Naturally-Occurring Nuclear Polyhedrosis Virus for the Biological Control of Douglas-fir Tussock Moth, *Orgyia pseudotsugata*, McDunnough, (Lepidoptera: Lymantriidae)

by

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ABSTRACT

Studies were conducted to investigate the interaction of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae), and its naturally-occurring nuclear polyhedrosis virus.

Egg masses were collected from 42 locations in the Kamloops Forest Region of British Columbia in the fall of 1991 and 1992. Regression equations for the number of eggs per egg mass against the weight of the egg mass with and without the cocoon, area and volume of the egg mass, showed that weight without the cocoon is the most closely correlated with the number of eggs. The best estimate of the number of eggs per egg mass can be obtained from the weight without the cocoon, although all regression equations can be used but they are less accurate. The regression equations were created for each age of the outbreak (age 0, age 1 and age 2) and for each collection year (1991 and 1992). Since the regression equations were significantly different, except for weight without the cocoon in 1991, the area and volume of the egg mass in 1992 and the volume of the egg mass for age 1 when the two collection years were compared, they cannot be combined. The regression equations do not pass through the origin.

The incidence of NPV infection was higher in 1992 than in 1991, and higher in age 1 of the outbreak than age 2 of the outbreak. It was hoped that a

threshold level of NPV infection could be identified and that would allow forest managers to treat only those sites not close to natural collapse with NPV. However a trend of increasing or decreasing NPV infection that would allow such a prediction has not been established in this study. To ensure that the incidence of viral infection is not raised artificially in the laboratory, tussock moth egg masses should be stored dry in paper bags for diapause development conditions.

Late instar, virus infected larvae can be used for extraction of viral DNA and genotypic variation studies. When Southern blot analysis of viral DNA was completed, polymorphisms were identified when digested with *Pst* I, *Sal* I, *Bgl* II and *Hind* III restriction enzymes. Five genotypes were obtained when viral DNA was digested with Pst I and Sal I enzymes. Three of the Pst I genotypes are unique and two are only found in the Kamloops geographic region.

When viral DNA extractions were completed, a coincident infection with a cytoplasmic polyhedrosis virus was noted. The CPV viral RNA is resistant to digestion with RNAase A at high ionic strengths, but digests under low ionic strength. The pattern of the RNA segments resembles those previously published and is within the reported size range for the DFTM sock cytoplasmic polyhedrosis virus. The viral extracts do not hybridize to chemiluminescent labelled Douglas-fir tussock moth viral DNA, when Southern blotted.

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Chapter 1

Introduction

The Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae), is a periodic defoliator of Douglas-fir trees, *Pseudotsugata menziesii* var. *glauca* (Beissn.) Franco. and *Abies* spp. in the forests of Western North America (Mason and Wickman, 1988). Since the pest was first reported in British Columbia in 1916, there have been eight major outbreaks of DFTM (Harris *et al.*, 1985; Sugden, 1957); the ninth outbreak of DFTM began in 1990 and collapsed by 1993. These outbreaks varied in intensity from high populations only, to high populations with heavy defoliation and tree mortality (Alfaro *et al.*, 1987; Harris *et al.*, 1985). Tussock moth outbreaks generally result in growth loss, top kill and tree mortality, and can also weaken the surviving trees making them susceptible to Douglas-fir beetle, *Dendroctonus pseudotsugae* (Hopkins), attack (Shepherd and Otvos, 1986). Outbreaks of DFTM occur at 7 to 12 year intervals and last 4 to 6 years before they suddenly subside (Harris *et al.*, 1985).

Among the early suspected reasons for the periodic nature of DFTM outbreaks were insect parasitoids and a nuclear polyhedrosis virus (NPV). In 1922, an outbreak of DFTM failed to develop even though egg-mass densities were high, and in 1949 and 1954-56 outbreaks of DFTM collapsed due to NPV infection (Morris, 1963). Steinhaus (1951) was the first to diagnose an NPV in tussock moth larvae. Two morphotypes of the virus, BV and SV were

identified in the DFTM (Hughes and Addison, 1970). BV is currently referred to as OpMNPV and SV is referred to as OpSNPV (O'Reilly, *et al.*, 1992). The OpMNPV morphotype has many nucleocapsids per virion envelope, where as OpSNPV has only one nucleocapsid enclosed in the virion envelope (Blissard and Rohrmann, 1991). The manipulation of this naturally occurring disease in DFTM populations provides foresters with a safe method for controlling developing DFTM outbreaks before their populations reach damaging levels (Otvos and Shepherd, 1991).

Defoliation of Douglas-fir trees by DFTM has occurred in the drier areas of South Central British Columbia. Infestations have been confined to the dry subzone of the Interior Douglas-fir Biogeoclimatic Zone and the Ponderosa Pine Zone of the Thompson, Okanagan, Similkameen and Kettle valleys of the Province. Some defoliation has also occurred near Lillooet in the interior, near the city of Vancouver, and on Vancouver Island (Harris *et al.*, 1985).

DFTM populations often reach outbreak proportions rapidly with little advanced warning of the impending damage to host trees. There may be localized outbreaks on ornamental and shade trees in residential areas a year or two before outbreaks occur in nearby forested areas, thus providing some warning to forest managers (Harris *et al.*, 1985). Defoliation occurs as distinct patches in the first year of the outbreak due to distribution of wind-borne first instar larvae. These patches then spread and coalesce in subsequent years of

the outbreak, creating a "halo-effect," where rings of discoloured trees surround inner, more defoliated trees (Shepherd and Otvos, 1986; Harris *et al.*, 1985). Stands which are severely defoliated during an outbreak appear reddish-brown as the foliage of the trees is partly consumed and dries out, and the twigs and tips of branches become exposed (Alfaro *et al.*, 1987; Harris *et al.*, 1985).

Millions of board feet of timber have been lost to DFTM and thousands of trees have suffered from top kill and growth reduction in British Columbia and the western United States (Alfaro *et al.*, 1987; Beckwith, 1978). Outbreaks of DFTM can also create problems in salvage of infested stands, forest regeneration and fire prevention at the infestation sites (Mason and Wickman, 1988). Due to these important factors, major advances have been made in understanding DFTM's life cycle and its natural control agents such as pathogens and parasitoids over the last two decades. A pest management system was developed and formulated for DFTM during the previous outbreak which includes monitoring of susceptible stands for population build up using pheromone baited traps and control of the outbreak through the early application of a naturally occurring virus (Otvos and Shepherd, 1991). During the most recent DFTM outbreak in British Columbia from 1990 to 1993, the pest management system was operationally used and a number of studies was also undertaken with the idea of investigating areas that might offer potential refinement or improvements of the management system.

This thesis describes three areas of investigation: 1) the potential change in egg-mass size as a function of age of the outbreak; 2) the incidence of the naturally occurring virus as it relates to age of the outbreak and as a potential index or "threshold" that may be used to forecast impending epizootics and population collapse, and 3) a survey of genotypic variation in the OpNPV in DFTM populations.

Results of the first two areas of investigation may be incorporated into the decision making process for DFTM control. Results from the third area of investigation could be used to look for more potent strains or a unique strain of OpNPV. Using a naturally occurring unique strain may provide the opportunity of determining the efficacy of treatment (i.e. what proportion of the overall virus caused mortality can be attributed to the effect of the virus treatment and what proportion of the mortality was caused by the naturally occurring strains. To date this has not been determined for any of the insect viruses used operationally. The use of naturally occurring, unique strains is environmentally more benign and more acceptable than using a genetically altered or marked virus.

Chapter 2

Literature Review

2.1 Life Cycle of DFTM

The Douglas-fir tussock moth is a univoltine insect that overwinters in the egg stage (Mason, 1976; Mason and Wickman, 1988). The female lays all her eggs on the cocoon from which she emerges. Egg masses may be found throughout the tree crown. Egg masses are usually located on the under side of host-tree branches, on tree trunks, or, at extremely high infestation levels, on rocks, nearby fence posts and logs (Wickman and Beckwith, 1978). As the infestation progresses and more defoliation occurs, the percentage of egg masses found in the lower crown increases (Luck and Dahlsten, 1967). In lower density populations, DFTM egg masses may be located in the outside part of the branches in the upper crown areas of sample trees (Condrashoff and Grant, 1962; Mason, 1970). The number of eggs is usually between 150 to 200 eggs per mass, but may vary depending on the phase of the outbreak, past defoliation, food quantity and quality, and larval stress (Wickman and Beckwith, 1978).

The DFTM eggs undergo diapause through the winter and hatch in late spring (late May or early June) about the time that buds break and new shoots begin to expand (Mason and Wickman, 1988; Erickson, 1990). Wickman (1976) found that bud burst and egg hatch were closely related to accumulated degree-days (days above 42^{O} F, 6^{O} C) and that peak egg hatch occurred when 77 - 97 %

of the new foliage had burst from the buds, which is at 354-414 degree days. The synchronization of bud burst and larval hatch provides an easily observed field event for monitoring DFTM populations (Wickman, 1976; Wickman and Beckwith, 1978). Hatching of first instar larvae occurs in late May to early June and is usually completed within seven days (Edmonds, 1979). Larvae chew through the chorion of the egg and remain on the egg mass for 1 to 2 days before dispersing. If temperature is cool and the weather inclement, larvae will tend to stay on the egg mass for longer periods than if the weather is warmer and dry (Wickman and Beckwith, 1978).

There is both within-tree and between-tree dispersal of DFTM larvae after hatching. Because the female DFTM has only rudimentary wings and cannot fly, the primary mode of dispersal is wind-blown first and second instar larvae. Once the newly hatched larvae begin to leave the egg mass, they crawl up and out on the branches to the soft, newly flushed foliage (Wickman, 1976). The first instar larvae which move towards the top and outer crown portions of the tree during this within tree dispersal, can easily become wind-born on silken threads and blown to neighbouring trees. There can also be some within- and between-tree dispersal of later instar larvae; when the host foliage is stripped in the tree top, larvae move to the lower branches or will drop from the severely defoliated trees and crawl to new trees in search of food (Wickman and Beckwith, 1978).

First instars feed exclusively on the underside of newly flushed foliage.

The damaged needles dry out and turn reddish-brown colour which is the first sign of a DFTM infestation and is visible from the air (Mason and Wickman, 1988). Once all new foliage is consumed, later instars can and will feed on older foliage resulting in large portions of the host tree crown being stripped of its foliage (Mason and Wickman, 1988). Larvae feed for about 60 days, approximately until late-July to mid-August. During this time DFTM male and female larvae progress through five and six instars, respectively (Wickman and Beckwith, 1978). Feeding activity in the field may be slowed as result of cool, wet weather. Pupation occurs from middle- to late-August, and will last approximately two weeks (Mason and Wickman, 1988).

Adults begin to emerge from the cocoons in late August and September. Males emerge about 1 week before females. The wingless female remains on her cocoon, attracts the winged male by her pheromone, mates and deposits all her eggs in a single mass (Wickman and Beckwith, 1978). The cycle is completed with the deposition of a new egg mass. Both males and females are capable of mating more than once, with egg fertility remaining the same regardless of the number of matings by an individual female (Swaby *et al.*, 1987). Oviposition usually occurs within one-half hour to three hours after mating (Swaby *et al.*, 1987). Unmated females will also lay their eggs but in these cases the egg masses are often laid in an aberrant pattern when compared to the deposition pattern of the fertile eggs (Swaby *et al.*, 1987).

2.2 Pest Management of DFTM

DFTM causes growth loss, top-kill and tree mortality in the infested stands, and due to the value of the host trees control measures for this pest are usually needed. The presence of natural epizootics in DFTM populations brought on by the naturally-occurring NPV, provide a natural means of controlling this pest. In 1977, the first field application of NPV against DFTM was completed jointly by Forestry Canada, the USDA Forest Service and the British Columbia Ministry of Forests (Otvos and Shepherd, 1991). These initial field trials, completed in the second year of the outbreak, tested the efficacy of three OpNPV sprays against DFTM larvae. The spray types were: a unicapsid OpSNPV and two multicapsid OpMNPVs. One OpMNPV was reared in *Orgyia leucostigma* , at Sault Ste. Marie, Ontario, Canada and the other reared in DFTM larvae at Corvallis, Oregon in the United States (Otvos and Shepherd, 1991). All treatments caused high larval mortality, but since the sites were not treated until the second year of the outbreak, the stands at all sites sustained considerable defoliation and the level of the naturally occurring virus was not measured. Because considerable damage to the host trees occurs in the first year of the outbreak, it is desirable to apply the virus spray at the beginning of the developing outbreak, which will protect the foliage (Otvos and Shepherd, 1991; Shepherd *et al.*, 1984b). After these initial spray trials, the multicapsid morphotype of OpNPV, produced in DFTM, was registered in the United States in 1976 under the name TM-BioControl-1^{R}.

The same virus, produced in the white-marked tussock moth, *O. leucostigma*, was also registered in Canada, in 1987, under the trade name Virtuss^{R}. The recommended dosage in polyhedral inclusion bodies (PIB) for both formulations is 2.5 X 10^{11} PIB/ha (Otvos and Shepherd, 1991; Shepherd *et al.*, 1984b)

In 1981, aerial and ground spray trials were conducted to investigate if an epizootic could be initiated in DFTM infested stands earlier than it normally would occur, causing population collapse before severe damage occurred, thus protecting the foliage within the treated stand (Shepherd *et al.*, 1984b). Application of 2.5 X 10^{11} PIB/ha to 19.8 ha resulted in 83 and 85% infection eight weeks after spraying and no egg masses could be found during the fall egg-mass survey at these sites (Shepherd *et al.*, 1984b). This experiment showed that virus can be introduced into a DFTM population by both ground and aerial application early in the outbreak cycle, prior to serious damage, and a viral epizootic can be initiated protecting the trees providing the outbreak is detected and treated early (Shepherd *et al.*, 1984b; Otvos *et al.*, 1987a). Further testing the following year with reduced dosages, showed that application of that 8.3 X 10^{10} PIB per hectare, i.e. one third the registered dosage, is sufficient to induce an epizootic in infested stands (Otvos *et al.*, 1987a). After the virus treatment has been completed, DFTM mortality may rise as high as 99% in some stands. When treated plots were examined in the fall following virus application in 1982, typically no egg masses were found

(Otvos *et al.*, 1987a)

Treatments should be applied to infestations approximately 10 days after egg hatch when larvae have dispersed or when most of the larvae are in the second instar. This will ensure maximum infection rates and increase the chance for secondary passages of the virus (Seltzer *et al.*, 1977).

Current work on DFTM and NPV during the last outbreak centered around the possible decrease in the amount of virus (PIBs) applied to start an epizootic and increasing the distance between flight lines. Instead of spraying a "blanket" coverage of NPV over an infested stand, it is thought that increasing the distance among flight lines to 100-200 m may be sufficient to "seed" the virus in the DFTM infested stands. Once "seeded," the virus will spread into untreated areas by older DFTM larvae dying and spreading NPV to the surrounding areas (I. Otvos, P.F.C., pers. comm.).

2.3 Population Monitoring and Sampling for DFTM

DFTM populations can rapidly change from endemic to epidemic levels with little advance warning (Shepherd *et al.*, 1984a). Most of the significant damage by these larvae occurs in the first 1 or 2 years of the outbreak (Alfaro *et al.*, 1987). Therefore, to minimize damage, forest managers need to be able to predict accurately where and when these outbreaks will occur and then treat the infested stands prior to severe DFTM defoliation (Shepherd *et al.*, 1985).

A pheromone monitoring system, which attracts males to pheromone-

baited sticky traps was developed as an easy way to monitor DFTM population trends over time. Shepherd *et al.* (1985) conducted a series of investigations using DFTM pheromone traps which showed that there was no direct relationship between the number of male moths caught in the traps and egg-mass numbers or subsequent defoliation. However, they found that 2 or 3 consecutive years of increasing numbers of males (25 or more) caught per trap is a signal of impending DFTM outbreak in that area within 2 years (Shepherd *et al.*, 1985; Shepherd and Otvos, 1986). The increasing trend is more important than the number of male moths caught. High male moth catches per trap are not always accompanied by high egg-mass populations. Therefore, once an outbreak is predicted, additional pheromone traps should be placed in the susceptible stands followed by ground surveys to confirm the presence and extent of the infestation and determine the level of egg-mass densities and to predict expected damage (Shepherd *et al.*, 1985).

As a result of these studies, permanent monitoring sites were established in susceptible Douglas-fir stands (Shepherd *et al.* 1985). Susceptible stands were identified by mapping the locations of past DFTM outbreaks and superimposing on these defoliation maps the forest type, plant community types and biogeoclimatic zones (Shepherd and Otvos, 1986). Permanent monitoring sites have been established about 30 km (range 15 to 57 km) apart in the areas defined by the overlays described above, and pheromone traps have been set up and monitored annually by the Forest Insect and Disease

Survey (FIDS) and the B.C Ministry of Forests, estimating the expected damage by DFTM. The pheromone trap system allows for the prediction of an impending outbreak, but does not provide its exact location or give an estimate of the insect density at the outbreak site. Once the increasing trend in DFTM has been established, additional pheromone traps need to be placed and ground surveys conducted around traps that caught 25 or more male moths for 2-3 consecutive years to identify the center of the infestation. During ground surveys sequential sampling is used to determine egg-mass densities and from which potential damage or defoliation in the area in the following year can be predicted (Shepherd *et al.*, 1984a). Egg-mass density and damage predictions aid in the decision whether or not to begin DFTM control measures (Shepherd and Otvos, 1986).

To adequately estimate the defoliation at a DFTM infestation site and the insect density, a sequential egg-mass sampling technique has been developed for DFTM (Shepherd *et al.*, 1984a). The sequential sampling technique permits the determination of the egg-mass density and from this one can predict the expected level of defoliation the following year (Shepherd *et al.*, 1984a). Once the center of the infestation is located, egg masses are counted on three branches from the lower whorls of 20 randomly selected sample trees or until a certain egg-mass density is reached. Counting the number of egg masses present at the center of the stand provides an accurate estimation of the maximum insect density and maximum defoliation that can

be expected at that site the following year. Sampling egg masses in the fall of the year, before an outbreak is expected, allows several months for planning control measures for the infested stands (Shepherd *et al.*, 1984a; Shepherd and Otvos, 1986).

The DFTM population is confirmed in the spring by the use of sequential sampling of the early instar larvae. If the number of larvae found on the tree is below the lower limit of the number of larvae for a given sample size (3.6 larvae per tree), then sampling can stop as there will be little defoliation that year. However, if the number of larvae is greater than the upper limit for that sample size (10.9 larvae per tree), then there will noticeable defoliation at that site in the summer and treatment of the infested stand should then be considered (Shepherd, 1985).

2.4 Nuclear Polyhedrosis Virus: Description and Infection

The present method of choice to minimize the damage caused by DFTM in infested stands is to spray the area early in the outbreak cycle with the naturally-occurring virus before the epizootic would occur naturally (Otvos *et al.*, 1987a, 1987b). The virus used is a nuclear polyhedrosis virus (NPV) (Family Baculoviridae) which are known exclusively from arthropods (Martignoni, 1984). NPVs are attractive as pest control agents because they only occur in arthropods, and individual NPVs or NPV strains have a limited host range, infecting only a few closely related species of the same genus or

family of insects. They do not interact with non-target organisms and, therefore, insect predators and parasitoids are not affected by NPV. These biological insecticides are non-polluting, provide biodegradable treatments for the control of forest or agricultural pests and because they occur naturally, they are considered environmentally friendly or "better" alternatives than chemical insecticides (Martignoni, 1984).

The Family Baculoviridae has four subgroups, A, B, and C, classified by differences in genome structure, number of nucleocapsids per virion and presence or absence of crystalline occlusion bodies (Blissard and Rorhmann, 1990). The baculovirus of DFTM belongs to subgroup A, the nuclear polyhedrosis viruses (NPV), in which the virions are occluded within singular intranuclear crystals called polyhedra, or polyhedral inclusion bodies (PIB) (Blissard and Rohrmann, 1990; Martignoni, 1984). The virions of this group consist of one or more cylindrical nucleocapsids enclosed within a proteinaceous envelope. The size of the nucleocapsid ranges from 30-40 nm to 200-350 nm (Martignoni, 1984). The nucleic acid is closed circular, double stranded DNA of 80-200 kilobasepairs (kb) in size (O'Reilly, *et al.*, 1992). The occlusion body or PIB, is composed of a 29 kd protein called polyhedrin (Blissard and Rohrmann, 1990; Rohrmann, 1986). The NPVs are often subdivided further into two groups depending on the number of nucleocapsids per polyhedron; some nucleocapsids are found singly in the polyhedra (SNPV) and others are found with multiple copies (usually two to

seven) of the nucleocapsid in each polyhedron (MNPV) (Blissard and Rohrmann, 1990; Martignoni, 1984). Initial work on *Orgyia pseudotsugata* NPV, or OpNPV, proved there are two strains of virus present in DFTM. The two strains are designated OpSNPV, for single occluded virus (single virions embedded in polyhedrin protein), and OpMNPV, for multiple occluded virions (several virions embedded in polyhedrin) (Hughes, 1976; Hughes and Addison, 1970).

In the environment, NPVs are often found in the soil, bark crevices and on foliage (Blissard and Rohrmann, 1990; Thompson *et al.*, 1981). NPV infections are acquired when the insect ingests PIBs along with their food. In the alkaline midgut (pH > 10) of the insect, the polyhedral protein is dissolved and the virions are released (Blissard and Rohrmann, 1990). Once free inside the insect gut, the virion's envelope fuses with the microvilli of the midgut epithelial cells. The first cycle of replication begins in the midgut cell nuclei, and progeny nucleocapsids can be detected as early as 8 hours post infection (p.i.). At 12 hr p.i. some progeny nucleocapsids begin to bud through the nuclear membrane (Blissard and Rohrmann, 1990). Once inside the cytoplasm, the nuclear envelope is lost and the nucleocapsid travels to the plasma membrane where it then buds through to infect neighbouring cells. These first virions which bud through both membranes are called the "budded virus" or BV phenotype. It is BV which is responsible for infecting the fat body, muscle, tracheal matrix, hemocytes and epithelial cells of the

infected insect. In the infected cells, the nucleocapsids formed in the nucleus have two fates: the first is to bud through both membranes (BV), the second is to be enveloped by only the nucleus and then be occluded within polyhedrin; this is called polyhedrin-derived virus or PDV (Blissard and Rohrmann, 1990; Martignoni, 1984). The rate of BV production reaches a plateau and then declines somewhat when the polyhedra appear about 48 hr p.i. (Bradford *et al.*, 1990). The life cycle of an NPV was investigated in the armyworm, *Pseudaletia unipuncta*, and with OpNPV in *Lymantria dispar* cells which shows the two forms of the virus being enveloped by the nuclear membrane in the manner typical of NPVs (Tanada and Hess, 1976; Bradford *et al.*, 1990).

The two phenotypes of NPV, BV and PDV, vary in virion morphology, protein composition, tissue specificity and mode of entry into host cells (Blissard and Rohrmann, 1990). The BV virions usually have one nucleocapsid with a loose fitting viral envelope, specific for interactions with the cells and tissues within the hemocoel. BV isolated from the hemolymph of infected insects is highly infectious in the hemocoel and in cell culture, but is less infective when taken up by midgut epithelial cells (Blissard and Rohrmann, 1990). The envelope of BV contains a viral-encoded glycoprotein, gp64, which is encoded by the virus. The gp64 protein is located on the cell surface and acquired by BV when it buds through the plasma membrane. The gp64 protein also appears to be located at the end of the virion and is required for the endocytosis of the virus into host insect cells (Blissard and Rohrmann,

1990; Blissard and Rohrmann, 1989). The PDV virions may have more than one nucleocapsid in a tight-fitting envelope. These envelopes are specialized for interacting with polyhedrin and for the infection of columnar epithelial cells of the midgut. PDV is very infectious to the midgut cells and less infective in the cells and tissues within the hemocoel. PDV enters the cell by fusion of the viral envelope with the microvilli. PDV does not have the gp64 glycoprotein (Blissard and Rohrmann, 1990).

The polyhedrin protein, in which the single or multiple nucleocapsids are embedded, provides two major functions for NPVs. The first function of the polyhedrin is the protection of the virions by the formation of the polyhedron crystal around the virions. The second function of this protein is resistance to solubilization except in the strongly alkaline midgut of the insect. These two factors allow NPV to remain viable in the environment outside the insect host for many years (Rohrmann, 1986; Leisy *et al.*, 1986).

NPVs affect the immature stages of insects particularly the larvae, though larvae infected in later instars may die while pupating. An infected larva will feed less than a healthy larva and often cease feeding completely, though, for some species, such as gypsy moth, *Lymantria dispar*, larvae feeding will not stop until immediately prior to death (Martignoni, 1984). As the disease progresses, the integument of the larva becomes fragile and easily ruptures, spilling the contents of the larva, including polyhedra, on the foliage or the forest floor below. Once the insect has acquired an NPV infection, the

infection is fatal. NPV has a short course of infection and can bring about larval death within 14 days depending on the environmental conditions i.e. the higher the temperature the shorter the infection cycle. Cooler field temperatures may delay death (Martignoni, 1984). It is interesting to note that a fully mature lepidopteran insect infected with NPV can produce up to 1.5 X 10^{10} polyhedra before dying, which may contaminate the surrounding foliage and infect other surviving larvae when they feed (Evans and Harrop, 1982). With such a large number of polyhedra in one insect it is easy to see how quickly infection can spread in a forest stand.

NPVs are a natural component of the host tree environment, persisting in high concentrations in the soil, litter, and on bark, for at least one year after a natural epizootic in gypsy moth (Podgwaite *et al.*, 1979) and 11 years in case of DFTM T(Thompson and Scott, 1977). Activity of *Lymantria dispar* NPV (LdNPV) in spray deposits on the foliage and bark was measurable for only 3-15 days after NPV treatment, and LdNPV liberated through larval cadavers was active into the following May after treatment of the site (Podgwaite *et al.*, 1979). The application of LdNPV at the rate of 2.5 X 10^{12} PIB/ha to an area already containing high levels of naturally occurring LdNPV did not cause an increase in the environmental LdNPV load (Podgwaite *et al.*, 1979).

OpNPV PIBs can remain active in the soil for up to 11 years between outbreaks and it is this reservoir of NPV which reinfects new DFTM populations with naturally occurring dust-borne virus (Thompson and Scott,

1977). PIBs which are not washed off into the duff layer by rain and snow will be degraded by ultraviolet radiation (Martignoni and Iwai, 1985). Griego *et al.* (1985) found that monochromatic wavelengths between 290 and 320 nm, the shorter wavelengths of sunlight, are effective in causing the inactivation of OpMNPV in the field. Since OpMNPV is composed mainly of DNA and protein which absorb maximally at 260 and 280 nm respectively, wavelengths longer than 400 nm would not have any effect on these substances, therefore the shorter wavelengths are sufficient to cause the inactivation of OpMNPV. It is possible that crude preparations of NPV (freeze-dried and ground up cadavers) may have a longer field life due to impurities which may partially absorb short-wave UV radiation (Greigo *et al.*, 1985).

Once NPV is released at a site, it can be horizontally transmitted among members of the same host insect from one generation to the next. NPVs packaged for commercial preparations are stable over time if kept out of direct light (Martignoni, 1984). For these reasons, NPVs are an attractive control option for forest or agricultural pests, providing that the cost to propagate the virus and its application in the field is economically feasible. At present few insect viruses are commercially available for pest control because they are highly species specific which narrows the host range for which virus treatment can be used for control. Also, the cost of researching and producing these viruses, even though they may be highly effective, needs considerable support and/or subsidy by Local, Provincial and Federal Government

agencies.

2.5 Influence of NPV on Population Dynamics of its Host

NPVs play an important role in the population dynamics of DFTM and other herbivorous insects, resulting in dramatic decreases in the pest populations in a short period of time. Initially there was a great deal of controversy over how the outbreaks arose and insect populations cycled. Were epizootics the result of environmental conditions or larval stress stimulating latent disease infection? There was little evidence to support these claims (Woods and Elkinton, 1987). Work with NPVs instead supported the notion that NPVs were spread from generation to generation by density-dependent interactions (Doane, 1970; Woods and Elkinton, 1987). Low levels of viral induced mortality among early instar larvae were thought to serve as inoculum for the remaining healthy larvae which, under high density conditions, resulted in high infection rates and mortality of late instar larvae. This hypothesis was confirmed to be true for several sawflies and gypsy moth, as well as for DFTM populations (Bird, 1961; Doane, 1970). Ten percent mortality among young sawflies resulted in an epizootic (Bird, 1961). Thompson (1978) indicated that an incidence of NPV as low as 0.1 to 1.0% in the first instar DFTM larvae successfully initiated an epizootic in two years and a 50% mortality in the first instar larvae could lead to a population collapse by the fourth instar of the same year. Dead larvae provide a source of

inoculum for the surviving larvae (Thompson, 1978). The early instar larvae acquire the virus when they chew through the chorion of the egg at eclosion (Doane, 1970; Thompson, 1978). The trans-ovum transmission is probably the most important source of generation to generation transmission in high density gypsy moth and DFTM populations (Thompson, 1978).

The rate at which epizootics develop depends on a combination of larval population densities, the prevalence of NPV at the infestation (the virus load) and temperature. Once the epizootic has developed to the point where NPV liberated from the dying larvae have thoroughly contaminated the host foliage, larval densities become less important and the incidence of NPV infection will peak when the population is declining. At the beginning of an epizootic, the incidence of infection may differ among localities, but is usually highest at locations where the defoliation (therefore population) was high in the previous year. As the epizootic progresses, the virus becomes more effective in peripheral areas and it may be spread to new locations by infected first instars carried by wind on silken threads, parasitoids among later instars or by avian predators. By the end of the season the areas of infection have coalesced and the epizootic is general (Thompson, 1978).

The incidence of virus increases late in the season as fifth and sixth instar DFTM larvae die "splattering" virus on the surrounding foliage. A large proportion of the late instars are infected with OpNPV as their consumption of NPV contaminated foliage is high, increasing the probability

of becoming infected (Woods and Elkinton, 1987). Late instar larvae hang head down, attached by the posterior abdominal prolegs when they die. Soon after death wind may release the grip of the prolegs and the larvae fall to the foliage below or to the forest floor. At this time, DFTM population densities may be so high throughout the host tree, particularly in the lower part of the tree crown, that any dying larvae will expose others to infection (Thompson, 1978). Increased temperatures in the field can increase the rate of development of the OpNPV epizootic and cool temperatures can slow its progress (Thompson, 1978).

DFTM females have six developmental instars, one more than males. During an epizootic the sex ratio may change from equal proportions of males and females, to a higher number of males. Females, as larvae, feed longer than males and this increases their chance of acquiring additional NPV infection (Mason, 1976; Thompson, 1978).

NPV may cause high mortality in the pupal stage of DFTM. Larvae which are infected late in their life may die of NPV infection after pupation i.e. during cocoon formation. These pupae can serve as a reserve of NPV which can be leached out of the cocoons during the winter by rain and snow, providing a source of NPV for the infection of the overwintering egg masses (Thompson, 1978). NPV can remain viable in the pupal remains for up to three years (Thompson, 1978; Woods *et al.*, 1989).

Rain is considered by Bird (1961) to be the most important agent for the

transmission of virus within the tree crown. Transmission of NPV from the top of a tree can result in the rapid spread of the virus among all insects and egg masses in the tree (Bird, 1961). Light rains following the mortality of young larvae can result in almost total contamination of foliage and a sudden upsurge of virus-induced mortality. In one case, the percent contaminated shoots of the current year's foliage increased from 12% the day before the rain, to 100% 2 days after the rain (based on 90 shoots from 30 trees measured by bioassay for each of the two sampling periods) (Thompson, 1978). During this time the percentage of larvae infected by NPV rose from 23% to 99.8% in the same period (based on 500 larvae collected from 30 trees at each of the sampling periods) (Thompson, 1978). Since rain does not often fall during the summer where DFTM outbreaks are located, epizootics may be slow to develop in the early instars, especially when the incidence of NPV in first instars is less than 10% (Thompson, 1978).

