THE OCCURRENCE AND PERSISTENCE OF NUCLEAR POLYHEDROSIS VIRUS IN FLUCTUATING POPULATIONS OF TENT CATERPILLARS

by

BARBARA KUKAN

B. Sc. (Biochemistry) University of Toronto
M. Sc. (Plant Science) University of British Columbia

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Department of

The University of British Columbia
Vancouver, Canada

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ABSTRACT

To determine the dynamics of infection by nuclear polyhedrosis virus (NPV) in western and forest tent caterpillars, *Malacosoma californicum pluviale* (Dyar) and *Malacosoma disstria* (Hbn), I developed and tested a DNA dot-blot hybridization assay for this system. The level of infection detected by the dot-blot assay was similar to that based on rearing field-collected tent caterpillars in the laboratory until viral death. Results based on testing crude caterpillar homogenates were the same as those in which DNA was extracted with phenol-chloroform. Experimental infection of second and third instar caterpillars showed that viral infection could be detected in 46% of the caterpillars two days after infection and 80 to 90% by four to five days post-infection.

I monitored the levels of viral infection in six populations of forest tent caterpillars in Prince George, British Columbia. I found that high host density populations tended to have higher levels of virus than low density populations, but exceptions occurred. Virus persisted in populations through low density and the population with the highest level of virus in the peak density year collapsed following decline.
Abstract

A temporal series of samples of caterpillars and tent material from four populations of western tent caterpillars were analyzed. Levels of infection were related to the density of populations but not to changes in density. Infection was not related to the size of the egg mass of the caterpillar family, but in one population tents were larger for families with some individuals positive for virus than for uninfected families. Experimental contamination of tent material showed that active virus persists and tent material could provide a source of infection to the next generation.

Pupae and moths from field populations of forest tent caterpillars and experimentally infected western tent caterpillars were rarely (pupae) or never (moths) positive for virus based on the sensitive polymerase chain reaction (PCR) detection assay. Approximately 2% of caterpillars reared from surface decontaminated egg masses were positive for virus based on DNA hybridization. A review of the literature revealed that low levels of viral infection are common among pupae of forest Lepidoptera and in caterpillars reared from surface decontaminated eggs. This could contribute to the persistence of virus in low density populations.
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LIST OF ABBREVIATIONS

AcNPV = *Autographa californica* nuclear polyhedrosis virus
ANOVA = analysis of variance
bp = base pairs
$\chi^2$ = chi-square
DNA = deoxyribonucleic acid
dNTP = deoxynucleoside triphosphate
ECL = enhanced chemiluminescence
EDTA = ethylenediaminetetraacetic acid
ELISA = enzyme linked immunosorbent assay
EGT = ecdysteroid UDP-glycosyl transferase
egt = ecdysteroid UDP-glycosyl transferase gene
FIDS = Forest Insect and Disease Surveys
G + C = guanine and cytosine
kb = kilobase pair
LB = Luria broth
Ld = *Lymantria dispar* gypsy moth
LdNPV = *Lymantria dispar* nuclear polyhedrosis virus
Md = *Malacosoma disstria* forest tent caterpillar
Mp = *Malacosoma californicum pluviale* western tent caterpillar
MdNPV = *Malacosoma disstria* nuclear polyhedrosis virus
MpNPV = *Malacosoma californicum pluviale* nuclear polyhedrosis virus

NPV = nuclear polyhedrosis virus

PCR = polymerase chain reaction

*pi* = post-infection

PIBs = polyhedral inclusion bodies

rpm = revolutions per minute

SDS = sodium dodecyl sulphate

SE = standard error

SSC = sodium chloride, sodium citrate

TE = 10mM Tris-HCl, pH 8.0, 1mM EDTA

Tris = tris(hydroxymethyl)aminomethane

U = unit

V = volts
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CHAPTER 1
GENERAL INTRODUCTION

1.1 NUCLEAR POLYHEDROSIS VIRUS

Nuclear polyhedrosis viruses (NPV) (Family: Baculoviridae) are occluded DNA viruses that can cause fatal disease in insects, mostly Lepidoptera. Because of their stability, limited host range, lack of mammalian toxicity, minimum impact on the environment, and pathogenicity to Lepidopterans and sawflies that are forest and agricultural pests, these viruses have been recognized as potential insect control agents.

Baculoviruses are characterized by a large rod-shaped, enveloped nucleocapsid, and a double-stranded supercoiled DNA genome of 88 to 160 kilobase pairs (Rohrmann 1986). NPVs are unusual among viruses in that they produce two phenotypes, one that transmits infection among hosts and one that spreads infection within the host. The first phenotype consists of one or more enveloped virions in a crystalline matrix composed primarily of the protein polyhedrin. This protein is soluble in alkaline solutions such as those in the midgut of caterpillars and infectious virions are released after ingestion by a caterpillar. These infectious virions enter midgut columnar epithelial cells by membrane
General Introduction

fusion (Engelhard et al. 1994, Adams and McClintock 1991). Infected epithelial cells produce a second phenotype, budded or extracellular virus, which frequently consists of a single nucleocapsid in an envelope derived from a viral-modified cellular plasma membrane acquired as virus buds out of a cell (Keddie et al. 1989). This phenotype systemically infects the host with the larval tracheal system providing a major conduit for viral spread (Engelhard et al. 1994).

1.2 HISTORY OF DETECTION METHODS

As early as 1808, the first scientific account of a disease caused by NPV was described as "jaundice" in silkworms, Bombyx mori, by Nysten (Bilimoria 1991). Formal demonstration of the association of polyhedral inclusion bodies (PIBs) with "jaundice" disease of silkworm was provided by Bolle in 1894-1898 (Entwistle and Evans 1985) and the first recorded recognition of a virus as a cause of insect disease was in 1909 when B. Wahl showed that an infectious entity of Nun moth, Lymantria monacha, could pass through an ultrafilter (Entwistle and Evans 1985).

NPV are routinely detected by rearing large numbers of caterpillars until death. Smears of dead caterpillars are examined microscopically with either stained or unstained preparations to confirm the presence of virus. Although
tissue smears are inexpensive and can be prepared quickly, positive identification of virus infection is limited to experienced researchers and is unreliable at low levels or early stages of infection (Kaupp and Ebling 1993). Also, microscopic diagnosis is tedious if large numbers of samples are to be examined (Kaupp et al. 1989).

Chemical techniques, mainly the enzyme-linked immunosorbent assay (ELISA), are more accurate than optical detection of virus but high background levels which interfere with spectrophotometric absorbance, and cross reactions to other species of NPVs are disadvantages.

Dot-blot hybridization assays have been developed to detect baculovirus infection in host larvae (Ward et al. 1987, Keating et al. 1989, Kaupp and Ebling 1993). In past surveys it has not been possible to assign a cause of death to as many as 30% of field-collected larvae by microscopic examination (Keating et al. 1989). While gross symptoms such as fragile cuticle and liquification of the body allow easy identification of individuals which die of virus, these symptoms are not always present in dead larvae. Researchers have found that using a highly specific DNA probe reduces much of the uncertainty and ambiguity of the microscopic diagnosis.
General Introduction

An even more sensitive technique is the polymerase chain reaction (PCR) which was developed by Saiki et al. (1988) and has been used on NPV of gypsy moth with a level of sensitivity as low as 5 viral genomes and DNA from 1 occlusion body equivalent (Burand et al. 1992).

1.3 ECOLOGY OF BACULOVIRUSES

Sensitive detection techniques are essential to investigations involving the ecology and epizootiology of a pathogen. With improved techniques in detecting and quantifying NPVs, progress can be made on the quantitative description of the ecology of viruses. Important ecological features to be examined include virus availability or infection levels, host density effects, transmission and persistence (Kalmakoff and Crawford 1982).

Virus availability and host density are important parameters affecting the ecology of the NPV. The amount of virus available for ingestion by larvae is quantitatively related to levels of infection in a host population (Young and Yearian 1988). Host density is important in determining the amount of inoculum maintained in a host population (Bilimoria 1991). Environmental breakdown of virus and limited dispersal avert a high level of infection if the host population is sparse. Higher host densities increase
the probability of virus-host interactions. The general consensus is that baculoviruses act in a delayed density dependent manner and induce maximum mortality some time after the host population has reached a peak (Bilimoria 1991, Anderson and May 1981, Berryman et al. 1987). Insect populations with one generation per year will have periods when no hosts are present and no inoculum will be produced and so the growth of infection will depend on the individual host generations. Few quantitative studies on how viral infection of the host changes as population densities fluctuate have been described.

Transmission is another important ecological feature of the host-pathogen interaction. Transmission is generally divided into horizontal (within or between generations) and vertical or parent to progeny transmission. Horizontal transmission of virus from one generation to the next is aided by the persistence of virus in the environment. It has been suggested that vertical transmission from moths to caterpillars in the field occurs from external contamination of newly emerged females (Murray and Elkinton 1989, Woods et al. 1989). However, unlike horizontal transmission which requires a minimum host threshold density, vertical transmission could be an important mechanism for maintaining virus in low density populations of hosts.
General Introduction

The most common means of virus transmission from one generation to the next is through the persistence of the virus, and some consider virus persistence to be the most important factor in the ecology and epidemiology of baculoviruses (Bilimoria 1991). Persistence may be biotic, in the host, secondary hosts, predators, and parasitoids or abiotic as in the soil or on the host plant.

1.4 PURPOSE AND SYNOPSIS OF CHAPTERS

The objective of this study was to investigate quantitatively host-virus interactions in NPV-tent caterpillar systems. This was done by adapting and evaluating the molecular techniques of dot-blot hybridization and PCR to quantify the incidence of NPV in tent caterpillars as population densities fluctuated. Incidence of NPV was tracked over several generations and in several populations of tent caterpillars. The important ecological features of levels of infection and host population density, vertical and horizontal transmission, and persistence of virus on tent material were examined.

The thesis is divided as follows. Chapter 2 describes experiments to test the specificity and sensitivity of the virus dot-blot hybridization assay and has been published in the Journal of Invertebrate Pathology (Kukan and Myers
General Introduction

1995). Dot-blot assay results were used to obtain comparative data among populations of forest and western tent caterpillars in different areas and in different phases of population fluctuations. The incidence of virus in field-collected tent caterpillars and tents was determined for samples from years over which population densities fluctuated. In Chapter 3, incidence of virus in forest tent caterpillars from peak and declining populations was quantified to determine the relationship between virus and host density. In Chapter 4, western tent caterpillars from low and increasing host densities were studied. A PCR assay was tested and used to examine the possibility of transmission of virus in tent caterpillar pupae and adults in Chapter 5. An overall summary of important results and concluding remarks are given in Chapter 6.
2.1 INTRODUCTION

Nuclear polyhedrosis virus (NPV) is frequently associated with declining populations of forest Lepidoptera (Tanada and Fuxa 1987). Viral disease could explain some of the mortality and fecundity characteristics of population cycling in the western or northern tent caterpillar Malacosoma californicum pluviale (Dyar)(Mp) and the forest tent caterpillar Malacosoma disstria (Hbn)(Md) (Lasiocampidae) (Myers 1988, 1990).

To quantify the role of virus in population fluctuations of forest Lepidoptera, it is necessary to have an efficient, reliable detection system for the virus. Historically NPV has been detected in field populations of caterpillars by rearing collected samples and identifying the virus through microscopic examination of stained smears of cadavers on slides. However, it is difficult to detect less than $1 \times 10^6$ polyhedral inclusion bodies (PIBs) per larvae microscopically (Kaupp and Ebling 1993), so researchers have turned to other methods. DNA hybridization assays, for example, have been used successfully in several studies for detecting NPV in caterpillars (Ward et al. 1987,
In this chapter, I describe the testing of a DNA dot-blot hybridization assay for use in monitoring the distribution of NPV in populations of tent caterpillars.

The assay was tested in three ways. (1) To determine whether DNA extraction is necessary, results using insect homogenate were compared to those from the more laborious phenol-chloroform extraction of DNA. (2) The development of infection in experimentally infected laboratory caterpillars was compared on various days post-infection to assess the sensitivity of the dot-blot assay. (3) The frequency of infection in field-collected caterpillars frozen on collection was compared to field-collected caterpillars reared to pupation or death; these caterpillars were also checked for viral infection using stained smears on microscope slides.

2.2 MATERIALS AND METHODS

2.2.1 Development of NPV DNA Probe

The development of the NPV DNA probe was done as part of my M.Sc. thesis and a brief summary is included here for clarity. Using protocols modified from Kalmakoff and Longworth (1980) and Ward et al. (1987), NPV DNA was
isolated from infected western tent caterpillars collected in the Vancouver area and fed MpNPV viral polyhedra (PIBs). Samples of MpNPV DNA, digested with BamHI, EcoRI, or PstI, were incubated with the Autographa californica virus NPV (AcNPV) polyhedrin gene (labelled with $^{32}$P), obtained from D. Theilmann (Agriculture Canada Vancouver), to identify fragments of the MpNPV DNA containing the homologous polyhedrin region (Southern 1975). Several fragments which were homologous to the AcNPV polyhedrin gene as well as fragments which were not homologous were cloned into the bacterial plasmid pGEM-3zf(+) after purification using the Geneclean kit from BIO 101 Inc. (Struhl 1985, Sambrook et al. 1989). Desired recombinants were screened by alpha-complementation and plasmid isolation (Sambrook et al. 1989).

Plasmids were purified using a modified alkaline lysis procedure (Zhou et al. 1990). These plasmids, containing pGEM-3zf(+) and the probe fragments, were inoculated into LB broth to amplify the DNA by a large scale plasmid preparation technique (Sambrook et al. 1989).

Since the polyhedrin region is known to be highly conserved (Rohrmann 1986), a non-polyhedrin gene, 1.6 kb probe, was chosen to avoid possible hybridization with other NPVs. Probe DNA was directly labelled with horseradish peroxidase, and enhanced chemiluminescence was used for
DNA Hybridization Assay
detection according to the procedure outlined in the Amersham Canada Ltd. ECL Direct Nucleic Acid Labelling Detection Kit RPN 3000/0001/3005.

