Research Article

Received: 27 July 2010 Revised: 11 October 2010 Accepted: 19 October 2010 Published online in Wiley Online Library: 11 January 2011

(wileyonlinelibrary.com) DOI 10.1002/ps.2093

Lethal and sublethal side-effect assessment supports a more benign profile of spinetoram compared with spinosad in the bumblebee *Bombus terrestris*

Linde Besard,[a] Veerle Mommaerts,[a,b] Gamal Abdu-Alla[a] and Guy Smagghe[a,b]*

Abstract

BACKGROUND: This study was undertaken to identify the potential side effects of the novel naturalyte insecticide spinetoram in comparison with spinosad on the bumblebee *Bombus terrestris* L. The potential lethal effects together with the ecologically relevant sublethal effects on aspects of bumblebee reproduction and foraging behaviour were evaluated. Bumblebee workers were exposed via direct contact with wet and dry residues under laboratory conditions to spinetoram at different concentrations, starting from the maximum field recommended concentration (MFRC) and then different dilutions (1/10, 1/100, 1/1000 and 1/10 000 of the MFRC), and compared with spinosad. In addition, the side effects via oral exposure in supplemented sugar water were assessed.

RESULTS: Direct contact of *B. terrestris* workers with wet residues of spinosad and spinetoram showed spinetoram to be approximately 52 times less toxic than spinosad, while exposure to dry residues of spinetoram was about 8 times less toxic than exposure to those of spinosad. Oral treatment for 72 h (acute) indicated that spinetoram is about 4 times less toxic to *B. terrestris* workers compared with spinosad, while exposure for a longer period (i.e. 11 weeks) showed spinetoram to be 24 times less toxic. In addition, oral exposure to the two spinosyns resulted in detrimental sublethal effects on bumblebee reproduction. The no observed effect concentration (NOEC) for spinosad was 1/1000 of the MFRC, and 1/100 of the MFRC for spinetoram. Comparison between the chronic exposure bioassays assessing the sublethal effects on nest reproduction, with and without allowing for foraging behaviour, showed that the respective NOEC values for spinosad and spinetoram were similar over the two bioassays, indicating that there were no adverse effects by either spinosyn on the foraging of *B. terrestris* workers.

CONCLUSION: Overall, the present results indicate that the use of spinetoram is safer for bumblebees by direct contact and oral exposure than the use of spinosad, and therefore it can be applied safely in combination with *B. terrestris*. Another important conclusion is that the present data provide strong evidence that neither spinosyn has a negative effect on the foraging behaviour of these beneficial insects. However, before drawing final conclusions, spinetoram and spinosad should also be evaluated in more realistic field-related situations for the assessment of potentially deleterious effects on foraging behaviour with the use of queenright colonies of *B. terrestris*.

Keywords: spinetoram; spinosad; microbial insecticide; spinosyns; bumblebees; *Bombus terrestris*; toxicity; direct contact; oral exposure; reproduction; foraging behaviour; IPM; ecotoxicity

1 INTRODUCTION

Naturalyte insecticides, which contain microorganisms or their byproducts, are more selective than conventional synthetic chemical pesticides because their side effects on non-target animals and humans are generally low or completely absent. As a consequence, these chemistries are used worldwide in environmentally friendly integrated pest management (IPM) programmes. One major class of naturalyte insecticides is the spinosyns, a novel class of natural products possessing a unique structure, a novel mode of action and commercial levels of insecticidal activity. Produced by the actinomycete *Saccharopolyspora spinosa* Mertz & Yao, the fermentation-derived spinosyns, and their insecticide activity, were discovered in the mid-1980s, eventually leading to the development and the 1997 registration of spinosad, a naturally occurring mixture of two of the most active spinosyns, A and D.[1–3] As demonstrated by

* *Correspondence to: Guy Smagghe, Department of Biology, Faculty of Sciences, Vrije Universiteit Brussel (VUB), Brussels, Belgium. E-mail: guy.smagghe@ugent.be*

a *Department of Biology, Faculty of Sciences, Vrije Universiteit Brussel (VUB), Brussels, Belgium*

