ANALYSIS OF SIDE EFFECTS OF DIFLUBENZURON AND TEBUFENOZIDE IN POLLINATING BUMBLEBEES *BOMBUS TERRESTRIS*

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ملخص

ينظر هذا البحث في مخاطر نوعين هامين من مبيدات الحشرات على حياة فصيلة النحل "الطنان" (bombusterrestris). اختبرنا منظمي نمو الحشرات (IGR) الديفلوبنزيون و التيبوفينوزيد الكثيرة الاستعمال في إبادة الحشرات بالبيوت البلاستيكية. تم عرض المبيد بواسطة ثلاث طرق (الملامسة / ماء وسكر بالفم / غبار الطلع) في المختبر وفي الظروف العملية. تظهر النتائج أن العاملة لم تتأثر كثيرا بالمبيدات بينما تقلص بصورة واضحة بيض الحضنة اثر المعالجة بالديفلوبنزيون، أما التيبوفينوزيد فلم يؤثر في تفريخ الحضنة. بصورة عامة، تبين النتائج انه يجب استعمال الديفلوبنزيون بحذر عند وجود الطنانات أما التيبوفينوزيد فهو آمن الاستعمال في الحالات المدروسة.

الكلمات المفتاحية: *منظمات نمو الحشرات؛ ديفلوبنزيون؛ تيبوفينوزيد؛ تسمم؛ نحل طنان.*

Résumé

Cette recherche a examiné les risques potentiels de deux insecticides importants à l'égard de la survie, la reproduction et la croissance larvaire des bourdons (*Bombus terrestris*). Nous avons testé deux régulateurs de croissance des insectes (IGRs): un inhibiteur de la synthèse de la chitine (diflubenzuron) et un agoniste de l'ecdysone (tebufenozide) qui sont utilisés dans le contrôle des insectes ravageurs des cultures sous serres. Les deux IGRs ont été appliqués selon trois voies d'exposition (contact par application topique et oralement par de l'eau sucrée et du pollen) dans les conditions de laboratoire et aux concentrations maximales recommandées en plein champ (MFRC). Dans un deuxième essai, une analyse de dose-réponse a été réalisée pour déterminer les concentrations létales CL_{50}. En général, les deux IGRs n'ont pas manifesté de toxicité à l'égard des ouvrières. Cependant, on note une réduction drastique sur la production des couvains après traitement oral avec le pollen et l'eau sucrée pour le diflubenzuron. En revanche, le tebufenozide n'a pas d'effet significatif. De façon générale, nos résultats suggèrent que le diflubenzuron doit être appliqué avec prudence en combinaison avec les bourdons, tandis que le tebufenozide est compatible avec le pollinisateur.

Mots clés : *Régulateurs de croissance des insectes; Diflubenzuron; Tebufenozide; Toxicité; Bourdon.*

Abstract

This research project examined the potential hazards of two important insecticides on survival, reproduction and larval growth of bumblebees *Bombus terrestris*. We tested two insect growth regulators (IGRs), the chitin synthesis inhibitor diflubenzuron and the ecdysone agonist tebufenozide; both important in the control of insect pests in greenhouses. The two IGRs were applied via three different routes of exposure (contact via topical application, and orally via the drinking sugar water and via pollen) under laboratory conditions and tested at their respective maximum field recommended concentrations (MFRC). In a second test, a dose-response assay was performed to calculate LC_{50} values. Generally, the two IGRs did not show acute toxicity on worker bumblebees. However, there was a dramatic reduction on brood production especially after diflubenzuron oral treatment with pollen and sugar water. In contrast tebufenozide did not exert a significant effect on brood production. Overall our laboratory results suggest that diflubenzuron should be applied with caution in combination with bumblebees, while tebufenozide is compatible with the pollinator.

Key words: *insect growth regulator; diflubenzuron; tebufenozide; toxicity; bumblebees*

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1. INTRODUCTION

The bumblebee *Bombus terrestris* L. (Hymenoptera) is one of the most important pollinators of wildflowers and glasshouse crops, such as sweet peppers, tomatoes and eggplants. Unfortunately, in the last decade declines in colonies have been reported, and this is probably due to habitat fragmentation, habitat loss and the use of pesticides [1, 2].

