Selection for improved virulence of Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) to False Codling Moth, *Thaumatotibia leucotreta*, by serial passage through a heterologous host

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By

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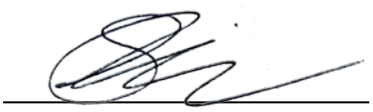
Declaration

I, Petrus Paulus Iita (19I8233) 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.



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Date

Abstract

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) is endemic to southern Africa, and strongly associated with citrus. As South African citrus production is mainly for export to foreign markets, the market access risk due to the phytosanitary status of this pest is considerable and its control is therefore imperative. Various control measures as part of a rigorous integrated pest management (IPM) programme targeted against *T. leucotreta* have been effective at suppressing the pest in citrus, but there is still a growing need for continued improvement of the programme and augmentation of the available control options. Of these control options, biological control, particularly the use of Cryptophlebia leucotreta granulovirus (CrleGV-SA), is a key component of IPM in citrus orchards and it has been very successful at reducing *T. leucotreta* populations in the field for almost two decades. There is however, a growing need for more baculovirus variants with an improved virulence against *T. leucotreta* for a more efficient pest management system. The newly identified insect virus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) offers a unique opportunity for an additional biopesticide in IPM for control of *T. leucotreta* in the field. This study aimed to conduct serial passaging of CrpeNPV through a heterologous host, *T. leucotreta*, in order to determine the potential for improved virulence or speed of kill against it.

In order to select for a variant of CrpeNPV with improved virulence against *T. leucotreta*, a high dose (LC_{90}) of the virus OBs was used to perform 12 serial passages through *T. leucotreta* larvae in surface-dose bioassays. Whole genome sequencing and analysis of the passaged virus, along with restriction endonuclease profiling *in silico* was performed to determine if the genetic identity of the virus had changed during serial passage, in relation to

the original virus. These analyses indicated that the dominant genotype of CrpeNPV was maintained following 12 serial passages through the heterologous host.

The biological activity of the passaged virus, along with the original virus was evaluated against neonate *T. leucotreta* in surface-dose bioassays and compared. Results from dose-response bioassays showed that the virulence of CrpeNPV did not improve after 12 serial passages. The LC_{50} values of the passaged virus and the original virus were estimated at 1.96×10^4 and 1.58×10^4 OBs/ml, respectively, whereas the LC_{90} values were estimated at 3.46×10^4 OBs/ml for the passaged virus and 3.68×10^4 for the original virus. Similarly, the results from time-response bioassays showed that the speed of kill of CrpeNPV did not improve after 12 serial passages. The LT_{50} values of the passaged virus and the original virus were 88.44 hours (3 days and 16 hours) and 83.74 hours (3 days and 12 hours), respectively, whereas the LT_{90} values were 115 hours (4 days 19 hours) for the passaged virus and 102 hours (4 days 6 hours) for the original virus. The virulence and speed of kill of the passaged virus decreased significantly, in relation to the original virus.

When the full genome of the passaged virus was sequenced and analysed, only a few SNPs were detected in the viral genome, in comparison to the original virus. No detectable difference in REN digestion patterns were observed following REN analysis of gDNA of the passaged virus with several restriction enzymes *in silico*. The results for this study suggest that CrpeNPV may already be optimally suited to the heterologous host as it persists under these conditions without significant changes to the genome. These results have positive implications for the genetic integrity of CrpeNPV as a potential biocontrol agent in the field.

This study is the first to report the virulence selection of CrpeNPV by serial passage through a heterologous host, and also the first to record bioassay data in terms of dose response (or lethal concentration) against *T. leucotreta* second instars. The data obtained have added to the

knowledge about interactions between CrpeNPV and its heterologous host, and may be fundamental to continued investigation into the effect of serial passage on pathogenicity and genetic diversity of CrpeNPV.

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List of Abbreviations

%	-	Percentage
×g	-	Time gravity
°C	-	Degrees Celsius
µl	-	Microliter
AGE	-	Agarose gel electrophoresis
ANOVA	-	Analysis of variance
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
BV	-	Budded virus
CTAB	-	Cetrimonium bromide
CrpeNPVpx0	-	Cryptophlebia peltastica NPV passage zero
CrpeNPVpx12	-	Cryptophlebia peltastica NPV passage twelve
D1	-	Dose 1
D2	-	Dose 2
D3	-	Dose 3
D4	-	Dose 4
D5	-	Dose 5
D6	-	Dose 6
DAFF	-	Department of agriculture, forestry and fisheries
DALRRD	-	Department of Agriculture, Land Reform and Rural Development
ddH_2O	-	Double distilled water DNA

DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
EPF	-	Entomopathogenic fungi
EPN	-	Entomopathogenic nematodes
EU	-	European Union
FCM	-	False codling moth
gDNA	-	Genomic deoxyribonucleic acid
gran	-	Granulin
GV	-	Granulovirus
HCl	-	Hydrochloric acid
IPM	-	Integrated pest management
Kb	-	Kilobase pairs
LC	-	Lethal concentration
LC_{50}	-	Lethal concentration (50%)
LC_{90}	-	Lethal concentration (90%)
LD	-	Lethal dose
LT	-	Lethal time
Ltd	-	Limited
lef	-	Late expression factor
LT	-	Lethal time
M	-	Molar
ml	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
MNPV	-	Multiple nucleopolyhedrovirus

mPCR	-	Multiplex polymerase chain reaction
NGS	-	Next Generation Sequencing
NEB	-	New England Biolabs
NPV	-	Nucleopolyhedrovirus
NTC	-	No template control
Na_2CO_3	-	Sodium Carbonate
OB	-	Occlusion Body
ODV	-	Occlusion derived virion
ORF	-	Open reading frame
P_1	-	Passage 1
P_2	-	Passage 2
P_3	-	Passage 3
P_4	-	Passage 4
P_5	-	Passage 5
P_6	-	Passage 6
P_7	-	Passage 7
P_8	-	Passage 8
P_9	-	Passage 9
P_{10}	-	Passage 10
P_{11}	-	Passage 11
P_{12}	-	Passage 12
PCR	-	Polymerase chain reaction
pif	-	*per os* infectivity factor
polh	-	Polyhedrin
Pty	-	Propriety

qPCR	-	Quantitative polymerase chain reaction
R	-	Rand
REN	-	Restriction endonuclease
RH	-	Relative humidity
SE	-	Standard error
Std Dev	-	Standard deviation
SDS	-	Sodium dodecyl sulphate
SIT	-	Sterile insect technique
SNP	-	Single nucleotide polymorphisms
SNPV	-	Single nucleopolyhedrovirus
TAE	-	Tris base, acetic acid and EDTA

Viruses:

AcMNPV	-	Autographica californica MNPV
AgMNPV	-	Anticarsia gemmatalis MNPV
CpGV	-	Cydia pomonella granulovirus
CfNPV	-	Choristoneura fumiferana
CrleGV	-	Cryptophlebia leucotreta GV
CrpeNPV	-	Cryptophlebia peltastica NPV
HearNPV	-	Helicoverpa armigera NPV
MbMNPV	-	Mamestra brassicae MNPV
PaflNPV	-	Panolis flammea NPV
SeMNPV	-	Spodoptera exigua NPV

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CHAPTER 1

Literature Review

1.1. Introduction

The South African agricultural sector plays a key role in the local economy, in terms of job creation, food security and foreign exchange. According to the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) (formerly Department of Agriculture, Forestry and Fisheries (DAFF)), the South African citrus industry represents one of the country's major contributors, by value, to the South African agricultural production. During the 2016/2017 production season the industry contributed over R19 billion to the total gross value of South African agricultural production (DAFF, 2018). Furthermore, South African citrus production is mainly for export to foreign markets and it is an important source of foreign currency.

However, the citrus industry faces severe pressure from insect pests and diseases to maximise quality yields for exports. The impact of insect pests is known to be one of the leading factors in any systematic decline in production of major crops and multi-million dollar losses (Oliveira, et al., 2014). Among the most economically important pests with potential threat to South African citrus production is the false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), a native of sub-Saharan Africa (Gilligan, et al., 2011; Maniania, et al., 2017). According to an early study by Moore et al. (2004), damage in citrus due to yield losses by *T. leucotreta* alone cost the southern African citrus industry over R100 million annually, and

the market access risk due to phytosanitary status of this pest is considerably more. However, these lossess are probably less now, due to improved *T. leucotreta* management systems (Hattingh, et al., 2020). However, as a result, the cost of *T. leucotreta* management has increased dramatically.

In order to safeguard the integrity of the multibillion-rand citrus industry, various control options are being used (e.g. chemical insecticides, mating disruption, attract and kill, sterile insect technique, cultural practices, and biological control) (Moore & Hatting, 2012; Malan, et al., 2018). According to Moore et al. (2017), the level of *T. leucotreta* induced by a single control measure is generally inadequate. Thus, various control measures are combined into a single strategic arsenal, an integrated pest management (IPM) programme to obtain maximum efficacy.

This chapter reviews previous studies to provide insights into the potential threat of *T. leucotreta* to South African citrus production and the economic impact related to the damage, and more importantly to the market access risk that it poses. It also gives an overview of the biology of the pest and available control measures used to mitigate its infestation in citrus orchards. Baculoviruses as biocontrol agents for the control of *T. leucotreta* in citrus will be discussed in depth. The chapter also reviews current knowledge on the newly described insect virus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) and its potential as a biocontrol agent. Finally, the chapter reviews some of the challenges of baculovirus application for pest control, with emphasis on *T. leucotreta*, and strategies for improving baculovirus efficacy in the field.

1.1.1. *Thaumatotibia leucotreta* as a threat

Thaumatotibia leucotreta is native to southern Africa, and strongly associated with citrus. The *T. leucotreta* larvae burrow into the citrus fruit, leading to feeding-lesions and fruit infestations (Moore, 2002; Malan, et al., 2018), and consequently poor quality fruit yields (Figure 1.1).

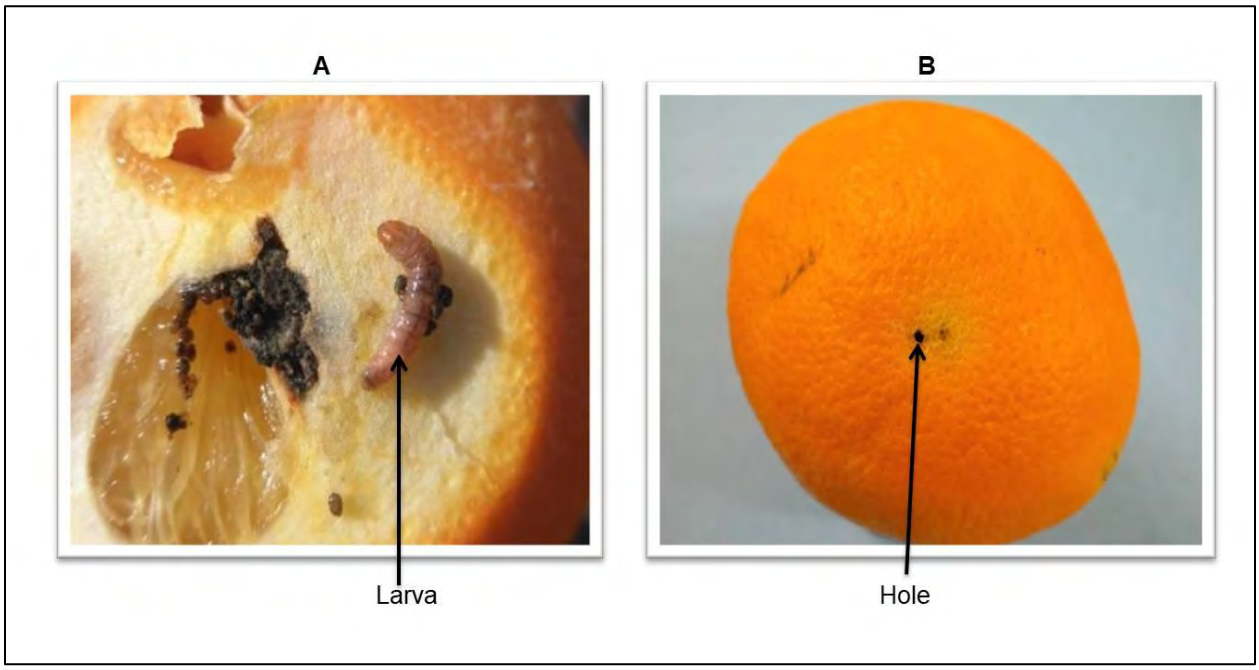

Figure 1.1: Examples of *T. leucotreta* damages in citrus, with A) a mature *T. leucotreta* larva in a fruit and B) an infested fruit with larval escape hole (Jansen, 2017; Hofmeyr, 2018).

However, *T. leucotreta* early instars in the fruits and eggs laid singly are difficult to detect (U.S Department of Agriculture (USDA), 2010). This is a serious concern and the spread of *T. leucotreta* to other parts of the world where it is not present through export of South African's citrus must be avoided. For example, interceptions of *T. leucotreta* have been made in South African citrus consignments in Italy (Mazza, et al., 2014). Additionally, *T. leucotreta* has been intercepted at United States ports of entry in both cargo and passenger luggage (USDA, 2010). As such, *T. leucotreta* has become a primary economic concern. Moreover, lucrative South

African citrus markets, especially the European Union (EU) and the Americas consider *T. leucotreta* a phytosanitary pest (Moore, 2021). Thus, detection of even a single *T. leucotreta* larva in fruit destined for export can result in total rejection of the entire consignment, resulting in massive financial losses (Moore, et al., 2004).

1.1.2. The biology of *Thaumatotibia leucotreta*

The biology of *T. leucotreta* was extensively studied by Daiber (e.g. Daiber, 1979a, 1979b, 1979c, 1980), by looking at individual life stages such as the egg, larva, cocoon, adult and generations during the year. Briefly, the life cycle of *T. leucotreta* begins with oviposition by an adult fertile female moth on the surface of a host plant, particularly the fruit (Figure. 1.2).

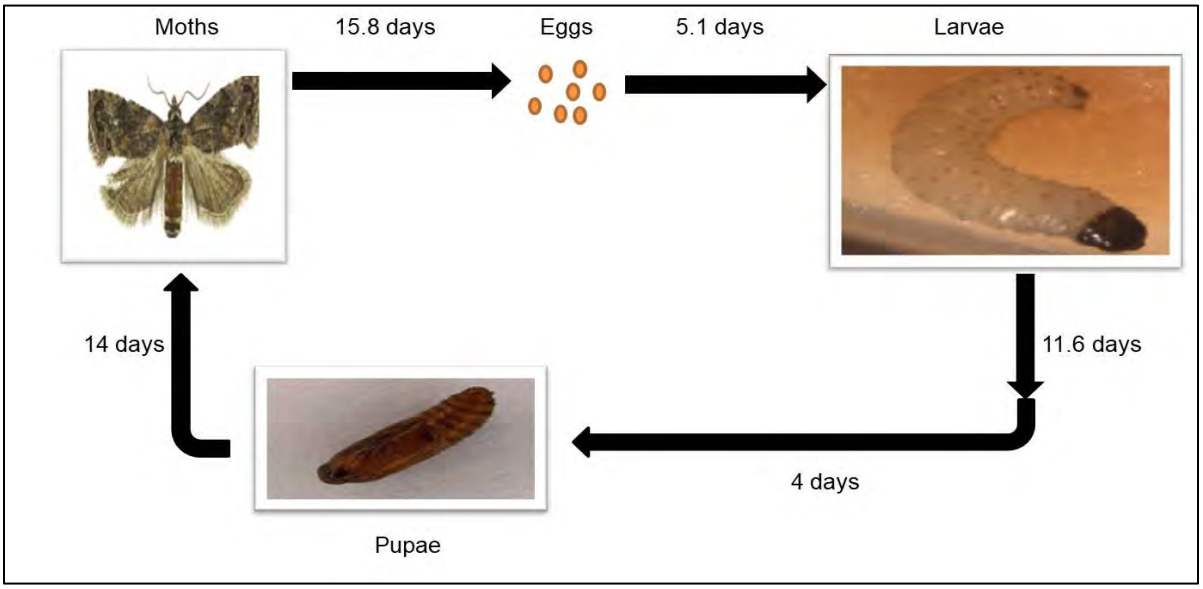

Figure 1.2: Different life stages of *T. leucotreta* reared at 25˚C, from the eggs to the adult moths, with arrows showing developmental direction along with duration in days (Daiber, 1979a, 1979b, 1979c, 1980).

1.1.2.1. The egg stage

Depending on temperature, *T. leucotreta* egg development ranges from 2 to 22 days (Daiber, 1979a). After egg development, *T. leucotreta* eggs hatch, upon which the newly hatched larvae must find a suitable host fruit to start feeding (Daiber, 1979b). The duration of *T. leucotreta* eggs from the time of development to hatching was found to be 5.1±0.04 days at 25˚C (Daiber, 1979a).

1.1.2.2. The larval stage

Generally, *T. leucotreta* passes through five larval instars, and each is differentiated by measuring the width of the head capsule, along with the duration at which development occur (Figure 1.3 and Table 1.1) (Daiber, 1979b; Hofmeyr, et. al, 2016b).

Table 1.1: Width measurements of the head capsule of *T. leucotreta* instars and the duration for each instar at ±25˚C (Daiber, 1979b; Hofmeyr, et. al, 2016b).

Instar	**Head capsule** (mm)*	**Head capsule** (mm)**	**Duration** (days)
1st	0.17 - 0.25	0.00 - 0.28	5.5
2nd	0.32 - 0.43	0.29 - 0.46	7
3rd	0.50 - 0.72	0.47 - 0.77	8
4th	0.82 - 1.07	0.78 - 1.16	10
5th	1.25 - 1.49	<1.17	13

*Daiber
** Hofmeyr, et al.

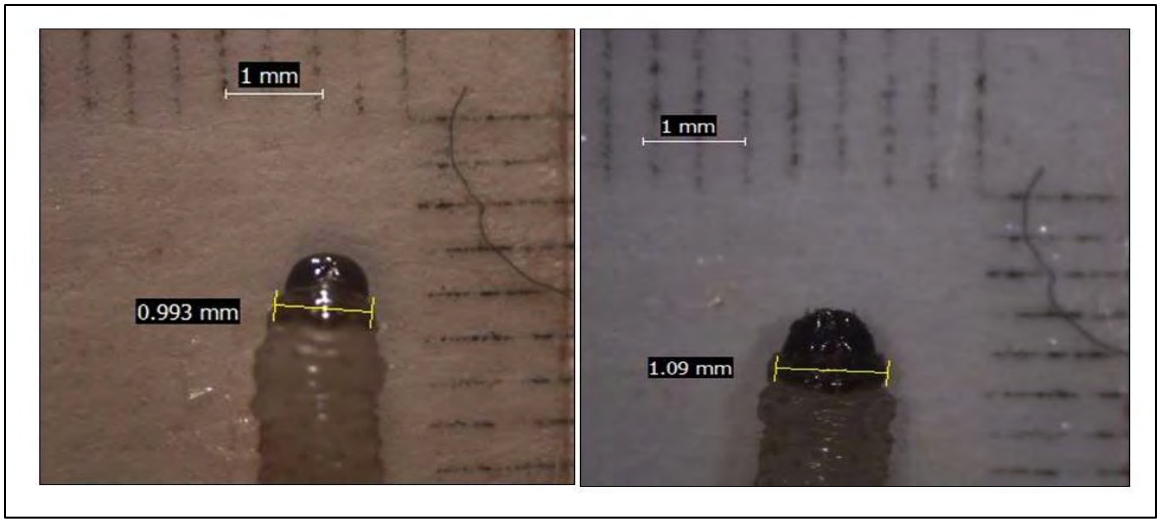

Figure 1.3: Measuring the width of the head capsule of two fourth instar *T. leucotreta*, 9 day old from the same batch reared at ±25˚C (Photo: P. Iita).

Hofmeyr et. al (2016b) sought to improve Daiber's head capsule range, as Daiber leaves gaps between instars. Moreover, temperature and food quality were found to greatly influence the duration of larval instars. At 25˚C the duration of the larval stage reared on artificial diet was found to be 11.6 days (Daiber, 1979b), at which time in nature the fruit quality would have deteriorated.

1.1.2.3. The pupal stage

Daiber (1979c) described the pupal stage of *T. leucotreta*, at which fifth instars form a cocoon from soil particles and silky body substances. The larva undergoes a prepupal stage and subsequently transforms into a pupa. The duration of the pupal stage observed, until eclosion of moths was about 45, 20 and 14 days at 15, 20 and 25°C, respectively (Daiber, 1979c).

1.1.2.4. The adult stage

Daiber (1980) recorded the life span and egg-laying of *T. leucotreta* at constant temperatures of 10, 15, 20 and 25°C. It was established that both male and female moths lived the longest at 15°C, whereas most of the oviposition occurred at 25°C and this increased.

1.2. Control options for *Thaumatotibia leucotreta*

Various control measures (e.g. mating disruption, attract and kill, sterile insect technique, cultural practices, and biological control) are being used in combination, along with chemical insecticides to reduce *T. leucotreta* infestations in citrus orchards. This strategy is part of an IPM programme which aims to combine available control measures into a single strategic arsenal to combat *T. leucotreta* efficiently, while also counteracting some of the problems of chemical pesticide dependency. Efficacy achieved in IPM equates to the sum of the efficacy of all control measures used, thus when measures are combined through the course of one season, over 95% reduction in *T. leucotreta* infestation may be achieved (Moore & Hattingh, 2012).