b *Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium*

spinosad, the spinosyns are primarily active against a wide range of lepidopteran and dipteran pest insects. To improve the biological efficacy of this novel chemistry, approaches have been explored to identify or derive new spinosyns with improved activity, an expanded spectrum of targets and an improved ecotoxicological profile. Next to the approach of searching for new naturally occurring spinosyns, synthetic modification of the naturally occurring spinosyns has proven to be very promising. Several semi-synthetic spinosyn analogues were explored, which resulted in the subsequent discovery of spinetoram (XDE-175), consisting of a mixture of chemically modified spinosyn J (major component) and spinosyn L.[3,4] Spinetoram provides both improved efficacy and an expanded activity spectrum while maintaining a benign environmental and toxicological profile.[3,4] Both spinosad and spinetoram have a broad-spectrum activity providing long-lasting control of a wide spectrum of insect pests (Diptera, Lepidoptera, Hymenoptera, Siphonaptera and Thysanoptera) in a variety of crops. Their mechanism of action is to cause hyperexcitation of the insect nervous system by activation of the nicotinic acetylcholine receptor (nAChR), specifically the subunit Dα6, and altering the function of GABA-gated chloride channels, resulting in involuntary muscle contractions and tremors, followed by paralysis and insect death.[1,3,5]

Bumblebees such as *Bombus terrestris* L. are well known for their high pollination capacity of wild flowers, and, over the last few decades, these insects have become widely exploited as pollinators of several economically important crops including tomatoes, sweet peppers and strawberries.[6] The use of bumblebees as natural pollinators of crops offers several advantages, including improved fruit quality, higher fruit weight and fewer malformed fruits. It is clear that the foraging activities of bumblebees have significant economic ramifications for crop production, and therefore it is extremely important that factors that lower their pollinating activity are minimised.[6,7] In practice, however, bumblebee workers are faced with the risk of being poisoned by applied plant protection products, such as insecticides, during their foraging activity, and it is clear that these products can cause harmful side effects on these beneficial insects.[6] Several studies have shown spinosad to be highly toxic to honey bees (*Apis mellifera* L.) and bumblebees. However, when dried residues of spinosad were tested, these were not found to be harmful towards adult bumblebees and larvae in laboratory studies, and this was also the case towards adults, brood and foraging rates in field studies.[8] However, greenhouse studies have suggested that the development of bumblebee brood may be impaired by spinosad.[9] In the case of spinetoram, the recent commercialisation of this product means that no data are available in the literature pertaining to its adverse effects on bumblebees. However, a few studies have been conducted on honey bees, and these have demonstrated that spinetoram is toxic to these pollinating insects when consumed orally or by direct contact. However, in field tests designed to mimic use conditions, spinetoram residues aged for 3 h after spray applications at 110 g ha^{-1} in alfalfa (*Medicago sativa* L.) caused no mortality of honey bees.[9–12]

In the assessment of side effects of pesticides, most studies have so far evaluated acute lethal effects or sublethal effects on the nest brood following chronic exposure via food. However, in addition there is a need to evaluate sublethal side effects on foraging behaviour to guarantee crop pollination. Recently, Mommaerts *et al.*[13,14] reported on a new reliable laboratory bioassay with queenless bumblebee microcolonies to evaluate such sublethal effects by pesticides and microorganisms on the foraging behaviour of bumblebee workers.

Table 1. Overview of the insecticides tested, spinosad and spinetoram, and the positive control insecticide imidacloprid, their commercial name, formulation type and percentage of active ingredient (AI) and the maximum field recommended concentration (MFRC) in % of formulation and corresponding amounts in mg AI L^{-1}

Insecticide	Commercial name	Formulation[a]	MFRC (% formulation)	MFRC (mg AI L^{-1})
Spinosad	Tracer	480 g L^{-1} SC	0.0048	400
Spinetoram	Delegate	250 g kg^{-1} WG	0.0025	25
Imidacloprid	Confidor	200 g L^{-1} SL	0.1	200

[a] WG = water dispersible granule; SC = suspension concentrate; SL = soluble liquid.

The present project aimed to produce an in-depth assessment of the hazards of spinetoram towards *B. terrestris*, and to compare the recorded effects with those elicited by spinosad. The potential lethal effects of exposure together with the ecologically relevant sublethal effects on aspects of bumblebee reproduction and worker foraging behaviour were evaluated. Spinetoram was used at different concentrations, starting from the maximum recommended concentration in the field (MFRC) and then different dilutions (1/10, 1/100, 1/1000 and 1/10 000 of the MFRC), and compared with spinosad. The bumblebees were treated via contact exposure to both wet and dry residues in order to obtain a better picture of the risks in practice when the spinosyns are sprayed. Following this, the side effects via oral treatment of the drinking sugar water were assessed. This study recorded acute toxicity towards workers and any sublethal effect on nest brood and reproduction (measured as production of males) and on worker foraging behaviour. It is evident that, if these three parameters are impaired, then pollination will be negatively affected, resulting in a loss of crop production. The results of this study are the first to present an in-depth assessment of the newly developed spinosyn spinetoram and the beneficial pollinator *B. terrestris*, and, as such, to evaluate its compatible use in integrated production systems in agriculture.

