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Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*)

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Abstract Bumble bees are important pollinators whose populations have declined over recent years, raising widespread concern. One conspicuous threat to bumble bees is their unintended exposure to trace residues of systemic neonicotinoid pesticides, such as imidacloprid, which are ingested when bees forage on the nectar and pollen of treated crops. However, the demographic consequences for bumble bees of exposure to dietary neonicotinoids have yet to be fully established. To determine whether environmentally realistic levels of imidacloprid are capable of making a demographic impact on bumble bees, we exposed queenless microcolonies of worker bumble bees, *Bombus terrestris*, to a range of dosages of dietary imidacloprid between zero and 125 μg L^{-1} and examined the effects on ovary development and fecundity. Microcolonies showed a dose-dependent decline in fecundity, with environmentally realistic dosages in the range of 1 μg L^{-1} capable of reducing brood production by one third. In contrast, ovary development was unimpaired by dietary imidacloprid except at the highest dosage. Imidacloprid reduced feeding on both syrup and pollen but, after controlling statistically for dosage, microcolonies that consumed more syrup and pollen produced more brood. We therefore speculate that the detrimental effects of imidacloprid on fecundity emerge principally from nutrient limitation imposed by the failure of individuals to feed. Our findings raise concern about the impact of neonicotinoids on wild bumble bee populations. However, we recognize that to fully evaluate impacts on wild colonies it will be necessary to establish the effect of dietary neonicotinoids on the fecundity of bumble bee queens.

Keywords *Bombus terrestris* microcolonies · Demographic toxicology · Fecundity · Neonicotinoid pesticides · Ovary development · Sublethal effects

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Introduction

Animal pollinators play an important role in global food production (Klein et al. 2007) and in maintaining wild plant communities (Kearns et al. 1998; Ashman et al. 2009). Wild and managed bees are important pollinators whose populations have declined over recent years (Goulson et al. 2008; van Engelsdorp et al. 2010; De la Rúa et al. 2009) raising widespread concern (Allen-Wardell et al. 1998; Potts et al. 2010). The detrimental factors affecting bee populations are likely to be multiple and interacting (Williams and Osborne 2009), but one conspicuous threat is their unintended exposure to agricultural pesticides that protect crops from pest herbivores (Desneux et al. 2007). Neonicotinoids, such as imidacloprid, are among the most effective and widely used pesticides employed to control common insect pests such as aphids and whiteflies (Elbert et al. 2008). They are synthetic neurotoxins that act as agonists of nicotinic acetylcholine

receptors to disrupt the nervous system of pests to lethal effect (Matsuda et al. 2001). Applied as a seed dressing or foliar spray, neonicotinoids are taken up by plants and distributed systemically (Sur and Stork 2003) to target pest herbivores that consume sap and plant tissues. Bees are non-target organisms that ingest dietary residues of neonicotinoids in the nectar and pollen of treated mass-flowering crops (Rortais et al. 2005). Bees are exposed to dietary trace residues, defined here as the range up to 10 μg insecticide kg^{-1} (=parts per billion or ppb), when foraging on several crops (reviewed in Blacquière et al. 2012). For example, imidacloprid residues ranging from 1.1 to 5.7 ppb were detected in pollen collected from French honey bee colonies (Chauzat et al. 2006). In treated sunflowers, *Helianthus annuus* L., imidacloprid residues in pollen averaged 3 ppb in field crops and reached 1.9 ppb in nectar and 3.3 ppb in pollen of greenhouse plants (Schmuck et al. 2001; Bonmatin et al. 2003). Pollen from imidacloprid-treated maize, *Zea mays* L., and oilseed rape, *Brassica napus* L., contained residues of 2.1 ppb (Bonmatin et al. 2005) and 1.0 ppb (Cresswell, pers. obs.) respectively, whilst the nectar of *Phacelia tanacetifolia* Benth. contained imidacloprid residues up to 10 ppb when sampled from the honey sac of foraging bees (Decourtye et al. 2003). Clothianidin, another major neonicotinoid, was detected in pollen from treated maize and in wild flowers growing near to treated fields at levels of 3.9 and 9.4 ppb, respectively; however, residues reached 88 ppb (nine times our defined field-realistic range) in pollen collected by honeybees foraging on treated crops (Krupke et al. 2012).

Quantifying population-level responses to a xenobiotic provides an important basis for assessing its potential for ecological impact (Walthall and Stark 1997; Forbes and Calow 1999; Herbert et al. 2004). In protecting the sustainability of a non-target species, we are particularly interested in establishing whether a realistic level of exposure to a pesticide is capable of causing the population to decline. In the past, certain pesticides have proven capable of causing population declines in non-target species and have been implicated as culprits by their evident detrimental effects on demographically relevant variables. For example, the insecticide dichlorodiphenyltrichloroethane (DDT) caused population decline in predatory bird species through reduced fecundity (Grier 1982). By analogy with such cases, some have asserted that neonicotinoids are a cause of bee declines (Hansard 2011), but in actuality the demographic consequences for bees of exposure to trace dietary neonicotinoids have yet to be fully established. In laboratory and semi-field trials on honey bees (*Apis mellifera* L.), trace dietary imidacloprid reduced performance on a variety of measures by between 6 and 20 % (Cresswell 2011), but uncertainty remains over the population-level implications of these effects. In field trials on honey bees, exposure to dietary neonicotinoids impacted on forager survival and colony dynamics in one recent study (Henry et al. 2012), whereas colony persistence was unaffected by neonicotinoids in other studies (Faucon et al. 2005; Cutler and Scott-Dupree 2007; but note, Cresswell (2011) showed these trials only had sufficient statistical power to detect severely detrimental impacts). We therefore further investigated the potential for neonicotinoids to make a demographic impact and, specifically, we examined the effects of trace dietary intake on reproduction in bees.

