Enhanced Pest Management of the Douglas-fir Tussock Moth,
Orgyia pseudotsugata (Lepidoptera: Lymantriidae)

by

Christine M. Thorne
BSc, University of Victoria, 2003

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

Enhanced Pest Management of the Douglas-fir Tussock Moth,
Orgyia pseudotsugata (Lepidoptera: Lymantriidae)

by

Christine M. Thorne
BSc, University of Victoria, 2003

Supervisory Committee

Dr. David B. Levin, (Department of Biology)
Supervisor

Dr. Imre S. Otvos, (Department of Biology)
Co-Supervisor

Dr. Peter Constabel (Department of Biology)
Departmental Member

Dr. Chris Lucarotti (Natural Resources Canada, Canadian Forestry Service, Atlantic Forestry Centre)
External Examiner

Supervisory Committee

Dr. David B. Levin, (Department of Biology)
Supervisor

Dr. Imre S. Otvos, (Department of Biology)
Co-Supervisor

Dr. Peter Constabel (Department of Biology)
Departmental Member

Dr. Chris Lucarotti (Natural Resources Canada, Canadian Forestry Service, Atlantic Forestry Centre)
External Examiner

Abstract

The incidence of baculovirus disease in susceptible insect hosts may be used to estimate future host population sizes and estimate the relative defoliation of these populations for economically important insects. We developed an Enzyme-linked immunosorbent assay (ELISA) that was specific to two native baculovirus pathogens of the Douglas-fir tussock moth (DFTM), that was more sensitive and accurate (R^2=0.99) than microscopic counts of virus polyhedron inclusion bodies, and more accurate than PCR or Southern hybridization for the direct detection of baculovirus disease. We also developed a field-based "dipstick" assay that was sensitive enough to detect virus disease prior to host mortality and did not require virus purification from macerated insect tissues, supporting its use for on-site field surveys. Finally, we used ELISA to establish, for the first time, a method to detect persistent sublethal baculovirus infections. Our work may contribute to more accurate understanding of the incidence and influence of persistent sublethal infections on insect hosts.

Table of Contents

List of Abbreviations

(ss/ds)DNA	single/double stranded deoxyribonucleic acid
ANCOVA	analysis of covariance
ANOVA	analysis of variance
bp	base pairs
BSA	bovine serum albumin
BV	budded virus
CfMNPV	*Choristoneura frugiperda* multinucleopolyhedrovirus
ChocGV	*Choristoneura occidentalis* granulosis virus
DAB	diaminobenzidine
DFTM	Douglas-fir tussock moth
ELISA	Enzyme Linked Imunosorbent Assay
EPA	Environmental Protection Agency
GV	Granulosis virus
HRP	horse radish peroxidase
IPM	Integrated Pest Management
LD_{50}	Lethal dose causing 50% mortality
LdNPV	*Lymantria dispar* nucleopolyhedrosis virus
LffNPV	*Lambdina fiscellaria fiscellaria* nucleopolyhedrosis virus
McplNPV	*Malacosoma californicum pluviale* nucleopolyhedrosis virus
MdNPV	*Malacosoma distra* nucleopolyhedrovirus
MNPV	multinucleopolyhedrovirus
NeabNPV	*Neodiprion abietis* nucleopolyhedrovirus
OaNPV	*Orgyia antiqua* nucleopolyhedrovirus
OlNPV	*Orgyia leucostigma* nucleopolyhedrovirus
OB	occlusion bodies (in reference to NPV PIBs and GV granules)
A	Absorbance
ODV	occlusion derived virion
OpNPV	*Orgyia pseudotsugata* nucleopolyhedrovirus (wherein morphotype is not known)
OpNPVs	*Orgyia pseudotsugata* nucleopolyhedroviruses (OpMNPV and OpSNPV)
OpMNPV	*Orgyia pseudotsugata* multinucleopolyhedrovirus
OpSNPV	*Orgyia pseudotsugata* single nucleopolyhedrovirus
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PBSTM/B	phosphate buffered saline with tween and BSA
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIB	polyhedron inclusion body
RNA	ribonucleic acid
SNPV	single nucleopolyhedrovirus
SPSS	statistical package for the social sciences
TBS	tris buffered saline
TBST	tris buffered saline with tween
TBSTM	tris buffered saline with tween and skim milk powder
TMB	tetramethylbenzidine
TM-Biocontrol-1	Registered product with OpMNPV as the active ingredient

List of Tables

List of Figures

Acknowledgments

This research was financially supported by Natural Resources Canada (Canadian forestry service Science and Technology Program, Canadian forestry service Youth Internship program, Pacific Forestry Centre Graduate Student Award) and the University of Victoria (Graduate Fellowship).

This research could not have begun or have been completed without the support of Dr. Imre Otvos and his support staff at the Pacific Forestry Centre. Most notably, Nicholas Conder was responsible for the design and implementation of the laboratory reared larvae bioassay used for the analysis of the lethal and sublethal OpMNPV infections and comparison of rapid detection methods. Janine Schieller should also be acknowledged for assisting Nicholas in maintenance of this population and maintenance of the field population reared for analyzing the persistence and development of OpNPV infections in field larvae.

I would also like to thank my supervisors David Levin and Imre Otvos for their support and encouragement throughout my Master of Science degree. I also appreciate their extensive support with the writing of scientific reports, papers and manuscripts.

Finally, I would like to thank my families, as without them I would have failed. My lab family (Beatrixe, Carlo, Dan, Dave, Elisa, John, and Simon (my mini-mentor)) encouraged me through debate, discussion, encouragement and humour. My immediate family (Mom, Dad, Sheryl and Michelle) championed me my entire life- and never let me doubt myself for very long. Lastly, my boyfriend Daryl provided daily inspiration, support, advice and balance.

Chapter 1 - General Introduction

1.1 Baculoviruses - an introduction

Viruses are obligate intracellular parasites that depend on their host's cellular machinery to replicate (Cann, 1997). Viruses are basic entities that at their simplest form consist of nucleic acids (RNA or DNA) enveloped by a protein/carbohydrate capsid. The presence of a capsid coat separates these obligate intracellular parasites from viriods.

The *Baculoviridae* is a large family of arthropod viruses that have large (82-180 Kbp), circular, dsDNA genomes (Hayakawa *et al.*, 2000; Lauzon *et al.*, 2004). The *Baculoviridae* consists of two genera: *Nucleopolyhedrovirus* (NPVs) and *Granulovirus* (GVs). NPVs have many virions (enveloped nucleopolyhedroviruses) embedded within crystalline polyhedron occlusion bodies (OBs) (Rohrmann, 1999) and GVs form crystalline ovoid OBs with usually only one virion embedded (Winstanley and O'Reilly, 1999). NPV OBs or polyhedrin inclusion bodies (PIBs) are composed of 95% polyhedrin protein, whereas GV OBs or granules are predominantly composed of granulin protein (Faulkner, 1981). The two genera are also distinguished by their location of replication. NPVs replicate in the nuclei of its host, whereas GVs may replicate in the nuclei and/or the cytoplasm of its host (Granados, 1980).

Whole genome phylogeny has been used to classify baculoviruses into three supergroups, which correspond to their host origin: dipteran NPVs, hymenopteran NPVs and lepidopteran NPVs and GVs (Herniou *et al.,* 2003; Garcia-Maruniak *et al.,* 2004). Within the lepidopteran host supergroup, baculoviruses are assembled into two clusters consisting of 3 groups. Cluster 1 consists of group I and group II NPVs, and cluster 2 consists of GVs. NPVs are also characterized by two strategies for primary viral

infection: SNPV and MNPV (Washburn *et al.*, 2003). Virions are embedded in the proteinaceous matrix as singly enveloped (SNPV) or multiply enveloped (MNPV) nucleocapsids.

Baculoviruses were first reported in Chinese poetry in 1521, which described a baculovirus 'jaundice' disease of the silk worm, *Bombyx mori* (Benz, 1986). Maestri and Cornelia (1856) first correlated the presence of refractive crystals (virus PIBs) with the symptoms of *B. mori* jaundice disease (Benz, 1986). Bergold (1947) was the first person to clearly demonstrate that the jaundice disease of *B. mori* was viral in nature.

Over 600 different baculoviruses have since been identified in insects, and several others have been identified in crustaceans and arachnids (Martignoni and Iwai, 1986a). Within insect hosts, baculoviruses have only been identified in holometabolus insects (insects which undergo complete metamorphosis) (Wood and Granados, 1991). MNPVs are found in the order Lepidoptera, where as SNPVs have been identified in all orders of insects that baculoviruses have been reported to infect including Lepidoptera, Hymenoptera, Diptera, Coleoptera, Thysanura, and Trichoptera (Rohrmann, 1986a; Federici, 1997). GVs are believed to only infect lepidopteran insects (Federici, 1997).

The host range of a given baculovirus is usually very restricted. Miller and Lu (1997) postulated that the *Baculoviridae* host range is significantly limited by the occlusion matrix, since only very alkaline environments may release infective virions from this protective barrier. Host range specificity is also controlled at the genetic level (Theim *et al.,* 1996; Wilson *et al.,* 2005). GVs are the most restricted, although they are also the least studied of the baculoviruses (Evans, 1986). SNPVs are moderately restricted and MNPVs are the broadest (although most are genera-specific) (Evans, 1986). An

exception to this rule is *Autographa californica* MNPV which is pathogenic to at least 32 species in 12 families (Volkman, 1997).

1.2 Lepidopteran insects - an introduction

The first insects appeared in the Devonian period, (410×10^6 years ago). Lepidoptera, (the butterflies and moths), an order of phytophagous insects, first appeared in the Cretaceous period ($65\text{-}144 \times 10^6$ years ago) during the late Mesozoic to Cenozoic eras (Labandeira and Sepkoski, 1993). Their phytophagous feeding pattern has led to their economic importance. Most lepidopteran larvae feed on plant material including agricultural crops and forest stands (Dow, 1986). Insects depend on plant sterols for membrane synthesis (Silberkang *et al.,* 1983), and therefore may be considered obligate parasites of plants. Phytophagous insects, however, only become parasitic *en masse*. In parallel, phytophagous insects only become economically important when their population densities are sufficiently high to reach an economic threshold.

Larvae feed extensively and as a result grow and accumulate biomass. In contrast, adult moths disperse and reproduce. Adult moths may feed on pollen, nectar, fruit juices or nothing at all (Dow, 1986). The labial and mandibular glands produce and excrete digestive enzymes (Dow, 1986), but digestion of food and adsorption of nutrients is mostly confined to the midgut of lepidopteran larvae (Volkman, 1997). Unlike the foregut and hindgut, which are lined by cuticle, the midgut is lined by a periotrophic membrane. The periotrophic membrane is composed of chitin, which directs adsorption of nutrients through pores in this barrier (Barbehenn and Martin, 1995).

Lepidopteran larvae are of particular importance in forest biology because many contribute to forest vigour through natural thinning and biomass recycling. Perhaps even

more important, a small fraction of lepidopteran larvae (1-2%) reach outbreak densities (Mason, 1970). Most of the lepidopteran larvae that reach outbreak densities, exhibit cyclic population dynamics (Myers, 1988) which forest managers can use to predict host populations to prevent economic losses (Mason, 1970). Many mathematical models predict that cyclic population patterns are caused by a time-delayed, density-dependent process (Berryman, 1978). These processes, however, have not been clearly defined. NPV pathogenesis has been postulated to cause the density-dependent time delay required to cause population cycles, but to date this hypothesis has not been confirmed (reviewed in Myers, 1988).

1.3 Baculovirus transmission and lepidopteran infection

Baculoviruses can be isolated from several environmental sources, including live insect larvae or cadavers, plant foliage, insect egg masses, plant debris and soil (Wood and Granados, 1991). OBs (NPV PIBs or GV granules) containing viral progeny can persist in soil and other UV-protected environments for years (Jaques, 1964, 1967; David and Gardiner, 1967; Thomas *et al.*, 1972; Thompson *et al.*, 1981). Insects become exposed to baculovirus infection upon ingestion of biomass contaminated with OBs. This is known as horizontal transmission of baculoviruses (intrageneration transmission), and is widely agreed to be the predominant mode of transmission (Federici, 1997). Horizontal transmission is believed to be the driving force of baculovirus transmission when larval densities are high, and is density-dependent (Anderson and May, 1981). At low larval densities other modes of transmission may be more important. For example, insects may also be exposed to baculovirus infection by vertical transmission (intergeneration transmission) from parent to progeny. The majority of this type of transmission occurs

when emerging larvae consume OBs on the surface of contaminated egg masses (transovum transmission) (Murray and Elkinton, 1989), but a small fraction of larvae may be exposed by internal infection (transovarian transmission) (Longworth and Cunningham, 1968; Hughes *et al.*, 1993, 1997; Burden *et al.*, 2002; 2003). Increasing evidence suggests that some baculovirus infections may be maintained in lepidopteran populations as low-level persistent infections (Hughes, 1997; Burden *et al.,* 2002, 2003; Cooper *et al.,* 2003). As well, insects may be infected upon viral injection by parasitoids (Harper, 1986).

While there are many potential modes of baculovirus transmission, only the infection of Lepidoptera by ingestion of baculovirus OBs has been well studied. Occlusion derived virions (ODVs) are released from OBs upon entry into the midgut of lepidopteran larvae due to alkaline pH (9-11.5) and enzymatic degradation of the proteins (Granados, 1978; Pritchett *et al.*, 1982). After ODVs are released, they pass through pores in the periotrophic membrane (Ferreria *et al.,* 1994; Barbenhenn and Martin, 1995). GVs and NPVs produce viral-encoded 'enhancins' to overcome this barrier (Popham *et al.,* 2001; Lauzon *et al.,* 2004). The presence of *enhancin* gene homologs are correlated with enhanced midgut infection (Derksen and Granados, 1988; Fazairy and Hassan, 1988; Gallo *et al.*, 1991). These viral encoded proteins appear to be ODV-specific (Volkman and Summers, 1977). Similar facilitate the fusion of ODVs into host midgut epithelial cells by mediating their binding to target midgut cells (Uchima *et al.*, 1988).

ODVs infect both mature and differentiating epithelial midgut cells (Engelhard *et al.*, 1994; Flipsen *et al.*, 1995, Washburn *et al.*, 1995) and may do so by a receptor-mediated fusion (Horton and Burand, 1993). Upon fusion of the ODV envelope with epithelial

midgut cells, the virus nucleopolyhedroviruses are released into the cell and move along the microvillus to the host cell nucleus (Federici, 1997). Within the host nucleus, virus genomes are released from their capsid and initiate replication (Granados and Lawler, 1981). Primary replication usually results in the directed production of budded virus, and thus the initiation of secondary or systemic infection (Volkman, 1997).

Budded virus (BV) is specialized for systemic infection of baculoviruses within the hemocoel of lepidopteran insects (Keddie and Volkman, 1985; Volkman, 1986). BV move via the insect tracheal system to reach the hemolymph and cause secondary infections in other tissues (Engelhard *et al.*, 1994). BV enters new cells by endocytosis (Volkman and Goldsmith, 1985; Volkman *et al.,* 1986) which is mediated by low pH and a BV-specific protein, gp64 (Blissard and Wenz, 1992). OBs usually begin to be produced in the nuclei of host cells (NPVs) or in the nuclei and cytoplasm (some GVs) once secondary infection commences.

Baculovirus infection can result in the production of as many as 10^9 OBs/late instar larva prior to host death (Wood and Granados, 1991; Otvos *et al.*, 1999) providing an infective source for susceptible larvae. After 7-14 days of infection, insect tissues are liquefied, the cuticle breaks, and baculovirus OBs are released into the environment. Baculovirus chitinase and cathepsin proteins are implicated in this release (Ohkawa *et al.,* 1994; Slack *et al.,* 1995). Diseased and dead larvae provide an infective inoculum to susceptible uninfected (and infected) larvae within a generation. Diseased and dead larvae also provide an infective source for subsequent generations. The deposition of egg masses onto virus-contaminated surfaces provides the infective inoculum for the primary viral epizootic of future host generations (Murray and Elkinton, 1989, 1990).

1.4 Baculoviruses in Integrated Pest Management programs

Host range specificity has encouraged the use of natural pathogens in Integrated Pest Management (IPM) programs as an alternative to broad range chemical pesticides, such as DDT. Despite this benefit, host range specificity has also contributed to the limited use of baculoviruses in IPM. In North America seven baculoviruses have been registered as viral insecticides (Wood and Granados, 1991). It is estimated that biological control methods contribute to only 1% of the total world pesticide sales (Jutsum, 1988) and only 0.2% of those sales are associated with baculoviruses (Wood and Granados, 1991). Baculoviruses are underutilized because of limited host range, high costs of registration and production, poor stability and slow speed of action, when compared to chemical counterparts (Falcon, 1976; Fuxa, 1987; Payne, 1988: Wood and Granados, 1991). To circumvent the limitations of baculoviruses in IPM programs, research in the 1990s was focused on developing recombinant viruses with greater virulence and broader host range (Wood and Granados, 1991; Black *et al.,* 1997).

1.5 Baculoviruses in Forestry IPM programs

Baculoviruses have been integrated into some IPM programs to control economically important insects (reviewed in Falcon, 1976; Fuxa, 1987; and Moscardi, 1999). There are four major categories of biological control: introduction, inundative, inoculative, and environmental manipulation. Introduction is the classical form of biological control wherein a control organism is introduced into a new environment or ecosystem to control an introduced pest, and its introduction generally results in the permanent suppression of the pest in the new environment (Fuxa, 1987; 1991). *Gilpinia hercyniae* NPV was inadvertently introduced to Canada probably via parasitoids introduced to control the

alien European spruce sawfly, *Gilpinia hercyniae*, resulting in permanent control of this forest pest (Cunningham, 1995). The success of the accidental introduction led to the application of the NPV to sites where it was not initially introduced and also resulted in permanent control (Balch and Bird, 1944; Cunningham, 1995).

Inundative augmentation, in contrast, is intended to provide rapid short-term control of pest populations. One common example used in forestry is the use of *Bacillus thuringiensis* (Fuxa, 1987; Moscardi, 1999). Environmental manipulation is more readily integrated into agricultural IPM programs, but, it has been integrated into experimental forestry practice. For example, experimental fertilization of Ponderosa pine *Pinus ponderosa* (Douglas ex Lawson) forests may have prevented defoliation by the western spruce budworm, *Choristoneura occidentalis* Freeman (Wickman *et al.,* 1988).

The fourth biological control method, inoculative augmentation, requires multiple releases of a native biological control agent. This strategy, when successful, can provide long-term control because the control agent recycles within and between generations until high pest population densities collapse (Fuxa, 1987; Moscardi, 1999). Most baculovirus biocontrol agents fall into the fourth category. The requirements of multiple releases and seasonal recycling highlight the necessity for accurate pathogen monitoring methods.

Inoculative biological control agents must be efficiently transferred between hosts and efficiently replicated for effective control of a pest population (Evans, 1986; Harper, 1987). This observation is evident in the biological control of the western spruce budworm, *C. occidentalis* with CfMNPV. Although this NPV was more virulent than its native pathogen, ChocGV, it did not act as an effective biological control agent because the limited larval feeding period prevented generational transmission (Otvos *et al.,* 1989).

In contrast, success stories utilizing NPVs in forestry are associated with virulent baculoviruses that recycle multiple times within a single host generation (Stelzer, 1977; Otvos *et al.,* 1987; Woods and Elkinton, 1987; Fuxa, 1991; Moreau *et al.,* 2005).

1.6 The Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough)

The Douglas-fir tussock moth (DFTM) is an ecologically important lepidopteran insect because it contributes to forest biomass recycling and natural thinning leading to forest vigour. DFTM are known to consume foliage from true firs *Abies spp.,* (Plin. ex Tourn.) Miller and interior Douglas-firs, *Pseudotsuga menziesii* var *glauca* Franco (Beckwith, 1978a; Shepherd *et al.*, 1984a; Harris *et al.*, 1985). DFTM larvae preferentially consume Douglas-fir foliage in its northernmost range, in the Kamloops-Cache Creek region of British Columbia, and true fir foliage from Washington to its southernmost region of New Mexico. In Idaho, DFTM population outbreaks are associated with sites where competition for moisture and nutrients is high, such as on upper slopes, ridge-tops, and over mature dense forests consisting of a mixture of Douglas-fir and white fir , *A. concolor* Gord. & Glend. (Lindl. ex Hildebr) (Stoszek and Mika, 1978). In contrast, in British Columbia all classes and sizes of Douglas-fir trees are susceptible to defoliation by DFTM larvae, and the most severely attacked trees may die when DFTM populations reach outbreak densities (Otvos *et al.,* 1998). Early instar larvae preferentially consume new growth, and late instar larvae are known to consume both new and old foliage (Beckwith, 1978b). During outbreak stages, severe defoliation results in top kill and some tree mortality in infested stands (Wickman, 1978; Alfaro *et al.,* 1987). Although defoliation by the tussock moth naturally thins stands and increases growth in the

surviving trees, this natural process is often interrupted by man to prevent economic losses.

The biology of the DFTM dictates its limited dispersal and patchy host defoliation patterns. DFTMs are univoltine insects that may only disperse from spring to fall when young larvae or adult male moths have emerged from eggs or cocoons, respectively. Larval eclosion from egg masses occurs from spring to early summer depending on geographical location and correspond with the timing of host tree flush (Wickman and Beckwith, 1978). Larvae normally undergo 5 (male) or 6 (female) instar stages which occur over approximately 6-8 weeks, depending on the environmental conditions (Beckwith, 1978a). First instar larvae have been observed to be wasteful eaters, consuming parts but not all of new growth needles. Later instar larvae can consume old and new foliage and contribute to the most biomass loss during the lifecycle of the tussock moth (Beckwith, 1978b). Adult moths devote their energy for reproduction and do not feed on foliage. Larvae pupate in silken-spun cocoons, and moths emerge in late summer. Female moths have rudimentary wings, and thus cannot fly, and remain on or near their cocoons where ultimately they mate and lay egg masses by August. Male moths, on the other hand, grow fully developed wings but their territory is limited by the female moth. Male moths are attracted by pheromones to return to female moths for late summer reproduction. It is thus believed that tussock moth dispersal is most significant during early instar stages when larvae balloon and can be moved by wind to maximal distances of approximately 300 meters (Wickman and Beckwith, 1978). Spatial limitations set by the biology of the flightless female moth, and early instar feeding

patterns, are believed to contribute to the patchy defoliation patterns observed when tussock moth population densities reach outbreak proportions.

The DFTM has been studied extensively because of the cyclic nature of its outbreaks and the severe defoliation it may cause. Harris and *et al.* (1985) reported that eight DFTM outbreaks occurred in the dry belt region of British Columbia between 1919 and 1985. DFTM outbreaks occurred every 9-13 years and were sustained for approximately four years each time. Since 1985, two additional outbreaks have occurred in this region which required virus treatment (Shepherd and Otvos, 1986; Canadian Forestry Service Report, 2001). In addition, there have been several reports of visible defoliation caused by the DFTM in western United States since 1932 (Stoszek and Mika, 1978; Myers 1988; Shepherd *et al.,* 1989; Mason *et al.,* 1998). Myers (1988) showed that the population patterns of the DFTM were significantly cyclic. She showed that the decline of population outbreaks were significantly correlated to spatially close DFTM population declines; whereas the initiation of outbreaks (or detection of visible defoliation) varied by years. In concordance, Mason (1996) found that DFTM populations from five ecosystems in the United States showed synchronous decline phases.

The cyclic DFTM outbreak population patterns observed over the past 85 years have been fitted into life tables to understand the life cycle of DFTM populations and to thus predict outbreaks (Mason, 1978; Mason and Wickman, 1991, Figure 1.1). The schematic illustration of outbreak populations shows that they are only sustained for 2-4 years before returning to endemic populations (Mason and Luck, 1978). The cause of the

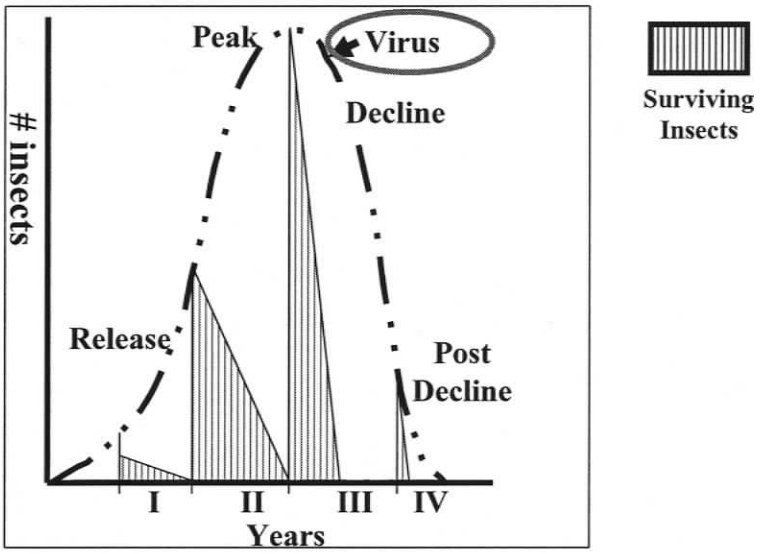


Figure 1.1. Generalized outbreak sequence of the Douglas-fir tussock moth. Schematic diagram of an outbreak sequence with generalized patterns of generation survivorship highlighting the release, peak, decline and post decline phases (modification of Mason and Luck, 1978). Native virus pathogens appear prevalent at the start of the decline phase of the outbreak cycle (Myers, 1988).

cyclic outbreaks is still unknown but one hypothesis is that the DFTM's natural pathogen, *Orgyia pseudotsugata* nucleopolyhedrovirus, may be responsible for periodic cyclic outbreaks (Berryman, 1978). Alternatively, Mason (1998) suggested that it is the biology of DFTM which contributes to a time-delayed negative feedback pattern that result in population cycles.

1.7 Discovery and characterization of Douglas-fir tussock moth viruses, *Orgyia pseudotsugata* nucleopolyhedroviruses

The DFTM viruses, *Orgyia pseudotsugata* nucleopolyhedroviruses (OpNPVs) were first discovered in tussock moths in 1947 from two distinct outbreak sites (Evenden and Jost, 1947; Steinhaus, 1951). At the time of discovery, it was understood that a virus was a major contributor to the collapse of tussock moth outbreak populations. The importance of this natural pathogen, however, did not reach its current magnitude until 1972, when the Environmental Protection Agency (EPA) in the United States banned the use of the chemical pesticide DDT. Since DFTMs were responsible for over 550,797 hectares of defoliation in the Western United States from 1944-1974 (Martignoni, 1999), the ban of DDT resulted in urgent research. The importance of the natural pathogen was amplified when the EPA temporarily raised its ban on DDT in 1974 to control a tussock moth population in Oregon and Washington because of a lack of viable alternatives (Stark, 1978). This controversy coincided with a major increase in the interest and research on alternative control strategies including the DFTM's natural pathogens.

Since its initial discovery, Hughes and Addison (1970) definitively determined that two species of NPV were responsible for the collapse of DFTM outbreaks, a single capsid baculovirus, OpSNPV and a multiple capsid baculovirus, OpMNPV. Transmission

electron microscopic evaluation of the two morphotypes revealed that the viruses differed by the number of nucleocapsids enveloped within the PIBs, and by host tissue location of ODV production. Furthermore, the two pathogens differed by geographical location. OpMNPV was geographically limited to British Columbia and Washington State, whereas OpSNPV was found throughout the geographical range of the host, from British Columbia to New Mexico (Hughes, 1976). Molecular characterization revealed that the polyhedrin protein of the two morphotypes were physically and antigenticly very similar (Rohrmann, 1977). Molecular sequencing revealed that the *polyhedrin* genes showed 76% sequence similarity (Leisy *et al.,* 1986) however whole genome-genome comparison revealed that the two pathogens had low genetic similarity (Rohrmann *et al.,* 1978). Furthermore, the two pathogens were characterized by their virulence to DFTM larvae (dose to mortality). While OpSNPV was more pathogenic at very low doses, infection by OpMNPV resulted in much faster mortality at moderate to high doses (Hughes, 1978). The host ranges of the pathogens were determined to be limited to members of the genus *Orgyia*. Two members of the genus, *O. cana*, the western tussock moth, and *O. antiqua,* the rusty tussock moth, occur within the geographical range of *O. pseudotsugata* (Hughes, 1976). Furthermore, Martignoni and Iwai (1986b) established that OpMNPV could infect and produce viable progeny in *Trichoplusia ni* larvae in a laboratory environment. The discovery and analysis of two pathogens responsible for the collapse of DFTM outbreak populations led to the integration of OpMNPV into the pest management program of the DFTM.

1.8 Integration of OpMNPV into the Integrated Pest Management of the DFTM

Laboratory and field trials of the tussock moth pathogens began soon after the discovery of the role of the pathogens in collapsing tussock moth populations. Successful laboratory studies in 1964 led to two small simulated field studies in 1965 in the United States, utilizing 4x 10^8 OpNPV PIBs/hectare (Tunnock, 1966) and 2 x 10^{10} OpNPV PIBs/hectare (Thompson and Maksymiuk, 1978). The higher dose successfully produced viral epizootics, and the EPA granted an experimental use permit in 1974 to allow researchers to begin field trials of the pathogen on natural outbreak populations (Martignoni, 1999). The first aerial spray trials in the field took place in British Columbia in the mid 1970s utilizing laboratory-propagated OpMNPV (Stelzer *et al.,* 1977). Previous work establishing the virulence of OpMNPV at moderate doses, and speed to mortality influenced the decision to utilize the multicapsid variety. Stelzer's study established that application of the pathogen early in the host's lifecycle resulted in pathogen transmission within a single generation. Furthermore, it has been established that pathogen application is required early in the outbreak cycle to prevent tree mortality caused by severe defoliation (Shepherd *et al.,* 1984b; Otvos *et al.,* 1999).

In 1976, the EPA registered OpMNPV as TM Biocontrol-1 for use in the United States, and by 1983 the same product was registered for use in Canada (Otvos *et al.,* 1999). Also in 1983, OpMNPV grown in the whitemarked tussock moth, *Orgyia leucostigma* was registered in Canada under the name Virtuss®. By 1991, both registered biological control agents were used operationally in Canada which led to aerial spraying of 1260 hectares of at-risk forests in Western Canada from 1991-1993 (Otvos *et al.*, 1999).

1.9 Methods for monitoring pest and pathogen populations

DFTM population models, from life tables to more complex mathematical models, have been constructed to understand its population dynamics and improve the pest management of the DFTM. The most basic (and successful) model used to predict DFTM population outbreaks have come from the development of accurate life tables generated from field monitoring tools. Egg mass densities (Dahlsten and Thomas, 1969; Mason, 1970) and larval densities determined from drop cloth collections (Mason, 1970; Mason and Wickman, 1991) and pheromone traps (Otvos and Shepherd, 1991) have been used to predict future host densities and have contributed to the development of lifetables and outbreak models. Furthermore, virus incidence in collected larvae (or emergent larvae from field-collected egg masses) has been utilized to estimate future pest populations (Stelzer, 1979). These approaches currently guide the pest management decisions of the DFTM (Otvos *et al.,* 1999).

Permanent pheromone monitoring programs have revealed patterns that have been used to predict host population densities. Successive years of upward population trends are better indicators than the average number of caught moths or larvae (Otvos and Shepherd, 1991). Mason and Wickman (1991) found that the survival of larvae from egg hatch to pupation was the best predictor of population trends during non-outbreak populations. Shepherd *et al.,* (1984a) found that fall egg mass density measurement could be used to predict population densities and expected defoliation in the following year, and would also provide the same survival clues during non-outbreak DFTM populations. These observations have been incorporated into lifetables or models for predicting populations (and thus potential biomass loss).

Mathematical models have been developed to assimilate the observations made in the field into predictive models. Two broad classes of mathematical models have been developed in order to predict and understand population dynamics between Lepidoptera and their pathogens (Cory, 1997). "Simple strategic analytical models" are generalized models that are used to ask broad questions about the dynamic between baculovirus pathogens and their hosts, such as the significance of horizontal transmission in epizootics at high and low host densities. In parallel, "complex tactical simulation models" are used to investigate one system in detail and therefore often include the base parameters defined by simple strategic analytical models, as well as ecosystem-specific abiotic and biotic factors. Complex tactical simulation models are required for accurate and detailed predictions for applied research, and with increased awareness of system interactions, the models are transformed and improve in their predictive value.

Several attempts have been made to assimilate the guidelines generated from field monitoring into predictive mathematical models. To date, mathematical models have failed to reproduce DFTM population patterns observed in the field. One exception was a simple strategic model developed by Berryman (1978), which utilized a time-delayed logistic model to generate periodic cycles seen in DFTM populations. By taking advantage of the engineering control theory that predicted that negative feedback time delays initiate cycles, he modeled the cyclic DFTM population density pattern observed in the field (Mason, 1974). The model produced the observed cycles, but did not define the cause of the time-delay (although he speculated viral disease of one generation would impact the population of the next, and thus produce a one year delay). McNamee and others (1981) also concluded that periodic cycles were the result of the interaction

between defoliators and pathogens, although failed to develop a mathematical model to predict outbreak cycles.