1.2.1. Chemical control

Chemical control for *T. leucotreta* has been effective against citrus loss in southern Africa. Some of the available chemicals for *T. leucotreta* control, in a review by Moore & Hattingh (2012) and Moore (2019) include synthetic pyrethroids (e.g. Cypermethrin and Fenpropathrin), Triflumuron (Alsystin), Teflubenzuron (Nomolt), Spinetoram (Delegate) and Rynaxypyr (Coragen). However, the use of chemical insecticides has been raising serious concerns about ecological, animal and human health risks arising from chemical residue exposure. Moreover, traces of chemical residues on fruit post-application are unacceptable to citrus markets (Moore, 2002;

Moore, et al., 2004; Malan, et al., 2018). The development of pest resistance to chemical pesticides has also become a major concern. For example, *T. leucotreta* populations have been reported to have developed resistance to the chemical pesticide, triflumuron (Alsystin) (Hofmeyr & Pringle, 1998). Consequently, several non-chemical based methods for *T. leucotreta* control are employed (and have been registered where necessary) in South Africa as alternatives in an IPM programme.

1.2.2. Cultural practices

Orchard sanitation is the main cultural practice employed. The aim is to regularly remove and destroy fallen fruit and hanging fruit that is suspected of *T. leucotreta* infestation. According to Moore & Kirkman (2008), a solid foundation of good orchard sanitation has the ability to improve the efficacy of all treatments directed against *T. leucotreta*. Thus, orchard sanitation forms the basis for all *T. leucotreta* control strategies and remains a highly effective pest and disease control option in citrus orchards (Moore & Hattingh, 2012).

1.2.3. Mating disruption

Mating disruption is another control option that is based on insect pest behavioural control, using synthesised female pheromones to confuse male moths so that they cannot find mates, which ultimately leads to *T. leucotreta* population suppression (Moore, 2002; Malan, et al., 2018). Currently, four products based on this technology, namely Checkmate FCM-F, Isomate FCM, Splat-FCM and X-Mate FCM have been registered in South Africa, and provide efficient control for *T. leucotreta* (Moore & Hattingh, 2012; Moore, 2019; 2021).

1.2.4. Attract and kill

Attract and kill is a similar technique to mating disruption, which uses a combination of female-pheromone along with synthetic a pyrethroid to attract and kill male moths (Malan, et al., 2018), thus suppressing *T. leucotreta* population growth. According to Moore & Hattingh (2012), one product based on this technique (Last Call FCM (Insect Science, South Africa)) is on the market for the control of *T. leucotreta* on citrus in southern Africa, and provides efficient control in low-pressure *T. leucotreta* regions.

1.2.5. Sterile insect technique (SIT)

Sterile insect technique (SIT) uses gamma radiation to sterilise mass-reared male moths, which are then released into an orchard to colonise the environment (Hofmeyr, et al., 2005; Carpenter, et al., 2007; Moore & Hattingh, 2012; Malan, et al., 2018). This provides the opportunity for female moths to mate with released sterile males, which then leads to infertile egg-laying, and eventually population growth suppression. However, this technique works best for area-wide control of *T. leucotreta* and has been effective both experimentally and commercially (Barnes, et al., 2015; Hofmeyr, 2016a).

1.2.6. Biological control

Biological control is a key component of IPM systems, aiming to reduce chemical pesticide usage and prevent insecticide-resistant pests (Bale, et al., 2008; Haase, et al., 2015). Several insect pathogens have been developed for use in the biological control of *T. leucotreta* in South Africa, particularly, parasitoids, entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) (Malan, et al., 2011; 2018; Coombes, et al., 2016; Hatting, et al., 2019; Moore,

2021). The most considered biological agents for efficient *T. leucotreta* suppression are the egg and larval parasitoids *Trichogrammatoidea cryptophlebiae* (Nagaraja) and *Agathis bishopi* (Nixon), which occur naturally in citrus-producing regions of South Africa (Malan, et al., 2018; Moore, 2019). Moreover, Malan et al. (2011) were the first to show the potential use of EPNs (belonging to species in Steinernematidae and Heterorhabditidae families) for *T. leucotreta* control in South Africa. These agents were found to be highly virulent against the soil-borne life stages of *T. leucotreta*, which includes the last instar larvae, pupae and emerging moths. It was also shown that emerging moths infected with nematodes could aid in pest control as well as pathogen dispersal. The use of EPNs in biocontrol is of great benefit due to their ability to reach insects in cryptic habitats, their high reproductive cycle and ease of mass production, as well as their safety in the environment (Gaugler, 2002; Georgis, et al., 2006).

The pathogenicity of several EPF isolates (e.g. *Beauveria bassiana* and *Metarhizium* species) against *T. leucotreta* has also been investigated (Goble, et al., 2011; Coombes, et al., 2013, 2016). These EPFs were found to be highly virulent against *T. leucotreta* fifth instars, both in the laboratory and the field, hence their potential to be formulated into a commercially viable mycopesticide. Like EPNs, the use of EPFs also has advantages in pest control, including their ability to leave no traces of toxic fungal residues on crops post-application, and posing no (or minimal) environmental risks (Goble, et al., 2011). Another major components of biological control used as part of an IPM programme in South Africa is baculoviruses (Moore, et al., 2004; Moore & Hattingh, 2012; Knox et al., 2015; Hatting, et al., 2018, 2019) which are dealt with in the following sections.

1.3. Baculoviruses and their role as biocontrol agents

Baculoviruses are DNA viruses with circular, double-stranded DNA genomes ranging from 80000 to 180000 base pairs (bp) in size (Slack & Arif, 2007; Rohrmann, 2013). The *Baculoviridae* family comprises four genera which are: *Alphabaculovirus* (lepidopteran-specific NPV), *Betabaculovirus* (lepidopteran-specific GV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV) (Jehle, et al., 2006). Nucleopolyhedroviruses (NPVs) have multiple virions occluded within single intranuclear crystals, while granuloviruses (GVs) have only a single virion within cytoplasmic crystals (Rohrmann, 1986, 2013) (Figure 1.4).

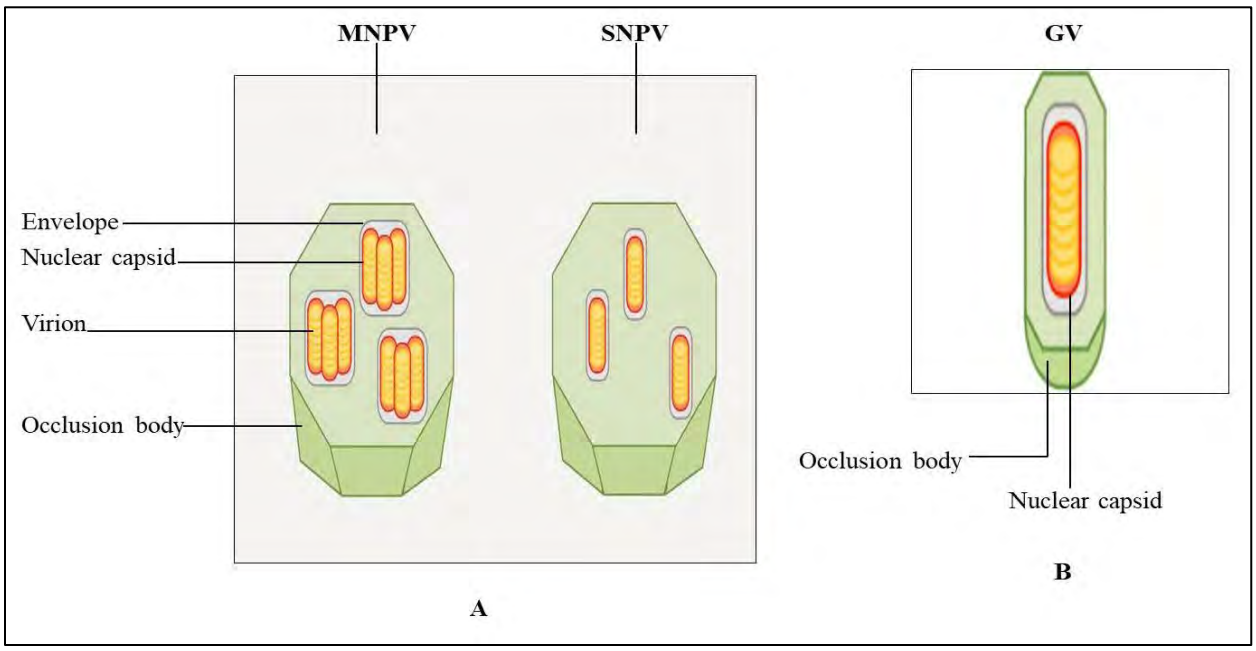

Figure 1.4: Diagram of baculovirus morphology, with A) representing the nucleopolyhedroviruses (NPVs) (NPV with single virions are termed SNPV, whereas those with multiple virions are termed MNPV), and B) the granuloviruses (GV) (Modified from Lee, et al., 2017).

Baculoviruses are characterised by occlusion bodies (OBs). These are proteinaceous structures, either polyhedrin (for NPV) or granulin (for GV) within an envelope which serves to stabilise the virus for many years in the environment, and contain infective virus particles (virions) (Rohrmann, 1986; 2013). The OBs act as the basic unit of infection for baculoviruses and are critical for spreading infection between hosts (Cory & Myers, 2003).

1.3.1. Baculovirus life cycle and infection process

Baculoviruses are known to cause lethal infections in insects, particularly the larval feeding stages of lepidopteran insects (Cory & Myers, 2003; Hitchman, et al., 2007). The replication cycle of lepidopteran-specific baculoviruses is characterised by two progeny virions, the budded virus (BV) and the occlusion-derived virus (ODV), embedded in OBs (Slack & Arif, 2007; Rohrmann, 2013; Hou, et al., 2016). Briefly, the primary infection by baculoviruses occurs when the susceptible insect host larva consumes OBs (polyhedra or granulin) in the environment while feeding (Figure 1.5A) (Harrison & Hoover, 2012). Ingested OBs then enter the alkaline midgut where they dissolve, releasing ODV which then infect midgut epithelial cells (Figure 1.5B). Subsequently, the virions bud out of the cells to form BVs and initiate a systemic infection (secondary infection). The virus spreads throughout the insect body, leading to insect behavioural change (e.g. climbing to the top of the plant), and subsequently death (Figure 1.5C). By the time the host dies new OBs have been formed and liberated over the host plant, thus serving as an inoculum for horizontal transmission to new insects (Figure 1.5D) (Burden, et al., 2003; Kroemer, et al., 2015). This exclusive life cycle and mode of virus transmission allows these viruses to be exploited for pest control. They have been isolated exclusively from insects and the application of molecular biology to their study made them popular research tools

(Harrison & Hoover, 2012). They are well known for biological control of pests in agriculture and forestry, as well as gene expression systems in biotechnology (Szewczyk, et al., 2011).

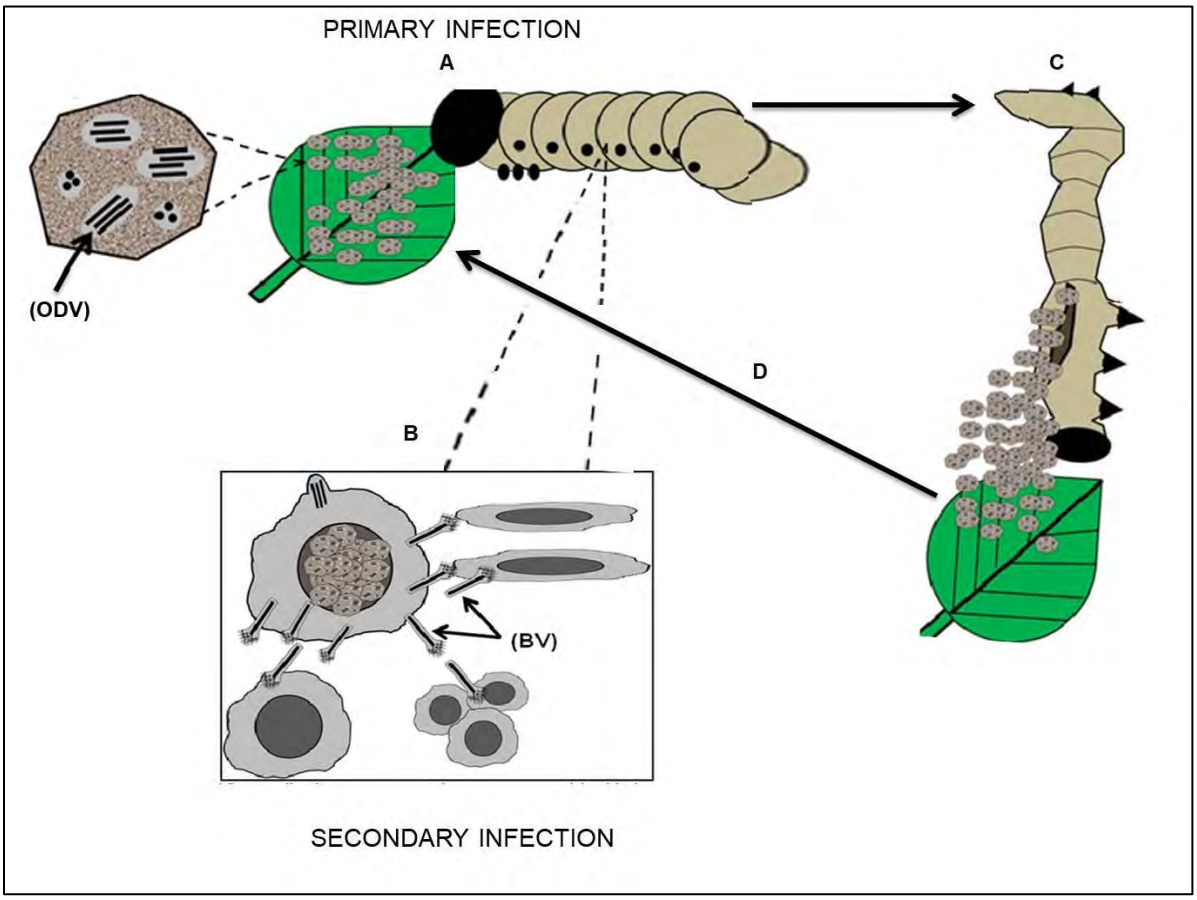

Figure 1.5: A representation of baculovirus primary and secondary infection process, and the pattern of virus transmission (Modified from Kroemer, et al., 2015).

1.3.2. Baculoviruses as biocontrol agents

Baculoviruses are popular components for integrated pest management (IPM) programmes for the control of crop pests in the field (Moscardi, 1999; Bale, et al., 2008; Haase, et al., 2015; Knox, et al., 2015; Hatting, et al., 2018, 2019). The use of baculovirus based pesticides in IPM are to a very great extent environmentally advantageous, in contrast to chemical pesticides due to host specificity, since they only target specific populations of insect (e.g. arthropods) (Cory & Bishop, 1997; Thiem, 1997). Their virulence and capability for causing epizootics also gives them great versatility for insect control by a variety of approaches in the field (e.g. sprays, dusts, spot introductions, host-insect auto-dissemination) (Fuxa, 1991). Although chemical pesticides are extensively used, baculoviruses are potentially alternatives to counter arising challenges of chemical pesticides in the pest management system. The comparative advantage of chemical pesticides is that control is fast, and in the absence of pest resistance, levels of mortality are normally guaranteed, high and predictable (Bale, et al., 2008).

The successful employment of baculoviruses for protection of citrus crops against *T. leucotreta* is exemplified by three biopesticides currently registered in South Africa, namely Cryptogran® (River Bioscience, South Africa), Cryptex®, and Gratham® (both Andermatt-Biocontrol AG, Switzerland) (Knox, et al., 2015, Hatting, et al., 2018, Moore, 2019; 2021). All three products contain Cryptophlebia leucotreta granulovirus (CrleGV-SA) as their active component (Moore, et al., 2011). Although CrleGV-SA has been successful in citrus orchards, a recently characterised insect virus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) has also shown efficacy against *T. leucotreta* in the laboratory (Marsberg, 2016).

1.3.3. The isolation and characterisation of CrpeNPV

Recently, a novel insect virus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) was isolated from a laboratory colony of *Cryptophlebia peltastica* (litchi moth) (Marsberg, et al., 2018). Morphological characterisation and analysis of OBs by transmission electron microscopy (TEM) indicated a SNPV varying in size from 421 to 1263 nm. Genetic characterisation of the isolate involving single restriction endonuclease (REN) profiling and whole genome sequencing revealed that the virus was indeed novel. The NPV genome was found to be 115 728 bp long and with a GC content of 37.2%. A total of 126 open reading frames (ORFs) were also identified in the virus genome. Moreover, the NPV was shown to be highly virulent against *C. peltastica*, *T. leucotreta* and *Cydia pomonella* (Marsberg, et al., 2016; 2018; Wennmann, et al., 2019) indicating a broad host range. These findings are of practical importance, suggesting that CrpeNPV could be a potential biocontrol agent against a range of pests and provide the basis for further research and development. The identification of CrpeNPV offers a unique opportunity for a new biopesticide that is more virulent than CrleGV-SA and has a broader host range. The novel NPV could also possibly represent a resistance management tool for *T. leucotreta*, as previously shown for Cydia pomonella granulovirus (CpGV) resistant codling moth (Wennmann, et al., 2019).

1.4. Challenges of baculovirus application for pest control

Despite the attractive nature of baculoviruses in pest control, they have challenges limiting their application as biocontrol agents. These include insect-specific resistance development, slow speed of kill and restricted host range (Fuxa, 1991; Moscardi, 1999; Knox, et al., 2015). Because of these drawbacks, the economic potential of baculoviruses as effective biological control

agents is at risk, and the need for continued research and development is endless. Some of the potential drawbacks of baculoviruses in pest control are described below.

1.4.1. Resistance

Much has been learned about the evolution of pest resistance to a baculovirus based pesticide in the field since it was reported in Europe where codling moth, *C. pomonella*, developed resistance to a commercial product containing the Mexican isolate of the Cydia pomonella granulovirus (CpGV-M) (Asser-Kaiser, et al., 2007; Berling, et al., 2009). However, genetic analysis and infectivity assays using a range of naturally occurring field isolates subsequently showed that some isolates other than CpGV-M were able to overcome at least one type of the resistances in the host. It was also shown that these isolates shared only a single common difference from that of CpGV-M, which is a mutation in the *p38* gene (Gebhardt, et al., 2014). For the past decade of application, CrleGV-SA has been very successful as a component of IPM on citrus in South Africa. It is not yet clear what the risk is of resistance development in *T. leucotreta* to either CrleGV-SA, which is regularly used in the field or to the novel CrpeNPV, which has been shown to be effective against *T. leucotreta.* However, the impact of other control options on *T. leucotreta*, along with some of the increased variability of factors (e.g. viral genetic variability, pest behaviour, dosage and duration of exposure) may provide an opportunity for reduced risk of resistance (Moore & Jukes, 2020).

1.4.2. Slow speed of kill

Speed of kill, defined as the time it takes the pathogen to replicate and kill its hosts from the period of infection, is an important characteristic in the use of baculoviruses for pest control.

According to numerous reviews (e.g. Fuxa, 1991; Moscardi, 1999; Bale, et al., 2008; Knox, et al., 2015) the slow suppression of pest populations by a pest control agent is the main limitation of biocontrol. Essentially, pathogen-infected insects may take several days or weeks to die (Bonning & Hammock, 1996; Cory & Franklin, 2012). For example, in dose-response bioassays, it took an average of 4 days and 22 hours for 50% of *T. leucotreta* neonates to succumb to CrleGV (Moore, et al., 2011). Such delays in killing the infected insects result in crop damage, relative to fast-killing chemical pesticides when used. This can potentially limit industry interest and acceptance of baculovirus based pesticides by the farmers.

1.4.3. Restricted host range

The host range of any virus is characterised by its ability to enter the cells and tissues of a host organism, replicate and release new infectious virus particles (Thiem, 1997). Most baculoviruses, particularly GV, have a narrow host range which is restricted to a few closely related species. NPVs, on the other hand, can have the ability to infect more than one species within a genus or family. Among well-known baculoviruses with a broader host range are Autographa californica NPV (AcNPV), Panolis flammea NPV (PaflNPV), Mamestra brassicae MNPV (MbNPV) and Helicoverpa armigera MNPV (HearMNPV) (Allaway & Payne, 1984; Doyle, et al.,1990; Maeda, et al., 1993; Hitchman, et al., 2007). CrleGV is only known to infect its ancestral host, *T. leucotreta.* But, Cydia pomonella granulovirus (CpGV) and the novel CrpeNPV whose ancestral host is not *T. leucotreta* can infect and kill *T. leucotreta* as well (Chambers, 2014; Marsberg, 2016). A narrow host range is an attractive quality for baculoviruses, as it means there is no chance of unwanted non-target effects, as is the case with chemical insecticides. On the other hand, a narrow host range could make baculoviruses unattractive for use in pest control because

additional resources would be required to sufficiently suppress pest populations in situations where insect pest complexes may be found on a particular crop (Thiem, 1997). Due to these challenges of baculovirus application for pest control, the following strategies have been proven to improve baculovirus efficacy in the field.

1.5. Strategies for improving baculovirus efficacy in the field

There is increasing demand for safer universal pesticides and chemical residue-free food and water. In addition to arising challenges of chemical pesticides, such as the increasing build-up of insect resistance to insecticides and elimination or restriction of various insecticides in South Africa, is increasing industry interest to adopt baculoviruses as pesticides (Moscardi, 1999; Hatting, 2018). As such, drawbacks of baculovirus application in pest control have provided exciting research opportunities, for example, bioprospecting for viruses with an enhanced speed of kill or broader host range. Other options, including baculovirus genotype isolation and mixed infections, and repeated virus passage (or serial passage), can potentially lead to improved virus efficacy in the field (Arrizubieta, et al., 2015; Graillot, et al., 2016).

1.5.1. Bioprospecting

The management of *T. leucotreta* cost the citrus industry substantial amount of money. However, as the world strives to become more environmentally responsible, and demands pesticide-free food and water, managing *T. leucotreta* exclusively with synthetic chemical pesticides becomes an unacceptable option. Added to that is an increasing need for novel baculovirus isolates to supplement CrleGV-SA, which is regularly used in the field. As such, bioprospecting effort of Opoku-Debrah et al. (2013) led to successful isolation of five novel variants of CrleGV, and all

being sufficiently virulent for use against *T. leucotreta*. In addition to the discovery of CrpeNPV, bioprospecting really offers a window of opportunity for unique isolates that could be potential biocontrol agents for *T. leucotreta* and other agricultural pests in South Africa.

