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ORIGINAL PAPER

Sublethal effects of kaolin and the biopesticides Prestop-Mix and BotaniGard on metabolic rate, water loss and longevity in bumble bees (*Bombus terrestris*)

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Abstract Kaolin is an inert material with a broad range of applications, e.g. as an insecticide and as a filling substance in the formulation of biopesticides. Hence, bees that dispense biopesticides to the field in the context of entomovectoring are exposed to elevated risks because of side-effects of those products. Here, we investigated with use of bumble bee workers of *Bombus terrestris* L. the lethal and sublethal effects of (i) pure kaolin, (ii) the biofungicide Prestop-Mix containing the parasitic fungus *Gliocladium catenulatum* and kaolin and (iii) the bioinsecticide BotaniGard containing the entomopathogenic fungus *Beauveria bassiana* and compared with wheat flour powder that we considered safe for bumble bees. As the most important result, treatment with kaolin increased the cuticular water loss and reduced the survival of treated bumble bees, while Prestop-Mix had no negative effect on longevity. BotaniGard caused mortality in the bumble bees because of the entomopathogenic spores it contained. In conclusion, our data indicated that substances used as 'inert materials' and in biocontrol agents which are used in IPM and organic farming systems may bring higher risks to bumble bees used in entomovector technology.

Keywords *Bombus terrestris* · Kaolin · Inert material · Biocontrol agent · Metabolic rate · Water loss · Survival

Key message

- Kaolin is not only known as an insecticide but also as an inert component of biopesticides. Therefore, kaolin in biopesticide formulations used in entomovector technology might affect vectoring bees.
- Kaolin increased water loss and decreased longevity in bumble bees, while the bioinsecticide Prestop-Mix had no effect on longevity. The bioinsecticide BotaniGard affected the longevity but not the water loss.
- Our data indicated that substances used in biocontrol agent formulations, which are used in IPM, may elicit deleterious effects on bumble bees.

Introduction

Kaolin is a white clay mineral which has found application in various agricultural practices. For instance, it is used to control warehouse pests (Golob 1997; Subramanyam and Roesli 2000; Kljajić et al. 2010) to protect plants against insects from different orders (Markó et al. 2008; Mikami et al. 2010; Muncha-Pelzer et al. 2010) and also against various plant diseases (Glenn et al. 1999; Glenn and Puterka 2005). It has a physical or deterrent mode of action (Subramanyam and Roesli 2000; Markó et al. 2008) which allows its use in organic and IPM programmes according to

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European IPM directive 2009/128. Besides, many microbial biocontrol agent preparations use inert materials like kaolin as a carrier substance (Ucar et al. 1999).

Pest management systems must be compatible with pollinators and other beneficial organisms which improve yields and quality of produce. In addition to their role as pollinators, bees are also used as entomovectors to achieve a better distribution of various microbial biocontrol agents in crops (Mommaerts et al. 2009; Mänd et al. 2010; Smagghe et al. 2012). Biofungicides like Prestop-Mix, which contains *Gliocladium catenulatum* Gilman and Abbott and is often used in entomovectoring technology, do not affect the honeybee *Apis mellifera* L. (Peng et al. 1992; Hokkanen and Menzler-Hokkanen 2009) or the bumble bee *Bombus terrestris* L. (Mommaerts et al. 2009, 2011, 2012). The bioinsecticide BotaniGard contains spores of *Beauveria bassiana* (Bals.-Criv.) Vuill., which may be hazardous to bees as to other insects, although it can be vectored by insects in certain conditions (Al-MazráAwi et al. 2007; Mommaerts et al. 2009; Shipp et al. 2012). In these microbial preparations, the biocontrol organisms are combined with inert materials to make a formulation. Kaolin is often used as a carrier substance in microbial preparations, as in Prestop-Mix.