Larvae can become infected with NPV for some time after an epizootic, even with low population densities, because the survivors acquire the virus from the heavily contaminated foliage. The amount of NPV on the foliage and bark and in the soil, litter or duff is substantial for at least 1 year following an outbreak. The amount of viable virus on the foliage and bark can decrease due to UV light inactivation (Woods *et al.*, 1989).

Doane (1969) showed that the major source of infection for newly hatched gypsy moth larvae is the overwintering egg mass. By disinfecting the

surface of eggs with sodium hypochlorite (bleach), the LdNPV was removed from the egg surface and no larvae were infected. Thus Doane positively identified trans-ovum transmission of LdNPV (Doane, 1969). The same method of disinfection can also be used on DFTM egg masses.

There are several possible theories on how the eggs acquire the virus. Gypsy moth eggs may acquire NPV from infected or externally contaminated female moths, who directly pass it on to the progeny, or by horizontal transmission by environmental contaminants (Murray and Elkinton, 1990). Larvae can acquire LdNPV from the hairs and setae deposited in the egg mass (Doane, 1975). As larvae chew their way through the egg masses at eclosion, they ingest virus from the egg surface and from the hairs surrounding the egg mass. In high gypsy moth populations, larvae congregate in shaded locations around trunks and under limbs of the host trees. Gypsy moth larvae will return to these natural shelters daily while feeding and will eventually pupate in the same location, creating a mat of pupal cases. When large larvae die in this area they can infect the cocoon mat with NPV. If egg masses are laid by healthy females in the same area, the egg masses will be surface contaminated and a higher proportion of hatching larvae will be infected with NPV the next spring (Doane, 1975). Since DFTM larvae also remain on the egg mass before dispersing, especially in cool or inclement weather, they may also become infected with NPV in this manner. The amount of time the larvae spend on the egg mass may increase the amount of NPV infections in larvae of both of

these species of insects.

Doane's (1969) work suggests that there is no maternal transmission of gypsy moth NPV; the source of the inoculum must be environmental. Further work by Murray *et al.*, (1990) indicated that LdNPV is not passed from parents which survive a "sub-lethal" dose of the virus. Scanning electron microscopy of the eggs from LdNPV infection surviving parents, showed no polyhedra on the surface, therefore contamination must be environmental (Murray *et al.*, 1990). Murray and Elkinton (1990) showed that contamination of gypsy moth egg masses occurs shortly after oviposition. Eggs sampled within three days of oviposition had the same amount of inoculum as eggs overwintered in the forest (Murray and Elkinton, 1990). However, this is not the case for DFTM egg masses (Otvos *et al.* (unpublished data), 1994). DFTM egg masses laid in the fall and collected in the fall had more infected first instar larvae than larvae reared from spring collected egg masses (Otvos *et al.*, (unpublished data), 1994). Also, gypsy moth larvae that hatch from egg masses that were laid on LdNPV treated bark showed a higher incidence of virus than those larvae hatched from untreated or bleached surfaces, suggesting that egg masses become infected by the contaminated surfaces they are deposited on (Murray and Elkinton, 1990). It is also interesting to note that larvae that hatched from eggs laid next to the contaminated substrate (i.e. in the "bottom" of the egg mass) had a higher mortality rate than the larvae that hatched from the outside surface (Murray and Elkinton, 1990). It is possible

that the virus from the contaminated surface is incorporated into the egg masses as they are deposited. The female DFTM and gypsy moth move their abdomens back and forth along the substrate depositing eggs and abdominal setae. This movement in the inner layers may distribute the virus among the lower layers in contact with the substrate (Murray and Elkinton, 1990). The outer layers of the egg masses may show a decrease in the incidence of virus due to UV light inactivation (Murray and Elkinton, 1990). The eggs laid against the contaminated substrate are more protected from adverse environmental conditions during the winter and show a higher incidence of virus. The dispersal of these contaminated first instar larvae to new, virus-free stands, can distribute the virus and begin population control (start an epizootic) in those areas as well (Murray and Elkinton, 1990).

Resistance to NPV was not observed in laboratory rearings or in the field (Thompson, 1978). DFTM larvae in each outbreak cycle are equally susceptible to NPV (Thompson, 1978). Thompson (1978) states that there was no increased resistance to NPV in a new outbreak population at a site in Idaho where there had previously been a DFTM outbreak whose collapse was attributed to an epizootic. There is also no difference in the development of epizootics in the distribution of the three main host tree species areas. Aerial applications of NPV have been equally effective on all host tree species (Thompson, 1978).

2.6 Food Quality, Number of Eggs Laid and Age of The Outbreak

In the field, feeding activity of the larvae is slowed by cool, wet weather, and larval development may take up to three months to complete (Wickman and Beckwith, 1978). It is during this long developmental period that two cycles of OpNPV epizootic can take place (Otvos *et al.* , 1987a). Other factors such as age and nutritional content of the foliage, may also affect larval development and fecundity (Mason and Baxter, 1970).

At the beginning of the growth season DFTM larvae are found concentrated in the tree tops and on branch tips where the new foliage is. As the season progresses and this new foliage is consumed the larvae move farther back on the branch nearer the older foliage (Mason and Baxter, 1970). These results suggest that new foliage is the preferred food of the young larvae but as the larvae mature, older foliage becomes acceptable and is utilized (Mason and Baxter, 1970). Laboratory studies showed that if there was no additional new foliage provided to the larvae, they would wander on the branch and only 12% of the larvae reared on older foliage survived. The mortality of these older foliage feeding larvae was due to starvation, indicating that some nutritional requirement was not met by the older foliage (Mason and Baxter, 1970).

Beckwith (1976) found similar results. The DFTM larvae concentrated on the new foliage at the tips of branches and "outer edge" in the top of the tree crown. This congregation may be due to geotropism (-) or the simple fact

that this is where there is a greater proportion of new shoots per unit area (Beckwith, 1976). If DFTM larvae were supplied only with new foliage they never fed on the older foliage, indicating a higher nutritive quality or moisture content in the new foliage (Beckwith, 1976). A higher moisture content provided by the new needles may be a developmental requirement to the young DFTM larvae. By forcing the larvae to feed only on old foliage, the time to pupation was extended but the overall duration of the pupal period remained constant. DFTM larvae feeding on old foliage produced more frass, indicating increased feeding to satisfy nutritional needs and possible poor utilization of the food consumed (Beckwith, 1976). Larvae may have to feed on more older foliage to provide the appropriate amount of moisture and/or nutrition (House, 1965). DFTM larvae can increase their foliage intake without serious problems, but if one nutrient is supplied in excess, the larvae may experience metabolic difficulties and decrease food consumption (House, 1965). Beckwith (1976) found that the number of DFTM eggs deposited per egg-mass in the lower crown portions was lower than the upper crown area. This indicates that when inadequate nutrition is provided by the older foliage, fecundity is reduced (Beckwith, 1976).

DFTM larvae are often forced to feed on older foliage during high population densities. Research by Beckwith (1976) indicates that when this occurs nutritional stress, mass starvation of the young larvae, delayed development, increased exposure to biological controls factors (parasitoids and

predators), reduced egg production and a general population quality decline will occur, contributing to the collapse of the outbreak. During these high population densities there may be an increase in the proportion of diseased insects, therefore crowding can increase the chances of transmission of the disease between healthy and infected insects (Steinhaus, 1958). In fact, the stresses of overcrowding, competition for food resources, poor food quality and malnutrition may weaken the larvae, making them more susceptible to NPV infection. Epizootics are often accompanied by malnutritional stresses that are thought to lower resistance to NPV infection, and allow the epizootic, once initiated, to develop more rapidly (Thompson, 1978).

The increased consumption of old foliage is of special interest to foresters who monitor defoliation; more older foliage needs to be consumed to provide adequate nutrition, resulting in higher defoliation. In the Douglas-fir tree, the last five years of foliage growth accounts for approximately 90% of the total foliage on the tree (Silver, 1962). The upper third portion of the tree contains a large portion of the current year's foliage (Silver, 1962). A complete loss of the new foliage by early instar feeding, represents a loss of about 28% of the total tree foliage (Silver, 1962). The lower two thirds of the tree has an average distribution of all five years of foliage (Silver, 1962). Defoliation of the host tree by DFTM is usually from the top down due to larval distribution within the tree and defoliating the current year's foliage can result in top-kill, progressing to tree mortality if the other years' foliage is also severely

damaged. Feeding patterns and nutrition content of the foliage are important parts of understanding DFTM outbreaks.

In heavily defoliated sites the egg masses of DFTM are located in the lower third of the crown while in sites with lighter defoliation, egg masses are located near the top of the crown (Luck and Dahlsten, 1967; Condrashoff and Grant, 1962). Mason *et al.* (1977) found that there were lower (fewer) numbers of DFTM eggs per egg mass in severely defoliated areas. It is thought that the fecundity of DFTM is influenced by the type of foliage (old or new) that the larvae are feeding on and by larval crowding. Certainly, the results of Mason *et al.* (1977) indicate that when larval populations are high and they are feeding on older foliage, the females are less fecund. This has also been shown in work by Leonard (1968) with the gypsy moth.

In the fall, egg-mass surveys for DFTM, which count and predict population size in the following spring based on the number of current year egg masses, are conducted in potential outbreak locations. Several attempts have been made to take simple measurements or weights of insect egg masses and relate them to the number of eggs per egg mass or to predicted population size (Beckwith *et al.* , 1978; Richerson *et al.* , 1978; Moore and Jones, 1992). Beckwith *et al.* , (1978) created a common regression equation for the number of DFTM eggs per egg mass based on weight of the egg masses collected at a number of different geographical locations. This common regression was then used by him to predict the number of eggs per egg mass based on weight

of the egg mass at a particular location. Since this sampling method involves taking weights, which involves bringing the egg masses back to the laboratory, in low population densities removing egg masses from the field may effect the actual number of larvae hatching at the site, and thus may decrease the population.

For gypsy moth egg masses, a simple measurement of the length of the egg mass can be used to predict fecundity of this insect (Jones *et al.* , 1990). Changes in the fecundity of an insect can contribute significantly to changes in population density. For both DFTM and gypsy moth, fecundity is assumed to be a constant and only the number of egg masses, not the number of eggs per mass, is considered when population and subsequent damage estimates are made from fall egg-mass surveys. Simple egg-mass dimensions can be measured in the field and predict more accurately the population size in the following year (Moore and Jones, 1987). Simple length measurements of the forest tent caterpillar (*Malacosoma disstria*, Hubner) egg mass provide an accurate prediction of the future population size (Goyer *et al.* , 1987). It may be possible to take length, area or volume measurements of DFTM egg masses in the field in the fall to predict the population size in the spring by regression of these values against number of eggs per egg mass. Monitoring the actual larval hatch could then be completed in the spring to check accuracy of the predictions. It has been postulated that the number of eggs laid by the females is influenced by quality and quantity of the foliage as well as age of the

outbreak (Beckwith, 1978). Larval stress from feeding on older foliage and larval competition, which is likely to occur at higher population densities, may cause a reduction in the size of the egg mass (i.e. the number of eggs per mass) as the outbreak progresses.

2.7 Regulation and Expression of NPV Genes

In an infected insect cell, the expression of NPV genes and DNA replication are believed to be the result of an ordered cascade of events, where the products of one phase are dependent on the products of the previous phase (Blissard and Rohrmann, 1990). The cascade of viral gene expression is regulated at the transcriptional level with gene products of one temporal class of baculovirus genes transactivating, either directly or indirectly, transcription of the genes in the next temporal class. Baculovirus gene expression is divided into two general phases, the early phase that precedes viral DNA replication and the late phase that occurs as or after viral DNA replication begins (Blissard and Rohrmann, 1990). The early phase is further divided into two phases. The first phase is the immediate early (IE) phase where the genes are those which can be transcribed by uninfected cells and require no viral gene products for their expression. The other phases of early genes are the delayed early (DE) genes which require other viral gene product(s) for their transcription (Blissard and Rohrmann, 1990). The late phase genes are genes which are first transcribed after or concurrently with the onset of viral DNA

synthesis and are transcribed from the late baculovirus promoter. The last phase of gene expression are the hyperexpressed late genes whose mRNA levels remain at high levels throughout the expression cycle (Blissard and Rohrmann, 1990).

Transcription events which have an effect on the synthesis of new gene products or a regulatory affect are common in the DFTM baculovirus. For example, the transcription of the delayed early gene (39k) is dependent on the gene product of a transactivating immediate early gene, IE-1 (Blissard and Rohrmann, 1990). The OpNPV IE-1 gene codes for a 520 amino acid protein with a molecular weight of 64,775 Daltons (Theilmann and Stewart, 1991). Transcriptional analysis of the OpNPV IE-1 gene identified two RNAs homologous to the IE-1 open reading frame that were 1.7 and 1.9 kb in size. The 1.7 kb transcript could be detected by 0 hr post infection (p.i.), increasing up to 48 hr p.i. (Theilmann and Stewart, 1991). The 1.9 kd transcript appears to be spliced and has peak expression between 4 to 6 hr p.i., but may still be active late in the infection cycle (Theilmann and Stewart, 1991). They also found that IE-1 of OpNPV can trans-activate its own promoter and that for maximal expression this requires sequences -420 and -330 relative to the IE-1 transcriptional start site.

A second immediate early gene has also been identified in OpNPV. The IE-2 gene codes for a protein 45,640 Da in size. This gene is expressed as a 1.3 kb transcript that was detected as early as 0.5 hr p.i and reached a maximum

steady state equilibrium at 6 hr p.i and declined after 48 hr p.i.. The IE-2 gene trans-activates the IE-1 promoter and is autoregulatory (Theilmann and Stewart, 1992).

For a number of the early genes like IE-1, IE-2, 39k delayed early and the gp64 envelope glycoprotein gene, transcription is initiated with a tetra-nucleotide "CAGT" motif which is perfectly conserved (Blissard and Rohrmann, 1990). A TATA box is also conserved in sequence and position (21-24 nucleotides) upstream of the CAGT transcriptional start site (Blissard and Rohrmann, 1990).

All late genes identified are transcribed from the consensus late promoter element, ATAAG or GTAAG. This core promoter functions as both the late gene promoter and mRNA start site (Blissard and Rohrmann, 1990). In several baculovirus genes, multiple copies of the ATAAG start site are located upstream of the open reading frame (ORF) and transcription may start at any or all of these upstream start signals (Blissard and Rohrmann, 1990). There are also two types of late baculovirus genes: late and hyperexpressed genes. Late genes are those genes in maximal abundance just after DNA replication and decrease thereafter. Hyperexpressed late genes are genes whose mRNAs and proteins are present at very high levels shortly after DNA replication and throughout infection (Blissard and Rohrmann, 1990).

2.8 Baculovirus Structural Proteins

In the baculoviruses there are three main structural proteins. The first class is the occlusion body proteins. The crystalline matrix of the occlusion body is made up of a 29 kd protein, polyhedrin (Blissard and Rohrmann, 1990; Vlak and Rohrmann, 1985). Originally it was thought that polyhedrin was produced by the host cell in response to NPV infection; now researchers have shown it is completely viral in origin (Vlak and Rohrmann, 1985). The polyhedrin protein can be detected at 12 hr p.i., but it becomes a major gene product only at late times p.i.. Formation of polyhedra themselves will not be apparent until 24 hr p.i. (Vlak and Rohrmann, 1985). The gene controlling this protein's production is hyperexpressed and highly conserved. Because of the high level of polyhedrin gene expression, foreign genes are often cloned behind the polyhedrin gene promoter in recombinant expression vectors (Mori *et al.*, 1992; Maeda, 1989). Current expression vector systems using the polyhedrin promoter include a shuttle vector which replicates in both *Bombyx mori* and *Spodoptera frugiperda* cells to express firefly luciferase (Mori *et al.*, 1992).

Belonging to another main structural protein class are the viral structural proteins like p6.9, a small histone-like protein which is viral encoded. Also the capsid protein, p39, which is assembled in the nucleus of infected cells is important in the viral structural protein group (Blissard and Rohrmann, 1990; Pearson *et al.*, 1988; Blissard et *al.*, 1989).

The last major structural protein group is the BV specific proteins,

among which the gp64, a 64 kd envelope glycoprotein of the BV phenotype, is the most important. The gene which codes for the envelope glycoprotein encodes a 509 amino acid protein. This protein is found in the cytoplasm of the cell soon after infection and then later is located in the plasma membrane where it is acquired by BV during the budding process (Blissard and Rohrmann, 1990).

2.9 Genotypic Variation in Baculoviruses

Several studies have compared NPV virus isolates obtained from geographically separate populations of a given host species. These studies include work on *Spodoptera littoralis* (Cherry and Summers, 1985), *Spodoptera frugiperda* (Shapiro *et al.*, 1991), *Phthorimaea operculella* (Vickers *et al.*, 1991), and *Autographa californica* (Lee and Miller, 1978; Miller *et al.*, 1980). Restriction endonuclease (REN) analysis is currently used to examine the strains of NPV from different locations. Isolating viral DNA from infected insects from many geographic locations and subjecting it to digestion with endonuclease enzymes provides distinct DNA patterns after electrophoresis on agarose gels. The banding patterns are reproducible each time the same DNA is cut with the same enzyme. The patterns can show similarities and differences in the virus infecting the same host insect in different areas and can provide information indicating evolutional changes in the virus, and aid in the identification of NPV strains with different

characteristics, such as virulence.

Studies involving geographical isolates from *Spodoptera littoralis* (SlNPV) larvae collected from 21 locations in Israel showed that there are two distinct, non-homologous strains of SlNPV (Cherry and Summers, 1985; Kislev and Edelman, 1982). In a geographical variant study of *Spodoptera frugiperda* NPV, larvae from 15 different locations showed the presence of 11 distinct isolates of SfNPV (Shapiro *et al.*, 1991). Other studies investigating geographical isolates of NPVs, including *Heliothis* spp. (Gettig and McCarthy, 1982), *Mamestra brassicae* (Vlak and Groner, 1980), *Phthorimaea operculella* (Vickers *et al.*, 1991) and *Autographa californica* (Miller *et al.*, 1980; Smith and Summers, 1978; Lee and Miller, 1978) have shown that the geographical isolates are closely related strains of the same NPV, differing only in the position of a few bands in the REN profile.

A study on the biological activities of three geographical isolates of NPV from the gypsy moth, *Lymantria dispar,* showed differences in the amount of PIBs needed to cause an epizootic (Shapiro and Robertson, 1991). These differences may allow a specific higher activity strain to be selected for control of gypsy moth in the field. Other genotypic variants have shown no differences in their biological activities or host range (Gettig and McCarthy, 1982).

A study on the genetic relatedness of OpSNPV and OpMNPV have shown no common patterns when restriction endonuclease fragments of both

DNA types were compared (Rohrmann *et al.*, 1978). Rohrmann *et al.* (1978) believe that the low level of homology between the two strains indicates the viruses may have developed from a common ancestor but have evolved to infect tussock moth under different environmental conditions, since the SNPV and MNPV have different geographical ranges and pathologic reactions (Rohrmann *et al.*, 1978).

While recent OpNPV research is centred around identifying and characterizing the genes of the virus, little research has been conducted to identify and compare geographical isolates of OpNPV within field populations or between provinces or states. Since there are two morphotypes of OpNPV present in B.C., it is thought that there may be geographical variants of the virus throughout the province. Identification of these strains may provide a genetically unique strain of virus which can be sprayed in the field and used to monitor and determine the proportion of mortality as a result of virus treatment in the infested stands. Also, identification of unique strains of OpNPV may lead to the selection and development of a more efficacious spray.

2.10 Cytoplasmic Polyhedrosis Virus

The cytoplasmic polyhedrosis viruses (CPVs), are composed of 10 equimolar segments of double stranded RNA (dsRNA) and belong to the family Reoviridae (Payne and Mertens, 1983). These viruses are known to

infect a number of insect species including DFTM. Since these viruses are pathogenic to many insect species, including Lepidoptera, they have the potential to be used as biological control agents. One CPV was commercially available in Japan for the control of the pine caterpillar, *Dendrolimus spectabilis* and work on DsCPV is currently ongoing in China (Payne and Mertens, 1983; Otvos, pers. Comm.). CPVs only cause chronic disease in the infected insect pest and the virus is biochemically similar to viruses which are known to infect vertebrates and plants, so they have received less attention as control agents than baculoviruses have (Payne and Mertens, 1983).

The CPVs are provisionally classified as a "type 1," "type 2" etc., on the basis of the hosts from which they were initially isolated and the electrophoretic mobilities of the viral genome segments. The DFTM CPV (OpCPV) is a type 5 CPV, composed of 10 bands of dsRNA with a molecular weight between 2.35 and 0.34 X 10^6 Daltons (Payne and Rivers, 1983). The distinct electrophoretic profiles of the types of CPVs allow for comparison of virus isolates from different geographical locations. Mixtures of two or more types of CPVs within the same host larva can be distinguished by the presence of submolar bands on the gel (Payne and Mertens, 1983). While the presence of OpCPV has been observed in some larvae (Payne and Mertens, 1983), little research has been conducted to determine the geographic regions where this virus is located naturally and its distribution within the DFTM populations.

2.11 Objectives of The Research

In surveying the considerable amount of DFTM and NPV literature, it is apparent that there are still several questions about their interactions which need to be investigated. The affect of NPV on the natural populations in the field during the course of an outbreak should be observed and may allow the prediction of an epizootic. The percentage of first and second instar larvae which are infected with NPV each year of the outbreak should increase, reach a "threshold" level which may be used to predict the impending epizootic. Determining the incidence of virus in each year of the outbreak may indicate this "threshold" level of infection, above which the population will collapse. If populations of larvae are sampled and the amount of NPV in the sample is high, treatment of the stand may not be needed since the population has enough virus present in the hatching first instar larvae to initiate an epizootic. These stands would not be treated, therefore the cost of spraying these stand will be saved.

The development of a new or modified sampling method which would allow the estimation of DFTM population size each year of the outbreak would be beneficial to forest managers. Currently the number of egg masses, not the number of eggs per egg mass, is used to predict population densities and this may over-estimate the actual larval hatch and subsequent damage. If one could take simple measurements of the egg masses in the field and use regression equations to estimate the number of eggs per egg mass in

that year of the DFTM outbreak, a better estimation of the next year's population may be achieved. This sampling method would be a more accurate estimation of the population in the infested stands since the actual size of the egg mass can be measured. Determining the population size in the fall through measurements would allow additional time for planning control strategies for the infested stands.

Investigation of the genotypic variation in the OpNPV in British Columbia is an important addition to DFTM research. There has been only a preliminary investigation of the variation between outbreak areas. If a strain is found that is genetically unique its efficacy could be investigated in the laboratory and the field for differences in biological activity . This unique strain would provide the proportion of the mortality due to the virus treatment at the site. At present we know that by applying virus sprays we can cause an epizootic but we cannot separate the mortality caused by the virus treatment from that caused by the naturally-occurring virus load.

This thesis will address the following points: a) To examine if there is any change in DFTM egg-mass size (number of eggs per egg mass) during the course of an outbreak, b) examine the level of percent infection of first and second instar larvae from field-collected egg masses during the course of an outbreak and, finally, c) a survey of genotypic variation of the OpNPV and OpCPV in several regions of British Columbia. Data from the above areas of research may allow refinement of the pest management strategy currently

used for DFTM in British Columbia.

Chapter 3

Egg Mass Size Change During a Douglas-fir Tussock Moth Outbreak

3.1) Introduction

The Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae) is a periodic, major defoliator of Douglas-fir, *Pseudotsugata menziesii* var. *glauca* (Beissn.) Franco, trees in the western United States and Canada (Sudgen 1957; Harris *et al.*, 1985). Current population estimates for DFTM are completed in the fall of each year using pheromone traps and sequential egg mass surveys to locate outbreak areas. Once outbreak areas are identified, population size and estimates of defoliation the following year at outbreak sites are made.

These population density measurements for DFTM accurately predict population size and defoliation, but they are labour intensive and do not take into account the total number of eggs per egg mass or the numbers of viable eggs. Regression equations to predict the number of eggs per egg mass based on simple measurements like length or weight of the egg mass, have been developed for *Lymantria dispar* (L.) (Jones *et al.*, 1990; Moore and Jones 1987) and for *Malacosoma disstria* (Hubner) (Goyer *et al.*, 1987).

Beckwith et *al.* (1978) developed a common regression equation for DFTM from four outbreaks locations with variable amounts of defoliation

(trace to heavy) which predicts the number of eggs per egg mass based on the weight of the egg mass without the cocoon attached. This common regression was used to predict population size in a DFTM outbreak in northeast Oregon and southeast Washington in 1972 and 1973 (Mason *et al.*, 1977). The common regression minimizes the effect of climatic conditions, site conditions, and phase of the DFTM outbreak since it incorporates data from several different outbreak conditions. This common regression is based on weight measurements that must be made in the laboratory, not in the field. If DFTM populations are at low levels removing any egg masses from the field to weigh them in the laboratory may decrease the population at the site, thus reducing the population. Length or width of the egg mass, could be taken in the field and if this would accurately predict the number of eggs in the mass, populations would not be depleted at the site, sampling time would be decreased and the number of eggs per eggs mass could then be incorporated into the egg-mass surveys which in turn may give a more accurate estimate of the expected population density and subsequent damage. This in turn could then be used to fine tune the DFTM management system currently in use.

The focus of this chapter of the thesis is to develop and examine regression equations based on the "age" of the outbreak in each of the 1991 and 1992 collection years and predict the number of eggs per egg mass from one of the following parameters: the weight with and without the cocoon attached, and area and volume of the egg mass. It is hoped that based on

these simple measurements, regression equations may be derived. These regression equations could then be used in the future to predict DFTM populations and subsequent damage more accurately by measuring the egg masses in the field and using these simple measurements and the regression equations to estimate the number of eggs per egg mass.

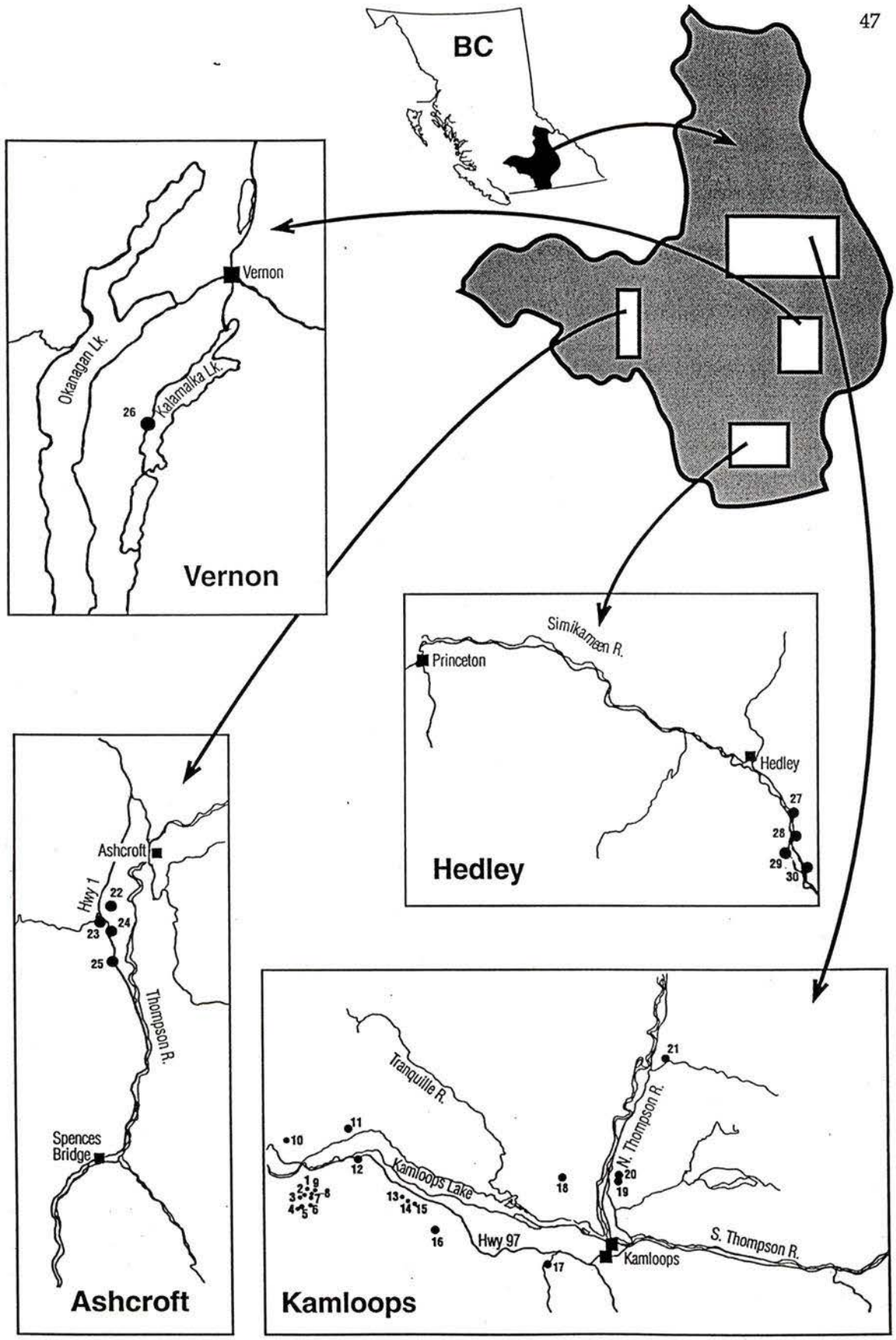
3.2) Materials and Methods

3.2.1) Egg Mass Collection and Processing

Forest Insect and Disease Survey (FIDS) rangers of the Canadian Forest Service, and others at the Pacific Forestry Center working with the DFTM, survey susceptible stands for the presence of DFTM egg masses (EM) in the fall of each year when male moth catches suggest impending outbreaks (Shepherd *et al.*, 1985). Canadian Forest Service personnel collected DFTM EM in the fall of 1991 and 1992 from 31 and 10 sites, respectively, in the Kamloops Forest Region of British Columbia during the fall EM survey (Figure 3.1). In fall 1992, EM were collected from one other site near Chilliwack B.C. (in the Vancouver Forest Region), during the fall EM survey in that region.

The EM collected during surveys served several purposes: 1) to locate DFTM infested stands which would be used for virus or pheromone treatment during the next summer, 2) develop regression equations to

Figure 3.1. The collection site in the Kamloops Forest Region were DFTM egg masses were collected in the fall of 1991 and 1992 for this study (sites names given in Appendix 1).


BC
Vernon
Okanagan Lk.
Kalamalka Lk.
26
Vernon
Simikameen R.
Princeton
Hedley
27
28
29
30
Hedley
Ashcroft
Hwy 1
22
23
24
25
Thompson R.
Spences Bridge
Ashcroft
Tranquille R.
N. Thompson R.
21
10
11
12
Kamloops Lake
18
19
20
1
2
3
4
5
6
7
8
9
13
14
15
16
Hwy 97
17
Kamloops
S. Thompson R.
Kamloops

estimate the number of eggs per mass, 3) determine the incidence of naturally occurring virus and 4) determine percent egg parasitism (a concurrent study conducted as part of a Msc program).

Current year EM were visually located on the underside of host tree branches and counted for the EM survey as described by Shepherd *et al.* (1985). Then the EM were removed from the branches with a small piece of the branch so that the EM were never touched and placed individually in plastic or paper bags. EM collection continued at the site for 1 hr or until at least 50 EM were collected. All individually bagged EMs from each collection site were placed in larger, labelled plastic bags and brought back to the Pacific Forestry Centre, Canadian Forest Service, Victoria B.C., where they were placed in cold storage at 4^{O} C, for at least three months to satisfy diapause requirements.

