Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees

Ian Laycock [a, *], Katie C. Cotterell [a], Thomas A. O'Shea-Wheller [a], James E. Cresswell [a]

[a] *College of Life & Environmental Sciences, Biosciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, EX4 4PS, UK*

[*] *Corresponding author (Ian Laycock), Email: il219@exeter.ac.uk; Tel: 01392 723779*

http://dx.doi.org/10.1016/j.ecoenv.2013.10.027

Abstract

Neonicotinoid pesticides are currently implicated in the decline of wild bee populations. Bumble bees, *Bombus* spp., are important wild pollinators that are detrimentally affected by ingestion of neonicotinoid residues. To date, imidacloprid has been the major focus of study into the effects of neonicotinoids on bumble bee health, but wild populations are increasingly exposed to alternative neonicotinoids such as thiamethoxam. To investigate whether environmentally realistic levels of thiamethoxam affect bumble bee performance over a realistic exposure period, we exposed queenless microcolonies of *Bombus terrestris* L. workers to a wide range of dosages up to 98 μg kg^{-1} in dietary syrup for 17 days. Results showed that bumble bee workers survived fewer days when presented with syrup dosed at 98 μg thiamethoxam kg^{-1}, while production of brood (eggs and larvae) and consumption of syrup and pollen in microcolonies were significantly reduced by thiamethoxam only at the two highest concentrations (39, 98 μg kg^{-1}). In contrast, we found no detectable effect of thiamethoxam at levels typically found in the nectars of treated crops (between 1 and 11 μg kg^{-1}). By comparison with published data, we demonstrate that during an exposure to field-realistic concentrations lasting approximately two weeks, brood production in worker bumble bees is more sensitive to imidacloprid than thiamethoxam. We speculate that differential sensitivity arises because imidacloprid produces a stronger repression of feeding in bumble bees than thiamethoxam, which imposes a greater nutrient limitation on production of brood.

Keywords

bee health; *Bombus*; field-realistic; imidacloprid; neonicotinoid; thiamethoxam

41 # 1. Introduction

42 The pollination services of wild bees help to maintain plant species in natural ecosystems and
43 are worth billions of dollars annually to agriculture (Williams and Osborne, 2009; Winfree,
44 2010). Evidence of declining wild bee populations (Biesmeijer et al., 2006) and the
45 extirpation of certain species (Burkle et al., 2013) are therefore issues of increasing concern
46 (Vanbergen and IPI, 2013). It is widely acknowledged that several factors are driving
47 declines in wild bees (Williams and Osborne, 2009; Potts et al., 2010). However, a group of
48 neurotoxic pesticides, the neonicotinoids, have specifically been singled out for blame
49 (Shardlow, 2012), which has lead to calls for restrictions on their use in agricultural (EFSA,
50 2013a; Maxim and van der Sluijs, 2013) that have recently been implemented across the
51 European Union (European Commission, 2013). The neonicotinoids, which include
52 imidacloprid, thiamethoxam and clothianidin, are systemic and so the pesticide is distributed
53 throughout plant tissues to control sucking insect pests (Elbert et al., 2008). Consequently,
54 trace residues can appear in nectar and pollen (Blacquière et al., 2012) and bees are exposed
55 to dietary neonicotinoids by foraging from the flowers of treated agricultural crops (Elbert et
56 al., 2008).
57
58 Bumble bees are important wild pollinators that are detrimentally affected by neonicotinoids
59 in laboratory studies, where dietary residues reduce food consumption and brood production
60 of *Bombus terrestris* L. workers (Tasei et al., 2000; Mommaerts et al., 2010; Cresswell et al.,
61 2012; Laycock et al., 2012), and in semi-field studies, where *B. terrestris* colonies under
62 exposure exhibit reduced production of brood, workers and queens (Gill et al., 2012;
63 Whitehorn et al., 2012). The majority of these studies focus solely on imidacloprid, which
64 has historical relevance because it was the first neonicotinoid in widespread use (Elbert et al.,
65 2008) and was identified publicly as a potential threat to bee health in 1999 (Maxim and van

66 der Sluijs, 2013). However, newer neonicotinoid varieties, such as thiamethoxam and its
67 toxic metabolite clothianidin, are increasingly preferred to imidacloprid in crop protection.
68 For example, in 2011 imidacloprid made up just 10 % of the total 80,000 kg of neonicotinoid
69 applied to UK crops (FERA, 2013). Consequently wild bumble bees are at increased risk of
70 exposure to these alternative neonicotinoids. We therefore chose to further investigate the
71 effects of dietary thiamethoxam on bumble bees.
72
73 Residues of thiamethoxam ranging from 1 to 11 μg kg^{-1} (= parts per billion or ppb) have been
74 detected in nectar from treated crops including alfalfa, oilseed rape, pumpkin, sunflower,
75 squash and *Phacelia tanacetifolia* (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer,
76 2012). In pollen, residues are typically higher, ranging from to 1 to 12 μg kg^{-1} in sunflower,
77 oilseed rape and squash, but reaching 39, 51 and 95 μg kg^{-1} in *Phacelia*, alfalfa, and
78 pumpkin, respectively (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). For
79 bees, exposure to residues such as these probably occurs in transient pulses; for example,
80 during the mass-flowering of treated oilseed rape that lasts for approximately one month and
81 peaks over a period of around two weeks (Hoyle et al., 2007; Westphal et al., 2009).
82 Detrimental effects on honey bees of dietary thiamethoxam at 67 μg L^{-1} have already been
83 demonstrated (Henry et al., 2012), but the effects on bumble bees in a similar dosage range
84 are unclear. For example, in one *B. terrestris* microcolony study 100 μg kg^{-1} thiamethoxam
85 presented to workers in sugar solution increased mortality and reduced drone production
86 while residues at 10 μg kg^{-1} had no detectable effect (Mommaerts et al., 2010). However, in
87 another study 10 μg kg^{-1} thiamethoxam reduced workers' production of drone brood (the
88 workers' eggs and larvae), while microcolony feeding rates were reduced at both 1 and 10 μg
89 kg^{-1} (Elston et al., 2013). With evidence of thiamethoxam's effects currently inconsistent, it
90 remains uncertain whether environmentally realistic residues are capable of having a