Since the mid 1950's, different classes of neurotoxic insecticides have been developed to control insect pests. Despite their economic success and broad-spectrum potency, these compounds have been in many cases harmful to non-target organisms, the environment and the health of producers and consumers. In addition, rapidly developing resistance and growing public concern towards these insecticides have stimulated the search of safer and more selective alternatives, especially, towards non-target and beneficial insects. A new class of more environmentally friendly insecticides is the insect growth regulators (IGRs), being an important group, the chitin synthesis inhibitors. These compounds are mainly larvicides and act through the inhibition of chitin formation resulting in abnormal endocuticular deposition that causes abortive moulting and death. In addition, some of these compounds such as diflubenzuron also possess strong ovicidal activity thereby causing a reduction in reproduction [3-5].

A second important class of IGRs is the ecdysone agonists or moulting accelerating compounds (MACs). MACs primarily work by ingestion, but also by contact. They become active by binding on the receptor site of the insect moulting hormone 20-hydroxyecdysone, the ecdysone receptor (EcR) [5]. Therefore a disturbance of the insect's endocrinology causes a cessation of feeding and a premature lethal moulting, preventing insect developping into the adult stage. The major compound of this class is tebufenozide for the selective control of Lepidoptera [6].

With the increasing employment of these novel IGRs in integrated pest management (IPM) programs, it is essential to perform a risk assessment of the hazards of these compounds towards non-target and beneficial insects such as bumblebees. Due to lack of data on bumblebees, frequently, data obtained for the honeybee (*Apis mellifera* L.) are used; however, the two bee species have several biological and morphological differences [1, 7], and their susceptibility towards pesticides is probably not the same. Therefore, studies to evaluate the acute and sublethal effects of insecticides on bumblebees are necessary for the joint use of pollinators and pesticides.

The purpose of this paper is to test the acute toxicity and the sublethal effects of two important IGRs, the chitin synthesis inhibitor diflubenzuron and the ecdysone agonist tebufenozide, when bumblebees are exposed through different routes, and at different life stages. With this information we provide the first extensive review of the combined use of these important IGRs with the different life stages of the bumblebee *B. terrestris*.

2. MATERIALS AND METHODS

2.1 Chemicals

The two IGRs that were tested in this study together with their respective type of formulation, amount of active ingredient (AI), and MFRC and the producing company name are listed in table 1 [6].

2.2 Insects

In all experiments, artificial nests with five *B. terrestris* workers each one, were used. The workers were obtained from colonies supplied by Biobest N.V. (Belgium). Nests were made of transparent plastic (15 cm wide, 15 cm deep, 10 cm high), and the drinking place and brood area were located in the middle.

They were kept under standardized laboratory conditions in the dark at 28 ± 2°C and 60 ± 10% RH, and under these conditions, after one week, the dominant worker started to produce eggs that develop into males. Commercial pollen and sugar water were provided as food [8, 9].

2.3 Treatment to assess insecticidal effects

The two IGRs were tested via three different routes of exposure. Adult worker bees were exposed via contact by topical application and orally via drinking sugar water or eating pollen [10]. For each insecticide 4 nests were treated, each containing 5 workers. The nests were followed during a period of 11 weeks and once per week the number of workers alive was scored to evaluate the life span, as optimized before [8, 9]. In addition, amount of brood, brood care, number of dead larvae removed from the nest and number of males were scored weekly as biological endpoints of effects on reproduction and larval growth [8, 9].

In the first series of tests, the different insecticides were applied as aqueous solutions at their maximum field recommended concentration (MFRC) (Table 1). For negative controls, workers were treated with water or fed on untreated diet (sugar water). For positive control, imidacloprid at its MFRC (200 mg AI/litre) was used. For a contact application, 50 µl of the aqueous concentration was topically applied to the dorsal thorax of each worker with a micropipette. In ingestion assays, worker bumblebees were provided with drinking sugar water treated with the IGRs. Hereto, each nest was exposed *ad libitum* to 500 ml of this concentration over a period of 11 weeks. Bumblebees were also exposed orally to the IGRs by spraying the pollen with the prepared concentrations of IGRs until saturation, which was supplied *ad libitum* to the nests. Each treatment consisted of 4 nests, each containing 5 workers. Then for the different routes of exposure, means ± SEM were analysed by one-way ANOVA and separated by a Tukey-Kramer *post hoc* test (P = 0.05) using SPSS 10.0 software.