We focus on bumble bees, *Bombus* spp., which are important pollinators of both agricultural crops (Goulson 2003a) and wild plants (Goulson et al. 2008). While declines among managed honey bee populations in some regions have received widespread recognition (van Engelsdorp et al. 2010; De la Rúa et al. 2009), evidence of population decline among bumble bees has also accumulated (Cameron et al. 2011). In the UK, for example, more than half of extant bumble bee species are rare or in decline (Williams and Osborne 2009). However, it is unclear whether dietary neonicotinoids could be implicated in bumble bee declines because the results of previous investigations are inconsistent. Following laboratory exposure to dietary imidacloprid at 6 or 12 ppb, colonies of the buff-tailed bumble bee, *Bombus terrestris* L., suffered reduced colony growth and queen production whilst developing under field conditions (Whitehorn et al. 2012). Under laboratory conditions, dietary imidacloprid in the range between 6 and 25 ppb affected the survivorship of *B. terrestris* in one study (Tasei et al. 2000), but not in another (Mommaerts et al. 2010). In colonies of the western bumble bee, *Bombus occidentalis* G., reproduction was unaffected by dietary imidacloprid (Morandin and Winston 2003), but in contrast imidacloprid reduced reproductive output in *B. terrestris* (Tasei et al. 2000; Mommaerts et al. 2010). Furthermore, these detrimental effects have been demonstrated principally at dosages above the range that bumble bees encounter in the nectar and pollen of imidacloprid treated crops, so it remains uncertain whether environmentally realistic exposures are capable of making a demographic impact on wild bumble bee populations. We therefore investigated the effect of dietary imidacloprid on brood production in bumble bees, and we tested a range of dosages that included the environmentally realistic range.

In order to investigate the influence of dietary neonicotinoids on brood production, we made use of the capacity of worker bumble bees to produce unfertilized eggs that mature into male drones (Amsalem et al. 2009). In this eusocial species, bumble bee queens normally dominate the reproductive output of the colony and the workers make only a small contribution (Alaux et al. 2004; Lopez-Vaamonde et al. 2004). However, bumble bee workers adaptively upregulate their reproduction in colonies rendered

queenless (Alaux et al. 2007). 'Microcolonies' are nests comprising a small group of worker bees that are allowed to develop, in the absence of a queen, until a worker becomes dominant and begins laying eggs while the others forage and care for brood (Blacquière et al. 2012). This method enables the convenient evaluation of both lethal and sub-lethal effects of dietary neonicotinoids using multiple replicates (Blacquière et al. 2012). In this study, we used queenless microcolonies to evaluate the effects of imidacloprid on ovary development and fecundity in *B. terrestris*.

Methods

Microcolonies

We obtained three domesticated queenright colonies of *B. terrestris*, each consisting of a single queen, approximately 150 workers, and brood at various stages of development (Natupol Beehive; Koppert B.V., Berkel en Rodenrijs, Netherlands). Groups of *B. terrestris* workers rendered queenless develop their ovaries and begin to oviposit after approximately 7 days (Alaux et al. 2007; Amsalem et al. 2009) and we made use of this reproductive plasticity in the laboratory by grouping together workers into queenless microcolonies. We placed 328 individual workers into microcolony boxes in groups of four (second and third trials) or five (first trial). The allocation of workers to microcolonies was randomised, but each microcolony contained workers from the same original queenright colony. Microcolonies were housed in a softwood box (internal dimensions: 120 × 120 × 45 mm) with a plywood base and a transparent acrylic cover with ventilation holes. A central wooden partition separated each box into two equal sized compartments, but workers had access to either compartment through a centrally drilled hole. Additional holes in the side of the box accommodated 2 mL microcentrifuge tubes (Simport, Beloeil, Canada) that were punctured so as to function as sugar syrup feeders. We maintained microcolonies for 14 days in a controlled environment (24–27 °C, 23–43 % relative humidity, 10:14 h light:dark period). Before exposure to imidacloprid, workers were given 24 h to forage ad libitum on control sugar syrup (Attracker: fructose/glucose/saccharose solution, 1.27 kg L^{-1}; Koppert B.V., Berkel en Rodenrijs, Netherlands). During this period we removed a small number of dead bees and replaced them with workers from the same original queenright colony.