2.2.2 Dot-Blot Hybridization Assay

The dot-blot assay was modified from the procedure which appeared in my M.Sc. thesis in order to improve the final autoradiogram. Samples were vacuum blotted onto nylon membrane (Zetabind) using a Bio-Rad Bio-dot microfiltration apparatus. DNA was denatured in 0.5 M sodium hydroxide + 1.5 M sodium chloride, incubated at 65°C for 30 min and neutralized in 0.5 M Tris-HCl + 1.5 M sodium chloride pH 7.5 for 5 min at room temperature. The membrane was washed in 10X SSC for 5 min and then dried at 80°C in a vacuum oven for 1 hr. The membrane was then washed for 1 hr in 0.1X SSC + 0.5% SDS at 65°C to minimize background signals. Membranes were prehybridized in hybridization buffer at 42°C for 1 hr. Labelled probe was added and the blot was incubated at 42°C overnight with agitation.

The blot was washed twice with 0.1X SSC + 0.4% SDS at 55°C for 10 min and twice with 2X SSC at room temperature for 5 min. These are the most stringent washing conditions allowed by the Amersham kit.
DNA Hybridization Assay

Detection solutions were added as outlined in the Amersham kit and blots were placed in a film cassette and exposed to film (Kodak XAR) for 10 min, then for 1 hr to provide two time exposures for analysis. The film was processed with an automated Kodak X-ray developing machine.

2.2.3 Testing the Assay System

2.2.3.1 Specificity of the Hybridization Assay

The specificity of the 1.6 kb probe isolated from western tent caterpillar NPV DNA was tested by hybridization to a blot containing DNA (phenol-chloroform extracted BamHI digested) from infected and uninfected western tent caterpillars and forest tent caterpillars identified by the dot-blot assay, as well as LdNPV infected and uninfected gypsy moth Lymantria dispar (Ld) (Lymantriidae) (provided by J. Elkinton, Univ. of Mass. Amherst).

2.2.3.2 Positive and Negative Controls

For all experiments, each dot-blotted membrane contained positive and negative controls. The positive controls were PIBs from both tent caterpillar species, as well as purified NPV DNA from western tent caterpillars. The negative controls in 1990 were laboratory-reared
DNA Hybridization Assay

*Trichoplusia ni* (Noctuidae) (provided by M. Milks, Zoology UBC) and *Peridroma saucia* (Noctuidae) (provided by M. Isman, Plant Science UBC). In 1993 and 1994, the negative controls were western tent caterpillars which had been reared from surface decontaminated egg masses.

### 2.2.3.3 Development of Infection in Laboratory-Reared Third Instar Caterpillars (1993 Experiment)

Western tent caterpillar egg masses were collected near Strathcona Park, Vancouver Island, B.C. (grid reference 115945, Canadian Dept. of Energy, Mines and Resources 1991) in the fall of 1992. These were overwintered outside and in April 1993 were decontaminated with a 2% solution of sodium hypochlorite (30% bleach, Domestic Miraclean) for 5 min, rinsed 2X with distilled water, and dried on paper towels. Each egg mass was placed in a separate plastic cup until hatch. Since this species is gregarious, larvae from the same egg mass were kept together in separate cups until the caterpillars were large enough to be infected (third to fourth instar). Insects that did not survive were discarded. Caterpillars were fed red alder (*Alnus rubra*) leaves which were washed with a 0.7% solution of sodium hypochlorite (10% bleach, rinsed 2X with distilled water and dried on paper...
towels. Caterpillars were kept in a controlled environment chamber at 26°C with an 18 hr photoperiod.

Caterpillars from 6 different egg masses were used to test the success of the assay in detecting virus at 5 and 10 days after infection. Fifteen to twenty caterpillars per egg mass were used per control group and 30 to 60 caterpillars per egg mass were used in the virus treatment. Numbers varied due to egg mass size and the number of larvae surviving to third instar.

Third and fourth instar caterpillars were fed virus on 1-cm-diameter leaf discs in individual cups. They were left for 24 hours and those that did not consume the leaf disc were discarded. Each viral-treated caterpillar was given a dose of $3.5 \times 10^6$ PIBs, from a stock solution of $3.5 \times 10^9$ PIBs/ml quantified using a haemocytometer (Kalmakoff and Longworth 1980). Control insects were given untreated leaf discs. After exposure, the caterpillars were fed red alder leaves. Half of the controls and half of the virus-treated caterpillars were frozen after five days and the remainder after 10 days. Larvae were examined daily and those that died between these dates were frozen immediately.

Samples for dot-blot were prepared by two different methods; a) phenol-chloroform extraction of DNA and b) crude homogenation.
2.2.3.4 Phenol-Chloroform Extraction

Caterpillars were macerated on ice with a sterile plastic pestle in 300 μl extraction buffer No.1 (100 mM Tris, 100 mM EDTA pH 8.0). Suspended material was centrifuged at 10,000 rpm for 5 min at 4°C in an Eppendorf microfuge. Each pellet was resuspended in 50 μl 0.1 M sodium carbonate pH 10.0 and was incubated at 37°C for 1 hr with agitation. Following the addition of 500 μl extraction buffer No.2 (0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.1% SDS, 100 μg Proteinase K), the tubes were incubated overnight at 37°C. Samples were then extracted with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). After ethanol precipitation, samples were resuspended in 50 μl TE pH 8.0. Samples were prepared for the Bio-rad Bio-Dot microfiltration apparatus by diluting 20 μl of each sample to 200 μl with TE pH 8.0.

2.2.3.5 Homogenate Preparation

Caterpillars were macerated on ice with a plastic pestle in 100 μl buffer (100 mM Tris, 10 mM EDTA, 0.1% SDS pH 8.0) and 10 μl of the resulting suspension blotted onto pretreated nylon membrane. Pestles were cleaned and autoclaved before reuse.
2.2.3.6 Development of Infection in Laboratory-Reared Second and Third Instar Caterpillars (1994 Experiment)

In 1994, western tent caterpillars were again reared from surface decontaminated egg masses. Egg masses had been collected in and around the city of Victoria B.C. on Vancouver Island. The objectives were to evaluate the detection assay on an earlier instar and at shorter time periods after viral infection. Caterpillars from 10 different egg masses were reared until they reached second instar and others left to reach third to fourth instar. Both groups were given a viral dose of $3.6 \times 10^6$ PIBs (same procedure as in 1993) and reared. Subsamples were frozen at 2, 4, and 6 days post-infection (pi). Larvae were examined daily and frozen immediately upon death. Crude homogenate preparations of caterpillars were made and 10 μl dot-blotted onto pretreated nylon membrane. Methods used are described in sections 2.2.3.3 and 2.2.3.5.

2.2.4 Comparison of Virus Detection Using Dot-Blot Assay and Rearing of Field-Collected Caterpillars

In June 1990, forest tent caterpillars were collected from a high-density population near Prince George B.C. and placed in plastic containers for transport. In the laboratory, caterpillars were either frozen for future
analysis by dot-blot hybridization (sample size n=134) or fed alder leaves until pupation, adult emergence or death (sample size n=180). Dead caterpillars were smeared on slides and triple stained or stained with naphthalene black (Kalmakoff and Longworth 1980). These slides were examined under a light microscope to check for the presence of PIBs.

Each caterpillar used in the dot-blot assay was individually cut into small pieces with sterile scissors and mashed with a clean toothpick in a 1.5 ml microtube containing 500 μl sterile distilled water. Samples were centrifuged at 1000 rpm for 5 min, and the supernatant centrifuged again at 14,000 rpm for 5 min. Pellets were resuspended in 50 μl sterile distilled water and 10 μl blotted on the pretreated nylon membrane.

2.3 RESULTS

2.3.1 Specificity and Sensitivity of the Assay

Specificity of the probe was tested by Southern blot hybridization against enzyme treated purified western tent caterpillar viral DNA as well as negative and positive controls composed of DNA from infected and uninfected insects. The sensitivity of the assay was tested using different concentrations of the purified NPV DNA in the dot-
DNA Hybridization Assay

blots. Results from the dot-blots indicate that the limit of detection was between 1 and 0.1 ng pure total viral DNA.

DNA from both the laboratory MpNPV-infected western (n=5) and field-collected MdNPV-infected forest tent caterpillars (n=6), as well as enzyme-treated purified viral DNA, hybridized to the probe. No hybridization was observed with DNA from uninfected tent caterpillar samples (Mp, n=8; Md, n=9). In addition, the probe did not hybridize to LdNPV-infected gypsy moth caterpillars (n=4) or to healthy gypsy moth caterpillars (n=3). Thus, the probe appears to be specific for MpNPV- and MdNPV-infected tent caterpillars.

DNA extracted from western tent caterpillars which had been reared from surface decontaminated egg masses were used as negative controls in the dot-blots and did not hybridize to the probe. Twenty six caterpillars from three different egg masses gave negative results. Negative results were also seen with T. ni larvae (n=5) and P. saucia larvae (n=2). At least two different caterpillars were used per membrane dot-blot. All positive controls (MpNPV and MdNPV PIBs, and pure MpNPV DNA) used in the dot-blots gave positive results (n=222). These observations add support to the Southern blot results that the probe is specific for NPV-infected tent caterpillars.
2.3.2 Experimental Infection of Laboratory-Reared Caterpillars from Decontaminated Egg Masses (1993)

In 1993, the sensitivity of the hybridization assay was also tested using experimentally infected western tent caterpillars reared from decontaminated egg masses. Figure 2.1 indicates that most exposed caterpillars died 7-8 days after infection. A few caterpillars died on Day 2 in both treated and control groups, which may have been due to stress in the handling of the caterpillars; these were not included in the subsequent analysis.

Some of these experimentally infected caterpillars had DNA extracted and were tested by dot-blot hybridization, some were used in dot-blots as crude homogenate, and some were kept to be used as controls on future dot-blots.

DNA was extracted from all caterpillars discussed in this section using the phenol-chloroform method and then tested by dot-blot hybridization. Only one control caterpillar, which died on Day 10, tested positive for NPV (Table 2.1). A significantly higher percentage were positive for virus in the caterpillars kept for 10 days than those kept for 5 days (Yates corrected $\chi^2 = 8.90$, 1df, 0.001 < p < 0.005). Eight of 253 insects (i.e. 3%) died before 5 days in the 5-Day group and of these 7 were positive and 1 negative for NPV.
As expected, more caterpillars had died by 10 days. On Day 10, 17 of 260 infected caterpillars (i.e. 7%) were still alive and 9 of these were positive and 8 negative for virus.

The intensity of the positive signal varied from strong (scored ++++) to weak (scored +) as shown in Figure 2.2 and in Table 2.2. The increase in signal intensity from 5 days to 10 days pi was probably due to an increase in viral DNA only and unlikely to be due to an increase in the genomic insect DNA in the sample. Samples of various intensities (n=12) were subjected to electrophoresis with lambda DNA of known concentrations on 0.7% agarose gels and there was no correlation between total amount of DNA (insect and viral) and signal intensity on dot-bLOTS.

All negative samples were tested to determine if the negative result was due to low amounts of DNA. Samples of 5 μl were subjected to electrophoresis with lambda DNA of known concentrations. Of 334 samples tested only 7 (i.e. 2%) had less than 5 ng DNA.

Caterpillars were checked daily and moulting was recorded. Of control caterpillars followed for 10 days, 107 of 111 (i.e. 96%) caterpillars moulted at least once, while 10 of 17 (i.e. 59%) infected caterpillars moulted at least once. This difference is statistically significant (Yates
corrected \( \chi^2 = 21.92, \text{df}=1, p < 0.005 \). It appears that moulting is delayed or stopped by virus infection.

2.3.3 Results Using Homogenized Caterpillars

Additional samples from the laboratory-reared western tent caterpillars from decontaminated egg masses were tested with the probe as homogenate without DNA extraction. Results were then compared to those obtained using DNA extraction. Fifteen 10-day control caterpillars were all negative, 24 of 30 (i.e. 80%) caterpillars tested 5 days after infection were positive, and 100% (n=28) of the 10-day pi group were positive. Results using homogenate and DNA extraction were not significantly different for any of the categories, controls, 5-day and 10-day post-infection samples or groups. However, there were fewer strong positives (+++) in the 5-day homogenate sample (Table 2.2). Again significantly more positive responses occurred by 10 days (Yates corrected \( \chi^2 = 4.28, \text{df}=1, 0.025 < p < 0.05 \)). Dot-blots of homogenates gave a ring rather than a dot as observed with phenol-chloroform extracted DNA. Similar observations of blotted insect macerates were made by Kaupp and Ebling (1993).
2.3.4 Experimental Infection of Laboratory-Reared Caterpillars from Decontaminated Egg Masses (1994)

In 1994, the sensitivity of the hybridization assay was tested on second and third instar caterpillars kept for different time periods $pi$ than had been used in 1993. The percentages positive for viral infection in the second and third instar groups are given in Table 2.3. There is no significant difference in viral infection in the controls for second instar 1994, third instar 1994 and third instar 1993. In the virus-treated groups, there is a significant difference in the percentage positive for viral infection between second and third instars at 2 days $pi$ with second instars having more infected individuals. In second instars infection increased between day 2 and 4 $pi$ and remained at a similar level on day 6. Infection of third instars also increased between 2 and 4 days $pi$.

A comparison of signal intensity (Table 2.4) in which homogenates were tested by dot-blot indicates that at 2 days $pi$ most of the positive signals were weak for both second and third instars. By 4 days $pi$ most of the second instar samples gave a strong positive signal while third instar samples vary in signal intensity. By 6 days $pi$, most of the positives were strong in both groups.
2.3.5 Comparisons of Viral Infection Based on DNA Hybridization vs Laboratory-Rearing of Field-Collected Caterpillars

To compare detection of virus by the dot-blot assay to identification of viral infection from death followed by microscopic examination for PIBs, field-collected caterpillars were either examined by dot-blot analysis or reared until death or pupation. Of 134 forest tent caterpillars collected from the field in 1990 and frozen immediately 32 (24%) were positive by dot-blot hybridization. Of the caterpillars that were reared individually in the laboratory until death or pupation, 33 of 180 (18%) died by Day 5 and were positive for virus. Another 69 of 166 caterpillars (42%) which were not parasitized by flies, died of virus by 10 to 13 days which gives a total observed mortality from virus of 29%. Therefore, the dot-blot hybridization technique reveals similar levels of infection to that observed from deaths of field-collected caterpillars reared in the laboratory.

