COMPARATIVE TOXICITY OF THE NONSTEROIDAL ECDYSONE AGONISTS TEBUFENOZIDE AND METHOXYFENOZIDE TO EARLY AND LATE LARVAL INSTARS OF THE WHITE-MARKED TUSSOCK MOTH, *ORGYIA LEUCOSTIGMA*

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Abstract

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Insecticidal toxicity can vary widely as a function of the developmental stage of the insect being targeted. Here the toxicity of the nonsteroidal ecdysone agonists tebufenozide and methoxyfenozide was evaluated against early and late instars of the white-marked tussock moth, *Orgyia leucostigma* (J.E. Smith) (Lepidoptera: Erebidae), using a droplet-feeding bioassay. On the basis of LD_{50} estimates, methoxyfenozide was ~9 and 22 times more effective than tebufenozide in inducing mortality in 1st and 4th instars of *O. leucostigma*, respectively. Unlike methoxyfenozide, tebufenozide was unable to cause more than 70% mortality in 4th instars, even at the highest dose tested (1 µg/larva). Analysis of the present data suggests that susceptibility of late instar larvae to tebufenozide is significantly compromised whereas that to methoxyfenozide is much less so, which suggests that the mechanism conferring resistance to tebufenozide in late instar larvae is either ineffective or less effective in the case of methoxyfenozide.

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Introduction

Nonsteroidal ecdysone agonists of the diacylhydrazine family comprise several insecticidal molecules that can induce a precocious and lethal larval molt. Their mode of action involves high-affinity binding to the ecdysone receptor and the persistent expression of molt-related transcription factors (Dhadialla *et al*. 1998; Nakagawa 2005; Retnakaran *et al*. 2001). Two such compounds, tebufenozide (RH-5992; Smagghe and Degheele 1994) and methoxyfenozide (RH-2485; Carlson *et al*. 2001), are highly selective for lepidopterans

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and, as such, are currently registered in Canada for the control of various lepidopteran pests; however, only tebufenozide has been registered for the suppression of forest insect pests. Because of their specificity to lepidopterans, these two growth regulators have minimal impact on non-lepidopteran organisms and are therefore considered reduced-risk insecticides.

Differences in susceptibility among insect life stages to a given insecticidal compound have often been reported, where early instars are typically more susceptible than later ones (Robertson *et al.* 2007). Such instar-associated variation in susceptibility to diacylhydrazines has been observed for several species, including examples where early instars were more susceptible than later instars (Moulton *et al*. 2000; Knight *et al*. 2001) and vice versa (Smagghe and Degheele 1994; Sáenz-de-Cabezón Irigaray *et al.* 2005), as assessed by comparing LC_{50} values estimated using leaf-dip or diet incorporation assays. The white-marked tussock moth (WTM), *Orgyia leucostigma* (J.E. Smith) (Lepidoptera: Lymantriidae), a highly polyphagous pest responsible for significant defoliation in deciduous and coniferous forest stands in Canada (Dedes 2009), may well represent an extreme example of developmental variation in susceptibility to tebufenozide. Indeed, first instars were reported to be highly susceptible to this compound, while 4th and 5th instars seemed largely resistant to it (Retnakaran *et al*. 2001, 2003). Following treatment with µg doses, 4th instars entered a moribund state from which they recovered after several days and moulted to the 5th stadium. It has been hypothesized (Retnakaran *et al*. 2003) that this late-instar resistance results from the cells' ability to pump out the analog using an ABC transporter homologous to the one identified in yeast (Hu *et al*. 2001).

In an effort to further explore the biochemical basis of this type of resistance and to identify genes whose transcription may be modulated differently by diacylhydrazines in different developmental stages, we undertook a study focusing on the transcriptional analysis of *O. leucostigma* larvae treated as 1st or 4th instars with either tebufenozide or methoxyfenozide. Given that the latter compound is typically effective at lower doses than the former (e.g., Carlson *et al*. 2001), we wanted to determine whether it was subject to the same type of developmental resistance reported for tebufenozide. Because LD_{50} toxicity of these two growth regulators to *O. leucostigma* has never been formally assessed, we estimated the LD_{50} values for the two compounds and developmental stages, so that we could later standardize treatments and dose acquisition for our transcriptional analyses. Here, we report on the results of these toxicity assessments.

Materials and Methods

Chemicals

Technical grade tebufenozide (N-tert-butyl-N-3,5-dimethylbenzoyl-N'-4-ethylbenzoylhydrazine; 95%) and methoxyfenozide (N-tert-butyl-N-3,5-dimethylbenzoyl-N'-3-methoxy-2-methylbenzoylhydrazine; 95%) were obtained from Dow AgroSciences (Indianapolis, USA). Analytical grade acetone was used in the preparation of the insecticidal solutions.

