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The Effects of Sublethal Doses of *Helicoverpa armigera* Single Nucleocapsid Nucleopolyhedrovirus on *Helicoverpa armigera* (Lepidoptera: Noctuidae)

Luisa Nardini

The Effects of Sublethal Doses of *Helicoverpa armigera* Single Nucleocapsid Nucleopolyhedrovirus on *Helicoverpa armigera* (Lepidoptera: Noctuidae)

Luisa Nardini

A dissertation submitted to the School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Master of Science.

Johannesburg, 2007

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Luisa Nardini

_____ day of _____ , 2007

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LIST OF ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
BV	budded virion
df	degrees of freedom
dpi	days post infection/inoculation
GV	granulovirus
hpi	hours post infection/inoculation
HearSNPV	*H. armigera* single nucleocapsid nucleopolyhedrovirus
LD	lethal dose
MS	mean of squares
NPV	nucleopolyhedrovirus
OB	occlusion body
ODV	occlusion-derived virion
RH	relative humidity
RR	resistance ratio
SDS	sodium dodecyl sulphate
SE	standard error
SS	sum of squares

ABSTRACT

The focus of nucleopolyhedrovirus (NPV) research has been directed toward lethal infections and the elimination of a pest population. Another aspect of NPV infection, and one that has received considerably less attention, is that surviving exposure to sublethal doses of NPV is frequently accompanied with a reduction in the fitness of the host. As such, sublethal effects of NPVs are thought to have an important impact on the ecology and population dynamics of a host population. Commonly observed sublethal effects include slower development time, reduced pupal mass and reduced reproductive capacity. The aim of this study was to investigate sublethal effects in third instar *Helicoverpa armigera* following exposure to sublethal doses of its NPV (HearSNPV). In addition, the impact of sublethal HearSNPV doses on the metabolic rate of the host was investigated. This parameter has not previously been used to characterise sublethal infections.

Sublethal effects were recorded in third instar *H. armigera* following exposure to an LD_{25}. This involved recording the duration of immature stage development and pupal mass of insects that survived exposure to HearSNPV. Fecundity was recorded in mating pairs in which treated male and female insects were paired with healthy partners of the opposite sex. The number of eggs laid by each mating pair was recorded daily and subsequently, the proportion of eggs that hatched determined. In addition, the sex ratio and survival of offspring of mating pairs in which one partner was treated were recorded. Both male and females that were exposed to HearSNPV exhibited a reduction in the duration of the immature stages, but no significant differences in pupal mass, when compared with untreated controls. Sex ratio and survival amongst offspring of these mating pairs were not significantly different to controls although the egg hatch amongst treated groups was notably lower than that of controls. Life tables were constructed for each population and jackknife estimates of relevant parameters were statistically compared to aid in the evaluation of the success of each population. Comparison of life table parameters indicated that the control population was, on the whole,

more successful than populations derived from mating pairs in which one partner was exposed to HearSNPV.

The metabolic rate was recorded in third instar *H. armigera* exposed to two sublethal doses, LD_{25} and LD_{75}, of HearSNPV. The metabolic rate was recorded daily for four days post treatment by closed system respirometry. The overall metabolic rate of treated insects was higher than that recorded in untreated controls. The metabolic rate of LD_{25} treatment survivors was maintained immediately after inoculation (i.e. the metabolic rate 1 and 2 days post treatment was similar to the metabolic rate of controls), but was significantly higher than that recorded in controls 3 and 4 days following treatment. Relative to controls, the metabolic rate of LD_{75} treatment survivors dropped significantly 2 days post inoculation, recovered 3 days post inoculation, and increased again 4 days post inoculation. The elevated metabolic rate observed in survivors may reflect the initiation of defence responses in the host, such as the mobilising of an immune response. The results suggest that exposure to sublethal HearSNPV doses are associated with significant metabolic costs.

Sublethal inoculation of *H. armigera* with HearSNPV was characterised by a number of changes in life-history and fitness characteristics. Specifically, these alterations included reduced development time, reduced fecundity, and elevated metabolic rates, relative to controls. Furthermore, the construction of life tables and the statistical comparison of life table parameters estimated by the jackknife technique proved to be a highly sensitive method for evaluating the impact of sublethal treatment on *H. armigera*. The results of the current study suggest that exposure to sublethal doses of HearSNPV has an important role in reducing the fitness, and consequently the success, of a pest population.

PRESENTATION OF RESEARCH

Chapter 4, entitled “Changes in the Metabolic Rate of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Larvae Exposed to Sublethal Doses of *H. armigera* Single Nucleocapsid Nucleopolyhedrovirus”, was presented as a poster at the 9th International Colloquium on Invertebrate Pathology and Microbial Control, 39th Meeting of the Society for Invertebrate Pathology, and 8th International Conference on *Bacillus thuringiensis*. The conference was held in Wuhan, China, from the 27th August – 1st September, 2006.

For my parents, Pierdomenico and Antoinette Nardini,
and siblings, Daniela and Marco

“We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.”

T.S. Eliot

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Chapter 1

General Introduction

1.1. Introduction

Biological control of crop pests has gained importance in recent years due to increased pressure to reduce the use of chemical pesticides and their residues in the environment (Mishra, 1998). The development of resistance to chemical pesticides, and subsequent reduction of pesticide efficacy, has also encouraged scientists to search for alternative control strategies (Metcalf, 1999). Pesticides typically target specific sites on an insect such that a single mutation in a pest genome is able to alter or remove a susceptible target site (Gullan and Cranston, 1994). This, combined with other factors, such as the overuse of pesticides, and the high frequency at which insects reproduce, have contributed to the emergence of pesticide resistance (Metcalf, 1999).

Members of the family *Baculoviridae* have great potential as biological control agents. They are highly pathogenic, but host specific, and therefore not harmful to non-target organisms (Falcon, 1982). Baculoviruses have been isolated from several insect orders including Lepidoptera, Hymenoptera, Diptera, Coleoptera, Trichoptera and Thysanura (Moscardi, 1999). Furthermore, baculoviruses occur in relatively stable infectious forms that improve survival and persistence outside the host (Cory and Hails, 1997).

Baculoviruses are a diverse group of large viruses with covalently closed, double stranded DNA genomes of approximately 80 – 180 kbp in size (Blissard and Rohrmann, 1990; Blissard et al., 2000). Currently, the family *Baculoviridae* is divided into two genera based on their morphology: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Bonning and Hammock, 1996; Blissard et al., 2000). The virions are rod-shaped and are made up of a lipoprotein envelope that surrounds the nucleocapsid (Funk et al., 1997). Nucleocapsids comprise a protein shell containing a DNA-protein core (Falcon, 1982), and range in size from 250 – 300 nm in length, depending on the size of the viral genome (Blissard et al., 2000). The virions in turn are surrounded by a protein matrix or occlusion body (OB) (Funk et al., 1997; Blissard et al., 2000). The OBs of NPVs are large polyhedron-shaped structures (called polyhedra or OBs) which contain many

virions. In NPVs, the virions vary in that the nucleocapsids may be enveloped singly (SNPV) or in multiples (MNPV) (Blissard et al., 2000). GVs on the other hand produce smaller ovocylindrical OBs called granules (but also sometimes referred to as OBs). These granules typically contain a single virion (Funk et al., 1997; Blissard et al., 2000).

Baculovirus disease primarily affects the larval stages (Federici, 1997). The progress of infection is dependent on several factors including the age at the time of inoculation (Cory and Myers, 2003), instar in which infection becomes apparent, infective dose, incubation temperature, degree of compatibility between the virus and the host, and the fitness of the larva (Federici, 1997).

Baculoviruses are identified and named according to the host from which they are initially isolated (King and Possee, 1992; Lange et al., 2004). Infection of different hosts with the same virus has resulted in double naming of identical viruses. For example, *Rachiplusia ou* NPV and *Anagrapha falcifera* NPV were shown to be the same virus (Harrison and Bonning, 1999). By the year 2004, 500 lepidopteran-specific baculoviruses had been described (Lange et al., 2004). Amongst these, the extent of double naming is unknown and therefore, true baculovirus diversity is unknown (Lange et al., 2004).

Recently, revision of baculovirus nomenclature and classification has been proposed (Jehle et al., 2006). As mentioned, baculoviruses have been classified using morphology as the primary criterion, however, sequence comparison of baculovirus genomes has shown that baculovirus phylogeny followed host classification more closely (Jehle et al., 2006). The proposed classification system includes four genera, namely the alphabaculoviruses, the betabaculoviruses, the gammabaculoviruses and the deltabaculoviruses. According to the proposal, the first group should include lepidopteran-specific S- or MNPVs. The second group should include the current lepidopteran-specific genus, *Granulovirus*. The gammabaculoviruses should include NPVs that are restricted to hymenopterans, such as *Neodiprion lecontei* NPV (NeleNPV). Finally, the deltabaculoviruses

would include the *Culex nigripalpus* NPV (CuniNPV) and other dipteran specific NPVs. This system groups baculoviruses based on phylogenetics and genome composition, in addition to morphological and pathological characteristics (Jehle et al., 2006).

1.2. Nucleopolyhedroviruses

Virions of the NPVs are occluded within polyhedral shaped OBs. The OBs range in size from 0.15 – 15 μm and are composed of a 29 kDa virally-encoded protein, polyhedrin (Blissard and Rohrmann, 1990). OBs are extremely stable, and as a result, are able to persist for long periods in the environment where they are typically found on plant surfaces and in soil (Blissard and Rohrmann, 1990). *Autographa californica* MNPV (AcMNPV) is the type species and is the most widely studied MNPV (Blissard and Rohrmann, 1990; Volkman, 1997). It has been investigated extensively as a pest control agent and is used widely as a vector for the expression of genes in insect cells (Volkman, 1997). *Heliothis zea* SNPV (HzSNPV) is the most widely studied SNPV, particularly as a biological control agent (Moscardi, 1999).

Despite their relatively large host range, it is important to note that NPV infections typically occur amongst species of Lepidoptera (Moscardi, 1999). Furthermore, individual NPVs have a limited host range in that they are typically restricted to a single host species or genus (with the exception of NPVs isolated from *A. californica, A. falcifera* and *Mamestra brassicae*) (Moscardi, 1999). The NPV infection cycle has been reviewed by several authors (Granados, 1980; Blissard and Rohrmann, 1990; Adams and McClintock, 1991; Federici, 1997; Volkman, 1997; Szewczyk et al., 2006); the following summary of the infection cycle has been based on these reviews.

1.2.1. NPV Infection Cycle

The insect gut is divided into three main regions: the foregut, the midgut and the hindgut (Gullan and Cranston, 1994). The foregut is mainly involved with ingestion, storage, grinding and transport of food to the midgut (Gullan and Cranston, 1994). In the midgut, digestive enzymes are produced and secreted and the products of digestion are produced (Gullan and Cranston, 1994). Material remaining in the gut lumen then enters the hindgut where water and salts are absorbed (Gullan and Cranston, 1994). The midgut epithelium in most insects is separated from the food by a thin sheath called the peritrophic membrane (Gullan and Cranston, 1994). This membrane consists of a network of chitin fibrils in a protein-carbohydrate matrix and is either produced by the whole midgut (Type I), or produced by cells at the start of the midgut (Type II) (Gullan and Cranston, 1994; Lehane, 1997). A Type I peritrophic membrane is found in most insect species, while a Type II peritrophic membrane is found in Diptera, Dermaptera, Embiodea and in some families of Lepidoptera (Lehane, 1997). Alternate membrane formation patterns are known as well where, for example, some Lepidoptera supplement the Type II peritrophic membrane with material from other parts of the midgut (Lehane, 1997).

As outlined by Volkman (1997), retention of gut contents and peritrophic membranes during moulting varies amongst lepidopteran larvae. Some larvae, like *Trichoplusia ni*, void both their gut contents and peritrophic membranes during moulting, while others, like *Heliothis virescens*, clear their gut contents, but retain their peritrophic membranes. Others do not shed their peritrophic membranes or clear their gut contents. These differences could have an effect on the efficacy of baculovirus infection in moulting larvae.

The main route of baculovirus infection is by ingestion of OBs and subsequent entry of virus through the midgut epithelium. The OBs dissolve rapidly in the alkaline (between pH 8 – 11) conditions of the insect midgut. This process is aided by proteases of the insect midgut. Virions released from the protein matrix

are referred to as occlusion-derived virions (ODVs). The ODVs initiate infection in the cells of the midgut as they pass through the peritrophic membrane and fuse with the microvillar membrane of the columnar epithelium, allowing nucleocapsids to enter the midgut epithelial cells. Nucleocapsids are transported to the nucleus and enter it via the nuclear pore where uncoating of the viral DNA occurs followed by gene expression and replication of viral DNA. Early viral gene expression occurs within 6 hours post infection (hpi) and results in the transcription of genes encoding proteins required for viral DNA replication and late gene transcription (Ramachandran et al., 2001).

Newly formed nucleocapsids assemble in and around a dense virogenic stroma that forms in the centre of the nucleus. The nucleocapsids leave the stroma and pass through the nuclear membrane, often acquiring an envelope from the membrane, although this is lost in the cytoplasm. These nucleocapsids then bud through the cytoplasmic membrane to become budded virions (BVs). In the budding process, the nucleocapsids acquire a new envelope derived from the plasmalemma. The envelope includes virion-encoded proteins such as gp64, a fusion glycoprotein essential for infection of other tissues (Rohrmann, 1992). It is important to note that although polyhedral crystals may be formed in the cells of the midgut epithelium during this stage of infection, the virions are not occluded in these cells in most lepidopterans. Infected cells are shed as the larva moults and are either replaced immediately by new cells, or later during larval moulting.

BVs cross the basal lamina, a fibrous matrix of glycoproteins secreted by epithelial cells, and enter the haemolymph. Alternatively, they accumulate between the basal lamina and midgut cells, passing the matrix and entering the haemolymph at a later stage. The BVs circulate throughout the body, causing secondary infections in the tracheal matrix, haemocytes, fat body and eventually in the nerves, muscles, pericardial cells, reproductive tissues and glandular tissues. While the ODVs are only infectious in the midgut of the host, the BVs are responsible for the spread of infection to other tissues of the insect. In this way, the infection cycle is dependent on two different viral phenotypes.

In permissive hosts, haemocytes are thought to play an important role in the baculovirus infection process by amplifying the virus (i.e. they support viral replication) and transporting it around the body (Trudeau et al., 2001). In semi-permissive hosts, the proportion of haemocytes that support viral replication is much lower than that of permissive hosts (Trudeau et al., 2001). Furthermore, the haemocytes in semi-permissive hosts have been shown to be involved in melanization and encapsulation of infected tracheae, although Trudeau et al. (2001) have shown that this response was not directly related to the presence of virus, but rather, melanization (of the infected tracheae) appeared to be the elicitor.

Little is know about the means by which BVs actually enter the secondary sites of infection, although endocytosis appears to be the predominant mode of entry (Funk et al., 1997). In these tissues, two different cycles of virion production occur. The first involves the production of BVs that migrate from infected cells and infect nearby cells. This results in the formation of concentric patterns of infected cells. These are typically observed 3 – 4 days after infection is initiated. In individual cells, BV production peaks at about 12 – 16 hpi.

The second cycle of virion replication involves the occlusion phase where occluded virions and OBs are produced. At this stage of infection, high levels of polyhedrin are produced (brought about by the *polh* gene). Maturation of the OBs involves the formation of a polyhedral envelope or membrane around each OB (this is not a true membrane as it is comprised mainly of carbohydrates and protein) (Adams and McClintock, 1991). Hundreds of OBs are produced and accumulate in the nucleus of infected cells causing hypertrophy of infected nuclei, and in turn hypertrophy of infected cells and tissues. The tissues of the fat body, epidermis and tracheal matrix produce the greatest number of OBs.

The formation of OBs and concomitant cellular hypertrophy lead to damage and weakening of the plasmalemma, causing the nuclei and cells to lyse. This process may be facilitated by the production of viral proteases as well as viral p10 protein

(assists in nuclear lysis) and cathepsins which aid cell lysis. As cell lysis progresses, millions of free OBs accumulate in the tissues. The basal lamina is weakened by viral enzymes, loses its integrity and ruptures, releasing OBs into the haemolymph. At this point, the cells lyse and the larva dies. During the occlusion phase, a viral-encoded chitinase is produced that, after larval death, likely aids in disruption of the chitin-rich cuticle, releasing OBs from the body of the dead caterpillar into the environment.

