ABSTRACT  We conducted laboratory experiments to examine the effects of single versus double exposures of spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) female larvae to various concentrations of a *Bacillus thuringiensis* variety *kurstaki* (Btk) commercial formulation (Foray 48B). Our main objective was to document the vulnerability to Btk and the sublethal responses of fifth-instar larvae that survived from a first ingestion of Btk during their fourth stadium and to compare them with insects treated either during their fifth or fourth stadium only. As reported in the literature, fifth-instar larvae were more vulnerable than fourth-instar larvae, but only at low and medium concentrations. Fifth-instar larvae that had survived Btk ingestion during their fourth stadium were more vulnerable to a high concentration of Btk and had a shorter feeding inhibition period than those that had not been exposed during their fourth stadium. Compared with a single treatment at the fourth stadium, a double exposure to Btk further reduced the population by 20–30%, depending on the concentration applied. The second treatment also induced another feeding inhibition period and increased larval development time by 14%. The impact of the different treatments on pupal weight depended on whether treated insects exhibited supernumerary instars. In the absence of developmental polymorphism, a higher concentration, a late, or a double exposure to Btk significantly reduced pupal weight.

KEY WORDS  Double application, *Bacillus thuringiensis*, *Choristoneura fumiferana*, spruce budworm, sublethal effects

*Bacillus thuringiensis* *VAR. kurstaki* Berliner (Btk) is the most common biopesticide used to suppress the spruce budworm (*Choristoneura fumiferana* (Clemens)) (Lepidoptera: Tortricidae), a major defoliator in boreal forests of eastern North America. Once activated by the alkaline digestive fluid of the insect, the toxin damages the midgut epithelium, which leads to a cessation of feeding (Percy and Fast 1983). Depending on the dose ingested, larvae may die from starvation or septicemia, or they may survive but suffer sublethal effects, such as feeding inhibition, reduced pupal weight, prolonged development time, and increased incidence of developmental polymorphism (Retnakaran et al. 1983, Fast and Régnière 1984, Alford and Holmes 1986, van Frankenhuyzen and Nystrom 1987, Ramachandran et al. 1993, Pedersen et al. 1997, Moreau and Bauce 2001).

Spruce budworm eggs usually hatch in early August. Neonate larvae do not feed but instead spin a hibernaculum, molt to second instar, and enter obligatory diapause that lasts until the following spring. In early spring, the larvae emerge and start mining the swelling buds of balsam fir (*Abies balsamea* L.), white spruce (*Picea glauca* (Moench) Voss), or other spruce species. Larvae feed on needles of developing shoots until the end of the sixth stadium and then they pupate. Adults eclose within 2 wk of pupation and lay eggs soon after.

Aerial applications of Btk against the spruce budworm usually target early fourth-instar larvae when developing shoots are sufficiently exposed to spray deposition, but before significant feeding has occurred. To ensure adequate foliage protection, a second application of Btk is often required when populations are high. Although the second application effectively reduces the amount of defoliation inflicted to trees when tree density is not too high, it is less lethal than the first (Régnière and Cooke 1998). However, Cadogan and Scharbach (1993) have shown that spruce budworm populations are reduced more efficiently by a late application than by a spray targeted against fourth instars and van Frankenhuyzen et al. (1997) reported that fifth and sixth instars were having a higher vulnerability to Btk as a function of weight.
than earlier instars. Régnière and Cooke (1998) proposed a number of possible hypotheses to explain the low efficacy of the second Btk application. First, spruce budworm larvae dying following the first exposure to Btk may be the most susceptible, leaving more resistant larvae as targets of the second application. Alternatively, surviving insects may have reduced rates of feeding because of midgut damage resulting in sublethal ingestion of toxins during their second exposure to Btk. Regardless, reduced efficacy is not a result of a difference in the behavioral response between fourth- and fifth-instar larvae (Moreau and Bauce, 2003).

In this paper, we test the hypotheses by Régnière and Cooke (1998) to explain why spruce budworm appears less vulnerable to a second Btk application. In addition, we document the vulnerability, dose acquisition, and sublethal responses of fifth-instar larvae surviving exposure to Btk during their fourth stadium.

