COMPARATIVE BIOLOGICAL AND BIOCHEMICAL STUDIES OF TWO ISOLATES OF NUCLEOPOLYHEDROVIRUSES INFECTING *HELICOVERPA ARMIGERA* (HÜBNER) (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

Two nucleopolyhedroviruses (NPVs) isolated from *Helicoverpa armigera* larvae, in South Africa and Kenya were compared for their pathogenicity to *H. armigera*. Bioassay on *H. armigera* first instar larvae (0.64 mg) showed that the LD_{50} values obtained for both the S. African isolate (20 occlusion bodies (OBs)/larva) and the Kenyan isolate (16 OBs/larva) were comparable, while the Gemstar® a commercial preparation of NPV had an LD_{50} four times as high. For the second instar larvae (4.08 mg) showed that the LD_{50} value obtained for the S. African isolate (20 OBs/larva) was about 125 times lower than that of Kenyan and 315 times lower than that of Gemstar® indicating that the S. African isolate was more virulent.

Ultrathin sections under transmission electron microscopy studies' revealed that the S. African virus isolate nucleocapsid rods were multiply embedded in the virus occlusion body, whereas the Kenyan virus isolate nucleocapsid rods were singly embedded in the virus occlusion body. The virus isolates were then assigned to MNPV and SNPV, subgenera of the Baculiviridae for the S. African and the Kenyan isolates, respectively.

Restriction endonucleases analysis of the virus genome with EcoR1 enzyme revealed that the HaNPV isolates are distinct with different genomic sizes of 107.2 kb and 81.5 kb for Kenyan and S. African isolates, respectively. Virion proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Each produced one identical polypeptide band of size 64 000 kDa indicating that the two isolates are closely related.

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DEDICATION

To my mum, Mrs. Peninah Aoko Ogembo, for her continuous support in prayers, advise and materially since childhood.

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CHAPTER ONE

1.0 INTRODUCTION AND JUSTIFICATION

1.1 INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is one of the most important economic insect pests of cotton, cereals, tobacco, legumes and vegetables in Africa, Australia, Asia, Southern Europe and America (Parson, 1940; Zalucki *et al.*, 1986; Nyambo, 1988; King, 1994). Females lay eggs on the flowering and fruiting structures of these crops, where voracious larval feeding leads to substantial economic loss (Reed and Pawar, 1982). Chemical pesticides are the primary means of controlling this pest on major agricultural crops in Zimbabwe, Kenya, South Africa and throughout the world (Reed and Pawar, 1982; Gunning *et al.*, 1984; Denholm and Rowland, 1992). Unfortunately, this practice has enhanced pesticide resistance, pest resurgence and other environmental hazards associated with use of chemical pesticides (Gunning *et al.*, 1984; Aggrey and Tekie, 1989; van den Berg *et al.*, 1997). There is need to use sustainable and ecologically acceptable pesticides. Viruses, particularly those belonging to the family Baculoviridae, have beer isolated and intensively investigated mainly to be used as biological control agents (Black *e al.*, 1997).

The family Baculoviridae comprises of arthropod-specific viruse {nucleopolyhedrovirus (NPV) and granulovirus (GV)} that can produce fatal infections i their susceptible hosts, primarily larval lepidopterans (Washburn, Wong and Volkman, 2001 These viruses occur naturally in a population and are capable of causing high mortality with a period of five to more days depending on their potency and the susceptibility of the hc (Washburn *et al.*, 1998; 2001).

Several single embedded (SNPV) and multiple embedded (MNPV) have been isolated in different geographical regions from *H. armigera* larvae (Ignoffo *et al.*, 1983; Williams and Payne, 1984; Hunter-Fujita *et al.*, 1998). Some of these isolates show different DNA restriction endonuclease (REN) profiles and also differ significantly in their insecticidal activity (Hughes *et al.*, 1983; Williams and Payne, 1984). As with many other lepidopteran pests, *H. armigera* larvae have also been recorded as hosts for several other NPVs including the *Autographa californica* MNPV (AcMNPV) (Scheepens and Wysoki, 1989), and the *Mamestra brassicae* MNPV (MbMNPV) (Wiegers and Vlak, 1984).

