The Establishment of a Virus Free Laboratory Colony of *Cryptophlebia leucotreta* (False Codling Moth) and Characterisation of *Cryptophlebia leucotreta* Granulovirus (CrleGV) Genes

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Abstract

Cryptophlebia leucotreta is an economically important agricultural pest throughout Sub-Saharan Africa. CrleGV has been considered as an alternative to chemical control of this pest due to its host specificity and innocuous nature towards vertebrates. A CrleGV free laboratory colony of *C. leucotreta* would be useful for the isolation of genotypically pure strains of the CrleGV and for virulence comparisons between isolates. It is preferable to have a full characterisation of CrleGV prior to its registration and release into the environment as a biopesticide.

A laboratory colony of *C. leucotreta*, set up at Rhodes University, containing a low level of infection indicated that CrleGV is vertically transmitted. To establish a virus free laboratory colony of *C. leucotreta,* a solution of 3.5% sodium hypochlorite and 1% Tween 20 was used to surface decontaminate *C. leucotreta* eggs for removal of transovum CrleGV from the laboratory colony. No apparent infection by CrleGV was induced by subjecting larvae to stress. PCR of DNA extracted from larvae using CTAB failed to detect virus in the laboratory colony. This detection protocol was able to detect down to 60 fg (480 genome copies of CrleGV). The possibility of low-level virus remaining in the colony requires monitoring of genotypic purity of virus manipulated in the colony.

Sequencing of *Bam* HI/*Kpn*I fragments produced a preliminary sequence of the granulin region of CrleGV. This preliminary sequence supports the trend that the gene organisation of the granulin region of the granuloviruses infecting the family Tortricidae is conserved.

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List of Abbreviations

AdorGV – Adoxophyes orana granulovirus

AcMNPV – *Autographa californica* multi nucleocapsids nucleopolyhedrovirus

AgMNPV – Anticarsia gammatalis multi nucleocapsids nucleopolyhedrovirus

AU- absorbance units

BLAST – Basic Local Alignment Search Tool

BmNPV – *Bombyx mori* nucleopolyhedrovirus

bp – nucleotide base pairs

BSA – Bovine serum albumin

BV – budded virus (budded virions)

CER – constant environment room

Cfu/ml - colony forming units per ml

ChfuGV – *Choristoneura fumiferana* granulovirus

CpGV – *Cydia pomonella* granulovirus

CrleGV- *Cryptophlebia leucotreta* granulovirus

CTAB – Cetyl trimethyl ammonium bromide/ Hexadecyltrimethyl ammonium bromide.

Da - Daltons

DE – delayed early genes (gene expression)

dNTPs – deoxyribonucleotide triphosphates

EDTA – ethylene diamine tetra-acetic acid

egt – gene coding for ecdysteroid glucosyl transferase

EsacGV – *Estigmene acrea* GV

FCM – false codling moth (*Cryptophlebia leucotreta*)

fg – femtogram

g – gram

GV – granulovirus

HabrGV – *Harrisina brillians* granulovirus

HaGV – *Heliothis armigera* granulovirus

HearNPV – *Helicoverpa armigera* nucleopolyhedrovirus

HCl – hydrochloric acid

hr – Homologous regions (in DNA sequence). Plural: *hr*s

IE – Immediate early genes (Gene expression)

IGR – Insect growth regulators

IPTG – isopropylthiogalactopyranoside

kPa – kilopascals

kV – kilovolts

LaolGV – *Lacanobia oleracea* granulovirus

LC90 - the concentration of virus at which 90 % of the host organisms die after exposure to this concentration

LydiNPV – *Lymantria dispar* nucleopolyhedrovirus

mA – milliamperes

mg – milligrams

ml – Millilitres

MNPV – Multi nucleocapsids nucleopolyhedrovirus

NCBI – National Centre for Biotechnology Information

NPV – nucleopolyhedrovirus

nt – nucleotides

ng – nanograms

nm – nanometers

PCR – Polymerase chain reaction

PBS – Phosphate buffered saline

pg – picograms

PhopGV- *Phthorimaea operculella* Granulovirus

PIB – Poly inclusion bodies (refers to the more than one nucleocapsid per occlusion body which occurs in NPVs)

PiraNPV – Pieris rapae nucleopolyhedrovirus

PlxyGV – *Plutella xylostella* Granulovirus

pmol – pico moles

PsunGV – *Pseudalatia unipuncta* Granulovirus

OB – Occlusion Bodies (Occluded virions)

ODV – Occlusion derived virus

ORF – open reading frame (of a gene)

RPM - Revolutions Per Minute

SDS - Sodium Dodecyl Sulphate

SctrGV – *Scotogramma trifolii* granulovirus

SNPV – Single nucleocapsids nucleopolyhedrovirus

SpfrGV – *Spodoptera frugiperda* granulovirusV

SpfrNPV – *Spodoptera frugiperda* nucleopolyhedrovirus

TAE buffer – Tris Acetate EDTA buffer

TE buffer- Tris-EDTA buffer

TnGV – *Trichoplusia ni* granulovirus

TnSNPV – *Trichoplusia ni* single nucleocapsids nucleopolyhedrovirus

UV- ultraviolet light

μg - micrograms

μl – microlitres

v/v – volume per volume

°C – Degrees Celsius

XecnGV – *Xestia c-nigrum* Granulovirus

X-Gal – 5- bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter 1: Review of Literature

1.1) Introduction

The existence of baculoviruses has been known for hundreds of years despite their having no role in human disease. Accounts of baculovirus infection have been found in ancient Chinese literature on silkworm culture. The first account in western culture of baculoviral symptoms in insect larvae was by an Italian bishop, Marco Vida. Research conducted during the early part of the 20th century revealed that baculoviruses are important in the natural control of insect populations. Granulosis viruses (GVs) were also described for the first time during this period (Miller, 1997).

Baculoviruses are virtually ubiquitous and infect arthropods in terrestrial and marine ecosystems. On land these viruses have been identified in hundreds of insect species inhabiting forests, fields, rivers and houses. They are specially designed to survive outside their host and can persist in soil or crevices for years. They are also present in large numbers in the food we eat and the air we breathe. The prevalence and diversity of baculoviruses can be attributed to the ubiquity and diversity of arthropod species. Over five hundred different insect species have been reported as susceptible to baculoviruses (Miller, 1997).

Baculoviruses are the most beneficial viruses known to humankind. Most other viruses are studied because they cause problems, while the basis of modern baculovirology was initiated by the potential utility of baculoviruses to control insect pests. Owing to the ability of baculoviruses to overexpress certain genes in their genome, they have potential in expression vector systems. Baculoviruses have also been used in studying the nature and function of apoptotic pathways. However, these viruses do cause certain problems such as infection of beneficial arthropod species such as silkworm colonies and shrimps that are now produced in aquaculture (Miller, 1997). Of specific interest to this project is the *Cryptophlebia leucotreta* granulovirus (CrleGV), considered to be an effective population control agent of ***Cryptophlebia leucotreta.*** This moth is a serious pest of many economically important crops in sub-Saharan Africa (Jehle ***et al***., 1992).

1.2) The *Baculoviridae*

The *Baculoviridae* are a family of large DNA viruses found only in arthropods, and holometabolous insects in particular (Blissard and Rohrmann, 1990). The name baculovirus is derived from ***baculum***, meaning rod or rod-shaped, which is used to describe the rod-shaped nucleocapsid that is characteristic of the family (Winstanley and O'Reilly, 1999).

Virions in this family characteristically have two different phenotypes involved in the infection process. The occluded virion phenotype (referred to as occlusion derived virus or ODV) is covered with a crystalline protein matrix to form an occlusion body (van Regenmortel ***et al.***, 2000). This form of the virus is responsible for horizontal infection of hosts and is protected from harsh environmental conditions by the occlusion body. This protein matrix makes the virus resistant to ultraviolet (UV) light inactivation as well as mechanical stress, thus maintaining the infectivity of these virions within the environment. Virions of the second virus phenotype are referred to as budded virions (BVs) and are mainly responsible for the cell-to-cell spread of the viral infection within the host organism (**Fig. 1.1**). The BVs are generated by nucleocapsids budding through the cellular membrane of infected cells and they normally contain only one nucleocapsid per virion (van Regenmortel ***et al.,*** 2000).

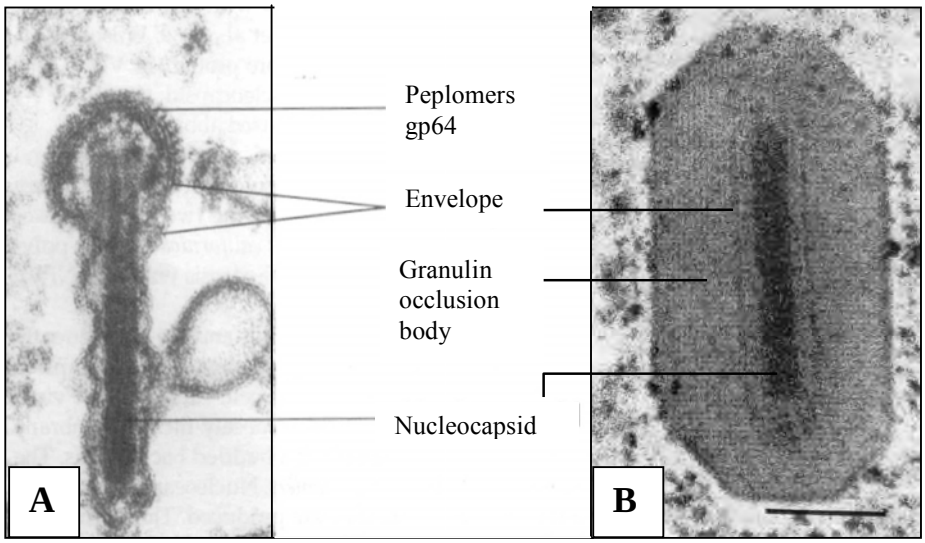


Figure 1.1: (A) longitudinal section of a budded virion. (O'Reilly ***et al***, 1992b) (B) Longitudinal section of an occluded virion. Note the thick protein coat enveloping the electron dense nucleocapsids (Winstanley and O'Reilly, 1999).

Taxonomically, the family is subdivided into 2 genera on the basis of occlusion body morphology and number of virions occluded into each occlusion body. These genera are the genus *Nucleopolyhedrovirus* and the genus *Granulovirus.* In nucleopolyhedrosis viruses (NPVs) the crystalline occlusion body has a polyhedral shape (0.15 μm to 15 μm) and contains multiple virions in each occlusion body. The granulosis virus occlusion body is ovicylindrical (0.3 μm x 0.5 μm) and contains one virion (van Regenmortel *et al.*, 2000). Nucleopolyhedroviruses may be further divided into multiple nucleocapsid NPVs (MNPVs) and single nucleocapsid NPVs (SNPVs). MNPVs have more than one nucleocapsid incorporated into a single virion, while SNPVs have only one nucleocapsid per virion envelope (**Fig. 1.2**). This further subdivision does not appear to be an official taxonomic classification (Winstanley and O'Reilly, 1999).

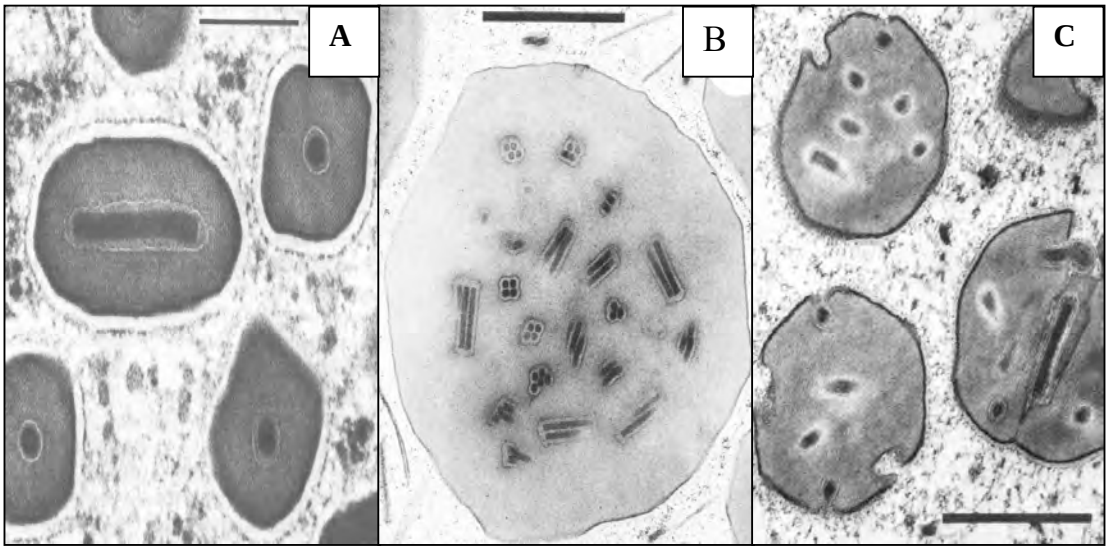


Figure 1.2: (A) a granulovirus clearly showing the single nucleocapsids per occlusion body. (Bar = 0.5 μm) (Tanada and Kaya, 1993) (B) A multiple nucleocapsid NPV virus (bar =1.0 μm) and (C) an SNPV with one nucleocapsid per virion embedded in the same occlusion body (bar =0.5 μm) (Maramorosch, 1977).

1.2.1) General structure of baculovirus virions

All viruses presently within the *Baculoviridae* have a similar basic structure. A rod-shaped nucleocapsid is enclosed in an envelope. There is an amorphous but definite layer between the nucleocapsid and the envelope (**Fig. 1.3**) (Tanada and Kaya, 1993). The occlusion-derived virus (ODV) has a tight fitting envelope around which a thick

crystalline protein matrix forms an occlusion body. The budded virus (BV) has a loose fitting envelope normally around single nucleocapsids. A glycoprotein (gp64) is embedded in the BV envelope on the cap side of the nucleocapsid, where the membrane is more loosely fitting (Blissard and Rohrmann, 1990) (**Fig. 1.1**). In nucleopolyhedrosis viruses the polyhedrin capsule may be enveloped by a protein and polysaccharide complex called the calyx (O'Reilly *et al.*, 1992b).

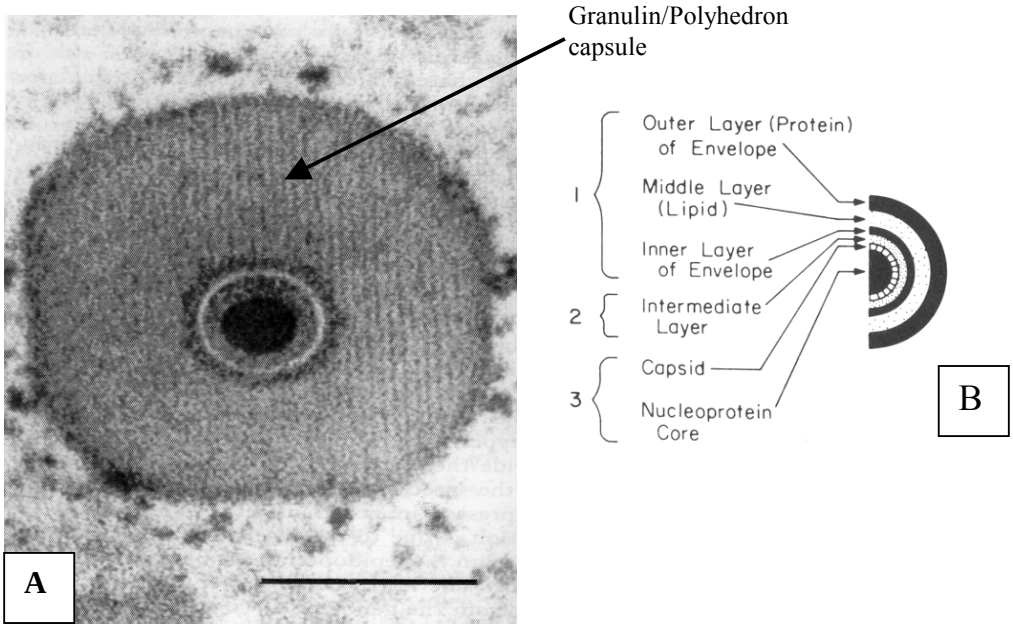


Figure 1.3: (A) Transverse section of baculovirus. Bar = 100 nm (Winstanley and O'Reilly, 1999) (See page 2 (**Fig. 1.1)** for longitudinal section of baculovirus). (B) Diagrammatic representation of the lipid and other layers of the nucleocapsid (Tanada and Kaya, 1993)

1.2.1.1) The nucleocapsid structure

The nucleocapsid structure in both GVs and NPVs appears to be the same. They are 30 nm to 60 nm in diameter and 200 nm to 300 nm long. A recent model suggests that the cylindrical portion of the nucleocapsid is a helical stacked ring structure that is repeated every third ring (Winstanley and O'Reilly, 1999). The ends of this cylindrical structure are closed by cap structures that are made up of proteins different from the cylinder as well as each other. These caps also differ morphologically. The one has a "claw and nipple" structure, while the other has a "claw" structure. The presence of different caps on either end of the nucleocapsid structure implies the existence of polarity in orienting

the nucleocapsid for viral envelopment, attachment, penetration through nuclear pores, exocytosis (budding of BV from the cell) and entry of the virus through the cell membrane (Tanada and Kaya, 1993).

Three proteins have been found to be part of the cylindrical section of the nucleocapsid: vp39, p80 and p24 (**Fig 1.4**). The protein making up the cap structure of one of the ends of the nucleocapsid has been designated p78 or p83 (depending on phosphorylation status). A DNA binding protein has also been discovered to be part of the nucleocapsid structure (Funk ***et al.***, 1997). It is designated p6.9 and allows for efficient packaging of the large baculoviral genomes into the nucleocapsid structure. The nucleocapsid serves as a protective wrapping for the double stranded DNA genome of both NPVs and GVs (Funk ***et al.***, 1997).

1.2.1.2) Baculovirus envelopes

The nucleocapsids of NPVs and GVs are enclosed in 2 membranes designated the intimate (inner) membrane and the outer membrane. The intimate membrane contains the virus nucleocapsid and is enclosed by the outer membrane. Intimate membrane formation occurs after the formation of the outer membrane. Subsequent to formation of the outer membrane, the material between the outer membrane and the virus nucleocapsid appears to become more concentrated and eventually forms the intimate membrane. This does not appear to be a true unit (bilipid layer) membrane, and it has been suggested that the material it is made up of originates from the outer membrane (Smith, 1971).

The outer lipid membrane is a true lipid membrane. In NPVs, multiple nucleocapsids may be included in each outer membrane with each nucleocapsid having its own intimate membrane. The outer membrane of ODVs originates from the host smooth endoplasmic reticulum (Smith, 1971).

The BV and the ODV envelopes are different in structure. The ODV envelope structure appears to be important in the association of granulin or polyhedrin with it and may also

contribute to virion stability in the harsh conditions of the insect gut subsequent to dissociation of the occluded protein crystal (Funk *et al.*, 1997).

In *Autographa californica* MNPV (AcMNPV), the BV envelopes contain 39% phosphatidylcholine and ODVs contain 50% phosphatidylserine (Funk *et al.*, 1997). These two lipids represent the major lipids found in the respective membranes. The ODV envelope contains more cholesterol and more protein than the BV envelope. There is also evidence to suggest that baculoviruses can selectively modify the lipid composition of host cell membranes (Braunagel and Summers, 1994). The differences in protein and lipid composition of ODV and BV is most likely due to the different functions that they are required to perform (**Fig. 1.4**). An example of differences in protein composition is the gp64 protein. This protein is found in BV envelopes, but is not present in ODV and is essential for entry of BV into host cells (Funk *et al.*, 1997).

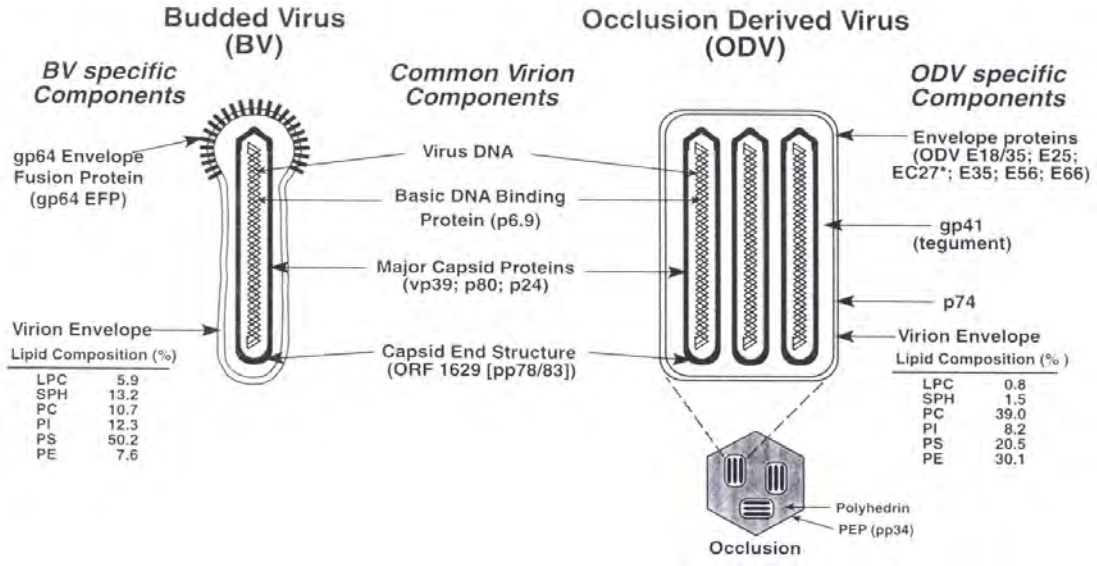


Figure 1.4: The protein and lipid compositions of BV and ODV baculovirus phenotypes. LPC – lysophosphatidylcholine; SPH – sphingomyelin; PC – phosphatidylcholine; PI – phosphatidyl inositol; PS – phosphatidylserine; PE – phosphatidylethanolamine. (Funk *et al.*, 1997).

1.2.2) Baculovirus genome structure and organisation

Virions of the *baculoviridae* have a circular double stranded DNA genome of between 90 and 180 kb (Hayakawa *et al.,* 1999; Hashimoto *et al*., 2000). This wide variation in the size of baculovirus genomes indicates that some baculoviruses may lack many of the

genes present in other members of the family (Possee and Rohrmann, 1997). The size of the AcMNPV genome (C6 isolate), the type virus of the NPV genus, is 133 894 bp and has been found to code for 154 open reading frames (ORFs) (Ayers ***et al.,*** 1994), while *Cydia pomonella* GV, the type species for granuloviruses, has a genome size of 123 500 bp and contains 143 ORFs of greater than 150 nucleotides. 82.5 % of the CpGV ORFs are homologous to genes previously found in other baculoviruses (Luque ***et al***., 2001). Complete genome sequences for both CpGV (Luque ***et al.,*** 2001) (**Fig. 1.5**) and AcMNPV (Ayers ***et al.,*** 1994) have been generated and illustrate the genetic complexity of this virus family.

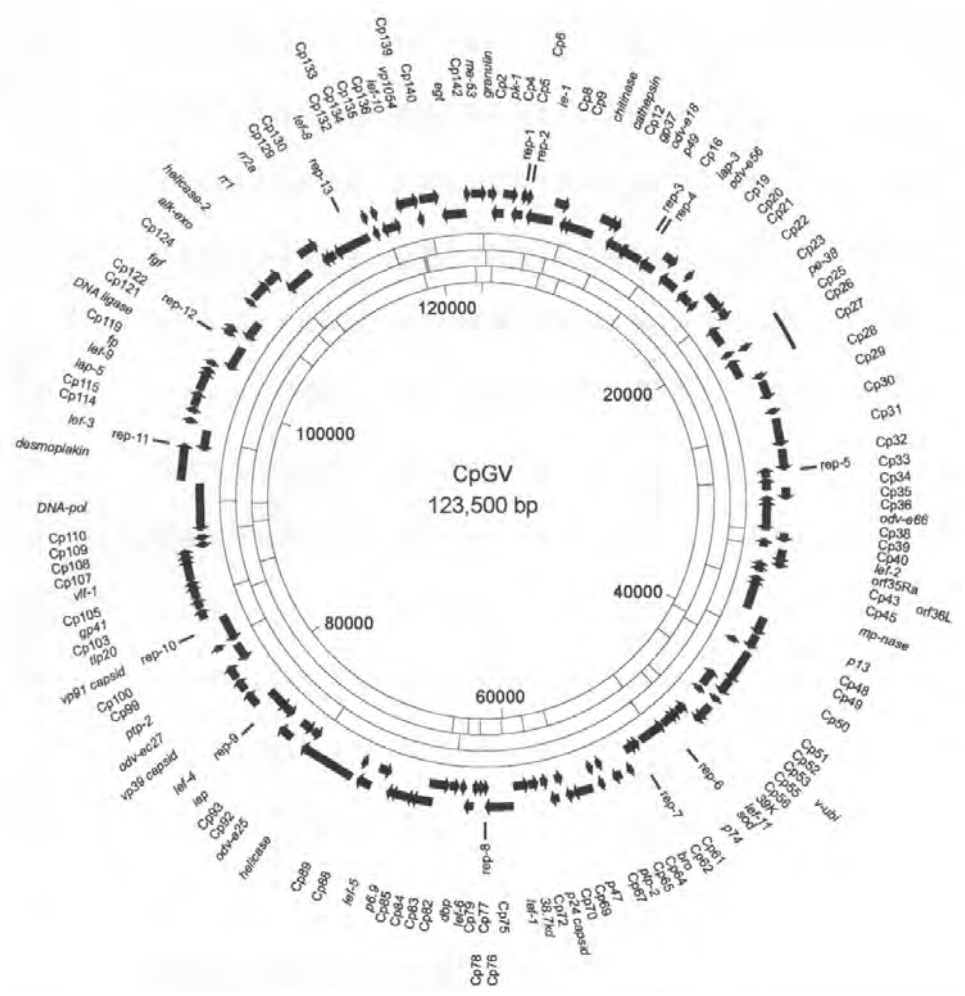


Figure 1.5: Circular map of the *Cydia pomonella* granulovirus genome. The inner set of circles indicate the positions of cleavage sites for the following enzymes: inner circle, ***Sal***I; Middle circle, ***Eco***RI and outer circle, ***Bam***HI. CpGV ORFs are indicated outside these circles with arrows indicating the direction of transcription. The positions of repeat sequences are also shown. A scale in bp is provided in the centre of the figure (Luque ***et al.,*** 2001).

A novel feature of many baculovirus genomes is the presence of homologous regions (*hr*s) that are located thoughout the genome. These *hr*s are composed of repeated sequences consisting of both direct repeats and imperfect palindromic sequences. Closely related counterparts are normally present elsewhere in the genome. It has been suggested that they function as transcriptional enhancers and viral replication origins (Possee and Rohrmann, 1997).

1.2.3) Baculovirus regulation of gene expression and DNA replication

In the baculovirus infected insect cell, the expression of viral genes and DNA replication are believed to occur in an ordered cascade of events in which each successive phase is dependent on the previous phase. Most evidence suggests that the viral gene expression is regulated at the transcriptional level with gene products of one class of baculovirus gene transactivating (directly or indirectly) transcription of the genes of the next class. Baculovirus gene expression is divided into an early phase and a late phase. The late phase occurs as DNA replication begins (Blissard and Rohrmann, 1990).

1.2.3.1) The early phase of gene expression

The early phase has been subdivided into immediate early and delayed early phases that are functionally defined stages. Immediate early (IE) genes are genes that can be transcribed by uninfected cells and no viral gene products are required for their expression. Delayed early (DE) genes require other viral gene products for their transcription. The baculovirus immediate early and delayed genes appear to have promoter elements similar to those from eukaryotic organisms, which is expected for promoters that must be recognized by the host cells. The immediate early genes such as IE-1 (immediate early gene 1) have been found to have a transactivating effect on other genes (such as the delayed early genes). The five homologous region sequences in the genome of AcMNPV are *cis*-acting and enhance the rate of utilization of promoters by RNA polymerase. It has been speculated that the activity of these *hr* enhancers may be stimulated by the IE-1 gene (Blissard and Rohrmann, 1990).

1.2.3.2) The late phase of gene expression

The late phase of viral expression is categorized as viral transcription that occurs subsequent to or during viral DNA replication. All late genes so far identified are transcribed from the consensus late promoter element ATAAG or GTAAG. This short promoter at the transcription initiation site is unusual in structure when compared to those recognised by nuclear eukaryotic RNA polymerases and bacterial RNA polymerases. Nuclear eukaryotic and bacterial promoters have multiple non-contiguous blocks of information. This short promoter bears more similarity to those in yeast mitochondrial DNA and certain bacteriophages. The transcription of baculovirus late genes may result from a viral-encoded or modified host RNA polymerase that recognizes the ATAAG promoter. If this polymerase is viral-encoded, the RNA polymerase could be similar to that of yeast mitochondrial DNA or phage T7 or T3 polymerases (Blissard and Rohrmann, 1990).

Baculovirus late genes can be divided into late genes and hyper expressed late genes. Late genes produce mRNAs in maximal abundance just after DNA replication initiation and decrease thereafter. Hyper expressed late genes are late genes that begin production of mRNA at high levels shortly after DNA replication of the virus genome and continue to do so throughout infection. Differences in transcription between the late and late hyper expressed genes could be due to the rate of transcription initiation from the two classes or the mRNA half-life. It has also been hypothesized that hyper expressed genes have a 12 nucleotide consensus sequence (A*ATAAG*TATTTT) surrounding the short promoter core (ATAAG) that may be important for elevated gene expression. Higher eukaryotes have 4 different RNA polymerase types (alpha, beta, gamma and delta), which are specialized either in function or their location in the cell. DNA polymerase delta is stimulated by proliferative cell nuclear antigen (PCNA), which is a cyclin. PCNA is associated with the S (DNA synthesis) phase of the cell cycle. A virally coded PCNA analogue protein has been suggested to function by stimulating an S-phase environment even in cells where host DNA replication is not occurring or shut down in order to allow viral DNA replication (Blissard and Rohrmann, 1990).

1.2.4) Techniques of distinguishing between different baculovirus strains

Amido black staining can be used to identify the presence of occlusion bodies within the infected host and would therefore be able to identify the pathogen as a baculovirus (Evans and Shapiro, 1997). Electron microscopy would be able to classify the baculovirus as an NPV or a GV and perhaps what subtype they are of each group (Griffith, 1985). All isolates of baculoviruses can be clearly distinguished by restriction enzyme analysis. To distinguish between different strains, a physical map of the genome of the isolated virus must be constructed. Strains can often be distinguished due to discrepancies in the restriction maps of the viral genome (Jehle *et al.*, 1992).

1.3) Granuloviruses

The presence of minute granules in virus-infected cells has led to the infection caused by these viruses being referred to as granulosis. The virus is called granulosis virus and the occlusion body is referred to as the capsule or granule. Advances in GV molecular biology and replication cycle have lagged behind those of NPVs due to difficulties in setting up a tissue culture system that supports GV infection. The GVs are similar to NPVs in terms of morphology, biophysical and biochemical properties. They differ from NPVs in terms of 1) number of nucleocapsids per virion and per occlusion body, 2) size and shape of occlusion body, 3) the process of occlusion of the enveloped nucleocapsids by the capsule matrix protein (granulin/polyhedron), 4) cytopathology and 5) to a lesser extent, tissue specificity (Tanada and Kaya, 1993).

1.3.1) Granulovirus host range and tissue tropism

The host range of GVs is generally narrow, with infection being confined to one or more species within the same family as the original host. They are also known only to infect insects in the order Lepidoptera (Federici, 1997). However, at least four GVs are known to have a relatively wide host range: CpGV (*Cydia pomonella* GV), HaGV (*Heliothis armigera* (cotton bollworm) GV), SpfrGV *(Spodoptera frugiperda* (fall armyworm) GV) and XecnGV (*Xestia c-nigrum* (spotted cutworm) GV) (Winstanley and O'Reilly, 1999). NPVs have been found to infect Lepidoptera as well as insects of the orders Hymenoptera, Diptera, Crustacea, Trichoptera and Thysanura. With the exception of one

crane fly disease, NPV infections within these other invertebrate orders are restricted to the midgut epithelium. In lepidopteran infections with both NPV and GV, the larval stage is the most susceptible to infection (Federici, 1997). Single nucleocapsid NPVs (e.g. *Heliothis zea* SNPV) appear to have a similar host range to GVs, while MNPVs (such as *Autographa californica* MNPV) appear to have a wider host range (Payne, 1982).

Three different types of GV disease have been distinguished on the basis of their tissue tropism (Federici, 1997). Type 1 has a relatively narrow tissue tropism, with infection occurring only in the midgut as well as the fat body. Since other important tissues such as the tracheal matrix and epidermis are not attacked, the infected larva may live as much as a week longer than a larva of the same stage infected with a similar amount of NPV (which has a wider tissue tropism). When infected by TnGV in the fourth instar, larvae of *Trichoplusia ni* can survive for 10 to 14 days and may maintain their appetites and grow much larger than healthy larvae. They often only become sluggish and slow to respond within a day of death. The type 2 GV infection exhibits a wider tissue tropism involving midgut, fat body, epidermis, tracheal matrix and possibly the malpighian tubules. An example of a GV in this group is CpGV and the viral infection and gross pathology parallel that of typical lepidopteran NPV infections. This is probably due to similar tissue tropisms. After invasion of the midgut, type 2 GVs attack most of the major body tissues and this results in a very acute disease. It typically lasts 5 to 10 days in larvae infected in the fourth instar. As the disease progresses larvae swell and distend and develop white to yellow irregular patches beneath the cuticle. Liquefaction of the body after death most likely occurs due to epidermis infection. The type 3 GV disease is unique to HabrGV (*Harrisina brillians* GV). This group is characterized by a tissue tropism restricted to the midgut epithelium. HabrGV replicates only in this tissue and produces virions and occlusion bodies in both larvae and adults. The disease is acute and larvae normally die within 4 to 7 days. Infection causes diarrhoea and faeces contain infectious granules and cells sloughed from the infected gut. The larvae begin to shrivel after the fourth or fifth day post infection and do not liquefy (Federici, 1997).

1.3.2) Granulovirus structure

The budded virion phenotype of NPVs and GVs as well as the nucleocapsid structure is the same. The GV occlusion bodies are generally referred to as capsules or granules and have a roughly oval morphology. They have a major axis diameter of 300 nm to 500 nm and a minor axis diameter of 120 nm to 300 nm (Winstanley and O'Reilly, 1999). This is much smaller than the 0.5 μm to15 μm size of the polyhedra (occlusion bodies of NPVs) (Tanada and Kaya, 1993) (**Fig. 1.2**). GV occlusion bodies each typically contain only one nucleocapsid and very rarely more than one. The capsule matrix protein of GVs is 25 kDa to 30 kDa in size and is called granulin. The granulin amino acid sequence is related to the NPV polyhedrin at the amino acid sequence level (Winstanley and O'Reilly, 1999).

In certain instances, capsules may occlude cellular components such as membranes or vacuoles. Electron micrographs of GV capsules indicate there could be an outer layer surrounding the capsule that is similar to the calyx that surrounds NPVs (Winstanley and O'Reilly, 1999). CrleGV virions have been found to be 50 nm to 80 nm x 200 nm to 400 nm in size and are occluded by a 29 kDa granulin protein (Jehle *et al.,* 1992).

1.3.3) Granulovirus genome structure and organization

As with all baculoviruses, the GV genome consists of a single double stranded DNA molecule between 90 to 160 kilobases in size (Tanada and Kaya, 1993). A number of granulovirus sequencing projects have been completed: XecnGV [178 733bp] (Hayakawa *et al*., 1999), PlxyGV [100 999 bp] (Hashimoto *et al.,* 2000), CpGV [123 500 bp] (Luque *et al*., 2001) and PhopGV [119 217 bp] (Croizier *et al*., 2002). Comparisons of the gene organisation in PlxyGV, XecnGV (Hashimoto *et al.,* 2000) and CpGV (Luque *et al.,* 2001) revealed that gene organisation is well conserved between these granuloviruses. In comparison, the gene arrangement of AcMNPV was very different, displaying only small areas of homologous gene arrangement (Luque *et al.,* 2001; Hashimoto *et al.,* 2000). The genome sequences of XecnGV, PlxGV and CpGV indicate that the ORFs discovered in these genomes are closely spaced but with little overlap between them (Luque *et al.,* 2001; Hashimoto *et al.,* 2000 and Hayakawa *et al.,* 1999), as is the case for NPVs (Winstanley and O'Reilly, 1999). Homologous region sequences have been discovered in

XecnGV, PlxyGV and CpGV, but these sequences (especially those in CpGV) are different to those found in NPVs (Luque *et al.,* 2001). Field isolates of many GVs display considerable genomic heterogeneity. A typical GV infection in the field is likely to consist of multiple genomic variants co-infecting the host insects. At least some of this genomic heterogeneity has been attributed to the insertion of transposable elements from the lepidopteran host genome into the viral DNA. These transposable elements belong to the family of TC1-like transposons also found in *Drosophila* and *Caenorhabditis* species. Transposition can play a large role in GV diversity (Winstanley and O'Reilly, 1999).

Although a number of GV sequencing projects have been completed [(*Cydia pomonella* granulovirus (Luque *et al*., 2001), *Plutella xylostella* granulovirus (Hashimoto *et al.,* 2000) *Xestia c-nigrum* granulovirus (Hayakawa *et al.,* 1999)] or are presently being conducted, the majority of GV genes have not been identified. Tentative identifications have been made based on NPV and other GV homologues (Luque *et al.,* 2001; Hashimoto *et al.,* 2000 and Hayakawa *et al.,* 1999). Only the *granulin, enhancin, iap, ODVP-6E, egt* and *cathepsin* genes have been subjected to any form of functional characterization within GVs. Granulin is the major protein of GV capsules and it has been discovered that occlusion body morphology is not an intrinsic property of the polyhedrin/granulin genes alone (Winstanley and O'Reilly, 1999).

Enhancin genes code for a metalloproteinase that appears to facilitate digestion of the peritrophic membrane lining the midgut of the insect host, to facilitate the initiation of the infection process (Lepore *et al*., 1996). This gene has been found in a range of GVs. The *iap* gene produces a product that acts to block apoptosis (programmed cell death) in infected cells and is present in most baculoviruses (Possee and Rohrmann, 1997). The *ODVP-6E* is a structural protein found in the envelope of virions contained within occlusion bodies (Possee and Rohrmann, 1997). *Egt* codes for the insect ecdysteroid moulting hormone inhibitor protein (ecdysteroid glucosyltransferase), and prevents insect development by preventing the occurrence of moulting (O'Reilly *et al.,* 1992a). This allows the diversion of energy, required for growth and moulting of the larva, into virus production. Phylogenetically, *egt* of CrleGV is closely related to *Lacanobia oleracea* GV

(Smith and Goodale, 1998) and *Cydia pomonella* GV (Wormleaton and Winstanley, 2001). GV cathepsin is a cysteine protease involved in degradation of host tissues and cuticle to facilitate progeny virus release (Kang *et al.,* 1998)). The *chitinase* gene of baculoviruses is also implicated in virus release from host cadavers for dissemination into the environment (Possee and Rohrmann, 1997).

As with NPVs, GV gene expression occurs in a cascade fashion. Three broad transcriptional classes (early, late and very late) can be identified based on the time of expression during viral infection. GV gene expression studies have been limited due to the lack of highly permissive cell lines as well as the complication of *in vivo* studies by the involvement of different tissues and asynchronous infection. Studies using recombinant BmNPV (*Bombyx mori* NPV) that included the *cathepsin* gene of CpGV indicated similar transcriptional control mechanisms (Winstanley and O'Reilly, 1999).

1.3.4) Granulovirus replication cycle and pathogenesis

The pathogenesis of GV diseases has not been studied in nearly as much detail as that of NPVs mainly due to the lack of success of growing GVs in cell culture. Most of the information obtained for the replication and cytopathology of GVs has been from *in vivo* studies using electron microscopy. The initial stages of host infection appear to be similar for both NPVs and GVs. However, there are major differences between NPVs and GVs in terms of tissue tropism, cytopathology and histopathology (Federici, 1997).

As is the case with NPVs, GV infection is initiated by the ingestion of occluded GV particles with contaminated food. The alkaline pH of the lepidopteran midgut promotes the dissociation of the granulin proteins of the capsule, thus causing the release of the virions into the midgut lumen. Enhancin is a capsule associated protein that catalyses the partial disruption of the peritrophic membrane lining the midgut, which allows easier access of the midgut epithelial cells to the virus. Virus entry into the epithelial cells is thought to occur through fusion of the viral membrane with the microvilli of the cells (Winstanley and O'Reilly, 1999). The viral nucleocapsids then migrate to the nucleus and attach to the nucleopores. Uncoating of the nucleic acid may occur at the nucleopore (e.g.

Trichoplusia ni GV [TnGV] and *Estigmene acrea* GV [EsacGV]) or in the nucleus (*Scotogramma trifollii* GV [SctrGV]). A virion-associated kinase is thought to be responsible for initiation of uncoating. The infection process up to this point generally occurs within 2 to 6 hours post infection (Tanada and Kaya, 1993). Once in the nucleus, nucleocapsid production begins and is divided into two phases. The first phase occurs prior to nuclear membrane fragmentation and the second occurs subsequent to this degradation. This is very different to NPV development, which occurs completely in the nucleus. The early stages of GV infection resemble the prophase stage of mitosis. Nuclear clearing occurs as a result of the nuclear pore complex and chromatin becoming marginated. The granular and fibrillar components of the nucleolus separate and the nuclear pores become larger (Winstanley and O'Reilly, 1999). During this time virogenic stroma forms and viral nucleocapsids are formed at about 6 to 12 hours post infection. These nucleocapsids are filled with the viral nucleoprotein and mature nucleocapsids have been observed at 12 to 18 hours post infection. Shortly after the appearance of the nucleocapsids, the nuclear membrane fragments and virogenesis continues in the cytoplasm of the cell (Tanada and Kaya, 1993).