91 detrimental impact on bumble bee populations. We therefore present an experiment designed
92 to test the performance of bumble bees presented with dietary thiamethoxam at a wide range
93 of concentrations, including dosages within the field-realistic range for nectar.
94
95 In this study, we made use of the reproductive capacity of *B. terrestris* workers in queenless
96 microcolonies to investigate the effects of thiamethoxam on bumble bee performance. In
97 microcolonies, small groups of bumble bee workers are maintained in the absence of a queen
98 and, over a period of days, a dominant worker lays eggs that will develop into drones while
99 the others forage and care for brood (Tasei et al., 2000). In a recent guidance document for
100 risk assessment of plant protection products on bees (EFSA, 2013b), the use of microcolonies
101 was recommended as part of 'higher tier' risk assessment studies in bumble bees. Using *B.*
102 *terrestris* microcolonies, we characterised dose-response relationships that described
103 thiamethoxam's effects on brood (eggs and larvae) production, food consumption and days
104 survived by workers (Laycock et al., 2012) over an exposure lasting 17 days. Following
105 laboratory exposure periods of similar length, imidacloprid produced substantive sublethal
106 effects on feeding and brood production in *B. terrestris* microcolonies (Laycock et al., 2012)
107 and reduced colony growth and production of new queens in queenright colonies allowed to
108 develop for a further six weeks in pesticide-free conditions (Whitehorn et al., 2012). Here we
109 applied dosages and some endpoints that were adopted in the imidacloprid microcolony study
110 (i.e. Laycock et al., 2012) to enable us to compare the relative sensitivity of bumble bees to
111 the two neonicotinoids.
112

2. Materials and methods

2.1 Microcolonies

We obtained four colonies of *B. terrestris* (subspecies *audax*) (Biobest, Westerlo, Belgium) each consisting of a queen and approximately 150 workers. One hundred queenless microcolonies were established by placing 400 individual workers (100 from each queenright colony) into softwood boxes (120 × 120 × 45 mm) in groups of four. The allocation of workers to boxes was randomized, but each microcolony contained workers from the same queenright colony. Each box was fitted with two 2 mL microcentrifuge tubes (Simport, Beloeil, Canada) that were punctured so as to function as syrup (artificial nectar) feeders. We maintained microcolonies for 18 days under semi-controlled conditions (23–29 °C, 20–40 % relative humidity) and in darkness except during data collection. Specifically, all microcolonies were acclimatised to experimental conditions by feeding *ad libitum* on undosed control syrup (Attracker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for 24 h prior to 17 days of exposure to thiamethoxam. A single bee that died during acclimatisation was replaced with a worker from its queenright source colony.

2.2 Thiamethoxam dosages

To produce a primary thiamethoxam stock solution (10^5 μg thiamethoxam L^{-1}), we dissolved 5 mg thiamethoxam powder (Pestanal®; Sigma-Aldrich, Gillingham, UK) in 50 mL purified water. Primary stock solution was further diluted (to 10^4 μg L^{-1}) in purified water and an aliquot of diluted stock was mixed into feeder syrup to produce our most concentrated dietary solution of 125 μg thiamethoxam L^{-1} (or 98.43 μg kg^{-1} = ppb). By serial dilution from the highest concentration we produced nine experimental dosages at the following concentrations: 98.43, 39.37, 15.75, 6.30, 2.52, 1.01, 0.40, 0.16, 0.06 μg thiamethoxam kg^{-1}.

137 Following acclimatisation, microcolonies were fed *ad libitum* for 17 days with undosed
138 pollen balls (ground pollen pellets, obtained from Biobest, mixed with water; mean mass =
139 5.3 g, SE = 0.1 g) and either undosed control syrup (19 control microcolonies) or syrup dosed
140 with thiamethoxam (9 dosed microcolonies per thiamethoxam concentration, listed above).
141 This level of replication (i.e. a minimum of nine replicates per concentration) is consistent
142 with similar microcolony studies (Mommaerts et al., 2010; Laycock et al., 2012; Elston et al.,
143 2013). Pollen balls were weighed before and after placement into microcolonies to quantify
144 pollen consumption and syrup feeders were weighed each day to measure syrup consumption.
145 We corrected for evaporation of water from syrup and pollen based on the mass change of
146 syrup feeders and pollen balls maintained under experimental conditions, but not placed into
147 microcolonies. Additionally, where syrup or pollen was collected by bees but not consumed,
148 for example where syrup was stored in wax honey pots, its mass was determined and
149 subtracted from consumption accordingly. We monitored microcolonies daily for individual
150 worker mortality and the appearance of wax covered egg cells that indicate the occurrence of
151 oviposition. To assess brood production, at the end of the experiment we freeze-killed
152 workers in their microcolony boxes and collected all laid eggs and larvae from the nests. In
153 our previous microcolony study (Laycock et al., 2012), we also investigated the effect of
154 imidacloprid on ovary development because imidacloprid produced a dose-dependent decline
155 in workers' brood production. Except at the highest dosages, thiamethoxam had no effect on
156 brood production (i.e. microcolonies laid eggs at a statistically equivalent rate, see section 3)
157 and we therefore chose not to measure ovary development here. The experiment was
158 conducted in two replicate trials between October and December 2012. Each trial comprised
159 50 microcolonies and dosage groups were approximately equally represented in both. The
160 results of the two trials were qualitatively similar and so data were pooled for further
161 analysis.