Imidacloprid was obtained as a solution in acetonitrile (Dr. Ehrenstorfer GmbH, Augsburg, Germany, product code L 14283700AL). Acetonitrile was removed by evaporation in a vacuum concentrator (ScanSpeed MaxiVac Beta; LaboGene ApS, Lynge, Denmark) and the imidacloprid was resuspended in deionised water before being mixed into syrup. After feeding on control syrup for 24 h, each microcolony was provided with a pollen ball, which was not dosed with imidacloprid, and feeders containing either control syrup or a syrup with one of the following dosages of imidacloprid (units are μg imidacloprid L^{-1}): 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, 0.08. The level of replication was such that we had a total of 6, 3, 5, 5, 7, 17, 7, 5, 6, and 15 microcolonies treated with dosages of 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, 0.08, and 0 μg L^{-1} imidacloprid, respectively. Once dosing began, we monitored microcolonies daily for mortality and brood production and dead bees were no longer replaced. Feeders were weighed each day to measure the consumption of syrup and fresh syrup at the appropriate dosage was provided as required. Pollen balls were prepared by grinding pollen pellets collected from honey bee hives (Werner Seip Bioprodukte, Butzbach, Germany) into a powder and mixing the mass with water to form dough. The pollen balls (mean mass = 5.4 g, SE = 0.03 g) provided workers with a protein source and a substrate for nest building, and they were weighed before and after the experiment to assess pollen consumption. In our analysis, we corrected for evaporation of water from syrup and pollen based on the mass change of several feeders and pollen balls kept in empty microcolony boxes under identical experimental conditions. Three trials each comprising 14 days (1 day of acclimatisation and 13 days of imidacloprid exposure) were conducted between November 2010 and March 2011. Across the entire study, the number of microcolonies originating from a single queenright parent colony was distributed approximately evenly within dosage treatments and across trials.

To verify the concentration of imidacloprid in our doses, we prepared the usual range of experimental dosages, but in water rather than syrup to facilitate analysis. 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) and using methods adapted from Takino and Tanaka (2006). Specifically, samples and standards (10 μL) were injected onto an Eclipse Plus (Agilent Technologies, Santa Clara, CA, USA) C_{18} reverse phase column (150 mm × 2.1 mm, 3.5 μm). Mobile phase A was 2 % acetonitrile + 0.1 % formic acid. Mobile phase B was 95 % acetonitrile + 0.1 % formic acid. The elution conditions were: 0 min—0 % B, 1 min—70 % B, 10 min—80 % B, 10.2 min—100 % B, 12 min—100 % B; with a flow rate of 0.3 mL min^{-1} increasing to 0.45 mL min^{-1} at 10 min. The source N_2 gas temperature was held at 350 °C with a flow of 11 L min^{-1} and a nebulizer pressure of 35 psi. The

capillary voltage was 4 kV. Fragmentor and collision energy voltages were 40 and 20 V, respectively. Imidacloprid was identified and quantified by selected reaction monitoring (SRM) using the product ion *m/z* 209 derived from the precursor ion of *m/z* 256. Samples of each dosage were spiked with a reference standard of 100 mg L^{-1} [^{2}H]imidacloprid (Sigma-Aldrich, Gillingham, UK). The deuterated imidacloprid was detected using a precursor ion *m/z* of 260 and a product ion *m/z* of 213. Imidacloprid concentrations in the dilution series were quantified by comparing peak areas from [^{2}H]imidacloprid to peak areas of non-labelled imidacloprid in SRM chromatograms. The instrument response was linear over the range 0.061–125 μg L^{-1} imidacloprid and we found that all dosages contained appropriate levels of imidacloprid (*measured imidacloprid* = 1.14 × *nominal dosage* + 0.86: $r^2 > 0.99$).

Ovary development and fecundity

After 14 days, we killed the worker bees and removed their laid eggs and larvae. The bees were dissected in phosphate-buffered saline to remove their ovaries. Each worker ovary contained four ovarioles and in each ovariole there were several oocytes. Using image analysis software (ImageJ; http://rsbweb.nih.gov/ij/), we measured the length, width and area of each intact terminal (proximal) oocyte from each dissected ovary (mean number of oocytes measured per ovary = 3.8, SE = 0.04) and each laid egg. The mean size of all intact terminal oocytes per ovary and the size of the largest terminal oocyte per bee were taken as measures of ovary development. We removed a forewing from each worker and recorded the length of the radial cell as a proxy for body size (Medler 1962). We also dissected and measured the oocytes of 10 workers taken directly from their original queenright colony in order to calculate the change in ovary development of workers during their time in the microcolony. All microcolony dissections, ovary dissections, and oocyte measurements were performed by operators who were unaware of the imidacloprid dosage that corresponded to the specimens.

Statistical analyses

To test whether the fecundity of *B. terrestris* microcolonies responded to dietary imidacloprid, we fitted a relationship between brood production and dosage with a Poisson error structure and a 'random effects' term to account for overdispersion. Specifically, we fitted a Bayesian Hierarchical Model (BHM) as follows: $brood \sim \text{Poisson}(\mu)$; and $\log(\mu) \sim \alpha + \beta * \log(dosage) + \lambda$. Here, α and β are fitted coefficients, which are analogous to the conventional regression coefficients of slope and intercept; and the 'random effects' term, λ, has a normal distribution with a mean of zero. We fitted the BHM using WinBugs (Lunn et al. 2000) and obtained 95 % confidence intervals with 20,000 iterations of Bayesian inference using a Markov Chain Monte Carlo method with Gibbs sampling.

To estimate the number of workers in each microcolony with mature oocytes inside their ovaries, we compared the longest terminal oocyte inside each bee's ovaries to the mean length of eggs laid in microcolonies fed control syrup (mean length = 3.0 mm, SE = 0.02 mm, $N = 146$). A worker was deemed to have mature oocytes if the length of the longest terminal oocyte in its ovaries was at least 3 mm.