In a second series of experiments, we performed dose-response bioassays using dilutions of diflubenzuron (1/1, 1/10, 1/100, 1/1000 and 1/10000 of the MFRC) that showed a significant effect on production of males (drones) or/and larval growth. The bumblebees were treated with the compound as described above. The results obtained were analysed using a non-linear sigmoid curve fitting, and the activity of each treatment was evaluated based on the medium-response concentration (LC_{50} values and corresponding 95% fiducial interval) using GraphPad 4 software; the goodness of the fitting to the curve model was evaluated based on R^2 values [11].

3. RESULTS

3.1 MFRC

3.1.1 Effect on mortality of worker bumblebees

In the toxicity tests using the two IGRs at their respective MFRC, no significant mortality was observed after topical application, or exposure to treated sugar water or pollen. In all cases, the number of dead worker bees in the treated nests over a period of 11 weeks was not above that of the control groups using water (0-10%). For the positive controls with imidacloprid, 100% mortality was scored in every treatment at the first week (data not shown).

3.1.2 Sublethal effects on worker bumblebee reproduction assessed as the number of males produced

When examining the brood clumps, diflubenzuron caused via the three routes of uptake a 100% inhibition of egg hatching, resulting in no male production (Fig. 1). No males were produced during the entire experiment of 11 weeks in every uptake route.

In the nests exposed to tebufenozide via contact, sugar water and pollen, it was clear that this IGR exerted no negative effects as the numbers of males produced did not differ significantly (P > 0.05) from those of the controls (Fig. 1).

Table 1: *Characteristics of the insecticides tested.*

IGR	Commercial name	Formu-lation and %AI	Company	MFRC [b] (in %)	MFRC (in mg AI/litre)
Diflubenzuron	Dimilin®	48% SC [a]	Solvay-Duphar, the Netherlands	0.06%	288
Tebufenozide	Mimic®	24% SC	Rohm & Haas, USA; now Dow AgroSciences, USA	0.1%	240

[a] SC = suspension concentrate
[b] MFRC = maximum field recommended concentration

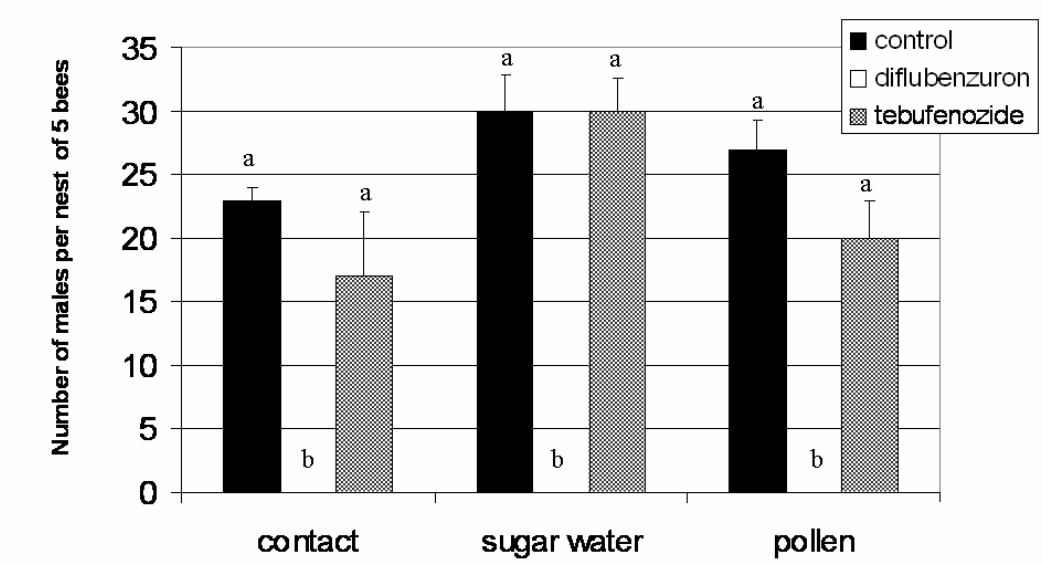

Figure 1: *Effect of diflubenzuron and tebufenozide on the reproduction in Bombus terrestris, when treated at their respective MFRC by topical contact and orally via sugar water and pollen. The mean number of males/drones produced per nest was scored after 11 weeks of treatment with IGR and also in control nests. The data are expressed as means ± SEM. Values per route of exposure that are followed by a different letter (a-b) are significantly different (Tukey-Kramer post hoc test with P = 0.05).*