Collection sites with less than 10 egg masses per site (Winter's Creek Upwind, Stemwinder, Dominic Lake, Plot 20 W. Pritchard, Duck Range, Monte Creek, Riffle Range, B.C. Ministry of Forests campsite, Guichon Junction and Plot 13), or sites which were geographically isolated and close to natural collapse (Chilliwack site) were not used in this study. Each DFTM collection site was given an estimated "age" of the outbreak (0, 1 or 2) based on the presence and amount of visible defoliation both on the current and previous year's growth at the site. Age 0 sites showed trace amounts of defoliation at the time of the EM survey, while age 1 site had light defoliation

and age 2 sites had moderate to severe defoliation. It was necessary to age the infestations this way because the B.C. Ministry of Forests made the operational decision to treat infested stands as soon as they were threatened with defoliation and the outbreak was not permitted to run it's natural course in any of the infested stands during this outbreak period.

Beginning in January 1992 and January 1993, EM were removed from cold storage and the EM collected from each site were examined; EM which were laid in bunches or had a surface contamination of an undetermined "penicillium-like" fungus were not included in this study. The EM used for this study were measured using forceps and electronic calipers, sterilized in a 2% sodium hypochlorite solution (commercial house-hold bleach); the length (longest part of the EM), width (widest part of the EM) and depth of each egg mass was measured in millimeters with the cocoon attached. The EM was transferred to a Sartorious electronic balance and the weight (mg) of the EM with the cocoon was taken. Using the forceps, the cocoon was removed and the EM was measured and weighed again. EM were then broken into small pieces with sterilized forceps and the number of eggs in each egg mass was counted.

3.2.2) Data Analysis

Using SAS Proc Means procedure, the mean number of eggs per egg mass, weight with and without the cocoon, area (length by width of the egg mass without the cocoon) and volume (length by width by depth of the egg

mass without the cocoon) was calculated for the total data, by collection year and by age of the outbreak (SAS Institute, 1989). SAS Proc ANOVA was used to compare: the mean number of eggs per egg mass; mean weight of the EM with and without the cocoon attached; and area and volume of the EM, for age of the outbreak in each collection year (1991 and 1992), all EM used in the study, and to compare the means between the collection years. SAS Proc GLM was used to create regression equations for the number of egg per egg-mass against weight of the EM with and without the cocoon, area and volume for each age of the outbreak, collection year and for the total data set (SAS Institute, 1989). SAS Proc GLM was used to complete one-way analysis of covariance for the regression lines, to compare their slopes and intercepts (SAS Institute, 1989; Bergerund and Sit, 1991).

3.3) Results

3.3.1) Number of Eggs per Egg Mass

During the DFTM EM collections in the fall of 1991 and 1992, 915 and 434 EM were collected, respectively, for a total of 1,349 EM from 42 sampling locations in the Kamloops Forest Region, B. C. The mean number of DFTM eggs per egg-mass was 150.97 for the combined collections (i.e. both years) and was 146.85 and 159.66 for 1991 and 1992 EM collections, respectively (Table 3.1).

When the mean number of eggs per egg mass was examined by "age" of the outbreak, age 0 had the highest number of eggs per egg mass (159.10)

Table 3.1. Mean weight of the egg mass (EM) with and without the cocoon attached and mean number of eggs per DFTM egg mass, by year of collection (1991 and 1992), age of outbreak (0, 1 and 2), and the total number of EM, Kamloops Forest Region, B.C.

Sample	Mean weight (mg) of EM with cocoon			Mean weight (mg) of EM without cocoon			Mean number of eggs per EM		
	Number of EM	Mean	Standard error	Number of EM	Mean	Standard error	Number of EM	Mean	Standard error
Age 0	243	118.68	2.29	243	103.94	2.09	243	151.62	2.54
Age 1	286	119.54	1.90	286	101.66	1.60	286	151.23	2.08
Age2	386	109.07	1.75	386	91.80	1.39	386	140.60	1.73
1991 EM	915	114.89	1.14	915	98.10	0.97	915	146.85	1.20
Age 0	141	125.21	2.83	141	112.77	2.60	141	171.99	3.74
Age 1	51	95.75	3.79	51	85.19	3.42	51	136.25	5.17
Age2	242	112.86	2.35	242	94.30	2.05	242	157.40	2.72
1992 EM	434	115.32	1.70	434	99.23	0.97	434	159.66	2.10
Age 0	384	121.07	1.79	384	107.18	1.64	384	159.10	2.17
Age 1	337	116.55	1.75	337	99.17	1.49	337	148.96	1.95
Age2	628	110.52	1.41	628	92.76	1.16	628	147.08	1.53
All EM	1,349	115.03	0.94	1,349	98.46	0.82	1,349	150.97	1.07

while age 2 had the lowest number of eggs per egg-mass (147.08) (Table 3.1). Analysis of variance showed that the mean number of eggs per mass in each age of the outbreak was significantly different (F-value 11.95, Pr>F 0.0001). The Duncan's multiple range test (Pr = 0.05) showed that age 0 and age 1 EM had a higher mean number of eggs than age 2 (Table 3.2).

The mean number of eggs per egg mass were examined in each of the collection years and a decrease in the number of eggs was observed as age of the outbreak increased. Analysis of variance for the 1991 EM collection showed that the mean number of eggs per mass for each age of the outbreak were significantly different (F-value 10.10, Pr>F 0.0001) (Table 3.2), and the Duncan's multiple range test (Pr = 0.05) showed that age 0 EM were the largest, and mean number decreased in age 1 and age 2. In 1992 EM collections, the mean number of eggs was less in age 1 sites than in age 2 sites. Analysis of variance showed that the mean number of eggs was significantly different (F-value 14.06, Pr>F 0.0001) and the Duncan's multiple range test showed that age 0 had the highest number of eggs per EM, but the age 1 had the smallest (Table 3.2). When the two collection years were compared, the number of eggs per EM were significantly different (F-value 32.05, Pr>F 0.0001) and the Duncan's multiple range test (Pr = 0.05) showed that the number of eggs was higher in 1992 than 1991.

3.3.2) Weight of the EM Without the Cocoon

The mean weight of all 1349 EM without the cocoon was 92.76 mg, and

Table 3.2. Anova table for mean number of eggs per egg mass, weight with and without the cocoon attached, for each collection year (1991 and 1992) and all EMs, Kamloops Forest Region, B.C..

	Source	df	MS	F-value	Pr>F
	Mean number of eggs per EM				
1991	Age	2	13035.98	10.10	0.0001
1992	Age	2	25309.52	14.06	0.0001
1991 vs. 1992	Coll. yr.	1	48278.48	32.05	0.0001
All EM	Age	2	18125.34	11.95	0.0001
	Mean weight (mg) of egg mass with cocoon				
1991	Age	2	11387.34	9.82	0.0001
1992	Age	2	11387.34	11.49	0.0001
1991 vs. 1992	Coll. yr.	1	53.96	0.04	0.8326
All EM	Age	2	13782.88	11.60	0.0001
	Mean weight (mg) of egg mass without cocoon				
1991	Age	2	13628.79	16.49	0.0001
1992	Age	2	20879.22	22.11	0.0001
1991 vs. 1992	Coll. yr.	1	373.11	0.41	0.5227
All EM	Age	2	24887.22	28.38	0.0001

in 1991 and 1992 the weight was 98.10 mg and 99.23 mg, respectively (Table 3.1). The weight of the EM without the cocoon showed a decrease in weight as the age of the outbreak progressed; age 2 had a mean weight of 92.76 mg, while age 0 EMs had a mean weight of 107.18 mg. This decrease in weight was significantly different in each age of the outbreak (F-value 28.38, Pr>F 0.0001) and the highest weights were found in age 0 EM (Duncan's multiple range test, Pr = 0.05) (Table 3.2).

When the age of the outbreak was used to examine the weight of the egg masses without the cocoon attached, in each collection year (1991 and 1992), the weights were significantly different (F-value 16.49 and 22.11, respectively, Pr>F 0.0001) (Table 3.2). The Duncan's multiple range test (Pr = 0.05) showed that the mean weight of the EM was significantly higher in age 0 than age 1 or age 2 in 1991. However, in 1992 the Duncan's test showed that age 1 outbreaks had the lowest means weight without the cocoon (Table 3.2). The mean weight without the cocoon was not significantly different when the two collection years were compared (F-value 0.41, Pr>F 0.5227).

3.3.3) Weight of the EM with the cocoon

The mean weight of the egg mass with the cocoon attached was 115.03 mg for all EM and in 1991 and 1992 the weights were 114.89 mg and 115.32 mg, respectively (Table 3.1). The overall mean weight of the egg mass with the cocoon was highest in year 0 and lowest in year 2 of the outbreak, 121.07 mg and 110.52 mg, respectively (Table 3.1) and analysis of variance showed that

the mean weights were significantly different (F-value 11.60, Pr>F 0.0001), with the age 2 EM having a significantly smaller weight than age 1 and age 0 (Table 3.2).

In 1991, the weight of the EM with the cocoon attached significantly decreased with age of the outbreak (F-value 9.82, Pr>F 0.0001) (Table 3.2) and the mean weight was lowest in age 2 EMs (Duncan's multiple range test, Pr = 0.05). The mean weight with the cocoon by age of the outbreak was also significantly different in 1992 (F-value 11.49, Pr>F 0.0001) (Table 3.2) but the smallest EM were found in age 1. The mean weight without the cocoon was not significantly different (F-value 0.41, Pr>F 0.5227) when the two collection years were compared overall (Table 3.2).

3.3.4.) Area of the Egg Mass Without the Cocoon

The mean area of the EM without the cocoon attached was calculated for the total data set and was 137.33 mm^2 (Table 3.3). The mean area of the EM was significantly smaller in age 2 outbreaks and was highest in the age 0 outbreaks (F-value 11.18, Pr>F 0.0001) (Table 3.4). In 1992 EM collections, the area of the EM without the cocoon decreased to 134.63 mm^2 from 138.62 mm^2 in 1991 (Table 3.3). The area of the EM by age of the outbreak was significantly different in 1991 and 1992 EM collections (F-value 11.12 and 13.01, Pr>F 0.0001). In 1991, the smallest EM were found in age 2 while in 1992, the smallest EM were found in age 1. The area of the EM was not significantly different (F-value 2.49, Pr>F 0.1152) (Table 3.4) between the two collection

Table 3.3. Mean area (mm^2) and mean volume (mm^3) of the EM without the cocoon attached, by collection year (1991 and 1992), age of the outbreak (0, 1 and 2), and the total number of EM, Kamloops Forest Region, B.C..

Sample	Area of the EM (mm^2)			Volume of the EM (mm^3)		
	Number of EM	Mean	Standard error	Number of EM	Mean	Standard error
Age0	243	143.09	2.90	243	331.39	8.48
Age 1	286	145.29	2.59	286	337.95	7.46
Age 2	386	130.84	2.07	386	307.01	6.43
1991 EM	915	138.61	1.43	915	323.15	4.25
Age 0	141	146.09	3.74	141	341.48	10.34
Age 1	51	111.59	4.93	51	238.46	13.63
Age2	242	132.80	2.72	242	295.55	7.85
1992 EM	434	134.63	2.08	434	303.76	5.93
Age 0	384	144.20	2.29	384	335.09	6.57
Age 1	337	140.19	2.41	337	322.89	6.93
Age 2	628	131.59	1.65	628	302.59	4.98
All EM	1,349	137.33	1.18	1,349	316.91	3.46

Table 3.4. Anova table for mean area and volume of the egg mass without the cocoon attached for each collection year (1991 and 1992) and all EMs, Kamloops Forest Region, B.C..

	Source	df	MS	F-value	Pr>F
	Mean area (mm^2) of EM without cocoon				
1991	Age	2	20483.89	11.12	0.0001
1992	Age	2	23209.63	13.01	0.0001
1991 vs. 1992	Coll. yr.	1	4678.84	2.49	0.1152
All EM	Age	2	20758.21	11.18	0.0001
	Mean volume (mm^3) of EM without cocoon				
1991	Age	2	89856.19	5.50	0.0042
1992	Age	2	217151.08	15.14	0.0001
1991 vs. 1992	Coll. yr.	1	110650.04	6.87	0.0089
All EM	Age	2	133852.82	8.36	0.0002

years.

3.3.5) Volume of the EM Without the Cocoon

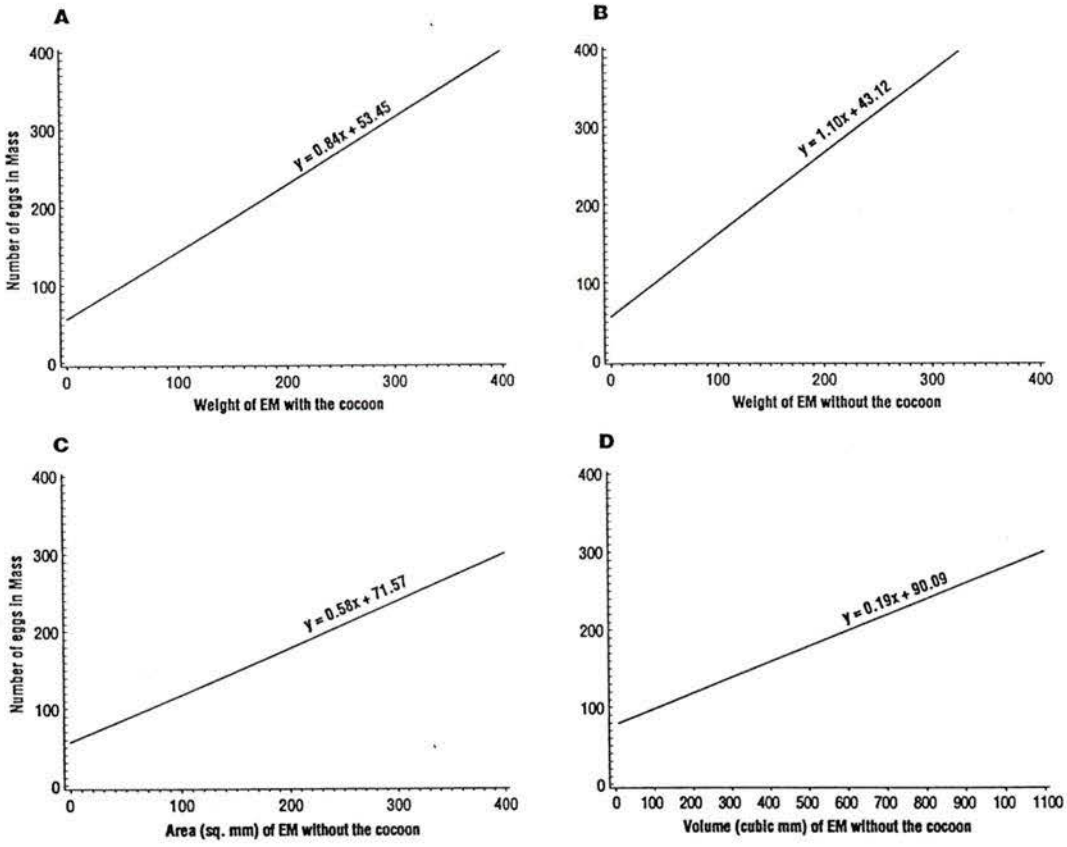
The mean volume of the egg-mass was larger in 1991 than in 1992 collected EM, 323.15 mm^3 and 303.76 mm^3, respectively (Table 3.3). The mean volume was found to 316.91 mm^3 and as the age of the outbreak progressed, the mean volumes were found to be significantly different (F-value 8.36, Pr>F 0.0001) with age 2 having the smallest mean volume (Duncan's multiple range test, Pr = 0.05).

When the volume of the egg mass without the cocoon was determined for each age of the outbreak in the 1991 and 1992 collection years the mean volumes were significantly different (F-value 5.50 Pr>F 0.0042 and F-value 15.14, Pr>F 0.001, respectively) (Table 3.4). In 1991 the mean volume was smallest among age 2 Ems , while in 1992, the smallest volume was found in age 1 (Duncan's multiple range test Pr = 0.05) (Table 3.4). Analysis of variance showed that the mean volume was significantly higher among 1991 collected EM than 1992 EM (F-value 6.87, Pr>F 0.0089, Duncan's multiple range test Pr = 0.05) (Table 3.4).

3.3.6) Linear Regression Equations

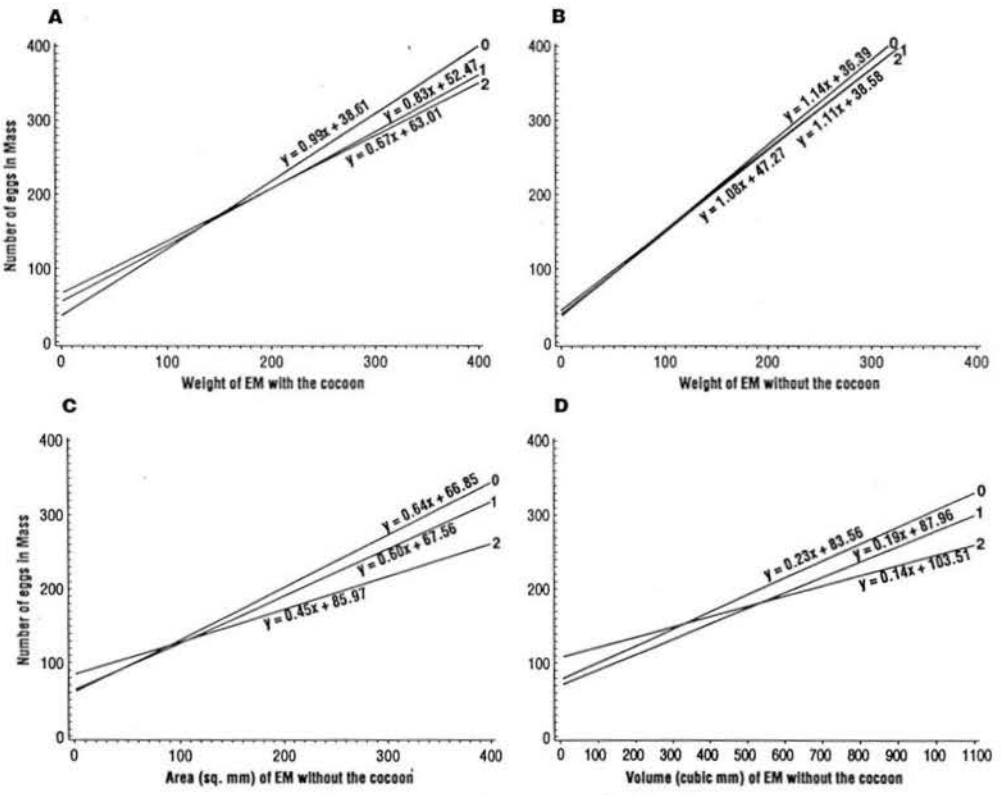
Linear regression equations were fitted to estimate the number of DFTM eggs per egg mass from the weight with and without the cocoon, area and volume of the egg mass for the total data combined (Figure 3.2 and 3.3), each collection year (Figures 3.4, 3.5, 3.6 and 3.7) and age of the outbreak

Figure 3.2. Regression equations for the total data set (1991 and 1992 collected EM combined) to predict the number of eggs per egg mass based on weight of the EM with and without the cocoon, area and volume of the EM.



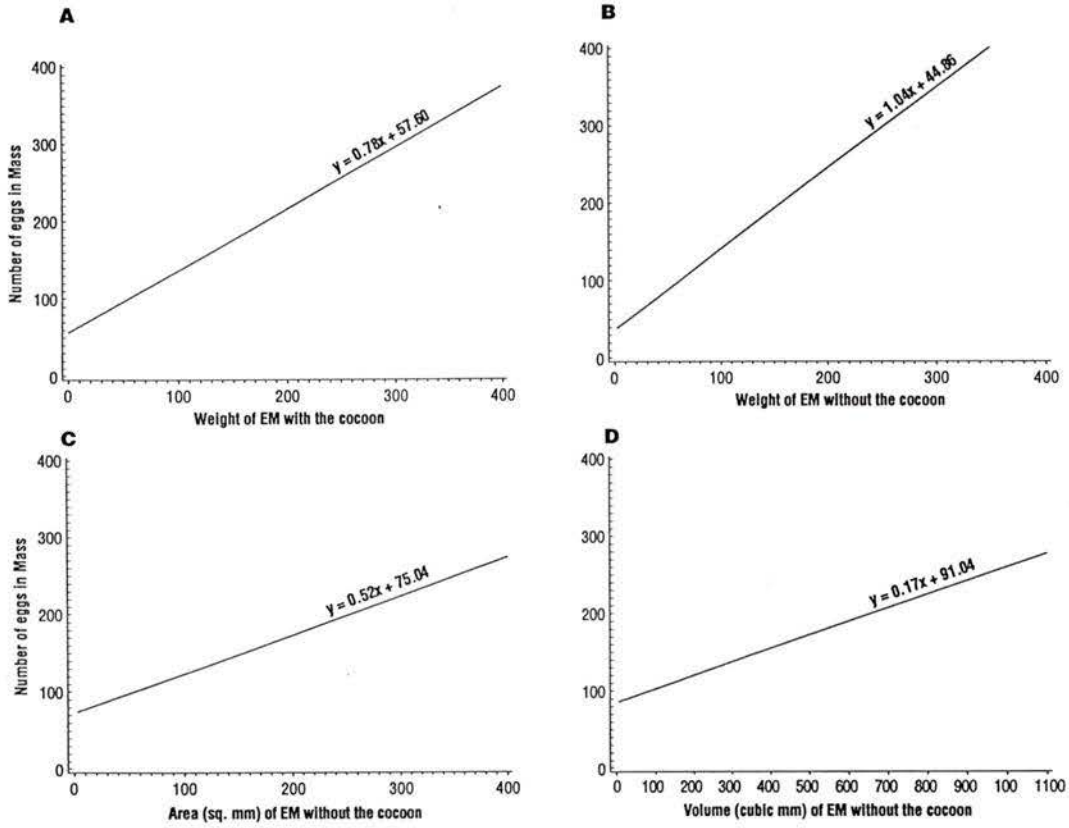
Total Egg Mass Data

Figure 3.3. Regression equations for each age of the outbreak (0, 1, and 2) for the total data set to predict the number of eggs per egg mass based in weight of the EM with and without the cocoon, area and volume of the EM.



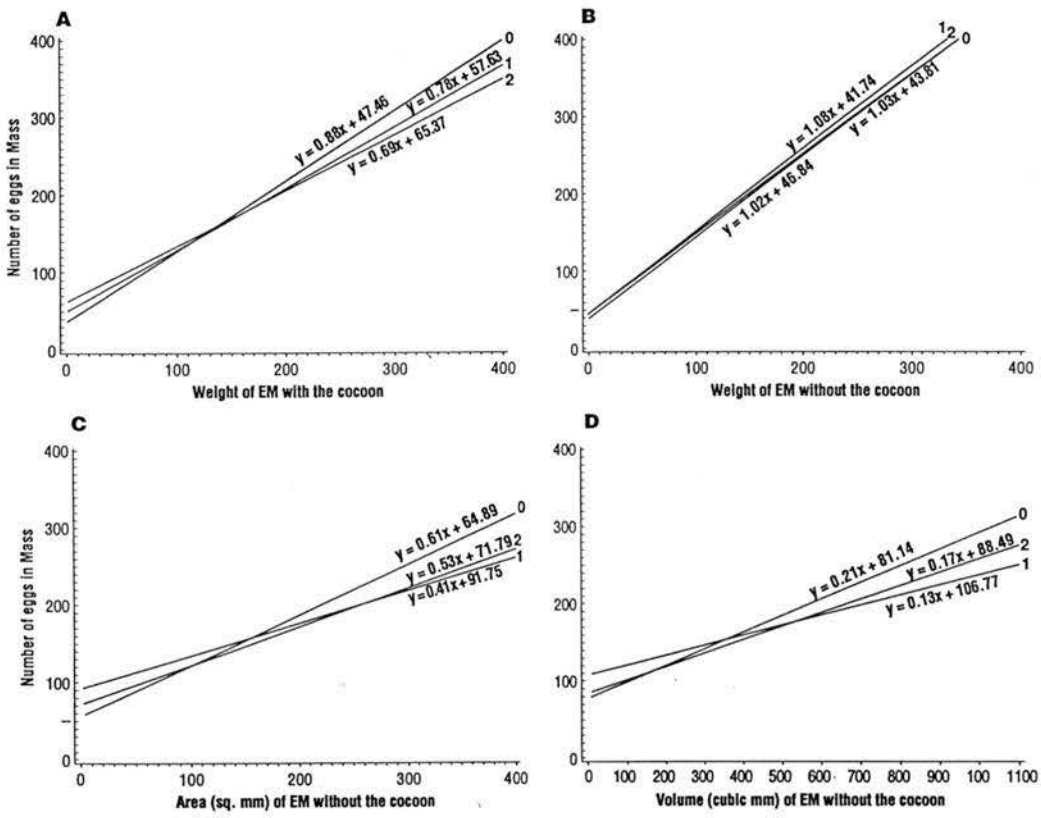
Total Egg Mass Data by Age of Outbreak

Figure 3.4. Regression equations for the 1991 collected EM to predict the number of egg per EM based on the weight of the EM with and without the cocoon, area and volume of the EM.



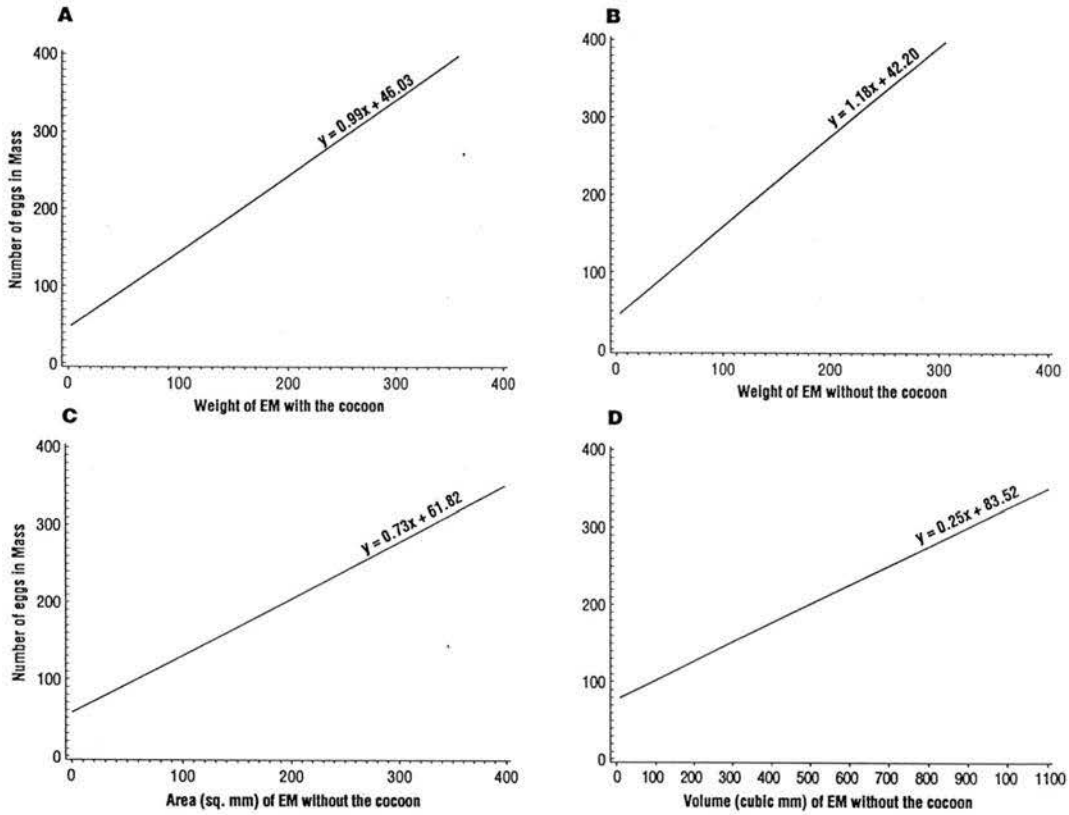
1991 Egg Mass Collection, Total Data

Figure 3.5. Regression equations for each age of the outbreak in the 1991 collected EM.



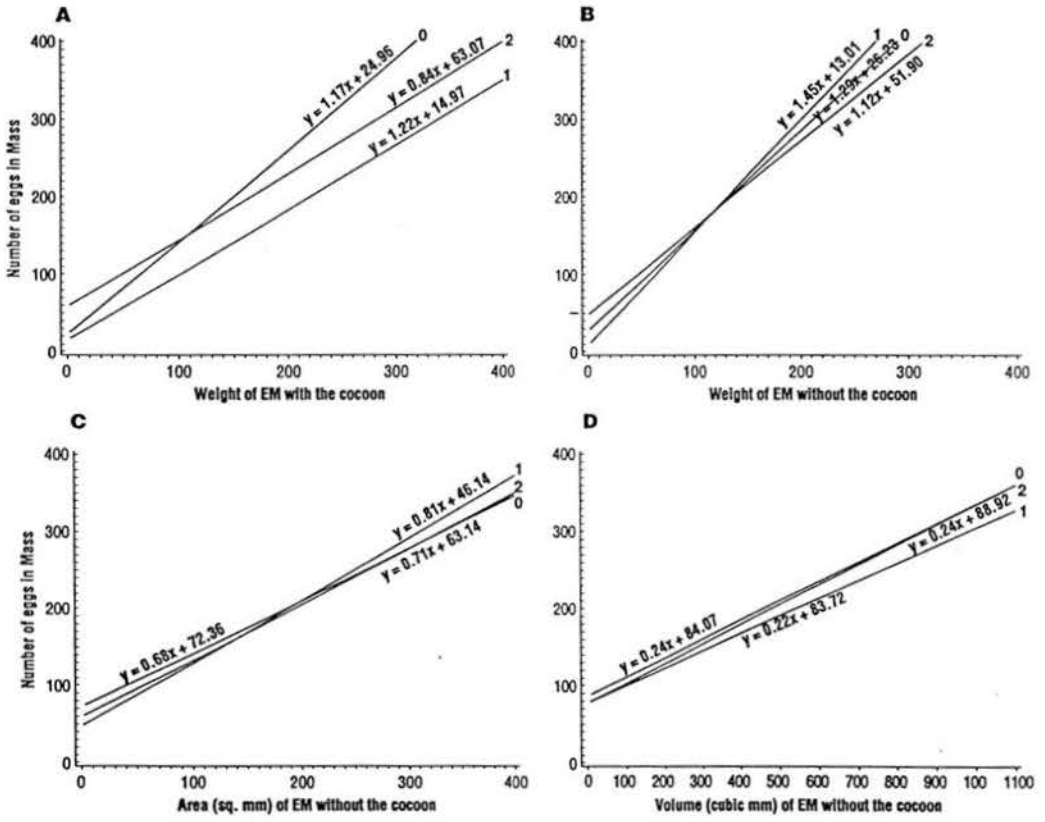
1991 Egg Mass Collection by Age

Figure 3.6. Regression equations for the 1992 collected EM to predict the number of eggs per EM based on the weight of the EM with and without the cocoon, area and volume of the EM.



1992 Egg Mass Collection, Total Data

Figure 3.7. Regression equations for each age of the outbreak in the 1992 collected EM.



1992 Egg Mass Collection, by Age of Outbreak

(Tables 3.5 and 3.6). The R-squared value was highest for the regression based on the weight of the EM without the cocoon, indicating that this variable is the best estimate of the number of eggs per egg mass in all cases (Table 3.5). Weight with the cocoon attached is also a good estimator of the number of eggs per egg mass in all cases (Table 3.5). Area of the egg mass and volume of the egg mass also estimate the number of eggs per egg mass but the over all R-squared values in all cases are lower than the weight R-squares (Table 3.6).