1.5.2. Mixed infections

Several studies have shown that baculovirus genotypes can be isolated and their mixtures lead to improved efficacy (e.g. increased speed of kill) or have synergistic effects compared to a single virus in isolation (Arrizubieta, et al., 2015; Biedma, et al., 2015). Given the high level of genetic diversity in these systems, novel isolates may be generated through recombination in mixed infections (Cory & Franklin, 2012). Jukes (2018) undertook an investigation for improved baculovirus efficacy by mixed infections in *T. leucotreta*. For this, CrpeNPV and CrleGV isolates were mixed in various proportions and their synergism resulted in improved virulence against *T. leucotreta* in the laboratory. Essentially, baculovirus mixtures, as opposed to individual virus species or isolates, could be considered as an active ingredient in future biopesticides to provide improved control for *T. leucotreta* in citrus orchards.

1.5.3. Serial passaging of a virus through a heterologous insect host

The serial passaging technique has been long harnessed to select for several baculovirus variants with an enhanced level of insecticidal efficacy against specific insect pests (Pavan, et al., 1981; Stairs, et al., 1981; Tompkins, et al., 1981; Kolodny-Hirsch & Van Beek, 1997; Graillot, et al., 2016). Attention has been on passaging baculovirus isolates through heterologous insect hosts (or alternate hosts) as a method for selecting a more efficient virus strain for pest control. Some

of the viruses known to have improved efficacy against specific insect pests after several passages through heterologous insect hosts are presented in Table 1.2.

Table 1.2: Examples of baculoviruses shown to have improved efficacy to specific insect species after several passages through a heterologous host.

Virus	Passages	Host	Target	Reference
CpGV	12	*G. molesta*	*G. molesta*	Graillot et al. (2016)
AcMNPV	20	*P. xylostella*	*P. xylostella*	Kolodny-Hirsch & Van Beek (1997)
AgNPV	10	*P. includes*	*A. gemmatalis*	Pavan et al. (1981)
CfNPV	Several	*G. mellonella*	*G. mellonella*	Stairs et al. (1981)
HearMNPV	Several	*T. ni, S. exigua*	*T. ni, S. exigua*	Tompkins et al. (1988)

In each case presented in Table 1.2, serial passaging resulted in significantly increased pathogenicity to the host and in most cases efficacy of the passaged virus to the homologous host was retained (e.g. Stairs, et al., 1981; Kolodny-Hirsch & Van Beek, 1997; Graillot, et al., 2016), which is useful from a commercial production perspective (Graillot, et al., 2016). Since *T. leucotreta* is the heterologous host of CrpeNPV, it is possible that serial passaging of this virus a number of times through it could also lead to enhanced efficacy against the pest in the field.

1.6. Motivation

Thaumatotibia leucotreta is a major threat to citrus production in South Africa with considerable economic impact and its control is therefore imperative. The primary control strategy for *T. leucotreta* involves a rigorous IPM programme that combines chemical, cultural, biological and

other control measures into a single strategic arsenal to sufficiently suppress this pest to the required degree. Biological control, particularly the use of CrleGV-SA, is a key component of IPM in citrus orchards and it has been very effective at reducing *T. leucotreta* populations in the field for almost two decades. Given the typical limitations of baculovirus application for pest control (e.g. insect-specific resistance, restricted host-range and slow speed of kill) there is need for continued improvement to the efficiency of IPM programmes in order to maintain the integrity of *T. leucotreta* control for sustainable citrus production. As such, a recent bioprospecting effort unearthed a novel baculovirus isolate, CrpeNPV, which was shown to infect and kill *T. leucotreta* larvae in the laboratory. The discovery of CrpeNPV offered the opportunity for improvement in *T. leucotreta* management. However, there is evidence that serial passaging of a baculovirus through a heterologous insect host can lead to improved virulence. Since *T. leucotreta* is the heterologous host of CrpeNPV (the homologous host being *C. peltastica*), it now offers a window of opportunity to investigate the potential for improved virulence or speed of kill against it in the laboratory. Improved efficacy of CrpeNPV to *T. leucotreta* has positive implications in future formulations of additional biocontrol agents in the citrus industry. This also offers a potential interchangeable alternative to CrleGV-SA which is regularly used in the field, should signs of resistance in *T. leucotreta* to this virus be observed in the future.

1.7. Research aims and Objectives

The overall aim of this study was to conduct serial passaging of CrpeNPV through a heterologous host, *T. leucotreta*, in order to determine the potential for improved virulence or speed of kill against it. The specific objectives were three-fold: (1) to conduct serial passaging

assays of CrpeNPV through *T. leucotreta* larvae, (2) to analyse the genome of the virus recovered after serial passage to determine potential changes in the genetic composition of the virus due to serial passaging, and (3) to evaluate the biological activity of the virus harvested after serial passage against *T. leucotreta* in laboratory bioassays, in order to determine whether virulence or speed of kill of the virus had changed

1.8. Overview of chapters

Chapter 2 evaluates the biological activity of CrpeNPV against second instar *T. leucotreta* in surface dose-response bioassays in preparation for serial passage experiments. It also describes the rearing of *T. leucotreta* larvae in the laboratory and confirms the integrity of the NPV before use by TEM and sequencing of the *polh/gran* gene region.

Chapter 3 performs serial passaging assays of CrpeNPV through *T. leucotreta*, the heterologous host to select for a variant of the virus with potentially improved virulence or speed of kill against the pest. Serial passage experiments were performed in surface dose bioassays using third or fourth instar *T. leucotreta* as hosts. The full genome of the passaged virus is sequenced in this chapter by next generation sequencing (NGS) and compared to the original virus to determine potential genetic changes due to serial passage through a heterologous host.

Chapter 4 evaluates the biological activity of CrpeNPV against neonate *T. leucotreta* following serial passage through the heterologous host. The results were compared to that of the original virus before passage to determine whether virulence or speed of kill of the virus had changed.

Lastly, **Chapter 5** is a general discussion of the results obtained from the previous chapters, with emphasis on the biological activity of the potential variant recovered after serial passage, along

with genome analysis of the virus. A discussion is also given for future work and recommendations for improving the results obtained in this study.

CHAPTER 2

Biological activity of CrpeNPV against *Thaumatotibia leucotreta* second instars

2.1. Introduction

The CrpeNPV was identified in diseased litchi moth larvae by Marsberg et al. (2018). Subsequent genetic characterisation revealed that it is a novel baculovirus with biological activity against not only its homologous host (litchi moth), but also against *T. leucotreta* (the heterologous host) (Marsberg, 2016; Marsberg, et al., 2018). This opened a window of opportunity for continued investigation into CrpeNPV as a potential biocontrol agent for *T. leucotreta* management in the field. Of interest to this study is the observation that CrpeNPV was more virulent against *T. leucotreta* than the homologous virus, CrleGV. Since there is evidence that virulence of a baculovirus can be improved by serial passage through a heterologous host (Tompkins, et al., 1988; Kolodny-Hirsch & Van Beek, 1997; Graillot, et al., 2016), the overall aim of this study was then to determine if this is true of CrpeNPV by passaging it a number of times through *T. leucotreta* larvae in laboratory bioassays, which is the subject of Chapter 3.

Before conducting serial passage assays, it is necessary to evaluate the base line biological activity of CrpeNPV against *T. leucotreta* through the use of bioassays. The base line bioassays were conducted to determine a dose-response relationship between the virus and the host, thereby enabling selection of a suitable dose for serial passage experiments. Bioassays are useful

tools in dose-mortality studies of baculoviruses that help to determine suitable application for pest control by giving an evaluation of the virus-host relationship, measured by mortality and the speed of kill (Cory & Bishop, 1997; van Beek & Hughes, 1998). Several bioassays have been developed (e.g. droplet feeding and surface dosage bioassays) (Hughes & Wood, 1986; Cory & Bishop, 1997; Jones, 2000; Sparks, et al., 2008). Surface dose bioassays are suitable for insect larvae that feed on the surface of the diet which has been contaminated with a known concentration of the virus, whereas droplet feeding bioassays allow larvae to consume a virus suspension containing a coloured dye which becomes easy to see in the insect's gut after ingestion through fluorescence spectroscopy and it is easy to know how much virus was ingested (Cory & Bishop, 1997; Jones, 2000; Pereira-da-Conceicoa, et al., 2012). By using these techniques, dose-response can be measured and quantified by estimating the amount of virus that would take to kill 50% of host population tested (LC_{50}) or the dose at which 50% of the host population exposed to the virus would die (LD_{50}) (Kooijman, 1981; Hughes & Wood, 1981; Cory & Bishop, 1997; Thomas & Elkinton, 2004). Of interest to this study is the estimation of a concentration resulting in 90% mortality (LC_{90}) for second instar *T. leucotreta*. These instars were selected in order to evaluate a suitable dose that could be used as inoculum for serial passage experiments and a concentration that is high enough to allow high OB yield in third or fourth instar *T. leucotreta* which were used as hosts for the serial passage assays (Chapter 3).

The overall aim of this chapter was first to confirm the integrity of the NPV by TEM and sequencing of the baculovirus *polh/gran* gene region, and then to evaluate the biological activity of the NPV against second instar *T. leucotreta*, by determining LC_{50} and LC_{90} using surface dose-response bioassays in preparation for serial passage experiments. The specific objectives were four-fold: (1) to rear *T. leucotreta* larvae in the laboratory for use in bioassays and identify

second instars by measurement of the head capsule width, (2) to prepare and identify the NPV morphologically by TEM, (3) to identify the NPV genetically by sequencing of the baculovirus *polh/gran* gene region to ensure that the virus used in this study is specifically CrpeNPV and (4) to determine the LC_{50} and LC_{90} values of the CrpeNPV against second instar *T. leucotreta*.

2.2. Material and Methods

2.2.1. Rearing of *T. leucotreta* larvae

Insects were provided by the Centre for Biological Control (CBC), Department of Zoology and Entomology, Rhodes University. They were continuously reared on FCM artificial diet described by Moore et al. (2014) in 250 ml honey jars fitted with cotton wool (Figure 2.1).

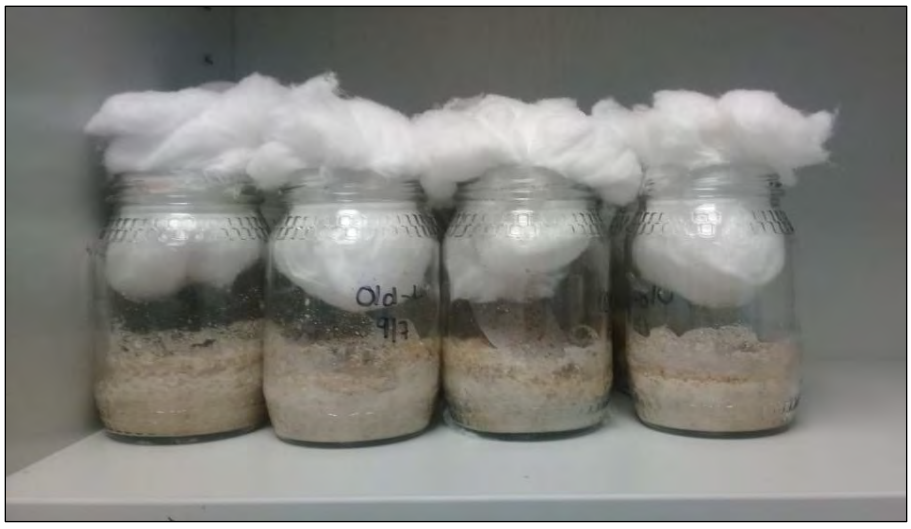

Figure 2.1: A *T. leucotreta* larval culture in artificial diet in 250 ml honey jars, stopped with cotton wool and maintained at ±25°C.

The larval culture was maintained at ±25°C, from which larvae used in this study were taken. Larval instars were identified by closely monitoring the duration of developmental life stages for

each *T. leucotreta* instar as per Daiber (1979b). Thus, for this chapter, seven day old larvae were used in the bioassays. For each assay (three replicates), a sample of six larvae (18 larvae in total from all replicates) was randomly taken from each insect batch used that day, frozen and the head capsule width was measured using Leica EZ4 D Stereo microscope (Leica Microsystems). From these measurements, the average width of all larval samples was determined and hence it was possible to confirm that the larvae used were indeed second instars. The relationship between *T. leucotreta* instars and the width of the head capsule was determined previously by Daiber (1979b) and Hofmeyr et al. (2016b). The particular instar category in table 1.1 in Chapter 1 (section 1.1.2.2) was used as reference to confirm the instar directly using the determined mean width of the head capsule.

2.2.2. Transmission Electron Microscopy (TEM)

The transmission electron microscopy (TEM) protocol was adapted from Abdulkadir et al. (2013) to confirm the morphology of CrpeNPV before use. For this, a droplet of the prepared sample (in ddH_2O) was placed on a Forvar carbon coated grid for 30 seconds. Filter paper was used to drain off the excess sample. A droplet of uranyl acetate was then placed on the grid and left for 20 seconds. Filter paper was used to remove the excess stain and the grid was observed using a Zeiss Libra 120 (Zeiss, Germany) TEM at 80 000 kV. The images were analysed using Mega View (G2) Olympus analysis software (Olympus, Japan).

2.2.3. DNA extraction from purified CrpeNPV OBs

A CTAB DNA extraction protocol was adapted from Aspinall et al. (2002). Using 200 µl of purified OBs in a 1.5 ml microfuge tube, 90 µl of 1M sodium carbonate (Na_2CO_3) was added and

incubated at 37°C for 30 minutes. A volume of 120 µl Tris-HCl (1M, pH6.8), 90 µl of 10% SDS and 25 µl proteinase K (25 mg/ml) were added and samples were incubated for a further 30 minutes at 37°C. A 10 µl aliquot of RNAse A (10mg/ml) was then added and the sample was incubated for another 30 minutes at 37°C. The suspension was then centrifuged at 12100 × *g* for 3 minutes. The supernatant was transferred to a new 1.5 ml tube and the pellet was discarded. A 400 µl aliquot of warm CTAB buffer was then added to the supernatant and the sample was incubated at 70°C for 60 minutes. A 400 µl aliquot of 4°C choloroform was added and centrifuged at 6500 × *g* for 10 minutes. The upper phase was transferred into a new 1.5 ml tube and 400 µl of -25°C isopropanol was added. The sample was left overnight at -25°C. The sample was then centrifuged at 12100 × *g* for 20 minutes and the supernatant discarded. A 1 ml aliquot of cold 70% ethanol was added to the pellet and the sample was centrifuged at 12100 × *g* for 5 minutes. The supernatant was discarded and the pellet was incubated at 50°C, until dry. The pellet was re-suspended in 20 µl of ddH_2O. The genomic DNA (gDNA) was analysed by 0.7% agarose gel electrophoresis (AGE) with ethidium bromide (final concentration of 0.4 µg/ml) and visualised in Chemidoc (Bio Rad, USA), with band size being estimating using a 1 kb gene ruler (Thermo Scientific). The successfully extracted gDNA was then used as template for PCR amplification of the baculovirus *polh/gran* gene region as described below.

2.2.4. PCR amplification of the *polh/gran* gene region and sequencing

In order to confirm that the virus was CrpeNPV, a set of degenerate oligonucleotide primers (prPH-1 and prPH-2) designed by Lange et al. (2004) were used to amplify the baculovirus *polh/gran* gene region using gDNA isolated from the virus OBs (Table 2.1).

Table 2.1: Degenerate primers for the partial amplification of the polh/gran gene region (Lange, et al., 2004).

Primer name	Sequence (5' to 3')
Polh/gran (prPH-1)	TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT
Polh/gran (prPH-2)	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT

*N = C, A, T or G; R = A or G; Y = C or T; D = A, G or T. Underlined nucleotides indicate standard sequencing primers M13 forward and M13 reverse.

A 25 µl PCR reaction was set up containing 12.5 µl Taq (Ampliqon). To this, 1 µl of prPH-1 and prPH-2 oligonucleotides (10µM) were added. About 2 µl of gDNA (50 ng) was added as template, and then topped up to a total volume of 25 µl with ddH_2O. The reaction set up included a negative control, which consists of no template DNA (template DNA replaced by ddH_2O). The negative control was included to show that equipment and reagents functioned optimally. The PCR parameters used were adapted from Lange et al. (2004) (Table 2.2).

Table 2.2: PCR cycles used for the amplification of the *polh/gran* gene region using degenerate primers (Lange, et al., 2004).

Stages	**Temperature** (˚C)	**Duration** (minutes)	**Cycles**
Pre-denaturing	95	03:00	1
Denaturation	95	00:30	
Annealing	50	01:00	36
Elongation	70	01:00	
Final elongation	72	10:00	1

The PCR amplification was performed in a thermocycler (Bio Rad®). The amplicon was analysed by 1% AGE with ethidium bromide (0.4 µg/ml), with band size estimated using a 1 kb gene ruler (NEB®). The successfully amplified product was sequenced by Inqaba Biotechnological Industries (Pty) Ltd. (South Africa) using M13F and M13R primers. The resulting nucleotide sequences were cleaned up in Geneious R10 (Biometters, New Zealand) and aligned to the full sequence of CrpeNPV *polh/gran* gene using ClustalW.

2.2.5. Virus preparation

A 100 µl aliquot of purified CrpeNPV OBs at a concentration of 2.6 x 10^{10} OBs/ml was provided by the Department of Microbiology and Biochemistry, Rhodes University. A dilution series of six concentrations was made from a stock of CrpeNPV OBs in sterile distilled water (ddH_2O) (Figure 2.2).

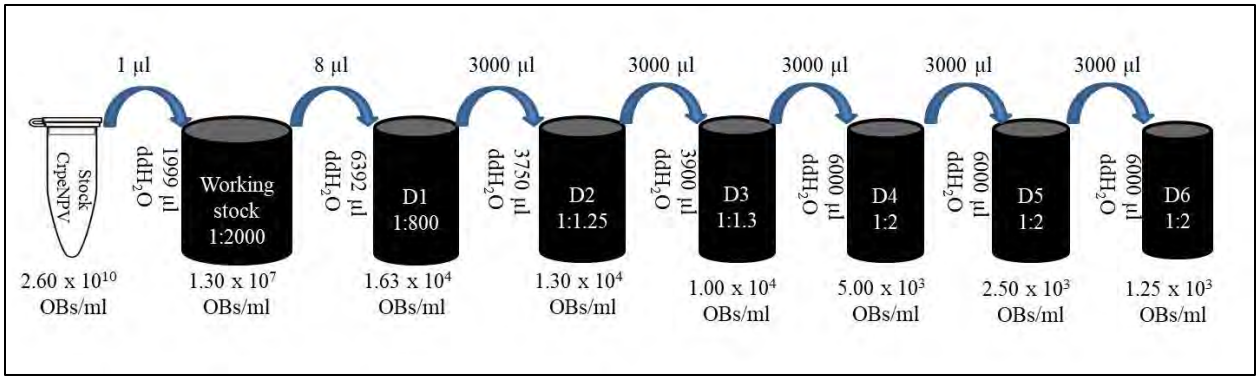

Figure 2.2: A dilution series of CrpeNPV for surface dose-response bioassays against *T. leucotreta* second instars.

The stock virus suspension was diluted 1:2000 to produce a working stock suspension (final concentration of 1.30×10^7 OBs/ml) from which bioassay doses were prepared. The working stock was further diluted 1:800 resulting in dose 1 (D1) at a concentration of 1.63×10^4 OBs/ml. The subsequent dose, D2, was prepared by a 1.25-fold dilution of D1 to a concentration of 1.30×10^4 OBs/ml. The subsequent dose, D3, was serially diluted 1.3-fold to a final concentration of 1.00×10^4 OBs/ml. The subsequent doses, D4 to D6, were each serially diluted 2-fold to final concentrations ranging from 5.00×10^3 to 1.25×10^3 OBs/ml.

2.2.6. Surface dose-response bioassays of CrpeNPV against *T. leucotreta* second instars

Surface dose-response bioassays were conducted in 24 well plates (Fisher Scientific, USA). The diet was prepared by adding 250 g of FCM artificial diet to a Pyrex dish (Moore, et al., 2014). Distilled water (300 ml) was added to the diet and mixed well. The diet was cooked in an oven at 200°C for 30 minutes and placed in the laminar flow cabinet to cool. In the laminar flow cabinet, about 1 g of the diet was cut out using a modified 10 ml syringe and then carefully pressed into each well of 24 well plates. The diet in the wells was properly packed and levelled evenly to

facilitate the flow of liquids. The plates were covered with glass lids immediately after to prevent the diet from desiccation. Bioassays were performed using six concentrations (D1 to D6) of purified OBs of stock CrpeNPV as dosages (Figure 2.2), along with ddH_2O as a control. A volume of 50 µl of each dose or control were pipetted onto the centre of a flat diet surface of each well using a sterile micropipette and spread uniformly over the surface by swirling. Each dose was vortexed several times (at least 10 seconds each time) between inoculations to ensure homogeneity of OBs in the suspension. Inoculated plates were closed immediately with glass lids to prevent the diet from desiccation and left for ±30 minutes, until they had dried. A single healthy second instar larva was carefully placed into each well using a sterile paint brush (R0). Assay plates were double checked (after about 30 minutes) for mobile larvae on the surface of the diet with the aid of a dissecting microscope (BestScope). At the time of inspection, glass lids were kept on to minimise the risk of possible contamination. The wells with larvae dying due to manipulation in the control and treatment were marked and the larvae were replaced with healthy ones prior to incubation, and no additional changes were made thereafter. The plates fitted with glass lids were further sealed with parafilm to prevent larvae from escaping and kept in a CE room at ±26°C and a relative humidity of 60 to 80%. After 7 days, the plates were opened and the diet was inspected with the aid of a dissecting microscope (BestScope) with sub-stage lighting, and larvae were recorded as either dead or live. Larvae that were marked dead and replaced at the onset of the experiment were not recorded. The assay was replicated three times at separate occasions under similar conditions. Dose-response data were subjected to probit analysis in R (version 3.6.1) using the package 'drc' (Ritz, et al., 2015) to determine the mean for all three replicates as LC_{50} and LC_{90}.