The new EFSA guidance document (EFSA 2013) requires that the compatibility of pest control agents and also of inert components of biopreparations with pollinators must be evaluated, especially at the sublethal level. Dispersing biopesticides to fields using bumble bees and honey bees might endanger them as pollinators. Although the insecticidal potential of kaolin is clear, its mechanism of action is not fully understood yet (Subramanyam and Roesli 2000; Cook et al. 2008). The sublethal effects of pesticides may sometimes be overlooked in behavioural or mortality studies. Small physiological changes (Kestler 1991) might not affect the longevity or behavioural aspects of bees under normal environmental conditions, but in extreme situations, these may lead to clear effects (Bryden et al. 2013). Metabolic rate (Kestler 1985, 1991; Zafeiridou and Theophilidis 2006) as well as water loss rate (Muljar et al. 2012) has been used as indicators for sublethal stressors like pesticides. Kaolin is suggested to affect the cuticular permeability (Cook et al. 2008) to water which is one of the limiting factors for insects.

This study aimed to examine potential adverse effects of kaolin, kaolin-containing biofungicide Prestop-Mix and bioinsecticide BotaniGard on bumble bees and compared this to wheat flour powder. We considered wheat flour to be safe to bees based on Mommaerts et al. (2012). Hereto, foragers of the bumble bee *B. terrestris* were used in two assays in standardized conditions. *B. terrestris* is a general pollinator in Europe and is used in modern agriculture for biological pollination, e.g. tomatoes in greenhouses (Velthuis and van Doorn 2006). First, we investigated the effects on metabolic rate and water loss rate after a single topical treatment. Second, bumble bee longevity and survival were followed in the different treatments, and this was done at two temperatures (18 and 28 °C); these temperatures were chosen to accord with those experienced by bees when foraging or inside the nest. Our results may also help to better understand the mechanism of action of kaolin and the two biopesticides.

Materials and methods

Insects

Bumble bees (*B. terrestris*) were purchased from Koppert Biological Systems (Berkel en Rodenrijs, the Netherlands). The hives were kept at room temperature (21–22 °C), and the bees were fed with sugar water, as provided originally by the supplier (Koppert Biological Systems), and honeybee-collected pollen (Taali Mesila, Roela, Estonia). We collected bumble bee foragers as they emerged naturally from the hive entrance tunnel. The mean body weight ±SD of these workers was 240 ± 54 mg.

Products

Natural kaolin ([$Al_2Si_2O_5(OH)_4$], particle size: 3 microns) was obtained from Bang to Bonsomer Estonia (Tallinn, Estonia), Prestop-Mix containing *G. catenulatum* J1446 strain from Verdera (Espoo, Finland) and BotaniGard containing *B. bassiana* GHA strain from BioWorks (Victor, NY). Wheat flour was obtained from Tartu Mill (Tartu, Estonia) and used as a negative control based on Mommaerts et al. (2012).

Treatment of bumble bees

Upon collection, foraging bumble bee workers were placed individually in vials containing 50 mg of one of the powders, and the vials were gently shaken to cover the workers with the powder. This method simulates the bee walking through the powder and getting covered with the formulations. Covering bumble bees with powders all over the body simulates the maximum dosage which occurs when bumble bees are used in purpose of vectoring biopesticides. Workers of the control group were treated in a similar manner but without powder in the vial. Per treatment, five or six workers were used to assess effects on metabolic rate and water loss rate and 15–20 workers for effects on longevity.

Assessment of effects on metabolic rate and water loss rate

Collected workers were placed individually into the respiration chamber (Eppendorf Tips 100–5000 µl, the bee was gently pushed into the narrow part the tube to prohibit active movement) connected to an LI-7000 differential CO_2/H_2O analyser (LiCor, Lincoln, NE) that allows parallel recording of CO_2 release (VCO_2, ml h^{-1}) and water loss rate (VH_2O, µl h^{-1}). The LI-7000 analyser was connected to a computer to record CO_2 and H_2O production using LiCor software. The air flow was set at 166 ml min^{-1} (10 l h^{-1}), and NaOH and $M(ClO_4)_2$ were used to eliminate CO_2 and H_2O from the air used in the flow-through system. Measurement of metabolic rate (expressed as rate of CO_2 release) and water loss rate lasted for 6 h per individual (3 h before and 3 h after treatment) and was conducted at 18 °C at a relative humidity of 2 % (see also Muljar et al. 2012; Schimpf et al. 2013). Mean metabolic rate and water loss rate were calculated automatically by averaging data over 3-h period after excess CO_2 and H_2O, which entered the system during handling, had left the system.