We verified the concentration of thiamethoxam in our doses using solid phase extraction (SPE) and liquid chromatography-mass spectrometry (LCMS) as follows. First, we dissolved our dosed syrups in LCMS-grade water (Fisher Scientific, Loughborough, UK). To extract thiamethoxam from syrup, the diluted samples were processed through 1 mL Discovery® DSC-18 SPE tubes (Sigma-Aldrich, Gillingham, UK) under positive pressure. Specifically, we conditioned the SPE tube with 1 mL LCMS-grade methanol (Fisher Scientific, Loughborough, UK) followed by 1 mL LCMS-grade water, prior to passing through a 1 mL diluted sample. The tube was washed with 1 mL LCMS-grade water and the thiamethoxam was eluted from the column with three separate, but equivalent, aliquots of LCMS-grade methanol, totalling 450 μL. Methanol was removed by evaporation in a ScanSpeed MaxiVac Beta vacuum concentrator (LaboGene ApS, Lynge, Denmark) and the remaining thiamethoxam was dissolved in 500 μL of LCMS-grade water. Extracted thiamethoxam samples were analysed in an Agilent 1200 series liquid chromatograph interfaced via an electrospray ionisation source to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), along with a calibration curve consisting of nine known thiamethoxam concentrations that ranged from 0.1 to 125 μg L^{-1}, using methods described in Laycock et al. (2012). The instrument response was linear over the range 0.1–125 μg L^{-1}, with the relationship of the calibration curve given by *instrument response* = 228.42 × *thiamethoxam concentration* + 265.87, $R^2 > 0.99$). We used the calibration equation to determine the concentration values of our extracted samples and found that all dosages contained appropriate levels of thiamethoxam (*measured thiamethoxam* = 1.16 × *nominal dosage* + 1.57, $R^2 > 0.99$).

2.3 Statistical analyses

187 In our experiments, endpoints responded only to the two highest dosages of thiamethoxam
188 (see section 3). We therefore analysed the variation in food consumption and days survived
189 by workers in microcolonies that was due to thiamethoxam using one-way ANOVA, with
190 *dosage* (dosage of thiamethoxam in $\mu g\ kg^{-1}$) treated as a categorical variable, and compared
191 the highest dosage groups to those below using orthogonal contrasts.
192
193 We tested whether the two highest thiamethoxam dosages were associated with an increased
194 frequency of oviposition failure (zero brood produced) using a 2×2 contingency table and
195 Pearson's Chi-squared test with Yates' continuity correction.
196
197 To determine whether brood production was dose-dependent below the two highest dosages,
198 we used zero-inflated Poisson regression (ZIP) because of an excess of zero counts in our
199 data (Lambert, 1992). We tested the appropriateness of the ZIP model by comparing it to a
200 standard Poisson model using a Vuong non-nested test and confirmed that the ZIP model was
201 the superior choice (*Vuong test statistic* = -5.17, $P < 0.001$).
202
203 In our analysis, the total number of eggs and larvae produced in microcolonies during the 17-
204 day exposure period represents *brood* (brood were not produced during pre-dose
205 acclimatisation). Where necessary, we log-transformed *dosage* to log(*dosage* +1) to meet test
206 assumptions. All statistical analyses were conducted in R v3.0.0 (Ihaka and Gentleman,
207 1996).
208

3. Results

Per capita consumption of syrup and pollen in microcolonies was significantly affected by thiamethoxam (ANOVA: *syrup consumption*, $F_{9, 90} = 9.29$, $P < 0.001$; *pollen consumption*, $F_{9, 90} = 15.14$, $P < 0.001$; Fig. 1). Specifically, a significant reduction in food consumption was evident only in microcolonies exposed at the two highest dosages, 39 μg kg^{-1} and 98 μg kg^{-1} (orthogonal contrast: *syrup consumption*, $F_{9, 90} = 9.29$, $t = -8.87$, $P < 0.001$; *pollen consumption*, $F_{9, 90} = 15.14$, $t = -11.22$, $P < 0.001$). No dose-dependent variation was detectable among microcolonies exposed to dosages ≤ 16 μg kg^{-1} (ANOVA: *syrup consumption*, $F_{7, 74} = 0.39$, $P = 0.91$; *pollen consumption*, $F_{7, 74} = 0.90$, $P = 0.51$). Despite consuming less syrup, microcolonies exposed to higher dosages nevertheless ingested larger amounts of thiamethoxam (Table 1).

In microcolonies, the frequency of oviposition failure at the two highest thiamethoxam dosages (94% failure) was greater than at lower dosages (48%) and these frequencies differed significantly (Chi-squared contingency table analysis: $X^2 = 11.33$, $df = 1$, $P < 0.001$; Fig. 2). Excluding the two highest dosages, thiamethoxam did not significantly affect the number of brood produced (ZIP regression: *brood count*, $z = -1.26$, $P = 0.21$; *zero brood production*, $z = 0.45$, $P = 0.65$; Fig. 1).