2.3. Results

2.3.1. Head capsule measurement

To confirm instars used in the three replicates of the bioassay, head capsule measuring was performed. The typical width of the head capsule of 7 day old larvae used ranged from 0.300 to 0.385 mm (Table 2.3), indicating second instars (Hofmeyr, et al., 2016b).

Table 2.3: Mean width of the head capsule of larvae used in the three replicates of dose-response bioassays (n = 6, total of 18).

Replicates	**1**	**2**	**3**
Mean width (mm)	0.34 ± 0.02	0.33 ± 0.01	0.34 ± 0.02

2.3.2. Transmission Electron Microscopy

Transmission electron microscopy was done to examine the purity and type of virus by morphology before it was used. Morphological observations showed OBs with irregular, spherical shapes and of variable sizes, typical of NPVs (Ishii, et al., 2003) (Figure 2.3). These OBs were also free of debris, indicating purity.

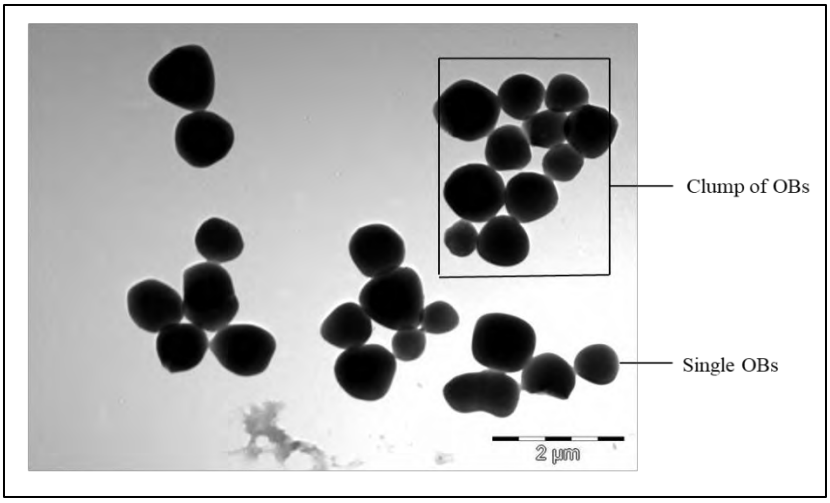

Figure 2.3: Transmission electron micrograph of CrpeNPV OBs.

2.3.3. DNA extraction, PCR amplification of the *polh/gran* gene region and sequencing

Genomic DNA was successfully extracted from purified OBs of stock CrpeNPV. The DNA was analysed by 0.7% AGE and found to be of high molecular weight (>10000 bp) (Figure 2.4).

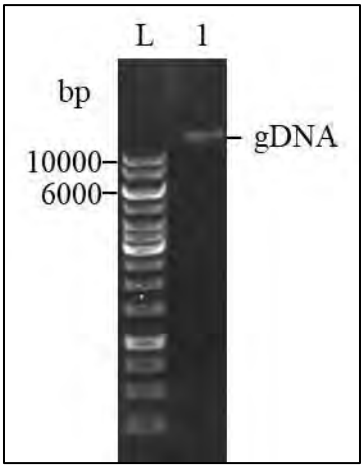

Figure 2.4: A gel image of gDNA extracted from purified OBs analysed by 0.7% AGE. Lane L: 1 kb gene ruler (Thermo Scientific), Lane 1: gDNA.

The PCR analysis of gDNA extracted from purified OBs produced an amplicon of approximately 500 bp, which matched the expected size for the target region in the *polh/gran* gene (Lange, et al., 2004) (Figure 2.5).

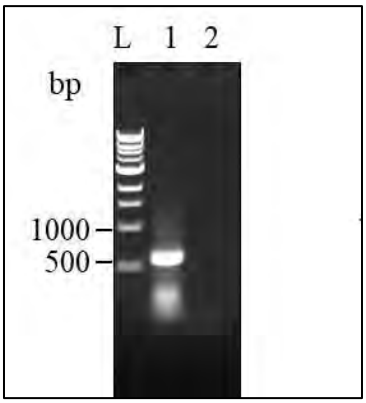

Figure 2.5: PCR amplification of the *polh/gran* gene analysed by 1% AGE. Lane L: 1 kb DNA ladder (NEB®), Lane 1: PCR product, Lane 2: No template control (DNA template replaced by ddH_2O).

To identify the virus genetically, the obtained PCR product was sequenced using two sets of sequencing primers, M13F and M13R. Following sequencing, a pair of sequences was obtained, cleaned up and trimmed in Geneious (version R11) (Biometters Ltd, New Zealand) from 5' to 3' ends to get rid of ambiguous nucleotides. Pairwise alignment of the two sequences using a ClustalW method produced a nucleotide consensus of about 700 bp. Pairwise alignment and BLAST of this consensus with the *polh* gene of CrpeNPV (GeneBank accession: MH394321.1) (Marsberg, et al., 2018) showed a 100% identity (Figure 2.6), confirming that the virus used in this study was indeed CrpeNPV.

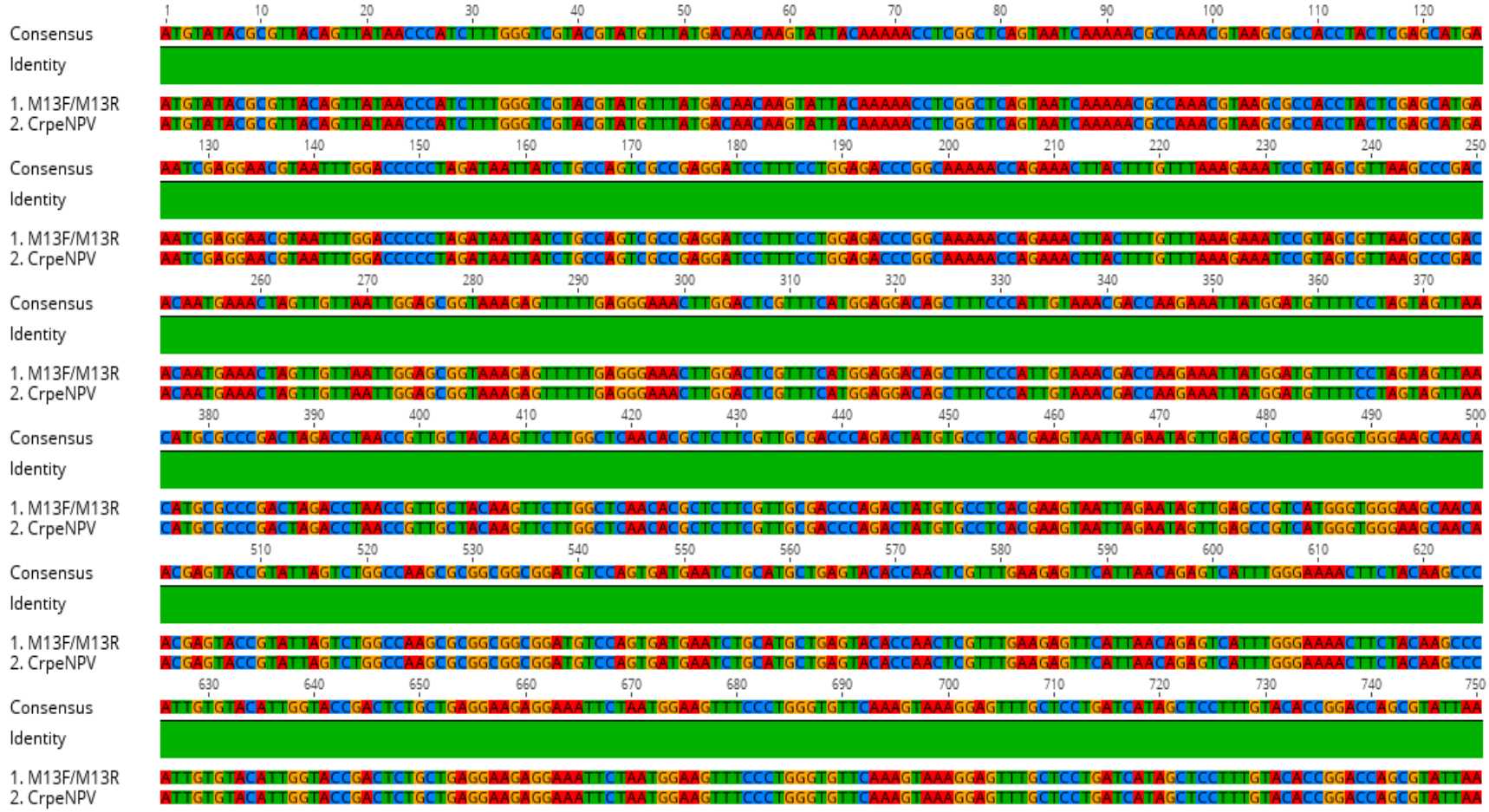

Figure 2.6: Pairwise alignment of the nucleotide sequence of the *polh/gran* gene amplicon to CrpeNPV *polh* gene region, with the green bar showing alignment identity.

2.3.4. Surface dose-response bioassays of CrpeNPV against *T. leucotreta* second instars

Three replicates of the surface-dose response bioassays of CrpeNPV were carried out against second instar *T. leucotreta* to determine the LC_{50} and LC_{90} of the virus. The recorded mortality for second instar *T. leucotreta* from dose-response bioassays with CrpeNPV is shown in Table 2.4 for each dose used.

Table 2.4: Mortality for second instar *T. leucotreta* in dose-response bioassays with six CrpeNPV dosages.

Dosage	**Concentration** (OBs/ml)	**Replicate 1** Mortality (%)	**Replicate 2** Mortality (%)	**Replicate 3** Mortality (%)
Control	0	0	0	0
D6	1.25×10^3	8.33	8.33	4.17
D5	2.50×10^3	20.8	25.0	62.5
D4	5.00×10^3	79.2	41.7	50.0
D3	1.00×10^4	91.7	66.7	54.2
D2	1.30×10^4	91.7	95.8	87.5
D1	1.60×10^4	100	100	91.7

Larval mortality ranged from 8 to 100% and no mortality was recorded in the controls, indicating that experimental conditions were working optimally. The highest concentration (D1) resulted in 100% larval mortality in replicate 1 and 2 and therefore was excluded from statistical analysis. The dose-response curve for this bioassay is shown in Figure 2.7.

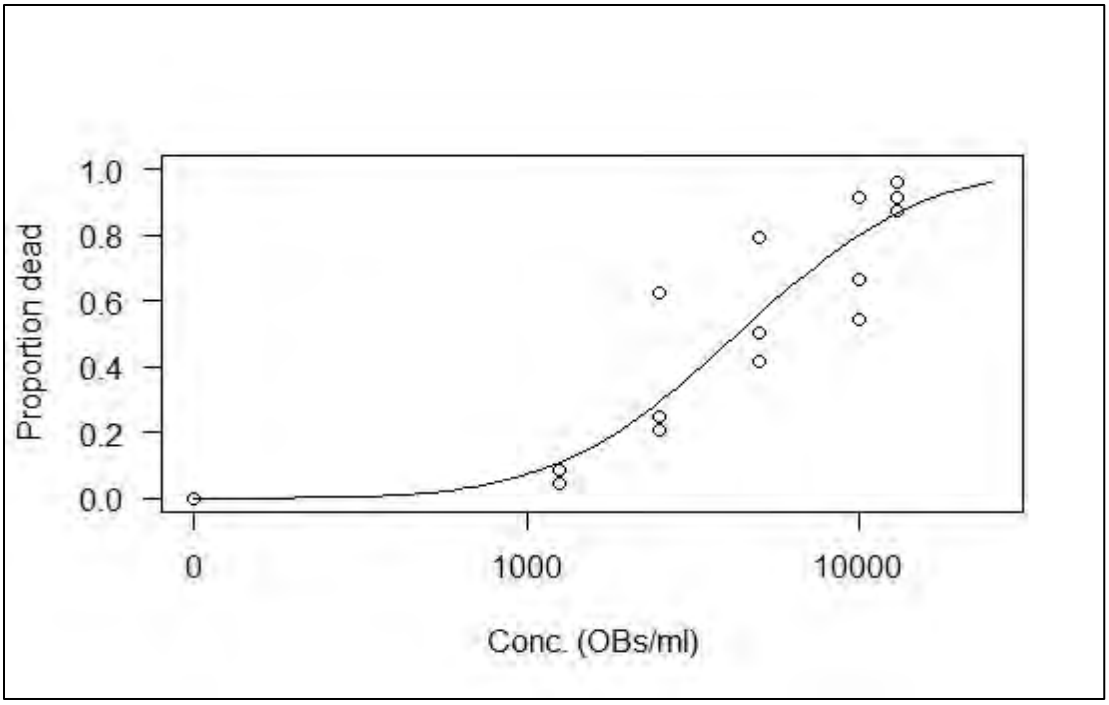

Figure 2.7: Dose-response probit plot for CrpeNPV against second instar *T. leucotreta.*

Probit analysis of data obtained from surface dose-response bioassays of CrpeNPV against *T. leucotreta* second instars indicated an LC_{50} value of 4.33×10^4 OBs/ml and LC_{90} of 1.55×10^4 OBs/ml with 95% confidence interval (Table 2.5).

Table 2.5: The LC_{50} and LC_{90} values of CrpeNPV obtained against second instar *T. leucotreta*.

Lethal concentration	Concentration OBs/ml	Standard Error (SE)	95% Confidence limits	
			Lower SE	Upper SE
LC_{50}	4.33×10^3	326.45	3.69×10^3	4.97×10^3
LC_{90}	1.55×10^4	2118.7	1.14×10^4	1.97×10^4

2.4. Discussion

In order to conduct serial passaging of CrpeNPV through *T. leucotreta* (described in Chapter 3), it was necessary to: (i) rear *T. leucotreta* in the laboratory and identify second instars, (ii) confirm the morphological and genetic identity of the virus to be passaged and, (iii) evaluate the base line biological activity of the virus against second instar *T. leucotreta*, to determine a suitable dose of the virus to be used as inoculum in serial passage experiments (Chapter 3).

With the rearing of *T. leucotreta*, the head capsule width of six larvae (7 day old) was measured per batch, for instar identification. These larvae were taken from each insect batch used that day for each bioassay replicate. As expected, the analysed head capsule width data in this study, matched a second instar category described by Hofmeyr et. al. (2016b), which indicates that the larvae used in this study were second instars.

To confirm the integrity and type of baculovirus in preparation for serial passage, OBs were subjected to TEM for morphological analysis. Results have shown high purity OBs with the same morphology of a NPV OBs first described by Marsberg (2016). The OBs had large,

irregular spherical shapes of variable sizes, and clumped together, which are typical characteristics of NPVs (Ishii, et al., 2003). Moreover, sequencing of the *polh* gene region showed a 100% nucleotide sequence identity to the *polh* gene region in CrpeNPV (GeneBank accession: MH394321.1) (Marsberg, et al., 2018), thus confirming that the virus is indeed CrpeNPV. Having identified second instars and confirmed the identity of the stock virus, the next objective was then to evaluate the biological activity of CrpeNPV, measured by LC_{50} and LC_{90} using a surface dose-response bioassay. Since *T. leucotreta* larvae are internal feeders and first penetrate the fruit surface, this method was the most suitable for this purpose, as it mimics the natural feeding process of the insect (Jones, 2000; Grout & Moore, 2015; Grové, et al., 2015). The doses used in the bioassay were obtained following several preliminary assays (data not shown) under similar conditions using a range of doses, starting with very high concentrations. This was done to assess a concentration resulting in at least 10 to 90% mortality, which is a prerequisite for statistical analysis used in this study. From these preliminary bioassay results, all concentrations exceeding 1.60×10^4 OBs/ml resulted in 100% larval mortality, whereas a poor response was recorded in the lowest dose, below 2.5×10^3 OBs/ml. In order to optimise the dose for the bioassay, OB concentrations were adjusted by dilution with ddH_2O using dilution factors in Figure 2.2. For a possibly fair evaluation of the biological activity of this virus towards second instars in the future, regular dilution rates can be used. Moreover, since this is the first time the biological activity of CrpeNPV against second instar *T. leucotreta* is being investigated, there is no data available for comparison.

Overall, the objectives of this chapter were achieved. *T. leucotreta* larval instars were successfully reared in the laboratory for use in bioassays with CrpeNPV. The virus to be passaged was also confirmed as CrpeNPV and its biological activity against second instar *T.*

leucotreta was evaluated. Finally, the LC_{50} and LC_{90} of the virus towards second instar *T. leucotreta* were estimated at 4.33×10^3 OBs/ml and 1.55×10^4 OBs/ml. This allowed serial passage experiments of CrpeNPV to be conducted using the evaluated LC_{90}. This LC_{90} was considered appropriate to allow high OB yield in third or fourth instar *T. leucotreta*, which were used as hosts in the serial passage experiments (Chapter 3).

CHAPTER 3

Serial passage of CrpeNPV through *Thaumatotibia leucotreta*

3.1. Introduction

In Chapter 2, the biological activity of CrpeNPV was evaluated against second instar *T. leucotreta* in dose-response bioassays to determine the LC_{90} which was found to be 1.55×10^4 OBs/ml. This enabled serial passage of the virus to be conducted using this value as a dose. The dose was selected with a view to allow high OB yield in third or fourth instars, which were selected as hosts for the serial passage experiments.

Serial passage refers to a process of performing a consecutive inoculation series of a virus into new generations of a suitable insect larval host. Typically, the inoculated virus would be recovered from diseased larvae that have been infected at each stage in the assay and used again to re-infect the new batch of insect (Stairs, et al., 1981; Gani, et al., 2014; Graillot, et al., 2016). Serial passage is a useful technique in virology that is commonly used in the development of vaccines, often by attenuation (Locher, et al., 2003; Hunt, 2014). Moreover, serial passage has been shown to improve baculovirus replicative fitness in cell culture (Sohi, et al., 1984) and virion yield per OB in insects (Tompkins, et al., 1981; Kolodny-Hirsch & Van Beek, 1997), which is important in the mass production and pathogenicity of these viruses for pest control. Serial passage of a virus has also been used in laboratory experiments that sought to understand

the development of insect resistance to baculoviruses (Briese & Mende, 1983; Fuxa, et al., 1988; Abot, et al., 1996; Nakai, et al., 2017) and the adaptation of baculoviruses to heterologous hosts for various purposes (Martignoni & Iwai, 1986; Graillot, et al., 2016).

Of relevance to this study is evidence that serial passage of a baculovirus through a heterologous host can lead to increased virus pathogenicity (e.g. virulence or speed of kill) against specific insects. As shown by Pavan & Ribeiro (1989), the serial passaging of Anticarcia gemmatalis NPV (AgNPV) through *Diatraea saccharalis* resulted in selection of a variant of the virus with increased virulence towards the heterologous host larvae. Moreover, in an attempt to select for a more virulent variant of AcMNPV, Kolodny-Hirsch & Van Beek (1997) serially passaged this virus through *Plutella xylostella*. After 20 generations of selection, they measured a 15-fold increase in virulence to *P. xylostella*. Evidence suggests that increased virulence of the passaged AcMNPV was based on changes in variant structure of the virus population, coupled with changes in virus morphology in response to passage pressure. Graillot et al. (2016) also employed serial passaging to select for an improved virulent strain of CpGV-M against *Grapholita molesta,* with pathogenicity of the virus population found to have improved significantly against this heterologous host.

Since *T. leucotreta* is a heterologous host of CrpeNPV, it is possible that serial passaging of this virus through it could also lead to changes in virulence or speed of kill towards it for improved *T. leucotreta* management in the field. However, when serially passaging a baculovirus through a heterologous host it is important to keep in mind possible risk of contamination with the homologous virus as a result of potential covert-overt infections in insects by baculoviruses (Burden, et al., 2003; Williams, et al., 2017). This is imperative as the contaminated virus sample

harvested in the process cannot be used as a dose for subsequent passaging. This observation was made when passaging CrpeNPV through *T. leucotreta* later instars (Jukes, 2018). In this case, CrpeNPV triggered a mixed infection with the homologous virus, CrleGV, and it is likely that this could also happen in this study. To ensure purity of the virus being passaged, Jukes (2018) developed a multiplex PCR (mPCR) method for detection and identification of either CrpeNPV or CrleGV DNA in a purified viral sample using specific oligonucleotides for amplification of the *per os factor* (*pif1*) gene in CrpeNPV and *late expression factor 4* (*lef4*) in CrleGV. Other studies employed REN profiling (Kolodny-Hirsch & Van Beek, 1997) and qPCR (Belda, et al., 2019) to ensure absence of contamination in samples to be used in the serial passage experiments.

Another relevant aspect that gained attention in recent years is the potential effect of serial passage on the genetic composition of the virus. A number of studies, using REN and genome sequencing, found substantial variations in NPV genomes that appeared to be selected during serial passage through heterologous hosts (Kolodny-Hirsch & Van Beek, 1997; Hitchman, et al., 2007; Arrizubieta, et al., 2015). These studies, supported by Erlandson (2009) and Hodgson et al. (2001) suggest that wildtype baculoviruses and in particular NPVs, can exist as multiple genotypes within the isolate. Since CrpeNPV is a wildtype virus, it is also possible that new variants in the genome may be generated over the course of serial passaging, due to possible genetic alterations resulting from potential mutations and recombination events, hence increasing genetic diversity in the viral population (Cory & Franklin 2012; Gilbert, et al., 2016).

