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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 began our study by conducting a field-based experiment to quantify the level of the neonicotinoid Thiamethoxam contamination in bee bread produced by workers that were foraging on canola crops in Western Australia. We assumed that local pesticide levels in pollen must be sublethal, given the absence of large-scale honey bee losses, even for colonies used for crop pollination. We quantified

pesticide concentrations in bee bread, which is a mixture of pollen and honey stored by bees in the hive and used to feed developing brood. We placed eight colonies next to flowering canola in Bindi Bindi, Western Australia (30.56° S, 116.34° E) and Three Springs (29.32° S, 115.43° E) in 2013. At both locations, no pesticide applications were made while our colonies were present and we identified a Thiamethoxam seed-treated canola planting, as well as a field with untreated plants. The distance of bee hives exposed to treated and untreated fields was 2.9 km in Three Springs and 1 km in Bindi Bindi. Although foraging ranges of honey bees can be several kilometres (Beekman and Ratnieks, 2000), they have been found to forage in close proximity to their hives if nectar and pollen sources are provided close to the hive and from a dominant plant in bloom such as canola (Sabbahi et al., 2005). Foraging ranges of honey bees in agricultural areas are therefore substantially smaller and range between 600-800 m (Visscher and Seeley, 1982).

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

2.5 Male breeding

Previous research revealed that honey bee males are particularly susceptible to environmental stress (Sturup et al., 2013) and we therefore decided to quantify effects of pathogen and pesticide exposure on males as well as female workers. We bred an age-matched cohort of males in each of our eight experimental colonies by restricting queens to one frame of male and two frames of worker comb for 3-6 days. Male brood was removed from the hives after 23 days and placed in an incubator at 32 °C, 60% humidity. We collected up to 180 newly eclosing drones per colony and inoculated half with 1 µl of 150% sucrose solution containing 10,000 *N. apis* spores with a pipette tip, and half with 1 µl of 150% sucrose solution as a control. After treatment, males were placed in

small cages of 30 each, separated by infection treatment (*N. apis* or control) and pesticide exposure (Thiamethoxam or control) and returned to their maternal colonies to allow them to sexually mature. When we retrieved the cages 15-18 days later to quantify encapsulation response, sperm viability and sperm number, we found that a large number of males had not survived, especially those exposed to pesticide; we therefore used survival data per cage to test for treatment effects.

2.6 Measuring immune response

To evaluate immunocompetence, we quantified encapsulation response, a cellular response commonly used to measure insect immunity. The process involves haemocyte recognition and attachment to a foreign particle. Haemocytes melanise and eventually form a capsule around the object. Encapsulation response correlates with pathogen resistance in bumblebees (Doums and Schmid-Hempel, 2000) and honey bees (Evans et al., 2006; Strand, 2008) and has been used to compare innate immune responses in bees and ants (Baer et al., 2006; Baer et al., 2005; Baer and Schmid-Hempel, 2003). We randomly selected 10 infected and 10 uninfected surviving workers per colony. Each bee was anaesthetised with CO_2 and placed into equipment normally used for artificially inseminating honey bee queens (Ruttner and Drescher, 1976). Two steel hooks were used to pull apart the terga and expose the inter-segmental membrane between the third and fourth tergites. A small hole was pierced into the membrane using a sterilized injection needle. We then implanted a 1 mm long piece of nylon, sterilized in 70% ethanol, into the bees' haemocoel. We allowed bees to recover and placed them in cages separated by treatment and colony, held in an incubator at 32°C, 60% humidity and with sucrose solution *ad libitum*. All bees were killed after 24 hours and stored at -20°C. Nylon implants were retrieved by dissection, embedded on a microscope slide with Eukitt (Sigma Aldrich) and protected with a cover slip. We photographed implants using a Canon EOS D 60 digital camera connected to a Leica 9.5 dissecting microscope. Photographs

were analysed with ImageJ (http://rsb.info.nih.gov/ij/download.html) to quantify grey values of implants and backgrounds. For statistical analyses, we calculated encapsulation response as the difference between the grey value of the implant minus the background.