2 MATERIALS AND METHODS

2.1 Insects

All experiments were undertaken with worker bumblebees obtained from a mass rearing culture (Biobest NV, Westerlo, Belgium) and conducted under standardised conditions of 28–30 °C, 60–65% RH and continuous darkness in the authors' laboratory at VUB (Belgium). The insects were provided *ad libitum* with commercial sugar water and pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) as energy and protein sources respectively.[15]

2.2 Chemicals

Table 1 gives the respective commercial name, type of formulation and percentage active ingredient (AI) and MFRC of the two products tested, spinosad and spinetoram, and the positive control insecticide imidacloprid. The products were used and stored in accordance with the manufacturers' guidelines.

2.3 Bioassay to assess acute side effects via dry and wet contact

Potential side effects of spinosad and spinetoram on bumblebee workers were assessed via contact exposure to wet and dry residues at different concentrations, starting from the MFRC (1/1) and then different dilutions (1/10, 1/100, 1/1000 and 1/10 000 of the MFRC). For contact exposure to wet residue, five bumblebee workers were collected from the mass rearing and placed in a transparent plastic round box (9 cm diameter, 4 cm height) with five air holes (1 cm diameter). Prior to this, the inert glass bottom plate of this box was immersed for 10 s in the corresponding concentration of spinetoram or spinosad (prepared in water). In the negative control, the glass bottom plate was immersed in water. For contact exposure to dry residue, the same experimental design was used, except that the glass bottom plates were first allowed to dry at room temperature before exposure to the bumblebee workers. For each treatment, four replicates were performed, each consisting of five workers, and each experiment was repeated 2 times.

In the boxes, the total numbers of dead workers were recorded after 6, 24, 48 and 72 h of exposure and expressed as percentage mortality. Where necessary, data were corrected using Abbott's correction[16] if there was mortality in the controls. In addition, mortality percentages were scored in accordance with the classification of the International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC): 'class 1' = <25% effect = non-toxic; 'class 2' = 25–50% effect = weakly toxic; 'class 3' = 50–75% effect = moderately toxic; 'class 4' = >75% effect = highly toxic.[13]

The median toxicity concentrations (LC_{50} values) together with the 95% confidence limits (95% CL) were calculated for the two products. For the dose–response curves of spinetoram and spinosad at 1/1, 1/10, 1/100, 1/1000 and 1/10 000 of their MFRC in the wet and dry contact treatments, non-linear sigmoid curve fitting using Prism v.4 software (GraphPad Software, La Jolla, CA) was used. The goodness of fit of the data to the curve model was evaluated on the basis of R^2 values.[13]

2.4 Bioassay to assess acute and chronic side effects via oral treatment

For these experiments, microcolonies were used, each consisting of five *B. terrestris* workers as previously optimised for oral exposure to pesticides.[13] Briefly, five newly emerged workers were put in an artificial transparent plastic nest box of 15 cm width, 15 cm depth and 10 cm height. These microcolonies were then provided with sugar water (energy source) via a container (500 mL) under the nest box and with commercial pollen (protein source) inside the nest box. In the centre of each nest box, an artificial brood area was made to stimulate egg laying and the building of brood cells. After 1 week, a hierarchy became established, the ovaries of the dominant worker developed and it started to lay haploid eggs that developed into drones. In the laboratory, microcolonies were kept under standardised conditions in the dark at 28–30 °C and 60–70% relative humidity.

Under these conditions, the adult workers were orally exposed to spinetoram and spinosad at their respective MFRC (1/1) (Table 1; prepared in water) and at different dilutions of the MFRC (1/10, 1/100, 1/1000 and 1/10 000) via the feeding of treated sugar water.[13] In the negative control, workers were fed on untreated sugar water. In a positive control, workers were exposed to imidacloprid at its MFRC (0.1% or 200 ppm, prepared in sugar water), which resulted in 100% mortality in all cases. For each treatment, four replicates were performed, each consisting of a microcolony of five bumblebee workers, and each experiment was independently repeated twice. The nests were exposed for 11 weeks *ad libitum* to 500 mL of sugar water contaminated with the product at its MFRC or a dilution. In these experiments, the sugar water and the pollen were refreshed weekly.

As described above, to assess the acute lethal effects, the total numbers of dead workers were recorded after 72 h of exposure, expressed as percentage mortality, and where necessary corrected using Abbott's correction,[16] and then scored in accordance with the IOBC classification. In addition, the chronic toxicity of spinetoram and spinosad was also scored on a weekly basis up to 11 weeks of exposure to treated sugar water.