2.4 DISCUSSION

Tracking the occurrence of viral infections through fluctuations in population density of forest Lepidoptera is important for the evaluation of the mechanisms behind
population dynamics. Laboratory rearing of field-collected caterpillars to determine viral infection is time and labour intensive, particularly for species that are not successfully reared on artificial medium. The opportunity for contamination is always present in the laboratory which can bias the results. I describe my protocol for using a dot-blot hybridization test for NPV of western tent caterpillars which allows identification of viral infection of field-collected caterpillars. Although the probe was developed from MpNPV (western tent caterpillar NPV), it was also effective in identifying MdNPV (forest tent caterpillar NPV). This allowed me to compare detection of the virus with DNA hybridization to detection through laboratory rearing until death of field collected forest tent caterpillars from peak to declining densities (Kukan and Myers unpublished).

My study agrees with those of Ward et al. (1987) with Wiseana spp., and Keating et al. (1989) with gypsy moth, Lymantria dispar. In all cases no differences were found for dot-blot results between using DNA extraction with phenol-chloroform and simply homogenating caterpillars. It is possible that some caterpillars in the very early stages of infection could be identified by DNA extraction but missed by using homogenates, since the former appears to be slightly more sensitive. On the other hand, DNA can poten-
DNA Hybridization Assay

tially be lost during extraction. In my study of 334 samples extracted, only 7 (2%) had less than 5 ng total DNA (caterpillar and viral). These could have appeared as false negatives if the amounts of DNA had not been quantified. Although there was slight qualitative variation in the dots after hybridization with extracted DNA and homogenates, this did not change the scores of positive and negative in tests with caterpillars actually exposed to virus. In a field study it is often important to increase the sample size. Therefore, the time saved by not extracting the DNA could be effectively used for sampling more times, more areas, or more caterpillars.

I found, however, that it was important to use stringent washing conditions for the dot-blot to eliminate false positive responses. This was particularly a problem with adults and pupae which seemed more prone to give weak positive results in initial studies (not presented here). Caterpillars that die of virus always give very strong signals when hybridized with the probe.

My comparison of virus detection using dot-blot assay to laboratory rearing and microscopic examination of cadavers also agrees with the study of Keating et al. (1991) with gypsy moth. Dot-blot hybridization of field-collected caterpillars shows the same levels of infection as revealed
by viral deaths in 5 or 6 days. In both cases, however, following larval death to 10 to 13 days allowed detection of more viral-infected insects. Early infections are not identified by the dot-blot assay and, therefore, measurements of the prevalence of virus in populations will be conservative. In Keating’s study, dot-blot assay of field samples of later instars were more similar to results from 10- to 13-day mortality. Therefore, using the dot-blot assay late in larval development could be the best way of obtaining estimates of viral infection in field populations. Sampling should be done during the second wave of viral infection that occurs in field populations (Woods and Elkinton 1987).

In my studies initiated with surface decontaminated eggs, I found an increase in the percentage of positive responses between the 5 day and 10 day samples in 1993, but not between the 4 day and 6 day samples in 1994. In the study by Keating et al. (1989), they found no difference in the proportion of individuals positive for virus between 6 and 10-13 days post-infection. In the 1993 experiment, the difference between results could be due to variation in the instars exposed to virus or rearing temperatures, either of which could influence the rate of virus replication (Johnson et al. 1982, Watanabe 1987, Benz 1987). In my 1994
experiment, infection developed more rapidly than in 1993. The more rapid the development of virus the more likely that virus will be at a sufficient level to be detected by the dot-blot assay.

Burand and Park (1992) in their study of L. dispar reported that NPV inhibited moulting through the activity of a gene which codes for an ecdysteroid UDP-glycosyl transferase (egt) (O’Reilly and Miller 1989). Expression of this gene interrupts the normal development of the host through EGT inactivation of ecdysteroid (moulting hormone). I found evidence that moulting was suppressed in western tent caterpillars infected with NPV, which suggests that MpNPV has the egt gene as well. If viral infection slows development of caterpillars in the field, samples taken late in the season may be biased in favor of those individuals that are infected and have not yet pupated.

In conclusion, the dot-blot assay gives an efficient technique for identifying viral infection in field populations of caterpillars. When used in a consistent manner, results can yield comparative data among populations in different areas and in different phases of the population fluctuations.
Table 2.1: Development of infection in laboratory-reared western tent caterpillars inoculated with NPV in 1993.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>VIRUS FED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0-5</td>
<td>Day 0-10</td>
</tr>
<tr>
<td>Percentage that died</td>
<td>0 (114)</td>
<td>2 (111)</td>
</tr>
<tr>
<td>Percentage +ve NPV</td>
<td>0 (30)</td>
<td>3 (31)</td>
</tr>
</tbody>
</table>

*(number tested)*

Note: Presence of NPV assessed by dot-blot hybridization.
Table 2.2: Signal intensity in a dot-blot assay of western tent caterpillars treated with virus in 1993 and reared for 5 days or 10 days after treatment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA EXTRACTION</th>
<th>HOMOGENATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity</td>
<td>#</td>
</tr>
<tr>
<td>+++</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>5 Day</td>
<td>++</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA EXTRACTION</th>
<th>HOMOGENATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity</td>
<td>#</td>
</tr>
<tr>
<td>+++</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>10 Day</td>
<td>++</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>138</td>
</tr>
</tbody>
</table>
Table 2.3: The percentage of caterpillars positive for viral infection in laboratory-reared 2nd and 3rd instar western tent caterpillars in 1994 and in 3rd instars in 1993. Presence of NPV assessed by dot-blot hybridization of insect homogenate. Sample size in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>1994 2nd instar</th>
<th>1994 3rd instar</th>
<th>1993 3rd instar</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROLS</td>
<td>0 (74)</td>
<td>2.5 (120)</td>
<td>2 (61)</td>
</tr>
<tr>
<td>VIRUS</td>
<td>2 day</td>
<td>46 (50) (^a)</td>
<td>19 (80)</td>
</tr>
<tr>
<td></td>
<td>4 day</td>
<td>90 (50) (^b)</td>
<td>90 (68) (^c)</td>
</tr>
<tr>
<td></td>
<td>6 day</td>
<td>83 (48)</td>
<td>94 (69)</td>
</tr>
<tr>
<td></td>
<td>10 day</td>
<td></td>
<td>100 (28)</td>
</tr>
</tbody>
</table>

Yates corrected $\chi^2$ for significantly different results:

\(^a\) 2nd and 3rd instars at 2 days $\chi^2 = 9.77$, 1 df, $p < 0.005$.

\(^b\) 2nd instars at 2 and 4 days $\chi^2 = 20.27$, 1 df, $p < 0.001$.

\(^c\) 3rd instars at 2 and 4 days $\chi^2 = 71.27$, 1 df, $p < 0.001$. 
Table 2.4: Signal intensity in a dot-blot assay of western tent caterpillars inoculated with virus and reared for 2 days, 4 days and 6 days after treatment (1994). Samples were dot-blotted as insect homogenate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intensity</th>
<th>2nd instar</th>
<th>3rd instar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#</td>
<td>%</td>
</tr>
<tr>
<td>+++</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2 Day</td>
<td>++</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>4 Day</td>
<td>++</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>6 Day</td>
<td>++</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>48</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 2.1: Number of western tent caterpillars dying per day after inoculation with $3.5 \times 10^6$ PIBs NPV per caterpillar. Caterpillars were laboratory-reared from decontaminated egg masses. Control and virus-fed 5 day results were the same as 10 day control.
Figure 2.2: Dot-blot assay of western tent caterpillars fed virus, then frozen after 5 days and the DNA extracted with phenol-chloroform. The membrane was probed with viral DNA and the autoradiogram was exposed for 1 hour. Note the different signal intensities observed.

Strong +++: A9, A12, B2, B3, B9, B10, C4, C6.
Medium ++: A5, A6, B4, B5, B6, B7, B8, C3.
Weak +: A2, A3, A7, A10, A11, B1, B12, C8, C10, C11.
Negative: A1, A4, A8, B11, C1, C2, C5, C7, C9, C12.
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CHAPTER 3

INCIDENCE OF NUCLEAR POLYHEDROSIS VIRUS IN FOREST TENT CATERPILLARS

3.1 INTRODUCTION

3.1.1 General Introduction

Infection by nuclear polyhedrosis virus (NPV) commonly occurs in forest Lepidoptera at outbreak densities, and may be associated with the fluctuating population dynamics characteristic of a number of species of forest caterpillars (Evans and Entwistle 1987, Myers 1988). Infection and spread of NPV are expected to increase with the density of the hosts (Anderson and May 1981), but little is known about the persistence of virus at low host density (Dwyer and Elkinton 1993). Possible reservoirs for virus are (1) polyhedra remaining infective in the environment for several years following population decline (Jaques 1975); (2) persistence of virus in an inactive form in the insects (Hughes et al. 1993); (3) low frequency infection in sparse caterpillar populations (Evans and Entwistle 1987).

To determine the proportion of caterpillars with viral infection, populations of forest tent caterpillars Malacosoma disstria (Hbn) were sampled once a year over 5
years from peak density through decline. I identified viral infection with a DNA dot-blot hybridization assay which allowed identification of virus in frozen samples of field collected caterpillars rather than having to rear the caterpillars in the laboratory until death or pupation.

3.1.2 Biology of Forest Tent Caterpillars

Forest tent caterpillars occur across Canada from British Columbia to Prince Edward Island and extend as far south as Louisiana (Stehr and Cook 1968). In the Prince George area (53° 55' 122° 45') populations have reached peak density in 1953-1954, 1962-1964, 1973-1975 (Turquist 1987) and 1989-1991 (personal observation). Trembling aspen (Populus tremuloides) is the predominant food plant of forest tent caterpillars.

Female moths each lay a single egg mass in July, and these overwinter to hatch in May synchronously with leaf development. There are five larval instars. In the vicinity of Prince George, B.C., pupation occurs in late June. Forest tent caterpillars congregate on silk pads on tree trunks when not feeding but do not actually form tents.
3.1.3 Regional Population Trends

General trends in the population density of forest tent caterpillars in the Prince George region are available from Forest Insect and Disease Surveys (FIDS) carried out by the Canadian Forest Service and summarized in Figure 3.1 (Wood and van Sickle 1990, 1991, 1992, 1993).

3.2 MATERIALS AND METHODS

3.2.1 Study Sites

Range Road. This site is on a hillside approximately 5 km west of Prince George town centre about 2 km south of Cranbrook Hill where egg masses were monitored by the Forest Insect and Disease Surveys, (Turquist and Ferris 1989, 1990, Humphreys and Ferris 1991). Caterpillars were collected from aspen trees along the edge of the clearing over a linear distance of approximately 0.5 km.

Truck Scale. This site is on a hill 4 km north of Prince George town centre about 2 km from Hart Highlands where egg mass densities were monitored by FIDS (Turquist and Ferris 1989, 1990, Humphreys and Ferris 1991). Caterpillars were collected along the edge of perpendicular tracks, one which went up a hill for approximately 1 km and the other which extended 0.5 km along the valley. In 1993
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and 1994 a wider area was searched but no caterpillars could be found.

Airport and Ellis Road. These sites are approximately 10 km southeast of Prince George town centre and are 2-3 km from the Airport Hill FIDS monitoring site (Turquist and Ferris 1989, 1990, Humphreys and Ferris 1992). At the Airport site caterpillars were collected from trees along perpendicular tracks through this aspen woodland over a distance of approximately 0.5 km in each direction. The Ellis site has a dense overstory of aspen trees approximately 5-7 m tall. Caterpillars were collected from the undergrowth along a transect of about 0.2 km into the woodland.

Westlake. This site is approximately 15 km southwest of Prince George and is about 2 km from the FIDS Fyfe Rd monitoring site (Turquist and Ferris 1990, Humphreys and Ferris 1992). Caterpillars were collected from a transect of approximately 0.5 km along Frenkel Road, and along a 1 km track from Muralt Road across a valley and up a hill. In 1994 when densities were low, trees along the edge of a pasture at the top of the hill were searched in addition to the original transect. This extended the search transect by approximately 1 km.
Blackburn Road. This site is approximately 10 km east of Prince George and was monitored in 1993 and 1994 only. Caterpillars were collected from the undergrowth along a transect of about 0.2 km into the woodland and another 0.2 km along a roadside.

3.2.2 Sampling Techniques

Caterpillars or pupae were collected along transects which were walked by one or two collectors. No more than 5 caterpillars were collected from one tree. Many trees were small enough to be pulled over to collect caterpillars from the tops and others were from undergrowth, low leaves or trunks. When densities were low, 1993 and 1994, almost all caterpillars seen were collected. In 1994 caterpillar collections were separated into those obtained in the first half of the collection period and those in the second half to determine if there was heterogeneity within study areas. In all years caterpillars were frozen within 8 to 48 hours of collection.

3.2.3 Estimating Population Densities

Budgetary constraints allowed only one short collection trip a year and so detailed estimates of caterpillar or egg mass density could not be made. However, at the time of
sample collection visual estimates of the degree of defoliation were made. These observations were recorded and ranked as follows: 0 = no damage and no caterpillars; 1 = no defoliation observed; 2 = little defoliation; 3 = patchy defoliation; 4 = moderate defoliation; 5 = heavy defoliation; and 6 = total defoliation (trees appeared gray from a distance because leaves were gone). Over the years of the study, 11 counts of egg masses on cut trees were made by FIDS in areas which were 1 to 3 km from the study sites. These could be related to the observations on defoliation levels. The relationship between the rank of caterpillar density based on observed defoliation and the egg mass counts made by FIDS is shown in Figure 3.2. The relationship was highly significant with Spearman rank correlation ($r = 0.87$, $p < 0.01$); Figure 3.2). Therefore, I have used the observations of defoliation levels as an indicator of caterpillar density in further analyses.

