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RESEARCH ARTICLE

Toxic effects of hexaflumuron on the development of *Coccinella septempunctata*

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Abstract Studying the toxic risk of pesticide exposure to ladybird beetles is important from an agronomical and ecological perspective since larval and adult ladybirds are dominant predators of herbivorous pest insects (e.g., aphids) in various crops in China. This article mainly deals with the long-term effects of a single application of the insect growth regulator hexaflumuron on *Coccinella septempunctata*. A 72-h and a 33-day toxicity test with hexaflumuron (single application) were performed, starting with the second instar larvae of *C. septempunctata*. Exposure doses in the long-term experiment were based on the estimated 72-h acute LR_{50} (application rate causing 50 % mortality) value of 304 g active ingredient (a.i.) ha^{-1} for second instar larvae of *C. septempunctata*. The long-term test used five hexaflumuron doses as treatment levels (1/50, 1/100, 1/200, 1/400, and 1/800 of the 72-h acute LR_{50}), as well as a solvent control and blank control treatment. The measurement endpoints used to calculate no observed effect application rates (NOERs) included development time, hatching, pupation, adult emergence, survival, and number of eggs produced. Analyzing the experimental data with one-way analysis of variance showed that the single hexaflumuron application had significant effects on *C. septempunctata* endpoints in the 33-day test, including effects on development duration (NOER 1.52 g a.i. ha^{-1}), hatching (NOER 3.04 g a.i. ha^{-1}), pupation (NOER 3.04 g a.i. ha^{-1}), and survival (NOER 1.52 g a.i. ha^{-1}). These NOERs are lower than the reported maximum field application rate of hexaflumuron (135 g a.i. ha^{-1}) in cotton cultivation, suggesting potential risks to beneficial arthropods.

Keywords Hexaflumuron · *Coccinella septempunctata* · Second instar · Acute toxicity · Long-term toxicity · Insect growth regulator · NOER

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Introduction

Without effective crop protection, losses in agricultural production owing to pests, weeds, and diseases have been estimated to vary from 10 to 50 % (Pimentel et al. 1992; Beddington 2010). Since chemical pesticides are intentionally released into the environment to protect crops from harmful insects, weeds, and pathogenic microorganisms, it is common practice to evaluate their side effects as part of the registration procedure when placing pesticides on the market (EU 1991). Ecological risk assessment is well advanced in developed regions of the world, which have adopted regulatory procedures to assess environmental risks of pesticides to nontarget terrestrial arthropods (e.g., Alix et al. 2012). The effects of pesticides on beneficial arthropods have been the subject of an increasing number of studies (Flexner et al. 1986; Sotherton et al. 1987; Yuxian et al. 2012). Among beneficial arthropods, natural enemies of pest organisms have received the greatest attention because of their value in integrated pest management (Haynes 1988; Dechaume-Moncharmont et al. 2003; Thompson 2003; Desneux et al. 2007). Integrated pest management in many agricultural ecosystems mainly depends on optimizing the control of pest organisms by predators and a prudent use of pesticides (Youn et al. 2003). As predators of aphids, ladybird beetles (and their larvae) have been reported to efficiently control aphids (Seo and Youn 2000; Seo and Youn 2002).

However, the relationship between the survival of and ecosystem services provided by predators and their exposure to pesticides is not clear. To date, acute toxicity effects of some pesticides on ladybird beetles have been reported (Wu et al. 2007; Ji et al. 2011), but only a few studies have considered the long-term side-effects of pesticide application on ladybirds (Tongxian and Philip 2004; Yuxian et al. 2012).

The seven-spotted ladybird, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), is common in a wide range of natural and agricultural habitats, with worldwide distribution. Both the adult and larval stages are known to be highly polyphagous, feeding on Aphidoidea, Psylloidea, Coccoidea, and mites found on a variety of plants (Dolling 1991). It is a generalist, widespread and representative beneficial arthropod predator in China (Zhang et al. 2011). In addition, *C. septempunctata* is easy to be raised in the laboratory, making this species an ideal test organism to study the potential impacts of pesticides on beneficial arthropods as part of the registration procedure for pesticides.