**Materials and Methods**

**Insects.** Postdiapause second-instar spruce budworm larvae were obtained from the Canadian Forest Service insect production unit, Sault Ste. Marie, Ontario, Canada. Larvae were reared on artificial diet adapted from McMorran (1965) to simulate balsam fir (Abies balsamea (L.) Mill.) foliage profile in sugar and nitrogen (8% sugar, 4% nitrogen; dry weight) in 30-ml plastic cups. Larvae were maintained in a growth chamber following the conditions reported by Robertson (1985) at 23°C, 55–65% R.H., and a photoperiod of 16:8 (L:D) h. Development of individual larvae was monitored daily, and the feeding substrate was changed every 10 d to maintain quality. Insects were sexed during the third stadium (Robertson 1985), and males discarded because mortality rates, development times, and pupal weights of male and female budworm are similarly affected by Btk treatment when exposed at either the fourth or sixth instar (Bidon 1999).

**Feeding Tests.** Feeding tests were conducted on individual larvae in a test arena adapted from Albert and Bauce (1994) and described in detail by Moreau and Bauce (2001). Insects were weighed before the first feeding test and distributed randomly among four Btk treatments as they molted to the fourth stadium (Table 1, columns 1–4). More larvae were assigned to treatments with the highest concentrations of Btk to obtain sufficient numbers of survivors for statistical analyses. Survivors of the first feeding test were subsequently randomly assigned to a second feeding test as they molted to the fifth stadium (Table 1, columns 5–8). Before each feeding test, insects were starved for 24 h to increase disk ingestion. A starvation period does not alter the response of spruce budworm to Btk because they do little feeding during the first days following a molt (van Frankenhuyzen et al. 1997).

In each feeding test, individual larvae were placed in an arena to feed for 24 h. Feeding arenas contained a cellulose disk that was previously impregnated with 5 μl of a sugar/nitrogen (8% sugar, 4% nitrogen; dry weight) solution to simulate the nutritive quality of balsam fir foliage, the preferred host of the insect. Bidon (1999) described the chemical composition of the disk. Cellulose disks were also impregnated with 3 μl of distilled water or with 3 μl of one of three different dilutions of Btk at concentrations of 4.25, 8.5 and 17 International Units (IU)/μl. At the end of feeding tests, larvae were transferred to the artificial diet.

Frass production, feeding activity, survival, development time, and larval weight were monitored. Larvae fed Btk that died before their next molt were considered to have been killed by Btk. Btk ingestion induced feeding inhibition periods after each feeding test. A feeding inhibition period was defined as the period between the end of a feeding test and the production of the first frass because failure to produce frass is a direct result of feeding inhibition (Retnakaran et al. 1983). To evaluate treatment effects on development time, three different periods were examined: development time after the first feeding test was defined as the period between recovery from feeding inhibition at fourth instar and the following molt, development time after the second feeding test was defined as the period between recovery from feeding inhibition at fifth instar and pupal molt, and overall development time was defined as the period from the beginning of the fourth stadium to the pupal molt, including feeding inhibition periods.

### Table 1. Concentration used, number of larvae exposed, and larval response in the first and second feeding test. Values reported are means ± SEM

<table>
<thead>
<tr>
<th>First test conc., IU/μl</th>
<th>n</th>
<th>Percentage of disk eaten</th>
<th>Dose ingested, IU</th>
<th>Second test conc., IU/μl</th>
<th>n</th>
<th>Percentage of disk eaten</th>
<th>Dose ingested, IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>210</td>
<td>9.9 ± 0.3a</td>
<td>0.00 ± 0.00a</td>
<td>4.25</td>
<td>46</td>
<td>54.3 ± 2.5a</td>
<td>0.00 ± 0.00a</td>
</tr>
<tr>
<td>4.25</td>
<td>126</td>
<td>1.6 ± 0.4b</td>
<td>0.21 ± 0.03b</td>
<td>8.5</td>
<td>46</td>
<td>7.9 ± 2.9b</td>
<td>2.01 ± 0.28ab</td>
</tr>
<tr>
<td>8.5</td>
<td>205</td>
<td>1.8 ± 0.3b</td>
<td>0.45 ± 0.02c</td>
<td>17</td>
<td>63</td>
<td>5.9 ± 2.5b</td>
<td>3.02 ± 0.24b</td>
</tr>
<tr>
<td>17</td>
<td>276</td>
<td>1.3 ± 0.3b</td>
<td>0.67 ± 0.02d</td>
<td>17</td>
<td>84</td>
<td>5.3 ± 2.1b</td>
<td>2.69 ± 0.21b</td>
</tr>
<tr>
<td>Total</td>
<td>817</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Means within a column followed by different letters are significantly different (*P* < 0.05; Bonferroni procedure).
Btk dilutions were prepared using Foray 48B (Abbott laboratories, North Chicago, IL) containing a mixture of crystals and spores of Btk at a potency of 12.7 billion International Units (BIU) per liter. Foray 48B was diluted with phosphate-buffered saline (pH 8.0), containing 0.01% Triton X-100 as a surfactant, to obtain concentrations of 4.25, 8.5, and 17 IU/\mu l, which were then sonicated to fully suspend particulates. These concentrations resulted in mortality rates of 25, 50, and 75%, respectively, for fourth instars and 75% for fifth instars in earlier experiments (Bidon 1999) and the amount of IU per area of disk treated corresponded to aerial sprays of 15, 30, and 60 BIU/ha. The presence of the expected dose of Btk on the disk was confirmed by quantifying the Btk toxins using the Abbott Assessment method (ADAM Kit, Abbott Laboratories, North Chicago, IL). An optical image analysis system (Monochrome AgVision System, Decagon Devices Inc., Pullman, WA) was used to measure the proportion of each cellulose disk eaten and to estimate the dose ingested by multiplying the eaten proportion of the disk by Btk concentration.