Anderson and May (1981) were the first researchers to attempt to model lepidopteran-pathogen population cycles after standard predatory prey models, and simultaneously directed future simple strategic analytical modeling of Lepidoptera. Vezina and Peterman (1985) tested Anderson and May's model (and four variations based on their basic model) to determine if empirical data on DFTM and OpNPV populations could be predicted using their model. The modifications introduced 1) density-dependent mortality, which corrected the basic model for food depletion, and action of predators or parasitoids; 2) an incubation period model, which corrected for the time required for an initial infection to develop into a fatal infection; 3) a vertical transmission model; and 4) a model that incorporated all three modifications to the basic model. They concluded that the observed changes in DFTM populations could not be attributed to OpNPV infections. The simple strategic model of Anderson and May and four variations of this model failed to predict DFTM populations which may suggest that complex tactical simulation models are more appropriate for population predictions of the Douglas fir tussock moth.

Experimental estimation of model parameters is required for accurate population models. For example, Vezina and Peterman (1985) determined a disease-induced mortality rate parameter from empirical observation of the reduction of a DFTM population where virus infection was assumed to cause the collapse (Mason and Thompson, 1971). Dwyer (1991) measured the impact of host density, patchiness of infected individuals, and host stage (instar) on virus transmission. Virus incidence was determined by mortality of the host (Vezina and Peterman, 1985) and supplemented by

microscopic analysis of dead larvae (Dwyer, 1991). To accurately define the parameters for model predictions, the measurement of virus incidence must be accurate.

1.10 Rationale for objectives

We currently lack accurate tools for detecting OpMNPV infection, which negatively impacts the operational pest management of the DFTM. Inaccurate measurement of OpMNPV incidence may also negatively impact parameters which define predictive population models. Presently, the incidence of OpMNPV infection is estimated by collecting egg masses or larvae (Dahlsten and Thomas, 1969; Mason, 1970; Stelzer, 1979; Shepherd and Otvos, 1984). The emergent larvae that die in the first 14 days in rearing are then assayed for infection by microscopic counts of virus PIBs (Stelzer, 1979). When egg masses are collected in the fall, larvae can only emerge after sufficient cold storage replicates overwintering requirements (a minimum of 3 months) (Cameron, 1970). This method is used as a standard technique for confirming and quantifying viral disease, but it is time consuming, labour intensive, non-specific (requires visual interpretation) and expensive. Furthermore, Kaupp and Ebling (1993) estimated that the method is not very sensitive, requiring a minimum of 1 million PIBs/larva for accurate quantification.

The greatest tree mortality due to DFTM occurs during the first year of severe defoliation, therefore it is important to utilize sensitive and accurate pathogen monitoring tools to accurately plan for forest protection when pathogen concentrations are below epizootic levels. The development of an alternative strategy for determining virus incidence that is specific, sensitive and appropriate for large field surveys would be advantageous for accurate assessment of disease dynamics. Refined diagnostic

techniques will allow us to accurately assess virus incidence, and therefore improve our understanding of the OpNPV-DFTM population dynamic.

Moreover, there is a distinct gap in our current knowledge of the effect of sublethal OpNPV infections. Although the pathogen was the first baculovirus registered to control a North American lepidopteran forest pest, its sublethal effects have not been studied. Sublethal infections are hypothesized to play an important role the transmission of pathogens when host densities are low (Anderson and May, 1981). Furthermore, sublethal infections may explain how pathogens persist when host populations decline. Sublethal OpNPV infections may also explain how natural populations of the pathogen explode at sites to collapse host populations where they were previously not detectable or infrequently detected. Thus, the development of a specific, sensitive and appropriate detection system for large field surveys of virus incidence may also provide the means to elucidate whether or not sublethal OpNPV infections are present in natural and manipulated DFTM populations.

1.11 Objectives of thesis

The primary objective of this research was to develop and validate a simple, sensitive and specific antibody-based detection system for OpNPVs. This would include a laboratory-based screening system and a novel on-site field detection system. The secondary objective of this research was to use these detection systems determine if OpMNPV infection of DFTM can result in sublethal effects to DFTM larvae.

Chapter 2 - Immunodiagnosis I: The development and analysis of an ELISA system to detect Orgyia pseudotsugata nucleopolyhedroviruses

2.1 Abstract

An antibody-based detection system was developed to identify and quantify a viral pathogen responsible for causing the collapse of Douglas-fir tussock moth outbreak populations. Monoclonal antibodies were raised against OpMNPV proteins, and incorporated into an ELISA format for viral detection. A significant linear relationship between OpMNPV polyhedron inclusion body (PIB) proteins and absorbance was observed (R^2=0.99). The ELISA method is sensitive to a minimum of 835 OpMNPV PIBs or 7.8 ng of virus protein. The ELISA method is specific to OpNPV proteins, and produces more than twice the absorbance to OpMNPV variants than the single-occluded variants, OpSNPV. The ELISA method detected all identified genotypic variants of the multiple-occluded variety, OpMNPV. The immunodiagnostic tool has the potential to improve the biological control of the DFTM because it allows timely control decisions and provides a method to accurately quantify OpMNPV infection. The ELISA method also has the potential to improve predictive models due to its sensitivity and specificity.

2.2 Introduction

Theoretical and empirical understanding of virus-insect population dynamics are used to predict pest population densities (Anderson and May, 1981). Predicted pest populations may also be correlated with predicted defoliation patterns. Natural epizootics of viral disease have resulted in the collapse of outbreak populations of forest pests including the nun moth, *Lymantria monacha* (Linnaeus) in Europe, and the Douglas-fir tussock moth (DFTM), *Orgyia pseudotsugata* (McDunnough) in North America, (reviewed in Martignoni, 1999). This relationship has been augmented in many pest management programs to prevent resource loss (Reviewed in Fuxa, 1987 and Moscardi, 1999).

DFTM populations are regulated by its native pathogens, *Orgyia pseudotsugata* multinucleopolyhedrovirus (OpMNPV) and *Orgyia pseudotsugata* single nucleopolyhedrovirus (OpSNPV) (Hughes, 1976). OpMNPV is highly pathogenic and is associated with the collapse of DFTM outbreaks. The incidence of OpMNPV is currently used as one measure to predict future DFTM population densities and anticipated levels of defoliation (Otvos *et al.,* 1999). At the beginning of DFTM outbreaks, when natural disease incidence is low (less than 15%) (Stelzer, 1979), supplementary virus load is applied as a biological control agent (Shepherd *et al.*, 1984b; Otvos and Shepherd, 1991). In contrast, when disease incidence is high (greater than 25%) (Stelzer, 1979), forest managers can allow the natural OpMNPV population to reduce DFTM population densities to levels below economic importance.

Predictive population models used in many pest management programs require accurate measurement of pathogen incidence. Anderson and May (1981) recognized that

due to the pathogenic nature of many lepidopteran diseases, host populations could be predicted using standard predator-prey models. DFTM populations have been fitted into several models to allow for predictions of their populations, and predictions of resource loss (Berryman, 1978; McNamee *et al.*, 1981; Vezina and Peterman, 1985; Dwyer, 1991). These studies did not result in a clear picture of the impact of OpNPV on DFTM populations due in part to the different models used in the analysis, and the various methods used to define parameters of their models. Their contradictory results highlight the necessity of accurate measurement tools to produce the most accurate population models.

We currently lack accurate tools for detecting OpNPV infection (Section 1.10). The development of an alternative strategy for determining virus incidence that is specific, sensitive and appropriate for large field surveys would be advantageous for accurate assessment of disease dynamics. Refined diagnostic techniques will allow us to accurately assess virus incidence, and therefore may aid in our understanding of the OpNPV-DFTM population dynamic.

An antibody-based detection system may be an excellent alternative detection system to microscopic counts of OpNPV PIBs. Monoclonal antibodies by definition show affinity to a single epitope. Through a strict selection process, antibodies can be identified that are highly specific and sensitive to the test subject. If antibodies are incorporated into a plate system, such as those used in an Enzyme-linked immunosorbent assay (ELISA) for high-throughput detection, one can take advantage of the sensitivity and specificity of antibodies, and take advantage of the plate system for analysis of 96

different samples on a single assay plate. Furthermore, the ELISA method can be utilized to quantify the infection in test subjects (Clark and Barbara, 1987; Parola *et al.,* 2003).

ELISA systems have been utilized for the quantification of baculoviruses in cell culture (Langridge *et al.,* 1981) and infected insect larvae (Longworth and Carey, 1980; Langridge *et al.,* 1981; Shamim *et al.,* 1994; Stark *et al.,* 1999; Parola *et al.,* 2003). Crook and Payne (1980) determined that the indirect ELISA method was the most sensitive version for the identification and quantification of baculovirus proteins. An ELISA-based detection system may provide the sensitivity and specificity required to accurately assess virus incidence and virus quantity, and thus estimate and predict pest population potential (*i.e.* the amount of defoliation).

The purposes of this study were to 1) develop an ELISA-based method for the detection and quantification of OpMNPV, and 2) to evaluate the detection system by determining its sensitivity and specificity to various baculoviruses.

2.3 Methods and Materials

2.3.1 Extraction of baculovirus polyhedral inclusion bodies from insect cadavers

Insect larval cadavers containing baculovirus infections (OpMNPV genotypic variants, including the variant used in the registered biocontrol agent, TM-Biocontrol-1, OpSNPV genotypic variants, *Orgyia antiqua* NPV (OaNPV), *Choristoneura fumiferana* MNPV (CfMNPV), *Lambdina fiscellaria fiscellaria* NPV (LffNPV), three variants of *Lymantria dispar* MNPV (LdMNPV) and *Neodiprion abietis* NPV (NeabNPV) were received from Dr. Imre S. Otvos, Natural Resources Canada, Canadian Forestry Service, Pacific Forestry Centre. Baculovirus PIBs were extracted from insect cadavers as described in Martignoni *et al.,* (1968). Larvae were macerated with sterile mortar and pestle in 500

µl/larva STE-C buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 10 mM cysteine) supplemented with 0.1% SDS. Baculovirus PIBs were separated from insect debris by centrifugation at 145 ×g, 4°C, four minutes. Supernatant was aspirated from insect pellet. Supernatant was washed 2x with 200 µl STE-C buffer and centrifugation of insect debris. Baculovirus PIBs were pelleted by centrifugation at 15,000 xg for 15 minutes at 4°C. Baculovirus PIB pellet was washed three additional times with one ml of STE-C buffer each and pelleted as described. Extracted PIBs were stored at -20°C for analysis. Semi-purified PIBs were assayed for protein concentration using the Lowry method (RC-DC BioRad Protein Assay, BioRad Corporation). OpMNPV PIBs were counted under the 40X objective of a compound microscope using a haemocytometer to correlate protein concentration and PIB number.

2.3.2 Production of monoclonal antibodies (ImmunoPrecise Antibodies)

Semi-purified OpMNPV PIBs (TM-Biocontrol-1 variant) (Section 2.3.1) were used as immunogens against four BALB/c mice. Initially, individual mice were immunized with 50µg of PIB antigen, diluted in Complete Freund's Adjuvant. Subsequently, the mice were similarily immunized with four additional doses of 50µg of PIB antigen in complete Freund's adjuvant over three weeks. Mice were bled to test serum on semi-purified PIB antigens using the ELISA method. Two mice producing the strongest response to OpMNPV antigen in ELISA format were further immunized with 10µg of OpMNPV antigen diluted in phosphate buffered saline, pH 7.4, (137 mM NaCl, 2.7 mM KH_2PO_4, 2.7 mM KCl, 2.7mM $Na_2HPO_4.7H_2O$, pH 7.4), (PBS). Mouse antibody-secreting cells were fused to engineered transformed cells (SP2/0 parental myeloma cells) to produce hybridoma cell lines as described by Kohler and Milstein (1975). The hybridoma cell

lines were amplified in tissue culture format in Clonacell-HY medium (Stemcell Technologies Inc.). Clones were picked 11 days post-fusion, and resuspended in 200 µl of enhanced D-MEM medium (Invitrogen) containing 1% hypoxanthine/thymidine, 20% fetal bovine serum, 2 mM GlutaMax I, 1 mM sodium pyruvate, 50 µg/ml gentamycin, 1% OPI and 0.6 ng/ml IL-6.

2.3.3 Selection and purification of monoclonal antibodies.

Four days after inoculation in enhanced D-MEM media, the supernatants were screened by ELISA for antibody activity on plates coated with 2 µg/well of semi-purified OpMNPV PIBs. Active sera (12 clones) were further analyzed for sensitivity to limiting dilutions of OpMNPV antigen, and insensitivity to tussock moth antigen. Monoclonal antibodies were purified by polyethylene glycol and resuspended in PBS- 1% BSA-0.02% sodium azide.

2.3.4 Enzyme linked immunosorbent assay (ELISA) method

Monoclonal antibodies were selected for sensitivity and specificity to OpMNPV PIBs using an indirect ELISA. Semi-purified virus antigens (1000 ng/well for specificity assays; 1000-1 ng/well for sensitivity assays) were applied to polystyrene plates in 100 µl of PBS, and incubated 18 hours at 4°C. Unbound sites were blocked with PBS-3% BSA for 30 minutes at 37°C. PEG-purified monoclonal antibodies were diluted optimally to 1/1000 in PBS-0.1% Tween-20 -0.05% BSA (PBSTB) and incubated one hour, with shaking at 37°C. Secondary antibodies, goat-anti-mouse IgG (Fc portion) horseradish peroxidase (Pierce), were diluted optimally (1/20,000) in PBSTB and incubated as above. Plates were washed 5X over 30 minutes with PBS-0.1% tween-20 after each antibody step, and washed 2X over 5 minutes after antigen and blocking incubations. Antibodies

were visualized with tetramethylbenzidine buffer (BioFX) at 50 µl/well and incubated up to one hour in the dark. Absorbance at 650nm was measured with a MicroTek plate reader.

2.4 Results

2.4.1 Sensitivity of the ELISA method

Optimal antibody concentrations were determined by titration (data not shown). We determined the relationship between protein concentration and number of PIBs by microscopic counts of OpMNPV PIBs and Lowry assay. We estimated that one ng of semi-purified OpMNPV protein corresponds to 107 +/- 4.7 PIBs (Table 2.1). We found that the ELISA system could detect as few as 835 PIBs or 7.8 µg of protein (Figure 2.1). The criterion for positive identification of OpMNPV PIBs was based upon values greater than two standard deviations above the mean absorbance value of negative control antigen (Parola *et al.,* 2003) established from uninfected DFTM larvae (n=8).

2.4.2 Quantification of OpMNPV PIB protein

A significant linear relationship (R^2=0.96) was established between absorbance values and semi-purified OpMNPV proteins between one ng and one µg (Figure 2.2.a). The strength of the linear relationship increased between one and 250 ng of OpMNPV protein (R^2=0.99) (Figure 2.2.b). Thus, we determined that samples must be diluted to this limited range for accurate quantification of unknown samples.

Table 2.1. Microscopic estimation of OpMNPV PIB number

Replicate	**1**	**2**	**3**	**4**	**5**	**6**
			(# PIBs/square)			
	34	49	39	43	50	51
	38	45	36	39	44	44
	46	36	41	40	44	39
	39	46	40	38	45	51
	44	43	39	51	41	46
Σ	**201**	**219**	**195**	**211**	**224**	**231**
SD	4.8	4.9	1.9	5.3	3.3	5.1

Σ [a]	**SD**	**mm^{2} [b]**	**mm^{3}**	**PIBs/ml**	**PIBs/ml Stock [c]**	**PIBs/ng**
213.5	**4.7**	1.07 x10^{3}	1.07x 10^{4}	1.07x 10^{7}	**1.07x 10^{9}**	**107**

[a] Macroscopic estimation of PIB number was made from 1:100 dilutions of extracted OpMNPV PIBs

[b] One µl of test solution displaces 25 squares or one mm^{2} on a haemocytometer grid, therefore the average sum of PIB number for five squares was multiplied by five.

[c] TM-Biocontrol-1 stock solution (containing OpMNPV PIBs) was estimated to be 10 mg/ml using bovine serum albumin as a standard by Lowry Assay (BioRad).

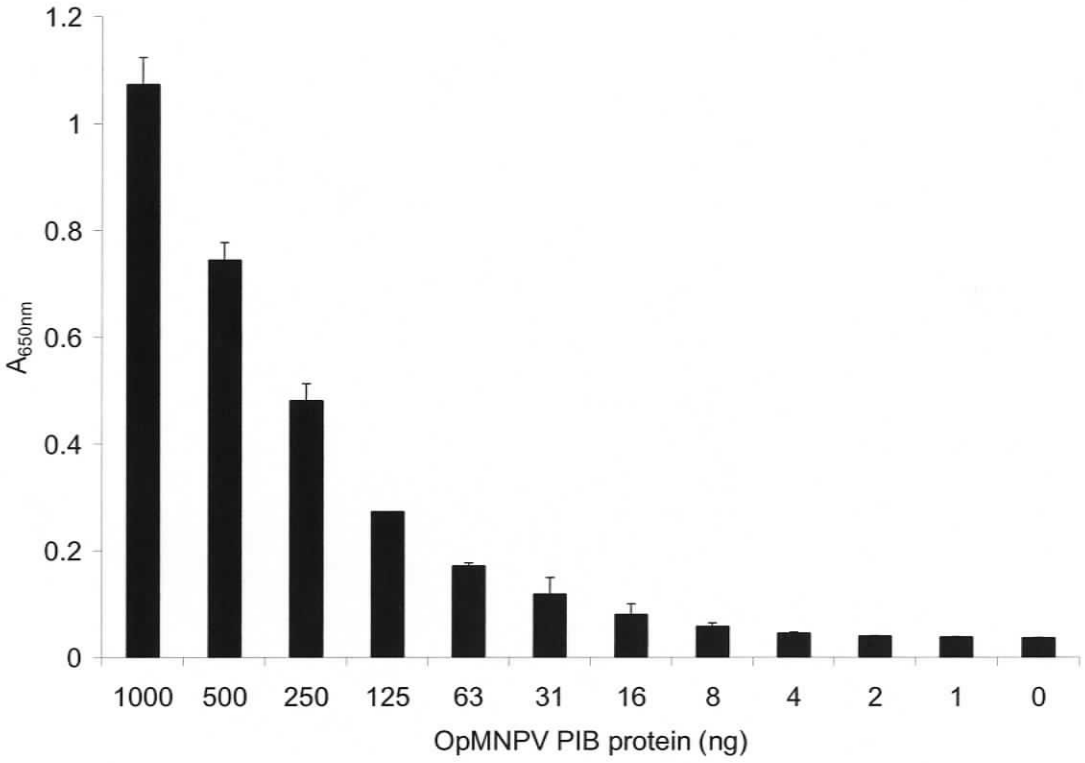
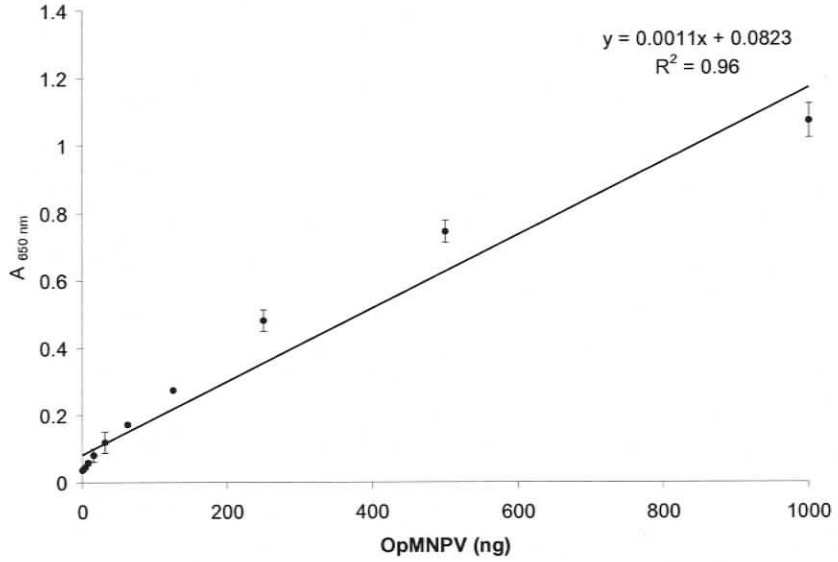


Figure 2.1. Sensitivity of the ELISA method to semi-purified OpMNPV PIBs. OpMNPV protein was detected in an indirect ELISA as described (section 2.3.4). Bars represent the standard deviation around the mean absorbance (n=4). The A650 to serial dilutions of semi-purified OpMNPV PIB protein was measured with a MicroTek plate reader. The limits of sensitivity were defined as two standard deviations above the mean absorbance of uninfected DFTM larvae (n=8).

(a)



(b)

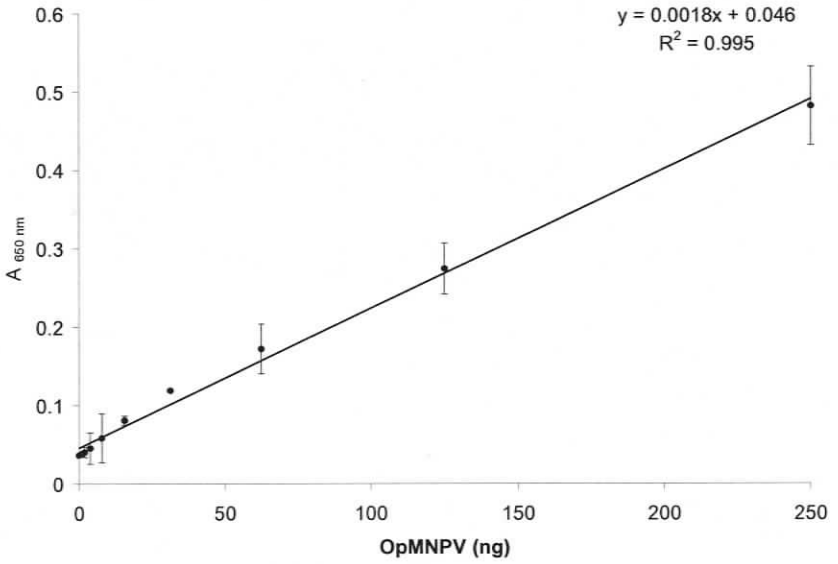


Figure 2.2. The linear relationship between absorbance and OpMNPV PIB proteins. OpMNPV was detected using an OpNPV-specific antibody in an indirect ELISA as described (Section 2.3.4). Bars represent the standard deviation around the mean absorbance, (n=4). (a) Linear relationship between A650 and 1000-one ng of semi-purified extracted protein. (b) Linear relationship between A650 and 250-one ng of extracted protein.

2.4.3 Selectivity to wild isolates of OpMNPV

We tested optimally diluted antibodies (determined by titration, data not shown) against one μg of extracted virus PIB protein to determine if the ELISA system could distinguish between field isolated genotypic variants of OpMNPV. We found that the antibodies raised against OpMNPV PIB proteins recognized all tested OpMNPV genotypic variants (Figure 2.3).

2.4.4 Specificity of the ELISA system

To determine the specificity of the monoclonal antibody raised against OpMNPV PIB proteins, we assayed semi-purified OpSNPV, OaNPV, CfMNPV, LffNPV, three variants of LdMNPV, and NeabNPV PIB proteins. We found a significant cross-reaction with OpSNPV PIBs, but the antibody did not cross-react significantly (Section 2.4.1) with other baculoviruses tested (Figure 2.4).

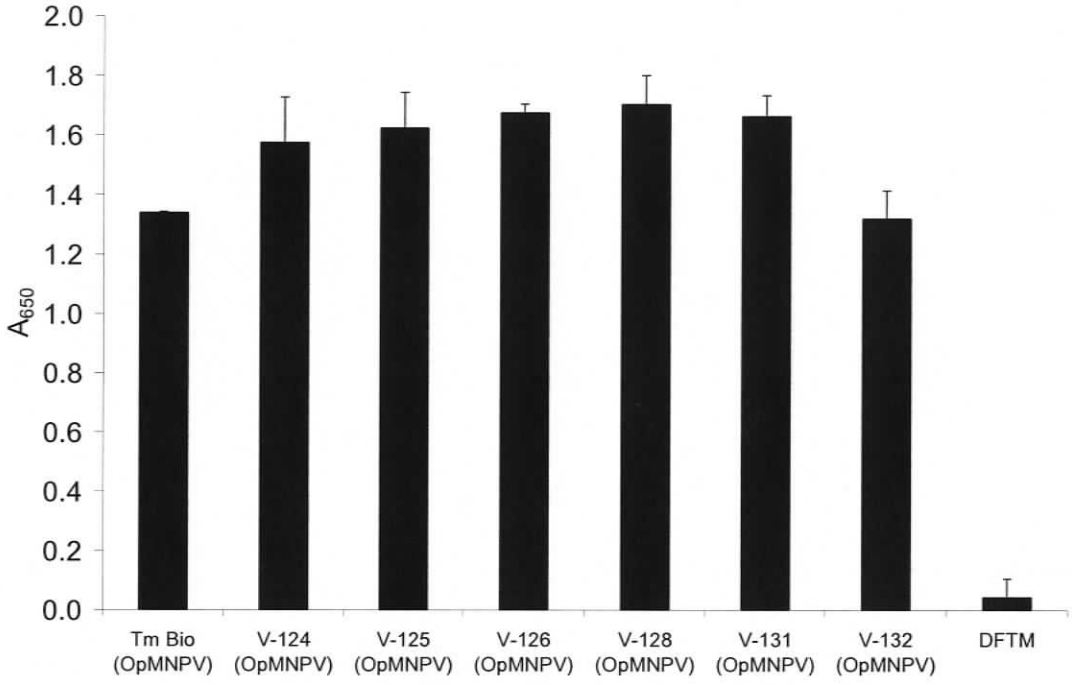


Figure 2.3. Selectivity of ELISA to field isolated OpMNPV genotypic variants Genotypic variants were isolated from British Columbia and OpMNPV variants in preparations of TM Biocontrol-1. TM Biocontrol-1 is a registered biological control agent containing multiple genotypic variants of OpMNPV. Genotypic variants V-124, V-126 and V-131 were isolated from Indian Gardens East, V-125 from Munroe, V-128 from Brussell Creek., and V-132 from Heffley Creek. OpMNPV PIBs were detected using an OpNPV-specific monoclonal antibody in an indirect ELISA (Section 2.3.4). Bars represent the standard deviation around the mean absorbance (n=8).

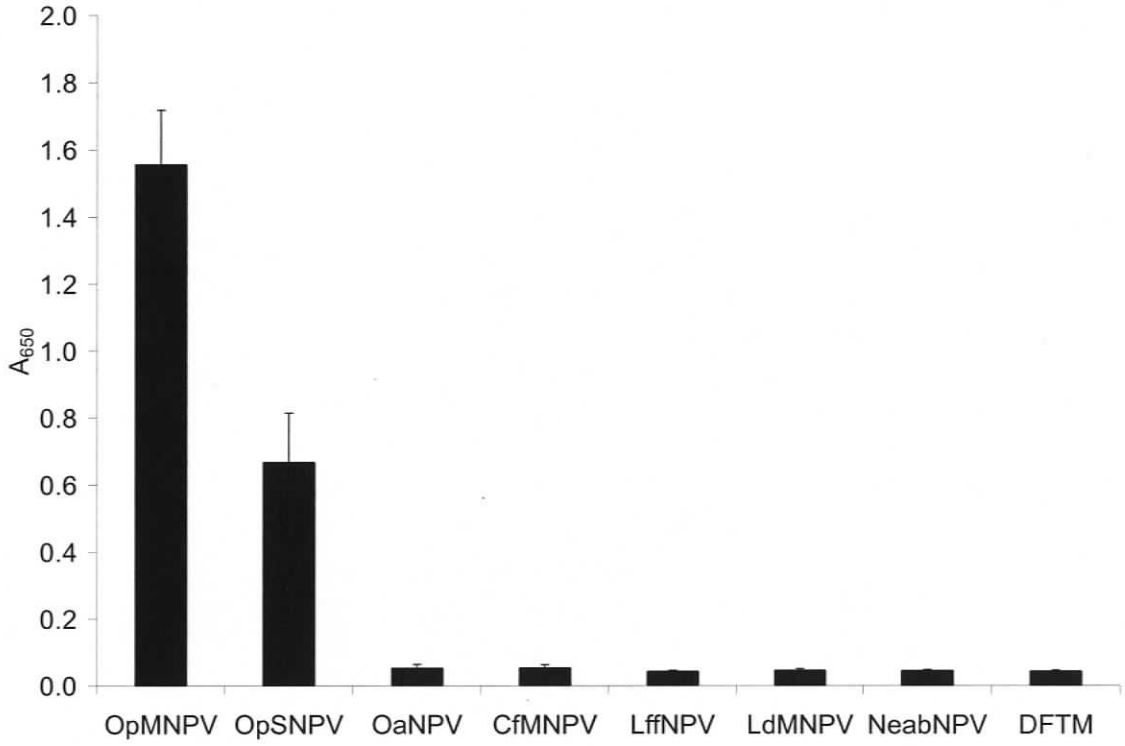


Figure 2.4. Specificity of the indirect ELISA to OpNPVs

Baculovirus antigens were extracted and semi purified by centrifugation from insect cadavers and quantified by Lowry Assay. One µg of the semi-purified protein was applied, and virus was detected in an indirect ELISA (Section 2.3.4). The A650 for OpMNPV antigen was established from the average of eight replicates of nine different genotypic variants including OpMNPV present in the biological control agent, TM Biocontrol-1. The A650 for OpSNPV was established from the average of eight replicates of two genotypic variants. The A650 for LdMNPV was established from the average of eight replicates of three genotypic variants. The absorbance values for the remaining baculoviruses were established from the average of eight replicates of one genotypic variant. Bars represent the standard deviation around the mean OD.

2.5 Discussion

Accurate measurement of baculovirus incidence and baculovirus quantity are essential for our understanding of their impact on insect populations. In pest management programs that utilize baculoviruses as control agents, the incidence of the naturally occurring virus is measured to determine if supplementary virus is required to prevent an emergent outbreak. Furthermore, predictive pest population models may depend on accurate measurement of virus incidence to define the effect of viral disease on pest populations. Predictive population models also depend on accurate measurement of virus incidence (and virus quantity) in the measurement of population model parameters such as transmission rate. Finally, accurate measurement of baculovirus incidence and quantity may improve quality control during production of baculovirus pesticides (Parola *et al.,* 2003).

The ELISA system in this study is sensitive to a minimum of 835 PIBs or 7.8 ng of virus protein based upon a minimum criterion of 2 standard deviations above the average absorbance of uninfected DFTM larvae. This standard is more conservative than previously defined (Parola *et al.,* 2003). For example, when using BSA as a negative control standard, the ELISA system detects as few as 1.95 ng of OpMNPV protein, or 209 PIBs. Due to the likelihood that insect proteins may contaminate extracted samples and that it would be advantageous to detect virus directly from macerated larvae, we feel that the conservative criterion will result in the most accurate evaluation of virus quantity. The sensitivity of the ELISA system described here is within range (100-2000 OBs or 0.53-30 ng/ml) of previous reports (Kelly *et al.,* 1978; Longworth and Carey, 1980; Crook and Payne, 1980; Shamim *et al.,* 1994; Stark *et al.,* 1999; Parola *et al.,* 2003).

The ELISA system is more sensitive than the current standard (microscopic analysis) (Kaupp and Ebling, 1993) used in the operational management system for controlling DFTM outbreaks (Otvos *et al.*, 1999). The use of the ELISA system in place of microscopic analysis will result in more sensitive analysis of dose-mortality experiments, and should result in more accurate understanding of the implication of virus incidence on insect populations, since previous studies depended on mortality and microscopic counts to measure virus pathogenicity (Stelzer, 1979; Dwyer, 1991; Martignoni, 1999). The sensitivity of the ELISA system may allow more accurate understanding of the effect of virus infections on DFTM larvae.

The ELISA method is also effective at accurately quantifying virus particles. Previous studies have demonstrated that the ELISA method can be used to accurately quantify baculovirus OBs (Clark and Barbara, 1987; Parola *et al.,* 2003). In this paper OpMNPV PIBs were accurately quantified through a linear regression between absorbance and OpMNPV PIB extracts. The demonstrated accuracy of the method may be useful for the quality control during the production of baculoviruses as biocontrol agents (Parola *et al.,* 2003) and estimating virus quantity for bioassays.

The ELISA system described here is specific to OpNPV. We found that the antibodies that were raised against OpMNPV proteins cross-reacted with the single-occluded variety, OpSNPV. This result is curious because Rohrmann *et al.* (1978) found that OpMNPV and OpSNPV share little sequence homology based upon restriction fragment map polymorphisms and southern hybridization. The ELISA method did not cross-react with any of the other baculoviruses tested including a baculovirus within the same host genus, OaNPV, and a baculovirus with the highest known genotypic similarity, CfMNPV

(Ahrens *et al.,* 1997; Herniou, 2001). Furthermore, we tested genotypic variants of OpMNPV identified in B.C. (Laitinen *et al.*, 1996; Williams and Otvos, 2005) and found that the antibody recognized all genotypes. This result is advantageous to our goal to use the ELISA system to accurately assess OpMNPV in field populations of the DFTM. The accuracy of the method ensures that the ELISA system will detect and quantify all known field variants of OpMNPV and OpSNPV, which are pathogenic to the DFTM (Hughes and Addison, 1970).

The ELISA system may therefore be the preferred method for quantifying virus infections because it is specific to OpNPVs, and is appropriate for large field surveys. The specificity of the assay ensures that the test cannot overestimate virus infection, unlike microscopic analysis, which requires visual interpretation of virus bodies from other round bodies such as urea crystals. The ELISA system was proven to be amendable to quantification of virus infection, like microscopic analysis. In contrast to microscopic analysis, the ELISA method also allows quick quantification of several hundred samples within a single day. The sensitivity, specificity and efficacy in large analyses suggest that the ELISA system would provide an accurate assessment of virus incidence and virus quantity. Our results support the integration of the ELISA system into the integrated pest management of the DFTM. The accurate measurement of virus infection may in turn increase our understanding of virus pathogenicity on DFTM populations.

