Response of Spruce Budworm (Lepidoptera: Tortricidae) Infected with *Nosema fumiferanae* (Microsporida) to *Bacillus thuringiensis* Treatments

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ABSTRACT Disease in spruce budworm, *Choristoneura fumiferana* (Clemens), caused by the microsporidian *Nosema fumiferanae* (Thomson), increased larval susceptibility to mortality by *Bacillus thuringiensis* (Berliner) treatments compared with larvae free of *N. fumiferanae* disease. The median lethal concentration (LC_{50}) of *B. thuringiensis*, as determined by the diet incorporation bioassay method, was significantly lower for larvae infected transovarially with *N. fumiferanae*, but the similar slope obtained for initially healthy larvae indicated an independent and additive interaction. The median lethal time (LT_{50}) for *B. thuringiensis* was shortest for the group diseased with *N. fumiferanae*. Results from separate and sequential oral treatments (horizontal transmission) of both microorganisms at fixed physiological stages support the findings from the transovarial studies. It also was determined that *N. fumiferanae*-free larvae surviving *B. thuringiensis* treatments were more susceptible to mortality from subsequent inoculations with *N. fumiferanae* than were larvae not previously exposed to *B. thuringiensis*.

KEY WORDS Insecta, *Choristoneura fumiferana, Nosema fumiferanae, Bacillus thuringiensis*

FORMULATIONS OF *Bacillus thuringiensis* (Berliner) var. *kurstaki* HD-1 isolate (*B.t.*) have recently replaced synthetic insecticides as the principal management tool used to suppress populations of spruce budworm, *Choristoneura fumiferana* (Clemens) in most outbreak areas. *B. thuringiensis*'s mode of action is primarily toxic, because individuals that survive exposure do not sustain infection.

Nosema fumiferanae (Thomson), an intracellular, microsporidian pathogen of spruce budworm, is capable of invading all tissues, but it is only weakly virulent. Vertical (transovarial) and horizontal (oral) transmission lead to a density-dependent increase of this pathogen over the duration of an outbreak (Thomson 1958, Wilson 1977a, Burke 1980). Intensity of the disease expression depends on the initial dose, age at inoculation, temperature, nutrition, and other factors that affect developmental rate (Bauer & Nordin 1988a,b). Spruce budworms infected with *N. fumiferanae* experience dose-dependent mortality, and those surviving take longer to complete larval development, are smaller and less fecund, and live shorter adult lives (Bauer & Nordin 1988a, 1989).

It is probable that the outcome of toxin-based suppression efforts is substantially influenced by chronic entomopathogenic infections within the pest population. The interaction may be independent and additive, antagonistic, or synergistic (Krieg 1971, Jaques & Morris 1981). In spruce budworm outbreaks where *B. thuringiensis* is likely to be sprayed, the incidence of *N. fumiferanae* infection in the overwintering population may be as high as 81.3% (Thomson 1960). Because ingestion of sufficient amounts of *B. thuringiensis* is necessary to cause mortality, factors that reduce consumption of *B. thuringiensis*-treated foliage could lessen toxicity of *B. thuringiensis*. However, survival at a dose of *B. thuringiensis* should be dependent on the health (vigor) of the larvae at the time of exposure. Smirnoff (1963) suggested that microsporidiosis in budworm populations may be an important factor in assessing the efficacy of *B. thuringiensis*. Additionally, horizontal transmission of *N. fumiferanae* becomes increasingly likely where spruce budworm is epidemic. This adds to the spore load of a population that is already infected (Wilson 1977b). Individuals recovering from a sublethal dose of *B. thuringiensis* may experience reduced tolerance for *N. fumiferanae*, leading to mortality or elevated sublethal effects. A better understanding of these interactions may ultimately increase the effectiveness of chemical and microbial control of spruce budworm populations. Our objectives were to determine the responses of spruce budworm infected horizontally or vertically with *N. fumiferanae* to oral treatments of *B. thuringiensis* and to determine the effect of *B. thuringiensis* exposure on larval susceptibility to *N. fumiferanae*.

