Short communication

No sub-lethal toxicity to bumblebees, *Bombus terrestris*, exposed to Bt-corn pollen, captan and novaluron

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Abstract Queenless microcolonies of *Bombus terrestris* were fed either mixed floral pollen, non-transgenic sweet corn pollen, transgenic Bt-sweet corn pollen, or pollen mixed with either the fungicide captan or the insect growth regulator novaluron. None of the treatments significantly affected survival of worker bees, rates at which they consumed pollen or sugar syrup, their ability to produce offspring, or the timing of events in the development of their offspring, compared with the appropriate controls. Drones from microcolonies fed 0.135 mg/kg⁻¹ a.i. novaluron were significantly shorter-lived than controls fed floral pollen. Drones from microcolonies fed corn pollen were significantly smaller than the controls, regardless of whether the corn pollen was transgenic or not. These pest control technologies are unlikely to affect *B. terrestris* bumblebees significantly in the field.

Keywords *Bombus terrestris*; transgenic Bt-corn pollen; captan; novaluron

INTRODUCTION

Recent concern about a general loss of pollinators in intensive agroecosystems worldwide (Allen-Wardell et al. 1998; Kevan & Phillips 2001) has prompted investigation of the impacts of a number of agrichemicals on bumblebees. Honey bees (*Apis mellifera*) are the standard test species for assessing the safety of these compounds to pollinators. Less is known about their impacts on bumblebees. The toxicity of organophosphate, carbamate and synthetic pyrethroid insecticides to bumblebees has been reviewed (Thompson 2001; van der Steen 2001) and more recently the effects of some newer insecticides, such as neonicotinoids and spinosad, have been examined (Tasei et al. 2000, 2001; Morandin & Winston 2003; Mayes et al. 2003; Franklin et al. 2004; Morandin et al. 2005).

There has been less research on the impacts on bumblebees of other pesticidal products, e.g., transgenic insect-resistant crop plants, fungicides, or insect growth regulators. Bumblebees have the potential to be exposed to pollen from transgenic Bt-sweet corn, the commonly-used fungicide captan, and the insect growth regulator novaluron, but there is little information on their possible impacts. Toxicity tests have shown that purified Cry1Ab has no effect on honeybees (USEPA 2001), but there has been no assessment of the effects of this toxin, or pollen from plants expressing it, on bumblebees. Purified Cry1Ac (at 11 µg/kg⁻¹, a dose representing potential exposure levels with transgenic Cry1Ac-crops), a related Bt toxin, was not toxic to *Bombus impatiens* or *Bombus occidentalis* (Morandin & Winston 2003). Captan is a dicarboximide contact fungicide which can be toxic when fed to honeybee larvae (Mussen et al. 2004) and to adult solitary bees, *Osmia lignaria*, in contact and oral toxicity tests (Ladurner et al. 2005) at doses calculated to reflect realistic field exposure levels, but its effects on bumblebees are unknown. Novaluron and other chitin synthesis inhibitors have been shown to delay egg development and cause larval mortality when fed to *B. terrestris* colonies (de Wael et al. 1995;

Gretenkord & Drescher 1995; Mommaerts et al. 2006), but sub-lethal impacts on adult offspring have not been investigated.

Here we examine the impacts of three different pesticidal technologies on the development and survival of *Bombus terrestris*: Bt-sweet corn pollen, genetically modified to express *Bacillus thuringiensis* (Bt) endotoxin Cry1Ab, the fungicide, captan, and the insect growth regulator, novaluron.

MATERIALS AND METHODS

Microcolony preparation

Colonies of *B. terrestris* were obtained from a commercial supplier (Biobees Ltd, New Zealand) and kept in a controlled temperature room (where all the experiments were carried out) at 25°C with a 16:8 hour light:dark regime. All workers present in purchased colonies were marked with white paint so that newly-emerged workers could be identified and harvested daily for use in experiments thereafter. Mixed floral pollen pellets, collected by honeybees foraging predominantly on white clover (*Trifolium repens*) and to a lesser extent on red clover (*Trifolium pratense*), lotus (*Lotus pedunculatus*), dandelion (*Taraxacum officinale*), and penny royal (*Mentha pulegium*), were obtained from a beekeeper in Hamilton, New Zealand, broken up in a coffee grinder, and kept frozen until required.