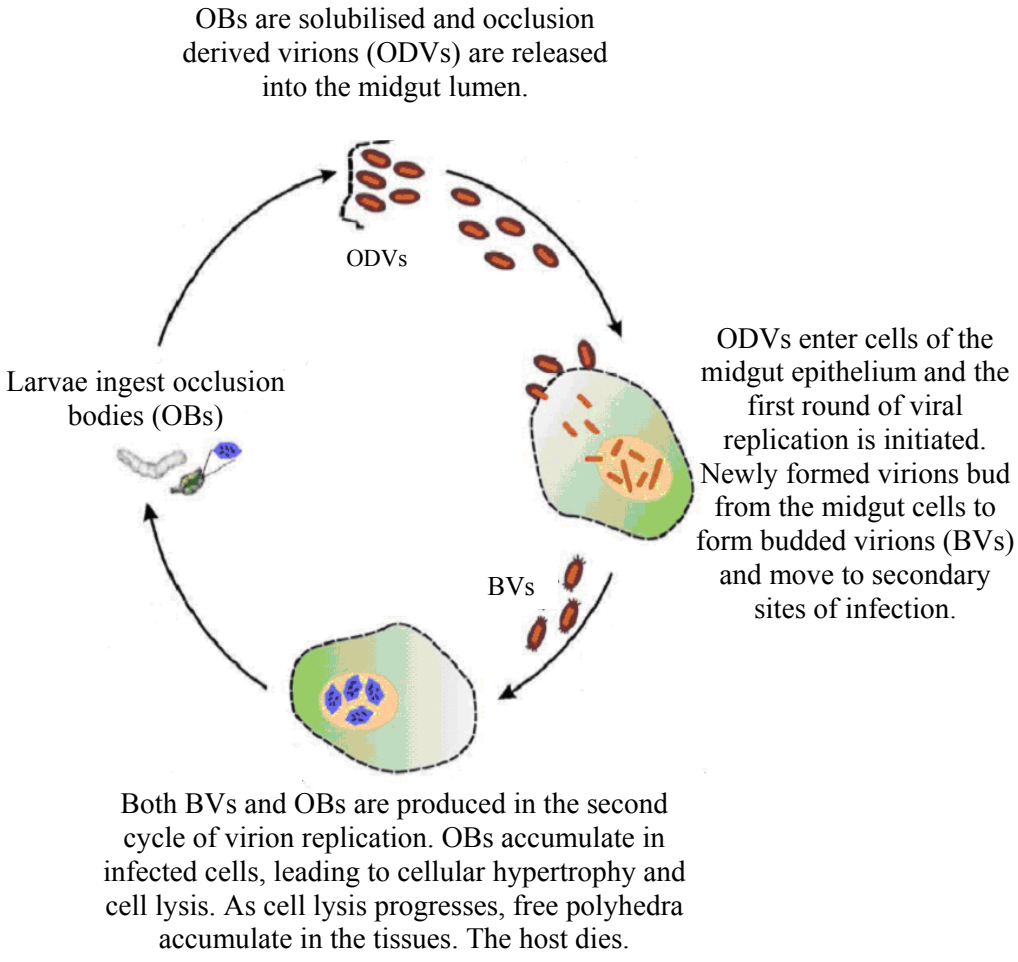


Figure 1.1. The natural infection cycle of an NPV (adapted from Szewczyk et al., 2006).

During NPV infection, more than a hundred viral genes are expressed in a cascade (Ramachandran et al., 2001). Viral expression can be separated into three stages: early, late and very late; and in each stage, a unique set of genes is expressed (Ramachandran et al., 2001). The system is extremely well regulated, where the products of one gene group are required for the expression of the next group. Early gene expression (0 - 6 hpi) occurs before viral DNA replication, and most of these genes encode proteins that regulate transcription. Expression of the late and very late classes of genes initiated by DNA replication. These encode proteins required for virion assembly and occlusion body formation. Interestingly, many insects cell host factors are important in late and very late viral gene expression too. An example is the transcription factor, *polh* promoter binding protein (PPBP), which acts an initiator binding protein involved in recruitment of the transcriptional machinery (Ramachandran et al., 2001).

1.2.2. Gross Pathology

The symptoms associated with NPV infections have been described in detail by Federici (1997). Insect larvae are most susceptible to viral infection during the early instars. In typical NPV infections such as those caused by AcMNPV, *T. ni* SNPV (TnSNPV), and *H. zea* MNPV (HzMNPV), the first symptoms are observed roughly four days following initial infection. Infected larvae respond more slowly than healthy larvae to tactile stimulation and their feeding begins to slow (by the sixth day, feeding typically stops completely). After 4 – 5 days the larvae look swollen and the cuticle appears glossy with small melanotic spots appearing in some species. In species in which the larval cuticle is translucent or lightly pigmented, the larvae develop a white to cream colour due to the presence of OBs accumulating in the epidermal and fat body nuclei. At this stage of infection, the haemolymph of infected larvae is murky due to the presence of infected haemocytes and polyhedra released from infected cells. Larvae at this stage of disease will die within 1 – 2 days. Just prior to this stage of infection, larvae crawl to the top of the vegetation on which they are feeding and die. These larvae are typically limp and flaccid and are attached to vegetation by their

prolegs. The cuticle eventually ruptures, releasing OBs from the lysed cells of the fat body, tracheal matrix, epidermis, and other tissues.

In some NPV infections, it has been found that the larvae fail to moult. This is due to the production of an ecdysteroid, UDP-glucosyltransferase (EGT), by the *egt* gene that is present in most baculoviruses (O'Reilly et al., 1998). EGT is produced by infected cells and is secreted into the haemolymph of infected larvae where it glucosylates the moulting hormone, ecdysone, thus preventing further moults (Federici, 1997). The time, energy and resources that normally go into moulting are instead directed to viral reproduction, effectively increasing the viral load.

1.3. *Helicoverpa armigera*

1.3.1. Pest Status

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae), commonly known as the African cotton bollworm, is one of the most serious crop pests worldwide (Fitt, 1989). It has one of the widest distributions of any agricultural pest, occurring throughout Africa, the Middle East, southern Europe, India, central and southeastern Asia and northern Australia, New Zealand and many eastern Pacific islands (Fitt, 1989). The pest is highly polyphagous, attacking a variety of agricultural and horticultural crops including cotton, beans, maize, sorghum, tobacco, tomato, sunflower, chickpea and soybean, amongst others (Hill, 1983; Fitt, 1989). Populations of *H. armigera* have developed resistance to all major insecticide classes (Srinivas et al., 2004). Economic losses from both direct yield reduction (damage to both flowering and fruiting structures) and from the cost of chemical insecticides and their application, are considerable (Fitt, 1989). As a result, alternative control strategies are required.

In addition to being polyphagous and exhibiting high resistance to pesticides, several other factors contribute to the success of *H. armigera* populations.

Helicoverpa armigera are multivoltine (more than two generations per year) with facultative diapause (the ability to arrest development combined with adaptive physiological changes), highly fecund, and capable of moving long distances as adults (Fitt, 1989; King and Coleman, 1989). As a result, they can rapidly exploit host crops. Another factor contributing to their pest status is the rapid rate at which the larvae consume food (King and Coleman, 1989). As a result insects develop rapidly (maturing from egg to adult in less than 30 days) and reach a relatively large size (King and Coleman, 1989).

1.3.2. Life Cycle

Helicoverpa armigera are holometabolous, undergoing complete metamorphosis. Each female moth is capable of laying between 1000 and 1500 eggs over the reproductive lifetime (8 – 10 days) (Fitt, 1989). Eggs are laid singly or in groups of two or three on or in the vicinity of young growing points or buds where larvae prefer to feed (Fitt, 1989). The eggs hatch 2 – 4 days after being laid (Hill, 1983). There are 5 larval instars and larval development lasts for a period of 14 – 24 days (Hill, 1983). The larvae cause extensive damage by boring holes in flower buds, cotton bolls and fruits (Hill, 1983). Pupation takes place in the soil and after 10 – 14 days, an adult moth emerges (Hill, 1983).



Figure 1.2. Late instar *H. armigera* larva.

1.4. Insect Protection against Viruses

1.4.1 Defence Mechanisms

Known mechanisms of insect defence against viruses include moulting and midgut cell sloughing (Cory et al., 1997), apoptosis (Clem, 2001), and the use of the host's immune system in melanization and encapsulation responses (Washburn et al., 1996). Resistance to baculovirus infection typically increases with age, both within and between larval instars. This developmental resistance has been observed in a number of lepidopteran larvae challenged with baculoviruses. Developmental resistance has important implications for the success of pest control programs based on baculoviruses where application levels may require adjustment according to the demographics of the pest population (Engelhard and Volkman, 1995). This type of resistance is related in part to an increase in larval mass as insects get older (Sait et al., 1994a). However, other mechanisms also appear to be involved. A study that compared oral with intrahaemocoelic inoculation demonstrated that developmental resistance did not occur when the virus was injected into the host, suggesting that this resistance is linked to events in the midgut (Teakle et al., 1986).

The mechanisms of developmental resistance were investigated in fourth instar *T. ni* larvae infected with a recombinant AcMNPV expressing a reporter gene (Engelhard and Volkman, 1995). In this study, four groups of fourth instar larvae were infected and each group differed in age by a few hours. Using a reporter gene to establish the early events of infection, it was found that mortality obtained in each test group corresponded with the ability of the virus to establish primary and secondary infection. The authors also observed that the sequence and timing of infection was similar in all four groups, although the proportion of infected larvae in each group was significantly different. Two mechanisms have been proposed to explain this: first, infected midgut cells are sloughed at each moult so that the later in an instar that a larva becomes infected, the less time the virus has to be transmitted to secondary target tissues (Engelhard and Volkman, 1995; Volkman, 1997). Second, the rate of establishing or sustaining infection in the

midgut appears to decrease as larvae get older within an instar. This effect may be a result of many factors including decreased susceptibility of midgut cells and/or increased cell sloughing (Engelhard and Volkman, 1995; Volkman 1997).

Baculoviruses are able to trigger apoptosis in host cells so that by sacrificing the few cells that are initially infected, the insect is able to avoid further infection (Clem and Miller, 1993; Clem, 2001; Clarke and Clem, 2003). It is thought that a number of processes are involved in inducing apoptosis. These include the initiation of viral DNA synthesis, the initiation of late viral gene expression, and the end of cellular RNA, protein and early viral gene product synthesis (Clem, 2001). When apoptosis occurs, NPV replication is effectively terminated, resulting in an overall reduction in the number of viral progeny (Clarke and Clem, 2003).

A number of studies have shown that the insect immune system plays a role in protection against viral infection (Washburn et al., 1996; Washburn et al., 2000; Trudeau et al., 2001). Infection of *H. zea* was tracked using recombinant AcMNPV containing a *lacZ* reporter gene. It was shown that the proportion of insects expressing *lacZ* decreased more than 50% between 20, and 48 to 72 hpi (Washburn et al., 1996). Examination of larval tissue revealed small areas of melanization colocalized with blue *lacZ* signals in the epidermis of tracheae associated with the midgut. The infected tracheae were surrounded by aggregations of haemocytes that often contained capsules encompassing the host cells expressing *lacZ*. These capsules were the same as those associated with insect cellular immunity (haemocytes surround, immobilise and kill invading pathogens). The authors also tracked *lacZ* expression in tissues following the application of chemical and biological agents that suppress the immune system in larval lepidopterans. In these insects, infection was more extensive, indicating that the cellular immune response is a significant factor in preventing the spread of infection.

1.4.2. Host Resistance

The development of resistance to NPVs has been recorded in both laboratory and field populations of insects. Laboratory selection for resistance to NPV was recorded first in *Spodoptera frugiperda* (Fuxa et al., 1988), although insect resistance to other groups of viruses had been recorded previously (Briese, 1981). Detection of the development of resistance during epizootics in the field is difficult but has been shown in populations of *S. frugiperda* (Fuxa, 2004). In this population, susceptibility to NPV decreased significantly during a single growing season, but generally, the population demonstrated an increase in heterogeneity without an overall change in susceptibility to infection (Fuxa et al., 1988). Resistance to NPV has also been associated with cross-resistance to other viruses. For example, NPV resistance in *T. ni* appeared to confer resistance to GVs as well (Milks and Myers, 2003). According to Fuxa (1993), insect resistance to NPVs is similar in many ways to insect resistance to chemical pesticides – these similarities occur in the context of their "dynamics, preadaptive nature, and genetics". The major difference exists in the mode of action, and in the fact that viruses, like their hosts, are able to change and adapt.

Resistance to NPV has been shown to carry costs in terms of environmental fitness and may also influence the degree of variation in observations of sublethal effects (Milks et al., 1998). For example, when compared with susceptible insects, NPV resistant *S. frugiperda* were characterised by reduced longevity, a reduction in the number of eggs produced by a female, and a reduction in the percentage egg hatch (Fuxa and Richter, 1989). In *Anticarsia gemmatalis*, resistance to NPV was associated with extended life spans, reduced larval survival, reduced pupal mass, a reduction in the number of eggs produced by a female, and reduced egg hatch. In some instances though, resistance to NPV does not incur fitness costs (Milks et al., 2002).

1.5. Sublethal Infections

Baculovirus research has focused predominantly on lethal baculovirus infections and the immediate reduction of pest populations (Goulson and Cory, 1995; Cory et al., 1997). However, in recent years, interest in sublethal infections as a means toward long-term suppression of pest populations has increased (Myers and Kuken, 1995). Sublethal infections may be latent infections, where minimal gene expression occurs; or persistent, where a range of viral expression occurs, but at a low level (Burden et al., 2003; Cory and Myers, 2003). Research has shown that individuals surviving baculovirus infections as larvae are frequently impaired relative to controls. Typical sublethal effects include changes in development time, reduced fecundity (reproductive output), reduced egg viability and changes in sex ratio (Rothman and Myers, 1996). Another important aspect of sublethal infection is the transmission of the virus between generations (vertical transmission) (Rothman and Myers, 1996).

The mechanisms by which sublethal effects occur are unclear. Sublethal effects may be a result of initial virus challenge, where host energy reserves are directed toward fighting infection (Cory et al., 1997). The use of the host's energy in defence against long-term persistent infection (Cory et al., 1997), or hormonal changes induced by the virus may also be physiologically costly (Rothman and Myers, 1996). For example, the process of midgut cell sloughing to avoid infection and eliminate virus requires the use of resources which could otherwise be used for host growth and development (Cory et al., 1997). These observations indicate that the mechanisms by which insects overcome sublethal infection are associated with a fitness cost.

Numerous studies of sublethal effects on a variety of insects have been performed. Goulson and Cory (1995) examined the sublethal effects of NPV in the cabbage moth, *M. brassicae*. They found that survivors of viral challenge exhibited extended development time as larvae and pupae, as the viral doses were increased. Pupal weight, sex ratio, fecundity and egg viability were not significantly different between infected and uninfected insects. It was also noted that a low

level of mortality occurred in offspring of adults that developed from larvae that were exposed to NPV. Furthermore, death in these offspring occurred mainly during the second instar.

Several studies have examined the influence of larval age on the type and extent of sublethal effects observed following exposure to NPV (Milks et al., 1998; Duan and Otvos, 2001). In larvae of the western spruce budworm, *Choristoneura occidentalis*, inoculated with NPV of the spruce budworm *Choristoneura fumiferana* (CfMNPV), it was found that sublethal effects were greater in insects treated as older larvae (Duan and Otvos, 2001). Larvae infected during the sixth instar demonstrated extended development time of males to pupation, decreased male pupal weight and decreased longevity of both male and female adults. A reduced proportion of female adults amongst survivors was recorded although more females than males died during the pupal phase. This significantly altered the sex ratio in favour of males, negatively affecting the ability of the population to increase. Fecundity and hatching success were not affected by treatment with the virus. In light of these results the authors concluded that in addition to mortality, sublethal effects should be examined when the efficacy of microbial pesticides is evaluated.

Milks et al. (1998) investigated the effect of larval age on sublethal effects of TnSNPV in the cabbage looper. Sublethal effects recorded included extended development time and reduced pupal weight, egg production and egg viability. The authors found that these effects were not dose-dependent but rather, differed with larval age where the effects were most prominent in larvae inoculated during the third and fourth instars, while larvae that survived exposure at the fifth instar exhibited no deleterious effects.

Myers et al. (2000) studied the effect of dose, time of infection and rearing temperature on the expression of sublethal NPV effects in *Lymantria dispar*. They considered infected females only, although infected males were used in mating experiments. When compared with controls, female insects that survived

inoculation in the fifth instar were smaller as pupae and laid fewer eggs as adults. Furthermore, the observed reduction in pupal and egg mass size increased with viral dose in larvae infected one day post moult. This dose effect was not observed in larvae infected five days post moult. Sublethal infection was induced in larvae reared at three different temperatures: 20, 25 and 28°C. Prolonging the larval period by rearing at cooler temperatures had no impact on the expression of sublethal effects as shown by pupal size, but egg masses of those reared at cooler temperatures were smaller. Vertical transmission of overt infection occurred in 15% of egg masses produced by females inoculated as larvae. The authors also investigated the effect of the *egt* gene in sublethal infection by infecting larvae with wild-type virus that contained the *egt* gene, and a genetically modified strain, lacking the gene. The pupal mass of insects infected with the different viral strains was reduced to a similar extent but only the pupal mass of insects infected with the wild-type strain was significantly lower than the pupal mass recorded for controls.