3.2.4 Sample Preparation and Dot-Blot Hybridization

In 1990, 1991 and 1994 caterpillars were dot-blotted as a homogenate preparation and in 1992 and 1993 samples were phenol-chloroform extracted before dot-blotted. Details of sample preparation and the DNA dot-blot hybridization
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...technique are described in Kukan and Myers (1995) and Chapter 2.

In all years bound probe was detected by enhanced chemiluminescence using an Amersham Kit. Positive controls for dot blots were pure DNA extracted from NPV, polyhedral inclusion bodies (PIBs) of NPV from tent caterpillars, and western tent caterpillars which had been fed virus and reared until death. Negative controls were laboratory cultures of Peridroma saucia caterpillars and Trichoplusia ni caterpillars in 1990 and 1991, and uninfected, laboratory reared western tent caterpillars in 1992, 1993, and 1994. Samples used for negative controls never produced positive results.

Before being used on field samples the dot-blot assay was tested for specificity and sensitivity (Kukan and Myers 1995). No difference was found between results using DNA extraction with phenol-chloroform and crude homogenates of caterpillars (Kukan and Myers 1995). Homogenizing samples without DNA extraction is more efficient.
3.3 RESULTS

3.3.1 Heterogeneity Among Instars

The predominant instars of caterpillars collected for analysis varied slightly among sites and years and ranged between late 4th and early 5th to late 5th and pupae. The dominant instars of samples from each site in each year were recorded at the time of collection. Westlake, Range Road, and Truck Scale tended to have slightly earlier instars than the sites near the airport. There was no relationship between the dominant developmental stage of the caterpillar samples and the percentage of caterpillars infected with virus when samples from all sites and years are compared (Spearman rank correlation $r = -0.23$, $p > 0.05$, df=20; Figure 3.3).

3.3.2 Heterogeneity Within Study Areas

In 1993 the percentage of infected caterpillars from the Blackburn site collected along the edge of the woods (4 or 13% N=30) did not differ significantly from that collected within the woods (1 or 8% N=12) (Yates corrected $\chi^2 = 0.15$, $p > 0.05$). A similar comparison in 1994 showed the same result, 13% (N=102) infected in the woodland and 13% (N=100) along the roadside. In 1994, two samples were
collected at each site and kept separate for analysis to test for heterogeneity within study areas. At 3 of 4 sites the proportion of infected caterpillars was not different between the two samples (Table 3.1). At Range Road however, one sample of 45 caterpillars had 6 infected individuals and the other of 71 caterpillars had no infected individuals. Therefore, when density and infection are low, some spatial heterogeneity of infected individuals within sites may occur.

3.3.3 Variation of Infection with Density

The relationships of density measured as severity of defoliation, and infection determined by DNA dot-blot hybridization are plotted for four study sites in Figures 3.4-3.7. Infection was related to density when data from all sites and years were considered (Figure 3.8), but this relationship was strongly influenced by two years, one of high density and high infection at Truck Scale in 1990, and the other of low density and low infection at Westlake in 1993. Temporal variation in the percentage of caterpillars infected showed a clear association at the Truck Scale site (Figure 3.5). Infection was highest at this site in 1990 and the population collapsed by 1993. The percentage of infected caterpillars at the Airport site (Figure 3.6) was relatively
low, less than 22%, and varied among years without an apparent relationship to density. This site increased in density in 1994 and other sites within several km had high caterpillar densities in 1993 and 1994. The percentage of infected individuals at these two sites, Ellis Road and Blackburn Road, were moderate when density was high, and defoliation was total (for Ellis Road 17%, n=24, in 1993 and 22%, n=134, in 1994; for Blackburn Road 12%, n=42, in 1993 and 13%, n=203, in 1994). The sample sizes in 1993 were small because many of the caterpillars had pupated by the time the samples were collected, but good collections in 1994 indicated viral infection at all sites that had caterpillars.

3.4 DISCUSSION

Infection by NPV is common in forest Lepidoptera, and is particularly interesting in those species that go through periodic outbreaks (Evans and Entwistle 1987, Myers 1988). Virus is spread by polyhedral inclusion bodies that contain virions which are released at the death of diseased caterpillars. At the simplest level, viral infection should increase with the density of the host since the exposure of increasing numbers of susceptible individuals to disease will result in more infection in the population (Anderson
Virus in Forest Tent Caterpillars

and May 1981). Models of the dynamics of NPV and caterpillar hosts have been developed which include other potential interactions in the transmission process besides numbers of infected and susceptible individuals (Hochberg et al. 1990, Bowers et al. 1993, Dwyer 1994). In these models the persistence of the virus between generations is crucial to the maintenance of realistic dynamics. Models point out the need to identify whether viral infection is maintained through periods of low host density.

My measurements of infection in tent caterpillar populations are conservative. The DNA probe identifies virus two days after infection (Chapter 2) and caterpillars die of infection 8 to 14 days pi. A sample taken at one time will identify only those individuals within that sample period (Kukan and Myers 1995). Since development time for caterpillars is four to six weeks in the field, total mortality from virus would be greater than the level of infection measured in one sample.

The general trend is for infection among forest tent caterpillars to be higher in years of high density, 1990 in 3 of the 4 initial study populations and 1993 and 1994 at Ellis and Blackburn sites, than at low density, 1993 and 1994 at Westlake and Range Road. There were exceptions to this trend however with only 4% of the sample being positive
for viral infection at the Airport site in 1991 and 7% at Westlake in 1990. I do not know how to interpret these observations. Even though the estimates of viral infection are conservative, they are similar to levels of infection reported by Otvos et al. (1987a) for Douglas fir tussock moth, Orgyia pseudotsugata. However, Keating et al. (1991), also with a dot-blot assay, found approximately 80% of late instar gypsy moth were infected with virus in high density populations and I found 35-70% of late instar western tent caterpillars to be infected with NPV (Chapter 4). These last values may have been biased however. If infected individuals have delayed pupation, samples of late instars may have abnormally high levels of infection (Burand and Park 1992). Cumulative death from viral infection in gypsy moth revealed by rearing varied from 10 to 15% in low density populations to over 80% in high density populations (Woods et al. 1991). These results agree with mine that viral infection is maintained in low density populations of Lepidoptera, and can increase with population density.

The level of infection in the two high density sites showed little increase in the second year of high density, 1994, but because many of the caterpillars had pupated by the time of collection in 1993, sample sizes were small and estimates are not robust. In general for other sites the
level of infection remained relatively high in 1992 when populations had begun to decline. The amount of inoculum could still be increasing as host density declines from viral deaths. The implication here is that increases in the amount of inoculum keep the level of infection high for the first two years of population decline and this helps bring about the collapse of the population outbreak. I observed that virus was maintained at low levels in sparse populations. This suggests that some infected caterpillars could act as a virus reservoir at low density which could provide inoculum to trigger an epizootic at high density.
Table 3.1: Comparison of the percentage of forest tent caterpillars infected with virus in independent samples collected from four areas in the vicinity of Prince George, B.C. in 1994.

<table>
<thead>
<tr>
<th>AREA</th>
<th>SAMPLE</th>
<th>%INFECTED</th>
<th>NUMBER</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range Road</td>
<td>A</td>
<td>0</td>
<td>71</td>
<td>7.5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>13</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airport</td>
<td>A</td>
<td>9</td>
<td>100</td>
<td>0.02</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Westlake</td>
<td>A</td>
<td>6</td>
<td>66</td>
<td>2.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ellis Road</td>
<td>A</td>
<td>16</td>
<td>74</td>
<td>2.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>28</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Hectares of aspen woodland defoliated by forest tent caterpillars in the region on Prince George British Columbia as reported by the Forest Insect and Disease Surveys.
Figure 3.2: Relationship between the rank of observed defoliation classification at the study sites (see text) and population estimates made by FIDS for nearby locations based on counting egg masses on cut trees. Spearman rank correlation coefficient $r = 0.87$, $P < 0.01$. 
Figure 3.3: The relationship between the percentage of the sample infected with NPV and the dominant developmental stage category of the caterpillars in the population at the time of collection. Caterpillars from the Westlake site were always slightly younger than at other sites and 3 points for 4th-5th instars are from this site. Spearman rank correlation using ranking of 1 (4th-5th) to 3 (late 5th) \( r = -0.19, p > 0.05, n=19. \)
Figure 3.4: Change in defoliation rank (see text) between years and the percentage of sampled caterpillars infected with NPV for the Range Road site. Sample sizes from 1990 to 1994 were 44, 80, 14, 9, 116.
Figure 3.5: Change in defoliation rank (see text) between years and the percentage of sampled caterpillars infected with NPV for the Truck Scale site. Sample sizes from 1990 to 1992 were 38, 76, 12.
Figure 3.6: Change in defoliation rank (see text) between years and the percentage of sampled caterpillars infected with NPV for the Airport site. Sample sizes from 1990 to 1992 were 25, 55, 14, 28, 194.
Figure 3.7: Change in defoliation rank (see text) between years and the percentage of sampled caterpillars infected with NPV for the Westlake site. Sample sizes from 1990 to 1994 were 27, 191, 35, 7, 184.
Figure 3.8: Relationship between the defoliation rank for each site and year and the percentage of caterpillars infected with NPV. Spearman rank correlation coefficient $r = 0.61$, $p < 0.01$, $n=21$. 
CHAPTER 4
INCIDENCE OF NUCLEAR POLYHEDROSIS VIRUS IN WESTERN TENT CATERPILLARS

4.1 INTRODUCTION

Western tent caterpillars are typical of a number of forest Lepidopterans in that they have fluctuating population densities. They also have a baculovirus pathogen which is usually associated with population declines (Tanada and Fuxa 1987, Entwistle and Evans 1985, Watanabe 1987). Tent caterpillar populations have been studied for a number of years in southwestern British Columbia and their population dynamics are well described (Myers 1990, 1993). A temporal series of samples of larvae and tent material has been collected from several sites over a range of insect densities, mainly from low to increasing density. Gregarious behaviour of tent caterpillars allows them to be found even at low density and therefore the material that has been collected provides a unique opportunity to track the incidence of virus. In this chapter, a dot-blot hybridization assay was used to determine the incidence of virus in field samples of western tent caterpillar larvae and tents from different years, sites, and densities. Four main questions are considered: 1) does viral infection vary
with host density? 2) does incidence of virus predict future population trends? 3) is virus maintained in low density populations? 4) are tents a source of viral contamination in the environment?

4.2 MATERIALS AND METHODS

4.2.1 Biology of Western Tent Caterpillars

Western tent caterpillars Malacosoma californicum pluviale, also called the northern tent caterpillar (Garbutt and Wood 1993), is a common defoliator of deciduous trees particularly red alder Alnus rubra (Bong.), common hawthorn Crataegus monogyna (Jacq.), and crab apple Malus diversifolia (Bong.) in coastal British Columbia. Outbreaks have occurred in at least one area in southwestern British Columbia about every 8 to 10 years since records began in 1933 (Garbutt and Wood 1993, Myers 1988, 1990, 1993). This species has one generation per year with first-instar larvae hatching from eggs in the spring in early to mid-April synchronously with bud burst of the host trees (Myers 1990). Young larvae feed gregariously and form a silken tent on the branch of the tree. These tents can be spotted even at low population density. Tents are enlarged as the larvae grow and provide protection during resting, bad weather,
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moulting, and serve as a surface on which caterpillars bask in the sun. Larvae feed for 5 to 6 weeks and pass through 5 instars before pupation. Adults emerge about 2 weeks later and females lay eggs (100-300) in a single mass on twigs. Old egg masses can be collected after hatching and the number of eggs per mass counted. Nuclear polyhedrosis virus (MpNPV) occurs in this species (Stairs 1965, Myers 1990).

4.2.2 Study Sites and Sampling

Western tent caterpillar larvae and tents were collected by Dr. Judith Myers starting in 1986 from four sites near Vancouver and the Gulf Islands in British Columbia. Cypress Provincial Park is about 15 km north of the University of British Columbia (UBC) campus at an elevation of 450 m. Mandarte Island is a 7-ha island in Haro Strait about 12 km east of Sidney, Vancouver Island. Galiano Island is one of the southern Gulf Islands and is about 40 km southwest of the UBC campus. Westham Island is a larger island in the delta of the Fraser River about 20 km south of the UBC campus.

At high host densities, 5-10 caterpillars per family were collected from a subsample of available families. At low densities only a few larvae were collected to reduce disturbance to the populations. Caterpillar sample sizes and
years collected are shown in Table 4.1. The majority of the
caterpillars collected were third-fifth instars (79%) but
the development stage varied slightly due to delays in
development or time available to collect. As many tents as
possible were consistently collected after all caterpillars
had left in late June.

4.2.3 Regional Population Trends

Information from Forest Insect and Disease Surveys
(FIDS) reports indicate that in 1986 the area defoliated by
western tent caterpillars had expanded for the fifth
consecutive year in the Vancouver region (Humphreys and
Ferris 1986) and in 1987 defoliation decreased (Humphreys
and Ferris 1987). In 1988 to 1991 populations were at
endemic levels (Humphreys and Clarke 1990). Populations
increased in the lower mainland until 1992, but declined in
1993 and 1994 (personal observation). On Vancouver Island
and the southern Gulf Islands populations increased from
1991 to 1994 (Figures 4.1-4.4) (Turquist and Clarke 1992,
Turquist and Wood 1993, 1994) and continued to increase to
1995 (personal observation).

Between 1990 and 1995, population trends became
asynchronous with two populations, Mandarte and Galiano,
increasing continuously and two populations, Westham and
Virus in Western Tent Caterpillars

Cypress, declining after 1992. The area adjacent to the Cypress site was sprayed with \textit{Bacillus thuringiensis} in 1992 as part of an Asian gypsy moth control program and the Westham site had a high density of the introduced winter moth \textit{Operopthera brumata} (L) and ermine moth \textit{Yponomeuta padella} (L).