Among microcolonies, we analyzed variation in mean oocyte size and number of workers with mature oocytes due to imidacloprid dosage using one-way Analysis of Variance (ANOVA) because the dose–response relationship was non-linear, which precluded the use of ANCOVA. Where ANOVA detected a significant response to dosage, we compared between selected dosage groups with orthogonal contrasts.

In order to test for dosage-independent effects on fecundity and ovary development of variation among microcolonies in feeding rate and body size, we used partial correlation analysis to control for imidacloprid dosage. All statistical analyses were conducted in R version 2.10 (Ihaka and Gentleman 1996).

Results

In our experiment, microcolonies began to lay eggs after approximately 7 days. After 14 days, microcolonies had laid up to 39 eggs and some offspring had progressed to a larval stage. In our analyses, we take the fecundity of a microcolony during the experimental period to be the total number of the brood it produced comprising both laid eggs and larvae. Levels of fecundity and the effects of dosage were highly similar in the three experimental trials and the data were pooled for analysis. During the 13 days of imidacloprid exposure, total mortality comprised one dead worker in a single microcolony exposed to imidacloprid at 125 μg L^{-1}.

Dosage effects

Worker fecundity declined significantly with increasing dosage of dietary imidacloprid (Spearman's $\rho = -0.61$, $N = 76$, $P < 0.001$; Fig. 1). Using BHM, the best description of the dose–response relationship was $brood = \exp[2.49 - 1.84 * \log(dosage)]$ and the overdispersion parameter was estimated as $\lambda = 1.39$. Based on this relationship, exposure to imidacloprid at an environmentally realistic level of 1 μg L^{-1} (=1.27 ppb) results in a

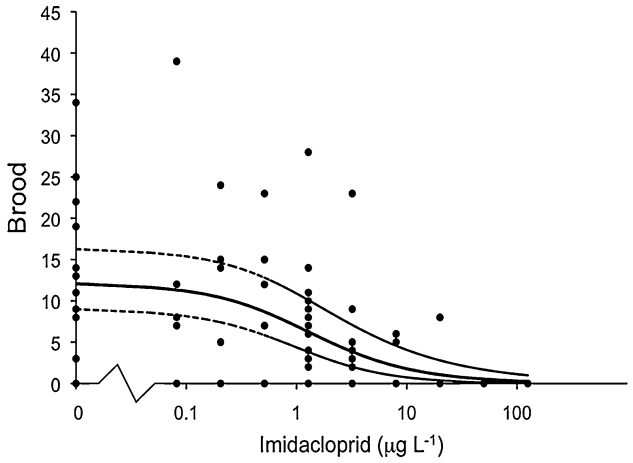

Fig. 1 Fecundity (*y*-axis: number of brood individuals per microcolony) of *B. terrestris* worker microcolonies ($N = 76$) after 14 days, including 13 days of exposure to dietary imidacloprid in dosed sugar syrups (*x*-axis: concentration of imidacloprid in syrup in µg L^{-1}). The *solid line* indicates the best-fit dose–response relationship and the *dashed lines* indicate the relationship's 95 % confidence intervals

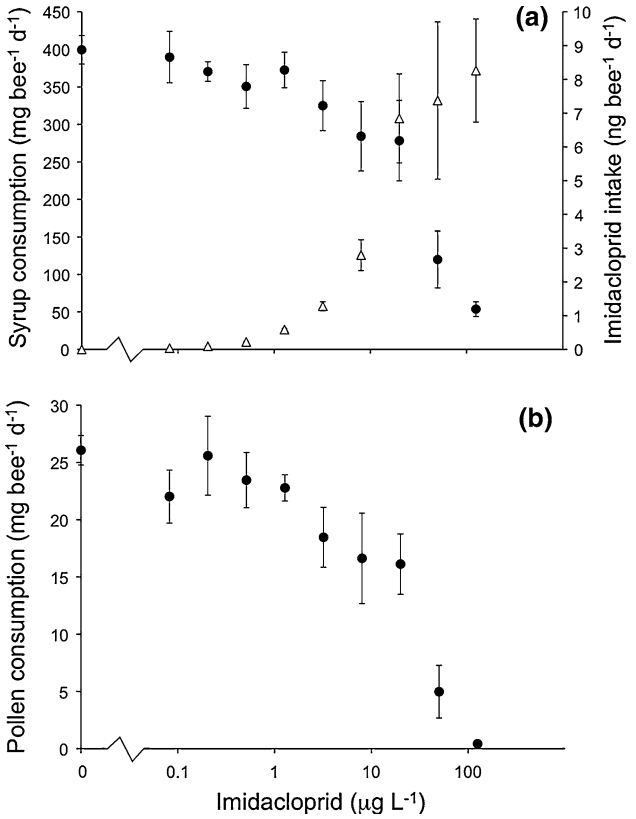

Fig. 2 **a** Daily syrup feeding rate (left *y*-axis: mean *per capita* feeding rate of microcolony in mg, denoted by *circular symbol*) and daily imidacloprid intake (right *y*-axis: mean *per capita* imidacloprid intake in microcolony in ng, *triangle*) in *B. terrestris* worker microcolonies ($N = 76$) fed for 13 days on imidacloprid-treated sugar syrup (*x*-axis: concentration of imidacloprid in syrup in µg L^{-1}) and untreated pollen. **b** Daily pollen feeding rate (*y*-axis: mean *per capita* feeding rate in microcolony in mg) in *B. terrestris* worker microcolonies ($N = 76$) fed for 13 days on imidacloprid-treated sugar syrup and untreated pollen. Error bars indicate 1 SE

42 % reduction in worker fecundity (95 % CI: 33 %, 51 %; Fig. 1). There was no effect of dosage on the number of days elapsing before the first oviposition was observed in a microcolony (mean elapsed days = 7.5, SE = 0.5; Spearman's correlation: elapsed days versus dosage, $\rho = -0.04$, $N = 57$, $P = 0.75$).