3.3.7) Analysis of Covariance to Compare the Regression Equations

One-way analysis of covariance was completed to compare the slopes and intercepts of the regression equations of egg number against weight with and without the cocoon, area of the egg mass and volume of the egg mass for each age of the outbreak in the collection year 1991 and 1992, and the total data set. Analysis of covariance by age of the outbreak for number of eggs per egg mass and weight of the egg mass without the cocoon for 1991 collected DFTM egg masses, show the regression lines are parallel, since the interaction term of age*weight without the cocoon was not significant (Table 3.7). The intercepts of the regression lines are not significantly different, so they may be combined to give one overall regression line for weight without the cocoon and number of eggs per egg mass in 1991.

Analysis of covariance of regression lines for weight with the cocoon, area and volume of the egg mass against number of eggs in the egg mass for age of the outbreak, showed in all cases a significant interaction term, which

Table 3.5. Regression equations for the weight with and without the cocoon attached against number of DFTM eggs per egg mass (egg no. = a + b(weight)), by age of the outbreak (0, 1 and 2), Kamloops Forest Region, B.C..

Sample	Number of EM	Weight of EM without cocoon			Weight of EM with cocoon		
		a	b	R^2	a	b	R^2
Age 0	243	43.81 + 4.45	1.03	0.7271	47.46 + 5.41	0.88	0.6263
Age 1	286	41.74 + 4.53	1.08	0.6877	57.63 + 5.62	0.78	0.5110
Age 2	386	46.84 + 3.45	1.02	0.6763	65.37 + 4.13	0.69	0.4876
1991 EM	915	44.86 + 2.30	1.04	0.7019	57.60 +2.83	0.78	0.5422
Age 0	141	26.32 + 6.31	1.29	0.8048	24.96 + 6.70	1.17	0.7879
Age 1	51	13.01 + 5.61	1.45	0.9142	14.67 + 8.93	1.22	0.8027
Age 2	242	51.90 + 4.61	1.12	0.7083	63.07 + 6.09	0.84	0.5246
1992 EM	434	42.20 + 3.33	1.18	0.7608	46.03 + 4.28	0.99	0.6414
Age 0	384	36.39 + 3.78	1.14	0.7505	38.61 + 4.78	0.99	0.6724
Age 1	337	38.58 + 3.89	1.11	0.7212	52.47 + 4.91	0.83	0.5535
Age 2	628	47.27 + 2.92	1.08	0.6725	63.01 + 3.58	0.76	0.4925
All EM	1349	43.12 + 1.96	1.10	0.7104	53.45 + 2.45	0.84	0.5628

Table 3.6. Regression equations for area (mm^2) and volume (mm^3) of the egg mass without the cocoon attached against the number of eggs per egg mass (egg no. = a + b(area or volume)), by age of the outbreak (0, 1 and 2), Kamloops Forest Region, B.C..

Sample	Number of EM	Area of EM without cocoon			Volume of EM without cocoon		
		a	b	R^2	a	b	R^2
Age 0	243	64.89 + 6.14	0.61	0.4766	81.14 + 4.86	0.21	0.5030
Age 1	286	91.75 + 6.20	0.41	0.2609	106.77 + 5.25	0.13	0.2233
Age 2	386	71.79 + 4.53	0.53	0.3973	88.49 + 3.54	0.17	0.3980
1991 EM	915	75.04 + 3.15	0.52	0.3839	91.04 + 2.57	0.17	0.3742
Age 0	141	72.36 + 9.45	0.68	0.4661	88.92 + 8.23	0.24	0.4527
Age 1	51	46.14 + 11.18	0.81	0.5925	83.72 + 11.34	0.22	0.3377
Age 2	242	63.14 + 6.35	0.71	0.5025	84.07 + 4.99	0.24	0.5129
1992 EM	434	61.82 + 4.74	0.73	0.5212	83.52 + 3.93	0.25	0.5030
Age 0	384	66.85 + 5.41	0.64	0.4550	83.56 + 4.44	0.23	0.4654
Age 1	337	85.97 + 5.39	0.45	0.3096	103.51 + 4.61	0.14	0.2510
Age 2	628	67.56 + 3.87	0.60	0.4253	87.96 + 3.09	0.19	0.4056
All EM	1349	71.57 + 2.73	0.58	0.4088	90.09 + 2.25	0.19	0.3876

Table 3.7. Analysis of covariance for the 1991 EM collections. The analysis is completed for each regression variable, weight with and without the cocoon, volume and area of the EM, Kamloops Forest Region, B.C..

Source	df	MS	F-Value	Pr>F
Age	2	162.49	0.41	0.6618
weight without	2	809025.79	2056.56	0.0001
wgt witho * Age	1	192.25	0.49	0.6136
Age	2	295.24	0.75	0.4720
weight without	1	818745.79	2083.6	0.0001
Age	2	2056.41	3.45	0.0322
weight with	2	625150.07	1048.55	0.0001
wgt with * Age	1	3280.78	5.50	0.0042
Age	2	1081.28	1.80	0.1666
weight with	1	628211.57	1043.38	0.0001
Age	2	6103.16	7.60	0.0005
Volume	2	426461.35	531.6	0.0001
Volume * Age	1	7205.69	8.97	0.0001
Age	2	4187.28	5.12	0.0061
Volume	1	432388.89	529.21	0.0001
Age	2	4605.86	5.76	0.0033
Area	2	438522.94	5458.23	0.0001
Area * Age	1	5131.23	6.41	0.0017
Age	2	1834.89	2.27	0.1042
Area	1	439365.18	542.83	0.0001

means the lines are not parallel, and the intercepts are all significantly different so that none of the regression lines can be combined (Table 3.7).

Analysis of covariance was also completed for regression equations created with the 1992 EM size data. Regression equations for weight of the egg mass with and without the cocoon and number of eggs per egg mass were compared using analysis of covariance for age of the outbreak, in both cases the regression lines were not found to be parallel and the intercepts were significantly different, giving three separate regression lines which cannot be combined (Table 3.8). The regression equations for volume and area of the egg mass against number of eggs per egg mass for each age of the outbreak were compared using covariance analysis and the regression lines were found to be parallel and the intercepts not significantly different, therefore the data can be combined to give one regression equation for area and volume of the egg mass for 1992 EM collections (Table 3.8).

The EM size data were combined for both collection years and analysis of covariance was completed for each regression variable (Table 3.9). Regression equations for weight of the EM without the cocoon against number of eggs per egg mass for each year of the outbreak were compared. Although the lines were found to be parallel but because the intercepts of the lines are significantly different, the lines cannot be combined (Table 3.9). The regression lines for weight without the cocoon, area and volume of the egg mass against number of eggs per egg mass for each age of the outbreak also

Table 3.8. Analysis of covariance for the 1992 EM collections. The analysis is completed for each regression variable, weight with and without the cocoon, volume and area of the EM, Kamloops Forest Region, B.C..

Source	df	MS	F-Value	Pr>F
Age	2	4009.26	9.25	0.0001
weight without	2	329077.49	758.85	0.0001
wgt witho * Age	1	2282.53	5.26	0.0055
Age	2	3728.82	8.43	0.0003
weight without	1	585503.36	1323.92	0.0001
Age	2	70352.88	10.86	0.0001
weight with	2	284562.77	439.51	0.0001
wgt with * Age	1	7353.03	11.36	0.0001
Age	2	2229.86	3.29	0.0384
weight with	1	483854.96	712.98	0.0001
Age	2	140.30	0.15	0.8627
Volume	2	176995.51	186.42	0.0001
Volume * Age	1	163.57	0.17	0.8418
Age	2	1976.49	2.09	0.1250
Volume	1	368972.94	390.11	0.0001
Age	2	1144.64	1.26	0.2856
Area	2	218884.4	240.34	0.0001
Area * Age	1	400.73	0.44	0.6443
Age	2	2504.45	2.76	0.0646
Area	1	385072.06	423.92	0.0001

Table 3.9. Analysis of covariance for the total, combined EM collections. The analysis is completed for each regression variable, weight with and without the cocoon, volume and area of the EM, Kamloops Forest Region, B.C..

Source	df	MS	F-Value	Pr>F
Age	2	1414.38	3.20	0.0410
weight without	2	1324699.97	2997.86	0.0001
wgt witho * Age	1	546.79	1.24	0.2905
Age	2	3501.66	7.92	0.0004
weight without	1	1446569.18	3272.50	0.0001
Age	2	5891.01	8.94	0.0001
weight with	2	1062220.22	1612.17	0.0001
wgt with * Age	1	8140.68	12.36	0.0001
Age	2	3571.21	5.33	0.0049
weight with	1	1139959.62	1701.43	0.0001
Age	2	5303.94	5.75	0.0033
Volume	2	710841.94	770.99	0.0001
Volume * Age	1	10718.94	11.62	0.0001
Age	2	6244.95	6.67	0.0013
Volume	1	781439.77	834.37	0.0001
Age	2	3978.71	4.44	0.0119
Area	2	764787.06	854.41	0.0001
Area * Age	1	7276.34	8.13	0.0003
Age	2	5702.30	6.30	0.0019
Area	1	824434.34	911.39	0.0001

cannot be combined as the lines are not parallel and the intercepts are significantly different in all cases (Table 3.9).

3.3.8) Analysis of Covariance to Compare the Collection Years

Analysis of covariance was used to compare the slopes and intercepts of the regression equations for each age of the outbreak in the two collection years (Table 3.10). For age 0 regression equations, analysis of covariance showed the interaction terms for weight without the cocoon and weight with the cocoon are significant, therefore the regression lines are not parallel. Also the intercepts of the two weight regression lines are significantly different, therefore, the lines are significantly different and cannot be combined. The analysis showed that the slopes of the area and volume of the EM regression equations for age 0 are parallel, and the intercepts are significantly different, also giving two separate regression equations.

The analysis of covariance for age 1 of the outbreak showed that in all cases, except volume of the EM, the regression lines were not parallel (Table 3.10), and the intercepts of the lines are not significantly different. The lines of each of these regressions cannot be combined and are therefore separate lines. For the volume of the EM regression equation comparing the collection years, the lines are parallel and the intercepts are significantly different, therefore the two regression lines are different and cannot be combined (Table 3.10).

The analysis of covariance for age 2 of the outbreak, comparing each

Table 3.10. Analysis of covariance for the two collection years (1991 and 1992), by age of the outbreak (0, 1 and 2) for each regression equation, weight with and without the cocoon, area and volume of the EM, Kamloops Forest Region, B.C..

Source	df	Age 0*			Age 1**			Age 2***		
		MS	F-Value	Pr>F	MS	F-Value	Pr>F	MS	F-Value	Pr>F
Coll year	1	2078.11	5.01	0.0258	2607.48	7.50	0.0065	354.19	0.82	0.3656
Weight without	1	475775.59	1146.16	0.0001	165840.12	476.88	0.0001	605178.60	1400.46	0.0001
Wgt witho * coll yr	1	5723.60	13.79	0.0002	3557.88	10.23	0.0015	1249.92	2.89	0.0895
Coll year	1	9590.12	22.35	0.0001	511.01	1.43	0.2326	29622.23	68.34	0.0001
Weight without	1	493098.73	1149.31	0.0001	300818.05	841.75	0.0001	604750.52	1395.25	0.0001
Coll year	1	3241.83	6.14	0.0137	5423.60	9.73	0.0702	73.23	0.11	0.7453
Weight with	1	439913.49	832.81	0.0001	130919.20	234.89	0.0001	440523.90	635.24	0.0001
Wgt with * coll yr	1	9194.64	17.41	0.0001	6246.79	11.21	0.0009	4040.27	5.83	0.0161
Coll year	1	17330.23	31.45	0.0001	91.20	0.16	0.6905	28908.87	41.37	0.0001
Weight with	1	446641.27	810.64	0.0001	228332.83	397.52	0.0001	438878.36	628.02	0.0001
Coll year	1	654.34	0.73	0.3931	3157.11	3.30	0.0702	421.38	0.55	0.4584
Volume	1	292860.25	327.13	0.0001	53135.44	55.55	0.0001	395801.62	517.18	0.0001
Volume * coll yr	1	1320.60	1.48	0.2253	3378.95	3.63	0.0611	13922.85	18.19	0.0001
Coll year	1	29277.03	32.66	0.0001	44.19	0.05	0.8306	53999.71	68.67	0.0001
Volume	1	315054.27	351.49	0.0001	98248.21	101.93	0.0001	384175.47	488.55	0.0001
Coll year	1	428.74	0.47	0.4933	7913.92	9.15	0.0027	1002.52	1.30	0.2552
Area	1	294174.54	322.57	0.0001	82338.86	95.21	0.0001	392428.85	507.64	0.0001
Area * coll yr	1	1023.38	1.12	0.2901	8815.41	10.19	0.0015	8683.33	11.23	0.0009
Coll year	1	30431.69	33.36	0.0001	1.36	0.00	0.9688	36277.78	46.17	0.0001
Area	1	308995.05	338.72	0.0001	123395.58	138.87	0.0001	384588.41	489.49	0.0001

* N = 384 ** N = 337 *** N = 628

collection year, showed that, for weight without the cocoon attached, the lines are parallel and the intercepts are significantly different, therefore the lines cannot be combined (Table 3.10). For age 2 of the outbreak weight with the cocoon, area and volume of the EM, all the regression equations are not parallel and the intercepts are also significantly different, and the lines are therefore, separate (Table 3.10).

3.4) Discussion

As the DFTM outbreak progresses the number of eggs per EM decreases. More eggs were found in age 0 EM than in the age 1 or age 2 EM (for the combined collection years). When the number of eggs was compared by collection years, 1992 EM had more eggs than 1991 EM, which is different than the decreasing age trend. 1992 was the third year of DFTM infestation in the Kamloops Forest Region and the number of eggs per mass should have decreased this year if it followed the age of the outbreak trend. There were only 434 EM collected in 1992, approximately half the number of EM collected in 1991. If more EM could have been located in 1992 the trend of decreasing size of the EM may have been apparent. Only one infestation in 1992 was in the age 1 class (51 EM), and the number of eggs per EM for this site was lower (136.25) than all others ages. Again if more EMs could have been collected, the trend of decreasing number of eggs as the outbreak progresses may have been more apparent. The 1991 collected EM did follow the trend of decreasing

size as the outbreak progressed.

Beckwith *et al.* (1978) collected 129 DFTM EM from a peaking population in the Kamloops area and found a mean number of eggs per EM to be 164.8 eggs. This number is higher than those found with this study. There has been variation in the number of DFTM eggs per mass in reports on tussock moth biology. Mason (1970) found an average of 160 eggs per EM from a small outbreak in northern California, while EM with 260 eggs were found in a pre-outbreak site in Arizona (Mason *et al.*, 1977). For some insects egg production can be influenced by a number of factors, especially the nutritional quality of the food source, different types of host tree and the ambient temperature at the infestation site (Mason, 1981). Either of these factors can vary by site and crown level in the host trees, affecting the fecundity of the female. In the present study, the range of outbreak conditions was sampled by choosing infestations with trace, light and moderate defoliation (this corresponds to the age classes) and eggs numbers were higher in trace defoliation sites, age 0, than in age 1 or 2 sites with more defoliation. When laboratory reared DFTM larvae are forced to feed only on older tree foliage, similar to when population densities are high in the field, the larvae feed longer and the time to pupation is increased (Beckwith, 1976). If larvae are given a choice between older and newly flushed foliage, they will always choose to eat new foliage (Beckwith, 1976; Mason and Baxter, 1970). The choice and preference by the larvae for new foliage suggest that the new

foliage provides the developing larvae with some beneficial factor, such as nutritional quality and perhaps more moisture. Depletion of the new foliage in high density populations will bring about nutritional stress, delay larval development, increase the exposure to biological controls agents (parasitoids and predators) and a decrease in egg production (Beckwith, 1976). Beckwith (1976) found that the mean number of eggs per mass was 143.2 in areas with heavy defoliation and the number of eggs increased to 195.14 in areas with light defoliation. A similar decrease in the number of eggs per egg mass was also observed in the 1971 and 1972 outbreak in El Dorado County (Dahlsten *et al.*, 1977). In 1972, the number of eggs per mass had decreased 34% from the fall of 1971 (Dahlsten *et al.*, 1977). It is clear that in a number of study sites, as the DFTM infestation progresses, the number of eggs per EM decreases. Since the DFTM population size was beginning to increase, the female's may have been feeding only on older foliage, leading to the decrease in EM size.

The weight of the egg mass with and without the cocoon, the area and volume of the egg mass are also indices of fecundity. These variables are related to the number of eggs in the mass and the weight of each egg. If the female DFTM has a lot of high quality food, the weight of the EM should be high, and as food decreases at the site due to competition among larvae, the weight should decrease. The weights reported in this study are lower than those reported by Beckwith *et al.* (1978), but as the outbreak progressed in Beckwith's study, the weight of the EM without the cocoon decreased. The

present study was completed during the first and second year of DFTM defoliation in the Kamloops area and Beckwith's study, was conducted in the Kamloops area, with DFTM collections being made in areas where the population was peaking. Since the populations were not exactly at the same stage of the outbreak, only the trend of decreasing EM size can be observed and no direct comparison of the weights should be made. Also Beckwith *et al.* (1978) only collected 129 EM which may have been too few to accurately observe the decreasing EM weight trends.

Of the regression equations created for the number of eggs per EM against the variables, weight of the EM with cocoon, weight without the cocoon, area of the EM and volume of the EM, the highest correlation was between weight without the cocoon and number of eggs per EM ($R^2 = 0.7104$ for all EM combined). Thus, weight of the EM without the cocoon is the best variable to estimate the number of eggs per EM. It would be possible to measure length and width of the EM in the field, calculate the area or volume of the EM in the laboratory, and use these variables to predict the number of eggs per EM, these predictions may be less precise, and underestimate the number of eggs present. Dimensions of the EM could be used if general estimates of the population were needed, but EM weight will result in a more precise population density estimate.

When the regression equations were examined, none of the lines were found to pass through the origin. The common DFTM regression created by

Beckwith *et al.* (1978) for weight without the cocoon to predict egg number, also does not go through the origin. The weights of the eggs themselves or the weight of the glue and abdominal hairs embedded in the EM as it is laid may be the reason the lines do not passing through the origin. Leonard (1970) found that gypsy moth, *Lymantria dispar* (L.), eggs varied in size when laid in the EM. Leonard (1970) weighed individual eggs from different parts of the EM, and found that eggs which were laid first in the mass, at the wider end of the EM, had a significantly higher weight then the eggs laid near the end of the EM. Leonard (1970) suggested that the weight of the egg is related to the amount of yolk present in the eggs and that the smaller, later laid eggs, had less yolk. Since the female DFTM also lays eggs in a mass similar to gypsy moth, the yolk may vary within the eggs as well, affecting the size and weight of the DFTM EM. This change in yolk size may account for the regression lines not passing through the origin. If the food quality is poor near the end of the outbreak and the female DFTM is forced to feed on older growth foliage this may affect the amount of nutrients available in the eggs in the eggs she lays, making the weights of the EM decrease. Also the poor quality of food may affect the female's overall fitness and when she lays the EM, she may have produced less glue and abdominal hairs, so that the weight of the EM may be smaller not only due to fewer eggs with less yolk, but also due to less glue and hairs.

The only regression equations which can be combined in this study are

the weight without the cocoon in 1991, the area and volume of the EM in 1992 and the volume of the EM for age 1, when the two collection years were compared. Since the equations cannot be combined, new regression equations should be created for each year of a DFTM outbreak. Sampling the EM at several sites early in the fall and measuring the size or weighing and counting the number of eggs per egg mass should be completed and then these values can be used to compute a regression for that year. Then other additional EM can be collected and the number of eggs in these additional EMs can be predicted from the regression. Beckwith *et al.* (1978) did create a common regression equation for DFTM by combining data from different collection conditions (trace to heavy defoliation). This common regression underestimates the size of EM in heavily defoliated sites and overestimates the EM size in lightly defoliated conditions (Beckwith *et al.*, 1978). Since it is time consuming to collect, weigh and count the number of eggs per EM to create the regressions, the common regression Beckwith *et al.* (1978) could be used to estimate the population size in general. To ensure accurate estimations of populations size using regression equations, larval counts should be made in the spring at the fall EM collection locations, to ensure the numbers of hatching larvae correspond to the predicted damage. During overwintering, egg viability may decrease due to adverse climatic conditions, or predation of the EM may have occurred, decreasing the population at the sites, so regression population predictions should be accompanied by spring

larval sampling.

3.5) Summary

Collections of DFTM egg masses in the fall of 1991 and 1992 in the Kamloops Forest Region show that the number of eggs per egg mass decrease during an outbreak. Of the parameters measured, the weight of the EM without the cocoon attached has the highest correlation with the number of eggs per egg mass and this regression can be used to predict DFTM population size. The other regression equations are also good predictors of the number of eggs per EM, but if they are used in population predictions, they may underestimate the population size. The regression equations cannot be combined in most cases, thus separate regressions must be created for each year of the outbreak. If the regressions were combined, they would underestimate the population size at high population densities and overestimate the size in low population densities.

Chapter 4

Incidence of Nuclear Polyhedrosis Virus In Early Instar Douglas-fir Tussock Moth Larvae

4.1) Introduction

The Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae) is a native, periodic defoliator in British Columbia's forests (Sudgen, 1957; Harris *et al.*, 1985). Current DFTM control measures in British Columbia include spraying infested stands with a naturally occurring nuclear polyhedrosis virus (NPV) prior to serious defoliation of the host trees.

Viral sprays of DFTM populations provide protection of the host foliage, but the only viable method of virus production at present is in living insects, and this procedure is costly. If the incidence of naturally-occurring NPV could be determined from fall collected egg masses and if the level of infection was naturally high, at a "thresh-hold level" after which the population would collapse naturally, then these stands could be excluded from treatment, reducing the overall cost of spraying. Dahlsten and Thomas (1969) were the first researchers to suggest this potential for predicting the onset of an epizootic by monitoring the incidence of virus from larvae hatched from DFTM eggs. By rearing all larvae from one egg mass in one petri dish on artificial diet, it was shown that the percent mortality in the laboratory may indicate the mortality in the field (Dahlsten and Thomas,

1969; Thompson and Peterson, 1978). It was found for the Gypsy moth that mortality greater than 20% caused by LdNPV among larvae reared from field-collected egg masses was predictive of impending epizootic, but when mortality was below 5% no epizootic resulted (Woods *et al.*, 1991). If a similar relationship between DFTM larvae in the field and laboratory rearings can be shown it could be used to indicate an impending epizootic in the field.

The focus of this study was to examine the percent incidence of NPV infection from fall collected DFTM egg masses from various sites in the field with different amounts of defoliation. By aging the outbreaks at the collection sites on the basis of visible defoliation at the site it was hoped that a "threshold level" of NPV infection could be identified above which sites will not need to be treated with NPV since the stands will have enough NPV inoculum in the hatching larvae to initiate an epizootic. If a "threshold" level can be identified the cost of treating stands close to a natural collapse can be saved.

4.2) Materials and Methods

4.2.1) Egg-mass Collection, Rearing and Determination of Infection

DFTM egg masses were collected by Canadian Forest Personnel as described in section 3.2.1.

Each of the 42 collection sites was grouped into one of six geographical regions. The geographical regions were determined by examining the

location of the collection sites and grouping them according to region by the closest Canada Post Office in the closest settlement center in the area. The six geographic regions are: Chilliwack, Ashcroft, Savona, Kamloops, Vernon and Hedley.

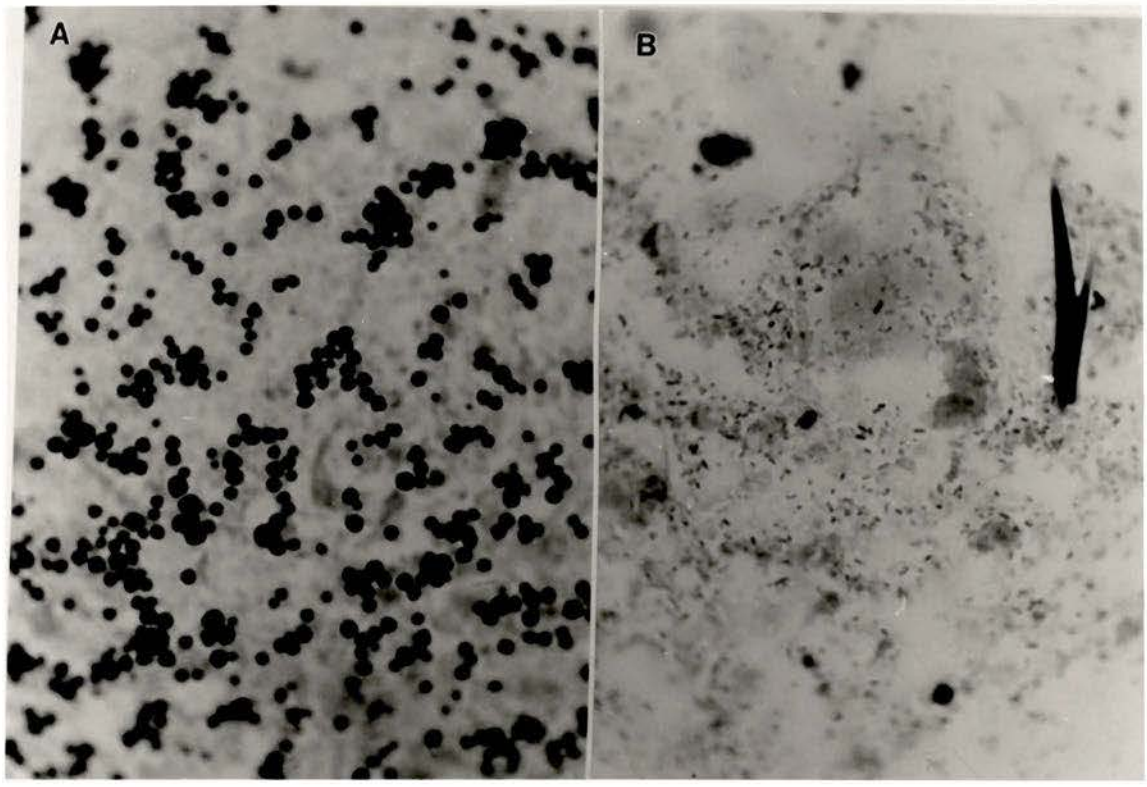
After the number of eggs per egg mass were counted (section 3.2.1), each EM was placed in a small petri dishes (9 X 50 mm) and placed into rearing at 25^{O} C, 60 % RH and 12 hours light: 12 hours dark to allow for hatching. On the second day of larval emergence, 25 randomly selected larvae were placed on artificial diet (Thompson and Peterson, 1978) and reared for 14 days, in 15 X 90 mm petri dishes, at the above conditions. If fewer than 25 larvae emerged from the egg mass, all larvae that did emerge were reared on artificial diet. After 7 days of rearing, the old diet was exchanged with fresh diet and any dead larvae were counted removed from the dish and stored individually in microfuge tubes at -20^{O} C until examination of the cadavers for the presence of NPV. After 14 days of rearing, any additional dead larvae were also removed and stored in microfuge tubes as above. If any larvae remained alive after 14 days of rearing, they were then used in the study of genotypic variation of OpNPV (Chapter 5 of this thesis).

When more than 50 EM had been collected from a study the site they were placed into rearing in two sets (Set A and Set B). 15 of 21 collection sites had two sets of rearings in 1991. Two sets of rearings were necessary because of the large amount of handling time required to rear 25 larvae from each

EM, and it would have been impossible to rear from all 50 EM all at once. When 30 or fewer EM were collected from a site, all EM from that site were reared as one set at one time. The first set of EM rearings (25 EM from 15 sites and from the 6 sites with less than 30 EM) were completed between January and April 1992 and the second set of rearings (next set of 25 EM from the same 15 sites) were completed between May and July 1992. All the EM from the fall 1992 collections were reared at one time because fewer DFTM infestation sites were present in 1992.

The dead DFTM larvae were stored individually in microfuge tubes at -20° C until all rearings were completed. When all the rearings from the fall collected EM were complete, the stored dead larvae were removed from their microfuge tube and smeared individually on a microscope slide in a drop of distilled water with a fresh toothpick for each insect cadaver. Slides were allowed to air dry. The slides were then stained with Napthalene Black stain at 40-45° C for 10 minutes (Napthalene Black stain: 4.8 g Napthalene Black 12B, 140 ml glacial acetic acid, 260 ml distilled water). Slides were removed from the stain and gently rinsed in distilled water to remove excess stain and then allowed to air dry. Microscopes slides were then examined for the presence of dark stained polyhedral inclusion bodies (PIB) under oil immersion 1000X magnification (Figure 4.1).

Figure 4.1. Positive (A) and negative (B) OpNPV infections of early instar DFTM larvae (1000X magnification).


A
B

4.2.2) Analysis

Mean and standard error of the incidence of NPV among insects from each EM, were calculated using SAS Proc Means procedure, for each site and year of the outbreak (SAS Institute, 1989). SAS Proc Anova procedure was used to compare the incidence of NPV among sites, age of the outbreak, collection years, geographic regions and between the first and second set of 1991 egg masses reared (SAS Institute, 1989).

4.3) Results

From the 1991 and 1992 collected EM, 21 sites (11 sites were not used since too few EM were collected from each site) and 10 sites, respectively, were used in the determination of NPV from early instar larvae. A total of 30,074 DFTM larvae, from 1,245 EM, were reared for the determination of incidence of NPV among early instars. Of the reared insects, 8,534 died and 4,810 of those that died had acquired NPV infection during eclosion and died of the infection during the first 14 days of rearing; this represents a 15.99% incidence of NPV. The incidence of NPV was determined for each of the 31 collection sites and each set of rearing (Set A or Set B), as applicable. Percent incidence of NPV ranged between 0.16% at Indian Gardens East 3 in the Savona geographic region to 84.25% at Brussels Creek in the Kamloops geographic region (Table 4.1, Table 4.2, Table 4.3 and Table 4.4).

Of the 21 sites with DFTM EM in 1991, 15 sites had two sets of rearings

Table 4.1. Incidence of NPV for each site and set (Set A or Set B) of egg masses reared from fall 1991 EM collections, age 0 infestations.

Site	Set	Number of egg masses	Number of larvae reared	No. dead larvae	No. larvae with NPV	Percent	Age	Region*
Larcan Cr. Tr.	A	19	466	47	42	9.01	0	H
Larcan Cr. up.	A	23	525	96	48	9.14	0	H
Bradshaw Cr.	A	22	507	116	59	11.64	0	H
Guichon 1.3E	A	25	624	78	71	11.38	0	S
	B	24	601	479	395	65.72	0	S
Guichon 2.2W	A	24	600	145	32	5.33	0	S
	B	22	536	41	8	1.49	0	S
Rattle Snake	A	25	610	78	4	0.66	0	Ver
	B	25	454	335	270	59.47	0	Ver
Shoemaker up.	A	26	639	131	106	16.59	0	H
Sub-total Set A		164	3,971	691	362	9.12		
Sub-total Set B		71	1,591	855	673	42.30		
Total		235	5,562	1,546	1,035	18.61		

* Geographic regions are: H=Hedley, Ver= Vernon and S= Savona.

Table 4.2. Incidence of NPV for each site and set (Set A or Set B) egg masses reared from fall 1991 EM collections, age 1 infestations.

