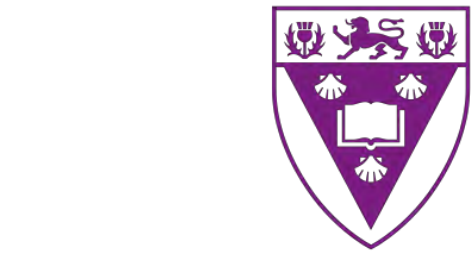

RHODES UNIVERSITY

Where leaders learn

Evaluating baculovirus mixtures against false codling moth *Thaumatotibia leucotreta* Meyrick. (Lepidoptera: Tortricidae)

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

By

Siviwe Tole

May 2024

Declaration

I, Siviwe Tole (g22T0002) hereby declare that the thesis submitted is my own work. It is being submitted for the degree of Master of Science at Rhodes University. It has not been previously submitted for assessment of any degree at any other university or other body, organisation outside of the university.

Author's signature

21 May 2024

Date

Abstract

False codling moth (FCM), *Thaumatotibia leucotreta*, is an important pest of citrus, stone fruit, avocados, peppers, and other important agricultural crops in southern Africa. Baculovirus-based biopesticides are components in an integrated pest management (IPM) programme to manage the pest in the field. Cryptogran™ and Cryptex™ which are CrleGV-SA based-biopesticides have been effective in the control of *T. leucotreta* for the past 15 years. Recently, CrpeNPV-based Multimax™ and Codlmax™ have been commercialised to control *T. leucotreta* and other important agricultural pests. Despite these viruses being relatively host-specific and safe to humans and animals in comparison to chemical insecticides, their application is hindered by their slow speed of kill, sensitivity to UV light, and the potential for insect resistance. Research investigating the effects of mixed baculoviral interactions against target pests has been a growing field of interest due to their potential to overcome such shortcomings. Previous studies using a combination of CrleGV-SA and CrpeNPV against *T. leucotreta* observed a reduction in lethal concentration in laboratory bioassays, indicating that such mixtures may have the potential for application in the field. This has led to the motivation to investigate further interactions between CrleGV-SA in combination with CrpeNPV, CpGV-M, and HearNPV-Au to understand better how these viruses interact and to determine whether synergistic, additive, or antagonistic interactions can occur against *T. leucotreta*. The outcome of these interactions will inform researchers and farmers about best practices concerning these viruses should they be combined against *T. leucotreta* in the future.

Prior to performing mixed baculovirus infections in laboratory bioassays, oligonucleotides targeting unique regions in the viral genomes of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were designed using Primer-BLAST. The specificity of these oligonucleotides was further tested in *silico* using Geneious R11 software (11.1.5). The stocks of CrpeNPV, CpGV-M, and HearNPV-Au were purified using crude OB extraction from diseased *C. peltastica*, *C. pomonella*, and *H. armigera* larval cadavers provided by River Bioscience (Pty) Ltd (Gqeberha, South Africa). The stock of CrleGV-SA was purified using crude OB extraction from infected *T. leucotreta* cadavers. Subsequently, the unique oligonucleotides were used in PCR assays to detect if the samples contained the baculoviruses of interest. Amplicons of the expected sizes were generated indicating the presence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in each of the samples. The OBs were counted using

darkfield microscopy and a counting chamber before the single and mixed infections were initiated against *T. leucotreta* neonate larvae.

Surface-dose biological assays were used to evaluate the relative virulence in terms of lethal concentration of CrleGV-SA, CrpeNPV, and CpGV-M, alone against *T. leucotreta*. After 7 days, the dose mortality data was analysed using "drc" in R studio and the LC_{50} and LC_{90} were compared amongst each virus. The CrleGV-SA treatment was estimated to be the most virulent in comparison to CrpeNPV and CpGV-M. A dose discriminate assay confirmed that HearNPV does not cause mortality in *T. leucotreta*.

Similarly, the relative virulence in terms of lethal concentration of CrleGV-SA in various ratios in combination with CrpeNPV, CpGV-M, and HearNPV-Au was determined using 7-day surface dose biological assays. The CrleGV/CrpeNPV was the most virulent mixture with lower LC_{50} and LC_{90} values measured in comparison to CrleGV/CpGV and CrleGV/HearNPV, respectively. The Tammes Bakuniak graphic method confirmed the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixtures to be antagonistic against *T. leucotreta* neonate larvae in terms of lethal concentration.

The last aspect of the study was to determine the probable cause of larval death. A modified CTAB protocol was used to extract genomic DNA from neonate-sized *T. leucotreta* cadavers collected in single and mixed infection assays. The gDNA served as templates in PCR assays using the unique oligonucleotides. In single infections, the presence of CrleGV-SA in CrpeNPV and HearNPV inoculated larvae was observed. The results suggest possible covert infections of CrleGV-SA in the *T. leucotreta* colony which may be caused by virus infection or an unknown stress factor. The results from the mixed infections showed the presence of each virus in all replicates except for the CrleGV/CpGV and CrleGV/HearNPV mixtures. In the CrleGV/CpGV mixture, only CrleGV-SA was present in the last replicate, suggesting a possible competition for host resources. In the CrleGV/HearNPV mixture, only CrleGV-SA was detected in all 3 replicates, suggesting that HearNPV did not have any effect and the larvae died of the CrleGV-SA infection.

This is the first study to report mixtures of CrleGV-SA in combination with CpGV-M and HearNPV-Au against *T. leucotreta* neonate larvae. Despite the antagonistic interactions observed in the evaluated mixtures, this study has laid a foundation to further investigate how these viruses interact in dual infections for the improved control of *T. leucotreta*. This may be

done by evaluating different ratios and combinations of baculoviruses to those used in this study.

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List of Abbreviations

$	-	US dollars
%	-	Percentage
× g	-	Times gravity
°C	-	Degree Celsius
µl	-	Microlitre
µM	-	MicroMolar
µm	-	Micrometre
AcMNPV	-	Autographa californica multiple nucleopolyhedrovirus
AGE	-	Agarose gel electrophoresis
AgMNPV	-	Anticarsia gemmatalis multiple nucleopolyhedrovirus
AgseGV	-	Agrotis segetum granulovirus
AgseNPV-B	-	Agrotis segetum nucleopolyhedrovirus B
ANOVA	-	Analysis of Variance
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
Bt	-	Bacillus thuringiensis
BV	-	Budded virus
CDS	-	Coding sequence
CE	-	Controlled environment
CM	-	Codling moth
CpGV-M	-	Cydia pomonella granulovirus, Mexican isolate
CrleGV-SA	-	Cryptophlebia leucotreta granulovirus, South African isolate
CrpeNPV	-	Cryptophlebia peltastica nucleopolyhedrovirus
CTAB	-	Cetyltrimethylammonium bromide
D1	-	Dose 1
D2	-	Dose 2
D3	-	Dose 3
D4	-	Dose 4
D5	-	Dose 5
DALRRD	-	Department of Agricultural, Land Reform and Rural Development

ddH_20	-	Double distilled water
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediamine tetraacetic acid
egt	-	ecdysteroid-UDP glucosyl transferase
EpapGV	-	Epinotia aporema granulovirus
FCM	-	False codling moth
g	-	Grams
gDNA	-	Genomic deoxyribonucleic acid
GM	-	Genetic modification
GV	-	Granulovirus
HearGV	-	Helicoverpa armigera granulovirus
HearNPV-Au	-	Helicoverpa armigera nucleopolyhedrovirus, Australian isolate
Hz	-	Hertz
IPM	-	Integrated pest management
Kb	-	Kilobase
Kbp	-	Kilobase pair
LC	-	Lethal concentration
LC_{50}	-	Lethal concentration (50%)
LC_{90}	-	Lethal concentration (90%)
LD	-	Lethal dose
LD_{50}	-	Lethal dose (50%)
LD_{90}	-	Lethal dose (90%)
LM	-	Litchi moth
LT	-	Lethal time
M	-	Molar
MbMNPV	-	Mamestra brassicae multiple nucleopolyhedrovirus
mg	-	Milligrams
ml	-	Millilitre
mm	-	Millimetre
mM	-	millimolar
mPCR	-	multiplex PCR
n	-	Number

NCBI	-	National Center for Biotechnology Information
ng	-	nanograms
nm		nanometre
NPV	-	Nucleopolyhedrovirus
NTC	-	No template control
NW	-	Needleman-Wunsch
OB	-	Occlusion body
ODV	-	Occluded derived virus
orf37	-	open reading frame 37
orf39	-	open reading frame 39
PCR	-	Polymerase Chain Reaction
pif/74	-	per os infectivity factors/74
PM	-	Peritrophic membrane
polh	-	polyhedrin gene
qPCR	-	Qualitative polymerase chain reaction
R	-	Rands
RefSeq	-	Reference Sequence
SA	-	South Africa
SADC	-	South African Development Community
SDS	-	Sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
SIT	-	Sterile insect technique
SNPs	-	Single nucleotide polymorphisms
SpfrGV	-	Spodoptera frugiperda granulovirus
SpfrMNPV	-	Spodoptera frugiperda nucleopolyhedrovirus
SpliGV	-	Spodoptera littoralis granulovirus
SlNPV	-	Spodoptera litura nucleopolyhedrovirus
SpliNPV	-	Spodoptera littoralis nucleopolyhedrovirus
SporGV	-	Spodoptera ornithogalli granulovirus
SporNPV	-	Spodoptera ornithogalli nucleopolyhedrovirus
TAE	-	Tris base, acetic acid and EDTA
TEM	-	Transmission Electron Microscopy
TnGV	-	Trichoplusia ni granulovirus

Tris-HCl	-	Hydroxymethyl aminomethane hydrochloride
USA	-	United States of America
V	-	Volts
w/v	-	weight per volume
W1	-	Working solution 1
XecnGV	-	Xestia c-nigrum granulovirus
XecnNPV	-	Xestia c-nigrum nucleopolyhedrovirus

Research Outputs

Tole, S., Knox, C., Jukes, M., Moore, S. & Hill, M. 2023. Evaluating baculovirus mixtures against false codling moth *Thaumatotibia leucotreta* Meyrick. (Lepidoptera: Tortricidae). Oral presentation at the 23rd Congress of the Entomological Society of Southern Africa (ESSA), Stellenbosch University, Stellenbosch, South Africa (**11-14 July 2023**).

Tole, S., Knox, C., Jukes, M., Moore, S. & Hill, M. 2023. Evaluating baculovirus mixtures against false codling moth *Thaumatotibia leucotreta* Meyrick. (Lepidoptera: Tortricidae). Oral presentation at the Annual Postgraduate Conference, Rhodes University, Makhanda, Eastern Cape (**8-9 September 2023**).

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- Lastly, I would like to thank my **Most High God**. I would not have made it without His faithfulness, mercy, and overwhelming grace. His guidance and strength have made it possible for the completion of this study – *Jeremiah 33:3* - Call to me, and I

will answer you; I will tell you wonderful and marvellous things that you know nothing about. *Psalms 116:7,12* – [7] Be confident, my heart, because the LORD has been good to me. [12] What can I offer the LORD for all his goodness to me? 1 *Thessalonians 5:16-18* – [16] Be joyful always, [17] pray at all times, [18] be thankful in all circumstances. This is what God wants from you in your life in union with Christ Jesus.

I show my gratitude to all those I did not mention, your contribution will always be highly appreciated!

Chapter 1
Literature review

1.1 The importance of the citrus industry in South Africa

The citrus industry in South Africa is well known locally and internationally due to its export orientation and role in job creation (Chisoro-Dube & Roberts, 2021). The citrus industry has made a significant financial contribution to the South African agricultural industry. According to DALRRD, 2020, the citrus industry's gross value is the third largest in the horticultural industry behind deciduous fruits and vegetables. This was significantly indicated in the 2018/2019 production season where the citrus industry contributed over US$957 000 (R16.1 billion) to the gross value of the agricultural production. South Africa is the second largest citrus exporter in the world behind Spain, emphasising the importance of the citrus industry as great investments are made (Citrus Growers' Association, 2022). South Africa exports 77% of citrus with Europe (34%), the Middle East (18%), and South East Asia (14%) being the major destinations (Citrus Growers' Association, 2022).

The citrus-producing regions in South Africa and the Southern African Development Community (SADC) such as Zimbabwe and Eswatini are indicated in **Figure 1.1**. The Limpopo, Eastern Cape, and Western Cape provinces are the largest citrus-producing regions in South Africa.

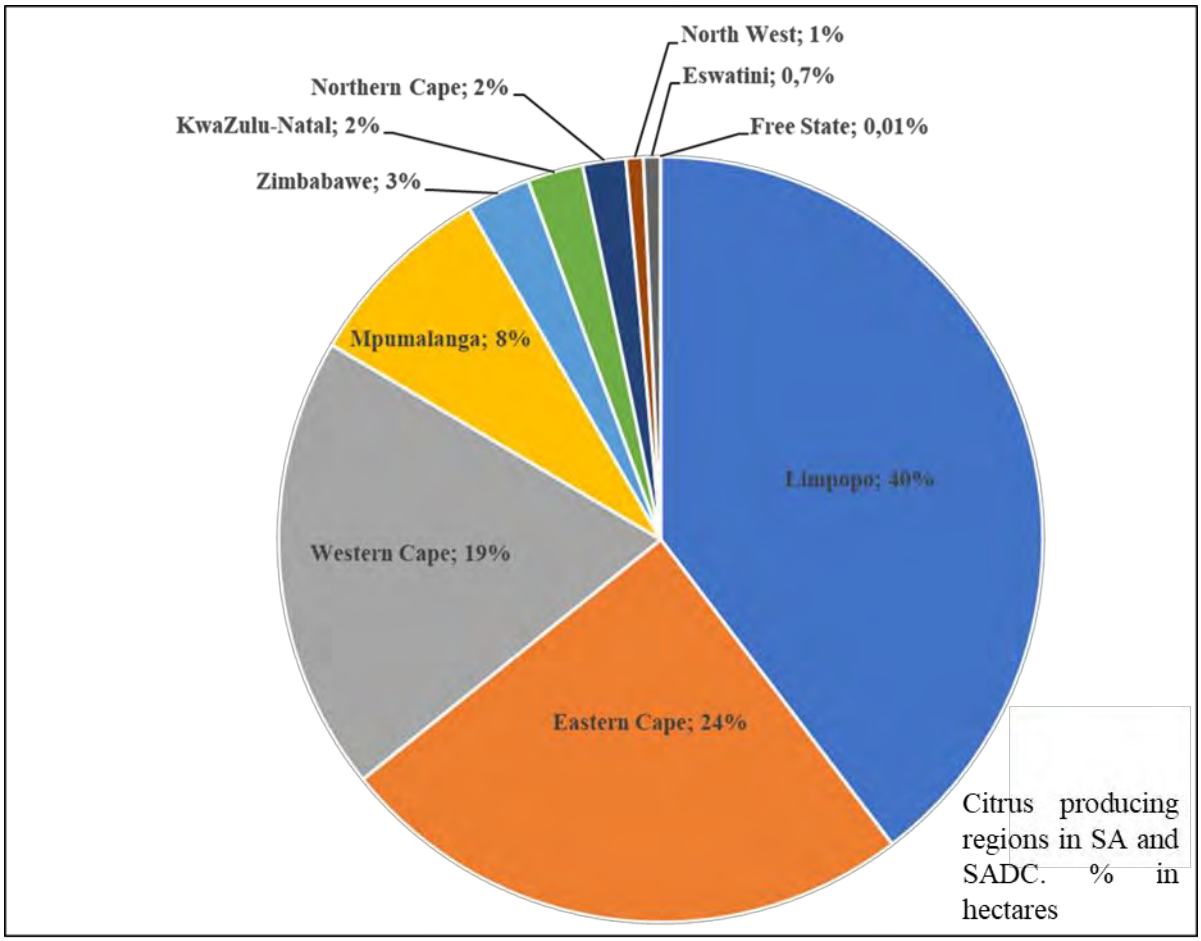


Figure 1.1 Citrus-producing regions in South Africa and the South African Development Community (Citrus Growers' Association, 2023)

1.2 Threats to the citrus industry

Harmful organisms can significantly reduce crop yields. Animal pests (insects, mites, nematodes), plant pathogens (viruses, bacteria, fungi), and weeds are examples of harmful organisms responsible for reducing crop production (Oerke, 2006). The damage caused by these harmful organisms results in poor fruit quality, making it non-marketable thus having negative implications on the export industry. It is important to protect the citrus industry from pathogens and pests that may cause economic losses. One threatening insect pest to the citrus

industry is the false codling moth (FCM), *Thaumatotibia leucotreta* which when uncontrolled, causes severe damage to citrus resulting in economic losses **Figure 1.2**.

Fruits infested by *T. leucotreta* show minimal symptoms on the surfaces since the larvae are internal feeders. Larval entry points are detected by brown spots or dark brown residues. The rind of the citrus fruits turns yellowish-brown due to decaying because of *T. leucotreta* infestation (Ladaniya, 2023). **Figure 1.2** illustrates the damage caused by *T. leucotreta* infestation on oranges.

Figure 1.2 The damage caused by *Thaumatotibia leucotreta* infestation on oranges (Pictures by David Taylor)

1.3 The pest: *Thaumatotibia leucotreta*

1.3.1 *T. leucotreta* taxonomy

The pest of interest in this study is *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) also known as the false codling moth. An image of an adult *T. leucotreta* is indicated in **Figure 1.3**.

Figure 1.3 *Thaumatotibia leucotreta* adult moth. (Image captured by David Taylor)

The lepidopteran moth was initially taxonomically described as *Argyroploce leucotreta* (Meyrick) and was later changed to *Cryptophlebia leucotreta* by Clarke, 1955. In the late 1990s, it was changed to *Thaumatotibia leucotreta* by Komai, 1999 and the genus and species names are presently used.

The current taxonomy of *T. leucotreta* (Meyrick), a significant pest of citrus and other important agricultural crops in Southern Africa (**Table 1.1**)

Table 1.1 Taxonomic classification of *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae)

i ngdom	Animalia
hylum	Arthro oda
Class	nsecta
rder	Le ido tera
amily	ortricidae
Genus	
ecies	
inomial name	(e yric , 2)

There are other lepidopteran pests related to *T. leucotreta* that are of economic importance. *Helicoverpa amigera*, commonly known as the African bollworm is a pest of cotton and other important agricultural crops (Fitt, 1989). Closely related tortricids such as the codling moth, *Cydia pomonella,* and litchi moth, *Cryptophlebia peltastica* will be further discussed later in this study emphasizing their economic importance and the control of these pests (Follett & Lower, 2000; Barnes, 1991; Manrakhan et al., 2008; Waite & Hwang, 2002).

1.3.2 *T. leucotreta* distribution

Thaumatotibia leucotreta is endemic to sub-Saharan Africa and has been established in many African countries namely: Angola, Benin, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Chad, Congo Democratic Republic, Côte d'Ivoire, Eritrea, Ethiopia, Gambia, Ghana, Kenya, Madagascar, Malawi, Mali, Mauritius, Mozambique, Niger, Nigeria, Rwanda, Réunion, Saint Helena, Senegal, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia, and Zimbabwe (Stibick, 2006). *Thaumatotibia leucotreta* establishment has been reported in Israel (Wysoki, 1986) **Figure 1.4** shows the geographical distribution of *T. leucotreta*.

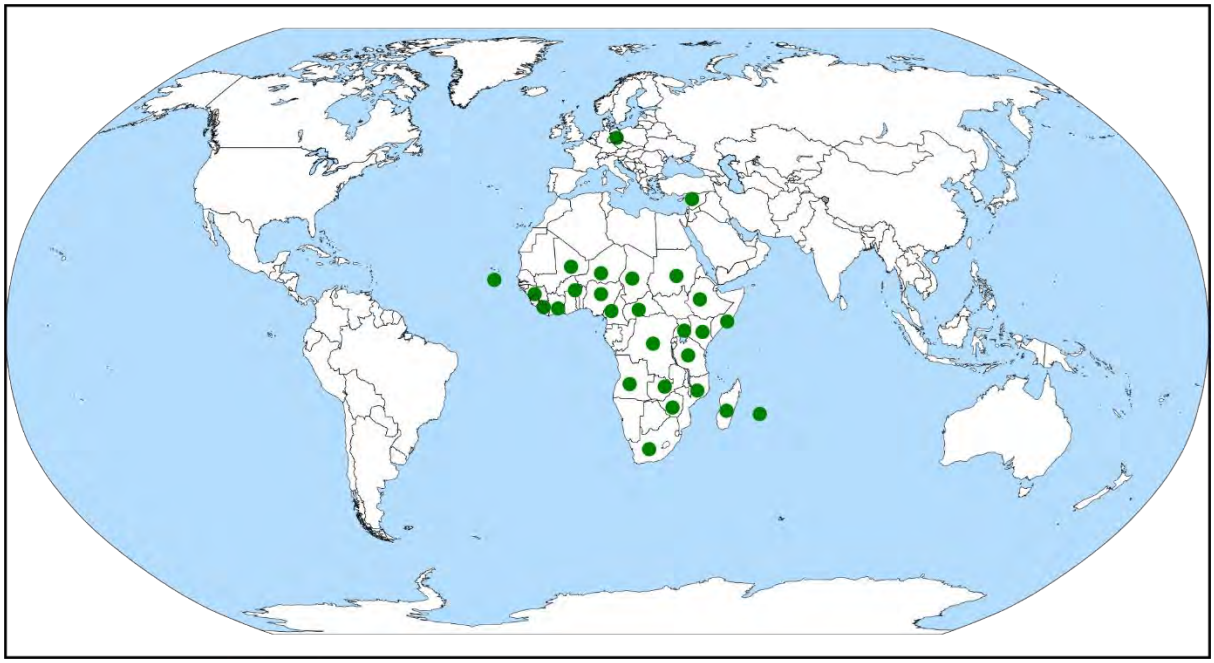


Figure 1.4 The distribution of *Thaumatotibia leucotreta*. *Thaumatotibia. leucotreta* is known to be endemic to sub-Saharan Africa but cases outside the native regions have been reported (Israel). (Picture adapted from https://www.cabi.org/isc/datasheet/6904)

Thaumatotibia leucotreta is considered a significant pest of citrus (Moore, 2002; Newton, 1998). The pest has a very wide range of host plants with over 70 species recorded and is adapted to many different plant families including cultivated and wild crops (Kirkman & Moore, 2007). Although known to be a key pest of citrus, *T. leucotreta* is also a significant pest of avocadoes (Grové et al., 1999), cotton (Reed, 1974), macadamias (Venette et al., 2003), and other important agricultural crops produced in southern Africa such as peaches, pomegranates, and peppers (Grout & Moore, 2015). Other secondary host plants include roses, hibiscus and other soft flowers (CABI, 2020). Due to its wide host range of crops, *T. leucotreta* is suited to various ecological environments allowing it to occur throughout the year (Newton, 1989).

1.3.3 Biology and Lifecycle

On citrus, *T. leucotreta* females lay eggs (**Figure 1.5 A**) on the surfaces of fruits that are initially cream in colour. The eggs change to red over time and then turn black before hatching (Daiber, 1979a). When the eggs hatch, neonate larvae (**Figure 1.5 B**) burrow through the fruit where feeding and larval development takes place. The width of the head capsule differentiates the 1st, 2nd, 3rd, 4th, and 5th larval instars (Daiber, 1979b). Poor food quality in the form of immature fruits and lower temperatures can hamper larval development (Daiber, 1979b). Upon reaching late larval instars, the larvae drop to the soil, spin a cocoon of silk, and pupate. The larval stage causes the most damage due to feeding on the pulp of the host plants' fleshly fruits, seeds, and pods (Djieto-Lordon et al., 2014; Schulthess et al., 1991; Vaissayre, 1995). Fruit infestation results in premature fruit drop (Daiber, 1979c, 1979b). Feeding damage caused by *T. leucotreta* can lead to secondary infections by fungi and bacteria causing a further reduction in crop quality, making it nonmarketable (Newton, 1989). Male pupae are differentiated from female pupae by being smaller in size and having two knobs on the ventral side of the abdomen. The pupa stage is regarded as the most sensitive (Adom et al., 2020) (**Figure 1.5 C**). Depending on the season, the adults (**Figure 1.5 D**) eclose after 4-33 days (Daiber, 1979a). Male and female adults are distinguished by multiple characteristics such as overall size, wing shape, and secondary sexual characteristics. Life spans of adults range from 13.7 to 48 days, dependent on the temperature and sex, and females can lay from 5 to 804 eggs under optimal conditions as recorded by Daiber, (1980).

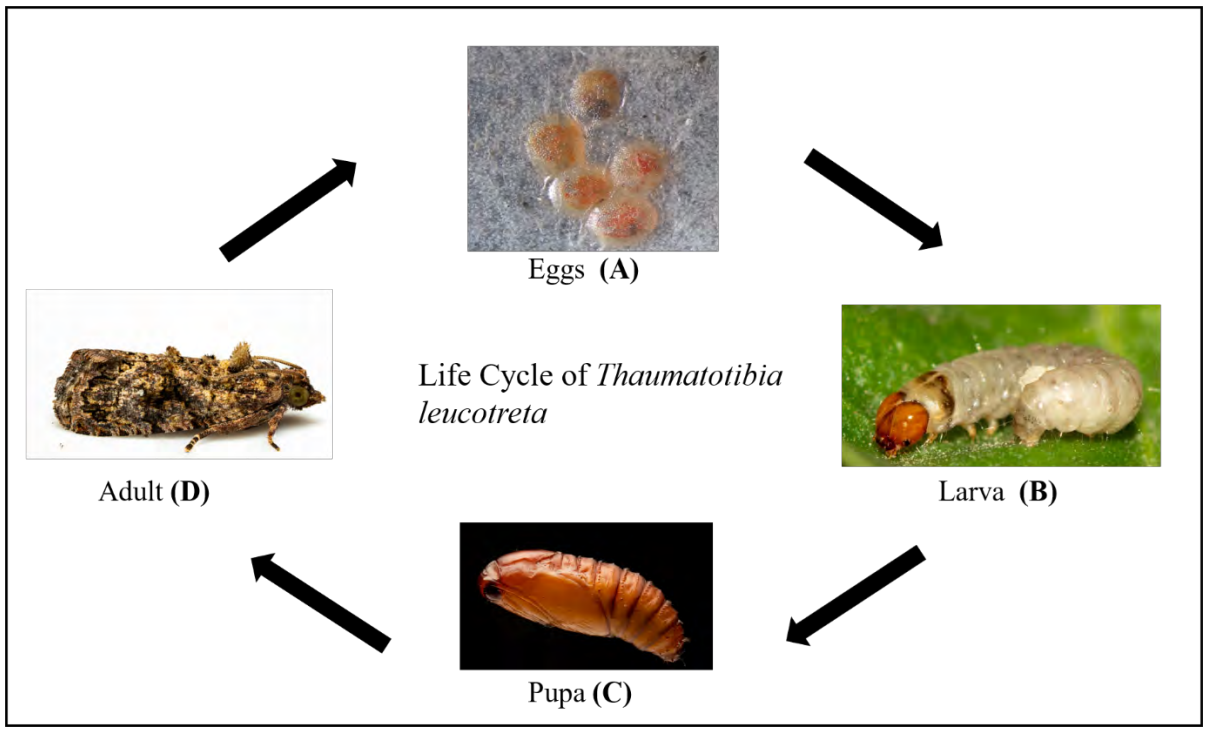


Figure 1.5. *Thaumatotibia leucotreta* lifecycle as summarised by Daiber, (1980, 1979a, 1979b, 1979c)

Since *T. leucotreta* is native to sub-Saharan Africa, it is regarded as a quarantine pest, giving it a high pest status (Grout & Moore, 2015; Hattingh et al., 2020). According to Follett & Neven, (2006) a quarantine or phytosanitary pest is a pest with the potential of establishing outside its native region making it of economic importance. Export markets have introduced bans on the export of fruits from where the pest has been established (Adom et al., 2020). This negatively impacts the export of fresh fruits to international markets because regulations need to be put in place to regulate the risks of establishment in regions outside sub-Saharan Africa. With the regulations set in place, *T. leucotreta* needs to be controlled effectively before fruits are exported to avoid any repercussions and economic losses (Moore, 2021).

1.4 Management strategies of *T. leucotreta*

1.4.1 Integrated pest management

Due to the growing human population, a foreseeable approach to reduce and control pests to increase crop production is required. *Thaumatotibia leucotreta* is controlled using the integrated pest management (IPM) program (Moore, 2021). The control measures of this pest are reviewed by Moore & Hattingh, (2012). Integrated pest management includes a combination of methods such as chemical insecticides, sterile insect technique (SIT)

(Hofmeyr et al., 2005), orchard sanitation (Moore & Kirkman, 2008), attack and kill, mating disruption (Hofmeyr et al., 2005), cultural and biological control. In the past, the control of pest populations (insects, nematodes, diseases, and weeds) relied heavily on the use of chemical insecticides. The use of chemical insecticides to control agricultural pests still plays a major role in most developing countries (Hajjar et al., 2023). Although chemical insecticides are effective in suppressing insect populations in a short space of time, in recent years there has been a decline in the use of chemical insecticides due to the negative impact on the environment, and living organisms, and the growing resistance of the pest populations to chemical insecticides (Gangwar et al., 2021; Ramlee, 2015). Although chemical insecticides are still part of the integrated pest management, research on biocontrol particularly baculoviruses as biopesticides has the potential to reduce or replace chemical insecticides used in the control of agriculture and forestry pests. Baculoviruses have been well exploited for use as biopesticides in the field due to their advantages such as being safe to human, animal, and plant life, highly specific to target pests, and hence play a role in IPM programs in the control of insect pests globally (Haase et al., 2015; Raj et al., 2022)

1.4.2 Baculovirus taxonomy and morphology

Baculoviruses belong to the family *Baculoviridae*, have circular, large double-stranded DNA genomes typically ranging from 80 to 180 kbp in length (van Oers & Vlak, 2007). The genomes are packaged in rod-shaped nucleocapsids that are generally 30 – 60 nm in diameter and 250 – 300 nm in length (Jehle et al., 2006). The genomes encode 90 to 180 genes with 38 of those genes being highly conserved in all baculovirus species that have been sequenced to date (Rohrmann, 2019). Two virion phenotypes typically exist namely: occlusion-derived virus (ODV) (**Figure 1.6 A**) and budded virus (BV) (**Figure 1.6 B**) (Blissard, 1996). Although ODVs and BVs have similar nucleocapsid structures, there are differences including where the virions are produced in the cell, envelope composition, and function in the baculoviral lifecycle (Blissard, 1996). ODVs are occluded in the protein matrix forming an occlusion body (OB) (Jehle et al., 2006). The crystalline protein matrix protects the virions from unfavourable conditions allowing for survival and persistence in the environment (Grzywacz, 2017; Hutchinson, 2021). The two types of viruses namely: nucleopolyhedrovirus (NPV) and granulovirus (GV) are distinguished based on their morphology and protein composition. Polyhedral OBs of NPVs are larger and shaped irregularly and are typically 0.6 to 2 um in size containing multiple virions that are occluded in polyhedrin (Bonning, 2005). Granular OBs, in contrast, are smaller in size (about 0.2 – 0.4

um), ovoid-shaped, and have a single virion that is occluded in granulin (Rohrmann, 2019). Baculoviruses are one of the largest and most diverse groups of viruses with over 600 species being described (Herniou & Jehle, 2007; Moscardi, 1999). Baculoviruses are divided into 4 genera namely: *Alphabaculovirus* (lepidopteran specific NPVs), *Betabaculovirus* (lepidopteran specific GVs), *Gammabaculovirus* (hymenopteran NPVs) and *Deltabaculovirus* (dipteran NPVs) with majority of species isolated from the insect orders Lepidoptera, Diptera and Hymenoptera (Harrison et al., 2018; Jehle et al., 2006). *Alphabaculovirus* is divided into two lineages, Group I and Group II based on phylogenetic analysis. Group I NPVs use GP64, a BV-specific protein fusion protein whereas Group II NPVs use F protein (Bulach et al., 1999; Herniou et al., 2001, 2003). Furthermore, *Betabaculoviruses* are also divided into clades a and b (Miele et al., 2011). The current taxonomy of baculoviruses is based on genome sequence analysis and host classification and overrides the previous system which was based on morphological traits. The new classification systems have placed the nuclear arthropod large DNA viruses (NALDVs) from the family *Baculoviridae* in the class *Naldaviricetes* and order *Lefavirales* (van Oers et al., 2023). Baculoviruses were initially named according to the insects from which they were isolated but currently a new Linnean binomial naming system is adopted although the common names and abbreviations still stay the same (van Oers et al., 2023). For this study, common names will be used throughout.

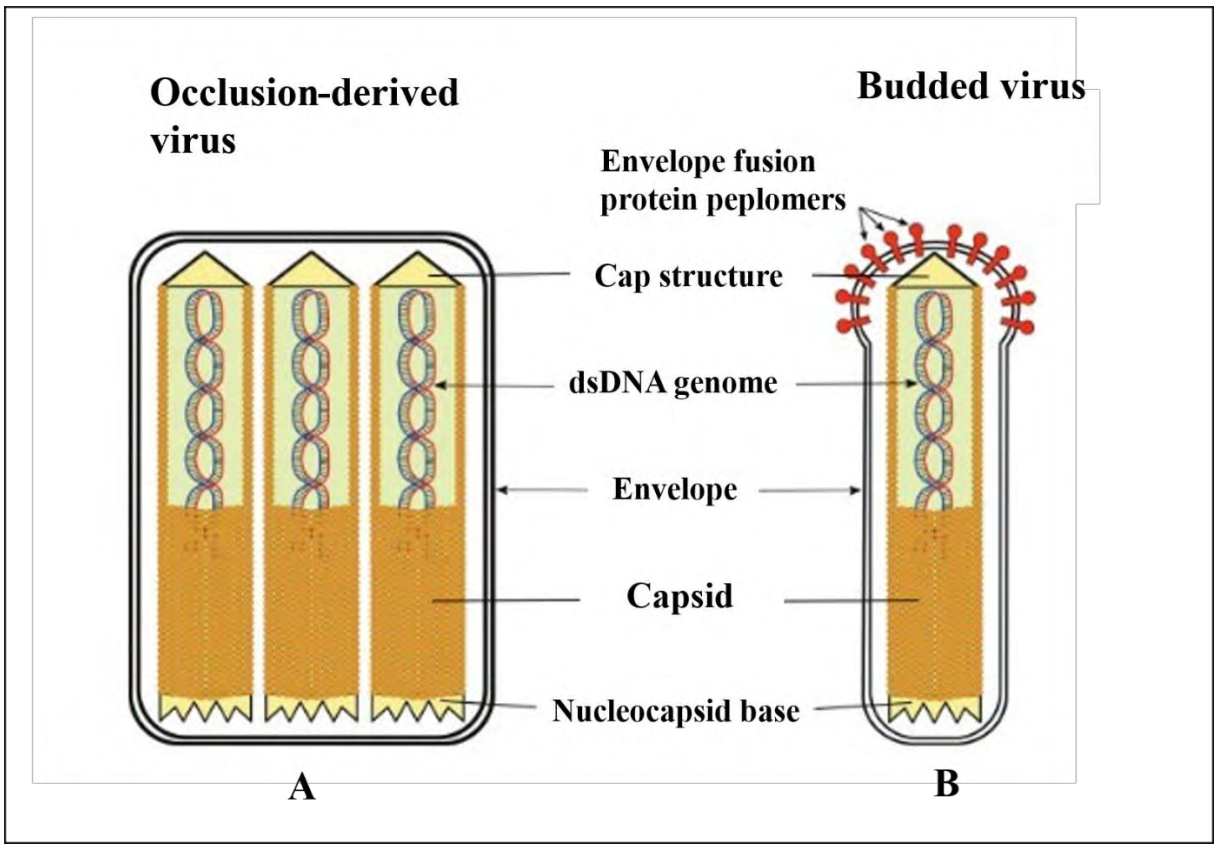


Figure 1.6 Two baculovirus virion phenotypes. (A) Occlusion-derived virus and (B) Budded virus (Harrison et al., 2018)

1.4.3 Baculovirus lifecycle

Baculoviruses are characterised by their biphasic replication cycle that is mediated the ODV and BV. The virions are produced at different stages of the infection cycle and have different roles as previously mentioned. As reviewed in Moscardi et al., (2011), primary infection and horizontal transmission are initiated by the ingestion of OBs from contaminated plant material by the insect larvae (**Figure 1.7 A**). The OBs dissemble upon reaching the insect midgut due to the high alkalinity conditions (**Figure 1.7 B**). The ODVs are then released and pass through the peritrophic membrane (PM) to infect the midgut epithelial cells of the insect (**Figure 1.7 C**). Nucleocapsids travel to the nucleus where the virions initiate replication. ODVs spread the infection from insect to insect. The secondary infection is initiated when the progeny virions (BVs) move toward the base of the cell and bud out through the basal lamellar membrane acquiring their envelopes (**Figure 1.7 D**). BVs are responsible for cell-to-cell and tissue-to-tissue transmission within an infected animal (**Figure 1.7 E, F**). The secondary infection ends in a massive production of OBs where progeny virions are

accumulated and ODVs acquire an envelope in the nucleus, encasing the virion within a crystalline protein matrix to form an occlusion body (**Figure 1.7 G**). Upon the death of the larvae, OBs are released back into the environment to initiate another infection cycle (**Figure 1.7 H**). As previously mentioned, baculoviruses are safe for humans as they do not produce any toxins or metabolites (Gelaye & Negash, 2023). When compared with other biocontrol agents, baculoviruses are highly favourable because they are fast-acting and are pathogenic to many important insect pests and hence explored as biopesticides (Beas-Catena et al., 2014; Haase et al., 2015; Lacey et al., 2015; Moore & Jukes, 2019) which are discussed in section 1.5.

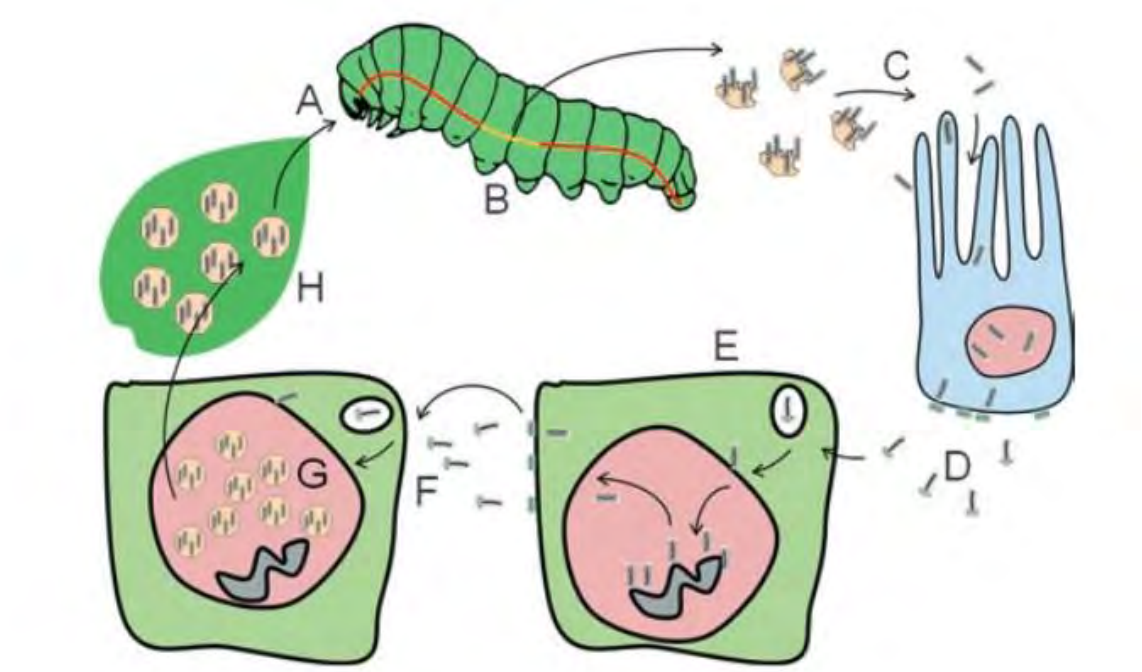


Figure 1.7 Baculovirus lifecycle (Moscardi et al., 2011)

1.5 Baculoviruses as biopesticides against important agricultural pests

There are many examples of baculoviruses that are used against pests globally. For this study, the focus will be on Cydia pomonella granulovirus, Cryptophlebia leucotreta granulovirus, Cryptophlebia peltastica nucleopolyhedrovirus, and Helicoverpa armigera nucleopolyhedrovirus. Currently, there are 11 commercially available baculovirus products for use in Africa (Moore & Jukes, 2023). Many of these products use baculoviruses isolated from Africa which will be further discussed in the section below.

1.5.1 *Betabaculovirus cypomonellae*, Cydia pomonella granulovirus (CpGV)

The codling moth (CM), *Cydia pomonella* (L), (Tortricidae: Lepidoptera) is a global pest in apples, pears, and walnut plantations (Barnes, 1991). The larvae burrow in the fruits and cause destructive damage leading to economic losses (**Figure 1.8 A)**. The application of Cydia pomonella granulovirus (CpGV) has been an effective biological control agent against this pest (Hunter-Fujita et al., 1998; Lacey et al., 2008). CpGV has a narrow host range, characterised by being highly virulent to larval stages of *C. pomonella* and closely related species in the order Lepidoptera (Cross et al., 1999; Gebhardt et al., 2014). An example of such a related species is *T. leucotreta* as CpGV can infect it (Fritsch et al., 1990). CpGV, a type 2 granulovirus, is fast-killing, has wide tissue tropism, and is considered one of the most virulent granuloviruses (Federici, 1997; Tanada & Hess, 1991). CpGV was first discovered in infected *C. pomonella* larvae from Mexico in 1963, and the Mexican isolate (CpGV-M) is an active agent in CpGV-based commercialised products in most countries where pome fruit is produced (Crook et al., 1997; Hunter-Fujita et al., 1998; Lacey et al., 2008; Tanada, 1964). In addition to CpGV-M, different naturally occurring CpGV isolates from various geographical regions have been discovered and have been classified in genome groups A – G based on single nucleotide polymorphisms (SNPs) in highly conserved genes (Arneodo et al., 2015; Eberle et al., 2009; Fan et al., 2020; Gebhardt et al., 2014; Wennmann et al., 2017). With the first cases of *C. pomonella* resistance to CpGV-based products having been reported in Germany and France (Fritsch et al., 2005; Sauphanor et al., 2006) and other countries in Europe (Schmitt et al., 2013) research has been ongoing in search of novel CpGV isolates to combat such shortcomings. Recently, CpGV-SA was isolated by Motsoeneng, (2016) and determined to have similar virulence as CpGV-M and could potentially be commercialised and used as an alternative to CpGV-M (Motsoeneng et al., 2019). Currently CpGV-SA is not commercialised in South Africa but is under investigation for registration (Hatting et al., 2019). In South Africa, commercialised CpGV-M-based products Madex® (Andermatt-Biocontrol AG, Switerzland) (**Figure 1.8 B**) and Carpovirusine® (Arysta Life Science, France) have been in the control against *C. pomonella* (Knox et al., 2015).