In the microcolonies, *per capita* daily rates of feeding declined with increasing dosage of imidacloprid for both syrup and pollen (Spearman's correlation: syrup feeding rate versus dosage, $\rho = -0.63$, $N = 76$, $P < 0.001$; pollen feeding rate versus dosage, $\rho = -0.63$, $N = 76$, $P < 0.001$; Fig. 2). Despite consuming less syrup, bees exposed to higher dosages nevertheless ingested larger amounts of imidacloprid (Fig. 2).

Compared to bees collected from the queenright colony at the beginning of the experiment, individuals examined after the 14-day experimental period had ovaries with larger oocytes at all but the highest dosage of imidacloprid (Fig. 3). All measures of oocyte size in microcolonies (mean length, width and area) and the number of workers with mature oocytes per microcolony were affected significantly by imidacloprid dosage (one-way ANOVA: oocyte size, $F_{9,42} \leq 7.7$, $P < 0.001$; number of workers with mature oocytes, $F_{9,42} = 3.7$, $P < 0.01$). By any measure, oocytes were smaller in bees from microcolonies exposed to imidacloprid at 125 µg L^{-1} (159 ppb) when compared to all other dosages (orthogonal contrast: $t \leq -6.6$, $P < 0.001$; Fig. 3). However, no dose-dependent variation in oocyte size was evident among bees exposed to dosages below 125 µg L^{-1} on any measure (one-way ANOVA: $F_{8,37} \leq 1.5$, $P \geq 0.20$; Fig. 3). There were fewer workers with mature oocytes in microcolonies at 125 µg L^{-1} compared to all other dosages (orthogonal contrast: $t = -4.2$, $P < 0.001$), but no significant difference was detectable among the numbers of workers with mature oocytes in microcolonies exposed to imidacloprid at dosages below 125 µg L^{-1} (mean number of workers with mature oocytes per microcolony = 1.39, SE = 0.11; one-way ANOVA: $F_{8,37} = 1.12$, $P = 0.38$).

Dosage-independent effects

After controlling statistically for the effects of imidacloprid and mean body size in a microcolony, fecundity increased significantly in microcolonies with higher *per capita* daily rates of feeding for both syrup and pollen (Pearson's partial correlation: fecundity versus syrup feeding rate, $r = 0.36$, df = 50, $P < 0.01$; fecundity versus pollen feeding rate, $r = 0.40$, df = 50, $P < 0.01$). There was no effect of mean body size on fecundity, independent of imidacloprid dosage and daily feeding rates (Pearson's partial correlation: $r = -0.10$, df = 50, $P = 0.50$).

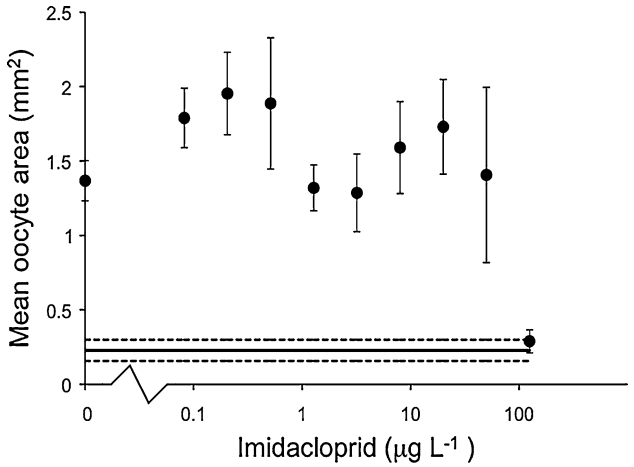

Fig. 3 Terminal oocyte area (*y*-axis: mean area of terminal oocytes in individuals from a microcolony) of *B. terrestris* workers ($N = 231$) in microcolonies ($N = 76$) after 14 days, including 13 days of exposure to dietary imidacloprid in dosed sugar syrup (*x*-axis: concentration of imidacloprid in syrup in μg L^{-1}). Error bars indicate 1 SE. The *solid horizontal line* indicates the mean terminal oocyte area of workers ($N = 10$) in the queenright colony before the microcolony experiment was conducted and the associated dashed lines indicate 1 SE

Daily rates of feeding did not significantly affect the mean size of terminal oocytes in a microcolony (Pearson's partial correlation: mean oocyte area versus syrup feeding rate, $r = 0.24$, df = 50, $P = 0.08$; mean oocyte area versus pollen feeding rate, $r = 0.26$, df = 50, $P = 0.06$), nor was there a correlation between mean terminal oocyte size and body size of individual bees (Spearman's $\rho = 0.01$, $N = 231$, $P = 0.89$).