Materials and Methods

Pathogens. *Nosema fumiferanae* was cultured in spruce budworm larvae to produce fresh spores

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(Wilson 1976). The triangulation method (Cole 1970) provided a pure spore suspension. Spore concentration in the stock solution was obtained using a Petroff-Hausser counting chamber (Improved Neubauer, C.A. Hausser & Sons, Philadelphia), and dosages were prepared using serial dilutions made with sterile distilled water. *B. thuringiensis* var. *kurstaki* HD-1 was formulated as Dipel 4 L (Abbott Laboratories, North Chicago, Ill.) wettable powder with a potency of 16,000 IU/mg.

Insects. Laboratory colonies of diapausing and nondiapausing spruce budworm larvae were used for the horizontal and vertical transmission studies, respectively. Larvae were reared at a density of eight larvae per 30-ml clear polystyrene cup on standard spruce budworm diet (McMorran 1965) without the antibiotic aureomycin at 20 ± 1.5°C and a 16:8 (L:D) photoperiod. Following experimental treatments, larvae were reared individually. Pupae and adults were maintained at 22 ± 2°C. Diapausing larvae were reared from second instars on diet following emergence from hibernacula. Maintenance of nondiapausing spruce budworm was similar, except that first instars were transferred directly to diet after egg hatch. Before experiments were begun, larvae from each colony were sampled and examined microscopically to verify the absence of microsporidia, nuclear polyhedrosis virus, and cytoplasmic polyhedrosis virus.

Bioassays. All bioassays were performed within the first 24 h of the larval stadia to minimize developmentally induced variability (Bauer 1987). The *N. fumiferanae* bioassay procedure for fourth and fifth instars was modified from that described by Nordin (1976). To accommodate these smaller and more active larvae, a 3-mm inner diameter glass assay tube was fitted with a rubber serum bottle stopper at one end. Standard spruce budworm diet, minus antimicrobial agents, was poured in a thin layer (5 mm deep) into plastic trays. By pressing an assay tube into the surface, an area of diet was delineated. Spore doses were applied to this area in a 2-μl droplet of suspension and allowed to air dry in a sterile hood. Sterile distilled water was used for controls. During the inoculation period, larvae were constrained within the assay tube on the contaminated diet and maintained at 22 ± 2°C with constant light. Only those larvae that consumed the entire dose within a 24-h period were included in the assays.

A cohort of larvae, infected transovarially with *N. fumiferanae*, was obtained in the F_1 generation from the nondiapausing colony. The bioassay procedure described above was used to inoculate 120 female larvae with a sublethal spore dosage (1×10^4 spores/fifth instar) (Bauer & Nordin 1988a). Following dose consumption, these females were reared individually and after adult eclosion they were mated with healthy males. The F_1 progeny were sampled and evaluated microscopically for the presence of *N. fumiferanae* spores. An infection rate of 100% was determined. A cohort of uninfected females was reared simultaneously, and their F_1 progeny were used as uninfected controls. A sample of uninfected F_1 progeny was also examined microscopically and no *N. fumiferanae* spores were detected. Cohorts of uninfected and transovarially infected F_1 progeny were then reared for determinations of median lethal concentrations of *B. thuringiensis* (LC_{50}'s).

The *B. thuringiensis* bioassay used to determine LC_{50} was modified from Dulmage et al. (1976). Dipel serial dilutions (0–500 IU *B.t.*/ml diet) were incorporated into spruce budworm diet without the antimicrobial agents formalin, methyl paraben, and aureomycin. Larvae were exposed to the contaminated diets for 24 h at a density of five larvae per 30-ml cup.

Experiment 1: Horizontal *N. fumiferanae* Infection. The interaction of *B. thuringiensis* with the horizontal infection process of *N. fumiferanae* was investigated by sequential exposure in the fourth instar to one agent, followed by the other agent at fifth. The applied dosages were the empirically determined fourth-instar *N. fumiferanae* LD_{50} (2×10^4 spores/larva) (Bauer & Nordin 1988a) and *B. thuringiensis* LC_{50} (400 IU *B. thuringiensis*/ml diet) (Bauer 1987). Following both pathogen treatments, larval mortality (during and after fifth stadium) was recorded. Sublethal effects measured for survivors included pupal weight (24 h after pupation) and adult longevity. Adults were maintained individually in 30-ml cups at 22 ± 2°C. This experiment was duplicated with 20 and 24 larvae per treatment. The results from these duplicate assays did not differ significantly ($P \leq 0.05$) and were pooled within each treatment.