Among microcolonies that produced brood, there was no effect of dosage on the number of brood produced or on the timing of first oviposition (Spearman's correlation: *brood vs. dosage*, $\rho = -0.03$, $N = 44$, $P = 0.85$; *days until oviposition vs. dosage*, $\rho = 0.08$, $N = 44$, $P = 0.63$; Table 1).

232 The number of days survived by workers in microcolonies varied significantly with
233 thiamethoxam dosage (ANOVA: $F_{9, 90} = 27.43$, $P < 0.001$; Fig. 1), but it was reduced only at
234 98 μg kg^{-1} (orthogonal contrast: $F_{9, 90} = 27.43$, $t = -15.44$, $P < 0.001$) and did not differ at
235 lower dosages (ANOVA: $F_{8, 82} = 1.25$, $P = 0.28$).
236

4. Discussion

4.1 Thiamethoxam effects

We found that thiamethoxam reduced feeding and brood production in *B. terrestris* microcolonies that fed on syrup with a dietary concentration of 39 μg kg^{-1} or above for 17 days. At lower dosages, microcolonies consumed syrup and pollen at normal control rates and brood production was not detectably dose-dependent. These results are consistent with those of a previous *B. terrestris* microcolony study in which dietary thiamethoxam produced an EC_{50} for drone production of 35 μg kg^{-1} and had no observable effect on workers at 10 μg kg^{-1} (Mommaerts et al., 2010). However, another recent study reported that 10 μg kg^{-1} thiamethoxam was capable of reducing syrup feeding and brood production in microcolonies (Elston et al., 2013). These contrasting results may have arisen because bumble bees consumed different amounts of thiamethoxam in nominally equivalent treatment groups, with Elston et al. (2013) having dosed both syrup and pollen at 10 μg kg^{-1}, whereas Mommaerts et al. (2010), like us, dosed only syrup. Additionally, our results correspond with studies of clothianidin, which is thiamethoxam's primary toxic metabolite and becomes active during thiamethoxam exposure (Nauen et al., 2003). Specifically, dietary clothianidin at 38 μg kg^{-1} negatively influenced honey bee foraging behaviour (Schneider et al., 2012), but lower dosages had no adverse effects on colonies of *Bombus impatiens* Cresson bumble bees (Franklin et al., 2004).

Where thiamethoxam was presented to microcolonies at 39 μg kg^{-1} or above, we observed an association between impaired feeding on syrup and pollen and failure to produce brood. A similar association was observed in *B. terrestris* microcolonies fed imidacloprid across a range of dosages (Laycock et al., 2012). The hypothesis proposed by Laycock et al. (2012), that nutrient limitation imposed by an imidacloprid-induced reduction of feeding may be

261 responsible for repression of brood production in bumble bees, can also be applied in our
262 current study to explain thiamethoxam's detrimental effect on brood production at higher
263 dosages. We therefore postulate that the capacity to impair bumble bee feeding behaviour is
264 common amongst neonicotinoids, particularly at high dosages, and this may provide a
265 general mechanism for reduced brood production (Gill et al., 2012; Laycock et al., 2012;
266 Elston et al., 2013).
267
268 Consistent with previous findings (Mommaerts et al., 2010), the number of days survived by
269 workers was significantly reduced in microcolonies fed approximately 100 μg kg^{-1}
270 thiamethoxam. For honey bees, relatively large dosages of thiamethoxam (67 μg L^{-1}) also
271 impact on worker survival (Henry et al., 2012). Apparently, these relatively high
272 concentrations of dietary thiamethoxam are highly toxic to bees in general.
273
274 *4.2 Differential sensitivity of bumble bees to thiamethoxam and imidacloprid*
275 In other toxicology studies the biological efficacy of thiamethoxam is said to be comparable
276 to other neonicotinoids (Nauen et al., 2003), but relative toxicity is somewhat inconsistent
277 among studies and species. For example, the LD_{50} for bees was lower for imidacloprid than
278 thiamethoxam in topical and oral toxicity studies (Iwasa et al., 2004; Mommaerts et al.,
279 2010), but higher when other beneficial arthropods and pest species were tested (Magalhaes
280 et al., 2008; Prabhaker et al., 2011). Our study indicates that bumble bees may be less
281 sensitive to thiamethoxam than imidacloprid at dosages in the realistic range typically found
282 in nectars of treated crops (approximately 1–11 μg kg^{-1}; Dively and Kamel, 2012; EFSA,
283 2012; Stoner and Eitzer, 2012). Whereas we found no detectable effect on *B. terrestris*
284 microcolonies of thiamethoxam in this range, a previous study conducted under
285 approximately identical conditions found that dietary imidacloprid was capable of