The overall aim of this chapter was to conduct serial passaging of CrpeNPV through *T. leucotreta* larvae to determine the potential for improved virulence or speed of kill against this insect, for improved *T. leucotreta* management in the field. The first objective was to conduct serial passage bioassays of CrpeNPV through third or fourth instar *T. leucotreta* using the LC_{90} determined previously as a dose. Secondly, mPCR analysis on gDNA extracted from OBs at each passage was performed to screen for possible CrleGV contamination. Finally, next generation sequencing (NGS) of the full viral genome recovered after serial passage was performed, to detect potential genetic changes in the virus population, particularly single nucleotide polymorphisms (SNPs). The availability of NGS data also allowed further analysis by *in silico* digestion of the viral genome to create REN profiles of the genome using several restriction enzymes.

3.2. Materials and Methods

3.2.1. Head capsule measurement

The larvae used in the bioassays were reared as described previously (Chapter 2, section 2.2.1). To confirm the instar used, six larvae were taken from each batch used that day for each assay, frozen and their head capsule width was measured as described previously (Chapter 2, section 2.2.1).

3.2.2. Serial passage bioassays of CrpeNPV through *T. leucotreta*

In order to select for a variant of CrpeNPV with improved virulence against *T. leucotreta*, a serial passage procedure was adapted from Graillot et al. (2016). The first step before initiation of serial passage bioassays through *T. leucotreta* was evaluating the biological activity of

CrpeNPV against second instar *T. leucotreta* in order to determine a suitable concentration of the virus (LC_{90}) to be used as a dose for the serial passage experiments (Chapter 2). The LC_{50} and LT_{50} of the original virus suspension against neonate *T. leucotreta* were evaluated before passage in order to compare virulence and speed of kill of the virus following passage 12, which is the subject of Chapter 4. The stock suspension was first used to infect the host larvae, hence passage one (P_1). The infected larvae that died first (or diseased larvae collected first) in the assay served as source of OBs for the inoculum to re-infect a new healthy batch of insects in the subsequent passage (P_2) and this process continued consecutively through to P_{12}. The virus was initially passaged through third instar *T. leucotreta* (8 day old) from P_1 to P_2 and later through fourth instars (9 day old) from P_3 to P_{12}. After each passage, an mPCR analysis developed by Jukes (2018) was carried out to screen for possible contamination with CrleGV. Following P_{12}, the recovered virus was stored and evaluated against neonate *T. leucotreta* in bioassays, which is the subject of Chapter 4. This was done in order to determine whether virulence (as LC_{50}) or speed of kill (as LT_{50}) of the virus had changed in relation to the original virus suspension before passage. Genome analysis of the virus recovered after P_{12} was also carried out to determine whether genetic changes in the virus population have occurred in relation to the original virus before passage. The general overview of this study (including Chapter 2 and 4) is illustrated in Figure 3.1.

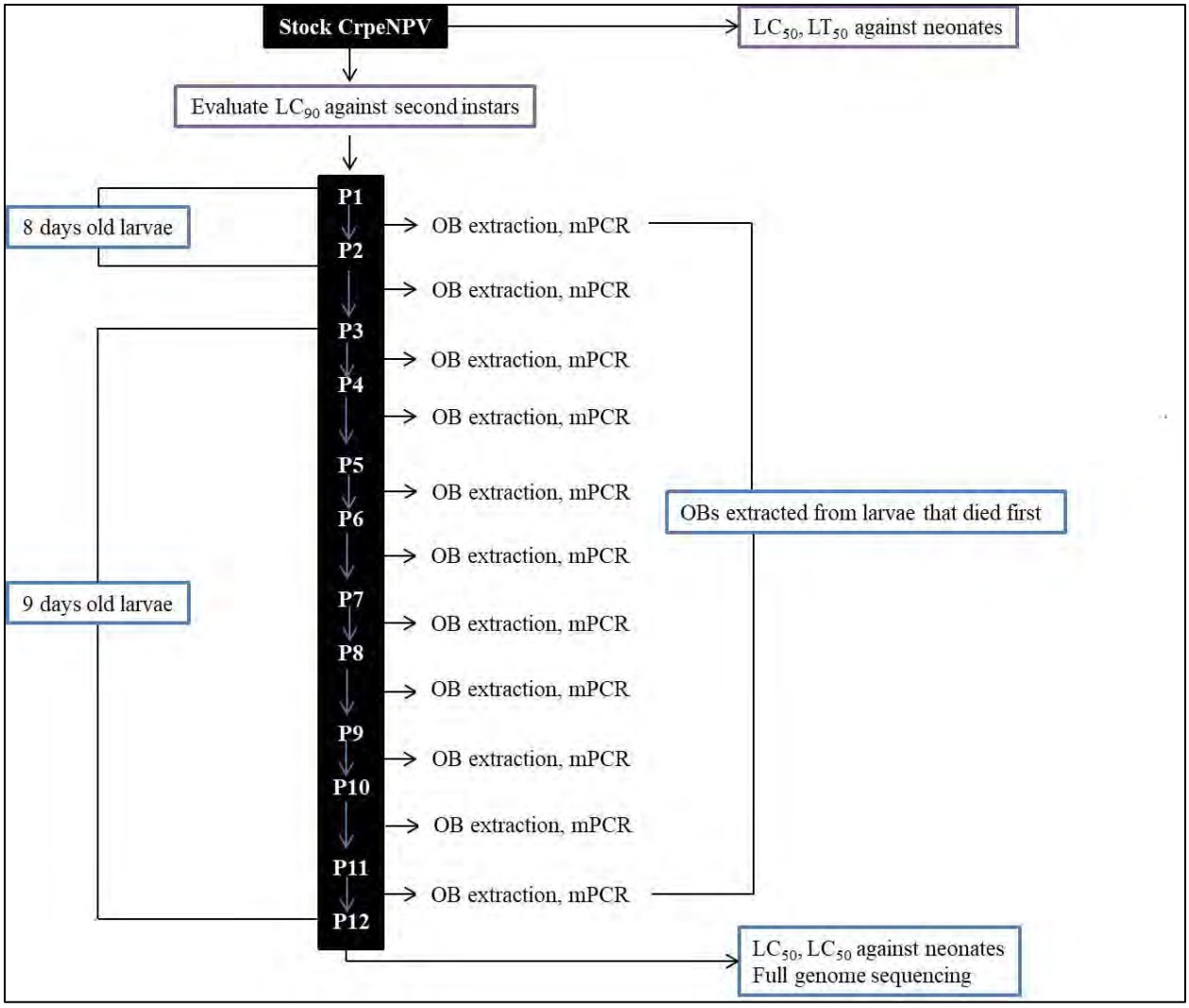

Figure 3.1: An illustration of the serial passage process of CrpeNPV in *T. leucotreta* from passage one to passage twelve (P_1 to P_{12}), with LC_{50} and LT_{50} being determined before and after passage, along with genomic analysis.

Serial passage was conducted using a surface dose bioassay in glass vials of the following dimensions: height (50 mm), diameter (23 mm). The diet was prepared as described previously (Chapter 2, section 2.2.6) and placed in the laminar flow cabinet to cool. In the laminar flow cabinet, about 1 g of the diet was cut out using a modified sterile 20 ml syringe. The diet was carefully pressed into each glass vial and levelled well using the syringe. A 100 µl aliquot of the

corresponding LC_{90} of CrpeNPV OB suspension (at 1.6 x 10^4 OBs/ml) was then deposited onto the diet surface in each vial using a micropipette and spread evenly by swirling. The diet was left for ±30 minutes, until it had dried adequately. A single larva was then placed in each vial and the vials were capped with plastic lids (Figure 3.2).

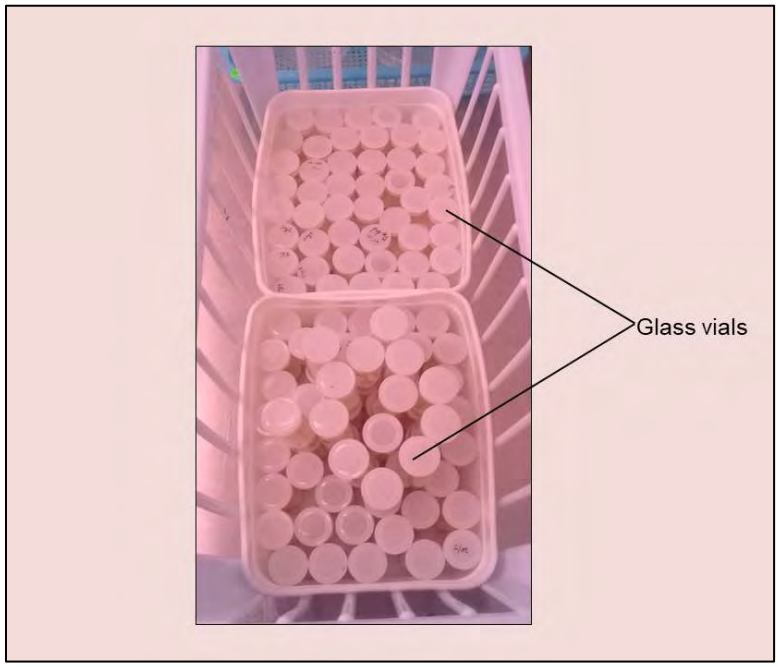

Figure 3.2: Packed glass vials for serial passage assay of CrpeNPV in *T. leucotreta*.

The vials were incubated in the CE room at ±26°C and a relative humidity of 60 to 80% for about 7 days. Vials were monitored daily (at an arbitrary time) for any symptomatic larvae that were either alive (with clear sign of viral infection) or dead. Thirty larvae were treated in the initial passages (P_1 to P_3), and the sample number subsequently increased to 100 larvae from P_4 to P_{12}. After each passage, the first diseased larvae that were infected were collected in microfuge tubes prior to liquefaction of larval cadavers. Ideally, the larval cadavers were collected individually or in groups of two to three (where necessary) prior to OB extraction or stored at -20˚C. The time interval at which each diseased larva was collected (or presumed dead)

post inoculation was recorded as an indication of trends in virulence during the passage process. Occlusion bodies were then extracted from these larvae at each passage and purified as described in the following section.

3.2.3. OB extraction and purification

Occlusion body extraction and purification was done using a sucrose cushion centrifugation method adapted from Wennmann & Jehle (2014). A 100 µl aliquot of 0.1% SDS was added to each virus sample in microfuge tubes. The sample was macerated with a micropipette tip and then sonicated four times at 60 Hz for 15 seconds each. The samples were then centrifuged three consecutive times at low centrifugation speed of 500 × *g* for 15 seconds each to remove larger debris. After these centrifugation steps, the supernatant was collected in new tubes, followed by centrifugation at 12100 × *g* for 15 minutes to pellet OBs with the resulting supernatants discarded and each pellet suspended in 100 to 200 µl TE buffer (10 mM Tris, 1mM EDTA, pH 7.2). For virus purification, sucrose cushions were prepared in new 2 ml microfuge tubes containing 1 ml of 50% sucrose (w/v) in TE buffer, and then cooled to 4°C for 5 minutes. The suspended samples were carefully loaded atop each sucrose cushion and centrifuged at 12100 × *g* for 30 minutes. The supernatant from each cushion was discarded and each pellet was washed twice by suspension in 2 ml of 0.1% SDS (w/v) followed by centrifugation at 12100 × *g* for 10 minutes. The supernatant was again discarded, and the final pellet was dissolved in 100 µl of ddH_2O and stored at -20°C until further use. Purified OBs were then subjected to mPCR screening before being used as inoculum for subsequent passage as described in the following section.

3.2.4. Multiplex PCR analysis

To ensure purity of the virus being passaged, purified OBs harvested at each passage were subjected to mPCR analysis for amplification of gDNA extracted from these OBs (Jukes, 2018). Genomic DNA was extracted using a CTAB DNA extraction method described in Chapter 2 (Section 2.2.3). To detect the presence of either CrpeNPV or CrleGV DNA in a sample, a set of oligonucleotide primers, CpNPV-pif1 and ClGV-lef4, were used for the partial amplification of the *per os factor* (*pif1*) gene in CrpeNPV and *late expression factor 4* (*lef4*) in CrleGV, respectively (Table 3.1).

Table 3.1: Oligonucleotide primers for the partial amplification of the *per os factor* (*pif1*) gene in CrpeNPV and *late expression factor 4* (*lef4*) in CrleGV (Jukes, 2018).

Primer name	**Sequence** (5' to 3')	**Amplicon size** (bp)
CpNPV-pif1F	ATCGGGATGGTTGGTCAAGT	187
CpNPV-pif1R	CAACGCATGTATTCGTCCGT	
ClGV-lef4F	TTCGCTTTCTAAACCGCTGTC	378
ClGV-lef4R	AGGATGACGTTCCTAATGACGG	

A 25 µl PCR reaction was set up for each sample containing 12.5 µl Taq (KAPA Biosystems, USA). To this, 1 µl of CpNPV-pif1F and ClGV-lef4F oligonucleotides (10µM) and 1 µl of the CpNPV-pif1R and ClGV-lef4R oligonucleotides (10µM) were added. A 2.5 µl aliquot of gDNA (about 100 ng) was added as a template, and then topped up to a total volume of 25 µl with ddH_2O. The reaction set up included a negative control and a positive control, of which the negative control consisted of no template DNA (template DNA replaced by ddH_2O), while the

positive control contained concentrated DNA of CrpeNPV and CrleGV. The negative control was included to eliminate the possibility of reagent contamination as no amplicon is expected. The positive control served as reference for amplification of two DNA samples in a single reaction. The PCR parameters used for amplification are shown in Table 3.2.

Table 3.2: Cycle parameters for mPCR using CpNPV-pif1 and ClGV-lef4 oligonucleotides (Jukes, 2018).

Stages	**Temperature** (˚C)	**Duration** (minutes)	**Cycles**
Pre-denaturing	95	03:00	1
Denaturing	95	00:30	
Annealing	55	00:30	30
Elongation	72	00:45	
Final elongation	72	03:00	1

The PCR amplification was performed in a Thermocycler (Bio Rad®, USA). The PCR products were analysed by 1% AGE with ethidium bromide (final concentration 0.4 µg/ml) and visualised in Chemidoc (Bio Rad®, USA). The captured gel images were cropped and edited in Microsoft PowerPoint. Following mPCR analysis, viral OB concentration was determined as described in the following section.

3.2.5. OB enumeration

The concentration of OBs recovered from diseased larvae at each passage was determined using the light microscopy protocol adapted from Smith (2009). A stock suspension of purified OBs

was diluted 1:5 with ddH_2O. This was further diluted 1:5 using 0.07% SDS. The SDS suspension was then sonicated for 60 seconds at 15 second time intervals to avoid particle damage. Further dilutions were made from this suspension depending on the OB concentration, ranging from 1:10 to 1:80. A Marienfeld counting chamber (0.02 mm depth, Marienfeld Superior®, Germany) was used to count the OBs under the light microscope (Olympus CX21LED) at 400X magnification. Before placing the suspension on the counting chamber, the cover slip and counting chamber were cleaned with 70% ethanol and observed under the microscope at 200X magnification to ensure cleanliness. The cover slip was then placed three quarters over the counting chamber, leaving a small space in which to apply the suspension. A volume of 5 µl of the virus suspension was pipetted onto the space left by the cover slip to allow for the suspension to completely fill the counting chamber through capillary action. The cover slip was then slid across to cover the counting chamber. The slide was then left to stand for 5 minutes to allow for Brownian motion of non-virus particles to cease. Five large squares were then chosen (top left, top right, bottom right, bottom left and random middle) and moving OBs were counted. The protocol was repeated three times using the same sample. The mean number of OBs was then calculated to determine the concentration of the virus, using the following formula:

***Equation* 3. 1**: Equation for the determination of OBs.ml^{-1} using a counting chamber.

$$\boldsymbol{OBs\ per\ ml} = (\boldsymbol{D} \times \boldsymbol{x}) \div (\boldsymbol{N} \times \boldsymbol{V})$$

Where D = dilution factor, x = Average No. of OBs counted, N = Number of small squares and V = Volume.

3.2.6. Whole genome analysis

To get a more accurate and comprehensive picture of potential genetic changes in the P_{12} virus (or CrpeNPVpx12); approximately 100 ng of gDNA was extracted from purified OBs, and fully sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South Africa) using the MiSeq platform (Illumina, USA). The resulting paired reads were analysed in Geneious (version R11) (Biometters Ltd, New Zealand). The reads were assembled into a full viral genome using the mapping to reference method, as per the software's manual. The full genome of CrpeNPV (GeneBank accession: MH394321.1) (Marsberg, et al., 2018) was used as reference. Prior to assembly, the ends of the reads were trimmed using the BBDuk Trimmer function. Once the paired reads were trimmed, error correction and normalisation were performed. A reference assembly was run with the data and a contig was produced. The contig was used to reassemble the genome of CrpeNPV, with single nucleotide polymorphisms (SNPs) being identified on the assembled contig. The resulting assembly consensus was used as template for restriction digestion as described in the following section.

3.2.7: Restriction endonuclease analysis

The availability of full genome sequence of CrpeNPVpx12 allowed *in silico* digestion of the viral genome to create REN profiles of the genome using the following restriction enzymes: *Kpn*I, *Sal*I, *Sma*I, *Xba*I, *Xho*I and *Bgl*I, as per the software's manual. The generated RE profiles were compared to the reference (GeneBank accession: MH394321.1) (Marsberg, et al., 2018).

3.3. Results

3.3.1. Head capsule measurement

The widths of head capsule for 8 day old larvae used from P_1 to P_2 ranged from 0.455 to 0.565 mm, indicating third instars (Hofmeyr, et al., 2016b) (see Chapter 1, Table 1.1). The typical width of head capsule for 9 day old larvae used from P_3 to P_{12} ranged from 0.626 to 1.13 mm, indicating third or fourth instars (Hofmeyr, et al., 2016b) (see Chapter 1, Table 1.1). The mean widths of the head capsule of these larvae at each passage are shown in Table 3.3.

Table 3.3: Mean widths of the head capsule of six *T. leucotreta* instars taken from each insect batch used that day for each passage (n = 6, total of 72).

Passage	P_1	P_2	P_3	P_4	P_5	P_6
Width (mm)	0.45-0.58	0.51-0.54	0.67-0.91	0.73-1.0	0.75-1.1	0.74-1.0
Mean (mm)	0.53±0.04	0.53±0.01	0.77±0.11	0.90±0.12	0.90±0.12	0.86±0.12

Passage	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}
Width (mm)	0.66-0.90	0.62-1.0	0.71-1.0	0.69-1.1	0.61-0.91	0.66-0.98
Mean (mm)	0.77±0.10	0.84±0.13	0.85±0.11	0.77±0.10	0.92±0.02	0.80±0.12

3.3.2. Serial passage assay of CrpeNPV through *T. leucotreta*

A total of 12 serial passages of CrpeNPV were carried out through *T. leucotreta* using OBs extracted from virus-infected larvae that died first at each stage in the assay. Some observations made during serial passage are summarised in Table 3.4.

Table 3.4: A summary of some observations made during the serial passage process.

Passage	Time of kill (Hours)	Number of larvae pooled	Estimated OB concentration OBs/ml	CrleGV detected (Yes/No)
P_1	96	9	2.7×10^9	No
P_2	96	12	3.3×10^9	No
P_3	140	1	1.4×10^9	No
P_4	148	2	4.2×10^9	No
P_5	180	2	7.7×10^9	Yes
P_6	164	6	2.7×10^{10}	No
P_7	164	2	7.4×10^9	No
P_8	150	3	2.3×10^{10}	No
P_9	130	3	2.3×10^{10}	No
P_{10}	130	6	5.2×10^{10}	No
P_{11}	125	3	2.3×10^{10}	Yes
P_{12}	125	6	2.3×10^{10}	No

As indicated in the table, sampling began 96 hours post inoculation from P_1 to P_2. Following the use of fourth instars from P_3, the first diseased larvae were collected 140 hours post inoculation and this time increased to 148 hours at P_4. Diseased larvae at P_5 were collected 180 hours post inoculation, with some samples shown to have CrleGV contamination following mPCR analysis. Contamination was also detected in some P_{11} samples, which were also discarded and continued with CrleGV free samples as inoculum for the subsequent passage. The first diseased larvae from P_6 and P_7 were each collected 164 hours post inoculation, which was 16 hours earlier than samples collected at P_5. Sampling was done 150 hours post inoculation at P_8 and this time gradually decreased through to 125 hours with passage pressure in the final passages (P_9 to P_{12}).

The OB concentration estimated at each passage is also shown in Table 3.4 along with the respective number of larvae from which OBs were extracted. The trends in observed time of collection (or time of kill) of diseased larvae used as source of OBs for each passage are shown in Figure 3.3.

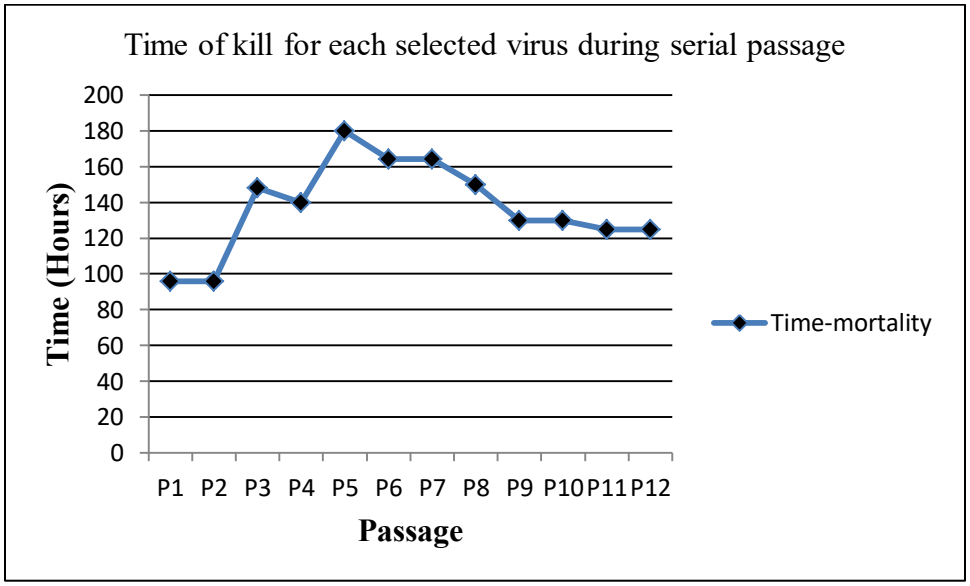

Figure 3.3: Time of kill plot for the diseased larvae collected first and served as source of OBs during 12 passages of CrpeNPV through *T. leucotreta*.