The nucleocapsids migrate to the periphery of the cell and align with their conical end perpendicular to the thickened area of plasma membrane where budding occurs. During budding, the nucleocapsids acquire a loose fitting envelope containing the peplomers characteristic of the BV phenotype. Further proliferation of nucleocapsids continues in the area of the cell where the nucleus was, while budding occurs. This stage of infection is normally attained at about 24 hours post infection (Tanada and Kaya, 1993). Little or no occlusion occurs in the midgut epithelium, with the exception of HabrGV, which is the sole member of type 3 GVs (Federici, 1997).

Viruses of the BV phenotype move through the basal lamina of the gut and enter the hemolymph of the infected insect. They then spread to other tissues by direct penetration through the basal lamina or through tracheal junctions with these tissues. In GVs that infect the tracheal matrix such as CpGV (and therefore most likely CrleGV, due to the relatedness of these 2 virus species), the virus may bud directly into the tracheoblasts

from the midgut cells (Federici, 1997). Unlike the ODV virion entry into midgut epithelial cells, the BV entry into cells is believed to be through receptor-mediated endocytosis. This involves the entire virion being endocytosed into an intracytoplasmic vesicle (endosome) that then fuses with acidic vesicles in the cytoplasm. The low pH in the endosome triggers fusion of the viral membrane with the endosomal membrane, thus releasing the nucleocapsid into the cytoplasm. It has been hypothesized that the BV gp64 is active in the fusion of the two membranes (Blissard and Rohrmann, 1990).

The ODV phenotype is produced mainly in the secondary sites of GV infection, such as the fat body. The initial phase of infection within these cells is similar to that of the infection within the midgut epithelial cells. The cell nucleus hypertrophies as the nucleoli enlarge and move toward the nuclear membrane along with the chromatin. As the nucleus enlarges it ruptures and the cytoplasm and nucleoplasm mix. Numerous small virogenic stromata are formed as dense granular areas throughout the cell and are interconnected by thin nucleoprotein strands. The nucleocapsids begin to assemble soon after this and are enveloped by intracytoplasmic membranes outside of the stroma. Granulin is also produced in large quantities at about this time and associates with the virions to produce occlusion bodies (Federici, 1997). The virions are occluded from one side, presumably by attachment of granulin to receptors on the viral envelope (**Fig. 1.6.)** (Winstanley and O'Reilly, 1999).

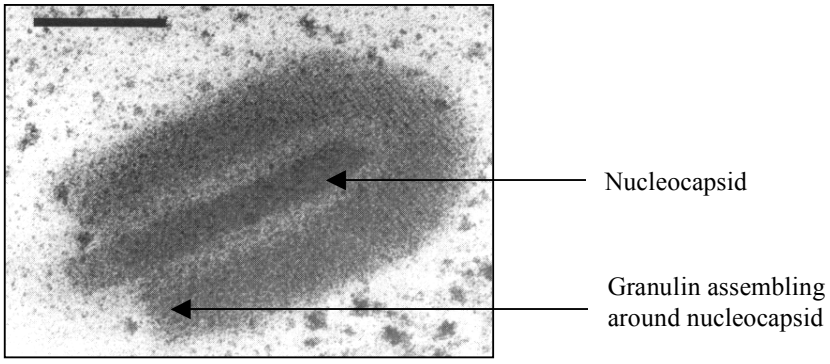


Figure1.6: Granulin assembling around nucleocapsids to form an occlusion body. Bar = 100 nm (Winstanley and O'Reilly, 1999).

The occlusion bodies accumulate in the cytoplasm, first in distinct masses and then completely filling the cells. Capsules are released from the cells by the rupture of the cellular plasma membrane and they accumulate in the centre of the fat body, the main site of occlusion body production, and in the hemolymph. This results in the infected larva becoming a milky or paler colour, due to the accumulation of capsules (Winstanley and O'Reilly, 1999). The features of granulovirus assembly are summarised in **Fig. 1.7.**

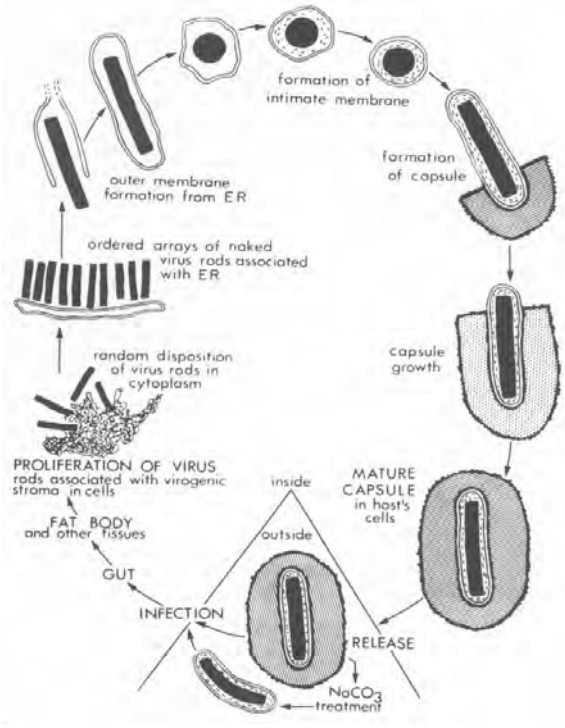


Figure 1.7: A diagrammatic representation of the granulovirus infection cycle, indicating the aspects that occur outside and within host tissues (Smith, 1971).

1.3.5) *Cryptophlebia leucotreta* granulovirus (CrleGV)

Cryptophlebia leucotreta granulovirus was first described and isolated from infected larvae originating from the Ivory Coast. Subsequently, isolates have been obtained from the Cape Verde Islands and from South Africa, and these isolates have been clearly distinguished by restriction analysis (Jehle *et al.,* 1992). CrleGV infects *Cryptophlebia leucotreta* (false codling moth) and is thought to have a narrow host range, due to the asymmetric cross infectivity observed with CrleGV and CPGV (Jehle *et al*., 1992).

CrleGV has the same general structure as that of all granuloviruses. Virions of the virus occluded in the granulin matrix protein (29 kDa) vary in size from 50 nm to 80 nm x 200 nm to 400 nm (Jehle ***et al.,*** 1992). Singh (2001) estimated a South African isolate of CrleGV (CrleGV-SA) (from Citrusdal in the Western Cape) to have a genome size of 112 kilobases and Jehle ***et al.*** (1992) determined the Cape Verde isolate of CrleGV (CrleGV-CV3) to have a genome size of 112.4 kbp. Although a complete genome sequence is not yet available for CrleGV, fragments of the genome have been sequenced (Jehle, 2002; Jehle and Backhaus, 1994 a, b) and a physical map has been constructed for CrleGV-CV3 (Jehle ***et al.,*** 1992) and CrleGV-SA (Singh 2001). Similarities in the protein patterns produced by SDS electrophoresis have been observed for CrleGV and CpGV and hybridisation experiments revealed similarities in regions of the genomes of the two viruses using stringent conditions (Jehle ***et al.,*** 1992).

1.4) *Cryptophlebia leucotreta*: The Host of CrleGV

Cryptophlebia leucotreta, or the False Codling moth (FCM), is the host of CrleGV. Taxonomically, it belongs to the order Lepidoptera, family Tortricidae (Annecke and Moran, 1982).

The majority of insects in the order Lepidoptera are the moths and butterflies. Adults are easily recognized by the scales that cover their bodies and wings. They have mouthparts which form a suctorial proboscis that is coiled beneath the head when not in use. It is used to drink nectar and water. The larval stages of these insects are notorious for their damage to plants when feeding, using their powerful jaws or mandibles. The larvae generally have six thoracic legs and may have a varied number of fleshy prolegs attached to the abdomen. A feature unique to Lepidoptera is that these prolegs end in a circle of hooks known as crochets (Annecke and Moran, 1982).

The adult of *Cryptophlebia leucotreta* is an inconspicuous dark brown to grey moth with black markings and a wingspan of 16-20 mm **(Fig. 1.8)**. The forewings have a mottled pattern ("leucotreta" means with white perforations), while the hind wings have a more uniform, lighter colour and are fringed with hairs. Adult males are smaller than females

and are distinguished by anal tuft scales, a scent organ near the anal angle of each wing and a collection of densely packed elongated scales on the hind tibia (Newton, 1998).

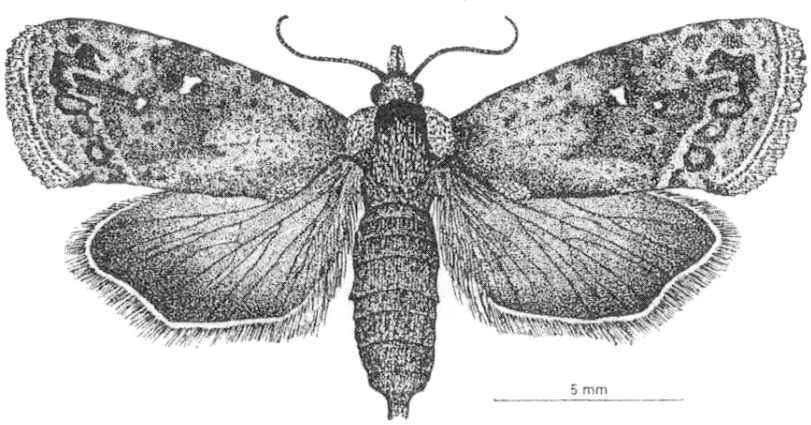


Figure 1.8: A *Cryptophlebia leucotreta* moth (Pinhey, 1975**).**

C. leucotreta is found throughout sub-Saharan Africa and the neighbouring islands of the Indian and Atlantic Oceans, including Mauritius. It has a wide range of indigenous host plants and has also adapted to attack many cultivated crops, particularly many deciduous, subtropical and tropical fruits (Newton, 1998) (See list of some susceptible species of cultivated crops in **Appendix 1**). Despite the wide host range, the major economic effect of the moth appears to be on the citrus and cotton crops and under South African conditions, it is most injurious to the commercial citrus crop. Other countries in which *C. leucotreta* has been found to be a pest on citrus are Mozambique, Zimbabwe and Swaziland (Newton, 1998).

1.4.1) Life history of *Cryptophlebia leucotreta*

The eggs of *C. leucotreta* are flat, oval and translucent with a flanged surface. They are approximately 1 mm in diameter and are most commonly laid on the surface of fruit. Up to 12 eggs are normally found per fruit (Pinhey, 1975). As population sizes increase, more eggs are laid per fruit. These eggs have a high rate of fertility and take a week to hatch (Newton, 1998).

There have been reports of newly hatched larvae being cannibalistic towards other eggs or larvae. This decreases competition among individual larvae and has been suggested as

the reason for normally only one larva completing development per fruit (Annecke and Moran, 1982). On hatching, the larva goes through five larval instars before it pupates. The first instar as well as the eggs are very delicate and suffer high mortality in cases of low humidity in laboratory cultures or low winter temperatures in the field. The fruit is often entered through an injury point or the navel end (in the case of oranges) and the last instar leaves the fruit through a frass-filled exit hole from which it drops to the ground on a silken thread, or emerges when the fruit has already fallen from the tree due to abscission. Early instar larvae are cream coloured with a black head capsule, while late instar larvae (instar 4 or 5) have a characteristic pinkish-red colour, which is less intense ventrally. Fully-grown larvae (**Fig. 1.9B**) are 15 to 20 mm in length (Newton, 1998).

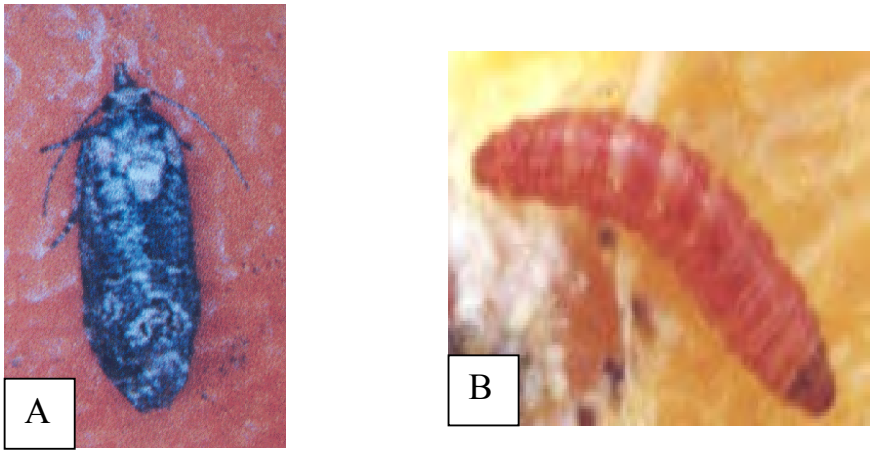


Figure 1.9: (A) *Cryptophlebia leucotreta* moth and (B) A 5th instar larva (Moore, 2001). (See text for dimensions).

Subsequent to a light beige prepupal stage, the larva pupates in loose soil, beneath surface debris or in cracks in the soil. It camouflages its silken cocoon by incorporating bits of leaf litter and soil particles into it (Newton, 1998).

On emergence of the adults (**Fig.1.9A**), females go through a 1 to 22 day pre-oviposition period, depending on the temperature. At 25°C pre-oviposition periods of 1 day have been recorded, while at 10°C pre-oviposition periods of 22 days have been recorded. Multiple mating occurs both in males and females. Egg laying capacities are temperature dependent and females have been recorded to have a fecundity up to 460 eggs or as few

as 0.6 eggs at 25°C and 10°C respectively. Egg laying may reach a peak at about 3-5 days of age. At constant temperature, adults in captivity may live from 13 to 34 days depending on their gender. Males, on average, live a shorter time than females do. Average survival of the moths in the field was recorded as two to three weeks (Newton, 1998).

1.4.2) Overall life cycle of *Cryptophlebia leucotreta* on citrus

Egg incubation times of 9 to 12 days in winter and 6 to 8 days in summer have been recorded. Larval development occurs over 35 to 67 days in winter and 25 to 35 days in summer (Newton, 1998). The larvae normally feed on the inner rind of the fruit and to a lesser extent on the juicy inner flesh (Pinhey, 1975). Pupation occurs over 29 to 40 days in winter and 21 to 24 days in summer. Total development times are 2.5 to 4 months and 1.5 to 2 months in winter and summer respectively. There are normally 5 to 6 poorly defined overlapping generations per year. Generation time is heavily dependent on temperature, as was shown by generations times (on artificial media) of 32 and 114 days at average ambient temperatures of 26.3°C and 13.7°C respectively. Under constant conditions of 27°C and 70% relative humidity (RH), *C. leucotreta* requires 23 to 26 days from egg to adult emergence. No diapause has been recorded for *C. leucotreta* in South Africa (Newton, 1998).

1.4.3) Economic importance of *Cryptophlebia leucotreta*: effect on citrus fruit

As was mentioned previously, under South African environmental conditions *C. leucotreta* preferentially attacks ripening citrus fruits. The navel cultivar of citrus appears to be the most heavily attacked of those grown in Southern Africa. Grapefruits and mandarins are less susceptible to FCM infestation and larval development is rarely completed in lemons and limes. This may be a result of their greater acidity and excessive juiciness (Newton, 1998).

On emergence of larvae from eggs laid on the fruit surface, it burrows into the fruit. In citrus this leads to premature ripening, premature abscission as well as fruit decay, primarily caused by fungal infection. This abscission of infested fruit occurs in early

November and some fruit is lost due to the lesions caused by exploring larvae, even though they may not survive long enough to burrow into the fruit**.** The larvae feed on the pith just below the citrus fruit surface or bore their way to the core of the fruit through the pith (**Fig.1.10**). This feeding causes the rind to take on a yellowish brown colour at the region of infestation and the tissue collapses as it decays (Newton, 1998). A single larva can therefore destroy an entire orange and the moth produced approximately 23 days later, can then produce further larvae that will destroy more fruit. Without control, the moth can thus cause a large amount of damage within one season. The degree of fruit damage is highly variable from orchard to orchard and between seasons, but it can be as much as 90 % of the citrus crop (Begemann and Schoeman, 1999).

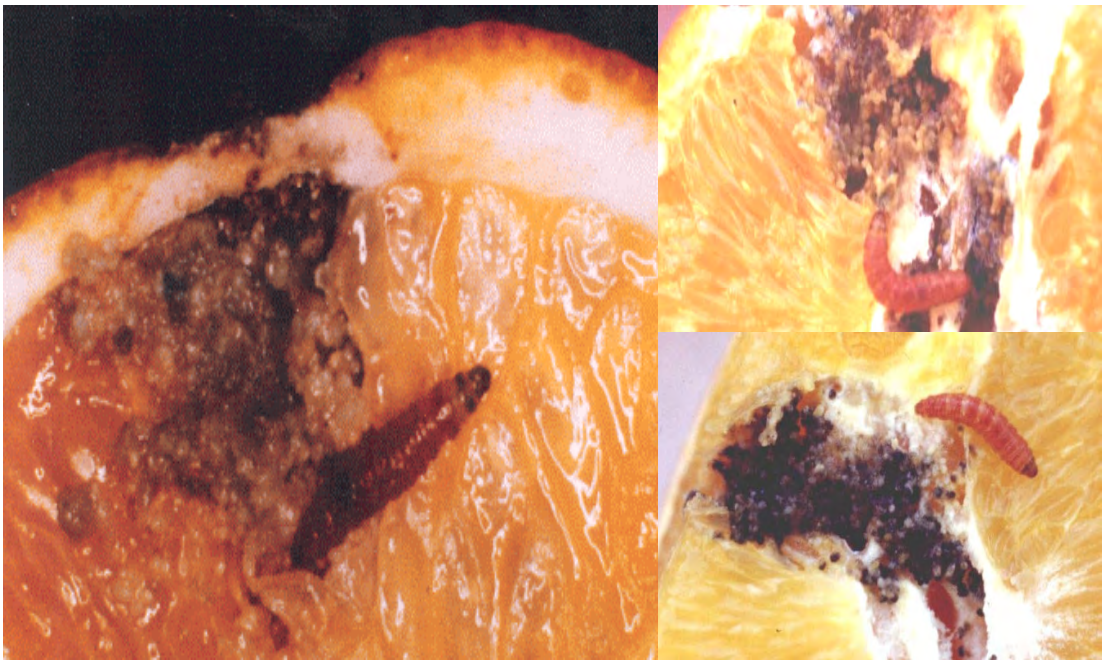


Figure 1.10 : The effect of the *C. leucotreta* larva on citrus fruit. Note the frass filled tunnels, as well as the initiation of decay visible in the left hand photo especially visible on the surface of the orange rind (Moore, 2001).

The broad host range in conjunction with mild tropical and subtropical winters, enable this moth to be a continual threat to its host plants all year round. The large number of indigenous host species also increases chances of the pest spreading from these indigenous plants to cultivated host plants (Newton, 1998). The moth has been estimated

to cause an annual loss of revenue in the citrus industry of R100 million. A large portion of the citrus produced are directed to exportation and many of the recipient countries are hesitant about accepting shipments that may contain *C. leucotreta* larvae for fear of the moth being introduced into that region of the world. The discovery of a single larva in a shipment of citrus may therefore condemn that entire shipment (Moore, 2001). Inadequate control of this pest may harm the growth of the citrus industry.

Other than citrus in South Africa, *C. leucotreta* causes huge infestations in sorghum in Central Africa and cotton in equatorial Africa. Macadamia nuts have also become a target host in Malawi and South Africa (Newton, 1998).

1.5) The Effect of Granuloviruses on Their Insect Hosts: Gross Pathology

GVs are mainly pathogenic towards the larval stages of the lepidopterous hosts. Symptoms are similar to those produced by polyhedroses, but differ due to infection of different tissues (Tanada and Kaya, 1993).

The first indication of infection in the larva is a loss of appetite and a progressive colour change from the normal colour to a pale whitish or milky-yellow appearance, particularly on the ventral side. This whiteness is due to the accumulation of occlusion bodies within the hypertrophied fat body of the insect. In cases where the infection is limited to mainly the fat body, the larva often increases in size, becomes white, opaque, and mottled at an advanced stage of infection, and later has a brownish to black discolouration often caused by the invasion of the body by gut flora. Melanotic spots may appear on the cuticle, indicating infection and necrosis of the epidermis (Tanada and Kaya, 1993; Federici, 1997). Such a larva may live longer and may become bigger than an uninfected one. The larva normally becomes progressively weaker, sluggish and flaccid with the onset of this colour change. The cuticle also takes on a glossy appearance and becomes increasingly fragile (Tanada and Kaya, 1993).

The hemolymph is often milky white and turbid, owing to the presence of large numbers of viral occlusion bodies discharged from infected tissues during advanced stages of infection. Under the light microscope, virus infected cells are yellow to light brown and opaque. As opposed to NPV infection within Lepidopterans, which is systemic, many GV infections are confined to one or a few tissues. In some granuloses, such as predominantly type 1 GV infections, mitotic proliferation of uninfected cells in order to replace the cells killed by infection occurs in the fat body. This produces a bloated enlarged larva during the late stages of infection that is bigger than uninfected larvae. In contrast, systemic granuloses normally cause the host larva to die off in a brief period, much shorter than an infection involving predominantly the fat body. Such systemic infections result in infected larvae being smaller than healthy larvae. The larval integument is fragile just prior to death and the dead larva is wilted. Systemic or type 2 GV infection is similar to that observed in NPV infections. Some examples of systemic GV infections are those caused by ArGV (*Artogeia rapae* GV) and CpGV (*Cydia pomonella* GV) (Tanada and Kaya, 1993). CrleGV therefore most likely also causes a systemic granulosis infection, due to its close similarity to CpGV (Jehle *et al.*, 1992). Type 3 GV infections (e.g. that caused by *Harrisinia brillians* GV) only infect the larval and adult gut. Susceptible insects develop chronic diarrhoea and die from dehydration (Tanada and Kaya, 1993).

Cryptophlebia leucotreta larvae infected with CrleGV in the advanced stages have a flaccid appearance, are sluggish and unresponsive and also hang inverted from their abdominal prolegs (Moore, 2001). In **Fig. 1.11** (**A**), the milky white colouration brought about by the accumulation of occlusion bodies is clearly visible and distinguishable from the example of the healthy larva **(Fig. 1.11 (D)).** Note that this milky white colouration is visible more easily on the ventral side of the larvae, due to the pink colouration of the larval cuticle. In **Fig. 1.11 (A), (B) and (C)**, the shiny condition of the cuticle is distinct in comparison to the healthy larva. **Fig. 1.11 (B)** clearly shows the development of dark necrotic spots on the epidermis, indicating tissue breakdown and death. **Fig. 1.11 (B)** and **(C)** show dark discolouration below the surface of the cuticle. This could indicate the

invasion of the body by gut flora. **Fig. 1.12** shows a flaccid larval cadaver hanging from an elevated position.

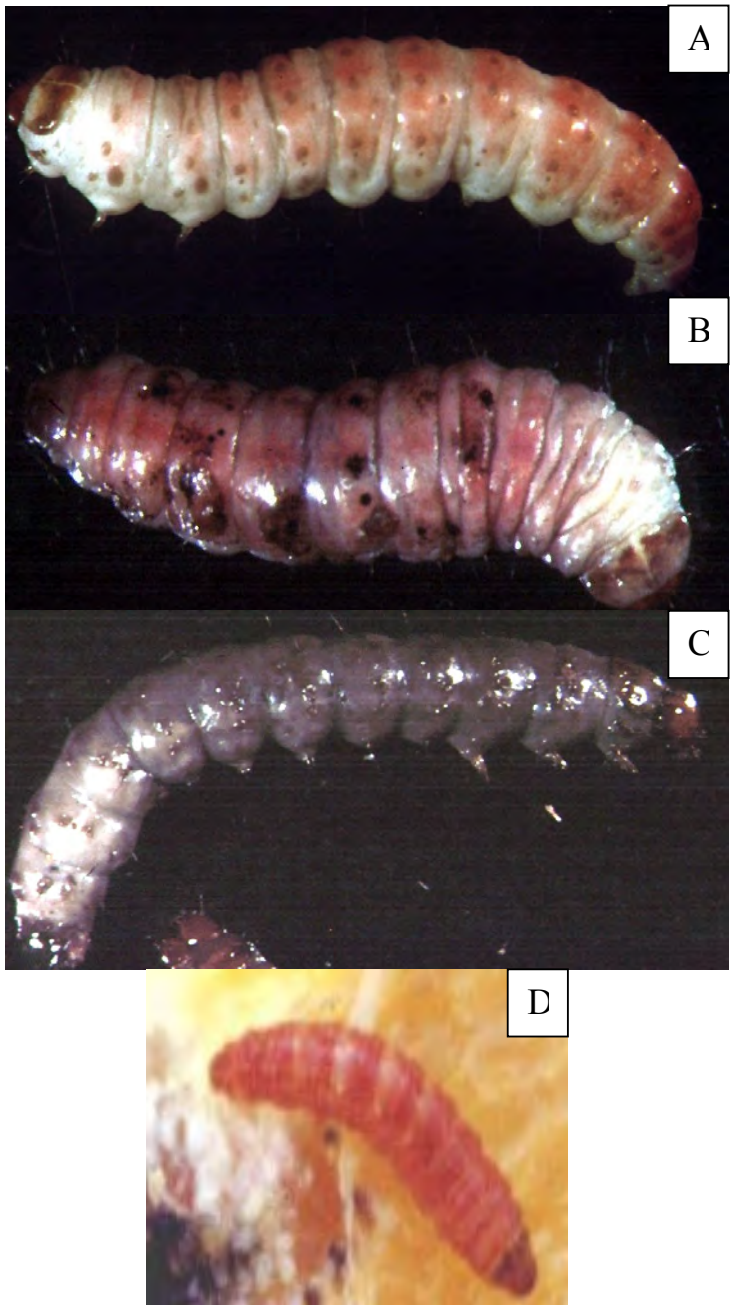


Figure 1.11: (A), (B) and (C) showing the macroscopic symptoms of CrleGV in *Cryptophlebia leucotreta* larvae. (D) An asymptomatic *C. leucotreta* larva (Moore, 2001).

Figure 1.12: A larval cadaver showing the distinct inverted hanging from an elevated position that is characteristic of many GV and NPV infections in larvae of the order Lepidoptera. Note the limp, flaccid appearance (Moore, 2001).

1.6) Baculovirus Ecology : Transmission and Persistence

Though baculoviruses remain one of the most studied groups of insect viruses, surprisingly little is known about their ecology. This involves the investigation of the interaction between the virus, its host and its natural environment. More emphasis appears to have been placed on the molecular characterisation of these viruses, even though a detailed understanding of their ecology is the key to their effective use as biological control agents and knowledge of the potential risks associated with their use. It would help us to make predictions about how genetically modified viruses designed for a faster kill rate and therefore production of a lower viral progeny yield may influence the viral transmission ecology. Baculoviruses can have considerable phenotypic variation between different isolates. How would selection for specific isolates by mass application of these isolates in the environment affect virus and host population dynamics? Two important and intimately related aspects of viral ecology are virus transmission between hosts and viral persistence in the environment (Cory *et al.*, 1997).

The spread and persistence of a pathogen, as well as its influence on host population dynamics, is determined by the transmission processes. In field populations of insects, pathogen transmission is often a combination of horizontal and vertical transfer.

Horizontal transfer refers to the transmission of pathogens among host individuals within a generation and between generations as environmental contamination. Vertical transmission is the transfer of pathogens from parent host individuals to progeny (Kukan, 1999). Vertical transmission can be further divided into transovum (passage of the pathogen to progeny on the surface of the egg) and transovarian (passage of the pathogen to progeny through the female parent's ovary as infectious virions or as latent infections). In the case of latent infections, the virus is in a noninfective and nonreplicative state and does not cause visible symptoms, but it can later be transformed into an infective and actively replicating state if the host is subjected to stress (Fuxa *et al.*, 2002). Stress factors that have been known to induce an active replication of latent virus are crowding, unsuitable diet, non-optimal environmental temperatures and co-infection with a different virus (Longworth and Cunningham, 1968). Relative humidity and physical stress (e.g being reared on a shaker) have been known to activate latent viral infections and predispose insects to infectious diseases (Fuxa *et al.*, 1999). Definitive evidence to distinguish between transovarian and transovum transmission has been difficult, due to the uncertain effectiveness of the total removal of virus from the egg surface (Fuxa and Richter, 1991). However, there is evidence for vertical transmission in some baculoviruses. For example, 14.3% mortality was observed in *Spodoptera frugiperda* progeny of individuals dosed with SpfrNPV, while under the same conditions, *Anticarsia gemmatalis* NPV (AgMNPV) was not vertically transmitted (Fuxa and Richter, 1993). Fuxa *et al.* (2002) also found that *Trichoplusia ni* SNPV (TnSNPV) was vertically transmitted at a prevalence rate of 15.4%.

Vertical transmission has potential advantages in bio control, but it does not appear to be critical in maintaining viral prevalence in nature, especially considering the ability of baculoviruses to remain viable outside of the host for long periods. However, it appears to be a mechanism of pathogen spread within an ecosystem (Fuxa *et al.*, 2002). Vertical transmission appears to be a feature of those viruses that replicate predominantly within the gut epithelium. Viable virions are often excreted in the moth faeces, which may contaminate the ovipositors of females (Payne, 1982).

Vertical transmission studies involving recombinant baculoviruses intended for pest management are important for risk assessment. Environmental harm is difficult to predict and if these recombinant viruses are vertically transmitted, they are more likely to be transported from their release sites and persist in the environment. Risk of environmental harm would therefore increase (Fuxa *et al.*, 2002). According to Fuxa *et al*. (2002), vertical transmission of recombinant AcMNPV (AcMNPV.AaIT – AcMNPV into which an insect specific scorpion toxin (AaIT) from *Androctonus australus* has been inserted) was minimal. This implies that the spread of the recombinant virus in nature may be more easily controlled (Fuxa *et al.*, 2002).

Sub-lethally infected individuals have been found to display a decrease in rate of development, fecundity and percentage hatch, resulting in decreased reproductive rate. These may result from the diversion of host energy reserves to support or combat the pathogen, disruption of oocyte development or hormonal changes induced by the pathogen. These effects appear to be primarily a feature of less pathogenic diseases. A better understanding of population dynamics of Lepidoptera may reveal that limiting virus-based biological control to mortality alone is greatly underestimating the capability of virus-based pesticides. However, certain researchers have argued that these debilitating effects could be an artefact produced in the rearing of laboratory colonies (Rothman and Myers, 1996).

Horizontal transmission of baculoviruses can occur when occlusion bodies from infected cadavers are deposited on plant foliage. Some of this virus is removed by abiotic factors, such as rain, and settles in the soil. As the virions adsorb to soil particles they remain in the upper layers of the soil. The soil therefore acts as a virus reservoir that maintains virus viability due to protection from UV light (Summers *et al.*, 1975). Ultraviolet light has been found to be a major cause of loss of virion viability in nature, due to the damage it causes DNA in the form of pyrimidine dimers (Mathews and van Holde, 1996). These dimers accumulate in the virions when they are not in host cells, as they do not possess the required cellular repair machinery. In spite of the inactivating effect of UV, occluded virus activity can still be detected as much as 10 days after virus application to fields

where it is exposed to sunlight. In an experiment involving the application of *Trichoplusia ni* NPV and *Pieris rapae* NPV occlusion body suspensions to cabbage leaves in field plots, inactivation of virus by sunlight caused about 50% of the infectious activity to be lost in 2 days after application, and most of the remaining activity was lost in the subsequent 8 days. **Fig. 1.13** shows a typical loss of infectious activity in occluded virions exposed to direct sunlight.

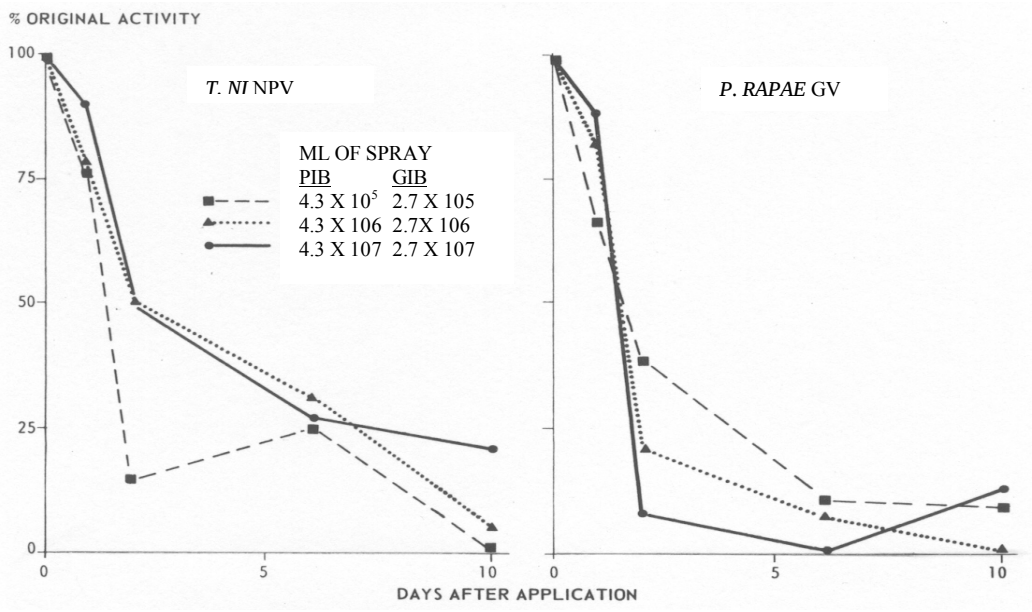


Figure 1.13: The infectious activity of *Trichoplusia ni* NPV and *Pieris rapae* GV after application of occlusion body suspensions to leaves of cabbage plants in field plots (Summers *et al*., 1975). PIB = poly inclusion/occlusion bodies (NPV), GIB = Granulin inclusion/occlusion bodies (GV).

The virions are resistant to strong acids and other chemicals, but they are susceptible to degradation by even reasonably weak alkalis (Summers *et al.*, 1975). This implies that virus would persist longer in more acidic soils. It has been hypothesised that virus is translocated from soil back onto plants through the same abiotic factors (rain splashing or wind blowing virus-containing dust (Cory *et al.*, 1997). Other horizontal transmission involves direct contact of individuals with virus-contaminated faecal matter or virus in the habitat. Eggs can be contaminated by contact with contaminated faeces, as they are laid (Hamm and Young, 1974). Prevalence of virus infections in adults is far lower and often undetectable (Kukan, 1999). Other factors that have been implicated in virus persistence and transmission are alternate insect hosts and other organisms such as

parasitoids, insectivorous birds and mammals. Persistence is an important factor to consider when evaluating a virus as a potential biocontrol agent (Payne, 1982).

1.7) Techniques of Controlling *Cryptophlebia leucotreta*

Cryptophlebia leucotreta is an extremely difficult pest to control as it lays eggs continuously throughout the fruiting period of citrus. The larva also bores into the fruit within hours of hatching, after which it is difficult to reach with control measures without damaging the fruit (Newton, 1998).

1.7.1) Chemical insecticides

Protection of crops by spraying them with insecticide 3 times in 2.5 to 6 weeks before harvesting serves to control *C. leucotreta*. However, the cost and side effects of applying chemical sprays at intervals throughout the growing season have prevented extensive use of chemical sprays for insect control in citrus orchards (Annecke and Moran, 1982). Chemical control of FCM is difficult and expensive due to the inaccessibility of many of the life stages and persistent pest pressure throughout the fruiting season. Use of dichlorodiphenyltrichloroethane (DDT) and parathion has reduced fruit infestation by 70%, but poison residue problems and other pest resurgence have occurred. More recently, three insecticides have been commonly used for FCM control. Insect growth regulators (IGR), e.g. triflumuron, work well only if tree coverage is thorough and most growers are unable or unwilling to apply the volumes of IGR. Resistance to triflumuron has developed in some areas. Microencapsulated methyl parathion is detrimental to other insects that may be beneficial (such as natural enemies of FCM) and its pre-harvest interval exceeds its period of residual efficacy, resulting in a period where the fruit is not protected. The pyrethroid, Fenpropathrin, is also non-specific in the insects it affects and can only be applied four weeks before harvest (Moore, 2001).

1.7.2) Techniques of control other than chemical pesticides

Orchard sanitation involving removal and disposal of fruit that falls from the trees, often due to larval infestation, has been found to cause a 40 % decrease in *C. leucotreta* populations and a decrease in crop losses (Newton, 1998). Egg parasitoids (e.g.

Trichogrammitoidea lutea [Trichogrammitidae]) and larval parasitoids (e.g. *Apophua leucotretae* and *Agathis bishopi*) of *C. leucotreta* can be released into citrus orchards to control FCM populations (Newton, 1998). Adhesive sex pheromone traps, impregnated with female FCM sex pheromones to attract male FCMs, can be used to monitor and control *C. leucotreta* populations within orchards. Fungal and viral pathogens that attack and reduce *C. leucotreta* populations are being considered as microbial-based control agents (Newton 1998).

1.8) Baculoviruses as Insect Pesticides

The potential of baculoviruses to be utilized as biopesticides has long been recognized, due to their host selectivity as well as their highly virulent nature. Their selectivity is an important safety aspect as they only attack their host species. This has far less impact on the environment and also does not affect humans. In the 1970s, the first baculoviral-based pesticide (*Elcar Helicoverpa zea* NPV) was released into commercial production and proved effective due to its high specificity and infectiousness (Tracey and All, 1996; Payne, 1982). However, at the onset of the 1990s baculovirus pesticides were only part of narrow niche markets and even in the present day, viral insecticides still represent a small part of the pesticide industry. Owing to a large amount of research being initiated into baculoviral use as pesticides, the proportion of viral pesticides in relation to chemical pesticides has the potential to increase substantially in the future (Black *et al.*, 1997). **Table 1.1** shows a list of baculovirus pesticides that have entered commercial production for the purposes of pest control.

The majority of these viruses have been isolated from Lepidoptera and Hymenoptera, where the active feeding stages are larval and the mode of viral entry is ingestion. The larval stage is therefore the most frequent target of viral based pesticides in biological control. Larval susceptibility to many NPVs and GVs decreases rapidly as larvae age. Older larvae can also cause more feeding damage and therefore many viral pesticides are aimed to control pests at the early larval instars (Payne, 1982).

Table 1.1: *Baculoviruses that have been used as biopesticides (Weeden, 1996).*

COMMODITY	INSECT PEST	VIRUS USED	VIRUS PRODUCT
Apple, pear, walnut and plum	Codling moth	Codling Moth granulosis virus (CpGV)	Cyd-x
Cabbage, tomatoes, cotton, potatoes	Cabbage moth, American bollworm, diamondback moth, potato tuber moth, and grape berry moth	Cabbage army worm nuclear polyhedrosis virus	Mamestrin
Cotton, corn, tomatoes	*Spodoptera littoralis*	*Spodoptera littoralis* nuclear polyhedrosis virus	Spodopterin
Cotton and vegetables	Tobacco budworm (*Helicoverpa zea*), and cotton bollworm (*Heliothis virescens*)	*Helicoverpa zea* nuclear polyhedrosis virus	Gemstar LC, Biotrol, Elcar
Vegetable crops, greenhouse flowers	Beet armyworm (*Spodoptera exigua*)	Spodoptera exigua nuclear polyhedrosis virus	Spod-x
Alfalfa and other crops	Alfalfa looper (*Autographa californica*)	*Autographa californica* nuclear polyhedrosis virus	Gusano Biological Pesticide
Forest habitat, lumber	Douglas Fir tussock moth (*Orgyia pseudotsugata*)	*Orgyia psuedotsugata* nuclear polyhedrosis virus	TM Biocontrol
Forest habitat, lumber	Gypsy moth (*Lymantria dispar*)	*Lymantria dispar* nuclear polyhedrosis virus	Gypchek

The ability of baculoviruses, applied to the foliage of crops, to control insect pests is dependent on two fundamental issues: 1) effective dose acquisition and 2) the speed of action of the acquired dose (Black ***et al.***, 1997). Effective dose acquisition is important to the success of any insecticide. The effective dose of contact poisons can be achieved easily when the target insect walks across a treated leaf surface. An effective dose for baculovirus pesticides requires that the targeted insect ingests a dose of virus that is capable of initiating a productive systemic infection. Factors that therefore influence effective dose acquisition in baculovirus pesticides are: the rate of baculovirus application, the stability (residual activity) of the applied dose on treated leaf surface over

time, the feeding behaviour of pests that need to be controlled and how well the host range of the baculovirus matches the composition of the pest complex at the time of application (Black *et al.*, 1997; Payne, 1982).

The occluded form of baculoviruses permits them to remain viable within the environment for long periods due to the protection from UV light provided by the granulin occlusion capsule (Blissard and Rohrmann, 1990). Rate of application of the insecticide is therefore lowered in comparison to that of other potential virus pesticides. Viruses within the *Baculoviridae* have extraordinary host range diversity. The chances of finding a baculovirus with the necessary host range for a particular pest complex are therefore good (Black *et al.*, 1997).

