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Research article

The development of larval resistance to a nucleopolyhedrovirus is not accompanied by an increased virulence in the virus

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Abstract. Two laboratory experiments were conducted to examine the possible coevolution of cabbage loopers (*Trichoplusia ni*) and their S nucleopolyhedrovirus (TnSNPV). At the conclusion of Experiments 1 and 2, *T. ni* had respectively evolved 4.4× and 22× resistance to TnSNPV. The higher level of resistance achieved in Experiment 2 could be due to marginally stronger selection, possibly greater genetic variability in larval resistance to TnSNPV, or both. However, the evolution of resistance was not accompanied by an increased virulence of TnSNPV or a change in the restriction profile of the viral DNA when digested with *BamHI*, *EcoRI*, *HindIII*, *PstI*, *SalI*, *SstI* or *XhoI*. Little genetic variability for virulence in the initial TnSNPV stocks, low mutation rates and possibly weak selection on the virus are some factors that may have constrained the evolution of TnSNPV. We discuss our results in light of the geographic mosaic theory of coevolution and their implications for the use of TnSNPV as a biological control agent against *T. ni*.

Key words: biological control, caterpillar, coevolution, geographic mosaic theory, nucleopolyhedrovirus, resistance, *Trichoplusia ni*, virulence

Introduction

Pathogens are believed to play an important role in the evolution of sexual reproduction, in the maintenance of some genetic polymorphisms and in the population dynamics of their hosts (e.g., Dobson and Hudson, 1986; Hamilton *et al*., 1990; Antonovics and Thrall, 1994). This coupled with the need to manage plant and animal (including human) diseases (Ewald, 1994), and with the increasing use of pathogens as biocontrol agents (Entwistle, 1983; Payne, 1988) has spurred a great deal of theoretical interests in host–pathogen interactions. These include modelling the effect of horizontal transmission (May

and Anderson, 1983), vertical transmission (Yamamura, 1993; Lipstich *et al*., 1995a), intra-host competition among pathogens (e.g., Levin and Pimental, 1981; Bremmerman and Pickering, 1983; Nowak and May, 1994; May and Nowak, 1995; van Baalen and Sabelis, 1995), host population structure (Lipstich *et al*., 1995b) and the immune system of hosts on the virulence of pathogens (Anitia *et al*., 1994) (for a review of models on virulence see Bull, 1994; Ewald, 1994; Garnett and Anitia, 1994; Frank, 1996). Other models have concentrated on the host and have examined the effect of fitness costs on the evolution of resistance (e.g., Simms and Rausher, 1987; Boots and Haraguchi, 1999). Finally, some models such as the geographic mosaic theory of coevolution (Thompson, 1994, 1999) concurrently examine the evolution of the host and the pathogen. The geographic mosaic theory examines the effect of population structure, genetic landscape and spatially/temporally varying selection pressure on the outcome of coevolution.

Although it is possible to find descriptive evidence supporting each of the previous models, the predictions and the assumptions of these models have generally not been tested experimentally. For example, the well accepted assumption that interaction between hosts and pathogens leads to reciprocal genetic changes has only been examined in one system, *Escherichia coli* and T4 phage (Lenski and Levin, 1985). A thorough test of this assumption consists of conducting controlled, replicated laboratory experiments in which the host and pathogen are allowed to coevolve (Kraaijeveld *et al*., 1998).

Nucleopolyhedroviruses (NPVs) are naturally occurring, lethal entomopathogens that mainly infect lepidopteran larvae. Caterpillars become infected when they ingest foliage contaminated with occlusion bodies (OBs) which contain the viral particles of NPVs (Volkman, 1997). The disease appears to predominantly be transmitted at death when the cadavers lyse and release a large number of OBs in the environment. Hence, selection should favour NPV genotypes that are sufficiently virulent to kill host caterpillars. However, selection will concurrently favour larvae that are resistant to the disease. Several studies have found genetic variation for NPV virulence and for caterpillar resistance to NPVs (e.g., Reichelderfer and Benton, 1974; Shapiro and Robinson, 1991; Laitinen *et al*., 1996; Fuxa *et al*., 1998) suggesting that lepidopteran–NPV systems may have all the predispositions necessary for coevolution to occur. However, this possibility has not yet been explored experimentally.

In this study, cabbage loopers (*T. ni*: Lepidoptera: Noctuidae) and their NPV (*T. ni* S nucleopolyhedrovirus, TnSNPV) were used as a model system to experimentally examine host–pathogen coevolution. This system was chosen because (1) TnSNPV occurs naturally in field populations of *T. ni* and therefore is relevant to the biology of cabbage loopers (Lindgren and Greene, 1984), (2) Milks (1997) found that resistance to TnSNPV varied among *T. ni* popu-

lations, and (3) cabbage loopers can easily be cultured in the laboratory and they have a relatively short generation time (about 25 days at 26 °C). Thus, controlled, replicated coevolution experiments of several generations in duration can be conducted within a reasonable period of time.

Materials and methods

General protocol for rearing and infecting T. ni

Trichoplusia ni eggs were surface-sterilized using a 0.2% sodium hypochlorite solution as described in Milks (1997). After hatch, neonates were placed individually in 25-ml plastic cups containing high wheat germ diet (Jaques, 1967), and reared at 26 ± 1 °C with a 16:8 (L:D) photoperiod. At 168 h of age (fifth instar), caterpillars were transferred to a second cup containing only a plug of diet which had been inoculated with 10 μl of the appropriate dilution of TnSNPV or mock infected with distilled water. Larvae that consumed the entire plug within 24 h were returned to their original cup while those failing to do so were discarded (typically about 3%). Caterpillars were subsequently checked daily until adult emergence or death. Diagnosis of viral mortality was based on gross pathology (discoloration and liquefaction of the larva; Volkman, 1997). Typically, >95% of larvae inoculated with TnSNPV that died exhibited these symptoms. None of the mock infected *T. ni* that died during this study showed signs of nucleopolyhedrosis.

Selection experiment

Experiment 1

A TnSNPV sample derived from naturally infected cabbage loopers collected near Harrow, Ontario was obtained from Dr R.P. Jaques. The sample was passed once in our *T. ni*, and the OBs were purified from the cadavers as described in Potter *et al.* (1978). The final pellet which contained the OBs was resuspended in distilled water, quantified 10 times using a Neubauer hemacytometer and divided in two halves called TnSNPV1-G0 and TnSNPV2-G0 (viral stocks used to infect generation 0 caterpillars of selected lines 1 and 2, respectively; see below).