This research was designed to determine to what extent the NPV isolates from Kenya and S. Africa were related through DNA restriction enzyme analysis and sodium dodecyl sulphate polyacrylamide gel SDS-PAGE by comparing DNA and protein profiles. Field trials to evaluate the efficacy of NPV infecting *H. armigera* (HaNPV) in South Africa and Kenya indicated that there was less damage and fewer fruits rejected for export in HaNPV-treated plots compared to fruits from plots treated with *Bacillus thuringiensis* and lambdacyhalothrin (Karate®) (Moore, Bouwer and Pittway, 1998; Baya, 2000). The biological efficacy of these two isolates was also compared with Gemstar®, a commercial HaNPV preparation.

1.2 JUSTIFICATION OF THE STUDY

NPVs comprise an important class of pathogens with potential to be used as biological control agents (Hunter-Fujita *et al.*, 1998; Langewald and Cherry, 2000; Jenkins and Grzywacs, 2000). NPVs are considered to be ecologically safe, non-toxic, non-polluting, stable and a highly sustainable pest control option (Fuxa, 1987; Cunnigham, 1998; Miller, 1997; Nordin, 1997).

HaNPVs are currently being used successfully in Kenya, S. Africa and Botswana to control *H. armigera* in sorghum, cotton, citrus and vegetables (Roome, 1979; Moore *et al.*, 1998; Baya, 2000). However, their biological and biochemical characteristics have not been determined. This project was designed to compare the biological and biochemical relatedness of the Kenyan and S. African isolates of HaNPV. Biological efficacy of the two isolates was compared with Gemstar®, a commercial NPV preparation based on laboratory studies of dose and time mortality relationships. Biological comparison of the isolates with Gemstar® was important, as Gemstar® a commercial preparation provided a standard baseline for selection of virulent isolates to be used in *H. armigera* control. Profiles from RENs and SDS-PAGE analyses of both isolates were also compared to determine their genetic relatedness.

1.3 **OBJECTIVES**

The main objective of the study was to compare biological efficacy and biochemical profiles of *Helicoverpa armigera* NPV isolates from South Africa and Kenya.

The specific objectives were: -

(i) To determine the lethal dose (LD_{50}) and lethal time (LT_{50}) of HaNPV isolates from Kenya, S. Africa and Gemstar®, a commercial preparation in laboratory biological assays against first and second instars of *H. armigera.*

(ii) To compare DNA restriction enzyme profiles of S. African and Kenyan HaNPV isolates.

(iii) To compare the protein profiles of Kenyan and S. African HaNPV isolates using polyacrylamide gel electrophoresis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The American bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

2.1.1 Taxonomy and distribution

The genus *Helicoverpa* contains six described species of which *H. armigera* has the widest crop host range (Zalucki *et al.*, 1986; King, 1994). Until recently, controversy existed concerning the correct nomenclature of the most common and widely occurring bollworms, *H. armigera* (Hübner) and *Helicoverpa zea* (Boddie) (King, 1994). In 1955 the name *Heliothis zea* became accepted for the New World species. The generic name *Helicoverpa* was introduced by Hardwick (1965), to distinguish members of the bollworm complex which are of old World distribution and are morphologically distinct from *Heliothis* species. Hardwick's nomenclature has now been generally accepted as a result of recent taxonomic revision of the genus (King, 1994). Of the several species belonging to *Helicoverpa* and *Heliothis* genera, *Helicoverpa armigera, Helicoverpa zea, Helicoverpa punctigera* (Wallengren) and *Heliothis virescens* (Fabricius) have achieved major pest status (King, 1994).