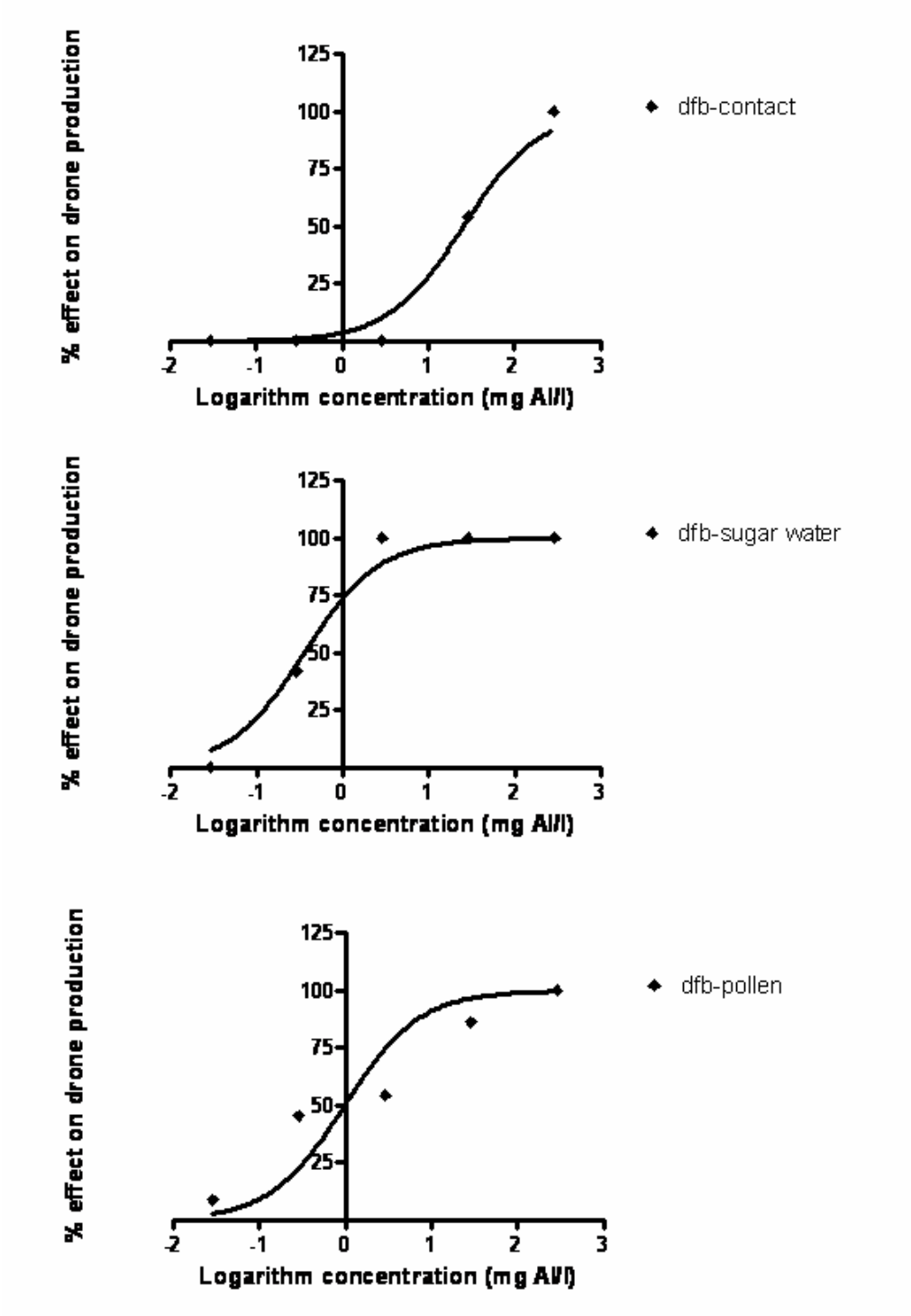

Figure 2: *Dose-response sigmoid curves expressing the effect of a series of concentrations of diflubenzuron (1/1, 1/10, 1/100, 1/1000, 1/10000 of the MFRC) on the production of drones/males in Bombus terrestris by topical contact and orally via sugar water and pollen. The goodness of curve fitting is based on the R^2 value; contact: 0.97, sugar water: 0.97, and pollen: 0.79.*