The following manipulations are summarized in Figure 1. Experiment 1 consisted of two control (lines C1 and C2) and two selected lines (lines S1 and S2) of 500 larvae. In an attempt to ensure some genetic variability for resistance to TnSNPV, each generation 0 (G0) line (line C1-G0, line C2-G0, line S1-G0 and line S2-G0) was established by taking 100 neonates from each of the

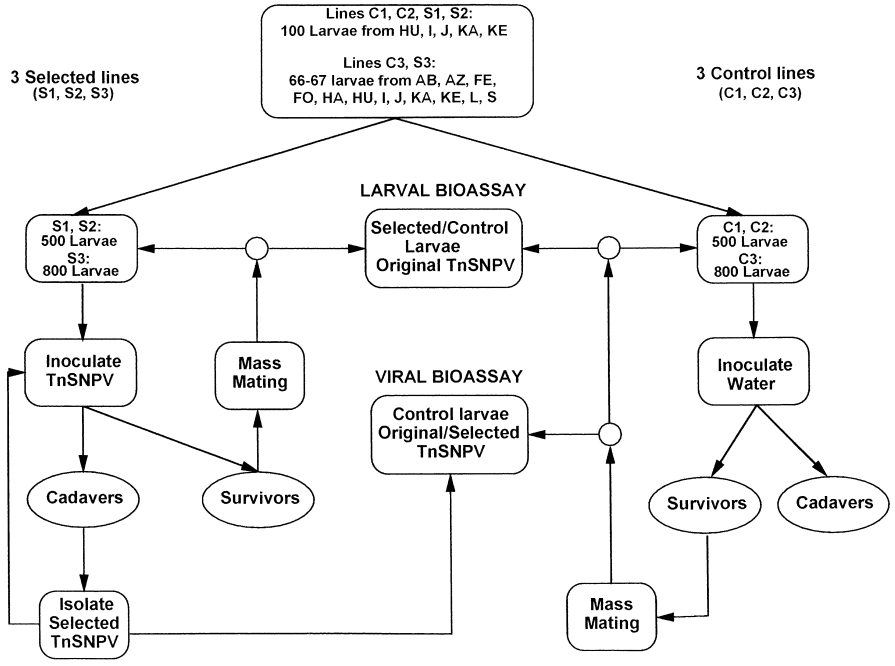

Figure 1. Flowchart summary of experimental protocol. Control and selected lines 1 and 2 (lines C1, C2, S1 and S2, respectively) were established by taking 100 larvae from each of the Hughes (HU), Ignoffo (I), Jaques (J), Kaupp (KA) and Keddie (KE) populations (n = 500 caterpillars per line); control and selected lines 3 (lines C3 and S3, respectively) were established by taking 66 or 67 larvae from each of the Abbotsford (AB), Arizona (AZ), Federici (FE), Fournier (FO), Hazelmere (HA), HU, I, J, KA, KE, Langely (L) and Sandoz (S) populations (n = 800 caterpillars per line). At 168 h of age (fifth instar), larvae of lines S1 and S2 were respectively infected with 11,000 OBs of TnSNPV1-G0 or TnSNPV2-G0; the caterpillars of line S3 were infected with 14,000 OBs of TnSNPV3-G0. Within a given selected line, the survivors were mass-mated while cadavers were collected, pooled and the virus purified from the corpses. This 'selected' TnSNPV (TnSNPV1-G1, TnSNPV2-G1 or TnSNPV3-G1) was used to inoculate the progeny (n = 500 for lines S1 and S2; n = 800 for line S3) of the survivors of that line. Cabbage loopers in the control lines were treated identically with the exception that they were mock infected with distilled water. For lines 1 and 2, this selection treatment was conducted for generations 0–8, discontinued between generations 9–14 and resumed from generations 15–19. For lines 3, selection was conducted for 26 generations without interruption. LARVAL BIOASSAY: The evolution of cabbage looper resistance to TnSNPV was monitored by inoculating control and selected larvae with the original stocks of TnSNPV. VIRAL BIOASSAY: The evolution of TnSNPV virulence was monitored by infecting control caterpillars with original or selected TnSNPV.

Hughes, Ignoffo, Jaques, Kaupp and Keddie populations (see Milks, 1997; only those populations were available at that time). At 168 h of age, the larvae of the selected lines (line S1-G0 and line S2-G0) were respectively infected with 11,000 OBs of TnSNPV1-G0 or TnSNPV2-G0, whereas those of the control lines (line C1-G0 and line C2-G0) were mock infected with distilled water. The cadavers of the selected lines were placed at 4 °C on the day they died. Once

the fate of all individuals was determined (dead or survived to adulthood), all the cadavers of a given selected line were pooled, the OBs were purified and quantified (as previously described), and the samples were designated TnSNPV1-G1 (OBs isolated from cadavers of line S1-G0) and TnSNPV2-G1 (OBs isolated from cadavers of line S2-G0). All of the surviving adults of a given line were placed in a mating cage (see Ignoffo, 1963), on the day they emerged, and 500 of their offspring were used to establish generation 1 of each respective line i.e. line C1-G1, line C2-G1, line S1-G1, line S2-G1. At 168 h of age, these larvae were treated with distilled water (line C1-G1 and line C2-G1) or with 11,000 OBs of TnSNPV1-G1 (line S1-G1) or TnSNPV2-G1 (line S2-G1). This selection treatment was carried out for eight generations, it was discontinued between G9–14 to examine if resistance to TnSNPV was stable, and resumed from G15–19. During G9–14, the caterpillars were reared individually but they were not infected or mock treated.

Experiment 2

In this experiment, we wanted to determine if increasing the amount of larval genetic variability for susceptibility to TnSNPV, the dose of virus fed to larvae and the duration of selection would lead to greater caterpillar resistance and increase the probability of TnSNPV evolving greater virulence.

At the conclusion of Experiment 1, a second TnSNPV sample was obtained from Dr Jaques. This sample had also been purified from naturally infected cabbage loopers, but different ones than those from which the first sample was derived. The sample was passaged in our *T. ni*, purified and quantified as previously described, and it was designated TnSNPV3-G0 (viral stocks used to infect generation 0 caterpillars of selected line 3; see below).