In addition, we measured cuticular and total water loss rate during the periods when bumble bees showed discontinuous respiration. To understand the causes of changing water loss rate, cuticular and respiratory water loss must be analysed separately. Discontinuous gas exchange (DGE) is a pattern of insect respiration (Kestler 1985) involving the constriction of the spiracles of the insect tracheae (constriction phase, C) during which no CO_2 release occurs; the C phase is followed by fluttering (flutter phase, F), during which spiracles partly open and close in rapid succession keeping the tracheal oxygen at a certain level. Finally, the opening of spiracles (open phase, O) allows the maximum exchange of gases: CO_2, H_2O and water vapour. During the C phase, water loss occurs only through the insect's cuticle. As not all bumble bees displayed DGE at 18 °C as was noted earlier also by Karise et al. (2010), only three individuals of each treatment were used in the analyses of cuticular and respiratory water loss rate. The mean amounts of H_2O lost during three consecutive C+F (cuticular water loss) and O (total water loss) phases were measured. C and F phases were combined due to the difficulties in accurately differentiating the F phase. Respiratory water loss was calculated as the difference between total water loss and cuticular water loss (Gibbs and Johnson 2004).

The effects on longevity

We measured the effects of the treatment on the longevity of individual workers at 2 different temperatures, 18 and 28 °C, at a relative humidity of 60 % (SANYO—Versatile Environmental Test Chamber, MLR-351, Japan). These temperatures were chosen to accord with those that a foraging worker meets outside (18 °C) or inside the nest (28 °C). Normal air humidity in a bumble bee nest is 50–65 % (Yoon et al. 2002). All the bees (15–20 per treatment) were placed individually in 50 ml transparent vials with perforated walls so they could hear and smell each other to avoid the stress from being alone. Each bumble bee was provided with 30 % sugar solution as food. The bees were checked daily until all were dead; death was defined when they did not move antennae or legs and did not respond to tactile stimulation.

Statistics

As the metabolic rate and water loss rate had a right skewed distribution, the generalized linear models with logarithmic link function were fitted with SAS procedure GLIMMIX to test the effect of treatment (i.e. compare values before and after treatment) on metabolic rate and water loss rate. The models considered effects of treatment, time and treatment by time interaction and repeated measurements of the same individuals. To test the statistical significance of the changes by treatments and to compare changes between treatments, the properly defined contrasts were used. Analogical models were used also in analyses of respiratory, cuticular and total water loss rate during periods of DGE.

In the longevity experiment, the Kaplan–Meier survival curves were calculated for both temperatures and all treatments, and the treatments inside the temperatures were compared with a Wilcoxon rank sum test. The overall treatment effect on median longevity of bumble bees at both of the temperatures was evaluated using the Kruskal–Wallis test.

All results were considered statistically significant at $P < 0.05$, and all analyses were performed with the statistical package SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

Effects on metabolic rate

The main effect of treatment on the metabolic rate was statistically significant ($P = 0.007$), while the effect of time was not ($P = 0.109$). Also, there was no statistically significant time by treatment interaction effect ($P = 0.116$). However, study of more specific contrasts revealed a treatment based significant change ($P = 0.012$) in the metabolic rate of the bumble bees in the control group (Fig. 1a). In contrast, the mean metabolic rate did not change after the