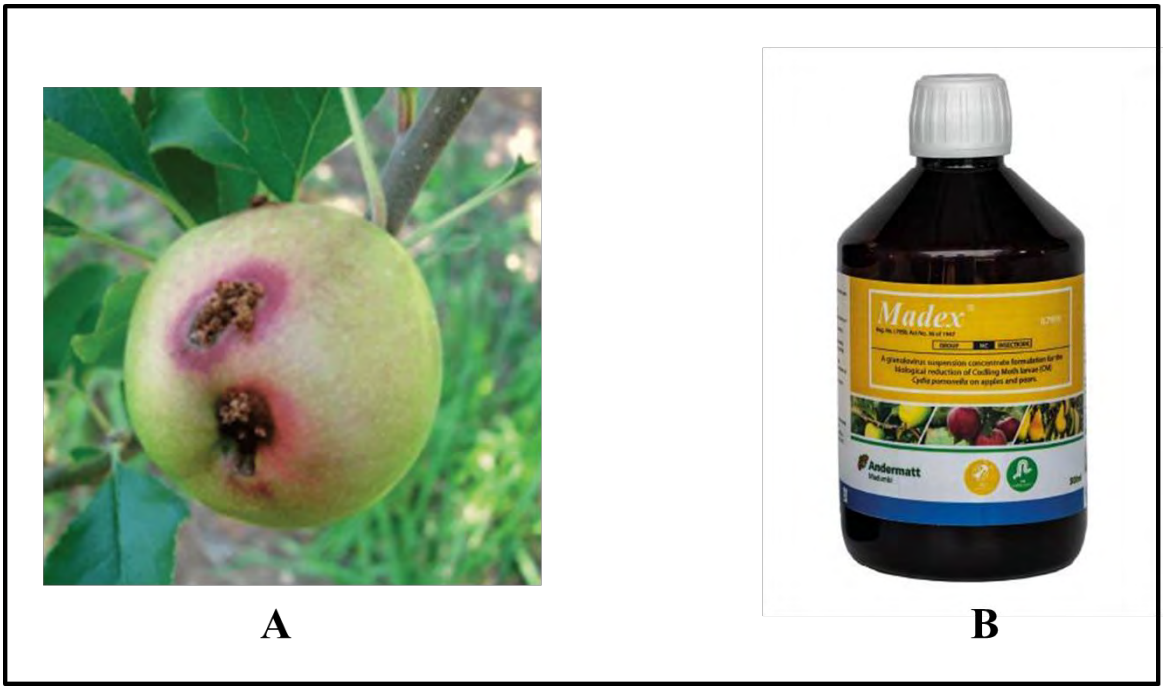


Figure 1.8 (A) Apple infested by *C. pomonella* (Picture captured by Jeanne de Waal) (B) Madex® CpGV-M based commercialised product (Knox et al., 2015).

1.5.2 *Betabaculovirus cryleucotretae*, Cryptophlebia leucotreta granulovirus (CrleGV)

The application of Cryptophlebia leucotreta granulovirus (CrleGV) is one of the control measures used against *T. leucotreta* (Moore et al., 2004; Moore, 2002; Moore et al., 2011) CrleGV was first described by Angélini et al., (1965) from infected *T. leucotreta* larvae in the Ivory Coast. The original name of the virus has not changed despite the genus of the host changing from *Cryptophlebia* to *Thaumatotibia* (Komai, 1999). Since then, additional isolates have been recovered and isolated from Cape Verde Islands (CrleGV- CV3) (Mück, 1985) and laboratory-reared insects originating from South Africa and are housed at the Hoechst Corporation in Germany (Jehle et al., 1992). The South African isolate, the Cape Verde isolate and the Ivory Coast isolate are different strains and can be distinguished by restriction analysis (Fritsch, 1989; Jehle et al., 1992). A novel South African isolate (CrleGV-SA) was described from diseased *T. leucotreta* larvae held in a laboratory colony at Citrus Research International in Port Elizabeth (Moore et al., 2011). CrleGV-SA was characterised by Singh et al., (2003) and later sequenced by van der Merwe et al., (2017) and the same isolate was formulated under the trade name Cryptogran® (River Bioscience, South Africa) (**Figure 1.9**) for the control of *T. leucotreta* on citrus and avocadoes (Moore et al., 2004; Moore, 2002; Moore et al., 2011). In addition, Cryptex® (Andermatt-Biocontrol AG Switzerland) has also been registered and used in South Africa (Kessler & Zingg, 2008).

CrleGV-SA has commercially been used for the past 15 years and the repeated field applications led to the bioprospecting of novel isolates to mitigate the risks of potential *T. leucotreta* field resistance as seen by the resistance of *C. pomonella* to CpGV-M (Knox et al., 2015). Opoku-Debrah et al., (2013) isolated five novel CrleGV-SA isolates from different geographic *T. leucotreta* populations. The discovery of the novel isolates are potential alternative to the formulations should *T. leucotreta* develop resistance to the current commercial products used (Opoku-Debrah et al., 2013).

Figure 1.9 Commercially available Cryptogran® (River Bioscience, Gqeberha) for the control of *T. leucotreta* (Moore et al., 2004; Moore, 2002; Moore et al., 2011)

1.5.3 *Alphabaculovirus crypeltasicae*, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV)

The litchi moth, *Cryptophlebia peltastica* (Meyrick) (Tortricidae: Lepidoptera) is an economic pest of litchis and macadamias in South Africa, Mauritius, Seychelles, Reunion Island, and Madagascar (Follett & Lower, 2000; Manrakhan et al., 2008; Waite & Hwang, 2002) (**Figure 1.10 A**). Recently a novel baculovirus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) was isolated from *C. peltastica* larvae and characterised by Marsberg et al., (2018). CrpeNPV is also the first NPV described from the Grapholitini tribe (Marsberg et al., 2018). In the study by Marsberg, (2016) and Taylor, (2021), lower LC_{50} (the concentration it takes to kill 50% of the individuals) and LC_{90} (the concentration it takes to kill 90% of the individuals) CrpeNPV values were measured against *T. leucotreta* neonates in

surface dose biological assays indicative that CrpeNPV is highly virulent against the pest. In addition, biological assays confirmed the virulence of CrpeNPV against *C. pomonella* resistant strains, offering additional microbial control agent against *C. pomonella* (Wennmann et al., 2019). Recently, CrpeNPV has been registered as a biocontrol agent under the name Multimax™ and Codlmax™ (River Bioscience, Gqeberha) in South Africa and is used on pome fruit, macadamias, and litchis against *T. leucotreta* and other important agricultural pests (Thackeray, Personal Communication, 2023) (**Figure 1.10 B**)

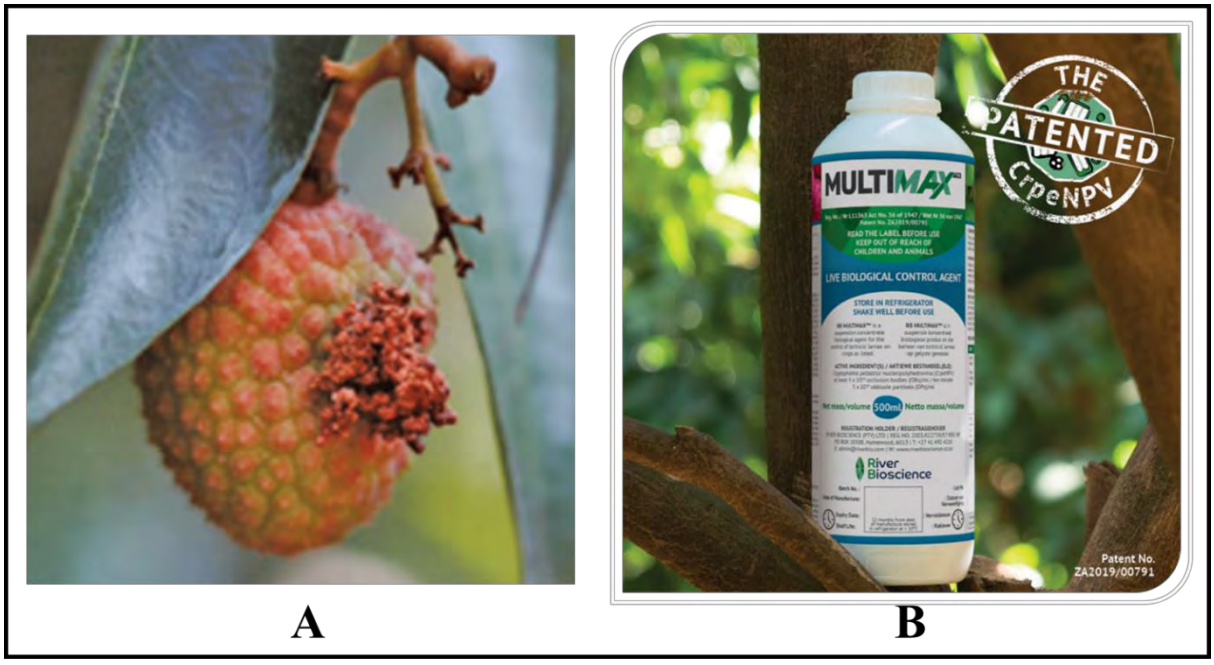


Figure 1.10 (A) Damage caused by *C. peltastica* infestation on litchis (Picture by T. Grove, ARC) (Thackeray, Personal communication, 2023). (**B**) Multimax™ a registered CrpeNPV biopesticide (River Bioscience, South Africa)

1.5.4 *Alphabaculovirus helarmigerae*, Helicoverpa armigera nucleopolyhedrovirus (HearNPV)

The African bollworm, *Helicoverpa armigera* (Huber) (Lepidoptera: Noctuidae) is a global pest of citrus, tomatoes, cotton, sorghum, peppers, maize, and other important agricultural crops (Fitt, 1989). African bollworm infestation can destroy up to 80% of the setting fruit as a result of the larvae feeding on the blossoms and small fruitlets (**Figure 1.11 A**) (Bedford, 1968). In South Africa, *H. armigera* is a pest of citrus and is considered one of the most important lepidopteran pests (Bell & McGeoch, 1996; Moran, 1983; Vermeulen & Bedford, 1998). Biocontrol products developed with Helicoverpa armigera nucleopolyhedrovirus (HearNPV) are compatible in an IPM programme and used in the control of *H. armigera*

(Hunter-Fujita et al., 1998). Whitlock, (1974) first discovered HearNPV in South Africa. Laboratory assays determined an estimated concentration of 3.5×10^4 OBs/ml resulted in 50% neonate larval mortality and the LT_{50} (time to kill 50% of the individuals in a sample) was determined to be 7 days (Whitlock, 1974, 1978). Laboratory assays performed by Ogembo, (2002) further showed the potential for the application of the HearNPV isolate as biopesticide where results indicated the dosage required to kill 50% of the individuals to be 20 OBs/ml. Moore et al., (2004) conducted field trials against African bollworm in citrus in different provinces of South Africa using the HearNPV isolate (Whitlock, 1974). The results showed a total of 100% elimination of African bollworm infestation where rates of between 1 and 5×10^{12} OBs/ha were applied on mature citrus trees (Moore et al., 2004; Moore & Kirkman, 2010). The fruit damage was reduced by up to 84% and the rejection for export was reduced by 96% (Moore et al., 2004; Moore & Kirkman, 2010). This led to the registration of HearNPV as a biopesticide under the trade name Helicovir™ (River Bioscience, Gqeberha) **(Figure 1.11 B)**. Although limited by the slow speed of kill, it is highly effective when applied on newly hatched larvae as part of IPM programmes (Moore & Kirkman, 2010). Another HearNPV-based product, Bolldex® (Andermatt-Biocontrol AG, Switzerland) is also commercially available in South Africa (Knox et al., 2015). To combat the potential insect resistance by *H. armigera* populations, research is ongoing to isolate and characterise novel HearNPV isolates that differ in virulence against *H. armigera.*

Figure 1.11 (A) An African bollworm larva feeding on a young orange. (B) Helicovir™ (River Bioscience, (Pty), Ltd) (Moore & Kirkman, 2010)

1.6 Disadvantages of baculoviruses as biocontrol agents

Although about 60 baculoviruses are commercialised or under development as biopesticides (Reid et al., 2023), the expansion of the use of baculoviruses as biopesticides in the field has been hampered by various shortcomings from an agricultural point of view. These include the slow speed of kill, sensitivity to UV light, narrow host range, and pest resistance.

1.6.1 Slow speed of kill

Firstly, as reviewed in Beas-Catena et al., (2014), baculoviruses are hampered by their slow speed of kill compared to synthetic chemical insecticides. Baculoviruses take from 5 days to even 2 weeks to get the desired outcome compared to chemical insecticides which typically take hours to control pests at high levels. The slow speed of kill allows pests enough time to potentially infest crops before succumbing to virus infection.

1.6.2 Sensitivity to UV light

Sunlight UV radiation poses the most destructive environmental factor as it hampers the persistence of baculoviruses in the field (Ignoffo, 1992). Baculovirus biopesticides break down in the presence of direct sunlight due to the sensitivity to UV light degradation.

1.6.3 Narrow-host specificity

The narrow host specificity of baculovirus also presents a limitation as high production costs are acquired in virus production (Hutchinson, 2021). The products are specific to one pest thus targeting a small market. In vivo production still plays a huge role in large-scale of baculovirus biopesticides. This is due to the difficulties of mass-producing viruses (Grzywacz, 2017). This occurs by the infection of healthy larvae. Due to the high costs and technical difficulties associated with in vitro production, alternative methods to produce baculoviruses are still sought after. The production of dual pesticides has the potential to reduce the high production costs associated baculovirus biopesticides (Sanches et al., 2021). This alternative option offers biopesticides to target two or more pests which may occur simultaneously in the field. The effects of mixed infections in the potential to improve the use of baculovirus biopesticides is discussed in the section below.

1.7 Strategies to improve the efficacy of baculoviruses

1.7.1 Bioprospecting

The research is ongoing to find new measures to improve the control strategies against *T. leucotreta*. One way to combat the shortcomings of baculoviruses is by searching for novel baculovirus isolates. New control measures are required to supplement CrleGV-SA, which has been used as an effective biocontrol agent in the field for more than 15 years against *T.*

leucotreta (Moore et al., 2004; Moore, 2002; Moore et al., 2015). The work by Opoku-Debrah et al., (2013) led to the isolation of five CrleGV-SA isolates which are sufficiently virulent for the use against *T. leucotreta*. The recent isolation and characterisation of CrpeNPV have offered additional control measures against *T. leucotreta* and other important agricultural pests (Marsberg, 2016). CrpeNPV was determined to be more virulent against *T. leucotreta* in comparison to CrleGV-SA and has now become commercially available under the trade names Mutlimax™ and Codlmax™ for the control of *T. leucotreta* and other agricultural pests (Marsberg, 2016; Taylor, 2021; Thackeray, Personal communication, 2023).

1.7.2 Genetic modification

One way to combat the slow speed of baculoviruses is through genetic modification. This can be done by adding genes from different organisms or deleting or inactivating genes which effectively reduce the speed of kill and thus improve the virulence of baculoviruses. In the study by Chang et al., (2003), the addition of *Bacillus thuringiensis* (Bt) Cry1Ac toxin improved the virulence of AcMNPV against diamondback moth, *Plutella xylostella*. In the study, the Bt-modified AcMNPV mutant reduced the lethal dose and lethal time.

Another alternative option in genetic modification of baculoviruses is the deletion or activation of genes such as the baculovirus-encoded ecdysteroid-UDP glucosyl transferase (*egt*) gene (’Reilly & il ler,) . Ecdysteroids are larval hormones responsible for feeding and moulting. The *egt*-encoded enzyme inactivates the larval hormones and thus prevents the insect from moulting. Post baculovirus infection, the larval growth and viral load is increased (Hughes, 2013). The deletion or inactivation of the *egt* gene in baculovirus genomes increases the speed of kill but the viral load is decreased (Moore & Jukes, 2019; Simón et al., 2012). This poses limitations for the application in the field.

1.7.3 Serial passage assays through a heterologous host

There have been cases reported where the efficacy of baculoviral isolates was improved by serial passages in the heterologous host. For example, Graillot et al., (2016) enhanced the virulence of CpGV in *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), a heterologous host of CpGV. After 12 passages, the study improved the LC_{90} by 450-fold. The use of serial passages provides alternative methods to select for more efficient baculovirus strains for the greater management of pests in the field. Since *T. leucotreta* is a heterologous host of CrpeNPV, serial passage assays can be conducted to improve its virulence.

1.7.4 Baculovirus mixed infections

The research on evaluating the effects of baculovirus mixed infections offers the potential to overcome the above-mentioned shortcomings. There are many cases in which dual infections lead to synergistic effects in terms of lethal concentration and lethal time. The section below further discusses the potential of dual infections in improving the efficacy in the management of *T. leucotreta* or other agricultural pests in the field.

1.8 Co-infections involving baculoviruses.

1.8.1 Effect of baculovirus interactions on insect hosts

The potential to improve the use of baculoviruses through dual infections is a promising field of research as reviewed by Ferrelli & Salvador, (2023). Viral interactions can be categorised based on the point of view of the viruses involved in the interaction or the point of view of the infected host. Dual infections from the point of view of the viruses investigate the effect on virus-virus interactions (DaPalma et al., 2010). Co-infections from the point of view of the host analyses the effect of the interactions on the host, for example how it affects host mortality in comparison to single infections (Ferrelli & Salvador, 2023). In this study, the interactions will be characterised from the point of view of the insect host. These interactions are categorised as synergistic, antagonistic, or additive (Koppenhöfer & Kaya, 1997). Synergy is defined as the interactions between two or more viruses resulting in a greater combined effect in comparison to the sum of the viruses when applied individually. Antagonism occurs when the interaction of the viral mixture results in a lower effect compared to the sum of the individual applications of the viruses involved. Additive occurs when the interaction of the viruses has an equal or the same effect as the sum of the viruses when applied individually. These categories classify the effect of viral mixtures and can be applied when one or both of the viruses are infectious to the host (Ferrelli & Salvador, 2023). Ideally, from a biological point of view, a synergistic effect is sought after due to the potential to improve the lethal concentration and lethal time in the management of pests.

1.8.2 Interactions where the host is susceptible to the NPV and GV

Several studies reviewed by Ferrelli & Salvador, (2023) reported mixtures of NPV/GV where both viruses are homologous to the host in various combinations and outcomes are different. In the study by Whitlock, (1977), an antagonistic effect was observed when *H. armigera* larvae were simultaneously infected with Helicoverpa armigera granulovirus (HearGV) and HearNPV at equal concentrations. The mortality rates of the mixtures were lower in comparison to the single infections concluding that the HearNPV/HearGV mixture had an antagonistic effect on the host. Goto, (1990) observed detrimental effects in mixed infections

of Xestia c-nigrum granulovirus (XecnGV) and Xestia c-nigrum nucleopolyhedrovirus (XecnNPV) against 4th instar *Xestia c- nigrum* larvae. In the study, increased GV proportions in the GV/NPV mixture were suggested to interfere with the NPV infection. More recently, Wennmann et al., (2015) evaluated the interaction between Agrotis segetum nucleopolyhedrovirus B (AgseNPV-B) and Agrotis segetum granulovirus (AgseGV) against *Agrotis segetum* neonate larvae. They demonstrated an additive or neutral effect when combining the AgseNPV-B and AgseGV at various concentrations. At higher AgseNPV-B concentrations compared to the AgseGV, the mortality was a result of the NPV infection and at low NPV concentrations, the larvae died of the AgseGV infection. Wennmann et al., (2015) concluded both viruses acted independently as the sum of the combined effect was the same as the individual viruses when applied separately. On the other hand, Cuartas-Otálora et al., (2019) and Barrera et al., (2021) reported synergistic effects between mixtures of Spodoptera frugiperda nucleopolyhedrovirus (SfMNPV) and Spodoptera frugiperda granulovirus (SpfrGV) against *Spodoptera frugiperda* larvae and Spodoptera ornithogalli nucleopolyhedrovirus (SporNPV) and Spodoptera ornithogalli granulovirus (SporGV) against *Spodoptera ornithogalli* neonate larvae respectively. The synergistic effect in both studies was observed when the GVs proportion in the mixtures did not exceed 2.5%. Lower GV proportions in GV/NPV mixtures where the host is susceptible to the GV and NPV are often associated with synergistic effects (Ferrelli & Salvador, 2023).

Research has been conducted to evaluate combinations of CrleGV and CrpeNPV against *T. leucotreta* (Jukes, 2018; Taylor, 2021). Both CrleGV and CrpeNPV are known to infect *T. leucotreta,* as mentioned above. In the study by Jukes, (2018), 1:3 and 3:1 GV/NPV ratios were evaluated against *T. leucotreta* neonate larvae. In the GV dominant mixture, an additive effect in terms of lethal concentration was measured at LC_{50} but at LC_{90}, a synergistic effect was observed. However, the 1:3 GV/NPV mixture was measured to be synergistic at LC_{50} and LC_{90}. Taylor, (2021) observed a synergistic effect in a decrease in lethal concentration in a 1:1 GV/NPV mixture against *T. leucotreta* but there was an increase in lethal time. The increased lethal time is not beneficial for management strategies as the slower speed of kill allows for larval infestation in agricultural crops.

1.8.3 Interactions where the host is susceptible to the NPV and not the GV

Several studies were conducted investigating the effects of baculoviral mixtures where the host is susceptible to the NPV and not the GV. The work of Tanada & Hukuhara, (1971) initiated the research in identifying synergistic factors present in baculoviruses. Enhancin

genes are associated with synergistic factors in terms of lethal concentration as these genes often lead to the enhanced infectivity of other baculoviruses. These enhancin genes are common in GVs but are also found in NPVs as reviewed in Ferrelli & Salvador, (2023). Guo et al., (2007) evaluated the effect of XcenGV in combination with Spodoptera litura nucleopolyhedrovirus (SlNPV) against *Spodoptera litura* larvae. Xestia c-nigrum granulovirus does not infect *S. litura* but the dual infection demonstrated a synergistic effect in the decrease in the LC_{50} values. In the study, it was concluded that XcenGV had an enhancing effect on the SlNPV infection. Laboratory assays performed by Biedma et al., (2015) observed a similar effect when combining a 1:120 mixture of Autographa californica multiple nucleopolyhedrovirus (AgMNPV) and Epinotia aporema granulovirus (EpapGV) OBs against *Anticarsia gemmatalis* larvae. Also, in this case, *A. gemmatalis* is not susceptible to EpapGV but a synergistic interaction was observed in the combination. In another example, Lara Reyna et al., (2003) reported synergistic effects between Autographa californica multiple nucleopolyhedrovirus (AcMNPV) in combination with Trichoplusia ni granulovirus (TnGV) against *Trichoplusia ni* larvae. In some of the above-mentioned cases the hosts are not susceptible to the GVs. Higher GV proportions in mixtures where the host is not susceptible to the GV infection are associated with synergistic outcomes.

Ideally from a biocontrol perspective, a decreased lethal concentration and decreased lethal time are the synergistic effects that are sought after in baculoviral mixtures. The reduction in lethal concentration decreases the cost of OB production as less virus dosage is required to elicit larval mortality in the shortest amount of time. A reduction in lethal time prevents larval infestation in a short period of time. Should any of the viruses in this study be used in combination against *T. leucotreta*, the alternative option of using mixtures offer the application in a broader spectrum where more than one pest can be targeted under field conditions (Ferrelli & Salvador, 2023).

1.9 Motivation

The integrated pest management (IPM) has been implemented for the control of *T. leucotreta* and one important component of this is the use of baculoviruses. Although baculoviruses are highly effective in suppressing insect populations, their field application has been hindered by some shortcomings. One exciting field of research is investigating the effects of mixed infections against the target pest. In several cases mentioned above, synergistic effects in terms of lethal concentration and lethal time have been observed. For example, synergistic interactions were observed by Biedma et al., (2015) and Guo et al., (2007) where interactions

were evaluated between two baculoviruses but one was non-infectious to the target host. The knowledge of how viruses interact in mixed infections is not well understood. There have only been two mixed infection studies reported against *T. leucotreta*. Jukes, (2018) and Taylor, (2021) observed synergistic effects in terms of lethal concentrations but an antagonistic effect in increased lethal time was encountered. This has led to the motivation of the study to investigate mixed infections of CrleGV-SA in combination with CrpeNPV, CpGV-M, and HearNPV-Au to determine whether synergistic, additive, or antagonistic interactions can occur against *T. leucotreta*. The outcome of this study will equip researchers and farmers with the knowledge of how these viruses interact should they be used in combination against *T. leucotreta*.

1.10 Aim and objectives

The project aims to evaluate mixtures between CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au to determine whether synergistic, antagonistic, or additive interactions can occur against *T. leucotreta* neonate larvae. The project aims to determine whether any of the combinations tested can provide enhanced lethal concentrations for improved management of *T. leucotreta* in the field.

The specific objectives were to: 1) design oligonucleotides targeting unique regions in CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences for PCR amplification, 2) obtain viral stock samples (CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au) and perform occlusion body purification, DNA extraction, and PCR amplification for the selected genome regions, 3) evaluate the virulence of each virus alone and in comparison with mixtures against *T. leucotreta* neonate larvae by conducting laboratory biological assays and 4) determine the presence of target viruses by performing PCR amplification from the collected *T. leucotreta* cadavers from both single and mixed infections.

1.11 Overview of chapters

Chapter 2 describes the design of oligonucleotides targeting unique regions in CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences. Diseased *C. peltastica*, *C. pomonella,* and *H. armigera* larval cadavers infected with CrpeNPV, CpGV-M, and HearNPV-Au were received from River Bioscience (Pty) Ltd (Gqeberha, South Africa). CrleGV-SA was quantified in the laboratory by infecting 3rd instar *T. leucotreta* with CrleGV-SA using surface-dose biological assays. A crude extraction was used to obtain the OBs, and this was followed by DNA extraction. The unique oligonucleotides were tested on

each genomic DNA template (CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au) to determine if the samples contained the desired viruses.

Chapter 3 reports on the enumeration using darkfield microscopy and counting chamber of each viral stock (CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au) and the biological activity of each virus alone against *T. leucotreta* neonate larvae.

Chapter 4 reports on the biological activity of CrleGV-SA in combination with CrpeNPV, CpGV-M, and HearNPV-Au against *T. leucotreta* neonate larvae. The lethal concentrations from the single and mixed infections were evaluated and compared.

Chapter 5 reports on the presence of the target viruses using PCR amplification. A modified CTAB DNA extraction method was used to extract DNA from neonate-sized *T. leucotreta* larval cadavers collected from the biological assays in both single and mixed infections. The unique oligonucleotides designed in **Chapter 2** were tested against each DNA template to determine the presence of the target viruses. The purpose of this chapter was to investigate the cause of larval mortality using molecular analysis.

Chapter 6 is an overall general discussion of the results obtained in the previous chapters and the implications it has on the integrated pest management (IPM) program in the control of *T. leucotreta*. This chapter also includes a discussion for future work and recommendations to improve the results obtained from this study.

Chapter 2
Design of oligonucleotide sets specifically targeting genome regions of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au isolated from diseased larval cadavers

2.1 Introduction

Registered products such as Cryptogran® (River Bioscience, South Africa) and Cryptex® (Andermatt-Biocontrol AG, Switzerland) use the Cryptophlebia leucotreta granulovirus South African isolate (CrleGV-SA) as their active agent in the control of false codling moth, *Thaumatotibia leucotreta* (Kessler & Zingg, 2008; Moore et al., 2004). Recently Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) has been commercialised under the trade names Multimax™ and Codlmax™ (River Bioscience, South Africa) to control *T. leucotreta* and other agricultural pests (Thackeray, Personal communication, 2023). Although commercialised products are available against *T. leucotreta*, improvements are required to increase their effectiveness. The interaction of two viruses in mixed baculovirus interactions against the same larval host offers the potential to improve the application of baculoviruses as biopesticides in the field (Ferrelli & Salvador, 2023). Here the effect of mixed baculovirus interactions between CrleGV, CrpeNPV, Cydia pomonella granulovirus (CpGV), and Helicoverpa armigera nucleopolyhedrovirus (HearNPV) against *T. leucotreta* was evaluated.

The overall aim of this chapter was to develop a PCR-based method to identify the presence of baculoviruses in single and mixed infections. Therefore, oligonucleotides specifically targeting unique regions in the baculoviral genomes of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were designed. The unique oligonucleotides were used in PCR reactions to detect the baculoviral sequences and determine if the viruses that were used in this study were present in the samples provided. These unique oligonucleotides were used alongside universal *polh* oligonucleotides, which served as a positive control. The *polh* oligonucleotides target the polyhedrin/granulin gene which is one of the highly conserved genes in all sequenced baculovirus species (Lange et al., 2004).

Oligonucleotides can be designed manually or using available software tools through bioinformatics (Ye et al., 2012). Several online software tools aid in oligonucleotide design, a commonly used application is the Primer3 program which uses a diversity of parameters in designing oligonucleotides (Rozen & Skaletsky, 2000). Although widely used, the Primer3 program lacks target analysis thus users must additionally check for target specificity. Ye et

al., (2012) designed Primer-BLAST to address such shortcomings. Unlike the BLAST program, which uses a local alignment algorithm tool, Primer-BLAST incorporates a global alignment algorithm tool which allows for the complete return of matching information between oligonucleotides and target sequences. The Primer-BLAST program uses 2 modules for functionality namely: Primer3 to generate candidate oligonucleotide pairs dependent on the input sequence and the BLAST program in addition to the Needleman-Wunsch (NW) global alignment algorithm to check for specificity (Needleman & Wunsch, 1970; Ye et al., 2012). Geneious software is an additional bioinformatics tool that users can use to design and test oligonucleotides for specificity. The software provides functionality for obtaining data from various databases, the ability to analyse data, and tools to visualise results (Kearse et al., 2012).

The genome sequences of the baculoviruses which were tested in both single and mixed infections against *T. leucotreta* were obtained from the NCBI Genbank (Clark et al., 2016). CrleGV-SA, the South African isolate, was characterised by Singh et al., (2003) and later sequenced by van der Merwe et al., (2017) (GenBank accession number: MF 974563.1) CrpeNPV was characterised and sequenced by Marsberg et al., (2018) (GenBank accession number: NC_055500.1). CpGV-M (Lacey et al., 2008; Tanada, 1964) the Mexican isolate, which was discovered and isolated in Mexico in 1963 has been used as an active agent in CpGV-based products in many countries and was later fully sequenced by Gebhardt et al., (2014) (GenBank accession number: KM217575.1). HearNPV-Au, was isolated in Australia with the complete genome sequenced by Zhang et al., (2014) (GenBank accession number: JN584482.1), and was also used for this study.

Diseased larval cadavers infected with the CrpeNPV, CpGV-M, and HearNPV were provided by River Bioscience (Pty) Ltd (Gqeberha, South Africa). OBs can be extracted using the sucrose or glycerol centrifugation method but for this study, a crude OB extraction method that involves low and high-speed centrifugation was used (Grzywacz et al., 2004). Before the PCR amplification, the genomic DNA template for each virus was extracted using the CTAB method although various methods such as commercial kits could have similarly been used. Post PCR amplification, the amplicons were sequenced, and manually trimmed using Geneious R11.1.5, although automatic trimming using ABI base recall can be used. To confirm the identity of the sequences, the trimmed sequences were submitted to BLAST analysis and further aligned using ClustalW alignment. ClustalW has been widely used to perform accurate pairwise alignments within a short period (Chenna et al., 2003).

This chapter aimed to design a set of oligonucleotides specifically targeting unique regions in the baculoviral genomes of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au to detect the presence of the viruses in the samples provided. To achieve the aim, the initial specific objectives included (i) retrieving genome sequences from NCBI Genbank for CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au to use as templates, (ii) designing oligonucleotides that target unique regions in the genome sequences of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au using Primer-BLAST and (iii) to test for target specificity using Geneious R11 software (11.1.5). Subsequent objectives were (iv), crude OB extraction from the samples provided, (v) genomic DNA extraction and (vi) testing the set of unique oligonucleotides on the gDNA templates by standard PCR.

2.2 Methods and Materials

2.2.1 Oligonucleotide design targeting unique regions in CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences using Primer-BLAST

The genomic sequences for Cryptophlebia leucotreta granulovirus (CrleGV-SA) (MF 974563.1), Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) (NC_055500.1), Cydia pomonella granulovirus (CpGV-M) (KM 217575.1) and Helicoverpa armigera nucleopolyhedrovirus (HearNPV-Au) (JN 584482.1) were retrieved from NCBI GenBank. Oligonucleotides targeting unique regions within the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genomes were designed using Primer-BLAST (Ye et al., 2012). On the Primer-BLAST interface (**Figure 2.1**), the NCBI accession number of each baculovirus was inserted in the PCR template (**Figure 2.1 A**). The oligonucleotide design range was limited to the first 50 000 bp of each genome sequence (**Figure 2.1 B**). The size of each PCR product for each baculovirus genome sequence was designed to fall within the 600 to 800 bp range (**Figure 2.1 C**). The database was changed to RefSeq representative genomes, and the organism was changed to each respective baculovirus species as the oligonucleotide design was performed separately for each genome sequence (**Figure 2.1 D**). Primer-BLAST provided candidate oligonucleotide pairs for each search and one oligonucleotide pair was selected for each baculovirus species.

The Primer-BLAST interface allows users to design new oligonucleotides by inserting input sequences or testing pre-existing oligonucleotides for their specificity. The Primer-BLAST user interface is highly flexible as it allows users to change various parameters depending on their requirements (**Figure 2.1**).

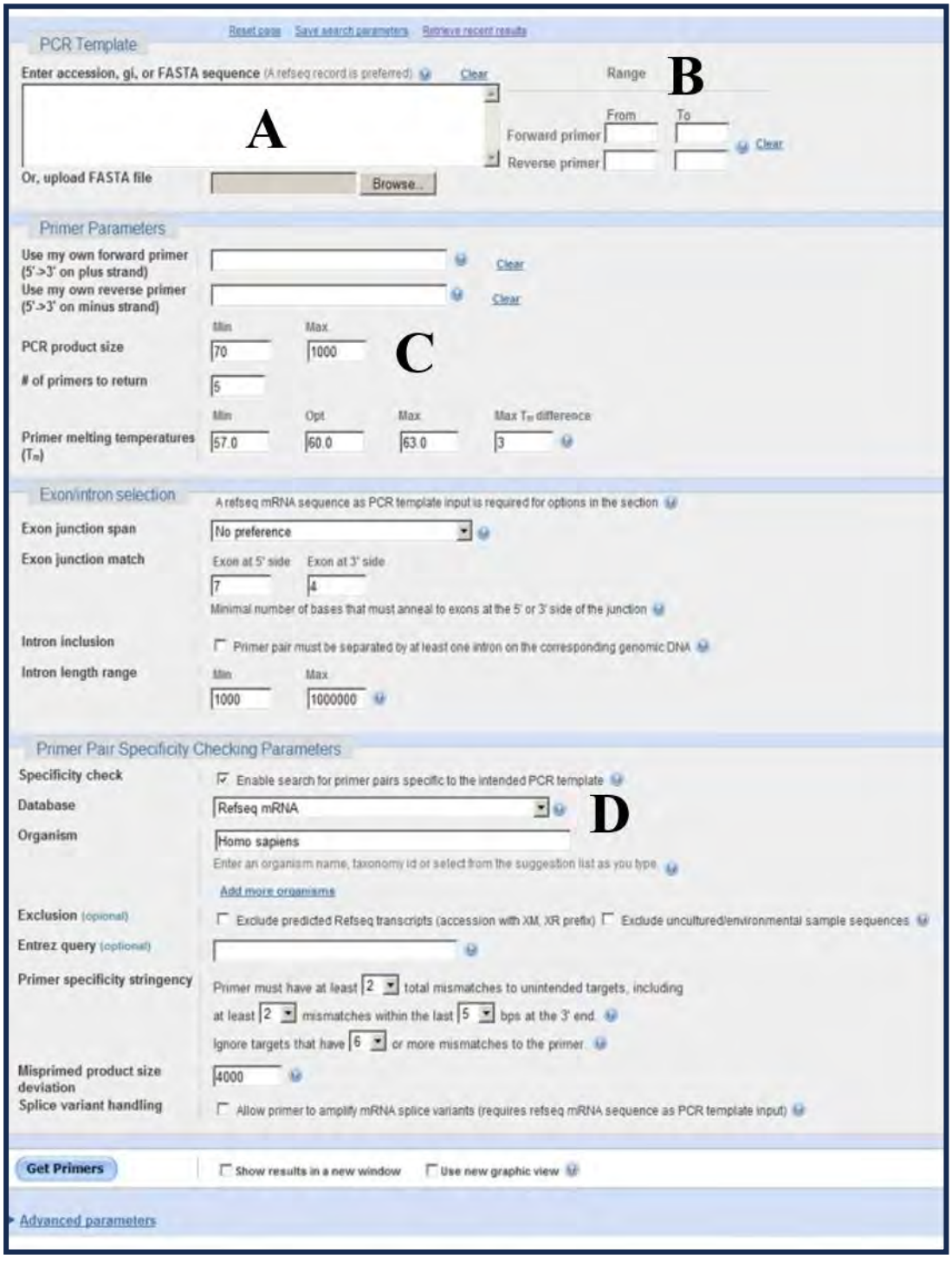


Figure 2.1 The Primer-BLAST interface (Ye et al., 2012)

The CrleGV oligonucleotide pair target the region within a *hypothetical protein* CDS, amplifying a 687 bp amplicon (**Table 2.1**). The CrpeNPV oligonucleotide pair amplify a 756 bp amplicon targeting the *p74/pif0* CDS/*orf* 14 CDS region within the CrpeNPV genome sequence. The CpGV oligonucleotide pair target regions within the *orf37* CDS/*orf39* CDS gene, amplifying a 764 bp amplicon. The HearNPV oligonucleotide pair amplify a 794 bp amplicon which target regions within a *hypothetical protein* CDS of the HearNPV-Au genome sequence. Geneious R11 software (11.1.5) was used to test the oligonucleotides *in silico* for specificity.

Table 2.1 Oligonucleotides targeting unique regions in CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences. prPH-1 and prPH-2 oligonucleotides which target the polyhedrin/granulin gene in baculovirus genome sequences (Lange et al., 2004).

Name	Length (bp)	qu (5'- 3')	Amplicon size (bp)	Tm
CrleGV-F	37	GTAAAACGACGGCCAGTTTGTGACGGGTGTTACAGGT	687	59.17
CrleGV-R	37	CAGGAAACAGCTATGACTCTGGGACGTGACCAAAAAC		58.33
CrpeNPV-F	37	GTAAAACGACGGCCAGTCTCCTTCTCGTGTGGCCATT	756	60.04
CrpeNPV-R	37	CAGGAAACAGCTATGACTCGACTTCGCGATTAGGTCG		59.97
CpGV-F	35	GTAAAACGACGGCCAGTTTGCGCAAACTGGGTGGT	764	60.44
CpGV-R	37	CAGGAAACAGCTATGACTGTGTTCCACATGTACCCCG		59.96
HearNPV-F	37	GTAAAACGACGGCCAGTTCGCCGCTTGTTGTTTGAAG	794	59.97
HearNPV-R	37	CAGGAAACAGCTATGACACTGCTTTCGCCGTTTTCAC		59.97
prPH-1	33	TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT	507-510	38 – 54
prPH-2	33	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT		38 – 52

*The underlined sequences are M13 universal forward and reverse adapter sequences that were engineered into the unique and *polh* oligonucleotides to allow for sequencing in the forward and reverse direction.

2.2.2 Sample collection

Diseased *C. peltastica, C. pomonella, and H. armigera* larval cadavers infected with CrpeNPV, CpGV-M, and HearNPV-Au were supplied by River Bioscience (Pty) Ltd (Gqeberha, South Africa) from which OBs were extracted using a crude OB extraction method detailed below. Surface-dose laboratory bioassays were set up infecting third instar *T. leucotreta* larvae with CrleGV-SA (2×10^5 OBs/ml). The biological assays were set up using 24-well plates with the *T. leucotreta* diet placed in each well. 50 µl of the virus suspension was used to inoculate each well. A total of 24 3rd instar *T. leucotreta* larvae were infected.

Eleven infected *T. leucotreta* larvae were collected after 7 days but only 4 were used in crude OB extraction.

2.2.3 Virus Crude Extraction

The virus crude extraction protocol was taken from Marsberg, (2016), which was adapted from Grzywacz et al., (2004) with some minor modifications. For each virus, a total of 3-4 infected larval cadavers were transferred to a sterile 1.5 ml microcentrifuge tube and homogenised in 1 ml of 0.07% (w/v) sodium dodecyl sulphate (SDS) using a sterile pipette tip. The virus samples were vortexed until the solution was homogenous before centrifugation at 100 ×g for 30 seconds using a MiniSpin® desktop centrifuge (Eppendorf, Germany). For each sample, the supernatant was transferred to a clean 2 ml microcentrifuge tube and the pellet was resuspended in 1 ml of 0.07% (w/v) SDS. The sample was centrifuged at 100 ×g for 30 seconds and the supernatant was again collected and pooled with the supernatant in the 2 ml microcentrifuge tube. The pooled supernatant was centrifuged at 10 000 ×g for 10 minutes after which the supernatant was discarded, and the pellet was resuspended in 1 ml of 0.07% (w/v) SDS. The resuspended pellet was centrifuged at 10 000 ×g for 10 minutes after which the supernatant was again discarded. The pellet was resuspended in 1 ml of ddH_2O and centrifuged a third time at 10 000 ×g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in a final volume of 200 µl of ddH_2O. The occlusion bodies for each virus were stored at -20°C for long-term storage.

2.2.4 Genomic DNA Extraction

The DNA extraction was performed using the CTAB method (Aspinall et al., 2002) with minor modifications. For each virus, a dilute sample of OBs totalling a volume of 200 µl (50 µl occlusion bodies and 150 µl ddH_2O) was prepared and transferred to a 2 ml microcentrifuge tube. The sample was vortexed and 90 µl Na_2CO_3 (sodium carbonate) 1 M was added after which the sample was incubated at 37°C for 30 minutes. Following, a volume of 120 µl Tris-HCL (1M; pH 6.8), 90 µl 10% (w/v) SDS, and 25 µl proteinase K (25 mg/ml) was added before incubating at 37°C for 30 minutes. A volume of 10 µl RNase A (10 mg/ml) was added to the sample and incubated at 37°C for 30 minutes. After the incubation period, the sample was centrifuged at 13 148 ×g for 3 minutes using a MiniSpin® desktop rotor (Eppendorf, Germany). A volume of 400 µl (70°C) CTAB (2% w/v CTAB, 10 mM Tris (pH 8.0), 20 mM Na_2EDTA, 1.4 M NaCl) was added and the sample was incubated at 70°C for 45 minutes and inverted regularly. 400 µl of pre-cooled chloroform (4°C) was added, inverted regularly, and centrifuged at 6 708 ×g for 10 minutes. The supernatant was

transferred into new 1.5 ml microcentrifuge tubes. To this, 400 µl of ice-cold isopropanol (-20°C) was added and the sample was incubated overnight at -20°C. The sample was centrifuged at 13 148 ×g for 20 minutes and the supernatant was discarded. A volume of 1 ml ice-cold ethanol (70%) was added to the pellet before centrifuging at 13 148 ×g for 5 minutes. The supernatant was discarded, and the pellet was air-dried before it was resuspended in 20 µl of autoclaved ddH_2O. Microcentrifuge tubes were labelled with the appropriate details and the genomic DNA (gDNA) was stored at -20°C for long-term storage.

2.2.5 Agarose gel electrophoresis

The gDNA was visualised using 1% agarose gel electrophoresis (AGE). To prepare the gel, 1 g Agarose LE powder (Benchmark, USA) was dissolved in 100 ml 1 × TAE (40mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA) before staining with 4 µl ethidium bromide (10 mg/ml). For each virus, 3 µl of extracted gDNA (the concentration ranged between 4,9 ng/µl – 47,2 ng/µl) were run on the agarose gel at 90 V for 30 minutes alongside 6 µl GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, USA). The gel was visualised using a ChemiDoc™ XRS+ (BioRad, USA), and the images were captured by the Image Lab™ software (BioRad, USA).