Chapter 3 - The development and comparison of rapid methods to detect baculoviruses within their insect hosts.

3.1 Abstract

Knowledge of the natural incidence of *Orgyia pseudotsugata* multinucleopolyhedrovirus (OpMNPV) is required for pest management decisions for the Douglas-fir tussock moth (DFTM). Three OpMNPV detection methods were compared. A modified two-step baculovirus DNA extraction protocol which included PCR confirmation of virus infection was developed for rapid detection of OpMNPV. A rapid hybridization protocol was developed which required maceration of test insects and application to a solid phase support, followed by hybridization of an OpMNPV-specific probe. An ELISA method using OpNPV-specific antibodies was developed previously (Chapter 2). Southern hybridization was the least sensitive method with detection limits of approximately 6000 OpMNPV PIBs and the rapid PCR method was the most sensitive method with detection limits of approximately 8 PIBs. Direct detection of OpMNPV infections within infected DFTM hosts by dot blot analysis resulted in 31% false negatives, whereas the PCR method resulted in 2.5% false negatives. In contrast, the ELISA method did not produce false negatives, although it had a detection limit of 854 PIBs. We found that the rapid PCR and ELISA methods are advantageous for the identification of OpMNPV in infected hosts because of specificity, sensitivity, fast analysis and infrequent false negative results. The ELISA method may be preferable in some applications because virus titers can be quantified (R^2= 0.99) and because the method did not produce false negative results.

3.2 Introduction

The incidence of pathogenic microorganisms can be used to predict host populations because infection usually results in host death and reduced host population densities (Stairs, 1972; Anderson and May, 1981). Baculoviruses are entomopathogens that play an important role in controlling many insect pest populations (Myers, 1988). Baculoviruses appear to be most prevalent after pest populations reach outbreak proportions, and especially during the declining phase of the outbreak cycle (Tanada and Fuxa, 1987). The correlation between baculovirus incidence and pest population is consistent for some host-pathogen interactions therefore one may monitor pathogen populations to predict host populations. Thus, Pest Management decisions are directed by monitoring both host and pathogen populations.

Accurate monitoring methods are required for appropriate pest management decisions (Section 1.10), yet the current detection methods used in the operational pest management of the Douglas-fir tussock moth (DFTM) are not highly specific, sensitive, or accurate (Sections 1.9, 1.10). Pathogen monitoring tools must be sensitive enough to detect pathogens at early development of disease when pathogen concentrations are low (prior to extensive replication). It is also important that the detection methods produce fast results for timely management decisions because of the speed of defoliation that occurs during outbreak populations and the incubation time of disease required to cause the collapse of an outbreak population. Furthermore, to ensure fast (but accurate) results, the detection methods should be amendable for large scale analysis. To address the limitations of the current detection systems and to fulfill the requirements for accurate

monitoring of cyclic pathogens, three virus detection systems were developed and compared including PCR, Southern hybridization and ELISA.

Polymerase chain reaction (PCR) is an excellent candidate to enhance the pest management program for the DFTM because it is extremely sensitive, and through careful design, can also be highly specific. Several studies have utilized the sensitivity of PCR to show that viral pathogens may be maintained in host populations as persistent infections (Hughes, 1993, 1997; Fuxa *et al.,* 2002; Burden *et al.,* 2002, 2003; Cooper, 2003). Modifications to PCR protocols have been developed to improve extraction of microbial DNA from environmental samples (Tebbe and Vahjen, 1993; Straub *et al.,* 1994; de Moraes *et al.,* 1999; Ebling and Holmes, 2002). PCR, however, may not be appropriate for large field analyses because the procedure is time consuming and requires expensive laboratory equipment.

Hybridization is also an excellent candidate method to improve the pest management program of the DFTM because it is amendable for large field screening projects that may be required to correctly assess pathogen incidence in pre-outbreak DFTM field populations. Hybridization has been used previously to effectively identify other lepidopteran pathogens (Ward *et al.,* 1987; Kaupp and Ebling, 1993; Kukan and Myers, 1995; Ebling *et al.,* 2001). These studies have demonstrated that dot blot methods are more sensitive than standard microscopic analysis of infected larvae, and are useful for large population analyses. Hybridization methods, however, are limited by sensitivity, when compared to PCR and ELISA methods.

Enzyme-linked immunosorbent assay (ELISA) is a third candidate method for detecting viral pathogens because it is sensitive, can be designed to be specific, and is

appropriate for large screening assays. Previously, we developed a sensitive, specific and accurate ELISA method for quantifying *Orgyia pseudotsugata* nucleopolyhedrovirus (OpNPV) PIBs. An ELISA system may also be used to identify viral infection from crude extracts. Mowat and Dawson (1987) demonstrated that an ELISA method was effective at identifying plant viruses from crude extracts of plant material. The ELISA method however may not lend well to accurate quantification of baculoviruses from crude samples (Clark and Barbara, 1987). ELISA methods, may also be limited because of sensitivity (when compared to PCR) and because of the requirements of expensive equipment for accurate quantification of the test analyte.

The objectives of this chapter were to 1) compare PCR, Southern hybridization, and ELISA for the identification of OpNPV infection in the DFTM, and 2) evaluate the methods based on sensitivity, specificity, speed of assay, and efficacy in large field surveys.

3.3 Methods and Materials

3.3.1 Sensitivity to semi-purified OpMNPV PIBs

OpMNPV PIBs (TM-Biocontrol-1 strain) were extracted from DFTM larvae cadavers as described (Section 2.3.1) and were used to determine the limits of sensitivity of PCR, Southern dot blot and ELISA to semi-purified sources of virus.

3.3.1.1 Sensitivity of PCR

Virus PIBs (3.06×10^7 PIBs) were lysed in an alkaline lysis buffer (0.02 M Na_2CO_3, pH 11, final concentration) to release virions. Lysis was encouraged for 20 minutes at 25°C with gentle mixing. The solution was neutralized with of 0.16 M NaAcetate, pH 5.0 (final concentration). Enveloped virions were lysed by heating for 10 minutes at

94°C (incorporated into the PCR). The presence of virus was confirmed with baculovirus-specific primers specific to a 750 bp region of the *polyhedrin* gene (Table 4.1). Lysed solution (306,000-7.65 PIBs /reaction) were added to a PCR master mix (10 mM Tris HCl, pH 9.0, 60 mM KCl, 2.5 mM $MgCl_2$, 200 µM each dNTP, 1 mM each primer, 0.045 % Triton-X, 1.45 % Tween 20, 1 mg/ml BSA, 1 U Taq polymerase). The PCR reactions were subjected to 94°C for 10 minutes, 30 cycles of (94°C, 30 s; 65°C, 45s; 72°C, 2 minutes), 72°C for 7 minutes, and held at 4°C. PCR-amplified products were separated on 1% agarose-TBE gels (Sambrook and Russell, 2001).

3.3.1.2 Sensitivity of Southern dot blot

OpMNPV DNA was released from PIBs by alkaline and boiling lysis as described above with one modification. After enveloped virions were released and pH was neutralized, viral DNA was released by heat lysis for 10 minutes at 94°C. Serial dilutions of the lysate (7.65×10^5-5.98×10^3 PIBs) were denatured and filtered to nitrocellulose (HydroBond +) membrane by vacuum (BioDot microfilter, BioRad Corporation) following a standard protocol (Sambrook and Russell, 2001). Unbound sites were blocked with 10 µg/ml herring sperm.

Production of a radioactive probe: OpMNPV DNA was isolated from OpMNPV infected DFTM cadavers following a modified procedure of Martignoni *et al.* (1968). Larvae were macerated by mortar and pestle as described (Section 2.3.1) however STE-C, 0.1% SDS buffer was supplemented with 0.6 µg/ml DNase I (Invitrogen) for extraction of baculovirus PIBs from insect tissues. The mixture was incubated at room temperature (21°C) for 20 minutes with gentle shaking. Insect debris was removed by

Table 3.1. Oligonucleotide sequence of *polyhedrin*-specific primers

	Sequence [1]
polHF	5' TCG ATT TAA TAC GCC GGG CCG 3'
polHR	5' TGC CAG ATT ACT CGT ACC GGC CG 3'

[1] Primers were targeted to a 750 bp region of the *polyhedrin* gene that showed insignificant levels self-complimentarity and primer dimer, but optimal melting temperatures for PCR, using OLIGO software.

centrifugation at 145 ×g, 4° C for four minutes. Supernatant was washed with STE-C buffer, and virus PIBs were separated by centrifugation at 15,000 ×g, 4°C for 15 minutes. PIB pellets were washed three additional times with STE-C buffer. Baculovirus virions were released from their polyhedron matrix by alkaline lysis. Baculovirus PIBs were resuspended in an alkaline buffer (0.1 M Na_2CO_3) and incubated for 20 minutes with gentle agitation to release enveloped virions. Enveloped virions were disrupted by incubation in TES buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% SDS) supplemented with 0.2 mg/ml proteinase K (Invitrogen). Virions were incubated overnight at 55°C. DNA was isolated from contaminating proteins by phenol:chloroform extraction and DNA was purified by glassmilk (DNA extraction kit K0513, Fermentas). A 750 bp amplicon of OpMNPV's *polyhedrin* gene was produced following the PCR protocol described above with 10 ng of purified OpMNPV DNA template. Amplified DNA was separated on a 0.6% TAE gel (Sambrook and Russell, 2001), gel purified (QiaPrep spin minprep kit, Qiagen), and cloned into pBlueScript KS II +. After sequence confirmation of the 750 bp *polyhedrin* insert, the purified clone (QIAprep Spin Miniprep Kit, Qiagen) was labelled with dCTP-α^{32}P (Bench Top Labelling, Gibco). Lysed DNA samples were probed with the radiolabelled clone (Sambrook and Russell, 2001) and exposed to a phosphoimager screen for 4 hours, and then scanned (STORM).

3.3.2 Specificity of detection methods

Baculovirus PIBs were isolated from insect cadavers and the extract was quantified as described (Section 2.3.1). Viral DNA was extracted following the procedure of Martignoni *et al.,* (1968) and further purified by glass milk (Fermentas) as described above. For identification by PCR, baculovirus DNA (10 ng) was amplified by PCR as

described (Section 3.3.1.1). For identification by Southern dot blot, baculovirus DNA (100 ng) was denatured, filtered onto nitrocellulose, and probed as described (Section 3.3.1.2).

3.3.3 Detection of OpMNPV infection in treated DFTM larvae

3.3.3.1 Laboratory reared DFTM larvae

DFTM larvae were reared from a virus-free laboratory strain (Goose Lake) DFTM egg masses, which were decontaminated with 2% bleach solution (Javex) and hatched in 150 x 15 mm petri plates, at 25 °C, 50% RH and 16:8 photoperiod. Upon hatching (approximately 2 weeks after removal from cold storage), larvae were reared in a population maximum of 10 in 100 x 15 mm petri dishes containing a piece of artificial diet (Thompson and Peterson, 1978). Larvae were reared to 2nd instar, and then moved to fresh petri dishes and starved for 24 hours.

3.3.3.2 Viral infection method

Fresh OpMNPV PIBs were produced and purified as previously described (Section 2.3.1). Diet plugs were made from a sheet of DFTM diet (Kaupp and Ebling, 1990), and each plug (4.4±1 mg) received one LD_{50} dose of OpMNPV (9.5 PIBs/larva) in a total volume of one µl. Control larvae received one µl of distilled water to their diet plug. Larvae were reared in individual wells of a 24 well culture plate with a diet plug containing OpMNPV (treated) or distilled water (control). Larvae (12 control, 12 treatment) were randomly selected and frozen individually (day one post-ingestion) for analysis. This procedure was repeated daily to day 10. Larvae that died prior to selection were collected each day, frozen individually in their solo cups and assayed later for cause of mortality.

3.3.3.3 Identification of OpMNPV infection by Polymerase Chain Reaction

Virus DNA was extracted from macerated DFTM larvae by a novel alkaline lysis-boiling lysis procedure that allowed for quicker extraction for application to large field surveys (modification of Martignoni *et al.,* 1968). Larvae were macerated with individual, sterile 1000µl pipette tips. Homogenization was encouraged with an equal volume of STE-C buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 10 mM cysteine) to insect weight (1 ml/gram insect, wet weight). Homogenates were lysed in an alkaline lysis buffer (0.02 M Na_2CO_3, pH 11, final concentration) for 20 minutes, at 25°C with gentle mixing to release enveloped virions. The solution was neutralized with 0.16 M NaAcetate, pH 5.0 (final concentration). Enveloped virions were lysed to free viral DNA by heating for 10 minutes at 95°C (incorporated into the Polymerase Chain Reactions). Infection was confirmed by PCR amplification of the *polyhedrin* gene as described above (Section 3.3.1.1).

3.3.3.4 Identification of OpMNPV infection by Southern Dot blot

Insect tissue homogenate (Section 3.3.3.3) (100 µl/insect) were denatured, filtered onto nitrocellulose and probed as described (Section 3.3.1.2).

3.3.3.5 Identification of OpMNPV infection by ELISA

OpMNPV infection was confirmed by an indirect ELISA as previously described (Section 2.3.4) with the following modification. Tissue homogenate from macerated larvae (Section 3.3.3.3) was diluted 1:10 in PBS and 100 µl of the dilution was incubated in polystyrene ELISA plates overnight at 4°C. The remainder was stored at -20°C for later use in PCR reactions or ELISA replicates.

3.4 Results

3.4.1 Sensitivity of PCR, Southern dot blot and ELISA

The rapid DNA extraction/PCR protocol was the most sensitive method for detecting OpMNPV from semi-purified OpMNPV PIBs. The method was sensitive to a minimum of 7.8 OpMNPV PIBs (Figure 3.1). In contrast, the ELISA method was previously demonstrated to be sensitive to a minimum of 854 PIBs (Chapter 2, Figure 2.1). The Southern dot blot method was the least sensitive method, detecting a minimum of approximately 6000 OpMNPV PIBs (Figure 3.2).

3.4.2 Specificity of PCR, Southern dot blot and ELISA

Several baculoviruses were tested to determine the specificity of the methods employed to identify OpMNPV. The PCR method was not specific to OpMNPV because the primers were designed to amplify a 750 bp region of the *polyhedrin* gene (Figure 3.3), which is highly conserved within nucleopolyhedroviruses (Rohrmann, 1986b; Zanotto *et al.,* 1993). The Southern dot blot was most sensitive to baculoviruses with the highest sequence identity to OpMNPV (Figure 3.4). The 750 bp probe, which targeted OpMNPV's *polyhedrin* gene, cross-reacted with CfMNPV (87% identical, de Jong *et al.,* 2005), moderately cross-reacted with LdNPV and LffNPVs (76% identical, Kuzio *et al.,* 1999; Levin *et al.,* 1997), was minimally reactive to OpSNPV (76% identical, Leisy *et al.,* 1968), and did not react with NeabNPV (55% identical, GI:22651831).

In contrast to the PCR and Southern dot blot methods, the ELISA method was demonstrated to be specific to *Orgyia pseudotsugata* NPVs (including the single and multiple occluded varieties) (Chapter 2, Figure 2.4). The antibody method did not cross react with any other baculoviruses above the criteria for positive identification

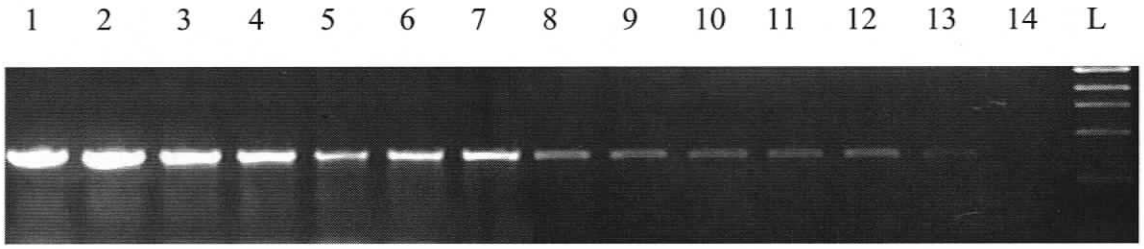


Figure 3.1. Sensitivity of PCR to alkaline lysed OpMNPV PIBs.

Lanes 1-13: Serial dilutions of OpMNPV viral DNA (extracted 3.06×10^5 - 7.65 PIBs); lane 14: Negative Control; L: 200 ng 1 Kb ladder (New England Biolabs).

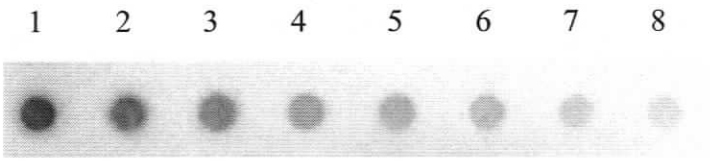


Figure 3.2. Sensitivity of Southern dot blot to alkaline lysed OpMNPV PIBs. Lysate was titrated (7.65×10^5-5.98×10^3 PIBs) and probed with a radioactive probe containing a 750 bp OpMNPV *polyhedrin* insert.

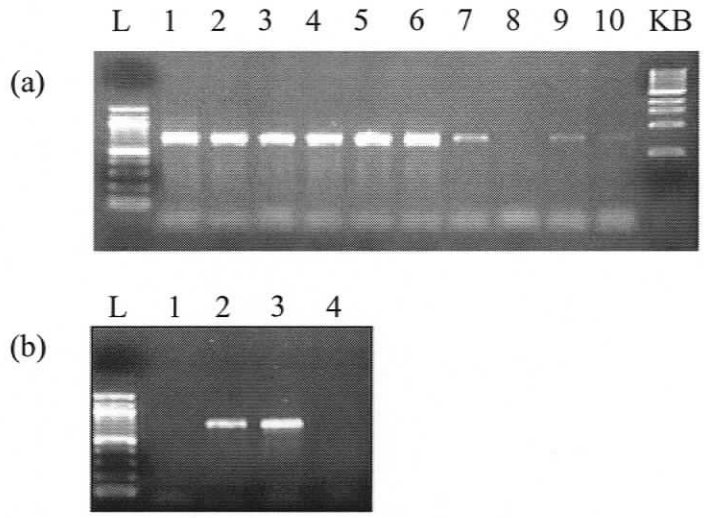


Figure 3.3. Specificity of PCR primers designed to OpMNPV's *polyhedrin* gene Amplified DNA products from baculovirus genomic DNA was subjected to gel electrophoresis (1% agarose, 1X TBE). L: 200 ng of 100 bp ladder, Kb: 200 ng 1Kb ladder (New England Biolabs). PCR amplification of the polyhedrin gene was not specific. (a) Lanes 1-6 OpMNPV variants: v-124, v-126, v-128, v-131 and v-132 accordingly; lane 7 CfMNPV, lanes 8-10 LdNPV geographical variants. (b) Lane 1 LffNPV, lane 2 NeabNPV, Lane 3 OpSNPV, Lane 4 negative control.

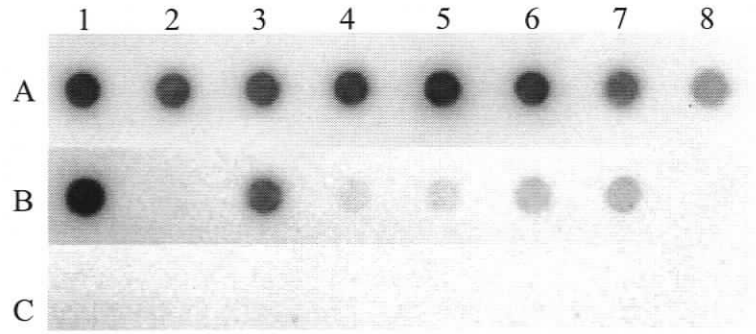


Figure 3.4. Specificity of Southern dot blot to baculovirus DNA.
Row A, 1-8: OpMNPV genotypic variants 124, 125, 126, 128, 131, 132, OpMNPV-TM Biocontrol-100 ng, 50 ng. Row B, 1-8: OpMNPV, OpSNPV, CfMNPV, LdNPV-d, LdNPV-h, LdNPV-j, LffNPV and NeabNPV. Row C, 1-8: No DNA, DFTM DNA, Uninfected Controls 3.2, 4.1, 6.4, 8.5, 10.2, 10.2; No DNA.

(2 standard deviations greater than the mean absorbance of uninfected DFTM larvae, n=8; Chapter 2).

3.4.3 Identification of OpMNPV infection in DFTM larvae

The initial infective dose (9.5 PIBs/larva) was greater than the minimum detection level of PCR for semi-purified PIBs (7.8 PIBs) (Figure 3.1), but individual larvae were divided for detection by PCR and ELISA. Thus, the detection of OpMNPV in test larvae by all methods was indicative of infection and not initial ingestion of 9.5 PIBs/larva. PCR amplification of the *polyhedrin* gene confirmed OpMNPV infection in 5% of survivors of LD_{50} doses of OpMNPV (Figure 3.5). Lethal OpMNPV infections were confirmed in 95% of DFTM larvae (Figure 3.6). OpMNPV infection was detected as early as day three post-ingestion in 12.5% of individuals tested (n=8). In contrast, the Southern dot blot method did not identify OpMNPV infections earlier than day six post-ingestion. OpMNPV infections were detected by the Southern dot blot method in 2.5% of surviving insects and in 71% of larvae that were killed (Figure 3.7).

The ELISA method consistently detected OpMNPV infections as early as day three post-ingestion (Table 3.2; replicates=4). The criterion for positive infections (A650=0.079) was defined as an absorbance equal to or greater than 2.5 standard deviations above the mean absorbance of uninfected DFTM larvae (n=80). OpMNPV infection was identified in 7.5% of larvae that survived ingestion of a LD_{50} dose of OpMNPV. Lethal infections were confirmed in 95% of larvae that died between days six to 10 post-ingestion (Table 3.2). Furthermore, the ELISA method was utilized to quantify OpMNPV infections in DFTM larvae. Absorbance values of survivors were highly variable on a given day reflecting that OpMNPV infections were infrequent in

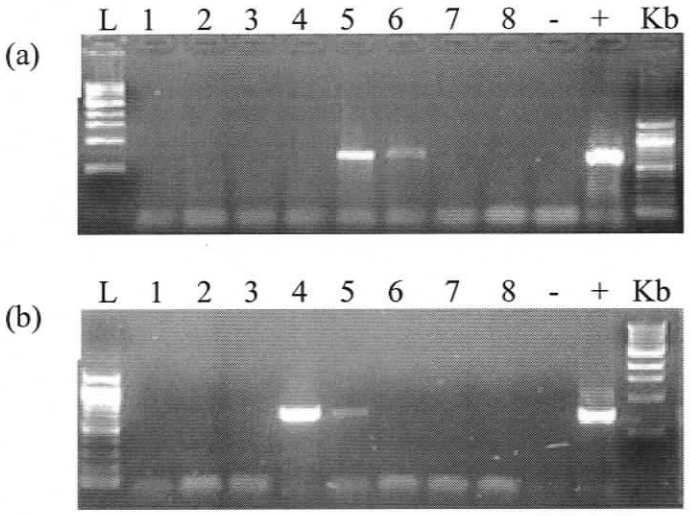


Figure 3.5 PCR amplification of the *polyhedrin* gene from sacrificed DFTM larvae. Second instar DFTM larvae were infected *per os* with LD_{50} dose of OpMNPV PIBs (9.5 PIBs/larva) and were reared in a sterile laboratory environment. Larvae were sacrificed from day 1-10 post ingestion, and frozen until analysis. Larvae were assayed individually. PCR amplification of a 750 bp region of the baculovirus *polyhedrin* gene was used to confirm virus infection as the division of the larva for detection by multiple methods reduced the initial dose to levels below their minimal detection levels. Kb: 200 ng 1 Kb ladder (New England Biolabs), Lanes 1-8: Larvae 1-8, (-): No DNA, (+): alkaline-lysed OpMNPV DNA, L: 200 ng 100 bp ladder (New England Biolabs). (a) Day 6 post-ingestion, (b) Day 8 post-ingestion.

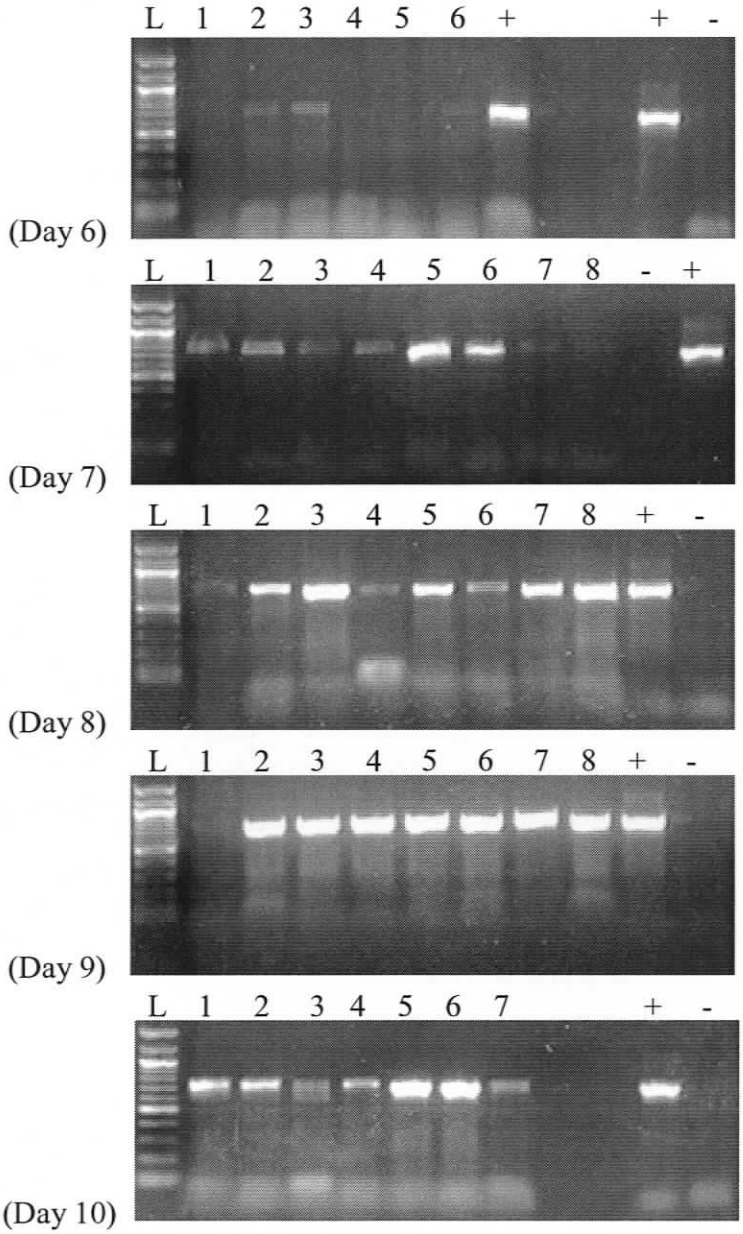


Figure 3.6 PCR amplification of the *polyhedrin* gene from dead DFTM larvae. DFTM larvae were fed a LD_{50} (9.5 PIBs/larva). Larvae that were killed were frozen and OpMNPV infection was confirmed by PCR amplification of a 750 bp region of the *polyhedrin* gene. L: 200 ng of 100 bp ladder (New England Biolabs), (+): alkaline lysed OpMNPV PIBs, (-): No DNA, 1-8: Larvae 1-8.

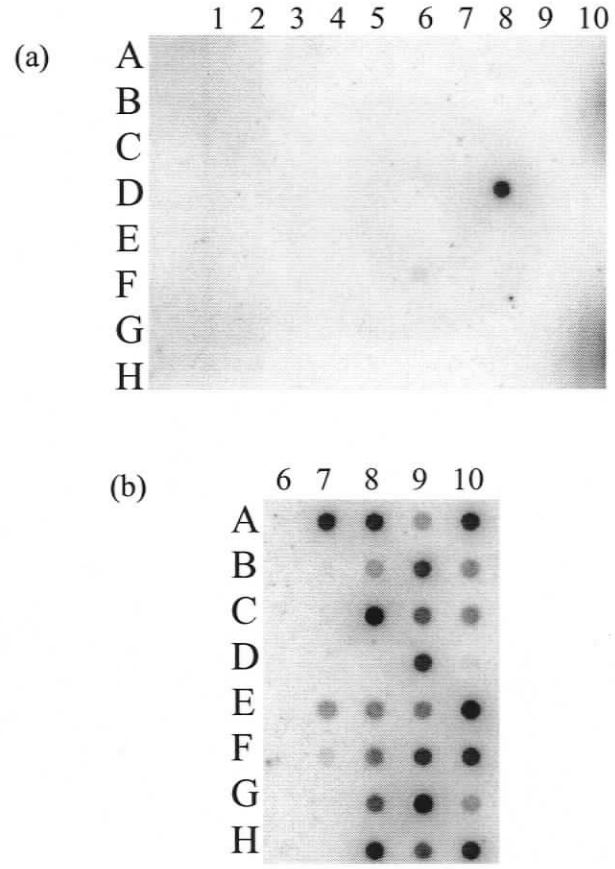


Figure 3.7. Southern dot blot identification of OpMNPV infection in DFTM larvae Larvae were treated with LD_{50} dose of OpMNPV from day one to ten post-ingestion. Columns 1-10: day post-ingestion; rows A-H: larval samples 1-8. Sensitivity of the Southern dot blot method to OpMNPV infection in DFTM larval homogenates. OpMNPV infection was identified with a radioactive clone containing a 750 bp amplicon of the OpMNPV *polyhedrin* gene. (a) sacrificed larvae, and (b) putatively lethally infected DFTM larvae.

Table 3.2. Detection of OpMNPV infection in treated DFTM larvae using ELISA

		1	2	3	4	5	6	7	8	9	10
Live	% Infection[1]	0	0	12.5	0	0	25	0	25	0	12.5
	Mean OD[2]	0.06	0.06	0.06	0.06	0.05	0.25	0.05	0.19	0.05	0.05
Dead	% Infection	n/a	n/a	n/a	n/a	n/a	67	100	100	100	100
	Mean OD						0.42	0.75	1.14	1.08	1.13

1. Absorbance values great than or equal to 2.5 standard deviations above the mean absorbance of uninfected DFTM larvae were considered positive. Percent infection was determined from a population of DFTM larvae, n=8/day or n=6 for day 6-post-ingestion (lethal infections).

2. Mean A650 of DFTM larvae, where n=8/day

survivors (Figure 3.8.a). Absorbance values of larvae killed in the experiment increased linearly from days six to eight post-ingestion (R^2=0.97), after which the quantity of infection was greater than the upper limit of the ELISA method (Clark and Barbara, 1987; Chapter 2; Figure 3.8.b).

3.4.4 Frequency of false negative and false positive results

We compared the frequency of OpMNPV detection to assess the accuracy of each approach for identifying OpMNPV infection in DFTM larvae (Table 3.3). ELISA and PCR shared equal sensitivity for detecting virus in infected larvae (Table 3.3) even though the PCR protocol was significantly more sensitive to OpMNPV DNA from semi-purified PIBs (Figure 3.1). The southern dot blot method in contrast produced 31% false negatives (Table 3.3). There were no occurrences of false positive results for any of the methods tested.

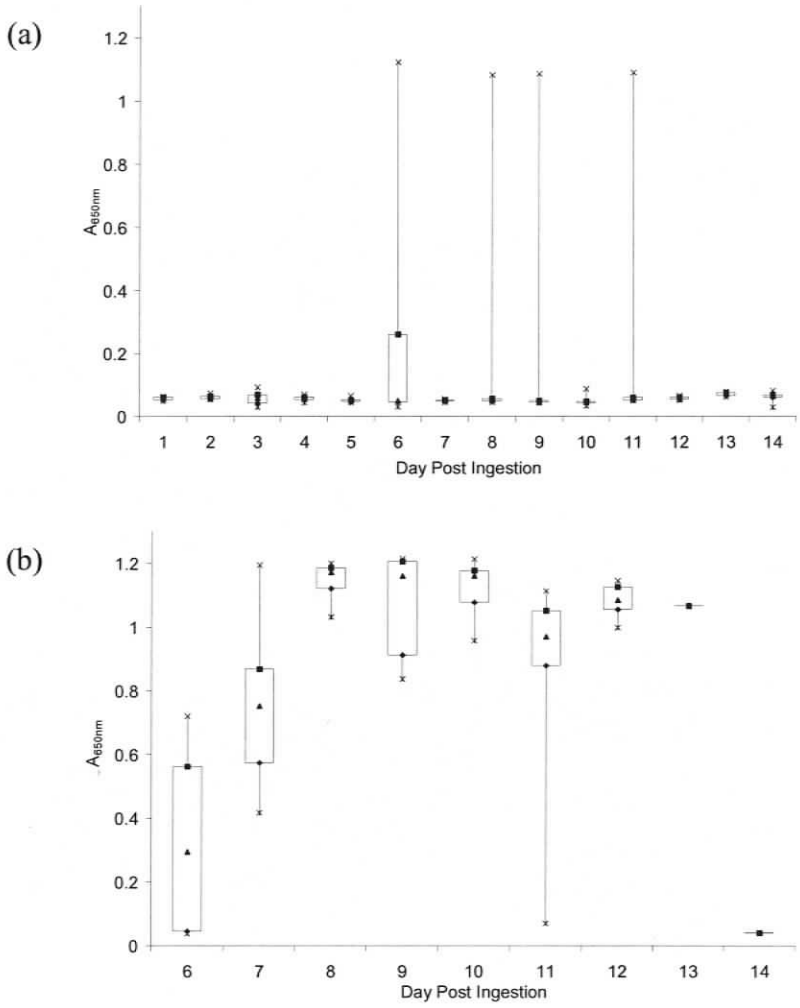


Figure 3.8 Identification and quantification of OpMNPV in DFTM larvae by ELISA Virus was detected with an OpNPV-specific antibody in an indirect ELISA (Section 2.3.4). Absorbance values that were 2.5 standard deviations greater than the median A650 of uninfected DFTM larvae (n=80) were considered positive. (a) Sacrificed larvae treated with OpNPV, from day 1-10 post-ingestion, (b) Putatively lethally-infected larvae, from day 6-10 post ingestion.