Vertical transmission of NPV is an interesting feature of sublethal infection and is thought to be important for the maintenance of the virus in a host population (Hughes et al., 1997; Burden et al., 2003), particularly when population densities are low (Kukan, 1999). Vertical transmission of sublethal infections represents a complicating factor in the study of virus-pest population dynamics where virus can be transmitted to offspring via eggs (either on or within the eggs) of sublethally infected female moths.

A number of recent studies have been devoted to investigating the persistence and transmission of NPVs in a host population (Fuxa et al., 2002; Khurad et al., 2004; Zhou et al., 2005). For example, between 50 and 100% of *M. brassicae* larvae, collected from 10 geographically distinct sites in England, were positive for the presence of virus as indicated by PCR of the NPV polyhedrin gene (Burden et al., 2003). The number of larvae collected for three of the populations was sufficient to rear insects for several generations. In these populations, polyhedrin specific PCR products were amplified in subsequent generations, confirming the

transmission of virus to subsequent generations. Furthermore, reverse transcription (RT) PCR analysis of these populations indicated that polyhedrin gene expression occurred, suggesting that the virus was actively replicating at a low level.

Fifth instar *Bombyx mori* larvae were infected with *B. mori* NPV (BmNPV) in order to investigate vertical transmission of the virus in its host (Khurad et al., 2004). Female moths that survived exposure to BmNPV as larvae, paired with healthy males, showed reduced fecundity, and reduced egg hatch, indicating transovarial transmission. Reduced egg hatch was also obtained when healthy females were mated with males that survived exposure to BmNPV as larvae, indicating venereal transmission. It was further noted that transovarial transmission caused death of offspring in the first and second instars, while venereal transmission produced lethal infections at the end of the third or fourth instar. PCR of the immediate early-1 (*ie*-1) gene of BmNPV isolated from both adults and offspring confirmed vertical transmission of the virus.

From these studies it is clear that sublethal effects of NPV infections on different hosts vary considerably and no consistent trends emerge. A range of factors can affect the outcomes observed following exposure to sublethal viral doses, including the age at which insects are inoculated, or the dose used (Goulson and Cory, 1995). Generally, sublethal effects are more obvious in older insects (from about third instar), and with higher sublethal doses (Goulson and Cory, 1995; Rothman and Myers, 1996; Milks et al., 1998).

Despite the variability in the type and extent of sublethal effects, the studies described here confirm that sublethal infections have an important impact on insect development and reproduction. Furthermore, sublethal infections are considered to be an important factor in the dynamics of plant and animal populations (Sait et al., 1994b). Invertebrate host-pathogen interactions have generated a great deal of interest as an aid to understanding how diseases regulate populations and in evaluating the potential of pathogens as pest control agents

(Sait et al., 1994b). From a pest control perspective, vertical transmission of virus and sublethal effects could be extremely important as they offer a "density-independent" mechanism for maintaining the virus in the pest population when numbers are low, as well as providing control benefits in terms of reduced fitness within the pest population (Burden et al., 2002).

1.6. Life Table Analysis in the Evaluation of Biological Control Agents

Life tables are useful for studying population dynamics of arthropods, and allow us to estimate parameters related to population growth potential (Maia et al., 2000). Life tables are valuable tools for analyzing the impact that an external factor has on growth, survival, reproduction and rate of increase of an insect population (Bellows et al., 1992; Wittmeyer and Coudron, 2001). The construction of life tables is particularly useful in the study of biological control agents, not only from a control perspective, i.e. to assess the impact of a control agent on the pest population, but also to assess the environmental impact of such control agents on other insects (Maia et al., 2000).

A number of parameters can be estimated from life tables; these are:

- *The net reproductive rate (R_o):* the mean net contribution per female to the next generation, expressed as the total number of offspring females per female, during the entire oviposition period (Maia et al., 2000).
- *The intrinsic rate of increase (r_m):* a constant value used to determine the population increase under specified physical conditions in an unlimited environment (Dent, 1997; Wittmeyer and Coudron, 2001). This provides a summary of an insect's life-history traits (Dent, 1997), and is essentially the difference between birth rate and death rate (Wittmeyer and Coudron, 2001).
- *The mean generation time (T):* the mean time span between the birth of individuals of a generation and that of the next generation (Maia et al., 2000).
- *The doubling time (Dt):* the time span necessary for doubling the initial population (Maia et al., 2000).

- *The finite rate of increase (λ):* this is a multiplication factor of the original population at each time period. The decimal part of the finite rate of increase corresponds to the daily rate of increase expressed as a percentage (Maia et al., 2000).

1.7. Respirometry and Metabolism

Metabolic rate (measure of the total energy metabolized by an animal in unit time) is one of the most commonly measured physiological variables and is valuable in comparative studies of animal adaptation and performance (Wilmer et al., 2000). Metabolic rate can be determined in one of four ways: by measuring the energy value of food ingested, against that of the waste excreted; measuring the amount of oxygen used up or carbon dioxide produced; measuring the amount of heat produced; or by measuring the amount of metabolic water produced (Wilmer et al., 2000).

In insects, metabolic rate varies depending on activity, size and temperature (Terblanche et al., 2004). Other factors that affect metabolic rate are age, sex, feeding status, season and time of day (Terblanche et al., 2004). Numerous studies indicate that metabolic rate also varies in an adaptive manner (Terblanche et al., 2004). For example, many insects from colder environments have higher metabolic rates than those from warmer climates, at the same temperature (Terblanche et al., 2004). This conservation of metabolic rate is thought to enable insects to complete growth, development and reproduction at relatively low temperatures (Chown and Gaston, 1999).

According to Djawden et al. (1997), exposure to stressful conditions, such as exposure to toxins, disease or adverse environmental conditions, are sufficient to induce changes in the metabolic rate of an insect. Few studies have focussed on the effects of parasites or pathogens on the metabolic rate of insects. Variation in metabolic rate was observed in a study of the effects of *Bacillus thuringiensis* (Bt) Cry1C toxin on the metabolic rate of Cry1C-resistant and susceptible *Spodoptera*

exigua (Dingha et al., 2004). The authors hypothesised that mechanisms of resistance to the Bt toxin may be associated with a metabolic cost which may be measured as an increase in metabolic rate compared with Bt susceptible insects. It was found that the metabolic rate of third instar Cry1C-resistant larvae reared continuously on diet containing toxin was significantly higher than the metabolic rate of the control populations. According to the authors, reduced toxin binding was not the major mechanism of resistance in *S. exigua* and therefore the increase in metabolic rate was probably associated with other mechanisms. For example, the destruction of midgut cells and the concomitant rapid increase in the number of stem and differentiating cells requires energy. The production of detoxifying enzymes induced by the presence of toxin is another process that likely requires energy, and in turn contributes to the observed increase in metabolic rate. Therefore, increased metabolic rate could indicate reduced fitness, as energy is diverted from everyday activities, and used for counteracting the effects/presence of the toxin. Similarly, where an increase in the metabolic rate of *Pieris brassicae* pupae 'infected' with a nylon filament was recorded (Freitak et al., 2003), the authors concluded that the activation of the immune system in insect pupae could be expressed in "energetic currency".

The common thread in these studies is that a response to the presence of a toxin, or invading microorganism, is associated with a fitness cost. In these examples, this was reflected as changes in the metabolic rate of challenged insects. In the case of sublethal baculovirus infections, it is well-known that fitness costs, in the form of for example, reduced fecundity, are incurred. The effects of sublethal baculovirus infection on the metabolic rate of larvae has however, not been explored.

1.8. Aims and Objectives

The safe and effective use of baculoviruses is dependent on a thorough knowledge of their biology and interactions with their hosts (Cory and Hails,

1997). The main objective of this study was to examine the interaction between *H. armigera* and its NPV. Specifically, the aims of the study were to:

- Obtain dose-mortality data for infections of *Helicoverpa armigera* SNPV (HearSNPV) in *H. armigera*.
- To investigate the effects of exposure of *H. armigera* to sublethal HearSNPV doses by recording alterations in a range of life-history characteristics.
- To investigate whether the exposure to sublethal HearSNPV doses carries costs that might be reflected in the metabolic rate of the host; this parameter has not previously been used to characterise sublethal infection.

Chapter 2

Determination of the Dose-Mortality Relationship between *Helicoverpa armigera* Single Nucleocapsid Nucleopolyhedrovirus and its host, *Helicoverpa armigera* (Lepidoptera: Noctuidae)

2.1. Abstract

Bioassays are essential for the characterisation of an insect pathogen and to assess its biological activity. Ranging assays were performed initially in order to distinguish lethal versus sublethal *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) doses, in neonate, second, third and fourth instar *H. armigera* larvae. From this, replicate bioassays were set up in order to obtain comprehensive dose-response/mortality information. Probit analyses indicated that median lethal dose (LD_{50}) values for the various instars, from the youngest to oldest, were 3, 249, 2.27×10^4 and 7.35×10^4 occlusion bodies/larva. The remainder of this thesis is based on studies of third instar larvae. For these larvae, the estimated LD_{25}, LD_{75} and LD_{99} values were found to be 6.61×10^3, 7.82×10^4 and 1.61×10^6 occlusion bodies/larva respectively.

2.2. Introduction

The genus *Nucleopolyhedrovirus* (NPV) is a group of large, arthropod-specific viruses that are able to produce lethal infection in their hosts (Washburn et al., 2001). The rod-shaped viruses comprise covalently closed, double stranded DNA genomes that are enclosed in a protein capsid; together, the DNA core and capsid are referred to as a nucleocapsid (Blissard et al., 2000). The viruses are designated as SNPV or MNPV, depending on whether the nucleocapsids occur singly (S) or in multiples (M) in the virion envelope (Blissard and Rohrmann, 1990). The virions in turn are surrounded by a protein matrix, or occlusion body (OB), that improves persistence of the virus in the environment (Cory and Hails, 1997). NPVs have been investigated for use as biological control agents of phytophagous insects, mainly those belonging to the orders Lepidoptera, Hymenoptera and Diptera (Chen et al., 2001).

Helicoverpa armigera has been described as one of the most "infamous of heliothine moths" (Szewczyk et al., 2006). The pest has an extensive world-wide distribution and attacks a range of agricultural and horticultural crops including cotton, soybean, sunflower, pepper and maize (Fitt, 1989; Szewczyk et al., 2006). Populations of *H. armigera* have developed resistance to a range of insecticides including members of the endosulfans, pyrethroids, carbamates and organophosphates (Fitt, 1989; Srinivas et al., 2004). *Helicoverpa armigera* SNPV (HearSNPV) is a host-specific pathogen and shows a high degree of virulence to its host. HearSNPV is currently produced on a large scale as a viral pesticide in China and is used for the protection of cotton fields against *H. armigera* (Chen et al., 2001). It was first registered in 1993 and has been used to treat 100 000 hectares of cotton (Szewczyk et al., 2006). Successes such as this promote NPVs as biological control agents and indicate that they are competitive alternatives to chemical pesticides for pest control.

Bioassays are essential for determining the infectivity of a particular virus or viral preparation, and for the comparison of different isolates or batches of the same or

different viruses (Evans and Shapiro, 1997). Initial bioassays are typically carried out in the laboratory so that one can maintain as much control as possible over the variability that might affect the results (Evans and Shapiro, 1997). In the absence of good dose-mortality data, it is necessary to carry out ranging assays before one initiates intensive bioassays (Evans and Shapiro, 1997). This approach was used in the current study; following analysis of the bioassay data, sublethal doses of HearSNPV against *H. armigera* could be selected for further experiments.

2.3. Materials and Methods

2.3.1. Insect Rearing

Eggs of *H. armigera* were originally obtained from the Agricultural Research Council, Pretoria, South Africa. A permanent culture was set up in the laboratory where larvae were reared on an artificial diet (Bot, 1966) at 28°C, 70% relative humidity (RH), with a 12:12 L:D photocycle. Oviposition took place on nets that covered glass cages containing moths. Eggs and pupae were sterilised using 0.2% (m/v) and 0.25% (m/v) sodium hypochlorite respectively, to maintain the sterility of the culture. Egg batches used for experiments were not sterilised.

2.3.2. Virus Propagation and Purification

HearSNPV was propagated *in vivo* using third instar *H. armigera* larvae. Larvae were inoculated by means of the diet contamination method (Evans and Shapiro, 1997), and were harvested just prior to liquefaction. Larvae were homogenised in 1% (w/v) SDS using a stomacher (Lab-Blender 400 BA 6021, Seward Medical House), followed by filtration through 2 layers of muslin cloth. The purification process is a modified version of the protocol described by Crook and Payne (1980). The filtered suspension was subjected to low speed centrifugation in order to remove insect debris. The supernatant was removed and run through a glycerol [20 – 90% (v/v), 13 000 g, 40 min.], and then sucrose gradient [35 – 65% (w/w),

46 000 g, 1.5 hours]. The gradients were prepared using sterile distilled water. Following each rate-zonal centrifugation step, the band containing OBs was removed and washed in sterile distilled water. The purified OB sample was run through two final washes in sterile distilled water. The OBs were resuspended in sterile distilled water and quantified using a haemocytometer. The sample was stored at -20°C.

2.3.3. Bioassays and Dose-Mortality Relationship of HearSNPV in Different Instars of H. armigera

All bioassays were performed in growth rooms set at 28°C, 70% RH at a 12:12 L:D photocycle. Neonates were used within 6 hours of hatching, and were infected by droplet-feeding (Hughes and Wood, 1981) using solutions containing 0.5 mg/ml erioglaucine, a blue dye, and virus, diluted in sterile distilled water. In this instance, dose calculations were based on the volume of liquid imbibed by neonate larvae (Bouwer and Avyidi, 2006). The age of older larvae was determined by measuring their head capsule size. Second, third and fourth instar larvae were infected by presenting them with cubes of artificial diet inoculated with virus. Larvae that ate the entire cube within 24 hours were transferred to glass vials containing the artificial diet, while those that did not eat the entire cube in the given time were excluded from the experiment. Controls for the experiment were treated in the same manner, but sterile distilled water was used to 'inoculate' the larvae/diet cubes.

Larvae of each instar were infected with a range of trial doses (usually 6) that produced between 10 and 90% mortality. Where necessary, the doses were adjusted until the desired range was obtained. Once suitable doses were determined, twenty-four larvae were infected for each dose tested against a particular instar, and the entire set of infections was repeated 6 times for each instar. Mortality was recorded after 7 days. LD_{50} values were estimated by probit analyses of mortality data using the LdP Line software program (Ehab Mostafa Bakr, 2000). In addition to transforming the raw data, the LdP Line program used

for analyses calculated the slope of each probit regression line, and the Chi-squared (χ^2) statistic (to test for goodness-of-fit).

2.4. Results

The results of the dosage-mortality studies are shown in Table 2.1 and Figure 2.1. The relative LD_{50} values for each instar differed considerably; they were estimated as 3, 249, 2.27 x 10^4 and 7.35 x 10^4 OBs/larva for neonate, second, third and fourth instars respectively. A comparison of the LD_{50} values indicates that neonates are significantly more susceptible to infection than second, third and fourth instar larvae. The slope values for neonate and the second instar were similar, while the slope values for the third and fourth instar were considerably lower. The χ^2 value for each regression line (values not shown) was not significant at 95% probability level indicating no systematic heterogeneity of response in any of the instars. Probit analysis of third instar data was of particular interest as this data was used for subsequent experiments. For these insects, the estimated LD_{25}, LD_{75} and LD_{99} values were 6.61 x 10^3, 7.82 x 10^4 and 1.61 x 10^6 OBs/larva respectively.

2.5. Discussion

Bioassays are essential for the characterisation of isolates that have potential as control agents. The most commonly used method for analysis of dosage-mortality data is probit analysis which involves log normal transformation of data so that the typical sigmoidal curve obtained in dose-mortality experiments, can be linearized and compared using LD_{50} and slope values (Evans and Shapiro, 1997). According to Whitlock (1977), comparison of the slopes can be used to demonstrate resistance of different instars to virus infection. The values of the slopes obtained for third and fourth instar larvae were similar, and both were lower than those obtained for younger insects. The lower slope obtained in older

larvae indicates a less uniform response from the insects to HearSNPV. In other words, greater variability in susceptibility is observed in older larvae.