Discussion

The key result emerging from our work is that ingestion of imidacloprid at environmentally realistic levels substantively reduced the fecundity of worker bumble bees. This finding is consistent with those of previous studies, which have shown that exposure of *B. terrestris* workers to dietary imidacloprid at 10 ppb in feeder syrup reduced larval production by 43 % (Tasei et al. 2000) and drone production by between 41 and 62 % (Tasei et al. 2000; Mommaerts et al. 2010). However, wild bees are probably exposed to imidacloprid residues lower than 10 ppb when they consume the nectar and pollen of treated crops (Bonmatin et al. 2003, 2005; Chauzat et al. 2006). We have now demonstrated that dietary trace residues of imidacloprid in the range of 1 ppb can reduce worker fecundity by at least one third.

Our methodology is likely to have produced realistic exposures to dietary imidacloprid. The amount of imidacloprid ingested by foraging honey bees in nectar and pollen is estimated to be between 152 and 610 pg per day (Rortais et al. 2005). In our experiments, *B. terrestris* workers ingested on average 587 pg of imidacloprid per day when feeding on syrup dosed with imidacloprid at 1.63 ppb (1.28 μg L^{-1}), which is in the lower range of field-realistic concentrations. In actuality, individual bumble bees probably consume more nectar in a day than honey bees (Thompson and Hunt 1999); therefore, our observations may be reasonably used as a minimum estimate of the effects on the fecundity of worker bumble bees that feed exclusively on real nectars containing imidacloprid residues.

Our findings raise further concern about the impact of systemic neonicotinoids on wild bumble bee populations. A recent review summarising 15 years of research on the hazards of neonicotinoids to bees highlighted the sub-lethal effects of exposure in the laboratory to neonicotinoids ≥6 ppb on reproduction and behaviour in bumble bees (Blacquière et al. 2012). We have now shown that dietary neonicotinoids in the range <6 ppb can cause substantive sub-lethal effects on bumble bee reproduction. However, we recognize that to fully evaluate impacts on wild colonies it will also be necessary to establish whether the fecundity of bumble bee queens is as sensitive to a dietary neonicotinoid as that of workers. Whitehorn et al. (2012) demonstrated that exposure of young *B. terrestris* colonies to dietary imidacloprid at 6 ppb for 14 days reduced colony growth after 8 weeks by 8 % and queen production by 85 %. The underlying mechanism was not studied, but we speculate that reduced fecundity in queens during imidacloprid exposure could account for these observations. Additionally, it will be necessary to evaluate the capacity of bumble bees to recover from the short-term pulsed exposure to dietary neonicotinoids that is likely to occur during the synchronous bloom of a mass-flowering neonicotinoid-treated crop. Consider, for example, the interaction between bumble bees and neonicotinoid-treated oilseed rape, which probably provides the most widespread exposure of bees to dietary neonicotinoids in Europe. In the UK, a field of winter-sown oilseed rape blooms for around 28 days with approximately 75 % of the flowering occurring over a peak period of about 18 days in April and May (Hoyle et al. 2007). A bumble bee colony is initiated in spring; it develops over several months and typically delays the production of new queens and drones until its latter stages (Goulson 2003b), which are therefore likely to emerge after oilseed rape has flowered. Other insects, such as aphids, whitefly and midges, are able to recover once a neonicotinoid disappears from their diet (Nauen 1995; Azevedo-Pereira et al. 2011; He et al. 2011). If the fecundity of a bumble bee colony recovers as the levels of dietary neonicotinoid diminish, the impact on reproduction and colony growth may be less severe than otherwise, but this speculation awaits further research.

In our study, the strongly detrimental effects of imidacloprid on fecundity at dosages of 63.5 ppb (50 μg L^{-1}) or lower were not due to impaired ovary development. Similarly, ovary development in the Eastern bumble bee, *B. impatiens* C., was sensitive only to very high dietary concentrations of the alkaloid gelsemine, which occurs naturally in the nectar of *Gelsemium sempervirens* L. (Carolina jessamine) (Manson and Thomson 2009). We therefore speculate that ovary development in bumble bees may be somewhat resilient to dietary toxins in general.

Except at relatively high dosages (i.e. above 150 ppb), the detrimental effect of imidacloprid on worker fecundity also cannot be explained by delayed brood production. When brood was produced, we observed egg cells in microcolonies after approximately 1 week, regardless of imidacloprid dosage, and this timescale is entirely typical of *B. terrestris* workers in queenless colonies (Alaux et al. 2007; Amsalem et al. 2009).

Indeed, the precise toxicological mechanisms that caused the detrimental effects of imidacloprid on bumble bee fecundity at dosages below 159 ppb are not revealed by our study. An individual bee's physiological function is tightly integrated with its nervous system and therefore the effects of a dietary neurotoxin are probably manifold. However, we observed that dietary imidacloprid reduced feeding on both syrup and pollen and that microcolonies that consumed more syrup and pollen produced more brood. Carbohydrates (Murphy et al. 1983; Boggs 1997; O'Brien et al. 2000) and protein (Webster et al. 1979; Wheeler 1996) are essential components for brood production in insects and we therefore speculate that reduced feeding imposed nutrient limitation on reproduction.