2.2.6 PCR amplification targeting unique regions in the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences

A polymerase chain reaction (PCR) was set up to test the four oligonucleotide sets (**Table 2.1**) against gDNA extracted from each of the four viruses namely CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au to confirm specificity. Each PCR reaction consisted of 12.5 µl of Taq DNA Polymerase 2× Master Mix Red (Ampliqon, Denmark), 1 µl of 10 µM of forward and 1 µl of 10 µM reverse oligonucleotides and 1 µl of gDNA template. The PCR reactions were made up to 25 µl using ddH_2O. A no template control (NTC) reaction was prepared which consisted of additional ddH_2O instead of the gDNA template and a positive control was prepared using the *polh* universal oligonucleotides (**Table 2.1**) (Lange et al., 2004). The PCR reaction had an initial denaturation step at 95°C for 3 minutes. Following, the samples underwent 30 cycles of 95°C for 1 minute, 56°C for 30 seconds, 72°C for 1 minute, and lastly a final elongation step at 72°C for 3 minutes. The PCR amplicons were visualised using 1% AGE (section 2.2.5) with 5 µl of each PCR amplicon loaded into each well, respectively.

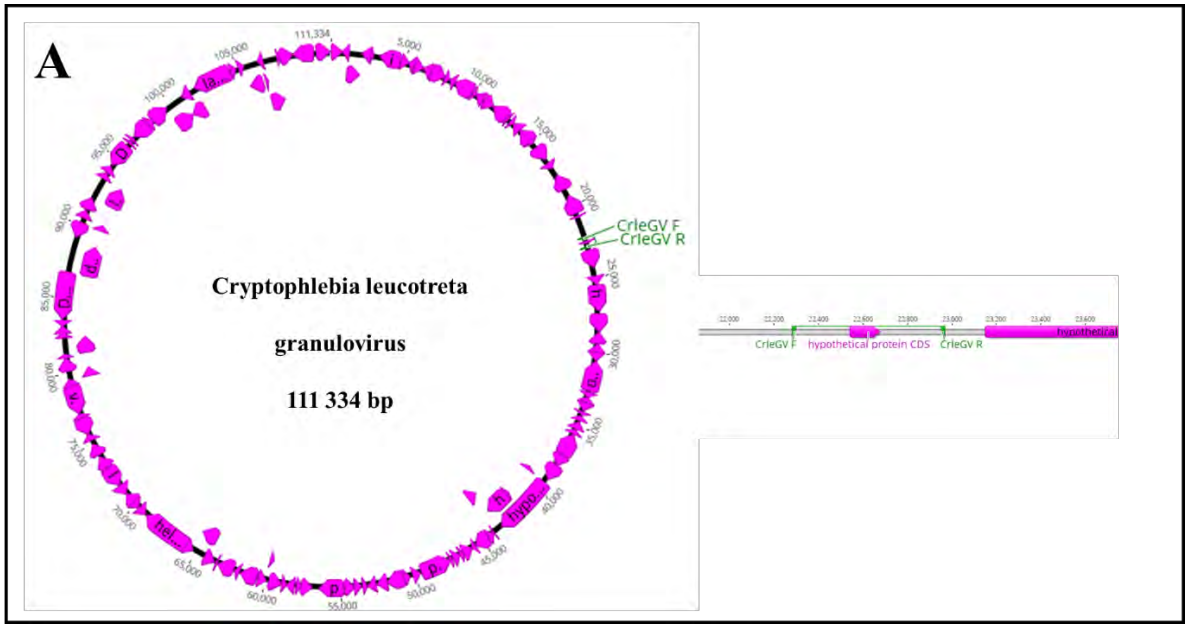
2.2.7 Sanger sequencing of the PCR amplicons and BLAST

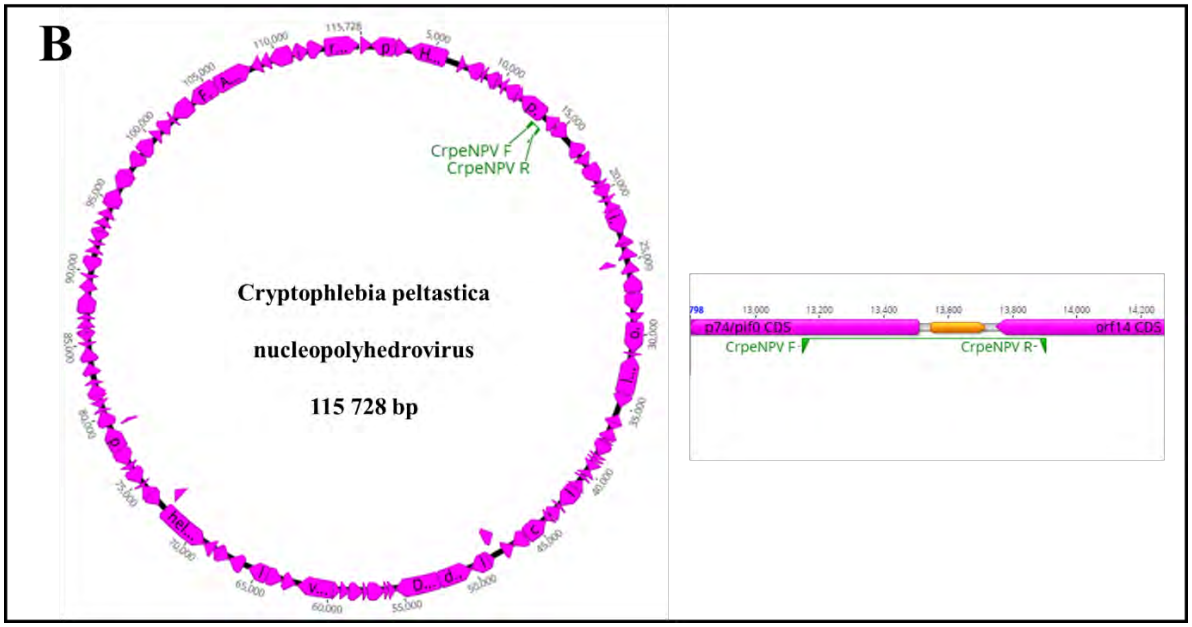
The PCR amplicons were sequenced by Inqaba Biotechnical Industries (Pty) Ltd in the forward and reverse direction using the M13 forward and M13 reverse sequencing oligonucleotides, which bind to the respective M13 sequences engineered into the oligonucleotides listed in **Table 2.1**. The first 10 base pairs at the beginning of each sequence and the last 10 base pairs at the end of each sequence were manually trimmed off using the Geneious R11.1.5 software. Ambiguities were manually corrected based on sequence chromatograms after which each sequence was submitted to NCBI BLAST and optimised to search against highly similar sequences (Megablast). This was followed by a ClustalW alignment in Geneious R11.1.5 of each sequence against the respective target region, which was extracted from each reference sequence obtained from GenBank.

2.3 Results

2.3.1 Oligonucleotide *in silico* target specificity test using Geneious R11.1.5 software

The position where each oligonucleotide pair binds on the circular genome and the resulting target region (indicated in green) are shown in **Figure 2.2.** CrleGV-F/CrleGV-R oligonucleotides target the region within a *hypothetical protein* CDS of CrleGV-SA genome sequence amplifying a 687 bp amplicon between 22283 bp – 22969 bp in the genome sequence (**Figure 2.2 A**). CrpeNPV-F/CrpeNPV-R oligonucleotides target regions within the *p74/pif0* CDS/*orf* 14 CDS of the CrpeNPV genome sequence amplifying a 756 bp amplicon between 13148 bp – 13903 bp in the genome sequence (**Figure 2.2 B)**. CpGV-F/CpGV-R oligonucleotides target the regions within the *orf37* and *orf39* CDS of CpGV-M genome sequence amplifying a 764 bp amplicon between 33026 – 33789 bp in the genome sequence (**Figure 2.2 C**). Lastly, the HearNPV-F/HearNPV-R oligonucleotides target the region within the *hypothetical protein* CDS of HearNPV-Au genome sequence amplifying a 794 bp amplicon between 5158 – 5951 bp in the HearNPV-Au genome sequence (**Figure 2.2 D)**.


A
Cryptophlebia leucotreta
granulovirus
111 334 bp
CrleGV F
CrleGV R
CrleGV F
hypothetical protein CDS
CrleGV R


B
Cryptophlebia peltastica
nucleopolyhedrovirus
115 728 bp
CrpeNPV F
CrpeNPV R
p74/pif0 CDS
orf14 CDS
CrpeNPV F
CrpeNPV R

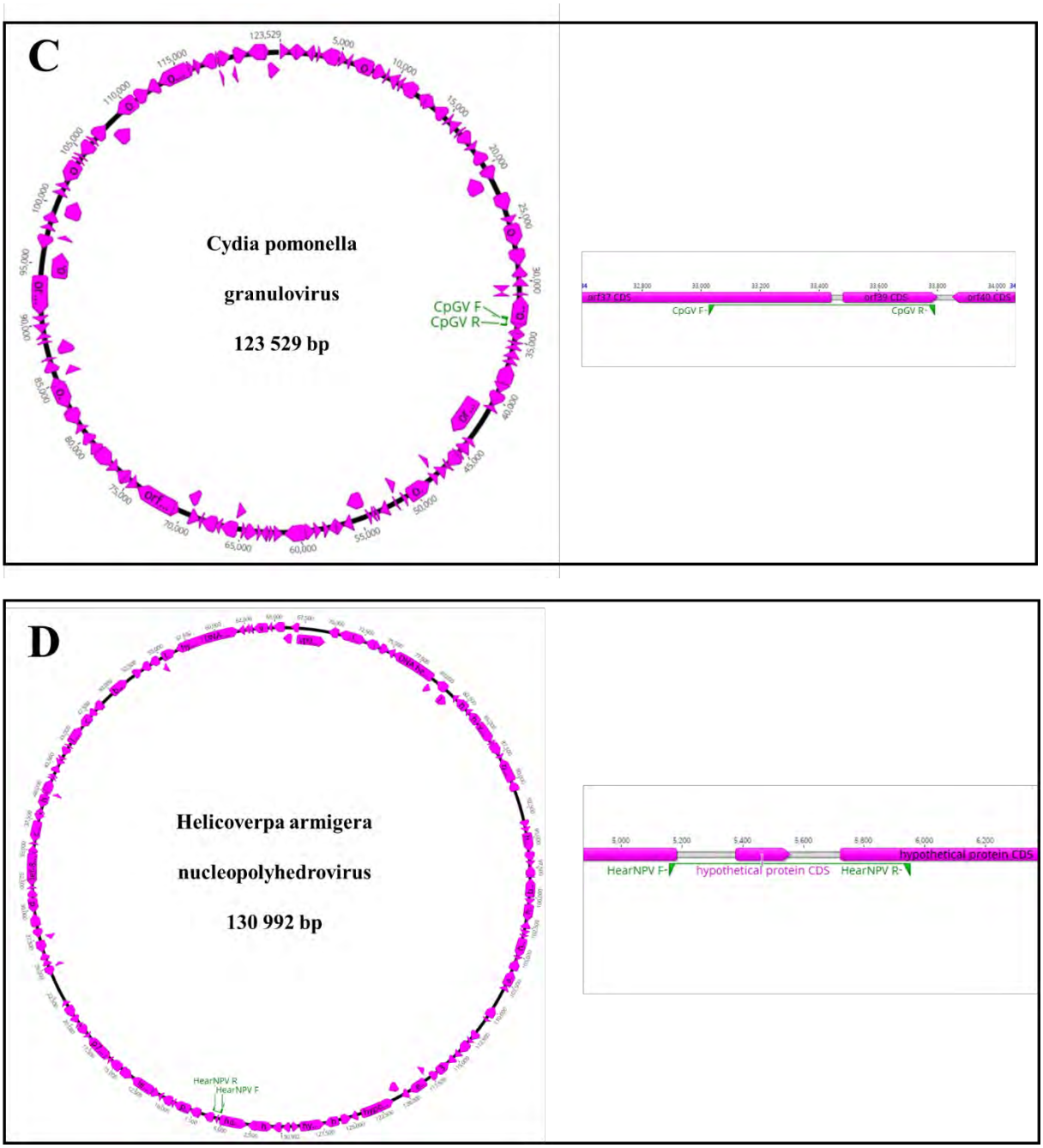


Figure 2.2 Visual representation of oligonucleotides targeting unique regions within the CrleGV-SA genome sequences A (MF 974563.1), CrpeNPV genome sequences B (NC 055500.1), CpGV-M genome sequences C (KM 217575.1) and HearNPV-Au genome sequences D (JN 584482.1). The target specificity test was performed using Geneious R11 software (11.1.5). The oligonucleotide binding sites are indicated in green.

2.3.2 Genomic DNA extraction

Following the CTAB gDNA extractions from CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au OBs, 1% AGE was used to resolve the bands, and images were captured. All 4 gDNA bands are above the 10 000 bp as shown in lanes 1, 2, 3, and 4 for each virus, respectively (**Figure 2.3**).

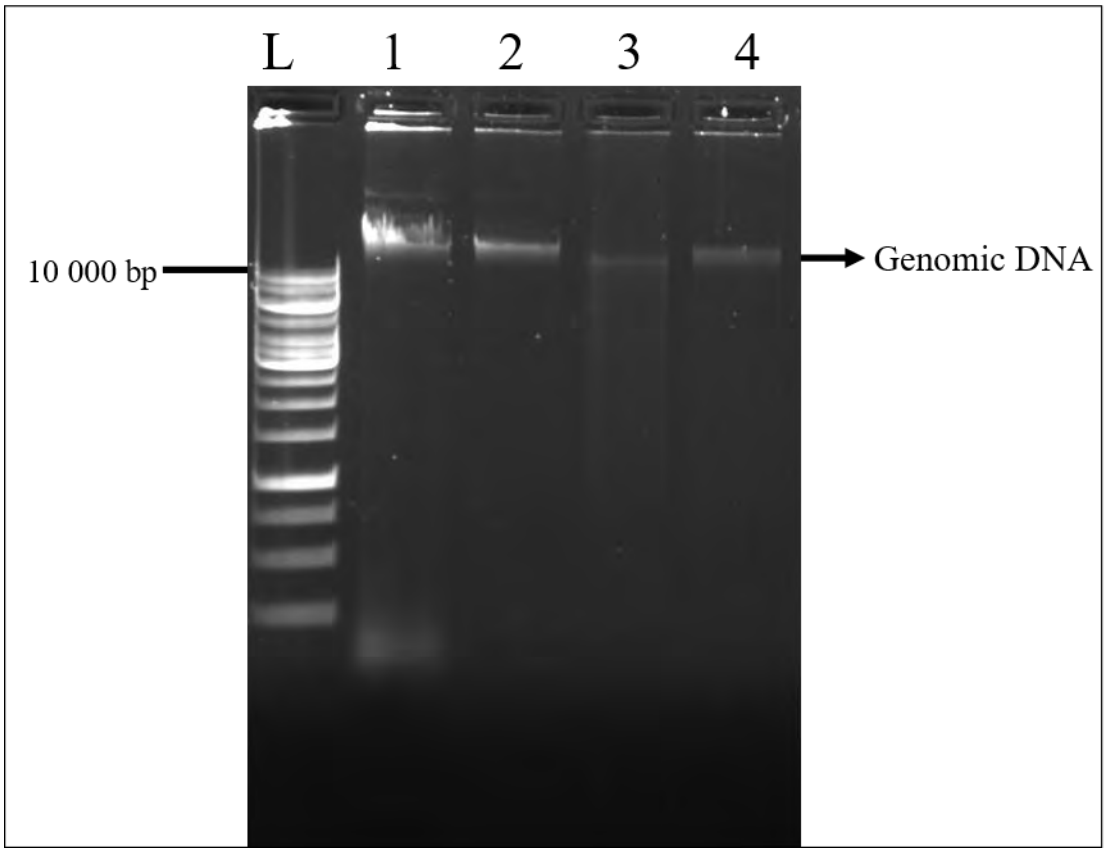


Figure 2.3 Genomic DNA extracted from purified occlusion bodies. Lane L – GeneRuler 1 kb DNA ladder; Lane 1 – CrleGV DNA template; Lane 2 – CrpeNPV DNA template; Lane 3 – HearNPV DNA template; Lane 4 – CpGV DNA template

2.3.3 PCR amplification targeting unique regions in the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genomes

Following DNA extraction from CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au OBs, the gDNA of each virus was used as a template to test the unique oligonucleotide sets (**Table 2.1**). The results are presented in **Figure 2.4**. The CrleGV template, CrleGV F/R oligonucleotides amplified a band between 500-750 bp (lane 1), and the *polh* positive control amplified a band slightly larger than 500 bp (lane 6) (**Figure 2.4 A**). No amplicons were observed in the no template control (lane 5). No PCR products were amplified using the

CrpeNPV F/R, CpGV F/R, and HearNPV F/R oligonucleotide sets shown in lanes 2, 3, and 4 respectively. For the CrpeNPV gDNA template (**Figure 2.4 B**) the CrpeNPV F/R oligonucleotides amplified a PCR product roughly 750 bp in length (lane 2) and the *polh* positive control amplified a faint band slightly larger than 500 bp (lane 6). No amplification was observed in the no template control (lane 5). No PCR products were amplified using the CrleGV F/R, CpGV F/R, and HearNPV F/R oligonucleotide sets shown in lanes 1, 3, and 4 respectively.

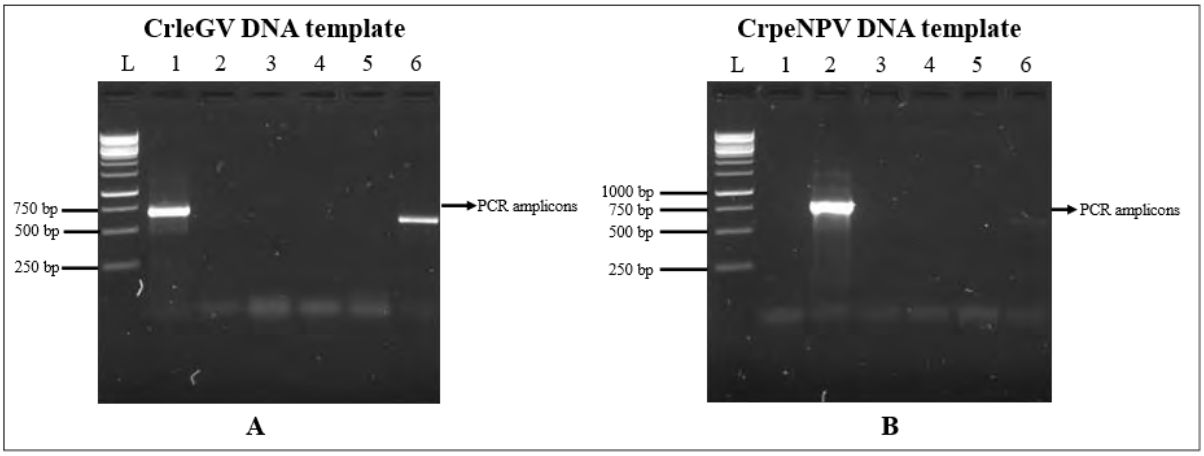


Figure 2.4 (A) PCR amplification using CrleGV gDNA as a template. Lane L – GeneRuler 1kb DNA ladder; Lane 1 – CrleGV oligonucleotides (687 bp); Lane 2 – CrpeNPV oligonucleotides; Lane 3 – CpGV oligonucleotides; Lane 4 – HearNPV oligonucleotides; Lane 5 – NTC; Lane 6 – polh oligonucleotides. (**B**) PCR amplification using CrpeNPV gDNA as a template. Lane L – GeneRuler 1kb DNA ladder; Lane 1 – CrleGV oligonucleotides; Lane 2 – CrpeNPV oligonucleotides (756 bp); Lane 3 – CpGV oligonucleotides; Lane 4 – HearNPV oligonucleotides; Lane 5 – NTC; Lane 6 – polh oligonucleotides

In the CpGV template, CpGV F/R oligonucleotides amplified a PCR product within the 750 bp range (lane 3) and the *polh* positive control amplified a band slightly larger than 500 bp (lane 6) (**Figure 2.5 A**). No amplicons were observed in the no template control (lane 5). No PCR products were amplified using the CrleGV F/R, CrpeNPV F/R, and HearNPV F/R oligonucleotide sets shown in lanes 1, 2, and 4 respectively. For the HearNPV gDNA template (**Figure 2.5 B**) the HearNPV F/R oligonucleotides amplified a PCR product slightly larger than 750 bp (lane 4) and the *polh* positive control amplified a faint band of roughly 500 bp (lane 6). No amplification was observed in the no template control (lane 5). No PCR

products were amplified in lanes 1,2 and 3 using the CrleGV F/R, CrpeNPV F/R, and CpGV F/R oligonucleotide sets respectively.

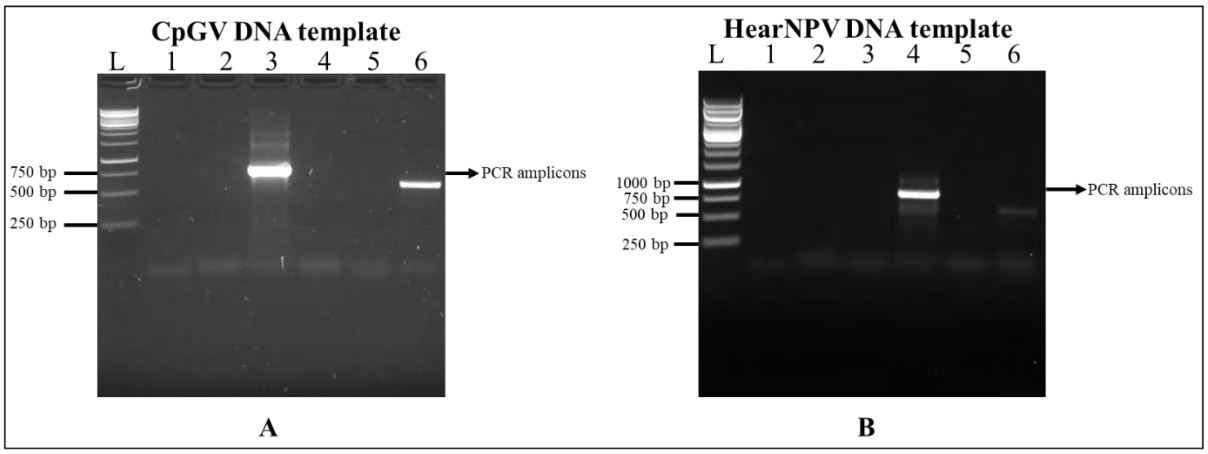


Figure 2.5 (**A**) PCR amplification using CpGV gDNA as a template. Lane L – GeneRuler 1kb DNA ladder; Lane 1 – CrleGV oligonucleotides; Lane 2 – CrpeNPV oligonucleotides; Lane 3 – CpGV oligonucleotides (764 bp); Lane 4 – HearNPV oligonucleotides; Lane 5 – NTC; Lane 6 – polh oligonucleotides. (**B**) PCR amplification using HearNPV gDNA as a template. Lane L– GeneRuler 1kb DNA ladder; Lane 1 – CrleGV oligonucleotides; Lane 2 – CrpeNPV oligonucleotides; Lane 3 – CpGV oligonucleotides; Lane 4 – HearNPV oligonucleotides (794 bp); Lane 5 – NTC; Lane 6 – polh oligonucleotides.

2.3.4 Sanger sequencing of the PCR amplicons and BLAST

The CrpeNPV and CpGV-M PCR products were sequenced in the forward direction using the M13 forward sequencing oligonucleotide, and the CrleGV-SA and HearNPV-Au PCR products were sequenced in the reverse direction using the M13 reverse sequencing oligonucleotide. After the sequences were manually trimmed, the sequences were submitted to BLAST and searched against highly similar sequences (megablast). The top 3 matches (if available) from the BLAST search are indicated in **Table 2.2, 2.3, 2.4** and **2.5** for CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au respectively. Following this, the sequences were aligned against the target regions using the ClustalW alignment tool in Geneious R11.1.1. The results from the alignments are included in Appendix A.

Table 2.2 The top matches resulting from the CrleGV-SA BLAST search

Description	Scientific name	Query Cover	Percentage Identity	Accession number
1. Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	*Cryptophlebia leucotreta granulovirus*	94%	97.34%	MF974563.1
2. Cryptophlebia leucotreta granulovirus, complete genome	*Cryptophlebia leucotreta granulovirus*	94%	83.94%	NC_005068.1

Table 2.3 The top matches resulting from the CrpeNPV BLAST search

Description	Scientific name	Query Cover	Percentage Identity	Accession number
1. Cryptophlebia peltastica nucleopolyhedrovirus, complete genome	*Cryptophlebia peltastica nucleopolyhedrovirus*	98%	100%	NC_055500.1
2. Spodoptera littoralis nucleopolyhedrovirus isolate SpliNPV-Tun2, complete genome	*Spodoptera littoralis nucleopolyhedrovirus*	8%	86.89%	MG958660.1
3. Spodoptera littoralis NPV isolate AN1956, complete genome	*Spodoptera littoralis nucleopolyhedrovirus*	8%	86.89%	NC_038369.1

Table 2.4 The top matches resulting from the CpGV BLAST search

Description	Scientific name	Query Cover	Percentage Identity	Accession number
1. Cydia pomonella granulovirus isolate CpGV-M, complete genome	*Cydia pomonella granulovirus*	98%	98.64%	KM217575.1
2. Cydia pomonella granulovirus isolate CpGV-ZY, complete genome	*Cydia pomonella granulovirus*	98%	98.64%	MN696171.1
3. Cydia pomonella granulovirus isolate CpGV-KS2, complete genome	*Cydia pomonella granulovirus*	98%	98.64%	MN696168.1

Table 2.5 The top matches resulting from the HearNPV BLAST search

Description	Scientific name	Query Cover	Percentage Identity	Accession number
1. Helicoverpa SNPV AC53, complete genome	*Helicoverpa SNPV AC53*	98%	96.88%	KJ909666.1
2. Helicoverpa armigera nucleopolyhedrovirus strain WV104, complete genome	*Helicoverpa armigera nucleopolyhedrovirus*	98%	96.62%	MT810812.1
3. Helicoverpa armigera nucleopolyhedrovirus G4, complete genome	*Helicoverpa armigera nucleopolyhedrovirus*	98%	96.62%	NC_002654.2

2.4 Discussion

This chapter aimed to detect CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in the samples provided using unique oligonucleotides in standard PCR reactions. While oligonucleotides have been designed by other researchers against the viruses used in this study (Jukes 2018, Motsoeneng 2016), they were not necessarily designed to be used in the presence of non-target baculoviral DNA and may exhibit non-specificity binding. Additional optimisation and testing would have been required to increase specificity. Therefore, the advantage of designing genomic specific oligonucleotides does not require additional PCR optimisation, thus reducing time and costs. Oligonucleotides targeting unique regions in baculoviral genomes of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were designed using Primer-BLAST. Geneious R11 software (11.1.5) was used to further test the oligonucleotides for specificity *in silico*. The genome sequences for CrleGV-SA (MF 974 563.1), CrpeNPV (NC_055500.1), CpGV-M (KM 217575.1), and HearNPV-Au (JN584482.1) were retrieved on the NCBI Genbank and used as reference sequences. When using Primer-BLAST, the template length in the oligonucleotide design is limited to 50 000 bp (Ye et al., 2012). Since baculovirus genomes range from 80 to 180 kbp (van Oers & Vlak, 2007), any region limited to 50 000 bp could have been used. For this research project, the first 50 000 bp upstream of the granulin or polyhedrin gene was chosen. Primer-BLAST generates multiple candidate oligonucleotide pairs for each sequence of which any pair could have been selected to target different unique regions in each genome sequence. The genes within the target regions were not of interest and did not influence the decision in oligonucleotide selection. The main purpose was to select oligonucleotide pairs that bind to the respective target genome and not any other genomes from the viruses tested in this study. For this research, oligonucleotide pairs of CrleGV F/R targeting regions of a *hypothetical protein* CDS of the CrleGV-SA genome sequence were chosen. The CrpeNPV F/R oligonucleotide pairs target the *p74*/*pif*0 CDS/*orf* 14 CDS region within the CrpeNPV genome sequence were chosen for this study. Oligonucleotide pairs of the CpGV F/R and HearNPV F/R target the region within the *orf37* and *orf39* CDS of the CpGV-M genome sequence and a *hypothetical protein* CDS of HearNPV-Au genome sequence were chosen for this research, respectively.

The OBs of CrpeNPV, CpGV-M, and HearNPV-Au were extracted using the crude extraction protocol from diseased *C. peltastica*, *C. pomonella,* and *H. armigera* larval cadavers that

were received from River Bioscience (Pty) Ltd. The OBs of CrleGV-SA were extracted from *T. leucotreta* cadavers after being infected with CrleGV-SA by inoculating an artificial diet with the virus using a surface dose methodology in the laboratory. Similar studies involving the bulk up of baculovirus using surface dose biological assays have been performed (Bennett, 2022; Mwanza, 2019). Following the virus extractions, the OBs were subjected to the CTAB method to extract the gDNA. All extracted gDNA samples were observed to run above 10 000 bp marker as expected since baculoviruses have large genomes ranging from 80 to 180 kbp in length (van Oers & Vlak, 2007). These results are consistent with those obtained by Jukes, (2018) and Taylor, (2021) where DNA was extracted from CrleGV and CrpeNPV occlusion bodies.

To determine if the samples contained the viruses of interest, each gDNA template was tested against all four unique oligonucleotide sets. For the CrleGV-SA template, the AGE showed a band smaller than 750 bp, consistent with the expected size of 687 bp. Results for the CrpeNPV template produced a band of approximately 750 bp which was clearly visible and is consistent with the expected size of 756 bp. The CpGV-M template and HearNPV-Au templates produced bands larger than 750 bp which is consistent with the expected sizes of 764 bp and 794 bp, respectively. The *polh* oligonucleotides target regions within the *polyhedrin/granulin* gene which is one of the highly conserved genes in all sequenced baculovirus species (Lange et al., 2004). The *polh* oligonucleotides are degenerate. Some positions in the sequence have several possible bases characterising these oligonucleotides as degenerate (Kwok et al., 1994). The *polh* oligonucleotides amplify a 507 – 510 bp PCR product. The positive control for both CrleGV-SA and CpGV-M showed visible bands slightly larger than 500 bp, consistent with the expected sizes. However, the positive control for the CrpeNPV and HearNPV-Au produced faint bands, which could be due to differences in template DNA resulting in poor oligonucleotide binding and/or the PCR cycling parameters may not have been ideal which allowed less efficiency in the amplification of the regions in the *polyhedrin/granulin* gene (Ye et al., 2012). In the study by Lange et al., (2004), an annealing temperature of 50°C was used to amplify regions in the *polh* gene which is different from this study. Although a valuable tool in biology, PCR has its limitations. The results of this study do not conclude that other baculoviral sequences were not present in the samples. To design the oligonucleotides, sequence data is required, and thus PCR can only detect the presence of the target genes based on the oligonucleotide design (Garibyan & Avashia, 2013). Since the oligonucleotides were designed to target regions in the CrleGV-

SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences, only the target regions in those baculoviral sequences were amplified. Furthermore, the minimum and maximum detection limits of the PCR technique developed in this study should be evaluated since the viral DNA may be present at low concentrations. But for the interest of this study, the PCR-based techniques are sufficient for downstream experiments as the purpose was to detect these viruses which will be used in the single and mixed infections against *T. leucotreta*.

The PCR amplicons were all sent for sequencing. The presence of the desired viruses was confirmed in each viral stock for the samples provided, as indicated by the BLAST results. It is not uncommon that naturally occurring baculoviruses are found as mixtures of different genotypes. As seen in **Table 2.5** different isolates of HearNPV each with high percentage identity scores were observed from the BLAST search. The mixed populations of the same baculovirus show genetic variation as a result of mutations such as insertions/deletions as well as single nucleotide polymorphisms (SNPs) (Chateigner et al., 2015; Crook et al., 1985; Larem et al., 2019; Thézé et al., 2014; Wennmann et al., 2017). The sequences were also successfully aligned with the targeted regions of each baculovirus as indicated in Appendix A.

This chapter aimed to design oligonucleotides targeting unique regions in the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genomes for PCR amplification to detect the presence of these viruses in the samples provided. The PCR results showed that CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were present in the samples provided, with no cross-contamination of these viruses detected between the samples (i.e., CrleGV-SA was not detected in the CpGV-M sample and so on for the other samples). These results show that the oligonucleotide pairs generated can be used to specifically target each respective genome and do not bind non-specifically to gDNA from any of the other baculoviruses used in this study The BLAST results and the ClustalW alignments further confirmed the presence of the viruses in the samples provided. The following chapter describes the bioassay analysis of single infections of each virus against *T. leucotreta* neonate larvae.

Chapter 3
Evaluating and comparing the lethal concentrations of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au against *T. leucotreta* neonate larvae

3.1 Introduction

The virulence of each virus alone against *T. leucotreta* neonate larvae needs to be determined before comparing the effects of the mixed infections. Consequently, the OBs of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au extracted as described in Chapter 2 were enumerated to determine the concentration of each sample before initiating the biological assays. There are various techniques used for virus quantification. Each technique has its advantages and disadvantages. The quantitative polymerase chain reaction (qPCR) method is highly sensitive with the ability to distinguish between different viruses and is beneficial in quantifying viruses in mixed infections (Jukes, 2018; Wennmann & Jehle, 2014). However, qPCR is limited by the high costs and is accurate for the enumeration of GVs with one nucleocapsid (Dhladhla et al., 2018; Roldão et al., 2009). Scanning electron microscopy (SEM) is a time-efficient technique that offers high resolution and accuracy in counting (Dhladhla et al., 2018). Scanning electron microscopy is limited by the costs and availability of SEM facilities. In addition, the necessary experience is required to prepare the samples and knowledge to operate the microscopes (Dhladhla et al., 2018). Dark-field microscopy is an alternative and commonly used virus enumeration method (Eberle et al., 2012). A haemocytometer is used to count the OBs under dark field microscopy. The drawback of using darkfield microscopy is the difficulty of quantifying the OBs due to their small size, especially for GVs, and can be time-consuming (Dhladhla et al., 2018). Additionally, it is quite subjective if two different people count the same sample, the results obtained can be different. One can overestimate the OB count due to a lack of training and expertise (Grzywacz & Moore, 2017). Despite the above-mentioned disadvantages, darkfield microscopy can be used as an alternative to SEM as both methods were found to be statically comparable (Dhladhla et al., 2018). In this study, darkfield microscopy was the chosen method for virus quantification due to the availability of the equipment, expertise, and it is the most common method used for counting baculoviruses.

The use of biological assays is one way to evaluate the relative virulence of baculoviruses against lepidopteran pests. Virulence is the measure of the ability to cause harm or disease in an infected host (Shapiro-Ilan et al., 2005). Baculoviruses need to be ingested to initiate rima ry infection, thus a lyi ng the virus to the host's feeding sites maximises the opportunities for viral infection (Cory & Bishop, 1997). The three classified types of dose-response biological assay methods are droplet feeding, diet incorporation, and surface dose assays. Droplet feeding was first described by Hughes & Wood, (1981) and later developed for *T. leucotreta* neonate larvae by Pereira-da-Conceicoa et al., (2012). The method incorporates a brilliant blue dye in the virus suspension and the larvae feed on the virus in the form of droplets. This technique increases the probability of insect pests feeding on the virus and offers the opportunity to identify the insects that have consumed the virus suspension due to the dye. This accurate droplet feeding assay allows for the reporting of the lethal dose as the number of OBs consumed by the larvae can be controlled and remains constant. The diet incorporation method involves mixing the diet and virus suspension and allowing the insect to feed (Fritsch et al., 2007; Huber, 1981). The number of OBs ingested by the insect can be quantified as the amount of diet consumed can be measured thus the lethal dose can be determined. For this study, the surface dose assay was used to determine the relative virulence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au as this method has been extensively used against *T. leucotreta* neonate larvae (for example Jukes, 2018; Moore, 2002; Taylor, 2021). With this method, a virus suspension of a known concentration is applied on the surface of the diet, upon which the insect feed. Therefore, the number of OBs consumed by the larvae is unknown, hence the dose-response is determined in terms of the concentration applied (Hughes & Shapiro, 1997). It is also important to consider the feeding behaviour of different insects, for example, *T. leucotreta* larvae are cryptic feeders (Daiber, 1979b). The application of the virus on the surface enables infection during the initial feeding stages by neonate larvae, after which further infection is prevented as the larvae will burrow beyond the rind of fruits. Surface dose biological assays are therefore an accurate reflection of how biopesticides are applied in the field against insect pests, further motivating the choice of assay for this study.

Dose-response mortality curves are used to describe the relationship between the virulence of the applied virus and the treatment concentration against a given insect (Cory & Bishop, 1997). This is often expressed as LD_{50} (lethal dose required to kill 50% of the population) or LD_{90} (lethal dose required to kill 90% of the population). Due to the choice of biological

assay, the biological parameters were expressed as LC_{50} (lethal concentration required to kill 50% of the insect population) and LC_{90} (lethal concentration required to kill 90% of the insect population). A probit analysis is a commonly used model to determine LD and LC values based on dose-mortality data, but other models are also used (Finney, 1978; Hughes & Wood, 1986).

This chapter aimed to determine the relative virulence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in single infections against *T. leucotreta* neonate larvae before performing mixed infection assays. To achieve this aim, the first objective of this study was to enumerate each viral stock using a haemocytometer and darkfield microscopy. The second objective was to determine the LC_{50} and LC_{90} of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in single infections against *T. leucotreta* neonate larvae using surface dose biological assays.

3.2 Methods and Materials

3.2.1 Occlusion body enumeration

The viral stocks of each sample were vortexed to ensure that it was homogeneous. A 5 × dilution of each virus suspension was prepared by adding 40 µl sterile water to 10 µl of virus to make up a total volume of 50 µl. A homogenous suspension was obtained by shaking the virus suspension. A further 5 × dilution was made by adding 200 µl 0.07% SDS (w/v), bringing the suspension to a total of 25 × dilution. Each virus sample was sonicated at 60 Hz for 60 seconds, but this was divided into 4 rounds of 15-second bursts to prevent the samples from overheating. A further 10 × dilution was prepared by dissolving 100 µl of each virus suspension in 900 µl sterile water in new 1.5 ml tubes, bringing the suspension to a total of 250 × dilution. Using the 250 × dilutions of each sample, 2000, 1750, 1500, and 1250 × final dilutions were prepared for counting using sterile water. Each sample was vortexed to ensure that it was homogenous. A 0.02 mm Thoma bacterial counter chamber (Hawksley Helber Bacteria 1 Cell Thoma Z30000) was prepared by cleaning the slide and cover slip on both sides using 70% ethanol and lens tissue paper. The cover slip was placed over the counting chamber allowing a small space to pipette 5 µl of the 1:2000 suspension. The cover slip was secured by totally covering the counting chamber and the slide was allowed to stand for 5 minutes before counting commenced. The counting chamber was examined under darkfield microscopy at 400 × magnification. A total of 5 large squares were counted, one at each corner of the counting chamber and one random square in the middle of the counting chamber. The large squares each had 16 small squares and the entire grid was counted. Each dilution, starting from the most dilute suspension to the most concentrated suspension was

counted to determine which dilution had ±7 occlusion bodies per small square (**Figure 3.1**). Upon achieving this, the dilution which had ±7 occlusion bodies was used for counting. Each sample was counted 3 times, using 5 µl of sample for each count. The concentration for each viral stock was calculated using **Equation 3.1**.

The OBs under darkfield microscopy are visible as points of light (**Figure 3.1**). Each large square has 16 small squares. A total of 5 large squares were counted. The counting continued for each virus until the dilution containing ± 7 occlusion bodies per small square was achieved.

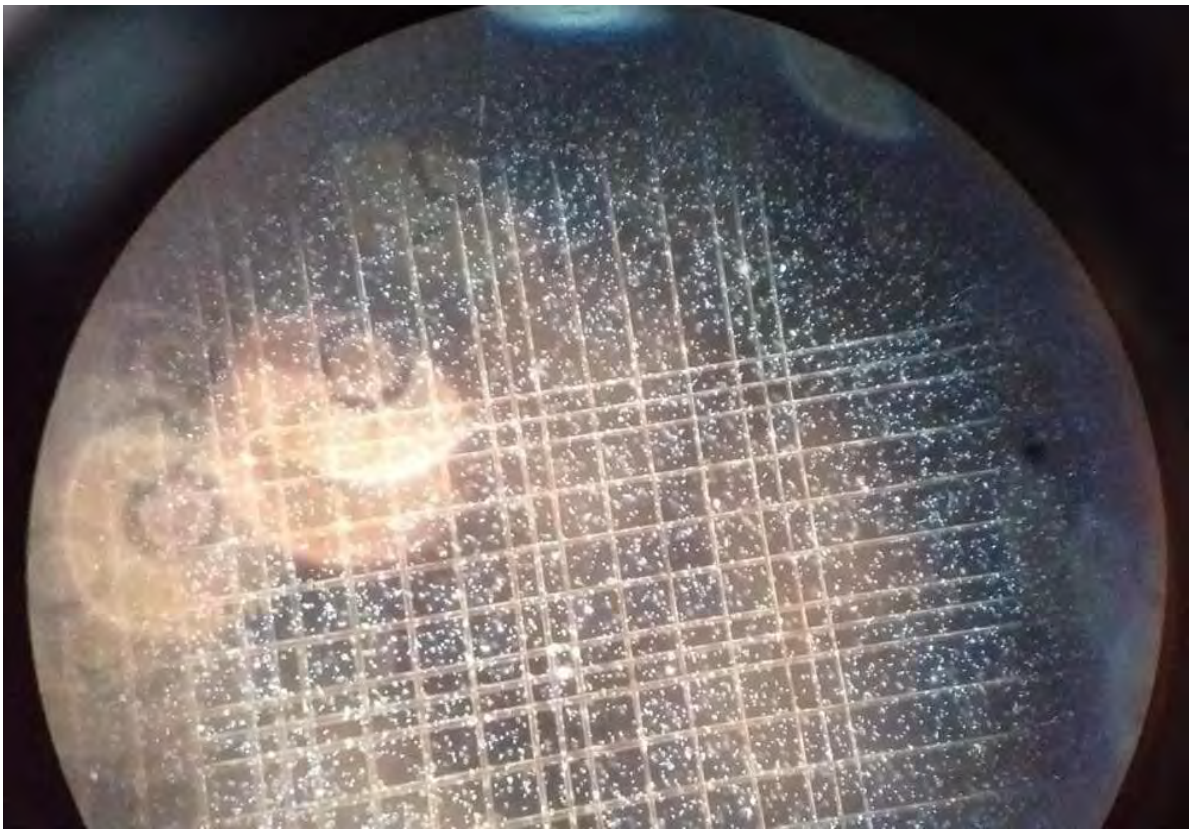


Figure 3.1 CrleGV SA OBs under darkfield microscopy. Picture captured by Kurhula Luphondo

Equation 3.1 Equation for determining the concentration of OBs per viral stock using the counting chamber.

$$\mathbf{OBs/ml = (D \times X) / (N \times V)}$$

Where: D = dilution of suspension, X = number OBs counted, N = number of small squares counted, V = volume per small square in ml

3.2.2 *T. leucotreta* egg sheet preparation

The *T. leucotreta* egg sheets were obtained from the laboratory colony housed at the Department of Zoology and Entomology, Rhodes University. The *T. leucotreta* egg sheets were rinsed in 0.15% sodium hypochlorite for 1 minute followed by rinsing in sterile water for 5 minutes. The egg sheets were air-dried in a laminar flow cabinet before placing them in sterile Petri dishes. The Petri dishes were sealed using Parafilm™ (Bemis Company Inc., Wisconsin) and left to incubate at 25°C in a controlled environment (CE) room until hatching.

