

1 **Field Realistic Doses of Pesticide Imidacloprid Reduce Bumblebee Pollen Foraging Efficiency**

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12 **Abstract**

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14 Bumblebees and other pollinators provide a vital ecosystem service for the agricultural sector. Recent
15 studies however have suggested that exposure to systemic neonicotinoid insecticides in flowering
16 crops has sub-lethal effects on the bumblebee workforce, and hence in reducing queen production.
17 The mechanism behind reduced nest performance, however, remains unclear. Here we use Radio
18 Frequency Identification (RFID) technology to test whether exposure to a low, field realistic dose
19 (0.7ppb in sugar water and 6ppb in pollen) of the neonicotinoid imidacloprid, reduces worker foraging
20 efficiency. Whilst the nectar foraging efficiency of bees treated with imidacloprid was not
21 significantly different than that of control bees, treated bees brought back pollen less often than
22 control bees (40% of trips versus 63% trips, respectively) and, where pollen was collected, treated
23 bees brought back 31% less pollen per hour than controls. This study demonstrates that field-realistic
24 doses of these pesticides substantially impacts on foraging ability of bumblebee workers when
25 collecting pollen, and we suggest that this provides a causal mechanism behind reduced queen
26 production in imidacloprid exposed colonies.

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28

29 **Introduction**

30 Around a third of all human food is thought to depend on insect pollination (McGregor 1976) and
31 many crops benefit from this service, with adequate pollination increasing yields and improving crop
32 quality (Klein et al. 2007). Recently, however, there has been growing concern over the use of
33 neonicotinoid pesticides in agriculture and the sub-lethal effects they can have on pollinators
34 (Decourtye et al. 2004; Desneux et al. 2007; Yang et al. 2008; Aliouone et al. 2009; Henry et al. 2012;
35 Whitehorn et al. 2012, Gill et al. 2012; Williamson & Wright 2013; Di Prisco et al. 2013; Matsumoto
36 2013), which has culminated in an EU-wide restriction on the use of three neonicotinoid pesticides.
37 The ban comes into place in December 2013 and is a temporary, two year measure preventing the use
38 of imidacloprid, clothianidin and thiametoxam until further research can clarify the impact these
39 pesticides are having on bees (European Commission 2013). Sub-lethal effects do not bring about
40 direct mortality but impair an organism's ability to function in some other way, for example by
41 impacting on activity, fecundity, neurophysiology, learning performance or other aspects of behaviour
42 (Desneux et al. 2007; Laycock et al. 2012). There is mounting evidence that neonicotinoid pesticides,
43 formulated to target neurotransmitter receptors in insects, are negatively impacting the foraging
44 behaviour of bees by inducing memory and learning dysfunctions, and impairing navigational skills
45 (Henry et al. 2012; Aliouane et al. 2009; Yang et al. 2008; Decourtye et al. 2004). The continuing
46 decline of pollinators such as bumblebees and honeybees, coupled with an increased focus on
47 sustainable food production means that a greater understanding of the wider impacts of pesticides on
48 pollinators is required.

49 Imidacloprid is the second most widely used agrochemical in the world (Pollak 2011), and is
50 commonly used as a seed dressing to protect crops from insect pests. The pesticides used in these
51 seed dressings are transported throughout the plant via the sap, ending up in both pollen and nectar at
52 concentrations typically ranging from <1 to 10 ppb (parts per billion) (Cresswell 2011; EFSA 2012).
53 Oil seed rape is the second most abundant arable crop grown in the UK in terms of area (Garthwaite et
54 al. 2010) and its flowers are known to attract bumblebees, honeybees and other pollinating insects
55 (Hayter & Cresswell 2006). The majority of growers that produce oilseed rape do so using dressed

56 seeds, with recent figures suggesting that only around 4% of seed sown in the UK remain untreated
57 (Garthwaite et al. 2010). Fields of rape offer a mass flowering crop that blooms for several weeks and
58 bees that forage on these plants are thus exposed over large scales to trace dietary insecticides such as
59 imidacloprid.