The first larval mortality during the initial passages (P_1 and P_2) was recorded 96 hours post inoculation (Figure 3.3). Time recorded for larvae that died first at P_3 increased following the use of fourth instars and gradually decreased with passage pressure from P_7 to P_9, with some stability observed towards the final passages at 125 hours.

3.3.3. OB purification and multiplex PCR

To ensure that the virus being passaged was not contaminated with CrleGV, a 50% sucrose cushion was used for the purification of OBs extracted from diseased larvae collected at each

passage. Genomic DNA was then extracted from these OBs using CTAB (Chapter 2, section 2.2.3) and amplified by mPCR using CpNPV-pif1 and ClGV-lef4 oligonucleotides designed by Jukes (2018). The results are shown in representative gel images (Figures 3.4 to 3.6). Multiplex PCR analysis of gDNA extracted from a sample taken from purified OBs at P_1 (Figure 3.4) showed an individual band of approximately 200 bp, which matched the expected size for CpNPV-pif1 amplicon (Table 3.1), indicating purity. As expected, no band was observed in the negative control in Lane 2 while two individual bands were detected in the positive control in Lane 3, matching the correct size of both CpNPV-pif1 and ClGV-lef4 amplicons (Table 3.1).

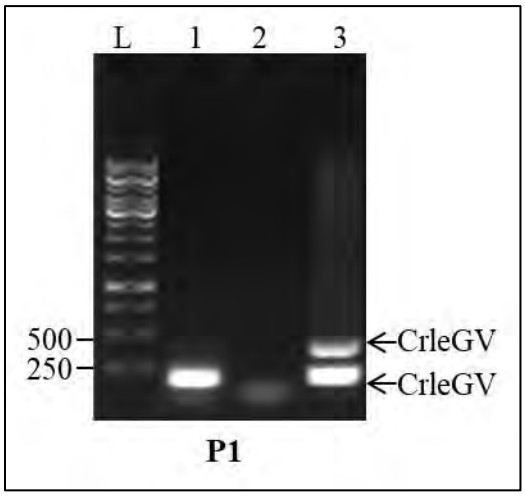

Figure 3.4: Amplified gDNA from purified OBs from a pool of P_1 samples, with (L): 1 kb DNA ladder (Thermo Scientific), Lane 1 (analysed sample), Lane 2 (negative control), Lane 3 (positive control).

The gel image shown above is typical of P_2, P_3, P_4, P_6, P_7, P_8, P_9 and P_{10} samples and hence serves as a representative for these samples. Amplification of gDNA from P_5 and P_{11} individual samples showed two individual bands in Lanes (3, 4, 5, 6 and 7) and Lanes (2 and 4), respectively (Figure 3.5). These bands were approximately 400 bp, which matched the expected

size for ClGV-lef4 (Table 3.1), indicating CrleGV contamination.

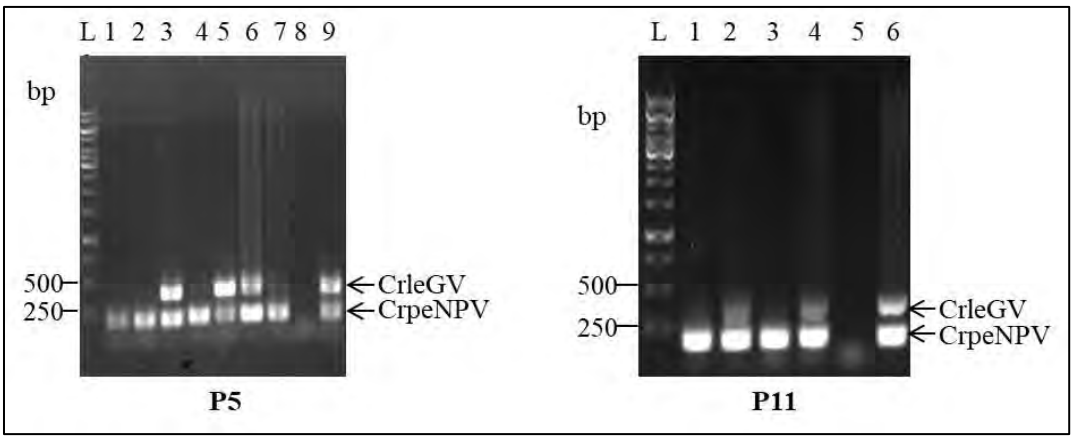

Figure 3.5: Amplified gDNA from purified OBs of P_5 and P_{11} individual samples, with (L): 1 kb DNA ladder (Thermo Scientific), Lanes 1 to 7 (analysed samples), Lanes 8 in P_5 and 5 in P_{11} (negative control), Lanes 9 in P_5 and 6 in P_{11} (positive control).

Amplification of gDNA from purified OBs of P_{12} individual samples (Figure 3.6) showed clear individual bands in Lanes 1 to 6 of approximately 200 bp, which matched the expected size for CpNPV-pif1 amplicon, indicating purity in CrpeNPVpx12. No band was observed in the negative control in Lane 7 while two individual bands were detected in the positive control in Lane 8, indicating optimum amplification of the samples.

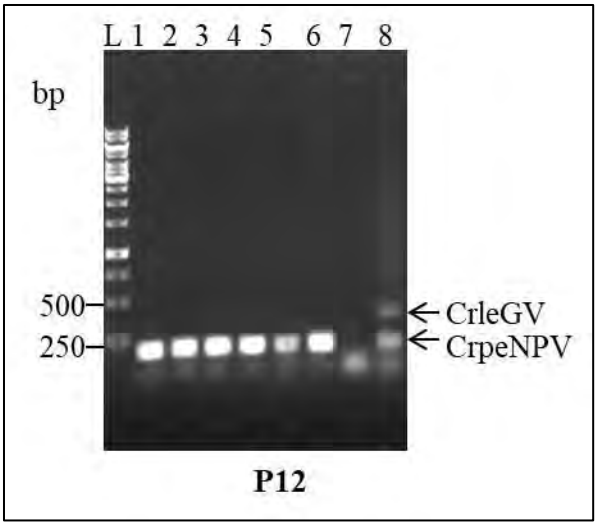

Figure 3.6: Amplified gDNA from purified OBs of P_{12} samples, with (L): 1 kb DNA ladder (Thermo Scientific), Lanes 1 to 6 (samples), Lane 7 (negative control), Lane 8 (positive control).

3.3.4. Whole genome analysis

A total of 229544 reads were produced by NGS of CrpeNPVpx12 gDNA. These reads were assembled in Geneious (version R11) (Biometters Ltd, New Zealand) using the map to reference method. The full genome sequence of CrpeNPV (GeneBank accession: MH394321.1) (Marsberg, et al., 2018) was used as the assembly reference to reconstruct the original viral genome, with the assembly results shown in Table 3.5.

Table 3.5: Assembly results of CrpeNPVpx12 genome against the reference.

Length	Pairwise % identity	Coverage						
		Min	Max	Mean	St Dev	Q20	Q30	Q40
115805	99.8%	53	163	108.4	12.8	99.9%	99.8%	97.4%

As shown in Table 3.5, the NGS data assembled were of good quality, as indicated by the expected Q-value (Q > 97%). The reads mapped to the reference with a total length of 115805 bp and a 99.8% pairwise identity, and a mean coverage of 108.4. The resulting contig of the assembly was annotated, and SNPs were identified inside and outside of coding regions of the genome (Table 3.6).

Table 3.6: SNPs with gaps, found on the mapped data of CrpeNPVpx12 against the reference CrpeNPV.

Polymorphism type	Amino acid change	Coding region	Protein effect	Length	Codon change
Deletion		ac26-like protein	Frame Shift	17	
Insertion (tandem repeat)				1	
SNP (transition)	Gly->Asp	dUTPase	Substitution	1	GGT->GAT
SNP (transition)		p87/vp80	None	1	TAC-> TAT
SNP (transversion)	Gly->Cys	p45/p48	Substitution	1	GGC->TGC

From the analysis (Table 3.6), a significant change was the 17 bp deletion in the ac26-like protein gene, between 11262 and 11278 bp on the reference sequence, which resulted in a frame shift. This frame shift appeared to shorten the ac26-like ORF from 342 bp to 325 bp resulting in a shortening of the coded protein by 5 amino acids. A single nucleotide was inserted at position 5916 on the reference sequence, which had no change on the protein sequence. Another synonymous SNP occurred at position 77828 on the reference in the p87/vp80 gene. A non-

synonymous SNP occurred at position 77206 on the reference sequence in the p45/p48 gene, which resulted in an amino acid substitution (Glycine for Cysteine). Another non-synonymous SNP occurred at position 81065 on the reference in the dUTPase gene, which resulted in an amino acid substitution (Glycine for Asparagine).

3.3.5: Restriction endonuclease analysis (REN)

To further investigate potential genetic change in the genome of CrpeNPVpx12, *in silico* digestion of the assembled genome with several restriction enzymes was performed. As seen in representative profiles using *Kpn*I and *Sal*I, the banding pattern and fragment size were the same as the reference sequence, an indication that the genome had not changed. The results for this analysis are shown in Figure 3.7.

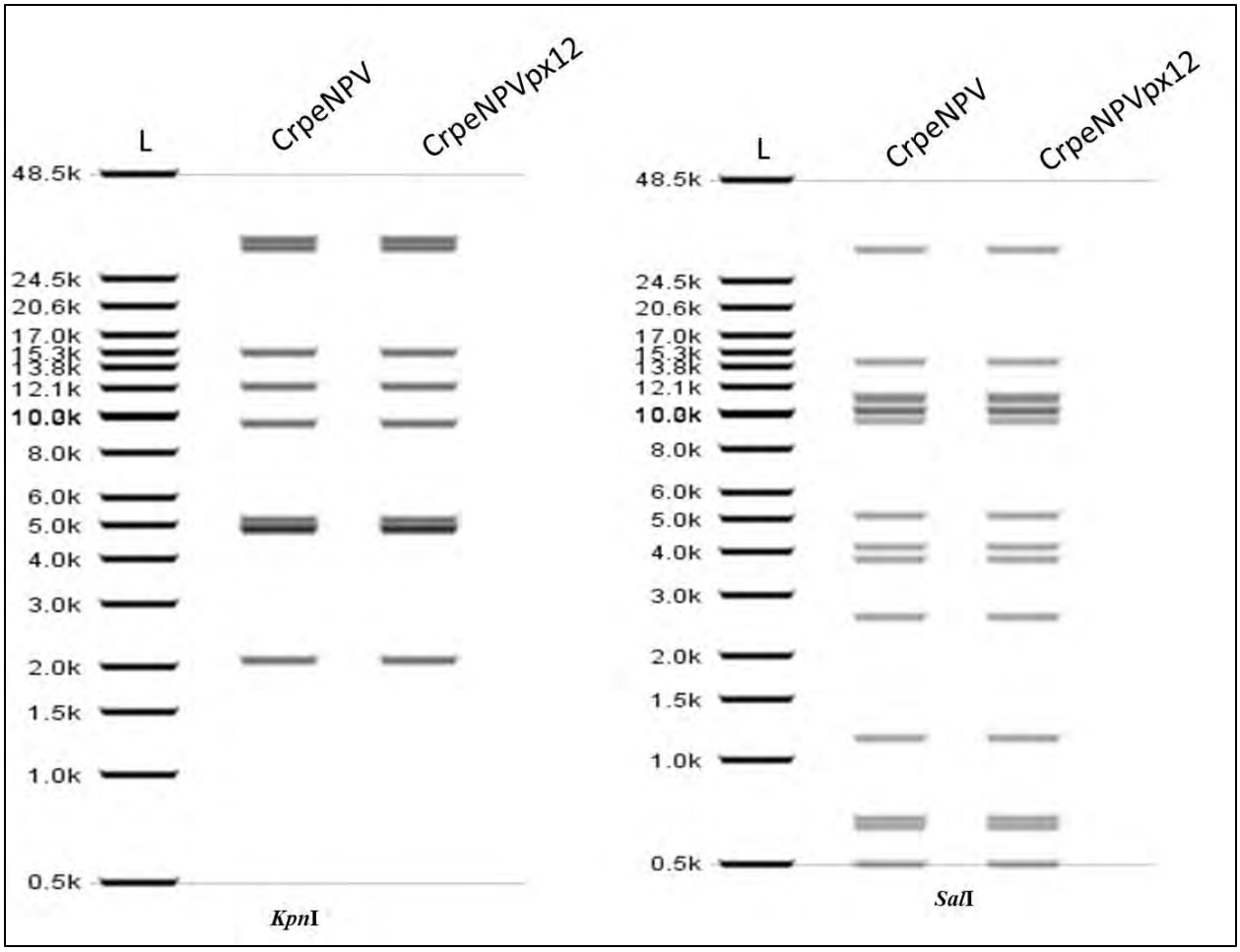

Figure 3.7: Schematic REN profiles of CrpeNPV and CrpeNPVpx12 genomes with *Kpn*I and *Sal*I digested *in silico*, with L representing a gene ruler.

3.4. Discussion

The overall aim of this chapter was to conduct serial passaging of CrpeNPV through *T. leucotreta* larvae to determine the potential for improved virulence or speed of kill against this insect, for improved *T. leucotreta* management in the field.

The LC_{90} of CrpeNPV was evaluated previously against second instar *T. leucotreta* to determine an appropriate dose that would yield high numbers of OBs in third or fourth instars, which were

used as hosts in the serial passage experiments. This viral dose was then fed to third or fourth instar *T. leucotreta*, due to their capacity to produce high OB yields for subsequent passage and downstream experiments. These larvae were identified by measuring the width of the head capsule of six larvae taken from each batch used that day at each passage. It was therefore, shown that larvae used in this study were indeed third or fourth instars (Hofmeyr, et al., 2016b). As per OB enumeration performed after each passage, a high OB yield was recorded in 9 day old larvae as compared to 8 day olds. Following each passage, larval cadavers were collected, with the time of collection (or time of kill) being recorded prior to OB purification. Several of these virus-infected larval cadavers died at various time intervals during the experiment and those that died first at each passage served as the source of OBs to re-infect a new batch of healthy larvae. Since wildtype baculoviruses, and in particular, NPVs, isolated from natural populations co-exist as multiple genotypes in a single virus population (Hodgson, et al., 2001; Erlandson, 2009) it is possible that the fastest killing or more virulent variant of the virus could be selected through serial passaging (Pavan & Ribeiro, 1989). Interestingly, the recorded time of kill during passage decreased with successive passages, which may indicate possible selection for the faster killing genotypes potentially present within the CrpeNPV population.

A multiplex PCR analysis was performed on gDNA extracted from purified OBs recovered at each passage to detect the presence of CrleGV and ensure purity in samples to be passaged. As expected, CrleGV was detected in some samples. The emergence of CrleGV could be an indication of possible reactivation of covert-overt infections of baculoviruses in insects resulting from several stress factors (Burden, et al., 2003; Murillo, et al., 2011; Williams, et al., 2017). For example, larval exposure to the heterologous virus (e.g. CrpeNPV), overcrowding, high temperatures, starvation and humidity have been suggested as possible stress factors that may

trigger covert infections in insects into lethal states (Hughes, et al, 1993; Fuxa, et al., 1999; Williams, et al., 2017; Kasianov, et al., 2017; Akhanaev, et al., 2020). This process was shown previously in *T. leucotreta* larvae by Opoku-Debrah et al. (2013) using overcrowding as an induction method, which led to several CrleGV isolates being recovered. Furthermore, similar observations of CrleGV contamination in *T. leucotreta* larvae were also made by Jukes (2018) following five serial passages of CrpeNPV in this host. Contamination was also reported with other viruses in heterologous hosts during serial passage. For example, a homologous GV associated with AcMNPV was detected following infection of *P. xylostella* (Kolodny-Hirsch & Van Beek, 1997) and Spodoptera exigua MNPV (SeMNPV) following MbMNPV infection of *S. exigua* (Belda, et al., 2019). To minimise the risk of contamination further in this study, it was necessary to increase the number of larvae treated in the experiment from 30 to 100 larvae in order to increase sample size. It was also necessary to collect diseased larvae of interest individually or in small groups prior to OB purification. In previous studies, the contaminated virus suspension was back-passaged through a selective host which is only permissive to the virus of interest so that a contaminant free virus could be isolated and purified (Kolodny-Hirsch & Van Beek, 1997). Others, like Belda et al. (2019) replaced the entire insect population suspected of harbouring a latent infection with new virus-free insects after detection of contamination in the samples.

The last section of this chapter investigated potential genetic changes in the viral genome due to serial passaging through the heterologous host, using NGS and REN profiling. No variations were observed in the genome of the passaged virus following *in silico* digestion of gDNA with several restriction enzymes, relative to the original virus. Similarly, Graillot et al. (2016) found no difference in REN profiles of CpGV following 12 passages through *G. molesta* larvae,

suggesting virus adaptation to the heterologous host and maintenance of genetic diversity. It is likely that a high concentration (LC_{90}) used as a dose in this study could have potentially reduced the genetic variability of the passaged virus, and maintained the dominant genotype which was then sequenced and found to resemble the original virus (Baillie & Bouwer, 2013; Kitchen, 2017). It is also possible that additional passages could be required to bring change to the genotypic composition of the virus. For example, Kolodney-Hirsch & Van Beek (1997) found substantial variations in the genome of AcMNPV following 20 serial passages through *P. xylostella.*

When the genome of the passaged virus was sequenced, several synonymous and non-synonymous SNPs were detected (Table 3.6), relative to the genome of the original virus. Based on the quality of coverage (Table 3.5), it is unlikely that these SNPs are a result of sequencing or assembly errors. Of these SNPs is a 17 bp deletion in the ac26-like protein gene, which resulted in a frameshift. The ac26-like protein gene encodes a protein of unknown function (GeneBank accession: YP_717553) (Zhu, et al., 2009), hence it is not clear what effect it would have on the life cycle of the virus. Furthermore, a single nucleotide substitution occurred in the p45/p48 gene, resulting in an amino acid change (from Glycine to Cysteine). This is a highly conserved gene in baculoviruses and it is essential for BV production and ODV envelopment (Yuan, et al., 2008). Another single nucleotide substitution occurred in the dUTPase gene, resulting in an amino acid change (from Glycine to Asparagine). This gene encodes the enzyme dUTPase, which plays a crucial role in the replication cycle of the virus (Rohrmann, 2013).

Overall, the objectives of this chapter were achieved. A high dose of CrpeNPV was serially passaged through *T. leucotreta* larvae, the heterologous host in order to determine the potential

for improved virulence or speed of kill. A more virulent virus would be desirable for even more effective control of *T. leucotreta*, which is of importance for continued improvement of the IPM programme targeted against this pest. The virus recovered following 12 successive passages was CrleGV-free or at least at levels that were undetectable using mPCR assay. No difference was observed in the *in silico* REN profile between the passaged and original virus and only minor polymorphisms were detected in the genome of the virus following serial passage. These results suggest that 12 passages did not change the dominant genotype of the virus. The next objective was then to determine whether virulence or speed of kill of the virus recovered after 12 passages had changed in comparison to the original virus before serial passage (Chapter 4).

CHAPTER 4

Bioassays of CrpeNPV with neonate *Thaumatotibia leucotreta*

4.1. Introduction

In Chapter 3, CrpeNPV was serially passaged 12 times through *T. leucotreta* larvae to determine the potential for improved virulence of the virus. Following serial passaging, the next objective was to evaluate the biological activity of the virus harvested after passage 12 against neonate *T. leucotreta* by performing surface dose bioassays. This was done to determine whether virulence of the virus had changed during serial passage, in relation to that of the original virus. Surface dose bioassay was considered an appropriate tool for determining a dose or time-response relationship between the virus and the host, thereby enabling the estimation and quantification of virulence and speed of kill of the virus.

Studies have shown that pathogenicity of a baculovirus can be enhanced by serial passaging through a heterologous host. For example, a 12 to 15-fold increase in virulence of HearMNPV was measured following several passages through *Trichoplusia ni* and *S. exigua* (Tompkins, et al., 1988). Twelve serial passages of a mixture of various CpGV-M isolates through *G. molesta* also resulted in a significant increase in virulence against the host larvae (Graillot, et al., 2016). The LC_{90} measured against CpGV-M neonate larvae was 450-fold lower than that of the original mixture before passage, and 120-fold lower than that of the wildtype CpGV-M isolate alone. In

addition, efficacy in the homologous host, *C. pomonella* was also retained (Graillot, et al., 2016). Although serial passage has shown increased pathogenicity in the above studies, in a recent study by Belda et al. (2019), MbMNPV demonstrated a decrease in virulence, while the speed of kill increased significantly after six passages through this host. In the same study, they found no significant changes in the insecticidal efficacy of HearMNPV after six passages through either *S. exigua* or *S. littoralis*.