2.7 Data analysis

All statistical analyses were performed using SPSS version 21 for Macintosh. To compare pesticide concentrations between canola fields in Experiment 1 we used a Generalised Linear Model (GLM) with location (Bindi Bindi and Three Springs) and seed treatment (Thiamethoxam versus control) as independent factors. To compare survival in both sexes and encapsulation responses in workers in Experiment 2, we used GLMs with gamma distributions and log Link functions. Pesticide exposure and pathogen infection were used as independent factors, and colony was nested within pesticide treatment. To test for significant effects of co-exposure to both stressors, we 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.

3.2 Effects of Thiamethoxam exposure during development on workers

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

3.3 Effects of continuous Thiamethoxam exposure on males

A total of 1248 males (156 ± 17.49 (mean ± sem) individuals per colony) were available for the inoculation treatments. At 15 - 18 days after treatment, the majority of males had not survived in their maternal colonies (Figure 4). Mortality was significantly higher in males that originated from colonies fed with Thiamethoxam contaminated pollen patties compared to males from control colonies (GLM: Wald Chi square 113.28, $p < 0.001$, see Table 4 for statistical details). Mortality was also higher for *N. apis* infected males than for uninfected males (GLM: Wald Chi square 7.89, $p = 0.005$) but the pathogen x pesticide interaction term was not significant (GLM: Wald Chi square 1.737, $p = 0.188$ n.s.), implying that *Nosema* infections had no additional effects. However, because male mortality was high and was driven by pesticide exposure, any potential effects of co-occurring *N. apis* infections would have been difficult to detect in our data set (Figure 4). As a result of the low survival of Thiamethoxam-exposed males (no male survivors in two of four Thiamethoxam-treated colonies), the remaining sample sizes were too small to analyse other life history traits such as sperm number, sperm viability or encapsulation response.

4. Discussion

We conducted a field-based study of honey bees from a population where major losses or declines are absent in wild and managed stock. The bees were exposed to two different environmental stressors, a pathogen and a neonicotinoid pesticide. Our experimental setup exposed honey bees to a pesticide concentration significantly lower than levels we initially detected during our first experiment in the field. Our design for the second experiment therefore recreated a situation where

a cohort of workers and males was raised with pesticide-contaminated pollen and a exposure of some of these bees to a pathogen during adult life.

Overall, we found strong effects of these stressors on bee health. We confirmed the presence of synergistic effects of both environmental stressors on worker bee health and mortality was high in males exposed to very low levels of Thiamethoxam.

4.1 Effects on workers

We confirm significant synergistic effects of *N. apis* infection and pesticide exposure in honey bees; exposure to both stressors at the same time resulted in a significant increase in mortality and 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 exposure reduces the immune response capabilities of the affected bees. A reduced encapsulation response is known to correlate with other key responses and life history traits such as resistance to viral infections (Trudeau et al., 2001; Washburn et al., 1996), pathogen resistance (Doums and Schmid-Hempel, 2000), colony size (Baer and Schmid-Hempel, 2003), foraging activity (Doums and Schmid-Hempel, 2000; König and Schmid-Hempel, 1995) and the amount of sperm stored (Baer et al., 2006). A reduction in individual encapsulation response, therefore, may impact colony performance. It would be interesting to unravel the proximate factors responsible for these long-term effects and lag times of sublethal pesticide exposure during development, especially because previous studies reported delayed increases in mortality in response to pesticide exposure during larval development (Oliveira et al., 2014; Rondeau et al., 2014; Van den Brink et al., 2016). This may be a result of irreversible binding of the pesticide to insect nicotinic acetylcholine receptors (nAChR), resulting in continuous neuronal activity (Matsuda et al., 2001; Motohiro Tomizawa and John, 2003) and accumulation of the pesticide on neuronal synapses until it reaches a critical threshold and results in the death of the animal (Pisa et al., 2017). Two previous studies also confirmed immunosuppressive effects of neonicotinoids in honey bees (Brandt et al., 2016; Di

Prisco et al., 2013), which resulted from up-regulation of an inhibitor of a member of the gene family NF-jB within the TOLL pathway (Evans et al., 2006). More work is required to confirm the physiological suppression of individual immune pathways in response to pesticide exposure.