4.2.4 Preparation of Samples - Larvae

Larvae were homogenized for dot-blot hybridization assay as outlined in Chapter 2. Briefly, caterpillars were macerated on ice with a sterile plastic pestle in 100 μl buffer (100 mM Tris, 10 mM EDTA, 0.1 % SDS, pH 8.0) and 10 μl of the resulting suspension blotted onto a pretreated nylon membrane (Zetabind). Pestles were bleached and autoclaved before reuse.

4.2.5 Preparation of Samples - Tents

Tent material was extracted and prepared for dot-blotting in the following way. Tents were removed from twigs and the silk material was cut up and placed in 50 ml centrifuge tubes. Twenty-five ml of 0.1% SDS was added to completely cover the tent material and the sample was soaked overnight at room temperature. Each sample was sonicated in a Branson 2200 ultrasonic bath for 4 min and the contents of
the tube poured through 2 layers of cheesecloth. The liquid was collected in a 50 ml centrifuge tube. The tent material was washed with 10 ml of 0.1% SDS and the resulting liquid added to the 50 ml tube. The liquid samples were centrifuged in a IEC Centra 4B centrifuge at 3800 g for 10 min. Pellets were resuspended in 500 µl distilled water and frozen until use. Before blotting, samples were vortexed and 10 µl dot-blotted onto nylon membrane (Zetabind).

4.2.6 Viral Contamination of Tents

In 1993 a preliminary experiment was done to determine if tent material could be a source of virus infection for western tent caterpillars, and to see if virus on tent material could be extracted and detected with the dot-blot assay. Western tent caterpillars reared from surface decontaminated egg masses were given a lethal dose of virus in the laboratory and then placed outside on alder trees to complete their life cycle (20 infected caterpillars per tent). Control insects were not given the virus. Tent material was collected from 4 control and 4 virus infected tents, extracted and dot-blotted.

In October 1994, persistence of NPV on tents was again measured. Twelve tents were contaminated with $3 \times 10^9$ MvNPV PIBs (polyhedral occlusion bodies) and placed outside.
Sixteen tents were also treated by the addition of 8 caterpillars which were known to have died from virus infection. Four control tents with no treatment were also placed outside. On December 20, after 2 months, 7 of the virus contaminated tents and 9 of the tents with infected caterpillars were collected and frozen for storage. On February 19, after 4 months outside, the remaining tents were collected and frozen. Tent samples were extracted and dot-blotted for hybridization to the MpNPV probe.

4.2.7 Bioassay of Virus Contaminated Tent Material

In the spring of 1994, an experiment was carried out to determine if third instar western tent caterpillars could be infected by MpNPV extracted from tent material which had given a positive dot-blot reaction for viral contamination. Fifty third instar caterpillars reared from decontaminated egg masses were fed 10 µl of virus infected tent extract (moderately strong signal by dot-blot) from 1993 and 50 fed virus tent extract (weak signal by dot-blot) from 1993. Larvae were checked every day and frozen as they died or after 6 days. Dot-blot hybridization of insect homogenates was used to check for infection.
4.3 RESULTS

4.3.1 Viral Infection in Field-Collected Larvae and Tents

The percentages of caterpillars infected with virus are given in Table 4.1, and Figures 4.1-4.4 illustrate the relationship between density, as the number of tents, and the percentage infection. Based on the data collected from the 4 sites over the years 1990 to 1995, the average percent of caterpillars positive for virus (arcsin square root transformed) is significantly correlated to the number of tents counted at each site in each year ($r = 0.66$, $p = 0.01$ Figure 4.5). Of particular interest are the out of phase (with other sites) declines in both the level of infection and the population density in the Cypress population after 1992 which indicates an association between these two factors. I standardized the changes in numbers among years for the 4 areas by calculating $R = \ln N_{t+1} / \ln N_t$ ($N=$number of tents counted) for pairs of years for which data were available and related this to percentage of individuals positive for virus. There was no association between viral infection and change in population numbers ($r = 0.14$, df=12, $p > 0.05$).

The percentage of tents which were positive for viral contamination was generally below 25% (Table 4.2) with the
highest values occurring in 1987 during the early phase of the population decline. The percentage of the tents which were positive for virus contamination was not directly correlated to the population density over the range of densities covered in the analysis ($r = 0.02$, $p = 0.95$).

### 4.3.2 Characteristics of Infected Families

Many of the groups of caterpillars assayed for viral infection could be associated with an egg mass when they were collected from the field. It has been proposed (Myers 1993, Myers and Kukan 1995) that the decline in the fecundity of tent caterpillars with decreasing population densities could result from selection for individuals resistant to virus but having a lower fecundity as a cost of resistance. This hypothesis was tested by comparing the number of eggs in masses giving rise to families of caterpillars for which some individuals were found to be positive for virus, to those in which no individuals were infected. This disease resistance hypothesis predicts that egg masses of groups without virus would be smaller than egg masses of groups with virus. An ANOVA of the number of eggs per egg mass for families with some caterpillars testing positive for virus and families for which no caterpillars tested positive for virus with site and year as predictor
variables showed no significant variation or interaction. The average size of egg masses for families without virus was 195, SE = 4, n = 84; and with virus the average size was 200, SE = 4, n = 75.

The size of tents (length x width) in field populations is correlated to the number of late instar caterpillars in the family group (Myers 1990). I predicted that tents of families with virus would be smaller if early mortality reduced the number of caterpillars surviving to the late instars. An ANOVA of tent size of families with some caterpillars positive for virus and of families tested but with no positive caterpillars showed that year and site were also significant for the years in which data were available for all sites (year, F = 5.2, p = 0.023, and site, F = 4.1, p = 0.05). Therefore, data from each site were analyzed separately. Variation among years was not significant for any of the 4 sites although sample sizes differed markedly. The mean tent sizes for those families with virus and those without are presented in Table 4.3. The only population in which the sizes of the tents were significantly different was Cypress and most of the data were from 1991 during the population increase. Tents of families with virus were larger than those of families without virus. A similar trend was apparent in the data from Galiano collected in 1994.
which for this site was also during the phase of population increase, but data from Mandarte showed the opposite trend.

4.3.3 Variation Among Instars

Caterpillars were separated into two age groups; early instars, first to early third; and late instars, late third to fifth. Viral infection was assessed in years and sites which had both groups (Table 4.4). There was a significant difference between infection in early and late instars at Cypress in 1990 and 1995 ($\chi^2 = 4.52$, 1 df, $p < 0.05$), at Westham in 1992 ($\chi^2 = 5.95$, 1 df, $p < 0.05$), and at Galiano in 1994 ($\chi^2 = 16.34$, 1 df, $p < 0.001$) (all $\chi^2$ were Yates corrected). The trend was toward higher detection of infection in late instars.

4.3.4 Persistence of Virus on Experimental Tents

In the preliminary experiment, all 4 controls were negative when hybridized to the MpNPV DNA probe and all 4 virus infected tents were positive. In the subsequent experiment all 4 control tents were negative; the 16 tents collected after 2 months were all positive; and 10 of 12 of the samples kept outside for 4 months (i.e. 83%) were positive. Signal intensities of the positives varied from 13 strong, 7 moderate and 6 weak.
Virus in Western Tent Caterpillars

Ninety-eight percent of larvae (n=100) inoculated with extracts from tents which had given a positive signal (moderate and weak) when testing with the dot-blot hybridization assay became infected with virus, while only 2.5% of control caterpillars were positive for NPV (n=120). This is significantly different (Yates corrected $\chi^2 = 196.5$, df=1, p< 0.001).

4.4 DISCUSSION

The incidence of virus in fluctuating populations of tent caterpillars was quantified to gain insights into the relationships between the pathogen, NPV, and the host, tent caterpillars. Detection of infection generally increased from early to late instars. In other studies, increasing or high density caterpillar populations have had levels of infection from 5 to 28 percent in early instars and 53 to 91 percent in late instars (Table 4.5). I found levels of infection in this range for high density years (1985, 1986, 1994, 1995) of tent caterpillar and somewhat lower levels in low host density years (1988, 1990, 1991) (Table 4.1).

Does viral infection vary with host density? Population density of tent caterpillars is determined by the number of family groups indicated by the tents and the number of caterpillars in the families which is indicated by
Virus in Western Tent Caterpillars

the size of the tent (length x width). The percentage of larvae positive for virus appears to be related to the density of the populations measured by the number of tents. Also, more caterpillars were positive for virus as tent size increased at Cypress with a similar trend occurring at Galiano. This result is surprising since one might expect that deaths of early instars caused by virus would reduce the family size and therefore the tent size of late instars. Egg mass size did not vary with the occurrence of virus so it is unlikely that infected families started with more individuals. More likely explanations are that virus infection spreads more efficiently among individuals in large groups than in small groups or that the presence of virus causes infected individuals to be more active or produce more silk which enlarges the tents.

Other studies have had mixed results when examining the relationship between host density and levels of infection. There are examples of a positive relationship between the pathogen and host density (Jaques and Harcourt 1971 with Trichoplusia ni and Pieris rapae, Carter et al. 1983 with Tipula paludosa, and Kaupp 1983 with Neodiprion sertifer) and examples where there is no correlation between virus prevalence and the host density (Benz 1987).
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Does incidence of virus predict future population trends? Although levels of infection were higher in populations at high density, the percentage infection was not related to changes in population numbers between years when data from all sites and years were analyzed. For example, 40% of tested individuals from Cypress were positive for virus in 1992 and the population declined while 50% of the sample from the population at Mandarte were infected in 1994 and the population numbers and percentage infection increased in 1995. It could be that the errors in estimating the proportion of caterpillars infected are so large that predictions cannot be robust or that factors causing changes in population density vary between sites so that mortality from viral infection makes different contributions to population change in different areas.

Is virus maintained in low density populations? Caterpillars infected with virus occurred in the 2 years of lowest density (1990, 1991) in 4 of the 6 samples collected. Therefore virus appears to be maintained at low levels in years of low host density. The maintenance of virus at low host density is important for transmission to the next generation.

Are tents an environmental source of virus? Virions are protected by their proteinaceous occlusion bodies and have
been shown to persist in the soil or duff (Thompson et al. 1981, Weseloh and Andreadis 1986, Olofsson 1988a), on the bark of trees (Podgwaite et al. 1979, Woods et al. 1989), and in host cadavers (Clark 1955), but viral activity is short lived on foliage due to breakdown by uv in sunlight (Jaques 1975, Richards and Payne 1982). There are very few studies that directly measure the environmental contamination of NPV since most use a bioassay of potential infected material against insects in the laboratory. While this is important for determining infectivity it may be compromised by differences in susceptibility of laboratory colonies versus field animals, and by different diets (Watanabe and Imanishi 1980).

Table 4.6 presents the results of studies in the literature recording the percentage of caterpillars which died from viral infection given extracts of environmental material. The levels of infection recorded for tent material in years of high tent caterpillar density varies within the range of values resulting from assays of bark or soil samples in other studies. At low host densities there appeared to be very little viral contamination of tents. The incidence of virus in tents is lower than that of caterpillars in the same years. This may be due in part to abnormal behaviour of infected caterpillars before death.
Virus in Western Tent Caterpillars

Infected insects have been observed to climb to the tops of trees and plants (Watanabe 1987) and to cause abnormal dispersal in gregarious insect species (Smirnoff 1965). Smirnoff noted that infected insects could wander from their usual food plants, lose their gregarious habits and feed solitarily, and no longer return to a communal resting place. Therefore, tents would not be contaminated if caterpillars died elsewhere. Another bias could occur if viral infection killed the whole colony at an early stage. These small tents would not have been included in samples of tents associated with late instar caterpillars. Tents with caterpillars that have been killed by virus can be seen in field populations. This bias would also influence the proportion of infected caterpillars occurring in samples of late instars since whole families could have died as early instars.

It appears that virus on tent material can cause infection in caterpillars and contaminated tents are possibly a source of virus in high density populations. The number of infected tents was highest in the early stage of population decline (1987).

In chapter 3, forest tent caterpillars were monitored for viral infection for five years, from peak density to decline. Results indicated that generally viral infection
Virus in Western Tent Caterpillars

was higher in years of high host density than at low density. In addition, viral infection does seem to persist in low density populations in both species of tent caterpillar.

Pathogens and their host have a complex relationship. It involves both host infection levels and environmental contamination. Both of these are strongly influenced by the host population density. Although current evidence using models suggests that host-pathogen interactions alone do not generate forest insect cycles, virus disease can still have significant effects on caterpillar dynamics (Berryman 1996, Bowers et al. 1993). While other factors such as parasitoids, foliage quality, and maternal effects may play a significant role, viral diseases that infect a large portion of the host population can destabilize population through increasing mortality and reducing fecundity through sublethal effects (Myers and Kukan 1995).
Table 4.1: Percentage of caterpillars positive for viral infection in four study sites. Sample size in parentheses.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>CYPRESS</th>
<th>MANDARTE</th>
<th>GALIANO</th>
<th>WESTHAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>----</td>
<td>39 (52)</td>
<td>----</td>
<td>20 (20)</td>
</tr>
<tr>
<td>1985</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>23 (103)</td>
</tr>
<tr>
<td>1986</td>
<td>----</td>
<td>34 (170)</td>
<td>----</td>
<td>43 (110)</td>
</tr>
<tr>
<td>1987</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>1988</td>
<td>0 (11)</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>1989</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>1990</td>
<td>21 (61)</td>
<td>----</td>
<td>0 (5)</td>
<td>6 (69)</td>
</tr>
<tr>
<td>1991</td>
<td>15 (319)</td>
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</tr>
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<td>1992</td>
<td>40 (38)</td>
<td>27 (22)</td>
<td>----</td>
<td>38 (85)</td>
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<td>1993</td>
<td>11 (70)</td>
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</tr>
<tr>
<td>1994</td>
<td>20 (20)</td>
<td>49 (53)</td>
<td>48 (120)</td>
<td>----</td>
</tr>
<tr>
<td>1995</td>
<td>17 (233)</td>
<td>73 (11)</td>
<td>74 (39)</td>
<td>----</td>
</tr>
</tbody>
</table>
Table 4.2: Percentage of samples of tent material positive for viral contamination. Sample size in parentheses.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>CYPRESS</th>
<th>MANDARTE</th>
<th>GALIANO</th>
<th>WESTHAM</th>
</tr>
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<td>21 (43)</td>
</tr>
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<td>1988</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>0 (6)</td>
</tr>
<tr>
<td>1990</td>
<td>0 (16)</td>
<td>0 (5)</td>
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<td>0 (24)</td>
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<tr>
<td>1991</td>
<td>1 (77)</td>
<td>0 (12)</td>
<td>0 (4)</td>
<td>0 (7)</td>
</tr>
<tr>
<td>1992</td>
<td>2 (55)</td>
<td>13 (15)</td>
<td>3 (32)</td>
<td>0 (31)</td>
</tr>
<tr>
<td>1993</td>
<td>6 (16)</td>
<td>0 (15)</td>
<td>3 (65)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>1994</td>
<td>33 (6)</td>
<td>9 (33)</td>
<td>8 (38)</td>
<td>0 (3)</td>
</tr>
<tr>
<td>1995</td>
<td>-----</td>
<td>15 (20)</td>
<td>15 (20)</td>
<td>5 (20)</td>
</tr>
</tbody>
</table>
Table 4.3: ANOVA and least squared means of variation in tent size (length x width) for tent caterpillar families with some individuals testing positive for virus and those for which no caterpillars were positive.

