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Authors

Grassl, Julia
Holt, Shannon
Cremen, Naomi
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Synergistic effects of pathogen and pesticide exposure on honey bee (*Apis mellifera*) survival and immunity

Julia Grassl^{1,2}, Shannon Holt^{1,3}, Naomi Cremen^{1,3}, Marianne Peso¹, Dorothee Hahne⁴, Boris Baer^{2,5*}

¹ Honey Bee Health Research Group, ² ARC Centre of Excellence in Plant Energy Biology, School of Molecular Sciences, ³ Animal Biology, School of Biological Sciences, ⁴ UWA Centre for Metabolomics, Metabolomics Australia, The University of Western Australia, 6009 Crawley, Australia and ⁵ Centre for Integrative Bee Research (CIBER), Department of Entomology, University of California, Riverside CA 92521, United States of America

* Author for correspondence:
boris.bar@ucr.edu

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34 **Abstract**

35

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

57 1. Introduction

58

59 Pollination services of insects are of central importance for human food production and ecosystem
60 stability (Breeze et al., 2011; Ollerton et al., 2011; Potts et al., 2010). Non-managed and native
61 pollinators are especially important for flower pollination and increased fruit set (Garibaldi et al.,
62 2013) but substantial declines in both wild and managed insect pollinator populations have been
63 reported over recent years (Kosior et al., 2007; Nieto et al., 2014; Watanabe, 1994). The European
64 honey bee (*Apis mellifera*) is a key insect pollinator of global significance (Breeze et al., 2011) and
65 substantial losses in managed stock have been reported over recent years, especially in Europe and
66 North America (Aizen and Harder, 2009; Godfray et al., 2014; Goulson et al., 2015; Potts et al.,
67 2010). Because of their economic importance for managed pollination of agricultural crops, a
68 substantial number of studies have been conducted to investigate the impact of environmental
69 stressors on honey bee performance to quantify their effects on bee health. These include studies of
70 various pathogens (Goulson et al., 2015), pesticide exposure (Budge et al., 2015; Calatayud-
71 Vernich et al., 2016; Godfray et al., 2014; Samson-Robert et al., 2017; van der Sluijs et al., 2013;
72 Woodcock et al., 2017), habitat loss, malnutrition and climate change (Goulson et al., 2015), which
73 have all been proposed as possible contributors of declining pollinator health. No single factor
74 investigated so far fully explains the losses observed in the field, implying that combinations of
75 factors are responsible for observed declines (Bryden et al., 2013). There has been a substantial
76 increase in publications investigating the effects of neonicotinoids on honey bee health and
77 behaviour, as summarized in two recent reviews (Godfray et al., 2015; Pisa et al., 2017). More
78 recent work specifically tested for possible synergistic, additive or antagonistic effects of pesticides
79 and other stressors, in particular pathogens, on honey bee health, although most of these studies

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

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

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

103 activities towards younger bees (Lach et al., 2015) and reduce the length of foraging trips (Dosselli
104 et al., 2016). In honey bee males, infections reduce longevity (Peng et al., 2015) and spores can be
105 found in ejaculates of older males (Peng et al., 2015). Males respond to *Nosema* infections by a
106 systemic upregulation of immune proteins in their seminal fluid (Grassl et al., 2016), which can
107 efficiently kill *Nosema* spores (Peng et al., 2016). Nevertheless, surviving *Nosema* spores
108 transferred within ejaculates to queens during mating can trigger novel infections (Roberts et al.,
109 2015). The pathogen can impact colony performance, as previous research has shown that chronic
110 infections can reduce a colony's ability to regulate hive temperature (Wang and Moffler, 1970) or
111 even kill the entire colony (Fries, 1993).

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

126 2014; Tison et al., 2016) or impairment of the bees' capacity for learning and memory (Belzunces et
127 al., 2012; Blacquièrè et al., 2012; Decourtye et al., 2004a; Decourtye et al., 2004b; Han et al., 2010;
128 Henry et al., 2012; Palmer et al., 2013; Papach et al., 2017; Piiroinen and Goulson, 2016;
129 Williamson and Wright, 2013; Yang et al., 2012). More recently, increased mortality in honey bees
130 exposed to pesticides and a second stressor have been reported (Alaux et al., 2010; Di Prisco et al.,
131 2013; Doublet et al., 2015; Goulson et al., 2015; Papach et al., 2017). Consequently, neonicotinoid
132 pesticides are prime suspects for sublethal effects that negatively impact honey bees.

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

140

141

142 **2. Materials and Methods**

143

144 *2.1 Field relevant exposure levels of Thiamethoxam*

145 A number of previous studies have quantified the effects of neonicotinoid pesticides on bee health.
146 However, these studies were criticised for using pesticide exposure levels that were deemed too
147 high and therefore not field realistic (Australian Pesticides and Veterinary Medicines Authority,
148 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

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

183 To quantify Thiamethoxam concentrations in these enriched samples, we used an Agilent
184 1100 Series chromatograph coupled to a model 6430A triple quadrupole mass spectrometer
185 (Agilent Technologies), with a JetStream electrospray source in positive ionization mode, using the
186 same transition ions, as described in Takino (2006). Acetonitrile (ACN), methanol (LC-MS
187 Chromosolv, $\geq 99.9\%$), and HPLC water were obtained from Fluka (Sigma–Aldrich,).
188 Thiamethoxam and the isotope labelled ISTD Thiamethoxam-d3 (99.8 %) were purchased from
189 Sigma-Aldrich with purity of 99.7%. The initial stock standard solutions were prepared in
190 acetonitrile at a concentration of 100 $\mu\text{g}/\text{ml}$ and then stored in amber glass vials at $-20\text{ }^\circ\text{C}$ until use.
191 The calibration standards and working standards were prepared by dilution with HPLC water on the
192 day of analysis. Chromatographic separation was performed on a Poroshell 120 EC-C18 2.7 μm , 3
193 \times 100 mm column (Agilent Technologies). The mobile phase consisting of: (A) water, and (B)
194 methanol, both containing 10 mM of ammonium acetate, was used at a flow rate of 0.4 ml / min.