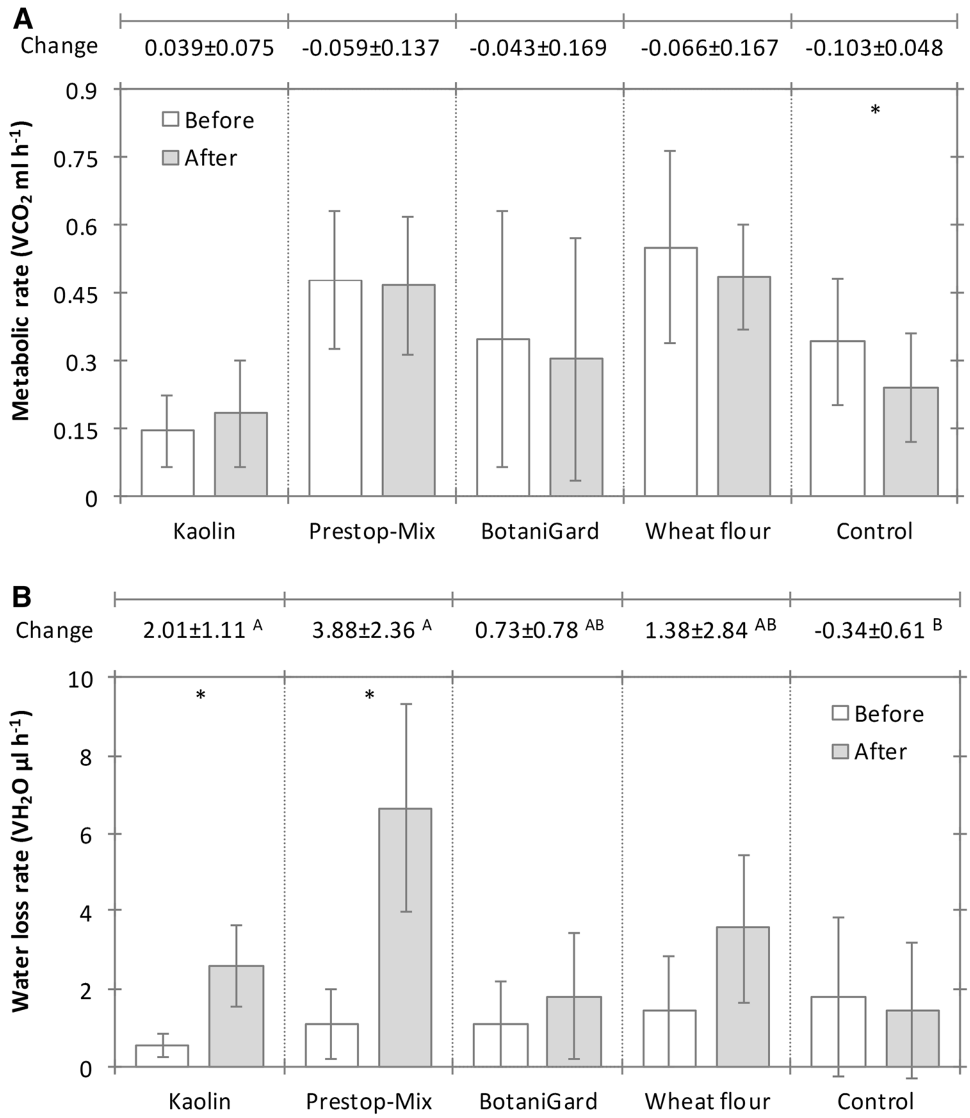

Fig. 1 Mean (±SD) meabolic rates (**a**) and water loss rates (**b**) of forager bumble bees exposed to kaolin, Prestop-Mix, BotaniGard and wheat flour. *Stars* indicate statistically significant ($P < 0.05$) intra-treatment differences before and after exposure, and different *letters* indicate statistically significant ($P < 0.05$) differences between treatments

treatment with kaolin, Prestop-Mix, BotaniGard and wheat flour.

Effects on mean water loss rate

The time affected the water loss rate significantly ($P < 0.001$), while the effect of treatment was not statistically significant ($P = 0.128$). The interaction effect of time and treatment was statistically significant ($P = 0.001$), indicating that the change in time is different at different treatments (Fig. 1b). In the control group, there was no decrease in water loss rate as was also the case with wheat flour and BotaniGard. In contrast, the treatments with kaolin and Prestop-Mix caused a significant increase (both $P < 0.001$) in water loss rate. In the latter bumble bees, the mean water loss rate had increased in each individual tested.

Effects on respiratory and cuticular water loss rate

Treatment significantly affected respiratory water loss ($P = 0.021$) but time did not ($P = 0.92$). Cuticular water loss and total water loss were significantly affected by treatment ($P < 0.001$ for both endpoints) and time ($P < 0.001$ for both endpoints) and the interaction of those factors ($P < 0.001$ for both endpoints). Both the cuticular water loss and total water loss increased significantly after treatment of bumble bees with kaolin and Prestop-Mix (both $P < 0.001$), whereas total water loss decreased in controls ($P = 0.048$; Table 1).