60 A recent study by Whitehorn et al. (2012) found that *Bombus terrestris* (Linnaeus 1758) colonies
61 exposed to field realistic doses of imidacloprid (0.7ppb in sugar water and 6ppb in pollen), produced
62 85% fewer queens than control colonies. Bumblebees have an annual cycle, with new queens single-
63 handedly founding the next generation of nests (Goulson 2010). There is evidence that only the most
64 successful nests produce new queens (Muller & Schmid-Hempel 1992) and reductions in the number
65 of queens produced one year will likely have knock on effects for the number of colonies founded the
66 next. However, Whitehorn et al. (2012) did not attempt to elucidate the mechanisms underlying
67 reduced performance of treated colonies, which might include reduced fecundity of the queen, or
68 reduced foraging efficiency of the workers. Gill et al. (2012) provide a possible explanation; they
69 found that exposure to imidacloprid at 10 ppb in sugar water reduced the foraging success of worker
70 bumblebees. However, the highest concentration of imidacloprid found in the nectar of seed-treated
71 oilseed rape to date is 0.8ppb (EFSA 2012). In this study we examined the effect of a lower, more
72 field realistic dose of imidacloprid to determine what effects it may have on the foraging ability of *B.*
73 *terrestris* workers.

74 **Methods**

75 Six commercial *B. terrestris* colonies (Biobest N.V., standard hives) were used to examine the effect
76 of field realistic doses of the pesticide imidacloprid on foraging activity over a four week period.
77 Upon arrival, nests were small, evenly aged and consisted of the queen and up to 65 workers. They
78 contained two internal tanks which supplied the bees with sugar solution during transportation, and
79 these tanks were sealed prior to controlled feeding to ensure that bees only had access to the ‘nectar’
80 provided as part of the experiment. Colonies of approximately equal weights were randomly
81 allocated to either a treatment or control group (three in each). Control colonies were fed ad libitum

82 (ad lib) with pollen and a sugar water solution for a period of 14 days in the laboratory. Over the
83 same period colonies assigned to the treated group were fed pollen and sugar water containing $6\mu\text{g kg}^{-1}$
84 1 and $0.7\mu\text{g kg}^{-1}$ imidacloprid respectively, thus mimicking levels of imidacloprid found in oil seed
85 rape (Bonmatin et al 2005). During the 14 day period bees were provided with no alternative forage.

86 After two weeks of controlled feeding in the lab, all colonies were placed out in the field and the
87 foraging behaviour of bees was monitored over a four week period (07.08.2012 – 04.09.12). The
88 study was carried out in domestic gardens in an urban area of Stirling in the Central-belt region of
89 Scotland. The nearest farmed area was over 1km away. Bees were allowed to acclimatise to their
90 surrounding for 24 hours. After this time the first 12 bees exiting each nest that had undamaged
91 wings were collected. In treated nests this first batch of bees would have been individuals that
92 consumed contaminated pollen and nectar during their adult life, however given the 9 day pupation
93 phase they are not likely to have been larvae reared on food contaminated with imidacloprid (Van Der
94 Steen 2008). We used Radio Frequency Identification (RFID) technology to monitor the foraging
95 duration of individual bumblebees, and an automated system to record the weight of bees entering and
96 exiting the nest. RFID technology is increasingly being used to study the behaviour of insects
97 (Robinson et al. 2009: ants; Streit et al. 2007; Molet et al. 2008: bumblebees; Sumner et al. 2007:
98 paper wasps), and allows an accurate and automated way of monitoring their activity (Ohashi et al.
99 2010). A small RFID tag (mic3@-AG64 bit RO, iID2000, 13.56MHz system, 1.0x1.6x0.5mm;
100 Microsensus GmbH, Erfurt, Germany) weighing 3mg (<3% of the weight of the smallest bee tagged)
101 was glued to the dorsal surface of each bee's thorax. The weight of these tags was small relative to
102 the average weight of nectar and pollen carried by bees; bumblebees are known to carry up to 90% of
103 their own body weight (Goulson et al. 2002). The tags were carefully positioned so that they would
104 not hamper wing movement and bees were then released and left to forage independently for a period
105 of four weeks. Treated and control colonies were randomly paired and each pair were monitored for a
106 24h period every third day (approximately). A fully automated system was set up to record the time
107 and weight of bees departing from and returning to the nest: in a set-up similar to that used by Stelzer
108 et al. (2010) a system of 2cm tubes were used to connect the entrance of each colony to a clear plastic

