Developmental polymorphism: a major factor for understanding sublethal effects of Bacillus thuringiensis

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Abstract

Developmental polymorphism in Choristoneura fumiferana (Clemens) (Lepidoptera: Tortricidae) was induced by Bacillus thuringiensis (Berliner) in the laboratory. The B.t. formulation concentration induced a quadratic response on the incidence of developmental polymorphism. A double application of Bacillus thuringiensis at fourth and fifth instar respectively resulted in a higher occurrence of developmental polymorphism than a single application at fifth instar. Smaller larval weight prior to B.t. exposure resulted in higher incidence of developmental polymorphism. However, larvae exhibiting developmental polymorphism exhibited larger pupae and an extended larval period. Thus, developmental polymorphism and Bacillus thuringiensis ingestion together prolonged the development time but induced opposite effects on pupal weight. These findings suggest that the calculation of sublethal effects on pupal weight and development time that include developmental polymorphism as an uncontrolled source of variation could lead to an inadequate understanding of insect response in any stress-induced experiments.

Introduction

Bacillus thuringiensis (B.t.), a biological insecticide, is widely used to suppress insect pests in North America. Toxicity of B.t. results from combined effects of crystalline toxins and spores. When B.t. is fed to insects, alkaline digestive juices activate the toxin which attacks and destroys the midgut epithelium (Percy & Fast, 1983). Feeding then stops and the larva dies from starvation or septicemia within a few days following ingestion of a lethal dose. However, if feeding stops before a lethal dose is reached, a larva can recover and resume feeding, albeit with sublethal effects on its growth and development (Retnakaran et al., 1983; Fast & Régnière, 1984; van Frankenhuyzen & Nystrom, 1987). Stressful natural events such as periods of food deprivation, cool temperatures and crowding can induce similar sublethal effects in other insects and also induce supernumerary instars (Wigglesworth, 1972; Dingle & Haskell, 1967; Leonard, 1968, 1970a). According to Leonard (1968), this phenomenon, called postembryonic developmental polymorphism or developmental polymorphism (Schmidt & Lauer, 1977) prolonged the development time and increased the pupal weight of gypsy moth larvae (Lymantria dispar (L.)). Few unnatural events have been reported to induce developmental polymorphism in insects, except for treatments with juvenile hormones, which produced nonviable supernumerary instars, presumably because they are intermediates between immature and
Materials and methods

Insects. Post-diapausing second-instar spruce budworm larvae were obtained from the Canadian Forest Service insect production unit, Sault Ste. Marie, Ontario. Larvae were reared on artificial diet adapted from McMorran (1965) to simulate a balsam fir (Abies balsamea (L.) Mill.) foliage profile in sugar and nitrogen (8% sugar, 4% nitrogen, dry weight) in 30-ml plastic cups at 23 °C, 55–65% r.h. and L16:D8 photoperiod (Robertson, 1985). Diet was changed every 10 days to maintain its quality. Development of individual larvae was monitored daily and the number and dates of molts were recorded. Insects were sexed during the third instar (Robertson, 1985) and males were discarded since previous work showed no apparent differential sexual dimorphism for the proportion of developmental polymorphism in spruce budworm populations (Schmidt & Lauer, 1977) and because adverse effects on fecundity are in a large part explained by changes in female pupal weight (Pedersen et al., 1997). More than 1000 female larvae were exposed to feeding tests at the beginning of the fourth and fifth instars, which are typically targeted for spray application in the field. Insects were weighed prior to feeding tests and randomly distributed among each treatment. Female pupae that survived following exposure to B.t. were weighed 16 h after the pupal molt to allow the pupal cuticle to harden.