Speed of action has historically been one of the largest weaknesses of baculovirus-based pesticides. Most naturally occurring baculoviruses require a period of 4 to 7 days to kill their hosts and some such as the gypsy moth (*Lymantia dispar*) NPV can take up to 18 to 21 days. During this time period, the larvae continue to feed and may cause substantial crop damage. It is therefore possible to apply enough virus to control the entire pest population and still lose the crop due to feeding damage. Several strategies have been developed in an attempt to increase the speed of action of baculoviruses. The two main ones involve gene insertion and gene deletion within the viral genome. Certain genes non-essential within the viral genome actually act to prolong the life of the host or halt it in certain stages of development in order to maximize virus production before host death. One such gene is the ecdysteroid glucosyltransferase (*egt*) gene first isolated from AcMNPV (Black *et al.,* 1997). It acts to inactivate the ecdysteroid hormones, which control moulting and pupation of the larval stages. Inactivation of the ecdysteroids effectively halts moulting of the larvae and keeps them in the early instar stages where viral production is at its highest (Wormleaton and Winstanley, 2001). It has been found in studies with AcMNPV that deletion of *egt* caused *Autographa californica* to die up to 30 % faster than when it was infected with egt^{+} strains. Insertion of insect-specific toxin genes or insect genes into the baculovirus genome has also been considered (Sun *et al.,* 2002; Hughes *et al.,* 1997; Stewart *et al.,* 1991). In the case of insect gene insertion, the

baculovirus is used to cause inappropriate regulatory gene expression within the insect and result in a disruption of homeostasis great enough to cause accelerated death of the host. Insect-specific toxin genes, particularly neurotoxins, are viewed as the most promising method of creating a commercially viable bio pesticide (Black *et al.*, 1997). A spider toxin gene inserted into the AcMNPV genome showed a reduction in feeding time of *Autographa californica* larvae of up to 39%. This indicates a significant decrease in feeding damage done to crops in comparison to the wild type virus (Hughes *et al.*, 1997). In another example, an insect-specific toxin from the scorpion *Buthus empeus* was inserted into AcMNPV and placed under the control of the polyhedrin promoter. This caused rapid death to the *Autographa californica* larvae through paralysis and therefore drastically reduced feeding damage (Stewart *et al*., 1991). Although the results obtained from recombinant viruses in terms of pest control have been better than wild type viruses, the general public opinion towards the release of genetically modified viruses is likely to be a huge hindrance to their application in the field. The viruses with inserted toxin genes are likely to be the most difficult to be accepted on grounds of having the name toxin associated with them (Black *et al.*, 1997).

There is reason to believe that most of the parameters affecting baculovirus performance as insecticides can be optimised, and once this is achieved they will be able to compete effectively with present chemical pesticides (Black *et al*., 1997). Baculovirus insecticides are already a reality on a small scale. An NPV pathogenic to the velvet bean caterpillar is used in Brazil to control outbreaks of this pest on the soybean crop. *Cydia pomonella* GV has proved to be an effective control of *Cydia pomonella* and has been produced commercially as a pesticide in Germany, Switzerland and France (Winstanley and Crook, 1993). The narrow host range of this virus has enabled the control of *C. pomonella* without affecting the populations of beneficial insects (Payne, 1982). The use of virus in controlling insect populations is favourable due to the rapid resistance of pests to insecticides. There is also concern due to the hazardous nature of chemical-based insecticides in the environment and therefore baculoviruses have been identified as a viable alternative (Doerfler and Bohm, 1986).

The safety of baculovirus pesticides has been a well-scrutinised subject. Humans and animals have long been exposed to baculoviruses through contact and contamination of their food, as a result of the widespread and persistent nature of these virions within their natural habitat, with no detrimental effect (Summers ***et al.,*** 1975). Inhalation, intramuscular and subcutaneous applications of viral preparations into humans have resulted in no pathogenicity and toxicity (Payne, 1982).

1.8.1) The advantages and disadvantages of baculoviruses as insect control agents

Baculoviruses occur naturally in the environment and serve as a natural form of insect population control. The use of wild type viruses as biopesticides would therefore cause far less ecological turmoil than the use of chemical insecticides. Many baculoviruses also have a narrow and specific host range. For example, CrleGV has been shown to infect only *Cryptophlebia leucotreta* larvae and not *Cydia pomonella*, a closely related insect (Jehle ***et al.***, 1992). Baculoviruses that have a narrow host range can therefore be chosen to prevent the removal of beneficial insect species from crops when broad-spectrum pesticides are used to control pest species. This has overall benefit for the crop or orchard, as pollinating species of arthropods are not affected and they may even protect plants against pathogens. Virus biopesticides therefore augment, rather than supplant, natural control of crop pests (Tracey and All, 1996). There has also been no evidence of a build up of resistance to viruses in the field, as happens with chemical pesticides (Payne, 1982; Eldridge ***et al.***, 1992). In laboratory tests, however, high selection pressure has produced virus resistant insects. This is a factor that will require monitoring in virus-based pest control (Payne, 1982). Baculoviruses have only been recorded to infect arthropods and are therefore non-toxic to other organisms. Virus infection in vertebrates including humans has been tested with 26 different baculoviruses. A dosage of 10 to 100 times the per acre field rate used in the bio pesticide administration was used to adjust to the weight of a 70 kg human. No infection was observed at all and therefore the baculovirus pesticides are far safer than many chemical pesticides that accumulate within biological tissues and increase in concentration with each successive step of the food chain (Black ***et al.***, 1997). Chemical sprays such as carbaryl, malathion and methyl parathion have also been shown to cause damage to crop plants and induce mutations

within these plants (Summers ***et al.,*** 1975). In this respect, therefore, baculoviruses appear to offer a safer alternative to chemical sprays that have a far larger ecological impact. **Table 1.2** shows the comparative toxicity of three commonly used chemical pesticides and *Heliothis* NPV on groups of vertebrates. It is evident that the baculovirus has far less effect on the rest of the environmental ecology (Summers ***et al.***, 1975).

Table 1.2: The comparative toxicity of a *Heliothis* NPV and three chemical pesticides to classes of invertebrates (Summers *et al*., 1975)

Material	**Response**		
	Mammals	**Birds**	**Fishes**
Heliothis NPV	Negative	Negative	Negative
Parathion	Toxic	Toxic Teratogenic Embryotoxic	Toxic
Malathion	Toxic	Toxic Teratogenic Embryotoxic	Toxic
Carbaryl	Toxic Teratogenic Embryotoxic	Toxic Teratogenic Embryotoxic	Toxic predisposition

Baculoviruses have a thick protein capsule forming an occlusion body in which the virions are embedded. This capsule provides environmental stability to the fragile virions that are provided protection from UV light as well as mechanical stress. The virions can therefore persist in the environment for a long period, thus decreasing the number of applications of pesticide required for effectiveness, which is economically suited for long term crops such as forestry plantations that create a lower UV environment (Black ***et al.***, 1997). The longevity of viral pesticides has been increased by adding sunshield additives such as carbon and fluorescent materials such as polyflavinoids, which shield the virus from ultra violet light (Payne, 1982).

In the case of *Cryptophlebia leucotreta*, larvae burrow into citrus fruit (Annecke and Moran, 1982). There is only a short time that these larvae would be sensitive to chemical sprays. Once inside the fruit, the sprays can't get to them easily without contaminating the interior of the fruit, which would make human or animal consumption dangerous. In contrast, the larva could ingest CrleGV virions, deposited on the surface of the fruit, as it

is burrowing into the fruit. The larva will then die as a result of the infection, even after it has burrowed into the fruit. However, a faster rate of kill is required in order to decrease feeding damage inflicted by the infected larvae prior to death. The population of the pest will therefore still be controlled. A faster rate of kill could be achieved by genetic modification (Black *et al.*, 1997). Owing to the small size of virions, virus pesticides can be applied to crops using the same machinery as for chemical pesticides (Moore, 2001). Conversion to virus pesticides by farmers will not mean extreme cost in capital equipment.

Baculovirus pesticides do have a number of disadvantages. As with all viruses, baculoviruses require host cells to replicate. This means that baculovirus pesticides would have to be produced using insect colonies, which is labour intensive, or tissue culture requiring a highly sterile environment, both of which would dramatically increase the cost of production (Black *et al.*, 1997). A disadvantage of the specificity of virus pesticides is that each pest requires the development of a new pesticide, which requires finance for research and development. This process could prove to be costly as a good understanding of the ecology of each virus is required before it is used as a pesticide. In addition the process may only prove to be commercially viable if there is a sufficient market for control of that particular pest. The complexity of viral action and its potential for genetic change through mutation requires constant monitoring and quality control of commercial virus pesticides (Payne, 1982). Monitoring of insect resistance to viruses must also be closely monitored and assays for the development of viral residue detection are also important (Doerfler and Bohm, 1986). This monitoring may be done using techniques such as the one described by Moraes *et al.* (1998), in which polymerase chain reaction is used to detect NPVs in field samples. The greater sophistication required to get good results with biocontrol may also make growers hesitant to convert to this form of insect control, unless their market demands it (Payne, 1982).

Chemical pesticides appear to be far cheaper to produce at present. Other microbial pesticides such as the toxin from *Bacillus thuriengensis* (a bacterium) are easier to

produce, since they just require the correct nutrients to reproduce themselves as opposed to host cells.

It may be useful to consider that the ideal biocontrol agent should have a wide range of host species (most of them pest species), each with similar susceptibilities and should pose no ecological hazard. The wide host range ensures a constant reservoir of the biocontrol agent in the environment (Payne, 1982). There is no control agent that can fulfil these requirements completely although viruses do pose a much lower ecological hazard than chemicals and don't affect useful invertebrate species. Ideally an integrated pest management approach to pest control should be employed, where all of the previously mentioned methods of control are used together in order to effectively control pests. However, such a programme requires careful monitoring and coordination of the various control measures in order to get the desired results.

1.9) Research Objectives

This project had two main research objectives. The first one was to establish a virus free laboratory colony of *Cryptophlebia leucotreta*. Latent or occult virus infections are common within insects and are normally difficult to eradicate. Most molecular genetic studies of baculoviruses are performed using plaque-purified strains in cell culture (Possee and Rohrmann, 1997). In the absence of a tissue culture cell line that supports CrleGV infection, another way of isolating a genotypically pure strain of the CrleGV would be to do so using whole larvae. However, these larvae would need to be free of virus infection. Virulence testing of different isolates of CrleGV, as well as the testing of genetically modified viruses, would be simplified if there was no latent virus infection within the laboratory colony used for these experiments.

The second objective was to identify and locate certain genes in CrleGV that are known to be present in related granuloviruses. If these genes were present, then the intention was to sequence them. The aim was to increase understanding of the CrleGV gene organisation as well as its phylogenetic placement.

Chapter 2: General Techniques Used in the Project

2.1) Source of Virus Material and Larvae for the Laboratory Colony

False codling moth larvae were kindly provided by Sean Moore (Citrus Research International, Port Elizabeth) from his laboratory colony, originally isolated in Citrusdal in the Western Cape, South Africa. He used the colony to produce a Western Cape isolate of the virus. When isolated, this colony had a natural infection of CrleGV and this was the source of virus used in this study.

2.2) Introduction

A number of baculoviruses have been considered and some used as viral based insecticides (Maeda and Majima, 1990). Much less is known about the molecular biology of granuloviruses than that of nucleopolyhedroviruses (Jehle ***et al.,*** 1992). In the context of this project, very little was known about the molecular biology of *Cryptophlebia leucotreta* granulovirus and most of its genome is still un-sequenced. In order for it to be used as a biological control agent a full characterisation of the virus is desirable, if not essential. Purified virus is required for the extraction of DNA necessary for genetic analysis of the virus. Purified infectious virus is also necessary for the infectivity bioassay studies.

Virus purification involves the physical separation of a virus in a concentrated form from the host milieu in which it propagates (Mahy and Kungro, 1996). Ultra centrifugation is the usual method of choice for virus purification due to their small size and low density, and in the case of the CrleGV purification protocol a swinging bucket rotor was required for density gradient centrifugation.

All the raw virus material obtained for this project was in the form of larval cadavers that had been collected in an advanced stage of infection. As the titre of budded virus is higher in the earlier stages of infection (Federici, 1997), the yields of budded virus that would have been obtained from these cadavers is unknown. Also, the budded virus is less

recalcitrant than occluded virus. Consequently it was decided to prepare a unoccludded form of virus by using purified occlusion bodies from which the granulin had been dissolved away. Budded virus was needed to ascertain the effectiveness of the DNA extraction protocol, used in detection, to detect virus in any form.

In order to characterise virus genes a known concentration of intact extracted viral genomic DNA is required. Treatment with phenol and chloroform:isoamyl alcohol (24:1) is a routine method used to remove proteins from nucleic acids. Removal of proteins from an aqueous solution is more effective if treated with two different organic solvents. An additional treatment with chloroform:isoamyl alcohol serves to remove any residual phenol that could disrupt subsequent *in vitro* enzymatic reactions (Farrell, 1993). An important technique for DNA analysis is gel electrophoresis. Agarose electrophoresis is a standard method of DNA analysis that separates DNA molecules or molecule fragments of varying size based on their mobility through the pore matrix in the agarose gel. Since DNA is negatively charged it migrates from negative to positive when subjected to an electric current in solution. Ethidium bromide is a convenient stain for DNA as it intercalates into the double helix structure and fluoresces when exposed to UV light. The DNA bands are therefore visible as orange bands in the gel if viewed directly (Maniatis *et al.*, 1989). Restriction endonuclease digests can be used as an easy way of distinguishing between viral strains or isolates by comparing the profile of fragments produced by digestion of the viral genomic DNA (Jehle *et al.*, 1992).

In many cases it is also necessary to know the quantity of DNA obtained from a batch of purified occlusion bodies. This is most easily done using the property of DNA to absorb light strongly at a wavelength of 260 nm. The ratio of the absorbance at 260nm/280nm gives an indication of the purity of the DNA solution. A pure preparation of DNA gives a ratio of about 1.8 (Maniatis *et al.*, 1989).

2.3) Methods and Materials

2.3.1) Occlusion body (OB) purification

Routinely, 2 g of virus-infected larval material was macerated in 4 ml of 0.1% sodium dodecyl sulphate (SDS) using a SORVAL homogeniser (model 17106) for about 2 to 3 minutes**.** The volume of the homogenate was then made up to 10 ml and filtered though cheesecloth in order to remove large debris such as the chitinous larval exoskeleton. The homogenate was then transferred to 2 clean Beckman JA-20 centrifuge tubes and centrifuged for 30 minutes at 10 000 rpm and 4°C in a Beckman JA-20 rotor. Each occlusion body pellet was re-suspended in 3 ml of 0.1% SDS. Two 30-80 % (v/v) glycerol gradients in 0.1% SDS were prepared in 35 ml centrifuge tubes compatible with the Beckman SW-28 swing out rotor, by adding equal volumes (16 ml) of the 30% and 80% glycerol solutions to the different mixing chambers of the BIORAD model 538 gradient maker. Glycerol was used, as it appeared to maintain a better continuous gradient than sucrose and produced clearer virus bands. It is also inert in relation to the virus. The 3 ml re-suspended OB pellet was carefully layered onto the gradient and the tubes were centrifuged at 15 000 rpm (4°C) for 15 minutes using the Beckman L-70 ultracentrifuge. The virus band was visualized by illuminating the tube from below and it was extracted with an autopipette into 6 Beckman JA-20 centrifuge tubes (kept on ice). The tubes were filled to the lid with distilled water and centrifuged for 14 minutes at 10 000 rpm (4°C). The pellets were re-suspended in distilled water and the spin repeated. They were again re-suspended in water and combined into two tubes, filled to the top with water and spun as before. Three washes were required to get rid of traces of glycerol. The two pellets from the final wash step were re-suspended in 1.0 to 1.5 ml of distilled water. These were stored at 4°C or in 500 µl aliquots at -20°C. **Appendix 2.1** gives the solutions used. The occlusion body concentration was determined.

2.3.2) The estimation of the occlusion body concentration

The purified occlusion body concentration was determined using the Beckman DU530 spectrophotometer. A 1/100, (or more if necessary) dilution was made of the virus. Absorbance readings were measured in a quartz cuvette at 350 nm and 260 nm and the number of occlusion bodies was calculated according to the following formulae (Dr S. Wormleaton, Horticulture Research International, U.K.):

OD_{350nm}/ 13 x dilution factor = mg/ml

OD_{260nm}/ 31 x dilution factor = mg/ml

1mg/ml = 3.83×10^{10} Capsules/ml

For example, if the absorbance (15/1000 dilution of OB solution) at the two wavelengths were 0.292 and 0.356 for OD_{350nm} and OD_{260nm} respectively, the occlusion body concentration would be calculated as follows:

OD_{350nm} : 0.292 / 13 x (1000 / 15) = 1.49 mg/ml

OD_{260nm} : 0.561 / 31 x (1000 / 15) = 1.21 mg/ml

Average = 1.35 mg/ml

Number of occlusion bodies = average x 3.83×10^{10} Capsules/ml

= $1.35 \times 3.83 \times 10^{10}$ Capsules/ml

= 5.18×10^{10}capsules/ml

2.3.3) Budded virus (BV) preparation

An adaptation of the budded virus purification from tissue culture cells as outlined in O'Reilly ***et al.*** (1992b) was used. As no tissue culture system was available for ***C. leucotreta***, 500 µl of occlusion bodies (4.3×10^7 OB/µl) were incubated at 37°C with 40 µl of 1 M sodium carbonate for 30 minutes in order to dissolve the granulin capsule surrounding the virus particle. If the solution did not clarify, it was incubated for a longer period.

A sucrose cushion was prepared in Beckman SW 41 centrifuge tubes. Six ml of phosphate buffered saline (PBS) pH 6.2 were added to each tube and 1 ml of 25 % sucrose solution was underlaid. A further 4 ml of PBS was then added to fill each of the tubes. The 500 µl of occlusion bodies were then added to each tube. The tubes were then centrifuged at 80 000 x *g* (25 600 rpm) for 80 minutes in the Beckman L-70 ultracentrifuge. The virus appeared as a translucent white pellet with a slight blue tinge around the edges. The supernatant was decanted off and sucrose traces removed with a rinse in 2 ml PBS. The pellet was re-suspended in 0.5 ml PBS overnight at 4° C. The virus solution was filter-sterilised using a 0.2 µm filter and stored at 4°C. See **appendix 2.2** for solution recipes.

2.3.4) DNA extraction of CrleGV genomic DNA

To extract DNA from 500 µl of purified occlusion bodies (concentration in the range of $1x10^{10}$ to $1x10^{15}$ OB/ml), 25 µl of 1 M sodium carbonate was added and the solution incubated for 30 minutes at 37°C. If the solution did not clarify, more sodium carbonate (Na_2CO_3) was added. The Na_2CO_3 raised the pH of the solution, which caused the granulin occlusion body to dissolve and release the virus particle (Harris, 1964). The pH lowered to between 8 and 9 using 30 μl or more of Tris EDTA (TE) buffer (10 mM, pH 8). 25 µl of RNase A (10 mg/ml) was then added and the solution was incubated at 37°C for one hour. 60 µl of 10% (w/v) SDS and 50 µl of Proteinase K (25 mg/ml) was added and incubated for an additional hour at 37°C. The SDS increased the activity of proteinase K and inhibited protein-protein or protein-DNA interactions by assigning a net negative charge to the surface of all proteins, thereby assisting in the disassociation of the viral nucleocapsids (Stryer, 1988). A volume of tris-buffered phenol (pH 8) (**Appendix 2.3**) equivalent to the volume of the aqueous virus solution was added and mixed by inverting the tube several times. The phenol caused the precipitation of proteins out of the aqueous layer of the solution and so resulted in the removal of proteins from the solution (Farrell, 1993). The phenol-virus solution emulsion was then centrifuged at 15 000 rpm in a bench top centrifuge for 3 minutes. If the upper aqueous phase was cloudy, the spin

was repeated. The aqueous phase was then extracted into a new clean eppendorf tube and an equal volume of phenol and of chloroform: isoamyl alcohol (24:1) mixture was added to the aqueous solution. The solution was then mixed and centrifuged as in the previous step. The aqueous phase was again transferred into a new eppendorf tube and extracted with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous solution was transferred into a clean tube and then final purification was performed in one of two ways. If viral DNA was being extracted from large volumes of virus solutions, dialysis was the method of choice. This technique involved boiling the dialysis membrane in 1 x TAE buffer for 5 minutes. The aqueous solution was then added to the dialysis bag and immersed in a beaker containing 1 litre of 1 x Tris-acetate EDTA (TAE) buffer and dialysed overnight at 4° C. The buffer was changed the following morning and then again after a subsequent 2 hour incubation. After an additional two-hour incubation, the DNA was transferred into eppendorf tubes and the concentration determined.

Alternatively, smaller volumes of DNA were precipitated using ethanol precipitation. One tenth of the volume (of the viral DNA suspension) of 7 M sodium acetate and twice the volume (of the viral DNA suspension) of 95% ethanol was added to the solution. This was mixed by inversion 2 to 3 times and incubated at -20° C overnight. The solution was then microfuged at 15 000 rpm for 30 minutes. The supernatant was discarded and the pellet washed with 1 ml 70% ethanol. The eppendorf tube was then drained of excess ethanol by inverting on paper towel for 3 to 4 minutes. The tubes were incubated in a heating block at 50 °C for a further 5 to 10 minutes in order to remove the remaining traces of ethanol. The pellet was then re-suspended in 25 to 50 µl of 10 mM Tris-HCl buffer (pH 8) by leaving at 4 °C overnight. The viral DNA was quantified and stored at 4°C. The advantage of the ethanol precipitation method was that it achieved concentration of the DNA into a small volume by re-suspending the pellet of DNA into a smaller volume of liquid. Tris-HCl buffer was used instead of Tris-HCl EDTA buffer, as the EDTA might have inhibited subsequent reactions in which the DNA was used (Innis ***et al.***, 1990).

2.3.5) DNA quantification

The purified DNA was either quantified using the Pharmacia Biotech Genequant or the Beckman DU 530 spectrophotometer.

2.3.5.1) Quantification of viral DNA using the Genequant

A 1/100 dilution of the CrleGV DNA was made using triple distilled water and triple distilled water used as a blank. The absorbance of the DNA was read at 260 nm, 280 nm, 320 nm and 230 nm and a concentration was calculated by the machine. This value was multiplied by 100 in order to get to the concentration in the original solution to allow for the 1/100 dilution. The advantage of using the Genequant machine was that only one microlitre of the sample was expended on the DNA quantification as the quartz cuvette only required a volume of 100 µl in order to take a reading.

2.3.5.2) Quantification of DNA using a conventional spectrophotometer

DNA absorbs light best at a wavelength of 260 nm. The DNA concentration can therefore be calculated using the formula that 1 absorbance unit at OD_{260nm} = 50 µl/ml. A 1/200 dilution was made and put into a 1 ml quartz cuvette and the reading at 260 nm was taken. A quartz cuvette is required as Perspex or glass cuvettes absorb a certain amount of light at 260 nm, while quartz absorbs a negligible amount of light at this wavelength. Purity of the DNA was estimated by measuring the absorbance at 260 nm and 280 nm in order to check for contaminating protein. The ratio OD_{260nm}/OD_{280nm} of 1.8 indicated a lack of proteins in the DNA solution (Mahy and Kungro, 1996).

2.3.6) Restriction endonuclease digestion of CrleGV genomic DNA

Restriction digests of CrleGV genomic DNA were carried out using 1 to 5 µg of viral DNA. Six µl of 10x restriction endonuclease digestion buffer and 1.5 µl (15 Units) of enzyme were added. The volume was made up to 60 µl (80 µl if 8 to 10 µg of DNA was used) with sterile distilled water. These reactions were incubated at the specified temperature for the enzyme (normally 37 ºC) for 5 hours. The digest was then analysed using agarose gel electrophoresis.

2.3.7) Agarose gel electrophoresis for the analysis of DNA

Agarose slab gels with a length of either 12 cm or 20 cm were used in this project. The long gels were used for restriction digestion analysis of CrleGV-SA DNA, while the shorter gel was used for polymerase chain reaction (PCR) analysis and other routine DNA analysis (see **Appendix 2.4**). DNA samples (15 to 30 µl) were mixed with 1 µl loading buffer and 2 µl glycerol before being loaded into the wells. Long gels in (TAE) buffer were run overnight for 16 hours at 30 volts and short gels (also in TAE buffer) were run for 1 to 3 hours at 70 volts. Gel photographs were then taken using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

2.4) Results

A creamy white band of occlusion bodies accumulated in the centre of the glycerol gradient (**Fig 2.1**). This band was extracted carefully by lowering an autopipette to the band. There was often a second band below the virus band, presumably containing some host cellular or tissue component. A typical yield of purified virus from 2 g of larvae was between 4 x 10^{10} and 8 x 10^{10} OB/ml, as determined spectrophotometrically.

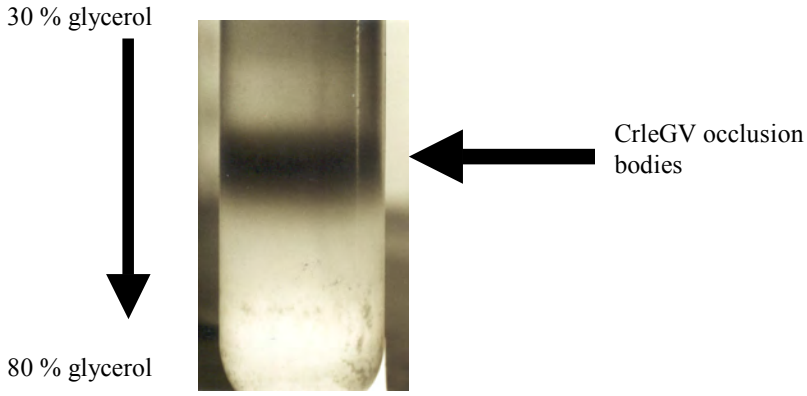


Figure 2.1: The CrleGV virus band on a 30% to 80% glycerol gradient.

The normal yield of DNA from 500 µl occlusion bodies (4 x 10^{10} OB/ml) was approximately 45 µg**.** A filamentous precipitate was normally visible in the eppendorf tube if the ethanol precipitation method was utilised as the final purification step.

A translucent white pellet with a bluish tinge around the edges was observed in the base of the centrifuge tubes used to isolate budded virus. **Figure 2.2** shows a typical gel analysis of CrleGV DNA digested with restriction endonucleases.

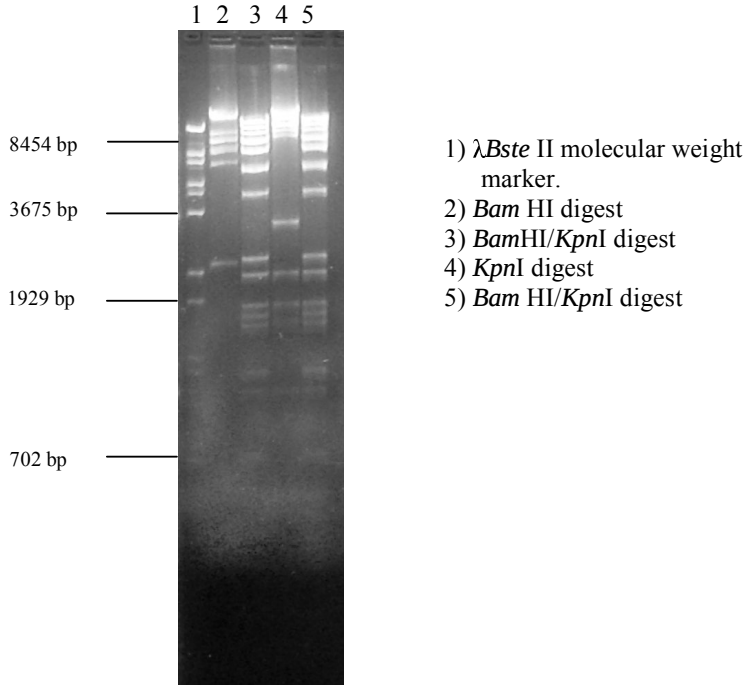


Figure 2.2: Restriction digestion profiles of CrleGV DNA for *Bam* HI, *Bam* HI/*Kpn*I and *Kpn*I.

2.5) Discussion

The technique used for occlusion body purification from *Cryptophlebia leucotreta* larvae and DNA extraction from the purified virus proved to be successful. Virus was purified using a simple protocol with low speed centrifugation and no dialysis required for removal of glycerol.

DNA extraction first required the release from the occlusion body capsule. These capsules dissolve in an alkaline environment (i.e. an environment containing excess carbonate ions). The nucleocapsid was then broken down by the addition of Proteinase K (isolated from *Tritirachium album*) and SDS (final concentration of 1% or less). SDS has been found to increase the activity of proteinase K up to seven-fold (Roche, 2001). SDS

also encases all peptides in the solution giving them a net negative charge, and therefore prevents reassociation of the nucleocapsid proteins. Phenol was then used to eliminate the proteins from the aqueous layer, leaving purified DNA behind. Ethanol precipitation was the routine final purification step used as it concentrates the DNA and it is an efficient technique. However, the pellet must not be allowed to dry for too long, as this will lead to problems with resuspension of the DNA, producing a jelly-like pellet. An alternative would be to use dialysis, but this will not concentrate the DNA as much.

The budded virus that was prepared in protocol **2.2.3** was not true budded virus. It was just occluded virus with the granulin coat removed. It would therefore not be suitable for use as budded virus in tissue bioassays or in tissue culture work and it is questionable as to whether the virus would still be infective. However, this form of virus was acceptable for the tests required in Chapter 5. 15 µl of the restriction digests of a final volume of 80 µl were analysed by electrophoresis. Due to the shorter DNA fragments intercalating less ethidium bromide, they appeared fainter than larger bands even though stoichiometrically there should be the same number of DNA pieces in each band. Ethidium bromide is also positively charged and has a tendency to migrate to the negative pole of the gel system. Thus if the fluorescent dye has been added to the gel during casting, it leaves one part of the gel darker than the other when viewed under UV light.

This purified DNA and virus material was then used for the experiments in the rest of this project, such as gene detection and bioassays.

Chapter 3: The Establishment of a Laboratory Colony of *Cryptophlebia leucotreta*

3.1) Introduction

In order to have a reasonably constant supply of insects that are produced under stable conditions for experiments, it is necessary to have a laboratory colony of those insects maintained on artificial diet. For the purposes of developing a virus free colony, it is essential to eliminate introduction of virus into the insect population through environmental factors. In the case of *Cryptophlebia leucotreta*, it would be difficult and inconvenient to rear insects on oranges due to the lack of availability throughout the year, microbial decay of the fruit and the possible introduction of insect pathogens with oranges. In the pursuit of a virus free colony, this latter possibility should be avoided. Contamination of larval food with fungi from decay can sometimes negatively influence larval health or even induce mortality. A sterile artificial diet that avoids the complications of microbial decay and introduction of pathogens from the environment is therefore desirable.

3.2) Materials and Methods

Two colonies were set up. The first colony was derived from larvae provided by Sean Moore (Citrus Research International, Port Elizabeth). The second colony used larvae collected from the Ferriera citrus orchards in the Sundays River Valley recorded as having no detectable incidences of CrleGV (Singh, 2001).

A modification of the insect rearing protocol used by Moore (CRI, Port Elizabeth) was used for the rearing of *C. leucotreta* in the laboratory. A range of larvae from first to fifth instar were put into bottles containing artificial diet. This diet contained wheat germ, maize meal, milk powder, brewers yeast, sorbic acid and p-hydroxybenzoic acid methylester – mixed with water and then sterilised at 121 °C, 110 KPa using an autoclave **(Appendix 3)**. The artificial diet was made in bottles with cotton wool bungs to keep it sterile. Contamination with certain fungal species can be detrimental to larval health

(Tanada and Kaya, 1993). The larvae developed on the artificial diet until they began pupation. At the beginning of pupation they emerged from the artificial diet and climbed to the cotton wool bung where they spun a cocoon in which they pupated. The cotton wool bungs containing the pupating larvae were transferred to an emergence box, consisting of a box with a transparent bottle attached at one end. When the moths emerged, they were attracted to the light and flew into the bottle. They were then transferred to an inverted sieve on a wax paper sheet through a hole in the top (normally plugged with cotton wool), using a funnel. In order to make the moths more controllable, the bottle of moths was left in the freezer (–20 ºC) for 2 to 5 minutes. This slowed down their activity and made them less likely to fly around. The cotton wool plugging the top of the sieve was kept moist in order to provide a drinking source for the moths. A tub of water was used to keep the atmosphere in the constant temperature room humidified. Droplets of 40% sucrose were applied to the upper surface of the inverted sieve to act as a nutrient source for the moths. Female moths laid their eggs on the sheets of waxed paper located under the inverted sieve. These sheets were removed every day or second day, depending on the density of the eggs on the sheet. The circular area of the egg sheet covered in eggs was separated from the rest of the sheet and then cut into squares about 4 cm x 4 cm. These were then put into a clean 400 ml bottle and fumigated with formaldehyde for 4 to 6 hours, by putting 25 µl of formaldehyde onto filter paper attached to the inner surface of the lid of the bottle. Fumigated egg sheets were then transferred to the autoclaved artificial diet using sterile technique and a bunsen burner. These eggs then developed into the next generation of larvae. **Fig. 3.1** shows a schematic diagram of the process. Larvae and moths were kept at a constant temperature of 28 ºC, found to be the optimum temperature for *Cryptophlebia leucotreta* (Moore, 2001).

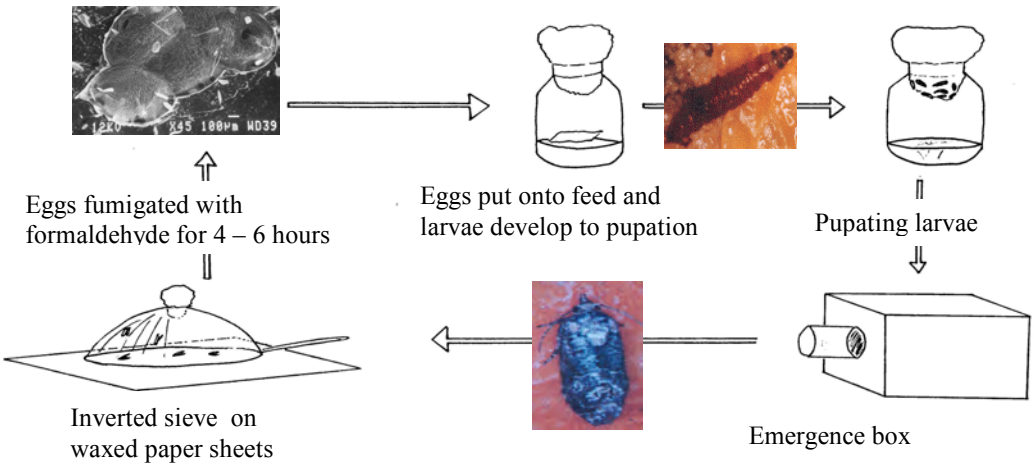


Figure 3.1: Diagram showing the method used for the establishment and maintenance of a laboratory colony of *Cryptophlebia leucotreta.*

3.3) Results

A generation cycle of *Cryptophlebia leucotreta* from egg to adult was completed in 24 to 27 days at 28 ºC. Relative humidity was difficult to maintain due to failure of the humidifying system in the designated constant environment room (CER). A tub of water appeared to suffice in maintaining relative humidity at an acceptable level.

After 5 complete generations, symptoms of a baculoviral infection developed in some of the bottles, indicating the possibility of conversion of a latent infection to an active one. Restriction enzyme analysis (with *Bam* H1, *Kpn* I and *Eco* R1) of virus DNA purified from individuals displaying symptoms produced the same profiles as those obtained by Singh (2001) for the CrleGV- SA isolate of the virus (**Figs. 3.2 and 3.3**).

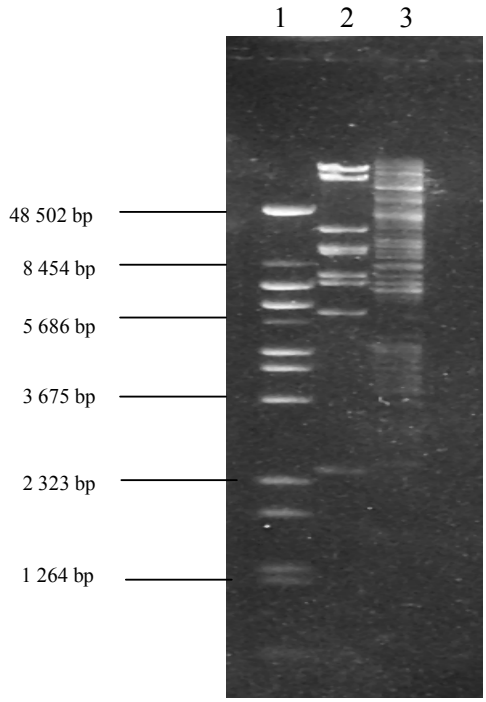


Figure 3.2: Restriction analysis of viral DNA extracted from CrleGV isolated from the FCM colony set up at Rhodes University. The digests were resolved using a 0.7 % agarose gel in TAE buffer.

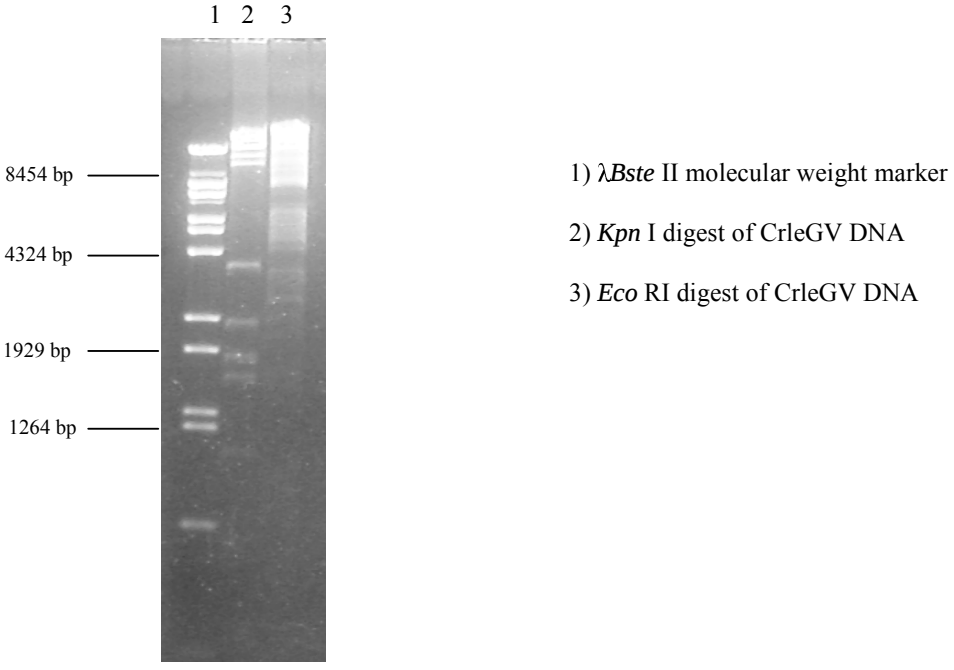


Figure 3.3: Restriction enzyme analysis of CrleGV DNA isolated from diseased larvae in the laboratory colony set up at Rhodes University. The digests were resolved using a 0.7 % agarose gel in TAE buffer was used.

The attempt to establish a colony from the Ferreira orchard with no virus detected in sampled larvae (Singh, 2001) failed due to a high infestation of collected larvae by the larval parasitoid *Agathis bishopii* and a very low fecundity in the subsequent generation.

3.4) Discussion

The appearance of virus symptoms in the colony indicated that the colony was not virus free. The activation of a latent infection may have been caused by an increase in the number of eggs deposited into each bottle, thus resulting in overcrowding stress (Longworth and Cunningham, 1968). The restriction analysis also indicated that the causative agent was the South African isolate of CrleGV (CrleGV-SA), as the restriction profiles matched those of the virus studied by Singh (2001). The comparison of the restriction profile of *Bam* HI in Chapter 2, **Fig. 2.2** with the restriction profile in **Fig. 3.2** also reveals the same pattern. DNA used in **Fig. 2.2** was from purified CrleGV-SA. **Fig. 3.3 and Fig. 3.2** show restriction digests in which purified DNA from the virus infecting the laboratory colony was used. **Fig. 6.3** showed the same restriction profiles for *Kpn* 1 and *Bam* H1 digests of CrleGV-SA DNA as were observed for virus isolated from the laboratory colony. The similarity of the virus isolates is not surprising as the larvae of this colony, as well as CrleGV-SA virus, both originated from the Western Cape (South Africa). There may therefore have been a latent or non-apparent infection in the original larvae. When the colony was initially started and larvae were still adapting to laboratory conditions, virus symptoms were recognised (Moore, 2001) indicating that virus was present. It is apparent that this infection has continued in a latent or non-apparent state, as symptoms had not been found in recent generations of the colony (Moore, 2001). Determination of various antiviral and sanitary treatments was therefore necessary for the purposes of establishing a virus free colony.

Owing to the persistent and ubiquitous nature of baculoviruses in the environment (Cory *et al.*, 1997), it is unlikely that field populations of insects would not contain a baculovirus specific for those insects. Therefore isolation of a virus free colony from the field is unlikely to succeed.