New, more selective chemicals are being developed to reduce the environmental side-effects of pesticides. Insect growth regulators (IGRs) seem promising because of their more specific mode of action towards pest insects and their lower toxicity to nontarget organisms when compared with many conventional insecticides (Tongxian and Philip 2004; Nedjoua and Noureddine 2011). Hexaflumuron is a commonly used IGR in China with 120 products with hexaflumuron as the active ingredient having been registered and a usage of about 36 t per year against insect pests in vegetables, fruit trees, forest trees, and other crops (till March 2013; http://www.chinapesticide.gov.cn/service/aspx). In the USA, hexaflumuron is registered only as active ingredient in termite bait products because of its potential high risks to nontarget aquatic and terrestrial organisms (http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_0886/0901b80380886a87.pdf?filepath=productsafety/pdfs/noreg/233-00932.pdf&fromPage=GetDoc). Hexaflumuron is a benzoylphenylurea derivative (1-[3,5-dichloro-4-(1,1,2,2 -tetrafluoroethoxy)phenyl]-3-(2,6-difluorobenzoyl)urea), which interferes with chitin synthesis at the time of molting and is effective in controlling immature stages of insects. IGRs are generally considered compatible with natural enemy (beneficial arthropod) conservation (Desneux et al. 2007).

The present study aimed to investigate the sublethal, long-term effects of a single application of hexaflumuron on *C. septempunctata*, when applied to the second instar larvae. It also intends to help to promote the protection of ladybirds and to provide reference values for their possible use as a standard test species in prospective risk assessment for pesticides.

Materials and methods

Pesticide

The hexaflumuron content of the product used in the toxicity tests, which was obtained from our institute, is 95 %. As its water solubility is low, stock solutions were prepared by dissolving the substance using 0.1 ‰ Polysorbate 80 as a surfactant, prior to each experiment.

Test species

Second instar larvae of *C. septempunctata* were reared in the toxicity tests performed. The test organisms were obtained from a laboratory culture characterized by complete metamorphosis, consisting of the egg stage, four larval stages, pupa, and adult, mainly feeding on black bean aphid, *Aphis craccivora* Koch, which lived on *Vicia faba* L. *C. septempunctata* was reared in conditions of 20±1 °C, 50–70 % relative humidity (RH) and 16:8 (light/dark (L/D)) photoperiod. The eggs hatched in 3 days at 25±1 °C, 70 % RH and 16:8 (L/D) photoperiod.

Acute toxicity test method

The test systems consisted of enclosures, each containing a pot filled with soil (11 cm in diameter) in which 10 broad bean plants (length 10 cm) were planted. In each test system, 10 second instar ladybird larvae were introduced before insecticide spraying. The stock solution of hexaflumuron was prepared by dissolving the active ingredient with Polysorbate 80. The hexaflumuron concentration measured in the stock solution approximated the intended concentration. Different test solutions were obtained by diluting the stock solution. The test solution was introduced to the test systems by spraying the plants, simulating the spray application on crops as closely as possible. An amount of 1.52 mL of test solution was sprayed toward the plants in each test system and the concentrations of active ingredient in the different test solutions used for the different treatments were 66, 99, 148, 222, 333, and 500 mg L^{-1}, respectively. The maximum field application rate normally used to control insect pests in cotton crops is 135 g active ingredient (a.i.) ha^{-1} (http://www.chinapesticide.gov.cn/service/aspx/B3X.aspx?aiid=GRHEX). The application rates in the acute toxicity test were equivalent to 106, 158, 237, 355, 533, and 800 g a.i. ha^{-1}. In addition, the experimental design included a blank control treatment, sprayed with distilled water, and a solvent control treatment, sprayed with a 0.1 ‰ Polysorbate 80 solution. Three replicates were used for each treatment. Mortality of ladybird larvae was recorded 72 h after treatment and the LR_{50} (the application rate causing 50 % mortality among the test individuals) was calculated. The

toxicity experiment was conducted in the laboratory at 20–25 °C, 50–70 % RH, and 16:8 (L/D) photoperiod.

