19.

Fries, I., et al., 2013. Standard methods for *Nosema* research. Journal of Apicultural Research. 52**,** 1-28.
Garibaldi, L. A., et al., 2013. Wild Pollinators Enhance Fruit Set of Crops Regardless of Honey Bee Abundance. Science. 339**,** 1608-1611.
Gerloff, C. U., et al., 2003. Effects of inbreeding on immune response and body size in a social insect, *Bombus terrestris*. Funct Ecol. 17**,** 582-589.
Godfray, H. C. J., et al., 2014. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proceedings of the Royal Society B: Biological Sciences. 281**,** 20140558.
Godfray, H. C. J., et al., 2015. A restatement of recent advances in the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proceedings of the Royal Society B: Biological Sciences. 282.
Goulson, D., 2013. REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides. Journal of Applied Ecology. 50**,** 977-987.
Goulson, D., et al., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347**,** 1255957.
Grassl, J., et al., 2016. Infections with the sexually transmitted pathogen *Nosema apis* trigger an immune response in the seminal fluid of honey bees (*Apis mellifera*). Journal of Proteome Research. 16**,** 319-334.
Han, P., et al., 2010. Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee *Apis mellifera* L. Ecotoxicology. 19**,** 1612.
Henry, M., et al., 2012. A common pesticide decreases foraging success and survival in honey bees. Science. 336**,** 348-350.
Henry, M., et al., 2015. Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees. Proceedings of the Royal Society B: Biological Sciences. 282**,** 20152110.
Johnson, R. M., et al., 2013. Effect of in-hive miticides on drone honey bee survival and sperm viability. Journal of Apicultural Research. 52**,** 88-95.
Kairo, G., et al., 2017. Assessment of the toxic effect of pesticides on honey bee drone fertility using laboratory and semifield approaches: A case study of fipronil. Environmental Toxicology and Chemistry. 36**,** 2345-2351.
Kairo, G., et al., 2016. Drone exposure to the systemic insecticide Fipronil indirectly impairs queen reproductive potential. Scientific Reports. 6**,** 31904.
Koh, I., et al., 2016. Modeling the status, trends, and impacts of wild bee abundance in the United States. Proceedings of the National Academy of Sciences. 113**,** 140-145.
Kosior, A., et al., 2007. The decline of the bumble bees and cuckoo bees (*Hymenoptera: Apidae: Bombini*) of Western and Central Europe. Oryx. 41**,** 79-88.
Krupke, C. H., et al., 2012. Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. PLoS ONE. 7**,** e29268.
König, C., Schmid-Hempel, P., 1995. Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. Proceedings of the Royal Society B: Biological Sciences. 260**,** 225-227.
Lach, L., et al., 2015. Parasitized honey bees are less likely to forage and carry less pollen. Journal of Invertebrate Pathology. 130**,** 64-71.
Matsuda, K., et al., 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends in Pharmacological Sciences. 22**,** 573-580.

672 Mattila, H. R., Seeley, T. D., 2007. Genetic diversity in honey bee colonies enhances productivity
673 and fitness. Science. 317**,** 362-4.
674 Milbrath, M. O., et al., 2015. Comparative virulence and competition between *Nosema apis* and
675 *Nosema ceranae* in honey bees (*Apis mellifera*). Journal of Invertebrate Pathology. 125**,** 9-
676 15.
677 Mitchell, E. A. D., et al., 2017. A worldwide survey of neonicotinoids in honey. Science. 358**,** 109-
678 111.
679 Mogren, C. L., Lundgren, J. G., 2016. Neonicotinoid-contaminated pollinator strips adjacent to
680 cropland reduce honey bee nutritional status. Scientific Reports. 6**,** 29608.
681 Motohiro, T., John, E. C., 2005. NEONICOTINOID INSECTICIDE TOXICOLOGY: Mechanisms
682 of Selective Action. Annual Review of Pharmacology and Toxicology. 45**,** 247-268.
683 Motohiro Tomizawa, a., John, E. C., 2003. SELECTIVE TOXICITY OF NEONICOTINOIDS
684 ATTRIBUTABLE TO SPECIFICITY OF INSECT AND MAMMALIAN NICOTINIC
685 RECEPTORS. Annual Review of Entomology. 48**,** 339-364.
686 Nieto, A., et al., 2014. European Red List of bees. Luxembourg: Publication Office of the European
687 Union. 98.
688 O'Donnell, S., Beshers, S. N., 2004. The role of male disease susceptibility in the evolution of
689 haplodiploid insect societies. Proc Biol Sci. 271**,** 979-83.
690 Oliveira, R. A., et al., 2014. Side-effects of thiamethoxam on the brain andmidgut of the africanized
691 honeybee *Apis mellifera* (*Hymenopptera: Apidae*). Environmental Toxicology. 29**,** 1122-
692 1133.
693 Ollerton, J., et al., 2011. How many flowering plants are pollinated by animals? Oikos. 120**,** 321-
694 326.
695 Palmer, M. J., et al., 2013. Cholinergic pesticides cause mushroom body neuronal inactivation in
696 honeybees. Nature Communications. 4**,** 1634.
697 Papach, A., et al., 2017. Larval exposure to thiamethoxam and American foulbrood: effects on
698 mortality and cognition in the honey bee Apis mellifera. Journal of Apicultural Research.
699 56**,** 475-486.
700 Peng, Y., et al., 2015. Consequences of *Nosema apis* infection for male honey bees and their
701 fertility. Scientific Reports. 5**,** 10565.
702 Peng, Y., et al., 2016. Seminal fluid of honeybees contains multiple mechanisms to combat
703 infections of the sexually transmitted pathogen *Nosema apis*. Proc Biol Sci. 283.
704 Peng, Y., et al., 2014. Quantifying spore viability of the honey bee pathogen *Nosema apis* using
705 flow cytometry. Cytometry A. 85**,** 454-62.
706 Pettis, J. S., et al., 2012. Pesticide exposure in honey bees results in increased levels of the gut
707 pathogen Nosema. Die Naturwissenschaften. 99**,** 153-158.
708 Piiroinen, S., Goulson, D., 2016. Chronic neonicotinoid pesticide exposure and parasite stress
709 differentially affects learning in honeybees and bumblebees. Proceedings of the Royal
710 Society B: Biological Sciences. 283**,** 20160246.
711 Pisa, L., et al., 2017. An update of the Worldwide Integrated Assessment (WIA) on systemic
712 insecticides. Part 2: impacts on organisms and ecosystems. Environmental Science and
713 Pollution Research.
714 Pisa, L. W., et al., 2015. Effects of neonicotinoids and fipronil on non-target invertebrates.
715 Environmental Science and Pollution Research. 22**,** 68-102.
716 Poquet, Y., et al., 2016. Modulation of pesticide response in honeybees. Apidologie. 47**,** 412-426.

