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

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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 $\mu g\,kg^{-1}$ 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 $\mu g\,kg^{-1}$). In contrast, we found no detectable effect of thiamethoxam at levels typically found in the nectars of treated crops (between 1 and 11 $\mu g\,kg^{-1}$). 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.

1. Introduction

The pollination services of wild bees help to maintain plant species in natural ecosystems and are worth billions of dollars annually to agriculture (Williams and Osborne, 2009; Winfree, 2010). Evidence of declining wild bee populations (Biesmeijer et al., 2006) and the extirpation of certain species (Burkle et al., 2013) are therefore issues of increasing concern (Vanbergen, 2013). It is widely acknowledged that several factors are driving declines in wild bees (Williams and Osborne, 2009; Potts et al., 2010). However, a group of neurotoxic pesticides, the neonicotinoids, have specifically been singled out for blame (Shardlow, 2013), which has led to calls for restrictions on their use in agricultural (EFSA, 2013a; Maxim and van der Sluijs, 2013) that have recently been implemented across the European Union (European Commission, 2013). The neonicotinoids, which include imidacloprid, thiamethoxam and clothianidin, are systemic and so the pesticide is distributed throughout plant tissues to control sucking insect pests (Elbert et al., 2008). Consequently, trace residues can appear in nectar and pollen (Blacquière et al., 2012) and bees are exposed to dietary neonicotinoids by foraging from the flowers of treated agricultural crops (Elbert et al., 2008).

Bumble bees are important wild pollinators that are detrimentally affected by neonicotinoids in laboratory studies, where dietary residues reduce food consumption and brood production of *Bombus terrestris* L. workers (Tasei et al., 2000; Mommaerts et al., 2010; Cresswell et al., 2012; Laycock et al., 2012), and in semi-field studies, where *B. terrestris* colonies under exposure exhibit reduced production of brood, workers and queens (Gill et al., 2012; Whitehorn et al., 2012). The majority of these studies focus solely on imidacloprid, which has historical relevance because it was the first neonicotinoid in widespread use (Elbert et al., 2008) and was identified publicly as a potential threat to bee health in 1999 (Maxim and van der Sluijs, 2013). However, newer neonicotinoid varieties, such as thiamethoxam and its toxic metabolite clothianidin, are increasingly preferred to imidacloprid in crop protection. For example, in 2011 imidacloprid made up just 10 percent of the total 80,000 kg of neonicotinoid applied to UK crops (FERA, 2013). Consequently wild bumble bees are at increased risk of exposure to these alternative neonicotinoids. We therefore chose to further investigate the effects of dietary thiamethoxam on bumble bees.

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Residues of thiamethoxam ranging from 1 to 11 μg kg^{-1} (=parts per billion or ppb) have been detected in nectar from treated crops including alfalfa, oilseed rape, pumpkin, sunflower, squash and *Phacelia tanacetifolia* (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). In pollen, residues are typically higher, ranging from 1 to 12 μg kg^{-1} in sunflower, oilseed rape and squash, but reaching 39, 51 and 95 μg kg^{-1} in *Phacelia*, alfalfa, and pumpkin, respectively (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). For bees, exposure to residues such as these probably occurs in transient pulses; for example, during the mass-flowering of treated oilseed rape that lasts for approximately one month and peaks over a period of around two weeks (Hoyle et al., 2007; Westphal et al., 2009). Detrimental effects on honey bees of dietary thiamethoxam at 67 μg L^{-1} have already been demonstrated (Henry et al., 2012), but the effects on bumble bees in a similar dosage range are unclear. For example, in one *B. terrestris* microcolony study 100 μg kg^{-1} thiamethoxam presented to workers in sugar solution increased mortality and reduced drone production while residues at 10 μg kg^{-1} had no detectable effect (Mommaerts et al., 2010). However, in another study 10 μg kg^{-1} thiamethoxam reduced workers' production of drone brood (the workers' eggs and larvae), while microcolony feeding rates were reduced at both 1 and 10 μg kg^{-1} (Elston et al., 2013). With evidence of thiamethoxam's effects currently inconsistent, it remains uncertain whether environmentally realistic residues are capable of having a detrimental impact on bumble bee populations. We therefore present an experiment designed to test the performance of bumble bees presented with dietary thiamethoxam at a wide range of concentrations, including dosages within the field-realistic range for nectar.