Long-term toxicity test

The test system of the long-term test was similar to that of the acute test, but the experimental design differed in that lower application rates (single application) were used, the observation period was extended to at least 33 days, several sublethal endpoints were monitored, and aphids (*A. craccivora*) were added to the test systems as food for the test animals every 2 days (until the prepupal stage of *C. septempunctata*). The hexaflumuron application rates were 1/50, 1/100, 1/200, 1/400, and 1/800 of the acute LR_{50} (derived from the acute toxicity test described above), and blank controls and solvent controls were again employed. Three replicates were used for each treatment, and 10 second instar larvae with the same size and morphological characteristics as those used in the acute test were introduced to each replicate test system. In each test system, an average of 1.52 mL of a solution containing different concentrations of hexaflumuron was sprayed onto the leaves of the broad bean plants. During spraying, the spraying device was located approximately 40 cm above the plants and care was taken that the solution of hexaflumuron was uniformly distributed over the leaves of the plants. After spraying, all the test systems were stored on the shelves in the climatized room at 20–25 °C, 50–70 % RH, and 16:8 (L/D) photoperiod. Measurement endpoints (development period, pupation percentage, hatching percentage, adult emergence, mortality, and egg production) were assessed and recorded at daily intervals until the second instar larvae of the next generation of *C. septempunctata* appeared. The total test duration was at least 33 days and covered the complete life cycle of *C. septempunctata*, from the second instar stage of the first generation (which was sprayed) to the second instar stage of the next generation. In the course of the experiment, the dates when the larvae became third instar, fourth instar, pupae, and adults were recorded as well as their numbers. At the end of the experiment, when surviving pupae had emerged, one male and one female adult ladybird were collected from each test system and placed in a plastic cage with a size of 20×10×7 cm, together with sufficient *A. craccivora* (aphids) as food, and the number of eggs produced was recorded daily. A number of eggs were then collected from each cage (representing a specific test system) and placed in another climatized room at the same condition. The percentage of egg hatching was calculated by observing the number of larvae in each replicate.

Data analysis

The results obtained from the acute toxicity test allowed us to calculate the application rate that was lethal to 50 % of the test animals, using prohibit analysis with SPSS software. The results of the long-term toxicity test did not allow an appropriate calculation of the LR_{50} (application rate leading to 50 % mortality among the test individuals), since only partial effects (usually less than 50 %) were observed at the highest treatment levels. The results did, however, allow for the calculation of the no observed effect rate of application (NOER) by means of one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) tests were used to separate the differing means (Zar 1996).

Results

Acute toxicity

The 72 h acute LR_{50} values for hexaflumuron and *C. septempunctata* are shown in Table 1. The regression equation was $y=-7.147+3.135x$, $r^2=0.987$. The LR_{50} was 304 g a.i. ha^{-1}, with a 95 % confidence interval of 280–333 g a.i. ha^{-1}.

Long-term toxicity

Effect of hexaflumuron on survival rate of C. septempunctata

Effects of a single application of hexaflumuron on the survival rate of larvae of *C. septempunctata* in the long-term toxicity test (test period 0–21 days) are reported in Fig. 1. There was no significant difference between the blank control and the solvent control. The survival started to decrease from the second day after treatment onwards at the two highest treatment levels. Compared with the blank control, the survival in test systems that had received 3.04 and 6.08 g a.i. ha^{-1} had decreased to 83.3 and 76.7 %, respectively, on day 6 after the insecticide application. At the end of the observation period for the larval stages (21 day), the survival in blank controls was 96.7 % while that in the 6.08 g a.i. ha^{-1} treatment was 53.3 %. The 21-day LR_{50} was calculated to be 6.08 g a.i. ha^{-1}.

Table 1 Total initial number of second instar larvae of *C. septempunctata* in the acute toxicity test, and number and percentage of dead larvae 72 h after application of hexaflumuron in test systems that received different application rates ($n=3$)

Application rate (g a.i. ha^{-1})	Total	72 h dead	Mortality (%)
0	30	0	0
106	30	2	7
158	30	5	17
237	30	11	37
355	30	19	63
533	30	24	80
800	30	26	87