Potts, S. G., et al., 2010. Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution. 25, 345-353.
Qin, F., et al., 2015. Enantioselective bioaccumulation and toxic effects of fipronil in the earthworm *Eisenia foetida* following soil exposure. Pest Management Science. 71, 553-561.
Retschnig, G., et al., 2014a. Thiacloprid–*Nosema ceranae* interactions in honey bees: Host survivorship but not parasite reproduction is dependent on pesticide dose. Journal of Invertebrate Pathology. 118, 18-19.
Retschnig, G., et al., 2014b. Sex-specific differences in pathogen susceptibility in honey bees (*Apis mellifera*). PLoS One. 9, e85261.
Roberts, J., et al., 2015. Upgrading knowledge on pathogens (particularly viruses) of Australian honey bees. Rural Industries Research and Development Corporation. <http://www.agrifutures.com.au/wp-content/uploads/publications/15-095.pdf>.
Rolff, J., 2002. Bateman's principle and immunity. Proceedings of the Royal Society B: Biological Sciences. 269, 867-872.
Rondeau, G., et al., 2014. Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. Scientific Reports. 4, 5566.
Rortais, A., et al., 2017. Risk assessment of pesticides and other stressors in bees: Principles, data gaps and perspectives from the European Food Safety Authority. Science of The Total Environment. 587-588, 524-537.
Rundlöf, M., et al., 2015. Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature. 521, 77.
Ruttner, F., 1966. The life and flight activity of drones. Bee World. 47, 93-100.
Ruttner, F., Drescher, W., 1976. The Instrumental insemination of the queen bee. Apimondia, International Beekeeping Technology and Economy Institute, Bucharest.
Sabbahi, R., et al., 2005. Influence of honey bee (*Hymenoptera: Apidae*) density on the production of canola (*Crucifera: Brassicacae*). Journal of Economic Entomology. 98, 367-372.
Samson-Robert, O., et al., 2017. Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development. PeerJ. 5, e3670.
Sánchez-Bayo, F., et al., 2016. Are bee diseases linked to pesticides? — A brief review. Environment International. 89–90, 7-11.
Sandrock, C., et al., 2014. Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure. PLoS One. 9, e103592.
Schmid-Hempel, P., 2005. Evolutionary ecology of insect immune defenses. Annual Review of Entomology. 50, 529-551.
Selman, M., Corradi, N., 2011. Microsporidia: Horizontal gene transfers in vicious parasites. Mobile Genetic Elements. 1, 251-255.
Somerville, D., 2005. Fat bees skinny bees : a manual on honey bee nutrition for beekeepers : a report for the Rural Industries Research and Development Corporation. Rural Industries Research and Development Corporation (Australia).
Strand, M. R., 2008. The insect cellular immune response. Insect Science. 15, 1-14.
Straub, L., et al., 2016. Neonicotinoid insecticides can serve as inadvertent insect contraceptives. Proceedings of the Royal Society B: Biological Sciences. 283, 20160506.
Sturup, M., et al., 2013. When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera*. Behavioral Ecology. 24, 1192-1198.
Takino, M., 2006. Determination of 44 pesticides in foodstuffs by LC/MS/MS. Agilent Application Note. Food Safety, 12.