In the same way as contact toxicity, median toxicity concentrations (LC_{50} values) together with their corresponding 95% CL were estimated using the dose–response curves of spinetoram and spinosad at 1/1, 1/10, 1/100, 1/1000 and 1/10 000 of their MFRC in the sugar water with the use of Prism v.4. In addition, the no observable effect concentration (NOEC) was indicated.

In addition to lethal effects, the sublethal effects on the reproduction of workers in the microcolonies treated by the two spinosyn products (MFRC and the different dilutions) were evaluated over a period of 11 weeks. On a weekly basis, the numbers of male offspring produced per nest were counted and compared with the negative controls. In the positive control, imidacloprid was used at its MFRC, as described above, which resulted in a complete loss of reproduction in all cases. For each treatment, four nests were used, each consisting of five workers, with experiments repeated independently twice. The data on reproduction were analysed by one-way ANOVA, and then means ± SEM separated by *post hoc* Tukey–Kramer tests ($P = 0.05$) using SPSS v.15.0 software (SPSS Inc., Chicago, IL). In addition, the sublethal effects of the two spinosyns tested were categorised into four groups in accordance with the IOBC on the basis of the percentage of effect, ranging from no effect (<25%), low effect (25–50%), moderate effect (50–75%) to high effect (>75%).[13]

2.5 Bioassay to assess acute and chronic side effects via oral treatment, including foraging behaviour

In a manner similar to that described above (Section 2.4), the acute and chronic side effects, including foraging behaviour, were assessed. Here, those concentrations of spinetoram and spinosad that did not show any harmful lethal or sublethal effects in the above-mentioned acute experiments (Section 2.4.) were investigated. This was done using the foraging behaviour test as developed by Mommaerts *et al*.[14] In brief, two artificial nest boxes (A and B) were connected to a tube of 20 cm length and 2 cm diameter. In box A, five newly emerged workers constructed their nest, and, after 2 weeks, when third- and fourth-instar larvae appeared in the nests, food was removed from box A and placed in box B. Before exposure to the insecticide, the workers were allowed a training period of 2 days to forage in box B for untreated sugar water and pollen. Subsequently, plain sugar water in box B was replaced with treated sugar water.

Worker survival and drone production were recorded weekly, in a manner similar to that described above, over a period of 7 weeks.[14] In the negative control, nest worker bees were fed on untreated sugar water and pollen; in the positive controls, workers were exposed to imidacloprid at 0.00001% or 20 ppb in the drinking sugar water, which negatively affected foraging behaviour in all cases.[14] For each treatment, four replicates were

Table 2. Lethal effects of spinosad and spinetoram on the survival of workers of *Bombus terrestris* by contact with wet and dry residues after acute (72 h) treatment

	LC_{50} (dilution of MFRC) (95% CL; R^2)	
Product	Wet residue	Dry residue
Spinosad	1/28 (1/52–1/15; 0.98)	1/10 (1/48–1/2; 0.92)
Spinetoram	2/1 (1/3–10/1; 0.92)	1/1.2 (1/3–2/1; 0.92)
Ratio spinetoram/spinosad	52	8

performed, each consisting of five workers, and each experiment was repeated twice. The data were analysed as described above.

3 RESULTS

3.1 Bioassay to assess acute side effects via dry and wet contact exposure

Based on the acute (72 h) LC_{50} values, wet contact exposure to bumblebees was highly toxic for spinosad, while spinetoram was 52 times less toxic (Table 2). Contact with a dry residue of spinosad at 1/10 of the MFRC killed 50% of the bumblebee workers (LC_{50}), whereas spinetoram was about 8 times less acutely toxic, with 50% worker mortality at full MFRC (1/1).

It is also to be noted that, for spinosad, dry contact exposure was 3 times less acutely toxic than exposure to wet residues. Specifically, wet and dry residues of spinosad at full MFRC killed all (100%) of the exposed workers; at 1/10 of the MFRC the respective mortalities were 80% (class 4) and 40% (class 2), and at 1/100 of the MFRC only 15 and 20% (class 1). In contrast, the acute toxicities of wet and dry contact residues of spinetoram were relatively similar, with 45% (class 2) and 55% mortality (class 3) at the full MFRC respectively. The residues of spinetoram at 1/10 of the MFRC were safe and gave 15 and 8% (class 1) mortality for the wet and dry residues respectively.

Typically, intoxicated bumblebee workers showed symptoms of tremors and paralysis, resulting in death. For spinosad, wet and dry exposure to the full MFRC killed 50% of the treated workers within 6 h, but at 24 h of treatment all were dead. With a lower concentration of 1/10 of the MFRC of spinosad, 50% of the workers exposed to wet residues were dead after 24 h, while this was only 20% with dry residues. In contrast, for spinetoram, the mortalities at full MFRC were observed later, after 3 days.