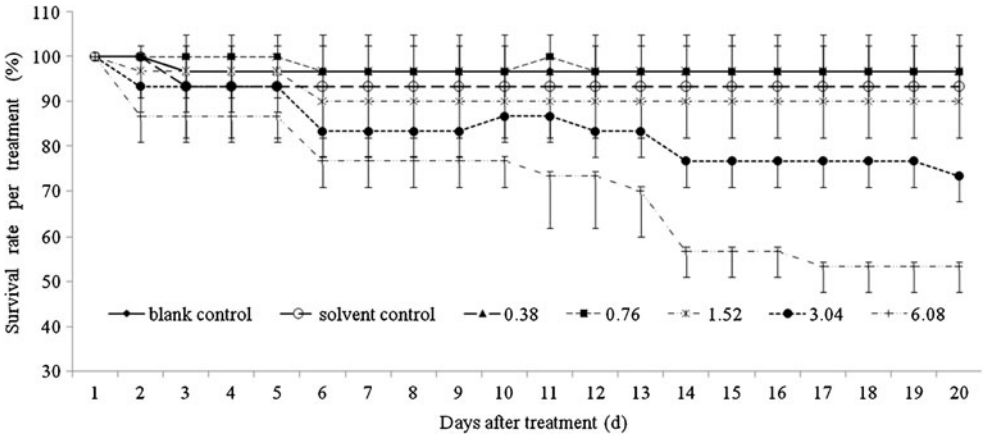

Fig. 1 Effects of a single application of hexaflumuron (gram per active ingredient per hectare) on the survival rate of *C. septempunctata* during a 21-day observation period in a laboratory toxicity test

Differences between blank controls and the two highest treatment levels were significant (ANOVA, $p<0.05$; 21-day NOER=1.52 g a.i. ha^{-1}).

Effect of hexaflumuron on the development time of C. septempunctata

The treatment-related effect of hexaflumuron on the development time of *C. septempunctata* (from second instar to second instar of the next generation) is presented in Table 2. The total developmental time in the controls was 28.43 days. Increasing hexaflumuron application rates were associated with larger delays in development. An NOER of 1.52 g a.i. ha^{-1} was calculated for the development time of the second instar. Treatment-related effects on development time were, however, relatively small, since the time at the highest treatment level (6.08 g a.i. ha^{-1}) was only approximately a factor of 1.15 longer than that in the controls. At the highest treatment level, the development times of the second, third, fourth instar larvae, the pupae and the total generation were 2.60, 2.92, 5.77, 6.80, and 32.75 days, respectively, all significantly longer than in the controls. A NOER of 1.52 g a.i. ha^{-1} was calculated for the development time of the second instar larvae, while that for the other instar larvae was 3.04 g a.i. ha^{-1}.

Effect of hexaflumuron on egg hatching of C. septempunctata

Observed treatment-related effects of hexaflumuron on the egg hatching percentage of *C. septempunctata* are presented in Fig. 2. The hatching percentage in the control test systems was 88.3 %. The lowest hatching percentage, 76.9 %, was observed in the test systems that received the highest dose, and was significantly different from that in controls (NOER=3.04-g a.i. ha^{-1}). The mean hatching percentages in the other treatments were, in order of increasing dosage, 88.0, 88.7, 86.1, and 83.57 %, but there was no significant difference compared to the controls.

Effect of hexaflumuron on pupation of C. septempunctata

Treatment-related effects of hexaflumuron on the pupation percentage of *C. septempunctata* are shown in Fig. 2. The pupation percentages in the control and hexaflumuron treatments up to 1.52 g a.i. ha^{-1} were 100 %. In the test systems that received 3.04 g a.i. ha^{-1}, the mean pupation percentage

Table 2 Effect of hexaflumuron on the time of development (in days) of *C. septempunctata* in a long term toxicity test

Treatment (g a.i. ha^{-1})	Time of development (mean ± SD, days)							
	Second instar	Third instar	Fourth instar	Pupae	Pre-oviposition	Egg	First instar	Entire generation
Blank control	2.03±0.06	2.33±0.12	4.7±0.53	6.03±0.12	8.33±0.58	3.00±0.00	2.00±0.00	28.43±0.21
Solvent control	2.08±0.14	2.325±0.04	4.6±0.50	6.23±0.31	9.33±1.15	3.00±0.00	2.00±0.00	29.61±1.63
0.38	2.07±0.06	2.43±0.06	4.83±0.21	6.37±0.32	9.00±1.00	3.00±0.00	2.00±0.00	29.70±1.01
0.76	2.20±0.10	2.50±0.10	4.37±0.06	6.40±0.20	9.00±1.73	3.00±0.00	2.00±0.00	29.47±1.60
1.52	2.12±0.06	2.50±0.00	4.83±0.67	6.37±0.55	9.33±0.58	3.00±0.00	2.00±0.00	30.17±1.63
3.04	2.30±0.10*	2.53±0.16	5.29±0.26	6.45±0.40	9.67±0.58	3.00±0.00	2.00±0.00	31.23±0.18*
6.08	2.60±0.10*	2.92±0.07*	5.77±0.31*	6.80±0.36*	9.67±1.53	3.00±0.00	2.00±0.00	32.75±1.43*