The LD_{50} values increased substantially between instars (from neonate to fourth instar larvae). Developmental resistance to NPVs in insects is well documented (Whitlock, 1977; Evans, 1983; Williams and Payne, 1984; Teakle et al., 1986; Sait et al., 1994a). In a study conducted by Whitlock (1977), a single viral dose was used to infect neonates and larvae that ranged in age from 4 to 13 days old (i.e. a batch of insects was infected daily from 4 days post hatch until 13 days post hatch). The mortality decreased from 100% in neonates and young larvae, to no mortality in 13 day old insects.

Initially, developmental resistance was thought to be related solely to an increase in the size of insects where, for example, an increase in the volume of the gut lumen as insects grow, reduces 'chance encounters' between the virus and the cells of the midgut (the presence of food in the midgut is also likely to reduced the chance of interaction between midgut cells and virus) (Engelhard et al., 1991). In experiments that used intrahaemocoelic injection as a method for inoculation, developmental resistance was not reported (Teakle et al., 1986). Such studies suggest that developmental resistance may be related to events in the midgut (Teakle et al., 1986). It appears that as insects get older within an instar, the ability to shed midgut cells increases, and the susceptibility of midgut cells to infection, decreases (Engelhard and Volkman, 1995).

A number of studies have investigated dose-mortality data of HearSNPV against *H. armigera* (Whitlock, 1978; Flattery, 1983; Williams and Payne, 1984; Tuan et al., 1989; Figueiredo et al., 1999; Herz et al., 2003; Guo et al., 2006). When the pathogenesis of wildtype Spanish and Chinese isolates of HearSNPV was compared in neonate *H. armigera*, LD_{50} values of 32 and 34 OBs/larva respectively were reported (Herz et al., 2003). These LD_{50} values are considerably higher than that reported in this study for neonate larvae, suggesting that the isolate used in this study may be more virulent. Williams and Payne (1984) also

investigated the biological activity of HearSNPV against neonate *H. armigera*; they obtained an LD_{50} of 15 OBs/larva, an estimate closer to the LD_{50} reported here, than that reported by Herz et al. (2003). In the present study, and that of Herz et al. (2003) and Williams and Payne (1984), the experimental conditions were similar, but not identical. For example, in the study conducted by Herz et al. (2003), the insects were maintained at 26°C and 70% RH with a 16:8 hour light/dark cycle. The insects were inoculated using contaminated leaf discs and were given 48 hours in which to consume the entire leaf disc. Such differences in protocol may account, in part, for the large discrepancy in LD_{50} observed between the different studies. Having said this, a considerable degree of variability is observed in the biological characteristics of NPV isolates from the same host species (Guo et al., 2006). Furthermore, it is likely that the biological characteristics, such as susceptibility to HearSNPV infection, of the insect populations used in different studies would differ. Other factors, such as the diet used for insect rearing, may also impact on the fitness of a population.

Guo et al. (2006) characterised the biological activity of two HearSNPV isolates in third instar *H. armigera* larvae. They reported LD_{50} values of 568 and 1584 OBs/larva for the two isolates tested. These values are much lower than the LD_{50} value for third instar larvae (2.27×10^4 OBs/larva) reported in this study, suggesting that our isolate is not as virulent as the those tested by Guo et al. (2006). The large discrepancy could, in part, be accounted for by the different approaches used for inoculation of the insects. While the rearing conditions before and during the bioassays were similar to those used here, the authors starved the test insects for 16 - 24 hours and allowed them to moult into third instars prior to inoculation. The starvation period may have compromised the insects (i.e. they may have been weakened to a degree), improving the potential of the virus to initiate infection. As no food was passing through the insect's system, the possibility for infection of midgut cells may have increased. Furthermore, infected midgut cells are shed at each moult so the later in an instar that an insect becomes infected, the less time the virus has to be transmitted to secondary sites of infection (Engelhard and Volkman, 1995; Volkman, 1997). Also, the rate of

establishing or sustaining infection in the midgut appears to decrease as larvae get older within an instar (Engelhard and Volkman, 1995; Volkman, 1997). If insects are inoculated early in an instar (i.e. just after moulting), the virus has more time to be transmitted to secondary sites of infection.

In the current study, larvae were infected the day after they had moulted to third instar, and head capsule slippage, indicating the onset of moulting to the fourth instar, had begun by the following day (i.e. on the day that they were returned to diet after having ingested the inoculated diet cube). Furthermore, no starvation period was included. This considered, in their study, Williams and Payne (1984) infected late third instar larvae using contaminated leaf discs which insects were allowed to consume over a 24 hour period. According to the authors, the larvae had moulted to the fourth instar within the 24 hour inoculation period. For these insects, they reported an LD_{50} of 670 OBs/larva as opposed to 2.27×10^4 and 7.35×10^4 OBs/larva reported here for third and fourth instar larvae respectively. Despite the moulting process, and the possible loss of some inoculum in shedded midgut cells, 50% mortality was obtained at a relatively low dose, suggesting that the isolate used by Williams and Payne (1984) is highly virulent.

The LD_{50} values obtained from a comparison of two Spanish and a Russian HearSNPV isolate, tested against third instar larvae, by Figueiredo et al. (1999), were closer to those obtained by Guo et al. (2006). Figueiredo et al. (1999) obtained values that ranged from 68 to 414 OBs/larva when the various HearSNPV isolates were tested against newly moulted third instar larvae. Flattery (1983) obtained slightly higher LD_{50} estimates for HearSNPV against third instar *H. armigera* (1.57×10^3 OBs/larva), and in the case of fourth instar larvae, obtained a higher LD_{50} estimate (2.67×10^5 OBs/larva) than that reported in the current study.

Comparison of the bioassay data reported in this study with previous studies suggests that there is a high level of variability in the biological activity of HearSNPV isolates from a range of geographic areas, and generally, the isolate

used in this study was not as virulent as those tested in similar experiments. While the exact time of inoculation within an instar, or approach used for inoculation vary, and probably impact on the observed outcome, these cannot account fully for the range of LD_{50} values reported in various studies for a particular instar. It is likely that insect populations used in different studies differ considerably (for example, slightly higher rearing temperatures may promote growth of the insects, or differences in the diets used for rearing may affect the quality of the host). Another important point to consider in comparison of bioassay data is the method of virus purification. Crude extracts have been found to be more virulent than highly pure samples. This is thought to be linked to the potential presence of pathogenic bacteria and other microorganisms which serve to further challenge the insect (Flattery, 1983). In all the studies mentioned here, the virus samples used were purified.

In the context of this thesis, bioassays were required to characterise the virulence of the HearSNPV isolate used for further experiments. Analyses of bioassay data provided estimates of LD_{25} and LD_{75} values that were used for subsequent studies of the effects of sublethal doses of HearSNPV on *H. armigera*.

Table 2.1. LD_{50} values obtained for neonate, second, third and fourth instar *H. armigera* larvae.

Instar	LD_{50} (OBs/larva)	RR[a]	Slope ± SE[b]
First (neonates)	**3**	1	1.88 ± 0.11
Second	**249**	83	2.03 ± 0.13
Third	**2.27×10^4**	7.6×10^3	1.26 ± 0.10
Fourth	**7.35×10^4**	2.5×10^4	1.18 ± 0.09

[a] Resistance ratio (RR) derived from comparison with neonates.

[b] Slope ± standard error (SE).

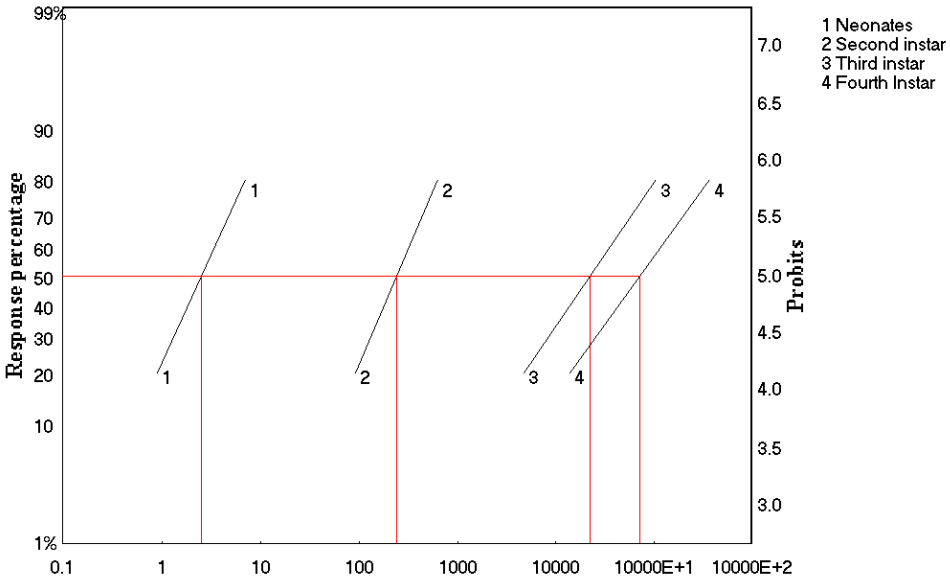


Figure 2.1. Probit analyses of bioassay data obtained when larvae of different age were infected with different HearSNPV doses. Response (mortality) percentages are indicated by the axis on the left, while the probits are depicted by the axis on the right. The LD_{50} value for each instar is indicated by the red line (estimated values are provided in Table 2.1).

Chapter 3

Evaluation of the Effects of Sublethal Nucleopolyhedrovirus Doses on *Helicoverpa armigera,* using Fertility Life Table Parameters

3.1. Abstract

Sublethal effects of nucleopolyhedroviruses (NPVs) are thought to have an important impact on the ecology and population dynamics of a host population. In addition, these effects are thought to enhance their value as potential pest control agents. In the current study, the effects of exposure to a sublethal dose of *Helicoverpa armigera* single nucleocapsid NPV (HearSNPV) on various life-history and fitness traits of third instar *H. armigera* were investigated. Larvae that survived inoculation with HearSNPV were monitored until pupation. Both male and female survivors exhibited a reduction in immature stage development time, but no differences in pupal mass, when compared with uninoculated controls. The effects of sublethal doses on fecundity were investigated by mating female and male inoculation survivors with healthy insects of the opposite sex. The number of eggs laid by each mating pair was recorded daily and subsequently, the proportion of eggs that hatched counted. Sex ratio and survival amongst offspring of these mating pairs were not significantly different to controls, although, the egg hatch amongst treated groups was lower than that of controls. Life tables were constructed for each population. Comparison of the estimated life table parameters indicated that the control population was, on the whole, more successful than populations derived from mating pairs in which one partner was exposed to virus. Life table parameters, estimated by the jackknife technique, proved to be a sensitive method for assessing the impact of sublethal treatment on a pest population.

3.2. Introduction

Nucleopolyhedroviruses (NPV) have been studied extensively as biological control agents. They are particularly advantageous as they are highly pathogenic to their insect hosts, are host-specific, and therefore do not pose a threat to other, non-target organisms (Moscardi, 1999), and are able to persist in the environment as the virus particles are protected in a protein matrix (Zhou et al., 2005). While NPV research has focussed on lethal infections of insects, where insects are killed during the larval stages (Goulson and Cory, 1995; Cory et al., 1997), sublethal infection for the long-term suppression of a pest population has gained importance (Myers and Kuken, 1995). Sublethal infections have a significant impact on the density of an insect population, and on the population dynamics of their hosts (Rothman and Myers, 1996). In the context of integrated pest management systems they are particularly valuable.

Sublethal NPV infections have been associated with changes in a variety of life-history traits and appear to cause a reduction in the fitness of their hosts. When compared with uninfected controls, insects that survive NPV infection often show alterations in larval development with respect to size, moulting and pupation (Burand and Park, 1992), changes in development time (Goulson and Cory, 1995), reduced fecundity, reduced egg viability (Milks et al., 1998) and changes in sex ratio (Duan and Otvos, 2001). Another interesting aspect of sublethal infection is the vertical transmission of the virus across generations, either transovum, or transovarially (Kukan, 1999; Myers et al., 2000; Zhou et al., 2005). These infections are therefore considered to be a means by which NPVs persist in the environment. Sublethal effects of viruses have been reviewed in detail by Rothman and Myers (1996). According to their survey of studies of sublethal infection and effects, the inclusion of deleterious effects caused by sublethal NPV infection could reduce the growth of a population by an additional 22%, over that of NPV-induced mortality alone.

Sublethal infections are either latent or persistent. In the first instance, no or minimal gene expression occurs, while in the latter, a range of genes are expressed, but at a very low level (Burden et al., 2003; Cory and Myers, 2003). The effects of sublethal infection are thought to occur when resources are diverted from everyday activities to defence following initial viral challenge, and then for defence against long-term persistent infection (Cory et al., 1997). For example, sloughing of midgut cells in the presence of virus is one mechanism by which insects avoid infection. This process requires energy that would normally be used for growth, development or reproduction (Cory et al., 1997). The actual presence of viruses in tissues and organs, as well as hormonal changes induced by the virus, are also thought to impact negatively on the insect (Myers et al., 2000).

Life tables are extremely valuable for analyzing and understanding how external factors affect the growth, survival, reproduction, and rate of increase of an insect population (Bellows et al., 1992). They have been used to obtain information regarding the life cycle of pests and effectively evaluate the quality of a laboratory reared pest population (Garcia et al., 2006), investigate the effect of temperature on reproductive capacity and longevity (Kivan and Kilic, 2005), evaluate the impact of diet on the development of insects (Valicente and O'Neil, 1995; Wittmeyer and Coudron, 2001), and for the evaluation of biological control agents, not only from a control perspective (Bellows et al., 1992), but also to evaluate the environmental impact of these control agents (Nascimento et al., 1998).

The parameters usually estimated from life tables are the net reproductive rate (R_o), the intrinsic rate of increase (r_m), the mean generation time (T), the doubling time (Dt), and the finite rate of increase (λ) (Maia et al., 2000). According to Maia et al. (2000), comparison of life table parameters of different groups or populations requires that we have information on the degree of uncertainty, or variance, associated with its estimates. In the case of 'measurable' or observable values, such as the number of eggs laid by a female, this is easy to calculate. In the case of artificial parameters, such as those used to construct life tables,

variances must be calculated by alternative methods. Maia et al. (2000) developed algorithms for estimating R_o, $r_{m.}$, T, Dt and λ with their respective jackknife variances and confidence intervals. Their program performs specific pairwise comparisons between populations using one- or two-tailed *t*-tests based on jackknife variance estimates (Maia et al., 2000). They further suggest that a multiple comparison approach be used when more than two test populations are involved.

Information of population growth potential and population dynamics is essential for evaluating alternative pest control agents (Maia et al., 2000), such as NPVs. In this study, life tables were used to assess the impact of exposure to sublethal doses of NPV on the development and rate of increase of *Helicoverpa armigera*, a significant pest of various horticultural and agricultural crops (Fitt, 1989).

3.3. Materials and Methods

3.3.1. Insect Rearing

Eggs of *Helicoverpa armigera* were originally obtained from the Agricultural Research Council, Pretoria, South Africa. Larvae were reared on an artificial diet in the laboratory at 28°C, 70% relative humidity (RH), with a 12:12 L:D photocycle. Eggs and pupae were sterilized using 0.2% (m/v) and 0.25% (m/v) sodium hypochlorite respectively to maintain the sterility of the culture. Eggs used for experiments were not sterilized.

3.3.2. Virus Propagation and Purification

Helicoverpa armigera single nucleocapsid NPV (HearSNPV) was produced *in vivo* in third instar *H. armigera* larvae. Larvae were infected using the diet contamination assay. Larvae were monitored daily and were collected just before they died. Details of the purification protocol are provided in Chapter 2. Briefly,

cadavers were macerated in 1% (w/v) SDS, filtered through two layers of muslin, and subjected to low speed centrifugation to remove large debris. The supernatant was removed, and run through a glycerol gradient. The band containing occlusion bodies (OBs) was removed, washed in sterile distilled water, and then run through a sucrose gradient. The OB-containing band was once again removed and washed, followed by two additional wash steps. The OBs were resuspended in sterile distilled water, counted using a haemocytometer, and stored at -20°C.

3.3.3. Inoculation of Larvae

Ranging assays were performed in order to establish sublethal and lethal doses, after which comprehensive bioassays were performed (as described in Chapter 2). Third instar larvae were infected with a single sublethal HearSNPV dose (LD_{25}). Larvae were infected by presenting them with diet cubes contaminated with virus. Larvae that ate the entire cube within 24 hours were transferred to numbered glass vials containing artificial diet while larvae that did not eat the entire cube within the given time period were excluded from the study. Mortality was recorded, and larvae that pupated were kept for further study.