<table>
<thead>
<tr>
<th>SITE</th>
<th>YEAR</th>
<th>F</th>
<th>P</th>
<th>NO VIRUS</th>
<th>VIRUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MEAN</td>
<td>SE. n</td>
<td>MEAN</td>
<td>SE. n</td>
</tr>
<tr>
<td>Galiano</td>
<td>1994</td>
<td>2.9</td>
<td>0.11</td>
<td>68  41  3</td>
<td>143 16</td>
</tr>
<tr>
<td>Mandarte</td>
<td>All</td>
<td>3.4</td>
<td>0.08</td>
<td>152 29  8</td>
<td>86  20</td>
</tr>
<tr>
<td>Westham</td>
<td>All</td>
<td>0.1</td>
<td>0.81</td>
<td>59  5  31</td>
<td>57   7  16</td>
</tr>
<tr>
<td>Cypress</td>
<td>All</td>
<td>17.0</td>
<td>0.00</td>
<td>66  7  44</td>
<td>101  6  55</td>
</tr>
<tr>
<td>Cypress</td>
<td>1991</td>
<td>17.0</td>
<td>0.00</td>
<td>79  7  29</td>
<td>120  7  27</td>
</tr>
</tbody>
</table>
Table 4.4: Percentage of tent caterpillars positive for NPV of those collected as early instars (first to early third) and late instars (late third to fifth). Sample size in parentheses. (* indicates significant difference between age groups).

<table>
<thead>
<tr>
<th>YEAR</th>
<th>SITE</th>
<th>EARLY INSTARS</th>
<th>LATE INSTARS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PERCENTAGE OF CATERPILLARS INFECTED</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EARLY INSTARS</td>
<td>LATE INSTARS</td>
</tr>
<tr>
<td>1990</td>
<td>Cypress</td>
<td>17 (30)</td>
<td>54 (13) *</td>
</tr>
<tr>
<td></td>
<td>Westham</td>
<td>2 (53)</td>
<td>19 (16)</td>
</tr>
<tr>
<td>1991</td>
<td>Cypress</td>
<td>16 (95)</td>
<td>15 (224)</td>
</tr>
<tr>
<td></td>
<td>Westham</td>
<td>0 (8)</td>
<td>0 (7)</td>
</tr>
<tr>
<td>1992</td>
<td>Mandarte</td>
<td>20 (5)</td>
<td>29 (17)</td>
</tr>
<tr>
<td></td>
<td>Westham</td>
<td>27 (55)</td>
<td>57 (30) *</td>
</tr>
<tr>
<td>1993</td>
<td>Cypress</td>
<td>5 (20)</td>
<td>14 (50)</td>
</tr>
<tr>
<td>1994</td>
<td>Galiano</td>
<td>0 (17)</td>
<td>56 (103) *</td>
</tr>
<tr>
<td>1995</td>
<td>Cypress</td>
<td>5 (43)</td>
<td>20 (190) *</td>
</tr>
</tbody>
</table>
Table 4.5: Percentage of field-collected caterpillars in studies reported in the literature from unsprayed areas infected with NPV. Sample size in parentheses.

Early instars: 1st to early 3rd  Late instars: late 3rd to 5th

<table>
<thead>
<tr>
<th>HOST</th>
<th>% LARVAE INFECTED</th>
<th>DETECTION METHOD</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EARLY</td>
<td>LATE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INSTAR</td>
<td>INSTAR</td>
<td></td>
</tr>
<tr>
<td>HIGH DENSITY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>5-28 (450)</td>
<td>53-91 (450)</td>
<td>Mortality</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Woods et al. 1990</td>
</tr>
<tr>
<td>Malacosoma californicum pluviale</td>
<td>0-5 (60)</td>
<td>20-74 (416) DNA Hybrid.</td>
<td>Kukan unpublished</td>
</tr>
<tr>
<td>LOW DENSITY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malacosoma californicum pluviale</td>
<td>0-17 (191)</td>
<td>0-54 (287) DNA Hybrid.</td>
<td>Kukan unpublished</td>
</tr>
</tbody>
</table>
Table 4.6: Survey of the percentages of infection in larvae inoculated with environmental material contaminated with NPV in samples collected from unsprayed areas. Virus was identified by bioassay except for tents which were assayed by DNA hybridization and shows percentage of tents infected with NPV. Samples size in parentheses.

**Lymantria dispar**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% INFECTION</th>
<th>DENSITY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bark</td>
<td>17-44 (150)</td>
<td>High</td>
<td>Woods et al. 1989</td>
</tr>
<tr>
<td>Soil</td>
<td>&lt;7 (89)</td>
<td>High</td>
<td>Weseloh and Andreadis 1986</td>
</tr>
<tr>
<td>Litter</td>
<td>&lt;7 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bark</td>
<td>11 (89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pupal exuviae</td>
<td>55 (90)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Orgyia pseudotsugata**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% INFECTION</th>
<th>DENSITY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>20 (75)</td>
<td>41 yr post outbreak</td>
<td>Thompson et al. 1981</td>
</tr>
</tbody>
</table>

**Malacosoma californicum pluviale**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% INFECTION</th>
<th>DENSITY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tents</td>
<td>0-50 (185)</td>
<td>High</td>
<td>Kukan</td>
</tr>
<tr>
<td></td>
<td>0-1 (145)</td>
<td>Low</td>
<td>unpublished</td>
</tr>
</tbody>
</table>
Figure 4.1: Number of tents counted and the percentage of western tent caterpillars infected with NPV (95% CI determined from the binomial distribution) for each year at the Cypress site.
Figure 4.2: Number of tents counted and the percentage of western tent caterpillars infected with NPV (95% CI determined from the binomial distribution) for each year at the Mandarte site.
Figure 4.3: Number of tents counted and the percentage of western tent caterpillars infected with NPV (95% CI determined from the binomial distribution) for each year at the Galiano site.
Figure 4.4: Number of tents counted and the percentage of western tent caterpillars infected with NPV (95% CI determined from the binomial distribution) for each year at the Westham site.
Figure 4.5: Relationship between the number of tents counted and the percentage of samples infected with NPV. Pearson correlation coefficient $r = 0.66$, $p = 0.01$. 
CHAPTER 5

OCCURRENCE OF VIRUS IN PUPAE AND ADULTS

5.1 INTRODUCTION

Transmission of disease determines the spread and the persistence of pathogens and their influence on host population dynamics. In field populations of insects, transmission of pathogens probably consists of a combination of horizontal transmission and vertical transfer (Fine 1984). In horizontal transfer, pathogens are transmitted among individual hosts within a generation and between generations as environmental contamination, while vertical transfer occurs from parents to offspring (Andreadis 1987). A potentially important mode of transmission in insects is transstadial transmission. This transfer of a pathogen from one host stage to the next through the life cycle can be a means of maintaining viral infection in a host population in subsequent generations. Such details of transmission as whether the pathogen is transferred from host to host, survives in the environment, or is passed through the life stages of the insect, could influence the effectiveness of pathogens in control programs for forest or agricultural pests. The timing, frequency, and patterns of spraying of
Occurrence of Virus in Pupae and Adults

virus as a bioinsecticide will be influenced by the spread and persistence of virus in the host population.

The least understood and yet most crucial stage in the dynamics of viral infection is persistence of virus between host generations (Beukema 1992). Many studies have been done on transmission of NPV and results have varied. To determine if any patterns emerged from these observations, I have surveyed the literature for studies which recorded the carryover of virus from one year to the next. This research was divided into studies of transmission from parent to progeny, transmission from generation to generation, and studies of transstadial transmission. Experiments which used laboratory colonies of insects experimentally exposed to virus were separated from those which used field insects.

In addition, I investigated the transstadial and parent to progeny transmission of nuclear polyhedrosis virus (NPV) in field populations of tent caterpillars. To do this, I employed a polymerase chain reaction (PCR) detection assay to screen for virus in pupae and moths which have survived infection. Western and forest tent caterpillars have not previously been studied for transstadial transmission using PCR for viral detection in pupae and adults.
5.2 MATERIALS AND METHODS

5.2.1 Viral DNA and PCR Primers

PCR primers were prepared based on the nucleotide sequence of the ends of the 1.6-kb MpNPV DNA probe prepared previously for dot-blot hybridization assay of tent caterpillars (Kukan and Myers 1995). The viral probe was prepared from a DNA fragment obtained by digestion of NPV DNA isolated from infected Malacosoma californicum pluviale (Mp) caterpillars and cloning into the bacterial plasmid pGEM-3zf(+). Nucleotide sequence analysis was done by the NAPS Unit at the University of British Columbia on a double stranded DNA plasmid template using the chain termination method of Sanger et al. (1977). Primers of 21 nucleotides were prepared which had 62% G+C composition:

forward primer: 5'- ACGCATCGATCGTTCCACCGC -3'
and reverse primer: 5'- TACAGGCACCTGGCCGTACAC -3'.

5.2.2 PCR Amplification of Viral DNA Sequences

PCR was used to amplify viral DNA sequences. Each reaction mixture consisted of 1.0 µl of sample (DNA template), 2 mM MgCl₂, 10 U Taq DNA polymerase, 100 µM of each of four dNTPs, 30mM Tris Base pH 9.1, and 1 µM of both the forward and reverse primers. To each reaction mix 1 µl
of 10% skim milk powder was added to reduce inhibition of the reaction (De Boer et al. 1995). Sterile, filtered, distilled water was added to achieve a final volume of 50 μl. Each reaction mixture was overlaid with 30 μl light mineral oil. PCR reactions were performed with a Hybaid Omnigene thermal cycler using conditions recommended by the manufacturer. Cycling parameters were an initial 3 min denaturation at 94°C and then 40 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. A final extension step of 10 min at 72°C was performed.

For each PCR set, a positive control of 1ng pure MpNPV DNA, and a negative control of no DNA template was used. In addition along with unknown samples, control caterpillars, pupae, and moths were tested as negative controls and samples of control insect DNA (from caterpillars, pupae and moths) to which 1ng pure MpNPV DNA had been added were used as positive controls.

5.2.3 Analysis of PCR Products

The products of each PCR reaction were analyzed by agarose gel electrophoresis. After amplification, 25 μl of each reaction mixture was loaded onto a 0.7% agarose gel and subjected to electrophoresis at 100 V for 1.5 hr. A positive
amplification resulted in a fragment of about 1000 bp in length (Figure 5.1).

5.2.4 Occurrence of Virus in Pupae and Adults

To determine if virus is carried over from late instar caterpillars to pupae and adults, caterpillars from 15 different egg masses were inoculated as fifth instars. Virus was presented on 1-cm-diameter discs of red alder leaves in individual cups and caterpillars were left for 24 hr. Those that did not consume the leaf disc were discarded. One group of 426 larvae was given a dose of $3.6 \times 10^4$ PIBs (low) within 24 hr of having moulted to fifth instar. Control insects (200) were given an untreated leaf disc. An additional 88 fifth instar larvae were given a dose of $3.6 \times 10^6$ PIBs (high) a week after moulting to fifth instar. Larvae were examined daily and survivors were allowed to pupate at which time some were frozen and the others were left for adult emergence and then frozen.

5.2.5 Evidence of Infection

To interpret results it is necessary to know if individuals experimentally inoculated and evaluated with PCR had been infected with virus. Infection is indicated if exposed individuals differ from controls in size of pupae,
differ in time to pupation, and if mortality of inoculated caterpillars occurs. There was no significant difference in percent successful pupation in the high dose vs low dose groups. The percentage of caterpillars which pupated successfully was higher for controls than for caterpillars receiving either a high or low dose of virus (Table 5.1). Rate of development did not differ.

A high dose of virus reduced the pupal weight of tent caterpillars as a sublethal effect (Table 5.1). For females and males, the mean weight of control pupae is significantly different from that of the high dose group but not from the low dose group. This indicates that at least the individuals that received a high dose of virus are likely to have experienced infection prior to pupation, and therefore should provide a relevant sample for transmission of virus to pupal and adult stages.

5.2.6 Sample Preparation for PCR

Crude homogenate preparations of western tent caterpillars were used in initial experiments to test the sensitivity of the PCR assay. Caterpillars were ground in a microfuge tube and the liquid portion was removed from the solid debris and centrifuged at 14,000 rpm. The supernatant was removed and the pellet was suspended in 50 µl 0.1M
Occurrence of Virus in Pupae and Adults

Na₂CO₃ pH 10 and incubated for 1 hr at 37°C. Two volumes of 100% ethanol were added and samples were frozen overnight. Samples were then centrifuged for 5 min at 14,000 rpm, the pellet dried under vacuum and resuspended in 100 µl sterile distilled water.