Experiment 2 consisted of one control (line C3) and one selected line (line S3). In an attempt to increase larval genetic variability, the number of individuals per line was increased to 800 from 500, and lines C3-G0, and S3-G0 were founded by taking 66 or 67 neonates from each of the Abbotsford, Arizona, Federici, Fournier, Hazelmere, Hughes, Ignoffo, Jaques, Kaupp, Keddie, Langley and Sandoz populations (see Milks, 1997). The same protocol as in Experiment 1 was used with the exception that the larvae of line S3-G0 were inoculated with 14,000 OBs of TnSNPV3-G0. The virus recovered from the cadavers of line S3-G0 was called TnSNPV3-G1 and was used to inoculate the progeny of the survivors of line S3-G0. This experiment lasted 26 generations without interruption (see Fig. 1 for a summary). In both Experiments 1 and 2, a first order autoregressive model (PROC AUTOREG; SAS Institute, 1990) was used to relate the % survival (arcsin square root transform) of control and selected *T. ni* to adulthood to the number of generations of selection. However, there was never any significant autocorrelation (data not shown) and so, the results from a simple linear correlation are presented.

Monitoring larval susceptibility to TnSNPV

The susceptibility of *T. ni* to TnSNPV was monitored by infecting control and selected lines with original (G0) TnSNPV (Fig. 1). Since all the caterpillars were treated with the same virus, differences in mortality among lines can be attributed to prior exposure to TnSNPV.

Experiment 1

For G1–6 and 12, single dose assays were conducted by infecting 50 larvae (168-h old) per line with TnSNPV-G0 (5500 OBs in G1; 11,000 OBs G2–6; 25,800 OBs in G12), and the mean % survival (arcsin square root transformed) of control and selected lines were compared using *t*-tests (2 df, one-tail probability). For G7–11 and 20 (offspring of the last generation of selection), multiple dose assays were done. Caterpillars of lines C1 and C2 were infected with 260, 1300, 2600, 5200 or 12,900 OBs of TnSNPV-G0 while those of lines S1 and S2 were infected with 1300, 2600, 5200, 12,900 or 25,800 OBs. Each dose was replicated 5–6 times and there was 7–10 larvae per replicate. PROC PROBIT (SAS Institute, 1990) was used to calculate the LD_{50} with 95% confidence intervals [CI] of each line, and the mean LD_{50} of lines of C1 and C2 was compared to that of lines S1 and S2 by *t*-test (2 df, one-tail probability). Furthermore, at G20, the slope and intercept of the dosage-mortality curve for the pooled control lines were compared to that for the pooled selected lines as described in Collett (1991). During each of the previous assays (single and multiple doses), cohorts of 25–50 larvae per line were mock infected with distilled water. There were no bioassays conducted between G13–19.

Experiment 2

For G1–3, 30 larvae of lines C3 and S3 were treated with 7,500 OBs TnSNPV3-G0 and a chi-square test corrected for continuity was used to compare the survival of control and selected *T. ni*. For G4–22, 30 larvae (3 replicates of 10) of lines C3 and S3 were infected with 1,500, 7,500, 15,000 or 35,000 OBs of TnSNPV3-G0. For G22–77, 30 larvae (3 replicates of 10) of lines C3 and S3 were infected with 1,500, 7,500, 15,000 or 35,000 OBs of TnSNPV3-G0, and 30 larvae (3 replicates of 10) of line S3 were infected with 7,500, 15,000, 35,000, 45,000 or 90,000 OBs of TnSNPV3-G0. Cohorts of 20–25 larvae per line were mock treated with distilled water at each generation. The LD_{50} of lines C3 and S3 were calculated using PROC PROBIT (SAS Institute, 1990) and were judged to be significantly different when their 95% CI did not overlap (e.g., Tabashnik *et al.*, 1994). The slopes and intercepts of the dosage-mortality curves of lines C3 and S3 for G27 were also compared as described in Collett (1991). In both Experiments 1 and 2, the mortality of mock infected larvae was low (typically $<5\%$) and thus the data used to calculate LD_{50}s were not corrected for control mortality.

Monitoring the virulence of TnSNPV

The evolution of the virulence of TnSNPV was monitored by inoculating control larvae with TnSNPV from various generations of the selection experiments. Since the different viral isolates were fed to caterpillars of the same line, differences in survival can be attributed to changes in the virulence of TnSNPV.

Experiment 1

Fifth instar larvae from line C1-G20 were infected with 1000 or 2000 OBs of TnSNPV1 or TnSNPV2 from various generations (G0, 1, 2, 5, 7, 15, 16 and 19; $n = 50$ larvae for each possible combination of dose/generation/virus for a total of 1600 larvae). Twenty-five larvae were mock infected with distilled water. This assay was repeated with larvae from line C1-G21 but this time the caterpillars were only infected with 2000 OBs of TnSNPV1-G0, TnSNPV2-G0, TnSNPV1-G19, TnSNPV2-G19 or mock infected with distilled water.

Experiment 2

Twenty five larvae from line C3-G27 were infected with 3500, 7000, 14,000 or 21,000 OBs of TnSNPV3 from various generations (G0, 4, 5, 6, 8, 10, 13, 16, 19, 22 and 26). Because of the availability of larvae, the virulence of TnSNPV3-G2 was only assessed at three doses (7000, 14,000 and 21,000 OBs) while that of TnSNPV3-G24 was examined at two doses (14,000 and 21,000 OBs). Twenty-five larvae were mock infected with distilled water. For each dose, a first order autoregressive model (PROC AUTOREG; SAS Institute, 1990) was used to relate the virulence of a TnSNPV isolate (i.e. arcsin square root % of control larvae surviving to adulthood) to the number of generations of selection. However, the autocorrelation was never significant (data not shown) and so, the virulence of a TnSNPV isolate was related to the number of generations of selection using simple linear correlation.

Analysis of viral DNA using restriction enzymes

DNA of TnSNPV1-G0, TnSNPV1-G20, TnSNPV2-G20, TnSNPV3-G0 and TnSNPV3-G26 were isolated as described in Hostetter *et al.* (1990) and 2 ug were digested with *BamHI*, *EcoRI*, *HindIII*, *PstI*, *SalI*, *SstI* or *XhoI* in the appropriate buffer for 1 h. The restriction endonuclease fragments were separated by electrophoresis on 0.7% agarose gel at 35 V for 24 h.