3.2.3 Surface dose biological assays

Surface dose biological assays were set up using methods adapted from Moore et al., (2011), Marsberg, (2016), and Jukes, (2018). The *T. leucotreta* diet described by Moore et al., (2014) was baked in a 1:1 ratio of raw diet to water. Two hundred and fifty grams of *T. leucotreta* diet was mixed well with 250 ml of sterile water. The diet was placed in an oven dish and baked at 200°C for 20 minutes. Once baked, a 10 ml syringe with the end cut off was used to place the diet into individual wells in a 24-well tray. The piston of the syringe was used to compress the diet to the bottom of the wells. The transfer of the diet to the 24-well trays was done in a laminar flow cabinet to prevent contamination. A volume of 100 µl of each virus dilution was placed in each well and each plate represented a single concentration. For the control, a volume of 100 µl sterile water was used instead of virus suspension. The method of preparing the serial dilutions is indicated in **Figure 3.2**. A dose-discriminate assay was performed using a single high concentration for HearNPV-Au. After applying each virus suspension on the surface of the diet, the 24-well plates were left to air dry for 30 minutes. A fine paintbrush was used to place one *T. leucotreta* neonate larvae per well. Each 24-well plate was covered with a glass sheet before applying the lid to prevent the neonates from escaping the wells. The 24-well plates were sealed using masking tape and incubated at 25°C for 7 days. A sterile toothpick was used to evaluate larval mortality. Larvae that did not respond to being nudged by the sterile toothpick were recorded dead. This was replicated three times for each virus treatment. The larval cadavers from each replicate were pooled together, placed in 1.5 ml sterile tubes, and stored at - 20°C for further evaluation.

The OB concentrations used for this study are shown in **Table 3.1**. For each virus, a total of 5 doses were prepared through a serial dilution procedure except for HearNPV-Au which was applied at a single concentration.

Table 3.1 The concentrations of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au OBs used in the surface dose biological assays (OBs/ml)

	Occlusion body concentrations (OBs/ml)			
	CrleGV-SA	CrpeNPV	CpGV-M	HearNPV-Au
Dose 1	2.96×10^7	1.25×10^7	6.43×10^7	3.44×10^6
Dose 2	2.96×10^6	1.25×10^6	6.43×10^6	
Dose 3	2.96×10^5	1.25×10^5	6.43×10^5	
Dose 4	2.96×10^4	1.25×10^4	6.43×10^4	
Dose 5	2.96×10^3	1.25×10^3	6.43×10^3	
Control	0	0	0	0

The doses for each virus were prepared separately using serial dilution (**Figure 3.2**). For CrleGV-SA and CpGV-M, initially, a 100 × dilution was prepared from the virus stock, and the tubes were labelled "Wor ing Solution ". Another 00 × dilution was used to re a re the highest concentration. This was followed by a series of 10 × serial dilutions to the lowest concentration (**Figure 3.2 A**). The CrleGV-SA concentrations ranged from 2.96×10^7 OBs/ml to 2.96×10^3 OBs/ml. On the other hand, the CpGV-M concentrations ranged from 6.43×10^7 OBs/ml to 6.43×10^3 OBs/ml. For CrpeNPV, initially, a 100 × dilution was prepared from the virus stock, and the tubes were also labelled "Wor ing Solution ". hi s was followed by a series of 10 × serial dilutions to prepare the highest concentration to the lowest concentration (**Figure 3.2 B**). For the CrpeNPV biological assays, concentrations ranged from 1.25×10^7 OBs/ml to 1.25×10^3 OBs/ml. For HearNPV-Au, a 100 × dilution from the virus stock was prepared and this was followed by another 100 × dilution to prepare the highest concentration. The concentration of 3.44×10^6 OBs/ml was used for the HearNPV-Au dose discriminate assays.

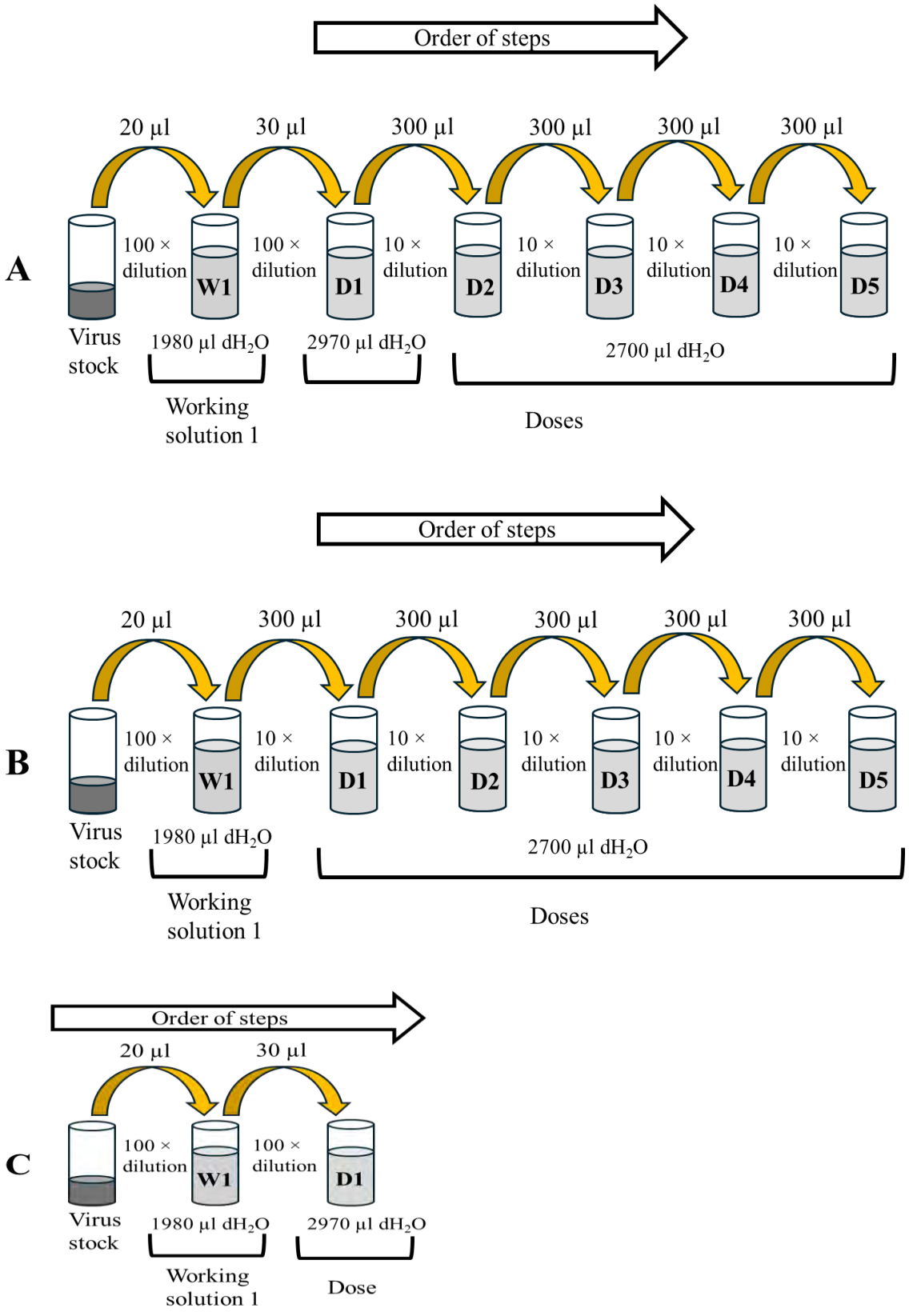


Figure 3.2 (A) The serial dilution method to prepare the CrleGV-SA and CpGV-M doses. **(B)** The serial dilution method to prepare the CrpeNPV doses. **(C)** The serial dilution method to prepare the HearNPV-Au dose for the dose-discriminate assays.

3.2.4 Statistical analysis of the dose-response biological assays

The data from the CrleGV-SA, CrpeNPV, and CpGV-M biological assays was analysed using the "drc" ac age (Ritz et al., 2015) in R version 4.3.3 and RStudio version 2023.03.1© 2009 – 2023 Posit Software, PBC. The control mortality was compensated for using Abbotts Correction (Abbott, 1925). The LC_{50} and LC_{90} values for each treatment were determined and com ar ed for any differences between each treatment using the "EDcom " function in the "drc" a c a ge. he mortality data from the HearN V -Au dose-discriminate assays were analysed using an ANOVA.

3.3 Results

3.3.1 Occlusion body enumeration of viral stocks

The viral stocks of CrleGV-SA and CrpeNPV contained 2.96×10^{11} OBs/ml and 1.25×10^{10} OBs/ml respectively. The concentration of the CpGV-M and HearNPV-Au viral stocks was 6.43×10^{11} OBs/ml and 3.44 x 10^{10} OBs/ml, respectively.

3.3.2 Percentage of larval mortality from each surface dose biological assay

Surface-biological assays were conducted using a range of doses for each virus except for HearNPV-Au. The highest HearNPV concentration (3.44×10^{6} OBs/ml) was used in the dose-discriminate assays. The experiment was replicated 3 times for each virus alone against *T. leucotreta* neonate larvae. After 7 days, the larval mortality was recorded for each viral dose in each treatment. The results are presented in **Table 3.2**. Sterile dH_2O was used for the control.

Table 3.2 The percentage larval mortality recorded from the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au dose-response biological assays.

Percentage Mortality (%)			
CrleGV-SA			
Dose (OBs/ml)	Rep 1 (n=24)	Rep 2 (n=24)	Rep 3 (n=24)
0	8.33	4.17	8.33
2.96×10^3	37.5	29.17	20.83
2.96×10^4	45.83	45.83	33.33
2.96×10^5	83.33	79.17	54.17
2.96×10^6	100	100	95.83
2.96×10^7	100	100	100
CrpeNPV			
0	4.17	8.33	12.50
1.25×10^3	12.50	16.67	8.33
1.25×10^4	25	12.50	16.67
1.25×10^5	41.67	25	37.50
1.25×10^6	83.33	66.67	79.17
1.25×10^7	100	91.67	100
CpGV-M			
0	8.33	4.17	12.50
6.43×10^3	25	20.83	16.67
6.43×10^4	33.33	20.83	20.83
6.43×10^5	33.33	37.50	33.33
6.43×10^6	58.33	54.17	62.50
6.43×10^7	75	70.833	83.33
HearNPV-Au			
0	4.17	0	4.17
3.44×10^6	4.17	4.17	0

The LC_{50} and LC_{90} values for CrleGV-SA were determined to be 2.94×10^4 OBs/ml and 1.21×10^6 OBs/ml respectively (**Table 3.3**). These LC_{50} and LC_{90} values are lower compared to

values obtained for CrpeNPV and CpGV-M. The LC_{50} values reported for CrpeNPV and CpGV-M were 1.52×10^5 OBs/ml and 2.02×10^6 OBs/ml and the LC_{90} values reported for CrpeNPV and CpGV-M were 8.02×10^6 OBs/ml and 3.03×10^9 OBs/ml respectively. However, the CpGV LC_{90} value is an extrapolation from the data as 90% mortality was not achieved.

Table 3.3 The LC_{50} and LC_{90} for the CrleGV-SA, CrpeNPV, and CpGV-M in biological assays against *T. leucotreta* neonate larvae.

	Concentration (OBs/ml)		
	CrleGV-SA	**CrpeNPV**	**CpGV-M**
LC_{50}	2.94×10^4	1.52×10^5	2.02×10^6
LC_{90}	1.21×10^6	8.02×10^6	3.30×10^9

3.3.3 Dose mortality curves

The data from the surface-dose biological assay generated a dose-mortality curve for CrleGV-SA, CrpeNPV, and CpGV-M against *T. leucotreta* as shown in **Figure 3.3**. No difference ($p = 1$) was observed between the control and larvae treated with HearNPV-Au at a concentration of 3.44×10^6 OBs/ml as indicated in **Figure 3.4**.

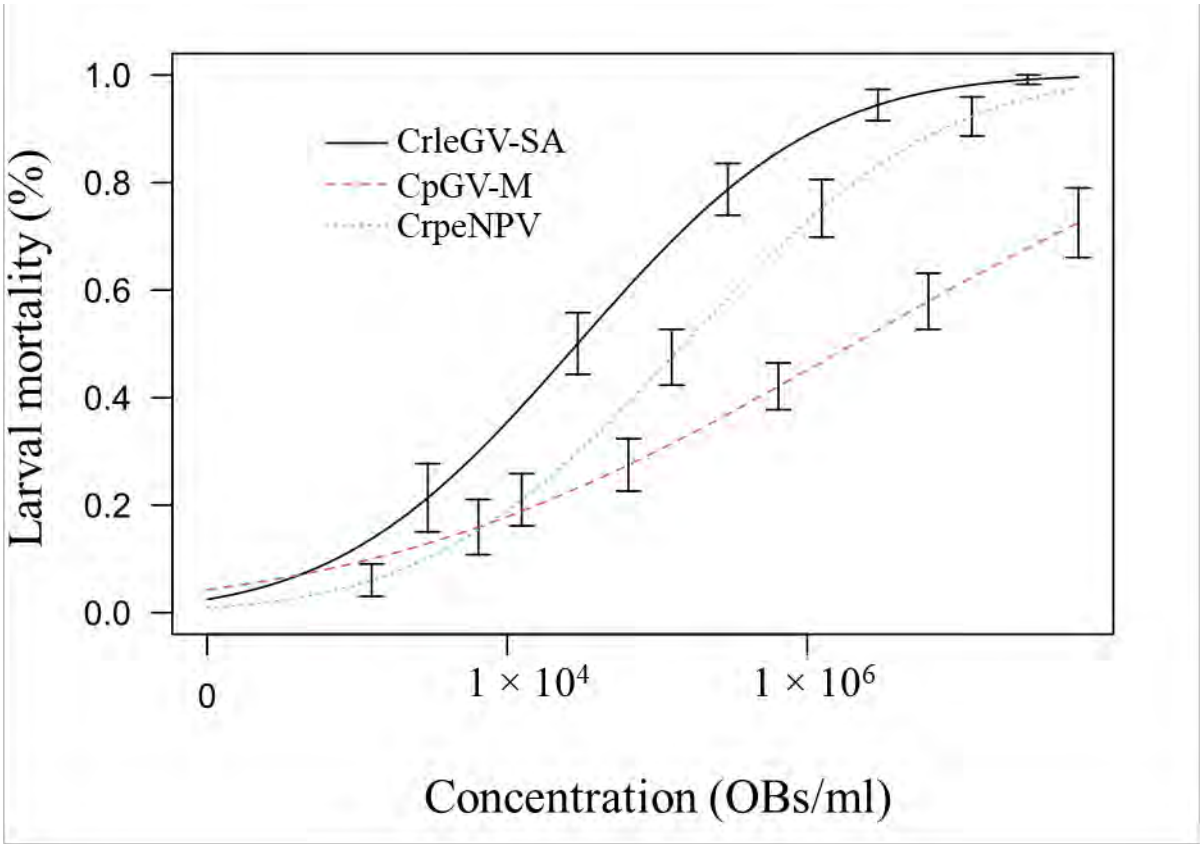


Figure 3.3 The dose-mortality curves for CrleGV-SA, CrpeNPV, and CpGV-M against *T. leucotreta* neonate larvae. The CrleGV-SA concentrations ranged from 2.96×10^7 OBs/ml to 2.96×10^3 OBs/ml. The CrpeNPV concentrations ranged from 1.25×10^7 OBs/ml to 1.25×10^3 OBs/ml. The CpGV concentrations ranged from 6.43×10^7 OBs/ml to 6.43×10^3 OBs/ml.

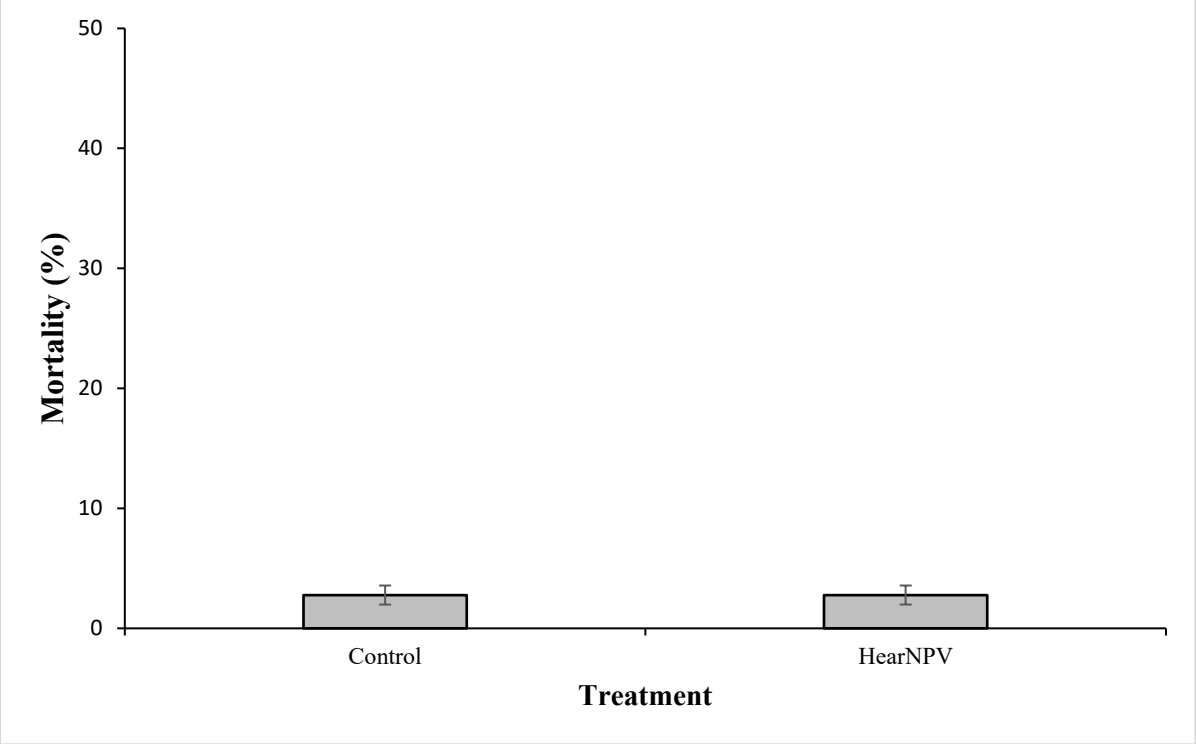


Figure 3.4 Analysis of the HearNPV dose discriminate assay against *T. leucotreta* neonate larvae

3.4 Discussion

This chapter aimed to determine the virulence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au alone against *T. leucotreta* neonate larvae by using surface-dose biological assays. Firstly, the concentration of the OBs of each virus was determined by counting using darkfield microscopy. The concentrations of the OBs required needed to be sufficiently high to estimate both the LC_{50} and LC_{90} of each virus against *T. leucotreta* neonate larvae. The LC_{90} values previously calculated by Jukes, (2018) were 3×10^6 OBs/ml and 2.75×10^6 OBs/ml for CrleGV-SA and CrpeNPV respectively against *T. leucotreta* neonate larvae. Similarly, LC_{90} values of 4.10×10^5 OBs/ml and 1.05×10^5 OBs/ml for CrleGV-SA and CrpeNPV respectively were previously reported by Taylor, (2021) against *T. leucotreta* neonate larvae. Based on these previous studies, the range of doses for CrleGV-SA and CrpeNPV were determined. Concentrations of 2.96×10^7 OBs/ml and 1.25×10^7 OBs/ml for CrleGV-SA and CrpeNPV respectively were chosen as the highest doses for biological assays for these two viruses. Chambers, (2014) reported concentrations of 9.78×10^6 OBs/ml for the highest LC_{90} value for CpGV against 5th instar *T. leucotreta* larvae. Therefore, a concentration of 6.43×10^7 OBs/ml was set as the highest dose for CpGV-M to conduct biological assays against *T. leucotreta* neonate larvae. Mtambanengwe, (2019) used the

highest concentration of 2.0×10^4 OBs/ml against 2nd instar Albany *H. armigera* larvae but did not manage to obtain mortalities above 90%. The study was conducted against the homologous host and Mtambanengwe, (2019) suggested that using higher doses would yield larval mortalities above 90%. Based on those suggestions, a concentration of 3.44×10^6 OBs/ml was chosen to conduct the dose discrimination assay against *T. leucotreta*.

Since *T. leucotreta* larvae are cryptic feeders, the larvae burrow into the fruit where larval development is completed (Daiber, 1979b). In field applications, it is imperative to apply virus treatments on the surface of the fruit to ensure the larvae encounter the virus and become infected before burrowing into the rind (Moore et al., 2011). Surface dose biological assays were chosen to reflect the field applications and to evaluate the relative virulence of each virus against *T. leucotreta* neonate larvae. A probit analysis was used to determine the LC_{50} and LC_{90} values of each treatment.

The LC_{50} and LC_{90} values were compared to determine which treatment was the most virulent against *T. leucotreta* neonate larvae. From this study, CrleGV-SA was the most virulent virus treatment with lower LC_{50} and LC_{90} values reported compared to CrpeNPV and CpGV-M. The CrpeNPV treatment was the second most virulent. Surface biological assays using CrleGV-SA and CrpeNPV against *T. leucotreta* neonate larvae have been previously evaluated by Jukes, (2018); Marsberg, (2016); Moore, (2002) and Taylor, (2021). These results are similar to those reported by Jukes, (2018) against *T. leucotreta* neonate larvae. Jukes, (2018) reported no improvement in LC_{50} values for the CrpeNPV treatment in comparison to the CrleGV-SA which was also observed in this study. However, these results differ from similar studies performed against *T. leucotreta* neonate larvae by Taylor, (2021), and Marsberg, (2016). There were improvements reported in the LC_{50} values for CrpeNPV treatment in comparison to CrleGV-SA as indicated by Marsberg, (2016) and Taylor, (2021) (**Table 3.4**). The LC_{50} and LC_{90} values for CrleGV-SA and CrpeNPV reported in Marsberg, (2016) were lower in comparison to Jukes, (2018), and Taylor, (2021) and the results obtained in this study. Furthermore, Moore, (2002) reported lower LC_{50} and LC_{90} values for CrleGV-SA against *T. leucotreta* neonate larvae in comparison to the results obtained in this study (**Table 3.4**). Although the above studies used the same viruses (CrleGV-SA and CrpeNPV) against *T. leucotreta* neonate larvae, the differences in the lethal concentrations in comparison cannot be easily explained. One can suggest that variations in genotype/isolates used, virus quantification, differences in viral dose preparation, colony health, environmental

conditions, and differences in data analysis can contribute to the inconsistent results observed between various studies.

Table 3.4 The LC_{50} and LC_{90} values for CrleGV-SA and CrpeNPV reported by Moore, (2002), Marsberg, (2016), Jukes, (2018) and Taylor, (2021) against *T. leucotreta* neonate larvae.

Study	Virus	LC_{50}	LC_{90}
Moore, (2002)	CrleGV-SA	4.095 × 103	1.185×10^5
Taylor, (2021)	CrleGV-SA	1.53×10^4	4.10×10^5
	CrpeNPV	1.15×10^4	1.05×10^5
Jukes, (2018)	CrleGV-SA	1.17×10^5	3.00×10^6
	CrpeNPV	1.23×10^5	2.75×10^6
Marsberg, (2016)	CrleGV-SA	4.09×10^3	1.18×10^5
	CrpeNPV	2.29×10^3	9.97×10^4

The CpGV treatment was the third most virulent among the viruses used in the single infections against *T. leucotreta* neonate larvae. A few studies have been performed using CpGV against *T. leucotreta*. The successful production of CpGV in *T. leucotreta* was shown by Reiser et al., (1993) but the larval percentage mortality from the study was not reported. Fritsch et al., (1990) reported that wild-type CpGV is 1000 times less virulent against *T. leucotreta* as compared to CrleGV. In this study, the LC_{90} value for CpGV-M was 2727.27 × higher compared to CrleGV-SA. This still shows that CpGV is less virulent against *T. leucotreta* in comparison to CrleGV-SA. Still, the variations in virulence may be due to differences in CrleGV isolates as Fritsch et al., (1990) used the CrleGV isolate from the Ivory Coast, and in this study, the South African isolate was used. In another study by Chambers, (2014), CpGV was produced in *T. leucotreta* 4^{th} and 5^{th} instar larvae. The LC_{90} was calculated to be 9.78×10^6 OBs/ml. The results obtained from this study cannot be compared to Chambers, (2014) as later instar larvae are known to be less susceptible to virus infection in comparison to early instars due to developmental resistance (Wang & Hu, 2019). Interestingly the LC_{90} is higher than what Chambers, (2014) measured which is unexpected. This study is the first to report the LC_{50} and LC_{90} values for CpGV-M against *T. leucotreta*

neonate larvae using surface dose biological assays and hence it is difficult to make comparisons with previous studies.

A dose discriminate assay was carried out to determine whether HearNPV-Au infects *T. leucotreta.* A concentration of 3.44×10^6 OBs/ml was used to evaluate the effect of HearNPV-Au against *T. leucotreta* neonate larvae. There was no difference between the control and the HearNPV-Au treatment. HearNPV isolates have a narrow host range and are known to infect species within the *Heliothis* and *Helicoverpa* genera (Gettig & McCarthy, 1982; Gröner, 1986). The results obtained from this study confirm HearNPV was non-infectious and did not result in larval death hence LC_{50} and LC_{90} values were not determined.

The overall aim of this chapter was to determine the relative virulence of each virus alone against *T. leucotreta* neonate larvae. The aim was achieved through surface-dose biological assays. The virulence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au alone against *T. leucotreta* will used to compare the effect of mixed infections. Chapter 4 will focus on the evaluation of mixed infections to detect whether there are improvements in lethal concentrations of mixtures against *T. leucotreta* in comparison to single infections.

Chapter 4
Evaluating and comparing the lethal concentrations of mixed infections of CrleGV-SA with CrpeNPV, CpGV-M, and HearNPV-Au against *T. leucotreta*

4.1 Introduction

In the previous chapter, single infections using CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were evaluated to determine the virulence of each virus alone against *T. leucotreta* neonate larvae. Cryptophlebia leucotreta granulovirus is an effective biological control agent and has been extensively used over 15 years in the control of *T. leucotreta* in South Africa (Moore et al., 2015; Moore, 2021). Although highly effective, the virus has a few shortcomings as previously reviewed in Chapter 1 (section 1.6). Research is ongoing to find novel ways to improve CrleGV infectivity and other commercialised baculoviruses to combat these shortcomings. One way to improve the efficacy of baculoviruses is through mixed infections. Research on dual infections involving baculoviruses is a growing field of interest due to their potential to improve their use as biopesticides in the field (reviewed in Ferrelli & Salvador, 2023).

Dual interactions from the point of view of the host insect can be characterised as synergistic, antagonistic, or additive (Ferrelli & Salvador, 2023; Koppenhöfer & Kaya, 1997). This classification system can be used when one or both baculoviruses are infectious to the host insect. Synergism describes an interaction where the viruses have a greater effect than the sum of the individual interactions. On the contrary, antagonism classifies an interaction where the viruses have a lower effect than the sum of the individual effects against the host. Additive is when the sum of the mixed and individual interactions is the same and thus has a "neutral" effect on the host (Ferrelli & Salvador, 2023; Koppenhöfer & Kaya, 1997). The outcome of viral interactions can be determined using various mathematical methods such as the ANOVA test for the LC_{50}, Tammes Bakuniak graphic method, and the Plackett and Hewlett joint-action rate test as previously used by Busvine, (1971) and Lara Reyna et al., (2003).

Tanada, (1956, 1959) first reported experimental mixed infections between two baculoviruses isolated from the same host. The synergism resulting from the study led to further interest in investigating double infections involving NPV and GVs isolated from the same host in

various combinations and the effects are reviewed in Ferrelli & Salvador, (2023). Much interest is taken in interactions that lead to synergism due to the advantages of improving lethal concentration and lethal time. Recently, Cuartas-Otálora et al., (2019) observed synergism when the Spodoptera frugiperda granulovirus (SpfrGV) proportion did not exceed 2.5% in combination with Spodoptera frugiperda nucleopolyhedrovirus (SpfrMNPV) against *Spodoptera frugiperda* larvae. Barrera et al., (2021) observed similar results in the mixed infections of Spodoptera ornithogalli nucleopolyhedrovirus (SporNPV) and Spodoptera ornithogalli granulovirus (SporGV) against *Spodoptera ornithogalli* neonate larvae. Work by Tanada, (1959); and Tanada & Hukuhara, (1971) led to the evaluation of synergistic factors present in GVs where the host is susceptible to the NPV and not the GV. Guo et al., (2007) reported synergistic interactions between Xestia c-nigrum granulovirus (XcenGV) in combination with Spodoptera litura nucleopolyhedrovirus (SlNPV) against *Spodoptera litura* larvae. Derksen & Granados, (1988); and Lara Reyna et al., (2003) also observed the enhancement in the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) infection in combination with Trichoplusia ni granulovirus (TnGV) against *Trichoplusia ni* larvae. In addition, Biedma et al., (2015) obtained similar results against *Anticarsia gemmatalis* larvae where Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) and Epinotia aporema granulovirus (EpapGV) mixed infections resulted in a synergistic interaction. In all the above-mentioned cases, the host is not susceptible to the GVs. As previously observed from the single infections, *T. leucotreta* was not susceptible to the HearNPV infection. Despite these findings, the study aimed to evaluate the effect of dual infections involving CrleGV and HearNPV against *T. leucotreta*. Mixed baculovirus interactions evaluating the effects where one virus does not infect the host have been well documented by Ferrelli & Salvador, (2023). This led to the motivation of the study to evaluate the effects of HearNPV in combination with CrleGV against *T. leucotreta* neonate larvae.

Baculoviral mixtures of CrleGV and CrpeNPV have been previously evaluated against *T. leucotreta* (Jukes, 2018; Taylor, 2021). Improvements in lethal concentrations were observed however an antagonistic effect in the increased lethal time was encountered. Ideally, a decrease in lethal concentration is sought after, as a less viral concentration is required to elicit larval mortality. In addition, a shortened lethal time is beneficial to pest control management as larval infestation is hindered within a short period. Based on the work initiated by Jukes, (2018) and Taylor, (2021), this research led to further interest in

investigating different combinations of baculoviruses against *T. leucotreta* neonate larvae to determine if the infectivity in terms of lethal concentration of CrleGV can be enhanced using mixed infections.

As reviewed in Ferrelli & Salvador, (2023) there are various factors that influence the outcome of baculovirus interactions such as the viruses used, proportions of the OBs in the mixture, and the larval stage of the host insect. There is no set criterion in determining which ratios to be used in baculovirus mixed infections. As previously studied, Jukes (2018) and Taylor (2021) evaluated 25:75, 75:25 and 50:50 CrleGV/CrpeNPV ratios against *T. leucotreta* which are different to those evaluated in this study. The same OB stocks of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au that were counted using darkfield microscopy as described in Chapter 3 were used in mixed infections. Similarly, the biological activity of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV in varied combinations was evaluated using surface dose assays against *T. leucotreta* neonate larvae.

This chapter aimed to evaluate baculoviral mixtures of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV against *T. leucotreta* neonate larvae to investigate whether any combination can result in the improvement in lethal concentrations of CrleGV for the better management of the pest in the field. To achieve this aim, the first objective was to determine the LC_{50} and LC_{90} of mixed interactions of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV against *T. leucotreta* using surface dose biological assays. The second objective was to determine the effect of the viral mixtures using the Tammes-Bakuniak graphic method in terms of lethal concentration.

4.2 Methods and Materials

4.2.1 *T. leucotreta* egg preparation

As previously described in Chapter 3 (section 3.2.2), *T. leucotreta* egg sheets were collected from the laboratory colony housed at the Department of Entomology and Zoology at Rhodes University. Initially, the *T. leucotreta* egg sheets were rinsed for 1 minute in 0.15% sodium hypochlorite before rinsing in sterile water for 5 minutes. The egg sheets were air-dried in a laminar flow cabinet before placing them in Petri dishes. The Petri dishes were sealed using Parafilm™ (Bemis Company Inc., Wisconsin) and left to incubate at 25°C in a controlled environment (CE) room until hatching.

4.2.2 Surface dose biological assays

The surface dose biological assays were set up using the methods adapted from Moore et al., (2011), Marsberg, (2016), and Jukes, (2018) as described in Chapter 3 (section 3.2.3). Two

hundred and fifty grams of *T. leucotreta* diet was mixed well with 250 ml of sterile water in a 1:1 ratio (Moore et al., 2014). The *T. leucotreta* diet was placed in an oven and baked at 200 °C for 20 minutes before allowing to cool down. A 10 ml syringe with the end cut off was used to place the diet in 24-well plates. The piston of the syringe was used to compress the diet to the bottom of the wells. To prevent contamination, the transfer of the diet was done in a laminar flow cabinet. Equal volumes of each virus were used to prepare the concentrations for the mixed infections of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV. For the CrleGV/CrpeNPV mixture, 150 µl of CrleGV at a concentration of 2.96×10^8 OBs/ml was mixed with 150 µl of CrpeNPV at a concentration of 1.25×10^8 OBs/ml. The mixture was vortexed and subsequent doses using 10-fold dilutions were prepared following the steps indicated in **Figure 4.1**. The CrleGV to CrpeNPV OB proportion was calculated to be 70:30 respectively. Each 24-well plate represented one virus concentration per mixture. A volume of 100 µl of virus suspension was inoculated in each well. The wells were allowed to air dry for 30 minutes using a laminar flow cabinet before placing one *T. leucotreta* neonate larva per well. The plates were covered with a glass plate to prevent the neonates from escaping the plates. The 24-well plates were sealed using masking tape and incubated at 25°C in a controlled environment for 7 days. Larval mortality was evaluated using a sterile toothpick. The larvae that did not respond to being nudged using a toothpick were recorded as dead. The experiment was replicated 3 times for each mixture. The larval cadavers from each replicate were pooled together and collected in 1.5 ml microcentrifuge tubes before storing at -20°C for further evaluation.

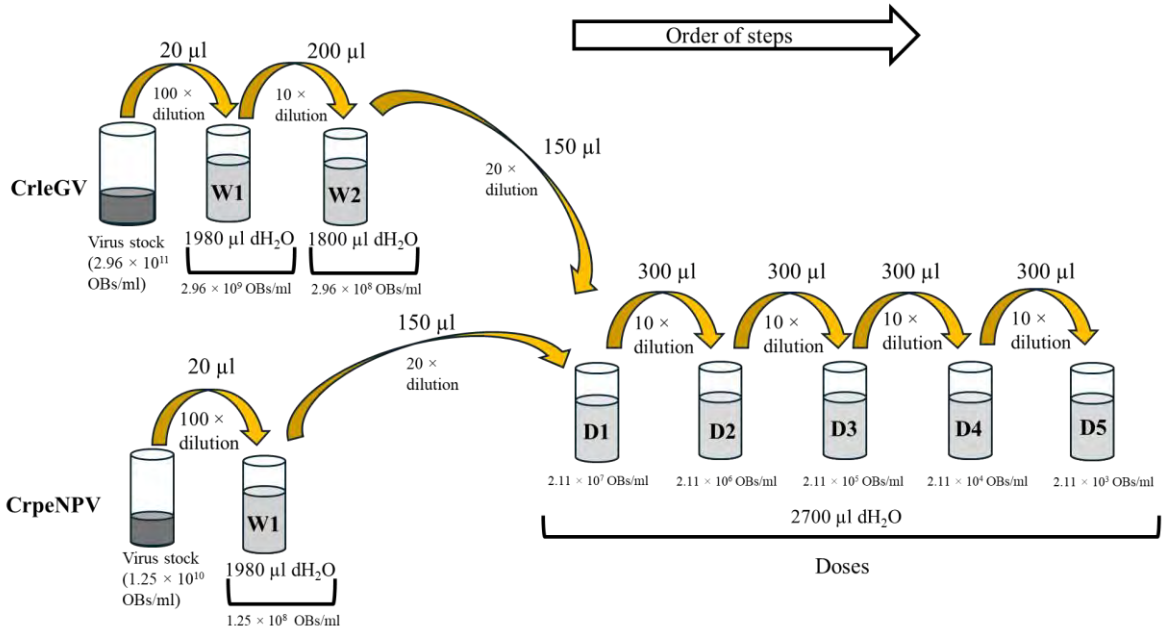


Figure 4.1 The serial dilution method to prepare the CrleGV/CrpeNPV mixed doses. The doses (D1-D5) ranged from 2.11×10^7 to 2.11×10^3 OBs/ml.

For the CrleGV/CpGV mixture, 15 µl of CpGV at a concentration of 3.43×10^9 OBs/ml was mixed with 15 µl CrleGV at a concentration of 2.96×10^9 OBs/ml. The mixture was vortexed thoroughly to ensure homogeneity and 10-fold dilutions were used to prepare the doses for biological assays as indicated in **Figure 4.2**. The CrleGV to CpGV OB proportion was calculated to be 46:54 respectively.

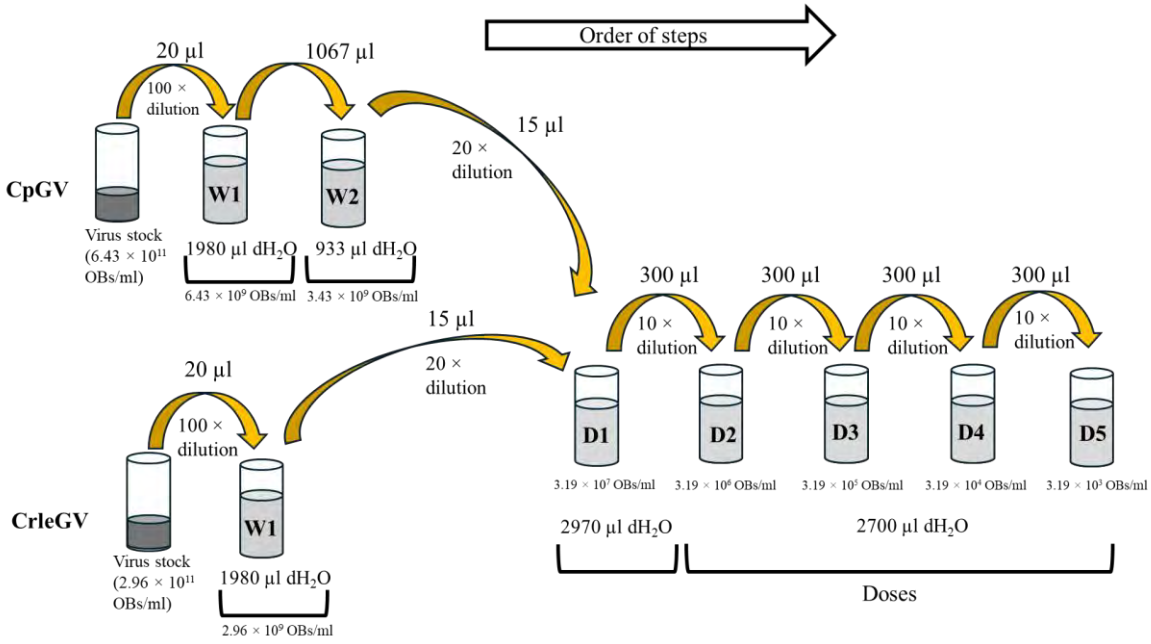


Figure 4.2 The serial dilution method to prepare the CrleGV/CpGV mixed doses. The doses (D1-D5) ranged from 3.19×10^7 to 3.19×10^3 OBs/ml.

A volume of 150 µl of HearNPV at a concentration of 3.44 × 10^8 OBs/ml was mixed with 150 µl CrleGV at a concentration of 2.96 × 10^8 OBs/ml. Doses 2 to 6 were further prepared using 10-fold dilutions as illustrated in **Figure 4.3**. The CrleGV to HearNPV OB proportion was calculated to be 46:54 respectively.

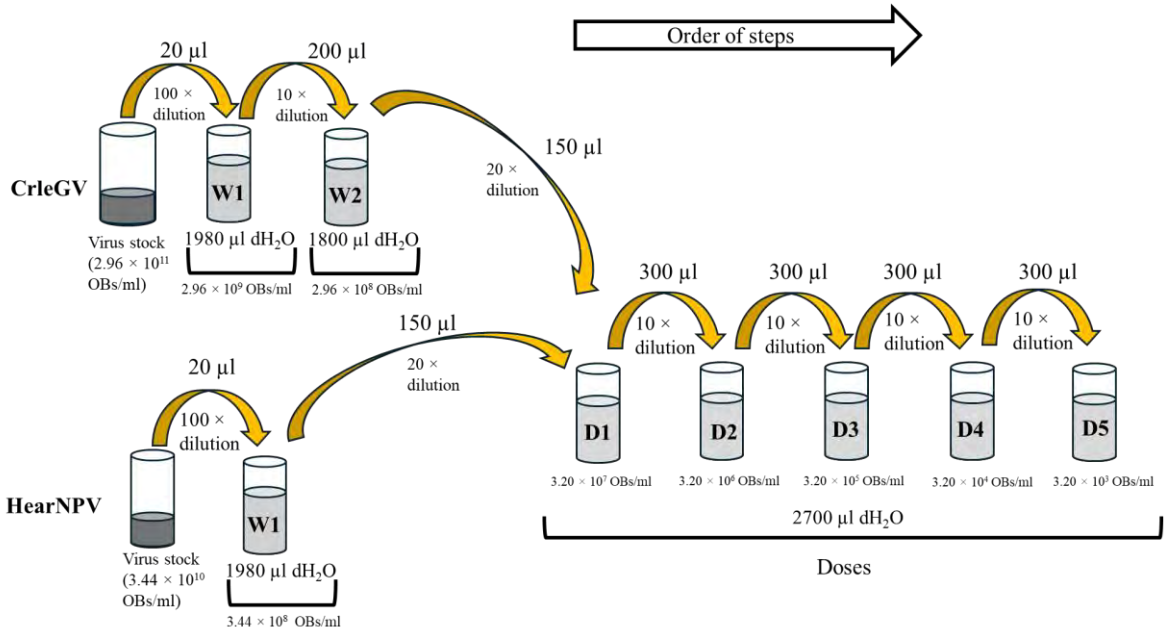


Figure 4.3 The serial dilution method to prepare the CrleGV/HearNPV mixed doses. The doses (D1-D5) ranged from 3.20 × 10^7 to 3.20 × 10^3 OBs/ml.

The OB concentrations for CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections against *T. leucotreta* neonate larvae are indicated in **Table 4.1.**

Table 4.1 The OB concentrations used for the mixed infections of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV against *T. leucotreta* neonate larvae. The concentrations were prepared as illustrated in the above figures (**Figure 4.1 – 4.3**)

	Concentrations (OBs/ml)		
	CrleGV-SA/CrpeNPV	CrleGV-SA/CpGV-M	CrleGV-SA/HearNPV-Au
Dose 1	2.11×10^7	3.19×10^7	3.20×10^7
Dose 2	2.11×10^6	3.19×10^6	3.20×10^6
Dose 3	2.11×10^5	3.19×10^5	3.20×10^5
Dose 4	2.11×10^4	3.19×10^4	3.20×10^4
Dose 5	2.11×10^3	3.19×10^3	3.20×10^3
Control	0	0	0

4.2.3 Statistical analysis of the biological assay data

The data from the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections was evaluated using the "drc" a c a ge (Ritz et al., 2015) in R version 4.3.3 and RStudio version 2023.03.1© 2009-2023 Posit Software, PBC, as described in Chapter 3 (section 3.2.4). The control mortality was compensated for using Abbotts Correction (Abbott, 1925). he "EDCom " function in the "drc" ac age determined and com a red the LC_{50} and LC_{90} values for each treatment. The synergistic, antagonistic, or additive interactions were determined by the Tammes-Bakuniak graphic method as described by Lara Reyna et al., (2003). The LC_{50} values of each virus in the combinations were plotted on each axis of the plane. The straight line between the LC_{50} values of both viruses is called the equitoxic line. The equitoxic line separates the antagonism zone from the synergism zone in the graph. The fiducial limits of each LC_{50} value are plotted in a buffer area around the equitoxic line which represents the additivity zone. The LC_{50} values of the mixed treatments were plotted on the graphs with their respective zones to determine the effect of each interaction.