Site	Set	Number of egg masses	Number of larvae reared	No. dead larvae	No. larvae with NPV	Percent	Age	Region*
Beaton Lk	A	18	450	152	9	2.00	1	K
	B	15	372	313	293	78.76	1	K
Shoemaker Tr.	A	21	481	156	134	27.86	1	S
IG Hydro line	A	24	601	67	17	2.83	1	S
	B	25	575	208	118	20.52	1	S
NE Lot 20	A	25	625	166	50	8.00	1	S
	B	24	565	143	97	17.17	1	K
Dome Fire 1	A	25	622	193	118	18.97	1	K
	B	24	604	487	441	73.01	1	K
Dome Fire 2	A	25	625	42	21	3.36	1	K
	B	23	577	231	178	30.84	1	K
Sub-total Set A		138	3,404	776	349	10.25		
Sub-total Set B		111	2,693	1,382	1,127	41.85		
Total		249	6,097	2,158	1,476	24.21		

* Geographic regions are: K=Kamloops and S=Savona.

Table 4.3. Incidence of NPV for each site and set (Set A or Set B) of egg masses reared from fall 1991 EM collections, age 2 infestations.

Site	Set	Number of egg masses	Number of larvae reared	No. dead larvae	No. larvae with NPV	Percent	Age	Region*
Brussels Cr.	A	18	450	53	50	11.11	2	K
	B	16	400	347	337	84.25	2	K
Brussels Lk.	A	25	602	87	6	0.99	2	K
	B	17	408	128	51	12.5	2	K
IG E1	A	29	676	195	36	5.33	2	S
IG E2	A	23	555	85	1	0.18	2	S
	B	17	405	60	13	3.21	2	S
IG E3	A	25	627	134	15	2.39	2	S
	B	25	591	225	173	29.27	2	S
IG W1	A	25	625	128	1	0.16	2	S
	B	44	1054	172	47	4.46	2	S
Munro	A	25	626	67	20	4.79	2	K
	B	25	626	127	96	15.36	2	K
Sabiston 4.3km	A	25	626	62	0	0.00	2	S
	B	25	625	89	17	2.72	2	S
Sub-total Set A		195	4,787	811	129	2.69		
Sub-total Set B		169	4,109	1,148	734	17.86		
Total		364	8,895	1,959	873	9.81		

* Geographic regions are: K=Kamloops and S=Savona.

Table 4.4. Mean percent NPV for the 15 sites in 1991 with two sets of rearings (Set A and Set B).

Set	Number of sites	Percent NPV	Standard error
Set A	15	4.81	1.4
Set B	15	33.25	7.83*

* Ducan multiple range test (p=0.05) showed significantly more NPV in Set B than Set A.

(Table 4.1, Table 4.2 and Table 4.3). The mean percent incidence of NPV for Set A (the first set of 25 EM reared) was 4.81% while Set B (the second set of EM reared) had a mean percent incidence of 33.25% (Table 4.4). The test of analysis of variance showed a significant difference in the incidence of infection of the early instar larvae with OpNPV between the two sets reared in 1991 (F-value 14.97, Pr>F 0.0007) (Table 4.6). A Duncan multiple range test (p=0.05) showed that the second set of rearings (Set B) had a significantly higher amount of NPV than Set A (Table 4.4).

The incidence of NPV infection among the 1992 EM ranged from 6.32% at Murray Creek in the Ashcroft geographic region to 33.81% in Iron Mask in the Kamloops geographic region (Table 4.5). The incidence of NPV infection was higher in age infestations (33.81%) than the age 0 or age 2 infestations (14.69% and 10.68%, respectively) (Table 4.5).

Since there is a significant difference in the amount of NPV in the two sets of rearings from 1991, these data can not be combined (Table 4.6). To compare the 1991 collected EM with the 1992 collected EM, Set A, from 1991 was used because EM in Set A were placed into rearing at the same time of the year as the 1992 EM and EM in 1992 were reared as one set. The mean incidence of NPV by age of the outbreak for each of the two collection years showed a similar trend (Table 4.7). The mean percent incidence of NPV Infection was higher for age 1 infestations, than for age 0 or 2 infestations (Table 4.7). The test of analysis of variance for each

Table 4.5. Incidence of NPV for each site and different age of the outbreak determined egg masses reared from fall 1992 EM collections.

Site	Number of egg masses*	Number of larvae reared	No. dead larvae	No. larvae with NPV	Percent	Age	Region**
Heffley Cr.	35	864	148	78	9.03	0	K
Murray Cr.	48	1,175	81	71	6.04	0	A
Sabistan 13km	53	1,311	403	343	26.16	0	S
Total yr 0	136	3,350	632	492	14.69		
Iron Mask	48	1,189	513	402	33.81	1	K
Total yr 1	48	1,189	513	402	33.81		
Lac du Bois	20	483	93	41	8.49	2	K
Sarangetti	29	657	134	71	10.81	2	A
Mara Hill	51	1,287	214	108	8.39	2	K
Red Hill Rest Stop	38	847	82	60	7.08	2	A
Oregon Jack Rd.	37	868	736	199	22.93	2	A
Red Hill Rd.	38	839	467	53	6.32	2	A
Total yr 2	213	4,981	1,726	532	10.68		

* All egg masses from the various sites were reared as one set.

** Geographic regions are: A=Ashcroft, K=Kamloops and S=Savona.

Table 4.6. Anova values for the two sets of rearings (Set A and Set B) of the egg masses collected in the fall of 1991 and age of the outbreak (0, 1 and 2).

Source of variation	df	MS	F	Pr>F
Set	1	1.0869	14.97	0.0007*
Age	2	0.1290	1.78	0.1906
Set*Age	2	0.0216	0.30	0.7450
Set A Only				
Age	2	0.1281	1.46	0.2712**
Set B Only				
Age	2	0.1281	0.99	0.7450**

* Duncan muliple range test (p=0.05) showed a significant difference in the amount of NPV between sets but not among age of the outbreak (compares incidence of NPV between sets).

** Duncan muliple range test (p=0.05) showed no signifcant difference in NPV among age of the outbreak.

Table 4.7. Mean percent NPV for each age of the outbreak (0, 1 and 2) for 1991* (N=21 sites) and 1992 (N=10 sites).

Collection Year	Age	Number of sites	Mean percent NPV	Standard error
1991	0	7	9.11	1.91
	1	6	10.52	4.33
	2	8	3.12	1.36
		21	7.22	1.57
1992	0	3	13.74	6.27
	1	1	33.81	-
	2	6	10.67	2.53
		10	13.91	3.14

* Only data from Set A was included in the analysis.

collection year, age of the outbreak and collection year by age was performed (Table 4.8). Analysis of variance showed that the collection year was significantly different (F-value 10.86, Pr>F 0.0029) and age of the outbreak is also significantly different (F-value 4.85, Pr>F 0.0166) although a Duncan multiple range test failed to show any differences among the age of the outbreak at the p=0.05 significance level (Table 4.8). A Duncan multiple range test (p=0.05) did show a higher incidence of infection among 1992 collected EM than the EM collected in 1991. The mean incidence of NPV infection for 1991 was 7.22% and it nearly doubled to 13.91% in 1992 (Table 4.7). When the test of analysis of variance was completed for age of the outbreak in each collection year (1991 or 1992), incidence of NPV infection by age of the outbreak was significantly different among 1991 collected EM, while it was not significantly different among 1992 EM (Table 4.8). A Duncan multiple range test (P = 0.05) showed that there was less virus in a 2 year old outbreak than outbreaks with little defoliation (age 0) or a 1 year old outbreak for the 1991 collected EM (Table 4.8).

There were no significant differences in NPV among geographic regions (F-value 1.31, Pr>F 0.2921). The mean percent NPV by geographic region varied from 25.75 in Kelowna to 10.64 in Ashcroft (Table 4.9).

4.4) Discussion

In 1991, the EM were collected in early November following an

Table 4.8. Anova values for 1991* and 1992 collection years and age of the outbreak (0, 1 and 2).

Source of variation	df	MS	F	Pr>F
Collection year	1	0.1727	10.86	0.0029**
Age	2	0.0771	4.85	0.0166
Coll. Yr.* Age	2	0.0202	1.27	0.2981
1991 Only				
Age	2	0.0581	3.37	0.0572***
1992 Only				
Age	2	0.0376	3.02	0.1136****

* Only data from Set A was included in the analysis.

** Duncan multiple range test (p=0.05) showed that egg masses collected in 1992 had significantly more NPV than 1991 egg masses.

*** Duncan multiple range test (p=0.05) showed that age 2 has significantly less NPV than age 0 and age 1.

**** Duncan multiple range test (p=0.05) showed no significant difference between the ages of the outbreak.

Table 4.9. Mean percent incidence of NPV by geographic region.

Geographic region	Number of sites	Mean percent NPV	Standard error
Ashcroft	5	10.64	3.19
Savona	10	11.80	3.82
Kamloops	10	21.99	5.21
Hedley	5	14.84	3.53
Vernon	1	25.75	-

unexpectedly early and heavy snowfall. The EM were wet when collected and placed in plastic bags for transportation to Victoria for diapause. The first set of egg masses (Set A) were reared between January and April, while the second set of EM (Set B) were reared between May and July of 1992. During the longer period of cold storage the dampness on the surface of the EM may have spread the virus over the EM surface resulting in the higher incidence of NPV among the hatching larvae in the second set of rearings (Set B). Doane (1969) found that long refrigeration periods of the EM did not increase the Gypsy moth's susceptibility (higher incidence of NPV infection) to its NPV. In this study with DFTM, the EM were stored damp in plastic bags and the moisture in the bag could have condensed on the surface of the plastic bag and on the egg mass itself. This moisture then may have spread over the surface of the egg mass, which could have lead to a more even distribution of the virus on the EM surface, giving a higher infection rate among larvae which hatched from EM that were stored in plastic bags for about 4 months longer. To ensure accurate reporting of the percent incidence of NPV in hatching DFTM larvae, EM should be stored dry and in paper bags to prevent further contamination. If possible, all the EM from one site should be reared all at the same time, in one batch.

The incidence of NPV infection among the hatching larvae varied considerably among collection sites. In a 1973 DFTM outbreak in the Blue Mountains in Oregon, the overall incidence of NPV in 1973 (during the

whole field season) among the larvae sampled was an average of 7.2%; 0.0% in very light defoliated plots, and 7.0% in heavily defoliated plots when the larvae were sampled soon after hatching (Mason, 1976). In the spring of 1974, when the population was collapsing, the same plots showed an increase in NPV infection in early instar larvae; 22.6% of the larvae sampled in June and July of 1974 were infected (Mason, 1976). At two DFTM infestation sites in Nevada, (Iron Mountain and Plummer Ridge) where the population was just beginning to rise and the populations were considered to be at a sub-outbreak level (the researchers did not indicate the amount of visible defoliation) no field-collected, early instar larvae were found to be infected with OpNPV (Mason *et al.*, 1983). At three infestation areas in northeastern California, 50%, 55% and 71.3% incidence of NPV infection among hatching larvae, from field-collected EM, were observed in DFTM populations close to collapse (Dahlsten and Thomas, 1969). Larvae collected at the time of hatching in Modoc National Forest, California had a 10% infection rate in the 1966 DFTM outbreak, leading to a population collapse in the summer (Mason and Thompson, 1971). It is apparent that the incidence of NPV infection in early instar larvae varies considerably depending on the site of infestation and possibly age of the outbreak at these sites. Early outbreak sites tend to show a low incidence of NPV among the hatching early instar larvae while older outbreak infestations show a rise in the number of hatching larvae infected with the virus (Mason, 1976).

Mason (1976) created four DFTM life tables based on the amount of visible defoliation at sites which correspond to heavy, moderate, light and trace defoliation categories and the incidence of OpNPV infection in the first three instars in these four defoliation categories was found to be 5.0%, 7.0%, 1.3% and 0.0%, respectively, after field collection and laboratory rearing. The current study classified the age of the outbreak at a site based on the amount of visible defoliation: trace defoliation is age 0, light defoliation is age 1 and moderate to heavy defoliation is age 2. The incidence of OpNPV among hatching larvae was found to be higher in age 1 infestations, with light defoliation, then moderate and trace defoliated sites. Mason's (1976) study showed a higher incidence of infection in the more heavily defoliated stands, but in the present study moderate and highly defoliated stands had a lower incidence of infection. Even though Mason's study and the present study do not show increased virus in the same defoliation classes, both studies indicate the trend of increasing NPV with age of the outbreak.

Incidence of NPV infection is usually low at the start of DFTM outbreaks and as population density is increasing, the chance of larvae acquiring an infection will increase since the population is growing more dense and there will be more cadavers on the host trees to spread the infection to the surviving larvae. As the outbreak progresses, more NPV infections will be acquired as the larvae hatch from surface contaminated egg masses. These infected early instar larvae when they die will then infect the

other surviving larvae and the rate of NPV infections will increase as shown in this study. This trend can also be observed if ages are tentatively given to the other DFTM outbreak locations in Nevada, and northeastern California (Mason *et al.*, 1983; Dahlsten and Thomas, 1969). Greater than 50% infection rates were observed in the heavily defoliated sites (age 2) of northeastern California, and in sites which were at a sub-outbreak level (age 0) in Nevada no OpNPV infections were observed. From the current study it can be seen that the incidence of NPV in early instar larvae increases with increasing defoliation within 1 season or between seasons.

The range of NPV infection among collection sites in the B.C. population during the outbreak studied is not surprising since different sites in different outbreak areas show a variety of infection rates (Mason, 1976; Mason *et al.*, 1983; Dahlsten and Thomas, 1969). The geographic region where the EM were collected did not affect the incidence of NPV among the hatching larvae. Though there are a range of infection rates depending on the region collected, this was not significantly different, indicating that the infection rates in different regions can be combined to give an overall rate of infection for the whole area.

FIDS collection data on DFTM populations predicted outbreaks of this pest beginning in 1990 in the Kamloops Forest Region of British Columbia (Koot and Hodge, 1990). Populations of DFTM were also at outbreak levels in the region in 1991 and 1992, the first and second years of the outbreak. The

incidence of infection was higher in 1992 than in 1991 (13.91% and 7.22%, respectively). The incidence of NPV among the hatching larvae in this study is similar to the 1973 rate of infection found in the Blue Mountain outbreak but is not as high as in the collapsing northeastern California infection rates (Mason, 1976; Dahlsten and Thomas, 1969). In the summer of 1991, 1992 and 1993, B.C. Ministry of Forests and the Canadian Forest Service applied OpNPV experimentally to stands where light or higher defoliation was predicted in the Kamloops area, resulting in the collapse of the DFTM outbreak completely in the summer of 1993. If some sites had not been treated with virus further increases in the infection rate may have been observed.

When sites were aged according to the amount of visible defoliation, a significant difference in incidence of NPV infection was observed only among the infestations in 1991. It was thought that the amount of NPV infections in the early instar larvae would increase as the population of DFTM increased during the outbreak. The incidence of infection actually rose from age 0 to age 1, but decreased in age 2 infestations. Aging the infestations in this manner and not observing the population at the same locations in the spring, may not allow for an accurate representation the true incidence of infection among the hatching larvae. It may be necessary to observe one or two infestation sites during the course of the next outbreak and monitor both the population trends and incidence of NPV infection as the population begins to rise and eventually decline due to the epizootic (this would eliminate the

need to age the infestations as the age of the outbreak would already be known). This may indicate more clearly if there is a "threshold level of infection," after which the population will collapse and treatment of the site with additional NPV through spraying would not be needed. The identification of a "threshold level of infection" would allow forest managers to treat only those infested stands which are not close to natural collapse, which in turn would save the cost of treating those DFTM infested stands where the infestation would collapse anyway without human intervention. Aging the infested stands on the basis of visible defoliation has allowed researchers to determine the amount of NPV in early instar larvae, but has not indicated a "threshold level of infection" as hoped. The rise in the number of NPV infections from 1991 to 1992, indicated that if the infestations were allowed another year without treatment, another increase in NPV infections may have been observed and the threshold level of infection may have been indicated.

4.5) Summary

DFTM EM from one site should be reared all at the same time to prevent over-estimating the amount of NPV infection at the outbreak area. If the EM are damp at the time of collection, they should be allowed to dry before cold storage or be stored in paper bags to prevent moisture on the surface of the EM from potentially spreading the NPV over the EM surface,

thus inflating the incidence of infection. The rate of NPV infection increased at sites which were in the first year of an outbreak, but decreased at sites which were in the second year of the outbreak. The incidence of infection increased in the fall 1992 collected DFTM EM, showing that as the outbreak progresses, an increasing rate of infection can be seen at all the infestation sites in the area. If a site could be monitored from the pre-outbreak period and left untreated until the population crashes during the next outbreak, the incidence of NPV infection could be monitored for several years and may clearly indicate the threshold level of infection above which treatment would not be needed.

Chapter 5

Genotypic Variation Among Wild Isolates of Nuclear Polyhedrosis Virus isolated From *Orgyia pseudotsugata* Larvae

5.1) Introduction

The Douglas-fir Tussock Moth (DFTM), *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae), is a native, periodic defoliator of British Columbia's forests. The main reason for the sudden DFTM population collapse is the presence of a naturally-occurring, species-specific nuclear polyhedrosis virus (NPV), *Orgyia pseudotsugata* NPV (OpNPV), in the host population.

Nuclear polyhedrosis viruses belong to family Baculoviridae and are characterized by circular, double stranded DNA genomes ranging from 75 to 230 kilobases (Wilson, 1991; Cherry and Summers, 1985). The DNA consists of one or more rod-shaped, electron-dense nucleocapsids enclosed in a single viral envelope (Wilson, 1991). The virions are then embedded in a crystalline protein viral occlusion, which maybe polyhedral in shape, containing one or more virions (Wilson, 1991). Two distinct NPV's have been isolated from DFTM; the SNPVs have one nucleocapsid per virion envelope while the MPNVs have more than one nucleocapsid per envelope.

Investigations of NPV genotypic variation in different geographical locations have been undertaken to understand the evolution of baculoviruses and to characterize more virulent strains for pest control (Cherry and Summers, 1985). Using restriction endonuclease (REN) analysis, two distinct virus types, with no homology, have been found to infect *Spodoptera littoralis* larvae collected from 21 sites in Israel (Cherry and Summers, 1985; Kislev and Edelman, 1982). In a study with *Spodoptera frugiperda*, 15 isolates of NPV from Louisiana were examined and 11 isolates were found to be distinct (Shapiro *et al.*, 1991). Recent studies investigating geographical isolates of some NPVs, including *Heliothis* spp. (Gettig and McCarthy, 1982), *Mamestra brassicae* (Vlak and Groner, 1980), *Phthorimaea operculella* (Vickers *et al.*, 1991) and *Autographa californica* (Miller *et al.*, 1980; Smith and Summers, 1978; Lee and Miller, 1978) have shown that the geographical isolates are closely related strains of the same insect virus, differing only in the position of a few bands in the REN profile.

While it has been established that the SNPV and MNPV infecting DFTM (referred to as OpSNPV or OpMNPV) occur in different geographical regions of the pest's host range (Hughes, 1976; Hughes and Addison, 1970), little work has been done on the genotypic variation of virus in one state or province or even within an infested area. Miller and Dawes (1978) compared the OpMNPV isolated from a site near Kamloops, B.C. with the OpNPV sprayed in the United States to control DFTM. The REN profiles of the two

OpNPV extracts were virtually the same except for a few submolar bands in the U.S. spray preparation (Miller and Dawes, 1978). Most of the work involving OpNPV has centered on describing the similarities and differences between the SNPV and MNPV morphotypes types (Rohrmann *et al.*, 1978; Rohrmann and Beaudreau, 1977; Rohrmann *et al.*, 1977), and has shown that the two virus types are at most 1% homologous (Rohrmann *et al.*, 1978). Recent research has focused on identifying and characterizing the genes of OpMNPV (Theilmann and Stewart, 1991; Theilmann and Stewart, 1992a; Theilmann and Stewart, 1992b).

The objective of this section of the thesis is to examine the genotypes of OpNPV in different geographic locations in B.C. from insects reared from field collected EM. The purpose of this part of the work is to determine if a genetically unique strain of OpNPV might be identified. One of these efficatious strains could then be used in the field applications to determine the proportion of DFTM mortality resulting from naturally occurring infection verses DFTM mortality arrising from field application of NPV. Currently, host mortality after field application of NPV is monitored, but mortality caused by the virus treatment and by the naturally-occuring virus infection cannot be separated.

5.3) Materials and methods

5.3.1) *Collection of infected larvae.*

DFTM egg masses were collected from 42 sites, in 6 geographical locations, in British Columbia in the fall of 1991 and 1992 as outlined in section 3.2.1.

After the cold storage period, the egg masses were placed in a growth chamber (25 ^{O}C: 60% R.H.: 12 hours light: 12 hours dark) and allowed to hatch. Thirty (30) first instar larvae were taken randomly from each egg mass and reared on artificial diet in 9 X 1.75 cm petri dishes (Thompson and Peterson, 1978). Once a week dead larvae were removed from each petri dish. Half of the number of dead larvae (cadavers) removed from the dishes were frozen individually in microfuge tubes at -20 ^{O}C. The other half of the dead larvae were ground in a mortar and pestle with 1 ml of distilled water. Fresh diet was then dipped in this NPV slurry and the surface infected diet was placed in the petri dish to infect the remaining live larvae as they fed during the next week. The rearing continued until all larvae from an egg mass died or the larvae had pupated.

For these genotypic variation studies, only two sites from each geographic region were chosen to extract viral DNA from, since time constraints did not allow a survey of all the 42 collection sites.

5.3.2) Collection of Non-infected DFTM Larvae and Extraction of Host DNA

Three disease-free DFTM egg masses were provided from the Forest Pest Management Institute in Sault St. Marie, Ontario. These larvae were reared by the method outlined above on non-infected artificial diet in a

separate rearing room, to prevent NPV infection. These larvae were then used for the extrattion of non-infected DFTM DNA.

Two groups of DFTM larvae were pooled (group 1= 4 insects, 0.114g; group 2= 6 insects, 0.128g) and were separately ground to a fine powder in a liquid nitrogen cooled mortar and pestle in PTE buffer (50 mM Tris-HCl, 10 mM EDTA pH 7.8). Tween 20 (0.56%) and Proteinase K (1 mg) were added to the pestle and left to freeze. When the buffer thawed slightly, the whole slurry was transferred to a weigh boat, the mortor was rinsed with PTE and the solution was transferred to a 5 ml graduated tube with a screw cap lid, SDS (Sodium Dodecyl Sulfate) and Ethidium Bromide were added to the samples at a final concentration of 10 mg/ml and 0.8 mg/ml, respectivley. Cesium chloride was then added to each tube (final concentration of 0.38 mg/ml) and the refractive index was adjusted to fall between 1.3870 and 1.3900. The samples were transferred to 5 ml quick seal tubes and spun in a Beckman Ultra centrifuge at 8,800 g, V Ti 65 rotor, 15 ^{o}C, for 16 hours. One DNA band was removed from each tube after illumination with ultra violet light. Two volumes of cesium chloride saturated isopropanol were added to each tube to extract the ethidium bromide. The bottom phase was transferred to dialysis tubing and dialyzed for 24 hours with three changes of 1 X TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.8). The total genomic DNA was then collected and run on a 0.6% agarose in 1 X TBE (0.09 M Tris-HCl, 0.09 M boric acid, 2 mM EDTA, pH 8.3) gel to check the quality of the extracted DNA. DNA

concentration was determined spectrophotometrically at 260 nm.

5.3.3) Extraction of NPV From Infected DFTM Larvae

Infected larvae were ground in a liquid nitrogen cooled mortar and pestle with 0.5 ml of STE-C buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 10 mM cystiene). The powder was transferred to 2 ml microfuge tubes and 1 ml STE-C containing 0.98 mg/ml SDS and 0.066 mg/ml DNAase was added to each sample. The tubes were then incubated on a rotary shaker for 20', at room temperature and then centrifuged at 145 g to pellet large pieces of insect debris. The supernatant was transferred to new tubes and spun at 15,000 g, for 10' to pellet the PIBs. Each PIB pellet was washed twice in STE-C containing SDS at a final concentration of 0.99 mg/ml. The final pellet was resuspended in buffer containing 11.8 mg/ml sodium carbonate and 5.3 mg/ml sodium thioglycolate, pH >14 and the tubes were again incubated on a rotary shaker for 20', at room temperature. The solution was neutralized by the addition of 100 mM Tris-HCl pH 7.5, 1.0 mM 0.5 M EDTA pH 8.0, followed by the addition of 1% Sodium Lauryl Sarcosine (SLS) and 0.5 mg/ml Proteinase K, and incubated overnight at 55 ^{o}C. The samples were then subjected to two phenol and two chloroform isoamyl-alcohol (24:1) extractions. Sodium acetate (pH 5.2) was added to a final concentration of 0.27 M and the nucleic acids were precipitated by the addition of 2 volumes of absolute ethanol followed by centrifugation 15,000 g, 30'. The DNA pellet was washed with 70% ethanol, dried under a vacuum, and then resuspended

in sterile TE. The DNA was then subjected to electrophoresis on 0.6% agarose gels (10 X 20 cm) in 1 X TBE buffer to check the DNA concentration and presence of a high molecular weight viral DNA band.

5.3.4) Total Genomic Probe DNA Preparation

To obtain OpNPV DNA for use as probe for Southern blot analysis, 0.5 g of Virtuss^R powder was resuspended in 20 ml of sterile distilled water. Virtuss^R is a registered OpNPV product and is used in Canada to control DFTM in the field. The samples were centrifuged 1,300 g, for 10' to pellet the PIBs. The supernatant was removed and the pellet was resuspended in 25 ml sterile distilled water, centrifuged at 1,300 g, for 10'. The upper white layer of the pellet was resuspended in sterile distilled water and removed with a pasteur pipette. The PIBS were then centrifuged at 15,000 g for 10' in a microcentrifuge. Each pellet was resupended in 10 mM Tris-HCl, pH 7.5 and 2 mg/ml DNAase and incubated at 37 ^oC, for 10'. The samples were then centrifuged, 15,000 g, 10', and the pellets were washed three times with TE. The final pellet was resuspended in TE, carbonate buffer (final concentration of 11.8 mg/ml sodium carbonate and 5.3 mg/ml sodium thioglycolate) was added, and the samples were incubated on a rotary shaker, 20', at room temperature. The solution was then neutralized by the addition of 100 mM Tris-HCl, pH 7.5, and 1.0 mM EDTA, followed by the addition of SLS and Proteinase K to final concentrations of 1% and 0.5 mg/ml, respectively, and then incubated overnight at 55 ^oC. Each sample was then subjected to two

phenol and two chloroform isoamyl-alcohol (24:1) extractions. The nucleic acids were precipitated and analyzed by gel electrophoresis as described above (section 5.3.3). The Virtuss^{R} DNA was then labelled with digoxigenin according to the AMPPD Detection Kit instructions (Bohringer Mannheim Inc.).

5.3.5) ie-1 gene Probe DNA Extraction

Plasmid pIE-1 was generously provided by Dr. David Theilmann (Pacific Agriculture Research Center, Vancouver, B.C.) for use as a single locus probe. *E. coli* strain NM522 was grown in Luria Broth (LB) media overnight at 37 ^{O}C with agitation. The following day, fresh LB was inoculated with 0.1 ml of the overnight culture and the growth of the culture was monitored until the *E. coli* had reached mid-log phase (3 hours). The cells were then incubated on ice for 10' and centrifuged for 10', 1300 g at 4 ^{O}C. The supernatant was removed and the pellets were incubated with 0.1 M calcium chloride on ice for ten minutes. The cells were then centrifuged again for 10' at 1,300 g, 4 ^{O}C. Fresh 0.1 M calcium chloride was added to the pellet and 1 ug pIE-1-Sal plasmid was added to competent cells and incubated on ice for 30'. The cells were heat shocked at 42 ^{O}C for 90 seconds and then placed on ice for 2'. Glucose LB (800 ul) was added and the cultures were incubated at 37 ^{O}C for 1 hour. Cells were then grown overnight on LB-ampicillin plates (10 mg/ml) at 37 ^{O}C. The next day, single colonies were picked from the plates and inoculated into fresh LB-ampicillin media. The cultures were then

grown overnight at 37 ^{o}C.

Stock culture tubes of the transformed cells containing recombinant plasmids were made by placing 0.5 ml of culture in 50% glycerol and freezing at -80 ^{o}C until needed. The remaining overnight culture was centrifuged for 5' at 1,300 g. The supernatant was poured off and the pellet was resuspended in Buffer I (1 mM EDTA, 50 mM glucose, 25 mM Tris-HCl, pH 8.0). Ice cold Buffer II (0.2 M NaOH, 0.3 M SDS) was then added and tubes were inverted gently. Buffer III (3 M NaOAc, pH 4.8) was then added and the samples were centrifuged at 8,800 g for five minutes. The supernatant was removed to fresh tubes and an equal volume of phenol:chloroform was added. The samples were centrifuged for 2' at 8,800 g and the upper aqueous layer was transferred to fresh tubes. Two volumes of absolute ethanol was added to each tube and the samples were left at -20 ^{o}C overnight. The samples were then centrifuged at 8,800 g for 15' and the pellet was washed with 85% ethanol and dried under a vacuum. The resulting pellet of DNA was then resuspended in sterile TE.

Three microliters of the plasmid DNA was digested with a three fold excess of New England Biolab (NEB) *Sal* I enzyme for 5 hours in 1X NEB *Sal* I buffer as supplied by the company. The digestion was then electrophoresed on 0.6% agarose in 1 X TBE for 16 hours at 40 volts. The restriction pattern was observed after staining the agarose gel with 1 ug/ml ethidium bromide in distilled water for 10'. The gels were placed on a UV transilluminator and

photographed using Polaroid Land Film.

Once the presence of the plasmid was determined, LB-ampicillin plates were streaked with glycerol-*E. coli* stock cultures of transformed cells containing the plasmid. The plates were incubated at 37 ^{o}C overnight. Single colonies were inoculated into Luria Broth (LB) containing 100 mg/ml ampicillian and allowed to grow overnight at 37 ^{o}C. The overnight culture was then centrifuged at 1,300 g , for 10'. The supernatant was discarded and the pellet was resuspended in Buffer I. Buffer II was added and the tubes were set on ice for 5'. Buffer III was then added and the tubes inverted gently and centrifuged at 1,300 g, for 10'. The supernatant was removed and 1/2 volume of ice cold isopropanol was added to each tube. Plasmid DNA was precipitated for 2 hours at -20 ^{o}C. The DNA was then centrifuged for 10' at 15,000 g and the supernatant was discarded. The pellet was resuspended in 1 ml of PTE buffer and this was transferred to a 5 ml graduated tube. The volume was adjusted to 5 ml with PTE containing 0.77 g/ml cesium chloride, 0.38 mg/ml of ethidium bromide. The refractive index was adjusted to between 1.3880-1.3900. The samples were transferred to quick seal tubes and centrifuged 16 hours at 260,000 g, 15 ^{o}C in Beckman VTi 65 rotor. The lower band was removed from the tubes after illumination with ultra-violet light. Two volumes of cesium chloride saturated isopropanol was used to extract the ethidium bromide from the plasmid DNA. The plasmid DNA was then dialyzed for 24 hours with three changes of TE. The plasmid DNA was then

digested with NEB *Sal* I enzyme, in 1X NEB *Sal* I buffer, for three hours and loaded on a 0.6% low melting point agarose gel (10 X 20 cm), which was electrophoresed for 15 hours at 40 volts. The resulting band of the ie-1 gene was removed from the gel, and the DNA was removed from the agarose following the Gene Clean Kit instructions. The ie-1 probe DNA (75 ng) was then labelled with digoxigenin according to the AMPPD Detection Kit instructions (Bohringer Mannheim Inc.).