Effects on longevity

Exposure to the products at the two selected temperatures of 18 and 28 °C resulted in a reduction of the lifespan of

Table 1 Mean (±SD) change in time of total, cuticular and respiratory water loss rate (WLR) during periods of discontinuous gas exchange of bumble bees with exposure to kaolin, Prestop-Mix, BotaniGard and Wheat flour. Statistically significant ($P < 0.05$) changes are presented in *bold*, and different *letters* in columns indicate statistically significant ($P < 0.05$) differences among treatments

Treatment	Change in time		
	Respiratory WLR	Cuticular WLR	Total WLR
Kaolin	0.001 ± 0.011	**0.14 ± 0.05**^{A}	**0.14 ± 0.06**^{A}
Prestop-Mix	−0.009 ± 0.016	**0.51 ± 0.14**^{A}	**0.50 ± 0.14**^{A}
BotaniGard	−0.004 ± 0.008	0.04 ± 0.01^{B}	0.03 ± 0.02^{B}
Wheat flour	−0.001 ± 0.027	0.04 ± 0.05^{B}	0.03 ± 0.05^{B,C}
Control	−0.001 ± 0.005	−0.01 ± 0.02^{B}	**−0.02 ± 0.03**^{C}
Treatment by time interaction	$P = 0.95$	$P < 0.001$	$P = 0.001$