286 substantively reducing brood production and food consumption in microcolonies at
287 concentrations as low as 1.0 and 2.5 μg kg^{-1}, respectively (Laycock et al., 2012). Similar
288 differences in sensitivity have been demonstrated in aphids, *Myzus spp*., with imidacloprid
289 repressing feeding at concentrations as low as 6 μg L^{-1} (Nauen, 1995; Devine et al., 1999)
290 and thiamethoxam failing to repress feeding even at higher dosages (Cho et al., 2011).
291 However, we note that the *B. terrestris* microcolony studies offer only an approximate
292 comparison. For example, in the present study brood production was lower overall than that
293 observed by Laycock et al. (2012), perhaps because of the intrinsic variation in reproductive
294 success that exists between bumble bee colonies (Müller and Schmid-Hempel, 1992). In
295 future work it will be important to compare the sensitivity of bumble bees from the same
296 colony.
297
298 Differential sensitivity may be due to imidacloprid producing a stronger repression of feeding
299 in bumble bees than thiamethoxam at field-realistic dosages (Cresswell et al., 2012; Laycock
300 et al., 2012). Such differences perhaps arise because of thiamethoxam binding to target sites
301 that are distinct from those of imidacloprid (Kayser et al., 2004; Wellmann et al., 2004;
302 Thany, 2011) or because imidacloprid has a greater affinity for insect nicotinic acetylcholine
303 receptors (nAChRs) (Wiesner and Kayser, 2000). However, while imidacloprid is only a
304 partial agonist of native nAChRs in several insects including honey bees (Déglise et al.,
305 2002; Brown et al., 2006; Ihara et al., 2006), clothianidin is a 'super' agonist of *Drosophila*
306 nAChRs (Brown et al., 2006) and has a higher agonist efficacy than imidacloprid in
307 cockroach nAChRs (Ihara et al., 2006). We assume that thiamethoxam is metabolised to
308 clothianidin in bumble bees as it is in other organisms (Nauen et al., 2003), but whether the
309 metabolite is a superior agonist of bumble bee nAChRs is currently unknown. If clothianidin
310 has the higher agonist efficacy in bumble bees, the differential sensitivity we observe may be

311 attributable to the superior hydrophobicity of imidacloprid (Ihara et al., 2006), which could
312 determine the neonicotinoids' accessibility to the receptor and therefore its insecticidal
313 potency (Ihara et al., 2006). While our results show that differential sensitivity of bumble
314 bees to neonicotinoids is possible, further research is required to understand the mechanistic
315 basis of this phenomenon.
316
317 *4.3 Environmental relevance*
318 In our study, realistic dietary residues of thiamethoxam between 1 and 11 μg kg^{-1} had no
319 detectable effect on the performance of bumble bee workers in microcolonies. We extrapolate
320 our results to wild bumble bee populations with caution because additional work is clearly
321 necessary to determine the impact of thiamethoxam on bumble bee queens and their colonies.
322 We also note that our study considers only the effects of dietary thiamethoxam in nectar and
323 not pollen. Furthermore, we test an exposure period of 17 days, whereas environmental
324 exposure could extend across a month or more as bumble bees forage on mass-flowering
325 crops throughout their bloom (Westphal et al. 2009). Consequently, we may underestimate
326 the effects of field-realistic exposures. However, our failure to detect an effect in this range is
327 consistent with a recent field study in which *B. terrestris* colonies reproduced new queens
328 successfully despite being found to contain stored forage comprising thiamethoxam at an
329 average of 2.4 μg kg^{-1} in nectar and 0.7 μg kg^{-1} in pollen (Thompson et al., 2013).
330
331 Our findings suggest that environmentally realistic residues of imidacloprid have the
332 potential to make a greater impact on bumble bees than residues of thiamethoxam, which
333 could have important implications for future neonicotinoid usage in agriculture. However,
334 further research is required to establish thiamethoxam's impact on queenright colonies in
335 wild populations.

Acknowledgements

Ian Laycock was supported by a studentship from the Natural Environment Research Council (NERC).

Disclosure statement

Research into bee health in the J.E.C. laboratory, unrelated to the work presented here, is funded in part by Syngenta.

References

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D, Settele, J., Kunin, W.E., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313**,** 351–354.

Blacquière, T., Smagghe, G., Gestel, C.A.M., Mommaerts, V., 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21**,** 973–992.

Brown, L.A., Ihara, M., Buckingham, S.D., Matsuda, K., Sattelle, D.B., 2006. Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J. Neurochem. 99, 608–615.

Burkle, L.A., Marlin, J.C., Knight, T.M., 2013. Plant-pollinator interactions over 120 years: loss of species, co-occurrence and function. Science 339, 1611–1615.

Cho, S.-R., Koo, H.-N., Yoon, C., Kim, G.-H., 2011. Sublethal effects of flonicamid and thiamethoxam on green peach aphid, *Myzus persicae* and feeding behavior analysis. J. Korean Soc. Appl. Biol. Chem. 54**,** 889–898.

Cresswell, J.E., Page, C.J., Uygun, M.B., Holmbergh, M., Li, Y., Wheeler, J.G., Laycock, I., Pook, C.J., Hempel de Ibarra, N., Smirnoff, N., Tyler, C.R., 2012. Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). Zoology 115, 365–371.

Déglise, P., Grünewald, B., Gauthier, M., 2002. The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. Neurosci. Lett. 321, 13–16.

Devine, G.J., Harling, Z.K., Scarr, A.W., Devonshire, A.L., 1999. Lethal and sublethal effects of imidacloprid on nicotine-tolerant *Myzus nicotianae* and *Myzus persicae*. Pestic. Sci. 48**,** 57–62.