Table 3.3 The frequency of false positive and false negative results

	% incidence[1]		False Positives	False Negatives[2]	Agreement[3]
PCR	5%	Live larvae	0%	5%	95%
	95%	Dead larvae			
Southern Dot Blot	2.5%	Live larvae	0%	31%	69%
	71%	Dead larvae			
ELISA	7.5%	Live larvae	0%	Not observed	100%
	95%	Dead larvae			

[1] Second instar larvae were fed 9.5 PIBs/larva and virus infection was detected by PCR, Southern hybridization and ELISA. A total of 80 live larvae and 37 dead larvae were used for each method.

[2] False negative results were confirmed where a method failed to produce an unambiguous signal indicative of detection, and at least one other method had produced a positive signal. Where only one method produced a positive signal, true positive results were confirmed with three additional replicates.

[3] Agreement to the most sensitive method for detection, which in this experiment was ELISA.

3.5 Discussion

In this paper we developed and compared three methods for measuring the OpMNPV incidence within DFTM larvae. Microscopic counts of virus particles, the standard currently used in the operational management of the pest is limited, because it is labour intensive and lacks sensitivity. Furthermore, we were interested in evaluating alternative identification strategies that were appropriate for large analysis to improve the accuracy of population predictions of economically important insect pests.

3.5.1 Sensitivity to semi-purified sources of OpMNPV PIBs

The modified PCR method was the most sensitive method for identifying OpMNPV DNA from semi-purified OpMNPV PIBs (10X more sensitive than ELISA). The level of sensitivity reported here is less than the full potential of PCR method because of the modification of the procedure. Previously, it has been reported that PCR can be used to amplify fentogram to picogram quantities of baculovirus DNA (Kukan, 1999; Burden *et al.,* 2002), and has been predicted to be sensitive enough to detect a single virus genome (Burden *et al.,* 2002). Our motivation, however, was not to develop the most sensitive methods for detecting baculoviruses in environmental samples, as has been extensively reported in the literature (for example Lupiani *et al.,* 1999; de Moraes *et al.,* 1999; Richards and Christian, 1999; Christian *et al,* 2001; Ebling and Holmes, 2002). In contrast, we were motivated to develop the most sensitive, yet rapid methods for identifying baculovirus infections in lepidopteran insect field populations. To this end, the rapid PCR method described here may be appropriate for analysis of large populations because the DNA extraction protocol has been simplified for efficient analysis in a single day. The sensitivity of the method, however, may have been reduced

because of contaminants within the macerated insect that may inhibit PCR reactions or inefficient release of viral DNA from nucleopolyhedroviruses.

The Southern dot blot method was also relatively sensitive at detecting OpMNPV DNA from centrifuge-purified PIBs (approximately 6000 PIBs). This level of sensitivity is consistent with previous reports. *O. leucostigma* NPV, LdNPV, CfMNPV, and CfGV occlusion bodies (OBs) were identified by enzyme or radiolabelled probes, and minimum detection limits reported were 7800, 5000, 2000, and 4000 OBs, respectively (Ebling *et al.,* 2001; Kaupp and Ebling, 1993). Furthermore, we previously demonstrated that the ELISA method was sensitive to a minimum of 854 PIBs when defining a stringent criteria for positive identification (two standard deviations greater than the average A650 value of uninfected DFTM larvae, n=8) (Chapter 2).

Our results indicate that all three methods are appropriate for identifying OpMNPV infection, because all of the methods are more sensitive than the current standard, microscopic counts. Previously, the least sensitive method, dot blot hybridization assays were compared to light microscopy (Ward *et al.,* 1987; Keating *et al.,* 1989; Kukan and Myers, 1995; Ebling *et al.,* 2001). These studies concluded that dot blot hybridization was more sensitive and more accurate for detecting baculovirus infections than light microscopy. Thus, one may predict that the incorporation of dot blot analysis may improve understanding of the effect of baculovirus infections on insect population densities.

The ELISA method, has been shown to be more sensitive than dot blot methods of whole PIB lysate, and can be utilized to accurately quantify semi-purified PIBs (R^2=0.99, Chapter 2), thus one may predict that the ELISA method may be more useful for

understanding the population dynamics of insect pest populations that are modified by baculovirus pathogens. The PCR method is the most sensitive to semi-purified sources of baculovirus PIBs, and the modified alkaline-lysis procedure described in this paper has improved its application to large field assays. Thus, based upon sensitivity to semi-purified sources of OpMNPV PIBs, the PCR method shows the most promise for future analyses of virus incidence in populations of the DFTM.

3.5.2 Specificity

Targeted amplification of a 750 bp region of the *polyhedrin* gene resulted in amplification of *polyhedrin* sequences from all nucleopolyhedroviruses tested. We acknowledge that species-specific primers could have resulted in specificity to OpMNPV. In contrast, the Southern dot blot method produced selectivity to the baculoviruses which had the highest *polyhedrin* sequence similarity to OpMNPV. Previous reports have utilized more specific probes to identify genus-specific baculovirus infection (Kukan and Myers, 1995). The specificity of the Southern hybridization may have improved with the use of OpNPV-specific primers although we have not confirmed this. In contrast, the ELISA method was specific to OpNPV baculoviruses, by selecting an antibody that was specific and sensitive to OpNPV PIBs (Chapter 2).

In natural field populations of lepidopteran insects, few larvae would be naturally exposed to multiple baculoviruses. However, the DFTM is infected by two baculoviruses, OpSNPV and OpMNPV (Hughes and Addison, 1970). Furthermore, in the field, DFTM co-consume Douglas-fir trees with another defoliating insect, the western spruce budworm, *C. occidentalis* (Otvos *et al.,* 1999; Chapter 5) and therefore could potentially be exposed to CfMNPV and CfGV infection. Recently, Cooper *et al.,*

(2003) demonstrated that when field populations of the forest tent caterpillars, *Malacosoma disstria* (Hubner) were infected with McplNPV, a baculovirus which is pathogenic to the western tent caterpillar, *Malacosoma californicum pluviale* (Dyar), a covert infection of MdNPV, a native pathogen to *M. disstria* became overt and caused mortality in 88% of the larvae tested. Their study showed that covert infections were prevalent in field populations, and that experimental baculovirus infection may initiate the disease process, but may not be the causative force of mortality. Thus, these examples provide a rationale to use specific methods when determining the incidence of baculoviruses in natural field populations.

3.5.3 Sensitivity to OpMNPV infection in DFTM larvae

ELISA and PCR were the most sensitive methods for identifying OpMNPV infections from crude larval homogenates. ELISA was the most accurate method because it did not produce false negative or false positive results (replicates= 4). In contrast, initial PCR reactions resulted in 5% false negatives. Repeated amplifications resolved some, but not all of the discrepancies. This error may be due to the method in which DNA was extracted from insect cadavers. Previous studies have identified sources of DNA inhibition from field sources of microbial pathogens and have resolved many of these problems (Tebbe and Vahjen, 1993; Straub *et al.,* 1994; de Moraes *et al.,* 1999; Ebling and Holmes, 2002). In contrast, we were interested in developing detection methods that would lend well to large surveys such as determining the incidence of OpMNPV infection in field populations of the DFTM. The rapid PCR method described here, unlike standard techniques, allows researchers to identify virus infection in hours, as

opposed to a minimum of 3 days for standard DNA extraction and PCR amplification of baculovirus DNA (Martignoni *et al.,* 1968).

Southern dot blot was the least sensitive method, and resulted in 31% false negatives. The reduction in sensitivity may have been due to competition of insect DNA for binding sites on the membrane. Previous studies have reported ambiguous results when using whole tissue homogenates (Kaupp and Ebling, 1993; Kukan and Myers, 1995). In contrast, we found that infected DFTM tissue homogenates produced consistent reactions with probes, although at lower sensitivity than PCR and ELISA. Previous studies have suggested that the method is applicable for analyses of large number sample numbers, and have reported high sensitivity compared to microscopic counts (Keating *et al.,* 1989; Ward *et al.,* 1987; Kukan and Myers, 1995; Ebling *et al.,* 2001). Thus, the Southern dot blot method could improve the pest management program for the DFTM because it is more sensitive than microscopic counts, but the ELISA and modified PCR methods described here show more potential because of their sensitivity (ELISA and PCR) and accuracy (ELISA).

3.5.4 Development time

The rapid PCR and ELISA methods could be used to identify virus infections in a single day after insects were homogenized. The Southern dot blot method could be used to identify virus infections in two days after insects were macerated and a specific labelled DNA probe was prepared. Thus, the ELISA and PCR methods described here are more appropriate for large field assays because analysis may occur within a single day. Thus, based upon development time, the ELISA and PCR methods show the most promise for large field analyses of OpMNPV incidence in the DFTM.

Conclusions

The comparison of PCR, Southern dot blot, and ELISA methods, indicates that the ELISA system is the most appropriate method for identifying OpMNPV incidence in large field surveys of the DFTM because it is specific, sensitive and is amendable for large analyses. Furthermore, ELISA can be used to accurately quantify virus present in host populations, unlike PCR or Southern dot blot. We believe that the modified PCR technique has merits in covert or sublethal studies where some infections would be missed by the ELISA approach.

Chapter 4 - Immunodiagnosis II: The development of an in-field detection system to measure the incidence of *Orgyia pseudotsugata* nucleopolyhedrovirus in field populations of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough).

4.1 Abstract

In this chapter, we describe the development of a novel field detection system for the identification of *Orgyia pseudotsugata* nucleopolyhedroviruses (OpNPVs) and OpNPV infections utilizing the antibodies in a dipstick immunoassay. The dipstick method is sensitive to a minimum of 10 ng of virus protein, or 1070 viral particles, and is sufficiently sensitive to detect OpNPV infections prior to mortality. In addition, the method can also be used effectively to detect virus in infected larvae without purification of the test sample. This research provides a novel tool for on-site assessment of the incidence of OpNPV in field populations of the Douglas-fir tussock moth and has the potential to improve the biological control of the pest by facilitating on-site pest management decisions.

4.2 Introduction

Several studies have demonstrated that rapid dipstick immunoassays (Figure 4.1) are effective tools for diagnosis (Horton *et al.,* 1991; Snowden and Hommel, 1991; Rossi *et al.,* 1991; Nataraju *et al.,* 1994; Zhu *et al.,* 2002). Previous work in this field has shown that dipstick immunoassays are viable for identifying baculoviruses (Nataraju *et al.,* 1994). That study, however, did not include unpurified insect samples or field collected samples. In a related field, James and Mukerji (1996) demonstrated that the cherry mottle leaf virus could be identified from crude plant extracts, but immunodiagnosis was not rapid. Rossi *et al.,* (1991) developed and demonstrated that a rapid dipstick immunoassay was capable of detecting Schistosomiasis and demonstrated that diagnosis could be made from whole blood. Their study demonstrated that rapid dipstick immunoassays could be used for field-based studies or low-technology laboratories with little manipulation of the test substrate.

The demonstration and use of monoclonal antibodies for identification of immunoreactive diagnostic proteins and carbohydrates (Kohler and Millstein, 1975) have led to many improvements in immunodiagnostic designs. Monoclonal antibodies are preferred over polyclonal antibodies because of characteristics that lend to more accurate detection systems. First, monoclonal antibodies are significantly smaller than polyclonal antibodies, and therefore are not limited spatially in immunofiltration studies to the extent that polyclonal antibodies may be. Second, monoclonal antibodies, by definition, recognize single epitopes. This characteristic is essential for dipstick immunoassays which require sterically distinct antibodies that are specific to the test analyate for optimal sensitivity (Figures 4.1, 4.2).

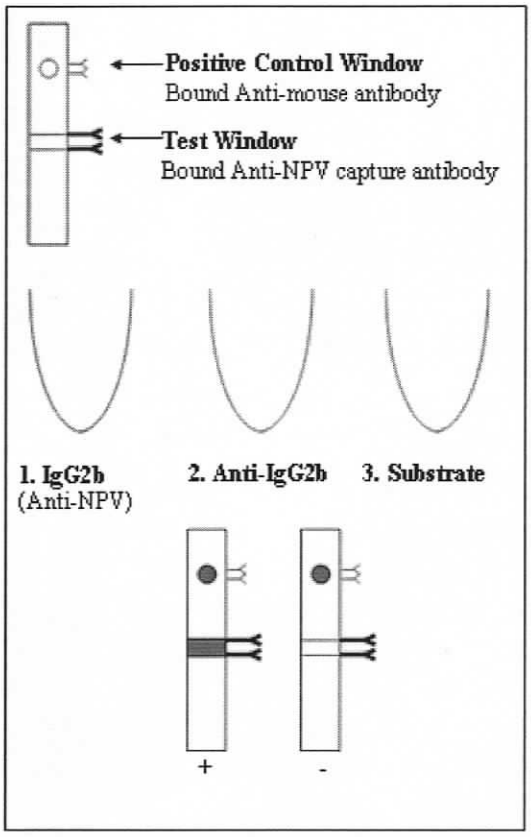


Figure 4.1. Schematic diagram of the dipstick immunoassay for OpNPV detection. After sensitizing membrane to test analyate (baculovirus polyhedron inclusion bodies or macerated test insect) for 15 minutes, sensitized membranes are incubated in (1) mouse IgG2b anti-OpNPV antibody, (2) HRP-labelled goat anti mouse IgG2b and (3) DAB substrate for 15 minutes each. Positive identification is confirmed where positive control and test windows produce a clear brown line and circle, respectively. True negative results are confirmed where reaction occurs at the control window exclusively. Permanent records are prepared by 3 washes in sterile ddH_2O. Dipsticks may be scanned for accurate quantification using a standard curve between the inverse of luminosity and known PIB numbers.

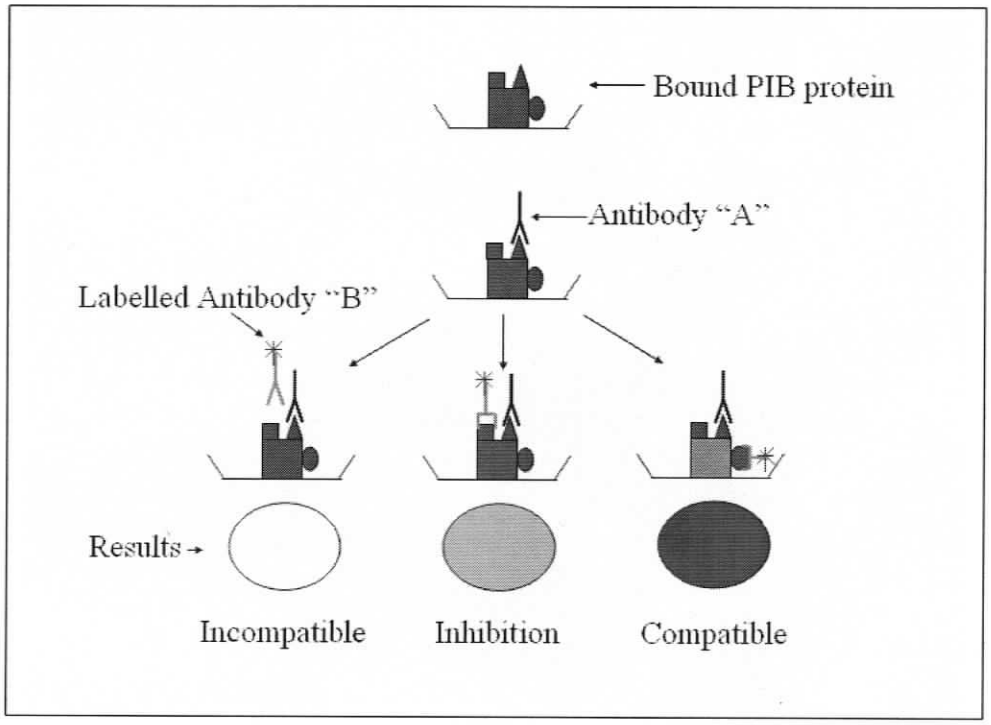


Figure 4.2. Schematic diagram of the competitive inhibition ELISA.
Dipstick immunoassay tests require two test-specific antibodies that are specific and sensitive to the test analyate, but recognize sterically distinct protein epitopes for optimal sensitivity.

The dipstick immunoassay pioneered by Glad and Grubb (1978) has since been modified to incorporate dye-labelled antibodies (Snowden and Homel, 1991; Brinbaum *et al.,* 1992; Nataraju *et al.,* 1994) or colloidal gold or silver labelled antibodies (Horton *et al.,* 1991), reducing the time of the assay. Nataraju *et al.* (1994) found that enzyme-labelled antibodies were more sensitive than dye-labelled antibody. Shyu *et al.* (2002) reported that even with silver enhancement, gold labelled antibodies were less sensitive than enzyme labelled antibodies in immunodiagnostic tools. Although enzyme-labelled antibodies are more sensitive than other visual tags, they are not as efficient because of extra incubation steps (Paek *et al.,* 2000).

In this chapter we described the development and use of a simple dipstick immunodiagnostic tool to detect *Orgyia pseudotsugata* nucleopolyhedroviruses (OpNPVs) and OpNPV infections in Douglas-fir tussock moth larvae. This is the first known paper to demonstrate rapid immunodiagnosis of baculovirus infection from unpurified insect homogenates.

4.3 Methods and Materials

4.3.1 Identification of specific and sensitive antibodies

Monoclonal antibodies were amplified in tissue culture format following a rapid-prime™ proprietary method (ImmunoPrecise Antibodies) and hybridoma supernatants containing excreted antibodies were screened for sensitivity and specificity by indirect ELISA (Section 2.3.4) with the following modification. Neat (undiluted) primary antibodies (hybridoma supernatants) were applied to polystyrene-bound virus antigens.

4.3.2 Selection of compatible antibodies

All OpNPV-specific antibodies were assayed for steric compatibility by Western blot analysis and solid phase competitive inhibition ELISA to determine which two antibodies could be used in a field-appropriate detection system.

4.3.2.1 Western Blot Analysis

OpNPV Polyhedral Inclusion Bodies were extracted from DFTM larvae following the protocol of Martignoni *et al.,* (1968). Viral PIBs were prepared for discontinuous SDS-polyacrylamide electrophoresis (SDS-PAGE) in Laemmli buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% Beta-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue), and heated for 10 minutes at 95°C. Viral proteins were separated on 4% stacking gel, 12.5% resolving gel at 45 volts and 120 volts, respectively until the dye front was 1 centimeter from the bottom of the gel. Gels were equilibrated for 10 minutes in chilled transfer buffer (25mM Tris Base, 192 mM Glycine). Gels were transferred to PVDF using a Mini Electroblot system (BioRad Corporation) in transfer buffer. PVDF membrane was prewetted in 100% methanol, and then equilibrated in transfer buffer for 10 minutes. Proteins were transferred for 1 hour at 100 volts (to a maximum of 500 mA). Transfer was confirmed by washing membranes with 4, 5 minute washes with PBS, pH 7.4, then a 1 minute wash with reversible Ponceau S stain (0.2% w/v Ponceau S, 1% Acetic acid). Membranes were scanned, and stain was removed with several washes with PBS, pH 7.4. Unbound sites on the membrane were blocked for 1 hour at 37°C with Tris Buffered Saline (TBS: 137 mM NaCl, 2.68 mM KCl, 24.8 mM Tris-Base)-0.1% v/v Tween-20-5% w/v Skim milk powder. Primary antibody was diluted optimally to 1/500 (mAbs) in TBS-0.1% Tween-20 –0.5% Skim Milk Powder, or undiluted (sera) and incubated for 1

hour, 25°C, with gentle shaking. Secondary antibody (goat-anti-mouse F/c-Horse Radish Peroxidase) was diluted optimally to 1/20,000 in TBSTM, and incubated with the membrane for 1 hour, at 25°C. Membranes were washed 3X for five minutes after each blocking and antibody steps. Membranes were exposed to chemiluminescent substrate, SuperSignal West Pico (PIERCE) for 5 minutes, then removed from substrate, and placed in a sheet protector. Membranes were exposed to autoradiography film (Kodak BioMax MR) at various incubation times.

4.3.2.2 Solid phase competitive inhibition ELISA

An OpNPV-specific monoclonal antibody, mouse IgG2b- anti-OpNPV was modified with a biotin tag following a protocol outlined in Harlow and Lane (1988). Competitive inhibition ELISAs were preformed to interpret steric hindrance of antibody binding between OpNPV-specific antibodies following a modified protocol of Wagener *et al.,* (1984) and described in Harlow and Lane (1988) (Figure 4.2). OpMNPV antigen (2µg/well) was diluted in 100 µl PBS, pH 7.4/well, and incubated overnight at 4°C. Unbound sites were blocked with 200 µl/well PBS-3% BSA for 30 minutes at 37°C. Plates were washed 2X with PBST over 10 minutes after antigen and blocking steps. Plates were incubated with 50 µl/well undiluted NPV-specific sera for 30 minutes at 37°C with gentle shaking. Biotinylated NPV-specific monoclonal antibody was optimally diluted to 1/500 in PBS-0.1% BSA-0.05% Tween-20 (PBSTB) and added to plates containing 50 µl sera/well. Plates were incubated at 37°C for 1 hour with gentle shaking, then plates were washed 5X over 30 minutes with PBST. The level of bound biotinylated antibody was determined by binding a streptavidin-horse radish peroxidase probe (Spring Bioscience) diluted optimally to 1/2000 in PBSTB. Plates were incubated

1 hour at 37°C with gentle shaking. Biotin-Streptavidin-HRP binding was visualized by incubating plates with 50 µl/well tetramethylbenzidine (BioFX) for 30 minutes in the dark. A650 was measured with a MicroTek plate reader. The relationship between absorbance and biotinylated OpNPV-specific antibody dilution was analyzed graphically by Microsoft Excel.

4.3.3 Proof of concept- sandwich ELISA

Optimal antibody-antibody ratios and binding conditions were determined in a standard indirect ELISA. Dipstick immunoassays work on the concept of specific-capture of immunoreactive proteins, therefore we developed a sandwich ELISA to determine optimal binding conditions for optimal sensitivity using bound mouse IgG1 antibody (anti-OpNPV capture), 10H8, and an indicator antibody mouse IgG2b antibody (anti-OpNPV indicator), 1A1. The 10H8 capture antibody was titrated in PBS-0.1% BSA (PBSB) and bound to polystyrene plates overnight, 4°C. Unbound sites were blocked with PBS-3% BSA, 37°C, for 30 minutes. Semi-purified OpMNPV PIB antigen (2-0.0 µg in PBS-0.1% Tween) was added to sensitized plate for 1 hour, 37°C, shaking. Captured OpMNPV PIB antigen was probed with a second OpNPV-specific antibody, 1A1 mouse IgG2b, diluted optimally (1/500) in PBSB-0.1% Tween, 1 hour, shaking, 37°C. A labelled antibody, goat-anti-mouse IgG2b-HRP (Axygen) was titrated (1/1000 - 1/20,000) in PBST. Polystyrene plates were washed 5X over 30 minutes with PBST between each antibody step. The capture ELISA was visualized with TMB substrate (BioFX) using a Microtek plate reader. The optimal capture antibody concentration was determined by the minimal capture antibody concentration resulting in a positive dose (capture antibody concentration)-response (absorbance) curve using Microsoft Excel.

4.3.4 Preparation of immunodiagnostic device - Dipsticks

Dipsticks were assembled for specific capture of OpMNPV PIBs. The optimal capture concentration determined by capture-ELISA was applied to dipstick immunoassays. OpNPV-specific antibody 10H8 (mouse IgG1-anti-OpNPV stabilized in 0.1% BSA) was diluted in striping buffer (Shleicher and Schuell) (10 mM Sodium phosphate buffer, pH 7.4, 3% Sucrose, 0.1% BSA, final concentration) and two µg/dipstick was applied with a rapidograph pen (KOH-I-NOOR) to nitrocellulose (AE98 lateral flow membrane, Whatmann). It has been previously established that sucrose increases the shelf-life and sensitivity of immunoassays (Paek *et al.*, 2000) because sucrose molecules take the place of water molecules (during dehydration of capture antibody) preventing structural inactivation of the capture antibody (Carpenter and Crowe, 1997). The bound OpNPV-specific antibody acted as a capture line for positive identification of OpNPV (Figure 4.1). A mouse-specific antibody (goat-anti-mouse F/c portion, PIERCE) was applied 2 cm from the capture antibody (1:1000 in striping buffer) and acted as a positive control capture reagent (Figure 4.1). Unbound sites on nitrocellulose were blocked with PBS-1% BSA for 1 hour with gentle shaking. Membranes were washed with three changes of PBS and dried at room temperature. Membranes were stored at 4°C until use.

4.3.5 Dipstick immunoassay

Prepared membranes were exposed to known concentrations of semi-purified OpMNPV PIBs (Sensitivity assays) or to 10 µl of infected homogenized DFTM tissues, and incubated for 15 minutes. Membranes were washed 3X for 5 minutes in PBS. Bound OpMNPV PIBs were confirmed by incubating membranes with a second OpNPV-specific antibody (1A1 mouse IgG2b-anti-OpNPV) optimally diluted (1/500) in PBS-

0.1% Tween-0.1% BSA (PBSTB) for 15 minutes. Excess 1A1 antibody was captured by the mouse-specific control antibody. Unbound 1A1 was removed with 3 washes with PBST. The presence of 1A1 capture was confirmed by incubating membranes for 15 minutes with a horse radish peroxidase-labelled goat-anti-mouse IgG2b antibody (1/20,000 in PBSTB), and excess unbound antibody was removed with 3 washes in PBST. Antibody binding was visualized by incubating membranes in diaminobenzidine substrate up to 15 minutes (BioFX). Colour was fixed by washing membranes with 3 washes in ddH_2O, and membranes were scanned. (Figure 4.1)

4.4 Results

4.4.1 Identification of specific and sensitive antibodies

Hybridoma supernatants that showed reactivity of a minimum of 2 standard deviations (SD) greater than the mean background reactivity (preimmune mouse sera, n=8) were screened against various baculovirus PIBs in an indirect ELISA and results were summarized (Table 4.1). Only 10H8, 13D4, and 1A1 antibodies were appropriate for on-site evaluation of OpNPV incidence because these antibodies were sensitive to all

Table 4.1. Specificity of Hybridoma supernatants from OpMNPV-immunized mice

Antibody	TM Biocontrol-1 (OpMNPV)	OpMNPV (genotypic variants)	OpSNPV	*Oa*NPV	Other NPVs	DFTM
1A1	+	+	+	-	-	-
3C9	+	+	+	-	+ *Cf*MNPV	-
5C11	+	+	+	-	+ *Cf*MNPV	-
7D3	+	+/-	-	-	-	-
13D4	+	+	+	-	-	-
7D6	+	+/-	+	-	-	+
3E2	-	-	+	-	-	+
5F9	+ high (594 SD)	+ low (12 SD)	-	-	-	-
10H8	+	+	+	-	-	-

Hybridoma specificity was determined by indirect ELISA (Section 4.3.2). ELISA reactions contained one µg of semi-purified baculovirus PIB protein, neat hybridoma supernatant, and optimally diluted (1:20,000) goat-anti-mouse-HRP secondary antibody (PIERCE). Reactions were visualized with TMB substrate (PIERCE) and quantified at 650 nm with a LKB plate reader. Reactions 2 SD above the mean background (pre-immune sera, n=8) were considered positive. Baculoviruses tested included *Orgyia pseudotsugata* MNPV (9 variants), OpSNPV (2 variants), *O. antiqua* NPV, *Choristoneura fumiferana* MNPV, *Lambdina fiscellaria fiscellaria* NPV, *Lymantria dispar* NPV and *Neodiprion abietis*.

tested OpNPV genotypic variants, and did not cross-react with other baculoviruses. We also identified one antibody, 5F9 which may be appropriate for assessing the environmental impact of introducing the biological control agent, TM Biocontrol-1 into the forest ecosystem. The antibody 5F9 had a significantly greater affinity (13X more signal) to OpMNPV genotypic variants found in the biological control agent, TM Biocontrol-1, than other genotypic OpMNPV variants and did not cross-react significantly with other baculovirus PIBs (Figure 4.3).

4.4.2 Identification of compatible antibodies

Western blot analysis was used to characterize the antibodies for their use in dipstick immunoassays. Specifically, we hoped to identify OpNPV-specific antibodies that recognized distinct virus proteins. When virus PIB proteins were denatured and separated on SDS-PAGE, and then probed in a Western blot, all antibodies (10H8, 13D4, and 1A1) predominantly recognized a 16.5 KDa protein (Figure 4.4). The 1A1 antibody predominantly recognized a 6.5 KDa protein in the genotypic variants found in TM Biocontrol-1 and OpSNPV and multiple protein bands with longer exposure to autoradiography film (Figure 4.4).

To analyze the steric effects of paired antibodies, a single antibody (1A1) was modified with a biotin label for competitive inhibition ELISA. The most reactive ratio of Biotin: 1A1 mouse IgG2b-anti-OpNPV antibody was selected for competitive assays (Figure 4.5). The competitive inhibition ELISA indicated that two antibodies 10H8 (mouse IgG1-anti-OpNPV) and 13D4 (mouse IgG1-anti-OpNPV) did not result in inhibition of binding of 1A1 (mouse IgG2b-anti-OpNPV-Biotin) to OpMNPV PIBs (Figure 4.6). In

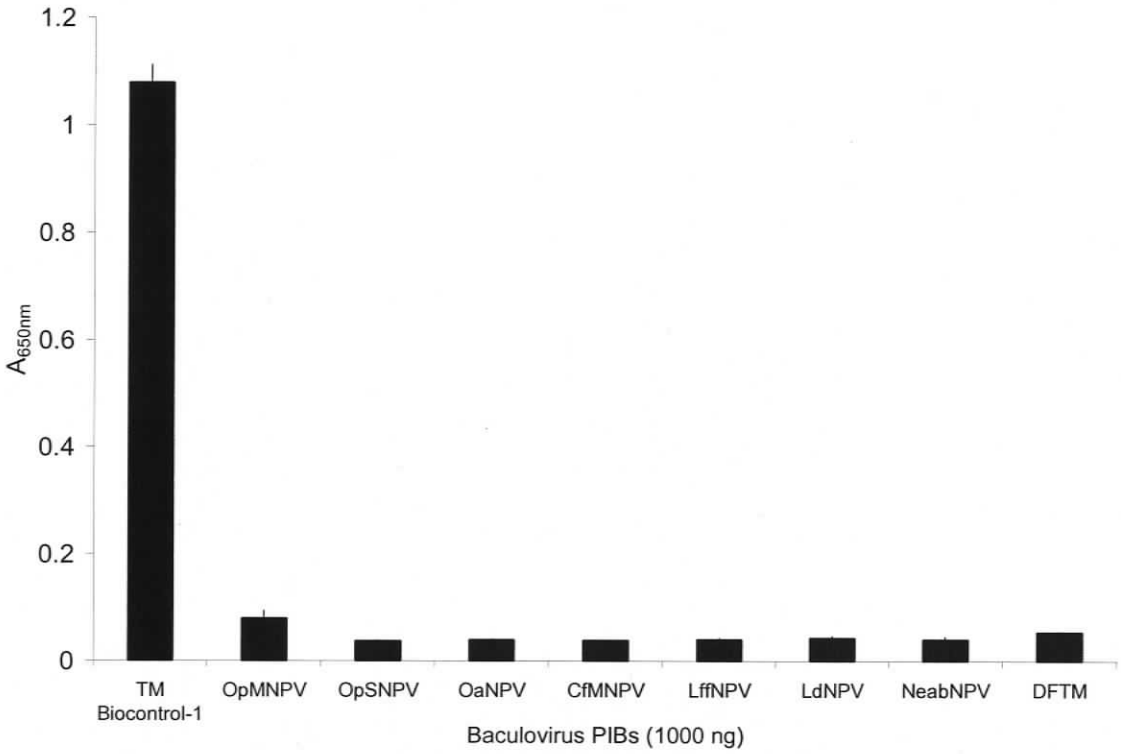


Figure 4.3. Specificity of 5F9 hybridoma supernatant to TM biocontrol-1. Baculovirus PIB antigens were isolated from insect cadavers, and 1 µg of each type was applied. Virus was detected in an indirect ELISA (Section 4.3.2). The A650 for OpMNPV was determined from the average of six genotypically distinct field variants. The absorbance values were established from the average of 8 replicates of per genotypic variant. Bars represent the standard deviation around the mean absorbance. Hybridoma supernatant 5F9 bound to OpMNPV PIBs found in the biological control agent, TM Biocontrol-1 with the highest affinity, but significantly cross-reacted with other OpMNPV field variants (A650 > 2 SD above mean A650 of uninfected DFTM larvae, n=8). No other baculoviruses cross-reacted with the antibody with significance.