4.3 Results

4.3.1 The percentage larval mortality from the mixed infections study

The surface dose biological assays of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections against *T. leucotreta* neonate larvae were replicated three times. The percentage of larval mortality was recorded after each replicate as indicated in **Table 4.2**. For the control, sterile dH_2O was used.

Table 4.2 The percentage larval mortality recorded from the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections using surface dose biological assays against *T. leucotreta* neonate larvae.

Percentage mortality (%)			
CrleGV/CrpeNPV			
Dose (OBs/ml)	Rep 1 (n=24)	Rep 2 (n=24)	Rep 3 (n=24)
0	12.50	12.50	12.50
2.11×10^3	8.33	12.50	16.67
2.11×10^4	25	29.17	37.50
2.11×10^5	62.50	41.67	58.33
2.11×10^6	83.33	83.33	91.67
2.11×10^7	100	100	100
CrleGV/CpGV			
Dose (OBs/ml)	Rep 1 (n=24)	Rep (n=24)	Rep (n=24)
0	12.50	12.50	8.33
3.19×10^3	12.50	8.33	29.17
3.19×10^4	25	16.67	25
3.19×10^5	50	62.50	45.83
3.19×10^6	100	100	83.33
3.19×10^7	100	100	100
CrleGV/HearNPV			
Dose (OBs/ml)	Rep 1 (n=24)	Rep (n=24)	Rep (n=24)
0	16.67	12.50	8.33
3.20×10^3	12.50	8.33	12.50
3.20×10^4	16.67	16.67	20.83
3.20×10^5	50	37.50	33.33
3.20×10^6	70.83	70.83	87.50
3.20×10^7	100	87.50	95.83

The LC_{50} and LC_{90} values for the CrleGV/CrpeNPV mixture were estimated to be 9.04×10^4 OBs/ml and 3.49×10^6 OBs/ml respectively (**Table 4.3**). These LC_{50} and LC_{90} values are lower compared to those obtained for CrleGV/CpGV and CrleGV/HearNPV viral mixtures. The LC_{50} values reported for the CrleGV/CpGV and CrleGV/HearNPV were 1.24×10^5 OBs/ml and 3.88×10^5 OBs/ml and the LC_{90} values reported for CrleGV/CpGV and CrleGV/HearNPV were 3.71×10^6 OBs/ml and 2.24×10^7 OBs/ml, respectively.

Table 4.3 The LC_{50} and LC_{90} values for the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infection treatments using surface dose biological assays against *T. leucotreta* neonate larvae. The 95 % confidence upper and lower confidence limits are also indicated except for the CrleGV/HearNPV mixture which was not able to be calculated.

Mixture	Lethal concentrations	Concentrations (OBs/ml)	95 % confidence intervals	
			Lower	Upper
CrleGV/CrpeNPV	LC_{50}	9.03×10^4	5.57×10^4	1.25×10^5
	LC_{90}	3.49×10^6	1.27×10^6	5.70×10^6
CrleGV/CpGV	LC_{50}	1.24×10^5	7.85×10^4	1.69×10^5
	LC_{90}	3.71×10^6	1.48×10^6	5.93×10^6
CrleGV/HearNPV	LC_{50}	3.88×10^5	2.29×10^5	5.47×10^5
	LC_{90}	2.24×10^7	5.89×10^6	3.89×10^7

4.3.2 Dose mortality curves for the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections against *T. leucotreta* neonate larvae

The CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mortality curves were generated from the data obtained from surface-dose biological assays. The CrleGV/CrpeNPV mixture performed best in comparison to CrleGV/CpGV and CrleGV/HearNPV respectively as seen in **Figure 4.4.**

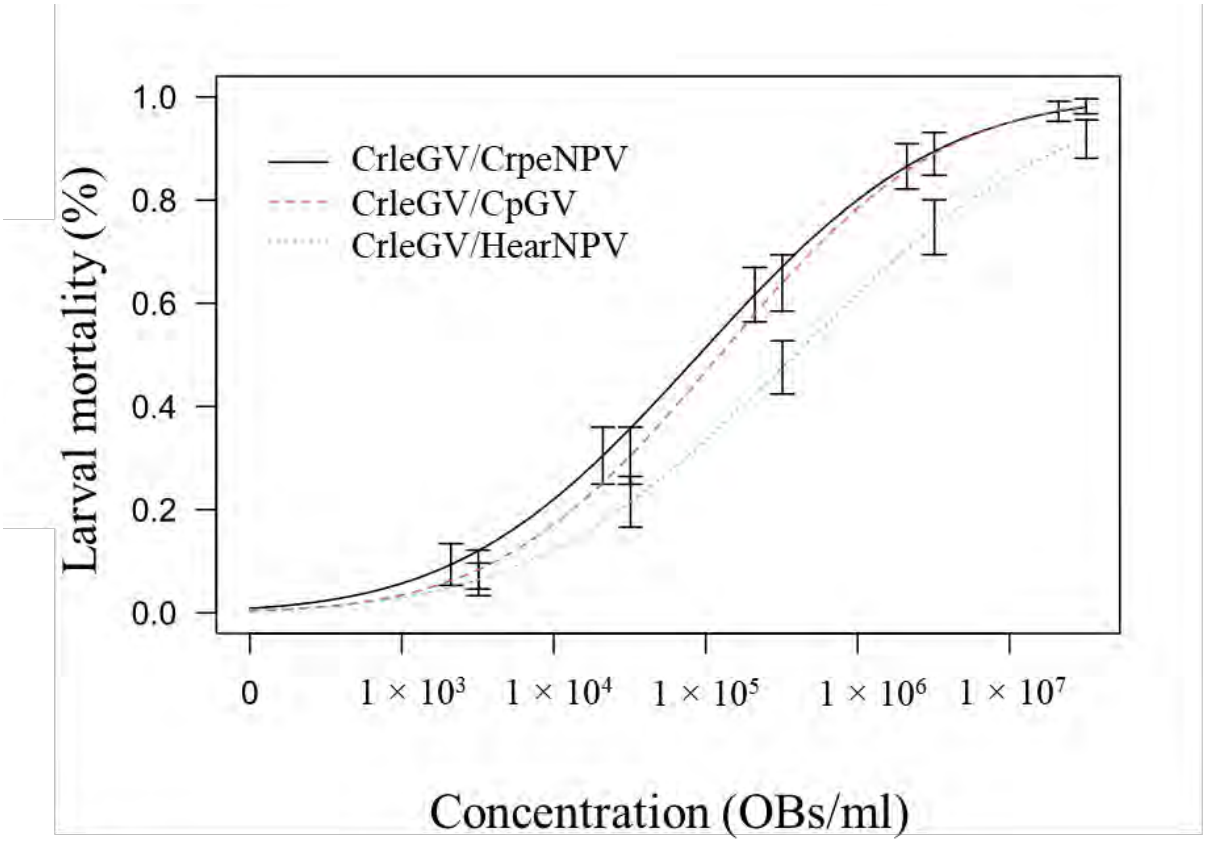


Figure 4.4 The dose-mortality curves for CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV against *T. leucotreta* neonate larvae. The CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV concentrations ranged from 2.11×10^7 OBs/ml to 2.11×10^3 OBs/ml, 3.19×10^7 OBs/ml to 3.19×10^3 OBs/ml and 3.20×10^7OBs/ml to 3.20×10^3 OBs/ml respectively.

4.3.3 Evaluation of synergism, antagonism, or additivity in the CrleGV/CrpeNPV and CrleGV/CpGV mixture against *T. leucotreta* neonate larvae using the Tammes-Bakuniak graphic method

The effect of viral interactions can be determined using the Tammes-Bakuniak method (Lara Reyna et al., 2003). The viral mixtures of CrleGV/CrpeNPV were observed to be antagonistic as indicated in **Figure 4.5**.

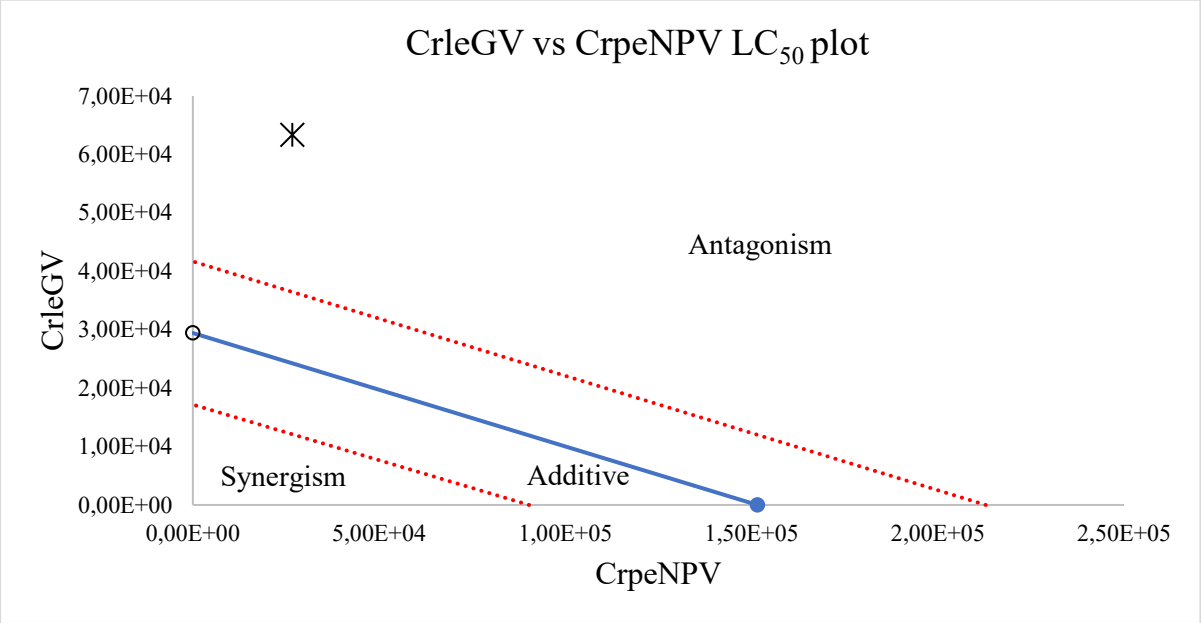


Figure 4.5 Tammes-Bakuniak plot representing the antagonistic interaction between CrleGV and CrpeNPV at LC_{50}. The solid line represents the equitoxic line which separates the antagonism zone from the synergism zone in the graph. The dotted lines indicate the LC_{50} 95% upper and lower fiducial limits. The area around the equitoxic line represents the additivity zone. The combination effect is indicated by a × symbol.

The additive zone was plotted using the individual confidence intervals for CrleGV and CrpeNPV, with the lower and upper limits for this additive zone having 9.03×10^4 OBs/ml and 2.13×10^5 OBs/ml values for the x-axis intercept and 1.72×10^4 OBs/ml and 4.17×10^4 OBs/ml values for the y-axis intercept. The LC_{50} line fell between these limits based on the single infections, which are 1.52×10^5 OBs/ml and 2.94×10^4 OBs/ml for CrleGV-SA and CrpeNPV, respectively. The combined CrleGV/CrpeNPV LC_{50} was determined to be 1.24×10^5 OBs/ml, with this position determined to fall at a position 2.67×10^4 OBs/ml, 6.33×10^4

OBs/ml on the x and y axis based on the ratio of each virus, falling within the antagonistic region.

The effect of the CrleGV/CpGV mixture was also determined using the Tammes-Bakuniak method (Lara Reyna et al., 2003). The viral mixtures of CrleGV/CpGV were observed to be antagonistic as indicated in **Figure 4.6**.

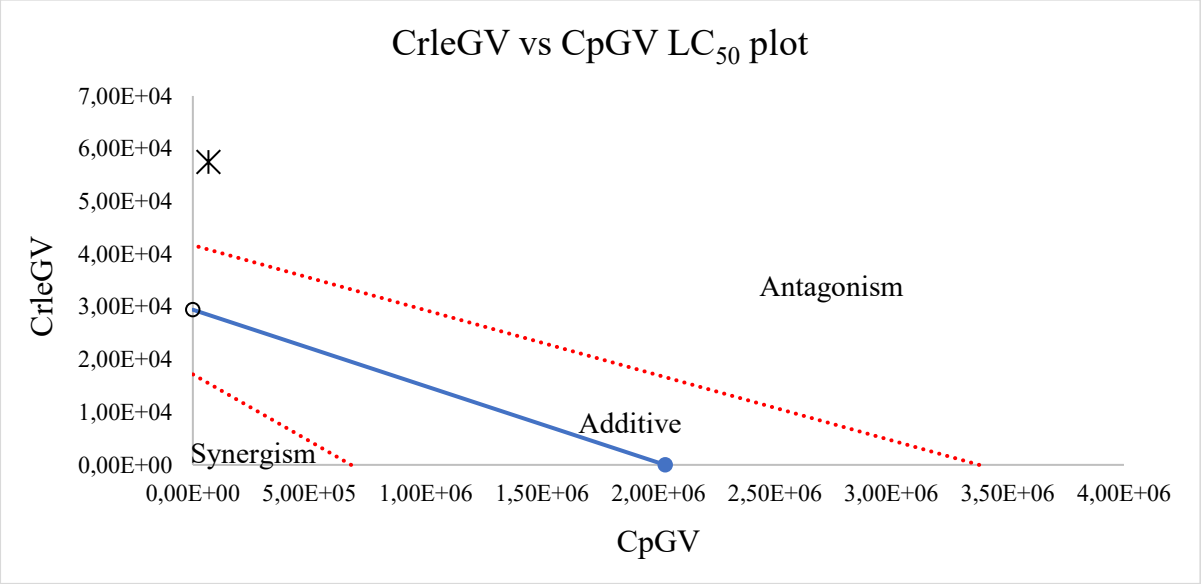


Figure 4.6 Tammes-Bakuniak plot representing the antagonistic interaction between CrleGV and CpGV at LC_{50}. The solid line represents the equitoxic line which separates the antagonism zone from the synergism zone in the graph. The dotted lines indicate the LC_{50} 95% upper and lower fiducial limits. The area around the equitoxic line represents the additivity zone. The combination effect is indicated by a × symbol.

The additive zone was plotted using the individual confidence intervals for CrleGV and CpGV, with the lower and upper limits for this additive zone having 6.80×10^5 OBs/ml and 3.38×10^6 OBs/ml values for the x-axis intercept and 1.72×10^4 OBs/ml and 4.17×10^4 OBs/ml values for the y-axis intercept. The LC_{50} line fell between these limits based on the single infections, which are 2.03×10^6 OBs/ml and 2.94×10^4 OBs/ml for CrleGV-SA and CpGV, respectively. The combined CrleGV/CpGV LC_{50} was determined to be 1.24×10^5 OBs/ml, with this position determined to fall at a position 6.68×10^4 OBs/ml, 5.74×10^4 OBs/ml on the x and y axis based on the ratio of each virus, falling within the antagonistic region. The interaction between CrleGV/HearNPV could not be evaluated using this method as the LC_{50} and LC_{90} values from the HearNPV single infection assays were not obtained.

4.4 Discussion

The chapter aimed to evaluate the biological activity of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections against *T. leucotreta* neonate larvae using surface dose biological assays. The purpose was to investigate whether the mixed infections could potentially enhance the infectivity of CrleGV in terms of lethal concentration against *T. leucotreta* neonate larvae. The LC_{50} and LC_{90} reported values from the CrleGV/CrpeNPV, CrleGV/CpGV and CrleGV/HearNPV mixed infections did not show any improvement compared to CrleGV when applied alone against *T. leucotreta* neonate larvae. However, the CrleGV/CrpeNPV and CrleGV/CpGV mixtures did show improvements compared to CrpeNPV and CpGV-M when the viruses were applied alone against *T. leucotreta* neonate larvae. The LC_{50} and LC_{90} values from the CrleGV-SA, CrpeNPV, and CpGV-M single infections as reported in Chapter 3 are indicated in **Table 4.4**. The Tammes-Bakuniak graphic method further confirmed the viral mixtures of CrleGV/CrpeNPV and CrleGV/CpGV to have an antagonistic effect at LC_{50} for both mixtures.

Table 4.4 The LC_{50} and LC_{90} for the CrleGV-SA, CrpeNPV, and CpGV-M against *T. leucotreta* neonate larvae

	Concentration (OBs/ml)		
	CrleGV-SA	**CrpeNPV**	**CpGV-M**
LC_{50}	2.94×10^4	1.52×10^5	2.02×10^6
LC_{90}	1.21×10^6	8.02×10^6	3.31×10^9

In the study by Jukes, (2018), a 1:3 GV/NPV mixture proved to be synergistic against *T. leucotreta* neonates at LC_{50} and LC_{90}. However, in the GV dominant mixture, Jukes, (2018) observed an additive effect at LC_{50}. In the study by Taylor, (2021) synergistic interactions were observed in a 1:1 GV/NPV against *T. leucotreta* neonate larvae. The CrleGV/CrpeNPV LC_{50} and LC_{90} values from studies performed by Jukes, (2018) and Taylor, (2021) are indicated in **Table 4.5**. It was intended to evaluate a 50:50 mixture but due to the method

used to prepare the viral dilutions, the resulting ratios deviated from this target. As reviewed in Ferrelli & Salvador, (2023), the OB proportions of viruses have a significant impact on mixed infections and thus different ratios of CrleGV in combination with CrpeNPV, CpGV, and HearNPV could have different outcomes. In this study, a 70:30 CrleGV/CrpeNPV mixture was produced while for the other two viral mixtures, it was closer to a 50:50 mixture. Given that 75:25, 25:75, and 50:50 mixtures of CrleGV/CrpeNPV have already been tested against *T. leucotreta* neonate larvae, this enabled another ratio to be evaluated. Reviewed literature does not clearly state a criterion for determining the doses of viruses used in viral mixtures (Ferrelli & Salvador, 2023). This makes it difficult to know the amount of virus or proportion to use before the assays are initiated. The ratio of the proportion of each virus in a mixture significantly affects the outcome of the interaction as observed in this study. As previously mentioned, the polyhedral OBs of NPVs have multiple virions in comparison to granular OBs of GVs which contain a single virion. Based on that, equal volumes of GV/NPV mixtures at equal concentrations are technically NPV-dominated due to the higher number of virions per OB. Previous studies by Barrera et al., (2021) and Cuartas-Otálora et al., (2019) observed synergistic interactions where the GV proportion in the GV/NPV mixture did not exceed 2.5%. Goto, (1990) observed detrimental effects in mixed infections of Xestia c-nigrum granulovirus (XecnGV) and Xestia c-nigrum nucleopolyhedrovirus (XecnNPV) against 4th instar *Xestia c- nigrum* larvae. In the study, increased GV proportions in the GV/NPV mixture were suggested to interfere with the NPV infection. Lower proportions of GVs where both the GV/NPV infect the same host are often associated with a synergistic outcome as seen with the above-mentioned mixed infection cases. However, when the host is not susceptible to the GV infection, higher GV concentrations are associated with a synergistic outcome. This was demonstrated in larvae that were treated in a 1:120 mixture of Anticarsia gemmatalis multiple nucleopolyhedrovirus (AngeMNPV) and Epinotia aporema granulovirus (EpapGV) OBs against *Anticarsia gemmatalis* larvae resulting in a synergistic interaction with improved lethal concentration and lethal time as reported by Biedma et al., (2015). On the contrary, Jukes, (2018) only observed a synergistic interaction in the 3:1 GV/NPV mixture at LC_{90} against *T. leucotreta* neonate larvae as opposed to an additive effect at LC_{50}. In this case, both the GV/NPV are known to infect *T. leucotreta* larvae (Jukes, 2018; Marsberg, 2016). However, in this study, a 70:30 CrleGV/CrpeNPV proportion did not result in an additive or synergistic effect as previously reported by Jukes, (2018) and Taylor, (2021). The antagonistic interaction observed could be due to variations in the genotype/isolates used, differences in proportions used in the viral mixture, colony health,

and environmental conditions which can have an impact on the outcome of the interaction. However, the CrleGV/CrpeNPV mixture was the most virulent in comparison to CrleGV/CpGV and CrleGV/HearNPV against *T. leucotreta* neonates. The negative interaction may suggest the need to potentially improve ratios and further study synergistic factors that are associated with OBs to gain a comprehensive understanding of the interaction if both viruses are applied in the field against *T. leucotreta* neonate larvae.

Table 4.5 The LC_{50} and LC_{90} for CrleGV and CrpeNPV mixed infections against *T. leucotreta* neonate larvae reported by Jukes, (2018) and Taylor, (2021).

Study	Ratio (GV: NPV)	LC_{50}	LC_{90}
Taylor, (2021)	1:1	4.38×10^3	4.19×10^4
Jukes, (2018)	1:3	7.95×10^4	7.18×10^5
Jukes, (2018)	3:1	8.55×10^4	1.07×10^6
This study	7:3	9.03×10^4	3.49×10^6

The CrleGV/CpGV viral mixture was the second most virulent mixture against *T. leucotreta* neonates. The cases that have been reported involving GV/GV interactions include natural or experimental mixtures of GV genotypes or assays of closely related GVs against insect hosts. Jehle et al., (2003) performed studies on the potential inter-specific recombination of CrleGV and CpGV in mixed infections against 4th instar *T. leucotreta* larvae. From the results, high larval mortalities were observed when CpGV was applied in higher proportions but at equal concentrations of both GVs, the larval mortality was below 50%. The results reported by Jehle et al., (2003) may suggest a competition for larval resources between the GVs in a 1:1 ratio but this cannot be concluded as a study to determine the cause of larval death was not performed. In addition, single infection assays from the study were not performed making it challenging to compare the effect of the mixed infections against the 4th instar *T. leucotreta* larvae. The efficacy of GV infections largely depends on the applied dose, susceptibility of the insect host, and the extent of infection based on their tissue tropism (Winstanley & ’ Reilly,) . C GV is nown to be a ‘fast’ acting or a ty e 2 GV. t has a wide tissue tropism, largely affecting the midgut, fat body, and epidermis of susceptible hosts

(Winstanley & ’ Reilly,) . But despite CpGV having a fast speed of kill, it is 1000 times less virulent against *T. leucotreta* compared to CrleGV (Fritsch et al., 1990). The lower susceptibility and perhaps competition for resources may be the result of why an antagonistic interaction from this mixture was observed. Since in this study, 46:54 CrleGV/CpGV OB proportions were evaluated in the GV/GV mixture, future work may include evaluating ratios where CpGV is applied at significantly higher concentrations.

As seen in Chapter 3, *T. leucotreta* was not susceptible to HearNPV infection preventing the determination of the LC_{50} and LC_{90} values. For this reason, the viral mixture of CrleGV/HearNPV could not be evaluated using the Tammes-Bakuniak graphic method as the LC_{50} and LC_{90} values from the single infections are required. The CrleGV/HearNPV viral mixture was the third most virulent mixture. The LC_{50} and LC_{90} values from the CrleGV/HearNPV mixed infection did not show any improvement in comparison to the CrleGV single infections. The result may suggest that the NPV did not have an effect, but PCR-based techniques will be used to confirm the cause of larval death (Chapter 5). This is first the study to report mixed infections on *T. leucotreta* where one virus does not infect the host thus making it difficult to make any comparisons.

The aims of this chapter in determining the relative virulence of the mixtures of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV were achieved using surface dose assays. The following chapter will determine and discuss the cause of larval death from single and mixed infections using PCR-based techniques.

Chapter 5
Investigating the cause of larval mortality in *T. leucotreta* cadavers using PCR-based methods

5.1 Introduction

The biological activity of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au against *T. leucotreta* in single and mixed infections was evaluated in Chapters 3 and 4. It is important to determine that the viruses used in the biological assays were in fact the cause of larval mortality and not due to other stress factors such as handling, contamination, or the desiccation of the diet. Molecular techniques can be used to determine the cause of mortality. This can be done by the collection of the larval cadavers and extracting total DNA using modified protocols. This is followed by PCR amplification of the viral DNA using genome specific oligonucleotides. Sequencing and BLAST analysis of the PCR amplicons would then reveal which viruses were present in the single and mixed infections thus determining the cause of larval mortality.

It is important to not assume that larval mortality is due to virus infection. One challenge associated with determining the cause of larval mortality in biological assays where single and/or mixed infections are involved is the possibility of inducing a covert virus which can then enter the infectious cycle. Covert infections can be induced as a result of baculovirus infection (Burden et al., 2002). Various stressors such as differences in diet, temperature, overcrowding, or infecting larvae with a heterologous virus can induce a covert infection (Hughes et al., 3; l'iny h & Ul'yanova, 2005; u a n, ; o u -Debrah et al., 2013; Smith, 1967). Covert infections are often associated with low virulence in host populations (Williams et al., 2017). Covertly infected insects often appear healthy and asymptomatic. Covert infections are typically transmitted from parent to offspring through vertical transmission, with the virus persisting longer within the generation and does not result in the death of the insect (Williams et al., 2017).

The detection of covert infections has been documented in *T. leucotreta* larvae. For example, it has been reported that infection of *T. leucotreta* larvae with CrpeNPV in biological assays resulted in the induction of CrleGV which was then detected in deceased larvae by molecular techniques (Jukes, 2018; Taylor, 2021). Therefore, it cannot be assumed that the cause of larval mortality in single or mixed infections is due to the viruses chosen for infection which makes it imperative to apply PCR-based techniques to examine the cause of death.

In this chapter, larval cadavers and symptomatic larvae were collected from the single and mixed infections and examined for the presence of baculoviruses using the PCR-based methods developed in Chapter 2. The unique oligonucleotides detect the presence of each of the baculoviruses used in the infection studies. The detection of viral genetic material could potentially determine the cause of larval mortality whether from the viruses applied in the biological assays or from a covert virus that was induced during infection. This information will assist in gaining insight into how the viruses interact in the mixed infections and will provide knowledge on both single and dual infections from an integrated pest management point of view.

This chapter aimed to use PCR-based techniques to detect which baculoviruses were present in *T. leucotreta* neonate-sized cadavers collected from the single and mixed infections evaluated in Chapters 3 and 4. To achieve this aim, the first objective was to collect the neonate-sized *T. leucotreta* larval cadavers from single and mixed infections. The second objective was to extract DNA from the *T. leucotreta* neonate cadavers using a modified CTAB DNA extraction protocol and test the four unique oligonucleotide sets designed in Chapter 2 on the gDNA templates by standard PCR. The third objective was to analyse the amplicons by Sanger sequencing and BLAST analysis. The fourth objective of this chapter was to perform alignments using ClustalW to confirm the presence of the baculoviruses detected in single and mixed infections.

5.2 Methods and Materials

5.2.1 *T. leucotreta* larval collection

Following the completion of each bioassay (Chapters 3 and 4), plates were meticulously examined for the presence of dead *T. leucotreta* neonate-sized larval cadavers which were carefully collected using sterile toothpicks. The symptomatic larvae from the HearNPV single infection biological assays were also collected using sterile toothpicks. The larvae were carefully picked to prevent the collection of surface diet. Larval cadavers were pooled together per replicate for each treatment across single and mixed infections and placed in labelled sterile 1.5 ml microcentrifuge tubes. The larval cadavers were stored at - 20 °C.

5.2.2 DNA extraction and PCR amplification

A modified CTAB DNA extraction method described by Taylor, (2021) was used to extract DNA from *T. leucotreta* neonate larvae to minimise the loss of DNA. DNA extractions were performed separately on pooled cadavers, with each representing one of three replicates from all single and mixed infections. The larval cadavers collected were homogenised in 5 µl ddH_2O. Following the homogenisation step, 2.5 µl Na_2CO_3 (sodium carbonate) 1 M was added and incubated at 37°C for 30 minutes. A volume of 3 µl Tris-HCL (1M; pH 6.8), 1.25 µl 10% (w/v) SDS, and 0.5 µl proteinase K (25 mg/ml) was added before incubating at 37°C for 30 minutes. A volume of 0.5 µl RNase A (10 mg/ml) was added to the samples and incubated at 37°C for 30 minutes. Following the incubation period, the samples were centrifuged at 12 100 ×g for 2 minutes using a MiniSpin® desktop centrifuge (Eppendorf, Germany). The supernatants were collected and added to new tubes before 10 µl (70°C) CTAB buffer (2% w/v CTAB, 10 mM Tris (pH 8.0), 20 mM Na_2EDTA, 1.4 M NaCl) was added and the samples were incubated at 70°C for 45 minutes and inverted regularly. Following, 50 µl of pre-cooled chloroform (4°C) was added to each tube, inverted regularly, and centrifuged at 6 700 ×g for 10 minutes. The aqueous layers were carefully transferred into new 1.5 ml microcentrifuge tubes. Fifty microliters of ice-cold isopropanol (-20°C) was added and the samples were incubated overnight at -20°C. Following the overnight step, the sample was centrifuged at 12 100 ×g for 20 minutes and the supernatant was discarded. A volume of 100 µl ice-cold ethanol (70%) was added to the pellet before centrifugation at 12 100 ×g for 5 minutes. The supernatant was discarded, and the pellet was air-dried before it was resuspended in 10 µl of autoclaved ddH_2O. The tubes were labelled with appropriate details, and the genomic DNA (gDNA) was stored at -20°C for long-term storage.

A PCR test was set up using the four oligonucleotide sets (**Table 2.1**) against the gDNA templates that were extracted from infected *T. leucotreta* neonate larvae in single and mixed infections. Each PCR reaction consisted of 12.5 µl of *Taq* DNA Polymerase 2× Master Mix Red (Ampliqon, Denmark), 1 µl 10 µM of forward and 1 µl 10 µM reverse oligonucleotides and 1.5 µl of gDNA template. The PCR reactions were made up to 25 µl using autoclaved ddH_2O. A no template control (NTC) reaction was prepared which consisted of an additional ddH_2O instead gDNA template. The PCR reaction had an initial denaturation step at 95°C for 3 minutes. Following, the samples underwent 30 cycles of 95°C for 1 minute, 56°C for 30 seconds, 72°C for 1 minute, and lastly the elongation step at 72°C for 3 minutes. The samples were kept at -20°C until being resolved using 1% AGE.

5.2.3 Agarose gel electrophoresis

The PCR amplicons were resolved using 1% AGE. As described in Chapter 2 (section 2.2.5), to prepare the gel, 1 g Agarose LE powder (Benchmark, USA) was dissolved in 100 ml 1 × TAE (40mM Tris-acetate, 20 mM acetic acid, 1mM EDTA) before staining with 4µl ethidium bromide (10mg/ml). A volume of 5 µl of each PCR amplicon was loaded in each well and run at 75 V for 45 minutes alongside 6 µl GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, USA). The gel was visualised using a ChemiDoc™ XRS+ (BioRad, USA), and the images were captured by the Image Lab™ software (BioRad, USA). The captured images were cropped to size.

5.2.4 Sanger sequencing and BLAST analysis of PCR amplicons

The PCR amplicons were sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South Africa) in the forward and reverse direction using the M13 forward and reverse sequencing oligonucleotides, which bind to the respective M13 sequences engineered into the oligonucleotides listed in **Table 2.1** as previously mentioned in Chapter 2. Similarly, the first 10 base pairs at the beginning of each sequence and the last 10 base pairs at the end of each sequence were manually trimmed off using the Geneious R11.1.5 software. Ambiguities were manually corrected based on sequence chromatograms after which each sequence was subjected to NCBI BLAST analysis and optimised to search against highly similar sequences (Megablast). This was followed by a ClustalW alignment in Geneious R11.1.5 of each sequence against the respective target region, which was extracted from each reference sequence obtained from GenBank as described in Chapter 2 (section 2.2.7).

5.3 Results

5.3.1 *T. leucotreta* larval collection

Each replicate per virus treatment had 120 *T. leucotreta* neonate-sized larvae excluding the control. Following the completion of the biological assays, not all dead *T. leucotreta* neonate larvae that were recorded in Chapters 3 and 4 could be collected. The number of larvae that died and those that were collected are indicated in **Table 5.1** and **Table 5.2**

Table 5.1 *Thaumatotibia leucotreta* larval collection from the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au single infections.

CrleGV Single Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	88	56
Rep 2	85	37
Rep 3	73	30
CrpeNPV Single Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	63	32
Rep 2	51	27
Rep 3	55	19
CpGV Single Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	54	21
Rep 2	49	29
Rep 3	52	32
HearNPV Single Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	1	22
Rep 2	1	23
Rep 3	0	24

Table 5.2 *Thaumatotibia leucotreta* larval collection from the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections

CrleGV/CrpeNPV Mixed Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	67	23
Rep 2	64	31
Rep 3	73	29
CrleGV/CpGV Mixed Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	69	36
Rep 2	69	28
Rep 3	68	32
CrleGV/HearNPV Mixed Infection		
	Number of dead *T. leucotreta* larvae	Number of *T. leucotreta* larvae collected
Rep 1	60	33
Rep 2	53	27
Rep 3	60	25

5.3.2 PCR amplification of the gDNA extracted from single infections

Following DNA extraction from the *T. leucotreta* neonate larval cadavers, each gDNA template from each replicate served as a template to test the four unique oligonucleotide sets designed in Chapter 2 (**Table 2.1**). The results from the single infections are presented in **Figure 5.1** and the results from the mixed infections are presented in **Figure 5.2**. The lanes labelled 'A' re resent the gDNA tested using the CrleGV oligonucleotide a ir from each re li cate. h e lanes labelled ' ' re r esent the gDNA tested using the Cr e N V oligonucleotide a ir from each re li cate. he lanes labelled 'C' re r esent the gDNA tested using the C GV oligonucleotide a ir from each re li cate. he lanes labelled 'D' re r esent the

gDNA tested using the HearNPV oligonucleotide pair from each replicate. The lanes labelled 'N' are the no tem l ate controls for each reaction.

Reporting on the results from the single infections, as seen in **Figure 5.1 (i)** for the CrleGV template, the CrleGV F/R oligonucleotide pair amplified PCR products slightly smaller than 750 bp as observed in lanes A in replicate 1, 2, and 3 respectively. Comparatively, the amplicon from replicate 2 is very faint and not as visible as those seen in replicates 1 and 3. No amplicons were observed in the no template control as observed in lane N. No PCR products were amplified using the CrpeNPV F/R, CpGV F/R, and HearNPV F/R oligonucleotide sets shown in lanes labelled B, C, and D respectively for all three replicates. For the CrpeNPV gDNA template **Figure 5.1 (ii)**, PCR products were amplified by the CrpeNPV F/R oligonucleotides as observed in lanes B for replicates 1, 2, and 3. The amplicons are slightly larger than 750 bp. A faint band amplified by the CrleGV F/R oligonucleotide pair was also observed which is smaller than 750 bp as observed in lane A in replicate 2. No PCR products were amplified in the no template control as seen in lane N. The CrleGV F/R oligonucleotide pair did not amplify PCR products as indicated in lanes A in replicate 1 and 3. The CpGV F/R and the HearNPV F/R oligonucleotide sets did not amplify any PCR products as observed in lanes C and D in replicates 1, 2, and 3 respectively.

As seen in **Figure 5.1 (iii)** for the CpGV template, the CpGV F/R oligonucleotides amplified PCR products slightly larger than 750 bp in all three replicates as seen in lanes C. No amplicons were observed in the no template control indicated in lane N. Furthermore, no PCR products were amplified using the CrleGV F/R, CrpeNPV F/R, and HearNPV F/R oligonucleotide sets as shown in lanes A, B, and D for all three replicates, respectively. For the HearNPV gDNA template **Figure 5.1 (iv**), very faint PCR products were amplified by the CrleGV F/R oligonucleotides as observed in lanes A for the replicates 2 and 3. The amplicons are slightly smaller than 750 bp. No PCR products were amplified in the no template control as seen in lane N. The CrleGV F/R oligonucleotide pair did not amplify a PCR product in replicate 1 as seen in lane A. The CrpeNPV F/R, CpGV F/R, and HearNPV F/R oligonucleotide sets did not amplify any PCR products in all three replicates as observed in lanes B, C, and D respectively.

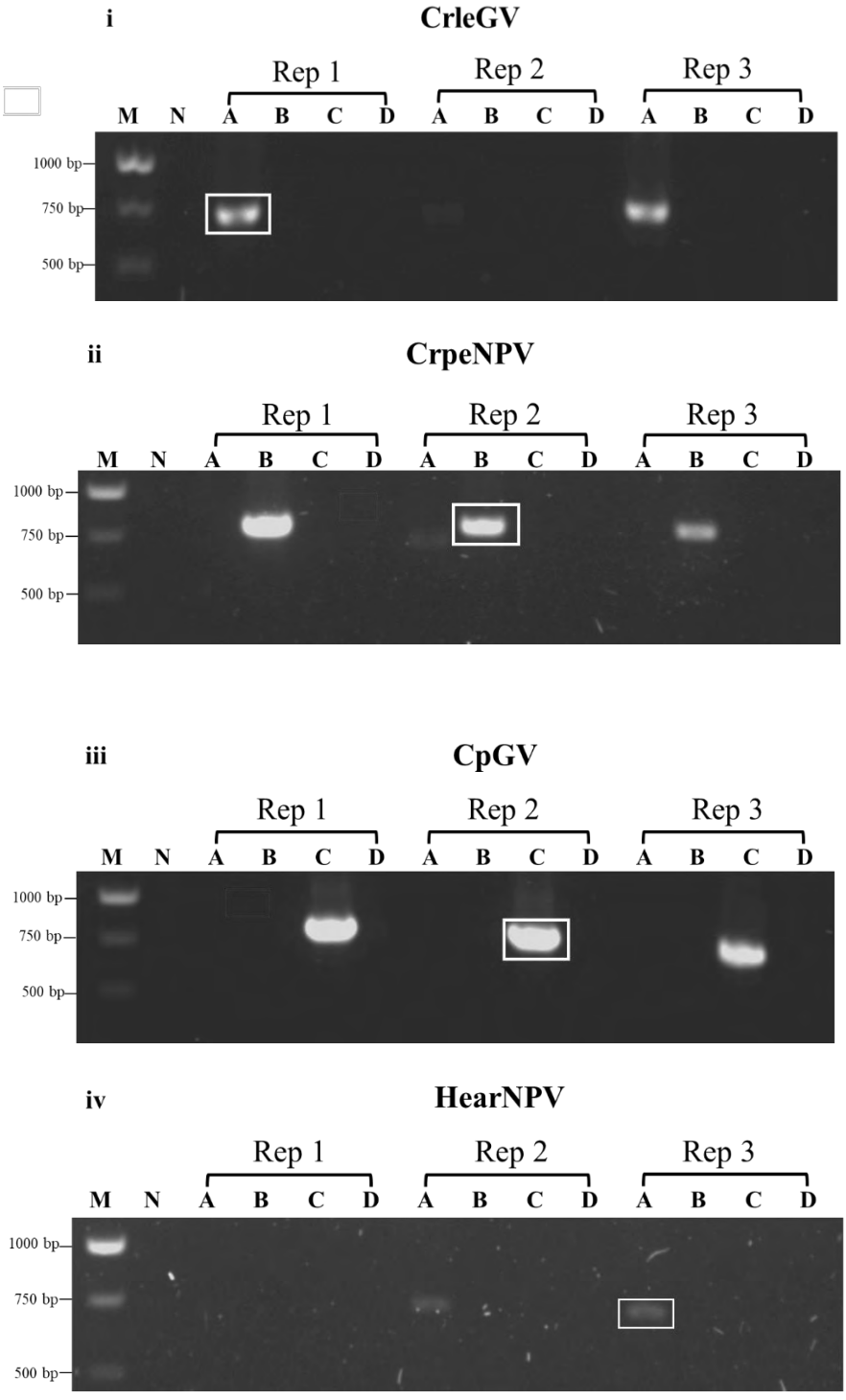


Figure 5.1 AGE analysis of PCR amplicons generated with gDNA extracted from larval cadavers collected for each replicate of the single infection bioassays, where M – GeneRuler 1kb DNA ladder; N – No template control; A – CrleGV oligonucleotides; B – CrpeNPV oligonucleotides; C – CpGV oligonucleotides; D – HearNPV oligonucleotides. **(i)** PCR amplification using CrleGV gDNA as a template from replicates 1, 2, and 3. (**ii**) PCR amplification using CrpeNPV gDNA as a template from replicate 1, 2, and 3. (**iii**) PCR

amplification using CpGV gDNA as a template from replicate 1, 2 and 3. (**iv**) PCR amplification using HearNPV gDNA as template from replicate 1, 2 and 3. White boxes indicate PCR samples sent for Sanger sequencing

5.3.3 PCR amplification of gDNA extracted from the mixed infections

The results from the mixed infections are shown in **Figure 5.2.** The CrleGV F/R and CrpeNPV F/R oligonucleotides amplified PCR products in all 3 replicates as observed in lanes A and B respectively in the CrleGV/CrpeNPV mixed infection gDNA template as seen in **Figure 5.2 (i**). The CrleGV F/R oligonucleotides amplified PCR products slightly smaller than 750 bp in replicates 1, 2, and 3 as seen in lanes A. The CrpeNPV F/R oligonucleotides amplified PCR products larger than 750 bp in replicates 1, 2, and 3 as seen in lanes B. No amplification of PCR products was observed in the no template control as observed in lane N. The CpGV F/R and HearNPV F/R oligonucleotide sets did not amplify any PCR products as indicated in lanes C and D respectively for all three replicates. Similar results were observed in the PCR amplification of the CrleGV/CpGV template where the CrleGV F/R and CpGV F/R oligonucleotides amplified products from the three replicates as observed in lanes A and C respectively (**Figure 5.2 ii**). Bands slightly smaller than 750 bp were amplified by CrleGV F/R oligonucleotides in replicates 1, 2, and 3 as seen in lanes A. The CpGV F/R oligonucleotides amplified PCR products larger than 750 bp in replicates 1 and 2 but did not amplify any PCR product in the third replicate as observed in lanes C. No amplification of PCR products was observed in the no template control as indicated in lane N. No PCR amplicons were amplified by the CrpeNPV F/R and HearNPV F/R oligonucleotide sets in all three replicates as indicated in lanes B and D respectively.

The PCR results of the last mixture between CrleGV/HearNPV are presented in **Figure 5.2 (iii)**. No amplification was observed in the no template control as indicated in lane N. The CrleGV F/R oligonucleotides amplified PCR products in all three replicates as observed in lanes A. The faint bands appear to be slightly less than 750 bp in size. The CrpeNPV F/R, CpGV F/R, and HearNPV F/R oligonucleotide sets did not amplify any PCR products in all three replicates as indicated in lanes B, C, and D respectively.