Reduced feeding on dosed syrup could be an indication that dietary imidacloprid is an aversive stimulus to workers or that imidacloprid reduced the bees' ability or need to feed. However, the initial reduction in feeding rate due to imidacloprid intensifies over successive days (Cresswell, pers. obs.), which suggests that it has a basis in toxicity rather than aversion. In our experiment, feeding on dosed syrup was accompanied by reduced feeding on untreated pollen. This phenomenon may be an adaptive response by workers that are attempting to maintain a constant protein to carbohydrate (P:C) ratio, because honey bee workers rendered queenless and fed a choice of diets are known to maintain strict P:C ratios (Altaye et al. 2010). In summary, we have shown dietary imidacloprid at levels up to approximately 65 ppb fails to prevent bumble bee workers from developing their reproductive organs and we hypothesize that its detrimental effects on fecundity emerge in whole or in part from nutrient limitation imposed by the failure of individuals to feed.

We found that bumble bee workers feeding on syrup at the highest dosage, 159 ppb, neither developed their ovaries fully nor laid eggs, and that microcolonies feeding on syrups at dosages of 25.4 ppb (20 μg L^{-1}) or less both developed ovaries to the same degree as those feeding on undosed syrup and were capable of laying eggs. However, the workers in microcolonies exposed to an intermediate dosage of imidacloprid, 63.5 ppb, developed their ovaries, but did not lay eggs. This situation is similar to that observed among isolated *B. terrestris* workers (Amsalem et al. 2009), who require a social stimulus to initiate brood production. We therefore speculate that imidacloprid at 63.5 ppb may have disrupted social interactions and thereby repressed oviposition in these workers; however, we acknowledge the possibility that imidacloprid may be involved in repression of egg laying at the individual level via a non-social mechanism. While dietary neonicotinoids are able to affect behavioural performance in honey bees (Lambin et al. 2001; Decourtye et al. 2003, 2004), further investigation is necessary to establish whether dietary neonicotinoids are capable of disrupting behavioural aspects of sociality in bees.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The work reported here conforms to the regulatory requirements for animal experimentation in the UK and has been approved by the Biosciences Ethics Committee at the University of Exeter.

References

Alaux C, Savarit F, Jaisson P, Hefetz A (2004) Does the queen win it all? Queen-worker conflict over male production in the bumblebee, *Bombus terrestris*. Naturwissenschaften 91:400–403

Alaux C, Boutot M, Jaisson P, Hefetz A (2007) Reproductive plasticity in bumblebee workers (*Bombus terrestris*)—reversion from fertility to sterility under queen influence. Behav Ecol Sociobiol 62:213–222

Allen-Wardell G et al (1998) The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. Conserv Biol 12:8–17

Altaye SZ, Pirk CWW, Crewe RM, Nicolson SW (2010) Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. J Exp Biol 213: 3311–3318

Amsalem E, Twele R, Francke W, Hefetz A (2009) Reproductive competition in the bumble-bee *Bombus terrestris*: do workers advertise sterility? Proc R Soc B 276:1295–1304

Ashman T-L et al (2009) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85:2408–2421

Azevedo-Pereira HMVS, Lemos MFI, Soares AMVM (2011) Behaviour and growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under imidacloprid pulse and constant exposure scenarios. Water Air Soil Pollut 219:215–224

Blacquière T, Smagghe G, van Gestel CAM, Mommaerts V (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21:973–992

Boggs CL (1997) Dynamics of reproductive allocation from juvenile and adult feeding: radiotracer studies. Ecology 78:192–202

Bonmatin JM, Moineau I, Charvet R, Fleche C, Colin ME, Bengsch ER (2003) A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens. Anal Chem 75:2027–2033

Bonmatin JM, Marchand PA, Charvet R, Moineau I, Bengsch ER, Colin ME (2005) Quantification of imidacloprid uptake in maize crops. J Agric Food Chem 53:5336–5341

Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF, Griswold TL (2011) Patterns of widespread decline in North American bumble bees. Proc Natl Acad Sci USA 108:662–667

Chauzat M-P, Faucon J-P, Martel A-C, Lachaize J, Cougoule N, Aubert M (2006) A survey of pesticide residues in pollen loads collected by honey bees in France. J Econ Entomol 99:253–262

Cresswell JE (2011) A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. Ecotoxicology 20:149–157

Cutler GC, Scott-Dupree CD (2007) Exposure to clothianidin seed-treated canola has no long-term impact on honey bees. J Econ Entomol 100:765–772

De la Rúa P, Jaffé R, Dall'Olio R, Muñoz I, Serrano J (2009) Biodiversity, conservation and current threats to European honeybees. Apidologie 40:263–284

Decourtye A, Lacassie E, Pham-Delegue MH (2003) Learning performances of honeybees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season. Pest Manag Sci 59:269–278

Decourtye A, Devillers J, Cluzeau S, Charreton M, Pham-Delegue MH (2004) Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. Ecotoxicol Environ Saf 57:410–419

Desneux N, Decourtye A, Delpuech JM (2007) The sublethal effects of pesticides on beneficial arthropods. Annu Rev Entomol 52: 81–106

Elbert A, Haas M, Springer B, Thielert W, Nauen R (2008) Applied aspects of neonicotinoid uses in crop protection. Pest Manag Sci 64:1099–1105

Faucon J-P et al (2005) Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies. Pest Manag Sci 61:111–125

Forbes VE, Calow P (1999) Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? Environ Toxicol Chem 18:1544–1556

Goulson D (2003a) Conserving wild bees for crop pollination. J Food Agric Environ 1:142–144

Goulson D (2003b) Bumblebees: their behaviour and ecology. Oxford University Press, Oxford

Goulson D, Lye GC, Darvill B (2008) Decline and conservation of bumble bees. Annu Rev Entomol 53:191–208