3.3.4. Sublethal Effects

The pupation date for larvae that survived exposure to HearSNPV was recorded. The pupae were weighed 48 hours post-pupation, sexed and placed on vermiculite in small trays. The trays were covered with a layer of clear cling wrap that was pierced several times with a needle to allow air to get into the trays. The same procedure was followed for uninoculated insects that were used as controls and as mating partners for treatment survivors. When treatment survivor moths emerged, the emergence date was recorded and moths were paired with uninoculated insects of the opposite sex that emerged on the same day. Mating pairs were kept in small plastic 540 ml containers and covered with netting that served as a removable substrate for egg laying. Moths were fed daily with cotton rolls soaked in sucrose (5% w/v) that were placed on the netting. When the first egg batches were laid,

nets and cotton rolls (moths often laid eggs on these as well) were removed and replaced. Eggs were counted, with the aid of a dissecting microscope, on the same day that a net and cotton roll were removed. Once the eggs were counted, the nets and cotton rolls were kept in clear plastic freezer bags at 28°C, 70% RH at a 12:12 LD photocycle. Larvae that hatched from these eggs were shaken off the nets daily until no further hatching was observed (this was done because larvae that hatch eat other eggs). Nets were then refrigerated and kept so that the number of unhatched eggs could be counted. This value was then subtracted from the number of eggs initially laid in that particular batch and the values recorded as the number of fertile eggs laid/female. Ten larvae of each mating pair were transferred to artificial diet. These larvae were monitored until emergence. The mating pairs were monitored until death, and the date on which each moth died, recorded. In some instances, males and females were unable to separate themselves after copulation – these pairs were excluded from the study.

3.3.5. Data Analysis

The effect of treatment on immature stage duration and pupal mass of females was compared between groups using ANOVA and in males using the *t*-test (Statistica, Version 6.0, Statsoft Inc., 2001). Immature stage survivorship and sex ratio data were analysed using the χ^2 test (with Yates' correction) for significant differences between test groups (Statistica, Version 6.0, Statsoft Inc., 2001). The net reproductive rate (R_o), intrinsic rate of increase (r_m), finite rate of increase (λ), mean generation time (T) and doubling time (Dt) were estimated using the SAS program developed by Maia et al. (2000). The program was executed using SAS, Version 9.1 (SAS Institute, Cary, NC). Details of the equations and algorithms used to calculate the various parameters are described by Maia et al. (2000).

3.4. Results

Immature stage duration of female insects differed significantly ($F_{2,76}$ = 21.6, $P < 0.05$) between groups where the duration of the immature stage of treated females was shorter than that of control females and females paired with treated males (Table 3.1). The pupal masses of control females and those from each of the treated groups were not significantly different ($F_{2,76}$ = 0.1, $P > 0.05$) (Table 3.1). Similarly, survival during the immature stages, and the sex ratio of offspring were not affected by exposure to sublethal HearSNPV doses (Table 3.1) ($P > 0.05$ in both instances). This said, the highest survival rate was observed for controls, followed by offspring of treated females, and then offspring produced by females fertilised by treated males. Finally, females exposed to HearSNPV as larvae produced, on average, the lowest number of fertile eggs. Control females, and females paired with treated males produced a similar number of fertile eggs (Table 3.1). The immature stage duration of male treatment survivors was significantly shorter (t = -7.8, df = 54, $P < 0.05$) than that of untreated males (Table 3.2). As in the case of females, pupal masses of males treated with HearSNPV were not significantly different to controls (t = -0.16, df = 54, $P > 0.05$).

The life table parameters are shown in Table 3.3. The Dt was significantly higher ($P < 0.05$) in treated populations than in controls, but no significant differences ($P > 0.05$) in T were recorded between the uninfected control population, and the treated groups. The R_o was highest in the control population (this was significantly higher, $P < 0.05$, than the R_o values obtained for the other test groups), followed by untreated females mated with males that survived treatment, and then female treatment survivors paired with uninfected males. Similarly, the r_m and λ of populations derived from treated males and females was significantly lower ($P < 0.05$) than in the control population. The lowest R_o, r_m and λ values were obtained for the population derived from females treated with HearSNPV.

The average daily oviposition patterns of females of different treatment groups are shown in Figure 3.1. The minimum female age at oviposition varied little between the different treatment groups. The earliest onset of oviposition was recorded in treated females that were paired with untreated males. The duration of the population oviposition period (the oviposition period of the population as a whole) did not vary much between treatment groups and was approximately 15 days for control and for untreated females paired with treated males, and 17 days for treated females paired with untreated males.

3.5. Discussion

In the current study, it was found that while the pupal mass of treated insects, and the sex ratios and survival of offspring produced by treated mating pairs were not significantly affected by exposure to sublethal doses of HearSNPV, the duration of the immature stage and fecundity were notably altered.

The duration of the immature stages of both male and female insects treated with HearSNPV were significantly shorter than those recorded in untreated male and female controls. Although cases of reduced development rates have been recorded (Rothman and Myers, 1996), sublethal infections are typically associated with an increase in development time (Goulson and Cory, 1995; Milks et al., 1998; Duan and Otvos, 2001; Prater et al., 2006). For example, the development time, from inoculation to pupation, of sublethally inoculated *Mamestra brassicae* was longer than that of controls, and as the dose increased, so too did the development time (Goulson and Cory, 1995). The increase in development time was attributed to the "establishment" of sublethal infection in a proportion of the surviving larvae (Goulson and Cory, 1995). If this were the case, the presence of a sublethal infection likely required that host resources that would normally be used for growth and development, be diverted and used in defence against the possibility of a lethal infection.

The decrease in development time recorded in this study was unexpected and the reason for this unclear. Magnoler (1974) reported reduced pupal development time in gypsy moth pupae infected with a cytoplasmic polyhedrosis virus. The author suggests that this may be related to hormonal imbalances caused by the virus (where hormone synthesis and distribution are affected). Fluctuations in environmental temperature are also known to affect the development rate of insects. Within a favourable range, a rise in environmental temperature results in an increase in the development rate of insects (Gullan and Cranston, 1994). This is unlikely to be a factor here due to the nature of the growth rooms used (growth rooms were relatively small so that temperature variation inside the chamber would be minimal), and the fact that all the insects were kept in the same area of the chamber throughout the experiment. It is also possible that changes in metabolic rate of the host in response to infection may affect the rate of host development. For example, immune system activation would likely be associated with a significant increase in metabolic rate (Freitak et al., 2003). This could lead to an increased rate of development, and concomitant reduction in the duration of the immature stages. While the above provide possible explanations for the observed reduction in immature stage duration, further studies are required in order to draw any conclusions.

The effects of sublethal doses on pupal mass are variable. In some instances, and as recorded in this study, no significant differences have been recorded in treated versus control populations (Perelle and Harper, 1986; Goulson and Cory, 1995), while other studies have shown significant decreases in pupal mass of inoculation survivors versus controls (Myers et al., 2000; Prater et al., 2006). Milks et al. (1998) reported significant decreases in the mass of both male and female pupae that survived exposure to virus as larvae. They found that the mass of pupae of larvae treated at a younger age (4 days old) was significantly lower than the mass of control pupae, but no significant difference in mass was recorded between pupae that developed from larvae treated at an older age (6 and 8 days old) and untreated controls. Duan and Otvos (2001) reported significant increases in pupal

mass of insects treated with lower sublethal doses, and significant decreases in pupal mass of insects treated with higher sublethal doses.

Pupal mass and fecundity are often correlated (Milks et al., 1998). However, in many studies that have investigated both parameters, the effects of treatment on fecundity are often greater than the effects of treatment on female pupal mass, or on the pupal mass of both sexes (Rothman and Myers, 1996; Milks et al., 1998). This has proven to be the case in the current study where, when compared with their respective controls, no significant differences in pupal mass were recorded for female or male pupae that were exposed to virus as larvae, despite a reduction in fecundity of females belonging to the different treatment groups.

No significant differences in the survival and sex ratio amongst offspring of mating pairs were recorded. Egg hatch and rate of survival of offspring was highest in the control population. This was consistent with trends observed in other studies (Goulson and Cory, 1995; Milks et al., 1998). In the current study, offspring of mating pairs in which one partner was treated generally died very early (i.e. before they reached second instar), such that it was difficult to confirm if death occurred with symptoms of viral infection, or as pupae. Previous studies have reported viral induced mortality in offspring of treated parents, but this occurred at very low levels i.e. about 0.55% mortality (Goulson and Cory, 1995; Myers et al., 2000).

In studies in which the sex ratio of infection survivors has been considered, the results have been inconsistent. Duan and Otvos (2001) recorded a significant decrease in the proportion of females amongst survivors treated at the sixth instar but no significant difference in the sex ratio of insects treated at the fourth instar. Studies by Perelle and Harper (1986) and Goulson and Cory (1995) report no significant difference in the sex ratio of treatment survivors. In the current study, of insects that were inoculated with HearSNPV, more males survived exposure than females (results not shown). Amongst offspring of mating pairs in which one partner was treated, the sex ratio favoured males (as opposed to females in the case of controls). In all instances though the results were not significant,

suggesting that males and females are more or less equally susceptible to sublethal infection, and that sublethal infections do not have a major impact on sex ratios of offspring. Sex ratio has a significant impact on insect population dynamics and sex ratios that favour males negatively affect the ability of a population to increase (Duan and Otvos, 2001).

A number of studies have shown that insects that survive exposure to NPV are compromised in a variety of fitness and life-history characteristics. Few studies have investigated these effects in *H. armigera*, and to our knowledge, the construction of life tables and the statistical comparison of life table parameters based on jackknife variance estimates has not been used to characterise the effects of exposure to sublethal doses of NPVs in their hosts.

Life tables are useful as they allow us to effectively compare different populations by combining information on survival, development and reproduction, into a single parameter (Bouwer, 2003). Jackknife estimates of life table parameters were compared statistically in order to determine the extent of treatment effects associated with sublethal doses. According to Rothman and Myers (1996), the frequency and extent of deleterious effects should not be used on their own to assess the potential growth of a population. Significant changes in the quality of an individual do not necessarily have biological significance and as a result, analysis of various deleterious effects on their own may lead one to underestimate the effects of a pathogen. The use of life tables provides a method by which various characteristics can be considered together and related to the potential growth of a population.

Life table analysis indicated that the T value was similar between groups and no significant differences were recorded. The Dt of the control population was however, significantly lower than that of populations derived from mating pairs in which either the male or female insect survived exposure to sublethal doses at the third instar. Reduced fecundity was illustrated clearly in the life table analysis where significant differences were recorded between the relevant parameters for untreated controls versus treated females paired with untreated males, and treated

males paired with untreated females. The R_o of controls was significantly higher than that of treated groups and female treatment survivors had the lowest R_o of all three groups. Similarly, the r_m and λ values were highest in controls. Research with other insects has shown that variation in fecundity, delay in oviposition, or an increase in developmental rate to adulthood will significantly decrease the λ value of an insect population (Wittmeyer and Coudron, 2001). Furthermore, the values for each life table parameter obtained by true calculations were almost identical to those calculated by the jackknife technique. As a result, the estimates obtained for this study were acceptable. Where a large discrepancy between true calculations and jackknife estimates occurs, it is considered preferable to consider values obtained from true calculations (Maia et al., 2000).

Calculation and statistical comparison of the various life table parameters show that the control population had the shortest Dt and highest R_o, r_m and λ values. These results suggest that the treated populations were not as successful as the control population, and that treatment with sublethal doses does contribute to a significant overall reduction in population size. Untreated females that were paired with males that survived treatment had a shorter Dt, and higher R_o, r_m and λ values than the population derived from female treatment survivors, although the differences were not significant. This would imply that the quality of females in a population affects the success of reproduction to a greater extent than if male quality is reduced. It would follow then, that if males and females are affected by treatment with sublethal doses to an equal degree, mating with a healthy female partner may compensate for any debilitating effects in males. Furthermore, it is likely that if males and females that survived exposure to virus were paired, as might occur in a natural population, the observed trend would be amplified. This was observed in sublethally infected *Bombyx mori* males and females that were mated (i.e. reduction in fecundity was more significant in these mating pairs than in those in which only one partner had been exposed to virus) (Khurad et al., 2004). The same study reported that the mating of sublethally infected *B. mori* females with uninfected males resulted in significantly reduced fecundity and lower egg hatch while the hatching success of eggs produced by untreated females

that were paired with male infection survivors, was severely reduced (Khurad et al., 2004). These results are consistent with those reported in this study.

The exact mechanisms by which sublethal effects occur are unclear, but a number of possible explanations have been suggested. The actual presence of virus in host cells and tissues is likely to impact on various aspects of host physiology. This is frequently used to explain reductions in reproductive output due to the infection of testes and ovaries with NPV (Santiago-Alvarez and Vargas-Osuna, 1988; Rothman and Myers, 1996; Milks et al., 1998; Khurad et al., 2004). For example, Khurad et al. (2004) observed OBs in cells of the testicular follicle, and in immature sperm cells such that spermatophytes that were about to undergo reduction division were carrying virions and at the adult stage, sperm were infected. Such observations might explain the results recorded in this study i.e. the observed drop in reproductive output of mating pairs in which one adult was exposed to HearSNPV.

Other mechanisms by which sublethal infections manifest themselves are linked to defence processes. It is thought that when insects are challenged, energy and resources that are used for everyday activities such as growth, development or reproduction are diverted and used for defence against viral challenge. Linked to this, is the question of whether insects escape infection entirely, or whether infection actually occurs. If insects are able to remove the virus from their systems before infection is established (as might occur if they are about to moult, or through midgut cell sloughing), any observed sublethal effects are likely to be minimal (but avoiding infection may have a fitness cost associated with it). If infection does occur, sublethal effects are likely to be more obvious and are probably brought about by virus-induced cell damage, or from the use of various defence mechanisms (e.g. the use of the immune system or virally-induced apoptosis) that require resources that would normally be used for general maintenance. These processes are also associated with a certain degree of damage to host cells.

The type and severity of reported sublethal effects varies considerably and no consistent trends emerge. Many factors can affect the observed outcome including the age at which insects are inoculated or the dose used (Goulson and Cory, 1995). Generally, these effects are more obvious in older instars (from about third instar), and with higher sublethal doses (Goulson and Cory, 1995; Rothman and Myers, 1996; Milks et al., 1998). In this study, a relatively low viral dose was used for inoculation of insects; it is likely that had a high dose been used, the type and extent of observed effects would be different to those observed here.

Despite the variability observed in studies of insects exposed to sublethal viral doses, it is clear that the occurrence of debilitating effects associated with such treatments have an important impact on population dynamics. Significant sublethal effects were recorded in this study despite the relatively low dose used The fact that individual quality was reduced, particularly in terms of reproductive output, lead to an overall reduction in the success of the population, as demonstrated by statistical analysis of life table parameters. Evaluation of non-integrative parameters (immature stage duration, pupal mass, fertility, and survival and sex ratio of offspring) suggested only a limited impact of treatment with sublethal doses where the duration of the immature stages was the only parameter that was statistically significantly affected. Evaluating the population using estimated life table parameters that integrate information on growth, reproduction and survival, suggested a significant impact of sublethal treatment on the population. The construction of life tables and the estimation of life table parameters proved to be a highly sensitive method for assessing the impact of the exposure of an insect population to sublethal doses of NPV.

Table 3.1. Life-history parameters of untreated female *H. armigera* paired with untreated males (control), untreated females mated with males that survived exposure to HearSNPV (M_F), and females that survived exposure to HearSNPV (F) paired with untreated males.

Group	Duration of immature stages (days) [1]	Pupal weight (mg) [2]	Fertility (eggs/female) [3]	Immature stages survival (%) [4]	Sex ratio (male:female) [5]
Control	23.8 ± 0.1 a (N = 36)	274.1 ± 8.0 a (N = 36)	152.8 ± 11.5	91.7 a	1:1.1 a
M_F	23.9 ± 0.1 a (N = 21)	284.9 ± 9.7 a (N = 21)	154.6 ± 7.7	83.5 a	1.2:1 a
F	22.8 ± 0.1 b (N = 22)	277.8 ± 13.4 a (N = 22)	126.7 ± 11.9	89.6 a	1.4:1 a

[1] Includes duration of egg, larval and pupal stages, mean ± standard error (SE). Significant difference (ANOVA, $F_{2, 76} = 21.6$, $P \leq 0.05$) between immature stage duration of controls and treated insects. Means with different letters are significantly different.

[2] Mean ± SE. No significant difference between pupal masses of insects from control and treated groups (ANOVA, $F_{2,76} = 0.1$, $P > 0.05$).

[3] Mean ± SE. Average number of fertile eggs laid per female, calculated by dividing the sum of the mean number of eggs laid by each female that hatched, by the total number of females.