4.2 Effects on males

Exposure of honey bee males to Thiamethoxam, at concentrations significantly lower than those measured in the field, resulted in high mortality whether or not the bees were infected with *N. apis*. 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

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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.

Figure 1

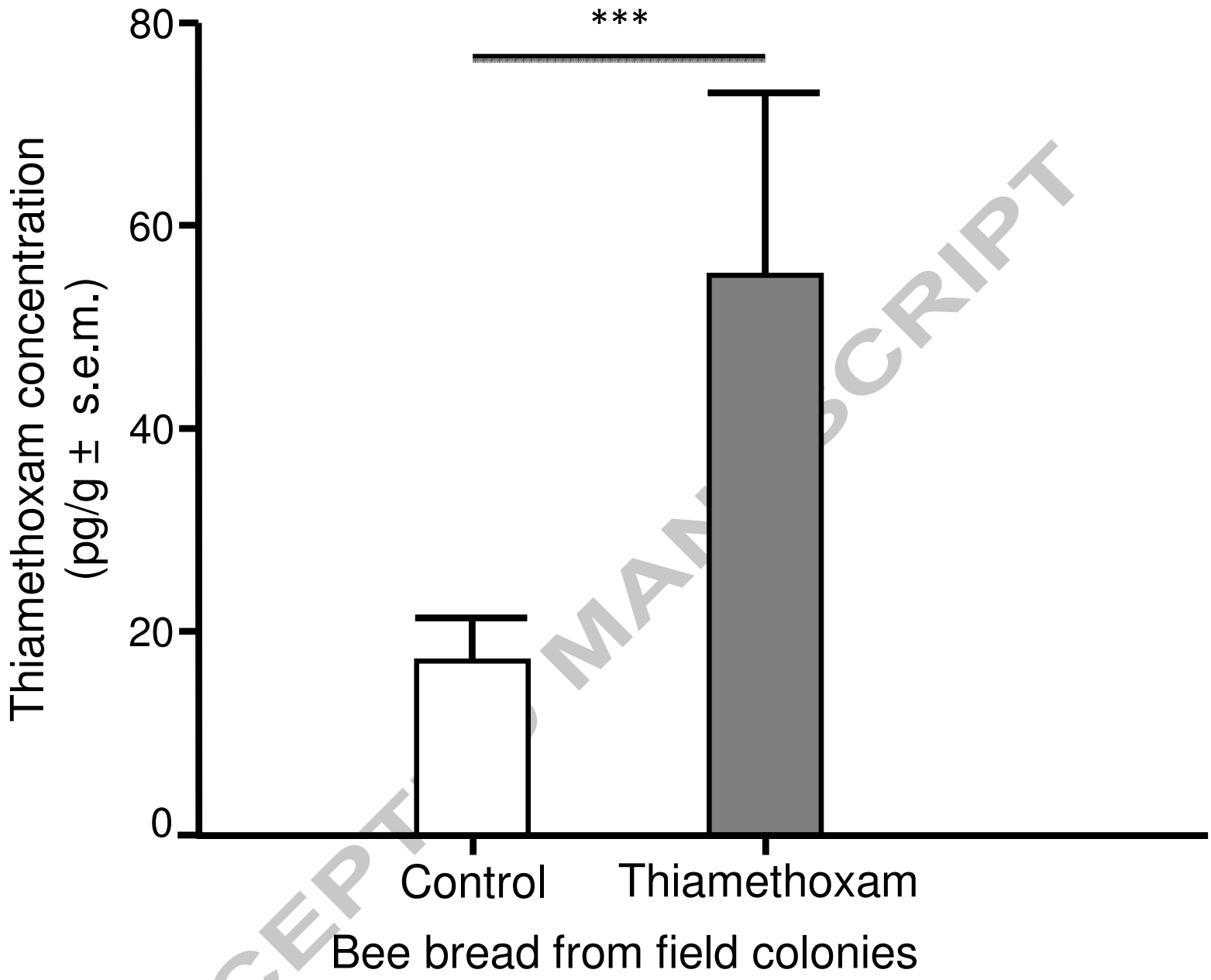


Figure 2

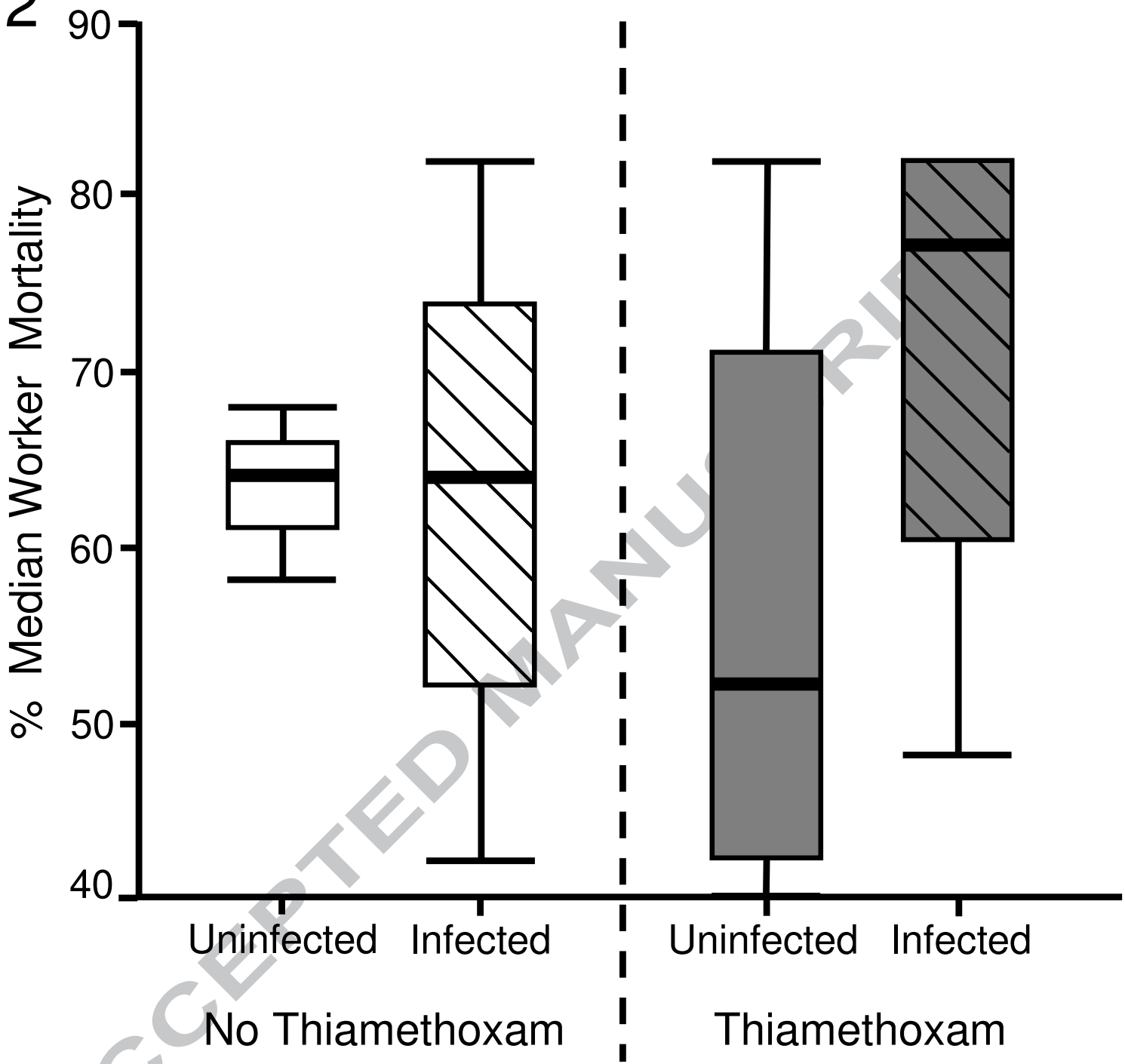


Figure 3

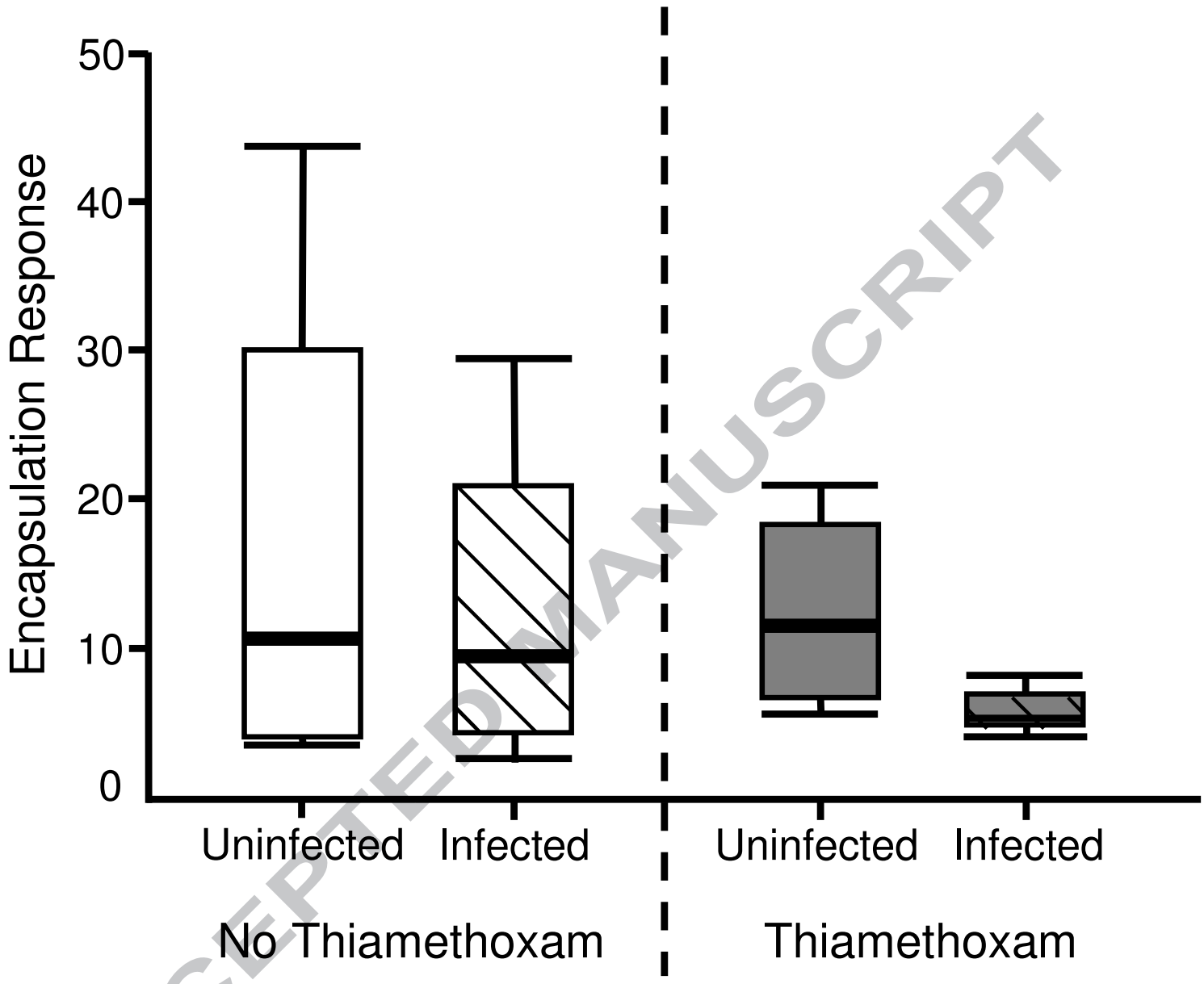