Grier JW (1982) Ban of DDT and subsequent recovery of reproduction in bald eagles. Science 218:1232–1235

Hansard (2011) Neonicotinoid pesticides. House of Commons Hansard Debates. http://www.publications.parliament.uk/pa/cm201011/cmhansrd/cm110125/halltext/110125h0002.htm#11012550000630. Accessed 7 Jan 2012

He Y, Zhao J, Wu D, Wyckhuys KAG, Wu K (2011) Sublethal effects of imidacloprid on *Bemisia tabaci* (Hemiptera: Aleyrodidae) under laboratory conditions. J Econ Entomol 104:833–838

Henry M et al (2012) A common pesticide decreases foraging success and survival in honey bees. Science 336:348–350

Herbert IN, Svendsen C, Hankard PK, Spurgeon DJ (2004) Comparison of instantaneous rate of population increase and critical-effect estimates in *Folsomia candida* exposed to four toxicants. Ecotoxicol Environ Saf 57:175–183

Hoyle M, Hayter KE, Cresswell JE (2007) Effect of pollinator abundance on self-fertilization and gene flow: application to GM canola. Ecol Appl 17:2123–2135

Ihaka R, Gentleman R (1996) A language for data analysis and graphics. J Comput Graph Stat 5:299–314

Kearns CA, Inouye DW, Waser NM (1998) Endangered mutualisms: the conservation of plant pollinator interactions. Ann Rev Ecol Syst 29:83–112

Klein A-M, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proc R Soc B 274:303–313

Krupke CH, Hunt GJ, Eitzer BD, Andino G, Given K (2012) Multiple routes of pesticide exposure for honey bees living near agricultural fields. PLoS ONE 7:e29268

Lambin M, Armengaud C, Raymond S, Gauthier M (2001) Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch Insect Biochem Physiol 48: 129–134

Lopez-Vaamonde C, Koning JW, Brown RM, Jordan WC, Bourke AFG (2004) Social parasitism by male-producing reproductive workers in a eusocial insect. Nature 430:557–560

Lunn DJ, Thomas A, Best N, Spiegelhalter D (2000) WinBUGS—a Bayesian modelling framework: concepts, structure, and extensibility. Stat Comput 10:325–337

Manson JS, Thomson JD (2009) Post-ingestive effects of nectar alkaloids depend on dominance status of bumblebees. Ecol Entomol 34:421–426

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol Sci 22:573–580

Medler JT (1962) Morphometric studies on bumble bees. Ann Entomol Soc Am 55:212–218

Mommaerts V, Reynders S, Boulet J, Besard L, Sterk G, Smagghe G (2010) Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. Ecotoxicology 19:207–215

Morandin LA, Winston ML (2003) Effects of novel pesticides on bumble bee (Hymenoptera: Apidae) colony health and foraging ability. Environ Entomol 32:555–563

Murphy DM, Launer AE, Ehrlich PR (1983) The role of adult feeding in egg production and population dynamics of the checkerspot butterfly *Euphydryas editha*. Oecologia 56:257–263

Nauen R (1995) Behaviour modifying effects of low systemic concentrations of imidacloprid on *Myzus persicae* with special reference to an antifeeding response. Pesticide Sci 44:145–153

O'Brien DM, Schrag DP, Martinez del Rio C (2000) Allocation to reproduction in a hawkmoth: a quantitative analysis using stable carbon isotopes. Ecology 81:2822–2831

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol 25:345–353

Rortais A, Arnold G, Halm MP, Touffet-Briens F (2005) Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36:71–83

Schmuck R, Schoning R, Stork A, Schramel O (2001) Risk posed to honeybees (*Apis mellifera* L. Hymenoptera) by an imidacloprid seed dressing of sunflowers. Pest Manag Sci 57:225–238

Sur R, Stork A (2003) Uptake, translocation and metabolism of imidacloprid in plants. Bull Insectol 56:35–40

Takino M, Tanaka T (2006) Determination of 44 pesticides in foodstuffs by LC/MS/MS. Agilent Technologies. http://www.chem.agilent.com/Library/applications/5989-5459EN.pdf. Accessed 21 March 2012

Tasei J-N, Lerin J, Ripault G (2000) Sub-lethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. Pest Manag Sci 56:784–788

Thompson HM, Hunt LV (1999) Extrapolating from honeybees to bumblebees in pesticide risk assessment. Ecotoxicology 8:147–166

van Engelsdorp D, Hayes J, Underwood RM, Pettis JS (2010) A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. J Apic Res 49:7–14

Walthall WK, Stark JD (1997) A comparison of acute mortality and population growth rate as endpoints of toxicological effect. Ecotoxicol Environ Saf 37:45–52

Webster RP, Stoffolano JG, Prokopy RJ (1979) Long-term intake of protein and sucrose in relation to reproductive behavior of wild and laboratory cultured *Rhagoletis pomonella*. Ann Entomol Soc Am 72:41–46

Wheeler D (1996) The role of nourishment in oogenesis. Annu Rev Entomol 41:407–431

Whitehorn PR, O'Connor S, Wackers FL, Goulson D (2012) Neonicotinoid pesticide reduces bumblebee colony growth and queen production. Science 336:351–352

Williams PH, Osborne JL (2009) Bumblebee vulnerability and conservation world-wide. Apidologie 40:367–387