[4] No significant difference in immature stages survival ($P > 0.05$; Yates' continuity corrected χ^2 test).

[5] No significant difference in sex ratios between treatment groups ($P > 0.05$; Yates' continuity corrected χ^2 test).

Table 3.2. Life-history parameters of untreated male *H. armigera* (control) and males that survived treatment as larvae (M)

Group	Duration of immature stages (days) [1]	Pupal weight (mg) [2]
Control	24.9 ± 0.1 a	291.4 ± 6.5 a
	(N = 29)	(N = 29)
M	24.0 ± 0.1 b	289.6 ± 9.1 a
	(N = 27)	(N = 27)

[1] Includes duration of egg, larval and pupal stages, mean ± SE. Significant interaction (t = -7.8, df = 54, $P \leq 0.05$) between immature stage duration and treatment. Means with different letters are significantly different.

[2] Mean ± SE. No significant interaction between (t = -0.16, df = 54, $P > 0.05$) pupal weight and treatment.

Table 3.3. Life table parameters, as determined using the jackknife technique, of *H. armigera* belonging to the following experimental groups: untreated female moths paired with untreated males (control), untreated female moths paired with males that survived treatment (M_F), and female moths that survived treatment, paired with untreated males (F). All values are given as mean ± SE, values with different letters are significantly different ($P \leq 0.05$). Dt, doubling time; T, mean generation time; R_o, net reproductive rate; r_m, intrinsic rate of increase; λ, finite rate of increase.

Group	Dt (days)	T (days)	R_o (female/female)	r_m (female/female/day)	λ (female/female/day)
Control	2.39 ± 0.02 a	23.60 ± 0.26 a	948.276 ± 55.301 a	0.291 ± 0.002 a	1.337 ± 0.003 a
M_F	2.47 ± 0.02 b	23.73 ± 0.12 a	777.913 ± 36.846 b	0.281 ± 0.003 b	1.324 ± 0.004 b
F	2.49 ± 0.04 b	23.59 ± 0.33 a	695.984 ± 34.034 b	0.278 ± 0.005 b	1.320 ± 0.006 b

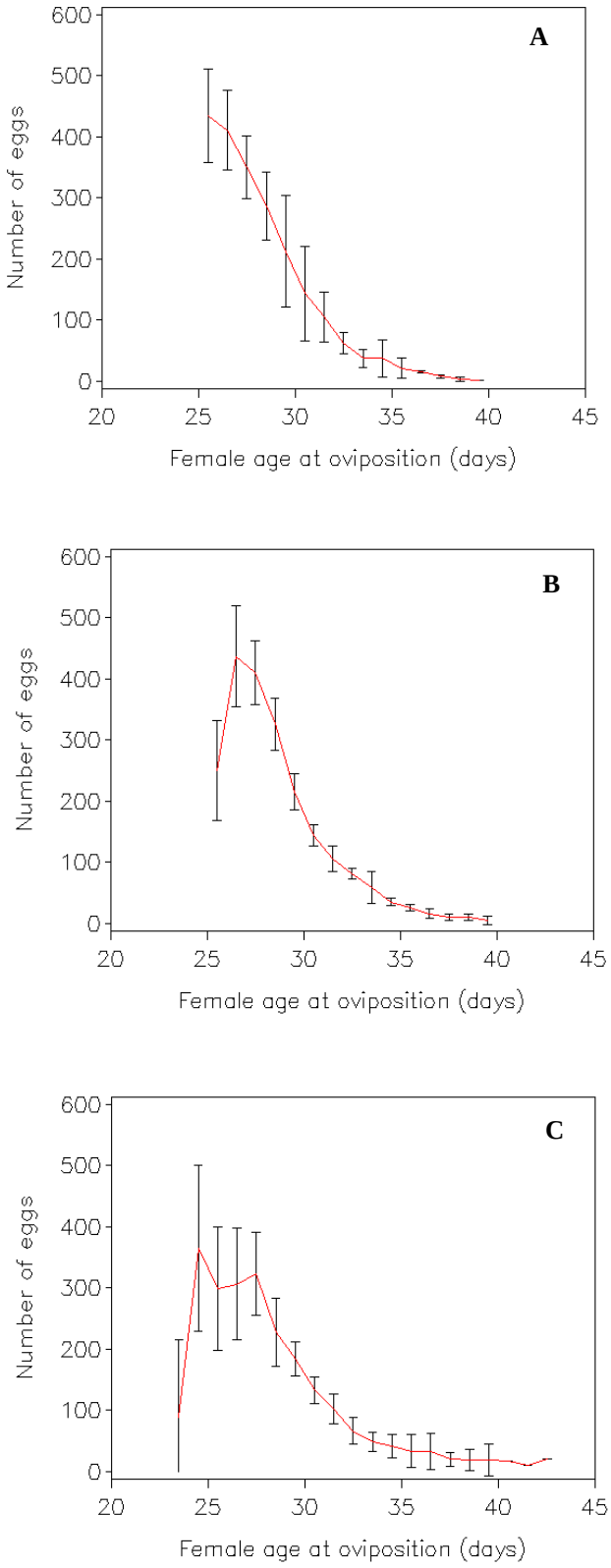


Figure 3.1. Average daily oviposition patterns of (A) untreated female *H. armigera* paired with untreated male moths, (B) untreated female *H. armigera* paired with treated males, and (C) female *H. armigera* that survived exposure to HearSNPV, mated with untreated males. The average daily oviposition is the average number of eggs laid per day by the population as a whole. Vertical bars join minimum and maximum oviposition values at each day.

Chapter 4

Changes in the Metabolic Rate of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Larvae Exposed to Sublethal Doses of *Helicoverpa armigera* Single Nucleocapsid Nucleopolyhedrovirus

4.1. Abstract

Sublethal nucleopolyhedrovirus (NPV) infections are known to affect a number of life history traits. Typical sublethal effects include altered development time, reduced fecundity, reduced egg viability and changes in sex ratio. To determine whether or not fitness costs are reflected in the metabolic rate of inoculated insects, the respiration rates of third instar *Helicoverpa armigera* larvae exposed to two sublethal doses, LD_{25} and LD_{75}, of *H. armigera* single nucleocapsid NPV (HearSNPV) were monitored. Respiration rates, measured as the rate of CO_2 production, were recorded daily for four days post inoculation (dpi) using closed-system respirometry. The overall metabolic rate of treatment survivors was higher than that recorded in untreated controls, although the result was significant in the case of LD_{25} treatment survivors only. In contrast to LD_{75} treatment survivors, the metabolic rate of LD_{25} treatment survivors was maintained immediately after infection (i.e. the metabolic rate 1 and 2 dpi was similar to the metabolic rate of controls), but was significantly higher than that recorded in controls 3 and 4 dpi. Relative to controls, the metabolic rate of LD_{75} treatment survivors dropped significantly 2 dpi, recovered 3 dpi, and increased again 4 dpi. The elevated metabolic rate observed in survivors may reflect a counteractive response in the insects, such as the mobilising of an immune response. The results suggest that significant metabolic costs may be associated with exposure to sublethal HearSNPV doses.

4.2. Introduction

Nucleopolyhedroviruses (NPVs) are promising alternatives to chemical insecticides as they are highly pathogenic to their target hosts, are able to persist in the environment, and are not harmful to non-target organisms (Cory and Hails, 1997; Moscardi, 1999). NPVs are large viruses with covalently closed, double stranded DNA genomes (Blissard and Rohrmann, 1990). The rod-shaped virions are made up of a lipoprotein envelope that surrounds a nucleocapsid (Blissard et al., 2000). The nucleocapsid consists of a DNA-protein core enclosed in a protein capsid (Blissard et al., 2000). Each virion may contain a single nucleocapsid (SNPV) or many (MNPV) (Funk et al., 1997; Blissard et al., 2000). The virions, in turn, are occluded in a protein matrix or occlusion body (OB) (Funk et al., 1997).

NPV research has focussed predominantly on lethal effects of infection and the immediate reduction of pest populations (Myers and Kuken, 1995; Rothman and Myers, 1996). In recent years, interest in sublethal infections as a means toward long-term suppression of pest populations has increased (Milks et al., 1998; Myers et al., 2000; Duan and Otvos, 2001). Sublethal infections may be latent infections, where no or minimal gene expression occurs, or persistent, where low level expression of a range of genes occurs (Burden et al., 2003; Cory and Myers, 2003). Research has shown that insects that survive inoculation with NPV are often impaired relative to uninoculated insects (Milks et al., 1998). Typical sublethal effects include changes in development time, reduced fecundity, reduced egg viability, and changes in sex ratio (Rothman and Myers, 1996). Furthermore, virus is often passed on to the next generation (Rothman and Myers, 1996).

The mechanisms by which sublethal effects occur are unclear. Sublethal effects may occur as a result of initial virus challenge, where mounting a defence response may be physiologically costly (Cory et al., 1997). The use of the host's energy in defence against long-term persistent infection (Cory et al., 1997), or hormonal changes induced by the virus may also impact negatively on the insect (Rothman and Myers, 1996). For example, one potential method for eliminating

invading virus involves shedding midgut cells; this process requires resources that would otherwise be used for host growth, and may reduce food intake (Cory et al., 1997; Cory and Myers, 2003). These observations suggest that the mechanisms by which insects overcome sublethal infection are associated with a fitness cost.

Variation in metabolic rate in insects is generated largely by activity, size and temperature, but also varies with age, sex, feeding status and time of day (Terblanche et al., 2004). Numerous studies have shown that insect metabolism is also altered in response to stress. According to Djawdan et al. (1997), environmental changes occur frequently, placing organisms under stress. Such conditions include exposure to toxins, disease, and adverse environmental conditions such as drought (Djawden et al., 1997). In addition, normal activity, such as reproduction or hatching, can place an insect under stress (Djawden et al., 1997). Metabolic rate is also thought to vary in adaptive manner. For example, reduced metabolic rate in xeric species is thought to contribute to the conservation of water which is lost from spiracles during gas exchange (Terblanche et al., 2004).

Few studies have focussed on the effects of host-parasite/pathogen interactions on insect metabolism. Parasitism of *Manduca sexta* by the braconid wasp, *Cotesia congregata,* was associated with a significant short-term reduction in metabolic rate, immediately after wasp ovipostion, and then following parasitoid emergence (Alleyne et al., 1997). Alterations in host metabolism in these host-parasite complexes is linked to hormonal changes; for example, changes in juvenile hormone and ecdysteroid titres in host insects may alter metabolism to suit the needs of the parasite (Beckage, 1997). Reduction in host activity and a severe reduction in feeding are characteristic of parasitoid infection and are likely to significantly contribute to the observed drop in metabolic rate just before parasitoid emergence (Alleyne et al., 1997).

Dingha et al. (2004) studied the effects of the *Bacillus thuringiensis* Cry1C toxin on the metabolic rate of both Cry1C-resistant and susceptible *Spodoptera exigua*

larvae. The authors found that the metabolic rate of resistant third instar larvae reared continuously on diet containing the toxin was significantly higher than that of resistant third instar larvae reared on toxin for 5 days only (followed by rearing on untreated diet), resistant larvae reared on untreated diet only, or the susceptible parent strain reared on untreated diet only. The authors suggest that the increase in metabolic rate may be due to the production of detoxifying enzymes which are induced in the presence of toxin. Furthermore, they suggest that the use of energy for enzyme production, and for the replacement of damaged midgut cells, may reduce the general fitness of the insect (as energy is diverted from 'everyday activities' to mechanisms that protect the host from the toxin). The authors note that differences in other fitness components such as pupal weight, fecundity, egg hatch, and mating success have been reported between *B. thuringiensis* resistant and susceptible lepidopterans, implying a trade-off between resistance to the toxin and other life-history traits.

Immune challenge in the white cabbage butterfly pupae was found to be energetically costly where individuals 'infected' with nylon implants were found to have a standard metabolic rate almost 8% higher than that recorded for controls (Freitak et al., 2003). The authors concluded that the activation of immune defence could be expressed in 'energetic currency'. According to Zuk and Stoehr (2002), evidence for costly immune response has been observed in a variety of invertebrate and vertebrate species. These costs can be considered from two perspectives: those associated with evolving immunity (and ultimately resistance to a particular pathogen/toxin), and those reflected as the physiological costs of maintaining and using the immune system when the host is challenged (Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003, 2005). The latter can be evaluated as a life-history trait where costs are reflected as the use of resources (such as energy or nutrients) or in the functional or structural aspects of an organism (Zuk and Stoehr, 2002). In addition to the use of the host defence mechanisms, the actual presence of a pathogen, parasite or toxin can impact on life-history traits (for example, reduced metabolic rate observed after parasitoid emergence was likely a result of cessation of feeding and a severe reduction in host activity, induced by

the parasite). As a result, mounting a counteractive response, in addition to an actual infection, induces alterations in life-history parameters.

The effect of exposure to sublethal NPV doses on the metabolic rate of lepidopteran larvae has not, to our knowledge, previously been studied. The aim of the current study was to investigate the impact of exposure to *Helicoverpa armigera* SNPV (HearSNPV) on the metabolic rate of *H. armigera* larvae, and specifically, to test whether exposure to sublethal doses of HearSNPV is associated with a metabolic cost. The data described in this study provides valuable insights into the impact of treatment with sublethal NPV doses on host metabolism.

4.3. Materials and Methods

4.3.1. Insect Rearing

Helicoverpa armigera larvae were originally obtained from the Agricultural Research Council, Pretoria, South Africa. The culture was reared on an artificial diet (Bot, 1966) in the laboratory. Insects were maintained at 28°C, 70% relative humidity, in a 12:12 L:D photocycle. Eggs and pupae were sterilised using 0.2% (m/v) and 0.25% (m/v) sodium hypochlorite respectively, to maintain the sterility of the culture. Eggs used for experiments were not sterilized.

4.3.2. Virus Propagation and Purification

HearSNPV was produced *in vivo* in third instar *H. armigera* larvae. Details of the purification protocol are provided in Chapter 2. Briefly, cadavers were macerated in 1% SDS, filtered through muslin cloth, and subjected to low speed centrifugation in order to remove insect debris. OBs were purified from the suspension by means of rate-zonal centrifugation through a glycerol, and then sucrose gradient. After each rate-zonal centrifugation, the OB-containing band

was removed and washed in sterile distilled water. Finally, the purified OBs were washed twice in sterile distilled water and sedimented by centrifugation. The OBs were resuspended in sterile distilled water, counted using a haemocytometer, and stored at -20°C.

4.3.3. Inoculation of Larvae

Ranging assays were performed in order to establish sublethal and lethal doses (refer to Chapter 2 for details). Third instar larvae were inoculated with three HearSNPV doses: LD_{25}, LD_{75} or LD_{99}. Larvae were infected by presenting them with diet cubes contaminated with virus. Larvae that ate the entire cube within 24 hours were transferred to numbered glass vials containing artificial diet while larvae that did not eat the entire cube within the given time period were excluded from the study.

4.3.4. Respirometry and Metabolic Rate Measurement

CO_2 production was measured 1 – 4 days post inoculation (dpi) by closed system respirometry. The basic set-up was as follows: incoming ambient air was scrubbed of CO_2 as it was drawn through a soda lime column and then bubbled through water. The moist CO_2-free air was drawn through the system to a $Mg[ClO_4]_2$ column where moisture was removed before the air entered the CO_2 analyser (LiCor LI6262). CO_2 measurements were recorded using the Datacan V (Sable Systems) data acquisition package. The flow-rate was maintained at 50 $ml.min^{-1}$ by a mass flow controller.

Respirometry chambers were constructed from 10 ml syringes that had a hole drilled through the side at the 8 ml mark. The syringes were covered with red cellophane in order to darken the environment inside the syringe and minimise the movement of the larvae (as they respond to light stimuli) during recording. The tips of the syringes were attached to three-way stopcocks, which in turn were attached, once the syringes had been flushed, to needles inserted into the system

(via tubing). Pre-weighed larvae were transferred to the syringes aseptically (using a brush sterilised in 0.35% w/v sodium hypochlorite and rinsed in sterile distilled H_2O) and then each chamber was flushed, via the drilled hole, with moist, CO_2-free air. Each chamber was flushed for 2 minutes after which the plunger was pushed down over the hole to the 7 ml mark, and the stopcock closed. Syringes containing untreated control larvae were prepared in the same way. Larvae were measured individually, but a control and two test larvae were recorded in a single run (i.e. one after the other). An empty syringe was included in each run as an additional control to account for any 'leakage' of CO_2 into the system.