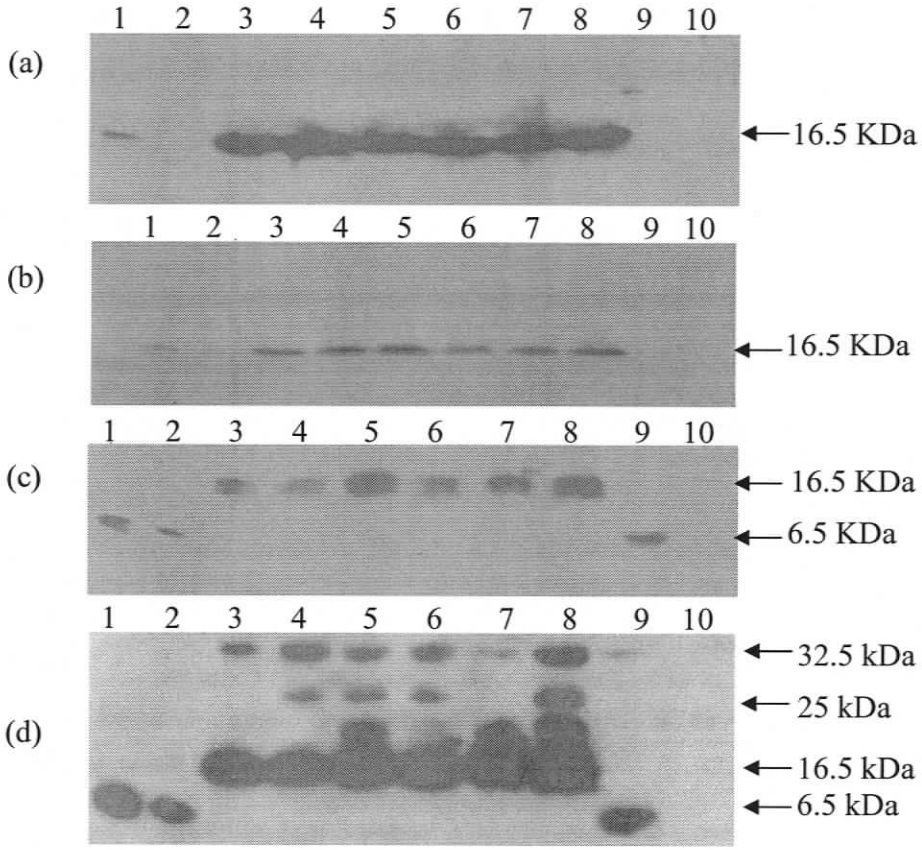


Figure 4.4. Western blot analysis: Compatibility of OpMNPV antibodies.
Western blot analysis was utilized to locate antibody-protein binding and thus reveal compatibility between OpNPV-specific antibodies. Total protein from infected DFTM larvae (100 µg) were separated on a discontinuous SDS-PAGE gel (4% stacking, 12.5% resolving gel). Proteins were transferred to PVDF and were probed with OpNPV-specific antibodies: (a) 10H8 (b) 13D4 and (c) 1A1. Lane 1,2: OpMNPV PIBs, virions, lanes 3-8: OpMNPV genotypic variants, Lane 9 OpSNPV, and Lane 10 uninfected DFTM. Size was estimated with a prestained protein ladder (BioRad Corporation). (d) Extended exposure (3 minutes) to autoradiography film (BioMax MR, Kodak) revealed that the monoclonal antibody 1A1 recognized an epitope that may be present in multiple OpNPV PIB proteins.

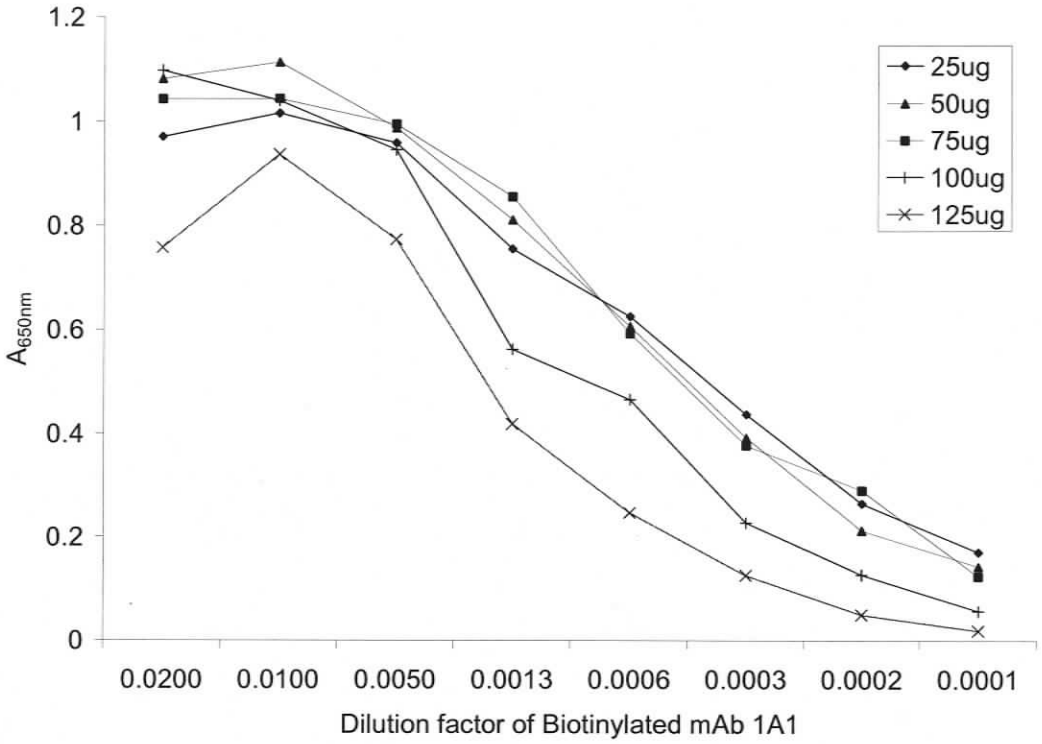


Figure 4.5. Empirical optimization of biotin labelled OpNPV-specific antibody. Dilutions of NHS-Biotin (25-125µg) were absorbed to 1 mg alkaline-dialysed 1A1 mouse IgG2b anti-OpNPV antibody. The optimal ratio was determined in an indirect ELISA with 1 µg of OpMNPV PIB antigen, serial dilutions of biotinylated 1A1 mAb, and optimally diluted (1/5000) streptavidin-HRP (Spring Bioscience). A650 was measured with a Microtek plate reader.

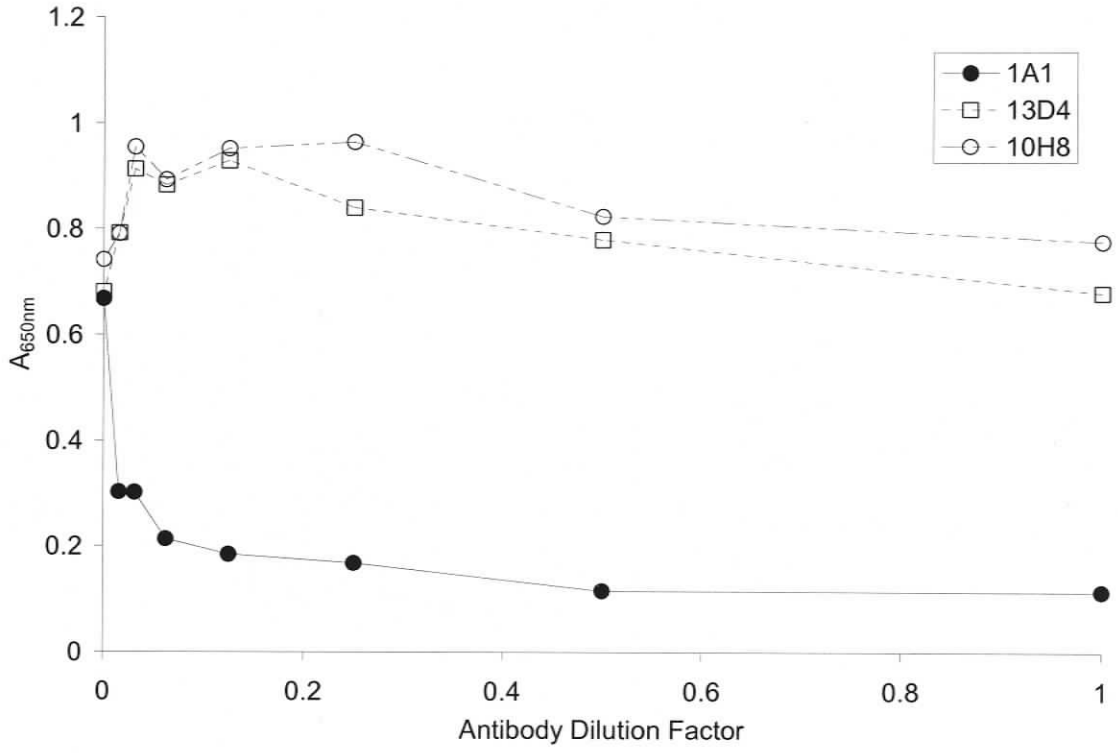


Figure 4.6. Competitive inhibition ELISA.

Unmodified hybridoma supernatants (1A1, 10H8, and 13D4) were bound to 2 μg of OpNPV antigen coated onto ELISA plates. A single antibody (1A1) was modified with NHS-Biotin and incubated with hybridoma supernatants. 1A1-Biotin was visualized with a streptavidin-HRP probe (Spring Bioscience). The reduction in A650 was indicative of competitive inhibition (*i.e.* 1A1 versus 1A1-Biotin). 10H8 and 13D4 antibodies were compatible with the biotinylated 1A1 antibody (Figure 4.2).

contrast, unmodified 1A1 inhibited the binding of the biotin-labelled 1A1 to OpMNPV proteins. The 1A1 antibody was selected for the dipstick assay because of its affinity to multiple OpNPV proteins (Figure 4.4) and the antibody 10H8 was selected due to compatibility with 1A1 antibody in non-denaturing conditions (Figure 4.6).

4.4.3 Sensitivity of OpNPV-specific antibodies to dilutions of semi-purified PIBs

Hybridoma supernatants that were selected for the dipstick assay were PEG purified and tested against limiting dilutions of OpNPV PIBs (Figure 4.7). We found that 1A1 was sensitive to a minimum of 835 PIBs while 10H8 was sensitive to a minimum of 1670 PIBs as per the criterion established for semi-purified PIBs (Figure 2.1).

4.4.4 Optimization and proof of concept by indirect and sandwich ELISA

An-enzyme labelled antibody specific to mouse IgG2b antibodies was used for dipstick immunoassays for optimal sensitivity (Nataraju *et al.,* 1994; Shuy *et al.,* 2002). Optimal dose-response with minimal background signal was determined by titration against OpMNPV-captured IgG2b antibody (dose-response) and OpMNPV-captured IgG1 antibody (background noise) (Figure 4.8a). The optimal capture antibody dilution (10H8 mouse IgG1-anti-OpNPV, 1/40) was determined by titration (Figure 4.8b). For future assays, bound capture reagent was defined by the highest positive signal where background signal was absent.

4.4.5 Sensitivity of the dipstick immunoassay to semi-purified OpMNPV PIBs

Positive identification was confirmed where samples produced a visible band at the capture line, and visible dot at the positive identification zone (*i.e.* double positive) (Figure 4.1). Initial assays were run in the absence of Tween-20 to reduce stringency of the assay due under time constraints of dipstick immunoassay. We determined, however,

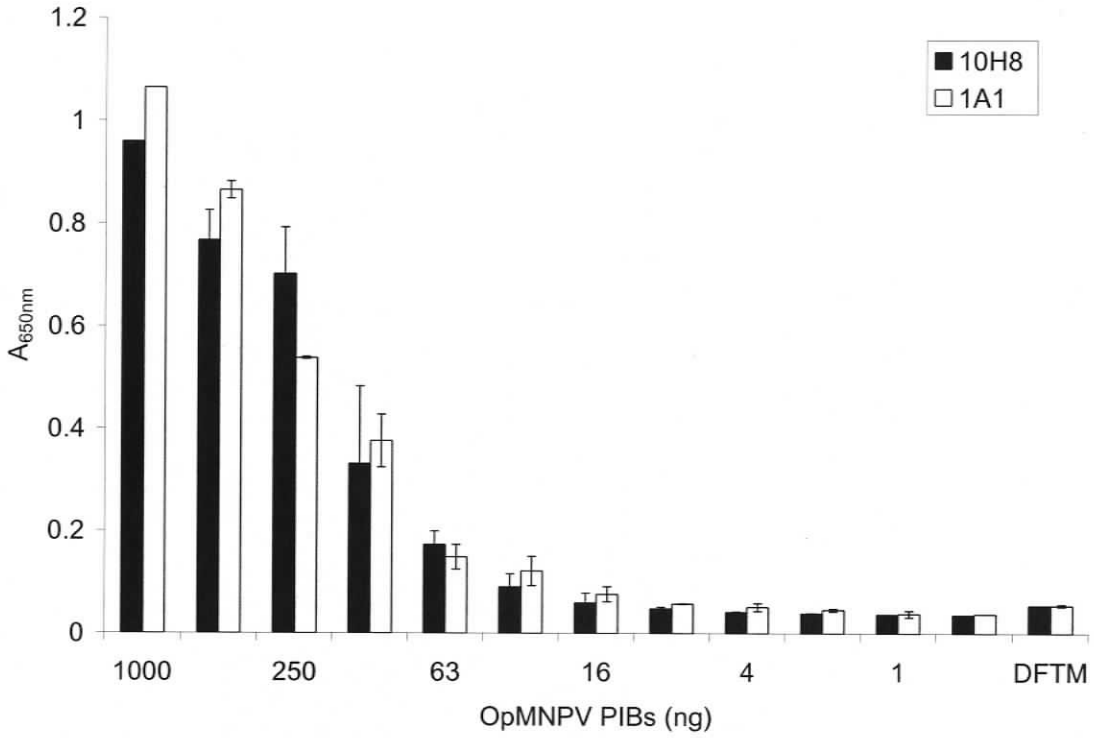


Figure 4.7. Sensitivity of two OpNPV-specific antibodies to OpMNPV PIB antigen. TM Biocontrol-1 (containing OpMNPV PIBs) was detected in an indirect ELISA as described (section 2.3.4). Bars represent the standard deviation around the mean absorbance (n=4). The A650 of serial dilutions of OpMNPV PIB protein was measured with a MicroTek plate reader. Antibody 1A1 was sensitive to a minimum of 7.8 µg of PIB protein or 835 PIBs and antibody 10H8 was sensitive to a minimum of 15.6 µg of PIB protein or 1670 PIBs based upon the criterion established (Chapter 2, Figure 2.1).

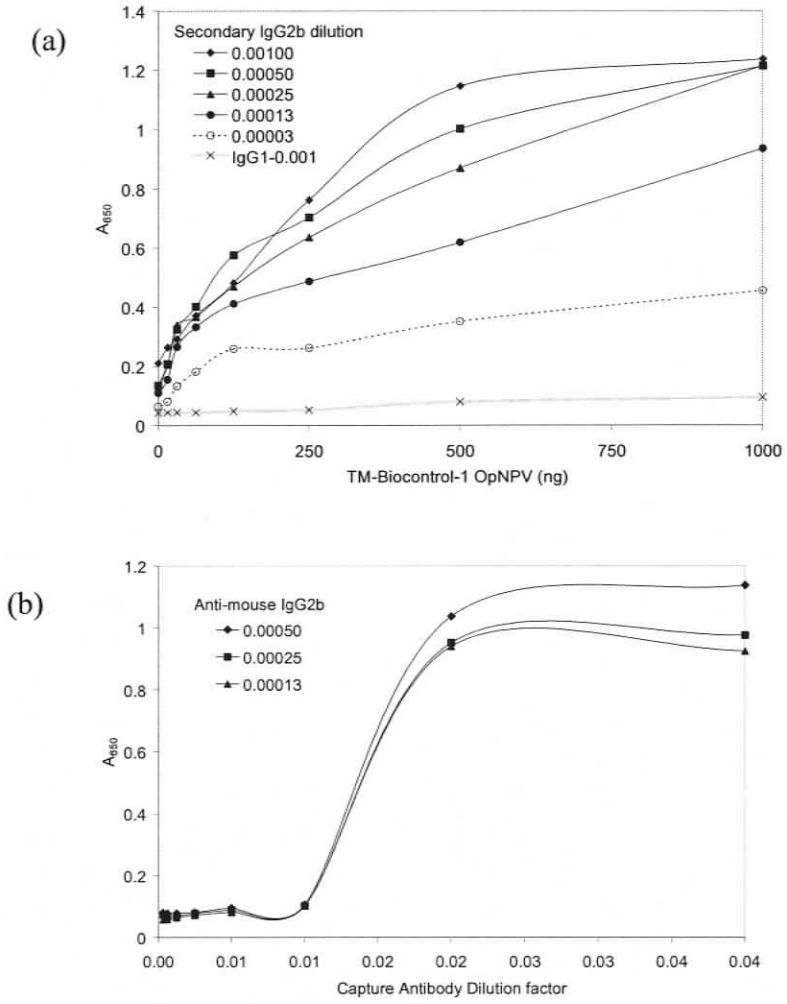
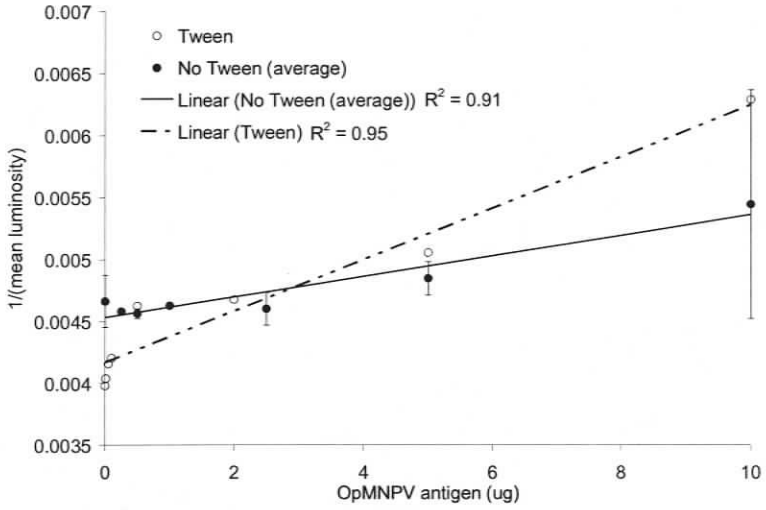


Figure 4.8. Optimization of dipstick immunoassay reagents.

(a) Optimization of indicator antibody, goat-anti-mouse IgG2b-HRP. OpMNPV antigen (1-0.0 µg/well) was bound to plates and acted as capture in an indirect ELISA. Mouse IgG2b antibodies were diluted optimally to 1/500 and captured by antigen. Antibody IgG1 (capture antibody in dipstick immunoassays) was used to confirm that the indicator antibody (goat-anti-mouse IgG2b) did not cross-react and bind with the antibody. The optimal concentration of goat-anti-IgG2b-HRP (GenTex Inc.) was determined by titration (1/1000-1/30,000). (b) Optimization of capture antibody (10H8) concentration. The optimal capture antibody concentration was determined by titration (1/20-1/2000) in a capture antibody ELISA. OpMNPV antigen was diluted to 1µg with PBS. Indicator antibody, mouse IgG2b-anti-OpNPV was diluted optimally (1/500). Secondary antibody, goat-anti-mouse IgG2b-HRP (GenTex Inc.) was optimally diluted (1/2000-1/8000), visualized with TMB substrate (BioFX) and measured at A650.

(a)



(b)

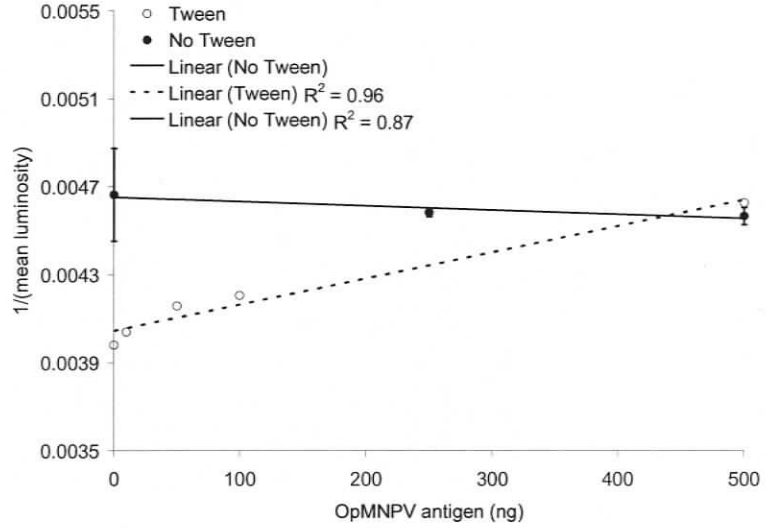


Figure 4.9. The effect of tween-20 on the dipstick immunoassay dose-response curve. Known concentrations of OpMNPV PIBs were incubated with prepared dipstick membranes (Figure 20). Bound PIBs at the capture line were visualized with an OpNPV-specific antibody 1A1 (mouse IgG2b) and visualized with a HRP-labelled goat-anti-mouse IgG2b antibody. Dipstick immunoassays were developed in the presence or absence of Tween-20. Dipstick immunoassays were scanned (Cannon) and the inverse of the mean luminosity (Adobe Photoshop) of the capture band was plotted against known PIB antigen. There was a negative dose-response curve in the absence of tween-20 when antigen concentrations were low. Dipstick immunoassays produced steeper positive dose-response curves in the presence of tween-20 at low and high concentrations of OpMNPV antigen. The dose-response curve of dipstick immunoassays exposed to (a) 0-10 µg of antigen and (b) 0-500 ng of antigen.

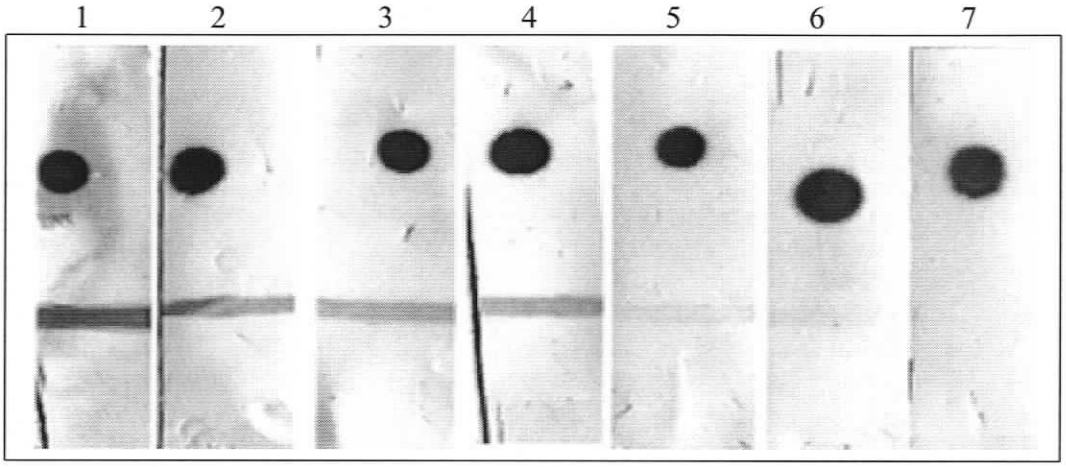


Figure 4.10. Dipstick Immunoassay: sensitivity to semi-purified OpMNPV PIBs. Known concentrations of OpMNPV PIBs were incubated with membranes (Strip 1-6: 2, 1, 0.75, 0.5, 0.05, 0.01 µg or 107,000-1070 PIBs, 6: no antigen). Bound PIBs at the capture line were visualized with an OpNPV-specific antibody 1A1 (mouse IgG2b). This antibody was also captured by the positive control mouse-specific antibody. A HRP-labelled goat-anti-mouse IgG2b-specific antibody bound specifically to antibody 1A1 and did not cross react with 10H8 (). HRP-enzyme was visualized with diaminobenzidine. The dipstick method was sensitive to a minimum of 10 ng or 1070 OpMNPV PIBs without background noise.

that the dose-response curve in the absence of tween-20 resulted in high background noise (Figure 4.9). Dose-response curves seen in the capture-ELISA were resolved in the presence of Tween-20 (Figure 4.9). True negative results were confirmed where there was no visible line at the capture line, but a clear visible dot at the positive identification zone (Figure 4.10). Single positive results (clear visible band at capture, no visible dot at positive zone) would be considered a failed test, but this was not observed. The dipstick method was sensitive to a minimum of 10 ng of semi-purified OpMNPV protein, or 1070 OpMNPV PIBs (Figure 4.10).

4.4.6 Detection of OpNPV infection in crude DFTM homogenates

Lethal quantities of OpNPV infection were confirmed in crude insect homogenates from laboratory controlled, infected populations and field-collected DFTM insects (Figure 4.11.a). Furthermore, OpNPV infections in larvae that were fed 9.5 PIBs/larva and were sacrificed prior to mortality for destructive sampling were unambiguously identified with the dipstick immunoassay (Figure 4.11.b).

4.4.7 Quantitative analysis of the dipstick immunoassay

The sensitivity assay demonstrated that the dipstick immunoassay was visually semi-quantitative (Figure 4.10). Furthermore, OpNPV infections that caused mortality, and OpNPV infections in larvae that were sacrificed for destructive sampling, were on average visually distinct (Figure 4.11). In addition, completed dipstick immunoassays were scanned for quantitative analysis during optimization of capture antibody concentration (data not shown) and optimization of binding conditions (Figure 4.9).

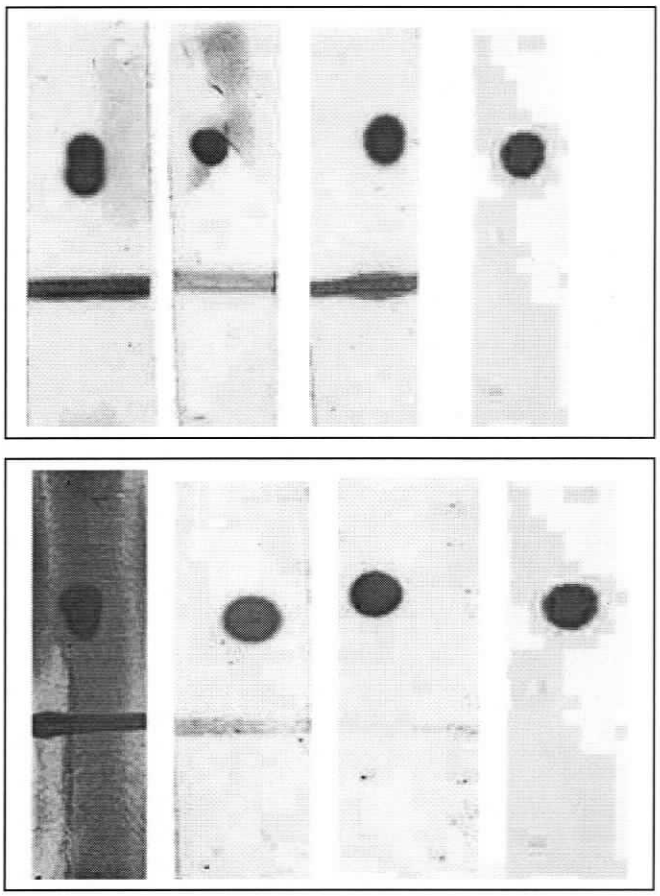


Figure 4.11. Dipstick Immunoassay: detection OpNPV infections in DFTM larvae Dipstick immunoassays were sensitive enough to detect lethal quantities of virus infection and virus infection in larvae sacrificed prior to mortality (initially detected by ELISA) in second instar DFTM larvae, and from mature larvae collected from the field. (a) DFTM larvae were infected with LD_{50} doses of OpMNPV PIBs, and larvae that were killed on day 6 post-ingestion were assayed for virus infection (strips 1, 2). Strip 3: homogenized OpNPV-infected dead DFTM larva collected from Scotty Creek, BC. Strip 4: uninfected DFTM larva. (b) DFTM larvae were infected *per os* with LD_{50} dose of OpMNPV PIBs (9.5 PIBs/larva) and sacrificed from days 1-10. Strip 1-3 DFTM larvae sacrificed days 6 and 8 post-ingestion. Strip 4: homogenized uninfected DFTM larva.

4.5 Discussion

4.5.1 Characterization of antibodies

Monoclonal antibodies were characterized to ensure that the dipstick immunoassay would be sensitive and specific to the baculoviruses responsible for the collapse of DFTM outbreaks. Several antibodies were initially selected for the dipstick immunoassay because they were sensitive to OpMNPV antigen, however, only the antibodies that were specific to OpNPV were selected for the final dipstick immunoassay. DFTM populations are reduced, in part, by two pathogenic baculoviruses, OpMNPV and OpSNPV (Hughes and Addison, 1970), thus we selected antibodies that were sensitive to all OpMNPV and OpSNPV genotypes tested. In the field, DFTM outbreaks can occur, at times, in mixed infestations with the western spruce budworm, *Choristoneura occidentalis* Freeman (Otvos *et al.,* 1999; Chapter 5), but its pathogens, CfMNPV and ChocGV are not pathogenic to the DFTM. Thus, the use of antibodies that are sensitive to CfMNPV, could potentially overestimate the incidence of DFTM pathogens. We selected antibodies for the dipstick immunoassay that showed specificity to genotypic variants of OpMNPV and OpSNPV so that the dipstick immunoassay would provide an accurate measurement of the pathogens responsible for the maintaining equilibrium populations of the DFTM.

The sensitivity of dipstick immunoassays was improved by employing two specific antibodies (one as a capture reagent, the other as an indicator antibody) that recognize distinct protein epitopes. Three monoclonal antibodies that were sensitive and specific to OpNPV PIB antigens were further characterized by competitive inhibition and western blot analysis to identify the two best antibodies for the dipstick immunoassay.

Competitive inhibition ELISA revealed that 10H8 and 13D4 antibodies were compatible with antibody 1A1. In contrast, when virus PIB proteins were first denatured and separated by SDS-PAGE prior to immunodetection by Western blot analysis, all antibodies showed the highest affinity to a viral protein close to 16 KDa in size. This analysis suggested that the antibodies may compete for binding position on the antigen. Previous work has revealed that protein epitopes can be lost due to unfolding of proteins bound to polystyrene (Kennel, 1982; Shields *et al.,* 1991) and epitope loss would be more significant during SDS-PAGE prior to Western blot analysis. Alternatively, the change in conformation of proteins by either treatment may have revealed new immunoreactive epitopes (Friguet *et al.,* 1984).

The dipstick immunoassay was run in conditions near to that of the ELISA, (*i.e.* test proteins were analyzed in their native conformation), and thus we were confident that the compatibility of antibodies in competitive inhibition ELISA would translate into compatibility in dipstick immunoassays. Less stringent Western blot conditions revealed that antibody 1A1 bound to multiple OpNPV proteins, suggesting that the antibodies were compatible regardless of binding conditions. Thus, two antibodies, 10H8 and 1A1 were selected for the dipstick immunoassay because the antibodies were compatible in both conditions tested.

We also characterized an antibody (5F9) that showed high affinity to the genotypic variants found in the biological control product, TM Biocontrol-1 (Martignoni, 1999) and low affinity to alternative OpMNPV genotypes. The antibody was not selected for the dipstick immunoassay because it would underestimate the natural incidence of OpNPV infection in field populations of the DFTM. Further characterization of the antibody by

Western blot analysis failed (replicates= 4) (data not shown). It is likely that the antibody recognized a topographical epitope in ELISA assays, which was lost due to the denaturing conditions of SDS-PAGE prior to Western blot analysis. This antibody may be useful for environmental fate studies of the biological control agent, TM Biocontrol-1, particularly in the southern United States where OpMNPV variants are not naturally present in the DFTM populations (Hughes, 1976). The application of the antibody for environmental impact may be limited in British Columbia because the genotypic variants found in the biological control product are also the most common variants found in field populations in B.C. (Williams and Otvos, 2005).

4.5.2 Demonstration of the dipstick immunoassay

The dipstick immunoassay was sensitive to a minimum of 10 ng of OpMNPV PIB protein or 1070 OpMNPV PIBs. Previously, dye-labelled and enzyme-labelled dipstick immunoassays were developed for the baculovirus pathogen of the silk worm, *Bombyx mori* Linnaeus (Nataraju *et al.,* 1994). Their work demonstrated that the dipstick immunoassay was sensitive to a minimum of 10 ng/ml of purified *B. mori* PIBs. The dipstick immunoassay described in this paper is as sensitive as the dipstick systems previously described for the identification of other baculoviruses.

The dipstick immunoassay described in this paper is semi-quantitative by visual interpretation and comparison to known PIB numbers. As well, completed dipstick immunoassays can be scanned upon return to the laboratory for quantitative analysis by utilizing the linear relationship between the inverse of luminosity and PIB number (R^2=0.95). Thus, the flexibility of the dipstick immunoassay allows semi-quantitative analysis for on-site field analysis, and quantitative analysis for lab-based analysis.

4.5.3 Conclusions

This is the first paper to my knowledge to demonstrate the use of a dipstick immunoassay to detect baculovirus PIBs directly from infected insects. The dipstick immunoassay was sensitive enough to detect OpMNPV infections prior to host mortality within both field collected and laboratory infected larvae. The results from the dipstick immunoassay were in 100% agreement with ELISA. Furthermore, this was the first known paper to demonstrate the use of a rapid immunoassay to identify baculovirus infections directly from macerated larvae.