$p<0.05$, significant difference between treatment and control (ANOVA, LSD test)

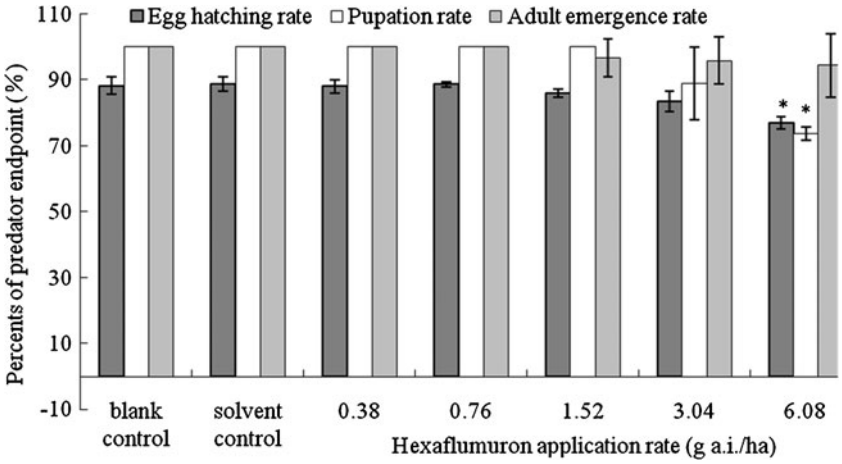

Fig. 2 Effect of different hexaflumuron doses on egg hatching rate, pupation rate and adult emergence rate of *C. septempunctata*. *$p<0.05$, significant difference between treatment and control (ANOVA, LSD test)

decreased to 88.9 % (although not significantly different from controls). At the highest treatment level, the mean pupation percentage was 73.80 %, which was significantly different from that in the controls (ANOVA, $p<0.05$; NOER=3.04 g a.i. ha^{-1}).

Effect of hexaflumuron on adult emergence of C. septempunctata

Treatment-related effects of hexaflumuron on adult emergence of *C. septempunctata* are shown in Fig. 2. The emergence of *C. septempunctata* in the controls and at the two lowest treatment levels was 100 %. Mean emergence percentages in the test systems at the three highest treatment levels were 96.7, 95.8, and 94.4 %, respectively, relative to controls, although the difference was not statistically significant (ANOVA, $p>0.05$).

Effect of hexaflumuron on egg production of C. septempunctata

Figure 3 shows the mean number of eggs produced per female of *C. septempunctata* after hexaflumuron treatment. Each female produced a mean of 1,042 eggs in the controls, and numbers decreased with increasing dosages of the active ingredient applied, particularly at the two highest treatment levels. The lowest mean number of eggs per female was recorded at the highest treatment level (736±86 eggs), although this number was not significantly different from control values.

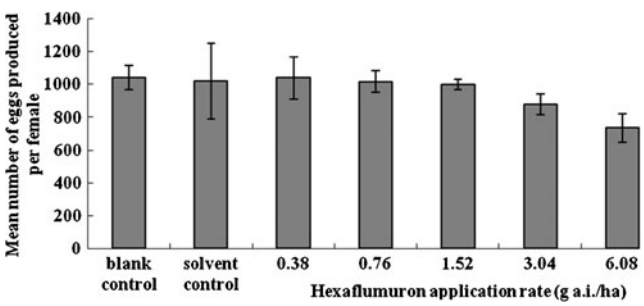

Fig. 3 Effect of different hexaflumuron doses on egg production per adult female of *C. septempunctata*

Discussion

Our 72-h acute toxicity test yielded an LR_{50} of 304 g a.i. ha^{-1} for second instar larvae of *C. septempunctata* exposed to the IGR hexaflumuron (single application). In the long-term toxicity test, the application rate causing approximately 50 % mortality among ladybird larvae 21 days after a single application of hexaflumuron was a factor of 50 lower (6.08 g a.i. ha^{-1}; Fig. 1). The long-term test lasted over 33 days and the NOERs for the endpoints of larval survival, development time for second instar larvae, and development time for the whole generation was 1.52 g a.i. ha^{-1}. These data not only illustrate a relatively high acute/chronic ratio for hexaflumuron and *C. septempunctata*, but also the occurrence of delayed effects after a single application of this IGR.