5.3.6) Restriction Enzyme Digests and Southern Blots

OpNPV DNA was extracted from single, late instar larva (between 10-20 ng), total genomic DFTM DNA (25 ng), Virtuss ^{R} and TM-BioControl ^{R} (the two products manufactured in Canada and the United States, respectively, for control of DFTM). OpNPV DNA to be probed with the IE-1 gene probe was digested with *Hind* I, *Hinf* II, *Ava* II, *Sau3 a* I, or *Rsa* I restriction enzymes for 5 hours at 37 ^{o}C. OpNPV DNA (20 ng) to be probed with total OpNPV DNA probe were digested with *Bgl* II, *Sal* I, *Pst* I or *EcoR* I enzymes for five hours at 37 ^{o}C. Each digest was electrophoresed in 0.6% agarose, containing 0.2% Synergel (SG) in 1 X TAE, 10 X 20 cm horizontal gels, at 20 volts overnight. Gels were stained with ethidium bromide (1 ug/ml) 10', placed on a UV transilluminator, and photographed with Polaroid Land Film.

Viral DNAs were then transferred to nitrocellulose membranes after, depurination (0.2 N HCl) for 10', followed by denaturization (1.5 M NaCl, 0.5 M NaOH) and neutralization (1.5 M NaCl, 1 M Tris-HCl) of the gels, for 30'

respectivley. Transfer was completed with 10 X SSC (3 M NaCl, 0.3 M Tri-sodium citrate pH 7.0). After transfer, the membranes were blotted dry and fixed with ultra-violet light for 1 minute.

5.3.7) Chemiluminescent Detections of Southern Blots

Pre-hybridizations and hybridizations with labelled ie-1 and total genomic probes were completed in 50 ml screw cap Corning tubes, two blots per tube. Blots were prehybridized for six hours, 42 ^{O}C, in 50% formamide, 5 X SSC, 2% Blocking reagent (Bohringer Mannheim Inc.), 0.1% sodium sarcosine and 0.02% SDS. Probe DNA was added to fresh hybridization solution and denatured at 95 ^{O}C for 10 minutes, then placed on ice for five minutes. Prehybridization solution was removed from the blots and the probe DNA was added. Hybridization was carried out at 42 ^{O}C, overnight, in a hybridization oven.

To remove the hybridization solutions, two 15 minute washes in 2 X SSC, 0.1% SDS at room temperature and two 15 minute washes at 65 ^{O}C in 0.2 X SSC, 0.1% SDS were completed. For digoxigenin detection, the membranes were washed for 5 minutes in wash buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20). Membranes were then incubated in Blocking Buffer (0.1 M maleic acid, 0.15 M NaCl, 1% sterile Blocking reagent) for 30 minutes. The Blocking Buffer was reduced to 0.2 ml/cm ^{2}, anti-digoxigenin alkaline phosphatase antibody was added (1:10,000), and the blots were incubated 30 minutes. The blots were then washed twice for 15 minutes in wash buffer as

above. The membranes were rinsed briefly in Buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) then sealed between plastic sheets with 0.1 mg/ml AMPPD substrate diluted in Buffer 3. Blot packages were incubated at 37 ^{O}C for 15 minutes and then exposed to X-Ray film (Kodak XAR film) for 20 minutes and 1 hour exposures without an intensifying screen. The ie-1 probe detections were exposed to X-Ray film for 20 hours.

5.4) Results and Discussion

5.4.1) *Genotypic Variation in OpNPV*

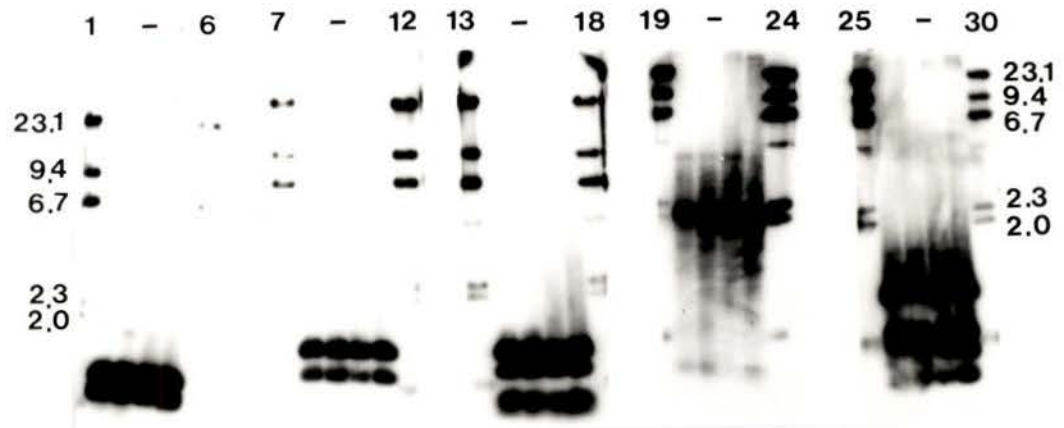
5.4.1.1) *Analysis of ie-1 probed Southern blots*

Detections with the IE-1 probe showed no polymorphisms with the five restriction enzymes (Figure 5.1). This gene is thought to be highly conserved and is important in the initiation of the viral regulatory cascade (Theilmann and Stewart, 1991) and therefore, may not be polymorphic in nature. A less conserved region of the genome is more likely to undergo point mutations, insertions, or deletions which result in polymorphisims.

5.4.1.2) *Analysis of total genomic probed Southern blots*

OpNPV was extracted from 90 larvae, from 10 locations, in six geographic regions of British Columbia. Previous genotypic variation studies involving *Spodoptera littoralis* (SlNPV) and *Spodoptera frugiperda* (SfNPV) were completed by collecting virus infected larvae from field locations and extracting the PIBs from the larvae. The PIBs were then used to infect

Figure 5.1. Southern blot of OpMNPV digested with *Ava* I (lanes 2 to 5), *Hind* III (lanes 8 to 11), *Hinf* II (lanes 14 to 18), *Sau* A I (20 to 23) and *Rsa* I (lanes 26 to 29) and probed with the ie - 1 probe. The first two lanes of each digest are from Beaton Lake and the second two lanes are from Chilliwack. Note that no polymorphisims are present.


1 – 6 7 – 12 13 – 18 19 – 24 25 – 30
23.1
9.4
6.7
2.3
2.0
23.1
9.4
6.7
2.3
2.0

laboratory reared insects, producing more viral DNA of a particular strain (Cherry and Summers, 1985; Shapiro *et al.*, 1991). These methods do not take into account the possible variation of the virus within the site or within an egg mass. The present study examined the genotypic variation between geographic regions, within sites in the region and among egg masses from that region. This study of OpNPV genotypic variation examines more insects per site and region than the earlier SlNPV and SfNPV studies.

Differences in DNA banding patterns, or Restriction Fragment Length Polymorphisims (RFLPs), can be seen when OpNPV is cut with *Hind* III, *Bgl* II, *Sal* I and *Pst* I restriction enzymes and hybridized with the total genomic probe. Digests of OpNPV DNA with the enzyme *BamH* I did not indicate any polymorphisms when probed with the total genomic probe (data not shown). Polymorphisms are said to occur in this study when the REN profile of the viral extract differs from the two control DNA samples, Vitruss ^{R} and TM-BioControl ^{R} DNA extracts, by at least one band.

5.4.1.3) *Analysis of Bgl II RFLPs*

OpNPV DNA from five sites were digested with *Bgl* II enzyme and 9 REN fragments were detected on the blots (Figure 5.2 and Table 5.1). Estimation of the fragment sizes with all restriction enzymes were made by comparing the mobility of the viral DNA fragments in 0.6% agarose/0.2% SG gels to the relative mobility of lambda *Hind* III DNA fragments of known size. Mobility of the DNA fragments is linearly related to the log of the

Figure 5.2. Southern blot of OpMNPV digested with *Bgl* II showing the two genotypes and banding patterns. Lanes 2, 3, 5, and 10 have a band at 2.5 kb that is not present in the other viral extracts from Guichon Rd. 1.3 km West or the Virtuss^{R} and BioControl^{R} lanes (lanes 14 and 15).

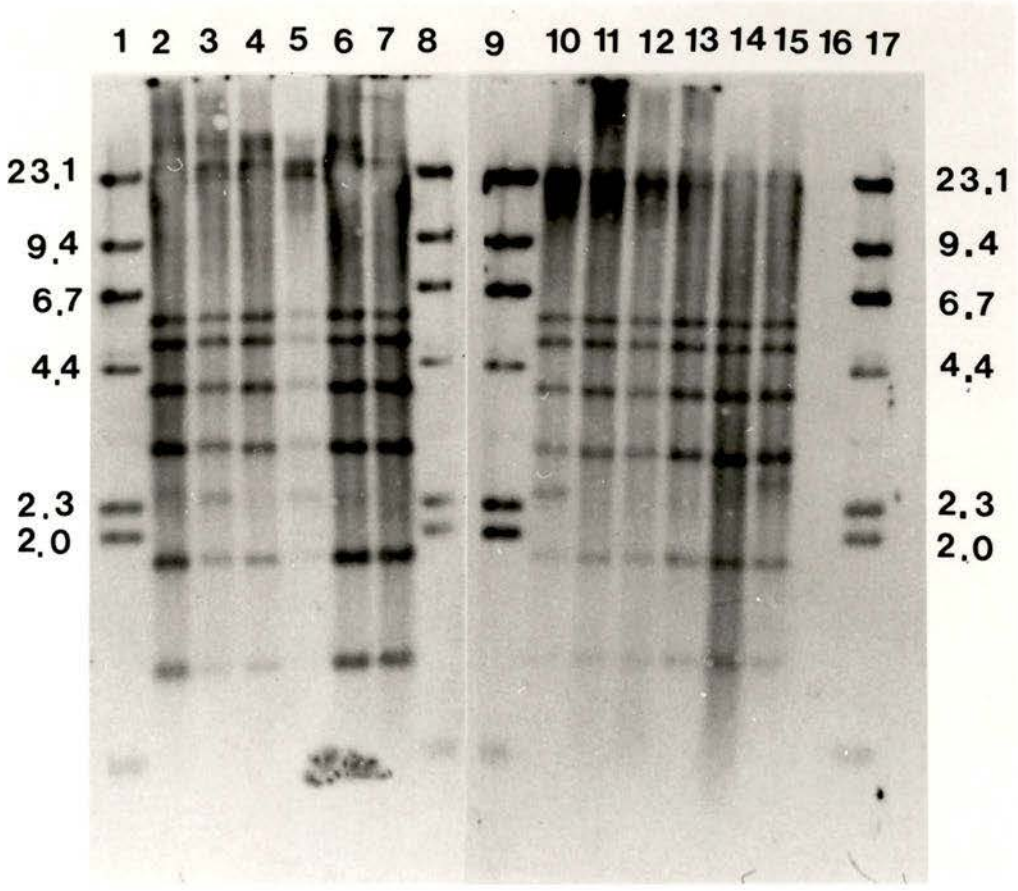

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
23.1
9.4
6.7
4.4
2.3
2.0
23.1
9.4
6.7
4.4
2.3
2.0

Table 5.1. REN fragment sizes (kb) generated by the digestion of OpNPV with *Bgl* II.

Genotype A kb	Genotype B kb
23.0 +	23.0 +
23.0 +	23.0 +
20.0	20.0
6.0	6.0
5.0	5.0
4.0	4.0
3.0	3.0
	2.5
1.8	1.8
0.9	0.9

molecular weight of the fragments in agarose gels (Miller and Dawes, 1978; Sambrook *et al.*, 1989). Restriction fragment sizes were estimated by determining the distance the fragment migrated and reading the size of the fragment from a graph of the log of the molecular weight of the lambda *Hind* III fragments. Only one polymorphism (at 2.5 kb) was observed with*Bgl* II in 4 viral DNA extracts from the Guichon Rd. 1.3 km West site in the Savona region (Figure 5.2, lanes 2, 3, 5 and 10). The other 39 viral extracts showed the same restriction pattern observed when the Virtuss^{R} or TM-BioControl^{R} DNA (Figure 5.2, lanes 14 and 15) were cut with *Bgl* II (Figure 5.2 and Table 5.1). The other (4) sites used for this study were not subjected to Southern blot analysis because digests with *Bgl* II often showed incompleted digestion of the viral DNA or did not cut at all with this enzyme. The cost of the enzyme per digest was too high to continue with restriction digests which were not complete. The DNA from these sites may be cut with *Bgl* II at a later date.

5.4.1.4) *Analysis of Hind III RFLPs*

Two OpNPV DNA extracts from eight sites were digested with *Hind III* (Figure 5.3 and Table 5.2) while the DNA from 4 viral extracts did not digest (Figure 5.3, lanes 9, 15, 16 and 17), the rest of the pairs digested well and 16 bands are resolved from 11 viral extracts. Miller and Dawes (1978) digested OpNPV with *Hind* III and were able to resolve 16 bands when digested OpNPV was electrophoresed on 0.7% agarose gels. The 16 *Hind* III bands shown in Figure 5.3 differ in position slightly from the ethidium bromide

Figure 5.3. Southern blot of OpNPV digested with *Hind* III showing the two genotypes. Note the presence of a band at 2.6 kb and at 8.5 kb of lane 4 that are not present in any other lane.

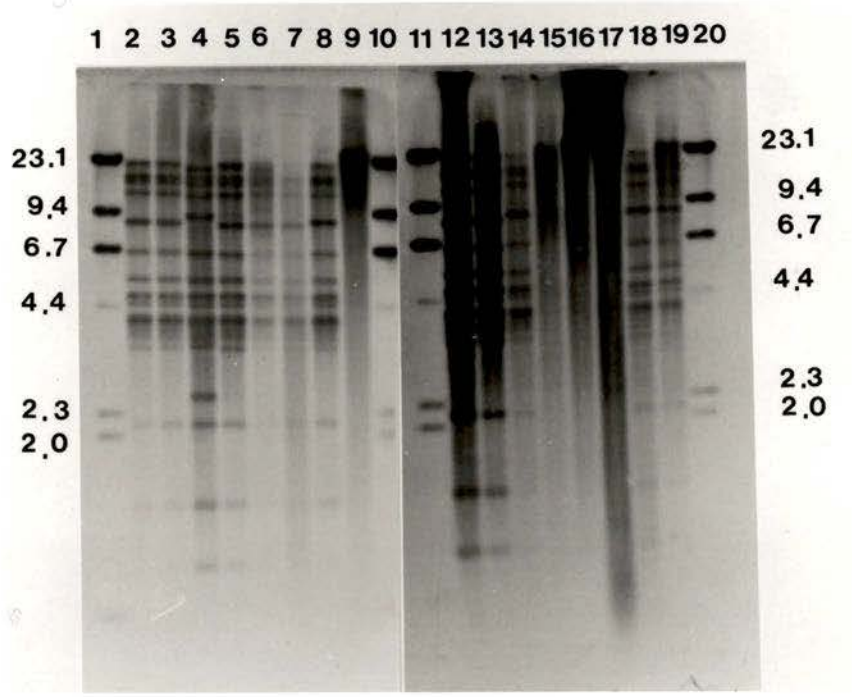

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
9.4
6.7
4.4
2.3
2.0
23.1
9.4
6.7
4.4
2.3
2.0

Table 5.2. REN fragment sizes (kb) generated by the digestion of OpNPV with *Hind* III.

Genotype A kb	Genotype B kb
23.0 +	23.0 +
18.00	18.00
15.00	15.00
14.00	14.00
8.00	8.5
7.00	7.00
5.60	5.60
4.90	4.90
4.30	4.30
4.00	4.00
3.90	3.90
3.80	3.80
3.50	3.50
	2.6
2.20	2.20
1.40	1.40
0.85	0.85

stained gel shown by Miller and Dawes (1978). The position differences may be due to the higher agarose concentration in the Miller and Dawes study (0.7% agarose; this study only used 0.6% agarose). However, the addition of Synergel to the agarose increases the sieving power of the gel and resolves the DNA bands present in the gel better than higher agarose concentrations (Perlman, 1991). Since the bands in Miller and Dawes (1978) were not sized, it is difficult to tell how different the sizes of the two sets of *Hind* III fragments are, but there are similarities between the two sets. Miller and Dawes (1978) show a set of four high molecular weight bands, and another set of five fragments that have migrated about half the gel distance. These two groups of bands are present in this study (Figure 5.3) in similar postions or groupings.

The viral DNA extract from Guichon Rd. 1.3 km West, when cut with *Hind* III, has a band at 2.6 kb and a shift in the fifth band to 8.5 kb, that is not present in any of the other viral DNA samples (Figure 5.3, lane 4, and Table 5.2). This 2.6 kb band may be a polymorphism, and more digests with*Hind* III should be completed to determine if this band is present in any other viral extracts from the Guichon Rd. site or in any other locations. Lane 5 of the *Hind* III digest also shows faint, lower intensity bands at the same location as lane 4. These bands may be submolar bands in the digest as a result of a mixture of two strains of OpNPV in the extract as observed with *Heliothis* spp. NPV (Gettig and McCarthy, 1982).

5.4.1.5) *Analysis of Eco RI RFLPs*

When the OpNPV DNA was cut with *Eco R* I, no polymorphisms were observed; all viral extracts showed the REN profile of the two control DNAs, Virtus^R and TM-BioControl^R. Digestion of OpNPV DNA with the enzyme *EcoR* I produced 6 fragments (Figure 5.4 and Table 5.3) of quite high intensity on the blots and in some instances longer exposure of the blots to the X-Ray film revealed the presence of 1 more band at 1.3 kb (Figure 5.4, lane 3). Miller and Dawes (1978) were able to resolve seven bands when OpNPV was digested with *Eco R* I. The lower band (1.3 kb) in lane 3 of Figure 5.4 is probably the seventh band that was observed in Miller and Dawes' (1978) study and does not represent a polymorphism. Seven bands were also observed by Rohrmann *et al.*, (1978) when OpNPV was cut with *Eco RI.* Though neither of these studies sized the restriction fragments, the REN profile of this study's *Eco R* I digest is consistant with those observed by Miller and Dawes (1978) and Rohrmann *et al.* (1978) (Figures 5.4 and 5.5).

5.4.1.6) *Analysis of Pst I RFLPs*

Eighteen (18) resolvable fragments, between 6.5 kb and 1.0 kb, were observed by Southern blot analysis when OpNPV DNA was digested with *Pst* I (Figure 5.6 and Table 5.4). Five different genotype patterns were observed when OpNPV was digested with *Pst* I (Figure 5.6 and Table 5.4). Of the 90 viral extracts made from infected larvae, 45.6% of the extracts had the same REN profile as control DNA, Virtus^R and TM-BioControl^R. The four other

Figure 5.4. Southern blot of OpNPV digested with *Eco R* I, showing seven bands in all lanes after five hour digestion.

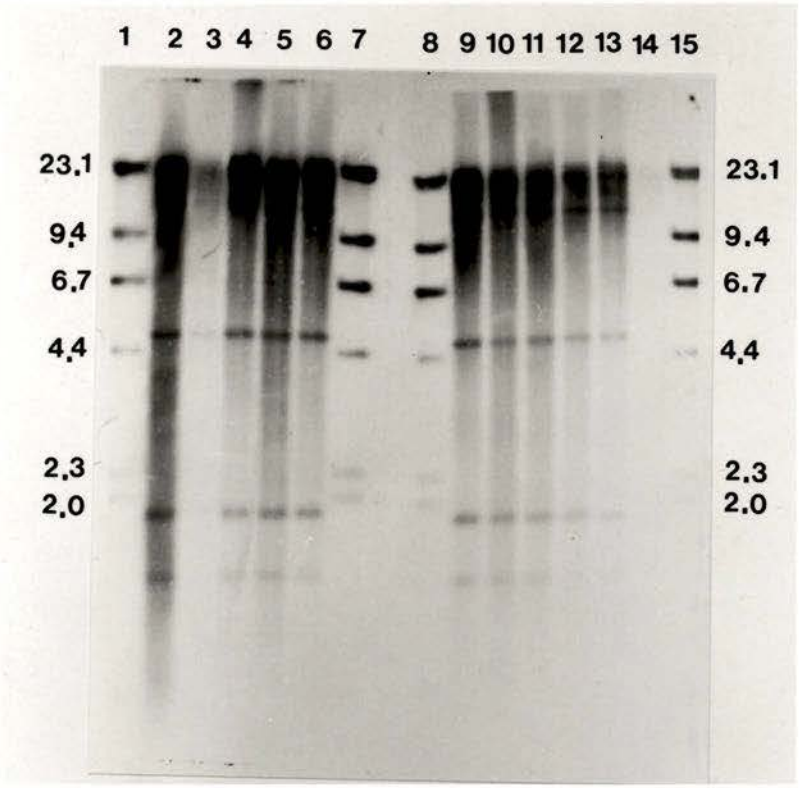

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
23.1
9.4
6.7
4.4
2.3
2.0
23.1
9.4
6.7
4.4
2.3
2.0

Table 5.3. REN fragment sizes (kb) generated by the digestion of OpNPV with *Eco R* I.

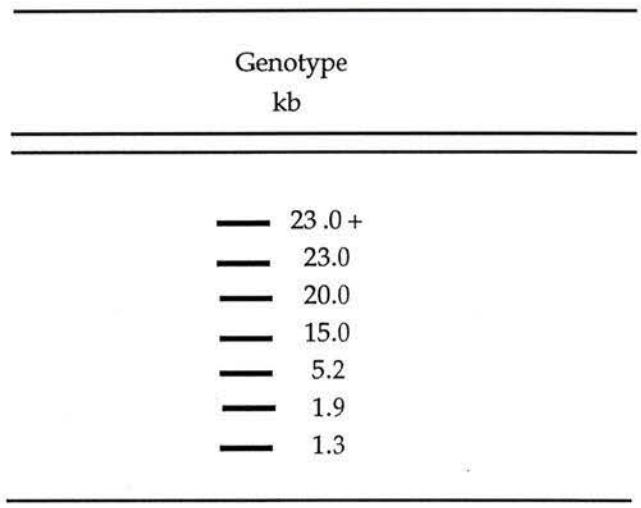

Genotype
kb
23 .0 +
23.0
20.0
15.0
5.2
1.9
1.3

Figure 5.5. Photograph of the ethidium bromide stained gel of OpNPV digested with *Eco RI* showing the three high molecular weight bands near 23 kb.

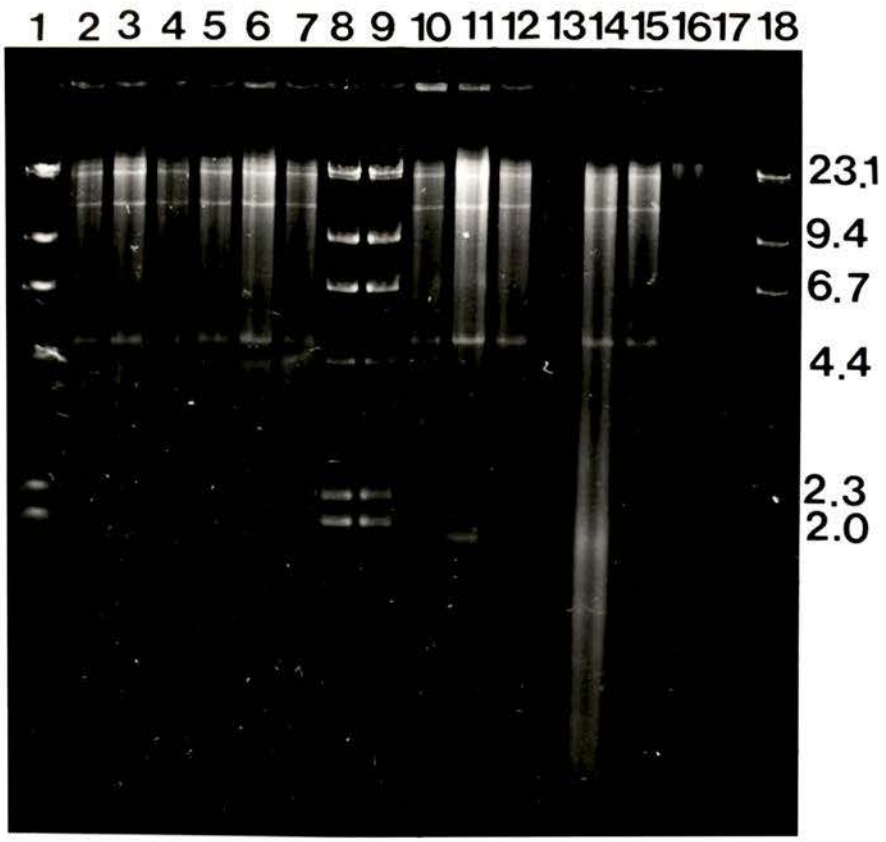

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23.1
9.4
6.7
4.4
2.3
2.0

Figure 5.6. Southern blot of OpNPV digested with *Pst* I. Note the presence of five genotypes in this digest. Genotype A, lanes 6, 10 and 11; Genotype B, lanes 3 and 4; Genotype C, lane 9; Genotype D, lane 2: Genotype E, lane 7. Lanes 1, 5 and 13 are Lambda *Hind* III molecular weight standards.

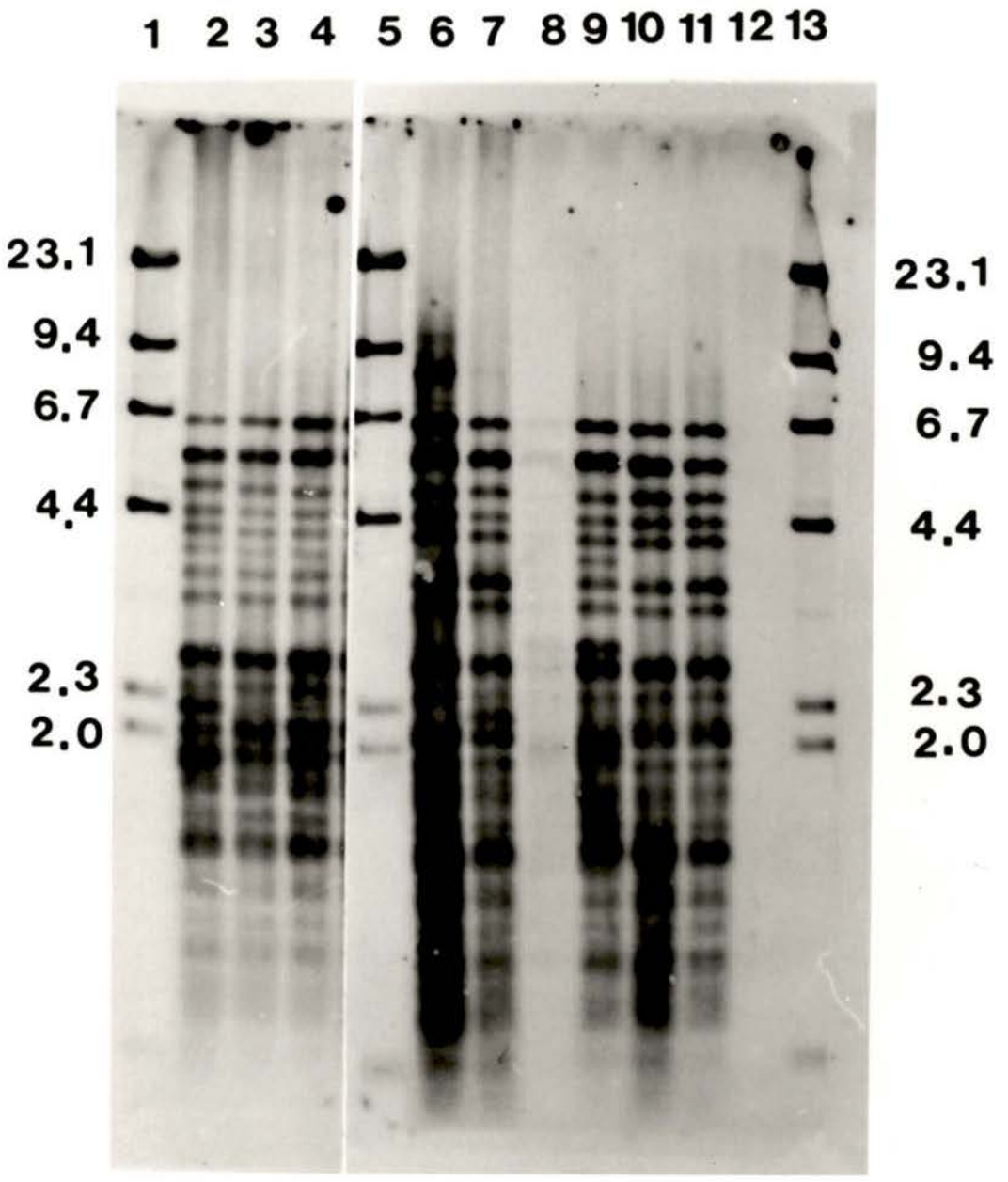
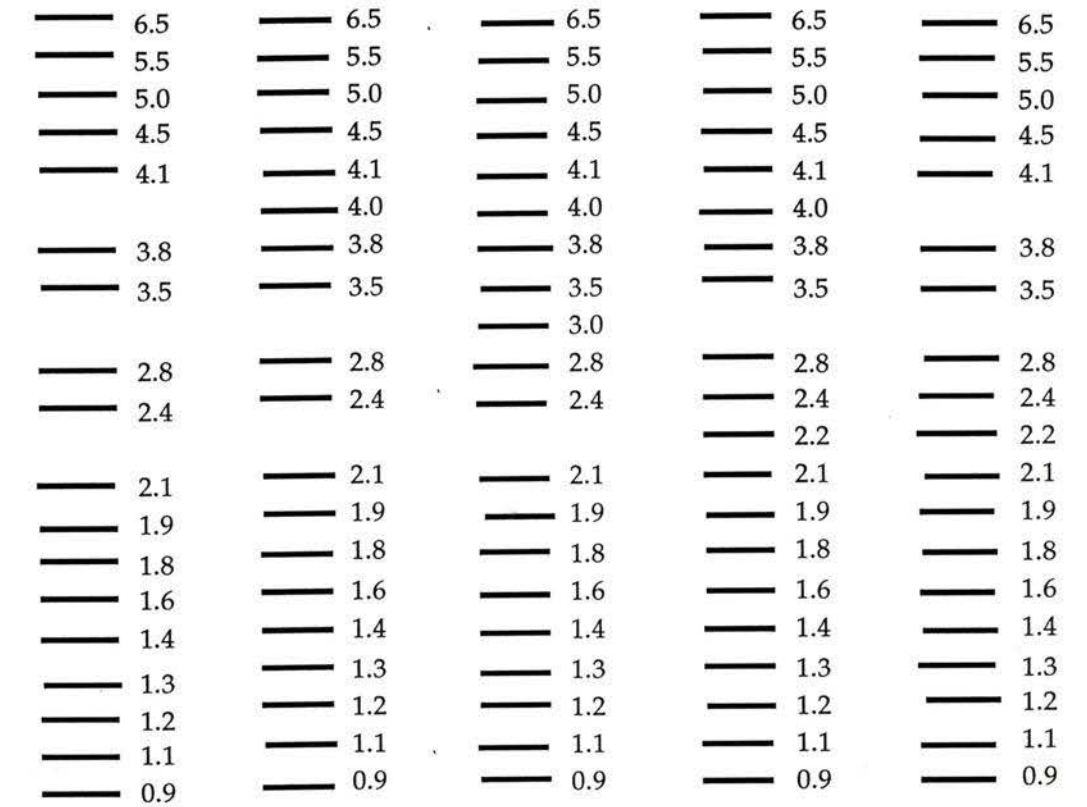

1 2 3 4 5 6 7 8 9 10 11 12 13
23.1
9.4
6.7
4.4
2.3
2.0
23.1
9.4
6.7
4.4
2.3
2.0

Table 5.4. REN fragment sizes (kb) generated by the digestion of OpNPV with *Pst* I.