Results

Survival of T. ni larvae during selection

When there was selection in Experiment 1 (G1–8 and G15–19) the mean survival of the control lines (lines C1 and C2) was, on average, 2.6 times greater than that of the selected ones (lines S1 and S2; Fig. 2(a)). The mean survival of the selected lines improve during that period ($r = 0.70$, $n = 14$, $p = 0.006$) but that of control lines did not ($r = -0.37$, $n = 14$, $p = 0.19$; Fig. 2(a)) When selection was discontinued (G9–14), the mean survival of control and selected lines were nearly identical (Fig. 2(a)).

The results of Experiment 2 were qualitatively the same. The survival of the control line (line C3) was, on average, 2.1 times better than that of the selected one (line S3; Fig. 2(b)). The survival of the selected line again improved during that period ($r = 0.72$, $n = 27$, $p < 0.001$) but that of the control line did not ($r = 0.10$, $n = 27$, $p = 0.63$; Fig. 2(b)).

Monitoring larval susceptibility to TnSNPV

Experiment 1

In the first generation, the assay sample of caterpillars from the control and selected lines did not differ significantly in their survival when challenged with 5500 OBs of TnSNPV1-G0 (mean ± SE survival of control lines: $35 \pm 1\%$; mean ± SE survival of selected lines $11 \pm 5\%$; $T = 1.73, p = 0.11$). During G2–6, the mean survival of caterpillars of the selected lines infected with 11,000 OBs of TnSNPV1-G0 was significantly higher than that of larvae from control lines at G2 and 5 (see Fig. 3(a)). The mean LD_{50} of the selected lines was significantly bigger (about 3×) than that of the control ones at G9–11. At G12, only 2% (SE = 1%) of *T. ni* from lines C1 and C2 survived a dose of 25,800 OBs compared to 27% (SE = 1%) from lines S1 and S2 ($T = -10.5, p = 0.005$). The slope and the *y*-intercept of the dosage-mortality curve for the control lines of G20 pooled differed from that for the selected lines (Fig. 4(a): slopes $\chi^2 = 7.55$, df = 1, $p < 0.01$: intercepts: $\chi^2 = 30.6$, df = 1, $p < 0.001$). Finally, the mean LD_{50} of selected lines for G20 was 4.4 times greater than that of the control ones (Fig. 3(b)).

Experiment 2

For G1–3, the survival of selected caterpillars inoculated with 7500 OBs of TnSNPV3-G0 (48, 17 and 20%, respectively) was greater than that of control larvae (36, 0 and 14%, respectively) but the difference was never significant (all Yates' $\chi^2 < 3.84$ and all $p > 0.05$). From G7 and on, the LD_{50} of the selected line was generally greater than that of the control line based on overlap of 95% CI (Fig. 5). The dosage mortality curves of lines C3 and S3 for G27 had the same

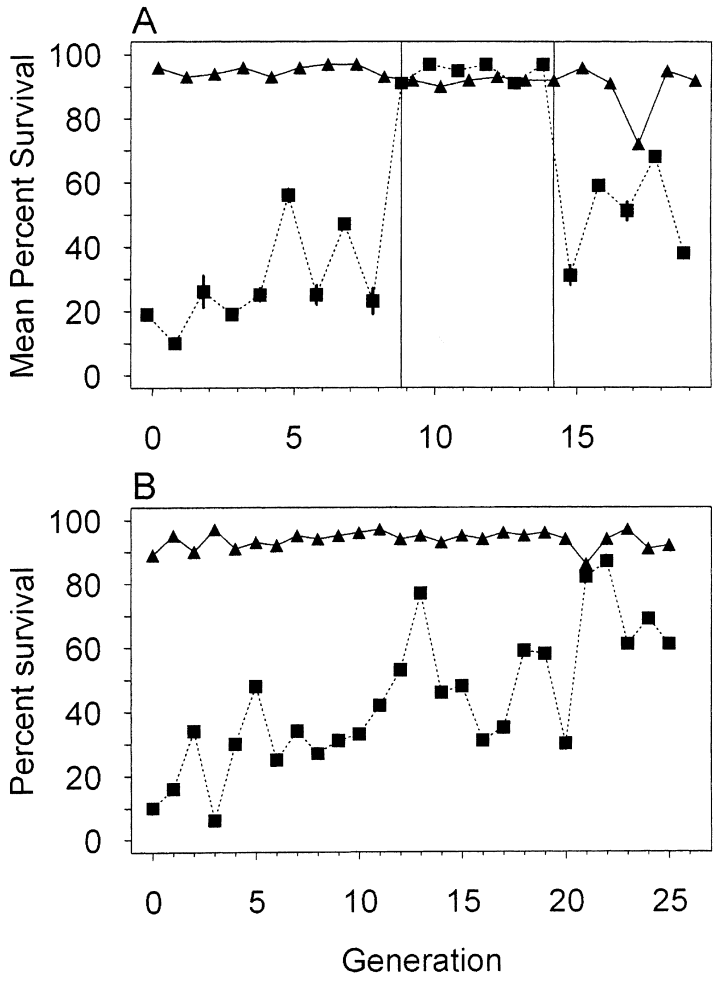

Figure 2. Percent survival of lines during selection. (a) Mean (±1 SE) percent survival of control (▲) and selected (■) lines 1 and 2. There was no selection between generations 9 and 14. The lower mean percent survival of the control lines at generation 17 was due to an outbreak of fungus on the diet. (b) Percent survival of control (▲) and selected (■) line 3.

slope (Fig. 4(b): $\chi^2 = 0.09$, df $= 1$, $p > 0.75$) but different intercepts (Fig. 4(b): $\chi^2 = 102$, df $= 1$, $p < 0.001$). The LD_{50} of the selected line at G27 (109,248, OBs) was 22 times greater than that of the control line (5059 OBs) (Fig. 5).