368 Dively, G.P., Kamel, A., 2012. Insecticide residues in pollen and nectar of a cucurbit crop
369 and their potential exposure to pollinators. J. Agric. Food Chem. 60**,** 4449–4456.
370 EFSA, 2012. Statement on the findings in recent studies investigating sub-lethal effects in
371 bees of some neonicotinoids in consideration of the uses currently authorised in Europe.
372 EFSA J. 10**,** 2752.
373 EFSA, 2013a. EFSA press release – EFSA identifies risks to bees from neonicotinoids.
374 European Food Safety Authority website, URL:
375 http://www.efsa.europa.eu/en/press/news/130116.htm. Accessed 11 September 2013.
376 EFSA, 2013b. EFSA Guidance Document on the risk assessment of plant protection products
377 on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA J. 11, 3295.
378 Elbert, A., Haas, M., Springer, B., Thielert, W., Nauen, R., 2008. Applied aspects of
379 neonicotinoid uses in crop protection. Pest Manag. Sci. 64**,** 1099–1105.
380 Elston, C., Thompson, H.M., Walters, K.F.A., 2013. Sub-lethal effects of thiamethoxam, a
381 neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in
382 bumblebee (*Bombus terrestris*) micro-colonies. Apidologie 44, 563–574.
383 European Commission, 2013. EUROPA press release – Bees & pesticides: commission to
384 proceed with plan to better protect bees. EUROPA website, URL:
385 http://europa.eu/rapid/press-release_IP-13-379_en.htm. Accessed 11 September 2013.
386 FERA, 2013. Pesticide usage statistics. Food and Environment Research Agency website,
387 URL: http://pusstats.fera.defra.gov.uk. Accessed 11 September 2013.
388 Franklin, M.T., Winston, M.L., Morandin, L.A., 2004. Effects of clothianidin on *Bombus*
389 *impatiens* (Hymenoptera: Apidae) colony health and foraging ability. J. Econ. Entomol.
390 97**,** 369–373.
391 Gill, R.J., Ramos-Rodriguez, O., Raine, N.E., 2012. Combined pesticide exposure severely
392 affects individual-and colony-level traits in bees. Nature 491, 105–108.

393 Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J.,
394 Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success
395 and survival in honey bees. Science 336**,** 348–350.
396 Hoyle M., Hayter K., Cresswell, J.E., 2007. Effect of pollinator abundance on self-
397 fertilization and gen flow: application to GM canola. Ecol. Appl. 17, 2123–2135.
398 Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. J. Comput.
399 Graph. Stat. 5**,** 299–314.
400 Ihara, M., Brown, L.A., Ishida, C., Okuda, H., Sattelle, D.B., Matsuda, K., 2006. Actions of
401 imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors
402 of American cockroach neurons and their relationships with insecticidal potency. J. Pestic.
403 Sci. 31, 35–40.
404 Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential
405 toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Prot. 23**,** 371–
406 378.
407 Kayser, H., Lee, C., Decock, A., Baur, M., Haettenschwiler, J., Maienfisch, P., 2004.
408 Comparative analysis of neonicotinoid binding to insect membranes: I. A structure–
409 activity study of the mode of [^{3}H] imidacloprid displacement in *Myzus persicae* and *Aphis*
410 *craccivora*. Pest Manag. Sci. 60**,** 945–958.
411 Lambert, D., 1992. Zero-inflated poisson regression, with an application to defects in
412 manufacturing. Technometrics 34**,** 1–14.
413 Laycock, I., Lenthall, K.M., Barratt, A.T., Cresswell, J.E., 2012. Effects of imidacloprid, a
414 neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*).
415 Ecotoxicology 21, 1937–1945.

416 Magalhaes, L.C., Hunt, T.E., Siegfried, B.D., 2008. Development of methods to evaluate
417 susceptibility of soybean aphid to imidacloprid and thiamethoxam at lethal and sublethal
418 concentrations. Entomol. Exp. Appl. 128**,** 330–336.
419 Maxim, L., van der Sluijs, J., 2013. Seed-dressing systemic insecticides and honeybees, in:
420 European Environment Agency, (Ed.), Late lessons from early warnings: Science,
421 precaution, innovation. URL: http://www.eea.europa.eu/publications/late-lessons-2.
422 Accessed 11 September 2013.
423 Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G., Smagghe, G., 2010. Risk
424 assessment for side-effects of neonicotinoids against bumblebees with and without
425 impairing foraging behavior. Ecotoxicology 19**,** 207–215.
426 Müller, C.B., Schmid-Hempel, P., 1992. Correlates of reproductive success among field
427 colonies of *Bombus lucorum*: the importance of growth and parasites. Ecol. Entomol. 17,
428 343–353.
429 Nauen, R., 1995. Behaviour modifying effects of low systemic concentrations of
430 imidacloprid on *Myzus persicae* with special reference to an antifeeding response. Pestic.
431 Sci. 44**,** 145–153.
432 Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V.L., Kaussmann, M., 2003. Thiamethoxam
433 is a neonicotinoid precursor converted to clothianidin in insects and plants. Pestic.
434 Biochem. Physiol. 76**,** 55–69.
435 Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010.
436 Global pollinator declines: Trends, impacts and drivers. Trends Ecol. Evol. 25**,** 345–353.
437 Prabhaker, N., Castle, S.J., Naranjo, S.E., Toscano, N.C., Morse, J.G., 2011. Compatibility of
438 two systemic neonicotinoids, imidacloprid and thiamethoxam, with various natural
439 enemies of agricultural pests. J. Econ. Entomol. 104**,** 773–781.