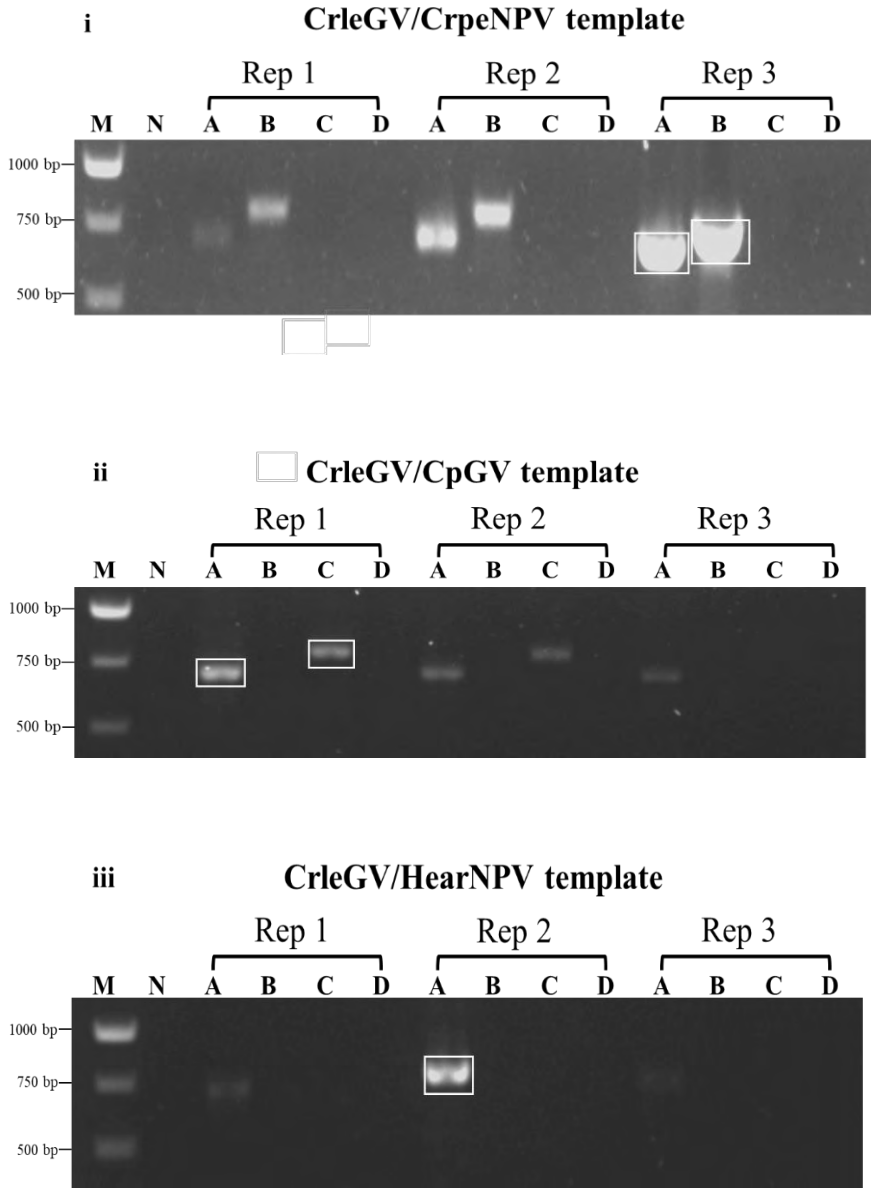


Figure 5.2 AGE analysis of PCR amplicons generated with gDNA extracted from larval cadavers collected for each replicate of the mixed infection bioassays, where M - GeneRuler 1kb DNA ladder; N - No template control; A - CrleGV oligonucleotides; B - CrpeNPV oligonucleotides; C - CpGV oligonucleotides; D - HearNPV oligonucleotides. **(i)** PCR amplification using CrleGV/CrpeNPV gDNA as a template from replicates 1, 2, and 3. (**ii**) PCR amplification using CrleGV/CpGV gDNA as a template from replicate 1, 2, and 3. (**iii**) PCR amplification using CrleGV/HearNPV gDNA as a template from replicate 1, 2 and 3. White boxes indicate PCR samples sent for Sanger sequencing

5.3.4 Sanger sequencing of PCR amplicons and BLAST analysis from the single infections

The PCR amplicons indicated with a white box in the AGE results from **Figure 5.1** were sent for sequencing. The CrpeNPV and CpGV PCR products resulting from the CrpeNPV and CpGV single infections respectively were sequenced in the forward direction using the M13 forward sequencing oligonucleotide. The CrleGV PCR products resulting from the CrleGV and HearNPV single infections were sequenced in the reverse direction using the M13 reverse sequencing oligonucleotide. Following, the sequences were manually trimmed and corrected, submitted to BLAST analysis, and searched against highly similar sequences (Megablast). The top three matches (if available) from the BLAST analysis search for each virus are indicated in **Table 5.3**. This was followed by the alignment of each sequence against the target regions using ClustalW alignment in Geneious R11.1.5. The results from the alignments are included in Appendix B.

Table 5.3. The top matches resulted from the BLAST analysis of selected samples from the CrleGV, CrpeNPV, CpGV, and HearNPV single infections

Single Infection	Description	Scientific name	Query Cover	Percentage Identity	Accession number
CrleGV	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	CrleGV	96%	96.02%	MF974563.1
	Cryptophlebia leucotreta granulovirus, complete genome	CrleGV	90%	83.94%	NC_005068.1
CrpeNPV	Cryptophlebia peltastica nucleopolyhedrovirus, complete genome	CrpeNPV	98%	99.86%	NC_055500.1
	Spodoptera littoralis nucleopolyhedrovirus isolate SpliNPV-Tun2, complete genome	SpliNPV	8%	86.89%	MG958660.1
	Spodoptera littoralis NPV isolate AN1956, complete genome	SpliNPV	8%	86.89%	NC_038369.1
CpGV	Cydia pomonella granulovirus isolate CpGV-M, complete genome	CpGV	97%	97.31%	KM217575.1
	Cydia pomonella granulovirus isolate CpGV-ZY, complete genome	CpGV	97%	97.31%	MN696171.1
	Cydia pomonella granulovirus isolate CpGV-KS2, complete genome	CpGV	97%	97.31%	MN696168.1
CrleGV	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	CrleGV	86%	96.19%	MF974563.1
	Cryptophlebia leucotreta granulovirus, complete genome	CrleGV	83%	86.25%	NC_005068.1
	Cryptophlebia leucotreta granulovirus isolate CV4 orf427 gene, partial cds	CrleGV	31%	88.39%	AY096243.1

5.3.5 Sanger sequencing of PCR amplicons and BLAST analysis from the mixed infections

The PCR amplicons indicated with a white box in AGE results from **Figure 5.2** were sent for sequencing. The CrleGV and CrpeNPV PCR products resulting from the CrleGV/CrpeNPV mixed infections were sequenced in the forward direction using the M13 forward sequencing oligonucleotide. The CpGV PCR product resulting from the CrleGV/CpGV mixed infections was sequenced in the forward direction using the M13 forward sequencing oligonucleotide. The CrleGV PCR product resulting from the CrleGV/HearNPV mixed infections was sequenced in the forward direction using the M13 forward sequencing oligonucleotide. The CrleGV PCR product resulting from the CrleGV/CpGV mixed infections was sequenced in the reverse direction using the M13 reverse sequencing oligonucleotide. The sequences were manually trimmed and corrected, submitted to BLAST analysis, and searched against highly similar sequences (Megablast). The top three matches (if available) from the BLAST search for each virus (**Table 5.4)**. A BLAST analysis search for the CrleGV PCR product from the CrleGV/CrpeNPV mixed infection could not be obtained. Exactly as mentioned in Chapter 2, this was followed by ClustalW alignment in Geneious R11.1.5 of each sequence against the respective target region, which was extracted from each sequence obtained from GenBank. The results from the alignments are included in Appendix B.

Table 5.4 The top matches resulted from the BLAST analysis of selected samples from the CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV mixed infections.

Mixed infection	Virus	Description	Scientific name	Query Cover	Percentage Identity	Accession number
CrleGV/CrpeNPV	CrpeNPV	Cryptophlebia peltastica nucleopolyhedrovirus, complete genome	CrpeNPV	98%	99.86%	NC_055500.1
		Spodoptera littoralis nucleopolyhedrovirus isolate SpliNPV-Tun2, complete genome	SpliNPV	8%	86.89%	MG958660.1
		Spodoptera littoralis NPV isolate AN1956, complete genome	SpliNPV	8%	86.89%	NC_038369.1
CrleGV/CpGV	CrleGV	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	CrleGV	94%	97.37%	MF974563.1
		Cryptophlebia leucotreta granulovirus, complete genome	CrleGV	91%	84.82%	NC_005068.1
	CpGV	Cydia pomonella granulovirus isolate CpGV-M, complete genome	CpGV	98%	98.25%	KM217575.1
		Cydia pomonella granulovirus isolate CpGV-ZY, complete genome	CpGV	98%	98.25%	MN696171.1
		Cydia pomonella granulovirus isolate CpGV-KS2, complete genome	CpGV	98%	98.25%	MN696168.1
CrleGV/HearNPV	CrleGV	Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	CrleGV	98%	92.44%	MF974563.1
		Cryptophlebia leucotreta granulovirus, complete genome	CrleGV	69%	85.53%	NC_005068.1

5.4 Discussion

The overall aim of this chapter was to detect the presence of the baculoviruses in the *T. leucotreta* neonate cadavers collected from the single and mixed infections. The purpose was to determine the cause of larval mortality using PCR-based techniques. To minimise the loss of DNA, a modified CTAB method was used to extract DNA from neonate-sized *T. leucotreta* cadavers. Genomic DNA extracted from samples in each replicate for the single and mixed infections served as a template to test for the presence of target baculoviruses using all four unique oligonucleotide sets designed in Chapter 2. Not all recorded dead *T. leucotreta* could be collected as infected individuals liquified upon handling, an indication of baculovirus infection (Clem & Passarelli, 2013). The *T. leucotreta* larvae collected from the HearNPV single infection biological assays appeared sluggish, feeding activity ceased, and the larval development was hindered which is also an indication of baculovirus infection (Harrison & Hoover, 2012). Infected individuals change their appearance and appear white or creamy due to the presence of occlusion bodies (OBs) (Federici, 1997). The symptomatic larvae were collected to determine which viruses were present. The AGE results from the CrleGV template in the single infections from replicates 1 and 3 show visible bands smaller than 750 bp amplified by the CrleGV F/R oligonucleotide pair. The bands are consistent with the expected size of 687 bp. The CrleGV F/R oligonucleotide pair amplified a very faint band in the second replicate also smaller than 750 bp, also consistent with the 687 bp expected size. The faint band could be due to low levels of viral DNA that could not be sufficiently amplified in the PCR reaction. The PCR amplification results from the CrleGV single infection show that CrleGV was the possible cause of larval mortality. The results are expected as CrleGV is highly pathogenic to *T. leucotreta* and likely the cause of death (Moore, 2002). The CrpeNPV templates from the single infections show visible bands of approximately 750 bp in all 3 replicates amplified by the CrpeNPV F/R oligonucleotide set, consistent with the expected size of 756 bp. CrpeNPV is known to infect *T. leucotreta* as studies have shown that CrpeNPV is highly virulent (Marsberg, 2016). A band smaller than 750 bp in the second replicate was amplified by the CrleGV oligonucleotide set, consistent with the expected size of 687 bp. Similar results have been reported where *T. leucotreta* inoculated with CrpeNPV, led to the detection of CrleGV-SA, possibly as a result of the presence of a covert to overt infection (Jukes, 2018). Sublethal or latent infections commonly exist in insect populations (Williams et al., 2017). PCR-based techniques are often used to detect covert infections targeting different genes in baculoviral genomes (Kemp et al., 2011;

Williams et al., 2017). The virus replicates in healthy tissue cells but at low levels, resulting in no visible signs of infection. The results from the PCR show that CrpeNPV is the likely cause of mortality. The AGE results from the CpGV single infections show visible bands amplified by the CpGV F/R oligonucleotide pair at approximately 750 bp in replicates 1, 2, and 3 respectively. The band sizes are consistent with the expected sizes of 764 bp. The detection of CpGV from the single infections is not surprising as CpGV is known to infect *T. leucotreta* (Fritsch et al., 1990). Chambers, (2014) also showed the successful production of CpGV in 4th and 5th instar *T. leucotreta* larvae. The results from the PCR show that CpGV is the likely cause of larval mortality. Although the HearNPV single infections did not result in larval mortality, as reported in Chapter 3, total gDNA was extracted from the symptomatic *T. leucotreta,* and PCR amplification using the four unique oligonucleotide sets was performed. The results from the AGE showed that the CrleGV F/R oligonucleotide set amplified faint bands smaller than 750 bp as indicated in replicate 2 and 3. These results are consistent with the expected size of 687 bp. HearNPV is not known to infect *T. leucotreta* as HearNPV isolates have a narrow host range, only known to infect species within the genera *Helicoverpa* and *Heliothis* (Gettig & McCarthy, 1982; Gröner, 1986). The PCR results suggest CrleGV covert infections in the *T. leucotreta* larvae. The HearNPV may have triggered a covert to overt infection or the covert to overt infection may have been triggered by another unknown stress factor. The latent infection appeared to allow the CrleGV infection to survive within the *T. leucotreta* larvae (Burden et al., 2003). But from a biological control perspective, covert infections are beneficial as insects with sublethal infections are susceptible to viral infections (Cooper et al., 2003; Kouassi et al., 2009).

The AGE results from the CrleGV/CrpeNPV mixed infections show visible bands amplified by the CrleGV and CrpeNPV oligonucleotide sets in all 3 replicates. Visible bands amplified by the CrleGV oligonucleotides are smaller than 750 bp, consistent with the expected size of 687 bp. The CrpeNPV oligonucleotide pair amplified PCR products approximately 750 bp in size, consistent with the expected size of 756 bp. The CrpeNPV bands were brighter compared to CrleGV except in the last replicate where the bands appeared to be equally bright. Since the number of virions per OB is higher for nucleopolyhedroviruses in comparison to granuloviruses, a 1:1 mixed infection between CrleGV and CrpeNPV is technically CrpeNPV dominated (Jukes, 2018; Marsberg, 2016). The results from the PCR show that both CrleGV and CrpeNPV in the mixed infections were the cause of larval mortality in all 3 replicates. For the CrleGV/CpGV gDNA template, the CrleGV

oligonucleotide sets amplified visible bands smaller than 750 bp in all three replicates, also corresponding to the expected size of 687 bp. The CpGV oligonucleotide sets amplified PCR products in replicates 1 and 2 but not in the third replicate. The AGE shows bands approximately 750 bp in length, corresponding with the expected size of 764 bp. The result from the PCR shows that CrleGV and CpGV in the mixed infections both played a role in the cause of larval death except for the third replicate where only the CrleGV oligonucleotide pair amplified a PCR product. The absence of an amplified CpGV PCR product may suggest a possible competition for host resources allowing the CrleGV to outcompete the CpGV infection (Wennmann et al., 2015). However, these results differ from studies by Wennmann et al., (2015) as the qPCR revealed the larvae died of the virus that was applied in a higher concentration in dual infections of AgseGV and AgseNPV-B against *A. segetum* larvae. The AGE results for the CrleGV/HearNPV show faint bands amplified by the CrleGV oligonucleotide pair in all 3 replicates. The faint bands are approximately smaller than 750 bp, also consistent with the expected size of 687 bp. The results from the PCR amplification show only the detection of CrleGV and not the HearNPV infection as no amplicons were amplified by the HearNPV oligonucleotide pair. CrleGV was the dominant virus as seen in the PCR results. HearNPV has not been reported to infect *T. leucotreta,* with only CrleGV detected and thus likely being the cause of larval mortality in the CrleGV/HearNPV mixed infections.

Only the PCR amplicons indicated with a white box were sent for sequencing. Some DNA sequences were sequenced in the forward direction using the M13 forward sequencing oligonucleotide and others in the reverse direction using the M13 reverse sequencing oligonucleotide and the results confirmed the presence of the target viruses using BLAST analysis (**Table 5.3** and **Table 5.4).** The BLAST analysis for the CrleGV PCR product from the CrleGV/CrpeNPV mixed infection could not be obtained. The DNA sequence could be sequenced in the M13 reverse direction using the M13 reverse sequencing oligonucleotide but due to time constraints, this was not performed. The sequences were also successfully aligned using ClustalW alignment in Geneious R11.1.5 against the respective target region, which was extracted from each reference sequence obtained from GenBank. The alignments are included in Appendix B. The results from the BLAST analysis and ClustalW alignment are similar to those obtained in Chapter 2 suggesting the viruses recovered from the bioassays were those that were extracted from the initial samples that were provided. The causes of the insertions and deletion in addition to the single nucleotide polymorphisms observed in the

alignments cannot be explained. The initial samples provided were thought to be CpGV-M however this has been multiplied in *C. pomonella* larvae which are locally sourced. There could be covert CpGV infections in these larvae which become overt when CpGV-M is inoculated. Other SNPs that were observed may be due to the use of standard *Taq* DNA polymerase. The use of a high-fidelity enzyme may reduce the number of SNPs. However, the results from the BLAST analysis and alignments confirm the presence of the target viruses that were used in the biological assays namely CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au respectively.

The results from the PCR do not conclude that there were no other baculoviral sequences present in both single and mixed infections, as PCR has its limitations in detection as previously discussed in Chapter 2. Another critique is the possible amplification of the virus as a result of surface contamination and not due to the larvae feeding. However, the results from the AGE show a strong indication that there was no surface contamination as seen from the CrleGV/HearNPV mixed infection, whereby no PCR products were amplified by the HearNPV F/R oligonucleotide pair. To further validate the results obtained, extensive sequencing needed to be done. Only the PCR amplicons indicated with a white box were sent for sequencing due to the reduction of costs and time. An ideal situation would be to increase the sample number by sending all PCR amplicons for sequencing including the faint bands. Furthermore, sequencing all samples in both the forward and reverse direction using the M13 forward and M13 reverse sequencing oligonucleotides. Future work may include evaluating the presence of baculoviruses in each individual larva collected from the single and mixed infections instead of pooling the larvae from each replicate. This may assist in the better understanding of how the individual larva react to the treatments. Additionally, to confirm the presence of the baculoviruses, morphology tests could have been done using transmission electron microscopy (TEM) which can be used to distinguish between the granuloviruses and nucleopolyhedroviruses based on morphology (Ackermann & Smirnoff, 1983; Bayramoglu et al., 2018; Grasela et al., 2008). Lastly, handling and collection of the *T. leucotreta* larval cadavers can be improved to increase the sample number as there is a difference between the number of dead larvae and those that were collected.

In conclusion, the overall aim of the study was achieved. The PCR-based techniques developed in Chapter 2 were used to detect the presence of baculoviruses in *T. leucotreta* cadavers collected from biological assays in single and mixed infections, to determine the possible cause of larval mortality.

Chapter 6
General Discussion

6.1 Thesis overview

The research investigating the effects of mixed baculoviral interactions against target pests is a growing field of interest. Synergistic interactions between CrleGV-SA and CrpeNPV against *T. leucotreta* were first reported by Jukes, (2018) and later by Taylor, (2021) which led to opportunities to further investigate other possible viral mixtures that could improve the control of *T. leucotreta*. The first objective of this study was to design oligonucleotides targeting unique regions in the genomes of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au, which was accomplished using Primer-BLAST software. Diseased *T. leucotreta, C. peltastica, C. pomonella,* and *H. armigera* larval cadavers were successfully obtained and this was followed by the crude extraction of OBs, from which DNA was subsequently extracted and used as a template for PCR amplification to confirm whether the samples contained the viruses of interest as described in Chapter 2. The enumeration of the stocks and dose-mortality of each virus alone against *T. leucotreta* neonates was evaluated in Chapter 3. The relative virulence of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV combinations against *T. leucotreta* neonates was determined (Chapter 4). Following this, the collected larval cadavers from the single and mixed infections were subjected to PCR-based methods to determine the cause of larval mortality. This chapter discusses the significant findings in the context of the published literature, highlights of this study, and proposes future research that can be done to improve further or further address knowledge gaps.

6.2 The development of PCR-based techniques to detect baculoviruses in viral stocks

In this study, single and mixed infections of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au were evaluated against *T. leucotreta* neonate larvae. Before the infection assays, the occlusion bodies were extracted from diseased *T. leucotreta*, *C. peltastica*, *C. pomonella*, and *H. armigera* larval cadavers. To screen if the samples contained the baculoviruses of interest, oligonucleotides targeting unique regions in the CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au genome sequences were designed using Primer-BLAST (Ye et al., 2012). The PCR-based methods successfully detected the presence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in the samples provided. However, it should be noted that the results do not imply that other baculoviruses were not present in the

samples as PCR has its limitations. PCR can only detect the presence of target regions based on oligonucleotide design (Garibyan & Avashia, 2013). Therefore, other viral genomes were probably in the samples but below the PCR detection limits. Since the oligonucleotides were designed to target regions of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au, only those target regions should be amplified. For this study, each species-specific oligonucleotide set was used for PCR on each sample. Each unique oligonucleotide set specifically detected the target virus in each sample with amplicons of the expected sizes generated. Jukes, (2018) developed a multiplex PCR (mPCR) assay to detect CrpeNPV and/or CrleGV-SA in viral stocks. In addition to this assay, the PCR-based described in this study offers a screening protocol to detect CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au should any of these viruses be in combination with commercially produced biopesticides. Importantly, as previously discussed it is necessary to evaluate the minimum and maximum detection limits of the PCR technique applied.

6.3 Single infections of CrleGV-SA, CrpeNPV, CpGV-M and HearNPV-Au against *T. leucotreta* neonate larvae

In this study, before the mixed infection assays were initiated, the relative virulence in terms of lethal concentration of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au alone against *T. leucotreta* was determined. For comparison, the single infection assays were essential to conduct to be able to evaluate the effects of the mixed infections.

Single infections evaluating the relative virulence of CrleGV-SA and CrpeNPV against *T. leucotreta* neonate larvae have been previously conducted by Jukes, (2018); Marsberg, (2016); Moore, (2002); and Taylor, (2021). In this study, CrleGV-SA was the most virulent treatment against the *T. leucotreta* neonates as lower LC_{50} and LC_{90} values were reported in comparison to the CrpeNPV treatment. Jukes, (2018) reported similar results where no improvements were observed in the LC_{50} values for CrpeNPV treatment in comparison to CrleGV-SA. However, Marsberg, (2016) and Taylor, (2021) observed the CrpeNPV treatment to be more virulent than the CrleGV-SA treatment. As previously discussed, variations in virus quantification, differences in viral dose preparation, colony health, environmental conditions, and differences in data analysis can contribute to inconsistent results. However, from a biological perspective, this study further substantiates that CrleGV-SA and CrpeNPV are effective control measures against *T. leucotreta* as both viruses are virulent against the pest.

CpGV is known to infect closely related lepidopteran species such as *T. leucotreta* although being less virulent in comparison to CrleGV (Fritsch et al., 1990). The CpGV treatment was the third most virulent treatment, further confirming the lower virulence against *T. leucotreta* in comparison to CrleGV-SA and CrpeNPV. Although Chambers, (2014) produced CpGV in fourth and fifth *T. leucotreta* instars, it is difficult to make comparisons as later instars are known to be less susceptible to virus infection (Wang & Hu, 2019). This is the first study to evaluate the biological activity of CpGV-M against *T. leucotreta* neonate larvae making it challenging to compare to previous studies.

HearNPV is known to infect species within the genera *Helicoverpa* and *Heliothis* thus having a narrow host range (Gettig & McCarthy, 1982; Gröner, 1986). A dose-discriminate assay confirmed that *T. leucotreta* is not susceptible to HearNPV. This is also the first study to evaluate the virulence of HearNPV against *T. leucotreta* neonate larvae.

6.4 Mixed infections of CrleGV-SA in combination with CrpeNPV, CpGV-M, and HearNPV-Au against *T. leucotreta* neonate larvae

6.4.1 CrleGV/CrpeNPV against *T. leucotreta* neonate larvae

The CrleGV/CrpeNPV mixture against *T. leucotreta* did not result in synergism in terms of lethal concentration as previously observed by Jukes, (2018) and Taylor, (2021). This may be due to the different combinations used in this study as compared to the previous studies. Lower GV proportions in GV/NPV mixtures where both viruses are known to infect the host are often associated with synergistic outcomes as reviewed in Ferrelli & Salvador, (2023). For example, Cuartas-Otálora et al., (2019) showed synergism when the Spodoptera frugiperda granulovirus (SpfrGV) proportion did not exceed 2.5% in combination with Spodoptera frugiperda nucleopolyhedrovirus (SpfrMNPV) against *Spodoptera frugiperda* larvae. Barrera et al., (2021) had similar results in the mixed infections of Spodoptera ornithogalli nucleopolyhedrovirus (SporNPV) and Spodoptera ornithogalli granulovirus (SporGV) against *Spodoptera ornithogalli* neonate larvae. On the contrary, higher GV proportions in GV/NPV mixtures often result in synergism where the host is not susceptible to the GV as reviewed in Ferrelli & Salvador, (2023). However, Jukes, (2018) observed a 3:1 CrleGV/CrpeNPV mixture to be more virulent in terms of lethal concentration than a 1:3 CrleGV/CrpeNPV mixture against *T. leucotreta* neonates. However, the synergistic effect in the 3:1 CrleGV/CrpeNPV mixture was reported at $LC_{90,}$ and at LC_{50}, an additive effect was observed. In a different study, Taylor, (2021) observed a synergistic effect in a 1:1 CrleGV/CrpeNPV ratio in terms of lethal concentrations but a prolonged lethal time was

encountered. Despite the antagonistic effect in a 7:3 CrleGV/CrpeNPV proportion as seen in this study; it is informative. The results obtained from this study are indicative that more research should be conducted to understand how these viruses interact in mixtures. The knowledge developed will equip researchers and farmers with a greater understanding of how these viruses interact in mixed applications and should both CrleGV and CrpeNPV be used in combination against *T. leucotreta*.

6.4.2 CrleGV/CpGV against *T. leucotreta* neonate larvae

The CrleGV/CpGV mixture resulted in an antagonistic effect against *T. leucotreta*. CpGV is known to infect *T. leucotreta* but is less virulent compared to CrleGV (Fritsch et al., 1990). In this study, CpGV-M was observed to be 2727.27 times less virulent in comparison to CrleGV-SA. The results from the GV/GV mixture allowed for possible improvements in the proportions of the viruses used in the mixture. Since a 46:54 CrleGV/CpGV OB proportion was evaluated in the GV/GV mixture, future work may include evaluating ratios where CpGV is applied at significantly higher concentrations since *T. leucotreta* is less susceptible to CpGV.

6.4.3 CrleGV/HearNPV against *T. leucotreta* neonate larvae

As observed in this study, HearNPV does not infect *T. leucotreta*. Mixed baculovirus interactions evaluating the effects where one virus does not infect the host have been well documented in Ferrelli & Salvador, (2023). For example, Guo et al., (2007) and Lara Reyna et al., (2003) reported synergistic effects between Xestia c-nigrum granulovirus (XcenGV) in combination with Spodoptera litura nucleopolyhedrovirus (SlNPV) against *Spodoptera litura* larvae and Autographa californica multiple nucleopolyhedrovirus (AcMNPV) in combination with Trichoplusia ni granulovirus (TnGV) against *Trichoplusia ni* larvae, respectively. In the above-mentioned cases, the insect hosts are not susceptible to the GVs. This led to the motivation of the study to evaluate the effects of HearNPV in combination with CrleGV against *T. leucotreta* neonate larvae. The CrleGV/HearNPV mixture did not show any improvements in terms of lethal concentration in comparison to CrleGV-SA when the virus was applied in isolation. The results suggest the proportion of the HearNPV did not have an effect and the larvae may have died due to the CrleGV infection in the mixture. The work of Tanada & Hukuhara, (1971) initiated the research in identifying synergistic factors present in baculoviruses. Enhancin genes are associated with synergistic factors in terms of lethal concentration as these genes often lead to the enhanced infectivity of other baculoviruses. These enhancin genes are common in GVs but are also found in NPVs as reviewed in Ferrelli

& Salvador, (2023). In a study by Hou et al., (2019) the ODV-E66 protein was found to have an important role in improving the oral infectivity of HearNPV. In the study, HearNPV ODV-E66 was suggested to play a role in the primary infection by degrading the peritrophic membrane of target pests. This is the first study to report dual infection assays where *T. leucotreta* is not susceptible to an NPV infection. The novelty of this study introduces the potential research in identifying and evaluating the effects of enhancin genes present in NPVs such as HearNPV in combination with other baculoviruses against *T. leucotreta.*

6.5 Molecular screening to investigate the probable cause of larval mortality

It is important to investigate the probable cause of larval death after the infection assays. This is to ensure that the viruses used in the biological assays were in fact the cause of larval mortality and not due to other stress factors such as handling, contamination, or the desiccation of the diet. The *T. leucotreta* cadavers collected from single and mixed infections were subjected to PCR analysis using the oligonucleotide sets described in Chapter 2. The purpose was to determine if the larval cadavers showed the presence of the viruses due to the treatment applied and to test for potential covert infections which may have become overt during the process of infection.

From the single treatment studies, each virus was present in the collected cadavers except in the cases where CrleGV-SA was detected in the CrpeNPV inoculated larvae as well as in the HearNPV single infection assays. This was also observed in studies performed by Jukes, (2018) and Taylor, (2021) where CrleGV-SA was detected in CrpeNPV-infected *T. leucotreta* neonate larvae. The detection of CrleGV-SA suggested possible covert infections in the *T. leucotreta* colony. The study by Hughes et al., (1993) demonstrated that covertly infected *Mamestra brassicae* insects harbouring the *M. brassicae* multiple nucleocapsid nucleopolyhedrovirus (MbMNPV) was activated by *Autographa californica* nucleopolyhedrovirus (AcMNPV), which is heterologous to the host, similar to what was observed in this study. This may pose a risk if CrpeNPV is produced in a covertly infected colony as CrleGV-SA may outcompete the infection or lead to unwanted contamination. In addition, the detection of CrleGV-SA covert infections in this study may suggest the need to screen mass reared colonies for CrleGV-SA and possibly CrpeNPV covert infections. This may be part of future studies as the detection of these covert infections may influence the development of biopesticides. Covert infections may reduce the colony health, resulting in the increase of costs in rearing the insect (Williams et al., 2017). As observed from the

results, *T. leucotreta* was not susceptible to HearNPV. Interestingly, the PCR results detected CrleGV-SA in the HearNPV inoculated larvae. Covert infections are frequent in host populations where the insects may seem healthy and asymptomatic (Williams et al., 2017). The CrleGV-SA covert to overt infection observed in this study cannot be explained. However, the covert infection may be triggered by the presence of HearNPV or an unknown stress factor.

From the mixed infection treatment studies, both CrleGV-SA and CrpeNPV were present in the collected cadavers. However, in the CrleGV-SA and CpGV-M mixtures, both viruses were present except in the last replicate where only CrleGV-SA was detected. This may suggest that the larvae predominantly died of the CrleGV-SA infection as CpGV has been reported to be 1000 times less virulent against *T. leucotreta* in comparison to CrleGV (Fritsch et al., 1990) In this study, the LC_{90} value for CpGV-M was 2727.27 × higher compared to CrleGV-SA. This still shows that CpGV is less virulent against *T. leucotreta* in comparison to CrleGV-SA. The variations in virulence may be due to differences in CrleGV isolates as Fritsch et al., (1990) used the CrleGV isolate from the Ivory Coast, and in this study, the South African isolate was used. The CrleGV-SA infection may outcompete the CpGV infection suggesting competition for host resources. Although the CpGV OB proportion was slightly higher, competition for host resources in dual infections has been documented by Wennmann & Jehle, (2014). From the study, qPCR analysis found that *Agrotis segetum* larvae died of the virus applied in the higher concentration in mixed infections between Agrotis segetum nucleopolyhedrovirus (AgseNPV) and Agrotis segetum granulovirus (AgseGV). This is different from what was observed in this study, but due to CpGV being less virulent, it is possible that CrleGV outcompeted the CpGV infection.

The PCR-based technique developed in this study can be used to detect baculoviral sequences of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in unknown samples and can be further used to determine the probable cause of larval mortality post single or mixed infections. A critique of this method of determining larval mortality is the possible amplification of viruses due to surface contamination and not due to larvae feeding on the virus. One way to avoid the possibility of surface contamination is using droplet-feeding assays to evaluate the biological activity of the viruses against *T. leucotreta*. In this way, when the insect is allowed to feed, the entire droplet of the virus is consumed before being placed on a fresh diet (Pereira-da-Conceicoa et al., 2012). This allows the determination of

the lethal dose and the larval death can be determined without the possibility of surface contamination.

6.6 Potential future work

In this study, the relative virulence of CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au was evaluated in single and mixed infections against *T. leucotreta* neonate larvae. Although the CrleGV-SA/CrpeNPV, CrleGV-SA/CpGV, and CrleGV-SA/HearNPV ratios used in this study resulted in an antagonistic effect, it has led to further research to investigate different combinations that may result in an improved virulence. Higher GV proportions in GV/NPV mixtures could be further evaluated as Jukes, (2018) observed the 3:1 CrleGV/CrpeNPV mixture to have a higher virulence compared to 1:3 against *T. leucotreta* neonate larvae. However, the synergistic effect was observed at LC_{90} in the 3:1 CrleGV/CrpeNPV mixture but at LC_{50} an additive effect was encountered. Biedma et al., (2015) also observed a synergistic effect in a 1:120 mixture of AngeMNPV and EpapGV OBs against *A. gemmatalis* larvae. With *T. leucotreta* known to be susceptible to all viruses used in this study with the exception of HearNPV, different combinations of CrleGV-SA, CrpeNPV, and CpGV-M in combination with HearNPV should be evaluated against *T. leucotreta*.

Additionally, mixed infection studies can be conducted to target other important agricultural pests of economic importance which include *Cydia pomonella*, *Cryptophlebia peltastica,* and *Helicoverpa armigera*. *Cydia pomonella* is a global pest of pome fruit and walnuts (Barnes, 1991). *Cryptophlebia peltastica* is a pest of litchis and macadamias in South Africa (Follett & Lower, 2000; Manrakhan et al., 2008; Waite & Hwang, 2002) and *H. armigera* is a pest of cotton, citrus, and other important agricultural crops (Fitt, 1989). Commercialised baculovirus biopesticides such as CpGV-based Madex® (Andermatt-Biocontrol AG, Switerzland) and Carpovirusine® (Arysta Life Science, France), HearNPV-based Helimax™ (River Bioscience, Gqeberha), and CrpeNPV-based Multimax™ (River Bioscience, Gqeberha) (Thackeray, Personal communication, 2023) are used to target the above-mentioned pests. Under natural conditions, when HearNPV is applied against *H. armigera* in pome fruit and citrus for example, there are increased chances of interactions with other commercialised baculoviruses such as CrleGV-SA, CrpeNPV, and CpGV in the field. The effect of CrleGV-SA, CrpeNPV, or CpGV on HearNPV against *H. armigera* is unknown thus research can be conducted to evaluate the types of interactions that may occur in the field.

Despite the effective biological control use of CpGV-M against *C. pomonella*, there has been reported insect resistance against the agent (Fritsch et al., 2005; Sauphanor et al., 2006;

Schmitt et al., 2013). Recently, CpGV-SA, a novel isolate has been discovered to have similar virulence to CpGV-M and can be used as an alternative in commercialised products (Motsoeneng et al., 2019; 2016). The use of baculovirus mixtures is also an alternative option to improve the virulence of CpGV against *C. pomonella*. CrpeNPV which has already been commercialised under the trade name Multimax™ (River Bioscience, Gqeberha) (Thackeray, Personal communication, 2023) is known to infect *C. pomonella,* thus combinations of CrpeNPV with CpGV and HearNPV against *C. pomonella* may prove to be effective in the control of the pest.

As previously mentioned above, the presence or absence of enhancin genes can influence the outcome of dual interactions as reviewed in Ferrelli & Salvador, (2023). Since the baculoviruses used in this study infect at least one of the above-mentioned pests, further research can be conducted evaluating GV/NPV, GV/GV, or NPV/NPV mixtures in different proportions to investigate whether the control of these agricultural pests can be improved.

This study only focused on the potential enhancement of mixed infections in terms of lethal concentrations, but it is also important to evaluate the effects of the infections in terms of lethal time. In mixed infections of CrleGV/CrpeNPV against *T. leucotreta*, an increased lethal time was encountered despite observing an improved lethal concentration (Taylor, 2021). It is thus important to evaluate the effects that may cause a slower speed of kill which are typically encountered with baculoviruses in comparison to chemical insecticides. Other studies by Jeyarani & Karuppuchamy, (2010) and Hatem et al., (2012) also showed the antagonistic effect in increased lethal times in dual infections of HearNPV and HearGV against *H. armigera* and Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) and Spodoptera littoralis granulovirus (SpliGV) against *S. littoralis* larvae, respectively. This shows the importance of performing lethal time response assays in dual infections as this is an important factor from a biocontrol point of view. A slower speed of kill allows larvae to cause fruit damage before succumbing to baculovirus infections. Thus, a lower lethal time indicative of synergism within the interaction and is sought after in the control of agricultural pests such as *T. leucotreta*.

6.7 Conclusion

The primary aim of the study was to evaluate mixtures of CrleGV-SA/CrpeNPV, CrleGV-SA/CpGV-M, and CrleGV-SA/HearNPV-Au to determine whether synergistic, antagonistic, or additive interactions can occur against *T. leucotreta* neonate larvae. The first objective of the study was to design oligonucleotides to target unique regions in the viral genomes of

CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au. This objective was achieved using Primer-BLAST software. The second objective of the study was to perform occlusion body purification on larval samples followed by DNA extraction, and PCR amplification for the selected genome regions. This objective was achieved and the results from the PCR analysis detected the baculoviruses of interest in each of the provided samples. The third objective of the study was to evaluate the virulence of each virus alone and in mixed infections against *T. leucotreta* neonate larvae. Surface-dose biological assays were used to determine the relative virulence in terms of lethal concentrations of single and mixed infections. The last objective of the study was to detect the presence of the target viruses from collected *T. leucotreta* cadavers in single and mixed infections. The purpose was to determine the cause of larval mortality from each infection assay. This objective was achieved using the unique oligonucleotides in PCR-based methods. Overall, the study aimed to determine whether any combination could provide an enhanced lethal concentration for the better management of this pest in the field. The surface dose biological assays of the mixed infections of CrleGV/CrpeNPV, CrleGV/CpGV, and CrleGV/HearNPV did not show any improvement in terms of lethal concentrations. However, the combinations of CrleGV/CpGV and CrleGV/HearNPV against *T. leucotreta* have never been performed before thus this study has laid the foundation to investigate the relationship and how these viruses interact in dual infections. Furthermore, this study has led to further insight into investigating the effect of different ratios that can lead to better management of *T. leucotreta* in the field. Lastly, this study developed a PCR assay that could be further utilised to discern between CrleGV-SA, CrpeNPV, CpGV-M, and HearNPV-Au in single and mixed infections, an important development given that all these baculoviruses are currently utilised as biopesticides in South Africa.

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, 18(2), 265–267.

Ackermann, H.-W., & Smirnoff, W. A. (1983). A morphological investigation of 23 baculoviruses. Journal of Invertebrate Pathology, 41(3), 269–280.

Adom, M., Fening, K., Billah, M., Wilson, D., Hevi, W., Clottey, V., Ansah-Amprofi, F., & Bruce, A. (2020). Pest status, bio-ecology and management of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) and its implication for international trade. Bulletin of Entomological Research, 111(1), 17-30.

Angélini, A., Amargier, A., Vandamme, P., & Duthoit, J. L. (1965). Une virose à granules chez le lépidoptère *Argyroploce leucotreta*. Coton et Fibres Tropicales, 20(2), 277-282.

Arneodo, J. D., De Anna, J., Salvador, R., Farinon, M., Quintana, G., & Sciocco-Cap, A. (2015). Prospection and molecular analysis of CpGV isolates infecting *Cydia pomonella* at different geographical locations in Argentina. Annals of Applied Biology, 166(1), 67–74.

Aspinall, T. V., Marlee, D., Hyde, J. E., & Sims, P. F. (2002). Prevalence of *Toxoplasma gondii* in commercial meat products as monitored by polymerase chain reaction – food for thought? International Journal for Parasitology, 32(9), 1193–1199.

Barnes, M. M. (1991). Tortricids in pome and stone fruits, codling moth occurrence, host race formation and damage. Tortricid Pests, Their Biology, Natural Enemies, and Control, Edited by L.P.S van der Geest & H.H. Evenhuis, Elsevier, Amsterdam, 313–327.

Barrera, G. P., Villamizar, L. F., Araque, G. A., Gómez, J. A., Guevara, E. J., Cerrudo, C. S., & Belaich, M. N. (2021). Natural coinfection between novel species of baculoviruses in *Spodoptera ornithogalli* larvae. Viruses, 13(12), 2520.

Bayramoglu, Z., Gencer, D., Muratoglu, H., Efe, D., Nalcacioglu, R., Demirbag, Z., & Demir, I. (2018). Characterization of a nucleopolyhedrovirus variant of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae) in Turkey. International Journal of Pest Management, 64(2), 119–127.

Beas-Catena, A., Sánchez-Mirón, A., García-Camacho, F., Contreras-Gómez, A., & Molina-Grima, E. (2014). Baculovirus biopesticides: an overview. JAPS: Journal of Animal & Plant Sciences, 24(2), 362–373.

Bedford, E. C. G. (1968). Biological and chemical control of citrus pests in the Western Transvaal. An integrated spray programme. South African Citrus Journal, 417, 9–28.

Bell, M., & McGeoch, J. (1996). An evaluation of the pest status and research conducted on phytophagous Lepidoptera on cultivated plants in South Africa. African Entomology, 4(2), 161–170.

Bennett, T. T. (2022). Genetic analysis and field application of a UV-tolerant strain of CrleGV for improved control of *Thaumatotibia leucotreta*. Rhodes University, Makhanda, South Africa.

Biedma, M. E., Salvador, R., Ferrelli, M. L., Sciocco-Cap, A., & Romanowski, V. (2015). Effect of the interaction between Anticarsia gemmatalis multiple nucleopolyhedrovirus and Epinotia aporema granulovirus, on *A. gemmatalis* (Lepidoptera: Noctuidae) larvae. Biological Control, 91, 17–21.

Blissard, G. W. (1996). Baculovirus--insect cell interactions. Cytotechnology, 20(1–3), 73–93.

Bonning, B. (2005). Baculoviruses: Biology, biochemistry, and molecular biology. Comprehensive Molecular Insect Science, 6, 233–270.

Bulach, D. M., Kumar, C. A., Zaia, A., Liang, B., & Tribe, D. E. (1999). Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. Journal of Invertebrate Pathology, 73(1), 59–73.

Burden, J. P., Griffiths, C. M., Cory, J. S., Smith, P., & Sait, S. M. (2002). Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctella*. Molecular Ecology, 11(3), 547–555.

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

Busvine, J. R. (1971). A critical review of the techniques for testing insecticides. 2nd edition, Commonwealth Agricultural Bureaux, England.

Centre for Agriculture and Bioscience International. *Thaumatotibia leucotreta*. Invasive Species Compendium. Available online: https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.6904 (accessed on 09 August 2024)

Chambers, C. B. (2014). Production of Cydia pomonella granulovirus (CpGV) in a heteralogous host, *Thaumatotibia leucotreta* (Meyrick) (False Codling Moth). Rhodes University, Makhanda, South Africa.

Chang, J. H., Choi, J. Y., Jin, . R., Roh, J. Y., lsz ews i, J. A., eo, . J., ' Reilly, D. R., & Je, Y. H. (2003). An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin. Journal of Invertebrate Pathology, 84(1), 30–37.