Microcolonies, each comprising three newly-emerged workers, were set up in clear plastic tissue culture pots (9.5 cm diam. × 6 cm tall) with the bottoms cut out and replaced with nylon tulle cloth secured with a rubber band. Each worker was weighed and then marked with a different coloured paint dot on the thorax before placing in the culture pot. The pots were then wedged into tall disposable waxed paper cups (9.5 cm diam. × 18 cm tall), so that the bees' faeces would pass through the tulle and into the cup which could then be replaced weekly. A gravity feeder supplied syrup (15% w:v fructose, 15% w:v D-glucose, 30% w:v sucrose in filtered autoclaved water) and a beeswax-coated ball of pollen (c. 15 mm diam.), that had been mixed to a thick paste with the syrup described above, was added to each pot. The relative humidity inside each container was $68 \pm 2\%$. Pollen balls and syrup feeders were weighed and replaced weekly, or more often if necessary. The bees and their brood were transferred to a fresh culture pot if and when fungal or faecal contamination was apparent. Any workers that died within the first week of the experiment were

classified as "handling deaths" and replaced with a fresh, newly-emerged worker.

Pesticide treatments

Corn pollen was collected by bagging tassels on non-transgenic sweet corn and Bt-sweet corn (ATTRIBUTE™, Syngenta Crop Protection, Bt 11 event) in Guelph and Fergus, Ontario, Canada, during August 2005, sent to New Zealand by air freight and kept frozen. Captan (Captan 80W, Nufarm Ltd, N.Z.) and novaluron (Rimon® 75WG, Mahkteshim-Agan distributed by Agronica NZ Ltd.) were purchased as formulated products. Rimon® 75WG granules were ground to a fine powder before dissolving in water for mixing with the pollen paste. Captan was already in powder form.

The concentrations of each pesticide were selected to reflect both realistic and unrealistically high exposure levels for the bumblebees. Captan residues between 0.04 and 18.97 mg kg⁻¹ have been measured in pollen collected by honeybees placed in apple orchards sprayed with this fungicide (Kubik et al. 2000). In the present study, 10 mg kg⁻¹ (the MRL for captan on fruit and vegetables in New Zealand (Anon. 2005)) and 6 times that dose were administered. In Canada, novaluron residues of 0.015 mg kg⁻¹ have been noted in corn pollen (Bailey et al. 2005). Here we used this as a "low" but realistic concentration of novaluron in pollen and also administered a "high" treatment 6 times that. An additional "very high" concentration of novaluron, 9 times the realistic concentration, was added as a positive control for the method used.

The pesticidal products were administered via pollen balls for 35 days; after that time, all microcolonies were maintained on plain mixed floral pollen until at least two of the three workers had died or at 70 days from the beginning of the experiment, whichever came first. There were eight different treatment groups, comprising bees given: (1) only honeybee-collected mixed floral pollen; (2) non-transgenic sweet corn pollen; (3) Bt11 transgenic sweet corn pollen; (4) floral pollen with 10 mg kg⁻¹ a.i. captan added; (5) floral pollen with 60 mg kg⁻¹ a.i. captan added; (6) floral pollen with 0.015 mg kg⁻¹ a.i. novaluron added; (7) floral pollen with 0.09 mg kg⁻¹ a.i. novaluron added; and (8) floral pollen with 0.135 mg kg⁻¹ a.i. novaluron added.

Long-term microcolony exposure assays

In total, 170 microcolonies were set up, each containing three worker bees: 30 microcolonies for the floral pollen control and 20 for each other treatment.