Feeding tests. Each insect was individually exposed to a cellulose disk impregnated with B.t. and placed in a feeding arena (adapted from Albert & Bauce, 1994). Feeding arenas consisted of 10-mm diameter holes drilled in pieces of 7-mm-thick plexiglas fixed on a styrofoam sheet, and covered with microscope cover glasses. A cellulose nitrate disk, 3.3 mm in diameter and punched from 0.45-μm pore size filter paper strips (Sartorius) and pinned in the center of each arena. All disks were impregnated with 5 μl of a sugar/nitrogen solution to stimulate feeding and to provide the insect with the same feeding substrate as the artificial diet used. Treated disks were then impregnated with 3 μl of one of three different formulations of Foray 48B (Bacillus thuringiensis Berliner variety kurstaki, Abbott Laboratories) at concentrations of 4.25, 8.5 and 17 International Units (UI) μl⁻¹. These are respectively equivalent to field concentrations of 15 BIU l⁻¹, 30 BIU l⁻¹ and 60 BIU l⁻¹, based on an aerial spray with Foray 48B at 30 BIU ha⁻¹ and 2.37 l ha⁻¹, an average deposit of 45 droplets of 80 μm in diameter per cm² of foliage and a droplet potency of 6.6 IU. We tested three types of exposure for each of the three concentrations used: exposure to B.t. at fourth instar and to distilled water at fifth instar (normal application), exposure to distilled water at fourth instar and to B.t. at fifth instar (late application), exposure to B.t. at fourth and fifth instars (double application). Control larvae were exposed to distilled water at fourth and fifth instars. Insects were starved for 24 h after molting to obtain significant disk ingestion and because a starvation period of 24 h does not alter the response of spruce budworm to B.t. (van Frankenhuyzen et al., 1997). Following this, one larva was placed in each arena and allowed to feed for 24 h. Larvae were transferred back to artificial diet at the end of the test period. An Optical Image Analysis System (Monochrome AgVision System, Decagon Devices Inc., Pullman, Washington, USA) was used to measure the proportion of each
cellulose disk that was eaten and estimate the dose ingested.

Data analysis. Statistical analyses were performed using Addelman’s methodology (1974) for factorial experiments with qualitative strategies (normal application, late application, double application) and quantitative concentrations including a zero level. The occurrence of B.t.-induced developmental polymorphism was tested on arcsin transformed data by $\chi^2$ tests (Steel & Torrie, 1980) and linear regression. Fourth-instar weight was used as a covariate in this analysis to eliminate the confounding effect of weight on the incidence of polymorphism. Computation of sublethal effects was achieved using GLM (SAS Institute, 1982) with polynomial contrasts to compare treatment differences for pupal weight and extended development time. Student $t$-tests were used to test the effect of developmental polymorphism on pupal weight and development time with all strategies and concentrations pooled.

Since larval weight is expected to influence the incidence of polymorphism, developmental polymorphism was logistically regressed against fourth and fifth-instar weight. Spearman correlations between mortality and instar weight were carried out to insure that developmental polymorphism was not induced by selective mortality. The effect of dose on developmental polymorphism was also analyzed with logistic regression. The covariance matrix was multiplied by a scaling factor based on deviance to account for sub-dispersion. Tests of goodness of fit showed no evidence of heterogeneity ($P \geq 0.10$).

Survival rate varied between 13% in the double application at 17 IU $\mu$L$^{-1}$ to 89% in the control. More than one-third of all larvae exhibited one additional instar over the usual six instars observed with female spruce budworm. Less than one percent exhibited more than seven instars. These two groups were pooled for statistical analyses.

Results

Influence of insect weight prior to B.t. exposure. In each feeding test, larval weight prior to B.t. exposure was negatively related to the incidence of developmental polymorphism, indicating that small larvae tend to undergo developmental polymorphism more often than large larvae. In normal application, we found a significant logistic relationship between fourth-instar weight and the incidence of polymorphism (Figure 1A). Data were pooled at fifth instar since the application strategy ($P = 0.81$), the concentration ($P = 0.34$) and their interaction ($P = 0.24$) did not influence the effect of weight on the incidence of polymorphism. The relationship between the latter and larval weight sharply increased after molting to fifth instar (Figure 1B), as showed by the comparison of $r$-square values (Figure 1).

Examination of the relationship between mortality due to B.t. exposure and larval weight prior to the treatment showed a negative correlation at fourth instar ($r = -0.25; n = 607; P < 0.01$) and no correlation at fifth instar ($P = 0.47$). In other words, B.t.-induced mortality is higher for small larvae at fourth instar and not related to weight at fifth instar. These results indicate that developmental polymorphism was not induced by lower mortality within susceptible instar-groups.