Chapter 4: The Determination of Suitable Anti-viral Treatments of *Cryptophlebia leucotreta* Laboratory Colony Eggs

4.1) Introduction

Understanding the mechanism by which a virus is transmitted is important in the establishment of a virus free colony. In the case of a laboratory colony, each generation must be kept separate (one cycle ends as the next begins), except for the transferral of eggs from one generation to the next in order to perpetuate the cycle. Bottles used for feed must be sterilised by autoclaving and then a second time when artificial diet is prepared. Therefore, if the virus is still being transmitted from one generation to the next, it must somehow be transported with the waxed paper sheets and the eggs. There are two forms of vertical transmission associated with the egg. These are transovarial transfer, where virus is present within the egg, and transovum transfer in which the virus is attached to the surface of the egg (Fuxa *et al*., 2002). Other avenues of vertical transmission could be through contamination of the waxed paper with virus or virus-containing moth faecal matter (Hamm and Young, 1974). Another important factor to remember is the persistent nature of the baculoviruses, which allows horizontal transfer to be an important transmission mechanism (Cory *et al.*, 1997; Payne, 1982).

If there is virus within the egg, as infective occluded virus, budded virus or viral genomic DNA, there is little that can be done to remove it without killing the embryo as well. Treatment of the egg sheets with an agent to inactivate or remove surface virus is a more viable option. Consequently, this chapter was dedicated to the establishment of a surface decontamination treatment for the egg sheets. Many of the treatments tested in this chapter were chosen on the basis of their extreme pH, which has been found to be a factor to which the virus is susceptible (Murphy *et al*., 1995). Another factor causing loss of infectivity is UV light (Cory *et al.*, 1997; Summers *et al.*, 1975).

4.2) Materials and Methods

4.2.1) Amido black staining of occlusion bodies

Smears of homogenised larvae or viral suspensions were applied to microscope slides and fixed through air-drying. The amido black staining solution **(Appendix 4)** was heated to between 40 and 45 ºC. The slide preparation was immersed in the staining solution and incubated at 45 ºC for 5 minutes. It was rinsed under running tap water for 10 seconds. The slide was allowed to dry and then examined under oil immersion for the presence of occlusion bodies using a light microscope (magnification = 1000 X) (Evans and Shapiro, 1997).

In order to determine the efficacy of virus disruption, 50 µl of 4.3 x 10^8 OB/ml virus stock was added to 200 µl of each treatment below. Samples were incubated at 25 ºC and 37 ºC for 30 minutes and 90 minutes. 5 µl of each sample was applied to different slides and fixed by drying. Treatments used were:

1) 1% (final concentration)Virkon pH 2.0 (commercial antiviral agent – active ingredient potassium peroxymonosulphate)
2) 1 M Na_2CO_3
3) 1 M Na_2CO_3 + 1% SDS
4) 15% formaldehyde
5) 2% sodium hypochlorite
6) 3.5 % sodium hypochlorite

Distilled water without added virus was used as the negative control. An untreated virus sample (diluted to equivalent concentration in water) was used as a positive control.
Slides were examined under the light microscope using oil immersion for the presence or absence of occlusion bodies.

4.2.2) Bioassay analysis of potential antiviral treatments

In this set of experiments, neonate larvae were used. The LC_{90} (i.e. the lethal concentration that produces death in 90 % of larvae) of CrleGV for *C. leucotreta* neonate larvae was calculated to be 2.36 x 10^5 OB/ml (Moore, 2001).

25 cell perspex trays with a lid (Sterilin, UK) were used as the bioassay rearing chambers. Each cell was 2 cm^3. See **Appendix 4** for tray preparation. The artificial diet had agar added to it in order to make the surface of the diet smooth. This ensured that the entire surface of the diet was covered when the virus preparation was added.

4.2.2.1) Potential antiviral treatments tested against CrleGV

1 µl of 4.3 x 10^8 OB/ml was incubated with 99 µl of treatment solution and incubated for one hour at room temperature. The solutions were microfuged at 15 000 rpm for 90 minutes to sediment budded virions and the pellet was resuspended in 1.5 ml of distilled water. 50 µl of this solution was aliquoted into each cell in the bioassay tray. 25 replicates were done per treatment (i.e. a new plate was used per treatment). The plates were tilted in every direction to ensure total coverage of the surface of the artificial diet and then allowed to dry under the laminar flow hood.

The treatments used were:

1) Tris-HCl pH 10 and 1% SDS
2) 15% formaldehyde
3) 1 M sodium carbonate
4) 2% Virkon (Commercial antiviral agent – aimed primarily at vertebrate viruses). Active ingredient is potassium peroxymonosulphate (pH 2.0).
5) 2% sodium hypochlorite
6) UV light (253 nm wavelength)

A 5-minute incubation of virus solution in 3.5 % sodium hypochlorite was also tested. In the UV treatment virus solution (4.3 x 10^8 OB/ml) was diluted 1 µl in 1500 µl distilled water, vortex mixed and 50 µl added to each bioassay cell in the tray. This tray was then exposed to UV light for 30 minutes. Thirty minutes was used as the incubation time, as an experiment to determine the sensitivity of *C. leucotreta* eggs to UV light revealed that exposure of eggs to UV for 35 minutes resulted in a 70% loss of egg viability, therefore risking the survival of the colony. At 30 minutes only 50 % loss of egg viability was observed. At 40 minutes of exposure, 2 % egg viability was observed.

Distilled water was used as a background virus control to quantify larval mortality caused by other factors than the virus treatment. Distilled water was dispensed onto the surface of the bioassay feed instead of the virus treatments. A virus solution treated with distilled water was used as the positive control in this experiment. The positive control should show mortality in 90 to 100 % of larvae.

Once the diet surface was dry a single neonate larva was deposited in each cell with a fine moistened paintbrush. The paintbrush enabled manipulation of the small delicate arthropods without injuring them. To ensure that neonate larvae were available for the bioassay, an egg sheet was collected and stored in a separate sterile bottle each day for 3 days prior to the bioassay experiment. Prior to closing the plate each larva was checked for signs of stress, such as being embedded in the agar during application. A wad of paper towel was placed over the cells, followed by a sheet of plastic and then the lid. The wad of paper was necessary to close a gap about a millimetre wide between the lid and the walls of the cells. The lid was secured in place with four clips (one on each side of the tray). Trays were then incubated at 28 ºC for approximately 14 days. Results were analysed by recording the number of live, dead and missing larvae. Neonate larvae are very small (less than a millimetre long). They can sometimes get out of their cell and into a neighbouring cell or their cadavers are lost in the granular texture and colour of the feed. The use of a dissecting microscope aided the location of neonate larval cadavers. Sometimes the larval diet required dissection before the larva was located.

4.2.3) Scanning Electron Microscopy of hypochlorite treated egg samples to establish the effectiveness of hypochlorite in the removal of virus from the surface of *Cryptophlebia leucotreta* eggs

Sodium hypochlorite was further evaluated for effectiveness in virus removal, using scanning electron microscopy. Samples of eggs were collected from egg sheets by cutting 6 mm x 6 mm squares that had similar numbers of eggs on them. Five replicates of each treatment were performed.

Table 4.1: Treatments used in electron microscope evaluation of antiviral properties of sodium hypochlorite.

Sample	**Treatment used**
Positive control	Virus added but no subsequent treatment
Test 1	Virus added with dH_2O wash (5 min)
Test 2	**Virus added + hypochlorite wash (5 min) + water rinse**
Negative control	No virus added and no other treatment
Test 1 negative control	No virus added + water wash (5 min)
Test 2 negative control	No virus added + hypochlorite wash (5 min) + water rinse

The amount of virus added to spike each sample was 10 µl of 4.3 x 10^8 OB/ml (i.e. 4.3 x 10^6 OB/µl). Once the virus sample was dry, the samples were immersed in the various treatments for 5 minutes and then dried. In the case of hypochlorite treatment, a brief distilled water rinse was used to remove residual hypochlorite as failure to remove this was found to decrease egg viability. Once the egg samples were dry, they were attached to scanning electron microscope (SEM) stubs with double sided carbon tape and coated with gold using a sputter coating technique (**Appendix 4**). Samples were then examined for the presence of intact virus under the Jeol JSM 840 scanning electron microscope.

4.2.4) Protocol for virus decontamination of the laboratory colony

The colony was maintained in a constant temperature room (28 ºC), separate from any virus work to reduce the chances of viral contamination. Egg sheets were processed in a laboratory separate from the virus research area. Colony work was performed prior to entering the virus research laboratory each morning to minimise chances of contamination with CrleGV.

The egg surface decontamination protocol was formulated using the results from the above experiments. After being cut into 6 cm x 6 cm pieces, egg sheets were first immersed in water for 1 to 2 minutes. This was to remove most of the moth faecal matter and moth scales from the surface of the eggs and waxed paper. The pieces were then immersed in 3.5% hypochlorite containing 1% (volume/volume) Tween 20 for 5 minutes. Slight agitation of the egg sheets during the 5 minutes lifted the majority of the eggs off

of the waxed paper sheets. The eggs were then further agitated by gently stirring the treatment solution in order to ensure the total surface of the eggs were exposed to the treatment. After the 5-minute treatment, eggs were collected onto filter paper using a Buchner funnel and vacuum pump. The eggs were then rinsed with distilled water while still in the Buchner funnel to remove any residual hypochlorite that could result in loss of egg viability. The eggs remained attached to filter paper sheets, which were then hung up to dry and were only handled using flame or autoclave sterilised forceps. Once the filter paper was dry it was transferred to a sterile (autoclaved) fumigation bottle using sterile forceps. 25 µl of formaldehyde were dispensed onto a piece of filter paper attached to the inner lid of the fumigation bottle and the bottle was closed for 3 to 4 hours. Subsequent to formaldehyde fumigation, the filter paper sheets were transferred to sterile artificial diet in 500 ml jars using sterile forceps and general sterile technique practices. Once the cotton wool bung of the bottles was replaced the bottles were transferred to the 28 ºC constant temperature room.

Once larvae had moved to the cotton wool bungs for pupation the cotton wool bungs were moved to a cardboard box that had no contact with the laboratory used for virus work. These served as the emergence boxes. The plastic bottles used to collect the moths were treated with 3.5 % hypochlorite for 24 to 48 hours to degrade any contaminating occlusion bodies and to dissolve eggs that may have been laid on the inner surface of the bottle. The bottles were then rinsed with distilled water and left under the germicidal lamp for 4 to 5 hours. At the stage of pupation of the larvae, the previous generation of moths had died off. Therefore the sieves were autoclaved for 20 minutes at 121 ºC (110 KPa of pressure) and the old cardboard emergence boxes were discarded. The CER surfaces were swabbed with hypochlorite, which was allowed to stand on these surfaces for 20 to 30 minutes. A fluorescent germicidal lamp (253 nm) was used to irradiate the surfaces of the CE room for 6 hours. Bottles containing artificial diet were recycled. They were therefore washed then autoclaved, and re-autoclaved when larval diet was prepared in them again. All apparatus used in the maintenance and processing of the laboratory colony were either sterilised by autoclaving or by UV irradiation at 253 nm, if the items could not be autoclaved. The rooms used for the colony were not entered again once the

virus laboratory had been entered on the same day, unless a shower and change of clothes had been organised.

4.3) Results

4.3.1) Amido black staining of occlusion bodies

Table 4.2: Results of the amido black staining of the various anti-viral treatments. The anti-viral treatments were incubated at 25 ºC and 37 ºC for 30 minutes and 90 minutes. A (+) indicates that occlusion bodies were still visible on the slide after anti-viral treatment. A (-) indicates that no occlusion bodies were visible on the slide.

Treatment	**Incubation at room temperature. (25 ºC)**		**Incubation at 37 ºC**	
	30 min	**90 min**	**30 min**	**90 min**
Negative control (no virus)	-	-	-	-
Positive control (virus added)	+	+	+	+
15 % formaldehyde	+	+	+	+
0.1 M Tris-HCl pH 10 + 1% SDS	+	+	+	+
2% Virkon	+	+	+	+
1 M Na_2CO_3	+	+	-	-
1 M Na_2CO_3 + 1% SDS	+	-	-	-
2 % sodium hypochlorite	-	-	-	-
3.5 % sodium hypochlorite	-	-	-	-

There was little difference in the results after incubating at 25 or 37 ºC, nor between the 30 and 90 minutes incubation times at both temperatures. Only sodium carbonate seemed to perform better when incubated at 37 ºC or for a longer incubation time at room temperature. The 15 % formaldehyde, 2% Virkon and 0.1 M Tris-HCl (pH 10)+ 1% SDS appeared not to be able to degrade occlusion bodies. 2% hypochlorite was able to degrade occlusion bodies under all the conditions used. Therefore, either 2% or 3.5 % sodium hypochlorite could be used, depending on the resistance of the *C. leucotreta* eggs to hypochlorite treatment. **Figure 4.1** shows micrographs of the results obtained for each treatment. The positive control (Fig. 4.1 (A)) illustrates a typical amido black stain of purified occlusion bodies.

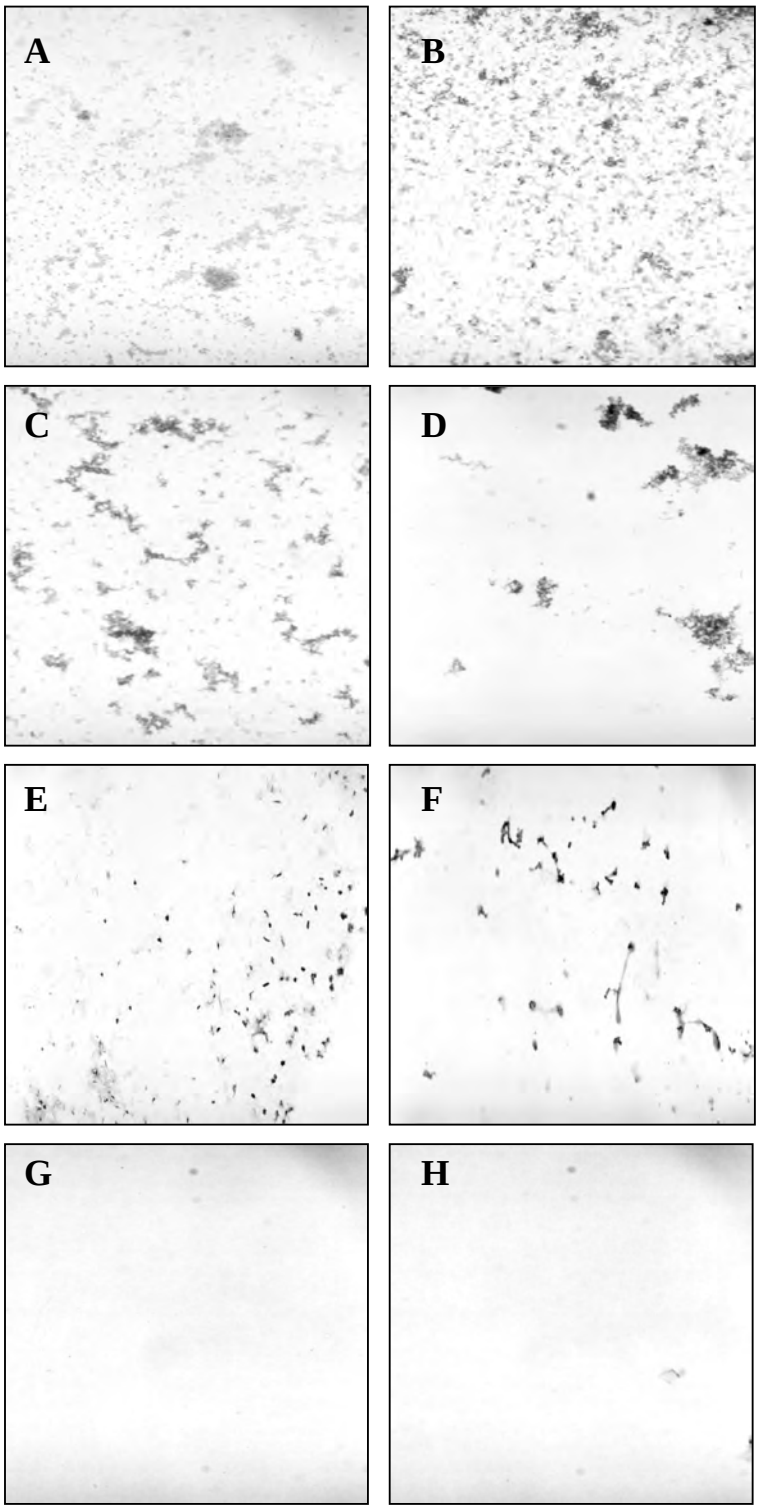


Figure 4.1: Photographs of the amido black staining of various antiviral treatments (25 ºC), showing their effect on occlusion body degradation. (A) Untreated Occlusion body stain. (B) 0.1 M Tris-HCl pH 10 + 1% SDS. (C) 2% Virkon. (D) 15% Formaldehyde. (E) 1M Sodium carbonate. (F) 1M Sodium carbonate + 1% SDS. (G) 3.5% hypochlorite (H) 2% hypochlorite. (Magnification 1000x).

No negative control is shown in **Fig 4.1**, as it would have been a blank photograph showing no occlusion bodies as indicated in the results summarised in **Table 4.2**. The 15% formaldehyde and 2% Virkon treatments appeared to cause aggregation of occlusion bodies, which were still clearly visible. The sodium carbonate treatments both eventually caused dissolving of the occlusion bodies. The granulin protein re-aggregated to form the smears that were stained by the amido black dye.

4.3.2) Bioassay analysis of potential antiviral treatments

Table 4.3: The results of the infectivity bioassays of the various anti-viral treatments to evaluate viral inactivation. The incubation time of anti-viral treatment was one hour unless otherwise stipulated.

Treatment	**Survival**	
	Number of larvae surviving (out of 25)	**% Survival**
Negative control (No virus)	20	80%
3.5 % sodium hypochlorite (1 hour)	20	80%
3.5 % sodium hypochlorite (5 min)	20	80%
1 M Na_2CO_3	20	80%
2% Virkon	19	76%
UV Light (30 minutes)	7	28%
15% Formaldehyde	14	56%
Tris-HCl pH 10 + 1% SDS	4	16%
Positive control untreated virus spike	0	0%

From **Table 4.3** it can be seen that most of the antiviral treatments were quite effective, except for the Tris-HCl pH 10 + 1% SDS and UV light. These treatments produced a very low insect survival rate, indicating that the virus was still very infective. The sodium hypochlorite treatments for 5 minutes and 1 hour both produced the same survival rate as the negative control, as did the 1 M sodium carbonate. This indicates that these treatments were effective in the inactivation of virus particles. Virkon was a little less effective, while formaldehyde was much less effective. The positive control produced

100% mortality. This could indicate that a slightly higher concentration of virus than the LC_{90} was used.

4.3.3) Scanning Electron Microscopy of hypochlorite treated egg samples to establish the effectiveness of hypochlorite in the removal of virus from the surface of *Cryptophlebia leucotreta* eggs

Figure 4.2 shows the effect of sodium hypochlorite treatment on the egg of *C. leucotreta.* An untreated egg (**Fig. 4.2 (A)**) has domed shape and has dimple-like impressions over its surface. Moth scales are also clearly visible. In contrast, an egg treated with sodium hypochlorite shows deeper dimple-like impressions and a distorted, shrunken surface, giving the impression of desiccation.

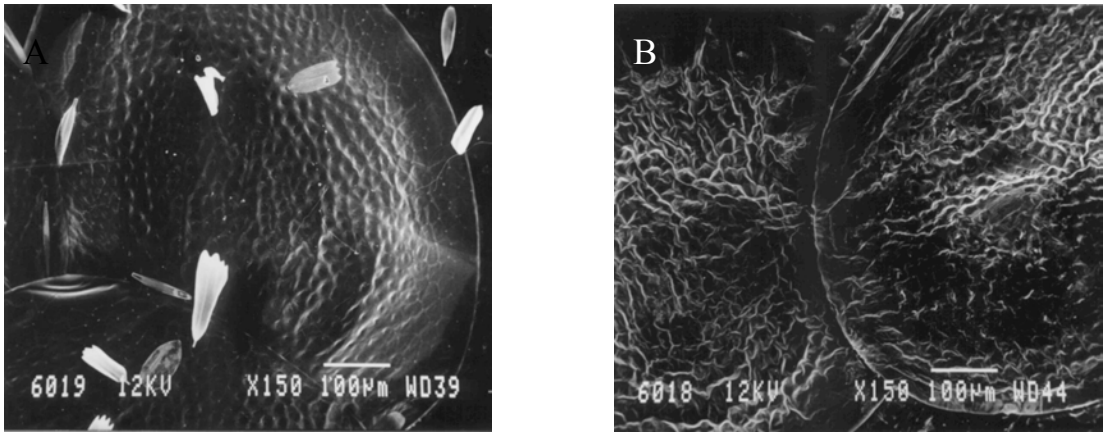


Figure 4.2: The effect of 3.5 % sodium hypochlorite treatment for 5 minutes on the eggs of *Cryptophlebia leucotreta*. (Bars = 100 µm). **(A)** An untreated egg and **(B)** egg treated with sodium hypochlorite

Fig. 4.3 shows a series of electron micrographs illustrating the effect of hypochlorite treatment on the degradation of virus attached to the surface of *C. leucotreta* eggs. **Fig. 4.3 (A)** shows an egg without added occlusion bodies and this served as a negative control. When compared with the positive control (**Fig. 4.3 (B)**), to which purified virus had been added, there is no visible virus in **Fig. 4.3 (A).** The ovicylindrical structures in **Fig. 4.3 (B)** are virus particles. **Fig. 4.3 (C)** was immersed in water for 5 minutes after the added virus solution had dried on the eggs. The micrograph shows that this treatment was

ineffective in removing intact virus. However, sodium hypochlorite is able to remove intact virus from the surface of the egg, as there is no intact virus visible (**Fig. 4.3 (D)**).

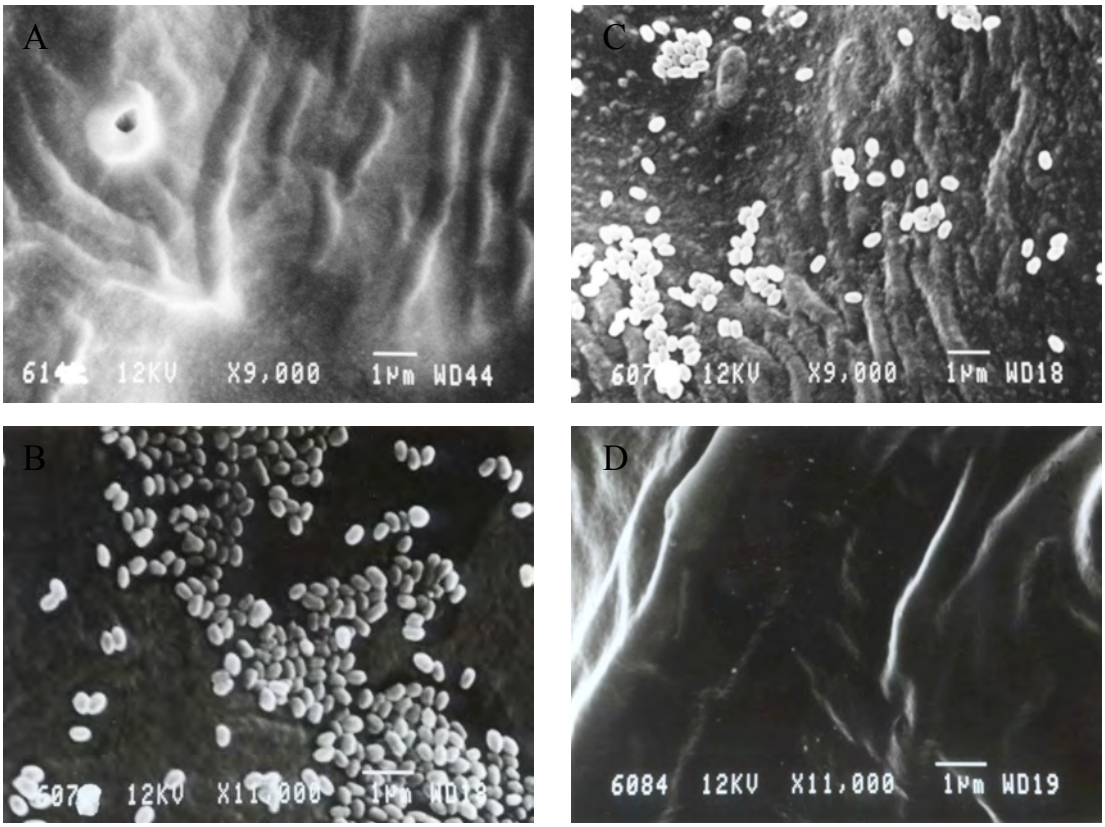


Figure 4.3: Scanning electron micrographs showing the effect of sodium hypochlorite treatment on occlusion bodies adhering to the surface of the *Cryptophlebia leucotreta* egg. Treatments were conducted at 25 ºC for 5 minutes. **(A)** The surface of an egg untreated with virus. **(B)** An egg treated with purified occlusion bodies. **(C)** A virus treated egg rinsed with distilled water. **(D)** The surface of a virus treated egg subsequently treated with sodium hypochlorite. All scale bars = 1 μm.

4.4) Discussion

Although the amido black staining of the 15 % formaldehyde, Tris-HCl pH 10 + 1% SDS and Virkon treatments did not appear to affect the occlusion bodies, this does not mean that they were still viable virions subsequent to treatment. The Virkon and formaldehyde treatments appeared to cause aggregation of the occlusion bodies. This may indicate that these agents cause cross-linking of the viral protein structures, which may inhibit OB dissolution in the larval midgut. It was unexpected that the pH 10 buffer + 1 % SDS did not degrade the granulin capsule, as the capsules are known to dissolve in the alkaline

conditions of the larval midgut. Only a little degradation of occlusion bodies was observed with this treatment after a 90-minute incubation at 37 ºC. The sodium carbonate treatments shown in **Fig. 4.1 (E)** and **(F)** showed smears across the slide that were stained by the amido black solution. As this is a protein specific stain (Evans and Shapiro, 1997), it implies that these smears consisted of the dissolved granulin from the occlusion bodies, thus indicating occlusion body degradation. However, this may have been an incomplete degradation. The hypochlorite treatments showed no occlusion body residues on the slides. The dots shown in the photos appear to be dirt on one of the microscope lenses as they appear in the same position in each photo. All that could be concluded from the preliminary amido black staining study was which treatments caused the partial or total degradation of occlusion bodies and which ones did not.

Bioassay analysis of the treatments revealed that most of the treatments were at least partially effective in inactivating occlusion bodies. However, there were some discrepancies when compared with the amido black staining. Although Virkon treatment showed what appeared to be intact occlusion bodies in the amido black assessment, it was almost as effective an inactivator as hypochlorite and sodium carbonate, indicating that some viral inactivation was occurring despite the presence of structures resembling intact occlusion bodies visible in the amido black stain. The negative control (no virus added) for the bioassay showed only 80 % survival. This is due to the fact that neonate larvae are very delicate and may die from causes other than viral infection. Also, due to the size of the larvae, they may be able to escape their bioassay cells or after death may be lost in the granular appearance of the artificial diet. Therefore, missing larvae were included in the mortality percentage and would add to lowering the % survival. A 100% survival in a *C. leucotreta* neonate bioassay is therefore unlikely.

The bioassay indicated that sodium hypochlorite and sodium carbonate produced 100 % inactivation of CrleGV occlusion bodies relative to the negative control, as all larval mortality appeared, statistically, to be from causes other than virus infection. However, the successful use of these antiviral treatments is also subject to their effect on the viability of *C. leucotreta* eggs. Ultraviolet light causes the inactivation of CrleGV virions.

However, it also causes an unacceptable loss of egg viability if egg sheets are exposed for longer than 30 minutes. Unfortunately the virus does not appear to be completely inactivated after a 30-minute exposure to UV light of 253 nm wavelength. Therefore UV light is not a good choice for an antiviral treatment used on the colony. Egg viability was observed to be severely lowered by treatment with both sodium hypochlorite and sodium carbonate for longer than 40 minutes. Therefore it was decided to try sodium hypochlorite treatments for 5 minutes as the bioassay showed this to cause complete virus inactivation. The advantage of hypochlorite acting as a sterilising agent was also taken into account in the choice.

The 5-minute treatment of sodium hypochlorite had a profound effect on the egg morphology. It is therefore not surprising that a longer treatment time caused severe losses in viability due to the high alkalinity (pH 13) and caustic nature of the sodium hypochlorite solution. Eggs would not normally be exposed to this pH extreme under normal environmental conditions. This treatment also removed moth scales that littered the surface of the eggs and waxed paper, possibly providing shelter for virions. The photographs in **Fig. 4.3** show that sodium hypochlorite treatment of egg sheets significantly decreases the amount of virus present on the surface of the eggs. It is difficult to conclusively say that virus is completely removed as small particulate matter that could be mistaken for occluded virions were sometimes found on the egg surface during analysis by electron microscopy. Occluded CrleGV virions (**Fig. 4.3 B and C**) were calculated to be 450 to 500nm long and 230 to 330 nm wide, which is considerably larger than the size for CrleGV occluded virions cited by Jehle *et al.* (1992) (50 to 80 nm x 200 to 400 nm). These virions may therefore be a similar size compared to some bacteria (range from 0.1 μm to 50 μm) (Madigan *et al.*, 1997). The few particles resembling occluded virus could have been contamination of the egg surface by dust or bacteria subsequent to the hypochlorite treatment.

Based on the tests discussed above, the protocol for virus decontamination of the surface of the egg and egg sheets described in Materials and Methods, section **4.2.4,** was formulated. In the antivirus processing of the egg sheets the Tween 20 detergent was used

in order to cut down surface tension. Owing to the convoluted surface of the egg, there was a possibility that tiny air bubbles attached to the egg surface would shelter occlusion bodies from the antivirus treatment. Tween 20 also had the advantage of removing the eggs from the waxed paper substrate, ensuring that the entire surface of the egg could be treated. Cardboard boxes were used as emergence boxes as they are easily disposable. This decreased the problems encountered with virus decontamination of emergence boxes. As UV treatment in the bioassay produced 28 % survival after a 30-minute exposure, it was decided that a 6-hour exposure of surfaces to UV (combined with hypochlorite swabbing and treatment for 20 minutes prior to UV exposure) for the purposes of virus inactivation was sufficient. This was supported by work done by Jehle *et al*. (1995). A 6-hour exposure of virus to UV-B (medium range UV light [290 nm -320 nm]) resulted in 99.97 % inactivation of the virus. Shorter wavelength UV (UV-C) is more damaging to DNA and therefore causes a more rapid inactivation. A combination of hypochlorite treatment and UV light (253 nm) exposure of 6 hours would be virtually guaranteed to inactivate any virus on these surfaces. The distilled water rinse was necessary to keep viability at an acceptable level. A combination of hypochlorite/Tween 20 treatment with a subsequent UV treatment of 5 to 10 minutes caused a severe loss in egg viability.

Chapter 5: Testing of the *Cryptophlebia leucotreta* Laboratory Colony for the Presence of Virus

5.1) Introduction

In order to determine whether an antiviral treatment used on the colony has succeeded in the eradication of virus, it is necessary to use a test sensitive for the presence of virus. Detection of virus in the colony indicates failure of the treatment in its objective. Detection of virus in the form of an active infection can normally be achieved through observation of the conspicuous symptoms in larval stages, which can be confirmed by a smear of the larval tissues on a slide for amido black analysis. Molecular techniques such as restriction digest analysis or PCR can be used for confirmation. In the case of a latent infection, the detection of virus is not as easy. There is less virus in individuals with a latent infection, as there is no active virus production. Virus is therefore more difficult to detect. The conversion of a latent infection to an active one would increase the amount of virus present in a single individual and would be likely to produce visible symptoms of virus infection in larvae. This can be done through the induction of stress in larval populations of the colony (Longworth and Cunningham, 1968; Fuxa *et al*., 1999). However, due to the sensitivity of molecular techniques such as PCR, the ultimate test of a colony for the presence of virus would be the use of these techniques for virus detection. PCR is an *in vitro* technique of nucleic acid synthesis used to amplify specific segments of DNA that are defined by oligonucleotide primers that flank the nucleotide sequence to be amplified (Saiki, 1990). Among its diverse applications is the ability to amplify a detectable signal from a single target DNA molecule, under optimal conditions (Innis *et al.*, 1990). As granuloviruses are mainly pathogenic towards the larval stages of their lepidopteran hosts (Tanada and Kaya, 1993), detection of virus in these stages would be more likely. The problems of moth scales and other exoskeleton debris contaminating the samples is also avoided using the larval stages. Most of the larval exoskeleton remains intact during homogenisation. The presence of contaminating DNA, which could interfere with the PCR detection, is also limited as these larvae are reared in a sterile environment.

The basic strategy used for testing the laboratory colony, based on the above information, was divided into two sections: the screening for symptoms of infection in larvae, and testing for the presence of virus using molecular techniques. The initial stage of screening for virus in the colony involved examination of larvae for the symptoms of infection. If none were observed, then attempts to activate a possible latent infection were investigated. If still no conclusive symptoms of virus were detected, then molecular testing in the form of PCR was used for virus detection.

5.2) Materials and Methods

The laboratory colony had successfully completed 10 generation cycles prior to any antiviral treatment. Subsequent to antiviral treatment, another 12 generations were completed without the detection of symptoms of CrleGV infection. The initial method of testing for virus in the laboratory colony was the visual examination of larvae for signs of symptoms. As the most dramatic symptoms are observed in the later instars (4^{th} and 5^{th}) when the epidermis has already turned a red colour, most emphasis was placed on the observation of these larvae for symptoms. The creamy white colour of the younger instars, as well as the lower yield of virus produced within them, makes it more difficult to establish the symptoms of virus infection.

5.2.1) Attempts to induce active viral infection through larval stress

5.2.1.1) Crowding of larvae

Larvae were reared in the same way as described in **section 4.2.4**, except that two to three times as many eggs were put into each bottle. Once larvae had hatched, the bottles were examined for larvae displaying symptoms of viral infection until the pupae had been transferred to the emergence boxes.

5.2.1.2) Mechanical Stress

The utensils used in this experiment were sterilised by autoclaving for 20 minutes or by UV sterilisation (253 nm) if they were unable to be autoclaved. The UV sterilisation cabinet was left on for 3 hours prior to commencement of the experiment. Bottles containing larvae ranging from third to fifth instar were sampled from the colony and the

exterior sterilised using 70 % ethanol. Working in the UV cabinet, the cotton wool bung was removed from the bottle and then a sterile knife or spatula was used to break up the diet. The contents of the bottle were emptied into a sterile, shallow dish and the diet was fragmented further until at least 80 % of the larvae had been removed from their tunnels. In some cases the feed was already so loosened by larval tunnelling that minimal manual fragmentation was required. The larvae and feed were then replaced in the bottle and the cotton wool bung restored. Bottles were put on an orbital shaker for 2 to 3 hours at 200 RPM and subsequently restored to the 28 ºC constant temperature room. Larvae were observed for symptoms of virus infection until pupation.

5.2.1.3) Alteration of larval diet to induce stress

A change in the artificial diet may often result in stress, which may induce virus infection (Moore, 2001). The main constituent and carbohydrate source in the artificial diet (i.e. maize meal) was replaced with an equivalent mass of chickpea or soya bean flour (**Appendix 5.1**). The routine number of eggs was put on these altered diets and incubated at 28 ºC. Larvae were observed for symptoms of infection from hatching until pupation.

5.2.1.4) Induction of stress through heat shock

When the majority of larvae within the bottles were in the third and fifth instar, bottles were removed from the colony and incubated at 65 ºC for 25 minutes. Most larvae could be observed emerging from their tunnels in the diet due to the heat. The bottles were allowed to cool and then replaced at 28 ºC and observed for symptoms of infection until pupation.

5.2.1.5) Induction of nutrient stress through overcrowding and rearing of multiple generations of larvae on the same feed

Two to three times as many eggs were put into each bottle and allowed to develop into moths. These moths laid eggs in the same bottle and another generation was then started in the same bottle. This was continued for approximately three generations. The result should be that stress is induced both through overcrowding and through nutrient shortage, as subsequent generations of larvae compete for the same food. In addition, no virus is

removed from the bottle and should build up from generation to generation under the stressed conditions. Chances of observing an active infection should therefore increase.

5.2.2) Molecular testing techniques

A PCR-based technique was used for testing in which total DNA, extracted from larvae using cetyl trimethyl ammonium bromide (CTAB) extraction, was screened for viral DNA.

5.2.2.1) Total DNA extraction for testing by polymerase chain reaction

A modification of the CTAB extraction protocol used by Aspinall *et al.* (2002) was used to extract total DNA from the sampled larvae.

To each larva, 600 µl of 0.1 M Tris-HCl (pH 8.0) buffer were added and the mixture homogenised in a clean mortar and pestle. The homogenate was filtered through cheesecloth to remove macroscopic debris such as the larval cuticle. 500 µl of this filtered homogenate were aliquoted into a sterile eppendorf tube, to which 25 µl of sodium carbonate (1M) were added. After mixing, the solution was incubated at 37 ºC for 30 minutes. The solution was neutralised with 60 µl of 1M Tris-HCl (pH 6.8). After the addition of 60 µl of 10 % SDS and 30 µl of proteinase K (25 mg/ml), the solution was further incubated at 37 ºC for 1 hour. The solutions were then microfuged for three minutes at 15 000 rpm to pellet any precipitates that may have formed. The supernatant was transferred to a new sterile microfuge tube and 500 µl of CTAB buffer (**Appendix 5.2**) was added. The tubes were mixed by inversion 3 to 6 times and then incubated at 70 ºC for 1 hour with occasional mixing by inversion. After transferral of the solution to a 2 ml tube, 1 ml of chloroform was added. The solution was mixed by inversion and then centrifuged at 4 ºC for 10 minutes at 9 300 x *g*. The aqueous layer was transferred to a fresh 1.5 ml microcentrifuge tube and 0.6 volumes of 100 % isopropanol was added. The solution was mixed by inversion and incubated at room temperature for 10 minutes. After another 10- minute centrifugation (9 300 x *g* at 4 ºC), the supernatant was discarded and the pellet resuspended in 1 ml of 70 % ethanol. The solution was microfuged at 16 000 x *g* for 13 minutes at 4 ºC after which the pellet was allowed to air dry by inverting tubes

on a piece of roller towel on the bench. The DNA pellets were not left to dry for too long, otherwise they became difficult to resuspend. Pellets were resuspended in 30 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) at 4 ºC for 16 hours.

5.2.2.2) Investigation of the sensitivity of virus detection in *Cryptophlebia leucotreta* using polymerase chain reaction

For titrating the sensitivity of the combination of the CTAB extraction and PCR detection protocols, differing amounts of viral genomic DNA (1 ng to 60 fg) were added to 500 µl aliquots of a uniform larval homogenate. One CrleGV genome copy is equivalent to 0.125 fg of virus DNA (**Appendix 5.3**). The uniform homogenate was prepared by taking one larva per sample of DNA to be tested and homogenising them together in a mortar and pestle with 0.1 M Tris-HCl (pH 8.0). After addition of the viral DNA aliquots the samples were subjected to CTAB total DNA extraction, followed by PCR.

5.2.2.3) Sampling of larvae from the laboratory colony to screen for CrleGV

Samples of 5 larvae were taken at random from different bottles. Each group of 5 larvae were homogenised together in 2.5 ml of 0.1 M Tris-HCl (pH 8.0) and 600 µl of each homogenate were used for CTAB extraction and CrleGV DNA detection by PCR.

5.2.2.4) Conditions for polymerase chain reaction detection of viral DNA

The Expand High Fidelity PCR Kit (Roche) was used for PCR reactions. Two primers designed by Spillings (2000) were used to amplify the CrleGV granulin gene and short segments of flanking DNA by PCR:

GRAN FOR 1: 5’ –TGGTGTGGATACCAGAAGAAA- 3’
GRAN REV 1: 5’ – GATGATGATTTAGACAACTTAGA- 3’

The amplicon containing the granulin gene had an anticipated size of 876 bp. Two master mixes were prepared, which were then subsequently aliquoted out into the different PCR tubes.

Table 5.1: reagents for polymerase chain reaction master mix1:

Reagent added to master mix 1	**Volume of reagent used per reaction**
DNTPs	1 µl (of 10 mM dNTPs)
Granulin forward primer 1	2 µl (of 10 µM stock)
Granulin reverse primer 1	2 µl (of 10 µM stock)
Distilled water	18.5 µl
Total volume	**23.5 µl**

Master mix 1 was vortex mixed and then 23.5 µl were aliquoted into each PCR tube. 1.5 µl of the DNA template from the CTAB extractions were added to the respective PCR tubes. 25 µl of master mix 2 were then aliquoted into the PCR tubes.

Table 5.2: reagents for polymerase chain reaction master mix 2:

Reagent added to master mix 2	**Volume of reagent used per reaction**
10 x Reaction buffer with $MgCl_2$	5 µl
High Fidelity DNA polymerase	0.3 µl
Sterile distilled water	19.7 µl
Total volume	**25 µl**

The master mix 2 was mixed with an autopippette.

25 µl of mineral oil were overlaid onto the surface of the aqueous reaction solution to prevent evaporation from the reaction solution during thermocycling.

Table 5.3: The thermocycling conditions used for the PCR detection of CrleGV in *C. leucotreta*.

Stage 1 step 1	1 minute 30 seconds at 95 ºC	**1 cycle**
Stage 2 step 1	30 seconds at 95 ºC	**30 cycles**
Stage 2 step 2	40 seconds at 54 ºC	
Stage 2 step 3	45 seconds * at 72 ºC	
Stage 3 step 1	5 minutes at 72 ºC	**1 cycle**
Holding temperature	4 ºC	

* The time for the elongation step (stage 2 step 3) of cycle two was increased by 5 seconds per successive cycle.