3.2 Bioassay to assess acute and chronic side effects via oral treatment

Table 3 shows that oral feeding of spinosad to bumblebees in sugar water for 72 h was highly toxic for bumblebee workers, with a mortality of 50% (class 3) at 1/5 of the MFRC. In detail, spinosad at 1/1 and 1/10 of the MFRC was highly toxic, causing 75% (class 4) and 40% (class 2) mortality respectively. Intoxicated workers showed tremors, leading to paralysis and rapid death as described above; there was about 50% mortality within the first 6 and 24 h of treatment with 1/1 and 1/10 of the MFRC treatments respectively. The other spinosad concentrations tested, i.e. 1/100, 1/1000 and 1/10 000 of the MFRC, did not cause any acute worker mortality (class 1). In parallel experiments, an LC_{50} of 1/1.2 of the MFRC was recorded for spinetoram, demonstrating that it was about 4 times less toxic than spinosad. In detail, spinetoram at 1/1 and 1/10 of the MFRC killed 55% (class 3) and 13% (class 1) of the workers respectively.

When the bumblebee workers were exposed for a longer period, i.e. 11 weeks, the chronic toxicity of spinosad resulted in an LC_{50} of 1/247 of the MFRC (Table 3). In detail, a high worker mortality of 100% was observed in nests exposed to 1/1 and 1/10 of the MFRC of spinosad (class 4), and 80% with 1/100 of the MFRC (class 4), while lower concentrations of 1/1000 and 1/10 000 of the MFRC were safe (class 1). In contrast to spinosad, spinetoram was less detrimental, with a chronic LC_{50} of 1/10 of the MFRC, demonstrating that spinetoram was about 24 times less toxic than spinosad. With the full MFRC of spinetoram, mortality was 100% (class 4) within 4 weeks. With a lower concentration of 1/10 of the MFRC, 54% mortality was scored after 11 weeks (class 3), and, interestingly, lower concentrations were safe (class 1). Based on these results, the NOEC for worker toxicity was 1/1000 of the MFRC for spinosad, and 1/100 of the MFRC for spinetoram.

In addition to the lethal effects, oral chronic exposure to the two spinosyns resulted in detrimental sublethal effects on bumblebee reproduction. Spinosad at 1/1, 1/10 and 1/100 of the MFRC resulted in a significant ($P < 0.05$) reduction in nest reproduction by 100% (zero drones, as all workers were dead), 95% (3 ± 2 drones) and 92% (5 ± 1 drones), respectively, as compared with the controls (62 ± 10 drones). Only when the concentration was lowered to 1/1000 and 1/10 000 of the MFRC was nest reproduction unaffected ($P > 0.05$) (class 1). For spinetoram, exposure to the full MFRC in the sugar water resulted in a total loss of nest reproduction, as all workers were dead after 4 weeks in these nests. However, when testing a lower concentration of 1/10 of the MFRC of spinetoram, the sublethal effect on drone numbers was significantly ($P < 0.05$) reduced to 44%, with only 25 ± 2 drones per nest as compared with 57 ± 4 drones in the control nests (class 2). Lower concentrations were safe (class 1). Based on these experiments, the NOEC for nest reproduction was 1/1000 of the MFRC for spinosad, while this concentration was 1/100 for spinetoram.

3.3 Bioassay to assess acute and chronic side effects via oral treatment, including foraging behaviour

In the foraging behaviour test, spinosad at 1/1000 and 1/10 000 of the MFRC in the drinking sugar water, which had no acute worker toxicity (as described above), also caused no significant worker mortality ($3 \pm 3\%$ and $16 \pm 5\%$ respectively) after 7 weeks of exposure (class 1) compared with the controls. For spinetoram, the concentrations of 1/100, 1/1000 and 1/10 000 of the MFRC (with no acute lethal effects, as described above) caused no significant mortality in seven-week-exposed bumblebee workers when foraging behaviour effects were included: $27 \pm 15\%$, $3 \pm 3\%$ and $5 \pm 5\%$ respectively. Here, the NOEC for worker toxicity was 1/1000 of the MFRC for spinosad and 1/100 of the MFRC for spinetoram.