Monitoring the virulence of TnSNPV

Experiment 1

The survival of control larvae (line C1-G20) did not vary significantly with the generation from which the virus was isolated (1000 OBs: $r = 0.61, p = 0.11$; 2000

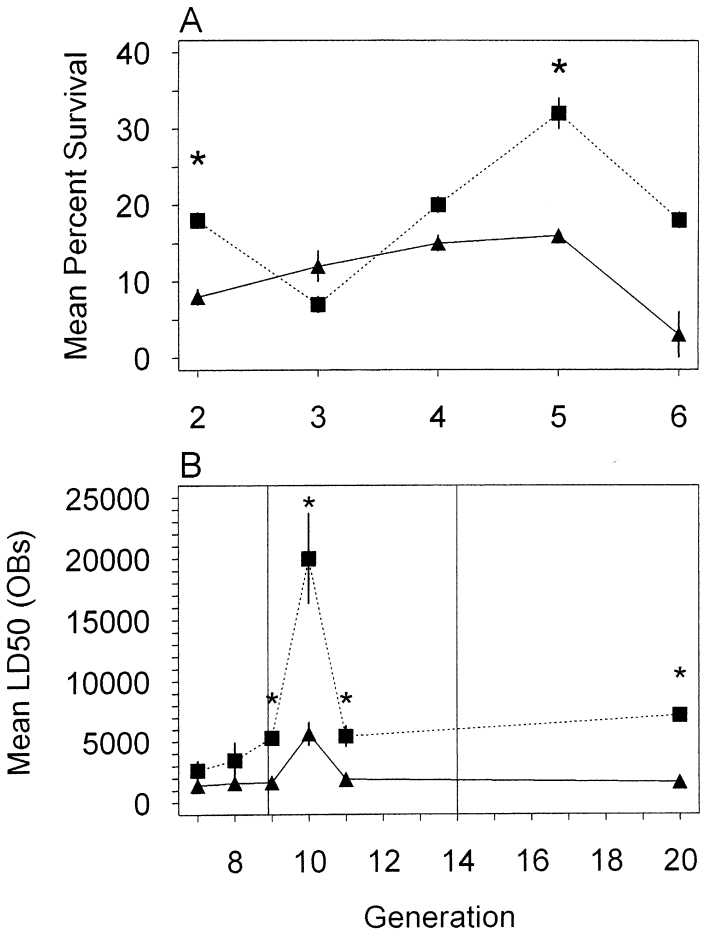

Figure 3. (a) Mean (±1 SE) percent survival of larvae from control (▲) and selected (■) lines 1 and 2 inoculated with 11,000 OBs of TnSNPV1-G0 for generations 2–6. (b) Mean (±1 SE) LD_{50} of control (▲) and selected (■) lines 1 and 2 for G7–11 and 20 infected with TnSNPV1-G0. * indicates that the means of the control and selected lines were significantly different by *t*-test, $p < 0.05$.

OBs: $r = 0.61, p = 0.11$, both $n = 8$; Fig. 6(a), (b)). The relationships were even weaker when the analyses were repeated without the viruses of G16, for which there was nearly no mortality (1000 OBs: $r = 0.51, p = 0.24$; 2,000 OBs: $r = 0.58$, $p = 0.18$, both $n = 7$). The mean survival of control larvae (line C1-G21) inoculated with 2000 OBs of original virus (mean ± SE: 53 ± 13%) did not differ from those treated with virus from G19 (mean ± SE: 64 ± 1%; $T = -0.92, p = 0.46$).

Experiment 2

The survival of control larvae (line C3-G27) inoculated with TnSNPV3 was not related to the generation from which the virus was isolated at all four doses

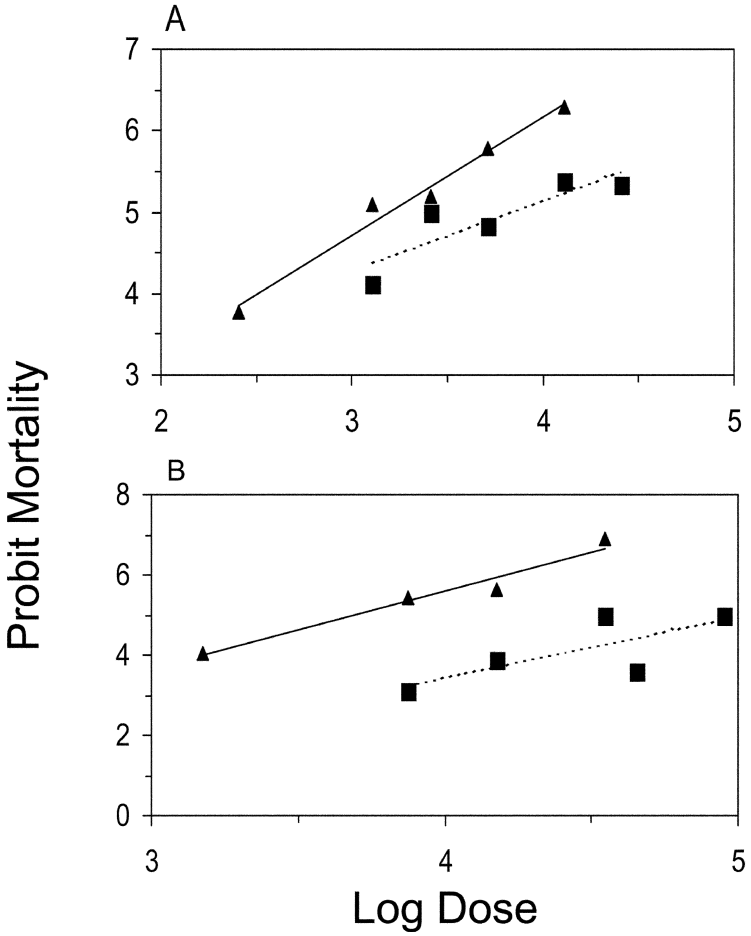

Figure 4. Dosage-mortality curve for control (lines C1 and C2 pooled; (▲)) and selected caterpillars (lines S1 and S2 pooled; (■)) at generation 20 infected with TnSNPV1-G0. (b) Dosage-mortality curve for *T. ni* caterpillars of control line 3 (▲) and selected line 3 (■) of generation 27 infected with TnSNPV3-G0.

tested (Fig. 7; 21,000 OBs; $r = -0.05$, $n = 13$, $p = 0.43$; 14,000 OBs: $r = -0.18$, $n = 13$, $p = 0.28$; 7000 OBs: $r = -0.17$, $n = 12$, $p = 0.30$; 3500 OBs: $r = -0.39$, $n = 11$, $p = 0.11$).