5.2.2.5) Detection of polymerase chain reaction products using agarose gel electrophoresis

A 1.5 % agarose gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA) was used to detect PCR products. A thick gel was poured to maximise the amount of PCR product that could be loaded (30 µl) as this would increase the sensitivity of the detection.

5.2.2.6) Precautions taken during the use of molecular techniques to test the *Cryptophlebia leucotreta* laboratory colony for the presence of virus

The glassware as well as the mortars and pestles (1 per group of 5 larvae) were baked at 200 ºC for 18 hours to degrade any DNA that may have been contaminating these items. A separate set of autopipettes was used for the positive controls in order to reduce chances of carry-over contamination with viral DNA to test samples. In addition, autopipette filter tips were used to reduce the chance of DNA carry-over between samples. Fresh solutions were made up for the experiments used to test the laboratory colony and only clean tips were used to extract volumes from these solutions. Autopippette tips were also packed using a clean pair of gloves that had not been exposed to viral or larval DNA. In each experiment, the positive controls were processed last in each step to reduce the risk of positive control DNA getting into test samples and negative controls. Cheesecloth used for filtering insect homogenate had no contact with the laboratory for virus work and was autoclaved in aluminium foil to reduce contamination of intact DNA.

Separate sets of gloves were used for processing the positive controls, negative controls and the test samples, to prevent contamination of the gloves causing cross contamination of DNA among samples. Owing to the sensitivity of PCR, it is important to be careful of false positives caused by cross contamination.

5.3) Results

There were no signs of virus within the colony prior to stress induction experiments. This indicates either a certain acquired resistance of the colony to viral infection, or that the sanitary procedures adopted had reduced the virus load below a threshold level for routine emergence.

5.3.1) Attempts to induce active viral infection through larval stress

Table 5.4: Detection of virus symptoms using stress induction techniques showing results prior to sodium hypochlorite treatment and subsequent to sodium hypochlorite treatment.

Stress Mechanism	Prior to sodium hypochlorite treatment of egg sheets	Subsequent to sodium hypochlorite treatment of egg sheets
Overcrowding	Symptoms detected	No symptoms observed
Mechanical stress	Symptoms detected	No symptoms observed
Heat shock	Symptoms detected	No symptoms observed
Diet alteration	No data*	No symptoms observed
Nutrient stress due to multiple generation rearing on the same feed	No data*	No symptoms observed

* no diet stress induction experiments were performed prior to sodium hypochlorite decontamination of egg sheets.

5.3.1.1) Crowding of larvae

Initial overcrowding of bottles with larvae prior to sodium hypochlorite decontamination of egg sheets caused noticeable outbreaks of virus. Up to 50% of the bottles contained larvae showing advanced stages of granulovirus infection. Any bottles showing symptoms were discarded from the colony. Subsequent to hypochlorite/Tween 20 treatment of egg sheets, overcrowding had no visible effect on the induction of an active viral infection as no symptoms were observed. Larval weight was observed to decrease as a result of overcrowding and cannibalism was also observed.

5.3.1.2) Mechanical stress

Mechanical stress also resulted in the induction of latent infection in a large number of larvae prior to egg surface decontamination with sodium hypochlorite/Tween 20 solution.

However, as with overcrowding, no symptoms were induced by mechanical stress in the colony generations in which egg surface decontamination had been used.

5.3.1.3) Alteration of larval diet to induce stress

The substitution of maize meal with both Soya flour and chickpea flour appeared to cause stress in larvae, as fewer larvae developed to pupation compared with the number of larvae reaching pupation on maize meal (the conventional diet). However, no symptoms of active CrleGV infection were observed.

5.3.1.4) Induction of stress through heat shock

Sodium hypochlorite/Tween 20 treatment of eggs stopped the occurrence of visible symptoms of active virus infection, previously induced by heating bottles containing larvae to 65 ºC for 25 minutes. Another attempt to induce stress by incubating bottles at 37 ºC for the duration of larval development resulted in 100 % mortality of larvae.

5.3.1.5) Induction of nutrient stress through bottle overcrowding and rearing of multiple generations in the same bottle

Again this attempt to stress larvae did not produce any visible symptoms of virus infection. Larvae were a smaller size in the fourth and fifth instars than those of the control, indicating that these larvae were under stress due to a lack of nutrients. Larval and pupal weight was visibly affected by this stressing mechanism. As with the overcrowding experiment in **5.3.1.1**, cannibalism was observed.

The final trend is that none of these stressing techniques produced an active viral infection displaying noticeable symptoms after egg surface decontamination with sodium hypochlorite/ Tween 20 had been performed.

5.3.2) Molecular-based tests of the colony for virus infection

Sensitivity tests of the PCR system conducted with purified CrleGV DNA showed that 1 fg of DNA was detectable using PCR (**Fig. 5.1**). The Expand High Fidelity PCR System (Roche) produced a greater detection sensitivity than either Roche Taq or Promega Taq PCR systems. A definite PCR product of about 870 bp was visible for each of the DNA dilutions, down to 1 fg. The PCR positive and negative controls confirmed that the reaction was authentic and that sensitivity had not been artificially enhanced through DNA carry-over in tip aerosols or contaminated solutions.

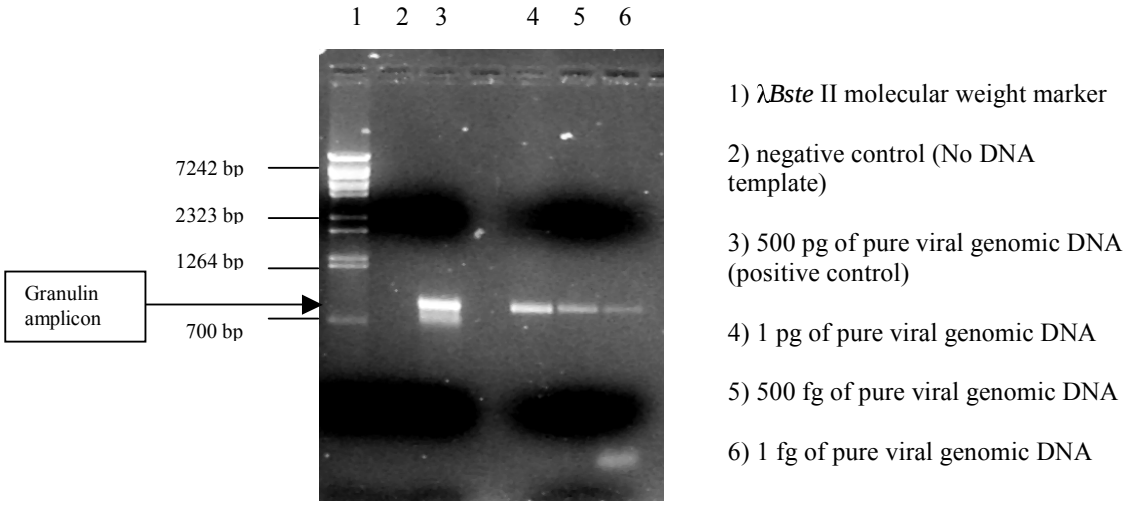


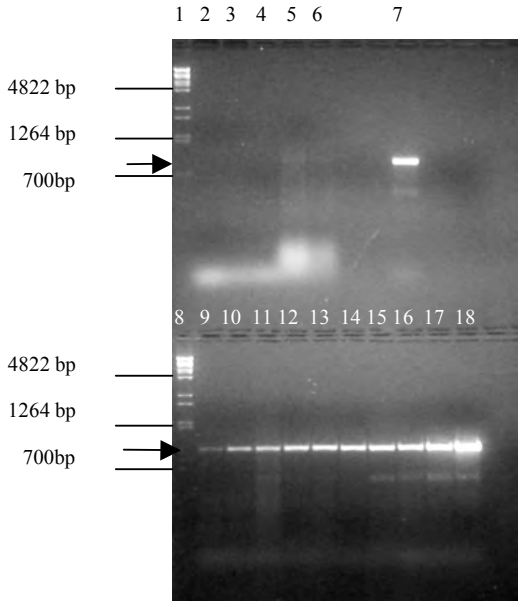
Figure 5.1: A titration of the sensitivity of PCR using purified CrleGV genomic DNA. Roche Expand High fidelity PCR kit produced the highest sensitivity.

5.3.2.1) Investigation of the sensitivity of virus detection in *Cryptophlebia leucotreta* larvae using polymerase chain reaction

The PCR detection technique in larval homogenate was able to detect down to 60 fg of added viral DNA (**Fig. 5.2**). Two negative controls were used for this experiment. The first was the control that contained no insect homogenate, but only the buffer used for homogenisation. This was used to check that all solutions were free from viral DNA. The second negative control consisted of larval homogenate to which no CrleGV DNA had been added. This was used to determine if there was a significant background virus signal. The PCR negative control ensured no virus DNA contaminated the PCR reagents

and the PCR positive control was used to determine that the PCR reaction worked. The larval homogenate containing virus DNA produced a decreasing signal with the decreasing amount of CrleGV DNA.

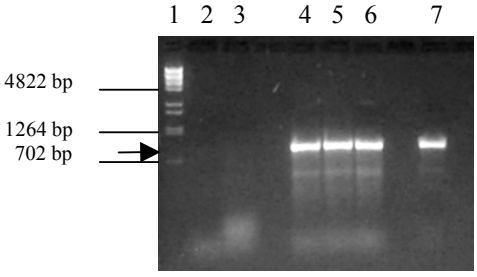
Fig. 5.3 shows that this virus detection technique was able to detect virus in the form of budded virus, occluded virus and naked DNA. The PCR negative and positive controls were used for the same purpose as in **Fig. 5.2**.



1) λ*Bste* II molecular weight marker
2) PCR negative control (no template)
3) negative control (only buffer added)
4) -ve control (larval homogenate only)
5) -ve control (larval homogenate only)
6) -ve control (larval homogenate only)
7) positive control (PCR control)

8) λ*Bste* II molecular weight marker
9) 60 fg viral genomic DNA
10) 125 fg viral genomic DNA
11) 250 fg viral genomic DNA
12) 500 fg viral genomic DNA
13) 750 fg viral genomic DNA
14) 10 pg viral genomic DNA
15) 125 pg viral genomic DNA
16) 250 pg viral genomic DNA
17) 500 pg viral genomic DNA
18) 1 ng viral genomic DNA

Figure 5.2: PCR sensitivity titration of CTAB extractions of larval homogenate containing decreasing amounts of added CrleGV DNA. Arrows indicate expected size of amplicon (870 bp).

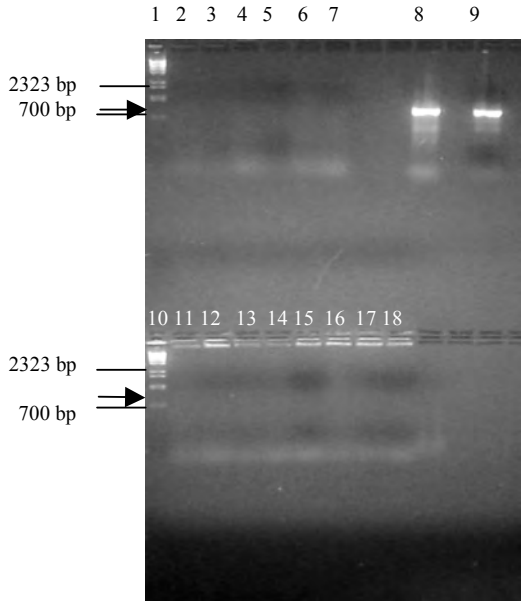


1) λ*Bste* II molecular weight marker
2) PCR negative control (no template)
3) Larval homogenate negative control (no viral DNA added)
4) Larval homogenate containing 1 ng of DNA as occlusion body
5) Larval homogenate containing 1 ng of DNA as unoccluded virus
6) Larval homogenate containing 1 ng of purified viral genomic DNA.
7) PCR positive control

Figure 5.3: The CTAB DNA extraction/PCR detection technique was able to detect virus DNA in any form (i.e. as occluded virus, unoccluded virus or naked DNA). Arrow indicates expected size of amplicon (870 bp).

5.3.2.2) The use of polymerase chain reaction to test for the presence of CrleGV in the laboratory colony

None of the test samples produced a definite band of 870 bp in any of the lanes in the gel **(Fig. 5.4)**. The PCR negative control (lane 2) showed no PCR product at the right size, while both the PCR positive control and the CTAB extraction positive control (both with 1 ng of viral DNA added) showed a strong signal of product at the right size. The negative controls that were used in the experiment in **Fig. 5.2** were also used for this experiment. The only change was that larvae of *Nudaurelia cytherea* (the pine emperor moth) (Lepidoptera: Saturnidae) were used instead of *C. leucotreta* larvae, as the former were known to be free of CrleGV which is specific for FCM (Jehle *et al*., 1992).



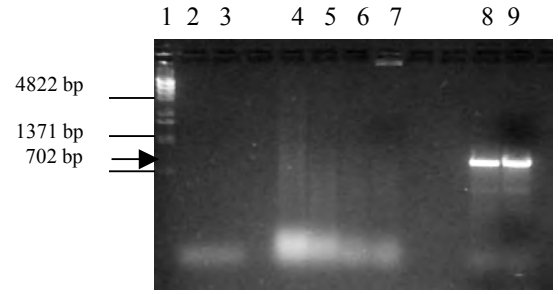
1) λ*Bste* II molecular weight marker
2) PCR negative control (no template)
3) Homogenisation buffer control (no larval homogenate)
4) Negative control (*Nudaurelia cytherea* homogenate)
5) Negative control (*Nudaurelia cytherea* homogenate)
6) Negative control (*Nudaurelia cytherea* homogenate)
7) Negative control (*Nudaurelia cytherea* homogenate)
8) CTAB positive control (1 ng of purified DNA added to larval homogenate)
9) PCR positive control (1 ng of purified DNA in a PCR reaction)

10) λ*Bste* II molecular weight marker
11) Test sample 1
12) Test sample 2
13) Test sample 3
14) Test sample 4
15) Test sample 5
16) Test sample 6
17) Test sample 7
18) Test sample 8

Figure 5.4: Gel analysis of PCR of total DNA extracted from *Cryptophlebia leucotreta* fifth instar larvae using CTAB to test for the presence of CrleGV within the laboratory colony. Test samples 1 to 8 each represent a batch of 5 pooled larvae used for CTAB extraction. Arrows indicate expected amplicon size of 870 bp.

The gel photograph in **Fig. 5.5** shows a repetition of the experiment in **Fig. 5.4**, but with fewer test samples. Both positive controls showed strong signals. The necessity for a pure PCR negative control was eliminated as the buffer control run with the CTAB extraction would also show if there was contamination being introduced from PCR reagents. The PCR negative control was left out in an attempt to conserve enzyme. A slight smearing

can be seen across the correct product size range from the PCR reaction in lane 4 and to a lesser extent in lanes 5, 6 and 7 of **Fig. 5.5**. The negative controls showed no smear in this region.



1) λ*Bste* II molecular weight marker
2) Homogenisation buffer negative control (no larval homogenate)
3) *Nudaurelia cytheria* homogenate as negative control
4) Test sample 1
5) Test sample 2
6) Test sample 3
7) Test sample 4
8) CTAB positive control (Larval homogenate containing 1 ng purified DNA.
9) PCR positive control (1 ng of purified viral DNA)

Figure 5.5: Gel analysis of a second PCR of total DNA extracted from *Cryptophlebia leucotreta* fifth instar larvae using CTAB, in an attempt to detect the presence of CrleGV in the laboratory colony. Test samples 1 to 4 each represent a batch of 5 pooled larvae used for CTAB extraction. Arrow indicates the expected size of amplicon (870 bp).

5.4) Discussion

The techniques used to stress larvae did not produce an active infection subsequent to surface decontamination of eggs with sodium hypochlorite and Tween 20. This indicated that the treatment reduced the viral load in the colony if it did not remove it completely. However, the possibility that subsequent generations of larvae had adapted to cope with the stress as a result of the short generation time (three to four weeks) cannot be ruled out. This could possibly have prevented these mechanisms from causing sufficient stress to stimulate an active viral infection. A counter-argument is that two of the stress mechanisms used were not used prior to the hypochlorite decontamination of eggs. Therefore, larvae would not have had time to adapt to these forms of stress. In addition, crowding of larvae were observed to have an effect on larval and pupal weight in a *Trichoplusia ni* laboratory colony (Fuxa, 1999). This same trend was observed in crowding experiments conducted with this colony of *C. leucotreta*, which implies that this form of stress was still effective. The observation of cannibalism in *C. leucotreta*

may not have been due to stress, as larvae normally develop in solitude (i.e. one larva per fruit) (Newton, 1998). Older larvae are less sensitive to virus infection than younger instars (Payne, 1982). This implies that younger larvae should be stressed to induce virus infection. However, younger instars are also more delicate in physiology and therefore are more inclined to die of the stress side effects instead of an active virus infection. For these reasons larvae ranging from third to fifth instar were used for the stressing experiments.

1 fg of viral DNA is the equivalent of 8 virus genome copies (see **Appendix 5.3** for calculations). The sensitivity of the PCR system that was used to detect pure CrleGV DNA was approximately equivalent to the system used for detecting pure *Malacosoma pluviale* NPV (MpNPV) DNA (Kukan, 1996). Kukan (1996) was able to detect down to between 1 fg and 5 fg of pure MpNPV DNA. Serological techniques were not chosen for detection purposes, as antibodies for any of the gene products were not readily available and the granulin proteins, in particular, have been found to be serologically cross reactive (Akiyoshi *et al*., 1985).

CTAB PCR detection could detect down to 60 fg of viral DNA when added to 500 µl of insect homogenate. This is equivalent to 480 CrleGV genome copies. The detection sensitivity decreased from that obtained using purified DNA, mainly due to the dilution effect of resuspending the DNA pellet from the CTAB extraction in a volume of 50 µl. 60 fg in 50 µl results in a DNA concentration of 1.2 fg/ µl, provided all of the added DNA is recovered. Therefore 1.5 µl (the amount of DNA solution used for PCR) of the DNA solution contained 1.8 fg of DNA, which is the equivalent of 14 genome copies of CrleGV. This indicated that the sensitivity of the PCR system was almost the same as for pure DNA, implying that the CTAB extraction was extracting the bulk of the DNA present, including the CrleGV DNA in a pure form. The limiting factor for the detection sensitivity here was therefore the proportion of the 50 µl of DNA that could be used in the PCR reaction. Adding a greater amount than 1.5 µl resulted in a large amount of smearing which may have resulted from the detection of background larval genomic DNA by the ethidium bromide in the gel.

Nudaurelia cytherea larvae were used as negative controls because they were known not to be infected by CrleGV and they were the only lepidopteran larvae available for use at the time of the experiment. *Cryptophlebia batrachopa* or *Cryptophlebia peltastica* may have been better controls as they are species within the same genus as *C. leucotreta*. However, if these species were infected with a granulovirus, there is a possibility that the conserved nature of the granulin gene (82-96% conservation of *Adoxophyes orana* granulin compared to other granulin sequences) (Wormleaton and Winstanley, 2001) may have led to a positive signal in these controls, even though the virus being detected was not CrleGV. There are no reports of *Nudaurelia cytheria* being infected by an NPV or a GV.

No virus was detected in the samples tested using the PCR detection protocol described in the Materials and Methods of this chapter. The sampling involved collection of 12 groups of 5 larvae from the laboratory colony of 15 bottles that each had an average of 80 larvae. Only 500 µl of the 2.5 ml of homogenate were processed for detection per group of 5 larvae to decrease the number of PCR reactions conducted. This resulted in 5 % of the larval population being tested. However, this could have affected the possibility of getting a signal because the virus DNA from a single infected individual among four other healthy individuals could have been diluted. It is uncertain what the smearing in the test samples of **Fig. 5.5** actually meant. It could have indicated a very low level of virus being detected or that the level of larval DNA was too high and is being detected as a background signal.

Virus DNA concentrations below 480 genome copies (i.e. 60 fg of DNA) could not be detected in larvae (see **Appendix 5.3**). This means that lower concentrations of virus DNA, if present, may go undetected. It is therefore still difficult to conclude that this laboratory colony of *C. leucotreta* is absolutely virus free. If more time had been available, sensitivity of this technique might have been extended to 20 fg (128 virus genome copies). However, detecting 1 virus genome copy among the large amount of larval DNA, as well as the limitation of the sampling technique used, would be unlikely

to produce consistent results. Theoretically, it should only take one virus particle to initiate a virus infection, and this would have tested the limits of detection using PCR even with pure CrleGV DNA.

With all the caveats considered, it can be concluded that sodium hypochlorite decreased the occurrence of viral infection in the laboratory colony of *C. leucotreta*. This observation supports previous work carried out by Doane (1969) and Kukan (1996) who also found sodium hypochlorite decontamination of eggs reduced the number of progeny with an observable viral infection. However, these workers also observed that infection was not completely eliminated by these treatments.

As this treatment would not have affected the interior of the egg, the implication is that at least part of the vertical transmission of CrleGV is by transovum transfer. As a high concentration of virus was effectively removed from the surface of an egg by hypochlorite treatment (see **section 4.2.3)**, it is likely that the far lower transovum virus titres normally present on egg surfaces would be removed or deactivated by this antiviral treatment. There is thus a strong possibility that any virus remaining in the colony would have been vertically transmitted through transovarial transmission. If this is so, the surface decontamination system will not produce complete removal of virus. Low levels of virus infection have been a common problem in work done with a range of laboratory colonies and little success has been recorded for the total removal of these persistent infections from these colonies (Kukan, 1999). This raises doubts as to the feasibility of complete removal of baculovirus infections from a population of insects. Instead control of these latent infections by surface decontamination may be the only alternative. In the final analysis, confirmation of freedom from virus will only be obtained if no symptoms have emerged in the colony after an extended period of handling and experimentation.

Possible applications of a virus free colony of *C. leucotreta* would be to isolate a genotypically pure strain of CrleGV and to compare virulence of various isolates of CrleGV as well as genetically modified viruses. However, if the colony developed in this study is to be used in such applications it is recommended that restriction enzyme

digestion analysis be conducted on all viruses isolated from the colony. Genotypic heterogeneity among virus populations and within virus isolates can be detected by the presence of sub-molar bands in restriction enzyme profiles (Smith and Crook, 1988). This would serve to detect any latent virus in the colony if infection with this latent virus reaches levels high enough to affect results of virulence bioassays.

Chapter 6: The Isolation of CrleGV Genes:

6.1) Introduction

The widespread commercial use of baculoviruses as biocontrol agents has been severely impaired by their slow rate of kill, taking from 4 to 14 days to kill host larvae (Chen *et al.,* 2000). During this lag period, severe feeding damage to crops may be inflicted. To enhance the rate of kill of baculoviruses genetic manipulation, use of gene deletion and insertion has been considered (Black *et al.,* 1997).

These genetic manipulations require an understanding of the gene organisation and content of these viruses. Characterisation of genes such as the *ecdysteroid UDP glucosyltransferase (egt), granulin, chitinase, cathepsin* and *enhancin* genes is also important before their manipulation could be used as a possible mechanism of virulence enhancement. The *egt* gene codes for the ecdysteroid UDP glucosyltransferase enzyme, which catalyses the conjugation of the ecdysteroid hormones. These hormones act as larval moulting signals (O'Reilly *et al.,* 1992a). This conjugation blocks the moulting signal and prevents moulting. Larval feeding times and survival times increase as a result, and energy obtained from feeding is diverted to maximise virus production (Sun *et al.,* 2002). Deletion of the *egt* gene has been found to decrease feeding and cause earlier mortality of infected larvae (Riegel *et al.*, 1994). Granulin is the matrix protein of granuloviruses and is produced in large quantities late in the infectious cycle. Occlusion of virions within granulin protein crystals stabilizes the virions and preserves their infectivity for years outside of host cells (Aikiyoshi, 1985). *Granulin* is the most characterised of the granulovirus genes and is highly conserved among granulovirus species, showing 82 to 96 % amino acid identity (Wormleaton and Winstanley, 2001). The *granulin* gene has received much attention for genetic modification studies as fusion of the promoter of this gene to other genes inserted into the viral genome will cause production of that gene product at high levels. It has been proposed that insertion of an insect toxin gene with this strong promoter will enhance the virus kill rate (Hughes *et al.,* 1997).

The enzymes produced by the *chitinase* (c*hi-A*) and *cathepsin* (c*at*) genes have been found to be associated with host tissue degradation at the end of the infection process. This degradation facilitates the dissemination of progeny virus into the environment (Kang *et al.,* 1998). The enhancin protein is proposed to enhance virus-host cell fusion and disruption of the peritrophic membrane of the larval gut (Popham *et al.,* 2001). Characterisation of these genes could be useful for the enhancement of viral biopesticide capabilities.

It was therefore decided to determine if the above mentioned genes were present, and then to isolate them to determine their nucleotide sequence. The sequencing of these genes was intended to further the genetic and phylogenetic understanding of the CrleGV genome. Two strategies were used to accomplish this objective. The first involved PCR amplification of the genes of interest and the second, restriction endonuclease digestion of the CrleGV genome. In both cases, fragments were cloned into plasmids for the purposes of sequencing.

6.2) Materials and methods

6.2.1) The isolation of genes from the CrleGV genome using polymerase chain reaction

6.2.1.1) Polymerase chain reaction primer design

PCR primers were designed using the consensus sequence of a Clustal W multiple sequence alignment of gene homologues found in other baculovirus sequences deposited in Genebank (Thompson *et al.,* 1994). The nucleotide and protein sequences were examined for consensus among the genes in the NCBI (National Centre for Biotechnology Information) database (Internet i). Areas of highest homology within the consensus sequence were used for primer design and GC clamps (5' end of the primer having a guanine or cytosine base) were also used. The annealing temperatures were checked using Gene Runner 3.04, a DNA sequence analysis programme. The same computer programme was used to check for dimer and hairpin loop interactions between the primers. Primers were synthesised by the DNA Synthesis Laboratory, University of Cape Town. A list of the primers used is presented in **Appendix 6.1.**

6.2.1.2) Polymerase chain reaction conditions

The Expand High fidelity PCR system (Roche) was used to attempt isolation of CrleGV genes through PCR. To each PCR reaction in a 0.2 ml PCR tube 200μM of each dNTP, 300 nm of each of the forward and reverse primer, 5 μl of 10 X buffer with 15 mM $MgCl_2$, 1 U of Expand High Fidelity PCR enzyme mix and 1 to 100 ng of template DNA were added. The volume was made up to a final volume of 50 μl with distilled water. Each 50 μl reaction was overlayed with 25 μl of mineral oil to prevent evaporation of the solution during thermocycling. The same general reaction cycle was used for each reaction with manipulations to the annealing temperature and the number of cycles for stage 2, depending on the primers used for each reaction

Table 6.1: PCR thermocycling conditions

Stage of cycle	**Temperature**	**Number of cycles**
Stage 1 step 1	95 ºC for 3 minutes	1 cycle
Stage 2 step 1	95 ºC for 30 seconds	*Y* cycles
Stage 2 step 2	*X* for 40 seconds	
Stage 2 step 3	72 ºC for 45 seconds *	
Stage 3 step 1	72 ºC for 5 minutes	1 cycle
Holding temperature	4.0 ºC	

X indicates a variable dependent on the primer pair used for a particular reaction. Y indicates the number of cycles of stage 2 that were carried out (up to 30 cycles)
* the elongation step in Stage 2 step 3 was increased by 5 seconds per successive cycle of stage 2.

The initial annealing temperature used was the lower melting temperature of the two primers and the annealing temperature was then increased if non-specific products occurred. In certain cases, a final concentration of 1 to 2 % dimethylsulfoxide or 0.1 % Tween 20 was added to the reaction to increase specificity of the primers. PCR reactions were analysed using agarose gel electrophoresis with TAE buffer. If a product was obtained at approximately the right size, the DNA band was extracted from the gel using the Nucleospin gel extraction kit (Machery-Nagel). The gel-extracted PCR product was then cloned into pGEM-T-Easy plasmid (Promega) for sequencing.

6.2.2) The isolation of genes from the CrleGV genome using restriction enzymes

Restriction enzyme digestion of the CrleGV genome with *Kpn*1 and *Bam* H1 restriction enzymes was conducted as an alternative attempt to isolate the *egt* gene. These enzymes were used based on information obtained by Singh (2001) to map the position of *egt* and *granulin* on the CrleGV genome which indicated that *egt* was flanked by *Kpn* I and *Bam* HI sites (**Fig. A1 Appendix 6.1**). Selected fragments were cloned into pBluescript II SK (Stratagene) for sequencing.

6.2.3) Cloning of CrleGV DNA fragments into plasmid vectors

6.2.3.1) Cloning polymerase chain reaction products into pGEM-T-Easy

The pGem-T-Easy Vector System (Promega) has been designed to clone PCR products with a 3' single adenosine overhang (that is generated by many polymerases independent of template). The pGEM-T-Easy Vector (**Appendix 6.2**) has been linearised using *Eco*RV and has a single 3' thymine overhang inserted at the ends which greatly improves the ligation into the plasmid of PCR products containing an A overhang.

Table 6.2: The ligation reactions for pGem T Easy was set up as follows:

	Standard reaction	**Positive control**	**Background control**
2 x Rapid ligation buffer	5 µl	5 µl	5 µl
pGEM-T-Easy vector (50ng/µl)	1 µl	1 µl	1 µl
PCR product	X µl	-	-
Control insert DNA	-	2 µl	-
T4 DNA ligase (3 Weiss Units/ µl)	1 µl	1 µl	1 µl
Sterile deionised water to a final volume of	10 µl	10 µl	10 µl

The ligation buffer was vortex-mixed before use. The reactions were incubated at 4 ºC for 16 hours. The ligated plasmid was transformed into *Escherichia coli* DH5α competent cells using the heat shock method (**Appendix 6.3**). Colonies were screened using blue/white screening on Luria agar containing ampicillin and X-Gal. The amplicon is

cloned into the plasmid at a point that interrupts the *lacZ* gene, preventing active β-galactosidase from being produced. The colourless X-Gal substrate is therefore not cleaved to produce a blue product. White colonies were picked into 5 ml of Luria broth containing 100 μg/ml ampicillin for plasmid purification using SMART preparations (**Appendix 6.3**). The SMART preparations of plasmid clones were screened for the desired inserts by digestion with *Eco*R1. 10 μl of plasmid were incubated with 2 μl of 10 X buffer H (Roche), 2 μl (20 Units) *Eco* R1 enzyme and 6 μl of sterile double distilled water for 2 hours at 37 ºC. These digests were analysed on a 1 % agarose gel in TAE buffer.

6.2.3.2) Cloning of restriction endonuclease fragments of the CrleGV genome into pBluescript II SK plasmid

Purified pBluescript II SK (**Appendix 6.2**) was digested with *Kpn* 1 and *Bam* H1 restriction enzymes. The linearised plasmid was run on a gel to separate the two fragments produced, and the larger fragment extracted. The linearised plasmid was treated with shrimp alkaline phosphatase (**Appendix 6.3**) to prevent the plasmid strands ligating. This was done to maximise the number of clones containing the desired insert.

Table 6.3: The ligation reactions for pBluescript II was set up as follows:

	Background control	**Typical reaction**
pBLUESCRIPT II (50ng/ μl)	3.75 μl	3.75 μl
Insert DNA	-	*X* μl
10 x ligation buffer (Promega)	1 μl	1 μl
T4 DNA ligase (Promega)	1 μl	1 μl
Sterile deionised water to a final volume of	10 μl	10 μl

X indicates a variable volume of insert, dependent on the concentration of the solution. A 1:1 insert to vector ratio was used.

Reactions were incubated at 4 ºC for 16 hours and then transformed into competent *E. coli* DH5α cells using heat shock at 42 ºC. Colonies were screened on Luria agar containing ampicillin and X-Gal (**Appendix 6.3**) for blue/white screening. White

colonies were picked into 5 ml of luria broth containing 100 μg/ml ampicillin for clone analysis using SMART preps and digestion with *Kpn* 1 and *Bam* H1.

6.2.4) Sequencing of cloned fragments

Selected clones were grown for 16 hours at 37 ºC in 5 ml of Luria broth containing 5 μl of 100 mg/ml ampicillin. 4 ml of this culture was then used for plasmid isolation using the High Pure Plasmid Purification Kit (Roche). The DNA was eluted in no more than 50 μl of sterile distilled water and quantified using the Pharmacia Biotech Genequant and the Unicam αHelios spectrophotometer (using absorbance$_{260nm}$). 200 to 600 ng of double stranded plasmid DNA were added to a tube with 3.2 pmol of either the forward (PUC F [**Appendix 6.1**]) or reverse sequencing primer (PUC R) and the volume made up to a total of 12 μl with sterile double distilled water. 8 μl of BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) was added to the tube, mixed with the 12 μl solution and put into the ABI GeneAmp PCR thermal cycler.

Table 6.4: Cycling conditions for the sequencing reactions.

Temperature and time	**Number of cycles**
96 ºC for 10 seconds	25 cycles
50 ºC for 5 seconds	
60 ºC for 4 minutes	
Hold at 4 ºC until DNA cleanup	1 cycle

The DNA from the sequencing was purified using the Zymogen DNA purification columns. The DNA was then dried at 37 ºC under vacuum and then resuspended for sequencing. Sequencing was carried out using the ABI 3100 Genetic Analyser, Rhodes University. Where possible, DNA was sequenced in both directions in order to confirm the sequence.

6.2.5) Analysis of sequence data

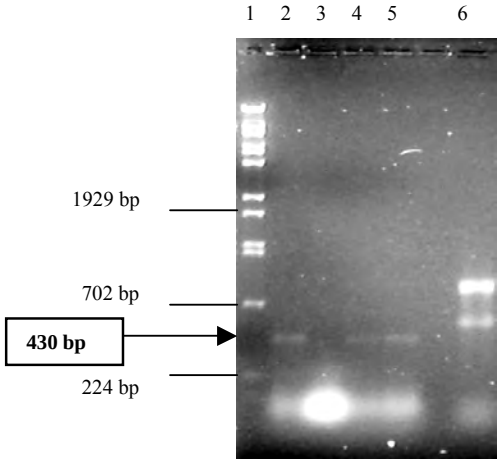
An initial BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) analysis of sequence data was performed to establish whether the sequence matched with high

similarity to any granulovirus sequences in the Genebank database at NCBI (Internet i). This served to confirm that the required gene was isolated and sequenced from the CrleGV genome. If the desired sequence was confirmed, the forward and reverse sequence were assembled and compared to correct any misallocation of nucleotide bases.

6.3) Results

6.3.1) The isolation of CrleGV genes using polymerase chain reaction

Despite the use of two sets of primers (**Appendix 6.1**) and attempts to optimise the PCR reaction, isolation of the *chitinase* gene by PCR was not successful. No definite product was produced and therefore no clone for sequencing could be made. After designing three sets of primers for the *cathepsin* gene (**Appendix 6.1**), a product of the right size (approximately 430 bp) was obtained using the primers CAT FOR 1 and CAT REV 2 (**Appendix 6.1**). The band obtained was very faint, indicating a less than optimum PCR reaction (**Fig. 6.1)**, but was gel purified and cloned for sequencing. However, a blast analysis of the sequence yielded hits with bacterial sequences only. The attempts to isolate *enhancin* using PCR yielded a product at approximately the expected size (600 to 700 bp) using ENHAN FOR 1 and ENHAN REV 1 (**Appendix 6.1**). This fragment was gel extracted and cloned into pGEM-T-Easy and sequenced in the forward and reverse orientation. BLAST analysis of this sequence data resulted in similarity being identified with the *arabinose C* and *cytochrome B* genes of bacterial species in the genus *Pseudomonas* and *Escherichia*.



1 – λ*Bste* II molecular weight marker
2 – PCR reaction with 2 % DMSO
3 – PCR negative control
4 – PCR with 0.1 % Tween 20
5 – PCR reaction with 100 μg/ml BSA
6 – PCR positive control (*granulin* primers)

Figure 6.1: 0.8 % agarose gel analysis of a PCR reaction attempting to isolate the *cathepsin* gene for sequencing. Primers CAT FOR 1 and CAT REV 2 were used. Lanes 2,4 and 5 show PCR reactions that contained different additives. These three lanes each have a faint band of the same size (approximately 430 bp).

Attempts to confirm the preliminary *egt* sequence of CrleGV-SA obtained by Singh (2001) resulted in production of the same-sized PCR fragments having the identical sequences. However, BLAST searches with these sequences produced no match with *egt* sequences of other viruses published in Genebank (NCBI database). The only matches found were vector sequences. A pairwise alignment of the *egt* sequence assembled by Singh (2001) showed 90.1 % identity with the pGEM-T-Easy vector (**Appendix 6.4**). Primers EGT FOR 3, EGT REV 3 and EGT FOR 2 were therefore discarded as being suspect, as they had been designed using this preliminary sequence of Singh (2001).

New primers were designed in an attempt to isolate *egt* from the CrleGV genome. These primers were EGT FOR 6, EGT FOR 5, EGT REV 4 and EGT REV 5 (**Appendix 6.1**). The original primers designed by Singh (2001) using alignments of *Lacanobia oleracea* GV and *Choristoneura fumiferana* GV (i.e. EGT FOR1, EGT REV 1 and EGT FOR 4) were also retained. Out of the various primer combinations, a definite product was produced when using EGT FOR 6 and EGT REV 1. This product of about 650 bp was cloned into pGEM-T-Easy, even though a product size far larger was expected for this pair of primers. A BLAST search with the sequence produced no hits with *egt* genes sequenced from other granuloviruses. Attempts to design additional primers were discontinued.

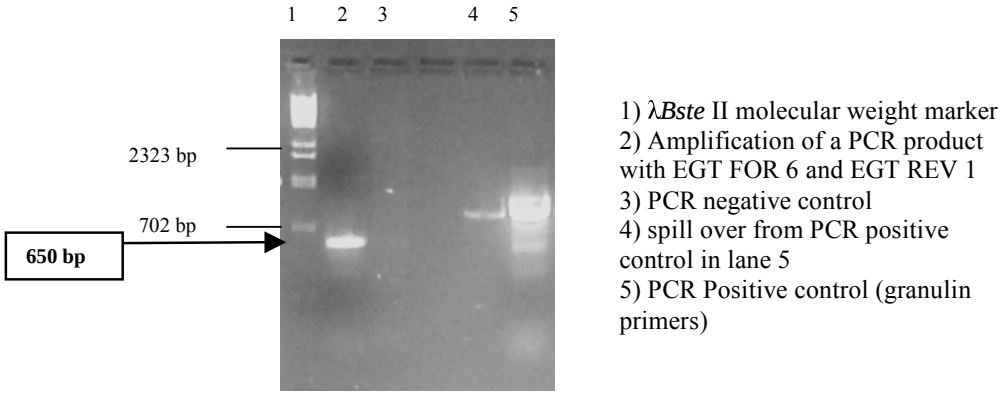


Figure 6.2: Attempt to isolate a segment of *egt* gene with oligonucleotide PCR primers EGT FOR 6 and EGT REV 1.

6.3.2) The isolation of genes from the CrleGV genome using restriction enzymes

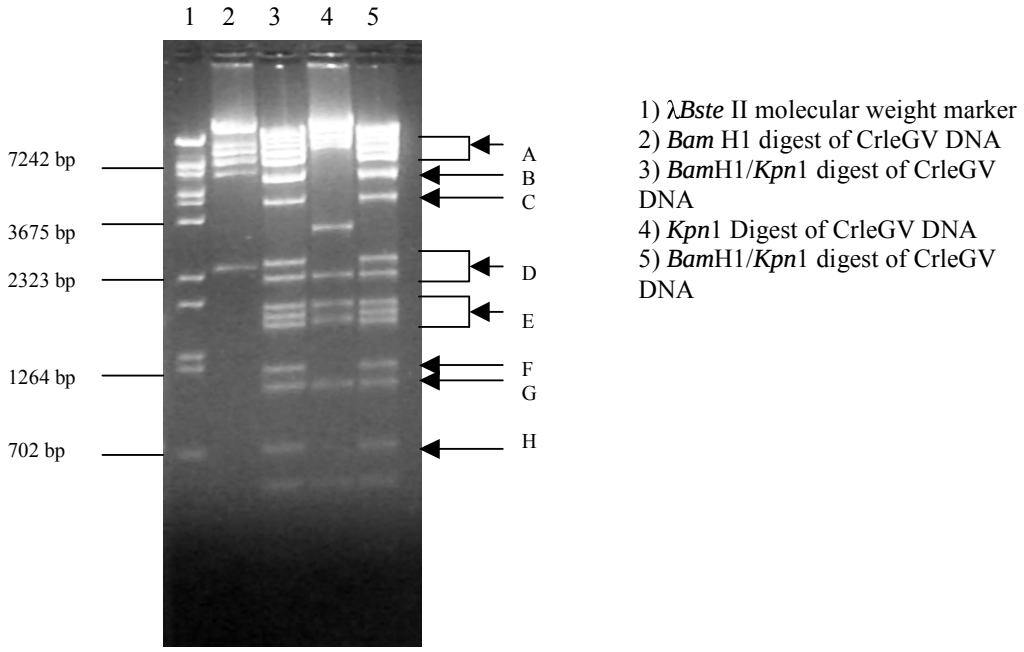
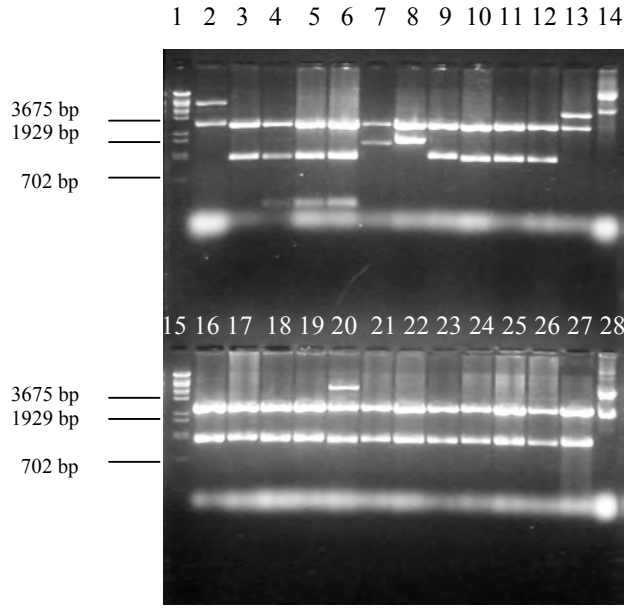


Figure 6.3: Restriction digestion of CrleGV DNA with *Bam* H1, *Kpn* 1 and *Bam* H1/*Kpn* 1 in an attempt to isolate a fragment containing *egt*.