In addition to reduced worker survival, tests were conducted to establish whether exposure to the two spinosyns in sugar water could provoke a reduction in reproduction, and, if this was the case, whether the effect was stronger when foraging behaviour was allowed for or prevented. Spinosad at 1/1000 and 1/10 000 of the MFRC did not reduce ($P > 0.05$) nest reproduction, and the numbers of drones produced were comparable with those in the negative control nests treated with water only. For spinetoram, the three tested concentrations of 1/100, 1/1000 and 1/10 000 did not cause a significant ($P > 0.05$) effect. In summary, for spinosad there were no observable effects (NOEC) for nest reproduction at 1/1000 of the MFRC, and this was also

Table 3. Lethal effects of spinosad and spinetoram on the survival of workers of *Bombus terrestris* by oral exposure via supplemented sugar water in situations that allowed for (with) and prevented (without) foraging behaviour after acute (72 h) and chronic (7 or 11 weeks) treatment

	LC_{50} (dilution of MFRC) (95% CL; R^2)			
	Acute		Chronic	
Product	without foraging	with foraging	without foraging	with foraging
Spinosad	1/5 (1/10–1/2.3; 0.97)	1/9 (1/16–1/4.6; 0.98)	1/247 (1/476–1/129; 0.98)	1/103 (1/206–1/52; 0.97)
Spinetoram	1/1.2 (1/1.4–1/1.1; 0.91)	1/1.8 (1/3–1/1.1; 0.90)	1/10 (1/9–1/11; 0.99)	1/13 (1/61–1/3; 0.94)
Ratio spinetoram/spinosad	4	5	24	8

the case for spinetoram at 1/100 of the MFRC. In contrast, for the positive control, imidacloprid at 1/10 000 of the MFRC (20 ppb), all nests showed a significant ($P < 0.05$) reduction of 50–55% in the numbers of drones produced as compared with the controls. Typically, these workers were less inclined to forage or feed, and the building up of the nest and their travel times were much longer when compared with workers in the control nests.

4 DISCUSSION

The present project performed for the first time an in-depth assessment in the bumblebee *B. terrestris* of the newly developed spinosyn spinetoram, which has been developed to deliver improved efficacy and an expanded activity spectrum. An assessment was made of the potential lethal and sublethal side effects of spinetoram in comparison with spinosad on this beneficial pollinator in the context of use in IPM. By the use of bumblebee microcolonies, bumblebee workers were exposed via three different routes of exposure: by contact with wet and dry residues and by oral exposure via the drinking of treated sugar water. In the past, diverse studies have reported on the high contact and oral (ingestion) toxicity of the microbial insecticide spinosad against several beneficial insects such as the honey bee *A. mellifera* and bumblebee *B. terrestris*, parasitoids such as *Telenomus remus* Nixon and predators such as the spider *Philodromus cespitum* (Walckenaer) and the earwigs *Doru taeniatum* (Dohrn) and *Forficula aericularia* L.[17–25] To explain the high sensitivity of bees to spinosad, Hsu *et al*.[26] concluded that this concurred with the low presence of hydrolase and monooxygenase detoxification. Similarly, Fernandes *et al*.[27] argued that the low levels of insecticide-detoxifying enzymes, such as hydrolytic carboxylesterases, P450 monooxygenases and conjugation glutathione-*S*-transferases (GST), are responsible for the high spinosad toxicity observed in the honey bee *A. mellifera*. An interesting observation is that the honey bee genome contains, as compared with *Anopheles* mosquitoes, *Tribolium* beetles, *Acyrthosiphon* aphids and *Drosophila* fruit flies, 30–50% fewer genes encoding carboxylesterase, P450 and GST enzymes, which are principally responsible for the metabolism of pesticides and in which the great majority of metabolic resistance mutations have been found in other species of invertebrates.[28–30] For the bumblebee *B. terrestris*, the whole genome sequence is planned to be available later in 2010–2011 (Schmid-Hempel P, private communication). The continuing research into improving the activities of the spinosyns has recently led to the development of spinetoram. While it should be mentioned here that honey bees (*A. mellifera*) are the representative pollinating insect in risk assessment studies, previous works have demonstrated that extrapolation of results to other pollinators, such as bumblebees, is not possible.[7,31] Although honey bees and bumblebees are closely related, they differ significantly in various parameters such as size, foraging behaviour and food consumption, which are determining factors in the risk of exposure and side effects. In particular, special attention needs to be paid to sublethal effects on nest reproduction and foraging behaviour, as any impairment of these subtle processes will lead to loss of pollination and a resulting loss of production.