The dipstick immunoassay method may enhance the pest management program for the Douglas-fir tussock moth because it has attributes that are not available with standard microscopic counts. The dipstick immunoassay is specific to OpNPV PIBs, and thus analysis will not over or under estimate pathogen incidence. As well, the dipstick immunoassay is sensitive to a minimum of 1070 PIBs, which can be equated to early-onset of OpNPV infection, since late instar larva produce up to 10^9 PIBs prior to mortality (Otvos *et al.*, 1999). Moreover, the dipstick immunoassay can be used to detect OpNPV infections prior to host mortality, which indicates that the method can be used to accurately measure virus incidence in field populations even when the pathogen infections are at earlier stages of disease development. Finally, we have demonstrated that larval samples require minimal preparation for unambiguous detection of OpNPV infection by dipstick immunoassay.

Chapter 5 – Virus load and persistence in baculovirus infected Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunough) (Lepidoptera:Lymantriidae) larvae.

5.1 Abstract

Sublethal infections by baculoviruses may play an important role in modifying host populations by affecting larval development and adult fecundity, and they may also significantly contribute to the persistence and transmission of baculoviruses when host populations are low. Currently, the quantity of virus that constitutes a sublethal infection is not known and therefore it is difficult to determine the incidence of these infections. In this chapter, we measured the differences in virus load that led to persistent sublethal infections and lethal infections. We defined the virus load as the number of OpNPV PIBs per tested larval homogenate. Virus loads in larvae that survived past the peak mortality wave had significantly different virus loads than larvae that had been sacrificed during the peak mortality wave (P=0.004, Welch's *t*-test, Mann-Whitney *U*-test). The virus load (1750-2350 PIBs) of these persistent infections had low variance (σ^2=0.000, n=5) even though they were detected over four instars. We used this limited range to estimate the incidence of 'persistent sublethal' infections. Persistent sublethal infections were detected in 2.6% of DFTM larvae challenged with 9.5 PIBs/larva, and 6.8% and 8.3% of DFTM sampled from a field population that was in the declining phase of an outbreak cycle. This is the first known attempt to define and differentiate virus loads that may persist in lepidopteran hosts without causing host mortality, from virus loads that may become lethal, and their differentiation may contribute to more accurate understanding of the incidence and influence of persistent sublethal baculovirus infections on host populations.

5.2 Introduction

The population densities of many forest defoliating insects are regulated by baculovirus pathogens (Stairs, 1972; Fuxa, 1987; Tanada and Fuxa, 1987; Myers, 1988; Moscardi, 1999). A small proportion of baculoviruses produce lethal infections that may be used to predict the fitness of future host populations (Anderson and May, 1981). Increasing evidence has suggested that sublethal infections also regulate pest populations through changes to larval behaviour, development, and fecundity (Sait *et al.,* 1994b; Goulson and Cory, 1995; Myers and Kukan, 1995; reviewed in Rothman and Myers, 1996; Myers *et al.,* 2000; Duan and Otvos, 2001). Several authors have argued that sublethal infections should be incorporated into predictive population models to more accurately predict pest populations (Régnière, 1984; Hochberg, 1989; Ginzberg and Taneyhill, 1994; Boots, 1999; Boots and Norman, 2000). Thus, the detection of sublethal and lethal infections (and the distinction of each) is important for predicting host populations.

The identification of sublethal infections may be used to understand baculovirus disease transmission. Viruses can be transmitted horizontally within a generation by feeding on contaminated foliage, or vertically between generations from parent to progeny (Swaine, 1966; Neelgund and Mathad, 1978; Fuxa and Richter, 1991; Hughes *et al.,* 1993, 1997; reviewed in Kukan, 1999; Fuxa *et al.,* 2002). When host populations are high, theory predicts that horizontal transmission of virus disease dominates. When host populations are low, however, it is predicted that vertical transmission prevails (Anderson and May, 1981). It is thought that some larvae are resistant to viral disease, develop into adults, and transmit the virus infection vertically to their progeny. Most progeny become infected upon eating through contaminated egg masses (Murray and Elkinton, 1989), but some progeny may receive viral infection transovarially (Longworth and Cunningham,

1968; Hughes *et al.,* 1993, 1997; Burden *et al.,* 2002; 2003; Cooper *et al.,* 2003). By identifying the frequency of sublethal infections, one may determine its impact on viral transmission and host population dynamics.

The identification of sublethal infections may also be used to understand baculovirus persistence. Baculovirus epizootics correspond with many lepidopteran pest population outbreaks (Fuxa, 1987; Myers, 1988; Moscardi, 1999), but baculovirus incidence between population outbreaks varies between infrequent to absent (Bird and Elgee, 1957; Sait *et al.,* 1994a; Kukan and Myers, 1997). This observation has led researchers to search for, and theorize about, mechanisms of baculovirus persistence. Baculovirus virions are protected from the environment within a proteinaceous matrix and are reintroduced into the pest population when insects consume virus-contaminated foliage. This 'sit and wait' mechanism of transmission is predicted to be supplemented by vertical transmission, particularly when larval host populations are very low (Rothman and Myers, 1996). The sublethal vertical transmission theory provides a mechanism for dispersal of disease, and a mechanism for baculovirus persistence in host populations.

To assess the relative impact of sublethal and lethal baculovirus infections on host population dynamics it is necessary to detect and distinguish them. PCR detection methods have been used to detect latent (hidden) baculovirus infections in some lepidopteran insects (Hughes *et al.*, 1993, 1997; Cooper *et al.,* 2003). PCR confirmation of infection, however, does not differentiate between infections that will result in mortality and infections that may persist as overt (sublethal) or covert (latent) infections. Phenotypic effects (larval growth rate, body mass, adult fecundity) may distinguish lethal, sublethal, and latent infections, but multiple factors may produce similar

phenotypes. It is therefore important to understand what level of virus infection results in latent, sublethal or lethal outcomes.

Antibody-based detection of baculovirus infection has been shown to be relatively sensitive and highly specific. Our work has shown that an ELISA system using OpNPV-specific antibodies is appropriate for measuring the incidence of virus infection because it is sensitive, specific, unambiguous, and can accurately quantify semi-purified OpMNPV PIB proteins (Chapter 2). The quantification of baculovirus PIB proteins may allow the distinction between lethal infections from those infections that may persist as sublethal infections. The ELISA method may also be appropriate because we have shown that it is appropriate for large-scale analysis necessary for empirical population studies or in studies that test theoretical models (Chapter 4).

The objectives of this study were to 1) quantify the virus load associated with persistent sublethal OpNPV infections and the virus load sufficient to cause a lethal infection, 2) estimate the incidence of persistent sublethal infections in laboratory and field DFTM populations, and 3) describe some of the sublethal effects of OpNPV infections.

5.3 Methods and Materials

5.3.1 Laboratory reared Douglas-fir tussock moth larvae

DFTM larvae were raised from virus-free, laboratory strain (Goose Lake) DFTM egg masses, which were decontaminated with a 2% bleach solution (Javex) and hatched in 150 x 15 mm petri plates, at 25 °C, 50% RH and 16:8 photoperiod. Upon hatching (approximately 2 weeks), larvae were reared in groups of 10 in 100 x 15 mm petri dishes containing a piece of artificial diet (Thompson and Peterson, 1978). Larvae were reared

to 2nd instar, and then moved to fresh petri dishes and starved for 24 hours prior to infection.

5.3.2 Viral Infection Method

Fresh virus (produced by infecting larvae with TM Biocontrol-1 containing OpMNPV PIBs) was produced, semi-purified (Section 2.3.1) and stored at –4 °C. Diet plugs were made from a sheet of DFTM diet (Kaupp and Ebling, 1990). Each plug (4.4±1 mg) received one LD_{50} dose (9.5 PIBs/larva, Kukan and Otvos, 2001) in a total volume of one µl. Equal distribution of infective PIBs was maintained by periodic vortexing of the infective solution. Second instar DFTM larvae were starved for 24 hours and transferred to individual wells of a 24-well culture plate with a diet plug containing OpMNPV (treatment) or distilled water (control). A total of 1260 larvae were used as controls and 2520 larvae were treated. Only larvae that consumed their entire diet plug within 24 hours were used in the current study. Twelve control and 12 treated larvae were randomly selected and frozen individually (day one) for analysis. This process was repeated until day 35 (until all larvae had been sampled, had died of viral infection or pupated). Larvae that died prior to selection were collected each day (frozen in their solo cups), and the mortality was recorded. Viral-induced mortality was confirmed or refuted by ELISA and PCR.

5.3.3 Field collection of DFTM larvae

Douglas-fir tussock moth defoliation is mapped by aerial surveys of stands deemed to be at risk based upon previous defoliation events (Otvos *et al.,* 1998). Visible defoliation of Douglas-fir trees was identified at Scotty Creek, BC (about 18.5 km north of Cache Creek) by surveys conducted in July/August 2003. During fall ground surveys in 2003, a

low density mixed infestation of DFTM egg masses and empty western spruce budworm pupal cases were observed on the trees with visible defoliation. The low density of DFTM egg masses, relative to the high level of defoliation, indicated that the DFTM population was in a declining phase, and therefore the virus incidence was predicted to be high the following year. Upon return to the site the following year on July 9th, 2004, there were no visual symptoms evident of nucleopolyhedrovirus disease in any of the larvae (*i.e.* no dead larvae were hanging from branches, therefore suggesting a lack of viral disease). Field larvae were collected (Sample one, n=300) and reared *en masse*, in a controlled laboratory setting for two weeks, then larvae were sacrificed on July 23, (Sample 1a, n=17) for identification and quantification of possible virus disease. A second collection was made on July 22nd (Sample two, n=81), two weeks after the first collection from the same site. In contrast to the first sample collection, there were clear visual symptoms of nucleopolyhedrosis disease in the field larvae collected on July 22nd (dead larvae were found hanging from branches, and some larvae appeared sluggish). Seventy-six (76) live and 14 dead larvae were collected, transported to the laboratory, and then frozen prior to quantification of virus load. In this study, virus load was defined as the number of OpNPV PIBs per tested individual larval homogenate. The virus load that resulted in mortality and the virus load of larvae that were sacrificed were compared.

5.3.4 Temporal influence on the virus load in field-collected DFTM larvae

Larvae from sample one were reared in a virus-free laboratory environment to analyze the development of virus load in DFTM larvae. The collected larvae were transported back to a laboratory at the Pacific Forestry Centre, Victoria, B.C. and reared *en masse* (at 25°C, 50% RH and 16:8 photoperiod) on foliage disinfected with 2% bleach solution

(Javex). Larvae were reared to July 23rd, n=17, July 29, n=7, and Aug 3, n=2. The objective of this experiment was to measure the incidence and virus load over time. Specifically, we wanted to determine if low-level virus loads persisted within the field population, or if low-level infections developed to levels indicative of lethal infections.

5.3.5 ELISA based detection of OpMNPV and definition of the virus load

DFTM larvae were macerated with autoclaved sea sand, sterile 1000 µl pipette tips, and an equal volume: larval weight, STE-C buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 10 mM cysteine). The tissue homogenate was diluted 1:10 with PBS and incubated (100 µl/well) in polystyrene plates overnight at 4°C and the remainder was stored at -20°C for later use in PCR reactions or ELISA replicates. ELISA-based detection was performed as previously described (Section 3.3.3.5). The measured A650 value and corresponding estimated PIB number (Figure 2.2) was used as a measure of 'virus load' of a given larva. Virus incidence was confirmed by PCR as previously described (Section 3.3.3.3).

5.3.6 Statistical analysis

5.3.6.1 Sublethal effects of baculovirus challenge on laboratory-reared DFTM larvae

Previously it has been reported that baculovirus infection that does not result in mortality may have detrimental effects on larval growth and adult fecundity (*i.e.* sublethal effects). Eight larvae were weighed individually per day from day one to 10 post ingestion (n=80). The effect of infection on the development of larvae that survived virus challenge was analyzed (Microsoft Excel) to view assumptions of normality and directionality of skew for analysis of covariance. Data was log transformed to conform

to assumptions of the Analysis of Covariance (ANCOVA). ANCOVA was performed to examine the effect of virus infection on the development of 2nd instar DFTM larvae between day 1-10 post-ingestion (SPSS).

5.3.6.2 Statistical analysis of the difference between the virus load in lethally infected and sacrificed larvae

OpNPV infections in sacrificed and dead larvae (greater than or equal to 2.5 standard deviations above the average absorbance of control DFTM larvae, n=80) were grouped and compared graphically using box plots to highlight skew and median absorbance values (Microsoft Excel). Appropriate statistical tests were determined from graphical observations of normality and skew and simple tests of data. Levene tests of homogenous variation were performed to confirm or refute homogenous variation for standard tests of variance, *i.e.* ANOVA (SPSS). Data with non-homogenous variance, *i.e.* data that failed Levene tests were analyzed by robust variance tests. Welch's *t*-tests were performed for data with non-homogeneous variance but balanced skew and Mann-Whitney *U* tests were performed for non homogenous data with bidirectional skew (SPSS).

5.3.6.3 The development of a criterion to differentiate between the virus loads in persistent sublethal infections and infections that result in mortality.

The virus load of OpNPV infections in larvae that were sacrificed during the peak mortality wave, and in larvae that were sacrificed prior to and after the peak mortality wave, were grouped and compared by appropriate statistical tests (Section 5.3.6.2). The criterion (virus load) that was present after the peak mortality wave was monitored for variance to confirm or refute that these infections persisted, and did not increase over

time. The incidence of "persistent sublethal" infections were determined by the number of larvae with virus loads within the defined criterion.

5.4 Results

5.4.1 Mortality of laboratory-reared DFTM larvae

Based upon viral dose, we expected 50% mortality of second instar larvae, but only 26% mortality (27.4%-1.4% control) was observed (Table 5.1). A bimodal pattern of mortality was present in larvae which ingested 9.5 OpMNPV PIBs (Figure 5.1). As observed with many lepidopteran insects, virus infection resulted in peak mortality (80% of total mortality observed) between 5-11 days post-ingestion (Figure 5.1). The second mortality wave coincided with the start of pupation.

5.4.2 Incidence of OpMNPV infection (controlled laboratory experiment)

A unique feature of our study was that we confirmed and quantified the virus load that resulted in larval mortality. We found that 91% of larvae that died from day 5-11 contained OpMNPV PIBs (Figure 5.2, Table 5.2). Furthermore, we found that 5% of sacrificed larvae were infected with OpMNPV. Infections in sacrificed larvae were detected as early as day 3, and as late as day 24 post-ingestion (n=14), but the majority of infections in sacrificed larvae (64%) were detected during the peak mortality wave (n=9) (Figure 5.2). In contrast, we did not detect virus infections in insects that died during the second wave of mortality that coincided with the start of pupation (Figure 5.1, Table 5.2).

Table 5.1. Mortality of DFTM larvae treated with LD_{50} dosage of OpMNPV

	# Assayed	# Dead	# Live	% Mortality
Treated Larvae	840	230	610	27.4%
Control Larvae	840	12	828	1.4%
Corrected Experimental Mortality				26%

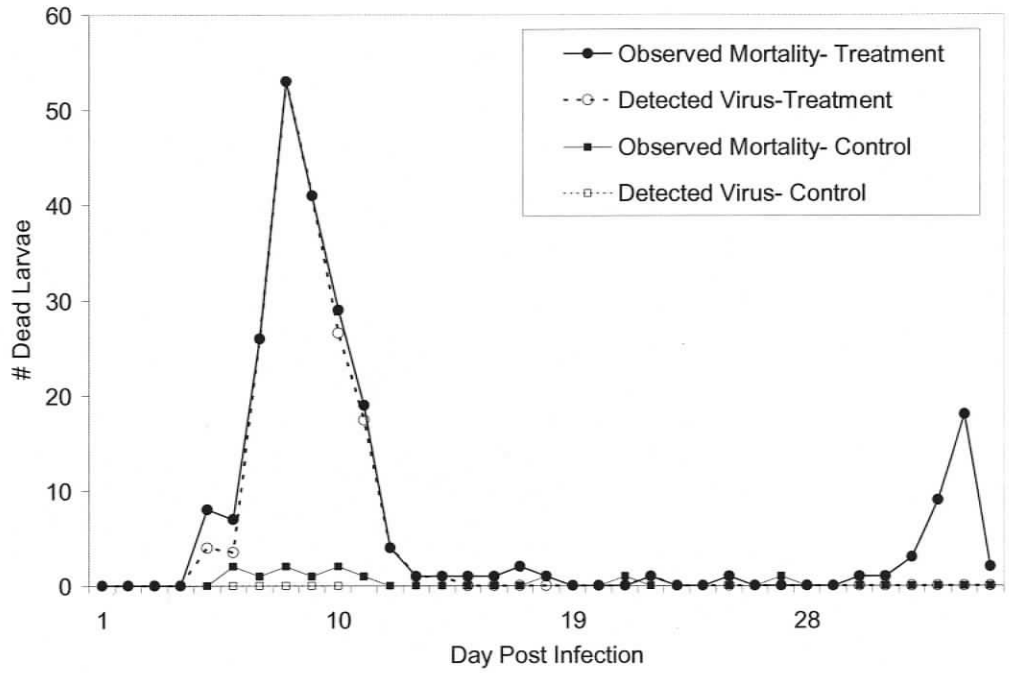


Figure 5.1. Bimodal mortality pattern of DFTM larvae treated with OpMNPV. DFTM larvae (n=840, 12/day) were infected with LD_{50} doses of TM Biocontrol-1 containing OpMNPV PIBs (9.5 PIBs/larva). Control DFTM larvae (n=840, 12/day) were mock infected with sterile ddH_2O. Larvae were assayed for OpMNPV infection by ELISA (Section 2.3.4).

Table 5.2. Detectable OpMNPV infections in DFTM larvae reared in the laboratory

	# Examined	# Infected	Incidence[1]	Incidence during peak mortality [2]	Incidence during pupation
Treated, Live	272	14	5%	64%	0%
Treated, Dead	90	55	61%	91%	0%

[1] Incidence of virus infection in sacrificed and dead larvae.

[2] Percentage of larvae with OpMNPV infections during the peak mortality wave (day 5-11 post-ingestion)

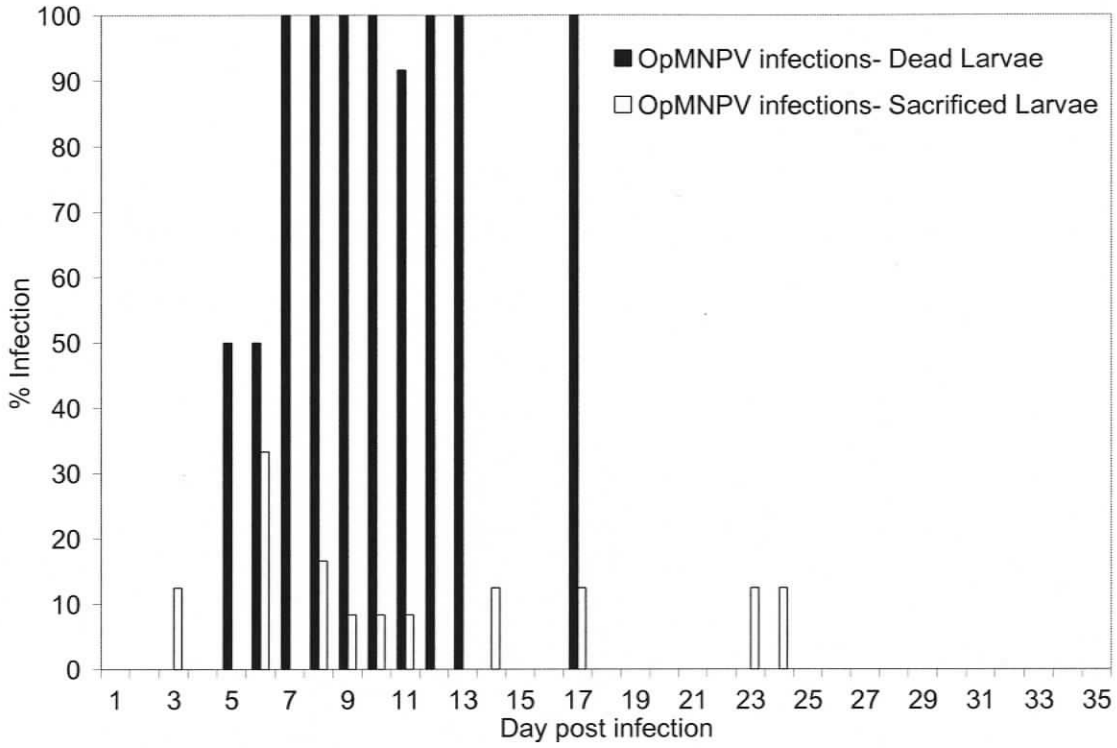


Figure 5.2. Incidence of detectable OpMNPV infection.

DFTM larvae were assayed by ELISA for the presence of OpMNPV PIBs (n=8/day sacrificed larvae; minimum n=6/day dead larvae). Positive infection was identified A650 values equal to or greater than 2.5 standard deviations above the mean A650 of uninfected DFTM larvae (n=80).

5.4.3 Effect of OpMNPV ingestion on DFTM larval weight

DFTM larvae (n=8/day) that survived the ingestion of 9.5 OpMNPV PIBs from days 1-10 post-ingestion were weighed to determine if OpMNPV infection altered larval development. We observed an increase in log larval weight over time for both control and treated larvae ($F= 397$, $P= 4.80 \times 10^{-41}$, ANCOVA) (Figure 5.3). Although control larvae appeared to exhibit a greater rate of growth than virus-treated larvae ($m= 0.1319$ versus $m=0.1115$, Figure 5.3) this difference was not statistically significant ($F=0.809$, $P=0.666$, ANCOVA).

5.4.4 Virus load in dead and sacrificed DFTM larvae- laboratory experiment

DFTM larvae that were positive for infection (had virus loads which measured greater than 2.5 SD than uninfected DFTM larvae) were analyzed. The virus load of dead and sacrificed larvae was compared to determine the minimum virus load required for mortality and to determine if virus loads were significantly different. The quantity of PIBs in larvae homogenates was estimated by the linear regression between A650 and PIB number ($R^2=0.96$) (Figure 2.2). The virus loads detected in sacrificed larvae were compared to the virus loads associated with larval mortality (Figure 5.4). This comparison highlighted significantly greater variance in the virus load of sacrificed larvae compared to the virus load associated with larval mortality (0.24 versus 0.045). The virus loads of sacrificed larvae were significantly lower than the virus loads that resulted in larval mortality ($P=0.004$, Welch's *t*-test, $P=0.002$, Mann-Whitney *U* test; Figure 5.4). A slight majority of these infections (57%) contained less than the minimum virus load required for mortality in laboratory reared larvae ($<1.86 \times 10^4$ PIBs).

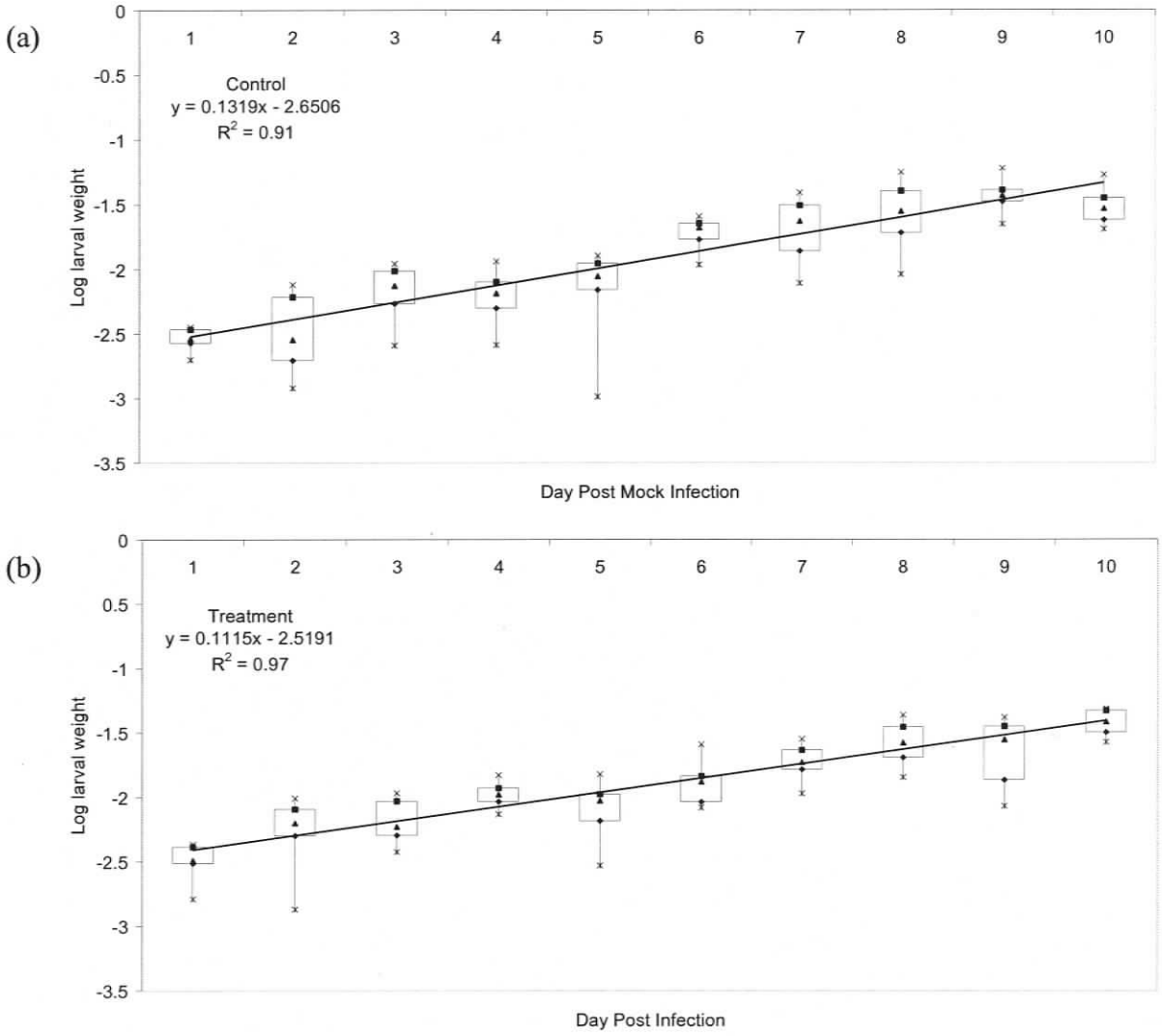


Figure 5.3. Linear regression of log DFTM weight over day post-ingestion. Median data points were derived from sample size (n=8), whiskers at maximum (x) and minimum (ж) log weight values represent direction of skew for each time point. Linear trend line was derived from log median scores and shows that control larvae had a faster growth rate than virus-treated larvae (m= 0.1319 versus m=0.1115), but this difference was not statistically significant (F=0.809, P=0.666, ANCOVA). (a) DFTM Larvae treated with LD_{50} dose of OpMNPV (b) DFTM larvae mock-infected with sterile ddH_2O.

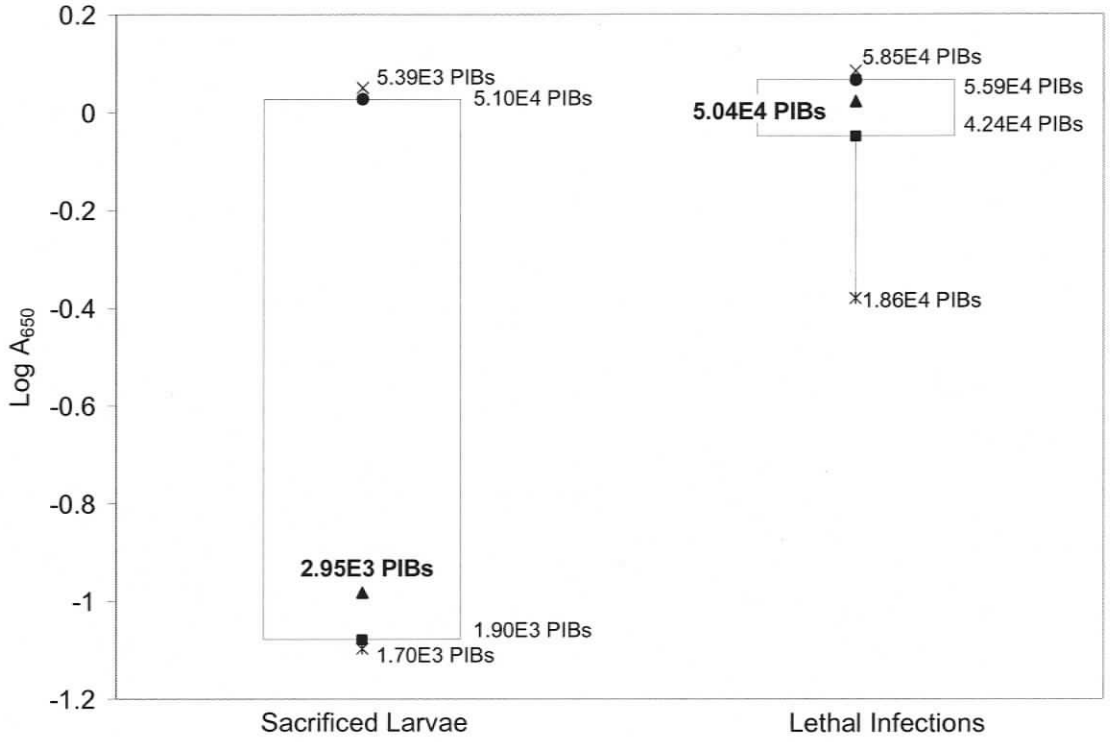


Figure 5.4. Comparison of the virus load in sacrificed and lethally infected larvae. Log transformation of data allowed comparison by robust analysis of variance. The virus load between sacrificed larvae and lethally-infected larvae was significantly different (P=0.004, Welch's *t*-test; P=0.002, Mann-Whitney *U* test). PIB numbers at the minimum (ж), 25th percentile (■), median (▲), 75th percentile (●) and maximum (x) were estimated using a linear regression model between A650 and OpMNPV PIBs (R^2=0.99, Figure 3b).

5.4.5 Virus load in sacrificed larvae at the peak mortality wave

Virus infections were grouped into 'peak' and 'non-peak' groupings because 67% of the larvae sacrificed during the peak mortality wave (day 5-11 post-ingestion) had virus loads theoretically sufficient to cause mortality. In contrast, no larvae that were sacrificed before or after the peak wave (day 3, 14-24) had virus loads sufficient to cause mortality. We thus hypothesized that these larvae contained 'persistent sublethal' infections. The virus load of larvae in the non-peak grouping was significantly lower (1750 PIBs versus 4.84×10^4 PIBs) and had lower variance (0.000 versus 0.231) than the virus load of larvae sacrificed during the peak mortality wave (Figure 5.5). The virus load of 'non peak' larvae indicated that they represented a distinct population from 'peak' larvae (P=0.004, Welch's *t*-test, Mann-Whitney *U*-test). Virus loads in larvae that persisted beyond the peak mortality wave ranged from 1700-2350 PIBs and did not increase over four instars unlike the virus load in larvae sacrificed during the peak mortality wave (Figure 5.6).

5.4.6 Incidence of OpNPV infection (field collected larvae)

Virus infection of field collected larvae was detected and quantified by ELISA (Figure 5.7). Virus infection was confirmed where the A650 value was greater than 2.5 standard deviations above the median A650 of laboratory propagated uninfected DFTM larvae (n=80). The incidence of OpNPV of sacrificed field larvae from sample one and two (larvae collected from an outbreak population on July 9th and 22nd), were 100%, and 66%, respectively, although the median virus load suggested that the viral disease was at earlier onset in sample 1 than in sample 2 (median= 9.24×10^3 PIBs versus 3.63×10^4 PIBs) (Table 5.3).