Insects

Larvae of *O. leucostigma* and the Bell artificial diet (Bell *et al*. 1981) on which they were reared were obtained from Insect Production Services, Canadian Forest Service, Sault Ste. Marie, Ontario (http://www.nrcan.gc.ca/forests/research-centres/glfc/13467). Larvae were reared individually in 22 mL plastic cups at 22°C, 50% relative humidity, and under a 12h:12h light:dark photoperiod. Male and female larvae were left unseparated. Larvae were transferred onto fresh Bell diet every 2 weeks.

Larval toxicity assays

LD_{50} values were estimated for both tebufenozide and methoxyfenozide in 1-day-old 1st and 4th instar larvae. Prior to treatment, larvae were starved individually for 24 h in empty diet cups. For dose acquisition, we used a droplet-feeding method (van Frankenhuyzen *et al*. 1997; Dallaire *et al*. 2004). Serial dilutions of the compounds were prepared in distilled water containing 1% acetone, 2% sucrose, and 2% red food colouring (Club House). Control larvae were fed the carrier only (i.e., the solution used for dilutions).

Five doses varying between 1.125×10^{-2} and 112.5 ng of tebufenozide or methoxyfenozide were administered to 1st-instar larvae in a volume of 0.25 µL of carrier deposited at the bottom of a Fisherbrand 0.25 mL microfuge tube, using a Cole-Parmer 74900 series microinjector mounted with a 0.25 cm^3 tuberculin syringe equipped with a 31-gauge Hamilton needle. A micro magnetic stirring bar (7 × 2 mm) was used to keep the insecticide in solution within the syringe. Five (methoxyfenozide) and six (tebufenozide) doses varying between 0.1 and 1125 ng of the same compounds were administered to 4th-instar larvae in a volume of 2.5 µL deposited at the bottom of a 12 × 75 mm culture tube (5 mL) using a P-10 Gilson pipette.

One larva of the appropriate stadium was transferred to each droplet-containing tube. Tubes were then held upside down under a fluorescent light for 4 h, and only larvae that imbibed the whole droplet during this period were used in the experiment. Thirty 1st and twenty 4th instars were processed in this way for each insecticide and each dosage, including control conditions. After imbibing the droplet, larvae were transferred to fresh Bell diet and held under the conditions described above. To assess mortality, larvae were monitored daily for up to 2 weeks following treatment. Insects that did not move after being probed with the tip of a fine paintbrush were considered dead.

Larval mass

To examine the relationship between larval mass and the estimated LD_{50} values, fifteen 1st- and ten 4th-instar larvae (1 day old) were weighed using a Mettler AE 100 balance.

Probit analysis

For probit analysis (Bliss 1934), we used the StatPlus Pro 2009 software from AnalystSoft Inc. (www.analystsoft.com). LD_{50} values (the amount of material, given all at once, that causes the death of 50% of a group of test animals) were estimated for each compound and each developmental stage.

Results

In comparing LD_{50} estimates obtained for the two test compounds, methoxyfenozide was ~9 and 22 times more effective than tebufenozide in causing mortality in 1st and 4th instars of *O. leucostigma*, respectively (Table 1). Strikingly, the 4th instar:1st instar LD_{50} ratio was more than twice as high for tebufenozide (~28) than for methoxyfenozide (~11), pointing to a difference between the two growth regulators with respect to the development of resistance in older instars.

Considering the existence of differences in size among larvae of different developmental stages, an increase in the dose required to achieve mortality in 4th instars relative to 1st instars was not completely unexpected. To examine the relationship between larval mass and LD_{50} estimates, the latter values were divided by the average mass of 1st- and 4th-instar larvae (0.316 ± 0.003 mg and 47 ± 8 mg, respectively). Interestingly, on a per mg basis, 4th instars appeared more susceptible than 1st instars to either compound, although this difference was greater for methoxyfenozide (14×) than for tebufenozide (5×), as determined from LD_{50} estimates (Table 1).

Discussion

In the present study, we conducted an assessment of the toxicity of two diacylhydrazine insecticides, tebufenozide and methoxyfenozide, against 1st and 4th instars of *O. leucostigma*. Originally meant as a preliminary step aimed at selecting appropriate doses (LD_{50}'s) for the treatment of larvae to be used in a transcriptional study, the work presented here already sheds some light on the phenomenon of developmental resistance to tebufenozide reported earlier for late instars of *O. leucostigma* (Retnakaran *et al.* 2001, 2003).