109 box mounted on top of a balance (weighing to 3 decimal places). A small clearance gap was left
110 between the tube system and the weighing box to ensure that only the weight of bees in the box was
111 recorded. In most cases the time it took for bees to traverse the box was sufficient to get a stable
112 reading. However on about a third of occasions there were multiple bees in the box at one time which
113 meant it was not possible to obtain an accurate weight of any one individual bee. If this occurred
114 when a bee was returning to the nest, the trip was excluded from the analysis of weight data.
115 However if it occurred when a bee was leaving the nest then an average weight of that bee was
116 obtained from other departures made during the monitoring period, and this was used as proxy in the
117 analysis. After traversing the box, bees then entered another length of tube leading them to the
118 outside. RFID readers were mounted between the nest entrance and the first length of tube, which
119 recorded the exact time bees entered and exited the nest. A motion detecting camera was set up to
120 record the weight of bees as they passed over the balance, and to determine if bees returned carrying
121 pollen. As in Stelzer et al. (2010), any trips that lasted for five minutes or less as well as those flights
122 where bees lost mass were excluded from analysis as the majority of these were likely to have been
123 orientation or defecation flights. These trips only accounted for a small number of the total trips
124 recorded and numbers were similar in treated and control colonies (only 15 trips in total, nine for
125 treated and 8 for control bees). Additionally any trips over four hours in duration (seven in total, three
126 for treated and four for control bees) were excluded from the analysis as these often occurred on rainy
127 days where bees may have been prevented from returning to the nest due to adverse weather.

128 Fourteen days into the four week data capture period a further 12 bees from each nest were tagged.
129 The development of *B.terrestris* workers in laboratory conditions includes ~14 day of larval
130 development during which larvae are frequency fed, followed by ~9 days as a pupa. Thus in treated
131 colonies, bees tagged at this point were likely to have been those reared on the pollen and nectar
132 dosed with imidacloprid. At the mid-way stage of the experiment one control nest and one treated
133 nest had to be removed from the experiment due to wax moth infestations; therefore no further bees
134 were tagged in each of these colonies.

135 All statistical analyses were conducted using R version 2.15 (R Development Team, 2012).
136 Generalised Linear Mixed Effects models (GLMM; fitted by maximum likelihood using the lme4
137 package) with Gaussian errors were used to test the effect of imidacloprid treatment on, trip duration,
138 weight of forage collected and the foraging efficiency (mg of forage collected per hour) of individual
139 bees. The time of day for each trip and the number of days since each bee was tagged were included
140 as covariates along with treatment as a fixed factor. Individual and colony I.D. were included as
141 random factors to account for pseudo-replication between and within colonies. 'Batch' (whether the
142 bees were tagged at the start of week one, or the start of week three) was also included as a fixed
143 factor. All two way interactions were included in the starting model. Factors that did not contribute
144 significantly to the model were removed in a stepwise manner, using $p=0.05$ as a threshold for factor
145 retention or removal. After each simplification step models were assessed using the Akaike's
146 information criterion (AIC; Akaike, 1974). When modelling both pollen and nectar foraging
147 efficiency the most parsimonious model determined using the stepwise approach matched the model
148 with the lowest AIC value.

149

150 A GLM, with quasibinomial errors to allow for over dispersion, was used to determine if there was a
151 significant difference in the proportion of trips in which treated and control bees returned with pollen.
152 The number of trips in which bees returned with pollen over the number of returns without pollen was
153 modelled as the response variable and treatment and batch were included as fixed effects. Nest was
154 included as a fixed effect in this analysis as, due to the relatively small sample size, models including
155 nest as a random effect were unable to correctly separate out nest effects that were not due to
156 treatment.

157

158 A Pearson's correlation was used to examine the relationship between time spent foraging and the
159 weight of the load collected during each bout. Unless otherwise stated all averages are means \pm
160 standard deviation.