Pupae and adults developing from caterpillars which had been fed virus were surface decontaminated by immersion in 10% bleach for 30 sec, rinsed 2X with distilled water, and dried on Kimwipes. This was done to eliminate any surface contamination of NPV. Control pupae and adults were not surface decontaminated. All samples were then cut into small pieces with scissors, and macerated (on ice) with a plastic pestle in 300 µl extraction buffer (0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.1% SDS). Scissors and pestles were cleaned in 10% bleach before reuse. The liquid portion was removed from solid debris and centrifuged for 5 min at 4°C and 14,000 rpm in an Eppendorf microfuge and the pellet resuspended in 200 µl 0.1 M Na₂CO₃ pH 10.0 and incubated at 37°C for 1 hr. Following the addition of 500 µl of the extraction buffer and 100 µg Proteinase K, the tubes were incubated overnight at 37°C. Samples were then extracted with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). After ethanol precipitation, samples were resuspended in 100 µl sterile
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distilled water and frozen until needed. DNA from pupae and adults was diluted 1/10 for use in the PCR reaction mix.

5.2.7 Field-Collected Pupae

In 1992 and 1993, pupae of forest tent caterpillar (Malacosoma disstria) were collected from a declining population near Prince George British Columbia. In 1992, 33 pupae were collected and frozen. In 1993, 411 pupae were collected and 163 frozen immediately while the others were left to emerge as adults. From these, 143 adults were obtained while the others did not emerge successfully or were parasitized. These samples were macerated and extracted as per the methods of sample preparation for PCR. After ethanol precipitation, samples were resuspended in 50 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and frozen until needed. Twenty µl of these samples was dot-blotted and hybridized to a MpNPV DNA probe as outlined by Kukan and Myers (1995).

5.2.8 Transfer of Virus from Surface Decontaminated Egg Masses to Hatching Caterpillars

Egg masses of western tent caterpillar (Malacosoma californicum pluviale)(Mp) were collected from a high density population near the inner harbour in the city of Victoria, British Columbia in the autumn of 1993. The next
Occurrence of Virus in Pupae and Adults

spring, these were surface decontaminated with a 2% solution of sodium hypochlorite (30% bleach) for 5 min, rinsed 2X with distilled water, and dried on paper towels. Each egg mass was placed in a separate paper cup until caterpillars hatched. Since this species is gregarious, larvae were kept together until they reached either third or fifth instar. Caterpillars were fed red alder (Alnus rubra) leaves which were washed with 10% bleach, rinsed 2X with distilled water, and dried. Caterpillars were kept in a controlled environment chamber at 26°C with an 18-hr photoperiod.

To determine if caterpillars from decontaminated egg masses carried virus, caterpillars were tested using the dot-blot hybridization assay when they reached third instar (Kukan and Myers 1995). At this time, 120 individuals from 12 families (10 per family) were frozen for later analysis.

5.3 RESULTS

5.3.1 Sensitivity of PCR Assay

Testing of the PCR assay indicated that the primers gave a sensitivity of between 1fg and 5fg with pure MpNPV DNA (Figure 5.1) as well as with pure MpNPV DNA added back to negative caterpillar DNA. Negative caterpillar DNA was obtained from western tent caterpillars reared from surface
decontaminated egg masses (Kukan and Myers 1995). Positive PCR reactions were obtained in 83% \( (n=42) \) of crude homogenate preparations of caterpillar samples known to be positive from the DNA hybridization assay (strong to weak). When samples were phenol-chloroform extracted, 98% \( (i.e. 41 of 42) \) gave a positive PCR reaction. The one sample which gave a negative result did not give a positive reading even when \( 1 \text{ng} \) of pure MpNPV DNA was added to it. This indicates the presence of inhibitory factors in this sample. A positive PCR reaction was obtained when \( 1 \mu l \) MpNPV PIBs was incubated for 1 hr at \( 37^\circ C \) in \( 0.1M \) Na\(_2\)CO\(_3\) pH 10 and diluted 1/1000. A positive PCR reaction was not obtained when pure MpNPV DNA was added to the reaction mix in the absence of polymerase \( (n=2) \); if primers were omitted \( (n=2) \); if one primer was left out \( (n=3) \); or when DNA from uninfected caterpillars was added \( (n=16) \).

5.3.2 PCR Assay on Western Tent Caterpillar Pupae and Adults

Of the 70 pupae which had been fed a low dose of virus as caterpillars, only 1 gave a positive PCR reaction. Of 20 pupae which had been fed a high dose of virus as caterpillars, 2 gave a positive PCR reaction. However, of the 50 control pupae, 3 also gave a positive PCR reaction.
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Thus, there is no significant difference between infection found in control and virus treated pupae.

No moths were positive for viral infection with PCR reactions among controls ($n=40$), those fed a low dose ($n=54$), nor those fed a high virus dose ($n=11$).

5.3.3 Field Samples of Forest Tent Caterpillar Pupae and Adults

Of 33 forest tent caterpillar pupae collected in the field in 1992 and tested by dot-blot hybridization, only one was positive for virus. In 1993, when the population density was low, none of the 163 forest tent caterpillar pupae tested were positive for virus. No virus was detected in 143 forest tent caterpillar adults emerging from pupae collected in 1993.

5.3.4 Virus in Larvae from Field-Collected Egg Masses

In 1993 (Kukan and Myers 1995) and 1994, third instar larvae from increasing populations (Chapter 2) were reared from field-collected egg masses which had been surface decontaminated to remove external NPV infection. These were tested for the incidence of virus using dot-blot hybridization. The levels of infection were 2% in 1993 ($n=61$) and 2.5% in 1994 ($n=120$).
5.4 DISCUSSION

To put my results in perspective with other studies, I have surveyed the literature and summarized results in Tables 5.2-5.4. Studies summarized in Table 5.2 determined the occurrence of virus in offspring of control insects in comparison to that of parents which were experimentally infected with virus. For the nine studies surveyed, the percentage of progeny with virus ranged from 0 to 8 for control insects and 0.36 to 94.3 in offspring of parents inoculated with virus as caterpillars. Two studies concluded that no transmission to progeny occurred (Doane 1969 and Young 1990). Murray and Elkinton (1989) interpreted the small amount of viral infection, less than 2% in both control and infected groups, as being due to inadvertent laboratory contamination.

Studying the transmission from parent to progeny in field insects is much more difficult because environmental contamination or transgenerational, horizontal transmission is nearly impossible to distinguish from vertical transmission. There were three studies where field collected egg masses were surface decontaminated and the progeny examined for incidence of virus (Table 5.3). The levels of infection were between 0 to 9.0%. The studies of field insects (Table 5.4) are examples of generation to generation
transmission since environmental contamination cannot be ruled out, but they may involve parent to progeny transmission. Without surface decontamination of eggs, the incidence of virus in progeny from control areas ranged from 0 to 82% and from virus treated areas from 0 to 81%.

Studies of viral infection in pupae and adults (Table 5.5) in laboratory reared insects indicate 0.03 to 0.7% of control pupae with virus (2 studies) and 0.23 to 0.5% of control adults with virus (1 study). Virus treated insects had 0.8 to 100% pupae with virus (6 studies) and 4.9 to 43.9% of adults with virus (3 studies). The other laboratory studies either did not measure incidence of infection or found none. There was only one transstadial study using field insects which reported no virus in pupae or adults. My results are included in the table for comparative purposes.

The majority of the studies of viral infection in pupae and adult moths, and of parent to progeny transmission, have measured infection by mortality and microscopic examination of cadavers for occlusion bodies (PIBs). The lowest level of detection microscopically is about 1x10^6 PIBs (Kaupp and Ebling 1993). DNA dot-blot hybridization can detect between 0.1ng and 1ng viral DNA which would be the equivalent of between 20-5000 PIBs (Ward et al. 1987, Kaupp and Ebling 1993, Keating et al. 1989, Kukan and Myers 1995). Using the
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PCR assay for virus detection increases the sensitivity to between 1fg and 5fg of pure MpNPV DNA. Therefore, if virus is present at these low levels it should still be detected. Also, these last two techniques can detect virus before polyhedra are formed.

In contrast to dot-blot methods, it was necessary to extract DNA from samples for use in PCR assay and samples of insects infected with virus had to be diluted to get a successful PCR result. This limits the number of samples that can be analyzed and greatly increases the cost of survey work.

Four percent of 140 tent caterpillar pupae, including both controls and virus inoculated specimens, were found to be positive for the presence of virus with the PCR assay. Only one of 196 (i.e. 0.5%) field-collected forest tent caterpillar pupae from a declining population was positive for NPV. Studies by other investigators (Table 5.5) have found that pupae developing from caterpillars treated with virus tend to have higher percentages of infection than controls.

The levels of infection in pupae determined by this study (0.5 to 4%) are similar to levels of infection of control larvae for both parent to progeny (Table 5.2) and transstadial (Table 5.5) studies (0 to 8%) in the
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literature. My results for infection levels in larvae that were reared from decontaminated field-collected egg masses were within this range (0 to 2.5%) and agree with field insect studies using decontaminated egg masses (0 to 9%, Table 5.3). In addition, the levels of infection in control larvae prior to spray (Table 5.4) in 5 of 7 studies are in the range from 0 to 10%. Low levels of infection tend to be dismissed as being due to contamination, but the fact that they appear in so many independent studies makes this explanation seem less likely. These results suggest that in the species of forest Lepidoptera that have been examined a low level of NPV infection persists. After spraying or in high density host populations, infection levels rose dramatically (from 1.6 to 28%, 0 to 78% and 0.5 to 17% in the studies by Otvos et al. 1989, Olofsson 1988b, and Olofsson 1989).

While no tent caterpillar moths in this study were positive for virus, approximately a third of the studies reviewed from the literature found virus in some adults. The incidence of infection in progeny supports the possibility of vertical transmission. It may be that virus is present in adults but in amounts below the detection levels of even the sensitive PCR assay. When 1ng of pure NPV DNA was added to a negative adult sample, a positive result was obtained
indicating that the PCR reaction was not inhibited by contaminants.

How much virus is necessary to cause an infection in an offspring? It may only take one PIB or one virion. Selection for *Spodoptera frugiperda* with an increased rate of vertical transmission (Fuxa and Richter 1991) suggests a genetic basis for vertical transmission. Viral infection could be maintained at a low level in the population if only a small number of infected individuals exist.

When larvae are infected with virus, some pupae and adults survive. How do the pupae and adults avoid infection and death? Stairs (1965) proposed that the development of disease could be interrupted by metamorphosis. This maturation immunity or hormonal control of virus replication as a result of changes in cell metabolism has been supported by others (Evans 1983, Whitlock 1977) but the mechanism is not understood. Recent work (O’Reilly and Miller 1989) has identified a viral gene which may influence host hormonal levels. Increased knowledge of the interplay of viral genes and host hormones will contribute to our understanding of maturation immunity.

Another method of avoiding infection may be linked to developmental resistance or the increased resistance of larvae to baculovirus infection with age (Stairs 1965,
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Whitlock 1977, Evans 1983, Engelhard and Volkman 1995). Resistance may be attributed in part to the sloughing off of infected midgut cells and the ability to clear infection in midgut epithelium by moulting (Engelhard and Volkman 1995). These processes may decrease the chances of virus becoming established as a systemic infection which would carry through to later life stages. Many insects may avoid infection in this way while others may become infected at low levels and pass the infection to their progeny.

Horizontal transmission of virus both within a generation and between generations as environmental contamination and contamination of eggs can dominate when host population densities are high. Even low levels of infection of adults associated with vertical transmission can allow the pathogen to persist in relatively low host densities and maintain a source of inoculum for epizootics at high host densities.

This and other studies indicate that virus may be maintained and transmitted from caterpillar to pupae at low levels but is very rarely found in adults. However, a high level of virus transmission between generations can occur with egg masses and virus sometimes even occurs in caterpillars hatching from surface decontaminated egg masses (<2%). It seems likely that persistence of virus on egg
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masses is the primary cause of transmission between
generations and future models of the dynamics of NPV in
populations of forest Lepidopterans should incorporate this
feature.
Table 5.1: Effect of MpNPV infection on pupation success (%) and pupal weight (g) of western tent caterpillars. 

\( n = \) sample size. \( SE = 0.01 \) for all means of pupal weights.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>LOW DOSE</th>
<th>HIGH DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUCCESS</td>
<td>56%</td>
<td>34%</td>
<td>39%</td>
</tr>
<tr>
<td>WEIGHT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEMALES</td>
<td>0.49</td>
<td>0.46</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>( n=26 )</td>
<td>( n=31 )</td>
<td>( n=15 )</td>
</tr>
<tr>
<td>MALES</td>
<td>0.27</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>( n=27 )</td>
<td>( n=39 )</td>
<td>( n=14 )</td>
</tr>
</tbody>
</table>

Pupation success: Yates corrected \( \chi^2 \); low dose compared to control = 24.47, \( df=1, \) \( p<0.001 \); high dose compared to control = 6.29, \( df=1, \) \( p<0.05 \).

Pupal weight of controls significantly greater than pupal weight of those infected with a high dose. 
Females \( F=36.90 \) (Tukey test \( p<0.05 \))
Males \( F=10.06 \) (Tukey test \( p<0.05 \))

Pupal weight of low dosed significantly different from pupal weight of those infected with high dose. 
Females \( F=36.90 \) (Tukey test \( p<0.05 \))
Table 5.2: Survey of the percentages of infected progeny of experimental moths infected with NPV as caterpillars and uninfected control moths. Viral detection was by death and microscopic examination. Number of offspring tested in parentheses.