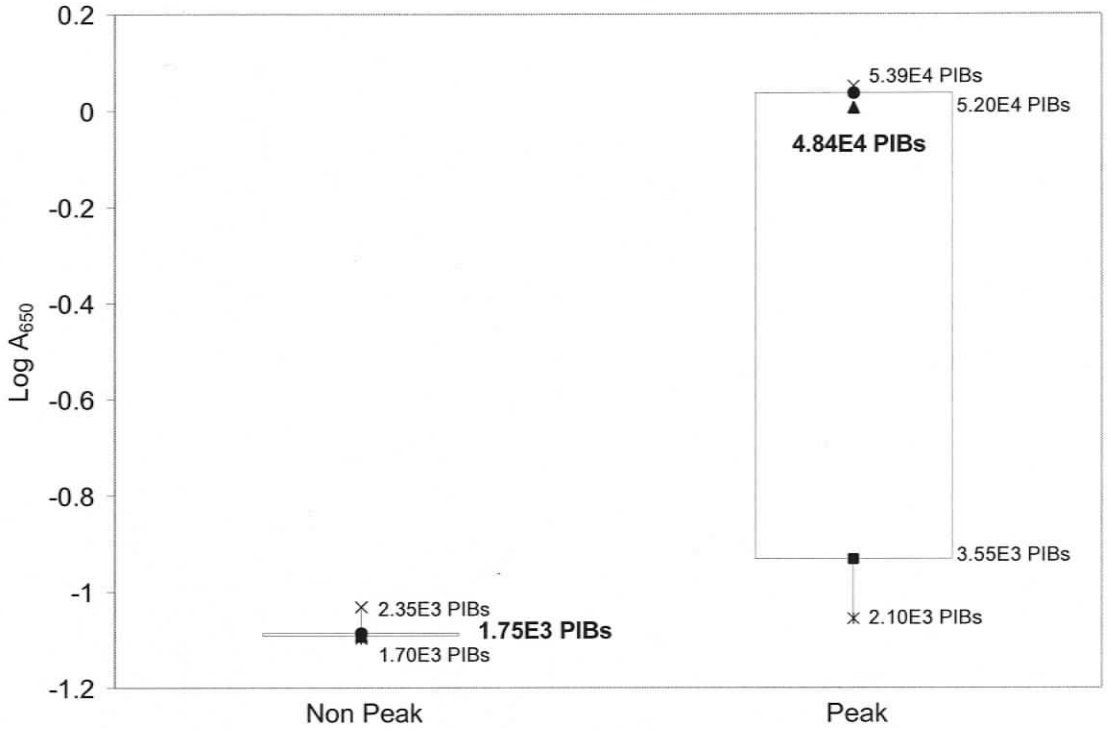


Figure 5.5. Virus load in larvae during peak and non-peak mortality periods Median values (▲) were determined from samples where absorbance values were > 2.5 standard deviations above the average DFTM control absorbance (n=80). Log transformation of data did not resolve bidirectional skew therefore comparisons were made by Mann-Whitney *U* tests. The virus load in larvae sacrificed during the peak mortality wave (day 5-11) was significantly different from the virus load in 'non-peak' larvae (P=0.004, Mann-Whitney U-test). PIB numbers at the minimum (ж), 25th percentile (■), median (▲), 75th percentile (●) and maximum (x) were estimated using a linear regression model between A650 and OpMNPV PIBs (R^2=0.99, Figure 3b).

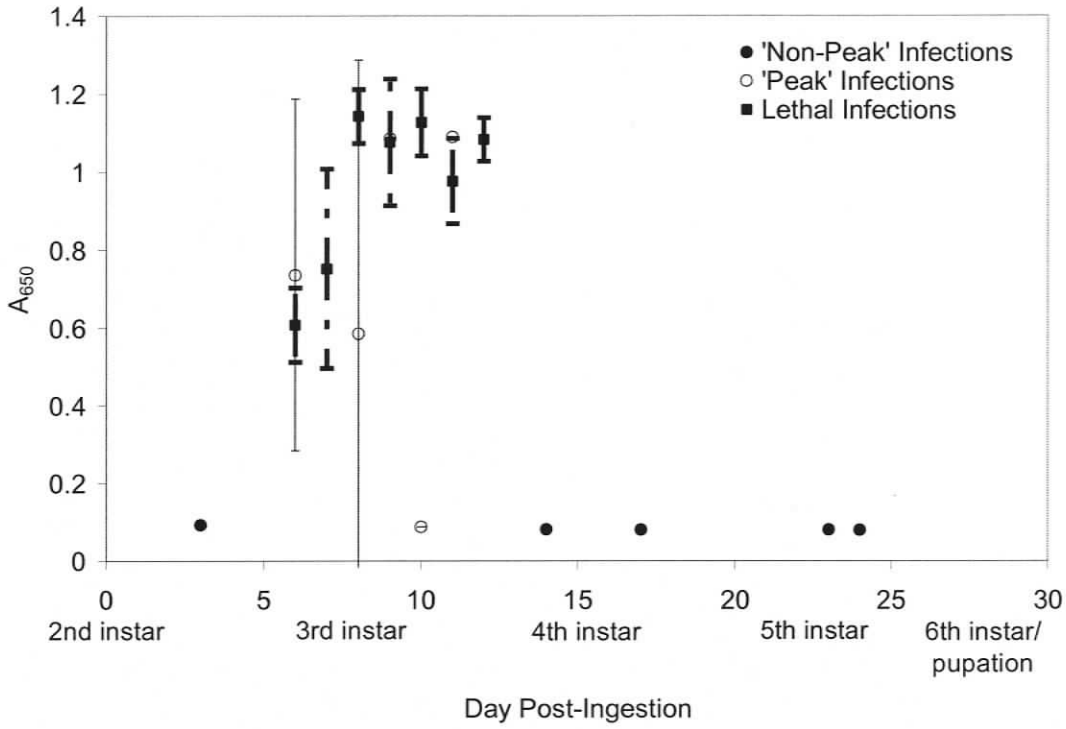


Figure 5.6. Virus load in larvae during peak and non-peak mortality periods over time Median values (●, ○, ■) were determined from larvae where OD values were > 2.5 standard deviations greater than the average DFTM control OD (n=80). Stage (instar) was estimated at 7 days/instar. Infected larvae were grouped into the 'peak' grouping if they were sacrificed during the peak mortality wave and 'non-peak' if they were sacrificed prior to or after the peak mortality wave (Figure 5.1). The virus load in 'non-peak' larvae persisted, and showed low variance (0.000), as opposed to 'peak' larvae that had virus loads with high variance (0.231) that significantly increased over time.

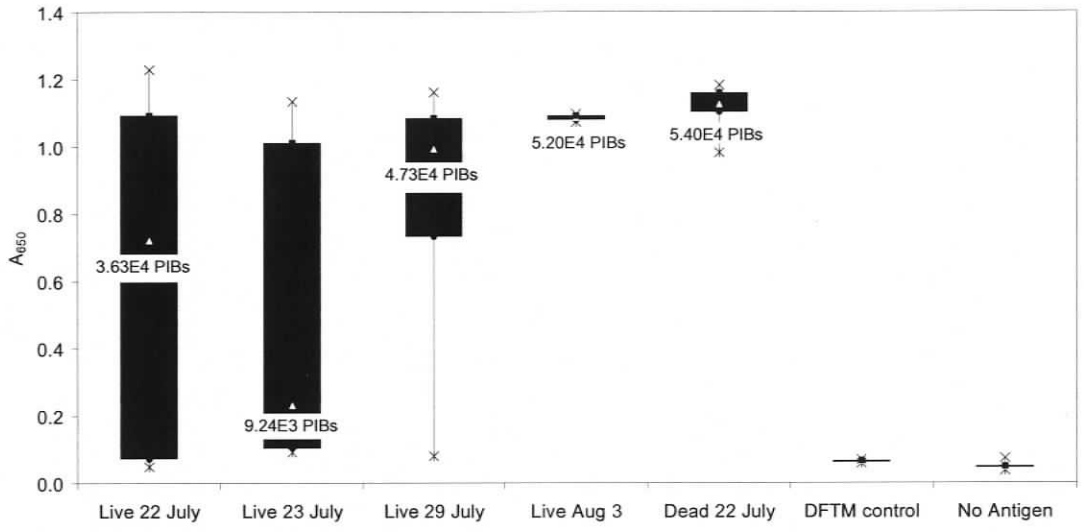


Figure 5.7. Relative quantity of OpNPV infection in field collected DFTM larvae. Virus infection was quantified with an OpNPV-specific antibody in an ELISA. Median values (Δ) represent OD values for a given day: July 22nd (n=67), July 23rd (n=17), July 29th (n=7), Aug 3rd (n=2), lethal infections (n=14), DFTM negative control larvae (n=7). Whiskers highlight direction of skew. PIB numbers at the median (Δ) were estimated using a linear regression model between A650 and OpMNPV PIBs (R^2=0.99, Figure 3b).

Table 5.3. Incidence and average virus load in field-collected DFTM larvae

Date	N		# Infected	Incidence	Median A650	Median PIBs
Field Sample 1						
July 22[1]	Live	67	44	66%	0.722	3.63×10^4
	Dead	14	14	100%	1.126	5.40×10^4
	Total	81	53	72%	N/A	N/A
Field Sample 2						
July 23[2]	Live	17	13	76%	0.231	9.24×10^3
July 29[2]	Live	7	6	86%	0.993	4.73×10^4
Aug 3[2]	Live	2	2	100%	1.086	5.20×10^4

[1] Field larvae collected on July 22nd (sample 1) were individually frozen prior to analysis by ELISA.

[2] Field larvae collected on July 9th (sample 2) were reared *en masse* in a controlled laboratory environment to July 23rd, July 29th or Aug 3rd, and then frozen individually prior to analysis by ELISA.

5.4.7 Comparison of the virus load in dead and sacrificed field DFTM larvae

The virus loads of field collected larvae that were sacrificed were compared to the virus loads that resulted in mortality in field collected larvae (Figure 5.8). The median virus load of sacrificed field larvae were significantly lower than the virus load that resulted in mortality (9.24×10^3 PIBs, Sample 1; 5.03×10^4 PIBs, Sample 2; versus 5.40×10^4 PIBs). The virus load in sacrificed larvae had OpNPV infections were significantly different from the virus load in larvae that died of OpNPV infections ($P=7.83 \times 10^{-05}$, P=0.025, Mann-Whitney *U* test, field sample 1, 2, respectively). Furthermore, 35% and 68% of the larvae from field sample 1 and 2 had virus loads which were sufficient to result in lethal infections (*i.e.* virus loads $>4.68 \times 10^4$ PIBs).

5.4.8 Temporal effect on the virus load in field populations of the DFTM

The median virus load in first field sample increased over time (9.24×10^3, 4.73×10^4, 5.20×10^4 PIBs) (Figure 5.9) and the variance in the virus load decreased over time (0.21, 0.14, 0.000) as the virus load of the majority of the larvae increased towards quantities sufficient for mortality (35%, 57%, 100%). As well, the differences between the virus load of sacrificed larvae and the virus load that resulted in mortality was reduced over time ($P=7.8 \times 10^{-5}$, 0.025, 0.200, Mann-Whitney *U*-test).

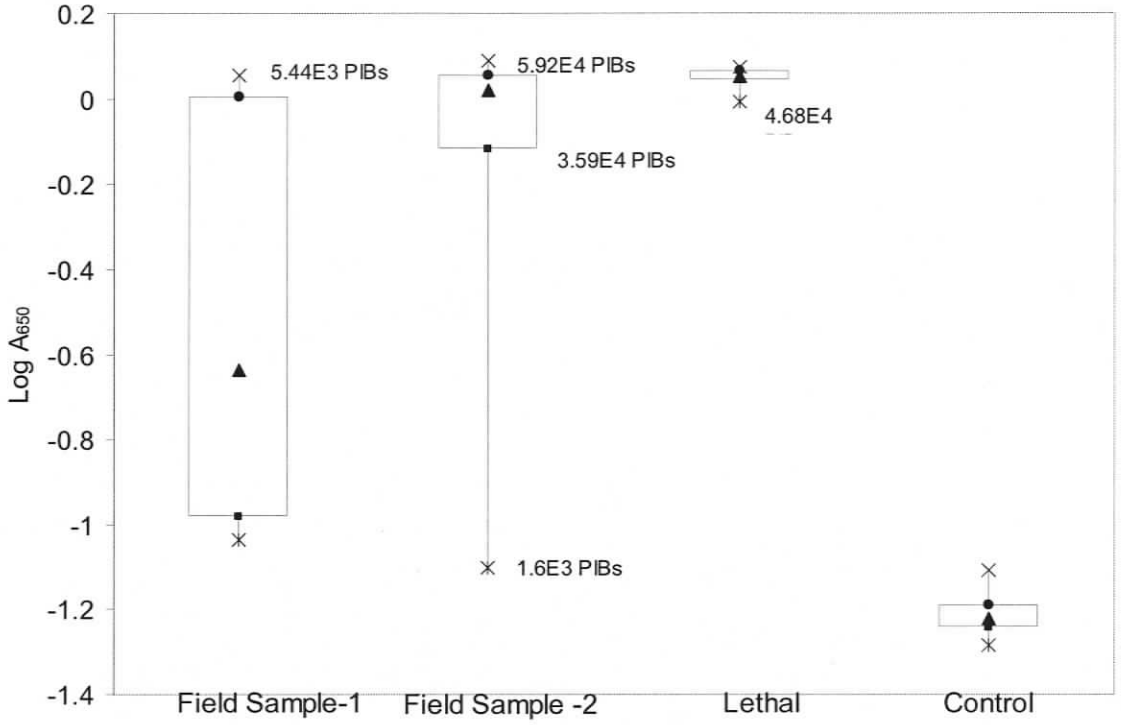


Figure 5.8. Comparison of the virus load in dead and live DFTM field larvae

Larvae were collected from Scotty Creek, B.C. on July 9th, 2004 and reared in the laboratory to July 23rd (Sample 1), and collected from the field on July 22nd (Sample 2). The virus load that resulted in death to larvae (n=14) and the virus load within sacrificed larvae (n=17, n=44, sample 1, 2, respectively) were compared. Log transformation of the data corrected skew for robust analysis of variance tests. The virus load within sacrificed larvae was significantly different from the virus load that resulted in mortality ($P=7.83 \times 10^{-05}$, P=0.025, Mann-Whitney *U* test, Sample 1,2 respectively). PIB numbers at the minimum (ж), 25th percentile (■), median (▲), 75th percentile (●) and maximum (x) were estimated using a linear regression model between A650 and OpMNPV PIBs (R^2=0.99, Figure 3b).

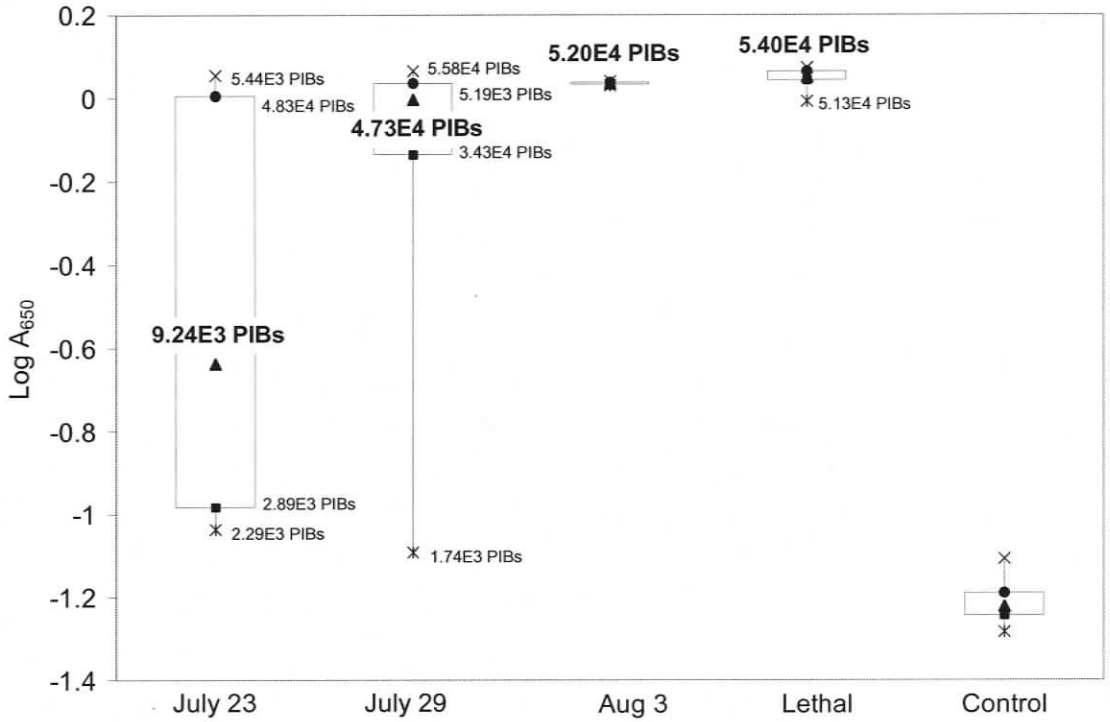


Figure 5.9. Comparison of virus load in dead and live DFTM field larvae over time DFTM larvae were collected on July 9th from the field and reared in a virus-free environment to July 23rd (n=17), July 29th (n=7) and Aug 3rd (n=2). Dead larvae with lethal infections were collected from the field on July 22nd (n=14). The significance of differences between the virus load in sacrificed larvae and virus load in larvae that died of OpNPV infection was reduced over time ($P=7.8 \times 10^{-5}$, 0.014, 0.200, Mann-Whitney *U*-test). PIB numbers at the minimum (ж), 25th percentile (■), median (▲), 75th percentile (●) and maximum (x) were estimated using a linear regression model between A650 and OpMNPV PIBs ($R^2=0.99$, Figure 3b) and highlight the progression of the virus load in sacrificed larvae to virus loads sufficient for DFTM mortality.

5.4.9 Estimation of "persistent sublethal" infections in DFTM populations

Given that the virus load in the larvae that were sacrificed from days 12-24 did not deviate from a limited range of virus load (1700-2350 PIBs) and because the virus load did not increase over time (Figure 5.6), we hypothesized that these larvae contained 'persistent sublethal' infections. Furthermore, since the virus load in larvae that were sacrificed during the peak mortality wave showed a significant increase in virus load over time (as seen with lethal infections), we used the range of virus loads present in 75% of the time (virus loads > 3.55×10^3 PIBs, Figure 5.5) to estimate the frequency of infections that were sufficiently large to result in mortality. Outliers in this group were considered putatively persistent infections. We have used these criteria estimate the incidence of 'persistent sublethal' infections that may be transmitted to future DFTM generations (Table 5.4).

Table 5.4 Persistent virus loads in laboratory and field populations of DFTM larvae

	Laboratory Day 1-30	Field Sample 1	Field Sample 2
% Persistent[1]	2.6%	6.8%	8.3%
Total Assayed	9	26	44

[1] The incidence of potential persistent infections was determined by applying measured virus loads in sacrificed larvae from laboratory and field populations against the criterion (range of virus loads) that were present in persistent infections in a controlled laboratory experiment. Virus loads were measured using an OpNPV-specific monoclonal antibody in an indirect ELISA.

5.5 Discussion

5.5.1 Persistent sublethal infections

We developed a method to quantify and identify persistent sublethal baculovirus infections for the first time. To date, sublethal infections of insect hosts by baculovirus pathogens have been measured by their phenotypic effects and not the infection themselves. The rationale for studying the effect and not the cause may be a lack of methods to accurately measure a 'sublethal' infection or because the phenotypic effects may contribute to more practical knowledge about the outcome of host populations. Burden *et al.* (2003) argue that the proof of sublethal infection is elusive because the infection may develop into an overt infection, which may result in mortality. Measurement of the effect rather than cause, however, may not accurately estimate the influence of a sublethal infection since multiple causes may contribute to similar phenotypic effects. Furthermore, a generalized pattern of sublethal effects is weakened by inconsistencies and variation in the effects both between hosts (Patil *et al.,* 1989; Perelle and Harper, 1986; Murray *et al.,* 1991; Myers *et al.,* 2000) and within hosts (Sait *et al.,* 1994b), and therefore calls for revised methods for the quantification of sublethal infections.

Predictive population models depend on accurate measurement of parameters such as mortality, disease transmission and sublethal infection. To contribute to our understanding of sublethal infections we have developed a method to differentiate between a 'sublethal persistent' infection from an infection that may result in mortality based upon their virus load. Furthermore, the ELISA method has allowed us to estimate the disease incidence which will result in mortality without waiting for this phenotypic

outcome. This may therefore contribute to more accurate measurement of baculovirus transmission within a generation and sublethal infections (and potential vertical transmission to future generations) which is used for predicting future host populations.

We found that virus loads consistent with persistent sublethal infections were present in laboratory and field populations of the Douglas-fir tussock moth larvae. DFTM larvae (2.6%) that were challenged with baculovirus ingestion in a controlled laboratory experiment maintained a low-level virus infection which did not significantly increase or decrease over four instars (2^{nd}-5^{th}). Virus loads consistent of persistent sublethal infections were also present in two field samples. Given that the frequency of these infections persisted in the field population reared in the laboratory (sample one) to July 29^{th}, and that only two larvae were tested for virus infection for August 2^{nd}, it appears the persistent sublethal infections can exist in field populations of the DFTM. It is important to note, however, that larvae with detectable virus infections from field sample two had significantly higher doses of infection (on average) than laboratory-reared field sample one, which may suggest that field larvae are subject to multiple infection events (Murray and Elkinton, 1989). This may limit the occurrence of persistent sublethal infections in natural environments (*i.e.* rearing field-collected larvae in the laboratory may have resulted in the overestimation of the natural frequency of persistent sublethal infections in DFTM larvae).

Our work has established a new method to study lethal and persistent sublethal infections caused by baculoviruses in lepidopteran larvae. We found that we can follow the progression of initial low-level infections to virus loads indicative of mortality. This method will allow other researchers to accurately determine the incidence of baculovirus

infections that result in mortality. More importantly, we have shown for the first time that there is a virus load that may be maintained in DFTM larvae without mortality.

5.5.2 Phenotypic effects of baculovirus challenge

We found that laboratory reared DFTM larvae that were challenged with a low dose of OpMNPV exhibited some of the phenotypic effects described previously in the literature. The general phenotypic effects of survivors of baculovirus challenge include lower larval and pupal weights, increased development time, reduced fecundity and increased mortality at pupation (reviewed by Rothman and Myers, 1996). We found that there was a significant increase in mortality at pupation in virus-treated larvae compared to control larvae. As well, we noted a decrease in the development rate of virus-challenged larvae compared with control larvae, although this difference was not statistically significant.

We did not identify OpMNPV infection in treated larvae killed at the start of pupation, although previous studies have suggested (but have not tested) that increased mortality at pupation is an effect of sublethal infections (Rothman and Myers, 1996). All samples were divided for detection by ELISA and PCR and this division reduced the initial ingested dose to levels below our detection (Chapter 4). It is possible that a low-level infection was present, but at levels lower than our minimum detection levels.

Alternatively, it is possible that we did not identify virus in these larvae because the insects resisted viral infection, and removed virus by releasing infected cells into the midgut during development (Keddie *et al.,* 1989). The energy required by insects' to clear virus infections may have resulted in their loss of fitness (Washburn *et al.,* 1996) and thus their mortality at pupation. This explanation suggests that the identification of a sublethal effect is distinct from a sublethal infection that may be transmitted to future

generations. If the frequency of sublethal effects does not represent the frequency of sublethal infections (and a potential source of virus for future generations) then the perceived influence of sublethal infections on host populations may be less than what was previously estimated.

Sublethal infections are hypothesized to provide a mechanism for the persistence of baculoviruses at low host populations and for vertical transmission of virus disease between generations. Previous studies have identified latent (covert) baculoviruses that persist in both laboratory host colonies (Hughes *et al.,* 1993, 1997) and in field populations (Burden *et al.,* 2002; Cooper *et al.,* 2003) and that may be selected for vertical transmission. Given that larvae were sacrificed for measurement of their virus load, our results are only an estimate of the potential inoculum for future generations, *i.e.* we do not know if larvae with these infections can produce viable progeny or if the virus disease would be transmitted to progeny. To determine if the virus load of the 'persistent sublethal' infections could be transmitted to future generations we would need to compare the incidence of these infections to the incidence of vertical transmission in a subset of larvae that were not sacrificed. This knowledge will further contribute to predictive population models of the Douglas-fir tussock moth.

Conclusions

Our work supports previous observations of sublethal effects and provides a novel method to estimate persistent sublethal infections that are significantly different from infections that have the potential to become lethal. Furthermore, our study suggests that sublethal effects may not be an accurate measurement of sublethal infections that may be transmitted to future generations. This is the first known attempt to define and

differentiate virus loads that may persist in lepidopteran hosts, from virus loads that may become lethal, and their differentiation may contribute to more accurate understanding of the incidence and influence of persistent sublethal baculovirus infections on host populations.

Conclusions

The theme of this research was to enhance the pest management program for the Douglas-fir tusscock moth (DFTM), *Orgyia pseudotsugata* McDunnough (Lepidoptera:Lymantriidae). The current pest management system is based upon monitoring developing outbreaks and applying naturally occurring (laboratory propagated) virus, *Orgyia pseudotsugata* multinucleopolyhedrovirus (OpMNPV) early in the outbreak cycle before significant defoliation occurs (Stelzer *et al.,* 1977; Shepherd *et al.*, 1984; Otvos *et al.*, 1987). An integral part of this pest management program involves monitoring the natural incidence of DFTM viruses in the expanding or declining insect population. The current monitoring system requires the collection of egg masses and larvae from at-risk field sites, followed by microscopic evaluation of virus particles in insect smears from emergent larvae. This system is time consuming and is only accurate to a minimum of 10^6 virus particles (Kaupp and Ebling, 1993). To enhance the current pest management strategy, we developed several detection methods to detect DFTM baculovirus pathogens.

We developed three different detection systems for determining the incidence of DFTM baculoviruses that were more sensitive than the current baculovirus detection system, microscopic counts of virus particles from macerated insect larvae (Stelzer, 1979; Kaupp and Ebling 1993). An ELISA system was developed that was specific to two DFTM pathogens, OpSNPV and OpMNPV, which are responsible for the collapse of outbreak population densities of the DFTM. The ELISA system was more sensitive and accurate than microscopic counts of virus particles.

Southern hybridization and PCR assays were developed and compared to the ELISA method by their specificity, sensitivity to semi-purified baculovirus PIBs, and sensitivity to baculovirus infections. We found that the rapid PCR assay was the most sensitive method for detecting OpMNPV PIBs, but the ELISA method was the most accurate method for identifying baculovirus infections directly from macerated insects. Southern hybridization was the least sensitive method; however, the method was more sensitive than microscopic evaluation.

An on-site detection method was developed for determining the incidence of DFTM baculovirus pathogens to allow for on-site pest management decisions. The dipstick immunoassay was as sensitive as what has been previously reported. We showed for the first time that dipstick immunoassays can be used to detect baculovirus infections directly from macerated larvae. Furthermore we demonstrated that the dipstick immunoassay is versatile, in that it can be used for qualitative or quantitative analysis of baculovirus infections. This work may contribute to enhanced pest management through on-site analysis.

Finally, we contributed to enhanced pest management of the Douglas-fir tussock moth by demonstrating that OpMNPV infections can result in sublethal effects to DFTM larvae and that sublethal infections can be identified by their virus load. Previously, it has not been noted that baculovirus infections can result in sublethal effects to DFTM. This new information provides clues to how DFTM pathogens persist and may be transmitted at low host densities. Previously, sublethal infections have only been studied by their effect rather than their infection. The novel approach for identifying sublethal infections,

described in this thesis, may contribute to a deeper understanding of sublethal infections and their effect to host populations.

In conclusion, to contribute to the theme of enhanced pest management of the Douglas-fir tussock moth, several detection methods were developed, compared and evaluated. As well, an on-site detection system was developed so that pest management decisions could be made in the field. Finally, we used ELISA to demonstrate that the incidence of sublethal infections could be measured and differentiated from lethal infections by the virus load of these infections.

Literature Cited

Ahrens C.H., Russell R.L.Q., Funk C.J., Evans J.T., Harwood S.H. and Rohrmann G.F. 1997. The sequence of the *Orgyia pseudotsugata* multinucleopolyhedrovirus nuclear polyhedrosis virus genome. *Virology.* 229:381-399.

Alfaro, R. I., Taylor, P., Wegwitz, E. and Brown, R. G. 1987. Douglas-fir tussock moth damage in British Columbia. *Forest Chronicles.* 63: 351-355.

Anderson, R. M., and May, R. M. 1981. The population dynamics of microparasites and their invertebrate hosts. *Philosophical Transactions of the Royal Society of London Ser. B.* 291:451–524.

Balch, R.E. and Bird, F.T. 1944. A disease of the European spruce sawfly *Gilpinia hercyniae* (Htg.) and its place in natural control. *Science Agriculture.* 25:65-80.

Barbehenn, R.V. and Martin, M.M. 1995. Peritrophic envelope permeability in herbivorous insects. *Journal of insect physiology.* 41:303-313.

Beckwith, R.C. 1978a. Chapter 2: Biology of the insect. pp. 25-37. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). *The Douglas-fir Tussock Moth: A Synthesis*. United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Beckwith, R.C. 1978b. Foliage damage. pp. 64-66. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). *The Douglas-fir Tussock Moth: A Synthesis*. United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Benz, G.A. 1986. Introduction: Historical perspectives. pp. 1-31. *In:* Granados, R. R. and Frederici, B.A. (Eds). *The biology of baculoviruses. Volume 1.* Boca Raton, Florida. CRC.

Bergold, G.H. 1947. Die isolierung des polyeder-virus und die natur der polyeder. *Ztschr Naturforsch Teil B.* 2:122-143.

Berryman, A. 1978. Population cycles of the Douglas-fir tussock moth (Lepidoptera:Lymantriidae): the time delay hypothesis. *The Canadian Entomologist* 110:513-518.

Bird, F.T. and Elgee, D.E. 1957. A virus disease and introduced parasites as factors controlling the European Spruce Sawfly, *Diprion hercyniae* (Htg), in central New Brunswick. *The Canadian Entomologist.* 89:371-378.

Black, B.C., Brennan, L.A., Dierks, P.M., and Gard, I.E. 1997. Commercialization of baculovirus insecticides. pp 342-354. *In* L.K. Miller (ed). *The baculoviruses.* Plenum Press, New York, NY.

Blissard, G.W. and Wenz, J. 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *Journal of General Virology.* 66:6829–6835.

Boots, M. 1999. A general host-pathogen model with free-living infective stages and differing rates of uptake of the infective stages by infected and susceptible hosts. *Research Population Ecology.* 41:189-194.

Boots, M. and Norman, R. 2000. Sublethal infection and the population dynamics of host-microparasite interactions. *Journal of Animal Ecology.* 69:517-524.

Brinbaum, S., Uden, C., Magnussion, C.G.M., and Nilsson, S. 1992. Latex-based thin-layer immunoaffinity chromatography for quantification of protein analytes. *Analytical Biochemistry.* 206:168-171.

Burden, J. P., Griffiths, C.M., Cory, J.S., and Sait, S.M. 2002. Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctella*. *Molecular Ecology.* 11:547-555.

Burden, J.P., Nixon, C.P., Hodgkinson, A.E., Possee, R.D., Sait, S.M., King, L.A., and Hails, R.S. 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. *Ecology Letters.* 6:524-531.

Cameron, E.A. 1970. Larval eclosion in the Douglas-fir tussock moth *Hemerocampa pseudotsugata*. *Annuals of the Entomological Society of America.* 63:1110-1113.

Cann, A.J. (1997). *Principles of molecular virology*, 2nd Edition. *pp.* 1 Academic Press, San Diego.

Christian, P., Gibb, N., Kasprzak, A. and Richards, A. 2001. A rapid method for the identification and differentiation of *Helicoverpa* nucleopolyhedroviruses (NPV *Baculoviridae*) isolated from the environment. *Journal of Virological Methods.* 96:51-65.

Clark, M. and Barbara, D. 1987. A method for quantitative analysis of ELISA data. *Journal of Virological Methods.* 15:213-222.

Cooper, D., Cory, J., Theilmann, D. and Myers, J. 2003. Nucleopolyhedrovirus of forest and western tent caterpillars: cross infectivity and evidence for activation of latent virus infection in high-density field populations. *Ecological Entomology.* 28:41-50.

Crook, N.E. and Payne, C.C. 1980. Comparison of three methods of ELISA for Baculoviruses. *Journal of General Virology.* 46:29-37.

Cunningham, J.C. 1995. Baculoviruses as microbial insecticides. *In* R. Reuveni (ed). pp. 261-292. Novel approaches to integrated pest management. Boca Raton, Florida. Lewis.

Dahlsten, D.L., and Thomas, G.M. 1969. A nucleopolyhedrosis virus in populations of the Douglas-fir tussock moth, *Hemerocampa pseudotsugata*, in California. *Journal of Invertebrate Pathology*. 13: 264-271.

David, W.A.L. and Gardiner, B.O.C. 1967. The persistence of a granulosis virus of *Pieris rapae* in soil and in sand. *Journal of Invertebrate Pathology.* 9: 342-347.

de Jong, J.G., Lauzon, H.A., Dominy, C., Poloumienko, A., Carstens, E.B., Arif, B.M., and Krell, P. 2005. Analysis of the Choristoneura fumiferana nucleopolyhedrovirus genome. *Journal of General Virology.* 86:929-43.

de Moraes, R., Maruniak, J., and Funderburk, J. 1999. Methods for the detection of *Anticarsia gemmatalis* nucleopolyhedrovirus DNA in soil. *Applied Environmental. Microbiology.* 65:2307-2311.

Derksen, A.C.G. and Granados, R.R. 1988. Alteration of lepidopteran periotrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology.* 167:242-250.

Dow, J.A.T. 1986. Insect midgut function. *Advanced Insect Physiology.* 19:187-389.

Duan, L. and Otvos, I.S. 2001. Influence of Larval Age and Virus Concentration on Mortality and Sublethal Effects of a Nucleopolyhedrovirus on the Western Spruce Budworm (Lepidoptera: Tortricidae). *Environmental Entomology.* 30: 136-146.

Dwyer, G. 1991. The roles of density, stage and patchiness in the transmission of an insect virus. *Ecology.* 72:559-574.

Ebling, P., Smith, P. and van Frankenhuyzen, K. 2001. DNA hybridization assay for detection of nucleopolyhedrovirus in whitemarked tussock moth (*Orgyia leucostigma)* larvae. *Pest Management Science.* 57:66-71.

Ebling, P.M. and Holmes, S.B. 2002. A refined method for the detection of baculovirus occlusion bodies in forest terrestrial and aquatic habitats. *Pest Management Science.* 58:1216-1222.