161

162 **Results**

163 Between 07 August 2012 and 04 September 2012 data were gathered from 256 foraging bouts; 21
164 foragers from control colonies were recorded making 113 foraging trips (5.4 ± 1.4 trips per bee), and
165 24 foragers from treated colonies made a total of 142 trips (5.96 ± 1.9 trips per bee). During the
166 course of the study two bees from the treated group and one bee from the control group failed to
167 return to the nest. There was no difference in the lifespan of bees from treatment and control groups,
168 with all tagged bees (with the exception of the three that failed to return to the nest) surviving until the
169 end of the study.

170 Control bees spent on average 25.44 ± 6.1 minutes foraging for nectar, with a mean weight of $42.6 \pm$
171 9.86 mg collected per bout, resulting in a nectar foraging rate of 101 ± 10.68 mg/hr⁻¹. This was not
172 significantly different from the nectar foraging rate of treated bees (GLMM: $\chi^2=0.534$, d.f=1,
173 $p=0.464$; Figure 1a) who spent on average 27.26 ± 8.4 minutes foraging for nectar, bringing back 44.7
174 ± 12.49 mg of nectar per bout resulting in a foraging rate of 99.24 ± 9.67 mg/hr⁻¹. Neither treatment
175 nor any of the other proposed explanatory variables; time of day, batch and number of days since the
176 bee was tagged, were significant in explaining trip duration, weight of nectar collected or nectar
177 foraging efficiency.

178 The average length of time spent on pollen foraging trips (trips in which the returning bee had visible
179 pollen loads) was 73.8 ± 14.38 minutes for control bees with a mean weight of 57.32 ± 11.22 mg
180 being collected per bout, resulting in a pollen foraging rate of 47.71 ± 7.62 mg/hr⁻¹. The mean length
181 of time spent on pollen foraging trips in treated bees was 77.85 ± 24.96 minutes, with the minimal
182 model for trip duration including treatment, the number of days since the bee was tagged and the
183 interaction between the two (GLMM: $\chi^2=9.99$, d.f=1, $p<0.01$). Trip duration in control bees remained
184 approximately constant throughout the experiment, however in treated bees the duration of trips
185 increased with time from tagging. The mean weight of pollen collected per bout by treated bees was
186 41.07 ± 12.72 mg, with treated bees bringing back significantly less pollen than control bees (GLMM:
187 $\chi^2=4.76$, d.f=1, $p <0.01$), with no other factors remaining in the minimal model. This resulted in a
188 mean foraging rate for pollen of 32.97 ± 9.43 mg/hr⁻¹, a 31% reduction compared to control bees
189 (GLMM: $\chi^2=18.06$, d.f=1, $p <0.001$; Figure 1b). There was no significant effect of time of day, batch

190 and number of days since individual bees were tagged in explaining pollen foraging efficiency.
191 Treated bees were also significantly less likely than control bees to return to the nest carrying pollen
192 (41% vs 65% of foraging bouts respectively; $t = -2.135$, $n=42$, $p<0.05$).

193 The positive correlation between time spent foraging for pollen and the amount of forage collect was
194 significant in both treated bees ($r=0.576$, $n=57$, $p<0.001$) and control bees ($r=0.729$, $n=71$, $p<0.001$).

195 In accordance with Whitehorn et al. (2012), treated nests gained less weight than control nests and
196 also produced fewer workers and queens, but the number of nests used in our experiment was too few
197 to permit meaningful statistical analysis of these differences.

198 **Discussion**

199 This study strongly corroborates the findings of previous studies, and shows that the neonicotinoid
200 imidacloprid can have sub-lethal effects on free-flying worker bumblebees, and thus is likely to
201 reduce colony success. Here we were able to quantify for the first time the change in bumblebee
202 foraging efficiency as a result of field-realistic measures of imidacloprid exposure showing that, on
203 pollen gathering trips, treated bees brought back 31% less forage per hour than controls, representing
204 a significant reduction in efficiency. This is in accordance with the findings of Gill et al. (2012) who
205 ranked the pollen loads of bumblebees returning to the nest as small, medium or large and found that
206 imidacloprid exposed bees brought back proportionally more small loads than unexposed bees. Gill et
207 al. (2012) also found that imidacloprid exposed bees collected pollen on 59% of their foraging bouts,
208 versus control bees that collected pollen on 82% of occasions (a 28% decrease). Using a lower, field
209 realistic dose we found that bees exposed to imidacloprid showed a 23% reduction in the frequency of
210 pollen-collecting trips, compared to controls.