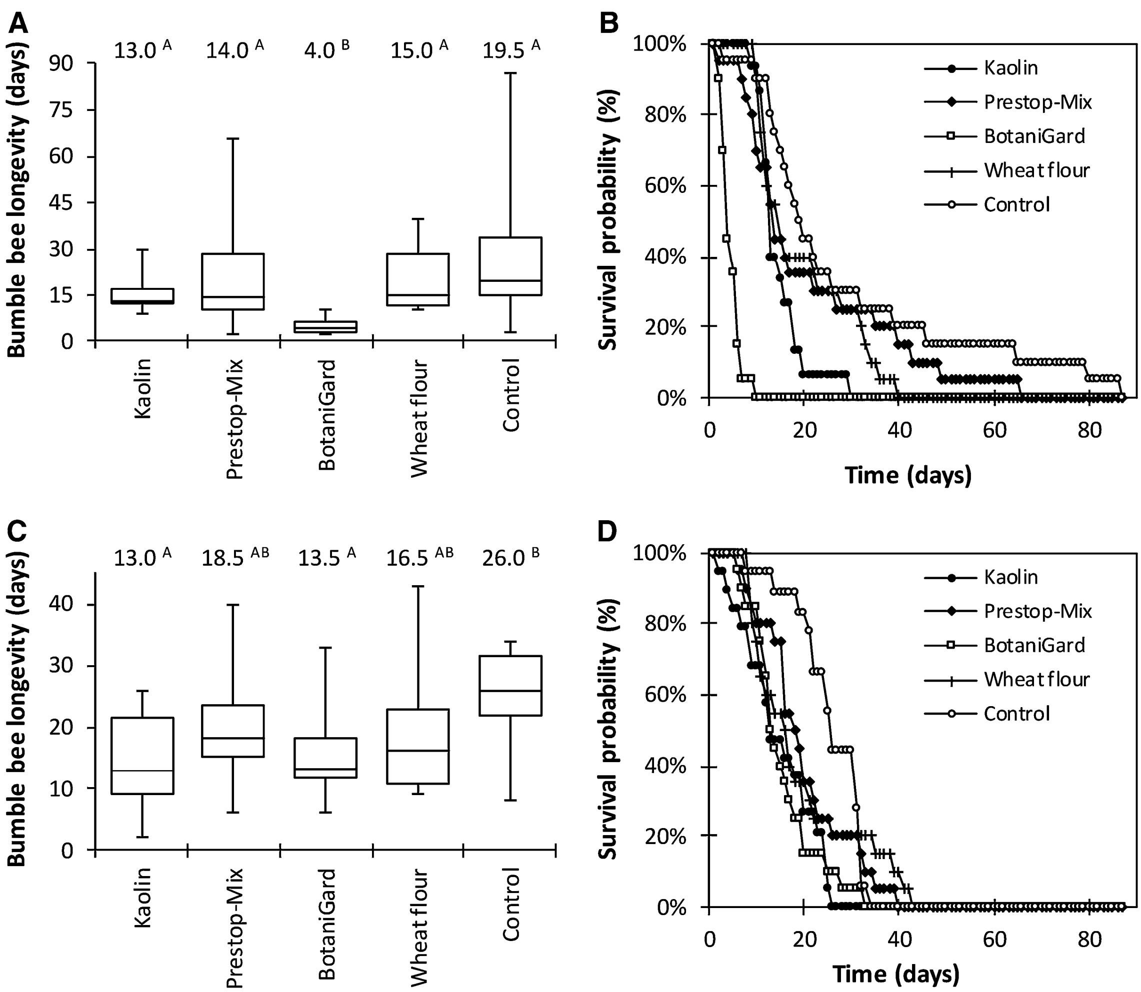

Fig. 2 Effects of exposure to kaolin, Prestop-Mix, BotaniGard and wheat flour on the longevity and survival probability of bumble bees at 18 °C (**a** and **b**) and 28 °C (**c** and **d**). Different *letters* above *box plots* indicate statistically significant differences between groups ($P < 0.05$)

the bumble bees, and the effect was depending on the temperature ($P = 0.017$). In the control groups, the median longevity (LT_{50}) was 19.5 days at 18 °C and 26.0 days at 28 °C (Fig. 2). In contrast, there was a significant decrease to 4.0 days with the treatment of BotaniGard at 18 °C, and at the higher temperature of 28 °C, there was a reduction to 13.5 days with BotaniGard and to 13.0 days with kaolin. In addition, with the treatment of BotaniGard, we observed that mycelia of *B. bassiana* were growing out from the body of the dead bumble bee workers only at 28 °C. For the other treatments, there were no negative effects on longevity as compared to the respective control group.

Discussion

As reported before (Kestler 1985; Mänd et al. 2005), the metabolic rate is a physiological factor which easily reveals minute changes in an organism. In the control group, we observed a clear decrease in metabolic rate and such decrease was expected since the mobility of the bumble bee worker was restricted (Zafeiridou and Theophilidis 2006; Karise et al. 2010). In contrast, with all powder treatments (kaolin, Prestop-Mix, BotaniGard and wheat flour), the metabolic rate remained unchanged or even slightly increased in some individuals. As a consequence, we expect that during the treatment with the powders, there must have been some irritation which increased the metabolic rate. In agreement, Israel and Boland (1992) reported strong grooming behaviour in honeybees after contact with silica-based powders. In addition, inert dust particles can enter the respiratory system or intestine, causing irritation (Subramanyam and Roesli 2000). The spores of *G. catenulatum*, the fungus in Prestop-Mix, have been tested against honey bees and bumble bees, and no negative effects were found on brood development (Van der Steen et al. 2003). However, commercial formulations of biopesticides may contain additives that may pose risks to bees. Indeed, kaolin is used in Prestop-Mix as carrier for the spores of *G. catenulatum,* and this preparation apparently prevented the normal decline of metabolic rate in tested individuals.