Analyses of viral DNA using restriction enzymes

The restriction endonuclease fragment profiles of the original TnSNPV isolates and those at the end of selection were identical when digested with *BamHI*, *EcoRI*, *HindIII*, *PstI*, *SalI*, *SstI*, and *XhoI* (see Fig. 8 as an example). The high

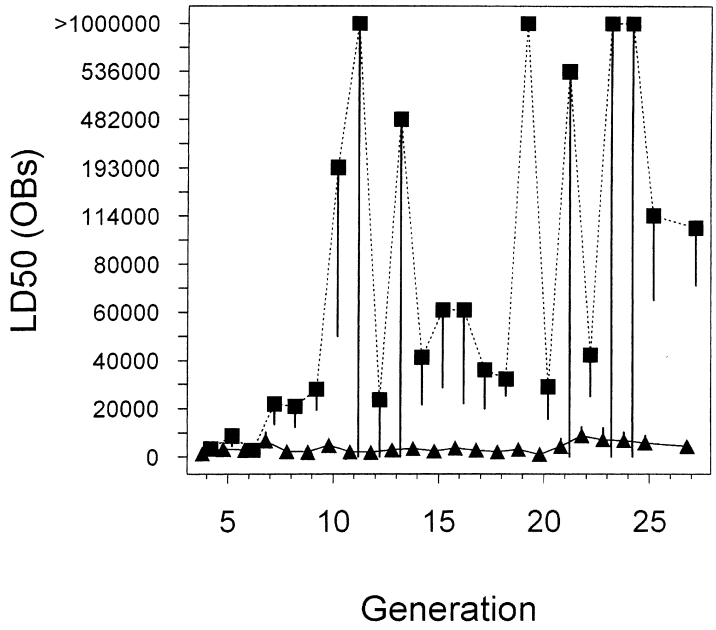

Figure 5. LD_{50} and 95% CI of control (▲) and selected (■) line 3 treated with TnSNPV3-G0. 95% CI could not be computed for the LD_{50} of line S3 at generations 11, 13, 16, 19 and 24 because PROC PROBIT failed to converge i.e. C = 0 (SAS Institute, 1990).

molecular weight fragment in the *EcoRI* digest of TnSNPV3-G27 was later showed to be a spurious band (data not shown).

Discussion

The present study is the first one to experimentally examine the coevolution of caterpillars and NPVs. The survival of *T. ni* inoculated with potentially co-evolving TnSNPV improved through time in both experiments (Fig. 2(a), (b)), due to selection for larvae resistant to the virus. At the end of Experiments 1 and 2, cabbage loopers had respectively evolved 4.4- and 22-fold resistance to the virus. Other studies have also successfully selected for resistance to NPVs or to their close relatives, the granuloviruses (GV). The LD_{50} of *Phthorimaea operculella* increased sixfold after selection with a GV for 10 generations (Briese and Mende, 1983). *Spodoptera frugiperda* evolved 4.5× resistance to an NPV in seven generations (Fuxa *et al.*, 1988). The LD_{50} of *Plodia interpunctella* was twice as high as that of control insects after being exposed to a GV for 2 years (Boots and Begon, 1993). *Anticarsia gemmatalis* developed >1000-fold resistance to an NPV within 15 generations of selection (Abot *et al.*, 1996). It is

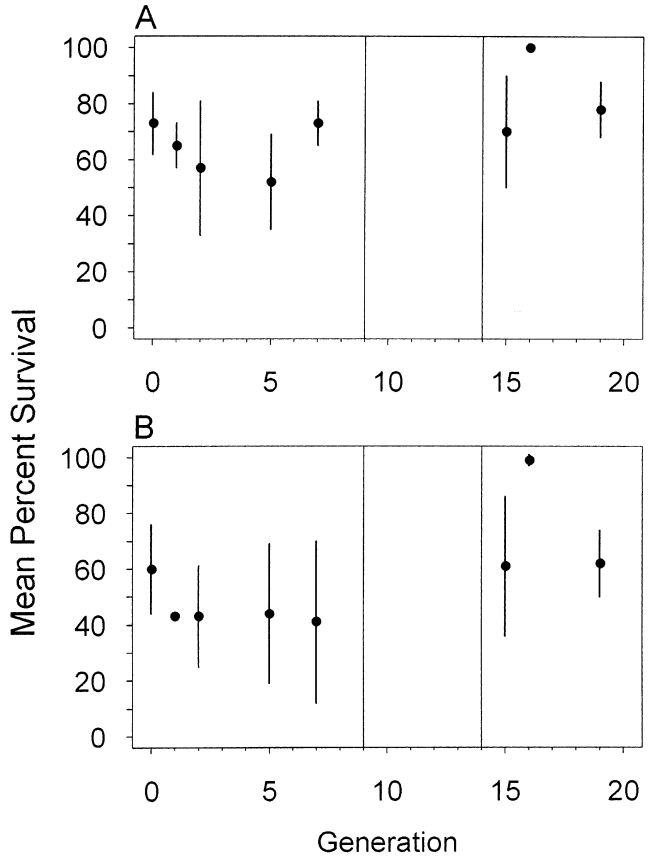

Figure 6. Mean (±1 SE) percent survival of larvae from control line 1 from G20 treated with (a) 1000 or (b) 2000 OBs of TnSNPV1 or TnSNPV2 from generations 0, 1, 2, 5, 7, 15, 16 or 19. There was no selection between generations 9 and 14. Note: The survival of mock infected caterpillars was 96% (24/25).

noteworthy that, in contrast to our study, the viruses were not allowed to evolve in any of those four selection experiments.

Three explanations may account for the greater resistance achieved in Experiment 2. First, Experiment 2 lasted longer (26 vs. 14 generations of selection in Experiment 1). However, the selected caterpillars of Experiment 2 (line S3) had still evolved greater resistance after 14 generations of selection (about 20×) than those of Experiment 1 (Fig. 5). Second, the larvae were selected with 14,000 OBs in Experiment 2 compared to only 11,000 OBs in Experiment 1. Third, the caterpillar lines used in Experiment 2 may have been more genetically variable and more likely to include a rare gene coding for resistance. In that experiment, the lines were established by taking individuals from 12 *T. ni* populations whereas only five populations were used to found the lines of