3.2. Dose-response assays

Diflubenzuron was detrimental to the bumblebees at the MFRC, so a series of dilutions were tested (1/1, 1/10, 1/100, 1/1000, 1/10000 of the MFRC), and they were found too also harm the pollinator irrespective the routes of exposure. In the nests exposed to diflubenzuron, reproduction was only statistically ($P > 0.05$) similar to that of controls at the relatively low concentrations of 1/100 in contact, 1/10000 in sugar water and 1/1000 in pollen. It should be noted that at higher concentrations there was no male production (Fig. 2). After sigmoid curve-fitting of the dose-response data, the LC_{50} values for diflubenzuron were low: 25 mg AI/litre in contact, 0.32 mg AI/litre in sugar water and 0.95 mg AI/litre in pollen, respectively (Fig. 2), which correspond to 1/12, 1/900 and 1/303 of the MFRC.

4. DISCUSSION

The impact of insecticides, such as IGRs, on beneficial organisms has been studied in different laboratories over recent years, and in most cases the effects depend on species and developmental stage tested, the application method, and the biological endpoint(s) used [4]. In the case of pollinators, previous experiments have demonstrated that several IGRs, particularly belonging to the class of chitin synthesis inhibitors, could be harmful for *B. terrestris* brood [12, 13]. In contrast, many are reported to be safe for the brood of honeybees, *A. mellifera* [14]. This agrees with the fact that it is not possible to draw a correlation for hazards of pesticides in bumblebees and honeybees [1, 7].

The present study gives more information on the hazards of a group of economically important IGRs, by testing direct toxicity and sublethal effects via different routes of exposure on workers of *B. terrestris*. When testing IGRs, it is essential to explore not only direct effects on the treated stage but also effects on a long term period, by studying reproduction because in literature there are many studies where detrimental effects on fecundity, fertility, etc have been found. Indeed our extensive tests confirmed strong larvicidal and ovicidal activities. Conspicuously in all our treatments large numbers of deformed first and second instar larvae were removed from the nest. For diflubenzuron, a reduction of egg hatching was observed. The dominant worker laid eggs but none of the eggs hatched. Therefore, it is likely that this chitin synthesis inhibitor is having a negative effect on the worker's ovaries. Also diflubenzuron is known to have effect on embryos. Somewhat similar effects were reported after 24 h of oral treatment of *B. terrestris* colonies with teflubenzuron (150 mg AI/kg) [12]. In a cage test where diflubenzuron was sprayed on *B. terrestris* colonies the same effects were observed. These workers also reported the occurrence of malformed cocoons that were spherical with abnormally brown dots on the surface [13]. Recently, the effect of four different IGRs on *A. mellifera* colony development, queen rearing and drone sperm production was investigated [15]. In the latter assays, diflubenzuron had severe short-term effects on brood mortality but also longer-term sublethal ones on emerging adults and queens. Taken together the current results of the eight compounds tested confirm their physiological mode of action as inhibitors of chitin synthesis in bumblebee larvae. In addition, the typical detrimental effects on reproduction concur with earlier reports for diflubenzuron and other chitin synthesis inhibitors in other pest and non-target insect species [3-5].

Our results also indicate that diflubenzuron, as chitin synthesis inhibitors in general, does not cause acute adult toxicity as it was already reported in other studies [12, 14]. In contrast, we observed a strong negative effect on the numbers of males produced. Diflubenzuron applied at its MFRC, caused a total inhibition of the adult

formation after topical and oral exposure, being very toxic with low LC_{50} values, making clear that this IGR is incompatible with *B. terrestris*.