Twelve treated larvae were recorded daily. Recordings were taken as follows: once the syringes had been flushed, closed, and attached to the system, the CO_2 analyser was allowed to reach a baseline. The stopcock of the control syringe was opened, 2 ml air was pushed into the system, and the stopcock closed. When the system returned to the baseline, the same procedure was repeated with syringes containing the second control, and test larvae, taking care to push air into the system each time at the same speed. The time at which each chamber was closed, and at which air from each chamber was pushed into the system was recorded. CO_2 emissions were recorded twice in each case. Each reading (area under the curve) was converted from parts per million to ml using the Datacan software program and corrected to standard temperature and pressure. These values were in turn calculated as a function of time. Following recording, larvae were returned to the original vial containing diet and monitored until pupation or death. Data was therefore collected from insects that survived inoculation with HearSNPV, larvae that eventually died from infection, and controls. The experiment was repeated for each dose and the data combined.

4.3.5. Data Analysis

Analysis of covariance (with mass as the covariate) using the General Linear Model (SAS, Version 9.1, SAS Institute, Cary, NC) procedure was used to analyse metabolic rate [rate of CO_2 production: $\dot{V}\,CO_2$ ($ml.h^{-1}$)]. Where the *F* test

was significant ($P \leq 0.05$), least squares means were subjected to multiple comparisons.

4.4. Results

4.4.1. Inoculation Survivors

The overall metabolic rate of treatment survivors was higher than that recorded for untreated controls, although the result was significant ($P = 0.0071$) in the case of LD_{25} treatment survivors only (Figure 4.1. A). In contrast to LD_{75} treatment survivors, the metabolic rate of LD_{25} treatment survivors was maintained immediately after inoculation (i.e. the metabolic rate 1 and 2 dpi was similar to the metabolic rate of controls), but was significantly higher than that recorded for controls 3 and 4 dpi ($P = 0.0029$ and $P = 0.0059$ respectively) (Figure 4.1. B). Relative to controls, the metabolic rate of LD_{75} treatment survivors dropped significantly 2 dpi ($P = 0.0178$), recovered 3 dpi, and increased significantly ($P = 0.0002$) again 4 dpi (Figure 4.1. B). The effect of dpi was found to be significant (Table 4.1), but was not considered further as it combines results for all treatment groups.

4.4.2. Larvae That Ultimately Died From Infection

The overall metabolic rate of larvae that ultimately died from LD_{99} infection was significantly lower than that obtained for larvae that developed lethal infections following exposure to the lower doses (LD_{25}: $P = 0.0329$; LD_{75}: $P = 0.0002$) (Figure 4.2. A). The metabolic rate decreased on each successive test day for all treatment groups, although there was no significant interaction between dose and dpi (Table 4.2). The metabolic rate of LD_{99} infected larvae decreased significantly each dpi (i.e. from the previous day), and was consistently lower on each day than the metabolic rate recorded for LD_{25} and LD_{75} infected larvae, although not significantly so (Figure 4.2. B). The metabolic rates of LD_{25} and LD_{75} infected

larvae 2, 3 and 4 dpi was significantly lower ($P < 0.05$) than that recorded 1 dpi, while the metabolic rate recorded 4 dpi was significantly lower ($P < 0.05$) than that recorded 1, 2 and 3 dpi.

4.5. Discussion

In the current study, we investigated the effects of exposure to sublethal HearSNPV doses on the metabolism of third instar *H. armigera* larvae, by recording the amount of CO_2 produced using closed-system respirometry. Third instar larvae provide an ideal age at which to begin recording. Smaller larvae would have to be recorded in groups (i.e. more than one insect in a syringe), as the amount of CO_2 they produce is extremely small. Third instar larvae are large enough to be measured on their own, and provide a reasonable time period for recording prior to pupation (as opposed to older larvae).

Analysis of the dose-dpi interaction in lethally infected insects indicated that the metabolic rate decreased on each successive test day for all treatment groups, suggesting that the insects were unable to cope with the infection. The daily decrease in metabolic rate reflects the severity of lethal infection as various tissues become infected, and eventually non-functional. Once infection has been initiated in the midgut epithelium, the first cycle of virion replication occurs and budded virions are produced (Federici, 1997). These enter the haemolymph and circulate around the body, causing secondary infections in the tracheal matrix, haemocytes, fat body and eventually nerves, muscles, pericardial cells, reproductive tissues and glandular tissues (Federici, 1997). Nucleocapsids are generally formed about 8 hours post inoculation (hpi) in infected midgut cells (King and Possee, 1992), and bud from membranes 10 – 24 hpi (Szweczyk et al., 2006). At high viral doses, infected haemocytes containing polyhedra can be detected as soon as 15 hpi (Granados and Lawler, 1981). Given the nature and extent of NPV infection, it is not surprising that larvae are unable to maintain their metabolic rate, particularly when the second cycle of virion replication occurs. Furthermore, insects that are lethally infected have reduced appetite until they eventually stop feeding, and also

become lethargic and do not respond to tactile stimulation (Federici, 1997); these factors are likely to contribute to the observed drop in metabolic rate.

The overall metabolic rate of treatment survivors was higher than that recorded for untreated controls. The elevated metabolic rate observed in insects that survived exposure to HearSNPV may reflect a counteractive response in the host, such as midgut cell sloughing, virally-induced apoptosis, and the use of the host's immune system. Therefore, an increase in metabolic rate may indicate reduced fitness of larvae as more energy and resources are directed toward eliminating the pathogen, or preventing the development of lethal infection, than toward growth, development and reproduction of the host.

Unlike LD_{75} treatment survivors, the metabolic rate of LD_{25} treatment survivors was maintained 1 and 2 dpi (relative to controls), but was significantly higher than that recorded for controls 3 and 4 dpi. Relative to untreated insects, the metabolic rate of LD_{75} treatment survivors dropped significantly 2 dpi, recovered 3 dpi, and increased again 4 dpi. This variation is probably due to the difference in dose ingested. The maintenance of their metabolic rates suggests that insects exposed to an LD_{25} coped more effectively in the initial stages following inoculation (as the viral load was lower).

The process of midgut cell sloughing may result in the complete elimination of HearSNPV from the host such that infection beyond the cells of the midgut does not occur (i.e. virally infected midgut cells are shed before budded virions are released from the midgut cells). The overall trends in the metabolic rate of insects treated with either the higher or lower sublethal dose suggest that early clearing of the virus is unlikely. One dpi, the metabolic rate of insects exposed to either the LD_{25} or LD_{75} was similar to that of controls suggesting that at this point in time, the insects were not stressed. An early response (i.e. within 24 hours) to the presence of virus, such as the shedding of midgut cells, requires energy and is likely to have had an impact on the metabolic rate recorded at this time. Beyond this time (i.e. after 24 hours), it is unlikely that the virus would still be localised in

the midgut cells (i.e. complete elimination of virus by shedding of midgut cells is unlikely). Furthermore, differences in the metabolic rate of inoculated insects versus uninoculated controls were recorded until 4 dpi; intuitively, it seems unlikely that early clearing of infection would be associated with prolonged effects on metabolic rate.

If infection was established in treated insects, a number of possible explanations could account for the observed changes in metabolic rate. It is likely that the early drop in metabolic rate (2 dpi) observed in insects that survived exposure to an LD_{75} corresponds with significant infection damage (for example, apoptosis in the presence of virus and virus-induced cellular damage) in the presence of high viral loads, and may indicate that the insects succumb to infection to a degree before they are able to counter infection effectively.

Although the metabolic rate of LD_{75} treatment survivors recovered 3 dpi, the initial drop in metabolic rate may explain, in part, why the overall (dose effect only) metabolic rate of insects exposed to an LD_{75} was not significantly different to that of the controls. The subsequent recovery and increase in metabolic rate of LD_{75} treatment survivors may reflect the point at which the insects overcome infection, and then contain the infection. The elevated metabolic rate recorded for treatment survivors until the end of the study period also suggests that it is unlikely, for either dose, that the virus is eliminated from the system entirely. Failure to eliminate the infection fits in with the theory that sublethal infection is a mechanism by which baculoviruses are able to persist in the environment by using their hosts as vehicles, either through latent/persistent infection (Hughes et al., 1997; Burden et al., 2003), or by vertical transmission (Myers et al., 2000; Khurad et al., 2004). In this context it may be useful to extend the study period to the pupal stage. Results obtained beyond 4 dpi, for late fifth instar larvae, would be difficult to interpret as larvae begin to prepare for pupation (where larval genes are suppressed and pupal genes become activated; Dingha et al., 2005).

Following entry of a pathogen into an insect host, recognition of the pathogen is the first step in the immune response where a number of proteins are involved in the recognition of surface characteristics of various microorganisms (Schmid-Hempel, 2005). Once a pathogen has been recognised, insects are able to respond to the pathogen through a variety of processes that include opsonization, phagocytosis, melanization, encapsulation and coagulation (Schmid-Hempel, 2005). In addition, insects release cytotoxic and reactive oxygen species and produce antimicrobial peptides and a range of defence molecules that include lysozyme, and proteolytic and hydrolytic enzymes (Nappi and Otaviani, 2000). In this way, immune response involves either cellular (e.g. haemocytes) or humoral (e.g. circulating molecules) responses in the haemolymph, or a combination of both.

Washburn et al. (1996) demonstrated that the insect's immune system is effective against viral pathogens. They infected *Helicoverpa zea* with recombinant *Autographa californica* MNPV (AcMNPV) containing the *lacZ* reporter gene. Larvae were sacrificed at various stages post inoculation, and the larval tissue examined for the *lacZ* signal. The proportion of insects expressing *lacZ* decreased from 96% 20 hpi, to 40%, between 48 and 72 hpi, indicating that some larvae were able to clear the infection. From 30 hpi, small brown areas (indicating melanization) co-localized with blue *lacZ* signals were seen in the epidermis of trachea associated with the midgut. The infected tracheae were surrounded by aggregations of haemocytes that often contained melanized capsules of host cells expressing *lacZ*. These capsules were the same as those associated with insect cellular immunity (haemocytes surround, immobilise and kill invading pathogens). The authors also tracked *lacZ* expression in tissues following the application of chemical and biological agents that suppress the immune system in lepidopteran larvae. In these insects, infection was more extensive, indicating that the cellular immune response is a significant factor in preventing the spread of infection in *H. zea* larvae. Immunity to baculoviruses involves other mechanisms as well. As mentioned, the shedding of infected midgut cells at the first moult following infection (Cory et al., 1997; Volkman, 1997), effectively clears the

virus before further infection occurs (i.e. before virions bud from the midgut cells and enter the haemolymph). In addition, baculoviruses are able to trigger apoptosis in host cells so that by sacrificing the few cells that are initially infected, the insect is able to avoid further infection (Clem, 2001).

Numerous studies have reported the negative impact of sublethal infection on an insect host including altered development time, reduced fecundity, reduced egg viability and changes in sex ratio (Rothman and Myers, 1996). The negative impact of infection on these characteristics, or life-history traits, suggests that living with sublethal infection carries a cost. The process of immunity considered, it is not unreasonable to assume that fighting infection carries costs, where resources that would be used for every-day activities, are diverted and used for the process of fighting infection. If resources are diverted, other aspects of insect physiology are likely to be affected. Despite evidence for trade-offs between immunity and life-history characteristics, it is not clear how the costs of immunity are paid.

In their study of *H. zea* immunity to AcMNPV, Washburn et al. (1996) reported a substantial decrease in the proportion of *lacZ*-expressing insects between 48 and 72 hpi, indicating the point at which most of the virus was eliminated by the host's immune system. This time frame is consistent with our study where the metabolic rate of LD_{25} treatment survivors was significantly higher than that recorded for controls, when measurements were taken 72 hpi. Similarly, for LD_{75} treatment survivors, a significant recovery in the metabolic rate was observed at 72 hpi.

In contrast to our results, the metabolic rate of *Pieris brassicae* pupae 'infected' with a nylon filament was higher than that recorded for 'uninfected' pupae shortly after the filament was inserted (i.e. 1 dpi) (Freitak et al., 2003). The metabolic rate continued to rise after the implants were removed. The authors suggest that this may be due to increased production of haemocytes following activation of the immune system – this process "may concur expenditure of extra energy". The

authors suggest that the increase in metabolic rate after removal of the implant may also result from the effect of oxidative stress in the presence of free radicals. Activation of the prophenol oxidase cascade following pathogen recognition results in melanization of the pathogen (Freitak et al., 2003). This process generates free radicals that are not only toxic to the pathogen, but also to host cells (Nappi and Ottaviani, 2000; Freitak et al., 2003). The processes of repair and removal of dead and damaged tissues caused by oxidative damage requires energy (Freitak et al., 2003). The authors suggest that the elevated metabolic rate observed after removal of the nylon filament is, in part, due to this process. Similar defence processes occurred in AcMNPV infections of *H. zea* larvae (aggregations of haemocytes surrounding infected cells and associated melanization) (Washburn et al., 1996), and it is likely that the repair and removal of dead and damaged tissues caused by oxidative damage occurred in treated insects in this study. In addition to these factors, it is likely that apoptosis, in response to the presence of virus, occurred. Cells that are in the process of apoptosis undergo numerous biochemical and morphological changes that are brought about by energy-dependent enzymatic processes (Kam and Ferch, 2000). Therefore, this process may have contributed to the observed increase in metabolism. In our study, it is possible that larvae that survived infection, if it occurred, with an LD_{25} or LD_{75} were able to clear the infection using these mechanisms and that the observed changes in metabolic rate are associated with continued costs of immune activation, in a similar manner to that observed in the study of *P. brassicae* described earlier. This argument is supported by the fact that, in their study of AcMNPV infections of *H. zea*, Washburn et al. (1996) recorded a substantial decline in the proportion of *lacZ*-positive insects, suggesting that some larvae had cleared the infection.

In the current study we investigated the effect of exposure to sublethal HearSNPV doses on the metabolic rate of *H. armigera* larvae. Larvae treated with an LD_{25} or LD_{75} and that ultimately died from infection were not able to recover their metabolic rate at any point during the study period and exhibited similar metabolic patterns to larvae treated with an LD_{99}. Larvae that survived treatment

with LD_{25} or LD_{75} appeared to invest energy to prevent lethal infection, suggesting that the mechanisms by which insects overcome sublethal infections are likely to be associated with a fitness cost.

Table 4.1. ANCOVA for $\dot{V}CO_2$ (ml.h^{-1}) of third instar *H. armigera* uninoculated controls and LD_{25} and LD_{75} inoculation survivors, with mass as the covariate.

Source	**d.f.**	**SS**	**MS**	***F***	***P***
Dose	2	1.1349	0.5675	4.40	0.0136
Dpi	3	3.5386	1.1796	9.14	<0.0001
Dose x dpi	6	3.3103	0.5517	4.28	0.0005

Table 4.2. ANCOVA for $\dot{V}CO_2$ (ml.h^{-1}) of third instar *H. armigera* larvae inoculated with an LD_{99}, and those that ultimately died from infection following inoculation with sublethal doses, with mass as the covariate.