As *egt* is flanked by *Bam* HI and *Kpn* I sites (**Fig. A1, Appendix 6.1)** it was reasoned that *egt* would be present in a restriction fragment of between 1 and 5 kbp present in the double digest and not in either of the single digests. The bands labelled A to H in **Fig. 6.3** were gel extracted individually using the Nucleospin Gel Extraction Kit (Machery-Nagel). Bands in regions A, D and E were too close to each other to extract separately, so fragments in these regions were extracted together. Running the gel for a longer time made the bands difficult to visualise using long wavelength UV light. It was decided to sequence fragments E and F in **Fig. 6.3.** *Egt* in other GVs is about 1.2 kb to 1.6 kb and therefore the fragment H was excluded as this would not have provided a complete *egt* sequence. As the available sequence analyser was not able to process more than 700 bp per sequencing reaction, fragments A, B and C would have been too big to be sequenced without designing additional PCR primers to access the middle part of the fragments for sequencing. Fragments D and G of the double digests produced fragments recognisable in the *Bam* H1 or the *Kpn*1 single-digest lanes. These two fragment regions were therefore excluded on the basis that they were most likely flanked by two *Kpn* 1 sites or two *Bam*

H1 sites. Restriction fragments E and F were cloned into pBluescript II SK (Stratagene) and transformed into *E. coli* DH5α and clones screened for inserts of the required sizes. **Fig. 6.4** shows a gel used to screen clones of restriction fragments in regions E and F of **Fig. 6.3**. Apart from lanes 7 and 8, all the digests contained an insert fragment of about 1.2 kbp. Therefore, clones 3, 4, 7 and 8 were selected for sequencing in both directions. As clones 7 and 8 were about 1.9 kb the sequencing of these fragments in both directions did not result in the overlapping of the sequences when analysed.



1& 15) λ*Bste* II molecular weight marker
2) Undigested cloned insert F (Figure 6.3)
3 to 6) digested cloned insert F.
7 to 12 & 16 to 27) Digested cloned from region E of figure 6.3.
28) Undigested clone of fragment in region E
13) Digested clone fragment C (Figure 6.3)
14) Undigested cloned fragment C.

Figure 6.4: Screening for cloned CrleGV-SA restriction fragments in pBluescript II SK using digestion with *Kpn* 1 and *Bam* H1.

BLAST analysis of sequences produced hits with the granulin, *Xestia c-nigrum* GV open reading frame (ORF 2) homologue and *protein kinase 1* (*pk*1) for *Cydia pomonella* GV and other granuloviruses (**Appendix 6.5**). A partial coding sequence was also found downstream of the *pk*1 gene and this showed similarity to ORF 4 of *Phthorimaea operculella* GV and *Cydia pomonella* GV as well as the ORF 7 of *Xestia c-nigrum* GV. The sequence data were assembled using the Gene Tools 1.0 computer programme (**Fig. 6.5**).

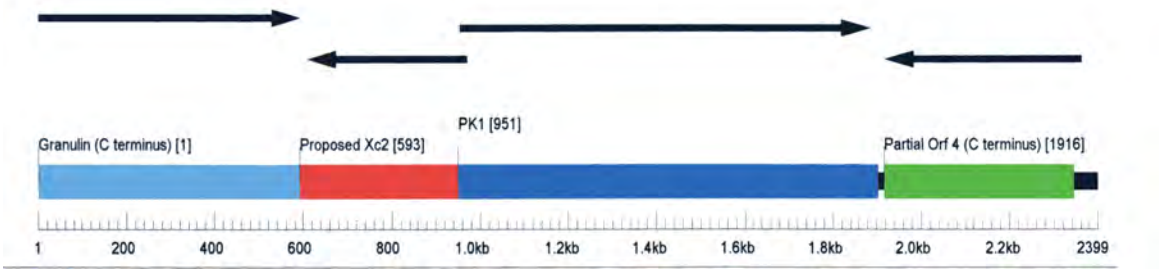


Figure 6.5: Diagram of the granulin region of the CrleGV genome showing the organisation of the genes in this region. The arrows indicate the orientation of the genes.

An alignment of the assembled sequence data and the *Cydia pomonella* GV granulin region is shown in **Appendix 7**. The preliminary sequence of the CrleGV granulin region is shown in **Fig. 6.6**. The various open reading frames identified through BLAST analysis are shown in different colours to facilitate identification. Gene Runner 3.04 was used to translate the sequence so that the various open reading frames could be identified through identification of start and stop codons.

In comparison to the granulin gene (Jehle and Backhaus, 1994a) in the NCBI database (accession number X79569), the granulin ORF shown in **Fig. 6.6** is only a partial coding sequence. The first 155 nucleotides of this gene are not shown in this preliminary sequence. The putative XecnGV *ORF 2* homologue has a region of overlap with the *pk-1* gene and is in the opposite orientation to the *granulin* and *pk-1* genes. A nucleotide identity of 59.0 % was observed between the preliminary CrleGV *XecnGV ORF 2* homologue and the *Cydia pomonella* GV *XecnGV ORF 2* homologue (**Table 6.5**). However, when the CrleGV-SA XecnGV ORF 2 homologue was translated no significant length of protein sequence was produced. The longest protein obtained from this region is about 35 amino acids long, which is quite small for a coding sequence of 473 bp. This irregularity could have been caused by a frameshift or a sequencing error. It can therefore only be classed as a putative ORF homologous to the *XecnGV ORF 2*. Comparison of the putative *XecnGV ORF 2* homologue in the preliminary sequence of CrleGV–SA with a partial sequence of the same region from CrleGV-CV3 (Jehle and Backhaus, 1994a) using a pairwise alignment (Pearson *et al.,* 1997) revealed a 96.7% identity. This confirms that the sequence in this region is mostly accurate. A few insertions were inserted into the CrleGV- SA sequence possibly due to sequencing error

```
BASE COUNT      759 a    507 c    359 g    774 t

    1 ggtggcggcc gctctagaac tagtggatcc atttcgtgga cccggcaaga atgtaaggat
   61 cacccttttc aaggaaatca gacgtgtcca cccagacaca atgaagctgg tatgcaactg
  121 gagcggtaaa gaattccttc gcgagacttg gacccgtttc atctctgaag aatttcccat
  181 caccacagac caagaaatta tagatttgtg gtttgagctt cagctacgac cgatgcaccc
  241 taaccgttgt tacaaattca ctatgcagta tgcgctctgt gcccatcccg attatgtcgc
  301 tcacgatgtg atccgccagc aggatcccta ttatgtagga cctaacaata tcgagcgtat
  361 caatctttcc aagaagggtt tcgctttccc actcacatgc ctacagtccg tctacaatga
  421 caactttgag aatttctttg atgacgttct gtggccgtat ttccaccgtc ccctggtgta
  481 tgtaggtact acgtctgccg aaattgagga gatcatgatt gaagtttctc tgttgttcaa
  541 gatcaaggag tttgcacccg acgtgcccct attcaccggt ccagcctatt aaataactat
  601 cttttaaaac taaaattttc taagttgtct aaatcatcat ctatccactc atgaacaata
  661 tcattgtcga tactattttt atcaatatca attttatcaa tatcagtttt atcattgtcg
  721 atatcgtttt tatcaacaac aatttttaac ttttcctcat catctatagt atccgcttaa
  781 tttattctcg tcacatacca ccactgattg atattgtttt cttgtaacgc attgttttag
  841 gttgcggcga ttccgctaca attctatcgg gttattttac tcctacacta atttatgcat
  901 cataaatata ctctcaaaag tctaccactt cttctattgt cataacgact atgtcgtcgc
  961 caattttgat gccaatttcc tttaagctca cgtcccactt catcagcttt ataatatcgc
 1021 gaacatctgc tttaaaacca gctctcttca tgaataccaa taaatccata tcgcgttttg
 1081 ctaaagaatt atcaaattat gaaattttaa aaaatttaga cgagaatgac tgtcaatctt
 1141 atagtaacgt atttttatgt aaaaaaaaag gtgaacaaaa aatgtatgtt tgcaaaatag
 1201 ttaaatcatc aacctttaat tctttagagt ttgatgttca catattaatg aaacacaata
 1261 taaattttat taaattctcc ttcgtattca acaatgaaga agatattttg ttgataatgg
 1321 attacgtaaa agatggtgat ttgtttgagt tggtaaaaaa gaatgacatc aaattggatg
 1381 aagctacgtg taaaaaactt attctaacac tagtaacagc tcttaacgat ctgcatagaa
 1441 aaaatattat acacaatgat gttaaattag aaaatttact gtatgatcgt aaaaaaaaac
 1501 gcctgtacgt gtgcgaggac ggattatcaa gtttttccgc acaccctcct tctacgacgg
 1561 caccacaccc tccttctacg acggcaccac agtatacttt cccacctgaa aagatccgcc
 1621 atgaaccata tcaggtctct ttcgattggt gggctgttgg agttgtctca tacgaaattt
 1681 tatcgtgcga atatccattt gatatagacg aagaaaacga agaggaaatg gacaatatgg
 1741 aaccagaaga tatgcttcct ctatattcca aaccgttgcc tcccatcaaa aatatttcaa
 1801 aaaaagccat gaattttgtg aaacaaatgt tggagttaga catcaacaac agactgagtt
 1861 cttacgatga aataataaaa catccatttt taaaattcta atatattttt tatttttaca
 1921 tcttcatatt ttgtaatatt atagcactag ctttatcgta cgcatggtaa gcctcagaca
 1981 atgacacaat atcatcaaag tgtttatggt gttcagccaa acacacaaat tttttcacaa
 2041 cgtccccgat tacttcgacg ttaggttgat ttgctagcat atattgttct aaaactttaa
 2101 aataaaaatt aaatgaggcg ctaatgcgcg tccttaattt acttttatac aattccaacc
 2161 agttatgatt atggtttaaa actactcgta aggtacaatt tactaacatc atttcaaatt
 2221 tatctttttc cccaaaacta ttgctatcta tcaaatccag tatcttcaac gacacataac
 2281 attcgtcaca tttcaacagc ctcaaatcga tatcaccacc gtttgtataa aacacctgat
 2341 catacacatt cacttaagct atccaaccaa ttctctatca ctgtcaacgc atctatcat
```

Figure 6.6: A preliminary sequence of the granulin region of the South African isolate of *Cryptophlebia leucotreta* granulovirus genome. The different genes are indicated by different colours: ***granulin***, proposed homologue to XecnGV ORF 2, ***protein kinase*** 1, partial coding sequence for a homologue to the CpGV ORF 4. The **light green** indicates a region of overlap between the XecnGV ORF 2 homologue and the ***protein kinase*** open reading frames. The **grey** section of the ***pk-1*** gene indicates an unsequenced section of the gene. When assembling the segments against CpGV, the sequences 6 and 4 (assembly in **Appendix 7**) did not overlap. Therefore a section of the CpGV ***pk-1*** gene homologue was used to act as a spacer for the purposes of the preliminary sequence.

and this is likely to have caused the translation problem. The gap in the *pk-1* gene sequence (**Fig. 6.6**) was caused by the fact that some of the fragments that were sequenced were 1.8 kb in size. Complete sequencing from both ends of this fragment would require over 900 bp of sequence data. The assembly was done with an alignment of the sequence data with CpGV. Therefore the gap was filled in with CpGV *pk-1* sequence to formulate the preliminary sequence until the centre section of the *pk-1* gene can be sequenced and the whole sequence confirmed. A homologue to the ORF 4 of *Cydia pomonella* granulovirus was found in this CrleGV preliminary sequence of the granulin region directly after the *pk-1* gene. Only a partial coding sequence was obtained and therefore further sequencing of this gene is required for clarity. A pairwise alignment CpGV ORF 4 homologue and the ORF 4 homologue of the CrleGV partial sequence yielded a 70.1 % identity (**Table 6.5**).

Table 6.5: The percentage identity produced by pairwise alignments (Pearson *et al*., 1997) of the preliminary gene sequences of the granulin region of CrleGV and gene homologues of other granuloviruses infecting Tortricidae deposited in the NCBI database (internet i). CrleGV= *Cryptophlebia leucotreta* granulovirus, CpGV= *Cydia pomonella* granulovirus, AdorGV= *Adoxaphyes orana* granulovirus, ChfuGV=*Choristoneura fumiferana* granulovirus.

Pairwise alignment of CrleGV- SA with:	***granulin*(592nt)**	***XecnGV ORF 2* homologue (473nt)**	***protein kinase 1* (852 nt)**	***CpGV ORF 4* homologue (429 nt)**
CrleGV-CV3	96.5% (597nt compared to 592 nt of CrleGV-SA granulin)	96.7 % (243 nt used for both sequences)	-	-
CpGV	82.3% (587nt)	59.0 % (525 nt)	70.1 % (840nt)	70.0 % (432nt)
AdorGV	78.3 % (592nt)	43.9 % (321nt)	63.1 % (828nt)	54.7% (429nt)
ChfuGV	78.1% (592 nt)	51.6 % (381nt)	66.1 % (834nt)	48.3% (429nt)

Pairwise alignments for CpGV and CrleGV-SA as well as alignments between CrleGV-CV3 and CrleGV-SA are shown in **Appendix 6.6.**

The pairwise alignments with the CrleGV sequence of CpGV especially showed good identities. These levels of identity appear to confirm the BLAST results that identified similarity between the *granulin*, *protein kinase-1 (pk-1)*, *XecnGV ORF 2* homologue and *CpGV ORF 4* homologue (also referred to as *XecnGV ORF 7* homologue) genes in other

granuloviruses and this sequence. **Table 6.5** shows the nucleotide lengths used for the pairwise alignments. A large difference in the lengths of the sequences being compared will result in the percentage identity decreasing due to the increasing amount of insertions in the sequences. This is important as some of the alignments performed involved partial coding sequences. A full sequence was available for the *XecnGV ORF 2* homologue in the CrleGV-SA preliminary sequence, and therefore no truncation was carried out on the gene sequences to which it was compared in spite of the differing coding region sizes. The full coding sequence of the CrleGV-SA *XecnGV ORF 2* homologue was not used in a comparison with the XecnGV ORF 2 homologue of the CrleGV-CV3 sequence as this was a partial sequence. The full coding sequence was also used for the comparison of *pk-1* genes with the CrleGV *pk-1*, although the 26 bp insertion of CpGV sequence (to act as a spacer until that part of the sequence can be finalised) was likely to have an effect on the percentage identity. However, this region only represents 3 % of the sequence being compared. Partial coding sequences of the genes for *granulin* and the *CpGV ORF4* homologue were extracted from the NCBI database, as only a partial sequence of these genes was present in the preliminary sequence of CrleGV-SA.

6.4) Discussion

The lack of BLAST matches for partial sequences isolated by PCR indicates that the sequences isolated were not partial sequences of the *cathepsin*, *enhancin* or *egt* genes. This is confirmed by the fact that complete genome sequences for CpGV, XecnGV, PhopGV and PlxyGV are located in the NCBI sequence database (internet i). Therefore BLAST matches should have been found for the sequences if they had contained the coding sequences for these genes. A partial DNA sequence of CrleGV-CV3 (Jehle and Backhaus, 1994a) (NCBI: X79569) contained the N-terminus to an *egt* homologue to *egt* in LAdorGV (Smith and Goodale, 1998). This indicates that there is an *egt* gene in the CrleGV genome. This would therefore indicate that the *egt* sequences used to design the primers for gene isolation in CrleGV may be too divergent to produce primers to recognise these genes in CrleGV. At the time of primer design, there were only two GV sequences that had *cathepsin* coding sequences identified and one GV sequence had a *chitinase* identified. Consequnetly, NPV *chitinase* sequences had to be used to produce

an indication of areas of sequence homology. It is possible that an alignment of these gene sequences did not accurately represent the degree of divergence among these genes, and a region of high homology for these two genes may have been revealed as a region of poorer homology had a larger number of GV sequences been available to compare.

There is also a possibility of bacterial DNA contaminating the viral genomic DNA used in the PCR reactions. Larval gut bacteria may invade the body tissues in the late stages of infection, and as some bacteria are similar in size to occluded virions there is a possibility that these bacteria could have co-purified in the glycerol gradient purification (Spillings (2000), found that coccus bacteria were co-purified with occlusion bodies even after glycerol gradient step). Subsequent DNA extraction would have extracted DNA from virus and bacteria. Even though there was no sign of bacterial contamination of the amido black stains of purified virus, the sensitivity of PCR may have been able to amplify out small amounts of DNA. This may explain why a faint amplicon band was obtained in **Fig. 6.1**. However, this would have to mean that the primers used were not sufficiently specific or that the PCR conditions used were not sufficiently stringent. The bacterial sequences should have been unlikely to bind the primers as these primers were designed using alignments of the virus genes of interest. The organisation of the *chitinase* and *cathepsin* genes in relation to one another on the baculovirus genomes appears to be conserved. A large portion of the baculoviruses on which these genes have been examined have these two genes juxtaposed in the opposite orientation (Kang *et al.*, 1998). This implies that if only one of these two genes had been isolated by PCR, the other could have been located (and then subsequently isolated) on the genome using southern blots with the isolated gene as a probe.

The percentage identity produced by the pairwise alignments appear to confirm the BLAST searches for putative open reading frames in the preliminary sequence of the granulin region of the CrleGV genome. The high percentage identity of the alignments of CrleGV-SA and CrleGV-CV3 serves to show that the sequence obtained for the granulin region of CrleGV-SA was accurate. The granulin gene is known to be conserved among granulovirus species and should therefore be virtually identical between isolates of the

same species, as was seen in the results. Nevertheless, a few base changes were observed by Singh (2001) when the CrleGV-SA and CrleGV-CV3 isolates were aligned. There was also some variability between the partial granulin sequences of CrleGV-SA and CrleGV-CV3 that were aligned in a pairwise alignment (**Appendix 6.6.1**). The fact that BLAST matches for the partial CrleGV-SA granulin sequence did not include the first 25 base pairs of this sequence (**Appendix 6.5**), indicates that this may be some vector sequence. This is supported by the lack of identity between the CrleGV-SA and CrleGV-CV3 in the pairwise alignment (**Appendix 6.6.1**). The variability in the length of the *XecnGV ORF 2* homologue (Wormleaton and Winstanley, 2001) may be responsible for the lower percentage identity between the sequences of the pairwise alignments for this gene, as a greater mismatch would have resulted from one gene being longer than the other.

A sequence of the CrleGV-CV3 granulin gene (Jehle and Backhaus, 1994a) also contained a homologue to the ME53 gene found in AcMNPV, the N-terminus of the *egt* gene (Smith and Goodale, 1998) and a partial sequence of the XecnGV ORF 2 homologue gene. From this sequence it can be determined that the ME53 gene occurs upstream of the granulin gene and the *egt* gene is next to the ME53 gene homologue, resulting in the putative gene organisation for CrleGV granulin region shown in the diagrammatic representation in **Fig. 6.7.**

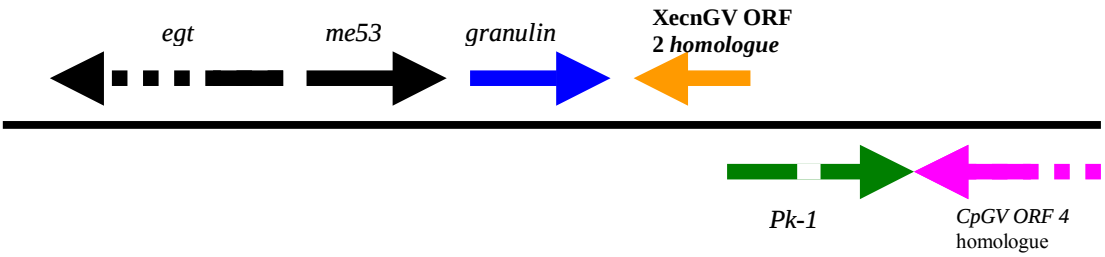


Figure 6.7: The positions of the different genes in the granulin region of *Cryptophlebia leucotreta* granulovirus based on the preliminary sequence of CrleGV-SA and sequencing done by Jehle and Backhaus (1994a). Dashed arrows indicate only part of the gene sequence was available. Colours correspond to those of the assembly shown in **Appendix 7**.

It has been observed that the gene organisation of the granulin gene region of the granuloviruses infecting moths of the family Tortricidae is quite conserved in comparison

to the arrangement of the same region within granuloviruses infecting moths of the order Noctuidae (Wormleaton and Winstanley, 2001). This trend is confirmed by the putative gene organisation observed on CrleGV-SA. This arrangement of CrleGV genes compares exactly to the arrangement for the granulin region of AdorGV (**Fig. 6.8**). The CpGV gene organisation of this region is also the same, except for the insertion of another open reading frame between the *egt* and the ME53 homologue gene. A similar trend is noticed in ChfuGV except an unknown ORF is inserted between the pk-1 and *CpGV ORF 4* homologue. AdorGV, CpGv, ChfuGV and CrleGV are all granuloviruses infecting moths of the family Tortricidae (Wormleaton and Winstanley, 2001). The *Cydia pomonella* GV ORF 4 homologue was found to be just downstream of the *pk-1* gene in CpGV in the genome sequence published on NCBI (internet i). The gene arrangement of CrleGV therefore confirms the conserved gene organisation of the granulin gene region of tortricid-infecting granuloviruses.

The overlap of the *XecnGV ORF 2* homologue and *pk-1* genes appears to be a conserved feature of the torticid-infecting granuloviruses. The NCBI sequence database showed that AdorGV (AF337646), ChfuGV (AF439352), CpGV (NC_002816) and CrleGV (X79569) all show a 16 to 20 bp overlap of the starting regions of the *pk-1* and *XecnGV ORF 2* homologue coding sequences. (Numbers refer to NCBI database accession number of sequence).

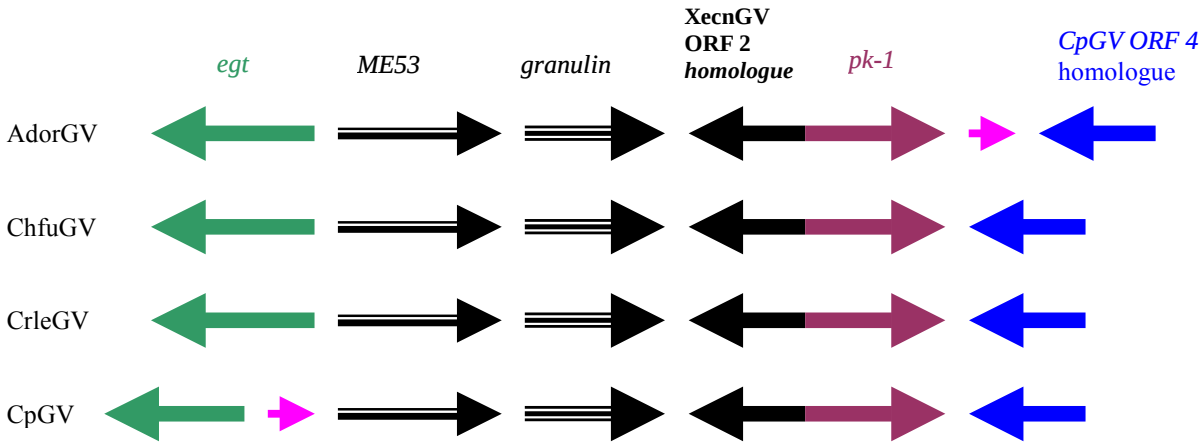


Figure 6.8: Diagrammatic representation of the gene order of four granuloviruses infecting moths of the family Tortricidae. Arrows are not to scale, but just show the ORF order and orientation. The short pink arrows indicate unidentified ORFs. (Information for diagram compiled using: Wormleaton and Winstanley, 2001; and NCBI sequence data [Accession numbers: Af43952, Af337646, NC_002816 and X79569]).

Although *egt* was not isolated from the CrleGV genome, sequence data about the granulin region gene organisation was obtained and it confirms the trend of the conservation of gene order of this region in viruses infecting the family Tortricidae. Further sequencing will be required to confirm the sequences before the genes that were identified can be used in phylogenetic analysis.

Chapter 7: Final Discussion and Future Research

7.1) The Establishment of a Virus Free Laboratory Colony of *Cryptophlebia leucotreta*

The emergence of virus within larvae in the laboratory colony established at Rhodes University indicates that CrleGV is vertically transmitted. The wax paper sheets onto which the eggs were layed were the only contact that each successive generation had with the previous one. This vertical transmission may have occurred as transovarian transfer, transovum transfer or as virus contaminating the wax paper surface (e.g. in moth faecal matter). The latter two forms of transmission are enhanced by the virus resilience provided by the granulin occlusion body. The persistent and ubiquitous nature of baculoviruses in the environment (Cory *et al.,* 1997), as well as evidence for vertical transmission, highlights the need for a laboratory protocol to establish a virus free colony, as isolation of a virus free colony from the field is unlikely.

A protocol for the development of a virus free insect colony is further complicated by the requirement that it should be able to inactivate virions while not killing the host individuals. Egg surface decontamination was used as the strategy of virus inactivation as the eggs went into the next cycle of the colony. Treatment of eggs to inactivate transovarian virus would most likely kill the embryo as well. Therefore surface decontamination treatments were used, even though there is no evidence for transovarial transmission of CrleGV in *C. leucotreta*. The presence of intact-looking virions did not necessarily mean that they were still viable. For example, Vircon did not appear to degrade occlusion bodies as judged by the amido black staining. However, bioassays with this treatment resulted in a low mortality indicating a good inactivation of virus. Sodium hypochlorite treatment was chosen, due to its effectiveness against virus after a 5 minute treatment, its easy availability as household bleach and its ability to inactivate any fungal spores that may be contaminating the eggs. Longer egg treatment times resulted in decreased egg viability. Virus proteins in the virus lipid envelope would probably also be degraded by the high pH of hypochlorite (pH 13).

No CrleGV virus was detected following larval stress or using PCR. This supports the hypothesis that it is possible to establish a CrleGV-free laboratory colony of *C. leucotreta*. However, the lack of CrleGV symptoms in the stressing experiment does not eliminate the possibility of latent infection. In addition, the PCR detection system was able to detect down to 1 fg (8 virus genome copies), which is equivalent to the system used by Kukan (1996). PCR detection of virus down to 60 fg (480 virus genome copies) of purified virus DNA was possible using total DNA extraction from larvae with CTAB. This loss in sensitivity was caused by the limited amount of template DNA that could be used in PCR reactions as a result of the high concentration of larval DNA. Theoretically, only one virion is required to cause an infection and even the PCR detection system, using purified viral genomic DNA, would be stretched to detect one virus genome copy. Therefore, routine monitoring of the laboratory colony for virus infection during a long period of handling and experimentation is required before it can be concluded that a lack of symptoms indicates that the colony is virus free. The lack of evidence for transovarial transmission of CrleGV in *C. leucotreta* also complicates formulation of a conclusion, as there is still the possibility that some CrleGV escaped inactivation by being inside the egg even if sodium hypochlorite had been 100% effective against transovum virus transfer.

As no CrleGV was detected in the laboratory colony, it is recommended that it be used for further experiments, as long as it is monitored for virus symptoms. Virus isolated from the colony during infectivity studies should be subjected to restriction endonuclease analysis to ensure the lack of sub-molar bands, which is an indication of contaminating virus isolates. This would indicate that latent virus is still present. This is particularly important when using the colony to bulk-up novel virus isolates.

7.2) Isolation and characterisation of CrleGV genes

Although *egt* was not isolated from the CrleGV-SA genome, there has been a report of the presence of the *egt* gene in the CrleGV-CV3 genome (Smith and Goodale, 1998). Further southern blotting experiments, using a probe designed from the CrleGV-CV3 *egt* N-terminus nucleotide sequence, are required to locate *egt* in restriction fragment profiles

of the CrleGV-SA genome. Isolation of *egt* based on positional mapping of *egt* in restriction fragments appears to be a better strategy, due to the variables associated with PCR isolation.

The failure to isolate the *chitinase* and *cathepsin* genes was most likely due to an insufficient number of gene sequences from other granuloviruses being available to establish regions of homology for the purposes of PCR primer design. The co-isolation of bacterial genomic DNA with virus genomic DNA could also have complicated gene isolation. This is indicated by the sequence BLAST analysis showing bacterial gene sequence matches.

Sequence data from the granulin region of CrleGV-SA supported the trend that the gene organisation of the granulin region of granuloviruses infecting moths of the family Tortricidae is conserved. Pairwise alignments of putative ORFs in the preliminary CrleGV sequence and these same ORFs from other granuloviruses infecting Tortricidae indicated a high level of nucleotide identity. This was especially the case for comparisons of CrleGV-SA sequences with CrleGV-CV3 sequences, which confirmed the sequence data obtained for CrleGV-SA. Further sequencing is required to confirm the preliminary sequence generated for the CrleGV-SA granulin region. PCR primers for the isolation and sequencing of the central region of the *pk-1* gene are required and further sequencing of the *CPGV ORF 4* homologue is required to produce a complete sequence for this gene. Confirmation of this preliminary sequence is necessary before it can be used for detailed phylogenetic comparisons of genes of this region with those of other granuloviruses.

7.3) Future Research

Despite the lack of absolute certainty of the virus free status of the laboratory colony of *C. leucotreta*, any virus remaining would be in titres too low to have a significant effect on bioassay experiments designed to compare the virulence of the different CrleGV isolates. Another important application of the virus free colony would be, prior to field studies, to test the effectiveness of genetically modified viruses with limited, if any, wild type virus present. However, field testing is still very necessary as testing in the

laboratory does not take into account factors such as recalcitrance of the virus in the environment and genetic heterogeneity of a larger host population that is not necessarily geographically isolated.

A further application of the virus free colony would be to isolate a genotypically pure strain of the virus, using a similar technique to that described by Smith and Crook (1988). It is preferable to work with a genotypically pure strain for genetic characterisation, as it eliminates variables that would complicate the genetic characterisation (e.g. slight variation in sequences or restriction enzyme profiles). A virus free colony could also be used for the "bulking up" of novel isolates of the virus that may be masked by the presence of a an active or latent infection within the laboratory colony.

However, if the laboratory colony is to be used for both these applications, it is necessary that virus manipulated within this colony be monitored for genotypic heterogeneity by restriction enzyme analysis of progeny virus. The presence of submolar bands would indicate the possibility of a residual background infection within the *C.leucotreta* colony, provided that the virus used to infect the colony was genetically pure. It would be beneficial to determine the exact mechanism of vertical transmission in CrleGV as this would enable extrapolations on the effectiveness of surface decontamination of eggs in the total removal of CrleGV from a laboratory colony.

The potential of *egt* in genetic enhancement makes it an important candidate gene for isolation and sequencing. Therefore, use of the N-terminal sequence of *egt* from CrleGV-CV3 (NCBI: X79569) to design a probe to determine the location of *egt* on restriction maps of CrleGV is required. This information can then be used to isolate and sequence the *egt* gene.

More sequencing of the granulin region of CrleGV-SA is required to confirm the preliminary sequence so that the sequence of the genes is completed. These genes could then be used for phylogenetic comparisons. Sequencing of the ends of each *Eco*RI fragment in the library of the CrleGV-SA genome generated by Singh (2001) would

generate a better understanding of gene organisation in CrleGV. The ideal would be to sequence the entire genome of CrleGV in the pursuit of complete genetic characterisation. This information would provide a better understanding of genetic organisation in the virus. ORFs identified through comparisons with other granulovirus sequences should also be used for phylogenetic studies.

In addition to this work adding to fundamental knowledge about the virus, it was also intended to provide tools for the generation of a biopesticide. Virulence studies of different isolates of the virus can be used to choose a virulent strain for the commercial production of the virus for use as a biopesticide. Monitoring of virus genetic change is simpler if the virus is produced in a colony lacking a background virus infection.

Appendix 1: Some of the Crops attacked by *Cryptophlebia leucotreta* include:

Citrus
Castor oil
Acorn
Guava
Plum
Avocado
Pomegranate
Tea
Pepper
Cotton
Pineapple
Peaches
Sorghum
Maize (mealies)
Walnuts
Olives
Almonds
Macadamia nuts

(From: Daiber, 1980; Newton 1998 and Pinhey, 1975)

Appendix 2: Virus purification and DNA extraction

2.1) Occlusion Body Purification

0.1 % SDS (100 ml)
0.1 g SDS in a 100 ml volumetric flask
Make up to the mark with water

80 % glycerol (100ml)
20 ml 0.1 % SDS
80 ml glycerol

30 % Glycerol (100 ml)
70 ml 0.1 % SDS
30 ml glycerol

2.2) Budded virus preparation

1M Sodium Carbonate (Na_2CO_3) (200 ml)
22 g sodium carbonate
200 ml water
Sterilise by autoclaving

25% Sucrose
25g sucrose
5 mM NaCl
10 mM EDTA
Make up to a final volume of 100 ml with distilled water.

Phosphate-buffered saline (PBS) pH 6.2

1 mM Na_2HPO_4. $7H_2O$
10.5 mM KH_2PO_4
140 mM NaCl
40 mM KCl

2.3) DNA extractions

1M Sodium Carbonate (Na_2CO_3) (200 ml)
22 g sodium carbonate
200 ml water
Sterilise by autoclaving

TE Buffer (10mM Tris-HCl, pH 8.0, 0.1 M EDTA) (1L)
121.1 g Tris base
1 ml 0.5M EDTA (pH 8.0)
Make up to 1 L with distilled water and adjust the pH to 8.0 with concentrated HCl. Sterilise by autoclaving. (Adjust the pH of the tris to 8.0 before adding the EDTA and top up to 1 L after checking pH).

RNase A (100 mg/ml)
Dissolve 0.1 g of RNase A powder in 1 ml of water

Chloroform:Isoamyl Alcohol (24:1) (100ml)
96 ml chloroform
4 ml isoamyl alcohol

0.01 M EDTA (100ml)
0.327 g EDTA
100 ml water
Autoclave for 15 to 20 minutes at 121°C, 119 kPa

95 % Ethanol (100ml)
Purchased as 95%

70% Ethanol (100ml)
70 ml Ethanol
30 ml water

10 % SDS (100 ml)
10 g SDS in a volumetric flask
Make up to 100 ml mark with water
May require warming to 65 °C in order to allow complete dissolving of SDS.

7 M Sodium Acetate (50ml)
28.7 g sodium acetate
50 ml distilled water

Mix and then autoclave

Proteinase K (25 mg/ml)
0.025 g Proteinase K
1 ml water
Store at – 20 °C

10 mM Tris-HCl (pH 8.0)
1.211 g of tris base in 800 ml of distilled water
Adjust to pH 8.0 with HCl
Make up to 1 L and autoclave

Tris- buffered phenol (pH 8.0)
Melt 100 g of phenol at 68 °C, add hydroxyquinoline (to a final concentration of 0.1 %) and then add an equal volume of 0.5 M Tris-HCl (pH 8.0) at room temperature. (Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase and a weak chelator of metal ions (Maniatis *et al*., 1989)). Stir the mixture on a magnetic stirrer for 15 minutes and then allow the solution to stand after switching the stirrer off. After the two phases have separated, aspirate as much of the upper aqueous phase off as possible. Add an equal volume of 0.1 M Tris-HCl pH 8.0 to the phenol and stir on magnetic stirrer for 15 minutes. Turn off the stirrer and allow solution to stand until phases have separated as before, then aspirate off aqueous phase. Repeat this procedure until phenolic phase has a pH of >7.8 (as measured with pH paper). After the phenol has been equilibrated and the final aqueous phase has been removed, add 0.1 volume (fraction of final phenol volume) of 0.1 M Tris-HCl (pH 8.0) containing 0.2 % *B*-mercaptoethanol. Phenol is highly corrosive and should be made up in a fume hood, with the use of latex gloves in order to avoid burns. Any areas of skin that come into contact with the phenol should be rinsed with a large volume of water and washed with soap and water. Do not use ethanol.

2.4) Agarose gel electrophoresis

1% Agarose (12 cm) gel
0.7% agarose powder
7 ml 10 x TAE buffer
63 ml distilled water
Heat in microwave until begins to boil. Then ensure all the agarose is dissolved by swirling. Reheat if necessary. Cool the gel to 50°C before adding 3 µl of 10 mg/ml ethidium bromide (0.4 µg/ml final concentration). Pour the agarose into a casting tray.

1.5 % Agarose (12 cm) GEL
Same as above except: 1 g agarose powder

0.7 % Agarose (20 cm) GEL
0.84 g Agarose powder
12 ml 10 x TAE buffer
108 ml distilled water
Follow same procedure as for 1 % agarose gel

DNA loading buffer (6x):
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol
50% glycerol

Ethidium bromide (10 mg/ml)
Dissolve 0.1 g ethidium bromide powder in 10 ml of distilled water. Aliqout the solution into 1 ml volumes and store @ 4 °C.

Tris acetate EDTA (TAE) buffer (1 L)
10 x stock : 48.4 g Tris base
11.42 ml glacial acetic aid
20 ml 0.5 M EDTA (pH 8.0)
make up to 1 L and autoclave

For a 1 x working solution, dilute 10 x Stock 1/10.

λ/*BstE* II Molecular weight marker
Digest 260 µl λ DNA (0.25 µg/µl) with 24 µl of 10 x Buffer D (Amersham) and 10 µl (100 U) enzyme for 3 hours at 60 °C. Check that the digest is successful by running 8 µl on a 1% TAE agarose gel. Add 650 µl TE buffer (10 mM, pH 8.0) and 150 µl loading buffer (6 x). Aliqout 300 µl volumes into eppendorf tubes and store at –20 °C.

Table A1: showing the fragment sizes in base pairs of the λ*Bste* II molecular weight marker.

Fragment	**Size of Fragment**
1 (closest to well of gel)	8 454
2	7 242
3	6 369
4	5 686
5	4 822
6	4 324
7	3 675
8	2 323
9	1 929
10	1 371
11	1 264
12	702
13	224
14	117

λ/*Hind* III Molecular weight marker

Same as for λ*BstE* II, but digest with Hind III enzyme and 10 x Buffer B (Roche) at 37 °C for 3 hours.

Appendix 3: The establishment of a laboratory colony of *Cryptophlebia leucotreta*.

Artificial diet for laboratory colony of *Cryptophlebia leucotreta*

Per 400ml honey jar:
- 40 g Maize meal
- 4 g Wheat germ
- 2 g Brewers yeast
- 0.73 g Milk powder (non-fat)
- 0.3 g P-hydroxybenzoic acid methyl ester
- 0.14 g Sorbic Acid
- 45 ml Distilled water

Mix and Autoclave at 121 ºC for 20 minutes after inserting a cotton wool bung and covering with foil.

Appendix 4: The Determination of Suitable Anti-viral treatments of Laboratory Colony Eggs.

Amido black/napthalene black staining solution (100ml)

1.5 g Napthalene black 12B
40 ml Glacial acetic acid
60 ml Distilled water

Preparation of bioassay trays:

Per tray:
2 g Bacteriological Agar
111 ml Distilled water
16.6 g Larval diet (ingredients mixed in the same ratio as for the laboratory colony artificial diet – see appendix 3).

Mix 2 g of bacteriological agar with 55 ml of distilled water in a conical flask. Autoclave agar solution for 15 minutes. Heat 55 ml of sterile distilled water to 90 º C on a hotplate in a laminar flow hood. The heated water was added to 16.6 g of artificial diet and mixed in a sterile mixing bowl using an electric cake mixer. Add the hot agar to the mixing bowl and mix with the artificial diet for a few seconds. Decant into a sterile beaker and fill each cell of the bioassay tray 1/3 to ½ full of diet. This should be done as fast as possible in order to prevent the agar from setting. It is also necessary to ensure that the diet surface is smooth (facilitated by the addition of the agar) so that the virus treatment covers the whole surface of the diet in each cell. The level of feed in the cells should be kept as uniform as possible in order to maintain uniformity of conditions for each bioassay cell. The trays are left to set and cool under the laminar flow hood, with their lids off in order to prevent condensation build up which otherwise may affect the larvae and increase chances of fungal and bacterial growth. Lids are then replaced once the plates are cool.

It is important to ensure that no feed is stuck to the sides of the cells, especially in neonate bioassays, since neonate larvae can survive for a long period on small pieces of food. As food on the walls of the cell would not be covered uniformly with virus, results may be affected. Therefore scrape off and remove any food stuck to the walls of the cells under the laminar flow hood to maintain sterility of food. The amount of diet added can be increased depending on the number of trays required. All equipment should be sterile. If items cannot be autoclaved, then place them under a germicidal lamp (253 nm) for 3 to 4 hours.