We tested three types of exposure for each of the three concentrations used: exposure to Btk at fourth instar followed by exposure to distilled water of the same larvae at fifth instar (normal application), exposure to distilled water at fourth instar followed by exposure to Btk of the same larvae at fifth instar (late application), and exposure to Btk at fourth instar followed by exposure to the same concentration of Btk of the same larvae at fifth instar (double application). Control larvae were exposed to distilled water at fourth and fifth instar.

Statistical Analyses. Because larvae were tested individually, they represent one replicate of their respective treatment. Analyses of lethal effects, feeding inhibition period, development time, and fifth-instar weight following the first feeding test were performed using analysis of variance (ANOVA). Analyses of lethal effects, feeding inhibition period, development time, and pupal weight following the second feeding test were performed using Addelman’s methodology (Addelman 1974) for factorial experiments with qualitative strategies (normal application, late application, double application) and quantitative concentrations including a zero level. Mortality was analyzed using main effect and interaction sum of squares in tables of proportions (Steel and Torrie 1980). ANOVA procedures followed by Bonferroni t-tests were used to compare values in Table 1. Student t-tests were used to compare weight before the treatment and dose ingested. A repeated measure ANOVA was carried out to compare feeding inhibition periods of fourth and fifth instars. Feeding inhibition periods were given logarithmic transformation to respect requirements for homogeneity and normality of residuals.

### Results

**Lethal Effects.** Larval mortality induced by the first feeding test increased linearly with Btk formulation concentration ($\chi^2 = 100.91; n = 817; df = 1; P < 0.01$) (Fig. 1A). Larvae that died during the feeding test were usually smaller (mean $\pm$ SEM: dead = 1.35 $\pm$ 0.02 mg; alive = 1.55 $\pm$ 0.02 mg; $df = 444; t = -6.03; P < 0.01$) and had ingested a higher dose than those that survived (mean $\pm$ SEM: dead = 0.56 $\pm$ 0.02 IU; alive = 0.47 $\pm$ 0.02 IU; $df = 475; t = 2.66; P < 0.01$).

Larvae in the normal application were only exposed to water during the second feeding test and suffered very little mortality. Consequently, a concentration by application strategy interaction was detected in the larval mortality induced by the second feeding test ($\chi^2 = 24.55; n = 522; df = 4; P < 0.01$) (Fig. 1B). The effect of Btk concentration on the slope of larval mortality was similar in the late and double application strategy ($P = 0.18$), with larval mortality increasing linearly with Btk formulation concentration ($\chi^2 = 84.99; n = 522; df = 1; P < 0.01$) (Fig. 1B). However, larval mortality was greater when larvae were treated for a second time (late versus double application; $\chi^2 = 5.23; n = 522; df = 1; P = 0.02$) (Fig. 1B), except at 8.5 IU/\mu l. When dose ingested in late and double application were compared using fifth-instar weight as a covariate, no differences were detected ($P = 0.88$). The use of a covariate was necessary to ensure that the
dose ingestion by fifth instars was not affected by reduced weight as a result of sublethal dose exposure at fourth instar. Larvae that survived the feeding test ingested a similar dose to those that died ($P = 0.92$).