Since *T. leucotreta* is the heterologous host of CrpeNPV, it is possible that a more virulent variant of the virus could be selected by serial passaging through it. This would be an advantage when the virus is applied as a component of the IPM programme to control the pest in the field. In order to investigate this, the overall aim of this chapter was to evaluate the biological activity of the virus harvested following passage 12 against neonate *T. leucotreta* in comparison to the original virus. For this reason, a surface dose method was used to determine a dose-response mortality curve, which enabled the estimation of lethal concentrations (LC_{50} or LC_{90}) and lethal time (LT_{50} or LT_{90}) of the virus towards the host. Furthermore, it must be noted that second instar *T. leucotreta* were previously used to evaluate the LC_{90} used as a dose for the serial passage experiments (Chapter 2), and then third and fourth instars were used as hosts for serial passage (Chapter 3). In this chapter, neonate larvae were selected, as this is the only larval stage that would be exposed to the virus in the field, penetrating the fruit shortly after hatching (Daiber, 1979b; Moore, 2021) and thus being protected against exposure to the virus thereafter. Therefore, it will be this life stage of the insect that will be targeted by the potential CrpeNPV-based biocontrol agent in the field. The objectives were two-fold: (1) to evaluate the LC_{50} and LC_{90} of both, the original virus (namely CrpeNPVpx0) and passage 12 virus (namely CrpeNPVpx12), and (2) to evaluate the LT_{50} and LT_{90} of both CrpeNPVpx0 and CrpeNPVpx12.

Each of these objectives was investigated alone and the subsequent results were compared to determine whether they differ significantly.

4.2. Material and Methods

4.2.1. Rearing of *T. leucotreta* larvae

Thaumatotibia leucotreta neonate larvae were obtained by cutting egg sheets into squares with approximately 200 to 400 eggs each as described by Moore et al. (2014). Egg sheets were surface sterilized by dipping in 33% bleach solution (household bleach, e.g. JIK) for about 10 seconds, followed by 10 seconds rinse in distilled water (dH_2O). The egg sheets were placed into petri dishes and allowed to air dry adequately. The petri dishes were then sealed with parafilm and kept in the CE room (±26°C) until larval development occurred.

4.2.2. Virus preparation

The doses used in this study were obtained following several preliminary bioassays with CrpeNPVpx0 and CrpeNPVpx12 (data not shown) under similar conditions using a range of doses, starting with very high concentrations. This was done to assess a concentration resulting in at least 10 to 90% mortality, which is a prerequisite for accurate and reliable statistical analysis used in this study. From these preliminary bioassay results, all concentrations exceeding 4.0×10^4 OBs/ml resulted in 100% larval mortality. In order to optimise the dose for the bioassays, OB concentrations were adjusted by 1.3-fold dilution with ddH_2O (Figure 4.1).

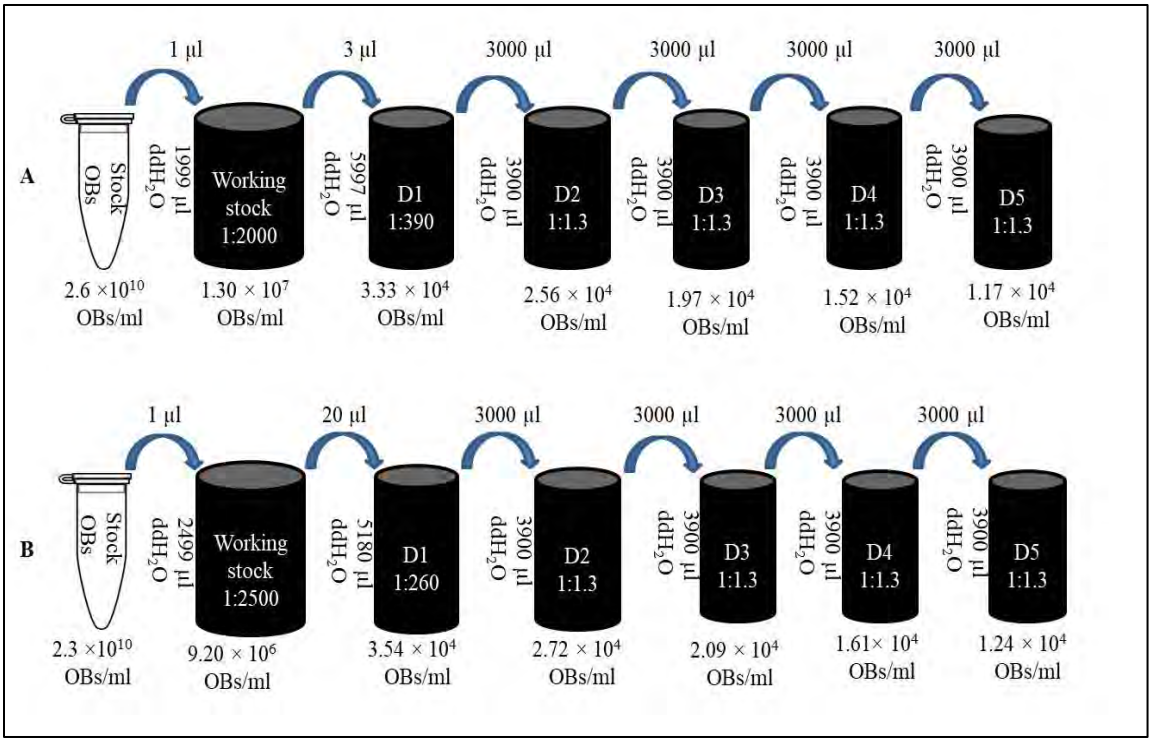

Figure 4.1: A dilution series of OBs for surface dose-response bioassays against neonate *T. leucotreta*, with (A) CrpeNPVpx0 and (B) CrpeNPVpx12.

The stock OB suspension of CrpeNPVpx0 (Figure 4.1A) was diluted 1:2000 to produce a working stock suspension (final concentration 1.30×10^7 OBs/ml), from which bioassay doses were prepared. The working stock was further diluted 1:390 resulting in dose 1 (D1) (final concentration 3.33×10^4 OBs/ml), and subsequent 1.3-fold dilutions: D2 (2.56×10^4 OBs/ml), D3 (1.97×10^4 OBs/ml), D4 (1.52×10^4 OBs/ml) and D5 (1.17×10^4 OBs/ml). Each of these doses was diluted from the previous one. The stock OB suspension of CrpeNPVpx12 (Figure 4.1B) was diluted 1:2500 to produce a working stock suspension (final concentration 9.20×10^6 OBs/ml), from which bioassay doses were prepared. The working stock was further diluted 1:260 resulting in dose 1 (D1) (3.54×10^4 OBs/ml), and subsequent 1.3-fold dilutions: D2 (2.72

$\times 10^4$ OBs/ml), D3 (2.09×10^4 OBs/ml), D4 (1.61×10^4 OBs/ml) and D5 (1.24×10^4 OBs/ml), with each of these doses diluted from the previous one.

4.2.3. Surface dose-response bioassays of CrpeNPVpx0 and CrpeNPVpx12 against neonate *T. leucotreta*

Surface dose-response bioassays were conducted in 24 well plates (Fisher Scientific, USA). The diet was prepared by adding 250 g of FCM artificial diet to a Pyrex dish (Moore, et al., 2014). Distilled water (300 ml) was added to the diet and mixed well. The diet was cooked in an oven at 200°C for 30 minutes and placed in the laminar flow cabinet to cool. In the laminar flow cabinet, about 1 g of the diet was cut out using a modified 10 ml syringe and then carefully pressed into each well of 24 well plates. The diet in the wells was properly packed and levelled evenly to facilitate the flow of liquids. The plates were covered with glass lids immediately after to prevent the diet from desiccation. Bioassays were performed, each using five concentrations of purified OBs of stock CrpeNPVpx0 and CrpeNPVpx12 as dosages (Figure 4.1A) and (Figure 4.1B), along with ddH_2O as a control. A volume of 50 µl of each dose and control were pipetted onto the centre of a flat diet surface of each well using a sterile micropipette and spread uniformly over the surface by swirling. Each dose was vortexed several times (at least 10 seconds each time) between inoculations to ensure homogeneity of OBs in the suspension. Inoculated plates were closed immediately with glass lids to prevent the diet from desiccation and left for ±30 minutes, until they had dried. A single healthy neonate larva that was actively moving was carefully placed into each well, at the centre using a sterile paint brush (R0). Assay plates were double checked (after about 30 minutes) for mobile larvae on the surface of the diet with the aid of a dissecting microscope (BestScope). At the time of inspection, glass lids were kept on to

minimise the risk of possible contamination. The wells with larvae dying due to manipulation in the control and treatment were marked and the larvae were replaced with healthy ones prior to incubation, and no additional changes were made thereafter. The plates fitted with glass lids were further sealed with parafilm to prevent larvae from escaping and kept in the CE room at ±26°C and a relative humidity of 60 to 80%. After 7 days, the plates were opened and the diet was inspected with the aid of a dissecting microscope (BestScope) with sub-stage lighting, and larvae were recorded as either dead or live. Larvae that were marked dead at the onset of the experiment were not recorded. The assay was replicated three times at separate occasions under similar conditions. Dose-response data were subjected to probit analysis in R (version 3.6.1) using the package 'drc' (Ritz, et al., 2015) to determine the mean for all three replicates as LC_{50} and LC_{90}.

4.2.4. Time-response bioassays of CrpeNPVpx0 and CrpeNPVpx12 against neonate *T. leucotreta*

Time-response bioassays were conducted in glass vials of the following dimensions: height (50 mm), diameter (23 mm). The diet was prepared by adding 250 g of FCM artificial diet (Moore, et al., 2014) to a Pyrex dish. Distilled water (300 ml) was added to the diet and mixed well. The diet was cooked in an oven at 200°C for 30 minutes and placed in the laminar flow cabinet to cool. In the laminar flow cabinet, about 2 g of the diet was cut out using a modified 20 ml syringe and then carefully pressed into the vials. The diet in the vials was properly packed and levelled evenly to facilitate the flow of liquids. Each vial was immediately capped with a plastic lid to prevent the diet from desiccation. Fifty glass vials were inoculated with 100 µl of sterile distilled water and another 50 vials were inoculated with the corresponding LC_{90} determined previously (section 4.2.3) at a concentration of approximately 3.7×10^4 OBs/ml for

CrpeNPVpx0 and 3.5×10^4 OBs/ml for CrpeNPVpx12. The suspension was vortexed several times (at least 10 seconds each time) between inoculations to ensure homogeneity of OBs. The diet was left for ±30 minutes, until it had dried adequately. A single healthy neonate larva that was actively moving was placed into each vial and the vials were sealed with plastic lids. Prior to incubation (about 30 minutes), each vial was double checked for mobile larvae on the surface of the diet, with the aid of a dissecting microscope (BestScope) with sub-stage lighting. The vials with larvae dying due to manipulation in the control and treatment were marked and the larvae were replaced with healthy ones prior to incubation, and no additional changes were made thereafter. Subsequently, the vials were kept in a CE room at ±26°C and a relative humidity of 60 to 80%. After 16 hours, the vials were inspected for any dead larvae. However, those larvae that died at the onset of the experiment due to manipulation and replaced were not recorded. The inspection of vials was done with the aid of a dissecting microscope (BestScope) with sub-stage lighting. The vials were checked every 8 hours thereafter until mortality had ceased. After a further 48 hours of no sign of mortality, the diet was then dissected to determine whether the remaining vials contained dead or live larvae. In addition, larvae showing no sign of infection after the additional 48 hours, relative to the controls were allowed to develop until pupation, hence recorded as survivors. Time-response data were analysed by logit regression in R (version 3.6.1) using package 'ecotox' (Hlina, et al., 2019) to determine the mean for all three replicates as LT_{50} and LT_{90}.

4.3. Results

4.3.1. Surface dose-response bioassays with CrpeNPVpx0 and CrpeNPVpx12 against neonate *T. leucotreta*

Three replicates of the surface-dose response bioassays of CrpeNPVpx0 and CrpeNPVpx12 were carried out against neonate *T. leucotreta* to determine the LC_{50} and LC_{90} of the virus. The recorded mortality for neonate *T. leucotreta* from dose-response bioassays with CrpeNPVpx0 is shown in Table 4.1 for each dose used.

Table 4.1: Mortality of neonate *T. leucotreta* in dose-response bioassays with five CrpeNPVpx0 doses.

Dose	**Concentration** OBs/ml	**Replicate 1** Mortality (%)	**Replicate 2** Mortality (%)	**Replicate 3** Mortality (%)
Control	0	0	0	0
D5	1.17×10^4	37.5	37.5	29.2
D4	1.52×10^4	50.0	50.0	29.2
D3	1.97×10^4	41.7	62.5	79.2
D2	2.56×10^4	58.3	79.2	83.3
D1	3.33×10^4	83.3	91.7	95.8

Larval mortality ranged from 29% to 96% and no mortality was recorded in the controls (Table 4.1). The dose-response curve for the bioassays of CrpeNPVpx0 against neonate *T. leucotreta* is shown in Figure 4.2.

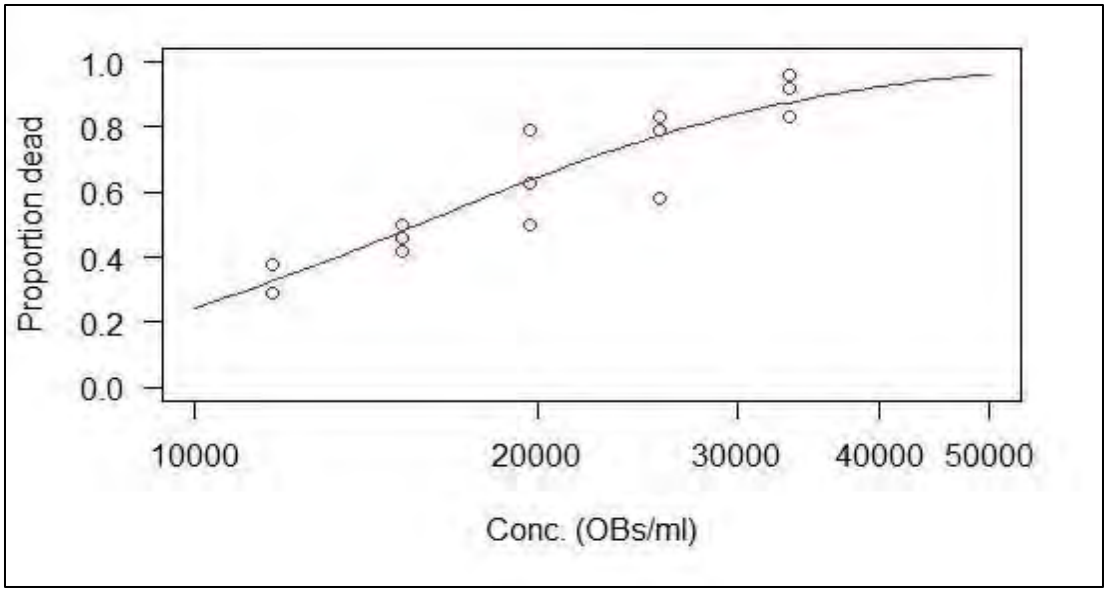

Figure 4.2: Dose-response plot for CrpeNPVpx0 against neonate *T.* leucotreta.

Probit analysis of data obtained from surface dose-response bioassays of CrpeNPVpx0 against neonate *T. leucotreta* indicated an LC_{50} value of 1.58×10^4 OBs/ml and LC_{90} of 3.68×10^4 OBs/ml with 95% confidence interval (Table 4.2).

Table 4.2: The LC_{50} and LC_{90} values of CrpeNPVpx0 against neonate *T. leucotreta*.

Lethal concentration	**Concentration** OBs/ml	**Standard Error(SE)**	**95% Confidence limits**	
			Lower SE	Upper SE
LC_{50}	1.58×10^4	824.04	1.41×10^4	1.74×10^4
LC_{90}	3.68×10^4	3735.6	2.94×10^4	4.4×10^4

The recorded mortality for neonate *T. leucotreta* from dose-response bioassays with CrpeNPVpx12 is shown in Table 4.3 for each dosage used.

Table 4.3: Mortality for neonate *T. leucotreta* in dose-response bioassays with five doses of CrpeNPVpx12.

Dose	Concentration OBs/ml	Replicate 1 Mortality (%)	Replicate 2 Mortality (%)	Replicate 3 Mortality (%)
Control	0	0	0	0
D5	1.24×10^4	12.5	16.7	12.5
D4	1.61×10^4	37.5	45.8	37.5
D3	2.09×10^4	41.7	54.2	50.0
D2	2.72 x 104	75.0	79.2	66.7
D1	3.54×10^4	95.8	95.8	91.7

Larval mortality ranged from 12% to 96% and no mortality was recorded in the controls. The dose-response curve for the bioassays of CrpeNPVpx12 against neonate *T. leucotreta* is shown in Figure 4.3.

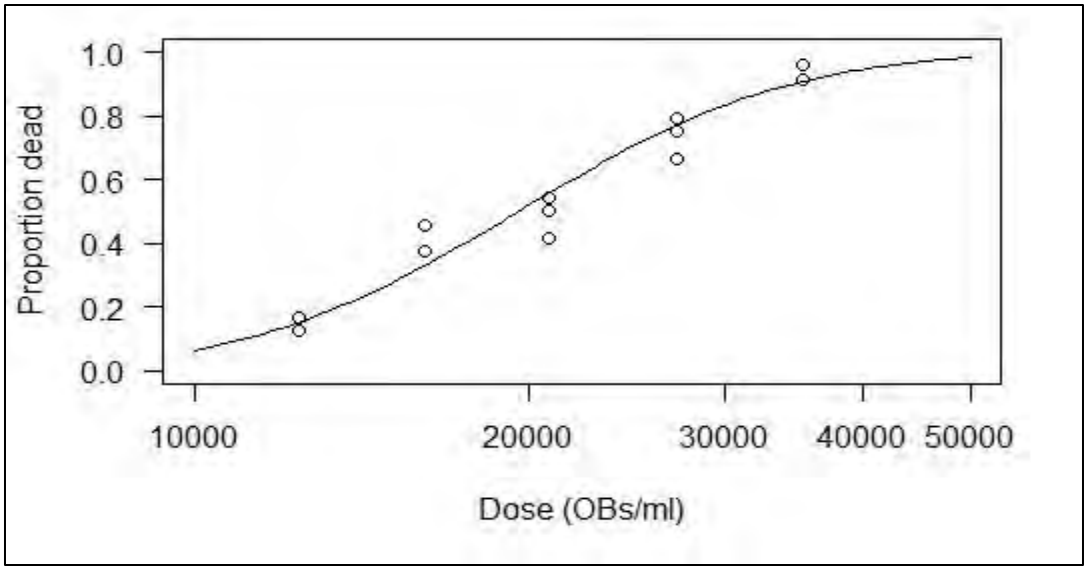

Figure 4.3: Dose-response plot for CrpeNPVpx12 against neonate *T. leucotreta*

Probit analysis of data obtained from surface dose-response bioassays of CrpeNPVpx12 against neonate *T. leucotreta* indicated a LC_{50} value of 1.96×10^4 OBs/ml and LC_{90} value of 3.46×10^4 OBs/ml with 95% confidence interval (Table 4.4).

Table 4.4: The LC_{50} and LC_{90} values of CrpeNPVpx12 against neonate *T. leucotreta*.

Lethal concentration	**Concentration** OBs/ml	**Standard Error(SE)**	**95% Confidence limits**	
			Lower SE	Upper SE
LC_{50}	1.96×10^4	649.82	1.83×10^4	2.09×10^4
LC_{90}	3.46×10^4	2162.8	3.03×10^4	3.88×10^4

The ratios from probit analysis at LC_{50} and LC_{90} for CrpeNPVpx0 and CrpeNPVpx12 were compared. Results showed that there was no significant difference between the LC_{90} for both viruses, whereas the LC_{50} differed significantly (Figure 4.4). The virulence of CrpeNPVpx12 was significantly lower than that of CrpeNPVpx0.

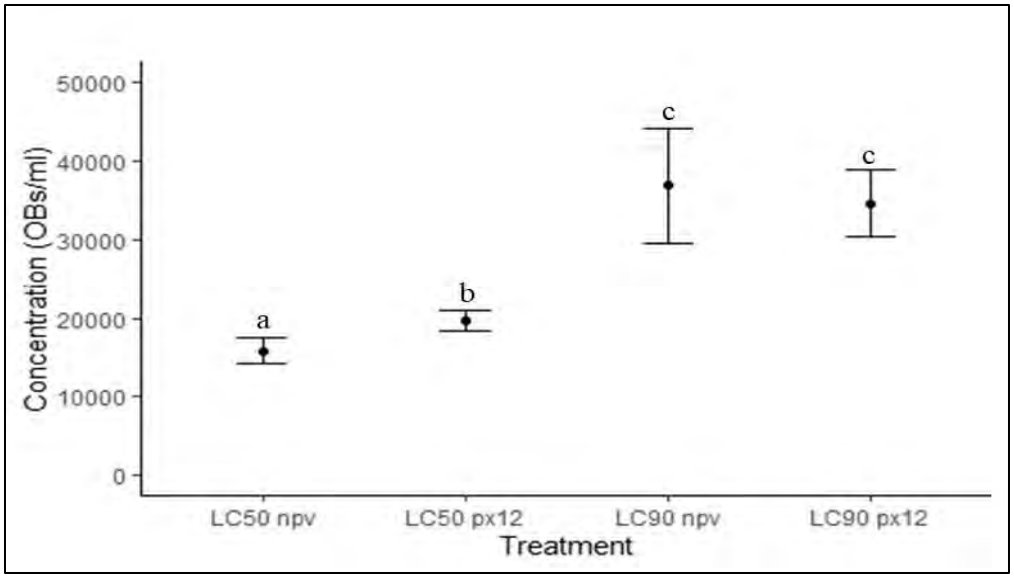

Figure 4.4: Relationship between probit ratios of *neonate T. leucotreta* at LC_{50} and LC_{90} with CrpeNPVpx0 and CrpeNPVpx12. The LC50npv and LC90npv represent the LC_{50} and LC_{90} values for CrpeNPVpx0, whereas LC50px12 and LC90px12 represent the LC_{50} and LC_{90} values for CrpeNPVpx12, respectively. Error bars with the same letter indicate no significance, whereas error bars with different letters represent significant difference.