Source	**d.f.**	**SS**	**MS**	***F***	***P***
Dose	2	2.8007	1.4004	11.71	<0.0001
Dpi	3	6.2546	2.0828	17.43	<0.0001
Dose x dpi	6	0.3078	0.0513	0.43	0.8583

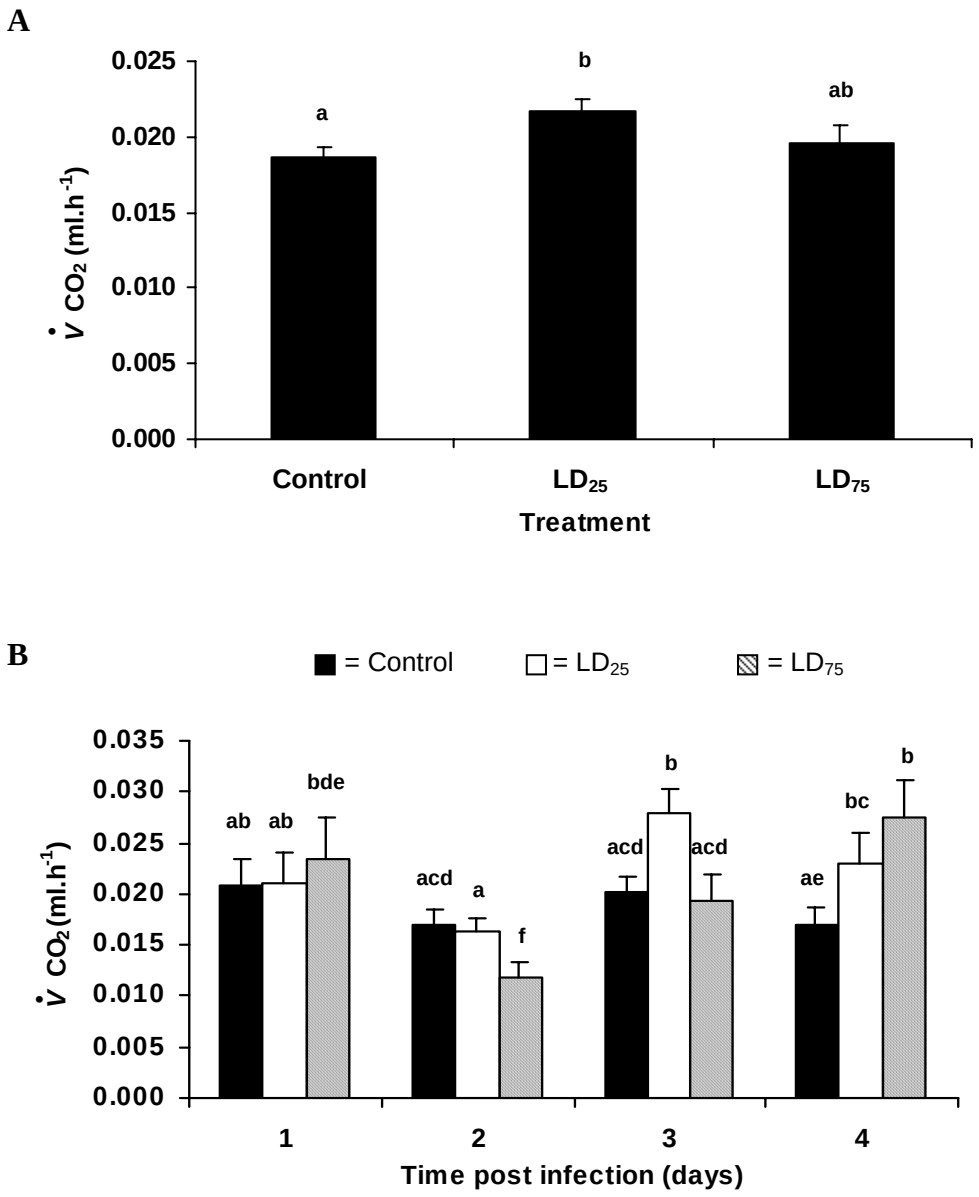


Figure 4.1. (A) The metabolic rate of uninfected controls and HearSNPV inoculation survivors, and (B) the metabolic rate of larvae that survived inoculation with HearSNPV, recorded on each day of the experimental period. Data are plotted as least square means ± standard error, values with different letters are significantly different, $P \leq 0.05$.

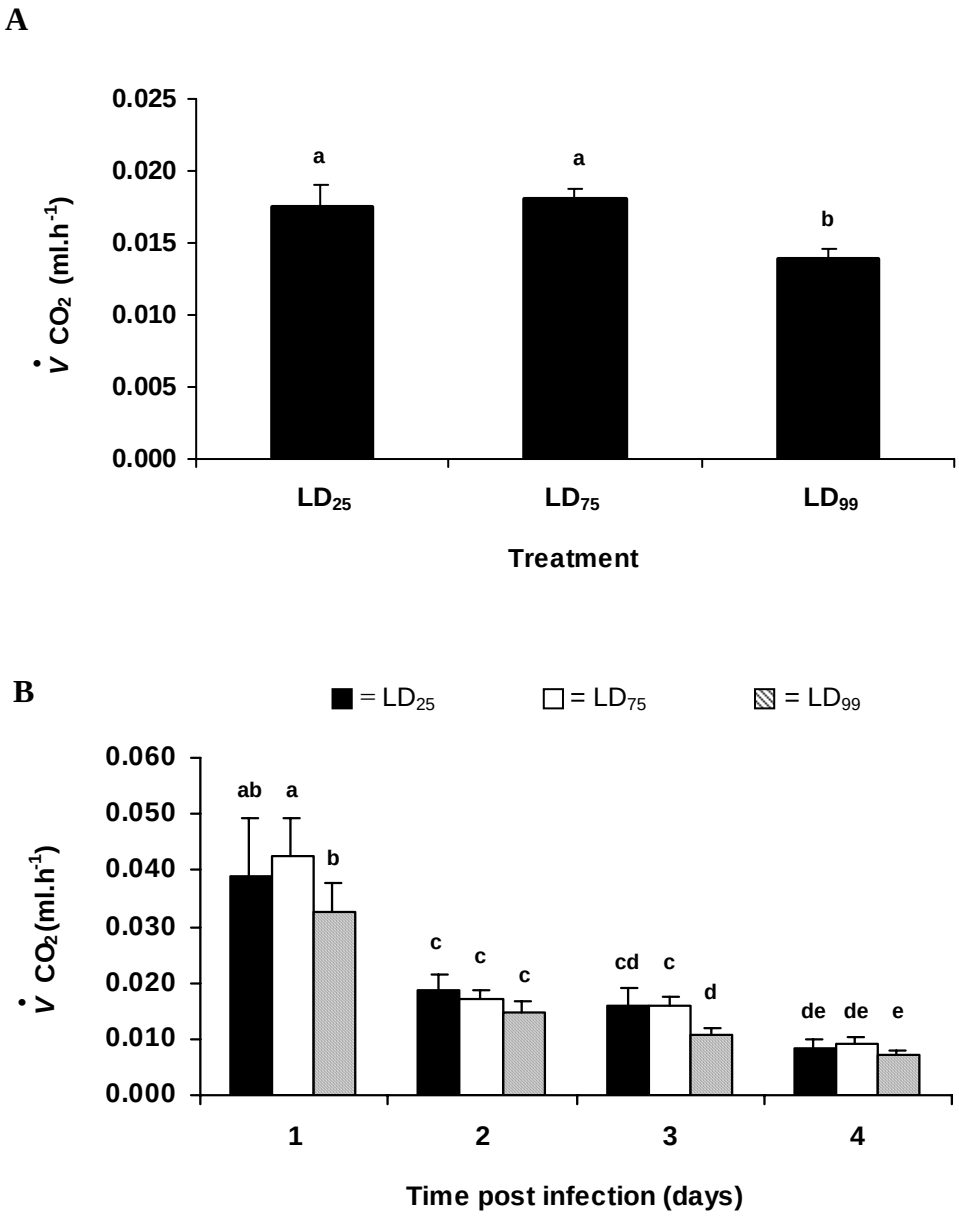


Figure 4.2. (A) The metabolic rate of HearSNPV inoculated larvae that ultimately died from infection, and (B) the metabolic rate of HearSNPV inoculated larvae that eventually died from infection, recorded on each day of the experimental period. Data are plotted as least square means ± standard error, values with different letters are significantly different, $P \leq 0.05$.

Chapter 5

General Discussion and Conclusion

5.1. Discussion

Helicoverpa armigera is a significant crop pest; it has an extensive world-wide distribution and attacks a variety of economically important crops (Fitt, 1989). The negative impact of chemical pesticides on the environment (Mishra, 1998), and the development of resistance of *H. armigera* to chemical insecticides (Srinivas et al., 2004), has prompted the search for alternative control strategies. The use of nucleopolyhedroviruses (NPVs) is an appealing alternative – they provide an environmentally friendly means for control, are highly host specific and are able to persist in the environment (Falcon, 1982).

Research on NPVs as microbial control agents has focussed on the elimination of pest populations by lethal infections (Rothman and Myers, 1996). More recently, interest in the effects of sublethal infections on pest populations has increased (Burand and Park, 1992; Kukan, 1999; Myers et al., 2000; Duan and Otvos, 2001; Burden et al., 2003). Sublethal infections are associated with a range of debilitating effects – most often observed are increased development time, reduced pupal mass and fecundity, and reduced adult longevity (Rothman and Myers, 1996). Debilitating sublethal effects associated with exposure to NPV are thought to play an important role in the ecology and dynamics of an insect population and as such, provide a means for the long-term control of a pest population (Goulson and Cory, 1995).

The mechanisms by which sublethal effects occur are unclear, but they are likely a product of many factors. Debilitating effects are thought to be associated with the actual presence of virus infection in cells, and the associated cell damage (Sait et al., 1994b; Khurad et al., 2004). It is thought that sublethal effects may also arise from the cost of eliminating a pathogen, or from preventing the development of lethal infection (Sait et al., 1994b). The processes of defence require resources that would normally be used for growth, development and reproduction. Insects protect themselves from viral infection in a number of ways. In the early stages of infection, larvae often shed infected midgut cells into the gut lumen preventing virus from establishing an infection (Milks et al., 1998). In subsequent stages of

infection, viruses are known to induce apoptosis (Clem, 2001), and initiate an immune response (Schmid-Hempel, 2005); these processes may result in the elimination of the virus, or may simply prevent lethal infection. The presence of latent or persistent infections, if they occur, are also likely to contribute to the occurrence of debilitating effects as they may disrupt normal metabolism, or alter hormone balance in the host (Burand and Park, 1992; Sait et al., 1994b).

The aim of this study was to investigate the effects of exposure of *H. armigera* to sublethal doses of *H. armigera* single nucleocapsid NPV (HearSNPV). This was done by evaluating a number of non-integrative life-history parameters including immature stage duration, pupal mass, fertility, sex ratio and survival. In addition, jackknife estimates of life table parameters for each population, such as net reproductive rate (R_o) and mean generation time (T), were statistically compared. Life table parameters integrate information on development, reproduction and survival into a single, descriptive parameter, and as such are particularly useful for evaluating the success of an insect population (Bouwer, 2003). The metabolic rate of insects exposed to sublethal HearSNPV doses was investigated as an additional method for explaining and characterising the effects of exposure to sublethal doses.

Despite the relatively low dose (LD_{25}) used to inoculate insects for the life-history study, significant changes in some host characteristics were observed. When compared with controls, male and female treatment survivors exhibited a reduction in immature stage development time, but no differences in pupal mass. The decrease in immature stage development time was unusual as most studies report extended development times (Goulson and Cory, 1995). Further research would be required to determine why this occurred. The effects of sublethal doses on fertility were investigated by mating treated female and male insects with untreated insects of the opposite sex. The number of eggs laid by each mating pair was recorded daily and subsequently, the proportion of eggs that hatched counted. Hatching was lowest in eggs produced by treated females mated with untreated males, while the rate of egg hatch in females mated with treated males was similar

to the rate reported for controls. Exposure to a sublethal HearSNPV dose had no significant impact on immature stage survival and sex ratio amongst offspring of treated mating pairs.

Statistical comparisons of life table parameters indicated that the control population was more successful than populations derived from mating pairs in which one partner was exposed to HearSNPV. Overall, the control population had the lowest doubling time (Dt), and the highest net reproductive rate (R_o), finite rate of increase (λ), and intrinsic rate of increase (r_m), relative to the treated populations. Exposure to the virus significantly affected treated populations and caused a reduction in the potential of the population to increase. Comparison of life table parameters provided information that was not obvious from non-integrative evaluations and as such, proved to be a valuable method for assessing the impact of a biological control agent on an insect population. Rothman and Myers (1996), in a survey of previous research on sublethal effects, suggest that through debilitating effects, sublethal infections contribute to a 22% reduction in the size of a pest population over that of virus-induced mortality alone. This value is probably far greater, but previous studies have mainly relied on the use of non-integrative parameters.

The impact of sublethal NPV treatment on the metabolic rate of insects has not previously been investigated. Insects that died following treatment with sublethal HearSNPV doses exhibited similar metabolic patterns to insects infected with a lethal dose of the virus (LD_{99}). In these insects the metabolic rate dropped on each consecutive day following inoculation and no recovery in metabolic rate was observed.

The overall metabolic rate of treatment survivors was higher than that recorded in untreated controls. When compared with controls, the daily metabolic rate of LD_{25} treatment survivors was maintained 1 and 2 days post inoculation (dpi), followed by a significant increase in metabolic rate 3 and 4 dpi. The metabolic rate of LD_{75} treatment survivors dropped significantly 2 dpi, recovered 3 dpi, and increased

again 4 dpi. The metabolic rate patterns of insects treated with the higher (LD_{75}) and lower (LD_{25}) sublethal dose were initially quite different, but from the third dpi, increases in metabolic rate were recorded in both instances suggesting that regardless of the dose, significant costs in the form of energy expenditure were incurred by the host.

The study of insect metabolism provided insight into the costs of viral challenge in the initial period following exposure to HearSNPV. The increase in metabolic rate observed in treated insects is probably linked to the increased energy requirements of the host for defence processes that either resulted in the elimination of the virus, or simply served to contain the infection such that lethal infections did not develop. Effectively, the allocation of resources shifted towards survival and away from 'non-essential' processes such as growth (Lochmiller and Deerenberg, 2000). This is probably true too for other defence processes (i.e. resources are diverted from everyday activities and allocated to the process of shedding midgut cells, or the activation of apoptotic pathways in the presence of virus).

The elevated metabolic rate may also reflect the expenditure of energy for processes that repair cell damage (either following exposure to cytotoxic chemicals associated with the immune system, or from actual viral damage). In insects treated with the LD_{75}, the severe drop in metabolic rate was followed by a sharp increase in metabolic rate. The period in which the metabolic rate was lowest was likely to be associated with a high level of viral replication and cell damage in the presence of higher viral loads.

It would be valuable to conduct the life-history study in insects exposed to an LD_{75}. The severe changes in metabolic rate observed in insects exposed to this dose may have a more significant impact on, for example, pupal mass (as resources are diverted away from growth and development and used for defence), and subsequently, on the reproductive output of female moths (as pupal mass and fecundity are strongly correlated; Milks et al., 1998). More significant

downstream effects would probably be observed because, in the presence of higher viral loads, a more significant defence response would be required, and therefore, a more significant contribution of resources for the processes of defence and cell repair. It would also be worth investigating whether energy costs are incurred at a later developmental stage (i.e during the pupal stage) in insects inoculated with an LD_{25} or LD_{75}.

The early expenditure of energy following viral challenge may be related to effects observed later in host development (e.g. reduced reproductive rate) but it is difficult to make this connection. Having said this, the use of a host's defence mechanisms has been linked to reduced fitness in a number of organisms (Zuk and Stoehr, 2002), implying a more long-term impact associated with defence against a pathogen. Studies of insects exposed to sublethal granulovirus (GV) and NPV doses provide evidence for costly defence responses where, following exposure to a particular virus, a range of debilitating effects have been reported (Rothman and Myers, 1996; Myers et al., 2000; Matthews et al., 2002). Several other examples exist in a range of vertebrate and invertebrate species. In the snail, *Lymnaea stagnalis*, the use of the immune system was associated with a reduction in reproductive success by reducing the number of eggs laid (Rigby and Jokela, 2000). In another study, immune challenge in bumblebees resulted in reduced survival compared with controls, provided the insects were not given access to food (Moret and Schmid-Hempel, 2000).

A complicating factor in the study of sublethal effects is the question of whether the virus is eliminated from the host, or is able to become established as a latent or persistent infection. The fact that significant downstream effects were observed in treatment survivors suggests that the virus was not eliminated from the host completely, but this can't be stated with complete certainty. The presence of a latent or persistent infection would probably have a significant impact on the reported changes in fitness and life-history characteristics. In this context, molecular studies become extremely valuable and could be used to test for the

presence of the virus in the pupal or adult stages, and then in eggs or offspring of treated parents.

5.2. Conclusion

HearSNPV inoculation of *H. armigera* would likely have activated a range metabolically costly defence responses, resulting in two possible outcomes – the elimination of the virus from the host, or the prevention of lethal infection (i.e. the infection was contained, but the virus remains in the host as a latent or persistent infection). In the event of secondary infection, the host would have had to contend with higher viral loads, more extensive cell damage, and the possibility of latent/persistent infections being established. In both cases, important resources that would normally be used for general growth and development would be diverted and used for the processes of defence, effectively reducing the overall fitness of the host. In this study, fitness costs associated with exposure to sublethal HearSNPV doses were reflected in alterations in host metabolism, and in a number of life-history characteristics.

Non-integrative parameters provided limited information on the extent of sublethal effects following exposure to HearSNPV. Comparison of life table parameters provided a more realistic and useful description of events. From a pest control perspective, these results suggest that the debilitating effects associated with sublethal treatment of insects, particularly those that affect reproductive success, enhance the value of NPVs as potential control agents.

Exposure of *H. armigera* to sublethal doses of HearSNPV was characterised in the early stages following inoculation, by significant alterations in metabolic rate, and then in later stages following inoculation, by a decrease in the duration of immature stage development, a reduction in hatching success of eggs produced by treated mating pairs, an increase the population doubling time, a decrease in the reproductive rate, intrinsic rate of increase and finite rate of increase. Effectively, the exposure of *H. armigera* to sublethal doses of HearSNPV led to a general decline in the success of populations derived from treated insects.

Chapter 6

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