Analysis of the overall mortality revealed two interactions between concentration and application strategies. The first was a result of mortality in single application being higher when larvae were treated later, but not at the highest concentration (normal versus late application $\chi^2 = 8.94; n = 392; df = 1; P < 0.01$) (Fig. 1C). The second interaction was due to mortality following late application being similar to the mortality in the double application treatment at low concentration but distinctly lower at high concentration (late versus double application $\chi^2 = 19.53; n = 392; df = 1; P < 0.01$) (Fig. 1C). No evidence of an interaction between the normal and double application was observed ($P = 0.15$). Larval mortality in these applications increased with concentration but was 27% higher when larvae were treated at fourth and fifth stadium instead of only once at fourth stadium (normal versus double application; $\chi^2 = 54.84; n = 392; df = 1; P < 0.01$) (Fig. 1C).

**Feeding Inhibition Periods.** After the first feeding test, the feeding inhibition period increased linearly with Btk formulation concentration ($F = 189.95; df = 1, 640; P < 0.01$) (Fig. 2A). After the second feeding test, a concentration by application strategy interaction was detected on the feeding inhibition period. The interaction was a result of decreased larval sensitivity in double application to an increase in Btk potency at the extreme concentration (late versus double application $\times$ linear effect of concentration; $F = 7.64; df = 1, 381; P < 0.01$) (Fig. 2B). Larvae in the normal application were only exposed to water during the second feeding test and did not exhibit feeding inhibition. The response of larvae subjected to a double application differed between feeding tests because the feeding inhibition period was 52% shorter in the fifth stadium than in the fourth stadium ($F = 116.54; df = 1, 333; P < 0.01$), with no evidence of an interaction with concentration ($P = 0.24$).

**Development Time.** Development time after the first feeding test increased linearly with Btk formulation concentration ($F = 32.82; df = 1, 632; P < 0.01$) (Fig. 3A). Similarly, development time after the second feeding test increased linearly with Btk formulation concentration ($F = 34.18; df = 1, 381; P < 0.01$) (Fig. 3B) and was longer for larvae exposed during fifth stadium than for larvae exposed during fourth stadium (normal versus late application; $F = 41.34; df = 1, 381; P < 0.01$) (Fig. 3C). No interaction was detected between these two factors ($P = 0.55$). Larvae that were treated a second time and larvae treated once during the fifth stadium developed at the same rate from the end of feeding inhibition at fifth instar to pupal molt (late versus double application; $P = 0.42$) (Fig. 3C).

A concentration by application strategy interaction was detected in the overall development time, stemming from the higher response of late application at 4.25 IU/μl (normal versus late application $\times$ quadratic effect of concentration; $F = 4.10; df = 1, 244; P = 0.04$) (Fig. 3D). There was no evidence of an interaction between normal and double applications and Btk formulation concentration ($P = 0.15$). However, a second application prolonged the overall development time by 14% (normal versus double application; $F = 45.60; df = 1, 244; P < 0.01$) (Fig. 3D).

**Insect Weight.** As Btk formulation concentration applied to fourth instars was increased, the weight of newly molted fifth instars decreased linearly ($F = 13.74; df = 1, 623; P < 0.01$) (Fig. 4). However, this linear weight reduction was not detectable after pupal molt if these larvae were treated only during the fourth stadium ($P = 0.53$).

More than one third of all larvae exhibited more than the usual six instars observed in female spruce budworm. Because the production of supernumerary instars has consequences for pupal weight (Moreau and Bauce 2001), analyses of the sublethal effects of
Btk on pupal weight were carried out for the presence or absence of polymorphism. Pupal weight of insects that did not produce supernumerary instars was affected significantly by Btk concentration and application strategy and no interaction was detected between these two factors \( (P = 0.13) \). Pupal weight of larvae that did not exhibit developmental polymorphism decreased linearly with Btk concentration \( (F = 5.26; \text{df} = 1, 244; P = 0.02) \) (Fig. 5A) and was reduced by 8% when larvae were treated later (normal versus late application; \( F = 5.56; \text{df} = 1, 244; P = 0.02 \)) (Fig. 5B) and 13% when they were treated twice (normal versus double application; \( F = 13.40; \text{df} = 1, 244; P < 0.01 \)) (Fig. 5B). However, for larvae that produce supernumerary instars, concentration \( (P = 0.42) \), later \( (P = 0.12) \), or double Btk exposure \( (P = 0.12) \) did not affect pupal weight.