211 Pollen is the main protein source for bumblebees and is particularly important for the rearing of young
212 to replace older workers (Harder 1990). It has been suggested that foraging for pollen is more
213 challenging than foraging for nectar (Raine & Chittka 2007), and it is usually restricted to dry, sunny
214 weather, whereas nectar can be collected in most conditions except heavy rain (Peat & Goulson
215 2005), so that pollen rather than nectar shortages are more likely to limit colony success (Goulson

216 2010). This is reflected in the lower foraging efficiency of bees when gathering pollen versus nectar
217 (Peat & Goulson 2005). Using the same concentrations of imidacloprid as the current study (6ppb
218 pollen and 0.7ppb nectar), and the same two-week exposure period, Whitehorn et al. (2012) found an
219 85% reduction in queen production in colonies exposed to imidacloprid. Developing queens are
220 known to require more food during their developmental period and thus queen production is likely to
221 suffer as a result of lower provisions of pollen. Whitehorn et al. (2012) also found that colonies
222 exposed to imidacloprid gained significantly less weight over time than control colonies. In previous
223 work studying *B. lucorum*, a species closely related to *B. terrestris*, a positive correlation was found
224 between nest size and queen production (Muller & Schmid-Hempel 1992). Hence our data provide a
225 simple mechanism for the dramatic declines in queen production described by Whitehorn et al.
226 (2012); a substantially reduced pollen supply to the colony.

227 In this experiment we made the assumption that if a bee returned with a visible pollen load then any
228 increase in weight recorded was due to the pollen it had collected whilst foraging. In some cases
229 however it is likely that bees foraged for a mixture of pollen and nectar. It is thus possible that the
230 lower weight of forage brought back by bees exposed to pesticide was due to reduced nectar
231 collection, or a combination of reduced pollen collection and reduced nectar collection. The former
232 seems less likely since bees which returned only with nectar showed no significant impact of pesticide
233 treatment.

234 It is worth noting that in the present study bumblebees were kept in the lab for two weeks and treated
235 colonies were given no alternative but to feed upon pollen and nectar dosed with imidacloprid. This
236 is perhaps unrealistic of field conditions as bees would normally be free to forage on a range of
237 contaminated and uncontaminated resources. However, oil seed rape is the third most abundant
238 arable crop grown in the UK (after wheat and barley) with the production area for this crop having
239 increased by 17.75% between 2010 and 2012, representing an increase of 114,000 hectares
240 (Garthwaite 2010; DEFRA 2012). Rape is known to flower for around 3-4 weeks providing an
241 abundant, if short-lived floral resource (Goulson et al. 2010). Both bumblebees and honeybees feed
242 on oil seed rape (Hayter & Cresswell 2006) and given the general decline in floral resources in the

243 countryside (Carvell et al. 2006) it is likely that, whilst in flower, oil seed rape constitutes a large
244 component of many bees' diets. A recent study by Thompson et al (2013) that examined the effects
245 of three neonicotinoids on bumblebee colonies in field conditions failed to establish a negative
246 control, demonstrating that these substances are widespread in agricultural environments.
247 Furthermore, substantial concentrations of neonicotinoids (up to 9ppb) have been found in
248 wildflowers growing near to treated crops (Krupke et al. 2012), suggesting that exposure to these
249 types of pesticide might not be restricted to bees foraging on the crops themselves. Hence we suggest
250 that the level of exposure used here is likely to approximate that experienced by some wild bumblebee
251 nests under field conditions.

252 Interestingly, we found no significant difference in foraging efficiency between the first batch of
253 foragers tagged and the second. This suggests that impaired foraging continued to be seen in bees
254 from treated colonies for at least four weeks after exposure. However, it is not clear whether this
255 occurred because bees continued to be exposed to imidacloprid in honey stored within the nests, or
256 whether the reduced performance of the second batch of foragers was due to exposure as larvae. Yang
257 et al. (2012) describe impaired learning in honeybees following exposure to imidacloprid as larvae.
258 Further studies could clarify the persistence of imidacloprid within the nest and the effects it may
259 have on subsequent generations of workers. Whatever the mechanism, our data suggest that exposure
260 to imidacloprid may reduce worker performance for at least four weeks after the source of exposure is
261 removed.