Our results do not support the idea that inert powders such as kaolin block the insect's spiracles (Subramanyam and Roesli 2000). Indeed, we observed an increase in water loss rate, and this was totally due to an increase in cuticular water loss and not due to respiratory water loss. If any of the studied substances would have blocked the spiracles, then the relative importance of respiratory water loss should have decreased after treatment. So, although the bumble bee workers in our experiments were covered with powders all over their body, we saw no significant decrease in respiratory water loss within the period of 3 h of treatment, suggesting that the insect spiracles were not blocked due to the treatments. While there was no effect on the respiratory water loss with kaolin and Prestop-Mix, we observed an increasing cuticular water loss in these treated bumble bees. The inert powder kaolin has different modes of action on insects. At the physiological level, the inert dusts have been seen to increase body water loss mainly due to cuticle impairment. Abrasive dusts between cuticular segments can damage the cuticle, but dusts can also absorb water and wax from epicuticular lipids leading to increased water permeability (Ebeling 1971; Golob 1997). It has been demonstrated that diatomaceous earth products can remove at least one epicuticular lipid tridecane (Cook et al. 2008). For Prestop-Mix, we know that kaolin is one of the components of this biofungicide, however, we do not know its exact content. Finally, it should be noticed for Prestop-Mix that the increase in water loss rate was even higher than with kaolin. Because the effect of *G. catenulatum* on the insect physiology is not well studied, we can only speculate about possible effects. Another intriguing observation is that beekeepers who have used honeybees to vector Prestop-Mix have noticed that these hives were in better condition than neighbouring ones (personal communication, Dr. H. Hokkanen, University of Helsinki, Finland). They suggest that Prestop-Mix can decrease the number of external microorganisms such as yeasts (Rosa et al. 2003) and entomopathogens (Yamoah et al. 2008) on honeybees.

For BotaniGard and wheat flour, we observed no changes in the water loss rate during the 3-h observation period or during DGE. It is likely that the wheat flour particle size or shapes do not perturb bumble bees. For BotaniGard, the fungus *B. bassiana* dissolves the insect's internal tissues, affecting their ability to use muscles (Amnuaykanjanasin et al. 2013). But in our experiment that was completed within a short period of 3 h, the germination process was only beginning and presumably no harm had yet been done. However, it should be remarked that to better understand the effects by BotaniGard, it would be useful to have information on the composition of the bioinsecticide formulation, especially on the filling products as kaolin.

On the lethal effects of the treatments against bumble bees, it was clear that BotaniGard had the strongest negative effects. Median longevity was shorter in all other treatments at 18 °C, although survival probability decreased more slowly than at 28 °C. The survival of bumble bees decreased significantly with the treatment of BotaniGard at both temperatures compared with the control. The lethal time for *B. bassiana* has been reported to be 6–7 days, but the first tissue damage emerges within 3 days (Fargues et al. 1997). We saw that the LT_{50} was 4 days at 18 °C. Probably, the bumble bees' capability to resist the pathogen was weaker at this low temperature. At the higher temperature of 28 °C, the bumble bees survived longer with an LT_{50} of 13.5 days. In addition, we observed that the mycelia of *B. bassiana* were growing out from the body of the dead bumble bee workers at that temperature. Probably, the elapsed time had been enough for mycelial and conidial growth at 28 °C.

Kaolin alone also reduced survival at 28 °C which might be due to the abrasive activity against the insect cuticle leading to increased water loss as discussed above. That this mortality was scored only at the higher temperature might be due the fact that the bumble bees were more active and their metabolic demands were higher which led to a faster exhaustion of the organism. We did not see a lethal effect by Prestop-Mix although it contains kaolin in its formulation. The abrasive activities of Prestop-Mix as product were not as aggressive as for pure kaolin; however, we have no information on the percentage of kaolin in the Prestop-Mix formulation. In addition, the period of exposure in our experiments was limited to a short period of 30 s. Mommaerts et al. (2012) reported that a strong worst case exposure to Prestop-Mix can be hazardous for bumble bees. This occurs with one-way dispensers where the bumble bees need to move in and out the hive through the powder and carry the formulation into the nest. With the use of two-way dispensers where bumble bees use separate pathways for in- and outward movement, as is the case in practice in entomovectoring tactics for the biological control of *Botrytis* in strawberry (Mommaerts and Smagghe 2011), the exposure of the biopesticide to bumble bees is much lower which reduces the risks. Indeed, no negative effects by Prestop-Mix have been reported when used in a two-way dispenser (Mommaerts et al. 2011).

In conclusion, our results demonstrated that kaolin and formulations of microbial pest control agents containing kaolin as Prestop-Mix and used in entomovectoring technology may pose a risk to vectoring bees.

Author contribution

RK, RM and MM conceived and designed research. RM, AK and GD conducted experiments. RK, RM, GS, TK, GD and MM analysed data. RK, RM, GS, TK, IHW and MM wrote MS. All authors read and approved the MS.

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