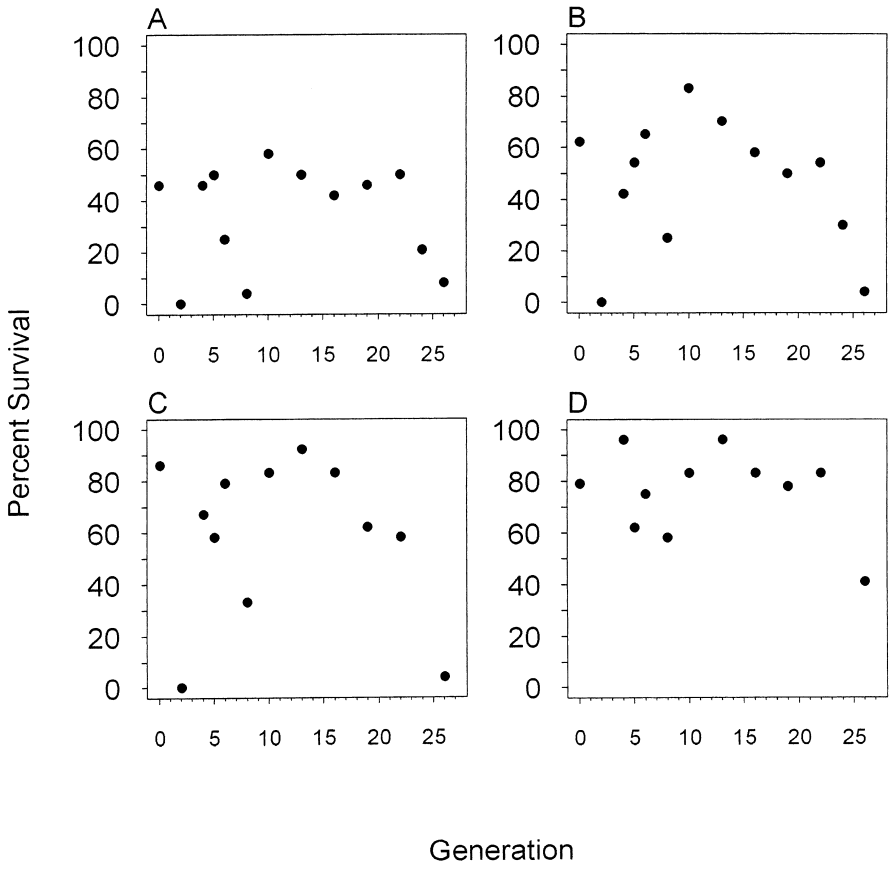

Figure 7. Percent survival of larvae from control line 3 from G27 infected with (a) 21,000, (b) 14,000, (c) 7000 or (d) 3500 OBs of TnSNPV3 from generations 0, 2 (only 21,000, 14,000 or 7000 OBs), 4, 5, 6, 8, 10, 13, 16, 19, 22, 24 (only 21,000 or 14,000 OBs) or 26. Note: The survival of mock infected infected caterpillars was 100% (25/25).

Experiment 1. Greater genetic variability may also explain why *A. gemmatalis* from Brazil (>1000×) evolved higher resistance to AgNPV (NPV of *A. gemmatalis*) than those from the United States (5×) (Abot *et al.*, 1996).

The resistance of cabbage loopers to TnSNPV appears to be stable. When selection was discontinued in Experiment 1 (G9–14), the resistance ratio (mean LD_{50} of selected/mean LD_{50} of control lines) only declined from 3.3 (G9) to 2.9 (G11). Also, the survival of selected larvae at generation 12 was still considerably higher than that of control caterpillars. In contrast, *S. frugiperda* and *A. gemmatalis* that were resistant to an NPV reverted to susceptibility in 1 and 3 generations respectively when selection was discontinued (Fuxa and Richter, 1989, 1998). It is believed that resistance was unstable in those two species because it entailed fitness costs (Fuxa and Richter, 1989, 1998). Resistant in-

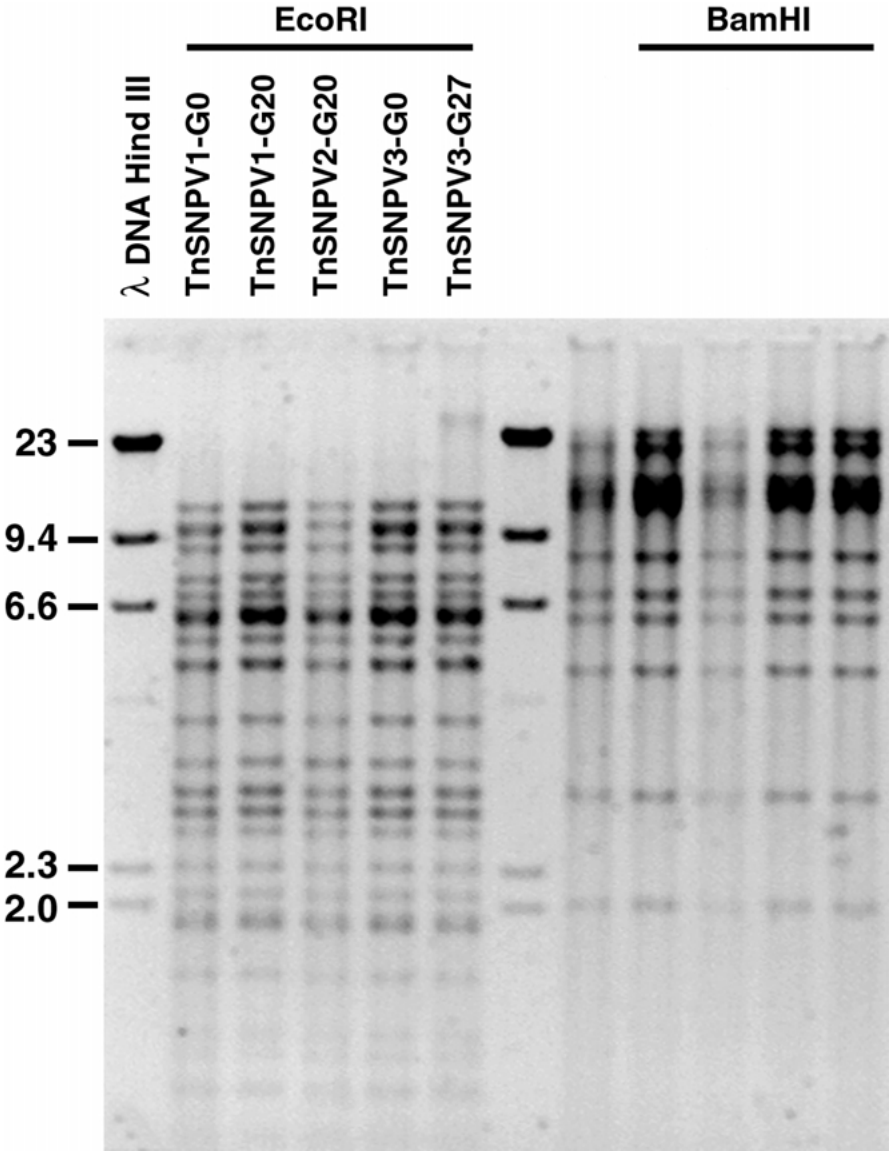

Figure 8. Restriction endonuclease profiles of the DNA of TnSNPV1-G0, TnSNPV1-G20, TnSNPV2-G20, TnSNPV3-G0, TnSNPV3-G26 digested with *EcoRI* or *BamHI* and electrophoresed on a 0.7% agarose gel at 35 V for 24 h. λ phage DNA *HindIII* restriction fragments were included as molecular weight markers. The size of bands is indicated in kilobase pairs.

dividuals had a slower development and lower reproductive success than susceptible ones when reared in the absence of their NPV, and may thus have been selected against when exposure to the virus stopped. Hence, *T. ni* resistance to

TnSNPV may be stable because it does not entail any costs (Milks and Myers, submitted MS). Alternatively, selection may have to be relaxed longer in *T. ni* than in *S. frugiperda* or *A. gemmatalis* for larvae to revert to susceptibility. Another explanation is that resistance to TnSNPV may involve an irreversible genetic change such as a deletion.