Experiment 2: Vertical *N. fumiferanae* Infection. *B. thuringiensis* interaction with the vertical (transovarial) infection process of *N. fumiferanae* was investigated by determining changes in the *B. thuringiensis* LC_{50} and LT_{50} in the fifth instars between larvae infected or uninfected with *N. fumiferanae*. Forty larvae were used for each treatment. Sublethal and lethal response variables were recorded. All experimental insects in the *N. fumiferanae* colony were examined microscopically after death for the presence of *N. fumiferanae* spores. Initial larval fresh weight was measured for a subsample of each cohort before exposure to *B. thuringiensis* to determine if mortality response was a function of weight.

Statistics. The χ^2 procedure was used to compare differences in percentage of mortality during and after the fifth stadium for horizontal treatments. Sublethal responses to pathogen treatments were analyzed using analysis of variance (ANOVA) procedures and the significance of paired multiple comparisons was based on Duncan's multiple range test. Maximum likelihood estimates of median lethal doses of *B. thuringiensis* and times for the vertical *N. fumiferanae* treatments were calculat-

Table 1. Lethal and sublethal responses ($\bar{x} \pm SE$) of spruce budworm larvae following sequential pathogen treatments with *N. fumiferanae* at fourth stadium and *B. thuringiensis* at fifth stadium

Response variables	Treatments (IV/V)				
	Controls			Sequential	Statistics
	Uninfected	0/*B.t.*	*N.f.*/0	*N.f./B.t.*	*F* (*P*)
	Lethal response variables				
Sexes pooled					
% Mortality[a]	2.5	39.5*	55.3§	79.2*§	—
n	80	43	38	48	—
	Sublethal response variables[b]				
Females					
Pupal fresh weight, mg	114.4 ± 4.2a	97.2 ± 8.5a	68.9 ± 7.0b	61.6 ± 7.2b	17.88 (0.000)
Adult longevity, days	7.9 ± 0.3a	7.3 ± 0.7a	4.3 ± 0.3b	3.2 ± 0.7b	20.28 (0.000)
n	33	10	14	10	—
Males					
Pupal fresh weight, mg	77.9 ± 1.7a	70.2 ± 3.7ab	65.3 ± 8.8b	45.9 ± 4.1c	18.03 (0.000)
Adult longevity, days	6.0 ± 0.2a	5.7 ± 0.6a	2.5 ± 0.4b	2.6 ± 0.5b	19.00 (0.000)
n	47	16	6	12	—

[a] Percentages are based on mortality occurring during or after the fifth stadium. Values with a symbol in common differ significantly at the $P \leq 0.05$ level (*, $\chi^2 = 14.7$; §, $\chi^2 = 5.6$).

[b] Within each sex and response variable, means followed by the same letters are not significantly different at the $P \leq 0.05$ level, Duncan's multiple range test.

ed with probit analysis (Finney 1971). The SAS system was used for all statistical analyses (SAS Institute 1982).

Results

Experiment 1: Horizontal *N. fumiferanae* Infection. Insects sequentially inoculated with *N. fumiferanae* at fourth stadium followed by *B. thuringiensis* at fifth (*N.f./B.t.*) experienced 1.4 times the mortality ($P \geq 0.05$) of the *N. fumiferanae* control (*N.f.*/0) and 2 times the mortality ($P \geq 0.05$) of the *B. thuringiensis* control (0/*B.t.*) (Table 1). The sublethal responses expressed by individuals that survived this sequential treatment were most similar to those expressed by larvae infected with *N. fumiferanae* alone (Table 1). These responses included reduced pupal weight and adult longevity in both sexes.