In our tests *B. terrestris* workers were exposed to the MFRC of diflubenzuron via three different routes. This easy to use test method is very stringent. In practice bumblebees will rarely be exposed to such high concentrations, but these experiments have been undertaken to evaluate with certainty the safety and compatibility of compounds with bumblebees. Furthermore, a dose-response assay was done to evaluate sublethal effects using a concentration series of the MFRC (1/1 up to 1/10000). Therefore, such effects on reproduction and foraging behaviour of bumblebees are crucial as they significantly affect the pollination capacity of a nest. In a next step of such tiered approach, it is important to validate the data that were obtained in the laboratory under more field-related conditions.

In addition and when applicable, special attention should be paid to plant systemic properties, which in the tests reported upon here may cause more severe effects after oral treatment of pollen.

Alongside evaluating the hazards of IGRs, another study [8] investigated the cuticular absorption profiles in adult workers for diflubenzuron and flucycloxuron in order to explain differences in their contact toxicity. Based on LC_{50} values, diflubenzuron (25 mg AI/litre) was 7 times more active than flufenoxuron (167 mg AI/litre) in workers of *B. terrestris*. In contrast to our expectations, our pharmacokinetic results showed that the penetration rate through the cuticle was 2 times lower for diflubenzuron. Therefore, no correlation could be drawn between toxicity and penetration for diflubenzuron and flufenoxuron, but as in a previous study, high retention of diflubenzuron was reported in pupae of the mealworm, *Tenebrio molitor* L., when injected with the chitin synthesis inhibitor. The highest activity could be due to the higher retention inside the body. Furthermore, then diflubenzuron was applied topically to *T. molitor* pupae, whilst the amount of compound incorporated was low, little degradation was observed over the duration of the pupal stage [16, 17]. Similarly, relatively low percentages of diflubenzuron penetrated the cuticle of larvae of *Spodoptera exigua* (Hübner) and *Spodoptera littoralis* (Boisduval) after topical contact (15-31%), but as metabolism was of minor importance, the two compounds were highly toxic [18]. Therefore, despite the low penetration that was most probably due to differences in solubility, we hypothesize that the low amounts of diflubenzuron may be highly toxic due to a high retention and/or a low degradation in the body tissues of *B. terrestris*. It is suggested that the toxicity of diflubenzuron is not only dependent on the degree of penetration into the insect body but also to the high retention and stability this compound exhibits. But we recommend here that more studies are necessary to verify the relationship between toxicity and the accessibility of the parent insecticides and their metabolites to the sites of action, before making a final conclusion on insecticide biological activity and insect selectivity for the chitin synthesis inhibitors in general.

In this project we can also suggest that diflubenzuron is transovarially transported into the deposited eggs when given to worker bumblebees via the pollen. This phenomenon is likely to be due to the high retention time of diflubenzuron in the female body, and as a consequence it is incorporated into the ovaries [8]. Accumulation of diflubenzuron in ovaries was also reported in other species [19-21]. As reported by Mommaerts et al. [8], the relatively high quantities of diflubenzuron recovered from the first egg batch of treated bumblebees, 4.48 ng per egg, is

indeed suggested to impair normal embryonic development, leading to a complete failure in egg hatch. Similarly, other authors found that females of *Chrysoperla carnea* (Stephens) lacewings accumulated diflubenzuron into the eggs, and although the amount of diflubenzuron was low (74-197 pg/egg), it was sufficient to cause egg mortality [20]. Hexaflumuron, another chitin synthesis inhibitor, was also found in the eggs after topical contact in female sugar beet beetles, *Aubeonymus mariaefranciscae* Roudier [22]. Moreover, the latter work also reported that the small amount of hexaflumuron in the deposited eggs was responsible for the detrimental effects in the embryos, causing abnormal cuticle formation and finally death [22]. On the mechanism of chitin inhibition by BPUs, recent assays in *Blattella germanica* (L.) and *Drosophila melanogaster* Meigen showed that such compounds work through the sulfonylurea receptor during chitin biosynthesis [23]. These latter studies confirm the embryocidal activity of chitin synthesis inhibitors. Therefore, based on previously published work, and the present study in *B. terrestris*, it can be concluded that such IGRs can cause strong negative effects on the next generation, and as such their use in IPM programs with beneficial insects should be considered with caution. However, before making final conclusions, it is necessary that our laboratory-based results are validated with field date for these insecticides because in literature there are many examples of harmfulness under laboratory conditions and harmlessness in the field. In this framework the application technique is also of great importance on the interpretation of toxicological data. We also believe that it is necessary to evaluate this aspect on a species-by-species basis, especially for IGRs that are used in IPM programs. For instance, an extrapolation of toxicity results from honeybees to bumblebees and vice versa is not possible, although both insects belong to the order of Hymenoptera [1, 7]. As such the generation of larger databases including economically important insects as bumblebees, is helpful in determining the impact of pesticides on our environment.