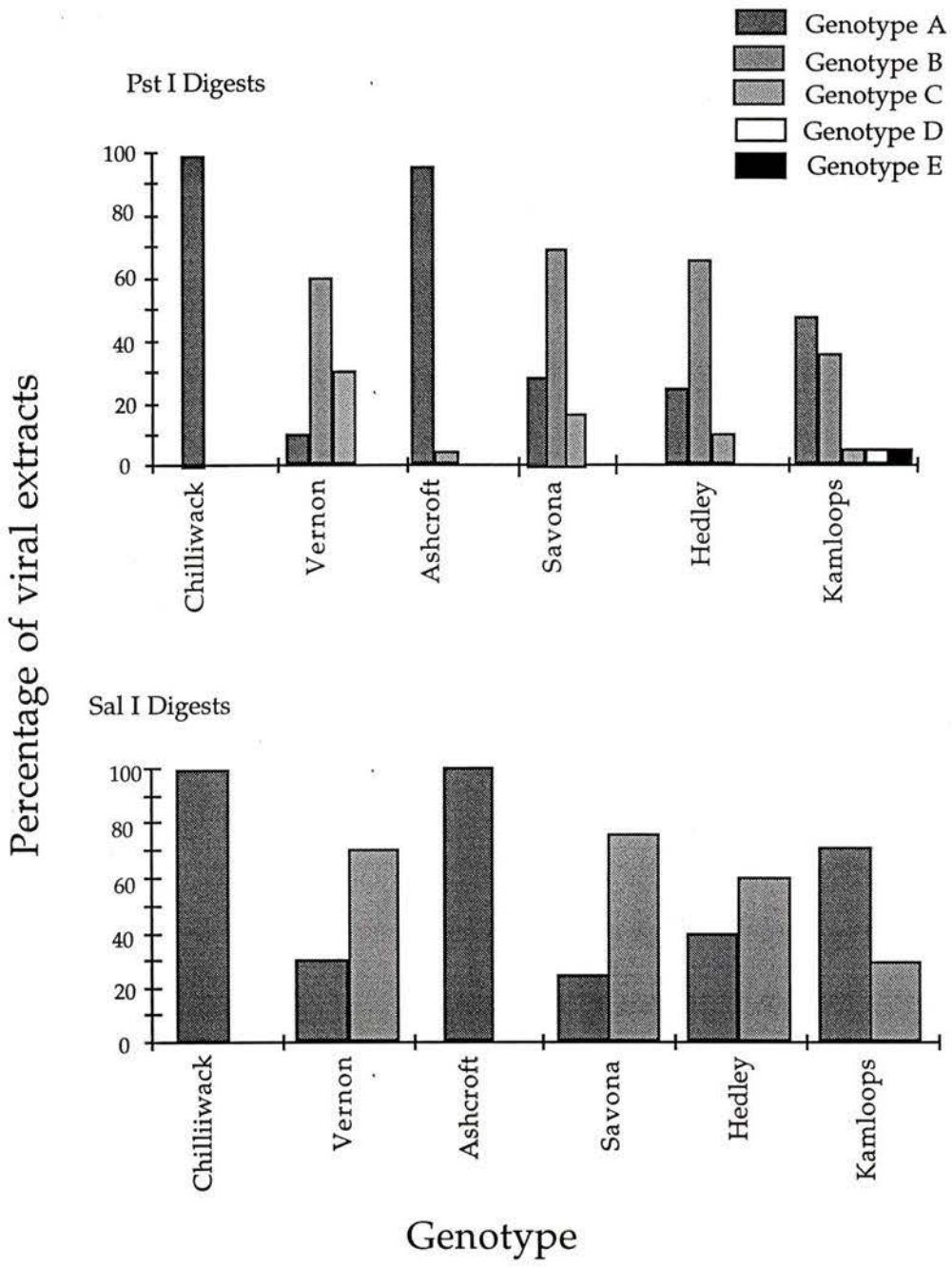

Genotype A
kb
6.5
5.5
5.0
4.5
4.1
3.8
3.5
2.8
2.4
2.1
1.9
1.8
1.6
1.4
1.3
1.2
1.1
0.9
Genotype B
kb
6.5
5.5
5.0
4.5
4.1
4.0
3.8
3.5
2.8
2.4
2.1
1.9
1.8
1.6
1.4
1.3
1.2
1.1
0.9
Genotype C
kb
6.5
5.5
5.0
4.5
4.1
4.0
3.8
3.5
3.0
2.8
2.4
2.1
1.9
1.8
1.6
1.4
1.3
1.2
1.1
0.9
Genotype D
kb
6.5
5.5
5.0
4.5
4.1
4.0
3.8
3.5
2.8
2.4
2.2
2.1
1.9
1.8
1.6
1.4
1.3
1.2
1.1
0.9
Genotype E
kb
6.5
5.5
5.0
4.5
4.1
3.8
3.5
2.8
2.4
2.2
2.1
1.9
1.8
1.6
1.4
1.3
1.2
1.1
0.9

REN profiles occur in different frequencies: 42.2% of the viral extracts showed the genotype B REN profile, but only 10.0%, 1.1%, and 1.1% of the viral extracts represent genotype C, D and E, respectivley. The D and E *Pst* I REN patterns occur only in the Kamloops geographical region. *Pst* I genotype C occurs in all the regions except the Chilliwack and Ashcroft regions.

Geographic isolates are said to occur when genomes in different locations can be distinguished by minor differences in their restriction enzyme digest profiles (Shapiro *et al.*, 1991). These *Pst I* genotypes C, D and E may be considered geographic isolates since they differ from one another and from the Virtuss^{R} DNA REN profile by the presence of additional DNA bands (Table 5.4).

The Kamloops geographic region was the only region to have all five *Pst* I REN profiles. Genotype A was the most common REN profile within this region. The Kelowna, Savona and Hedley regions had genotypes A, B and C and of the three patterns, genotype B was most common (Figure 5.7). The two unique genotypes (D and E) observed in the Kamloops geographic area were found only at one of the 10 sites sampled (Dome fire). This site was treated in the summer of 1993 with Vitrus^{R}. The genotype of the OpNPV infecting the insects during the next outbreak should be monitored to see if there are any changes in the genotypes at the site as a result of applying virus. There may be differences in the REN profile of OpNPV extracted from DFTM larvae or there may differences in the frequency of certain genotypes during

Figure 5.7. The percentage of *Pst* I and *Sal* I genotypes in the different geographic regions of British Columbia.


Genotype A
Genotype B
Genotype C
Genotype D
Genotype E
Pst I Digests
Sal I Digests
Percentage of viral extracts
100
80
60
40
20
0
Chilliwack
Vernon
Ashcroft
Savona
Hedley
Kamloops
Genotype

the outbreak following a virus application.

5.4.1.7)*Analysis of Sal I RFLPs*

When OpNPV was cut with *Sal* I enzyme, two genotypes were observed; genotype A (the REN profile of the control DNAs) and genotype B (an RFLP). Genotype B contains an extra band at 4.0 kb and there is a shift in the migration of the third band (7.0 kb to 7.5 kb; Figure 5.8 and Table 5.5). Of the viral extracts, 43.3% have the genotype B REN profile and 56.7% have genotype A REN profile. Genotype B is most common in the Kelowna, Savona and Hedley geographic regions while genotype A is most common in the Kamloops region. The Chilliwack and Ashcroft geographic regions contian only genotype A (Figure 5.7). Miller and Dawes (1978) also digested OpNPV with *Sal* I and resolved 18 bands in a 0.7% agarose gel.

5.4.2) *Common and Unique Genotypes*

When the *Pst* I and *Sal* I restriction profiles are combined for each viral extract, it is possible to determine the common and unique genotypes (Figure 5.9 and Figure 5.10). The genotypes EA and DB (the first letter refers to the *Pst* I genotype, the second letter refers to the *Sal* I genotype) only occur in 1.1% of the viral extracts, are only present in the Kamloops geographic region, and thus represent rare genotypes. Genotypes AA and BB are the most frequent, occurring in 43.3% and 35.6% of the viral extacts, respectively (Figure 5.9).

Figure 5.8. Southern blot analysis of OpNPV digested with *Sal* I, showing the two genotype REN profiles. Lanes 2 to 7 are Genotype B and have a band at 4.0 kb that is not present in lanes 10 to 15, Genotype A. Lanes 1, 8, 9 and 17 are Lambda *Hind* III molecular weight standards.

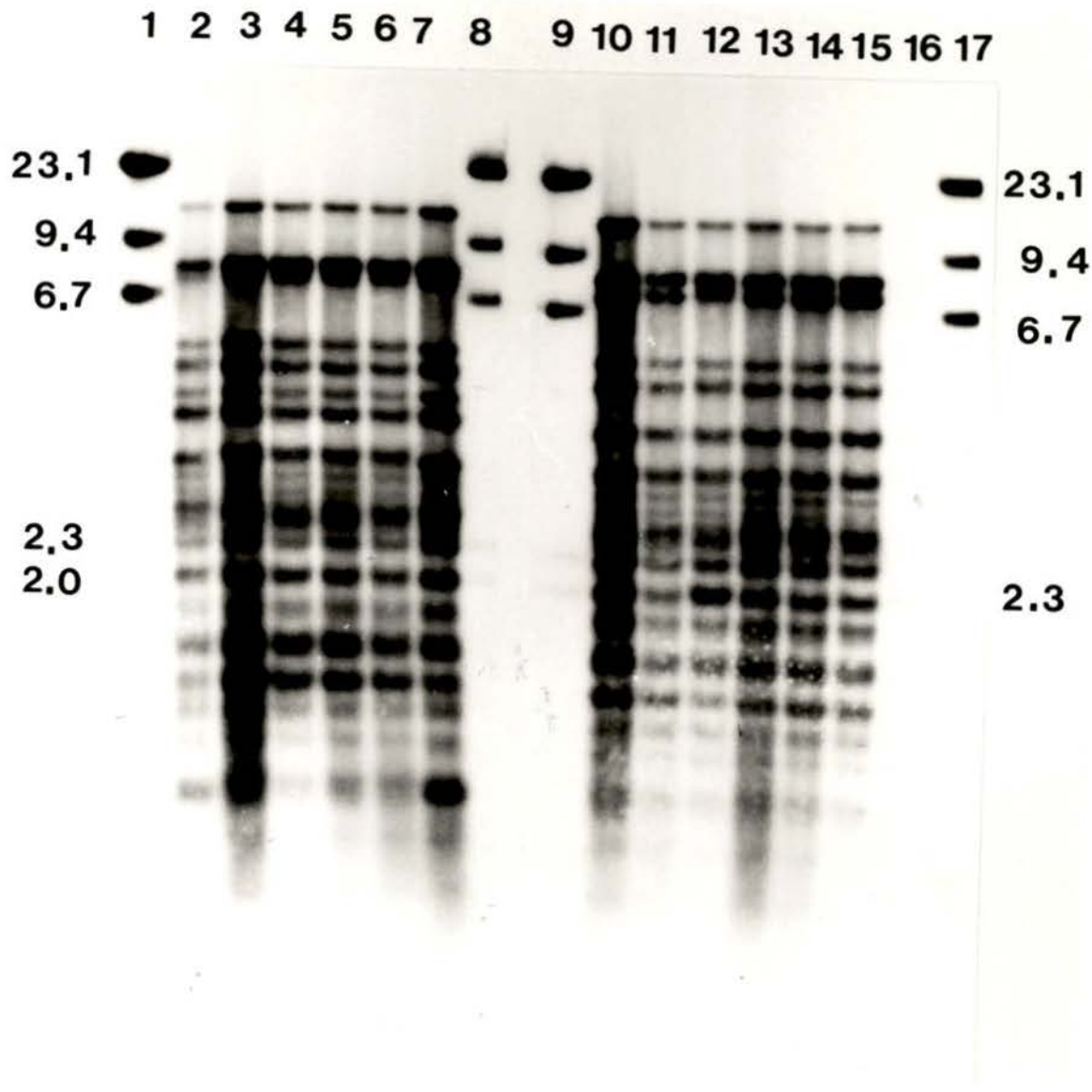

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
23.1
9.4
6.7
2.3
2.0
23.1
9.4
6.7
2.3

Table 5.5. REN fragment sizes (kb) generated by the digestion of OpNPV with *Sal* I. agarose, ethidium bromide stained gel.

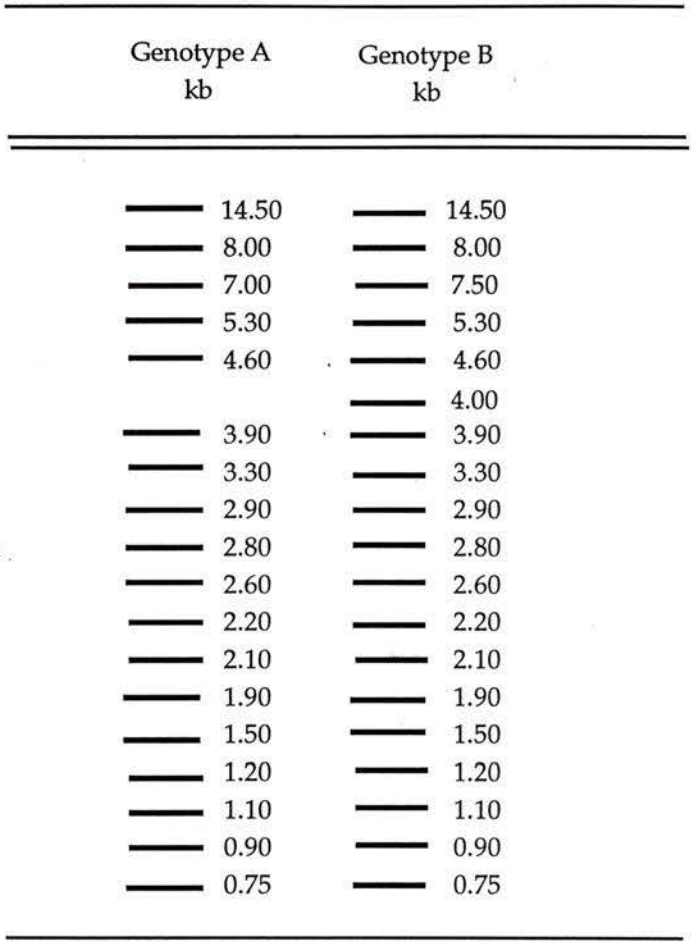
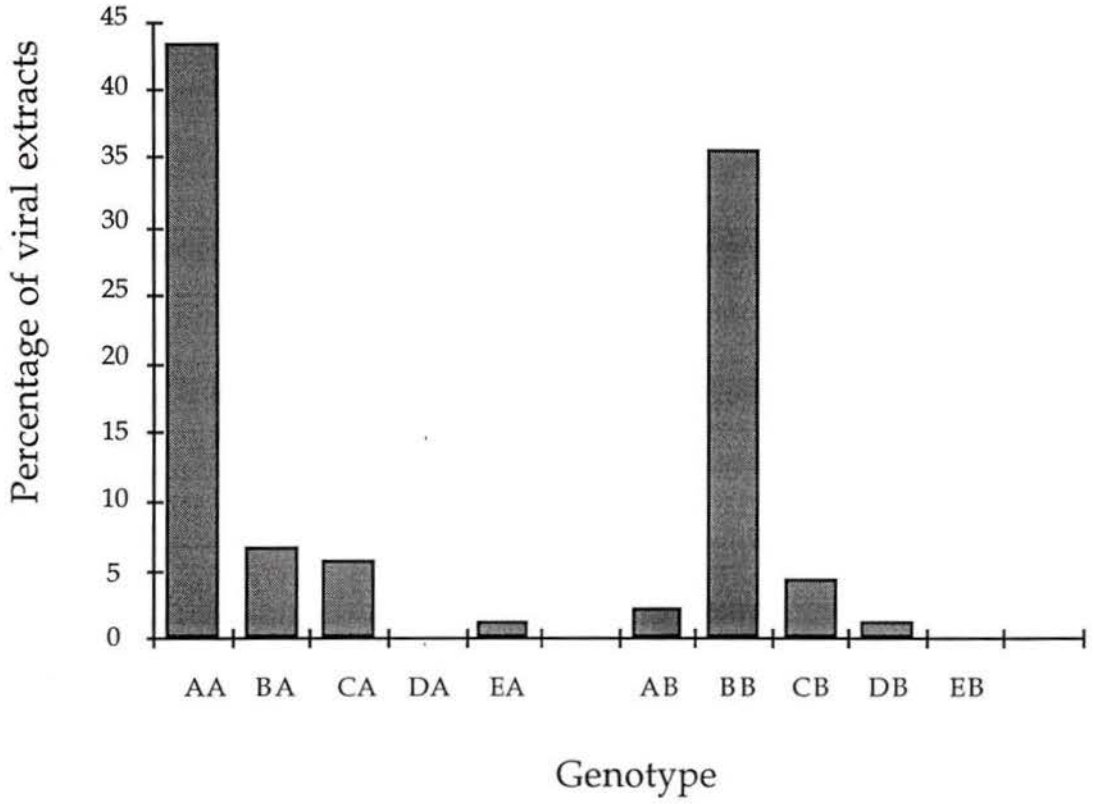

Genotype A
kb
14.50
8.00
7.00
5.30
4.60
3.90
3.30
2.90
2.80
2.60
2.20
2.10
1.90
1.50
1.20
1.10
0.90
0.75
Genotype B
kb
14.50
8.00
7.50
5.30
4.60
4.00
3.90
3.30
2.90
2.80
2.60
2.20
2.10
1.90
1.50
1.20
1.10
0.90
0.75

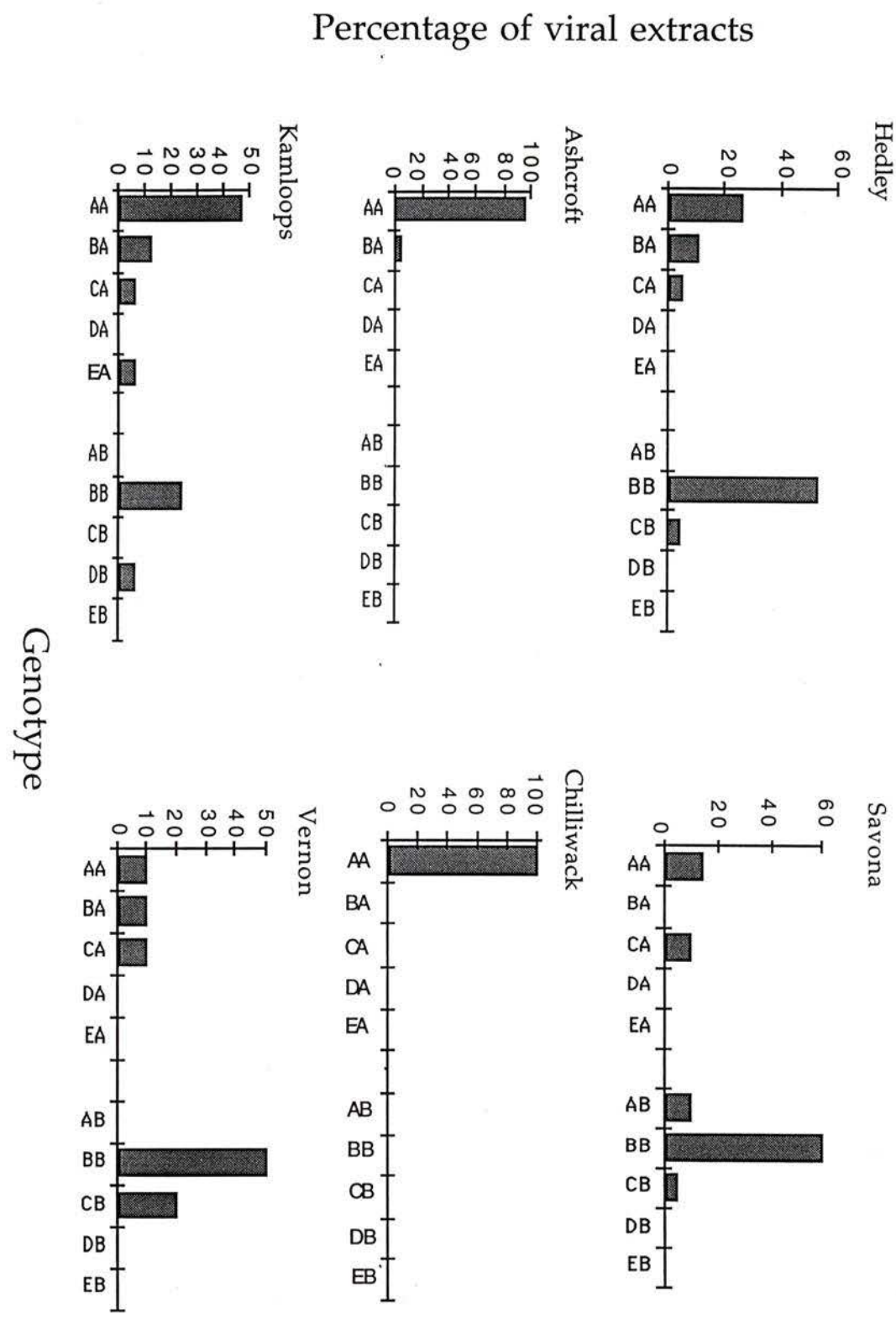
Figure 5.9. The percentage of viral extracts with the combined *Pst* I and *Sal* I genotypes. The first letter refers to the *Pst* I genotype of the extract and the second letter refers to the *Sal* I genotype of the extract.


Percentage of viral extracts
45
40
35
30
25
20
15
10
5
0
AA
BA
CA
DA
EA
AB
BB
CB
DB
EB
Genotype

The geographic regions contain one of two groups of common genotypes; AA genotype, which is most common in the Kamloops, Chilliwack and Ashcroft regions; and the BB genotype which is most common in the Vernon, Hedley and Savona regions. In total, five genotypes were obtained from the viral extracts in the Savona, Hedley and Vernon regions (Figure 5.10). The genotype CB was only present in these three regions. The genotypes DA or EB were never obtained from any viral extracts.

Since some genotypes are known to be more virulent than others (Vickers *et al.*, 1991), the incidence of NPV among early instar larvae was determined for the six geographic regions (see Chapter 4). The incidence of virus from the six regions ranged between 10.64% to 25.75% and did not differ significantly among regions ($F= 1.31$, $Pr>F$ 0.2921). None of the genotype patterns is associated with increased infection among early instars of DFTM larvae. The sites with predominately AA or BB genotypes did not differ in the infection rate among early instar DFTM larvae. Now that two rare genotypes have been determined, EA and DB, it may be possible to infect insect cell cultures with these OpNPV virus strains, observe the formation of polyhedra, and conduct plaque assays to determine if there are morphological or phenotypic differences among these isolates. Two strains of *Spodoptera littoralis* NPV (SlNPV-A and SlNPV-B) showed differences in plaque morphologies and growth characterisitcs when grown in cell culture (Cherry

Figure 5.10. The percentage of viral extracts for the combined *Pst* I and *Sal* I genotypes for each geographic region of British Columbia.


Percentage of viral extracts
Hedley
Ashcroft
Kamloops
Savona
Chilliwack
Vernon
AA
BA
CA
DA
EA
AB
BB
CB
DB
EB
Genotype

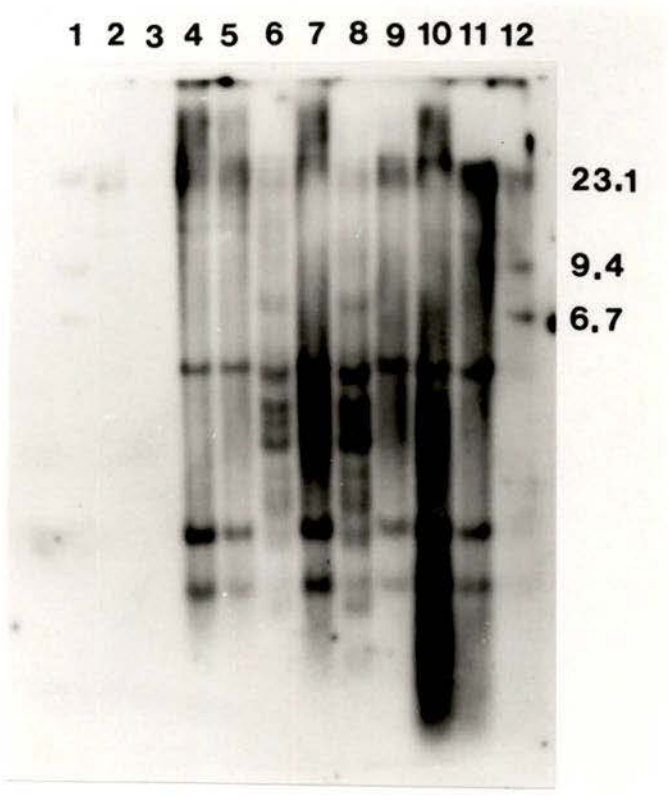
and Summers, 1985). SlNPV-A produced many, small polyhedra, while SlNPV-B produced few, large polyhedra under the same growth conditions (Cherry and Summers, 1985). Similar plaque morphology differences were also observed when *Spodoptera littura* NPV was grown in cell culture, and in combination with restriction enzyme digests, these morphological differences allowed the classification of wild SlNPV into four strains of genetic variants (Maeda *et al.*, 1990). Growing two apparently unique strains of OpNPV in cell culture may indicate similar morphological or phenotypic differences in the strains, which could lead to the development of one of these as a new, unique strain of OpNPV for field use.

5.4.3) *Possible Detection of OpSNPV*

In the preliminary digests with *Eco R* I, two of four viral extracts from the Chilliwack region showed a REN profile with 18 to 20 bands rather than seven observed in the other insects (Figure 5.11, lanes 6 and 8). Those DNAs hybridized strongly to the OpNPV DNA in Southern blots. The higher number of bands may be a result of digesting a SNPV rather than MNPV strain of virus. Rohrmann and Beaudreau (1977) digested OpSNPV with *EcoR* I and the REN profile showed 23 bands. The restriction profile from Rohrmann and Beaudreau (1977) resembles the pattern obtained from these two insects from the Chilliwack site, indicating that insects from Chilliwack may be infected with OpSNPV.

If the extra bands in the OpNPV actually represent the digestion of a

Figure 5.11. *Eco R* I digest showing extra bands in two viral extracts from the Chilliwack site (lanes 6 and 8).


1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.7

SNPV, it may be possible to separate the SNPV and MNPV genotypes and characterize the isolates within these groups, since the REN profiles are so different. REN digestions of *Heliothis* spp. NPV separated wild-type isolates into two major groups based on the virion morphology with geographic isolates occurring within these major groupings of the virus (Gettig and McCarthy, 1982). If these extra bands are a result of digesting a SNPV, the two morphotypes can easily be separated on the basis of their *EcoR* I digestion profile. It is surprising, however, that the OpMNPV probe from the Virtus^R strain of the virus hybridized to the putative SNPV, since Rohrmann *et al.*, (1978) indicated less than 1% homology between the two morphotypes of NPV.

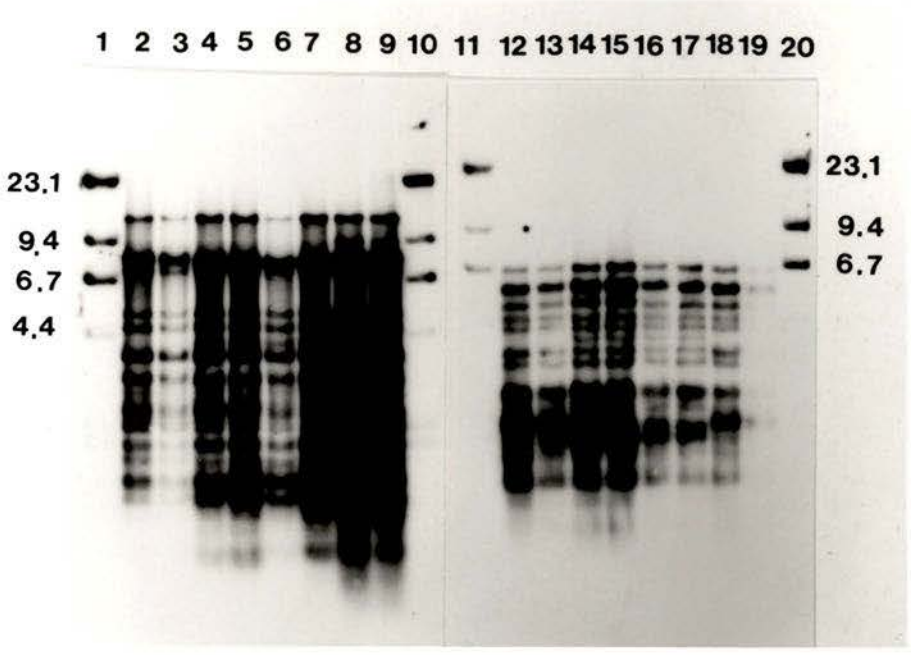
5.4.4) *NPV Infected Larvae From the Same Egg Mass*

Preliminary work with viral DNA extracts from late instar larvae reared from the same egg mass indicate these insects are infected with the same virus (Figure 5.12). However, further tests will need to be completed on viral extracts from insects from the same egg mass to confirm this observation. If the DNA is the same, then cadavers from the same egg mass can be pooled together and more viral DNA can extracted, allowing more extracts from the same site to be compared, and if needed, used for repeated cuttings with the same extract if a digestion or gel fails.

5.4.5) *Identification of Unique OpNPV Genotypes*

It has been shown that epizootics can be induced earlier than it would

Figure 5.12. Digests of viral extracts from the Rattle Snake Reserve site in Vernon with*Sal* I (lanes 2 to 9) and *Pst* I (lanes 12 to 19). Lanes 2, 3, 12 and 13, 4, 5, 14 and 15, 6, 7, 16 and 17 are viral extracts from the same egg mass. Note each pair of viral extracts from the same egg mass has the same genotype pattern.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
9.4
6.7
4.4
23.1
9.4
6.7

occur naturally by spraying the virus in the field (Otvos and Shepherd, 1991), but the proportion of the mortality caused by the virus treatment and the proportion of the mortality due to the naturally occurring virus increasing from one year to the next, presently cannot be separated. If a genetically unique strain is identified from this research and it is efficatious agianst its host, it could be multiplied in the laboratory and used in the field to determine the proportion of the mortality caused by the OpNPV treatment.

Genotypic differences in OpNPV may correlate with differences in the biological activity. Analysis of virulence of different OpNPV strains should be completed in the future, since small differences in the REN profiles of related strains of virus could result in large changes of virulence (Vickers *et al.*, 1991). Changes in the restriction profile have been correlated with differences in plaque morphology of *S. littoralis* NPV variants and differences in host range and virulence in *A. segetum* NPV variants (Vickers *et al.*, 1991; Cherry and Summers, 1985).

5.4.6) *Summary*

OpNPV can be extracted quickly from late instar, OpNPV infected DFTM larvae reared from egg masses or from the field collected larvae. REN/Southern blot analysis indicated that there are geographical isolates of OpNPV in British Columbia. Once the variation is fully investigated, by increasing the number of viral extracts examined, a genetically unique strain

of NPV may be selected in laboratory and field tested to examine its filed efficacy and monitor mortality as a result of the virus treatment using this unique strain.

Chapter 6

Infection Rates of Douglas-fir Tussock Moth Larvae (*Orgyia pseudotsugata*, McDunnough) in British Columbia with Cytoplasmic Polyhedrosis Virus

6.1) Introduction

Cytoplasmic Polyhedrosis viruses (CPVs) belong to family Reoviridae, and contain 10 eqimolar segments of double-stranded RNA (dsRNA) (Payne and Mertens, 1983; Payne and Rivers, 1976; Payne *et al.*, 1977). The 10 segments of dsRNA code for the virus structural and non-structural proteins (Payne and Mertens, 1983; Mertens *et al.*, 1989; Rohrmann *et al.*, 1980). The host range of CPVs is restricted to arthropods and these viruses are pathogens of many insects, infecting as many as 200 species from the orders, Lepidoptera, Hymenoptera and Diptera (Payne and Mertens, 1983; Payne and Rivers, 1976).