763 Tarpy, D. R., et al., 2013. Genetic diversity affects colony survivorship in commercial honey bee
764 colonies. Naturwissenschaften. 100**,** 723-728.
765 Thorbek, P., et al., 2017. Colony impact of pesticide-induced sublethal effects on honeybee
766 workers: A simulation study using BEEHAVE. Environmental Toxicology and Chemistry.
767 36**,** 831-840.
768 Tison, L., et al., 2016. Honey bees' behavior is impaired by chronic exposure to the neonicotinoid
769 thiacloprid in the field. Environmental science & technology. 50**,** 7218-7227.
770 Tofilski, A., Kopel, J. A. U., Krakow (Poland). Dept. of Bee Research), 1996. Influence of Nosema
771 apis on maturation and flight activity of honey bee drones. v. 40.
772 Trudeau, D., et al., 2001. Central role of hemocytes in *Autographa californica* M.
773 nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. Journal of
774 Virology. 75**,** 996-1003.
775 Vainio, L., et al., 2004. Individual variation in immune function in the ant *Formica exsecta*; effects
776 of the nest, body size and sex. Evolutionary Ecology. 18**,** 75-84.
777 Van den Brink, P. J., et al., 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a
778 mayfly species and some notes on seasonal differences. Environmental Toxicology and
779 Chemistry. 35**,** 128-133.
780 van der Sluijs, J. P., et al., 2013. Neonicotinoids, bee disorders and the sustainability of pollinator
781 services. Current Opinion in Environmental Sustainability. 5**,** 293-305.
782 Vidau, C., et al., 2011. Exposure to sublethal doses of fipronil and thiacloprid highly increases
783 mortality of honeybees previously infected by *Nosema ceranae*. PLOS ONE. 6**,** e21550.
784 Visscher, P. K., Seeley, T. D., 1982. Foraging strategy of honeybee colonies in a temperate
785 deciduous forest. Ecology. 63**,** 1790-1801.
786 Wang, D.-I., Mofller, F. E., 1970. The division of labor and queen attendance behavior of *Nosema*-
787 infected worker honey bees. Journal of Economic Entomology. 63**,** 1539-1541.
788 Washburn, J. O., et al., 1996. Insect protection against viruses. Nature. 383**,** 767.
789 Watanabe, M. E., 1994. Pollination worries rise as honey bees decline. Science. 265**,** 1170-1170.
790 Whitehorn, P. R., et al., 2011. Genetic diversity, parasites prevalence and immunity in wild
791 bumblebees. Proceedings of the Royal Society B: Biological Sciences. 278**,** 1195-1202.
792 Williams, G. R., et al., 2015. Neonicotinoid pesticides severely affect honey bee queens. Scientific
793 Reports. 5**,** 14621.
794 Williamson, S. M., Wright, G. A., 2013. Exposure to multiple cholinergic pesticides impairs
795 olfactory learning and memory in honeybees. Journal of Experimental Biology. 216**,** 1799-
796 1807.
797 Wood, T. J., Goulson, D., 2017. The environmental risks of neonicotinoid pesticides: a review of
798 the evidence post 2013. Environmental Science and Pollution Research. 24**,** 17285-17325.
799 Woodcock, B. A., et al., 2017. Country-specific effects of neonicotinoid pesticides on honey bees
800 and wild bees. Science. 356**,** 1393-1395.
801 Yang, E.-C., et al., 2012. Impaired Olfactory Associative Behavior of Honeybee Workers Due to
802 Contamination of Imidacloprid in the Larval Stage. PLOS ONE. 7**,** e49472.
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Figure legends

Figure 1

Thiamethoxam concentrations detected by LC-QQQ-MS analyses in bee bread collected from colonies placed in the vicinity of canola fields, either from untreated fields (white bar) or seed-treated fields (grey bars). Thiamethoxam was detected in all samples but levels were significantly higher in bee bread of colonies placed close to seed-treated crops. For statistical details see Table 1, bars show averages ± standard error of mean (s.e.m.).

Figure 2

Worker mortality was higher in individuals exposed to the pathogen *N. apis* and the neonicotinoid Thiamethoxam than in bees exposed to a single stressor or controls. For statistical details see Table 2, bars show median average mortalities (%) ± quartiles.

Figure 3

Encapsulation response was significantly reduced in individuals co-exposed to *N. apis* and Thiamethoxam compared to individuals exposed to each stressor alone or the control group. For statistical details see Table 3, bars depict median encapsulation responses ± quartiles.

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827 **Figure 4**

828 Mortality of honey bee males exposed to Thiamethoxam nearly tripled compared to non-exposed
829 males, independently of whether or not males where infected with *N. apis*. For statistical details see
830 Table 4, bars show median mortality (%) ± quartiles.

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