Chateigner, A., Bézier, A., Labrousse, C., Jiolle, D., Barbe, V., & Herniou, E. A. (2015). Ultra deep sequencing of a baculovirus population reveals widespread genomic variations. Viruses, 7(7), 3625–3646.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research, 31(13), 3497–3500.

Chisoro-Dube, ., & Roberts, . (202). nnovation and inclusion in outh Africa's citrus industry. Innovation and Inclusion in Agro-Processing Working-Paper, 1-51.

Citrus Growers' Association of Southern Africa. (2022). Key Industry Statistics for citrus growers, 2021 Export Season.

Citrus Growers' Association of Southern Africa. (2023). Annual Report, 2023.

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2016). GenBank. Nucleic Acids Research, 44(D1), D67–D72.

Clarke, J. F. G. (1955). Catalogue of the type specimens of Microlepidoptera in the British Museum (Natural History). Described by Edward Meyrick, 5.

Clem, R. J., & Passarelli, A. L. (2013). Baculoviruses: sophisticated pathogens of insects. PLoS Pathogens, 9(11), e1003729.

Cooper, D., Cory, J. S., Theilmann, D. A., & Myers, J. H. (2003). Nucleopolyhedroviruses of forest and western tent cater il lars: Cross-infectivity and evidence for activation of latent virus in high-density field o ul ations. Ecological Entomology, 28( ), 4 –50.

Cory, J. S., & Bishop, D. H. (1997). Use of baculoviruses as biological insecticides. Molecular Biotechnology, 7(3), 303–313.

Crook, N. E., James, J. D., Smith, I. R., & Winstanley, D. (1997). Comprehensive physical map of the Cydia pomonella granulovirus genome and sequence analysis of the granulin gene region. The Journal of General Virology, 78(4), 965–974.

Crook, N. E., Spencer, R. A., Payne, C. C., & Leisy, D. J. (1985). Variation in Cydia pomonella granulosis virus isolates and physical maps of the DNA from three variants. Journal of General Virology, 66(11), 2423–2430.

Cross, J., Solomon, M., Chandler, D., Jarrett, P., Richardson, P., Winstanley, D., Bathon, H., Huber, J., Keller, B., & Langenbruch, G. (1999). Biocontrol of pests of apples and

pears in northern and central Europe: 1. Microbial agents and nematodes. Biocontrol Science and Technology, 9(2), 125–149.

Cuartas-Otálora, P. E., Gómez-Valderrama, J. A., Ramos, A. E., Barrera-Cubillos, G. P., & Villamizar-Rivero, L. F. (2019). Bio-insecticidal potential of nucleopolyhedrovirus and granulovirus mixtures to control the fall armyworm *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae). Viruses, 11(8), 684.

Daiber, C. C. (1980). A study of the biology of the false codling moth *Cryptophlebia leucotreta* (Meyr.): the adult and generations during the year. Phytophylactica, 12, 187–193.

Daiber, C. C. (1979a). A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: The egg. Phytophylactica, 11(3), 129–132.

Daiber, C. C. (1979b). A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: The larva. Phytophylactica, 11(3), 141–144.

Daiber, C. C. (1979c). A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: the cocoon. Phytophylactica, 11(3), 151–157.

Department of Agricultural, Land Reform and Rural Development (2020). A profile of the South African citrus market value chain. 1–107.

DaPalma, T., Doonan, B. P., Trager, N. M., & Kasman, L. M. (2010). A systematic approach to virus-virus interactions. Virus Research, 149(1), 1–9.

Derksen, A. C., & Granados, R. R. (1988). Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology, 167(1), 242–250.

Dhladhla, B. I. R., Mwanza, P., Lee, M. E., Moore, S., & Dealtry, G. B. (2018). Comparison of microscopic and molecular enumeration methods for insect viruses: Cryptophlebia leucotreta granulovirus as a case study. Journal of Virological Methods, 256, 107–110.

Djieto-Lordon, C., Heumou, C., Elono Azang, P., Alene, C., Ngueng, A., & Ngassam, P. (2014). Assessment of pest insects of *Capsicum annuum* L.1753 (Solanaceae) in a cultivation cycle in Yaoundé. International Journal of Biological and Chemical Sciences, 8(2), 621–632.

Eberle, K. E., Sayed, S., Rezapanah, M., Shojai-Estabragh, S., & Jehle, J. A. (2009). Diversity and evolution of the Cydia pomonella granulovirus. The Journal of General Virology, 90(3), 662–671.

Eberle, K. E., Wennmann, J., Kleespies, R., & Jehle, J. (2012). Basic techniques in insect virology. In L.A. Lacey (Ed). Manual of techniques in invertebrate pathology. 2nd edition, 15–74, San Diego, USA.

Fan, J., Wennmann, J. T., Wang, D., & Jehle, J. A. (2020). Single nucleotide polymorphism (SNP) frequencies and distribution reveal complex genetic composition of seven novel natural isolates of Cydia pomonella granulovirus. Virology, 541, 32–40.

Federici, B. A. (1997). Baculovirus pathogenesis. In L. K. Miller (Ed). The Baculoviruses. 33–59, Springer, California, USA.

Ferrelli, M. L., & Salvador, R. (2023). Effects of mixed baculovirus infections in biological control: a comprehensive historical and technical analysis. Viruses, 15(9), 1838.

Finney, D. J. (1978). Statistical method in biological assay. In Charles Griffin & Company (Eds). 3rd edition, 508, London, UK.

Fitt, G. P. (1989). The ecology of *Heliothis* species in relation to agroecosystems. Annual Review of Entomology, 34(1), 17–53.

Follett, P. A., & Lower, R. A. (2000). Irradiation to ensure quarantine security for *Cryptophlebia spp.* (Lepidoptera: Tortricidae) in sapindaceous fruits from Hawaii. Journal of Economic Entomology, 93(6), 1848–1854.

Follett, P. A., & Neven, L. G. (2006). Current trends in quarantine entomology. Annual Review of Entomology, 51(1), 359–385.

Fritsch, E. (1989). Das granulosevirus des falschen apfelwicklers, *Cryptophlebia leucotreta* (Meyrick). Doctoral dissertation, 1–175, TU Darmstadt, Germany.

Fritsch, E., Huber, J., & Backhaus, H. (1990). CpGV as a tool in the risk assessment of genetically engineered baculoviruses. In Proceedings and abstracts of the 5th International Colloquium on Invertebrate Pathology and Microbial Control. 439–443, Adelaide, Australia.

Fritsch, E., Undorf-Spahn, K., Kienzle, J., Zebitz, C. P., & Huber, J. (2005). Apfelwickler-granulovirus: erste hinweise auf unterschiede in der empfindlichkeit lokaler apfelwickler-populationen. Nachrichtenblatt Des Deutschen Pflanzenschutzdienstes, 57(2), 29–34.

Fritsch, E., Undorf-Spahn, K., Kienzle, J., Zebitz, C. P., & Huber, J. (2007). Codling moth granulovirus: First indication of variations in the susceptibility of local codling moth populations. IOBC WPRS BULLETIN, 30(1), 181.

Gangwar, P., Trivedi, M., & Tiwari, R. K. (2021). Entomopathogenic bacteria. Microbial Approaches for Insect Pest Management, 59–79.

Garibyan, L., & Avashia, N. (2013). Research techniques made simple: polymerase chain reaction (PCR). The Journal of Investigative Dermatology, 133(3), e6.

Gebhardt, M. M., Eberle, K. E., Radtke, P., & Jehle, J. A. (2014). Baculovirus resistance in codling moth is virus isolate-dependent and the consequence of a mutation in viral gene *pe38*. Proceedings of the National Academy of Sciences of the United States of America, 111(44), 15711–15716.

Gelaye, Y., & Negash, B. (2023). The role of baculoviruses in controlling insect pests: A review. Cogent Food & Agriculture, 9(1), 2254139.

Gettig, R. R., & McCarthy, W. J. (1982). Genotypic variation among wild isolates of Heliothis spp nuclear polyhedrosis viruses from different geographical regions. Virology, 117(1), 245–252.

Goto, C. (1990). Enhancement of a nuclear polyhedrosis virus (NPV) infection by a granulosis virus (GV) isolated from the spotted cutworm, *Xestia c-nigrum L.*: Lepidoptera: Noctuidae. Applied Entomology and Zoology, 25(1), 135–137.

Graillot, B., Blachère-Lopez, C., Besse, S., Siegwart, M., & Lopez-Ferber, M. (2016). Host range extension of Cydia pomonella granulovirus: adaptation to oriental fruit moth, *Grapholita molesta*. Biocontrol, 1–9.

Grasela, J. J., McIntosh, A. H., Shelby, K. S., & Long, S. (2008). Isolation and characterization of a baculovirus associated with the insect parasitoid wasp, *Cotesia marginiventris*, or its host, *Trichoplusia ni*. Journal of Insect Science, 8, 1–19.

Gröner, A. (1986). Specificity and safety of baculoviruses. In R. R. Granados and B. A. Federici (Eds). The biology of baculoviruses, Biological Properties and Molecular Biology. Vol. I, 177–202, CRC Press, Boca Raton.

Grout, T., Moore, S. (2015). Citrus. In: G., Prinsloo and V. Uys (Eds). Insects of cultivated plants and natural pastures in Southern Africa. 447–501, Entomological Society of Southern Africa, Pretoria, South Africa.

Grové, T., Steyn, W., & De Beer, M. (1999). The false codling moth, *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) on avocado: A literature review. outh African Avocado Growers' Association Yearboo , 22, 3 –33.

Grzywacz, D. (2017). Basic and Applied Research: Baculovirus. In L. A. Lacey (Ed.), Microbial Control of Insect and Mite Pests. 27–46, Elsevier, Amsterdam.

Grzywacz, D., & Moore, S. (2017). Production, formulation, and bioassay of baculoviruses for pest control. In L. A. Lacey (Ed.), Microbial control of insect and mite pests. 109–124, Elsevier, USA.

Grzywacz, D., Rabindra, R., Brown, M., Jones, K., & Parnell, M. (2004). The Helicoverpa armigera NPV production manual. Natural Resources Institute, Chatham, 107.

Guo, H. F., Fang, J. C., Wang, J. P., Zhong, W. F., & Liu, B. S. (2007). Interaction of Xestia c-nigrum granulovirus with peritrophic matrix and Spodoptera litura nucleopolyhedrovirus in *Spodoptera litura*. Journal of Economic Entomology, 100(1), 20–25.

Haase, S., Sciocco-Cap, A., & Romanowski, V. (2015). Baculovirus insecticides in Latin America: historical overview, current status and future perspectives. Viruses, 7(5), 2230–2267.

Hajjar, M. J., Ahmed, N., Alhudaib, K. A., & Ullah, H. (2023). Integrated insect pest management techniques for rice. Sustainability, 15(5), 4499.

Harrison, R., & Hoover, K. (2012). Baculoviruses and other occluded insect viruses. In F. E. Vega & H. K. Kaya (Eds). Insect Pathology. 2nd edition, 73-131, Academic press, London, England.

Harrison, R. L., Herniou, E. A., Jehle, J. A., Theilmann, D. A., Burand, J. P., Becnel, J. J., Krell, P. J., van Oers, M. M., Mowery, J. D., Bauchan, G. R., & ICTV Report Consortium. (2018). ICTV virus taxonomy profile: Baculoviridae. Journal of General Virology, 99(9), 1185–1186.

Hatem, A., Faragalla, F., & Vargas-Osuna, E. (2012). Combination effects of Spodoptera littoralis nuclear polyhedrosis and granulous virus against larvae of the cotton leafworm. World Rural Observations, 4(4), 10–16.

Hatting, J., Moore, S., Malan, A., 2019. Microbial control of phytophagous invertebrate pests in South Africa: Current status and future prospects. Journal of Invertebrate Pathology 165, 54-66.

Hattingh, V., Moore, S., Kirkman, W., Goddard, M., Thackeray, S., Peyper, M., Sharp, G., Cronjé, P., Pringle, K., & Cha, D. H. (2020). An improved systems approach as a phytosanitary measure for *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in export citrus fruit from South Africa. Journal of Economic Entomology, 113(2), 700–711.

Herniou, E. A., & Jehle, J. A. (2007). Baculovirus phylogeny and evolution. Current Drug Targets, 8(10), 1043–1050.

Herniou, E. A., Luque, ., Chen, X., Vla , J. ., Winstanley, D., Cory, J. ., & ' Reilly, D. R. (2001). Use of whole genome sequence data to infer baculovirus phylogeny. Journal of Virology, 75(17), 8117–8126.

Herniou, E. A., lszews i, J. A., Cory, J. ., & 'Reilly, D. R. (2003). The genome sequence and evolution of baculoviruses. Annual Review of Entomology, 48(1), 211–234. Hofmeyr, J. H., Carpenter, J. E., & Bloem, S. (2005). Developing the sterile insect technique for *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae): Influence of radiation dose and release ratio on fruit damage and population growth in field cages. Journal of Economic Entomology, 98(6), 1924–1929.

Hou, D., Kuang, W., Luo, S., Zhang, F., Zhou, F., Chen, T., Zhang, Y., Wang, H., Hu, Z., Deng, F., & Wang, M. (2019). Baculovirus ODV-E66 degrades larval peritrophic membrane to facilitate baculovirus oral infection. Virology, 537, 157–164.

Huber, J. (1981). Apfelwickler-granulosevirus: produktion und biotests. Mitt. Dtsch. Ges. Allg. Angew. Entomol, 2, 141–145.

Hughes, A. L. (2013). Origin of ecdysosteroid UDP-glycosyltransferases of baculoviruses through horizontal gene transfer from Lepidoptera. Coevolution, 1(1), 1–7.

Hughes, D. S., Possee, R. D., & King, L. A. (1993). Activation and detection of a latent baculovirus resembling Mamestra brassicae nuclear polyhedrosis virus in *M. brassicae* insects. Virology, 194(2), 608–615.

Hughes, E., & Shapiro, M. (1997). Viruses. In L. A. Lacey (Ed). Manual of Techniques in Insect Pathology. 40–48. Academic Press, London.

Hughes, P. R., & Wood, H. A. (1981). A synchronous peroral technique for the bioassay of insect viruses. Journal of Invertebrate Pathology, 37(2), 154–159.

Hughes, P. R, & Wood, H. A. (1986). In vivo and in vitro bioassay methods for baculoviruses. In R. R. Granados and B. A. Federichi (Eds). The Biology of Baculoviruses, Practical Application for Insect Control. Vol. II, 1–30, CRC Press, Boca Raton.

Hunter-Fujita, F. R., Entwistle, P. F., Evans, H. F., & Crook, N. E. (1998). Insect viruses and pest management. Insect Viruses and Pest Management.

Hutchinson, M. (2021). Popular methods of biological control and the future of baculoviruses. Undergraduate Review, 16(1), 100–108.

Ignoffo, C. M. (1992). Environmental factors affecting persistence of entomopathogens. The Florida Entomologist, 75(4), 516–525.

l'iny h, A., & Ul'yanova, E. (2005). Latency of baculoviruses. iology ulletin, 32, 4 6 – 502.

Jehle, J. A., Backhaus, H., Fritsch, E., & Huber, J. (1992). Physical map of the Cryptophlebia leucotreta granulosis virus genome and its relationship to the genome of Cydia pomonella granulosis virus. The Journal of General Virology, 73 (7), 1621–1626.

Jehle, J. A., Blissard, G. W., Bonning, B. C., Cory, J. S., Herniou, E. A., Rohrmann, G. F., Theilmann, D. A., Thiem, S. M., & Vlak, J. M. (2006). On the classification and nomenclature of baculoviruses: a proposal for revision. Archives of Virology, 151(7), 1257–1266.

Jehle, J. A., Fritsch, E., Huber, J., & Backhaus, H. (2003). Intra-specific and inter-specific recombination of tortricid-specific granuloviruses during co-infection in insect larvae. Archives of Virology, 148(7), 1317–1333.

Jeyarani, S., & Karuppuchamy, P. (2010). Investigations on the enhancing efficacy of granulovirus on nucleopolyhedrovirus of *Helicoverpa armigera* (Hübner). Journal of Biopesticides, 3(1), 172-176.

Jukes, M. D. (2018). Baculovirus synergism: Investigating mixed alphabaculovirus and betabaculovirus infections in the false codling moth, *Thaumatotibia leucotreta*, for improved pest control. Rhodes University, Makhanda, South Africa.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647–1649.

Kemp, E. M., Woodward, D. T., & Cory, J. S. (2011). Detection of single and mixed covert baculovirus infections in eastern spruce budworm, *Choristoneura fumiferana* populations. Journal of Invertebrate Pathology, 107(3), 202–205.

Kessler, P., & Zingg, D. (2008). New baculovirus products offer solutions for the biological control of *Cydia pomonella* and *Cryptophlebia leucotreta*. Proceedings of the 23rd International Congress of Entomology, Durban, South Africa, 612.

Kirkman, W., & Moore, S. (2007). A study of alternative hosts for the false codling moth, *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* in the Eastern Cape. SA Fruit Journal, 6(2), 33–38.

Knox, C., Moore, S. D., Luke, G. A., & Hill, M. P. (2015). Baculovirus-based strategies for the management of insect pests: A focus on development and application in South Africa. Biocontrol Science and Technology, 25(1), 1–20.

Komai, F. (1999). A taxonomic review of the genus *Grapholita* and allied genera (Lepidoptera: Tortricidae) in the Palaearctic region. Entomologica Scandinavica Supplement, 55, 110.

Koppenhöfer, A., & Kaya, H. (1997). Additive and synergistic interaction between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control. Biological Control, 8(2), 131–137.

Kouassi, L. N., Tsuda, K., Goto, C., Mukawa, S., Sakamaki, Y., Kusigemati, K., & Nakamura, M. (2009). Prevalence of latent virus in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) and its activation by a heterologous virus. Applied Entomology and Zoology, 44(1), 95–102.

Kukan, B. (1999). Vertical transmission of nucleopolyhedrovirus in insects. Journal of Invertebrate Pathology, 74(2), 103–111.

Kwok, S., Chang, S. Y., Sninsky, J. J., & Wang, A. (1994). A guide to the design and use of mismatched and degenerate primers. PCR Methods and Applications, 3(4), S39-47.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology, 132, 1–41.

Lacey, L., Thomson, D., Vincent, C., & Arthurs, S. (2008). Codling moth granulovirus: A comprehensive review. Biocontrol Science and Technology, 18(7), 639-663.

Ladaniya, M. (2023). Preharvest factors. In M. Ladaniya (Ed). Citrus Fruit. 2nd edition, 113–144, Academic Press.

Lange, M., Wang, H., Zhihong, H., & Jehle, J. A. (2004). Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. Virology, 325(1), 36–47.

Lara Reyna, J., Del Rincón-Castro, M., & Ibarra, J. (2003). Synergism between the nucleopolyhedroviruses of *Autographa californica* and *Trichoplusia ni*. Acta Virologica, 47, 189–194.

Larem, A., Ben-Tiba, S., Wennmann, J. T., Gueli Alletti, G., & Jehle, J. A. (2019). Elucidating the genetic diversity of Phthorimaea operculella granulovirus (PhopGV). Journal of General Virology, 100(4), 679–690.

Manrakhan A., Abeeluck D., & Gokool A. (2008). Assessment of damage by *Cryptophlebia peltastica* (Meyrick) (Lepidoptera: Tortricidae) in litchi orchards in Mauritius. African Entomology, 16(2), 203–208.

Marsberg, T. (2016). The isolation and genetic characterisation of a novel alphabaculovirus for the microbial control of *Cryptophlebia peltastica* and closely related tortricid pests. Rhodes University, Makhanda, South Africa.

Marsberg, T., Jukes, M. D., Krejmer-Rabalska, M., Rabalski, L., Knox, C. M., Moore, S. D., Hill, M. P., & Szewczyk, B. (2018). Morphological, genetic and biological characterisation of a novel alphabaculovirus isolated from *Cryptophlebia peltastica* (Lepidoptera: Tortricidae). Journal of Invertebrate Pathology, 157, 90–99.

Miele, S. A. B., Garavaglia, M. J., Belaich, M. N., & Ghiringhelli, P. D. (2011). Baculovirus: molecular insights on their diversity and conservation. International Journal of Evolutionary Biology, 2011, 379424.

Moore, S. D. (2002). The development and evaluation of Cryptophlebia leucotreta granulovirus (CrleGV) as a biological control agent for the management of false codling moth, *Cryptophlebia leucotreta*, on citrus. Rhodes University, Makhanda, South Africa

Moore, S. D. (2021). Biological control of a phytosanitary pest (*Thaumatotibia leucotreta*): A case study. International Journal of Environmental Research and Public Health, 18(3), 1198.

Moore, S. D., Hendry, D. A., & Richards, G. I. (2011). Virulence of a South African isolate of the Cryptophlebia leucotreta granulovirus to *Thaumatotibia leucotreta* neonate larvae. BioControl, 56(3), 341–352.

Moore, S. D., Kirkman, W., Richards, G. I., & Stephen, P. R. (2015). The Cryptophlebia leucotreta granulovirus–10 years of commercial field use. Viruses, 7(3), 1284–1312.

Moore, S. D., Pittaway, T., Bouwer, G., & Fourie, J. G. (2004). Evaluation of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) for control of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on citrus in South Africa. Biocontrol Science and Technology, 14(3), 239–250.

Moore, S. D., Richards, G. I., Chambers, C., & Hendry, D. (2014). An improved larval diet for commercial mass rearing of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). African Entomology, 22(1), 216–219.

Moore, S., & Hattingh, V. (2012). A review of current pre-harvest control options for false codling moth in citrus in southern Africa. SA Fruit Journal, 11(4), 82–85.

Moore, S., & Jukes, M. (2019). Advances in microbial control in IPM: entomopathogenic viruses. In M. Kogan and E. Heinrichs (Eds). Integrated management of insect pests:

current and future developments. Burleigh Dodds Science Publishing, Cambridge, UK.

Moore, S., & Jukes, M. (2023). The History of Baculovirology in Africa. Viruses, 15(7), 1519.

Moore, S., & Kirkman, W. (2008). Citrus orchard sanitation with emphasis on false codling moth control. SA Fruit Journal, 7(6), 57–60.

Moore, S., & Kirkman, W. (2010). Helicovir™: A virus for the biological control of bollworm. SA Fruit Journal, 9(4), 63–67.

Moore, S., Kirkman, W., & Stephen, P. (2004). Cryptogran: a virus for the biological control of false codling moth. SA Fruit Journal, 56-60.

Moran, V. (1983). The phytophagous insects and mites of cultivated plants in South Africa: patterns and pest status. Journal of Applied Ecology, 20(2), 439–450.

Moscardi, F. (1999). Assessment of the application of baculoviruses for control of Lepidoptera. Annual Review of Entomology, 44(1), 257–289.

Moscardi, F., Souza, M., Castro, M., Moscardi, M., & Szewczyk, B. (2011). Baculovirus Pesticides: Present State and Future Perspectives. In Microbes and microbial technology. New York, Springer, 415–445.

Motsoeneng, B., Jukes, M. D., Knox, C. M., Hill, M. P., & Moore, S. D. (2019). Genome analysis of a novel South African Cydia pomonella granulovirus (CpGV-SA) with resistance-breaking potential. Viruses, 11(7), 658.

Motsoeneng, B. M. (2016). Genetic and biological characterisation of a novel South African Cydia pomonella granulovirus (CpGV-SA) isolate. Rhodes University, Makhanda, South Africa.

Mtambanengwe, K. T. E. (2019). Genetic characterization of a range of geographically distinct Helicoverpa armigera nucleopolyhedrovirus (HearNPV) isolates and evaluation of biological activity against South African populations of the African bollworm, *Helicoverpa armigera* (Hübner). Rhodes University, Makhanda, South Africa.

Mück, O. (1985). Biologie, Verhalten und wirtschaftliche Bedeutung von Parasiten schädlicher Lepidopteren auf den Kapverden. Bauer Exeter, NH, USA.

Mwanza, P. (2019). Development of a UV-tolerant strain of the South African isolate of Cryptophlebia leucotreta granulovirus for use as an enhanced biopesticide for *Thaumatotibia leucotreta* control on citrus. Rhodes University, Makhanda, South Africa.

Needleman, S. B., & Wunsch, C. D. (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. Journal of Molecular Biology, 48(3), 443–453.

Newton, P. J. (1989). The influence of citrus fruit condition on egg laying by the false codling moth, *Cryptophlebia leucotreta*. Entomologia Experimentalis et Applicata, 52(2), 113–117.

Newton, P. J. (1998). False codling moth *Cryptophlebia leucotreta* (Meyrick). In E. C. G. Bedford, M. A. Van den Berg and E. A. De Villiers, (Eds). Citrus pests in the Republic of South Africa. 192–200, Dynamic Ad, Nelspruit, South Africa.

Oerke, E. C. (2006). Crop losses to pests. Journal of Agricultural Science, 144(1), 31–43.

Ogembo, J. G. (2002). Comparative biology and biochemical studies of two isolates of nucleopolyhedroviruses infecting *Helivocoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Doctoral dissertation, University of Zimbabwe, Zimbabwe.

Opoku-Debrah, J. K., Hill, M. P., Knox, C., & Moore, S. D. (2013). Overcrowding of false codling moth, *Thaumatotibia leucotreta* (Meyrick) leads to the isolation of five new Cryptophlebia leucotreta granulovirus (CrleGV-SA) isolates. Journal of Invertebrate Pathology, 112(3), 219–228.

’ Reilly, D. R., & il ler, L. . ( ). m rovement of a baculovirus esticide by deletion of the *egt* gene. Bio/Technology, 9(11), 1086–1089.

Pereira-da-Conceicoa, L. L., Hill, M. R., & Moore, S. (2012). Development of a peroral, droplet-dose bioassay laboratory technique and its application on a granulovirus for *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). African Entomology, 20(1), 187–190.

Raj, M. N., Samal, I., Paschapur, A., & Subbanna, A. (2022). Entomopathogenic viruses and their potential role in sustainable pest management. In New and Future Developments in Microbial Biotechnology and Bioengineering, Elsevier, 47–72.

Ramlee, S. N. S. (2015). Studies of breeding habitats and seasonal occurrence of mosquitoes in Putrajaya and Kuala Selangor, with laboratory experiments of guppies and dragonfly nymphs as potential biocontrol predators against mosquito larvae. University of Malaya, Malaysia.

Reed, W. (1974). The false codling moth, *Cryptophlebia leucotreta* Meyr. (Lepidoptera: Olethreutidae) as a pest of cotton in Uganda. Cotton Growing Review, 51(3), 213–225.

Reid, S., De Malmanche, H., Chan, L., Popham, H., & Van Oers, M. M. (2023). Invertebrates and entomopathogens. In J. A. Morales-Ramos, M. G. Rojas, and D. I. Shapiro-Ilan, (Eds). Mass production of beneficial organisms: Invertebrates and entomopathogens. 2nd edition, 375–406, Academic Press.

Reiser, M., Gröner, A., & Sander, E. (1993). *Cryptophlebia leucotreta* (Lep.: Tortricidae) - a promising alternate host for mass production of the Cydia pomonella granulosis virus (CpGV) for biological pest control / *Cryptophlebia leucotreta* (Lep.: Tortricidae) - ein vielversprechender Ersatzwirt für die Massenproduktion des Cydia-pomonella-Granuiosevirus (CpGV) zur biologischen Schädlingsbekämpfung. Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz / Journal of Plant Diseases and Protection, 100(6), 586–598.

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-Response Analysis Using R. PLoS one, 10(12), e0146021.

Rohrmann, G. F. (2019). Baculovirus molecular biology. 4th edition, National Center for Biotechnology Information (US): Bethesda, MD, USA.

Roldão, A., Oliveira, R., Carrondo, M. J. T., & Alves, P. M. (2009). Error assessment in recombinant baculovirus titration: Evaluation of different methods. Journal of Virological Methods, 159(1), 69–80.

Rozen, S., & Skaletsky, H. (2000). Primer 3 on the WWW for general users and for biologist programmers. Methods in Molecular Biology, 132, 365–386.

Sanches, M. M., Guimarães, G. C., Sihler, W., & Souza, M. L. (2021). Successful co-infection of two different baculovirus species in the same cell line reveals a potential strategy for large in vitro production. Brazilian Journal of Microbiology, 52(4), 1835–1843.

Sauphanor, B., Berling, M., Toubon, J.-F., Reyes, M., Delnatte, J., & Allemoz, P. (2006). Carpocapse des pommes cas de résistance au virus de la granulose en vergers biologiques: Fruits et légumes. Phytoma, La Défense Des Végétaux, 590, 24–27.

Schmitt, A., Bisutti, I., Ladurner, E., Benuzzi, M., Sauphanor, B., Kienzle, J., Zingg, D., Undorf- a hn, ., ritsch, E., & Huber, J. (20 3). he occurrence and distribution of resistance of codling moth to Cydia pomonella granulovirus in Europe. Journal of Applied Entomology, 137(9), 641–649.

Schulthess, F., Bosque-Perez, N. A., & Gounou, S. (1991). Sampling lepidopterous pests on maize in West Africa. Bulletin of Entomological Research, 81(3), 297–301.

Shapiro-Ilan, D. I., Fuxa, J. R., Lacey, L. A., Onstad, D. W., & Kaya, H. K. (2005). Definitions of pathogenicity and virulence in invertebrate pathology. Journal of Invertebrate Pathology, 88(1), 1–7.

Simón, O., Williams, T., López-Ferber, M., & Caballero, P. (2012). Deletion of egt is responsible for the fast-killing phenotype of natural deletion genotypes in a Spodoptera frugiperda multiple nucleopolyhedrovirus population. Journal of Invertebrate Pathology, 111(3), 260–263.

Singh, S., Moore, S., Spillings, B., & Hendry, D. (2003). South African isolate of Cryptophlebia leucotreta granulovirus. Journal of Invertebrate Pathology, 83(3), 249–252.

Smith, K. M. (1967). Insect Virology. Academic Press, London, UK.

Stibick, J. (2006). New pest response guidelines: False codling moth *Thaumatotibia leucotreta*. USDA–APHIS–PPQ Emergency and Domestic Programs. Riverdale, Maryland.

Tanada, Y. (1956). Some factors affecting the susceptibility of the armyworm to virus infections. Journal of Economic Entomology, 49(1), 52–57

Tanada, Y. (1959). Synergism between two viruses of the armyworm, Psendaletia unipuncta (Haworth) (Lepidoptera, Noctuidae).

Tanada, Y. (1964). Granulosis virus of codling moth, *Carpocapsa pomonella* (Linnaeus) (Olethreutidae, Lepidoptera). Journal of Insect Pathology, 6, 378–380.

Tanada, Y., & Hess, R. T. (1991). Baculoviridae, Granulosis Viruses. In J. R. Adams and J. R. Bonami (Eds). Atlas of invertebrate viruses. 227–257, CRC Press, Boca Raton, Florida, USA.

Tanada, Y., & Hukuhara, T. (1971). Enhanced infection of a nuclear-polyhedrosis virus in larvae of the armyworm, *Pseudaletia unipuncta*, by a factor in the capsule of a granulosis virus. Journal of Invertebrate Pathology, 17(1), 116–126.

Taylor, D. G. (2021). Baculovirus synergism for improved management of false codling moth *Thaumatotibia leucotreta* Meyr. (Lepidoptera: Tortricidae). Rhodes University, Makhanda, South Africa.

Thackeray, S. (2023). Personal Communication. River Bioscience, Gqeberha, South Africa,

Thézé, J., Cabodevilla, O., Palma, L., Williams, T., Caballero, P., & Herniou, E. A. (2014). Genomic diversity in European Spodoptera exigua multiple nucleopolyhedrovirus isolates. Journal of General Virology, 95(10), 2297–2309.

Vaissayre, M. (1995). Ecological attributes of major cotton pests: Implications for management. In G.A. Constable, N.W. Forrester (Eds). Challenging the future, Brisbane 1994, Proceedings of the World Cotton Research Conference-1, Commonwealth Scientific Industrial Research Organisation. 499–510, Melbourne, Australia.

van der Merwe, M., Jukes, M. D., Rabalski, L., Knox, C., Opoku-Debrah, J. K., Moore, S. D., Krejmer-Rabalska, M., Szewczyk, B., & Hill, M. P. (2017). Genome analysis and genetic stability of the Cryptophlebia leucotreta granulovirus (CrleGV-SA) after 15 Years of Commercial Use as a Biopesticide. International Journal of Molecular Sciences, 18(11), 2327.

van Oers, M. M., Herniou, E. A., Jehle, J. A., Krell, P. J., Abd-Alla, A. M. M., Ribeiro, B. M., Theilmann, D. A., Hu, Z., & Harrison, R. L. (2023). Developments in the classification and nomenclature of arthropod-infecting large DNA viruses that contain pif genes. Archives of Virology, 168(7), 182.

van Oers, M. M., & Vlak, J. M. (2007). Baculovirus genomics. Current Drug Targets, 8(10), 1051–1068.

Venette, R. C., Davis, E. E., Dacosta, M., Heisler, H., & Larson, M. (2003). Mini Risk Assessment False codling moth, *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Meyrick) [Lepidoptera: Tortricidae]. University of Minnesota, Department of Entomology, CAPS PRA, 1-30.

Vermeulen, J., B., & Bedford, E. C. G. (1998). American bollworm, *Helicoverpa* (=*Heliothis*) *armigera* (Hübner). In M. A. Van den Berg, E. A. De Villiers, and E. C. G. Bedford (Eds). Citrus pests in the Republic of South Africa. 2nd edition, 217–221, Dynamic Ad. Pretoria, South Africa.

Waite, G. K., & Hwang, J. S. (2002). Pests of litchi and longan. In J. E. Peña, J. L. Sharp and M. Wysoki (Eds). Tropical Fruit Pests and Pollinators: Biology, Economic Importance, Natural Enemies and Control. 331–359, CABI Publishing, Wallingford, UK.

Wang, M., & Hu, Z. (2019). Cross-talking between baculoviruses and host insects towards a successful infection. Philosophical Transactions of the Royal Society B: Biological Sciences, 374(1767), 20180324.

Wennmann, J. T., Eigenbrod, M., Marsberg, T., Moore, S. D., Knox, C. M., Hill, M. P., & Jehle, J. A. (2019). Cryptophlebia peltastica nucleopolyhedrovirus is highly infectious

to codling moth larvae and cells. Applied and Environmental Microbiology, 85(17), e00795-19.

Wennmann, J. T., & Jehle, J. A. (2014). Detection and quantitation of Agrotis baculoviruses in mixed infections. Journal of Virological Methods, 197, 39–46.

Wennmann, J. T., Köhler, T., Gueli Alletti, G., & Jehle, J. A. (2015). Mortality of cutworm larvae is not enhanced by Agrotis segetum granulovirus and Agrotis segetum nucleopolyhedrovirus B coinfection relative to single infection by either virus. Applied and Environmental Microbiology, 81(8), 2893–2899.

Wennmann, J. T., Radtke, P., Eberle, K. E., Gueli Alletti, G., & Jehle, J. A. (2017). Deciphering single nucleotide polymorphisms and evolutionary trends in isolates of the Cydia pomonella granulovirus. Viruses, 9(8), 227.

Whitlock, V. H. (1974). Symptomatology of two viruses infecting *Heliothis armigera*. Journal of Invertebrate Pathology, 23(1), 70–75.

Whitlock, V. H. (1977). Simultaneous treatments of *Heliothis armigera* with a nuclear polyhedrosis and a granulosis virus. Journal of Invertebrate Pathology, 29(3), 297–303.

Whitlock, V. H. (1978). Dosage-mortality studies of a granulosis and a nuclear polyhedrosis virus of a laboratory strain of *Heliothis armigera*. Journal of Invertebrate Pathology, 32(3), 386–387.

Williams, T., Virto, C., Murillo, R., & Caballero, P. (2017). Covert infection of insects by baculoviruses. Frontiers in Microbiology, 8, 1337.

Winstanley, D., & ’ Reilly, D. ( ). aculoviruses (a culoviridae) | Granuloviruses. In R. G. Webster and A. Granoff (Eds). Encyclopedia of Virology. 2nd edition, 140-146, Academic Press, London.

Wysoki, M. (1986). New records of lepidopterous pests of macadamia in Israel. Phytoparasitica, 14(2), 147–147.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 13(1), 134–134.

Zhang, H., Yang, Q., Qin, Q.-L., Zhu, W., Zhang, Z.-F., Li, Y.-N., Zhang, N., & Zhang, J.-H. (2014). Genomic sequence analysis of Helicoverpa armigera nucleopolyhedrovirus isolated from Australia. Archives of Virology, 159(3), 595–601.

Appendix A

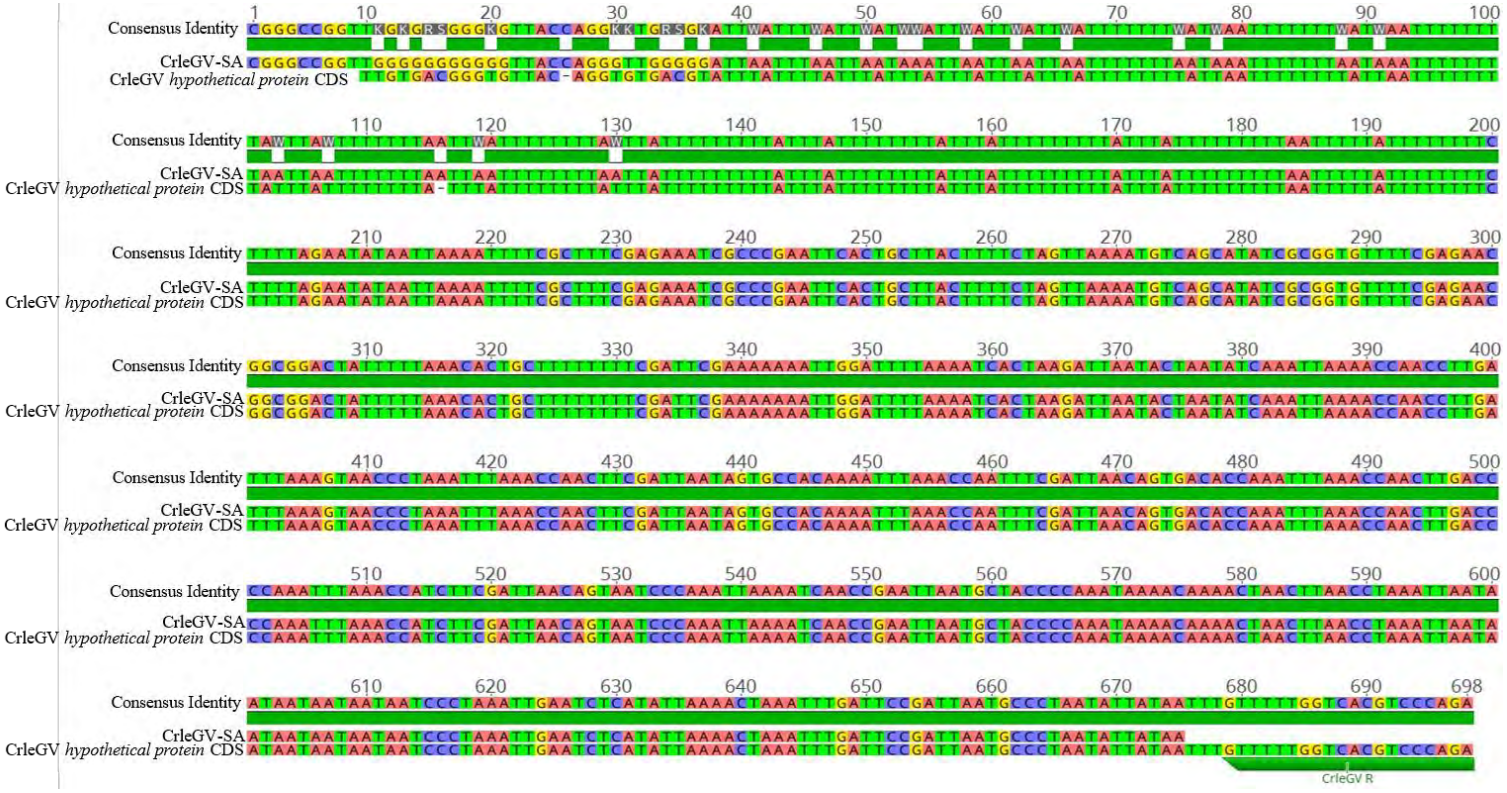


Figure S. 1. Sequence alignment of the CrleGV amplicon against the target region in the CrleGV-SA genome sequence.

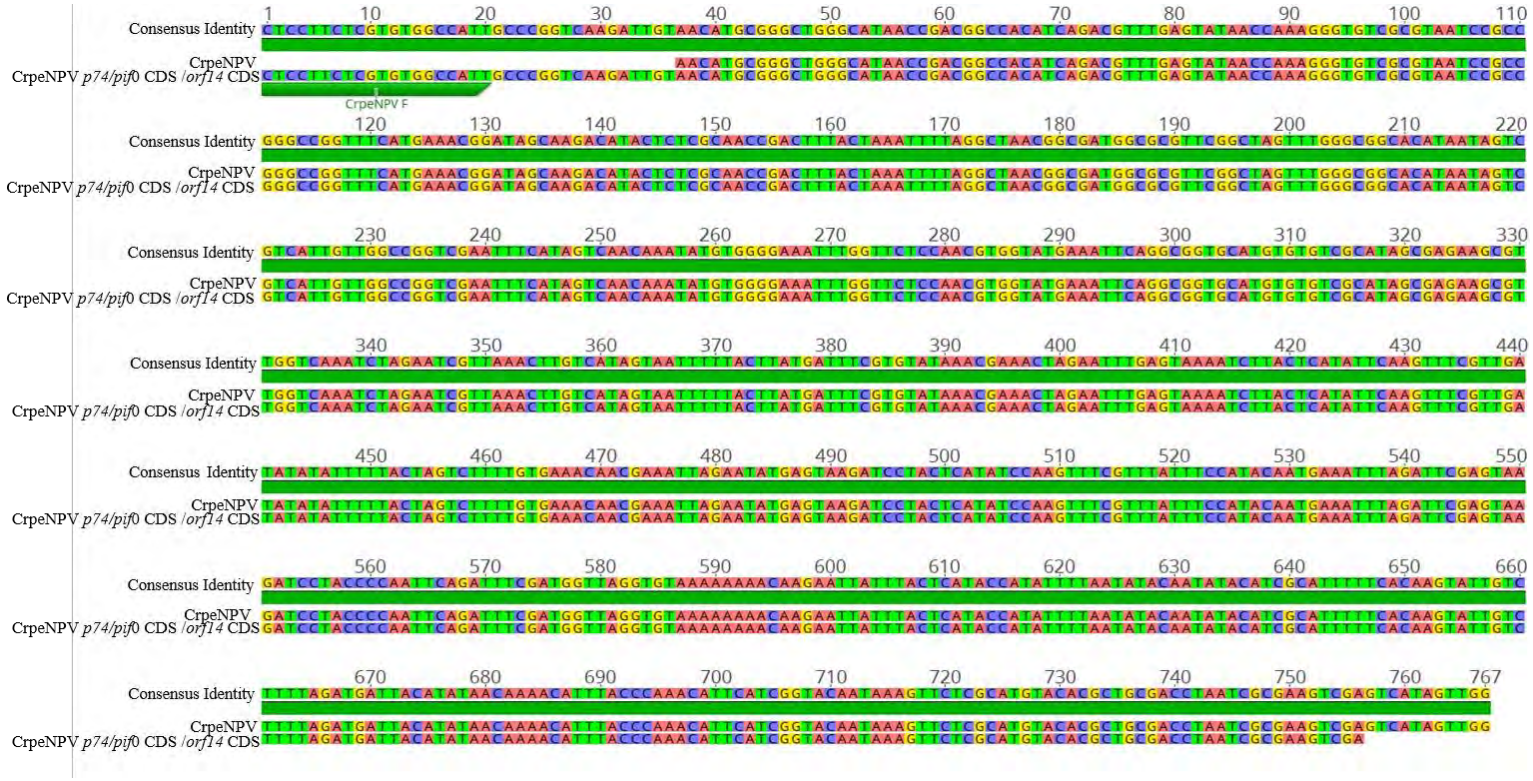


Figure S. 2. Sequence alignment of the CrpeNPV amplicon against the target region in the CrpeNPV genome sequence.