<table>
<thead>
<tr>
<th>HOST</th>
<th>CONTROL</th>
<th>EXPOSED TO NPV</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heliothis zea</em></td>
<td>0-7.1 (91)</td>
<td>0.4-94.3 (176)</td>
<td>Hamm and Young 1974</td>
</tr>
<tr>
<td>(adults fed $6.2 \times 10^5$-$5.9 \times 10^8$ PIBs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mythimra separata</em></td>
<td>2.0 (199)</td>
<td>16.5-57.1 (1128)</td>
<td>Neelgund and Mathad 1978</td>
</tr>
<tr>
<td>(6th instars fed $10^7$-$10^8$ PIBs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudoplusia includens</em></td>
<td>0.5-2.3 (?)</td>
<td>3.4-8.0 (?)</td>
<td>Young and Yearian 1982</td>
</tr>
<tr>
<td>(4,5,6th instars fed $4 \times 10^3$-$12 \times 10^5$ PIBs/mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera littoralis</em></td>
<td>1-8 (200)</td>
<td>9-48 (200)</td>
<td>Abul-Nasr et al. 1979</td>
</tr>
<tr>
<td>(3-5th instars fed $1.2 \times 10^6,10^7,10^8$ PIBs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera exigua</em></td>
<td>4.2 (60)</td>
<td>10.0-28.0 (60/group)</td>
<td>Smits and Vlak 1988</td>
</tr>
<tr>
<td>(5th instars fed $1 \times 10^5,2 \times 10^5,1 \times 10^6$ PIBs)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Spodoptera frugiperda**  0.3-0.8  3.6-23.1 (300/group)  Fuxa and Richter 1991
(3-5th instars fed $5 \times 10^4$-$3.5 \times 10^7$ PIBs)

**Lymantria dispar**  2.5-5.0 (540-720)  35.0 (340)  Murray and Elkinton 1990
(Bark drenched $5 \times 10^8$ PIBs /ml)

**Lymantria dispar**  <2.0  <2.0  Murray and Elkinton 1989
(2nd instars fed $5 \times 10^3$ PIBs)

**Lymantria dispar**  0 (1440)  4.7-11.5  Shapiro and Robertson 1987
(2nd instars fed 0.1-10 PIBs/mm$^2$)
Table 5.3: Survey of the percentages of infected larvae or egg masses with infected larvae in offspring of parents exposed to naturally occurring virus in the field as caterpillars. Egg masses were either untreated or surface decontaminated. Virus was detected by death and microscopic examination. Sample size in parentheses.

<table>
<thead>
<tr>
<th>HOST</th>
<th>% LARVAE INFECTED</th>
<th>COMMENTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Decontaminated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>&lt;10-80</td>
<td>0-0.1</td>
<td>% infected larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000/group</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1% bleach</td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>2-28</td>
<td>0-0.7</td>
<td>% infected larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 egg masses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cut in half</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% formalin</td>
</tr>
<tr>
<td>Malacosoma pluviale</td>
<td>22 (36)</td>
<td>9 (35)</td>
<td>% egg masses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50% bleach</td>
</tr>
</tbody>
</table>
Table 5.4: Survey of percentages of field-collected caterpillars infected with NPV in areas which were sprayed or unsprayed in the previous generation. Egg masses were not surface sterilized. Virus was detected by death or microscopic examination except for study by Keating et al. 1991 who used DNA Hybridization. (number of larvae in parentheses unless stated otherwise, a prespray sample).

<table>
<thead>
<tr>
<th>HOST</th>
<th>UNSPRAYED PARENTS</th>
<th>SPRAYED PARENTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Orgyia pseudotsugata</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1982 low-10 a</td>
<td></td>
<td></td>
<td>Otvos et al. 1987</td>
</tr>
<tr>
<td>1983 i) 2-8 (1252)</td>
<td>19-41 (690)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1983 ii) 74-82 (426)</td>
<td>39-81 (112)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(collected i=3-4th instars, ii=5-6th instars from population in early phase of outbreak, sprayed 1982 1.6x10^{10}-2.5x10^{11} PIBs/ha, 4 control and 4 treatment plots)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| *Choristoneura occidentalis* | 1982 2 a (313) |                   | Otvos et al. 1989 |
| 1983 6-9 (454)              | 20-28 (299)    |                 |                   |
| 1984 0-0.2 (752)            | 0-0.8 (357)    |                 |                   |
| (collected 4th instars from 1 year old infestation) (Sprayed 1982 5.4x10^{11} PIBs/ha, 3 control, 2 treatment plots) |

<p>| <em>Neodiprion sertifer</em>      | 1979 0 a (6000 colonies) |                   | Olofsson 1988b |
| 1980 0-12                  | 8-50                  |                 |                   |
| 1981 0-66                  | 0-78                  |                 |                   |
| (100-200 colonies/block)   |                       |                 |                   |
| (3,4,5th instars collected from outbreak population and % infected colonies determined, sprayed 1979 4x10^{11} PIBs/ha, 2 control, 1 treatment block) |</p>
<table>
<thead>
<tr>
<th>HOST</th>
<th>UNSPRAYED PARENTS</th>
<th>SPRAYED PARENTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neodiprion sertifer</td>
<td>1977-87 0.5 a</td>
<td></td>
<td>Olofsson 1989</td>
</tr>
<tr>
<td></td>
<td>1979 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1977-87 reared larvae from 950 colonies and % colonies infected determined, 1979 natural epizootic, % infected of 60 colonies)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>4-26 a (200)</td>
<td></td>
<td>Keating et al. 1991</td>
</tr>
<tr>
<td>(larvae reared 5 days, egg masses collected from high density population)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>6-33 (1000/plot)</td>
<td></td>
<td>Woods et al. 1990</td>
</tr>
<tr>
<td>(larvae reared 2 weeks from egg masses collected from 26 high density plots)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>0-1 a (1350)</td>
<td></td>
<td>Cunningham et al. 1991</td>
</tr>
<tr>
<td>(1st instar larvae collected and smeared)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5: Survey of the percentages of pupae and adults infected with NPV of insects experimentally exposed to NPV as caterpillars in the laboratory or potentially exposed to virus under field conditions. Virus was detected by death and microscopic examination unless indicated otherwise. (C= control insects, VT= virus inoculated insects and sample size in parentheses).

<table>
<thead>
<tr>
<th>LABORATORY COLONIES:</th>
<th>% PUPAE WITH NPV</th>
<th>% ADULTS WITH NPV</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C VT</td>
<td>C VT</td>
<td></td>
</tr>
<tr>
<td><strong>Galleria mellonella</strong></td>
<td>NA 45.0</td>
<td>NA 6.0</td>
<td>Stairs 1965</td>
</tr>
<tr>
<td>(50 5th instars fed $10^7$ PIBs/larva)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymantria dispar</strong></td>
<td>0 (146) 0 (1100)</td>
<td>NA NA</td>
<td>Magnoler 1974</td>
</tr>
<tr>
<td>(3rd instars fed $2.5 \times 10^2-2.5 \times 10^6$ PIBs/larva)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heliothis armigera</strong></td>
<td>NA 0</td>
<td>NA 0</td>
<td>Whitlock 1977</td>
</tr>
<tr>
<td>(4200 all age instars fed $6 \times 10^5$ PIBs/larva)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pseudoplusia includens</strong></td>
<td>0-0.7</td>
<td>1.5-15.2</td>
<td>Young and Yearian 1982</td>
</tr>
<tr>
<td>(4,5,6ths fed $2 \times 10^3-8.6 \times 10^5$ PIBs/mm$^2$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mamestra brassicae</strong></td>
<td>NA 5-100</td>
<td>NA 0</td>
<td>Evans 1983</td>
</tr>
<tr>
<td>(40 5-6th instars per dose, $10^3-10^8$ PIBs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymantria dispar</strong></td>
<td>0 (100) 6-25 (950)</td>
<td>0 (160) 5-44 (1034)</td>
<td>Shapiro and Robertson 1987</td>
</tr>
<tr>
<td>(2nd instars fed 0.1-10 PIBs/mm$^2$ diet, measured yield of PIBs produced)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOST</td>
<td>% PUPAE WITH NPV</td>
<td>% ADULTS WITH NPV</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>C (V)</td>
<td>VT (V)</td>
<td></td>
</tr>
<tr>
<td>Lymantria dispar</td>
<td>0 (22) 20 (71)</td>
<td>0 (17) 0 (44)</td>
<td>Murray et al. 1991</td>
</tr>
<tr>
<td>(4-5th fed 7-1.5x10^5 PIBs/larva, DNA Hybridization for detection)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spodoptera frugiperda</td>
<td>&lt;.05 0.8-2</td>
<td>0.2-0.5 8-10</td>
<td>Fuxa and Richter 1991</td>
</tr>
<tr>
<td>(approx. 300/category)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malacosoma neustria</td>
<td>0 0</td>
<td>0 0</td>
<td>Magnoler 1975</td>
</tr>
<tr>
<td>(C total=108) (VT total=633)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(from decontaminated egg masses 10% formalin, 3-4th instars fed 3-3x10^5 PIBs/larva)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malacosoma californicum pluviale</td>
<td>6 (50) 3 (90)</td>
<td>0 (40) 0 (65)</td>
<td>Kukan (unpublished)</td>
</tr>
<tr>
<td>(from decontaminated egg masses 30% bleach, PCR for detection, 5th instars fed 3.6x10^4, 3.6x10^6 PIBs/larva)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malacosoma disstria</td>
<td>0.5 (199) NA</td>
<td>0 (143) NA</td>
<td>Kukan (unpublished)</td>
</tr>
<tr>
<td>(collected pupa field samples, DNA hybridization for detection)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1: Products of the PCR amplification of western tent caterpillar NPV DNA using MpNPV primers separated by agarose gel electrophoresis. Lane 1 contains 1 Kb DNA ladder, Lane 2 contains no DNA template, Lanes 3-7 contain MpNPV DNA in the following concentrations; 0.1ng, 10pg, 1pg, 0.1pg, and 1fg.
CHAPTER 6
CONCLUSIONS

The objective of this thesis was to quantify interactions between tent caterpillars and their nuclear polyhedrosis virus. Dot-blot hybridization and PCR assays were adapted for detection of western and forest tent caterpillar NPVs and used to quantify the incidence of virus over several generations of varying host density. Persistence of virus in tent material and maintenance of low levels of host infection were examined. In addition, transmission of virus to pupae and adults was tested.

Dot-blot hybridization was successful in detecting virus in tent caterpillar larvae. In Chapter 2, no difference was found between results using DNA extraction with phenol-chloroform and crudely homogenating caterpillars. Using crude homogenates is more efficient. Caterpillars can be collected and frozen for later analysis which avoids laborious laboratory rearing. Dot-blot hybridization of field-collected caterpillars frozen immediately after collection showed the same levels of infection as rearing caterpillars in the laboratory until pupation or death. Infection could be identified early in second instar larvae and consistently in second and third
Conclusions

instars. I recommend using the dot-blot hybridization assay on tent caterpillar larvae but found it less useful when testing tent material. Tent material sample preparation was time consuming and levels of virus were low.

Testing of the PCR assay indicated the primers gave a sensitivity of between 1fg and 5fg with pure MpNPV DNA, as well as with pure MpNPV DNA added back to negative caterpillar DNA (Chapter 5). Positive PCR reactions were obtained 98% of the time when compared to dot-blot results. I recommend making primers for another part of the genome so that the second set could be used to confirm virus detection. While the PCR assay is a more sensitive technique, samples required phenol-chloroform extraction. Therefore, the dot-blot hybridization assay is a better overall technique when processing large numbers of samples.

Infection by NPV commonly occurs in outbreak populations in forest Lepidoptera but little is known about viral infection in populations following decline to low density. I used dot-blot hybridization to identify viral infection in western and forest tent caterpillars from years of increasing and declining host densities.

In Chapter 3, populations of forest tent caterpillars at 4 sites near Prince George B.C. tended to decline from
Conclusions

high to low density over the 5 years of the study although variation in this pattern occurred. Viral infection was highest among caterpillars from dense and declining populations but persisted at low levels in sparse populations. Populations at two sites which erupted in the last two years of the study had moderate levels of infection comparable to populations which were at high density at the beginning of the study.

In Chapter 4, a temporal series of samples of larvae and tent material of the western tent caterpillar, which had been collected mainly over years of low to increasing host densities, were analyzed for viral infection using the dot-blot hybridization assay. Infection increased within a generation with later instar caterpillars tending to have higher levels of infection. The percentage of larvae positive for virus was significantly correlated to density of the population measured by the number of tents, but there was no association between viral infection and change in population numbers. Also, in one population families with virus had larger tents than those without virus. The trend was for higher incidence of virus at higher host densities. During the two years of lowest density, viral contamination was detected in 4 of the 6 samples collected. At low host
Conclusions

densities very little virus was detected in tents, but the number of contaminated tents seemed to increase with higher numbers of tents. Virus on tent material caused infection in caterpillars in a laboratory experiment and so contaminated tents could be a source of infection in high density populations. There was no relationship between egg mass size and viral infection.

I found the highest incidence of virus in peak host population years and into the first years of population decline. In years of low host density, it appeared that virus was maintained in the host at low levels. These observations are consistent with predictions of models of viral dynamics. In cases where long-term population fluctuations are observed, most of the models predict that disease incidence will be low in most years (Dwyer 1995).

I investigated the transstadial and parent to progeny transmission of NPV in field populations of tent caterpillars. Using a PCR assay, I searched for low levels of virus in pupae and moths which had survived viral infection. Low levels of virus were found in pupae, but no moths were positive for virus. Approximately 2% of caterpillars reared to third instar from surface decontaminated egg masses were positive for virus. I
Conclusions

reviewed the literature to determine the extent of evidence for cross-generational horizontal and vertical transmission. There appeared to be persistent low levels of viral infection, similar to levels I found in tent caterpillars, in some forest Lepidoptera and in caterpillars reared from surface decontaminated eggs. This could contribute to the persistence of virus in low density populations, but environmental contamination is probably the dominant source of virus among generations.

Sensitive and reliable tests for the identification and precise monitoring of NPVs in the field are essential for ecological and biocontrol studies. The dot-blot hybridization assay is an ideal tool for such studies. By using this assay, quantitative data on field studies can be collected. An important aim of ecological studies on the NPVs is to identify key factors in the population dynamics of the virus and to use them to optimize the conditions for biocontrol programs. With the development of genetically engineered NPVs renewed emphasis must be placed on the importance and significance of basic ecological studies of these viruses. Future work on the ecology and epizootiology of NPVs should focus on improving the quantitative data base on all aspects of virus ecology.


References


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