The geographic distribution of *H. armigera* is mainly confined to the tropics and subtropics including regions in Africa, Asia, Australia and Southern Europe (Hardwick, 1965; Zalucki *et al.*, 1986). *H. armigera* is indigenous within the zone 40^0 North and South of the equator, while in higher latitudes in which records are cited, its presence could be attributed to migratory flights emanating from warmer tropical regions (Fitt, 1989).

2.1.2 **Pest Status**

H. armigera is considered to be one of the world's oldest destructive insect pests on cotton, tobacco, maize, sorghum, wheat, legumes and vegetables in America, Africa, Asia and many other parts of the world (Parsons, 1940; Pearson, 1958; Hardwick, 1965; Zalucki *et al.*, 1986; Fitt, 1989). The pest status of *H. armigera* is due in part to the polyphagous nature of its larvae (Fitt, 1989; King, 1994). Worldwide, *H. armigera* has been reported to attack at least 60 cultivated crops and 67 wild host plants (Zalucki *et al.*, 1986; Fitt, 1989). In Eastern and Southern Africa it has been recorded on cotton, tobacco, citrus, sunflower, maize, sorghum, millet, beans, pigeon pea, okra, carnations, tomatoes and other crops and weeds (Pearson, 1958; Greathead and Girling, 1985; Sithanantham *et al.*, 1997; van den Berg, Cock and Oduor, 1997). Extensive surveys carried out in Australia by Zalucki *et al.* (1994) reported 26 more species of host plants of *H. armigera*, suggesting that the host range of this pest is actually wider than what is known.

The importance of polyphagy to the population dynamics and pest status of *Helicoverpa* spp. is three-fold (Fitt, 1989). Firstly, populations may develop continuously on a number of cultivated hosts within a region. Secondly, populations may develop continuously during suitable periods by exploiting a succession of different cultivated and uncultivated hosts throughout the season. Thirdly, populations can persist at low density in seemingly unsuitable areas, since females have a high probability of locating a host able to sustain larval development. Although the larvae of *H. armigera* have a considerably wide host range, the rate of larval survival, development and subsequent adult fecundity varies greatly in different host plants (Zalucki *et al.*, 1986; Fujisaki *et al.*, 2000). Nevertheless, polyphagy offers *H. armigera* an avenue for population survival and persistence (Sithanantham *et al.*, 1997; Fujisaki *et al.*, 2000).

Cultivation of high valued crops with high input requirements, including fertilisers, is thought to provide ideal habitats for increased pest reproduction and survival (Zalucki *et al.*, 1986). For the last ten years, use of these high inputs to grow flowers, vegetables and fruits for exports have been expanding greatly in Zimbabwe, Kenya, S. Africa and other African countries (Sithanantham *et al.*, 1997; van den Berg *et al.*, 1997; Wilkinson, 2000). The practice increases the chances of *H. armigera* multiplying in large numbers (Zalucki *et al.*, 1986). However, no study on the magnitude of losses due to *H. armigera* on horticultural crops has been carried out in Zimbabwe (Zitsanza, 2000). Considerable losses arising from down grading and eventual rejection of produce from the smallholder farmers have been reported in Kenya, S. Africa, India, Pakistan, New Zealand and Australia (Greathead and Girling, 1985; Sithanantham *et al.*, 1997; Ahmad, Arif and Attique, 1997). In New Zealand, for example, up to 30% of tomatoes are destroyed in unsprayed late season crops (Cameron *et al.*, 2001).

H. armigera has the ability to undertake long-distance migratory flights (Fitt, 1989; Zalucki *et al.*, 1986), that may cover 1,000 to 1,500 km in two to three nights (Gregg *et al.*, 1993). This enables the ovipositing females to locate and utilize a wide range of host plants from a number of families (Zalucki *et al.*, 1986; King, 1994). The migratory nature is also believed to enable the insect to escape the adverse effects of weather, which provide poor conditions for reproduction (shortage of adult nectar sources or larval hosts) (Fitt, 1989). Evidence of migrating *H. armigera* has been reported in New South Wales, Australia, where attempts to manage the bollworm was believed by farmers to be a waste of resources because the moth is migratory and flew in from other cropping areas (Lawrence, 2000). Patterns of egg laying by *H. armigera* on cotton in the Sudan Gezira also suggest considerable mobility within the irrigation area (Haggis, 1981).