Engelhard, E.K., Kammorgan, L.N.W, Washburn, J.O. and Volkman, L.E. 1994. The insect tracheal system- a conduit for the systemic spread of *Autographa californica* nuclear polyhedrosis virus. *Proceedings of the National Academy of Science USA.* 91:3224-3227.

Evans, H.F. 1986. Ecology and epizootiology of baculoviruses. *In* R.R. Granados and B.A. Federici (Eds.). pp. 89-132. *The biology of baculoviruses,* Vol 2, *Practical applications for insect control.* Boca Raton, Florida. CRC.

Evenden, J. and Jost, E.J. 1947. A report of the tussock moth control, North Idaho. USDA Forest Service. 51 pp.

Falcon, L.A. 1976. Problems associated with the use of arthropod viruses in pest control. *Annual Review of Entomology.* 21:305-324.

Fazairy, A.A., and Hassan, F.A. 1988. Infection of termites by *Spodoptera littoralis* nuclear polyhedrosis virus. *Insect Science Application.* 9:37-39.

Federici. B. A. 1997. Baculovirus pathogenesis. *In* L. K. Miller (ed.). pp. 33-56. *The baculoviruses*. Plenum Press, New York, N.Y.

Ferreria, C., Capella, A.N., Sitnik, R., and Terr, W.R. 1994. Digestive enzymes in midgut cells, endo-and ectoperitrophic contents, and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. *Archives of Insect biochemistry and physiology.* 26:299-313.

Friguet, B., Djavadi-Ohaniance, L. and Goldberg, M.E. 1984. Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. *Molecular Immunology.* 21:673-677.

Fuxa, J.R. 1987. Ecological considerations for the use of entomopathogens in IPM. *Annual Review of Entomology.* 32:225-251.

Fuxa, J. R. and Richter, A. R. 1991. Selection for an increased rate of vertical transmission of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus. *Environmental Ecology.* 20:603-609.

Fuxa, J.R. 1991. Insect control with baculoviruses. *Biotechnology Advances.* 9:425-442.

Fuxa, J.R., Richter, A.R., Ameen, A. O., and Hammock, B.D. 2002. Vertical transmission of TnSNPV, TnCPV and AcMNPV and possibly recombinant NPV in *Trichoplusia ni*. *Journal of Invertebrate Pathology.* 79:44-50.

Gallo, L.G., Corasro, B.G., Hughes, P.R. and Granados, R.R. 1991. *In vivo* enhancement of baculovirus infection by the viral enhancing factor of a granulosis virus of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology.* 58:203-210.

Garcia-Maruniak, A., Maruniak, J.E., Zanotto, P.M. A., Doumbouya, D. E. Liu, J. C., Merritt, T.M. and Lanoie, J. S. 2004. Sequence Analysis of the Genome of the *Neodiprion sertifer* Nucleopolyhedrovirus. *Journal of Virology.* 78: 7036–7051.

Ginzberg, L.R. and Taneyhill, D.E. 1994. Population cycles of forest Lepidoptera: maternal effects hypothesis. *Journal of Animal Ecology.* 63:79-92.

Glad, C. and Grubb, A.O. 1978. Immunocapillary migration-a new method for immunochemical quantitation. *Analytical Biochemistry.* 85:180-187.

Goulson, D. and Cory, J. 1995. Sublethal effects of baculovirus in cabbage moth, *Mamestra brassicae. Biological Control.* 5:361-367.

Granados, R.R. 1978. Early events in the infection of *Heliothis zea* midgut cells by a baculovirus invasion and infection. *Virology.* 90:170-174.

Granados, R.R. 1980. Infectivity and mode of action of baculoviruses. *Biotechnology and Bioengineering.* 22:1377-1405.

Granados, R.R. and Lawler, K.A. 1981. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology.* 108:297-308.

Harlow, E. and Lane, D. 1988. *Antibodies: A laboratory manual.* Cold Spring Harbor Laboratory Press. 495 p.

Harper, J.D. 1986. Interactions between baculoviruses and other entomopathogens. pp 133-156. *In* R.R. Granados and B.A. Federici (eds), *Biology of Baculoviruses,* Vol 2, *Practical applications for insect control.* Boca Raton, Florida. CRC.

Harper, J.D. 1987. Applied epizootiology: microbial control of insects. *In* J.R. Fuxa and Y. Tanada (eds), *Epizootiology of Insect diseases.* New York, Wiley.

Harris, J.W.E., Dawson, A.F. and Brown, R.G. 1985. The Douglas-fir tussock moth in British Columbia, 1916-1984. Canadian Forest Service. Pacific Forestry Centre Information Report. BC-X-268. 16pp.

Hayakawa, T., G. F. Rohrmann, and Y. Hashimoto. 2000. Patterns of genome organization and content in lepidopteran baculoviruses. *Virology.* 278:1-12.

Herniou, E.A., Luque, T., Chen, X., Vlak, J.M., Winstanley, D., Cory, J.S., and O'Reilly, D.R. 2001. Use of whole genome sequence data to infer Baculovirus phylogeny. *Journal of Virology.* 75:8117-8126.

Hochberg, M. 1989. The potential role of pathogens in biological control. *Nature.* 337:262-265.

Horton, H.M. and Burand, J.P. 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells. *Journal of Virology.* 67:1860-1868.

Horton, J., Swinburne, S. and O'Sullivan, M. 1991. A novel, rapid, single-step immunochromatographic procedure for the detection of mouse immunoglobulin. *Journal of Immunological Methods.* 140:131-134.

Hughes, D. S., Possee, R.D., and King, L.A. 1993. Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. *Virology.* 194:608-615.

Hughes, D. S., Possee, R.D., and King, L.A. 1997. Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects. *Journal of General Virology.* 78:1801-1805.

Hughes, K.M. 1976. Notes on the nuclear polyhedrosis viruses of tussock moths of the genus *Orgyia* (Lepidoptera). *The Canadian Entomologist* 108:479-484.

Hughes, K.M. 1978. Virulence. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). pp 135-136. *The Douglas-fir Tussock Moth: A Synthesis*. United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Hughes, K.M. and Addison, R.B. 1970. Two nuclear polyhedrosis viruses of the Douglas-fir tussock moth. *Journal of Invertebrate Pathology.* 16:196-204.

Jaques, R. P. 1964. The persistence of a nuclear-polyhedrosis virus in soil. *Journal of Insect Pathology* 6: 251-254.

Jaques, R. P. 1967. The persistence of a nuclear polyhedrosis virus in the habitat of the host insect, *Trichoplusia ni* II. Polyhedra in soil. *The Canadian Entomologist.* 99: 820-829.

James, D. and Mukerji, S. 1996. Comparison of ELISA and immunoblotting techniques for the detection of cherry mottle leaf virus. *Annuals of Applied Biologists.* 129:13-23.

Jutsum, A.R. 1988. Commercial application of biological control: Status and prospects. *Philosophical transactions of the Royal Society of London, B.* 318:357-373.

Kaupp, W.J. and Ebling, P.M. 1993. Horseradish peroxidase-labelled probes and enhanced chemiluminescence to detect baculoviruses in gypsy moth and eastern spruce budworm larvae. *Journal of Virological Methods.* 44:89-98.

Kaupp, W.J. and Ebling, P.M. 1990. Response of third, fifth and sixth instar spruce budworm, *Choristonera fumiferana* (Clem.), larvae to nucleopolyhedrosis virus. *The Canadian Entomologist.* 122:1037-1038.

Keating, S.T., Burand, J.P., and Elkinton, J.S. 1989. DNA hybridization assay for detection of gypsy moth nuclear polyhedrosis virus in infected gypsy moth (*Lymantria dispar* L.) larvae. *Applied environmental microbiology.* 55:2749-2754.

Keddie, B.A. and Volkman, L.E. 1985. Infectivity difference between two phenotypes of *Autographa californica* nuclear polyhedrosis virus: importance of the 64K envelope glycoprotein. *Journal of general virology.* 66:1195-1200.

Keddie, B.A., Aponte, G.W., and Volkman, L.E. 1989. The pathways of infection of autographa-californica nuclear polyhedrosis-virus in an insect host. *Science.* 243:1728-1730.

Kelly, D.C., Edwards, M.L. and Robertson, J.S. 1978. The use of the enzyme-linked immunosorbent assay to detect a nuclear polyhedrosis virus in *Heliothis armigera* larvae. *Journal of General Virology* 40:465-469.

Kennel, S. 1982. Binding of monoclonal antibody to protein antigen in fluid phase or bound to solid supports. *Journal of Immunological Methods..* 55:1-12.

Kohler, G. and Milstein, C. 1975. Preparation of monoclonal antibodies. *Nature.* 25:256-259

Kukan, B. and Otvos, I.S. 2001. Comparison of laboratory response of second-instar and third-instar larvae of Douglas-fir tussock moth to TM Biocontrol-1 using the diet surface contamination and diet plug methods. *In*: B. Kukan and I.S. Otvos. pp. 57-77. Efficacy of Stored TM Biocontrol-1 and Research Related to its Use in Suppression Programs. Unpublished report.

Kukan, B. 1999. Vertical transmission of nucleopolyhedrovirus in insects. *Journal of Invertebrate Pathology.* 74:103-111.

Kukan, B. and Myers, J. 1995. DNA hybridization assay for the detection of nuclear polyhedrosis virus in tent caterpillars. *Journal of Invertebrate Pathology.* 66:231-236.

Kukan, B. and Myers, J.H. 1997. Prevalence and persistence of nuclear polyhedrosis virus in fluctuating populations of forest tent caterpillars (Lepidoptera:Lasiocampidae) in the area of Prince George, British Columbia. *Envrionmenal Entomology.* 26:882-887.

Kuzio, J., Pearson, M.N., Harwood, S.H., Funk, C.J., Evans, J.T., Slavicek, J.M., and Rohrmann, G.F. 1999. Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology*. 253:17-34.

Labandeira, C. C., and J. Sepkoski. 1993. Insect diversity in the fossil record. *Science.* 261:310-314.

Laitinen, A.M., Otvos, I.S., and Levin, D.B. 1996 Genotypic variation among wild isolates of Douglas-fir tussock moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *Journal of Economic Entomology.* 89:640–647.

Langridge, W.H.R., Granados, R.R. and Greenberg, J.F. 1981. Detection of baculovirus protein in cell culture and insect larvae by enzyme-linked immunosorbent assay (ELISA). *Journal of General Virology* 54:443-448.

Lauzon, H.A.M., Lucarotti, C.J., Krell, P.J., Feng, Q., Retnakaran, A., and Arif, B.M. 2004. Sequence and Organization of the *Neodiprion lecontei* Nucleopolyhedrovirus Genome. *Journal of Virology.* 78:7023-7035.

Leisy, D., Nesson, M., Pearson, M., Rohrmann, G., and Beaudreau, G. 1986. Location and nucleotide sequence of the *Orgyia pseudotsugata* single nucleopolyhedrovirus nuclear polyhedrosis virus polyhedrin gene. *Journal of General Virology.* 67:1073-1079.

Levin, D.B., Laitinen, A.M., Clarke, T., Lucarotti, C.J., Morin, B., and Otvos, I.S. 1997. Characterization of nuclear polyhedrosis viruses from three subspecies of *Lambdina fiscellaria. Journal of Invertebrate Pathology.* 69:125-134.

Longworth, J.F. and Carey, G.P. 1980. The use of an indirect enzyme-linked immunosorbent assay to detect baculovirus in larvae and adults of *Oryctes rhinoceros* from Tonga. *Journal of General Virology.* 47:431-438.

Longworth, J.F. and Cunningham, J.C. 1968. The activation of occult nuclear-polyhedrosis virus by foreign nuclear polyhedra. *Journal of Invertebrate Pathology.* 10:361-367.

Lupiani, B., Raina, A., and Huber, C. 1999. Development and use of a PCR assay for the detection of the reproductive virus in wild populations of *Helicoverpa zea* (Lepidoptera:Noctuidae). *Journal of Invertebrate Pathology.* 73:107-112.

Martignoni, M.E. 1999. History of TM Biocontrol-1: the first registered virus-based product for control of a forest insect. *American Entomology.* 45:30-37.

Martignoni, M.E. and Iwai, P.J. 1986a. A catalog of viral diseases of insects, mites and ticks. USDA forest service PNW-195. Washington, DC. USGPO. 50pp.

Martignoni, M. and Iwai, P. 1986b. Propagation of multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* in larvae of *Trichoplusia ni. Journal of Invertebrate Pathology.* 47:32-41.

Martignoni, M.E., Breillatt, J.P., and Anderson, N.G. 1968. Mass purification of polyhedral inclusion bodies by isopycnic banding in zonal rotors. *Journal of Invertebrate Pathology* 11: 507-510.

Mason, R.R. 1970. Development of sampling methods for the Douglas-fir tussock moth, *Hemerocampa pseudotsugata* (Lepidoptera:Lymantriidae). *The Canadian Entomologist.* 102:836-845.

Mason, R.R. 1974. Population change in an outbreak of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera:Lymantriidae), in central Arizona. *The Canadian Entomologist.* 106:1171-1174.

Mason, R.R. 1978. Population ecology. Outbreaks. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). pp. 43-47. *The Douglas-fir Tussock Moth: A Synthesis*. United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Mason, R.R. 1996. Dynamic behaviour of Douglas-fir tussock moth populations in the Pacific Northwest. *Forest Science.* 42:182-191.

Mason, R.R. and Luck, R.F. 1978. Population Growth and Regulation. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). pp. 41-47. *The Douglas-fir Tussock Moth: A Synthesis.* United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Mason, R.R. and Wickman, B. 1991. Integrated pest management of the Douglas-fir tussock moth. *Forest Ecology Management.* 39:119-130.

Mason, R.R., Scott, D.W., Loewen, M.D., and Paul, H.G. 1998. Recurrent outbreak of the Douglas-fir tussock moth in the Malheur forest: A case history. General Technical Report. PNW-GTR-402. USDA Forest Service, Pacific Northwest Research Station. 14pp.

McNamee, P., McLeod, J. and Holling, C. 1981. The structure and behavior of defoliating insect/forest ecosystems. *Research Population Ecology.* 23:280-297.

Millstein, J.A. 1988. *The population dynamics of the douglas-fir tussock moth: a study of multiple interactions.* (PhD dissertation). Washington State University.
Miller, L.K. and Lu, A. 1997. The molecular basis of baculovirus host range. *In* L.K. Miller (ed). pp.217-235. *The baculoviruses.* Plenum Press, New York.

Moreau, G., Lucarotti, C.J. Kettela, E.G., Thurston, G.S., Holmes, S., Weaver, C., Levin, D.B. and Morin, B. 2005. Aerial application of nucleopolyhedrovirus induces decline in increasing and peaking populations of Neodiprion abietis. *Biological Control.* 33: 65-73.

Moscardi, F. 1999. Assessment of the application of baculoviruses for control Lepidoptera. *Annual Review of Entomology* 44:257-289.

Mowat, W. and Dawson, S. 1987. Detection and identification of plant viruses by ELISA using crude sap extracts and unfractionated antisera. *Journal of Virological Methods.* 15:233-247.

Murray, K. D. and Elkinton, J.S. 1989. Environmental contamination of egg masses as a major component of transgenerational transmission of gypsy moth nuclear polyhedrosis virus (LdMNPV). *Journal of Invertebrate Pathology.* 53:324-334.

Murray, K.D, and Elkinton, J.S. 1990. Transmission of nuclear polyhedrosis virus to gypsy moth (Lepidoptera: Lymantriidae) eggs via contaminated substrates. *Environmental Entomology.* 19:662-665.

Murray, K.D., Shields, K.S., Burand, J.P. and Elkinton, J.S. 1991. The effect of gypsy moth metamorphosis on the development of nuclear polyhedrosis virus infection. *Journal of Invertebrate Pathology.* 57:352-361.

Myers, J.H. 1988. Can a general hypothesis explain population cycles of forest Lepidoptera? *Advances in Ecological Research.* 18:179-242.

Myers, J.H. and Kuken, B. 1995. Changes in the fecundity of tent caterpillars: a correlated character of disease resistance or sublethal effect of disease? *Oecologia.* 103:475-480

Myers, J.H., Malakar, R. and Cory, J. 2000. Sublethal nucleopolyhedrovirus infection effects on female pupal weight, egg mass size, and vertical transmission in gypsy moth (Lepidoptera: Lymantriidae). *Environmental Entomology.* 29:1268-1272.

Nataraju, B., Sivaprasad, V., Datta, R., Gupta, S., and Shamim, M. 1994. Colloidal textile dye-based dipstick immunoassay for the detection of nuclear polyhedrosis virus (BmNPV) of silkworm, *Bombyx mori* L. *Journal of Invertebrate Pathology.* 63:135-139.

Neelgund, Y.F. and Mathad, S.B. 1978. Transmission of a nuclear polyhedrosis virus in laboratory populations of the armyworm, *Mythimna (Pseudaletia) separata. Journal of Invertebrate Pathology.* 31:143-147.

Ohkawa, T., Majima, K. and Maeda, S. 1994. A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. *Journal of Virology.* 68:6619

Otvos, I. and Shepherd, R. 1991. Integration of early virus treatment with a pheromone detection system to control Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera:Lymantriidae), populations at pre-outbreak levels. *Forest Ecology Management.* 39:143-151.

Otvos, I. S., Cunningham, J. C., and Kaupp, W.J. 1989. Aerial application of two baculoviruses against the western spruce budworm, *Choristoneura occidentalis* Freeman (Lepidoptera:Tortricidae), in British Columbia. *The Canadian Entomologist.* 121:209-217.

Otvos, I.S., Cunningham, J.C. and Friskie, L.M. 1987. Aerial application of nuclear polyhedrosis virus against Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lepidoptera:Lymantriidae): I. Impact in the year of application. *The Canadian Entomologist.* 119:697-706.

Otvos, I. S., Cunningham, J. C., MacLaughlan, L., Hall, P. and Conder, N. 1999. The development and operational use of a management system for control of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera:Lymantriidae), populations at pre-outbreak levels. *In*: McMannus, M.L. and Liehhold, A.M. (Eds). pp. 143-154. *Proceedings: Population dynamics, impacts, and integrated management of forest defoliating insects.* USDA Forest Service General Technical Report. NE-27.

Otvos, I.S., MacLauchlan, L.E., Hall, P. and Conder, N. 1998. A management system to control Douglas-fir tussock moth, *Orgyia pseudotsugata*, using OpNPV. For. Res. App. Technical transfer note. No. 11.

Paek, S., Lee, S., Cho, J., and Kim, Y. 2000. Development of rapid one-step immunochromatographic assay. *Methods.* 22:53-60.

Parola, A.D., Sciocco-Cap, A., Glikmann, G. and Romanowski, V. 2003. An immunochemical method for quantification of *Epinotia aporema* granulovirus (EpapGV). *Journal of Virological Methods.* 112:13-21.

Payne, C.C. 1988. Pathogens for the control of insects: Where next? *Philosophical transactions of the Royal Society of London, B.* 318:225-248.

Patil, U. R., C. J. Savanurmath, S. B. Mathad, P. I. Aralaguppi, and S. S. Ingalhalli. 1989. Effects of nuclear polyhedrosis virus on the growth, development and reproduction in surviving generations of the armyworm *Mythimna (Pseudaletia) seperata* (Walker). J. *Applied Entomoloigy.* 108: 527-532.

Perelle, A.H. and Harper, J.D. 1986. An evaluation of the impact sublethal dosages of nuclear polyhedrosis virus on larvae on pupae, adults and adult progeny of the fall army worm, *Spodoptera frugiperda* (Lepidoptera: Lasiocampidae). *Environmental Entomology.* 23:864-869.

Popham, H.J.R., Bischoff, D.S. and Slavicek, J.M. 2001. Both *Lymantria dispar* Nucleopolyhedrovirus *Enhancin* Genes Contribute to Viral Potency. *Journal of Virology.* 75:8639-8648.

Pritchett, D.W., Young, S.Y. and Yearian, W.C. 1982. Dissolution of *Autographa californica* nuclear polyhedrosis virus polyhedra by the digestive fluid of *Trichoplusia ni* (Lepidoptera: Noctuidae) larvae. *Journal of Invertebrate Pathology.* 39:354-361.

Regniere, J. 1984. Vertical transmission of diseases and population dynamics of insects with discrete generations: a model. *Journal of Theoretical Biology.* 107:287-301.

Richards, A. and Christian, P. 1999. A rapid bioassay screen for quantifying nucleopolyhedroviruses (Baculoviridae) in the environment. *Journal of Virological Methods.* 82:63-75.

Rohrmann, G.F. 1977. Characterization of N-Polyhedrin of two baculovirus strains pathogenic for *Orgyia pseudotsugata. Biochemistry.* 16:1631-1634.

Rohrmann, G.F. 1986a. Evolution of occluded baculoviruses. pp. 203-215. *In:* Granados, R. R. and Frederici, B.A. (Eds). *The biology of baculoviruses.* Boca Raton, Florida. CRC.

Rohrmann, G.F. 1986b. Polyhedrin structure. *Journal of General Virology.* 67:1499-1513.

Rohrmann, G.F. 1999. Nuclear polyhedrosis viruses. *In* R.G. Webster and A. Granoff (ed.) *Encyclopedia of virology*, 2nd ed. Academic Press, England.

Rohrmann, G.F., McParland, R.H., Martignoni, M.E., and Beaudreau, G.S. 1978. Genetic relatedness of two nucleopolyhedrosis viruses pathogenic for *Orgyia pseudotsugata*. *Virology*. 84: 213–217.

Rossi, C., Tsang, V., and Pilcher, J. 1991. Rapid, low-technology field and laboratory-applicable enzyme immunosorbent assays for immunodiagnosis of *Schistosoma mansoni*. *Journal of Clinical Microbiology*. 29: 1836-1841.

Rothman, L., and Myers, J. 1996. Debilitating effects of viral diseases on host Lepidoptera. *Journal of Invertebrate Pathology*. 67:1-10.

Sait, S.M., Begon, M., and Thompson, D.J. 1994a. Long-term population dynamics of the Indian meal moth *Plodia interpunctella* and its granulosis virus. *Journal of Animal Ecology*. 63:861-870.

Sait, S.M., Begon, M. and Thompson, D.J. 1994b. The effects of a sublethal baculovirus infection in the Indian meal moth, Plodia interpunctella. *Journal of Animal Ecology*. 63:541-550.

Sambrook, J. and Russell, D. 2001. (Eds). *Molecular Cloning A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shamim, M., Baig, M., Datta, R.K., Gupta, S.K. 1994. Development of a monoclonal antibody-based sandwich ELISA for the detection of nuclear polyhedrosis virus infection in *Bombyx mori* L. *Journal of Invertebrate Pathology*. 63:151-156.

Shepherd, R.F. and Otvos, I.S. 1986. Pest management of Douglas-fir tussock moth: procedures for insect monitoring, problem evaluation and control actions. Pacific Forestry Centre. Information Report BC-X-270.

Shepherd, R.F., Otvos, I.S., and Chorney, R.J. 1984a. Pest management of the Douglas-fir tussock moth (Lepidoptera:Lymantriidae): sampling method to determine egg mass density. *The Canadian Entomologist*. 116:1041-1049.

Shepherd, R.F., Otvos, I.S., Chorney R.J., and Cunningham, J.C. 1984b. Pest management of Douglas-fir tussock moth (Lepidoptera: Lymantriidae): Prevention of an outbreak through early treatment with a nuclear polyhedrosis virus by ground and aerial applications. *The Canadian Entomologist*. 116: 1533-1542.

Shields, M., Siegel, J., Clark, C., Hines, K., Potempa, L., Gewurz, H. and Anderson, B. 1991. An appraisal of polystyrene- (ELISA) and nitrocellulose-based (ELIFA) enzyme immunoassay systems using monoclonal antibodies reactive toward antigenically distinct forms of human C-reactive protein. *Journal of Immunological Methods.* 141:253-261.

Shuy, R., Shuy, H., Liu, H. and Tang, S. 2002. Colloidal gold-based immunochromatographic assay for detection of ricin. *Toxicon.* 40:255-258.

Silberkang, M., Havel, C.M., Friend, D.S., McCarthy, B.J., and Watson, J.A. 1983. Isoprene synthesis in isolated embryonic Drosophila cells. I. Sterol-deficient eukaryotic cells. *Journal of Biological Chemistry*. 258:8503-8511.

Slack, J.M., Kuzio, J., and Faulkner, P. 1995. Characterization of *v-cath*, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *Journal of General Virology*. 76:1091-1099.

Snowden, K. and Hommel, M. 1991. Antigen detection immunoassay using dipsticks and colloidal dyes. *Journal of Immunological Methods.* 140:57-65.

Stairs, G. 1972. Pathogenic microorganisms in the regulation of forest insect populations. *Annunal Review of Entomology.* 17:355-372.

Stark, R.W. 1978. Historical aspects of tussock moth research. *In* Brookes, M.H., Stark, R.W. and Campbell, R.W. (eds). pp.1-2. *The Douglas-fir tussock moth: a synthesis*. USDA Technical Bulletin 1585.

Steinhaus, E.A. 1951. Report on the diagnoses of diseased insects 1944-1950. *Hilgardia.* 20:629-678.

Stelzer, R.F. 1979. How to determine the incidence of virus in egg masses. *In*: Douglas-fir Tussock Moth Handbook. USDA. Agriculture Handbook. No. 548.

Stelzer, M.J., Neiss, J., Cunningham, J.C., and McPhee, J.R. 1977. Field evaluation of baculovirus stocks against Douglas-fir tussock moth in British Columbia. *Journal of Econonomic Entomology.* 70:243-246.

Stoszek, K.J. and Mika, P.G. 1978. Population Ecology. Outbreaks, sites, and stands. *In* Brookes, M.H., Stark, R.W. and Campbell, R.W. (eds). pp. 56-59. *The Douglas-fir tussock moth: a synthesis*. USDA Technical Bulletin 1585.

Stark, D.M., Purcell, A.H. and Mills, N.J. 1999. Natural occurrence of Ametadoria misella (Diptera:Tachinidae) and the granulovirus of Harrisina brillians (Lepidoptera : Zygaenidae) in California. *Environmental Entomology.* 28:868-875.

Straub, T., Pepper, I., Abbaszadegan, M., and Gerba, C. 1994. A method to detect enteroviruses in sewage sludge-amended soil using the PCR. *Applied. Environmental. Microbiology.* 60:1014-1017.

Swaine, G. 1966. Generation-to-generation passage of the nuclear polyhedral virus of *Spodoptera exempta* (Wlk.). *Nature.* 5040:1053-1054.

Tanada, Y. and Fuxa, J.R. 1987. The pathogen population. *In:* J.R. Fuxa and Y. Tanada, (Eds). pp 113-158. *Epizootiology of Insect Diseases.* Wiley, N.Y.

Tebbe, C. and Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of a recombinant DNA from bacteria and a yeast. *Applied Environmental Microbiology.* 59:2657-2665.

Thiem, S.M., Du, X ., Quentin M.E. and Berner, M.M. 1996. Identification of baculovirus gene that promotes Autographa californica nuclear polyhedrosis virus replication in a nonpermissive insect cell line. Journal of Virology. 70:2221-2229. HJJjlsadjkf

Thomas, E. D., Reichelderfer, C. F. and Heimpel, A. M. 1972. Accumulation and persistence of a nuclear polyhedrosis virus of the cabbage looper in the field. *Journal of Invertebrate Pathology.* 20: 157-164.

Thompson, C.G. and Maksymiuk, B. 1978. Virus in biological control. Laboratory and simulated field tests. pp 147-149. *In*: M.H. Brookes, R.W. Stark and R.W. Campbell (eds.). *The Douglas-fir Tussock Moth: A Synthesis*. United States Department of Agriculture, Science and Education Agency Technical Bulletin 1585.

Thompson, C.G. and Peterson, L.J. 1978. *Rearing the Douglas-fir Tussock Moth*. USDA Agriculture Handbook No 520. Combined Forestry Pest Research and Development Program, Washington, DC. 20pp.

Thompson C.G., Scott D.W., and Wickman B.E. 1981. Long-term persistence of the nuclear polyhedrosis virus of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera:Lymantriidae), in forest soil. *Environmental Entomology.*10:254–55.

Tunnock, S. 1966. A pilot test to control Douglas-fir tussock moths by an aerial application of nuclear polyhedrosis virus during 1965. USDA. Forest Service. Division State and Private Forestry Region 1:1-17.

Uchima, K., Harvey, J.P., Omi, E.M., and Tanada, Y. 1988. Binding sites on the midgut cell membrane for the synergistic factor of a granulosis virus of the armyworm, *(Psuedaletia unipuncta). Insect Biochemistry.* 18:645-650.

Vezina, A. and Peterman, R. 1985. Tests of the role of a nuclear polyhedrosis virus in the population dynamics of its host, Douglas-fir tussock moth, *Orgyia pseudotsuga* (Lepidoptera:Lymantriidae). *Oecologia.* 67:260-266.

Volkman, L.E. 1986. The 64 KDa envelope protein of budded *Autographa californica* nuclear polyhedrosis virus. *Current topics in microbial immunology.* 131:103-118.

Volkman, L.E. 1997. Nucleopolyhedrovirus interactions with their insect hosts. *Advances in Virus Research.* 48:313-348.

Volkman, L.E. and Goldsmith, P.A. 1985. Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. *Virology.* 143:185-195.

Volkman, L.E and Summers, M.D. 1977. *Autographa californica* nuclear polyhedrosis virus: Comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. *Journal of invertebrate pathology.* 30:102-103.

Volkman, L.E., Goldsmith, P.A., and Hess, R.T. 1986. Alternate pathway of entry of budded *Autographa californica* nuclear polyhedrosis virus: Fusion at the plasma membrane. *Virology.* 148:288-297.

Wagener, C., Fenger, U., Clark, B. R., and Shively J. E. 1984. Use of biotin-labelled monoclonal antibodies and avidin-peroxidase conjugates for the determination of epitope specificities in solid-phase competitive enzyme immunoassay. *Journal of Immunological Methods.* 68: 269-274.

Ward, V.K., Fleming, S.B., and Kalmakoff, J. 1987. Comparison of a DNA-DNA dot blot hybridization assay with light microscopy and radioimmunoassay for the detection of a nuclear polyhedrosis virus. *Journal of Virological Methods.* 15:65-73.

Washburn, J. O., Kirkpatrick, B. A. and Volkman, L. E. 1995. Comparative pathogenesis of *Autographa californica* M nuclear polyhedrosis virus in larvae of *Trichoplusia ni* and *Heliothis virescens*. *Virology.* 209:561–568.

Washburn, J.O., Kirkpatrick, B.A. and Volkman, L.E. 1996. Insect protection against viruses. *Nature.* 383:767.

Washburn, J.O., Trudeau, D., Wong, J.F. and Volkman, L.E. 2003. Early pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus and *Helicoverpa zea* single nucleopolyhedrovirus in *Heliothis virescens*: a comparison of the 'M' and 'S' strategies for establishing fatal infection. *Journal of general virology.* 84:343-351.

Wickman, B.E. 1978. Tree mortality and top-kill related to defoliation by the Douglas-fir tussock moth in the Blue Mountains outbreak. United States Department of Agriculture Forest Service, *Research Paper PNW-233*. 39pp.

Wickman, B.E. and Beckwith, R.C. 1978. Life history and habits. *In* Brookes, M.H., Stark, R.W. and Campbell, R.W. (eds). pp.30-37. *The Douglas-fir tussock moth: A synthesis.* USDA Forest Service Technical Bulletin. 1585.

Wickman, B.E., Mason, R.R., and Savage, T.J. 1988. The effects of thinning and fertilization on western spruce budworm, *Choristoneura occidentalis* Freeman (Lepidoptera:Tortricidae) and grand for growth. *In* Proceedings of the 18th International congress of entomology, Vancouver, B.C. pp. 419.

Williams, H.L. and Otvos, I.S. 2005. Genotypic variation and presence of rare genotypes among Douglas-fir tussock moth multicapsid nucleopolyhedrovirus (OpMNPV) isolates in British Columbia. *Journal of Invertebrate Pathology.* 88:190-200.

Wilson, J.A., Forney, S.D., Ricci, A.M., Allen, E.G., Hefferon K.L., and Miller, L.K. 2005. Expression and Mutational Analysis of *Autographa californica* Nucleopolyhedrovirus HCF-1: Functional Requirements for Cysteine Residues 2005. 79: 13900-13914.

Winstanley, D., and O'Reilly, D. 1999. Granuloviruses. *In* R.G. Webster and A. Granoff (ed.), Encyclopedia of virology, 2nd ed. Academic Press, London.

Wood, A.H. and Granados, R.R. 1991. Genetically engineered baculoviruses as agents for pest control. *Annual review of entomology.* 45:69-87.

Woods, S.N. and Elkinton, S. 1987. Bimodal patterns of mortality from nuclear polyhedrosis virus in gypsy moth (*Lymantria dispar*) populations. *Journal of Invertebrate Pathology* 50:151-157.

Young, S.Y. and Yerian, W.C. 1982. Nuclear polyhedrosis virus infection of *Pseudoplusia includens* (Lep.: Noctuidae) larvae: Effect on post larval stages and transmission. *Entomophaga* 27: 61-67.

Zanotto, P.M., Kessing, B.D. and Maruniak, J.E. 1993. Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. *Journal of Invertebrate Pathology.* 62:147-164.

Zhu, Y., He, W., Liang, Y., Xu, M., Yu, C., Hua, W., and Chao, G. 2002. Development of a rapid, simple dipstick dye immunoassay for schisosomiasis diagnosis. *Journal of Immunological Methods.* 266:1-5.