Bumblebees were allocated to treatments in the order in which they emerged from the commercial colony, i.e., the first bee was allocated to treatment 1, the next to treatment 2, until all bees available that day had been assigned in sets of three to microcolonies. If one or two emergent bees remained after that, then these were discarded from that day's "harvest". As this continued for several rounds, each treatment included microcolonies set up on each of several emergence dates. Thus a blocking structure was formed where groups of eight colonies (each with one representing each treatment), as they were created, constituted the blocks.

A 35-day exposure period was chosen as a "realistic, high-exposure" scenario, based on the possibility that bumblebees could be exposed to sweet corn pollen for that long if four adjacent crops planted at weekly intervals shed their pollen over four 8-day periods in succession. This period was also realistic for flowering crops pollinated by bumblebees, such as kiwifruit orchards flowering at different times within a single region in New Zealand (McPherson et al. 1994) and for different varieties of cultivated blueberries flowering in New Zealand (Anon. 1999). Some of the microcolonies had begun to produce their first cocoons by the end of the 35-day exposure period, but no adult males had emerged (data not given).

For each microcolony, initial weights of each worker were recorded. Total consumption of pollen balls and syrup was recorded by weighing syrup feeders and pollen balls before and after they were given to the bees. To remove the obvious effects of worker size and longevity from the subsequent analysis of possible treatment effects on syrup or pollen consumption, each was calculated as grams consumed per live-bee-gram-days (a measure of worker biomass for the duration of the experiment calculated by multiplying each worker bee's longevity in days by its initial weight in grams). This would not remove the impact of brood rearing on these consumption figures, and therefore the influence of this parameter on food consumption would still be subject to the subsequent analysis of treatment effects. Microcolonies were checked daily for the entire experiment and dates of first oviposition, first discarding of larvae, first appearance of "larval balls" (i.e., separate spherical wax balls, measuring c. 8 mm in diam. or more and containing one larva each), first production of cocoons, and first emergence of progeny drones were recorded. Dead worker bees were removed daily and when two workers had died, the microcolony was terminated and the

remaining worker removed and killed by freezing. For subsequent analysis, "microcolony survival time" was defined as the number of days until at least two workers were dead. To measure individual worker longevity, workers that were killed because both their companions had died were assumed to have died that day, i.e., probably an underestimate of their longevity. Each week, syrup feeders and pollen balls were changed in each microcolony and larvae discarded by the bees were counted and removed. Emergent drones were recorded daily, removed from their microcolonies, weighed, given identifying tags and placed together in larger cages ($30 \times 22 \times 16$ cm; relative humidity = $62 \pm 1.0\%$), one per treatment group, supplied *ad libitum* with floral control pollen balls and syrup. These cages were examined daily and dead drones removed and recorded until all had died.

Data analysis

A mixed model was fitted to each of the 15 measured responses described above, with treatment as the fixed effect and individual microcolony within the 8-colony groups as the random effect (thus removing any effects owing to set-up date from the analysis). For the analyses of time to emergence of drones, average drone weight, and average drone longevity, the number of drones produced in each microcolony was used as a weighting factor, thus giving more emphasis to means calculated from a greater number of drones. The model assumes that the error terms are normally distributed with zero mean and constant variances. Where necessary, data were transformed before analysis using the logarithmic transformation to ensure that these assumptions were met. The analysis was adjusted to account for any overdispersion in the data. The data were analysed by the method of residual maximum likelihood (REML) (GenStat 2005). Model adequacy checks were performed by examining various plots (histograms, normal probability plots, scatter plots) of the residuals. Treatments were compared with the appropriate controls using Dunnett's LSD at the 5% level of significance.

RESULTS

There were no significant treatment effects on mean microcolony longevity, individual worker bee longevity, pollen or sugar syrup consumption, numbers of discarded larvae, numbers of drones produced, or times to first oviposition, first larval

ball, first discarded larva, first cocoon formation, or drone emergence.