TABLE 1: Probit statistics, including LD_{50} estimates (ng/larva), for the toxicity of tebufenozide and methoxyfenozide estimated for 1st and 4th instars of the white-marked tussock moth, *Orgia leucostigma*, using a droplet-feeding assay. The last column shows LD_{50} estimates expressed as ng/mg.

Insecticide	Instar	Slope ± SD	Intercept	LD_{50}	(95% CL)	LD_{50} (ng/mg)
tebufenozide	1st	1.3 ± 0.8	3.3	19.0	(0.7; 523)	60.2
	4th	0.7 ± 0.2	3.2	538.5	(163; 4713)	11.5
methoxyfenozide	1st	1.5 ± 0.4	4.4	2.2	(0.2; 45)	7.0
	4th	1.5 ± 0.3	3.0	24.2	(12.5; 47)	0.5

Although the 95% confidence limits determined for the LD_{50} estimates presented here point to a significant degree of variability in the responses of *O. leucostigma* to the test compounds (Table 1; more pronounced for tebufenozide than for methoxyfenoside, in part because of the poor response of 4th instars), our data indicate that the relationship between developmental stage and susceptibility to the test compounds differs for tebufenozide and methoxyfenozide. Not only was methoxyfenozide overall more effective than tebufenozide, as shown previously for other lepidopteran species (Charmillot *et al*. 2001; Trisyono and Chippendale 1998; Knight *et al*. 2001; Borchert *et al*. 2004; Moulton *et al*. 2000; Smagghe *et al*. 2000; Sundaram *et al.* 1998), it was considerably more potent against 4th instars than tebufenozide. For example, the L4:L1 LD_{50} ratio was 2.5 times higher for tebufenozide than for methoxyfenozide (see Results and Table 1). In addition, the highest tebufenozide dose tested against 4th instars (1 µg/larva) was insufficient to kill all or most larvae, which confirms earlier observations (Retnakaran *et al*. 2001, 2003).

Previous studies have reported differences in the toxicity of tebufenozide and/or methoxyfenozide as a function of the larval instar examined. While in two cases the authors noted a reduction in potency with advancing developmental stages of the test species (Knight *et al.* 2001; Moulton *et al.* 2000), in two other cases the authors observed the very opposite trend (Sáenz-de-Cabezón Irigaray *et al.* 2005; Smagghe and Degheele 1994). It is important to point out, however, that these conclusions were based on a comparison of lethal concentration (LC) estimates as opposed to the lethal dose (LD) estimates reported here. To estimate LC_{50} values with lepidopteran larvae, either foliage or artificial diet is dipped into solutions of the test compound prepared at different concentrations. While this approach has the advantage of being more representative of a field situation and provides more relevant information for the selection of application rates, larvae submitted to such an assay will consume increasing amounts of food as they advance in development and will therefore acquire vastly different doses of the test compounds, which are typically not reported. Therefore, differences in feeding rates among the insect species examined in the aforementioned studies could, in part, explain some inconsistencies in the trends that were reported. For the present work, we opted for the estimation of lethal dose values because our intention was to select doses (LD_{50}) that would enable a valid comparison of differential gene expression among larvae of distinct developmental stages and exposed to different test compounds. However, a simple comparison of LD_{50} values between different developmental stages does not take into account developmental differences in the ability of larvae to metabolize and/or excrete the test compounds, which should be related, at least in part, to larval mass. Recognizing that the ratios reported in Table 1 are not an accurate reflection of the true relationship between size and metabolism, the lower LD_{50}/mass ratios calculated for 4th instars nonetheless point in the same direction as two independent studies that reported an age-dependent rise in susceptibility to tebufenozide (Smagghe and Degheele 1994) and methoxyfenozide (Sáenz-de-Cabezón Irigaray *et al.* 2005) on the basis of LC_{50} values. Here, however, the developmental difference in LD_{50}/mass ratio was substantially smaller for tebufenozide than for methoxyfenozide (Table 1), again indicating a difference in the development-dependent response to the test compounds.

In conclusion, methoxyfenozide was, overall, more potent than tebufenozide in causing mortality in *O. leucostigma* larvae. In addition, analysis of the present data suggests that susceptibility of later instars to tebufenozide is significantly compromised

while that to methoxyfenozide is less so, which indicates that the mechanism conferring resistance to tebufenozide in older larvae is either ineffective or less effective in the case of methoxyfenozide. Transcriptional analysis should help pinpoint what these differences may be at the gene expression level.

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