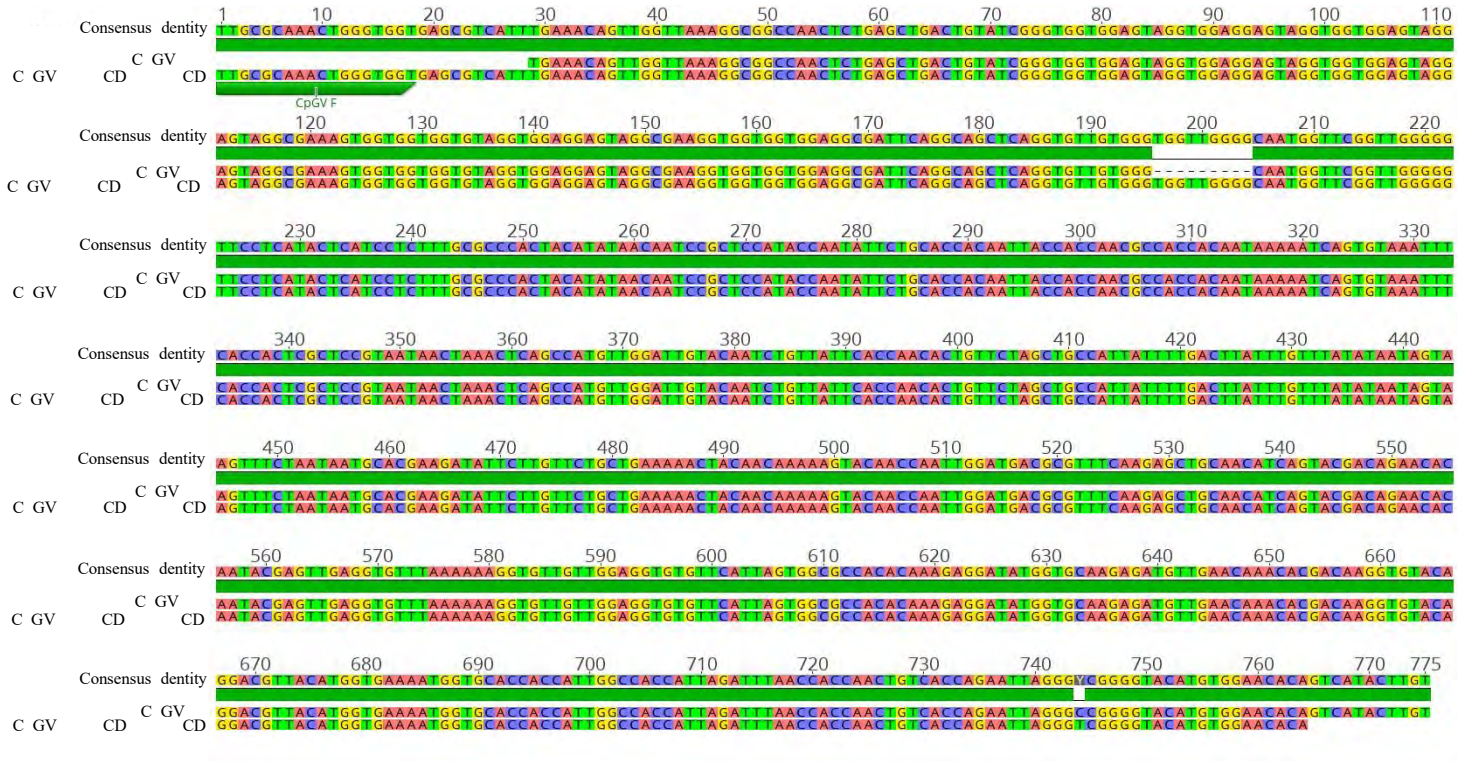


Figure S. 3. Sequence alignment of the CpGV amplicon against the target region in the CpGV-M genome sequence.

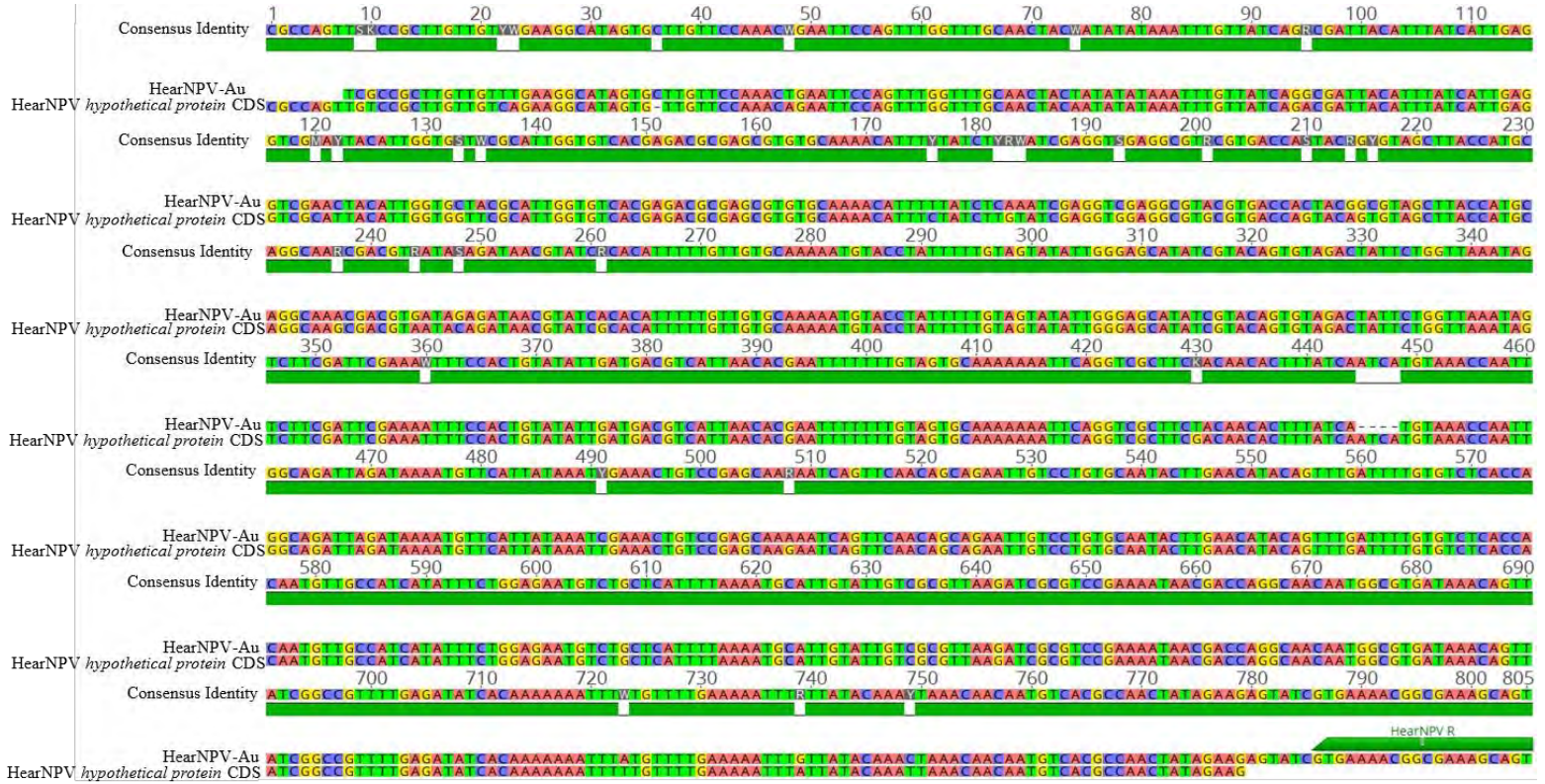


Figure S. 4. Sequence alignment of the HearNPV amplicon against the target region in the HearNPV-Au genome sequence.

Appendix B

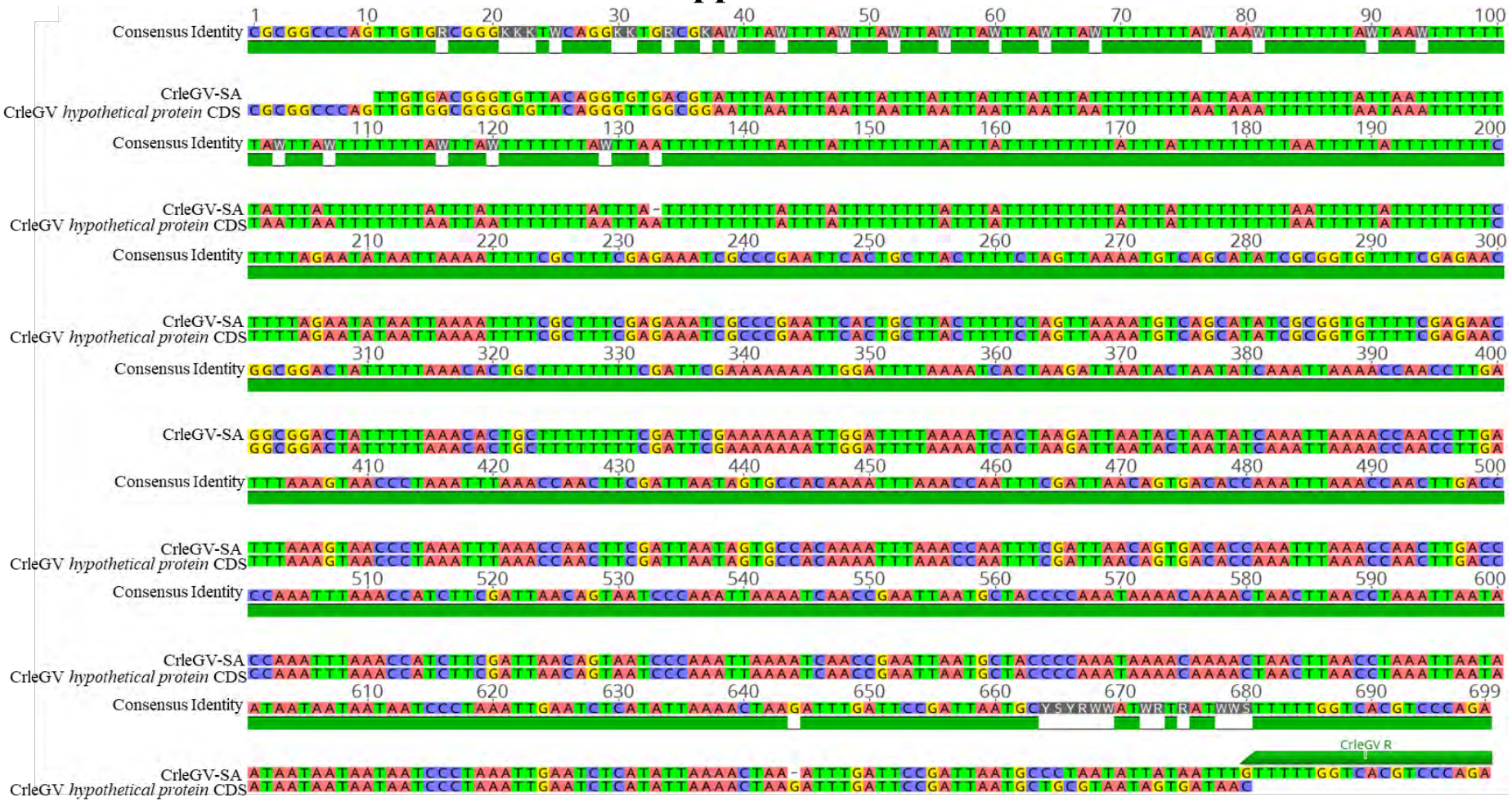


Figure S. 5. Sequence alignment of the CrleGV PCR amplicon from the CrleGV single infections against the target region in the CrleGV-SA genome sequence

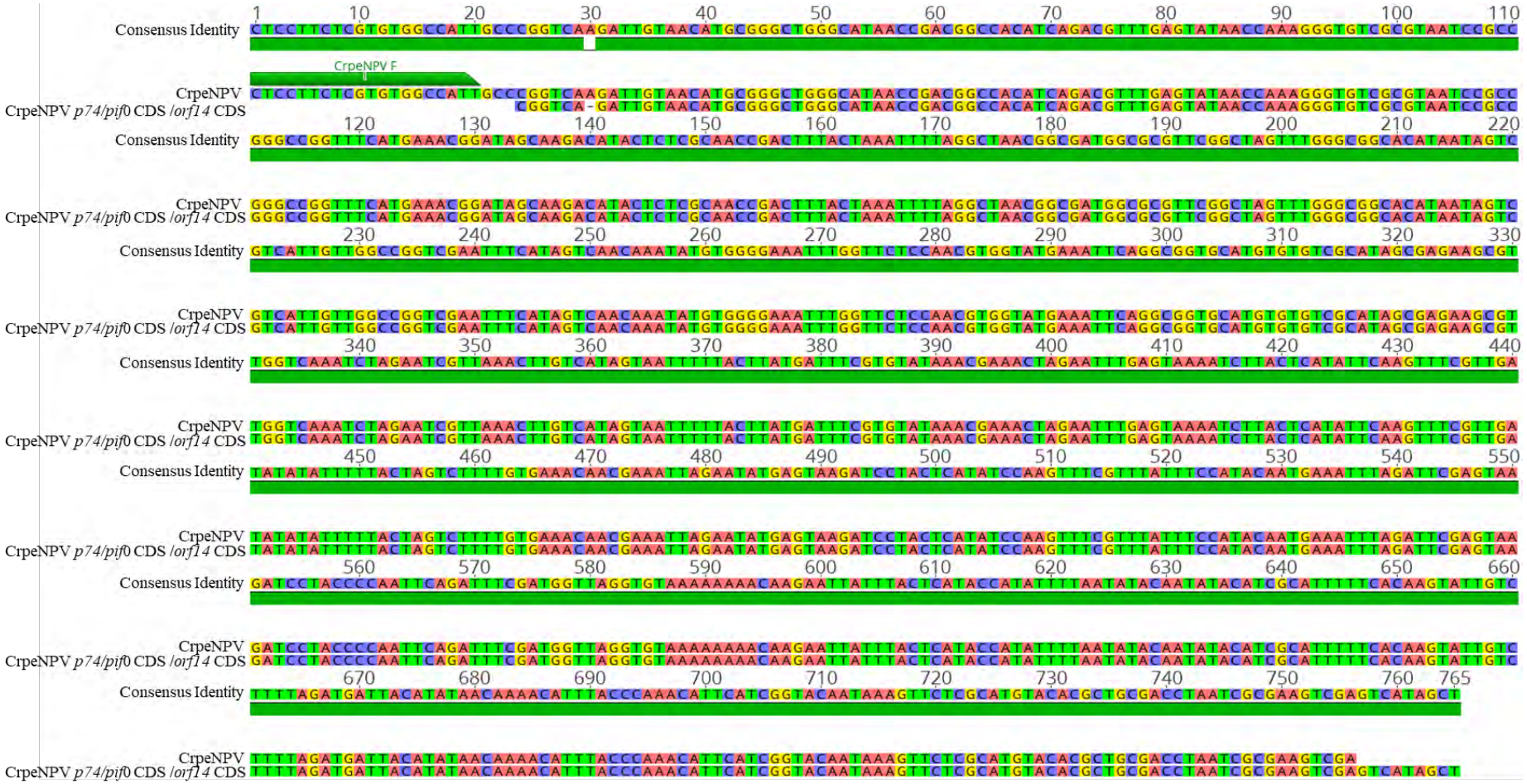


Figure S. 6. Sequence alignment of the CrpeNPV PCR amplicon from the CrpeNPV single infections against the target region in the CrpeNPV genome sequence.

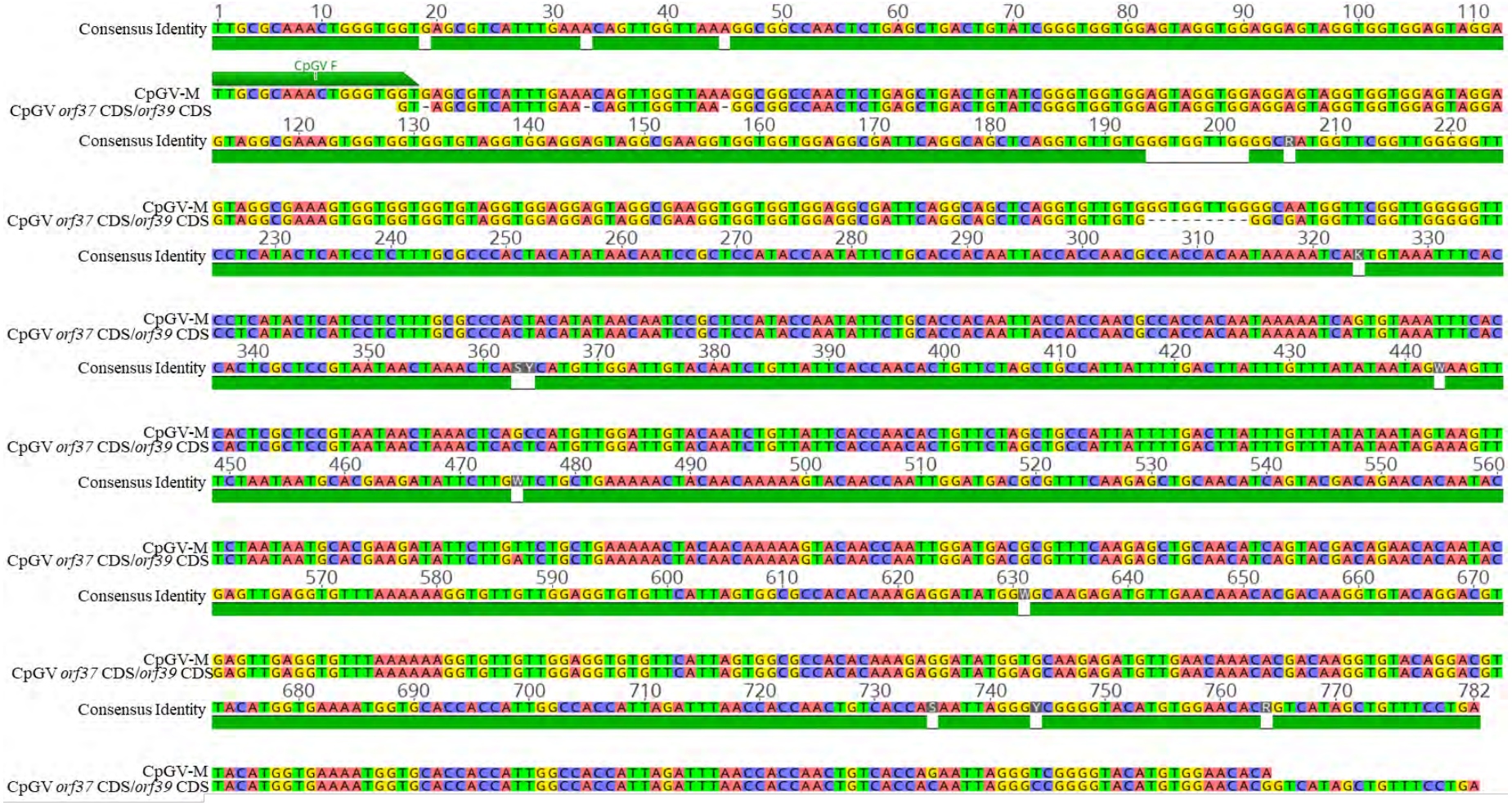


Figure S. 7. Sequence alignment of the CpGV PCR amplicon from the CpGV single infections against the target region in the CpGV-M genome sequence.

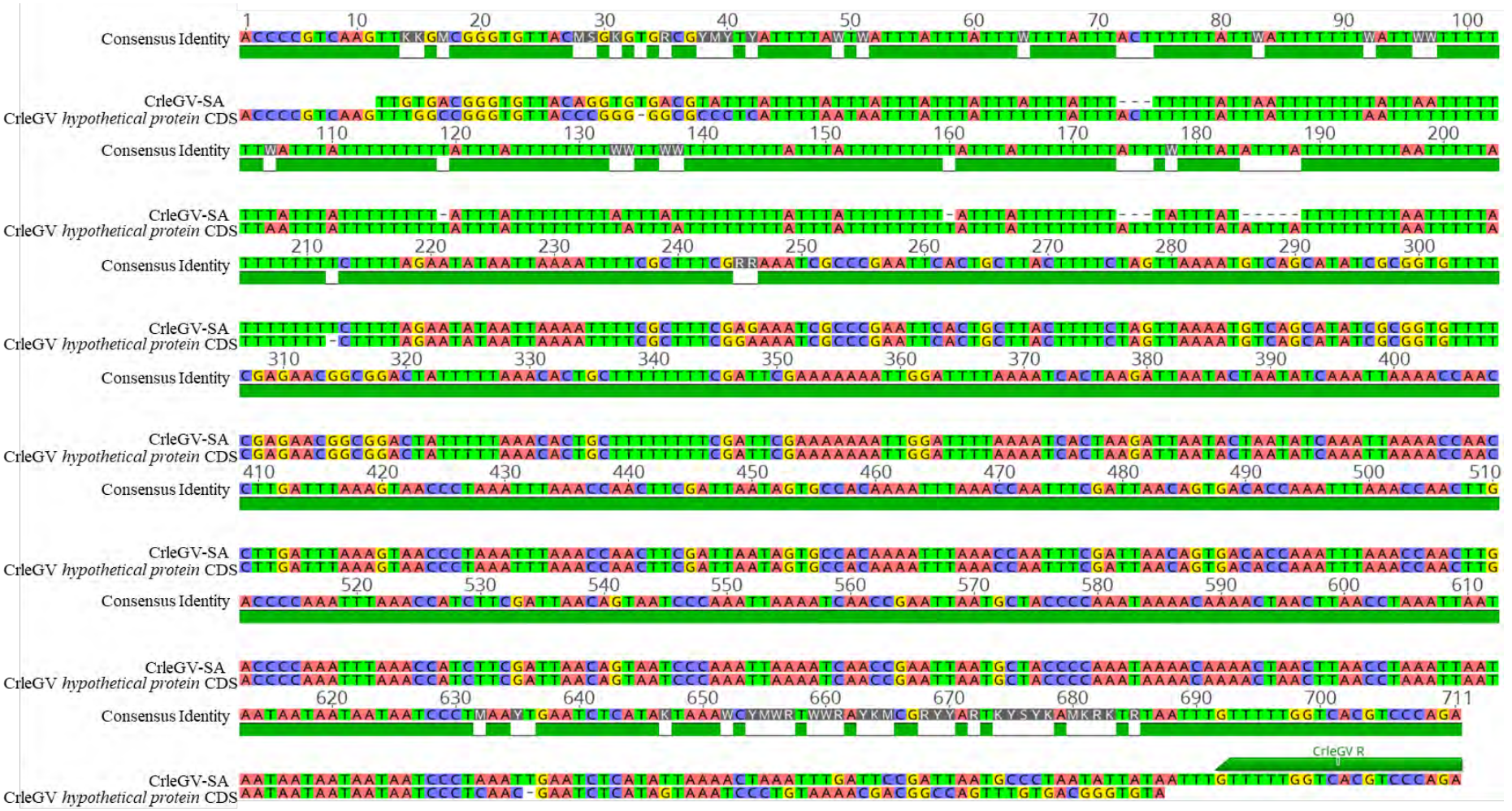


Figure S. 8. Sequence alignment of the CrleGV PCR amplicon from the HearNPV single infections against the target region in the CrleGV-SA genome sequence.

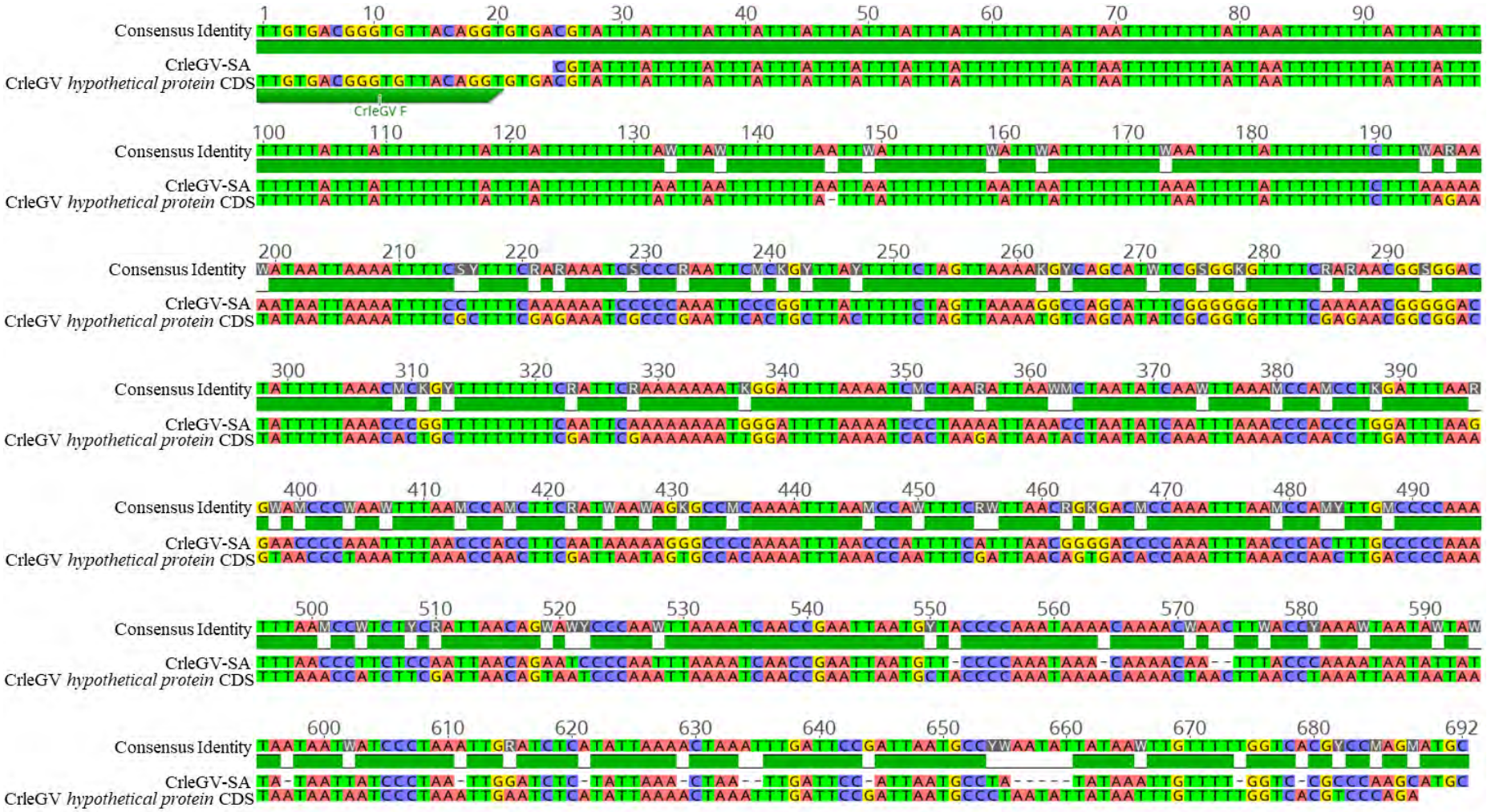


Figure S. 9. Sequence alignment of the CrleGV PCR amplicon from the CrleGV/CrpeNPV mixed infections against the target region in the CrleGV-SA genome sequence.

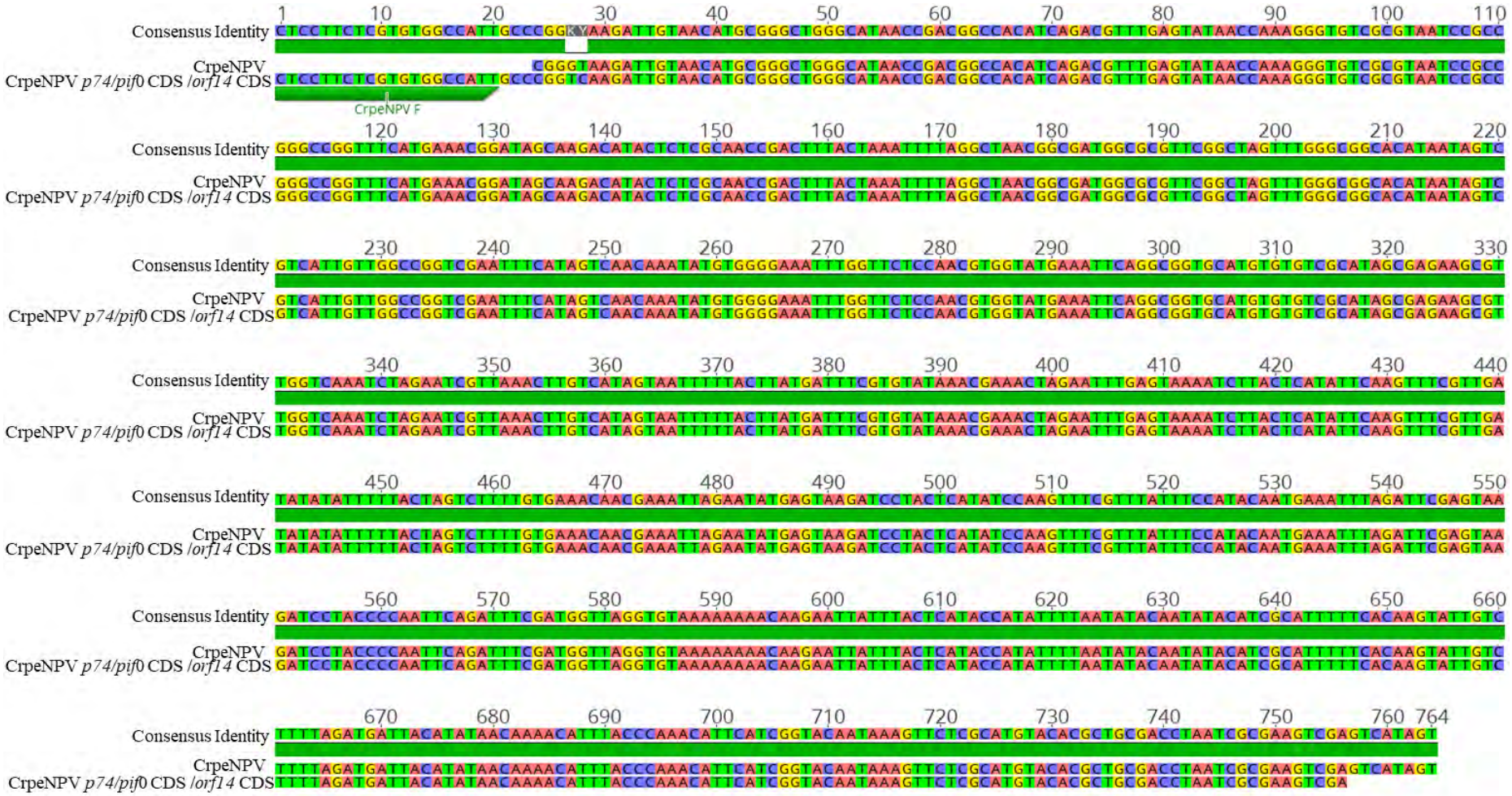


Figure S. 10. Sequence alignment of the CrpeNPV PCR amplicon from the CrleGV/CrpeNPV mixed infections against the target region in the CrpeNPV genome sequence.

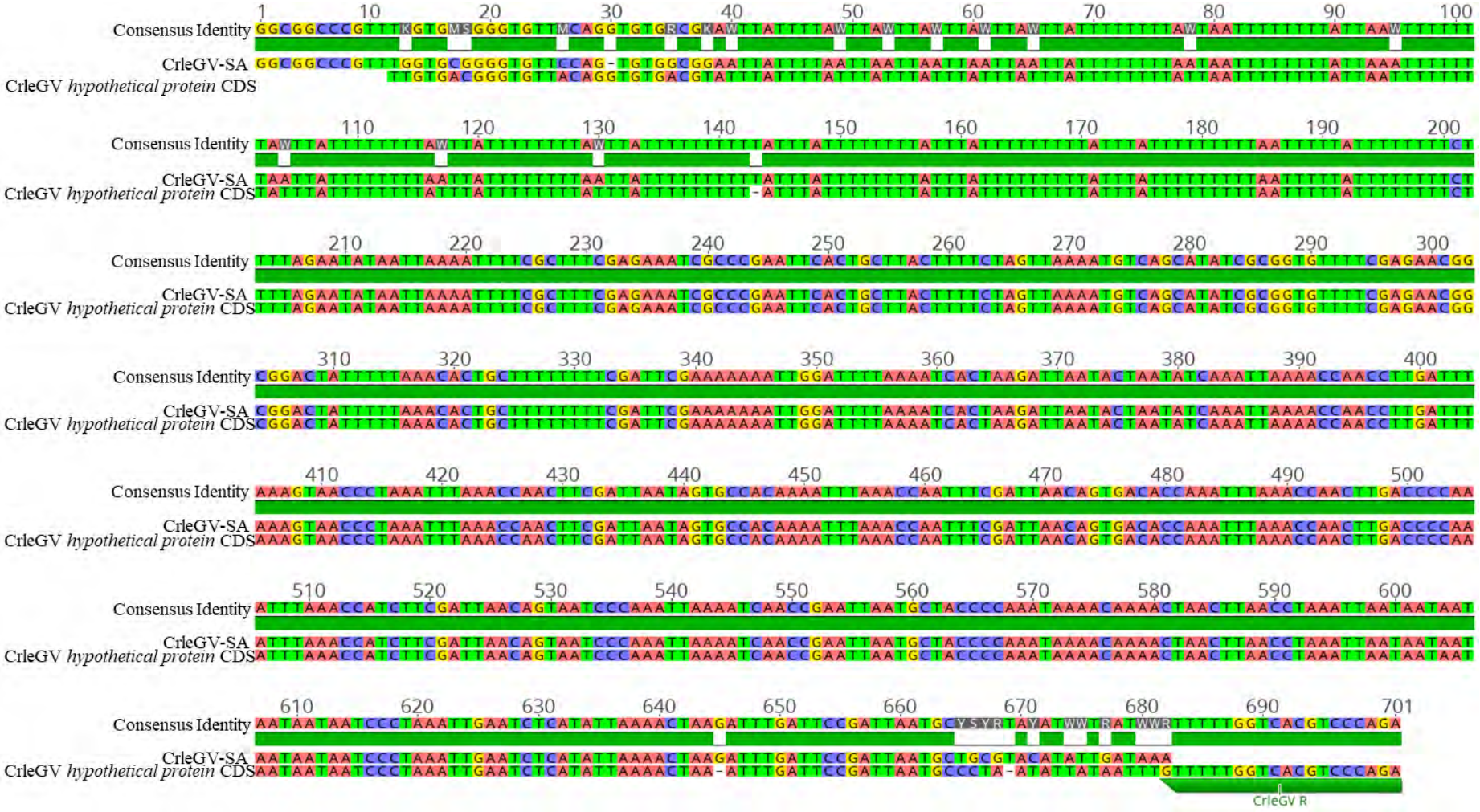


Figure S. 11. Sequence alignment of the CrleGV PCR amplicon from the CrleGV/CpGV mixed infections against the target region in the CrleGV-SA genome sequence

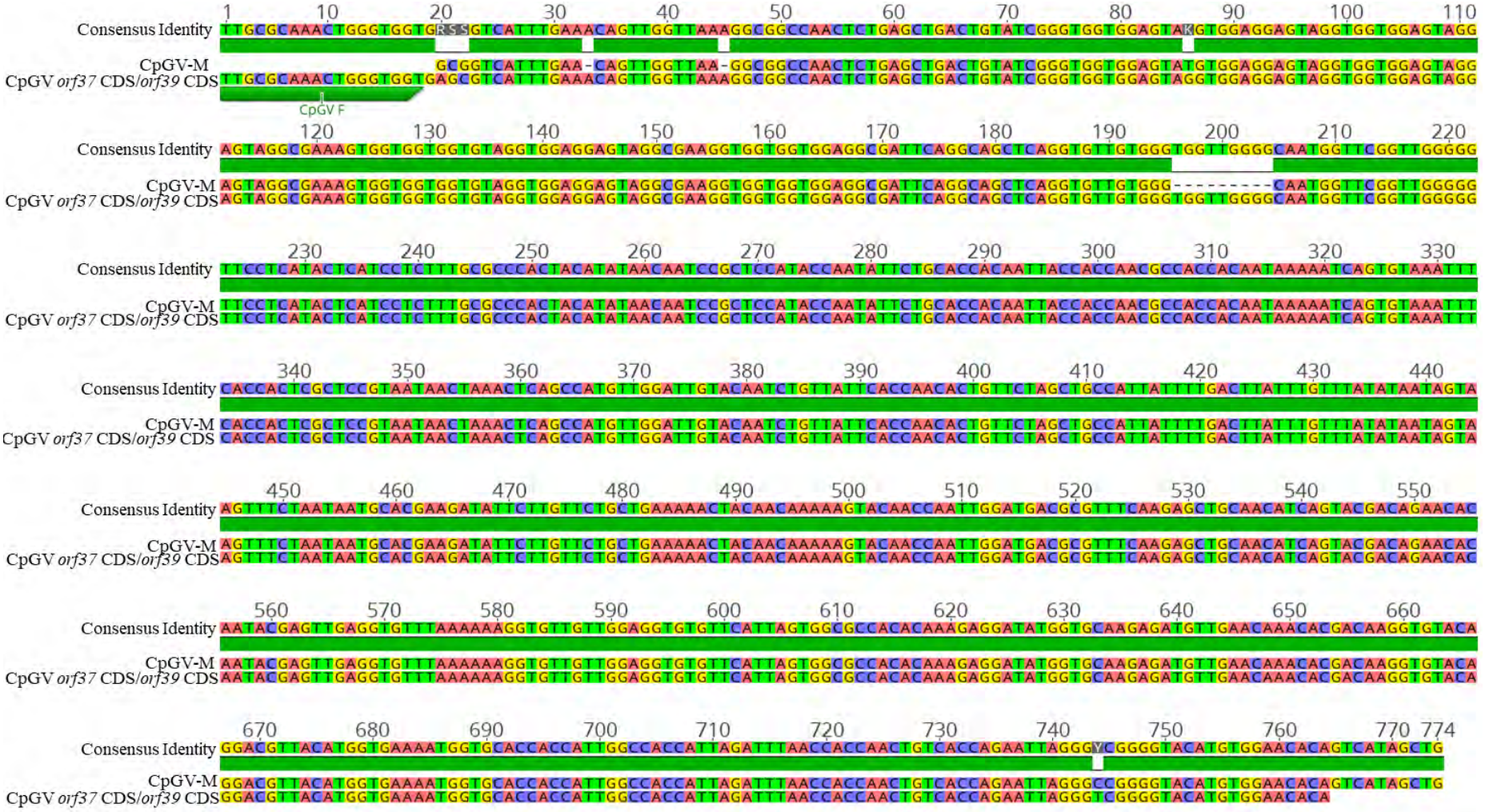


Figure S. 12. Sequence alignment of the CpGV PCR amplicon from the CrleGV/CpGV mixed infections against the target region in the CpGV-M genome sequence

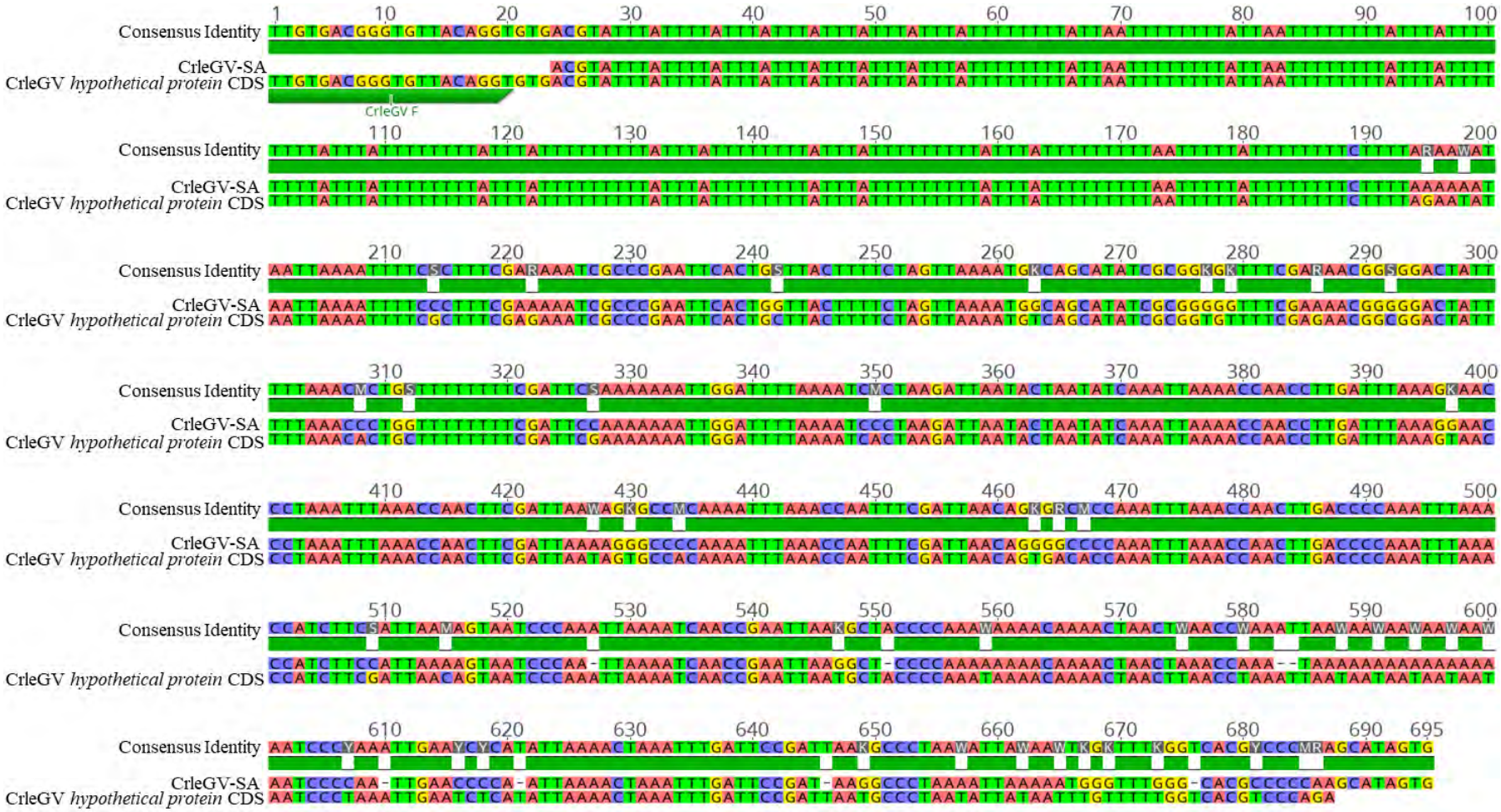


Figure S. 13 Sequence alignment of the CrleGV PCR amplicon from the CrleGV/HearNPV mixed infections against the target region in the CrleGV-SA genome sequence