Insecticides are regularly used to control *H. armigera*, particularly in cotton (Mathews and Tunstall, 1994). However, the indiscriminate use of these pesticides particularly during the 1980s and 1990s, contributed to the emergence of cotton bollworm as a primary pest on cotton and other crops (Gunning *et al.*, 1984; Denholm and Rowland, 1992; Anon, 1998a; Ahmad *et al.*, 2001). This practice also led to pesticide resistance. In the 1950s, 1960s 1970s dichlorodiphenyltrichloroethane (DDT) and methyl parathion were widely used on cotton and other food crops (Mathews and Tunstall, (1994), to control the cotton bollworm complex (*Helicoverpa/Heliothis* spp., *Pectinophora* spp., *Erias* spp. and *Diparopsis* spp.). These wide spectrum insecticides were initially very effective, but gave rise to increasingly severe attack by secondary pests as their natural control elements were destroyed (Mathews and Tunstall, 1994). Resistance to these pesticides was developed by *Helicoverpa* spp. as in Ord Valley, Australia (Wilson, 1974).

In many countries, DDT was phased out in the 1970s for environmental reasons, and the high mammalian toxicity of parathion led to the curtailment of its use in favour of less toxic insecticides. The most important of these was endosulfan, which was widely recommended and used against Heliothine bollworms from the mid 1970s and early 1980s (Mathews and Tunstall, 1994). *H. armigera* resistance to DDT was detected in 1972 in New Zealand, while resistance to endosulfan was subsequently reported (Wilson, 1974).

From late 1970s to date, the relatively inexpensive synthetic pyrethroids are increasingly relied upon to control bollworms, as they are very effective against many lepidopterous larvae. Currently, pyrethroids are still widely used to control Lepidopterans, although, there are reports of resistance to pyrethroids compounds like cypermethrin, deltamethrin, cyfluthrin, bifethrin and lambda-cyhalothrin (Ahmad *et al.*, 2001). Consequently, control failures in many cotton-growing regions such as Mississippi, Texas, Australia, Indonesia, Thailand, India and Turkey have been reported (Mathews and Tunstall,

1994). A notable exception to the appearance of resistance problems in the control of *H. armigera* applies to cotton in Zimbabwe, despite the entire crop having been treated with insecticides since the early 1960s (Mathews and Tunstall, 1994). The bollworm control in Zimbabwe is based on well rotational system of pyrethroids. Resistance to many groups of pesticides has contributed to the pest status of *H. armigera*.

2.1.3 **Biology of *Helicoverpa armigera***

Pearson (1958) described the life cycle of *H. armigera*. The average number of eggs laid by a fertile female ranges between 730 to 1600 over an oviposition period of 10 - 23 days (Pearson, 1958). The insect fecundity varies with host-plant type, probably as a result of differences in nutritional quality or previous experience of the insect with the host species (Parsons, 1940; Zalucki *et al.*, 1986; Cunningham, West and Wright, 1998; Cunningham, Zalucki and West, 1999). Pyke *et al.* (1987) carried out an investigation on the oviposition behaviour of *H. armigera* on cotton, and pigeon pea. Two strips of pigeon pea (200 m by six rows each) within a background of cotton (200 m by 84 rows) were planted. Egg sampling based on whole plant counts, on both cotton and pigeon pea over a period of three months revealed that a greater proportion of eggs were laid on pigeon pea than on cotton. Cunningham *et al.* (1999) observed a similar trend in their experiment, but stressed that host abundance regardless of 'innate' relative references could influence the choice of plants in the field. Previous experience of *H. armigera* with its host plant species was found to increase its relative attractiveness to that host (Cunningham *et al.*, 1998).