A number of physiological mechanisms have been suggested as possible factors determining resistance to NPVs. Fuxa and Richter (1990) showed that the mortality of susceptible and resistant *S. frugiperda* did not differ when virions were injected into the hemocoel and concluded that resistance was associated with the midgut. Resistance may be the result of a change in the pH of the midgut, increased activity of digestive proteases (e.g., Elam *et al.*, 1990), the presence of antiviral substances in the midgut fluid (e.g., Aizawa, 1962; Mukai *et al.*, 1969; Aratake and Ueno, 1973; Uchida *et al.*, 1984; Funakoshi and Aizawa, 1989), reduced pore size or increased integrity of the perithrophic membrane (see Derksen and Granados, 1988), absence of cellular receptors that are possibly required for the entry of virions into midgut epithelial cells, or sloughing of infected midgut cells into the lumen of the midgut (Keddie *et al.*, 1989; Washburn *et al.*, 1995, 1998; Hoover *et al.*, 2000). However, recent findings suggest that the hemocoel may also play a role in resistance to TnSNPV. Washburn *et al.* (1996) showed that infected cells were encapsulated by hemocytes and destroyed (see also Begon *et al.*, 1993).

The evolution of resistance in *T. ni* was not accompanied by a statistically significant change in the virulence of TnSNPV in either experiment. The restriction profiles of the initial and final TnSNPV were identical when digested with seven restriction enzymes. The virulence of the MNPVs of *T. ni* and *Mamestra brassicae* did not change when passaged in cabbage loopers for 15 and 25 generations, respectively (Potter *et al.*, 1978; Croizier *et al.*, 1985). However, the larvae were not concurrently selected for resistance in those two studies and thus there was little selection on the virus. The *Oryctes* virus (formerly believed to be a relative of NPVs) has been released to control the coconut palm rhinoceros beetle (*Oryctes rhinoceros*) on many small atolls in the South Pacific (Marschall, 1970; Zelazny *et al.*, 1990). Although the beetles may have evolved resistance to the virus, Crawford and Zelazny (1990) observed only three genomic changes in the 4 years after the virus had been released (an estimated 54,000 cycles of replication). However, Crawford and Zelazny (1990) did not compare the virulence of those isolates.

Three reasons can be given to explain why TnSNPV did not evolve greater virulence. First, there may have been little variation in the original virus samples. Although TnSNPV1-G0 and TnSNPV3-G0 were initially propagated from two different, non-clonal samples of TnSNPV, their restriction profiles were identical with seven enzymes and there were no submolar bands suggesting that the samples may have been monomorphic. Second, low mu-

tation rates may have constrained the evolution of the virus. Resistance to TnSNPV may only involve the loss of a receptor for the virus and could occur via a point mutation, deletion or insertion (Lenski and Levin, 1985). Conversely, increased virulence may require the synthesis of an entirely novel protein and more extensive genetic modifications. That is, it may be simpler for caterpillars to lose a function than for the virus to gain one. Third, the level of resistance that cabbage loopers evolved may not have been a sufficiently strong selective pressure on the virus.

Under the conditions used in this study, cabbage loopers evolved resistance to TnSNPV but the virulence of the virus did not change. Nevertheless, TnSNPV remains an effective and very important pathogen of cabbage loopers in the wild (Lindgren and Greene, 1984) suggesting that it is capable of eluding host defences that also evolve. In nature, there is likely to be more genetic variation for resistance and virulence than in our laboratory study and thus the possibility of coevolution could be greater in the wild. Conversely, in nature, there are factors not included in our study that could adversely affect cabbage looper-TnSNPV coevolution. For example *T. ni* are exposed to a variety of pathogens in the wild (Lindgren and Greene, 1984), and a negative correlation between resistance to TnSNPV and any of these pathogens could constrain cabbage looper-TnSNPV coevolution. TnSNPV might also have alternate hosts in nature with some of them being more or less susceptible to the virus. Currently, the prevailing hypothesis used to describe the coevolution of hosts and pathogens in nature is the geographic mosaic theory (Thompson, 1994, 1999). This hypothesis proposes that differences in biotic/abiotic factors (e.g., genetic landscape) among communities will produce selection mosaics and favour different evolutionary trajectories in different locales. The geographic mosaic theory also proposes that coevolution will only occur in a subset of communities and that there will be a continuous geographic re-mixing resulting from gene flow, drift and local extinction. Such selection mosaics have been found in at least two host-parasite systems including *Drosophila* and its parasitoid (Kraaijeveld and Godfray, 1999) and in a sterilizing trematode and its snail host (Lively, 1999). Cabbage loopers and TnSNPV may also fit this hypothesis and thus it may be necessary to study the interaction over a wide geographic scale for a prolonged period of time in order to obtain a thorough understanding of their coevolution (see Thompson, 1999).

Our results also have implications for the potential future use of TnSNPV as a biological control agent against *T. ni* (see Jaques, 1977; Entwistle, 1983). To reduce the risk of caterpillars evolving resistance we suggest using a spray consisting of several TnSNPV genotypes of different virulence and that the virus be used in rotation with other pathogens, preferably with ones that have a different mode of action. Fuxa and Richter (1990) showed that *S. frugiperda* that were resistant to an NPV were more susceptible to methyl parathion, and equally

susceptible to *Bacillus thuringiensis*. Hence, using TnSNPV in rotation with methyl parathion and/or *B. thuringiensis* might provide adequate control of cabbage loopers while minimizing the risk of resistance evolving. Finally, if part of the population were not sprayed, then the immigration of susceptible moths from the untreated plot(s) to the sprayed area might dilute any resistance that evolved.

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