Larvae that survived *B. thuringiensis* treatments as fourth instars and were inoculated at the fifth stadium with *N. fumiferanae* (*B.t./N.f.*) had 3 times the mortality of the *N. fumiferanae*-infected larvae that were not previously exposed to *B. thu-*

Table 2. Lethal and sublethal responses ($\bar{x} \pm SE$) of spruce budworm larvae following sequential pathogen treatments with *B. thuringiensis* at fourth stadium and *N. fumiferanae* at fifth stadium

Response variables	Treatments (IV/V)				
	Controls			Sequential	Statistics
	Uninfected	*B.t.*/0	0/*N.f.*	*B.t./N.f.*	*F* (*P*)
	Lethal response variables				
Sexes pooled					
% Mortality[a]	2.5	13.6§	11.9*	36.0*§	—
n	80	44	42	28	—
	Sublethal response variables[b]				
Females					
Pupal fresh weight, mg	114.4 ± 4.2a	98.8 ± 8.0a	102.2 ± 4.0a	87.8 ± 4.2a	2.31 (0.020)
Adult longevity, days	7.9 ± 0.3a	6.3 ± 0.3bc	7.6 ± 0.4ab	5.8 ± 0.7c	4.65 (0.000)
n	33	10	24	8	—
Males					
Pupal fresh weight, mg	77.9 ± 1.7a	70.4 ± 5.7a	69.0 ± 3.1a	58.9 ± 3.6b	8.57 (0.000)
Adult longevity, days	6.0 ± 0.2a	5.2 ± 0.6a	5.8 ± 0.4a	3.9 ± 0.3b	4.65 (0.000)
n	47	11	13	10	—

[a] Percentages are based on mortality occurring during or after the fifth stadium. Values with a symbol in common differ significantly at the $P \leq 0.05$ level (*, $\chi^2 = 5.7$, § $\chi^2 = 4.8$).

[b] Within each sex and response variable, means followed by the same letters are not significantly different at the $P \leq 0.05$ level, Duncan's multiple range test.

Table 3. Susceptibility of fifth-stadium spruce budworm, *Nosema*-free or transovarially infected with *N. fumiferanae*, to *B. thuringiensis* treatments

Treatment	LC_{50}[a]	95% Fiducial limits	Slope ± SE	Initial larval fresh weight (mg fw)[b]	LC_{50}/mg fw[b]
Nosema-free control	1,641.43	681.52–770.00 × 10^5	1.00 ± 0.44	10.04 ± 0.47	162.84
Nosema-infected	78.25	0.31–148.36	0.88 ± 0.37	5.48 ± 0.21	14.30

[a] LC_{50} = IU *B.t.*/ml diet, 24-h exposure period. Calculations were based on six Dipel concentrations with 40 larvae/concentration.
[b] fw, fresh weight.

ringiensis (0/*N.f.*) (Table 2), and 2.7 times the mortality of the controls treated with *B. thuringiensis* in the fourth stadium (*B.t.*/0). Individuals that survived this sequential treatment showed significant reduction in adult longevity compared with uninfected controls. Male pupal weight also was reduced significantly by this sequential treatment compared with all other treatments.

Transovarial *N. fumiferanae* Infection. The median lethal concentration (LC_{50}) for *B. thuringiensis* in spruce budworm, transovarially infected with *N. fumiferanae*, was >20 times lower than the LC_{50} for larvae uninfected with *N. fumiferanae* (Table 3). Despite the wide fiducial limits typical for *B. thuringiensis* assays, this difference is significant. The slope for each regression equation was similar for both populations. When the LC_{50} was corrected by initial larval weight, the value was 11 times lower in infected larvae.

The median lethal time (LT_{50}) for *B. thuringiensis* was 1.4 d shorter for insects infected with *N. fumiferanae* than for those not infected with *N. fumiferanae* (Table 4). This was 5 d shorter than the LT_{50} for insects that died of the *N. fumiferanae* infection alone.