In this study, we made use of the reproductive capacity of *B. terrestris* workers in queenless microcolonies to investigate the effects of thiamethoxam on bumble bee performance. In microcolonies, small groups of bumble bee workers are maintained in the absence of a queen and, over a period of days, a dominant worker lays eggs that will develop into drones while the others forage and care for brood (Tasei et al., 2000). In a recent guidance document for risk assessment of plant protection products on bees (EFSA, 2013b), the use of microcolonies was recommended as part of 'higher tier' risk assessment studies in bumble bees. Using *B. terrestris* microcolonies, we characterised dose–response relationships that described thiamethoxam's effects on brood (eggs and larvae) production, food consumption and days survived by workers (Laycock et al., 2012) over an exposure lasting 17 days. Following laboratory exposure periods of similar length, imidacloprid produced substantive sublethal effects on feeding and brood production in *B. terrestris* microcolonies (Laycock et al., 2012) and reduced colony growth and production of new queens in queenright colonies allowed to develop for a further six weeks in pesticide-free conditions (Whitehorn et al., 2012). Here we applied dosages and some endpoints that were adopted in the imidacloprid microcolony study (*i.e.* Laycock et al., 2012) to enable us to compare the relative sensitivity of bumble bees to the two neonicotinoids.

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 percent 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}. Following acclimatisation, microcolonies were fed *ad libitum* for 17 days with undosed pollen balls (ground pollen pellets, obtained from Biobest, mixed with water; mean mass=5.3 g, SE=0.1 g) and either undosed control syrup (19 control microcolonies) or syrup dosed with thiamethoxam (9 dosed microcolonies per thiamethoxam concentration, listed above). This level of replication (*i.e.* a minimum of nine replicates per concentration) is consistent with similar microcolony studies (Mommaerts et al., 2010; Laycock et al., 2012; Elston et al., 2013). Pollen balls were weighed before and after placement into microcolonies to quantify pollen consumption and syrup feeders were weighed each day to measure syrup consumption. We corrected for evaporation of water from syrup and pollen based on the mass change of syrup feeders and pollen balls maintained under experimental conditions, but not placed into microcolonies. Additionally, where syrup or pollen was collected by bees but not consumed, for example where syrup was stored in wax honey pots, its mass was determined and subtracted from consumption accordingly. We monitored microcolonies daily for individual worker mortality and the appearance of wax covered egg cells that indicate the occurrence of oviposition. To assess brood production, at the end of the experiment we freeze-killed workers in their microcolony boxes and collected all laid eggs and larvae from the nests. In our previous microcolony study (Laycock et al., 2012), we also investigated the effect of imidacloprid on ovary development because imidacloprid produced a dose-dependent decline in workers' brood production. Except at the highest dosages, thiamethoxam had no effect on brood production (*i.e.* microcolonies laid eggs at a statistically equivalent rate, see Section 3) and we therefore chose not to measure ovary development here. The experiment was conducted in two replicate trials between October and December 2012. Each trial comprised 50 microcolonies and dosage groups were approximately equally represented in both. The results of the two trials were qualitatively similar and so data were pooled for further 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 $instrument\ response = 228.42 \times thiamethoxam\ concentration + 265.87$, $R^2 > 0.99$). We used the calibration equation to determine the concentration values of our extracted samples and found that all dosages contained appropriate levels of thiamethoxam ($measured\ thiamethoxam = 1.16 \times nominal\ dosage + 1.57$, $R^2 > 0.99$).

2.3. Statistical analyses

In our experiments, endpoints responded only to the two highest dosages of thiamethoxam (see Section 3). We therefore analysed the variation in food consumption and days survived by workers in microcolonies that was due to thiamethoxam using one-way ANOVA, with *dosage* (dosage of thiamethoxam in