Gold sputter coating of samples for scanning electron microscope analysis:
Place the specimens in the sputter coater holder, clamp in position and close the chamber. Close argon leak valve and evacuate to a 10^{-1} Torr. Open main argon valve on cylinder and then argon leak valve until a pressure of 5 x 10-1 Torr. Allow the argon to flush out the residual air in this manner for 5 to 10 minutes depending on the number and size of

the specimens. Close the argon leak valve and allow the chamber to evacuate further. When a vacuum of better than 5 x 10-2 Torr has been achieved, turn on the HT (High tension referring to voltage) set switch and adjust field potential to 2kV. Open the argon leak valve slightly and allow the sputtering current to rise to about 15mA. Set the timer to 90 seconds and allow sputtering to proceed. To ensure delicate specimens are not damaged by heat build up, the field potential and sputtering current should be reduced and sputtering time increased. After coating is complete, turn off the HT and the vacuum pump and admit air to the chamber. If the coater has a cooled specimen stage, it is advisable to leak argon into the chamber first to prevent water vapour from accumulating on the specimens. Specimens are then examined under the scanning electron microscope (Cross, 1991).

Appendix 5: Testing of the laboratory colony of *Cryptophlebia leucotreta* for the presence of CrleGV.

Appendix 5.1: Recipes of alternative artificial diets:

1) Chickpea flour artificial diet:
Per bottle: 40 g chickpea flour
4 g wheat germ
2 g Brewers Yeast
0.73 g Milk powder (Fat free)
0.3 g P- Hydroxybenzoic acid methyl ester
0.14 g Sorbic acid

2) Soya flour artificial diet:
Per bottle: same as above, except add 40 g of Soya flour instead of chickpea flour.

Appendix 5.2: CTAB total DNA extraction solutions:

Sterile CTAB extraction buffer:
100 mM Tris-HCl (pH 8.0)
1.4 M NaCl
20 mM EDTA
2% (W/V) Hexadecyltrimethyl ammonium bromide (CTAB)
Autoclave the solution containing all reagents excluding the CTAB. When solution has cooled, add the CTAB powder to the solution and wrap bottle in foil to protect from light.

Tris EDTA Buffer:
10 mM Tris- HCl (pH 8.0)
1mM EDTA
Autoclave at 121 ºC for 20 minutes.

70 % Ethanol:
74 ml of 95 % ethanol
26 ml distilled water

10 % SDS:
10 g SDS
Make up to 100 ml of distilled water

25 mg/ml proteinase K:
0.025 g proteinase K
Add 1 ml of sterile distilled water and store at –20 ºC in aliquots of 300 µl.

0.1 M Tris-HCl (pH 8.0) (1L)
12.11g Tris base
add 500 ml distilled water
adjust pH to 8.0 using HCl
make up solution to 1L with distilled water and autoclave at 121 ºC for 20 minutes.

1M Tris HCl (pH 6.8) (500 ml)
Same as for 0.1 M Tris HCl above, except add 60.55 g Tris- Base and adjust pH to 6.8.

Appendix 5.3: Calculation of the number of CrleGV genome copies per 1 μg of DNA.

CrleGV genome size: 112 Kbp
= 112 000 base pairs

Assuming 1 nucleotide base pair has a molecular mass of approximately 660 Daltons.

Therefore, 112 000 x 660 Daltons = 73.9 x 10^6 Daltons,
which is approximately 7.4 x 10^7 Da, which is the approximate molecular weight of the CrleGV genome.

7.4 x 10^7 g of CrleGV DNA = 6.022 x 10^{23} molecules (genome copies of CrleGV).

Therefore: 7.4 g = 6.022 x 10^{16} genome copies
7.4 mg CrleGV DNA = 6.022 x 10^{13}
7.4 μg CrleGV DNA = 6.022 x 10^{10} genome copies of CrleGV
7.4 ng CrleGV DNA = 6.022 x 10^7 genome copies of CrleGV
7.4 pg CrleGV DNA = 6.022 x 10^4 genome copies of CrleGV
7.4 fg CrleGV DNA = 6.022 x 10^1 genome copies of CrleGV
= approximately 60 molecules of DNA

Therefore: 1 fg of CrleGV DNA = approximately 8 genome copies or molecules of CrleGV DNA.

1 μg of CrleGV DNA = 8 x 10^9 genome copies of CrleGV

(6.022 x 10^{23} = Avogadro's number)

APPENDIX 6: The isolateion of CrleGV genes
Appendix 6.1: Oligonucleotide primers used for extraction of genes:

Cathepsin gene:

CAT FOR 1: (16-mer) **Tm:** 60 ºC **Source:** XcGV and CpGV

5' -GTGCGGC(T/G)CGTGCTTGG- 3'

CAT REV 1: (20-mer) **Tm:** 57 ºC **Source:** XcGV and CpGV

5' –CGTA(T/A)CCCACCAGCA(A/G)CACC- 3'

CAT FOR 2: (20-mer) **Tm:** 55.1 ºC **Source:** XcGV and CpGV

5' –TGAACAAAAACGCTCTGCTG- 3'

CAT REV 2: (20-mer) **Tm:** 56.2 ºC **Source:** XcGV and CpGV

5' –ACGACGCGTACTCGTTCATC- 3'

CAT FOR 3: (15-mer) **Tm:** 49.1 ºC **Source:** XcGV and CpGV

5' –CCGTATCCCACCAGC- 3'

CAT REV 3: (14-mer) **Tm:** 49.0 ºC **Source:** XcGV and CpGV

5' –GAGTGCGGCTCGTG- 3'

Chitinase:

CHI FOR 1: (20-mer) **Tm:** 53 ºC **Source:** LydiNPV, HearNPV, AcMNPV, SpeiNPV, CpGV

5' –GC(C/G)TACTTTGT(C/G)GAGTGGGG- 3'

CHI REV 1: (18-mer) **Tm:** 54 ºC **Source:** LydiNPV, HearNPV, AcMNPV, SpeiNPV, CpGV

5' –TC(C/G)GCGTCAAT(C/T)TCCCAC- 3'

CHI FOR 2: (20-mer) **Tm:** 64.8 ºC **Source:** Xc-nGV, CpGV

5' –TTCCCGCIGACAAGGTGCCC- 3' (I = Inosine)

CHI REV 2: (20-mer) **Tm:** 65.9 ºC **Source:** Xc-nGV, CpGV

5' –CCGACCACCC(C/T)CTGCCGTAC- 3'

Enhancin:

ENHAN FOR 1: (20-mer) **Tm:** 55.2 ºC **Source:** ChfuGV, HaGV, PsunGV, XcGV

5' –GACCACACATACCGCT(A/G)CCC- 3'

ENHAN REV 1: (20-mer) **Tm:** 53.4 ºC **Source:** ChfuGV, HaGV, PsunGV, XcGV

5' –ACGAATGGTTGATGTTACGC- 3'

Egt:

EGT FOR 1: (16-mer) **Tm:** 52 ºC **Source:** LaolGV, ChfuGV

5' – ACACCTATGCCGCGTG – 3'

EGT REV 1: (17- mer) **Tm:** 52 ºC **Source:** LaolGV, ChfuGV

5' – GATTGAACACCTCCCTG – 3'

EGT FOR 2: (18-mer) **Tm:** 56 ºC **Source:** CrleGV (Singh, 2001)

5' – GATACCTGTCCGCCTTTC – 3'

EGT FOR 3: (20-mer) **Tm:** 60 ºC **Source:** CrleGV (Singh, 2001)

5' – GGTATCAGCTCACTCAAAGG – 3'

EGT REV 3: (20-mer) **Tm:** 60 ºC **Source:** CrleGV (Singh, 2001)

5' – GGTTGGACTCAAGACGATAG – 3'

EGT FOR 4: (21-mer) **Tm:** 66 ºC **Source:** LaolGV

5' – GATGACCGCTTCGAGCCATGA – 3'

EGT REV 4: (19-mer) **Tm:** 49.2 ºC **Source:** LaolGV, CpGV, ChfuGV

5' – AGA(T/G)CTCT(C/T)TGCGGAAACC – 3'

EGT FOR 5: (18-mer) **Tm:** 48 ºC **Source:** LaolGV, CpGV, ChfuGV

5' – TATTTTGTGTGTTTTCCC – 3'

EGT REV 5: (18-mer) **Tm:** 43.7 ºC **Source:** LaolGV, CpGV, ChfuGV

5' – CTGAAA(C/A)CCACC(C/T)TG(C/T)GT – 3'

EGT FOR 6: (19-mer) **Tm:** 54 ºC **Source:** LaolGV, CpGV, ChfuGV

5' – ATGTATAGTATCTTTGTTGTG –3'

Granulin: (for detection of CrleGV in the *C. leucotreta* laboratory colony)

GRAN FOR 1: (21-mer) Tm:58 ºC **Source:**CrleGV – CV3, CpGV, PuxyGV, Xc-nGV

5' –TGGTGTGGATACCAGAAGAAA- 3'

GRAN REV 1: (23-mer) Tm:50 ºC **Source:**CrleGV – CV3, CpGV, PuxyGV, Xc-nGV

5' – GATGATGATTTAGACAACTTAGA- 3'

Sequencing primers:

PUC F (Forward primer): (23-mer) **Tm:** 65.5 ºC

5' – CGCCAGGGTTTCCCAGTCACGAC –3'

PUC R (Reverse Primer): (22-mer) **Tm:** 56.2 ºC

5' – TCACACAGGAAACAGCTATGAC- 3'

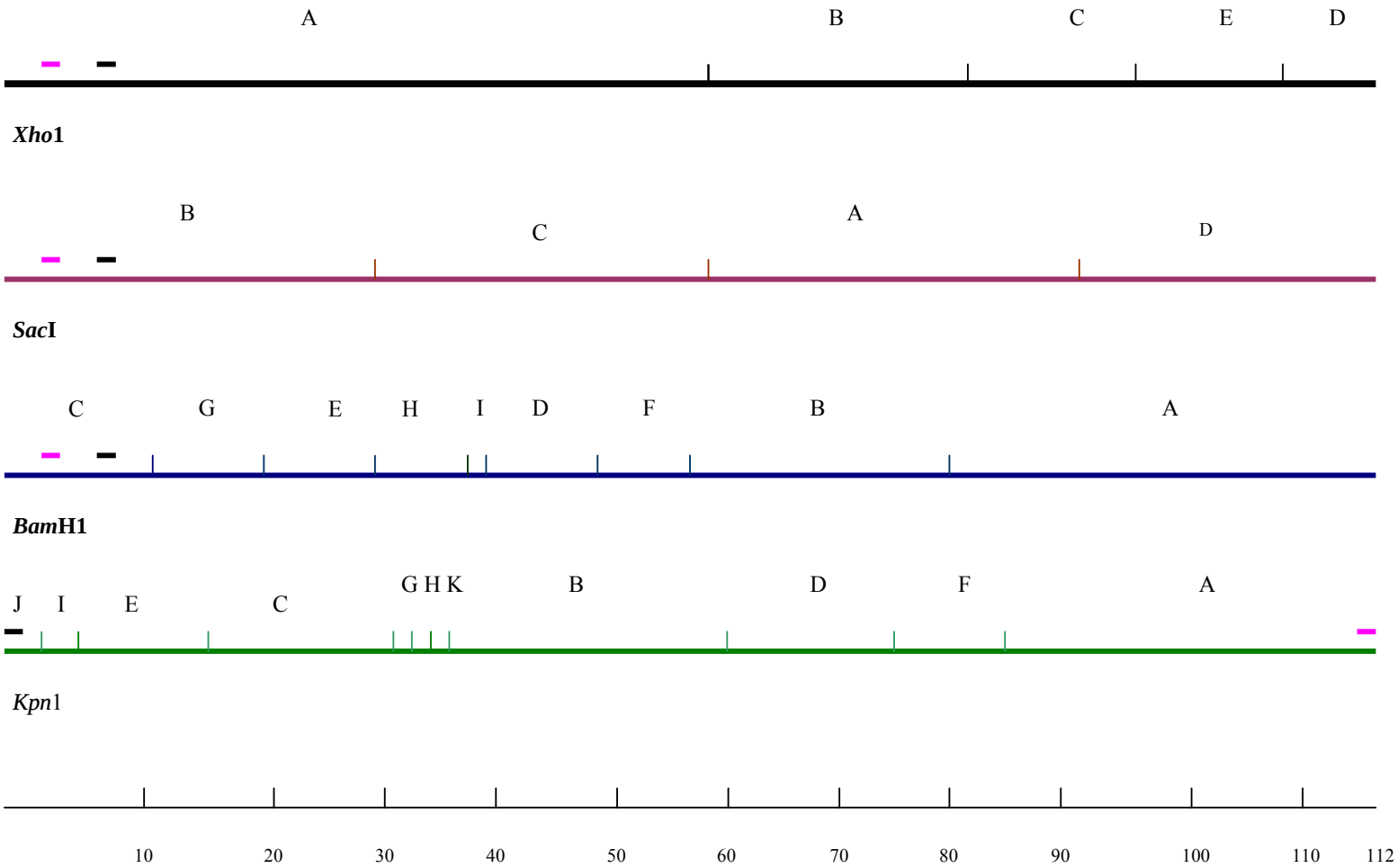
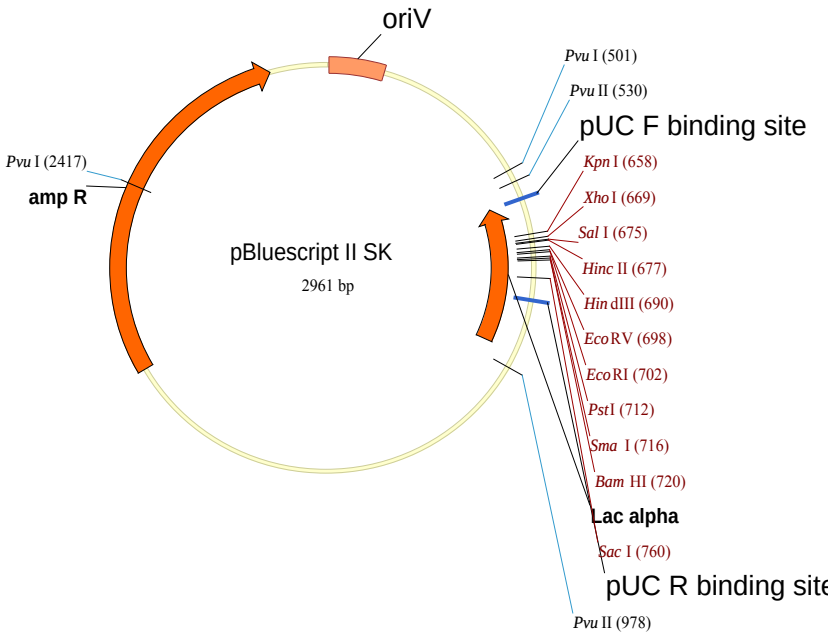


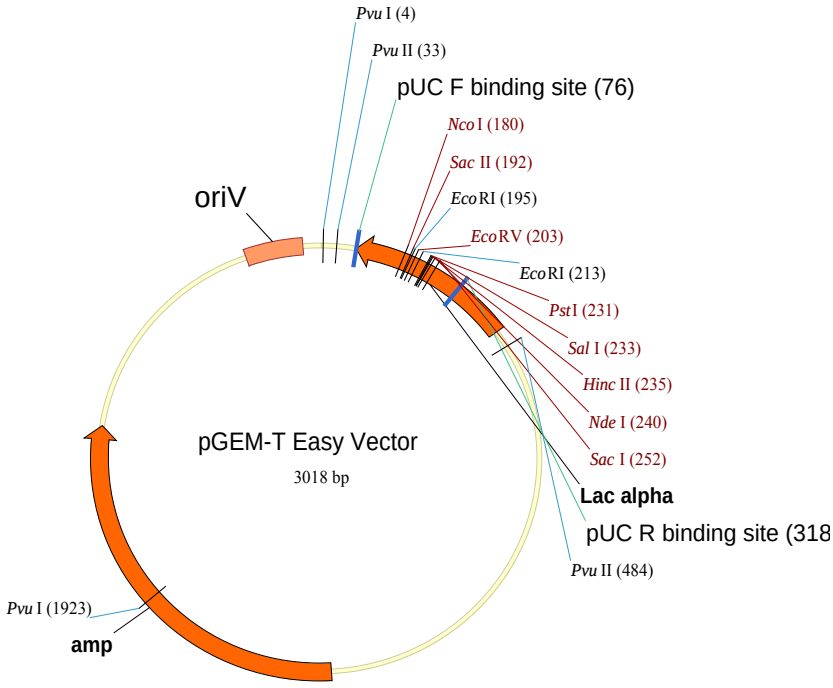
Figure A1: The position of the *granulin* and *egt* genes in the ClGV-SA genome. Pink = *egt*; black = granulin (Singh, 2001)

Appendix 6.2: CLONING VECTORS:

1) pBluescript II SK:



2) pGEM-T Easy Vector:



Appendix 6.3: Cloning protocols

Dephosphorylation of plasmid vectors with shrimp alkaline phosphatase:
General formula for the calculating of the picomoles of ends of linear double-stranded DNA is:

(µg DNA/kb size of DNA) x 3.04 = pmol of ends

X pmol of ends	linearised DNA
1.5 µl	10 x shrimp alkaline phosphatase buffer
1-2 µl	shrimp alkaline phosphatase (1U/pmol of ends)
x µl	sterile deionised water
15 µl	

- Incubate reaction for 15 minutes at 37 ºC
- Inactivate SAP enzyme for 15 minutes at 65 ºC
- Add another 1-2 µl SAP enzyme
- Incubate for 15 minutes at 37 ºC
- Inactivate SAP enzyme for 15 minutes at 65 ºC
- Store at –20 ºC until needed.

SMART buffer:
10 mM Tris-HCl (pH 8.0) *
1mM EDTA *
15 % (W/V) sucrose *
200 µg/ml DNAse free RNAse
100 µg/ml Bovine Serum Albumin (BSA)
2 mg/ml lysozyme

Store Aliqouts of 300 µl at -20ºC.

* indicates reagent solutions that should be sterilised by autoclaving prior to making up smart buffer.

DNAse free RNase:
Make a 4 mg/ml working solution of RNAse A in sterile triple distilled water. Boil tubes containing the stock solution in a boiling water bath for 15 minutes (Maniatis *et al*., 1989).

SMART preparations:

- Add 1.5 ml of culture to a micro-centrifuge tube.
- Microfuge for 60 seconds.
- Vacuum aspirate off the supernatant.
- Add more culture and repeat the procedure, if the pellet is not big enough (i.e. 2 to 3 mm deep).
- Resuspend pellet in 50 µl of SMART Buffer.
- Incubate at 37 ºC for 2 to 3 hours.
- Cool tubes on ice for 5 minutes.
- Boil the tubes at 100 ºC for 90 Seconds.
- Microfuge for 10 minutes.
- Transfer supernatant to a clean microcentrifuge tube.

Preparation of Tansformation-Competent *Escherichia coli* (DH5α) cells:

A pure culture of Escherichia coli (DH5α) was inoculated into 5 mls of luria broth (in a test tube) and incubated with shaking at 37 ºC for 16 hours. Inoculate 1.5 ml, 1 ml, 0.7 ml and 0.3 ml of the overnight culture into four flasks of 100 ml luria broth and shake for 2 to 3 hours until the flask inoculated with 1.5 ml of the overnight culture reaches an OD_{600} of 0.6 to 0.8 Absorbance Units (AU). The flasks with 100 ml of luria broth should be equilibrated to 37 ºC as this decreases the lag period of growth. Once the correct optical density has been reached, pool the 1.5 ml and 1.0 ml cultures together and the 0.7 ml and 0.3 ml cultures together into sterile 250 ml (Beckman JA 14 compatible) centrifuge tubes. Centrifuge in the Beckman JA 14 Rotor at 5000 RPM for 10 minutes at 4 ºC.

The pellets were resuspended in RF1 solution and incubated on ice for 20 minutes. Centrifuge the cells for 10 minutes in the JA 14 centrifuge rotor at 5000 RPM at 4 ºC. Pool the contents of the tubes together by resuspending them in a total volume of 4 ml of RF2 solution. Aliqout the cells into 300 µl amounts and store at – 70 ºC.

RF1: (Final volume 250 ml)

15 % (w/v; i.e. 37.5 g/250ml) glycerol pH 5.8
Make up to a final volume of 202.5 ml with distilled water.

Autoclave these stocks separately and then add:

25 ml 1 M KCl
12.5ml 1M $MnCl_2$
7.5 ml 1M CH_3COOK
2.5 ml 1M $CaCl_2$

RF2: (Final volume 150 ml)

15 % glycerol (w/v; i.e. 22.5 g/150ml) pH 6.8
Make up to a final volume of 135 ml with distilled water

Autoclave these stocks separately and then add:

1.5 ml 1M Mops
1.5 ml 1M KCl
11.25 ml 1M $CaCl_2$

Testing the competency of competent cells:
Add 2.5 ng, 100 pg and 50 pg of pBR322 plasmid DNA to different aliquots of competent cells. Use distilled water as a no DNA control. Incubate the DNA with the cells for 30 minutes on ice. Heat shock for 45 seconds at 42 ºC and cool on ice for 5 minutes. Add 1 ml of luria broth and incubate at 37 ºC for 1 hour. Spread plate 100 µl onto Luria agar plus ampicillin plates and incubate at 37 ºC over night.

Count the colonies on each plate and calculate the colony forming units per ml (Cfu/ml).

Transformation protocol:
Add 150 µl of thawed competent *E. coli* to ligated DNA and mix gently by pippetting once. Incubate on ice for 30 minutes and then heat shock at 42 ºC for 45 seconds. Cool on ice for 5 minutes and add 1 ml of cold luria broth. Incubate for 1 hour at 37 ºC and plate 200 µl of cells onto luria agar containing ampicillin and X-gal. Microfuge the remainder of the cells for 60 seconds and resuspend in 200 µl of luria broth. Plate this onto the same type of agar plates.

Luria broth (1L):
10 g Tryptone powder
5 g Yeast extract
5 g Sodium Chloride

Autoclave for 20 minutes at 121 ºC and 110 KPa.

Luria agar (1L):
10 g Tryptone powder
5 g yeast extract
5 g sodium chloride
15 g Bacteriological agar

For LURIA AGAR + ampicillin: cool agar to 55 ºC and add 1 µl (per ml of agar solution) of a 100mg/ml stock of ampicillin.

Luria agar + ampicillin + X-Gal
Make up luria agar + ampicillin plates and allow to set.

Add 100 µl 100 mM Isopropylthiogalactopyranoside (IPTG) and 20 µl of 50 mg/ml X-Gal. Spread onto the surface of the agar with a sterile spreader and incubate at 37 ºC for 30 to 60 minutes to allow the solutions to absorb into the agar.

100 mM IPTG stock:
1.2 g IPTG in a final volume of 50 ml of water. Filter sterilise the solution and aliquot into 1.5 ml volumes. Store at –20 ºC.

50 mg/ml X-Gal
100 mg 5-bromo-4-chloro-3-indolyl-β-D galactoside.
Dissolve in 2 ml of N,N'-dimethyl-formamide and cover with foil.
Store at –20 ºC.

DNA sequencing:

DNA clean up and concentration using zymo columns (Zymogen)

- Add 2 volumes of DNA binding buffer to sample
- Load onto a zymo column and place in a 2 ml collection tube.
- Centrifuge at 10 000g for 10 seconds. Discard the flow-through.
- Add 200 µl of wash buffer to the column and centrifuge for 30 seconds at maximum speed.
- Discard the flow-through and repeat the washing step.
- Add 8 µl of triple distilled water directly to the column matrix. Place in an eppendorf tube and centrifuge briefly to elute the DNA.
- Place the DNA into a vacuum centrifuge for 1 hour (until the liquid has evaporated).
- Store at –20 ºC until sequencing.

Appendix 6.4: Comparison of egt sequence (Singh, 2001) to partial sequence of pGem-T-Easy vector

```
>_ pGEM-T-Easy                                        1300 nt vs.
>_ CrleGV egt sequence (Singh, 2001)                  1376 nt
scoring matrix: , gap penalties: -12/-2
90.1% identity;        Global alignment score: 4586

                                  10              20
457676 GGG--------------------CGAATTGGGC-CCGACG-----TCGCATGC-------
         :                     ::  :::: : :::: :     : :  :::
_      ATGTTTGTGCCTATCCTACTCGTCGTCTTGGCCGCCGAAGCTTTTTTGTGTGCCCAATAT
               10        20        30        40        50        60

                     30                40              50
457676 -------------TCCCGGCCGC---------CAT--GGCGG----CCGCGGGAATTCGA
                    :::: :  :          :::  : ::     : :: : :   :::
_      TTTGTGTGTGTTTTCCCAGTTGAAGCGAATCTCATCAGTCGTTTTTCGGCAGTACGACGA
               70        80        90       100       110       120

      60              70                              80        90
457676 TAT------CACTAGTGAATTCGCGG--------------------CCGCCTGCAGGTC
       :::      ::::   : :  :: ::                     ::::::::::::
_      TATGTTAGGCACTTTGGGACACGAGGAAACTTTTTTGATGGCCAACCCCGCCTGCAGGTC
              130       140       150       160       170       180

            100       110       120       130       140       150
457676 GACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCAC
       ::::::::::::::::::  ::::::::::::::  ::::::::::::::::::::::
_      GACCATATGGGAGAGCTCCTCACGCGTTGGATGCATCACTTGAGTATTCTATAGTGTCAC
              190       200       210       220       230       240

            160       170       180       190       200       210
457676 CTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTC
       ::::::::::::::::::::::::::::::::::::::::::.::::::::::::::::
_      CTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGNGAAATTGTTATCCGCTC
              250       260       270       280       290       300

            220       230       240       250       260       270
457676 ACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      ACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
              310       320       330       340       350       360

            280       290       300       310       320       330
457676 GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTG
       :::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::
_      GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGACGGGAAACCTG
              370       380       390       400       410       420

            340       350       360       370       380       390
457676 TCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
              430       440       450       460       470       480
```

```
            400       410       420       430       440       450
457676 CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
       :::::::::::::::::::::::::::::: :::::::::::::::::::: :::::::
_      CGCTCTTCCGCTTCCTCGCTCACTGACTCGATGCGCTCGGTCGTTCGGCTGCAGCGAGCG
              490       500       510       520       530       540

            460       470       480       490       500       510
457676 GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGA
              550       560       570       580       590       600

            520       530       540       550       560       570
457676 AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG
       :::::::::::::::::::::::::::::: ::::::::::::::::::::::::::::
_      AAGAACATGTGAGCAAAAGGCCAGCAAAAG-CCAGGAACCGTAAAAAGGCCGCGTTGCTG
              610       620       630        640       650

            580       590       600       610       620       630
457676 GCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
     660       670       680       690       700       710

            640       650       660       670       680       690
457676 AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC
     720       730       740       750       760       770

            700       710       720       730       740       750
457676 GTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCG
     780       790       800       810       820       830

            760       770       780       790       800       810
457676 GGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
     840       850       860       870       880       890

            820       830       840       850       860       870
457676 CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC
     900       910       920       930       940       950

            880       890       900       910       920       930
457676 GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC
     960       970       980       990      1000      1010
```

```
          940       950       960       970       980       990
457676 ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGG
    1020      1030      1040      1050      1060      1070

           1000      1010      1020      1030      1040      1050
457676 TGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA
       ::::::::::::::::::::::::: ::::::::::::::::::::::::::::::::
_      TGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA
    1080      1090      1100      1110      1120      1130

           1060      1070      1080      1090      1100      1110
457676 GTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGC
    1140      1150      1160      1170      1180      1190

           1120      1130      1140      1150      1160      1170
457676 GGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGAT
    1200      1210      1220      1230      1240      1250

           1180      1190      1200      1210      1220      1230
457676 CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATT
    1260      1270      1280      1290      1300      1310

           1240      1250      1260      1270      1280      1290
457676 TTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGA---
    1320      1330      1340      1350      1360      1370

           1300
457676 TTTAAATC

_      --------
```

Appendix 6.5: Summary of BLAST matches between CrleGV-SA sequence assembly and other granuloviruses.

Table A2: Results of BLAST search involving preliminary sequence of CrleGV-SA granulin region.

Organism and sequence accession number in Genebank	Region of match on CrleGV-SA preliminary sequence	Region in matched organism	Score (Bits)	Expected (E) value	% Identity between matched sequences
Matches of preliminary CrleGV-SA sequence with *granulin* of other viruses.					
CrleGV-CV3 (X79569)	25 – 673 bp	1411 -2059	1247	0	99 %
Cydia pomonella GV (U53466)	25 - 593	180 –748	446	e-122	84 %
Epinotia aporema GV (AF473703)	25 - 596	249 – 820	325	2e-85	82 %
Phthorimaea operculella GV (AF499596)	92-594	247 – 749	307	4e-80	82%
Choristoneura fumiferana GV (AF439352)	25 - 471	2952 – 3398	260	9e-66	82%
Adoxophyes orana GV (AF337646)	101 -576	3529 - 4004	222	2e-54	80 %
Xestia c-nigrum GV (AF162221)	95 - 564	250 – 719	178	3e-41	79%
Harrisina brillians GV (AF142425)	68 - 360	479 – 771	153	1e-33	81 %
Trichoplusia ni GV (K02910)	87 - 596	380 – 889	147	9e-32	78 %
Pieris Brassicae GV (X02498)	25 –267 371 – 466 533 - 561	323 – 565 666 – 761 828 - 856	133 63.9 42.1	1e-27 1e-06 3.9	81 % 83 % 93 %
Plutella Xylostella GV (AF270937)	104 - 255	1203 – 1354	95.6	3e-16	82 %
Matches of preliminary CrleGV-SA sequence with *XecnGV ORF* 2 homologues in other viruses					
Cydia pomonella GV (U53466)	1007 - 1076	1211 - 1280	75.8	3e-10	88%
Matches of preliminary CrleGV-SA sequence with *pk-1* genes of other viruses					
Cydia pomonella GV (U53466)	1564 – 1625 1539 - 1567	1758 – 1817 1757 - 1785	69.9 58	2e-08 7e-05	90 % 100 %*
Choristoneura fumiferana GV (AF439352)	1866 - 1902	4675 - 4711	65.9	3e-07	82 %
Adoxophyes orana GV (AF337646)	1806 – 1891 1314 - 1359	5053 – 5138 4591 - 4636	60.0 44.1	2e-05 0.99	83 % 86 %
Matches of preliminary CrleGV-SA sequence with CpGV *ORF 4* and XecnGV *ORF 7* homologues of other viruses					
Cydia pomonella GV (U53466)	2268 – 2343 1837 - 1892	2528 – 2603 2029 - 2084	87.7 48.1	7e-14 0.063	89 % 85 %
Phthorimaea operculella GV (AF499596)	2064 - 2109	2508 - 2553	60	2e-05	91 %

* Due to insertion of 26 bp of CpGV sequence into preliminary CrleGV SA sequence, to act as a spacer until whole sequence can be obtained.

BLAST analysis indicated that the first 25 bp of the preliminary sequence of CrleG-SA may be vector as this part of the partial granulin gene sequence (CrleGV-SA) did not match granulin sequences in other granuloviruses.

APPENDIX 6.6: Pairwise alignments of CrleGV-SA genes and homologues in CrleGV- CV3 and CpGV

Appendix 6.6.1: Alignment of CrleGV-SA *granulin* partial sequence with partial CrleGV-CV3 *granulin* sequence (Jehle and Backhaus, 1994a).

```
>_ CrleGV - SA  partial granulin sequence          592 nt vs.
>_ CrleGV partial CDS from Genebank                597 nt
scoring matrix: , gap penalties: -12/-2
96.5% identity;        Global alignment score: 2232

               10        20              30        40        50
416303 GGTGGCGGCCGCTCTAGAACTAGTG------GATCCATTTCGTGGACCCGGCAAGAATGT
       : :  :: ::: :: : :  : :::      ::::::::::::::::::::::::::::
_      GATATCG-CCGATCAATACATGGTGACCGAGGATCCATTTCGTGGACCCGGCAAGAATGT
                10        20        30        40        50

           60        70        80        90       100       110
416303 AAGGATCACCCTTTTCAAGGAAATCAGACGTGTCCACCCAGACACAATGAAGCTGGTATG
       :::::::::::::::::::::: :::::::::::::::::::::::::::::::::::
_      AAGGATCACCCTTTTCAAGGAAATTAGACGTGTCCACCCAGACACAATGAAGCTGGTATG
      60        70        80        90       100       110

          120       130       140       150       160       170
416303 CAACTGGAGCGGTAAAGAATTCCTTCGCGAGACTTGGACCCGTTTCATCTCTGAAGAATT
       :::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::
_      CAACTGGAGCGGCAAAGAATTCCTTCGCGAGACTTGGACCCGTTTCATCTCTGAAGAATT
     120       130       140       150       160       170

          180       190       200       210       220       230
416303 TCCCATCACCACAGACCAAGAAATTATAGATTTGTGGTTTGAGCTTCAGCTACGACCGAT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
_      TCCCATCACCACAGACCAAGAAATTATAGATTTGTGGTTTGAGCTTCAGCTACGACCAAT
     180       190       200       210       220       230

          240       250       260       270       280       290
416303 GCACCCTAACCGTTGTTACAAATTCACTATGCAGTATGCGCTCTGTGCCCATCCCGATTA
       :::::::::::::::::::::::::::::::::::: :::::::::::::::::::::
_      GCACCCTAACCGTTGTTACAAATTCACTATGCAGTATGCTCTCTGTGCCCATCCCGATTA
     240       250       260       270       280       290

          300       310       320       330       340       350
416303 TGTCGCTCACGATGTGATCCGCCAGCAGGATCCCTATTATGTAGGACCTAACAATATCGA
       :::::::::::::::::::::::::::::::::: :::::::::::::::::::::::
_      TGTCGCTCACGATGTGATCCGCCAGCAGGATCCCTACTATGTAGGACCTAACAATATCGA
     300       310       320       330       340       350

          360       370       380       390       400       410
416303 GCGTATCAATCTTTCCAAGAAGGGTTTCGCTTTCCCACTCACATGCCTACAGTCCGTCTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GCGTATCAATCTTTCCAAGAAGGGTTTCGCTTTCCCACTCACATGCCTACAGTCCGTCTA
     360       370       380       390       400       410
```

```
          420       430       440       450       460       470
416303 CAATGACAACTTTGAGAATTTCTTTGATGACGTTCTGTGGCCGTATTTCCACCGTCCCCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CAATGACAACTTTGAGAATTTCTTTGATGACGTTCTGTGGCCGTATTTCCACCGTCCCCT
     420       430       440       450       460       470

          480       490       500       510       520       530
416303 GGTGTATGTAGGTACTACGTCTGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGTGTATGTAGGTACTACGTCTGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTT
     480       490       500       510       520       530

          540       550       560       570       580       590
416303 GTTCAAGATCAAGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GTTCAAGATCAAGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAA
     540       550       560       570       580       590
```

First 150 nucleotides of CrleGV (Genebank; Jehle and Backhaus, 1994a) *granulin* removed

Appendix 6.6.2: Partial alignment of *XecnGV ORF 2* homologue in CrleGV-SA with the same region in CrleGV-CV3.

Jehle and Backhaus (1994a) sequence 1983-2225bp corresponds to 586-827bp on preliminary CrleGV sequence.

```
>_ CrleGV XecnGV2 sequence                                  241 nt vs.
>_ Match for XecnGV2 (Jehle and backhaus sequence)          243 nt
scoring matrix: , gap penalties: -12/-2
96.7% identity;        Global alignment score: 890

              10        20        30        40        50        60
969338 CTATCTTTTAAAACTAAAATTTTCTAAGTTGTCTAAATCATCATCTATCCACTCATGAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CTATCTTTTAAAACTAAAATTTTCTAAGTTGTCTAAATCATCATCTATCCACTCATGAAC
              10        20        30        40        50        60

              70        80        90       100       110       120
969338 AATATCATTGTCGATACTATTTTTATCAATATCAATTTTATCAATATCAGTTTTATCATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AATATCATTGTCGATACTATTTTTATCAATATCAATTTTATCAATATCAGTTTTATCATT
              70        80        90       100       110       120

             130       140       150       160       170       180
969338 GTCGATATCGTTTTTATCAACAACAATTTTTAACTTTTCCTCATCATCTATAGTATCCGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GTCGATATCGTTTTTATCAACAACAATTTTTAACTTTTCCTCATCATCTATAGTATCCGC
             130       140       150       160       170       180

               190       200        210       220       230
969338 TTAA--TTTATTCTCGTCACATACCACCACT-GATTGATATTGTTTTCTTGTAA-CGCAT
       :: :  ::::: :::::::::::::::::: :::::::::::::::::::: :::::
_      TTTAATTTTATCCTCGTCACATACCACCACTTGATTGATATTGTTTTCTTGTAAACGCAT
              190       200       210       220       230       240
```

```
        240
969338 TGTTT
       :::
_      TGT--
```

Appendix 6.6.3: Alignment of CpGV *XecnGV ORF 2* homologue complete coding sequence with the preliminary sequence of CrleGV-SA *XecnGV ORF 2* homologue

```
>_ CrleGV XecnGV2 sequence reverse orientation          473 nt vs.
>_ CpGV XecnGV2                                         525 nt
scoring matrix: , gap penalties: -12/-2
59.0% identity;        Global alignment score: 456

               10        20        30        40        50        60
137246 ATGGATTTATTGGTATTCATGAAGAGAGCTGGTTTTAAAGCAGATGTTCGCGATATTATA
       ::::::::: ::: :::::::: ::: ::::::::::::::::: ::::::::: : :::
_      ATGGATTTACTGGGATTCATGAGGAGGGCTGGTTTTAAAGCAGACGTTCGCGATCTAATA
               10        20        30        40        50        60

               70        80        90         100        110
137246 AAGCTGATGAAGTGGGACGTGAGCTTAAAGAA--AATTGGCATCAAAATT-GGCGACGAC
       :::   :::::: :: ::  ::: :::::: :  ::::::: :: ::::: ::::: :
_      AAGAGAATGAAGCGGAACACGAGTTTAAAGGAGAAATTGGCGTCGAAATTTGGCGATGGT
               70        80        90       100       110       120

        120            130       140        150        160
137246 -ATA-----GTCGTTATGACAATAGAAGAAGTGGTAGA-CTTTTG-AGAGTATATT---T
        :::     ::  : ::::: :: :: :: :::   :: :: ::: ::::: :  :   :
_      GATACTGCTGTGTTGATGACGATCGAGGAGGTGACTGAGCTGTTGGAGAGTGTGGTGGTT
              130       140       150       160       170       180

        170        180        190         200       210
137246 ATGATGCATA-AATTAGTGTAGG-AGTAAAAT--AACCCGATAGAATTGTA---GCGGAA
       :::::::: : ::::::::: :: :: :  ::  :   : :::   :: :    :::::
_      ATGATGCACAGAATTAGTGTGGGTAGAATTATTGATGTCAATACTGTTATTAGTGCGGAT
              190       200       210       220       230       240

     220                                               230       240
137246 TCG------------------CCGCAAC--------------------CTAAAACAATG
        :                       : :: ::                    :: : ::: ::
_      ACCAATACTGTTACCACTAATACTGCTACTAGTAATACCACTCCTGTCGCTGAGACATTG
              250       260       270       280       290       300

               250       260        270       280           290
137246 CGT-TACAAGAAAACAATATCAATCAGTGG-TGGTATGTGACGAGAAT----AAATTAAG
       ::  : :: :: ::::::::::::  :  : :::: ::::  :    :    : ::::
_      CGGGTGCACGATAACAATATCAATTTGACGATGGTGTGTGGTGGTGGTGGGGAGATTATC
              310       320       330       340       350       360

          300       310       320           330         340
137246 CGGATACTATAGATGATGAGGAAAAG----TTAAAAATTGTTGTT--GATAAAAACGATA
        :::  :    ::::: ::::: :::    :::::: : :: :::  :    : :::: :
_      AGGA--CGGGGGATGAGGAGGACAAGGATATTAAAAGTGGTGGTTATGGGGTAGACGACA
                370       380       390       400       410
```

```
      350       360       370       380       390       400
137246 TCGACAATGATAAAACTGATATTGATAAAATT-GATATTGATAAAAATAGTATCGACAAT
        :::  : :: :: : :  :: ::: :::  : ::   : ::   ::  : :: : : :
_      ACGATGAAGAAAACAATC-TAGTGACAAACGTCGACGGTAATTCTAAACGCATTGCCGAA
      420       430       440       450       460       470

       410       420       430       440       450       460
137246 GATATTGTTCATGAGTGGATAGATGATGATTTAGACAACTTAGA-AAATTTTAGTTTTAA
        :  ::::        :::  ::::::::::      :::: :: ::::: :   :: :
_      CAGTTTGT--------GGAC-GATGATGATT------ACTTTGACAAATTGTCAGTTCA-
       480               490             500       510       520

        470
137246 AAGATAG
           :::
_      ---CTAG
```