440 Schneider, C.W., Tautz, J., Grünewald, B., Fuchs, S., 2012. RFID tracking of sublethal
441 effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. PLoS
442 One 7**,** e30023.
443 Shardlow, M., A review of recent research relating to the impact of neonicotinoids on the
444 environment. Buglife, URL: http://smallbluemarble.org.uk/wp-
445 content/uploads/2012/12/Buglife-A-review-of-recent-research-relating-to-the-impact-of-
446 neonicotinoids-on-the-environment.pdf.. Accessed 11 September 2013.
447 Stoner, K.A., Eitzer, B.D., 2012. Movement of soil-applied imidacloprid and thiamethoxam
448 into nectar and pollen of squash (*Cucurbita pepo*). PLoS ONE 7**,** e39114.
449 Tasei, J.-N., Lerin, J., Ripault, G., 2000. Sub-lethal effects of imidacloprid on bumblebees,
450 *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. Pest. Manag.
451 Sci. 56**,** 784–788.
452 Thany, S.H., 2011. Thiamethoxam, a poor agonist of nicotinic acetylcholine receptors
453 expressed on isolated cell bodies, acts as a full agonist at cockroach cercal afferent/giant
454 interneuron synapses. Neuropharmacology 60**,** 587–592.
455 Thompson, H., Harrington, P., Wilkins, S., Pietravalle, S., Sweet, D., Jones, A., (FERA)
456 2013. Effects of neonicotinoid seed treatments on bumble bee colonies under field
457 conditions. Food and Environment Research Agency website, URL:
458 http://www.fera.defra.gov.uk/scienceResearch/scienceCapabilities/chemicalsEnvironment/
459 documents/reportPS2371V4a.pdf. Accessed 11 September 2013.
460 Vanbergen, A.J., Insect Pollinators Initiative (IPI), 2013. Threats to an ecosystem service:
461 pressures on pollinators. Front. Ecol. Environ. 11, 251–259.
462 Wellmann, H., Gomes, M., Lee, C., Kayser, H., 2004. Comparative analysis of neonicotinoid
463 binding to insect membranes: II. An unusual high affinity site for [^{3}H] thiamethoxam in
464 *Myzus persicae* and *Aphis craccivora*. Pest Manag. Sci. 60**,** 959–970.

Westphal, C., Steffan-Dewenter, I., Tscharntke, T., 2009. Mass flowering oilseed rape improves early colony growth but not sexual reproduction of bumblebees. J. Appl. Ecol. 46, 187–193.

Whitehorn, P.R., O'Connor, S., Wackers, F.L., Goulson, D., 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336**,** 351–352.

Wiesner, P., Kayser, H., 2000. Characterization of nicotinic acetylcholine receptors from the insects *Aphis craccivora*, *Myzus persicae*, and *Locusta migratoria* by radioligand binding assays: relation to thiamethoxam action. J. Biochem. Mol. Toxicol. 14**,** 221–230.

Williams, P.H., Osborne, J.L., 2009. Bumblebee vulnerability and conservation world-wide. Apidologie 40**,** 367–387.

Winfree, R., 2010. The conservation and restoration of wild bees. Ann. N.Y. Acad. Sci. 1195**,** 169–197.

Table 1 Frequency of successful oviposition in *Bombus terrestris* bumble bee microcolonies, with the number of brood (eggs and larvae) produced by successful ovipositors and the time at which first oviposition occurred. Microcolonies ($N = 100$) were presented with thiamethoxam (TMX) in feeder syrup at given dosages for 17 days (replicates per dosage group: control, $N = 19$; dosage treatments, $N = 9$ per concentration). *Per capita* consumption of TMX in microcolonies is provided for each dosage treatment. Only data from the 44% (44/100) of microcolonies that produced brood is shown in successful oviposition, brood given oviposition and day of first oviposition columns. Except for successful oviposition, data represent the mean ± SE. We found no detectable effect of dosage on brood production or timing of oviposition in successfully ovipositing microcolonies (Spearman's correlation, $P > 0.05$).

TMX dosage ($\mu g\ kg^{-1}$ / ppb)	TMX consumed ($ng\ bee^{-1}\ day^{-1}$)	Successful oviposition (%)	Brood, given oviposition	Day of first oviposition
Control	0.000 ± 0.000	63	5.4 ± 1.1	10.7 ± 0.7
0.1	0.021 ± 0.002	67	4.3 ± 1.8	11.0 ± 0.5
0.2	0.051 ± 0.003	78	5.7 ± 1.6	13.1 ± 1.5
0.4	0.131 ± 0.004	22	11.0 ± 3.2	9.8 ± 3.5
1.0	0.324 ± 0.027	22	3.0 ± 0.0	9.5 ± 1.5
2.5	0.777 ± 0.068	33	5.3 ± 1.8	11.3 ± 3.6
6.3	1.809 ± 0.085	44	3.3 ± 1.0	12.8 ± 2.6
15.7	5.101 ± 0.509	67	5.0 ± 1.6	11.3 ± 0.6
39.4	7.379 ± 0.602	11	5.0 ± 0.0	12.0 ± 0.0
98.4	14.785 ± 2.076	0	–	–
	All ovipositing microcolonies		5.3 ± 0.6	11.4 ± 0.5