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

204

205 *2.2 Honeybee breeding*

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

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

228

229 *2.3 Collection, purification and inoculation of Nosema apis spores*

230 Sampling of microsporidian spores for subsequent infection of workers and males was done
231 according to a previously developed protocol (Peng et al., 2015; Peng et al., 2014). In the absence
232 of *N. ceranae* in Western Australia (Roberts et al., 2015), spore samples used for inoculations
233 contained only *N. apis*. We collected 20 foraging workers from the entrances of five non-
234 experimental hives with known *N. apis* infections. The midguts of 100 workers were dissected and
235 pooled in an Eppendorf tube along with 1 ml of DDI water and a 3 mm tungsten bead (Qiagen,
236 Australia). The sample was homogenized for 30 s in a mixer mill (Retsch MM301) at 25 Hz, and
237 0.5 ml was layered onto 1.5 ml of 100% Percoll (Sigma-Aldrich) in a 2 ml Eppendorf tube. The
238 sample was centrifuged at 18,000 x g for 60 min at 4 °C. After removing the supernatant, 1.5 ml of
239 DDI water was added before vortexing and centrifuging the sample 3 times at 20,700 x g for 5 min.
240 The pellet was resuspended in DDI water and spore concentration was determined using a Neubauer

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

247

248 2.4 Worker breeding

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

263

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 CO₂ 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

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

312

313

314 **3. Results**

315

316 *3.1 Determining field relevant exposure levels of Thiamethoxam*

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

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

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

398 Apart from increased worker mortality, we found an additional synergistic effect in
399 surviving workers; the encapsulation response was substantially lower in individuals exposed to
400 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- κ B 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

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

434 Although we did not quantify male or worker mortality during development, we found no
435 indication of increased larval or pupal mortality during the dual stressor experiment; all brood was
436 fully laid up on the frames with no apparent indication of developmental or eclosing failure such as
437 missing or patchy brood. The observed lethal effects of Thiamethoxam became evident during the
438 adult stage, similar to a recent study investigating the effects of co-exposure to neonicotinoid
439 pesticides and bacterial infections (Papach et al., 2017). Although our experimental setup did not
440 allow continuous quantification of individual survival over time, the majority of pesticide-exposed
441 males died prior to reaching sexual maturity (Ruttner, 1966; Tofilski and Kopel, 1996). The
442 observed mortality levels are expected to have substantial consequences because they reduce both
443 the reproductive success and fitness of colonies affected and ultimately impact bee populations by
444 reductions in gene flow and genetic diversity, two key components with known relevance to colony
445 health (Amiri et al., 2017; Baer and Schmid-Hempel, 2001; Mattila and Seeley, 2007; Tarpay et al.,
446 2013; Whitehorn et al., 2011).

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

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

462 Independently of the proximate factors that caused the observed high mortality in males, we
463 anticipate that males will make interesting study subjects for future research on effects and
464 interactions of environmental stressors on bee health. Previous studies have shown that miticide and
465 insecticide treatments of hives negatively impacts male fertility (Johnson et al., 2013; Kairo et al.,
466 2017; Kairo et al., 2016) and Chaimanee *et al.* (2016) recently reported a significant reduction in
467 sperm viability in drones exposed to the neonicotinoid, Imidacloprid, at doses as low as 0.02 ppm.
468 Straub *et al.* (2016) showed similar sperm viability reductions in honey bee males exposed to
469 Thiamethoxam at 4.5 ppb. If males are more sensitive to environmental stressors than female

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

477

478 *4.3 Thiamethoxam exposure under field conditions*

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

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

502

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508

509 **Tables**

510

511 **Table 1:** Generalised Linear Model (GLM) of Thiamethoxam concentrations in bee bread samples
 512 collected from colonies exposed to seed-treated as well as untreated canola fields at two different
 513 locations (Bindi Bindi and Two Springs). Thiamethoxam concentrations were significantly higher in
 514 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

515

516

517

518 **Table 2:** GLM analysis of effects of *N. apis*-infection and/or Thiamethoxam exposure on honey bee
 519 worker survival using colony as a nested factor within Thiamethoxam treatment. A significant
 520 interaction term indicates that animals exposed to both stressors experienced substantially higher
 521 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
<i>N. apis</i>	3.135	1	0.077
Colony (Thiamethoxam)	70.358	6	< 0.001
Thiamethoxam x <i>N. apis</i>	5.413	1	0.020

522

523

524

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

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

530

531

532

533 **Table 4:** GLM analysis of significant effects of Thiamethoxam and *N. apis* exposure on honey bee
534 male survival. Exposure to Thiamethoxam and infections with *N. apis* both reduced survival of
535 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
<i>N. apis</i>	7.898	1	0.005
Colony (Thiamethoxam)	458.296	6	< 0.001
Thiamethoxam x <i>N. apis</i>	1.737	1	0.188

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541

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804

805 **Figure legends**

806

807 **Figure 1**

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

813

814

815 **Figure 2**

816 Worker mortality was higher in individuals exposed to the pathogen *N. apis* and the neonicotinoid
817 Thiamethoxam than in bees exposed to a single stressor or controls. For statistical details see Table
818 2, bars show median average mortalities (%) \pm quartiles.

819

820

821 **Figure 3**

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

825

826

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 were infected with *N. apis*. For statistical details see
830 Table 4, bars show median mortality (%) \pm quartiles.

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