**Discussion**

Our results show that spruce budworm larvae exposed to sublethal doses of Btk are physiologically less resistant to a second application of Btk than untreated larvae of the same developmental stage. This leads us to reject the hypothesis that increased resistance could be responsible for reduced field efficacy of double applications. However, these results are in opposition to those of similar studies conducted in field conditions (Régnière and Cooke 1998). Differences between the results reported by Régnière and Cooke (1998) and our results can be explained by the fact that larvae in the field encounter Btk as discrete droplets and not as a product uniformly spread over an area as experienced on feeding disks. In the field, the probability of encountering a droplet containing a lethal
Dose is lower at fifth than at fourth instar because the LD_{50} increases with larval age (van Frankenhuyzen et al. 2000). Because feeding inhibition resulting from ingestion of a sublethal dose can hinder the acquisition of a lethal dose (van Frankenhuyzen et al. 2000), it is possible that Btk droplets in the field contain enough active elements to induce feeding inhibition but not to kill a fifth-instar larva. As a possible remedy, aircraft applicator nozzles could be adjusted to spray larger droplets that would contain more active elements during the second application without increasing the application rate.

A second Btk application to fifth-instar larvae enhanced lethal and sublethal effects of the first application applied at fourth stadium. The second exposure to Foray 48B reduced the population by an additional 20–30%, caused a second but shorter period of feeding inhibition, and increased larval development time by 14% at any concentration. In terms of pupal weight, the ability of spruce budworm to compensate for sublethal Btk effects was directly related to whether it produced supernumerary instars. Higher concentration and late or double Btk exposure reduced pupal weight of larvae that did not produce supernumerary instars but did not affect pupal weight of those that produced supernumerary instars. Other laboratory studies have shown that fourth-instar budworm larvae are able to recover from a single exposure to Btk without suffering reductions in pupal weight (Fast and Régnière 1984, Ramachandran et al. 1993). This can be explained by the capacity of stressed spruce budworm larvae to produce supernumerary instars (Moreau and Bauce 2001). Our study suggests that a second application at fifth instar impairs recovery, resulting in smaller female pupae. Larvae treated at 17 IU/μl in double application recuperated more rapidly from feeding inhibition than larvae treated once as fifth instars. Although this may be a response to selective pressure for more vigorous individuals caused by acute pesticide exposure, double application of Btk is unlikely to generate Btk-resistant strains of spruce budworm because selective pressure on populations is limited even during severe outbreaks (van Frankenhuyzen et al. 1995).

There are factors not accounted for in this laboratory assay that could influence insects in the field. Nevertheless, some implications for management can be drawn from this study. As suggested by Cadogan and Scharbach (1993), we observed that spruce budworm populations were reduced more efficiently by applying treatments when larvae were fifth instar rather than when they were fourth instar. However, a higher potency of Btk formulation resulted in similar mortality in normal and late applications. This suggests that an application targeted later for population reduction will not necessarily be more effective with high Btk concentrations. Our results also suggest that mortality caused by a simple application tend to reach a plateau that can only be increased by another application.

The conventional practice when using a double application is to apply the second spray 5 to 10 d after the first. However, if the second spraying occurs only 5 d after the first one, larvae may not have time to recover from feeding inhibition, therefore reducing the efficacy of the second spray. In the laboratory, after 2.5 d at 23°C, only 50% of the populations treated with a concentration of 8.5 IU/μl were observed feeding. This means that if field temperatures that usually average 16°C were simulated in the lab, then 50% of the populations would still be in feeding inhibition after 5 d (equation from van Frankenhuyzen and Nyström 1987).

Régnière and Cooke (1998) suggest that multiple applications may be the only way to ensure adequate foliage protection against high-density budworm populations. Double application could also increase the efficacy of Btk sprays in mixed conifer stands where variation in spruce budworm phenology induced by host trees may cause asynchrony between the timing of spray application and the development of the targeted insects (Morris 1984). Double application may also be favorable in years of balsam fir flowering because of diminished current-year foliage production (Carisey and Bauce 1997). The increased efficacy pro-
vided by a double application of Btk may also be needed if budworm outbreak is preceded by severe balsam fir sawfly (Neodiprion abietis (Harris)) outbreak because this sawfly feeding on old foliage can leave trees more sensitive to budworm damage (Cunningham 1984). However, because of increased cost, double applications should only be used when single applications are not providing the efficacy needed to suppress populations to desired levels.

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