A limitation of our study is that we could only express exposure–response relationships of hexaflumuron on *C. septempunctata* in terms of application rates, since we did not measure the fraction of the sprayed active ingredient that actually reached the bean plants, nor the dynamics in actual concentrations of hexaflumuron on the leaf surfaces and in the food items (aphids). The scientific literature reports that pesticide residuals both on surfaces (Hassan 1985) and in food (Angeli et al. 2000) may be important exposure routes for predatory insects. In our test systems, exposure of *C. septempunctata* probably occurred in the form of direct contact (spray droplets and residual contact activity on leaf surfaces) and ingestion of aphids (oral exposure). Although we did not investigate the different exposure routes in our test, our toxicity data clearly illustrate that potential effects of hexaflumuron on beneficial arthropods (including *C. septempunctata*) cannot be excluded

at field application rates higher than 1.52 g a.i. ha^{-1}. In China, the maximum field application rate of hexaflumuron to control pest insects on cotton was 135 g a.i. ha^{-1} in recent years (http://www.chinapesticide.gov.cn/service/aspx/B3X.aspx?aiid=GRHEX). Our study does not allow a detailed evaluation of the long-term ecotoxicological consequences of this field application rate on *C. septempunctata*, since the highest application rate tested in our long-term toxicity test was 6.08 g a.i. ha^{-1}.

Exposure to hexaflumuron is likely to perturb the development of the beneficial arthropod *C. septempunctata*, since it is an IGR that disrupts molting and cuticle formation and more generally acts on the insect's endocrine system (Dhadialla et al. 1998). In agreement with the results of our study, others have found adverse effects of hexaflumuron on the offspring of the beetle *Callosobruchus maculatus*, affecting its growth, development, and reproductive performance (Kellouche and Soltani 2006). Campiche et al. (2006) reported that hexaflumuron was highly toxic to a soil insect, *Folsomia candida*, with an EC_{50} of 0.6 mg kg^{-1} (dry weight). Wang et al. (2008) showed that IGRs like hexaflumuron have chronic oral toxic effects on longevity, fecundity, and offspring emergence in the hymenopteran *Anagrus nilaparvatae*. Some studies reported that hexaflumuron causes higher toxicity in leaf miner insects than among predatory beneficial arthropods (Josep-Anton and Garcia-Marí 2001).

Our long-term toxicity test used five measurement endpoints to assess the effect of hexaflumuron on *C. septempunctata*, viz., survival rate, development time, pupation rate, adult emergence, number of eggs laid, and hatching rate. These measurement endpoints covered the whole life cycle as well as specific stages within the life cycle of *C. septempunctata*. In our study, the effect on the survival of *C. septempunctata* larvae (NOER of 1.52 g a.i. ha^{-1}) was larger than the effects on pupation (NOER of 3.04 g a.i. ha^{-1}), adult emergence (NOER ≥6.08 g a.i. ha^{-1}) and hatching of eggs (NOER of 3.04 g a.i. ha^{-1}). It has been reported that pesticide impacts on the development of predatory beneficial arthropods typically depend on the biology of the experimental species (i.e., predators versus parasitoids). Studies using parasitoids have often reported effects on adult emergence from the pupal stage (Krespi et al. 1991; Schneider et al. 2003; Saber et al. 2005). Cônsoli et al. (1998) reported that *Trichogramma pretiosum* pupae (Hymenoptera) displayed a higher sensitivity to pesticides in terms of development time than eggs, larvae, or prepupae.

The intensive use of conventional insecticides has caused undesirable side effects on the environment (Frank 2009). The more novel IGRs are reported to be more selective in their mode of action (Isshaya 1990; Dhadialla et al. 2005) and to be less harmful to most nontarget organisms, including arthropods (Mulla 1995). Although there has been considerable research to evaluate the efficacy of IGRs against target pest population, relatively little information is available concerning their toxicity to beneficial arthropods. Our study demonstrates that long-term effects of the IGR hexaflumuron on ladybird beetles cannot be excluded at field application rates higher than 1.52 g a.i. ha^{-1}.

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