Incidence of developmental polymorphism. As hypothesized, we observed that strategies of B.t. exposure and B.t. concentration affected the occurrence of developmental polymorphism and no interactions were detected. The incidence of developmental polymorphism was not affected by an additional application (normal vs. double application; $P = 0.12$) but was 9.4% higher in double application than in late application ($\chi^2 = 7.48; df = 1; P < 0.01$). The B.t. concentration induced a quadratic response on the incidence of developmental polymorphism (Figure 2). On the other hand, the dose ingested by individual larvae and the incidence of polymorphism were poorly related in each treatment ($r^2 < 0.01$), which suggests that polymorphism was not dose-dependent.

Developmental polymorphism vs. B.t. sublethal effects on pupal weight. The observed tendency of larvae with supernumerary instars to weigh less at fourth and fifth instars was counteracted at pupation. The occurrence of additional molts resulted in a 10% higher mean pupal weight than that of the normally developed larvae ($t = -4.6821; df = 389; P < 0.01$) (Table 1). Since B.t. ingestion lowers pupal weight, this result means this negative effect was partly countered by developmental polymorphism. Consequently, analyses of the sublethal effect of B.t. on pupal weight were carried out separately, according to the presence or absence of polymorphism.

We found that the pupal weight of insects that did not exhibit developmental polymorphism was signif-
Figure 1. Expression of developmental polymorphism related to (A) fourth-instar weight in normal application ($n = 193, r^2 = 0.20, P < 0.01$). (B) fifth-instar weight in normal and late application ($n = 268, r^2 = 0.50, P < 0.01$).

Figure 2. Main effect of B.t. formulation concentration on the proportion of larvae expressing developmental polymorphism ($r^2 = 0.89, P < 0.01$).

Table 1. Pupal weight and development time from the start of fourth instar to pupation for spruce budworm that survived B.t. ingestion (all concentrations and strategies regrouped)

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>$n$</th>
<th>Pupal weight (mg fresh)</th>
<th>Development time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>138</td>
<td>97.4 ± 3.1a</td>
<td>27.64 ± 0.77a</td>
</tr>
<tr>
<td>No</td>
<td>254</td>
<td>88.2 ± 2.3b</td>
<td>24.41 ± 0.42b</td>
</tr>
</tbody>
</table>

Means ± s.e. followed by the same letters are not significantly different at $P > 0.05$ (t-test).

Developmental polymorphism vs. B.t. sublethal effects on development time. Larvae with supernumerary instars had a 3.2 day longer development time than those with six instars ($t = 8.6075; df = 389; P < 0.01$) (Table 1). In this instance, the effect of B.t. ingestion on development time was enhanced by developmental polymorphism. Thus, analyses of sublethal effects of B.t. on development time were done separately, according to the presence or absence of polymorphism. Extended development time was defined as the period exceeding the development time of control larvae, without the feeding inhibition period.
Figure 3. Effect of B.t. application strategies on mean pupal weight (A) for larvae with six instars (B) for larvae with more than six instars.

We found an interaction between application strategy and concentration on the extended development time of larvae that exhibited only six instars, which resulted from the stronger response of double application to an increase in B.t. concentration (normal vs. double application × linear effect of concentration; F1,243 = 4.43; P = 0.04) (Figure 4). No interaction was detected between normal and late application (P = 0.41) yet extended development time was significantly increased by 1.8 days when larvae were treated later (normal vs. late application; F1,243 = 13.59; P < 0.01). By comparison, extended development time of larvae that exhibited additional molts showed no evidence of an interaction between B.t. concentration and application strategy (P = 0.73). This effect was reduced to a mean increase of 2.5 days of the extended development time when larvae were treated twice (normal vs. double application; F1,128 = 8.37; P < 0.01). We did not find any evidence of a difference between normal and late application (P = 0.41).