262 Whilst this study has put forward a mechanism for reduced queen production in imidacloprid exposed
263 colonies (Whitehorn et al. 2012) it is not able to fully explain the mechanism behind the reduced
264 pollen foraging efficiency found in treated bees. In both treated and control bees a positive
265 correlation was found between time spent foraging and the weight of pollen collected and no
266 significant difference was found in the number of bees failing to return to the nest between treatments,
267 which, coupled with the fact that there were no differences found in nectar foraging efficiency,
268 suggests that navigation is not likely to have been the issue. Further studies are needed to clarify how
269 imidacloprid impairs bumblebee's ability to forage for pollen, with the evidence gathered in this study

270 suggesting that the pesticide may either reduce motivation to collect pollen, or impair the bees' ability
271 to collect pollen from flowers, rather than affecting their trips to and from their foraging sites.

272 Agricultural intensification has been proposed as a primary driver behind the decline of bumblebees,
273 with habitat loss as well as increased pesticide use believed to be two important causal factors
274 (Goulson et al. 2008). Whilst this study has focused on the effects of imidacloprid, the uses of other
275 neonicotinoids have also been called into question, since the three most commonly used compounds,
276 imidacloprid, thiamethoxam and clothianidin all have similar modes of action (Nauen et al. 2003). A
277 recent study has reported harmful effects of thiamethoxam on honeybee homing abilities (Henry et al.
278 2012), whilst another found no significant impact of thiamethoxam on colony initiation in bumblebees
279 (Elston et al. 2013). If ecologically and economically important pollinator populations are to be
280 maintained then the advisability of any future use of neonicotinoids on flowering crops must be
281 questioned and further work is needed to clarify their impacts.

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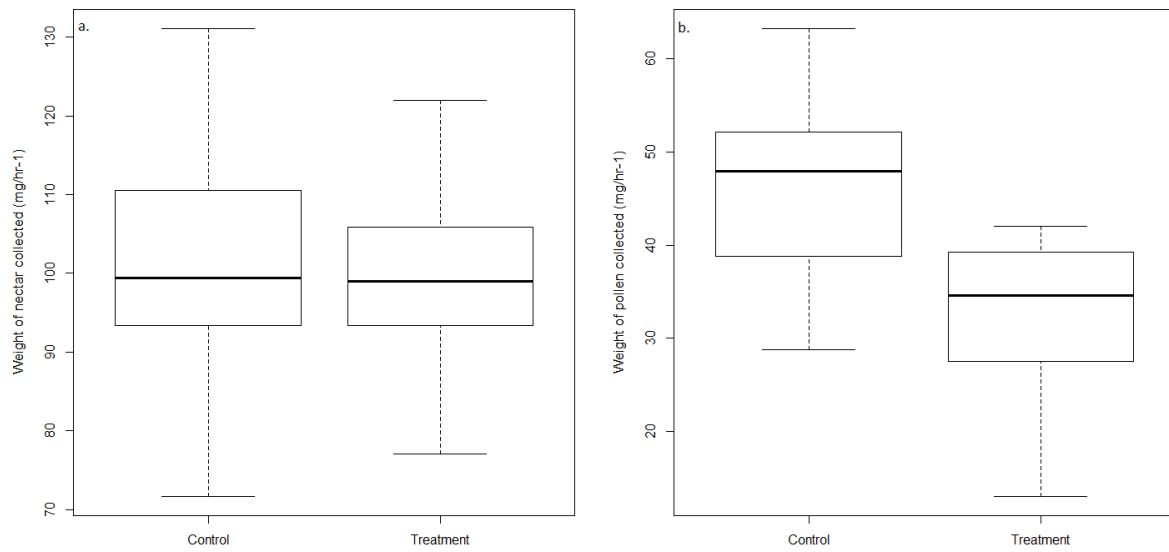
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 391 **Fig. 1** The efficiency of foraging on pollen gathering trips was significantly lower for treated bees
 392 than untreated bees, whilst no significant difference was found in foraging efficiency on nectar
 393 gathering trips. The box plots depict median and interquartile range, with the bars representing the
 394 means of treated and untreated bees and their 95% confidence interval.