Because CPVs infect many members of the order Lepidoptera, they have been considered as potential biological control agents. In Japan, one CPV is registered for the control of the pine caterpillar, *Dendrolimus spectabilis* (Payne and Mertens, 1983). However, CPVs are biochemically similar to viruses known to infect vertebrates and plants, and because they produce chronic rather than lethal infection in their hosts, CPVs have yet to be developed for biological control of other insect species besides the pine caterpillar (Payne and Mertens, 1983). At the present, even the CPV registered

in Japan is no longer commercially available but work on CPV is on going in China (Otvos, pers. comm., 1995).

A CPV infection of Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* McDunnough was first reported by Martignoni *et al.* (1969). The CPV infection was discovered in DFTM larvae reared in the laboratory from field collected DFTM egg masses (Martignoni *et al.*, 1969).

CPV infections, like those of nuclear polyhedrosis viruses (Baculoviruses) culminate in the occlusion of one to many hundreds of virions within a large, proteinaceous inclusion body (PIB), or polyhedra (Payne and Mertens, 1983; Payne and Rivers, 1976). Virus embedded in the polyhedral protein are highly stable in the environment and are the source of infection for the other susceptible members of the host. Once inside the insect midgut, the polyhedra is dissolved at high pH levels (above pH 10.5). The virions infect midgut epithelial cells and replicate in the cytoplasm (Payne and Harrap, 1977; Payne and Mertens, 1983). Larvae infected with CPV feed less than healthy larvae or may stop feeding entirely (Harrap and Payne, 1979; Payne and Mertens, 1983). Larvae grow slowly and overall development is delayed. Larval weight and body size decrease and diarrhea is common; feces are often contaminated with polyhedra (Payne and Mertens, 1983). Pupation of the host may occur but these pupae are smaller than uninfected pupae, and adults may fail to emerge or may be deformed or sterile if they emerge (Harrap and Payne, 1979; Payne and Mertens, 1983).

CPVs are provisionally classified on the basis of distinctive variations in the electrophoretic migration patterns of the genome segments (electropherotypes) (Payne and Rivers, 1976). Twelve distinct CPV electropherotypes have been defined (Payne and Rivers, 1976; Payne *et al.*, 1977), which differ from one another in the migration of at least three genome segments after electrophoeresis in 3% polyacrylamide gels (PAGE) with a tris-acetate buffer system (Payne and Rivers, 1976; Payne *et al.*, 1977; Payne and Mertens, 1983; Mertens *et al.*, 1989). The CPV infecting DFTM (OpCPV, for *Orgyia pseudotsugata* CPV) closely resembles the electropherotype obtained from the CPVs isolated from *Tricoplusia ni* and *Heliothis armigera* larvae and is placed within the "type 5" group of these viruses (Rohrmann *et al.*, 1980; Payne and Rivers, 1976). The total molecular weight of the OpCPV (type 5) is 14.82 X 10^6 Daltons (Payne and Rivers, 1976).

The type 1 and type 4 electropherotypes may have originated in Japan and the types 2, 3 and 6 share a predominately European or Palaearctic geographic distribution (Payne and Mertens, 1983). There are few studies of the incidence of CPV infection in insect populations, since the disease is normally at enzootic levels and rarely rises to epizootic levels (Payne and Mertens, 1983). The CPVs appear to persist in the environment even at low host densities. In surveys of the members of the Simuliidae (blackflies), the incidence of CPV infections ranged from 1 to 6.5% of the larvae (Payne and Mertens, 1983; Bailey, 1977).

While the presence of OpCPV has been identified in field and laboratory reared insects, little data on the field incidence of CPV infections in natural populaions has been reported. This chapter of the thesis reports the incidence of OpCPV in DFTM larvae reared from field collected egg masses. In this section the geographic regions of B.C. where CPV infections have been found are identified.

6.2) Materials and Methods

6.2.1) *Collection of Infected Larvae and Extraction of Viral DNA*

DFTM egg masses were collected from 42 sites, in 6 geographic locations in British Columbia in the fall of 1991 and 1992 (Figure 3.1) as described in section 3.2.1. Ten sites, two from each geographic region, except for Chilliwack and Vernon (only 1 site in these regions) were selected for viral extractions, since time contstraints did not allow a survey of all 41 collection sites.

Rearing conditions for the DFTM have been previously reported in section 5.3.1 of this thesis and the extraction of the CPV viral DNA was completed during the extraction for OpNPV. This procedure has also been previously reported in section 5.3.3 of this thesis.

6.2.2) *Number of Viral Extracts with CPV Infection*

The presence of several extra bands was noted on agarose gels when OpNPV viral extracts were electrophoresed to check the quality and quantity

of the viral DNA. These extra bands were a surprising result, and it was suggested that these extra bands might represent CPV infections in DFTM larvae. The number of viral extracts with these extra bands were counted for the ten sites examined and the percent incidence of putative CPV infection (number of viral extracts with extra bands divided by the total number of viral extracts completed for each site, multiplied by 100) was calculated.

6.2.3) *RNAase A digestion of CPV samples*

To verify the presence of a dsRNA virus in the samples, 7 - 10 ng of putative CPV extract (Heffley Creek, egg mass #30 extract), a sample of *Cryphonectria parasitica (Endothia)* isolate GH2, known to contain dsRNA (generously provided by Dr. Paul Spencer (PDF), Canadian Forest Service, Pacific Forestry Centre), and 25 ng of lambda DNA were digested with 0.5 unit/ml RNAase A at high ionic strength (2 X SSC) and low ionic strength (0.1 X SSC). The samples were also digested with 1 unit/ml RNAase free DNAase for 20 minutes at 37 ^{o}C. The digests were then electrophoresed for three hours at 60 volts, in 1% agarose containing 0.1ug/ml ethidium bromide (20 x 20 cm horizontal gel) with 1 X TAE buffer. After electrophoresis, the gel was then exposed to ultra violet light and photographed with Polaroid Land Film.

6.2.4) *Determination of the CPV Electopherotype*

Four viral extracts from the Heffley Creek site, two from the Red Hill

site and four from the Murray Creek site, were used to determine the electropherotype of the OpCPV. The viral extract samples (70-100 ng estimated on the basis of the intensity of the lambda-*Hind III* fragments when stained with ethidium bromide) and 250 ng of 1 KB ladder were electrophoresed at 20 volts overnight in a 0.5 % agarose/0.2% Synergel, 20 X 10 cm horizontal gel with 1 X TAE buffer. The electropherotype was visualized by staining the agarose gel with 1ug/ml ethidium bromide and visualizing the gel under ultra violet and photographing with a Polaroid Land Camera. The size (in kb) was determined for each dsRNA segment based on the fragment's mobility in 1% agarose when compared to the mobility of 1 kb fragments of known size.

6.2.5) *Digestion of Viral Extracts with EcoR I and Southern Blots*

Two viral extracts from each of the Heffley Creek, Red Hill and Murray Creek were digested with Promega *Eco RI* enzyme for five hours and electrophoresed, with two uncut viral extracts from each of the three sites, overnight at 20 volts in 1 X TAE with 0.5% agarose/0.2% Synergel. Gels were stained in distilled water containing 1 ug/ml ethidium bromide for 10 minutes. The digestion patterns were visualized with ultra violet light and photographed with a Polaroid Land Camera. Southern blots of the digests were completed as described in section 5.3.6 of this thesis. Chemiluminescent detections with the total genomic Virtuss^{R} probe were completed as outlined

in section 5.3.7 of this thesis.

6.3) Results and Discussion

6.3.1) *RNAase A Digestion of CPV Samples*

The putative dsRNA in the viral extracts containing CPV and the *C. parasitica* isolate GH2 were not hydrolysed by DNAase nor by RNAase A under high ionic strength (2 X SSC) (Figure 6.1, lanes 3, 5, 8 and 10). These dsRNAs, however, were hydrolysed by RNAase A under low ionic strength (0.1 X SSC) (Figure 6.1, lanes 4 and 9). The dsRNA of CPVs is, characteristically, highly resistant to degradation by RNAase A under high salt concentrations (high ionic strength), but degraded when the salt concentration is lowered (Payne and Mertens, 1983; Wakarchuk and Hamilton, 1985).

6.3.2) *CPV Electropherotype*

The agarose gel of viral extracts containing CPVs, as well as OpNPV, were electrophoresed overnight and stained with ethidium bromide (Figure 6.2). In 8 of the 10 lanes, 10 dsRNA segments were observed, while in the remaining two lanes only nine dsRNA segments were resolved (Figure 6.2). All CPV types contain 10 eqimolar genome segments, although all 10 are rarely resolved (Payne and Mertens, 1983; Payne and Rivers, 1976).

Figure 6.1. Digestion of putative OpCPV from Heffley Creek viral extract (lanes 2 to 5), *C. parasitica* (lanes 7 to 10), and lambda DNA (lanes 12 to 15) with RNAase A at high and low ionic strength (lanes 3, 4, 8, 9, 12 and 13) and DNAase (lanes 5, 10, and 15). Lambda Hind III fragments (lanes 1, 6, 11) are molecular weight standards.

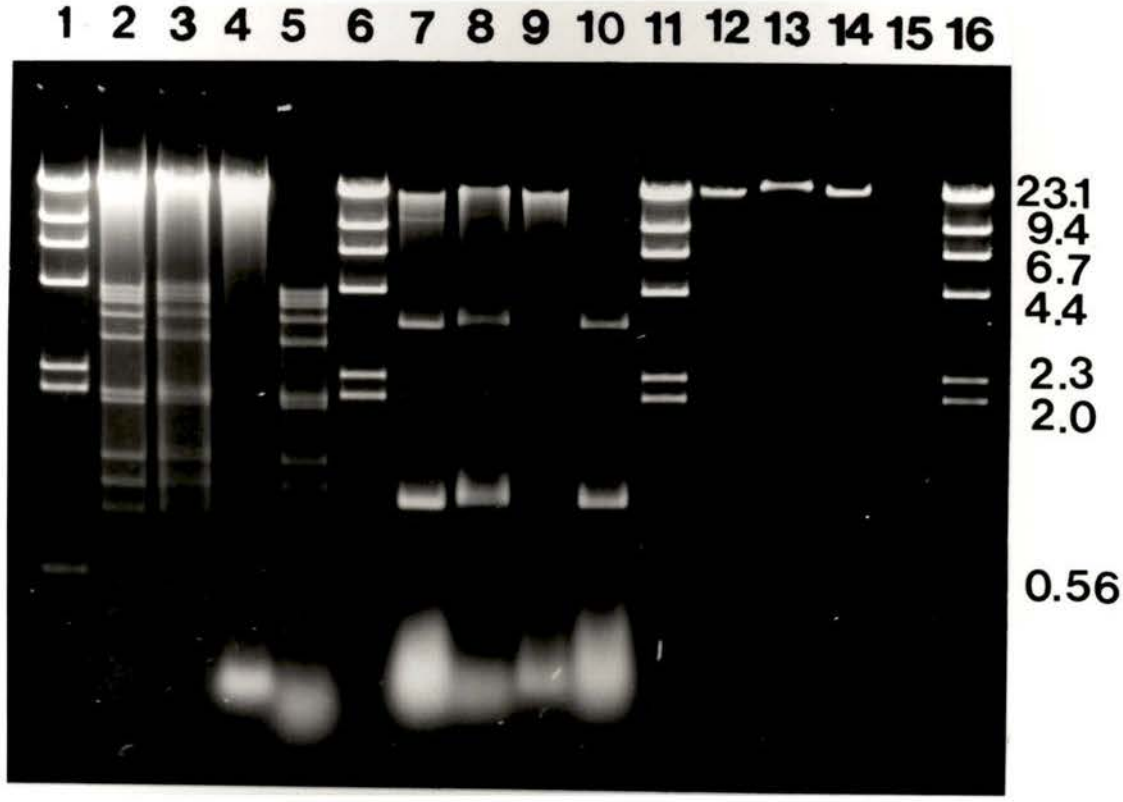
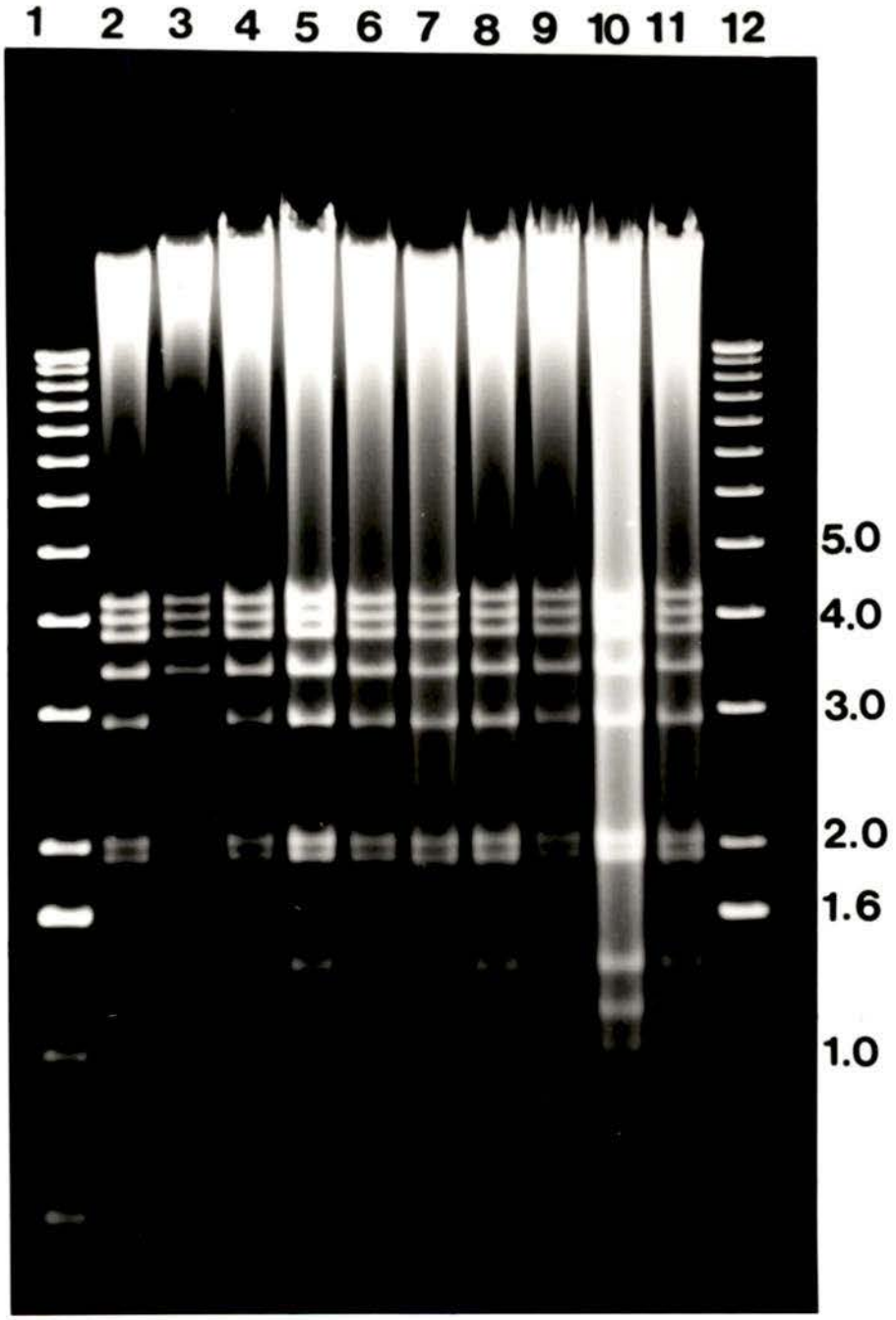

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
23.1
9.4
6.7
4.4
2.3
2.0
0.56

Figure 6.2. Electrophoretic profile of the putitive OpCPV from British Columbia. Lanes 2, 3, 4, 5 from Heffley Creek, lanes 6 and 7 from Red Hill, and 8, 9, 10, and 11 from Murray Creek sites. The upper smear in each lane is undigested OpNPV DNA, the lower bands are the 10 dsRNA segments of the putative OpCPV. Lanes 1 and 12 are 1 kb molecular size fragments.


1
2
3
4
5
6
7
8
9
10
11
12
5.0
4.0
3.0
2.0
1.6
1.0

Determination of only nine segments (Figure 6.2, lanes 3 and 9) resolved is not unusual. The molecular weight of the type 5 CPVs is reported in Payne and Mertens (1983), Payne and Rivers (1976) as 14.82 X 10^6 Daltons while Galinski *et al.* (1982) report the molecular weight of OpCPV to be 20.4 X 10^6 Daltons (29.8 kb). The apparent molecular weight of OpCPV from the viral extracts in British Columbia is 17.6 X 10^6 Daltons (25.85 kb) (Table 6.1). The dsRNA segments are larger in size than those reported in Payne and Rivers (1976) and Payne and Mertens (1983), but smaller than those reported by Galinski *et al.*, (1982). Payne and Rivers (1976) base the type 5 electropherotype size on the *H. armigera* CPV, not on OpCPV as reported by Galinski *et al.* (1982) and the difference in genome size may be due to comparing different CPVs or may be due to the method of size determination. In the report by Galinski *et al.* (1982) the segment sizes were determined in denaturing, glyoxal agarose gels rather than in non-denaturing agarose gels used in this study, or the 3% polyacrylamide gels used by Payne and Rivers (1976). In denaturing, glyoxal agarose gels, the dsRNA is free of secondary structure, and thus, size measurements are made when the dsRNA are independent of conformation, so segment sizes are presumably more accurate (Galinski *et al.*, 1982). The 1% non-denaturing agarose gels used in this study, underestimate the overall size of the OpCPV genome compared to that reported by Galinski *et al.*

Table 6.1. Molecular weight estimates for putative OpCPV determined by 1% agarose/SG electrophoresis for 20 hours at 20 volts.

Segment	Kilobases (kb)	Datons (10^6)
1	4.30	2.92
2	4.10	2.79
3	3.60	2.45
4	3.40	2.32
5	2.90	1.97
6	2.00	1.36
7	1.95	1.33
8	1.30	0.89
9	1.20	0.82
10	1.00	0.68
Total	25.85	17.60

(1982). However, size of the OpCPV genome, as estimated in this study, is within the general range of CPV genome sizes. The basis of altered relative migration of some segments in different gel systems is not clear but may be due to the different gel concentrations (Payne and Mertens, 1983).

6.3.3) *Southern Blots and Chemiluminescent Detections with OpNPV*

All six viral extracts containing both OpNPV and OpCPV showed digestion of OpNPV with *EcoR I* enzyme (Figure 6.3A). When the ethidium bromide stained agarose gel was photographed, before Southern blotting, the OpCPV segments can clearly be seen intact in both the uncut and the *EcoR I* digest lanes (Figure 6.3A and 6.3B). After Southern blotting and hybridization with the total genomic OpNPV probe, only the OpNPV digest bands are observed (Figure 6.3B), showing that the OpCPV dsRNA is not cleaved by *EcoR I* and is not homologous to OpNPV DNA.

6.3.4)*Incidence of CPV infection in British Columbia*

CPV infections were found in 27 out of 170 viral extracts from three geographic regions (four sites) in B.C. (Table 6.2). The Chilliwack, Kamloops and Ashcroft geographic regions of the Province showed CPV infection among the larvae reared from field-collected DFTM egg masses. The incidence of infection ranged from 86% in the Chilliwack

Figure 6.3. In part A the ethidium bromide stained Eco RI digestion of OpNPV and OpCPV viral extracts is shown. Lanes 2, 4, 6, 8, 12 and 14 are uncut OpNPV DNA showing the electropherotype of the putative OpCPV. Lanes 3, 5, 7, 11, and 15 are OpNPV digests with Eco RI, note the upper bands of of digested OpNPV. Lanes 16 and 17 are the Virtuss^{R} and BioControl^{R} viral DNA digested with Eco RI. Lane 18 is host (DFTM) DNA digested with Eco RI. Lanes 1 and 19 are the 1 kb molecular weight standards. In part B, total genomic OpNPV DNA probe is hybridized to the Southern bolts of the digests. Note no bands are present in the uncut OpNPV lanes.

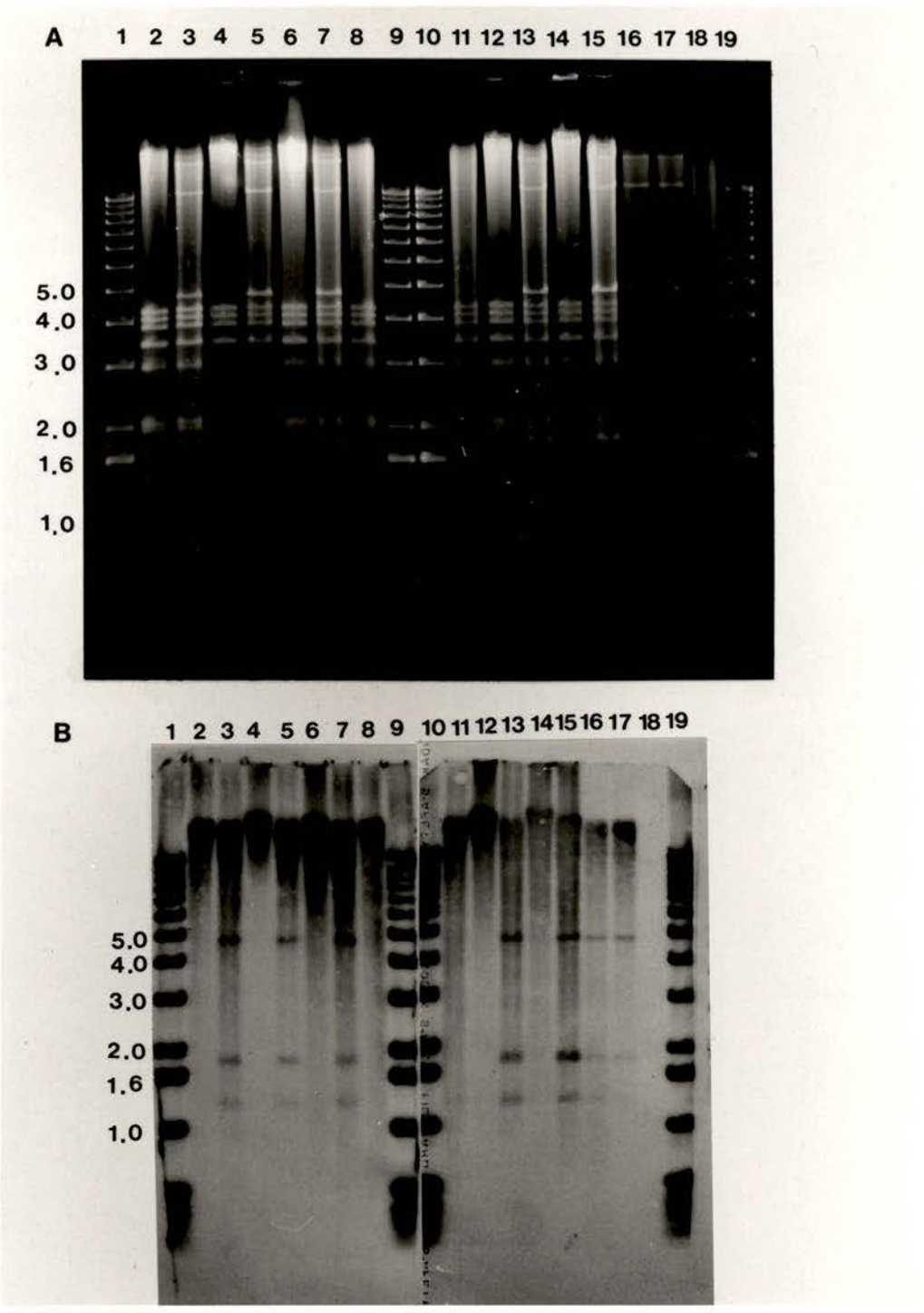

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
5.0
4.0
3.0
2.0
1.6
1.0
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
5.0
4.0
3.0
2.0
1.6
1.0

Table 6.2. Collection sites and geographic regions in British Columbia with OpCPV infections in fall 1991 and 1992.

Site	Geographic Region	Number of Viral extracts	Number of viral extracts with CPV	Percent Infection
Chilliwack	Chilliwack	7	6	86.00
Rattlesnake	Vernon	19	0	0.00
Dome fire	Kamloops	20	0	0.00
Heffley	Kamloops	19	12	0.00
Red Hill	Ashcroft	20	2	63.20
Murray Creek	Ashcroft	15	7	10.00
Sabiston 4.95 km	Savona	20	0	71.40
Guichon 1.3 km E	Savona	20	0	0.00
Bradshaw Cr.	Hedley	15	0	0.00
Larcan Tr.	Hedley	15	0	0.00
Total		170	27	15.90

site to 10% in the Red Hill site (Table 6.2). When Simuliidae larvae were collected from streams in Newfoundland, CPV infections were found in five species of black fly, *Cnepia mutata, Promismulium mixtum fuscum, Simulium tuberosum, Simulium venustum* and *Simulium vittatum* (Bailey, 1977). Of these insects, CPV infections were most common in *C. mutata* where up to 54% of the larvae were infected when the midgut was removed and examined for gross signs of infection (Bailey, 1977). Payne and Mertens (1983) state that CPV infections of the pine caterpillar are present in low levels in all areas of Japan.

6.4) Summary

By digesting putative dsRNA with RNAase A at high ionic strength and low ionic strength, it was shown that the extra bands in the OpNPV viral extracts were dsRNA. The electropherotype of the putative OpCPV was found to be similar to the type 5 CPV electropherotype published by Galinski*et al.* (1982) and Payne and Rivers (1976). Differences in the sizes of the dsRNA segments were seen in this study in comparison to those previously published, but fall within the reported range of CPV genome sizes. The putative OpCPV did not hybridize to OpNPV total genomic probe when digested with *EcoR I* enzyme, and is therefore not homologous to OpNPV. OpCPV

infections were found in DFTM larvae reared from field-collected egg masses from four of ten sites examined (three geographic regions) in British Columbia. An overall infection rate for the DFTM larvae was found to be 15.9% and infection rates ranged from 10% to 86% depending on the location of the site.

Chapter 7

Conclusion

The current pest management system of DFTM involves using pheromone traps placed in susceptible locations to monitor DFTM population size, and treatment of stands with the laboratory produced, naturally-occurring NPV if the damage warrants it. This system is effective at controlling DFTM outbreaks but, through the research undertaken in this study, it was hoped that refinements to the system might be made.

This study showed that the number of DFTM eggs per egg mass decreases with the age of the outbreak. This decrease in egg mass size may have been due to the lack of highly nutritional food sources at the infestation site. Regression equations created from the number of eggs per egg mass against weight of the egg mass with and without the cocoon attached, area and volume of the egg mass, showed that weight without the cocoon is most closely correlated to the number of eggs per egg mass. A regression using weight without the cocoon against number of eggs per eggs would best predict DFTM population size, since these R^2 values are the highest of all the regression equations. The other regression equations would also provide good estimates of DFTM population size, but at high population densities the regression equations may underestimate the population. None of the regression equations pass through the origin and this may be due to the amount of glue and abdominal hairs cemented in the egg mass or may be due

the weight of the eggs themselves. Only four of the regression equations from different years can be combined; the rest are significantly different. It was hoped that this study would generate regression equations which could be used to predict population size at different outbreak levels. Since the regression equations cannot be combined, new regression equations should be created for each outbreak. Incorporating the current year's defoliation of each tree, number of viable eggs per mass or spring population densities may provide another measure to incorporate into the regression equations and aid in developing common regressions to estimate DFTM populations.

The overall incidence of NPV among early instar DFTM larvae was 15.99%. In 1991, larvae were reared in two sets (Set A and Set B) of DFTM egg masses. The incidence of NPV was significantly higher in the second set of egg masses reared (Set B, 33.25% NPV) than Set A (4.81%). Set B egg masses were kept in cold storage longer than Set A, and since the egg masses were collected under wet conditions and were stored damp, in plastic bags, moisture in the bags may have spread the NPV over the surface of the egg masses, resulting in a higher rate of NPV infection. The incidence of NPV infection was highest in age 1 of the outbreak and decreased in age 2, in both the 1991 and 1992 egg mass collections. The overall rate of infection was highest among 1992 collected EM. 1992 was the third year in the current DFTM infestation and the incidence of infection may have been higher in 1992 because the population was beginning to peak in the Kamloops area.

Extractions of OpNPV viral DNA can be completed from late instar DFTM larvae. No polymorphisms were detected when the viral DNA was digested and probed with the ie-1 chemiluminescent probe. Polymorphisms were detected when the viral DNA was cut with *Pst* I, *Sal* I, *Hind* III and *Bgl* II restriction enzymes. Five genotypes of *Pst* I and two genotypes of *Sal* I were identified in this study. Three of the *Pst* I genotypes are unique and two of these genotypes occur only in the Kamloops geographic region. In preliminary studies of the viral DNA extracts from insects from the same egg mass, the banding pattern appears identical. Since there are genetically unique strains of OpNPV in British Columbia, more studies into the genotypic variation should be completed to determine a unique strain which could be used in field studies to monitor DFTM mortality.

During OpNPV extractions, insects were also found to be infected with a cytoplasmic polyhedrosis virus. This virus is composed of ten equimolar segments of double stranded RNA. The CPV bands were resistant to RNAase A digestion under high ionic strength, but degraded under low ionic strength. The electrophoretic profile of the CPV from the Kamloops area is similar in size to those previously published and the segments do not hybridize to chemiluminescent labelled OpNPV DNA. A total of 27 larvae from the Kamloops area were infected with the CPV.

This research project has addressed some of the interactions between DFTM and its naturally-occurring NPV, but further studies may need to be

completed. It is highly desirable, in fact essential, to leave infested stands untreated in the next outbreak to follow the level of a few viral infection sites during the course of the outbreak cycle so that a threshold of NPV may be more clearly indicated. The overall rate of NPV was rising during the outbreak, but observing the next year of the outbreak may have shown the infection rate and population to decrease. Identifying a threshold level of infection would allow forest managers to treat only those stands that are not close to natural collapse.

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Appendix I

List of the site where DFTM egg masses was sampled in the fall of 1991 and 1992, Kamloops Forest Region, B.C..

Savona Region

1. Indian Gardens W1
2. North East Lot 20
3. Indian Gardns Hydro Line
4. Guichon Rd. 2.2W
5. Guichon Indian Gardens Juction
6. Guichon Rd. 1.3E
7. Indian Gardens E3
8. Indian Gardens E2
9. Indian Gardens E1
10. Sabiston 4.3km
11. Sabistan 13km

Kamloops Region

12. Rifle Range
13. Plot 13
14. Munro Creek
15. Brussels Lake
16. Brussels Creek
17. Beaton Lake
18. Dominic Lake
19. Iron Mask
20. Mara Hill
21. Lac du Bois Rd
22. Dome fire 1
23. Dome fire 2
24. Heffley Creek
26. Monte Creek

Hedley Region

27. BCMOF Campsite
28. Stemwinder
29. Winter's Creek (up)
30. Bradshw Creek
31. Shoemaker Creek (up)
32. Shoemaker Creek (tr)
33. Plot 20
34. Larcan Creek (up)
35. Larcan Creek (tr)

Vernon Region

36. Rattlesnake Reserve

Ashcroft Region

37. Red Hill
38. Oregon Jack Rd.
39. Red Hill Rest Stop
40. Sarangetti
41. Murray Creek

Chilliwack Region

42. Chilliwack

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Naturally-occurring nuclear polyhedrosis virus for the biological control of the Douglas-fir tussock moth, *Orgyia pseudotsugata*, McDunnough, (Lepidoptera: Lymantriidae)

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July 28, 1995