Discussion

This study supports the hypothesis that the stress imposed by B.t. on spruce budworm development induces postembryonic developmental polymorphism. We could be tempted to consider this phenomenon as an individualistic compensatory response to an external stress, but this would be the wrong approach. Some larvae will never undergo developmental polymorphism, whatever the concentration or strategy used, while other untreated larvae exhibit supernumerary molts. Even if a smaller size induced developmental polymorphism (Wigglesworth, 1972) in the present study, not all the small larvae exhibited supernumerary instars and not all the bigger ones produced the normal six instars. Weight seems to be an important factor in determining which larvae will polymorph but an inherited propensity to produce additional molts (Key,
from the production of supernumerary instars. This extended larval period could force larvae to complete their development on older foliage of reduced nutritional quality (Bause & Carisey, 1996) and prolong their exposure to parasitoids and predators (Weseloh & Andreadis, 1982; Weseloh et al., 1983) but in the present study where larvae were fed on a constant diet, it clearly enhanced their opportunity to grow. This trade-off will not necessarily result in increased exposure to B.t. since the residual toxicity of treated foliage rapidly drops less than 2 days after spray (van Frankenhuysen & Nystrom, 1989).

B.t.-induced supplementary molts drastically affect sublethal effects of B.t. because developmental polymorphism and B.t. ingestion together prolonged the development time but induced opposite effects on pupal weight. The analysis of pupal weight showed two different responses depending on the presence or absence of developmental polymorphism. Without developmental polymorphism, a higher concentration, a late or a double treatment significantly reduced pupal weight. But for larvae that produce supernumerary instars, concentration, later or double treatments did not affect pupal weight. Moreover, the observed interaction between application strategy and concentration on development time not only shows the stronger response of double application to an increase in concentration, it also points out a singularity of double application that can only be observed with larvae that did not produce supernumerary instars. Clearly, developmental polymorphism complicates the comprehension of spruce budworm response by obscuring many treatment effects. The computation of sublethal effects including developmental polymorphism as an uncontrolled source of variation could then lead to an increased variance and insignificant results.

As was predicted, B.t. concentration and the application methodology employed influence the production of extra-instars. However, in normal and double applications, few clues can clearly indicate before the treatment which larva will produce additional molts. Head width cannot be used precisely as an indicator (Schmidt & Lauer, 1977) and no dose-dependent response is observed. Fourth-instar weight can only explain 20% of the occurrence of the phenomenon. However, prediction is easy in late application because insect weight prior to the treatment is strongly inversely related to developmental polymorphism. The window to insert additional molts is not very wide in late application and a strong predisposition is needed to polymorph and counter the negative effect of B.t. on pupal weight. Thus, additional molts could help to explain why female larvae treated before the fifth instar in laboratory experiments produce normal sized pupae while those treated later have reduced pupal weight (Alford & Holmes, 1986; van Frankenhuysen & Nystrom, 1987; Ramachandran et al., 1993; Pedersen et al., 1997).

Our understanding of the mechanisms underlying observed sublethal effects is far from complete and the exact role of developmental polymorphism in populations of spruce budworm is poorly documented, even if this phenomenon has many implications on important biological factors such as development time and fecundity. The gene or genes which control molting seem to respond to an increase in the development time (Leonard, 1970a) so any other stressful event with adverse effects on insect development could also see its response altered by the production of additional molts. Therefore, overlooking developmental polymorphism in stress-inducing experiments could lead to biases and misinterpretation of results.

**Résumé**

L’induction de polymorphisme de développement par le Bacillus thuringiensis (Berliner) chez Choristoneura fumiferana (Clemens) (Lepidoptera: Tortricidae) fut démontrée en laboratoire. La concentration en Bacillus thuringiensis de la formulation induisit une réponse quadratique sur l’incidence du polymorphisme de développement. L’application de Bacillus thuringiensis au quatrième et cinquième stade induisit plus de polymorphisme de développement qu’une simple application au cinquième stade. Un plus petit poids larvaire avant l’exposition au Bacillus thuringiensis eu comme effet d’augmenter l’incidence du polymorphisme de développement. Les larves qui
polymorphèrent ont cependant affiché un poids pupal supérieur et un temps de développement larvaire prolongé. Ainsi, le polymorphisme de développement et l’ingestion de Bacillus thuringiensis ont un effet additif sur le temps de développement mais opposé sur le poids des chrysalides. Ces résultats suggèrent que le calcul d’effets sublétaux sur le poids pupal ou le temps de développement incluant le polymorphisme de développement comme variable non-controllée pourrait conduire à une mauvaise compréhension de la réponse de l’insecte au sein de n’importe quelle expérience avec stress induit.

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