Appendix 6.6.4: Pairwise alignment of CpGV *pk-1* coding sequence with CrleGV-SA preliminary sequence of putative *pk-1* gene.

```
>_ Preliminary sequence of CrleGV pk-1 gene          852 nt vs.
>_ CpGV pk-1 gene                                    840 nt
scoring matrix: , gap penalties: -12/-2
70.1% identity;        Global alignment score: 1615

              10        20        30        40        50        60
294897 ATGAATACCAATAAATCCATATCGCGTTTTGCTAAAGAATTATCAAATTATGAAATTTTA
       :::::: ::: ::::::::: :::::: : ::  :::: :: :: :: :::::::: ::
_      ATGAATCCCAGTAAATCCATTTCGCGTGTGGCACAAGAGTTGTCCAAATATGAAATCTTG
              10        20        30        40        50        60

              70        80        90       100       110       120
294897 AAAAATTTAGACGAGAATGACTGTCAATCTTATAGTAACGTATTTTTATGTAAAAAAAAA
       :::::  : :: :: : ::::    : :::::::::  :: : :::::: :::::::::
_      AAAAAACTGGATGAAAGTGACACAGAGTCTTATAGTAGTGTGTATTTATGCAAAAAAAAA
              70        80        90       100       110       120

             130       140       150       160       170       180
294897 GGTGAACAAAAAATGTATGTTTGCAAAATAGTTAAATCATCAACCTTTAATTCTTTAGAG
       :: ::::: :::  :: ::: :: ::::: :::::: : :: ::::: :: ::  :::::
_      GGCGAACACAAACGGTTTGTGTGTAAAATCGTTAAACCGTCCACCTTCAACTCGCTAGAG
             130       140       150       160       170       180

             190       200       210       220       230
294897 TTTGATGTTCACATATTAATGAAACACAATATAAATTTTATTAAATTCTCC---TTCGTA
       ::::: :: :::::: : :::    ::::    :: ::::::::: :   :   :::::
_      TTTGACGTGCACATACTGATGCGCAACAACCCCAACTTTATTAAACTGCACAATTTCGTG
             190       200       210       220       230       240

       240       250       260       270       280       290
294897 TTCAACAATGAAGAAGATATTTTGTTGATAATGGATTACGTAAAAGATGGTGATTTGTTT
       :::::: :  : :  :: : : :  : :: ::::: ::::: :  ::::::::: : :::
_      TTCAACGACAATGGCGAGAGTCTACTCATCATGGACTACGTGAGCGATGGTGATCTCTTT
             250       260       270       280       290       300
```

```
       300       310       320                330       340
294897 GAGTTGGTAAAAAAGAATGACAT---CAAATTGG------ATGAAGCTACGTGTAAAAAA
       :: :: :: :: : ::: ::::    : : :::       : :::::  : :: ::::::
_      GATTTTGTGAAGATGAACGACACGCGCGAGTTGCGCCTCAACGAAGCCGCATGCAAAAAA
              310       320       330       340       350       360

      350       360       370       380       390       400
294897 CTTATTCTAACACTAGTAACAGCTCTTAACGATCTGCATAGAAAAAATATTATACACAAT
        : ::  : ::: : :: :: :: :: :::::::: :: : ::: :::::: : :::::
_      ATAATCATCACATTGGTGACGGCGCTCAACGATCTACACAAAAACAATATTGTGCACAAC
              370       380       390       400       410       420

      410       420       430       440       450       460
294897 GATGTTAAATTAGAAAATTTACTGTATGATCGTAAAAAAAAACGCCTGTACGTGTGCGAG
       :: :: ::: : ::::: ::  :::: :: :: ::::: ::::: ::::  :: :::::
_      GACGTCAAACTGGAAAACTTGTTGTACGACCGCAAAAAGAAACGACTGTTTGTATGCGAC
              430       440       450       460       470       480

      470       480       490       500       510       520
294897 GAC-GGATTATCAAGTTTTTCCGCACACCCTCCTTCTACGACGGCACCACACCCTCCTTC
        :: ::: : :: :   :                    ::  :::::   ::::::::::
_      TACCGGACTGTCGAAAAT-------------------CGTGGGCAC---ACCCTCCTTC
              490                           500          510

       530       540       550       560       570       580
294897 TACGACGGCACCACAGTATACTTTCCCACCTGAAAAGATCCGCCATGAACCATATCAGGT
       ::::::::::::::::: :::::::  ::: :: :::::::: :::::: : :: :::
_      TACGACGGCACCACAGTCTACTTTC--ACCGGAGAAGATCCGTCATGAAGCGTACCAGAC
       520       530       540         550       560       570

       590       600       610       620       630       640
294897 CTCTTTCGATTGGTGGGCTGTTGGAGTTGTCTCATACGAAATTTTATCGTGCGAATATCC
        :: ::::: :::::::: :: ::::: ::  : :: ::::: ::::::  ::: :: ::
_      ATCGTTCGACTGGTGGGCGGTGGGAGTGGTGGCGTATGAAATCTTATCGACCGAGTACCC
         580       590       600       610       620       630

       650       660       670       680       690       700
294897 ATTTGATATAGACGAAGAAAACGAAGAGGAAATGGACAATATGGAACCAGAAGATATGCT
        ::::: ::  :::: :: ::::: ::::: ::::::   :: :::::  :::: ::: :
_      GTTTGACATCAACGAGGACAACGAGGAGGAGATGGACGCCATTGAACCCAAAGACATGTT
         640       650       660       670       680       690

       710       720       730       740       750       760
294897 TCCTCTATATTCCAAACCGTTGCCTCCCATCAAAAATATTTCAAAAAAAGCCATGAATTT
        :: :: :: :: :::::: : ::  :::: :: :  : :: ::::: ::::   ::::
_      ACCCCTCTACTCTAAACCGCTACCCACCATTGAACACGTGTCCAAAAAGGCCAACGATTT
         700       710       720       730       740       750

       770       780       790       800       810       820
294897 TGTGAAACAAATGTTGGAGTTAGACATCAACAACAGACTGAGTTCTTACGATGAAATAAT
       ::: :  :  ::: : :   :::::::::: :  :::::::  : ::::::::::::::
_      TGTTAGGCGCATGCTAGCTCTAGACATCAATAGTAGACTGAGCACCTACGATGAAATAAT
         760       770       780       790       800       810
```

```
       830       840       850
294897 AAAACATCCATTTTTAAAATTCTAA
        ::::: :::::::::   :: :::
_      TAAACACCCATTTTTATGTTTTTAA
         820       830       840
```

Appendix 6.6.5: Pairwise alignment of partial sequence of CpGV *ORF 4* with partial sequence of *CpGV ORF 4* homologue in CrleGV-SA preliminary sequence.

```
>_ CrleGV ORF 4 Homologue partial CDS              429 nt vs.
>_ CpGV Orf 4 homologue                            432 nt
scoring matrix: , gap penalties: -12/-2
70.0% identity;        Global alignment score: 797

               10        20        30        40        50        60
744525 TATGATCAGGTGTTTTATACAAACGGTGGTGATATCGATTTGAGGCTGTTGAAATGTGAC
        :::: ::::::: :: :::::::::: :::::: ::::::::: :::::::: : :::
_      AATGAGCAGGTGTATTGTACAAACGGTGTTGATATGGATTTGAGGTTGTTGAAAGGCGAC
               10        20        30        40        50        60

               70        80           90       100       110
744525 GAATGTTATGTGTCGTTGAAGA---TACTGGATTTGATAGATAGCAATAGTTTTGGGGAA
       ::::::::::::::::  ::    :  :::: ::::: ::::::::::::::::: ::
_      GAATGTTATGTGTCGTTTGAGGGGGTGATGGAGTTGATGGATAGCAATAGTTTTGGAGAT
               70        80        90       100       110       120

       120       130       140       150       160        170
744525 AAAGATAAATTTGAAATGATGTTAGTAAATTGTACCTTACGAGTAGTT-TTAAACCATAA
       ::: :   : : :: :: ::: : :: :::::::: :: :: :: ::  :: ::::  ::
_      AAAAACTCACTGGAGATAATGATCGTGAATTGTACGTTGCGTGTGGTGCTTCAACCC-AA
              130       140       150       160       170

        180       190       200       210       220       230
744525 TCATAACTGGTTGGAATTGTATAAAAGTAAATTAAGGACGCGCATTAGCGCCTCATTTAA
       ::::   ::: :    :  :: ::    :  ::  : : :::::: : :::::: ::: :
_      TCATGCTTGGCTCACTTACTACAAGGAGAGGTTGCGAATGCGCATAAACGCCTCTTTTGA
     180       190       200       210       220       230

        240       250       260       270       280       290
744525 TTTTTATTTTAAAGTTTTAGAACAATATATGCTAGCAAATCAACCTAACGTCGAAGTAAT
       ::: :::::::::::: :: :: :: :: ::: : :: ::::: :: :: :: ::    ::
_      TTTCTATTTTAAAGTGTTGGAGCAGTACATGTTGGCCAATCAGCCCAATGTGGAGCGCAT
     240       250       260       270       280       290

        300       310       320       330       340       350
744525 CGGGGACGTTGTGAAAAAATTTGTGTGTTTGGCTGAACACCATAAACACTTTGATGATAT
       :::   :    : :  ::: :  :  : ::::: :: ::: : :: ::  :::: :::
_      CGGTCCCACGATTAGCAAACTCATTAGGTTGGCGGAGCACTACAAGCATCTTGACGATCA
     300       310       320       330       340       350

        360       370       380       390       400       410
744525 TGTGTCATTGTCTGAGGCTTACCATGCGTACGATAAAGCTAGTGCTATAATATTACAAAA
         : :: :::   :::::::  :: :: : :::::  :::::::::::  :: :::: ::
_      CATTTCGTTGATAGAGGCTTGTCACGCATTCGATAGGGCTAGTGCTATTTTAATACAGAA
     360       370       380       390       400       410
```

```
        420
744525 TATGAAGATGTAA
       ::: ::  :::::
_      TATTAAATTGTAA
     420       430
```

Appendix 6.6.6: Pair-wise alignment of Partial CpGV *granulin* sequence with partial *granulin* sequence of CrleGV preliminary sequence.

```
>_ CrleGV partial granulin sequence                592 nt vs.
>_ CpGV granulin gene                              587 nt
scoring matrix: , gap penalties: -12/-2
82.3% identity;        Global alignment score: 1628

               10        20        30        40        50        60
548164 GGTGGCGGCCGCTCTAGAACTAGTGGATCCATTTCGTGGACCCGGCAAGAATGTAAGGAT
          :     : :   : :     :::::: :::::::: ::::::::::::::::: ::
_      ACCAGTACATGGTGACGGA-----GGATCCCTTTCGTGGGCCCGGCAAGAATGTAAGAAT
               10             20        30        40        50

               70        80        90       100       110       120
548164 CACCCTTTTCAAGGAAATCAGACGTGTCCACCCAGACACAATGAAGCTGGTATGCAACTG
       :::::: :::::::: :: ::::: :::::::::::::::::::::::::::: ::::::::
_      CACCCTGTTCAAGGAGATTAGACGCGTCCACCCAGACACAATGAAGCTGGTGTGCAACTG
          60        70        80        90       100       110

              130       140       150       160       170       180
548164 GAGCGGTAAAGAATTCCTTCGCGAGACTTGGACCCGTTTCATCTCTGAAGAATTTCCCAT
       :::::: :::::::::::::: :: :: :::::::: ::::: :: ::::: :: :::::
_      GAGCGGCAAAGAATTCCTTCGTGAAACCTGGACCCGCTTCATTTCCGAAGAGTTCCCCAT
         120       130       140       150       160       170

              190       200       210       220       230       240
548164 CACCACAGACCAAGAAATTATAGATTTGTGGTTTGAGCTTCAGCTACGACCGATGCACCC
       :::::: :::::::: :: :: ::  ::::::: :::::::: :: ::::::::::::::
_      CACCACCGACCAAGAGATCATGGACCTGTGGTTCGAGCTTCAACTGCGACCGATGCACCC
         180       190       200       210       220       230

              250       260       270       280       290       300
548164 TAACCGTTGTTACAAATTCACTATGCAGTATGCGCTCTGTGCCCATCCCGATTATGTCGC
        :::::::: ::::: ::::: :::::::: :: ::: : ::::: :::::::::::: ::
_      CAACCGTTGCTACAAGTTCACCATGCAGTACGCTCTCGGCGCCCACCCCGATTATGTTGC
         240       250       260       270       280       290

              310       320       330       340       350       360
548164 TCACGATGTGATCCGCCAGCAGGATCCCTATTATGTAGGACCTAACAATATCGAGCGTAT
       :::::: :::::::::::: :: :: ::::: :: :: :: :: ::::: :: ::::: ::
_      TCACGACGTGATCCGCCAACAAGACCCCTACTACGTGGGCCCAAACAACATTGAGCGCAT
         300       310       320       330       340       350

              370       380       390       400       410       420
548164 CAATCTTTCCAAGAAGGGTTTCGCTTTCCCACTCACATGCCTACAGTCCGTCTACAATGA
       ::: :: ::::: ::::: :: :: ::::: ::::: :: :: ::::: :::::::: ::
_      CAACCTCTCCAAAAAGGGCTTTGCGTTCCCGCTCACTTGTCTTCAGTCTGTCTACAACGA
         360       370       380       390       400       410
```

```
              430       440       450       460       470       480
548164 CAACTTTGAGAATTTCTTTGATGACGTTCTGTGGCCGTATTTCCACCGTCCCCTGGTGTA
       :::::::::    ::::::::: :: :: :::::::: :: ::: :::: :: :::::::::
_      CAACTTTGAACGCTTCTTTGACGATGTGCTGTGGCCCTACTTCTACCGACCACTGGTGTA
         420       430       440       450       460       470

              490       500       510       520       530       540
548164 TGTAGGTACTACGTCTGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTTGTTCAA
        :: :: :: :: :: :::::::::: :::::::::::::: :: :: :: :::::::::
_      CGTTGGCACCACCTCCGCCGAAATTGAAGAGATCATGATTGAGGTGTCGCTCTTGTTCAA
         480       490       500       510       520       530

              550       560       570       580       590
548164 GATCAAGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAA
       ::::::::::::::::::::::: :::::::::::::: ::::: ::::::
_      GATCAAGGAGTTTGCACCCGACGTACCCCTATTCACCGGCCCAGCTTATTAA
         540       550       560       570       580
```

First 160 nucleotides of CpGV granulin were excluded for this alignment.

Appendix 7: Assembly of sequences of the CrleGV-SA granulin region of CrleGV-SA and comparison to *Cydia pomonella* GV granulin region.

(Rows labelled 4, 5, 6 and 7 are sequences from CrleGV)

```
Consensus CCTGCGCATGGAGTATGGACGCCAACACAATTTCGACAGGCCGCTACAGCTCAACGC 57
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom CCTGCGCATGGAGTATGGACGCCAACACAATTTCGACAGGCCGCTACAGCTCAACGC 57
        4 ------------------------------------------------------- 0

Consensus TCAACTCATCGCCGCCGTCAACAAACACTGCCAACTGGCCAAATATCATTTAGAGGT 114
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom TCAACTCATCGCCGCCGTCAACAAACACTGCCAACTGGCCAAATATCATTTAGAGGT 114
        4 ------------------------------------------------------- 0

Consensus GTACTACAAAGAATACGAAAAGTATTCACCGTTTGTGGTGTGTTACAATGTGAAGGA 171
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom GTACTACAAAGAATACGAAAAGTATTCACCGTTTGTGGTGTGTTACAATGTGAAGGA 171
        4 ------------------------------------------------------- 0

Consensus GGACAAGGAGTGTGTTTATTGCGAGGGAAAAATCATGCCGGACACCGGTCACCCTGT 228
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom GGACAAGGAGTGTGTTTATTGCGAGGGAAAAATCATGCCGGACACCGGTCACCCTGT 228
        4 ------------------------------------------------------- 0

Consensus GTTCAGTTGCAGTGTGTGCGGACCCACCAACCCCAACTACTTCACCAAAAGACACAC 285
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom GTTCAGTTGCAGTGTGTGCGGACCCACCAACCCCAACTACTTCACCAAAAGACACAC 285
        4 ------------------------------------------------------- 0

Consensus AATGATGTTTCCCTTCTGGACCTACTCTTACGATTACAACAAAGTTTACTGGAAAAC 342
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom AATGATGTTTCCCTTCTGGACCTACTCTTACGATTACAACAAAGTTTACTGGAAAAC 342
        4 ------------------------------------------------------- 0

Consensus GCTCAAGCGCAAGGGGTTGCTGAGGTGCGACGTGATGCTGTACGGAGTGGATGCTCG 399
        7 ------------------------------------------------------- 0
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom GCTCAAGCGCAAGGGGTTGCTGAGGTGCGACGTGATGCTGTACGGAGTGGATGCTCG 399
        4 ------------------------------------------------------- 0
```

```
Consensus AAGAAGTGTTTAGCCATATGACAATACTATATTGTATATTTTAGGTAATAAATGCCA 456
        7 ------------------------------------------------------------ 0
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom AAGAAGTGTTTAGCCATATGACAATACTATATTGTATATTTTAGGTAATAAATGCCA 456
        4 ------------------------------------------------------------ 0

Consensus TACTGTCAGATTTTATAAGGAATTTTAATTTTAATAACAACATCATGGGATATAACA 513
        7 ------------------------------------------------------------ 0
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom TACTGTCAGATTTTATAAGGAATTTTAATTTTAATAACAACATCATGGGATATAACA 513
        4 ------------------------------------------------------------ 0

Consensus AATCTTTGAGGTATAGCCGTCACGACGGCACCAGCTGTGTCATCGACAACCACCATC 570
        7 ------------------------------------------------------------ 0
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom AATCTTTGAGGTATAGCCGTCACGACGGCACCAGCTGTGTCATCGACAACCACCATC 570
        4 ------------------------------------------------------------ 0

Consensus TAAAGAGTTTGGGTGCTGTGTTAAACGATGTTAGGCGTAAAAAGGACCGCATCCGCG 627
        7 ------------------------------------------------------------ 0
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom TAAAGAGTTTGGGTGCTGTGTTAAACGATGTTAGGCGTAAAAAGGACCGCATCCGCG 627
        4 ------------------------------------------------------------ 0

Consensus AAGCCGAGTACGAGCCCATCATCGryrkCG-CCGmyC-AGwAC-TrGTG------GA 684
        7 ----------------------GGTGGCGGCCGCTCTAGAAC-TAGTG------GA 27
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom AAGCCGAGTACGAGCCCATCATCGACATCG-CCGACC-AGTACATGGTGACGGAGGA 682
        4 ------------------------------------------------------------ 0

Consensus TCCmTTTCGTGGrCCCGGCAAGAATGTAAGrATCACCCTkTTCAAGGArATyAGACG 741
        7 TCCATTTCGTGGACCCGGCAAGAATGTAAGGATCACCCTTTTCAAGGAAATCAGACG 84
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom TCCCTTTCGTGGGCCCGGCAAGAATGTAAGAATCACCCTGTTCAAGGAGATTAGACG 739
        4 ------------------------------------------------------------ 0

Consensus yGTCCACCCAGACACAATGAAGCTGGTrTGCAACTGGAGCGGyAAAGAATTCCTTCG 798
        7 TGTCCACCCAGACACAATGAAGCTGGTATGCAACTGGAGCGGTAAAGAATTCCTTCG 141
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom CGTCCACCCAGACACAATGAAGCTGGTGTGCAACTGGAGCGGCAAAGAATTCCTTCG 796
        4 ------------------------------------------------------------ 0

Consensus yGArACyTGGACCCGyTTCATyTCyGAAGArTTyCCCATCACCACmGACCAAGArAT 855
        7 CGAGACTTGGACCCGTTTCATCTCTGAAGAATTTCCCATCACCACAGACCAAGAAAT 198
        5 ------------------------------------------------------------ 0
        6 ------------------------------------------------------------ 0
Cydia pom TGAAACCTGGACCCGCTTCATTTCCGAAGAGTTCCCCATCACCACCGACCAAGAGAT 853
        4 ------------------------------------------------------------ 0
```

```
Consensus yATrGAyyTGTGGTTyGAGCTTCArCTrCGACCGATGCACCCyAACCGTTGyTACAA 912
        7 TATAGATTTGTGGTTTGAGCTTCAGCTACGACCGATGCACCCTAACCGTTGTTACAA 255
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom CATGGACCTGTGGTTCGAGCTTCAACTGCGACCGATGCACCCCAACCGTTGCTACAA 910
        4 ------------------------------------------------------- 0

Consensus rTTCACyATGCAGTAyGCkCTCkGyGCCCAyCCCGATTATGTyGCTCACGAyGTGAT 969
        7 ATTCACTATGCAGTATGCGCTCTGTGCCCATCCCGATTATGTCGCTCACGATGTGAT 312
        5 ------------------------------------------------------- 0
        6 ------------------------------------------------------- 0
Cydia pom GTTCACCATGCAGTACGCTCTCGGCGCCCACCCCGATTATGTTGCTCACGACGTGAT 967
        4 ------------------------------------------------------- 0

Consensus CCGCCArCArGAyCCCTAyTAyGTrGGmCCwAACAAyATyGAGCGyATCAAyCTyTC 1026
        7 CCGCCAGCAGGATCCCTATTATGTAGGACCTAACAATATCGAGCGTATCAATCTTTC 369
        5 ----------GATCCCTATTATGTAGGACCTAACAATATCGAGCGTATCAATCTTTC 47
        6 ------------------------------------------------------- 0
Cydia pom CCGCCAACAAGACCCCTACTACGTGGGCCCAAACAACATTGAGCGCATCAACCTCTC 1024
        4 ------------------------------------------------------- 0

Consensus CAArAAGGGyTTyGCkTTCCCrCTCACwTGyCTwCAGTCyGTCTACAAyGACAACTT 1083
        7 CAAGAAGGGTTTCGCTTTCCCACTCACATGCCTACAGTCCGTCTACAATGACAACTT 426
        5 CAAGAAGGGTTTCGCTTTCCCACTCACATGCCTACAGTCCGTCTACAATGACAACTT 104
        6 ------------------------------------------------------- 0
Cydia pom CAAAAAGGGCTTTGCGTTCCCGCTCACTTGTCTTCAGTCTGTCTACAACGACAACTT 1081
        4 ------------------------------------------------------- 0

Consensus TGArmryTTCTTTGAyGAyGTkCTGTGGCCsTAyTTCyACCGwCCmCTGGTGTAyGT 1140
        7 TGAGAATTTCTTTGATGACGTTCTGTGGCCGTATTTCCACCGTCCCCTGGTGTATGT 483
        5 TGAGAATTTCTTTGATGACGTTCTGTGGCCGTATTTCCACCGTCCCCTGGTGTATGT 161
        6 ------------------------------------------------------- 0
Cydia pom TGAACGCTTCTTTGACGATGTGCTGTGGCCCTACTTCTACCGACCACTGGTGTACGT 1138
        4 ------------------------------------------------------- 0

Consensus wGGyACyACsTCyGCCGAAATTGArGAGATCATGATTGArGTkTCkCTsTTGTTCAA 1197
        7 AGGTACTACGTCTGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTTGTTCAA 540
        5 AGGTACTACGTCTGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTTGTTCAA 218
        6 ------------------------------------------------------- 0
Cydia pom TGGCACCACCTCCGCCGAAATTGAAGAGATCATGATTGAGGTGTCGCTCTTGTTCAA 1195
        4 ------------------------------------------------------- 0

Consensus GATCAAGGAGTTTGCACCCGACGTrCCCCTATTCACCGGyCCAGCyTATTAAAnnnC 1254
        7 GATCAAGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAAATAAC 597
        5 GATCAAGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAAATAAC 275
        6 ------------------------------------------------------- 0
Cydia pom GATCAAGGAGTTTGCACCCGACGTACCCCTATTCACCGGCCCAGCTTATTAAA---C 1249
        4 ------------------------------------------------------- 0

Consensus TAkyknnnnAAmykAmAATTT-TCwAAGTnnnnnnAATCATCATCknTCCmCwnnnn 1311
        7 TATCTTTTAAAACTAAAATTT-TCTAAGTTGTCTAAATCATCATCTATCCCCTCATG 653
        5 TATCTTTTAAAACTAAAATTT-TCTAAGTTGTCTAAATCATCATCTATCCACTCATG 331
        6 ------------------------------------------------------- 0
Cydia pom TAGTG----AACTGACAATTTGTCAAAGT------AATCATCATCG-TCCACA---- 1291
        4 ------------------------------------------------------- 0
```

```
Consensus nnnnAwmTswTyGkCrATrCkwTTwkwATyAmyrTCrAy-TTTrTCAmTAknAkTkT 1368
        7 AACAATATCATTGTCGATACTATTTTTATCAATATCAAT-TTTATCAATATCAGTTT 709
        5 AACAATATCATTGTCGATACTATTTTTATCAATATCAAT-TTTATCAATATCAGTTT 387
        6 ----------------------------------------TTTATCAATATCAGTTT 17
Cydia pom ----AACTGTTCGGCAATGCGTTTAGAATTACCGTCGACGTTTGTCACTAG-ATTGT 1343
        4 ------------------------------------------------------- 0


Consensus TwTCwTyrTCGwTrTCGkyTwymyCA--ACmACmAyTTTTAAnnTwTCCTyrTCmTC 1425
        7 TATCATTGTCGATATCGTTTTTATCA--ACAACAATTTTTAACTTTTCCTCATCATC 764
        5 TATCATTGTCGATATCGTTTTTATCA--ACAACAATTTTTAACTTTTCCTCATCATC 442
        6 TATCATTGTCGATATCGGTTTTCCCA--ACAACAATTTTTAACTTTTCCTCATCATC 72
Cydia pom TTTCTTCATCGTTGTCGTCTACCCCATAACCACCACTTTTAA--TATCCTTGTCCTC 1398
        4 ------------------------------------------------------- 0

Consensus hwynrTmyCCssTymwkwTAwTCTCsyCACmnACCACCACws----------ATTG 1482
        7 ------------------------------------------------------- 764
        5 TATAGTATCCGCTTAATTTATTCTCGTCACATACCACCACTG----------ATTG 488
        6 AATAGTATCCGCTTAATTTATTCTCGTCACATACCACCACTG----------ATTG 118
Cydia pom CTC-ATCCCCCGTCCTGATAATCTCCCCACC-ACCACCACACACCATCGTCAAATTG 1453
        4 ------------------------------------------------------- 0

Consensus ATATTGTTwTCkTGyAmCssmwdkkky----------------wTTAs--GkwGCr 1539
        7 ------------------------------------------------------- 764
        5 ATATTGTTTTCTTGTAACGCATTGTGT----------------TTTAG--GTTGCG 526
        6 ATATTGTTTTCTTGTAACGCATGGT------------------TTTAG--GGTGCG 154
Cydia pom ATATTGTTATCGTGCACCCGCAATGTCTCAGCGACAGGAGTGGTATTACTAGTAGCA 1510
        4 ------------------------------------------------------- 0

Consensus GynATTms-GsTA-CA--ATT-sTATCynsrsTwATwwy----------------- 1596
        7 ------------------------------------------------------- 764
        5 GCGATTCC-GCTA-CA--ATT-CTATCTGGGGTTATTTT----------------- 560
        6 GCGATTCC-GCTA-CA--ATT-CTATC--GGGTTATTTT----------------- 186
Cydia pom GT-ATTAGTGGTAACAGTATTGGTATCCGCACTAATAACAGTATTGACATCAATAAT 1566
        4 ------------------------------------------------------- 0

Consensus wCT-CCyACmCTAATTy-rTGCATCAT-Amm--yAyACTCTCmAA-AGy-CwryCAC 1653
        7 ------------------------------------------------------- 764
        5 ACT-CCTACACTAATTT-ATGCATCAT-AAA--TATACTCTCAAA-AGT-CTACCAC 610
        6 ACT-CCTACCCTAATTT-ATGCATCAT-AAA--TATACTCTCAAA-AGT-CTACCAC 236
Cydia pom TCTACCCACACTAATTCTGTGCATCATAACCACCACACTCTCCAACAGCTCAGTCAC 1623
        4 ------------------------------------------------------- 0

Consensus yTCyTCkATyGTCAT-AAC----sAsTATswCs-TCGCCAAwTTT-GAyGCCAATTT 1710
        7 ------------------------------------------------------- 764
        5 TTCTTCTATTGTCAT-AAC----GACTATGTCG-TCGCCAATTTT-GATGCCAATTT 660
        6 TTCTTCTATTGTCAT-AAC----GACTATGTCG-TCGCCAATTTT-GATGCCAATTT 286
Cydia pom CTCCTCGATCGTCATCAACACAGCAGTATCACCATCGCCAAATTTCGACGCCAATTT 1680
        4 ------------------------------------------------------- 0

Consensus -TC-TTTAArCTCryGTyCCrCTTCATymkCTTTATwAkATCGCGAACrTCTGCTTT 1767
        7 ------------------------------------------------------- 764
        5 -TC-TTTAAGCTCACGTCCCACTTCATCAGCTTTATAATATCGCGAACATCTGCTTT 715
        6 -TC-TTTAAGCTCACGTTCCACTTCATCAGCTTTATTATATCGCGAACATCTGCTTT 341
Cydia pom CTCCTTTAAACTCGTGTTCCGCTTCATTCTCTTTATTAGATCGCGAACGTCTGCTTT 1737
        4 ------------------------------------------------------- 0
```

```
Consensus AAAACCAGCyCTCyTCATGAATmCCArTAAATCCATwTCGCGTkTkGCwmAAGArTT 1824
        7 ------------------------------------------------------- 764
        5 AAAACCAGCTC-----------------------------------------------  726
        6 AAAACCAGCTCTCTTCATGAATACCAATAAATCCATATCGCGTTTTGCTAAAGAATT 398
Cydia pom AAAACCAGCCCTCCTCATGAATCCCAGTAAATCCATTTCGCGTGTGGCACAAGAGTT 1794
        4 ------------------------------------------------------- 0


Consensus rTCmAAwTATGAAATyTTrAAAAAwyTrGAyGArArTGACwswsArTCTTATAGTAr 1881
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ATCAAATTATGAAATTTTAAAAAATTTAGACGAGAATGACTGTCAATCTTATAGTAA 455
Cydia pom GTCCAAATATGAAATCTTGAAAAAACTGGATGAAAGTGACACAGAGTCTTATAGTAG 1851
        4 ------------------------------------------------------- 0

Consensus yGTrTwTTTATGyAAAAAAAAAGGyGAACAmAAAmkGTwTGTkTGyAAAATmGTTAA 1938
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 CGTATTTTTATGTAAAAAAAAAGGTGAACAAAAAATGTATGTTTGCAAAATAGTTAA 512
Cydia pom TGTGTATTTATGCAAAAAAAAAGGCGAACACAAACGGTTTGTGTGTAAAATCGTTAA 1908
        4 ------------------------------------------------------- 0

Consensus AyCrTCmACCTTyAAyTCkyTAGAGTTTGAyGTkCACATAyTrATGmrmmACAAymy 1995
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ATCATCAACCTTTAATTCTTTAGAGTTTGATGTTCACATATTAATGAAACACAATAT 569
Cydia pom ACCGTCCACCTTCAACTCGCTAGAGTTTGACGTGCACATACTGATGCGCAACAACCC 1965
        4 ------------------------------------------------------- 0

Consensus mAAyTTTATTAAAyT-CwCm--TTCGTrTTCAACrAyrAwGrmGAkAkTyTryTsAT 2052
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 AAATTTTATTAAATT-CTCC--TTCGTATTCAACAATGAAGAAGATATTTTGTTGAT 623
Cydia pom CAACTTTATTAAACTGCACAATTTCGTGTTCAACGACAATGGCGAGAGTCTACTCAT 2022
        4 ------------------------------------------------------- 0

Consensus mATGGAyTACGTrArmGATGGTGATyTsTTTGAkTTkGTrAArAwGAAyGACAy--- 2109
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 AATGGATTACGTAAAAGATGGTGATTTGTTTGAGTTGGTAAAAAAGAATGACAT--- 677
Cydia pom CATGGACTACGTGAGCGATGGTGATCTCTTTGATTTTGTGAAGATGAACGACACGCG 2079
        4 ------------------------------------------------------- 0

Consensus CrArTTG-G-----AyGAAGCyrCrTGyAAAAAAmTwATymTmACAyTrGTrACrGC 2166
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 CAAATTG-G-----ATGAAGCTACGTGTAAAAAACTTATTCTAACACTAGTAACAGC 728
Cydia pom CGAGTTGCGCCTCAACGAAGCCGCATGCAAAAAAATAATCATCACATTGGTGACGGC 2136
        4 ------------------------------------------------------- 0

Consensus kCTyAACGATCTrCAyArAAAmAATATTrTrCACAAyGAyGTyAAAyTrGAAAAyTT 2223
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 TCTTAACGATCTGCATAGAAAAAATATTATACACAATGATGTTAAATTAGAAAATTT 785
Cydia pom GCTCAACGATCTACACAAAAACAATATTGTGCACAACGACGTCAAACTGGAAAACTT 2193
        4 ------------------------------------------------------- 0
```

```
Consensus ryTGTAyGAyCGyAAAAArAAACGmCTGTwyGTrTGCGAskAC-GGAyTrTC-AArw 2280
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ACTGTATGATCGTAAAAAAAAACGCCTGTACGTGTGCGAGGAC-GGATTATC-AAGT 840
Cydia pom GTTGTACGACCGCAAAAAGAAACGACTGTTTGTATGCGACTACCGGACTGTCGAAAA 2250
        4 ------------------------------------------------------- 0


Consensus TykTssGCACACCCTCCTTCTACGACGGCACCACAGTmTACTTTC--ACCkGArAAG 2337
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 TTTTCC---------------------------------------------------- 846
Cydia pom TCGTGGGCACACCCTCCTTCTACGACGGCACCACAGTCTACTTTC--ACCGGAGAAG 2305
        4 ------------------------------ACAGTATACTTTCCCACCTGAAAAG 25

Consensus ATCCGyCATGAAsCrTAyCAGrymTCkTTCGAyTGGTGGGCkGTkGGAGTkGTskCr 2394
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom ATCCGTCATGAAGCGTACCAGACATCGTTCGACTGGTGGGCGGTGGGAGTGGTGGCG 2362
        4 ATCCGCCATGAACCATATCAGGTCTCTTTCGATTGGTGGGCTGTTGGAGTTGTCTCA 82

Consensus TAyGAAATyTTATCGwsCGArTAyCCrTTTGAyATmrACGArGAmAACGArGAGGAr 2451
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom TATGAAATCTTATCGACCGAGTACCCGTTTGACATCAACGAGGACAACGAGGAGGAG 2419
        4 TACGAAATTTTATCGTGCGAATATCCATTTGATATAGACGAAGAAAACGAAGAGGAA 139

Consensus ATGGACrmyATkGAACCmrAAGAyATGyTwCCyCTmTAyTCyAAACCGyTrCCymCC 2508
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom ATGGACGCCATTGAACCCAAAGACATGTTACCCCTCTACTCTAAACCGCTACCCACC 2476
        4 ATGGACAATATGGAACCAGAAGATATGCTTCCTCTATATTCCAAACCGTTGCCTCCC 196

Consensus ATyrAAmAyrTkTCmAAAAArGCCAwsrATTTTGTkArrCrmATGyTrGmkyTAGAC 2565
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom ATTGAACACGTGTCCAAAAAGGCCAACGATTTTGTTAGGCGCATGCTAGCTCTAGAC 2533
        4 ATCAAAAATATTTCAAAAAAAGCCATGAATTTTGTGAAACAAATGTTGGAGTTAGAC 253

Consensus ATCAAyAryAGACTGAGywCyTACGATGAAATAATwAAACAyCCATTTTTAwrwTTy 2622
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom ATCAATAGTAGACTGAGCACCTACGATGAAATAATTAAACACCCATTTTTATGTTTT 2590
        4 ATCAACAACAGACTGAGTTCTTACGATGAAATAATAAAACATCCATTTTTAAAATTC 310

Consensus TAAkwk----------------------------------------ATTTTTT---- 2679
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom TAAGTGTATATATTGAATAGGTAACAATTAAACACAACAGCAACAAATTTTTTTTAC 2647
        4 TAATAT----------------------------------------ATTTTTT---- 323
```

```
Consensus ATTTyTACATCT--------------------TmATATTyTGTAwTAwwATAGCAC 2736
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom ATTTCTACATCTAAGTACATAAAACTTACAATTTAATATTCTGTATTAAAATAGCAC 2704
        4 ATTTTTACATCT--------------------TCATATTTTGTAATATTATAGCAC 359


Consensus TAGCyyTATCGwAyGCrTGryAAGCCTCwrwCAAyGAmAyrwkATCrTCAArrTGyT 2793
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom TAGCCCTATCGAATGCGTGACAAGCCTCTATCAACGAAATGTGATCGTCAAGATGCT 2761
        4 TAGCTTTATCGTACGCATGGTAAGCCTCAGACAATGACACAATATCATCAAAGTGTT 416

Consensus TrTrGTGyTCmGCCAAmCwmAyrArTTTkyTmAymryGksmCCGATkmsyTCsACrT 2850
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom TGTAGTGCTCCGCCAACCTAATGAGTTTGCTAATCGTGGGACCGATGCGCTCCACAT 2818
        4 TATGGTGTTCAGCCAAACACACAAATTTTTTCACAACGTCCCCGATTACTTCGACGT 473

Consensus TrGGyTGATTkGCyArCATrTAyTGyTCyAAmACTTTAAAATArAAATyAAAwGAGG 2907
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom TGGGCTGATTGGCCAACATGTACTGCTCCAACACTTTAAAATAGAAATCAAAAGAGG 2875
        4 TAGGTTGATTTGCTAGCATATATTGTTCTAAAACTTTAAAATAAAAATTAAATGAGG 530

Consensus CGyTwATGCGCrTyCkyAA--TyTmCTTkTAkw-AAkTs-ArCCArkyATGATTr-G 2964
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CGTTTATGCGCATTCGCAACCTCTCCTTGTAGT-AAGTG-AGCCAAGCATGATTG-G 2929
        4 CGCTAATGCGCGTCCTTAA--TTTACTTTTATACAATTCCAACCAGTTATGATTATG 585

Consensus GTTkAAr-ACyACwCGyAAsGTACAATTyACkAwCATyATyTCmArTkwrTyTTTwT 3021
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom GTTGAAGCACCACACGCAACGTACAATTCACGATCATTATCTCCAGTGAGTTTTTAT 2986
        4 GTTTAAA-ACTACTCGTAAGGTACAATTTACTAACATCATTTCAAATTTATCTTTTT 641

Consensus CyCCAAAACTATTGCTATCyATCAAmTCCAkyAyCy--TCAAACGACACATAACATT 3078
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CTCCAAAACTATTGCTATCCATCAACTCCATCACCCCCTCAAACGACACATAACATT 3043
        4 CCCCAAAACTATTGCTATCTATCAAATCCAGTATCT--TCAAACGACACATAACATT 696

Consensus CGTCrCmTTTCAACArCCTCAAATCsATATCAmCACCGTTTGTAyAAwACACCTGmT 3135
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CGTCGCCTTTCAACAACCTCAAATCCATATCAACACCGTTTGTACAATACACCTGCT 3100
        4 CGTCACATTTCAACAGCCTCAAATCGATATCACCACCGTTTGTATAAAACACCTGAT 753
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Consensus CAT----ACACmTwmwCTTAAG-CTrTC-CAA-CCAA----TTCTykwyCACTs--T 3192
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CATTAGCACACCTAATCTTAAGTCTGTCGCAAGCCAACACCTTCTTGTCCACTCGCT 3157
        4 CAT----ACACATTCACTTAAG-CTATC-CAA-CCAA----TTCTCTATCACTG--T 797


Consensus CAA---CGCAyCkA-TCATCTCACGGAGGATATCGTCAAAGTGATAGTAGACGGTGT 3249
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CAAACCCGCACCGAATCATCTCACGGAGGATATCGTCAAAGTGATAGTAGACGGTGT 3214
        4 CAA---CGCATCTA-TCAT-------------------------------------- 812

Consensus GCTCATCGAAAAATATGGACAACAT-TTTGGATTATGAGGAACAAGTGGAATGGAAC 3306
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom GCTCATCGAAAAATATGGACAACAT-TTTGGATTATGAGGAACAAGTGGAATGGAAC 3270
        4 ------------------------------------------------------- 812

Consensus CGTCTGCACGACGTCCACGTAAATATAACAAATAAGTGCGACATTTCCGAAGCGGAA 3363
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom CGTCTGCACGACGTCCACGTAAATATAACAAATAAGTGCGACATTTCCGAAGCGGAA 3327
        4 ------------------------------------------------------- 812

Consensus AAATTCTTCATAGACACCATTATGGCGCGTTGTGAAGAAATGATGACCAGAGAGAAC 3420
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom AAATTCTTCATAGACACCATTATGGCGCGTTGTGAAGAAATGATGACCAGAGAGAAC 3384
        4 ------------------------------------------------------- 812

Consensus AAGCAGCAAGTGTTTGGTATTGTACAACCGTTGTTGGAGGGTACAAAAAATGCTTAC 3477
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom AAGCAGCAAGTGTTTGGTATTGTACAACCGTTGTTGGAGGGTACAAAAAATGCTTAC 3441
        4 ------------------------------------------------------- 812

Consensus GACAAATTTGTGAGCGAAAACGGAAAATGACACAAAAAATCTGCACAAAATCATCGA 3534
        7 ------------------------------------------------------- 764
        5 ------------------------------------------------------- 726
        6 ------------------------------------------------------- 846
Cydia pom GACAAATTTGTGAGCGAAAACGGAAAATGACACAAAAAATCTGCACAAAATCATCGA 3498
        4 ------------------------------------------------------- 812

Consensus T                                                        3535
        7 -                                                        764
        5 -                                                        726
        6 -                                                        846
Cydia pom T                                                        3499
        4 -                                                        812
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