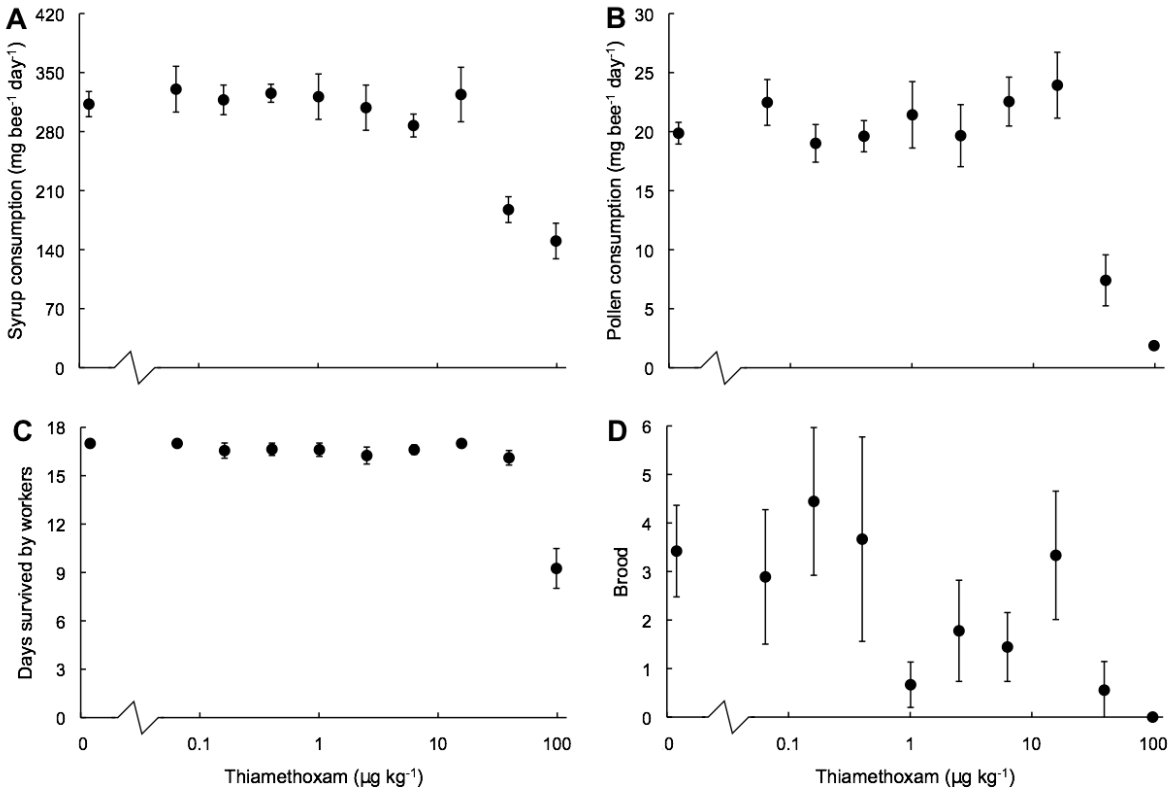
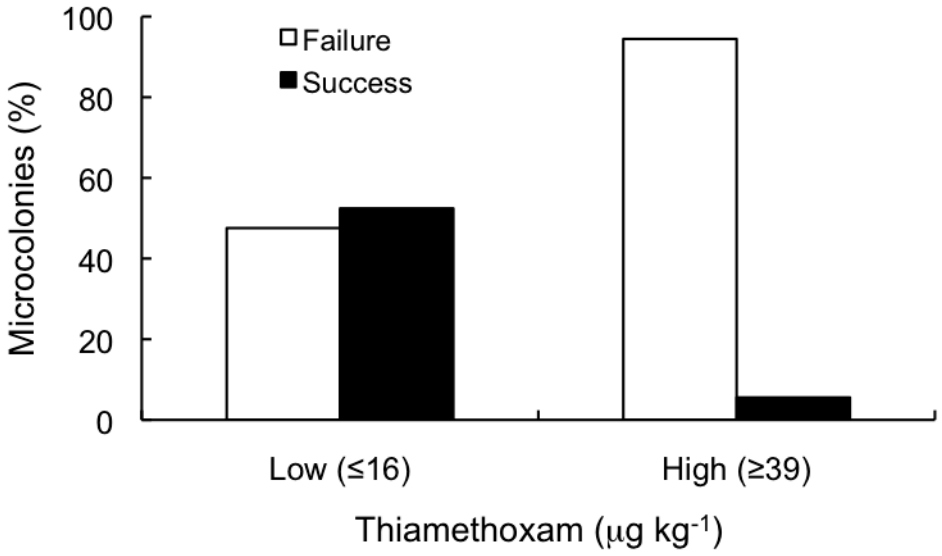


Fig. 1. Daily *per capita* feeding rates, days survived by workers and brood production in *Bombus terrestris* bumble bee microcolonies following 17 days of exposure to thiamethoxam in dosed syrup (µg kg^{-1} = parts per billion). (A) Daily *per capita* consumption of dosed syrup; (B) daily *per capita* consumption of undosed pollen; (C) number of days workers survived while under exposure (maximum = 17 days); and (D) brood production (eggs and larvae produced; data includes microcolonies that failed to oviposit). Data represent the means and error bars indicate ± SE (replicates per dosage group: control, N = 19 microcolonies; dosage treatments, N = 9 microcolonies per concentration). Control data (zero µg kg^{-1}) are displayed slightly displaced on the x-axis for ease of inspection.



500

501 **Fig. 2.** Frequency of oviposition failure and success in *Bombus terrestris* bumble bee
502 microcolonies presented for 17 days with thiamethoxam in dosed syrup (μg kg^{-1} = parts per
503 billion). Low dosage group (N = 82) and high dosage group (N= 18) consist of microcolonies
504 exposed to dietary thiamethoxam at concentrations of ≤ 16 and ≥ 39 μg kg^{-1}, respectively.
505 Open bars represent failure to produce brood (zero brood produced) and filled bars represent
506 success (≥ one brood individual produced). Frequency of oviposition failure in the high
507 dosage group (94%) differed significantly from that in low dosage group (48%; Chi-squared
508 contingency table analysis, $P < 0.001$).