Contact with residues of spinosad was highly detrimental for *B. terrestris* bumblebee workers, and this was most clear for wet residues, where the LC_{50} was 1/28 of the MFRC for exposure to wet residues and 1/10 for dry residues. Typically, intoxicated workers showed tremors followed by paralysis and insect death, which agrees with the known mechanism of action of spinosyns by activation of the insect nAChR. In addition, it should be noted that these detrimental effects in bumblebee workers were observed sooner, at a lower fraction of the MFRC, with spinosad than with spinetoram. Similarly, Sterk *et al*.[8] and Mayes *et al*.[9] have reported that wet residues of spinosad are highly toxic to bumblebees. The high contact toxicity of spinosad was also confirmed by Halsall and Grey[10], with a 50% lethal dose (LD_{50}) of around 0.12–0.16 µg per *A. mellifera* bee at 24–48 h after dermal dosage. In parallel, Aldershof[32] determined an LD_{50} value of 19.4 µg per bee at 48 h after dermal contact for *B. terrestris* workers, indicating that, although both pollinating insect species show high sensitivity to spinosad, the honey bees appear to be 100 times more sensitive than bumblebees. Interestingly, the discrepancy between *Apis* and *Bombus* could be mediated by differences at the target nAChR, specifically the subunit Dα6.[3] Interestingly, Watson *et al*.[3] could report on mutations in the nAChR ligand-binding domain conferring spinosyn A resistance in *Drosophila*, but to date the physical structure of the nAChR ligand-binding domain has not been directly determined for any insect species. In addition, it should be noted that, so far, no structure and docking data are available for spinosad/spinetoram with respect to the Dα6 subunit. Next to pharmacodynamics, it is evident that kinetics such as penetration rate through the insect cuticle, uptake and transport in the body tissues and metabolism can also play a crucial role in explaining differences in sensitivity. However, all data so far confirm the highly detrimental effects in honey bees/bumblebees by contact, especially with wet residues, of spinosad. As a consequence, it has been accepted that the spraying of this microbial insecticide onto crops at flowering and/or in combination with beneficial pollinating insects is not safe and therefore not recommended. Good examples include the period of apple and pear flowering in open field situations, while in greenhouses it is recommended to remove (or close) nests when spraying tomatoes. In contrast to the strong harmful effects for spinosad, it was of interest that lower toxicities were

recorded by contact with spinetoram, with exposure to the MFRC causing ≤50% mortality. Most paramount was that, based on LC_{50} values, exposure to wet residues of spinetoram was 52 times less toxic to *B. terrestris* workers than exposure to wet residues of spinosad. This is a strong indicator that a spray application of spinetoram on crops (flowers) to control pest insects has a higher safety compared with spinosad. The authors also believe that, potentially, this new chemistry could be employed in a pollinator-and-vectoring system with bumblebees to control flower pests; however, before this, adequate side-effect tests with a powder formulation of spinetoram need to be performed.

Following spraying, spinosad is partly absorbed into leaf tissue, after which it is transported throughout the plant and into nectar and, potentially, the pollen.[33] Given that nectar and pollen are the two main food sources of pollinators, it is likely that these beneficial insects are exposed to the risks of being poisoned by this insecticide during their foraging. In addition, Cheng-Dui and Li-Hui[34] found that spinosad is degraded by ultraviolet light irradiation; the degrading percentage was up to about 70% for a 484 min irradiation period. However, as spinosad residues inside the flowers are not exposed to UV irradiation, it is likely that high residues of spinosad will remain present inside the flowers. Besides, it has been reported that spinosad molecules remain stable in a water solution.[11] As a consequence, it is highly plausible that bumblebees and honey bees will be exposed to this insecticide, as flowers are the only site for pollination. Based on acute (72 h) LC_{50} values for spinosad when delivered via sugar water, a dilution of 1/5 of the MFRC caused 50% mortality with spinosad, while this effect was only observed with spinetoram at a concentration close to the MFRC (1/1.2), which indicates its compatible use in combination with bumblebees. The higher safety of spinetoram compared with spinosad was also confirmed during a chronic toxicity assay (7 weeks of oral exposure); its chronic LC_{50} was 1/10 of the MFRC, while that of spinosad was 1/247 of the MFRC. To date, the MFRC of spinosad has been set at 400 mg AI L^{-1}, while that of spinetoram is 25 mg AI L^{-1}. So, when expressed as mg AI L^{-1} in sugar water, it can be concluded that spinosad molecules (i.e. spinosyns A and D) are about 4 times less toxic in bumblebees than spinetoram molecules containing the chemically engineered spinosyns J and L as major and minor components. Otherwise, the lower MFRC for spinetoram indicates that these molecules possess a higher activity for the target site in pest insects, resulting in the use of lower application rates and, in turn, maintaining a higher selectivity and potential for use in IPM.