For the ecdysone agonists with a dibenzylhydrazine structure like tebufenozide, these IGRs are used specifically for the control of lepidopteran pests. In this study it was clear that this class of IGRs, as exemplified by tebufenozide, exerts no adverse effects on the different biological endpoints of adult survival, nest reproduction and larval growth in *B. terrestris*. Recently, Thompson et al. [15] reported on long-term effects of tebufenozide in honeybee colonies. In agreement with our current results, these authors also found that this IGR had no impact on honeybee colonies and queen development. As reviewed by Dhadialla et al. [5], an important process in the selectivity of this class of IGRs is the specific binding of the MAC molecules to the target EcR that is governed by a lock-and-key principle. For instance, in targeted Lepidoptera pests the binding affinity is high, whereas binding is low/not detectable in non-targeted insects [24]. Based on the current worst case exposure tests, it can be concluded that the use of the two tested MACs is compatible with bumblebees *B. terrestris*.

To explain the benign profile of tebufenozide in beneficial insects and other natural enemies, previous pharmacokinetic results showed that the MACs are accumulated to a very high percentage of 83% after cuticular administration; however, this class of MACs has no negative effect on *B. terrestris* when topically applied at the MFRC. Similar results were obtained for methoxyfenozide and tebufenozide in parasitic wasps [25]. These authors reported that after a topical application of these compounds on *Hyposoter didymator* about 60% was absorbed after 24h; but the

two MACs were not toxic for this beneficial parasitic wasp. In another beneficial insect the lacewing, *Chrysoperla carnea*, larvae had penetrated through the cuticle only 10% of tebufenozide at one day after topical contact and this low penetration helps in explaining its no-toxicity [20]. As is also suggested for these two other beneficial insects, we believe that the MACs are not able to bind on the insecticidal target site of the EcR of bumblebees and as such cause no adverse effects on *B. terrestris*. Although the so far available EcR sequences (http://www.ncbi.nlm.nih.gov) show a relatively strong conservation of the ligand-binding pocket, there exist divergent residues lining the binding pocket, namely 326, 368 and 379. These respective residues are isoleucine, methione and isoleucine in honeybee *A. mellifera* and also in other insects and non-insects/arthropods that show no/low susceptibility for tebufenozide. In contrast, in Lepidoptera (exemplified by *Heliothis virescens*, *Choristoneura fumiferana* and *Spodoptera frugiperda*, three important pest caterpillars in agriculture, horticulture and forestry) that show a high sensitivity for tebufenozide, these residues of Ile326, Met368 and Ile379 are replaced by a methionine and two valine residues, respectively. As also discussed by Wurtz et al. [26] especially the presence of a isoleucine at position 326 in non-sensitive species generates steric contacts between the γ-methyl group of the Ile-residue and the C5-methyl group at the B-ring of tebufenozide or the C4-ethyl group of its B-ring, depending on the orientation of tebufenozide. This can most likely account for the no toxicity of the MACs against honeybees and bumblebees. Nevertheless, we also suggest here in agreement with [26, 27] that in addition to the structure of the EcR ligand-binding pocket other factors like pharmacokinetics and metabolism may help in determining the toxicity spectrum of the MACs.

5. CONCLUSIONS

In general IGRs are considered to be relatively benign towards non-target and beneficial insects. However, our toxicity experiments in the laboratory via three different routes of exposure (contact via topical application, and orally via the drinking sugar water and via pollen) showed that this is only true for some of these compounds. Tebufenozide exerts no negative effect on *B. terrestris* when used at the recommended concentration, while diflubenzuron is not compatible with the pollinator *B. terrestris* because even at very low concentrations, it exhibited strong detrimental effects on brood production and reproduction.

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