4.3.2. Time-response bioassays of CrpeNPVpx0 against neonate *T. leucotreta*

Three replicates of time-response bioassays of CrpeNPVpx0 and CrpeNPVpx12 against neonate *T. leucotreta* were each carried out using their respective LC_{90} determined as inoculum to determine LT_{50} and LT_{90} of the virus. The recorded mortality for neonate *T. leucotreta* from time-response bioassays with CrpeNPVpx0 is shown in Table 4.5 for each replicate.

Table 4.5: Mortality for neonate *T. leucotreta* in time-response bioassays using the LC_{90} of CrpeNPVpx0.

Time (Hours)	**Mortality (%)**		
	Replicate 1 (n = 50)	**Replicate 2** (n = 50)	**Replicate 3** (n = 50)
16	0	0	0
24	0	0	0
32	0	0	0
40	0	0	0
48	0	0	0
56	0	0	0
64	2	0	0
72	30	22	12
80	48	54	30
88	52	74	58
96	80	90	78
104	86	94	86
112	92	96	98
120	96	96	100
128	96	100	
136	100		

The first larval mortality was recorded 64 to 72 hours post treatment, and observations continued until 100% mortality was recorded for each replicate (Table 4.5). No mortality was recorded in the controls. Logit regression analysis of time-mortality relationship for CrpeNPVpx0 against neonate *T. leucotreta* showed the means for the three replicates as LT_{50} = 83.74 hours (3 days 12 hours) and LT_{90} = 102 hours (4 days 6 hours). The recorded mortality for neonate *T. leucotreta* from time-response bioassays with CrpeNPVpx12 is shown in Table 4.6 for each replicate.

Table 4.6: Mortality of neonate *T. leucotreta* in time-response bioassays using the LC_{90} of CrpeNPVpx12.

Time (Hours)	**Mortality** (%)		
	Replicate 1 (n = 49)	**Replicate 2** (n = 50)	**Replicate 3** (n = 50)
16	0	0	0
24	0	0	0
32	0	0	0
40	0	0	0
48	0	0	0
56	0	0	0
64	0	6	6
72	22	30	34
80	28	30	38
88	38	44	54
96	52	60	68
104	74	74	72
112	80	92	92
120	86	96	98
128	90	100	100
136	96		
144	96		
152	98		

The first larval mortality was recorded 72 hours post treatment in replicate 1 and continued until 98% mortality was recorded, as one larva failed to respond to the treatment and pupationed, hence n = 49 (Table 4.6). Furthermore, the first larval mortality was recorded 64 hours post treatment in replicate 2 and 3, and observations continued until 100% mortality was recorded for each replicate (Table 4.6). No mortality was recorded in the controls. The time-mortality relationship for CrpeNPVpx12 against neonate *T. leucotreta* was analysed by logit regression. From the analysis, the means for the three replicates were calculated as LT_{50} = 88.44 hours (3

days 16 hours) and LT_{90} = 115 hours (4 days 19 hours). These results were compared to CrpeNPVpx0 using repeated measures ANOVA to determine whether the data differed significantly over the time intervals, with an interaction plot of the two treatments shown in Figure 4.5.

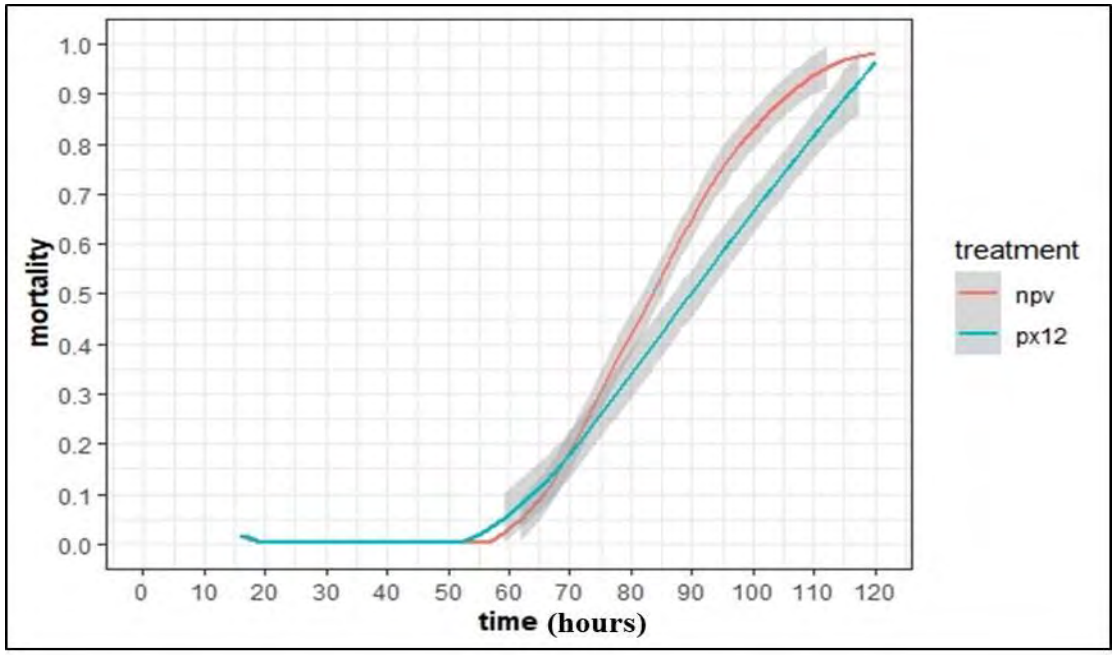

Figure 4.5: An interaction plot showing the relationship between CrpeNPVpx0 and CrpeNPVpx12 in time-response bioassays with neonate *T. leucotreta*. The shaded region represents 95% confidence interval. The keys, npv represents CrpeNPVpx0, whereas px12 represents CrpeNPVpx12.

The time series plot (Figure 4.5) showed an exponential increase in larval mortality for CrpeNPVpx12 between 56 and 72 hours in comparison to CrpeNPVpx0, indicating a potentially enhanced speed of kill of the virus at this point. However, ultimately the estimated LT_{50} and LT_{90} values for CrpeNPVpx12 were slightly, but significantly higher than that of CrpeNPVpx0, indicative of a decrease in the speed of kill.

4.4. Discussion

In this chapter, the biological activity of CrpeNPV was evaluated against neonate *T. leucotreta* to determine whether virulence or speed of kill of the virus had changed following serial passage, in relation to the original virus. For this reason, a surface dose method was used to determine a dose-response mortality curve, from which the LC_{50} and LC_{90} were calculated, and a time-response curve from which the LT_{50} and LT_{90} were calculated. As highlighted above, a more virulent variant would be desirable for efficient control of *T. leucotreta* as part of the IPM programmes used for the control of this pest in the field.

An interesting observation in this study, with emphasis on bioassays performed in Chapter 2 was the fact that zero control mortality was achieved in all bioassays performed. The possible basis for zero control mortality was the modifications made to the method. One of these modifications was ensuring proper packaging of the diet in the wells of the assay plates and the immediate covering of plates with glass lids thereafter to prevent the diet from desiccation, with a view to mimic the natural feeding process of the insect (Jones, 2000). Another modification was the removal of larvae dying from possible stress of handling at the onset of the experiment, and subsequent replacement with healthy ones. This may be a novel method in performing bioassays, but has the advantage that treatment mortality does not have to be corrected for control mortality. Consequently, all mortality recorded is a result of viral infection and thus accuracy of results is optimised. However, application of this method can be time consuming. Additionally, a repeat of this method would be worthwhile, particularly with other insects and viruses.

Due to the heterogeneity of genotypes observed in natural populations of NPVs (Hodgson, et al., 2001; Erlandson, 2009), it was expected that a more virulent variant of the CrpeNPV population

would be selected for during serial passage through *T. leucotreta*, the heterologous host. Conversely, analysis of dose-response data showed a significant increase in the LC_{50} of CrpeNPVpx12, relative to CrpeNPVpx0, indicating a lower virulence of the passaged virus. However, the LC_{90} values were not significantly different. This suggests that the virulence of CrpeNPV did not improve after 12 passages. A few studies have also shown that virulence of some NPVs can remain unchanged after several passages through the heterologous host. For example, an early study by Pavan et al. (1981) found that the virulence of AgNPV against *Pseudoplusia includens* remained constant after several passages through this host. Similarly, Belda et al. (2019) found that virulence of HearMNPV recovered after six passages through both *S. exigua* and *S. littoralis* did not change significantly. In the same study, they also found that MbMNPV demonstrated a decrease in virulence. It is unclear what the reason for this result is; however, Pavan et al. (1981) suggests possible heterogeneity within the host as opposed to the genetic structure of the virus population. It is also possible that a minimum number of serial passages may be required to potentially bring change in virulence. Twelve passages conducted in this study could be too few, as Pavan & Ribeiro (1989) and Kolodny-Hirsch & Van Beek (1997) recorded a significant increase in virulence of AgNPV and AcMNPV after 20 passages. It is also possible that *T. leucotreta* may not be a heterologous host for CrpeNPV as it was assumed since it was previously found covert in *T. leucotreta* laboratory cultures (Jukes, 2018). This suggests that CrpeNPV may be already in *T. leucotreta* populations and adapting to the host, hence no improvement in virulence.

Analysis of time-response data showed that the LT_{50} and LT_{90} values for CrpeNPVpx12 were 5.6-fold and 12.7-fold higher, respectively than those for CrpeNPVpx0, and statistically significant, indicating a slow speed of kill. This suggests that the speed of kill of CrpeNPV did

not improve during serial passage. This is a possible indication that a genotype (or mixture of genotypes) selected during serial passage may not be the fastest killing as it was assumed when selecting larvae that died first in Chapter 3. However, when the full genome of CrpeNPVpx12 was sequenced and analysed, only a few SNPs were detected (possibly due to sequencing and assembly errors) and no changes in the REN profiles were observed. As such, one would expect a positive change in virulence or speed of kill of the virus, as changes in REN profiles are strongly associated with significant changes in pathogenicity of NPVs (Kolodney-Hirsch & Van Beek, 1997; Cory, et al., 2005; Aguirre, et al., 2019). Belda et al. (2019) also reported that a six time passaged HearMNPV through *S. littoralis* and *S. exigua* showed no significant changes in the speed of kill towards these hosts. In the same study, they also found that the speed of kill of MbMNPV increased significantly after six passages through *S. littoralis*, suggesting the complexity of host-baculovirus interactions during serial passage (Pavan, et al., 1981; Belda, et al., 2019). Furthermore, the disparity in LT values calculated between CrpeNPVpx0 and CrpeNPVpx12 could possibly be attributed to the statistical analysis used, coupled with differences in doses used as inoculum (LC_{90}) for time-response bioassays. The use of different doses between the two viruses came due to a shift in dose-response to CrpeNPVpx12 in preliminary assays performed against neonate *T. leucotreta* (data not shown). These preliminary assays were aimed at assessing an appropriate dosage used in the dose-response bioassays in this study, and from which the LC_{90} used as inoculum for time-response studies came. The time response study can be performed with the same OB concentration for either virus (CrpeNPVpx0 and CrpeNPVpx12), rather than with the calculated LC_{90} for each virus. In this study, the latter was used.

Overall, the objectives of this chapter were achieved. Firstly, the biological activity of passage 12 virus against neonate *T. leucotreta* was evaluated in laboratory bioassays. Finally, the dose- and time-response relationship of the two viruses was determined, and the LC_{50}, LC_{90}, LT_{50}, and LT_{90} of the virus were calculated. These results were then compared between the original virus and the virus recovered after passage 12, to determine whether virus efficacy has changed during serial passage. The results showed that there was no significant improvement in virulence and speed of kill of CrpeNPV towards neonate *T. leucotreta* following serial passage.

CHAPTER 5

General discussion

5.1. Thesis overview

This study investigated the potential to select for a variant of CrpeNPV with an improved virulence against *T. leucotreta* by serially passaging the virus a number of times through *T. leucotreta* larvae, the heterologous host. Before the initiation of serial passage assays, the following objectives were achieved in Chapter 2: (1) *T. leucotreta* was reared in the laboratory, thereby enabling the identification of larval instars used in the bioassays, (2) the integrity of the virus being passaged was first assessed by evaluating the morphological and genetic identity of the virus through TEM and sequencing of the baculovirus *polh/gran* gene region and, (3) the LC_{90} for CrpeNPV against second instar *T. leucotreta* was evaluated and used as a dose for serial passage experiments. Once the viral dose was determined, serial passage assays were conducted, using third or fourth instar *T. leucotreta* as hosts (Chapter 3). During serial passage, monitoring of possible CrleGV contamination in samples being passaged was achieved by mPCR assays. Following serial passage, potential genetic changes in the viral genome were analysed by NGS of the full genome and REN profiling *in silico*. Lastly, the biological activity of the virus harvested after serial passage was evaluated against neonate *T. leucotreta* in surface dose bioassays (Chapter 4). This included determining the dose- and time-response relationship of the virus and calculating the LC_{50}, LC_{90}, LT_{50}, and LT_{90} of the virus.

5.2. Selection for a variant with improved virulence against *T. leucotreta*

It is well established that *T. leucotreta* is native to southern Africa and strongly associated with citrus. The pest poses substantial economic impact to the citrus industry, and more importantly to market access, due to its phytosanitary status (Moore, 2021). Various control measures as part of an IPM programme targeted against *T. leucotreta* have been effective at suppressing the pest in citrus, but there is still a growing need for continued improvement of the programme and augmentation of the available control options. Of these control measures, baculoviruses as biocontrol agents are a key component of IPM for effective *T. leucotreta* management in the field (Knox, et al., 2015; Moore & Jukes, 2020). More attention is paid to baculovirus isolates with improved virulence for additional biocontrol agents in the citrus industry to supplement CrleGV-SA which is regularly used in the field for control of *T. leucotreta*. The recent discovery and characterisation of CrpeNPV, and the observation that it infects and kills *T. leucotreta* (Marsberg, 2016; Marsberg, et al., 2018) created an opportunity that required further investigation. The virus is currently undergoing field trials as a potential alternative to CrleGV for control of the pest. Although CrpeNPV is more virulent against *T. leucotreta* than CrleGV (Marsberg, 2016), there is always room for improvement in the control measures for more effective pest control.

A number of studies have shown that serial passage of a NPV through a heterologous host can lead to increased virulence of the virus towards that host (Stairs, et al., 1981; Tompkins, et al., 1981, 1988; Pavan & Ribeiro, 1989; Kolodny-Hirsch & Van Beek, 1997). The observed increase in virulence of the virus during serial passage appeared to result from improved virus transmission and selection of certain genotypes within NPVs that are better adapted to the

heterologous host (Hitchman, et al., 2007; Arrizubiata, et al., 2015). Here, serial passage was achieved by feeding a high dose (LC_{90}) of CrpeNPV to healthy third or fourth instar *T. leucotreta* generations in iterations. Previous serial passage studies have also used high doses for third instar infections (Hitchman, et al., 2007; Kitchin & Bouwer, 2018), with the view to allow a high OB yield for the subsequent passage. As expected, third or fourth instar *T. leucotreta* used in this study demonstrated the capacity to produce sufficient OBs for serial passage. Therefore, third or fourth instar *T. leucotreta* may exhibit an ideal host system for the mass production of CrpeNPV, taking into account that CrleGV contamination is monitored throughout by mPCR (Jukes, 2018). CrleGV contamination in *T. leucotreta* was problematic in these experiments, resulting from possible baculovirus convert-overt infections due to several stress factors, including exposure to CrpeNPV (Burden, et al., 2003; Williams, et al., 2017; Akhanaev, et al., 2020). It was found that increasing sample size (i.e. number of larvae treated) during serial passage, OB purification from individual larvae prior to pooling and consistent mPCR screening potentially reduced the risk of CrleGV contamination in samples being passaged. Furthermore, with successive passaging, decreasing time of kill for the viral infected larvae was observed (Figure 3.3, Chapter 3), indicative of possible selection of genotypes with increased virulence or speed of kill within the CrpeNPV population. To investigate this further, the biological activity of CrpeNPV harvested after 12 passages was evaluated against neonate *T. leucotreta* in surface-dose bioassays, and compared to the original virus. The virulence of the passaged CrpeNPV (measured as LC_{50}) was similar to that of the original virus, but significantly lower. Likewise, the speed of kill of the passaged CrpeNPV (measured as LT_{50}) was similar to that of the original virus, but significantly slower. These results are clear indication that neither virulence nor speed of kill of CrpeNPV had improved after 12 passages through the heterologous host in these experiments. An early study

by Pavan et al. (1981) found that the LT_{50} of AgNPV against *P. includens* was constant following 10 passages through this host. Similarly, Belda et al. (2019) reported that HearMNPV retained pathogenicity of the original virus against *S. littoralis* and *S. exigua* after six passages through these hosts. The basis for these results, according to the authors was the heterogeneity in the host population and the complexity of host-baculovirus interactions during serial passage. It is unclear if *T. leucotreta* is really a heterologous host for CrpeNPV since it was previously found covert in *T. leucotreta* laboratory cultures by Jukes (2018). This suggests that CrpeNPV may be already in *T. leucotreta* populations and adapting to the host, hence no improvement in virulence or speed of kill after passage. While the observed LT_{50} in this study (i.e. 3 days 16 hours) was comparable to preliminary results reported by Marsberg (2016) (i.e. 3 days 8 hours), CrpeNPV has still demonstrated its potential as a biocontrol agent for *T. leucotreta* management in citrus. Even though virulence did not improve in this study, the data obtained may be important, particularly if the virus is to be mass produced in the field or for research purposes.

5.3. Genome analysis of the selected variant following serial passage

Another aspect of serial passage of NPVs that has attracted attention in recent years and was investigated in this study is the potential effect of serial passage on the genotypic composition of the virus population. Kolodney-Hirsch & Van Beek (1997), using REN analysis, found novel variants within the AcMNPV genome that appeared to be selected during serial passage through *P. xylostella*. Similarly, Hitchman et al. (2007) found different variants in the PaflNPV genome after several passages through four different heterologous hosts, suggesting that NPVs comprise a mixture of several genotypes within an isolate and their relative abundance may increase over the course of serial passage. It is apparent that during transmission and replication stages of the

infection, changes to the genotypic combinations of the virus population occur; resulting in selection for certain genotypes that are best suited to the host, and subsequently alter the viral genetic structure (Kolodney-Hirsch & Van Beek 1997; Hitchman, et al., 2007; Kitchin & Bouwer, 2018). To get a complete picture of the potential changes in the viral genetic structure, REN analysis of gDNA and sequencing are required. These techniques have been extensively used in the identification and characterisation of baculovirus genotypes for pest control (Cory, et al., 2005; Erlandson, 2009; Chateigner, et al., 2015; Aguirre, et al., 2019). Here, full genome analysis of the passaged CrpeNPV by NGS was performed to determine whether genetic changes have occurred in the viral genome during serial passage, in comparison to the original virus. Analysis of sequence data revealed several synonymous and non-synonymous SNPs in the coding regions of the passaged CrpeNPV genome. Baillie & Bouwer (2012) and Chateigner et al. (2015) noted that a significant number of synonymous and non-synonymous SNPs are prevalent across NPV genomes in all functional gene classes and significantly influence the genetic diversity of viral populations. The fact that SNPs as well as a distinct 17 bp deletion in the ac26-like gene of the passaged CrpeNPV (Chapter 3, section 3.3.4) was absent in the genome of the original virus is a possible indication that the genome had changed during serial passage. It could also be possible that the detected SNPs are false variations resulting from sequencing and assembly errors. Despite the detection of SNPs, no detectable difference in fragment digestion patterns was observed following REN analysis of gDNA of the passaged virus with several restriction enzymes *in silico*, which suggests that a dominant genotype of CrpeNPV population was maintained during serial passage. Belda et al. (2019) analysed the genomes of HearMNPV and MbMNPV after each passage through *S. littoralis* and *S. exigua* and found modifications in restriction fragments profiles at each passage. The results for this study suggest that CrpeNPV

may be suited to the heterologous host as it persists under these conditions without significant changes to the genome and this can have positive implications for maintenance of the genetic integrity of the biocontrol agent in the field (van der Merwe, et al., 2017).

5.4. Conclusions and future works

If possible, a repeat of serial passage experiments would be worthwhile to generate novel variants of CrpeNPV with improved pathogenicity against *T. leucotreta*. Future work could involve conducting serial passage of CrpeNPV through field-collected insects as opposed to an inbred laboratory colony which was used in this study. Field insects may challenge the fitness of the virus as opposed to laboratory insects and therefore serial passage may result in selection for different genotypes with modified pathological characteristics (Cory & Myers, 2003; Cory, et al., 2005). In addition, it would be interesting in future work to perform biological assays and genome analysis after each passage as opposed to the final passage only.

Further research projects can investigate the biological activity of the passaged virus against the homologous host, *C. peltastica*, to determine whether virulence has changed. If a more virulent variant of CrpeNPV is selected against *C. peltastica*, it would be important in control of the pest in the field.

In conclusion, the overall aim of this study was to conduct serial passaging of CrpeNPV through a heterologous host, *T. leucotreta*, in order to determine the potential for improved virulence or speed of kill against it. The biological assays of the virus recovered after serial passage, along with the original virus were conducted to evaluate the biological activity against *T. leucotreta* and enable comparison between the two viruses. Results from bioassays showed that the

virulence and speed of kill of CrpeNPV did not improve after 12 serial passages; however, CrpeNPV has demonstrated potential as a biological control agent for *T. leucotreta* management in the field. Potential genetic changes in the genome of the virus population during serial passage were also investigated, with only minor polymorphisms observed in the genome. These results may be fundamental to continued investigation into the effect of serial passage on pathogenicity and genetic diversity of CrpeNPV.

CHAPTER 6

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