Discussion

Field applications of *B. thuringiensis* against spruce budworm are currently timed to treat young budworms (third and fourth instars) (Dimond 1985). At these early stages, individuals infected with *N. fumiferanae* become infected primarily by the transovarial route (Thomson 1958). Therefore, experiments that use populations that are transovarially infected with *N. fumiferanae* more closely simulate field conditions. Similar slopes for the maximum-likelihood estimates of median lethal concentrations (LC_{50}) indicated that the *B. thuringiensis* mode of action was not altered by the presence of congenital *N. fumiferanae* infections. The pathogen interaction, therefore, can be considered as independent and additive. Additive effects also were reported for *B. thuringiensis* and *Vairimorpha necatrix* (Kramer) in *Heliothis zea* (Boddie) (Fuxa 1979) and for *B. thuringiensis* and nuclear polyhedrosis virus in *Trichoplusia ni* (Hübner) (McVay et al. 1977).

The actual dose of *B. thuringiensis* provided to larvae by the diet incorporation method is proportional to the rate of consumption. Spruce budworm larvae infected with *N. fumiferanae* experience a significant reduction in consumption index (Bauer & Nordin 1988b). Despite this reduction in the effective dose of *B. thuringiensis*, insects infected with *N. fumiferanae* were more susceptible to the lethal and sublethal effects of *B. thuringiensis* than were healthy insects treated with it.

Operational trials with *B. thuringiensis* during the early 1970s, performed during increasing spruce budworm populations, were plagued with variable and unpredictable results. Within the last few years, improved efficacy was attributed primarily to concentrated formulations. Dimond & Morris (1984), however, note that the use of these improved formulations coincided with peak population levels. They suggested that reduced vigor of spruce budworm populations due to microsporidiosis and starvation stress may have contributed to the success of the *B. thuringiensis* treatments during this time. The results of this study suggest that populations infected with *N. fumiferanae* will experience more mortality than healthy ones.

Individuals surviving the *B. thuringiensis* treatments expressed different sublethal responses depending on the presence of preexisting *N. fumiferanae* infections. Female and male survivors of *B. thuringiensis* from the *Nosema*-infected colony

Table 4. Median lethal time (LT_{50}) for fifth-stadium spruce budworm, *Nosema*-free or transovarially infected with *N. fumiferanae*, after exposure to *B. thuringiensis*

Treatment	*n*	LT_{50}[a]	95% Fiducial limits	Slope ± SE
Nosema-infected + *B. thuringiensis*	124	2.0	1.8–2.2	2.13 ± 0.10
Nosema-free control + *B. thuringiensis*	14	3.4	2.5–4.2	4.57 ± 0.87
Nosema-infected control	43	7.0	6.2–7.7	6.49 ± 1.15

[a] LT_{50} reported in days. Calculations were based on five pooled Dipel concentrations.

tended to achieve larger pupal weights with increasing concentrations of *B. thuringiensis* than those individuals not exposed. This suggests that *B. thuringiensis* killed the smaller individuals that were most stressed by *N. fumiferanae*. The low energy reserves accumulated by individuals infected with *N. fumiferanae* may have reduced their ability to recover from anorexia induced by *B. thuringiensis*. In contrast, *Nosema*-free survivors of *B. thuringiensis* treatments tended to have lower pupal weights. This suggests that spruce budworm larvae do not fully overcome exposure to *B. thuringiensis*. This finding is supported by the greater susceptibility of insects surviving *B. thuringiensis* treatments to mortality after inoculation with *N. fumiferanae*.

The smaller and shorter-lived adults of both sexes that emerged in response to the individual and dual treatments are important expressions of reduced vigor. Because males actively seek out females, the outcome may be fewer and lower quality matings. Females will not only have fewer fertile eggs, but less time for oviposition. Reduced adult vigor also may reduce survival during long-range migrations, limiting spread to distant areas. The premature mortality of females infected with *N. fumiferanae* caused by *B. thuringiensis* treatments, however, may reduce transovarial transmission and natural spread of this pathogen within and among populations.

Based on the results of this study, spruce budworm mortality caused by *B. thuringiensis* treatments is enhanced if the population is infected with *N. fumiferanae*. Quantification of the incidence and level of *N. fumiferanae* in spruce budworm populations would contribute to a better understanding of budworm responses to *B. thuringiensis* treatments in the field and laboratory.

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