Next to testing the potential lethal effects on survival of bumblebee workers, the present project also investigated the potential sublethal effects on bumblebee reproduction of spinosad and spinetoram via the drinking of treated sugar water. As described above, oral exposure to both spinosyns caused high worker mortality, and in these cases this resulted in loss of reproduction. Good examples are spinosad at 1/1, 1/10 and 1/100 of the MFRC, which resulted in a total (92–100%) reduction in nest reproduction, as all workers were dead in the chronic exposure test, and this (100% mortality) was also the case with spinetoram at its full MFRC. When spinetoram was lowered to 1/10 of its MFRC, the worker mortality was 55%, and this concurred with a reduction in the nest reproduction of 61%. Overall, it can be said that detrimental effects on bumblebee reproduction were observed sooner, at lower MFRC concentrations, with spinosad than with spinetoram. The NOEC for sublethal effects on nest reproduction by spinosad was 1/1000 of the MFRC, while this was 1/100 of the MFRC for spinetoram. Interestingly, these NOEC values for reproductive effects concurred with those for the lethal effects towards workers. As a consequence, the authors believe that the sublethal effects on reproduction as observed in the present experiments are due to the lethal effects causing worker mortality. However, it cannot be ruled out that sublethal effects can also occur after exposure to spinosyns. Indeed, Vayias *et al.*[35] reported a significant reduction in progeny production of two coleopteran pest insects, *Tribolium confusum* Jacquelin du Val and *Cryptolestes ferrugineus* (Stephens), after treatment with spinosad at 0.1 mg L^{-1}. Desneux *et al.*[36] extensively reviewed the sublethal effects in different beneficial insects, including honey bees, bumblebees, parasitoids and predators, by low doses of the major groups of neurotoxic insecticides such as pyrethroids, carbamates and organophosphates. The mode of action of the spinosyns is based on excitation of the insect nervous system by activation of nAChRs, which is similar to that of the neonicotinoids. Indeed, for imidacloprid, Nauen *et al.*[37] and Hu and Prokopy[38] reported that doses lower than those necessary to cause lethal effects are responsible for sublethal effects such as inhibition of feeding and also reproductive reduction. In summary, although attention should be paid to potential sublethal effects, such as effects on reproduction, the results of this project clearly show that spinetoram has a higher safety than spinosad and can be applied in combination with *B. terrestris* without loss of nest reproduction at low chronic concentrations of 1/100 of the MFRC.

Finally, the 'behaviour test' as developed by Mommaerts *et al.*[13,14] was performed to assess the potential foraging behaviour side effects of spinosad and spinetoram in bumblebees. Indeed, it is recommended that behaviour assays be included in risk assessment tests, because impairment of foraging behaviour can result in decreased or complete loss of pollination. Here, the concentrations that did not result in mortality or in effects on reproduction in the classical exposure bioassay were tested, so that any appearance of effects in the behaviour bioassay was indicative of an impairment activity by the treatment on the foraging behaviour. Overall, comparison between the two bioassays without and with the inclusion of foraging behaviour indicated that the respective NOEC values were similar for spinosad (1/1000 of the MFRC) and spinetoram (1/100 of the MFRC) over the two bioassays. These correlations for lethal and sublethal effects provide strong evidence that the two spinosyns, as evaluated at low rates, pose no negative risk to the foraging behaviour of *B. terrestris* workers. However, as for the sublethal effects on reproduction, the same remarks on potential hazards should be postulated here. Hence, before drawing final conclusions, spinetoram and spinosad should also be evaluated in more realistic field-related situations for the assessment of potentially deleterious effects on foraging behaviour, using queenright colonies of *B. terrestris*, and where bumblebee workers need to forage/fly for food that is placed at a distance (i.e. 3 m) from their hives. Clearly, a good knowledge of environmentally relevant concentrations of the two spinosyn products is also necessary.

In conclusion, the results of this study provide evidence that spinetoram shows a higher safety than spinosad by direct contact and oral exposure to bumblebees. Secondly, both spinosyns, as tested at low rates, have no negative effect on the foraging behaviour of *B. terrestris* bumblebee workers. However, before drawing final conclusions concerning compatible use in the field, spinetoram and spinosad should be evaluated in more realistic field-related situations for the assessment of potentially deleterious effects on foraging behaviour, with the use of queenright colonies of *B. terrestris*.

ACKNOWLEDGEMENTS

The authors are indebted to Dr Howard Bell (Fera, York, UK) for the careful editing of this manuscript, and acknowledge the gifts of bumblebees by Biobest NV (Westerlo, Belgium). This research was funded by the Special Research Fund of VUB (Belgium). GAA thanks the EMCW of the European Community Mobility Programme for a postdoctoral research scholarship in VUB (Belgium).

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