The quality of drones produced was significantly affected by some of the treatments. Microcolonies fed corn pollen (i.e., Bt- or non-transgenic) produced significantly smaller drones (means and standard errors of 177 ± 8.5 mg and 182 ± 7.8 mg, respectively) than those fed floral control pollen (238 ± 7.3 mg) (REML, $F = 4.78$, $P < 0.001$; Dunnett's test, $P < 0.05$). Drones from microcolonies fed with 0.135 mg kg^{-1} a.i. novaluron had significantly reduced longevity (14 ± 2.7 days) compared with drones from the floral controls treatment group (29 ± 2.3 days) (REML, $F = 3.18$, $P = 0.006$; Dunnett's test, $P < 0.05$).

DISCUSSION

Although corn pollen is not known particularly as a source of forage for bumblebees, they have been observed occasionally in significant numbers on pollen-shedding corn tassels (Gross & Carpenter 1991). The lack of effect of Bt-sweet corn pollen, when compared with non-transgenic sweet corn pollen, on *B. terrestris* suggests that the expression of Bt in this crop would not be detrimental to bumblebees in any way. These results support those obtained by Morandin & Winston (2003) from *B. impatiens* and *B. occidentalis* colonies fed a related, purified Bt toxin (Cry1Ac). Both studies indicate negligible risks to bumblebees from transgenic Bt-corn crops. However, *B. terrestris* microcolonies fed either transgenic or non-transgenic corn pollen, produced significantly smaller drones than those fed floral control pollen, suggesting that a diet composed entirely of corn pollen was sub-optimal for bumblebee development. This is probably owing to the lower protein content of corn pollen (14–15%) compared to white clover pollen (23–25%) (Stace 1996), which was the predominant pollen type in the mixed floral pollen used here. However, under realistic field conditions this is not of concern, since only in rare circumstances are honeybees or bumblebees confined to foraging on a single plant for their entire lifetimes.

Chitin synthesis inhibitors, such as novaluron, have previously been shown to adversely affect bumblebee larval development. Significant mortality of *B. terrestris* larvae has been recorded in colonies fed with 150 mg kg^{-1} teflubenzuron in diet (de Wael et al. 1995), 12.5 , 25 , and 250 mg kg^{-1} diflubenzuron in diet (Gretenkord & Drescher 1995), and 40 mg litre^{-1} novaluron sprayed onto pollen "until saturation" (Mommaerts et al. 2006).

Mommaerts et al. (2006) based their novaluron dosing regime on the Maximum Recommended Field Concentration for this compound (40 mg litre^{-1} a.i.), noting that bumblebees would rarely be exposed to such high concentrations and observing that colonies fed this concentration of novaluron in pollen produced significantly fewer male offspring and discarded more larvae, many of which showed signs of deformity. Further trials using a dilution series (40 , 4 , 0.4 , 0.04 , 0.004 mg litre^{-1} a.i.), allowed Mommaerts et al. (2006) to calculate an LC_{50} of 6.2 mg litre^{-1} a.i. and a NOEC of 4 mg litre^{-1} a.i. for novaluron in pollen with respect to *B. terrestris* larval mortality. These values are higher than the highest concentration of novaluron administered in the present study (0.135 mg kg^{-1} a.i. in pollen), which was chosen to represent a 9-fold increase in the concentration of this pesticide recorded from field-collected pollen (Bailey et al. 2005). The results confirm the NOEC for larval mortality demonstrated by Mommaerts et al. (2006), in that no such mortality was observed here, and demonstrate that even the subtle negative effect on drone longevity observed was eliminated when lower concentrations, representing field-collected pollen residues and a dose 6 times that (0.015 or 0.09 mg kg^{-1} litre^{-1} a.i.), were used. These results suggest that, if these novaluron concentrations are typical of those likely to be encountered by bumblebees in the field, then these pollinators are unlikely to be detrimentally affected by this pesticide.

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