Figure 4, with colony median plot

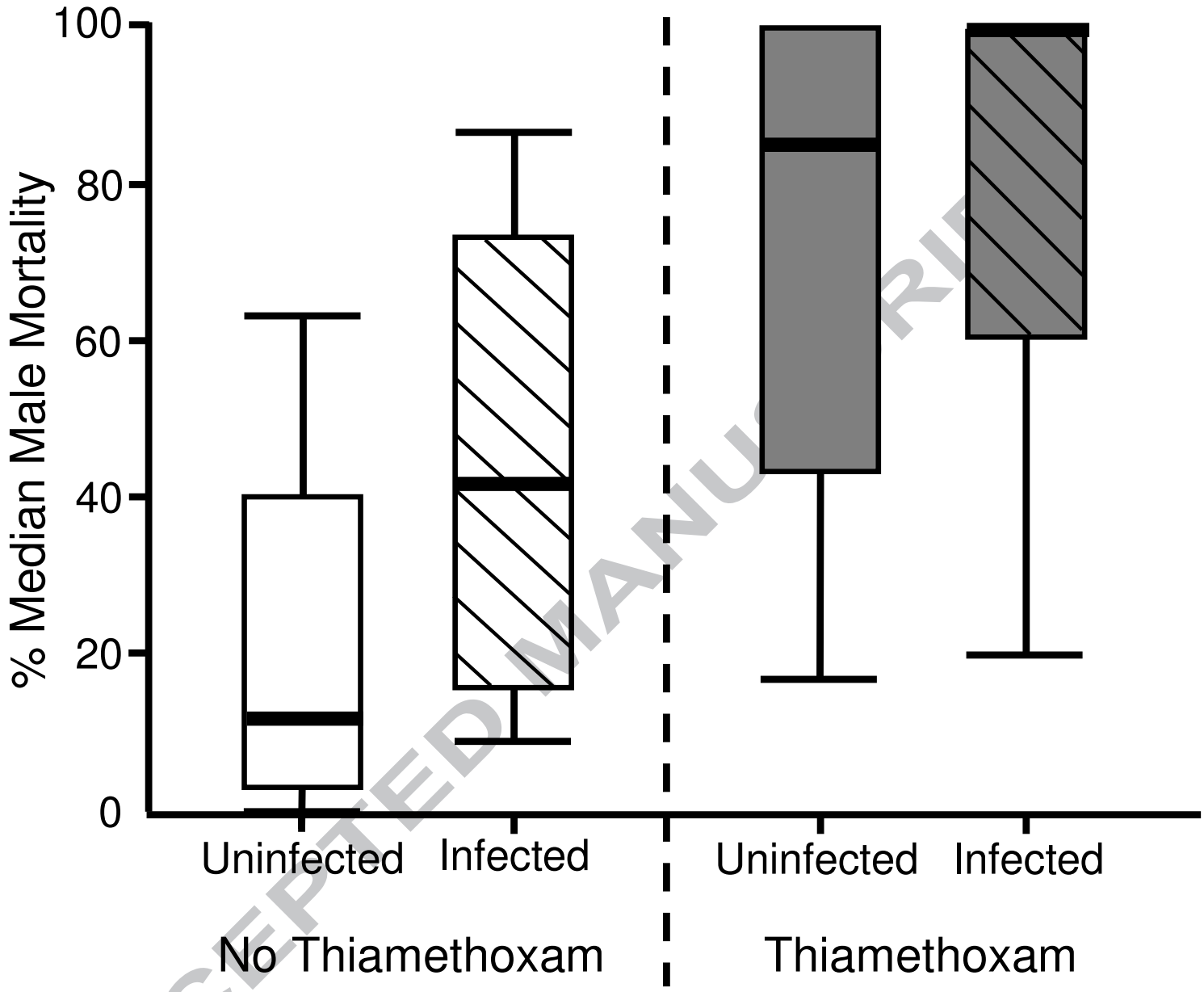
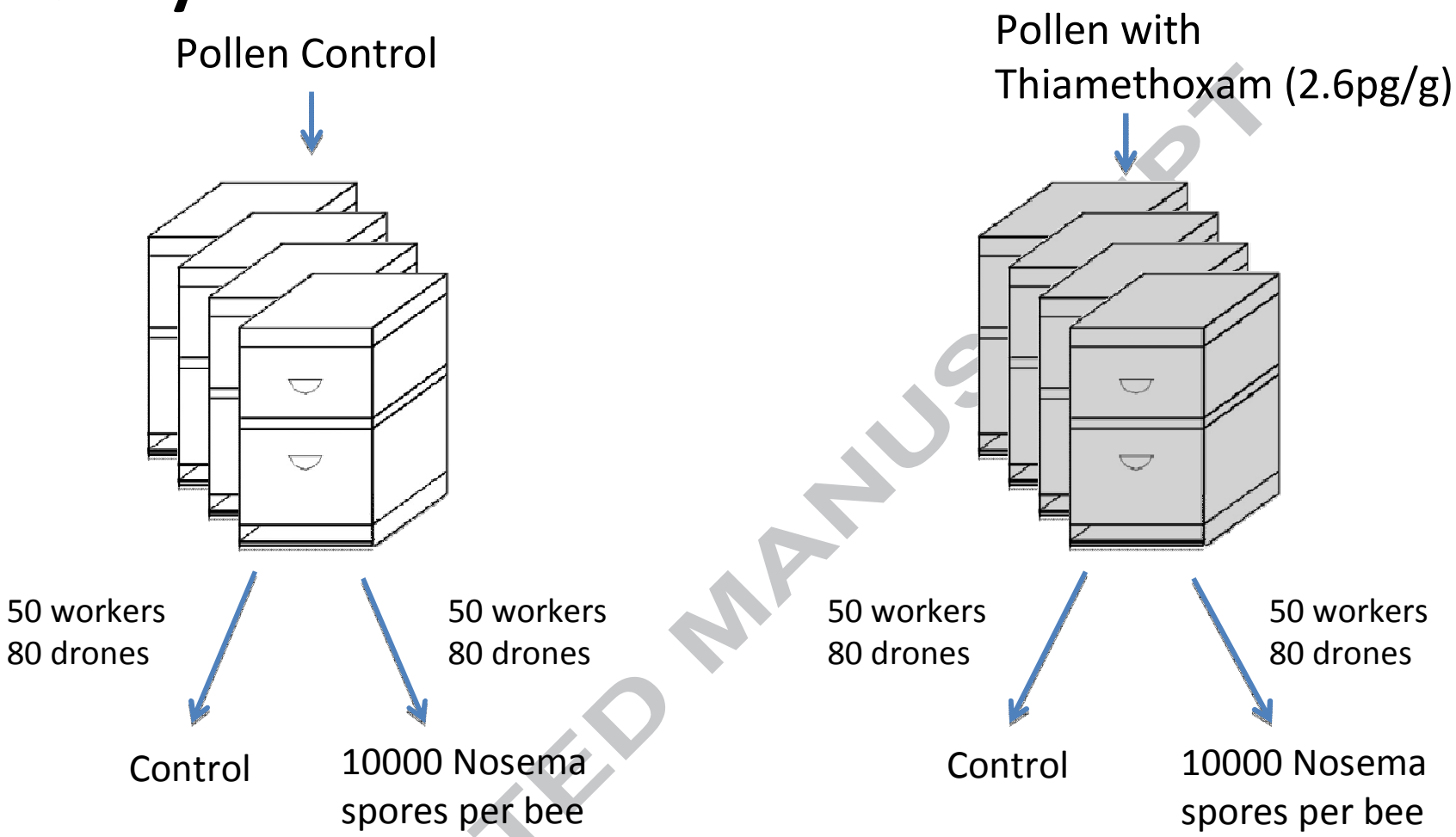


Figure 4

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

Synergistic effects of parasitism and pesticide exposure on honeybee (*Apis mellifera)* survival and immunity



Highlights

Sublethal exposure to the microspridial gut parasite *Nosema apis* and the insecticide Thiamethoxam had synergistic effects on mortality and immunocompetence of honeybee workers

Exposure of Thiametoxam to males at concentrations more than 20 times lower than found in the field substantially increased mortality, indicating that male honeybees are more sensitive to pesticide exposures compared to workers.

Our data support the idea that declines in insect pollinators are driven by a combination of stress factors