The female moths deposit eggs at dusk on the substrate, especially plant stems and leaves, with few eggs laid on flowers (Parsons, 1940). The eggs are yellowish-white in colour when freshly laid, and turn yellowish-brown as they develop. The eggs are dome-shaped with a diameter of 0.45 - 0.5mm, with distinct ridge structures and raised micropyles

on top (Pearson, 1958). The incubation period of eggs in warm weather (25 – 30 ^{0}C) takes between three to four days while in cooler temperatures (18 - 23 ^{0}C) this period is extended to eight days (Parsons, 1940; Pearson, 1958; Taylor, 1982; Zalucki *et al.*, 1986).

On hatching, the larvae consume part of the eggshell and then look for suitable food elsewhere, preferably the soft plant tissues such as buds, flowers and leaves (Hardwick, 1965). The larval period depends mainly on temperature and nutritional content of the diet. For temperature range between 25 - 30 ^{0}C, larvae take 12 - 24 days to reach pupal stage and up to 50 days in very low temperatures (Taylor, 1982; Baya, 2000; Fujisaki *et al.*, 2000).

Fujisaki *et al.* (2000) in their laboratory experiment reported mean larval duration range of 18.48, 15.04, 14.50, 13.19, 12.52, and 11.4 days, at 25 ^{o}C ± 1 on cotton leaf, tomato fruit, soybean seed, okra fruit, corn kernel and cotton boll respectively. Five to seven larval instars have been recorded, and the difference in the number of larval-instars is attributed to diet quality (Fujisaki *et al.*, 2000). Development is said to be optimal at 35 ^{0}C on artificial diet (Zalucki *et al.*, 1986).

The characteristic features used in identifying the *Helicoverpa/Heliothis* larval-instars are based on colour and width of the head capsule. The first three larval-instars have brown, dark brown or black heads, which average from 0.26 mm in *H. punctigera* to 0.29 mm in *H. zea* (Hardwick, 1965). The trunk is ivory when the larva hatches but becomes yellow or greenish-yellow as the larva increases in size (Hardwick, 1965). In the third instar, the two colour phases become evident. In *H. armigera*, *H. gelotopoeon*, and *H. zea*, a brown colour phase predominates. In *H. hawaiiensis* and *H. assulta*, green colour phase predominates (Hardwick, 1965). The fourth, fifth and sixth instars may be yellow, green, pink, orange, brown or black depending on the food substrate and temperature of the surroundings, but all the instars have characteristic light and dark stripe longitudinal lines on their trunk (Fig. 2.1).

Fig. 2.1: The longitudinal lines on a mature larva of *H. armigera*

When fully mature, the larvae burrow into the soil to pupate (Fig. 2.2). Pupation lasts two to four weeks in the tropics and around six months in temperate regions (Roome, 1979; Zalucki *et al.,* 1986). Diapause is thought to be rare in tropical environments but more common in sub-tropical regions with dry and cold winters (Roome, 1979; Fitt, 1989; Lawrence, 2000).

Fig. 2.2. The pupae of *H. armigera*

Unfavourably low temperatures (< 23 ^{0}C) and short day length (<12 hours) (Parsons, 1940; Pearson, 1958; Roome, 1979) mainly induce diapause. It has been observed that over 90 % of pupae will diapause when both pupae and larvae are exposed to a temperature of 19 ^{0}C and 12L: 12D photoperiods (Fitt, 1989). Diapausing pupae exhibit a lower rate of weight loss than non-diapausing pupae when the two are held at the same temperature (Roome, 1979). Presumably this reflects the enhanced respiratory rate of the non-diapausing pupa in which adult development is proceeding, even at low temperature (Roome, 1979). Diapause pupae of *H. armigera* produced adults in 123 days at 18 ^{0}C, 19 days at 27 ^{0}C and 10 days at 33 ^{0}C in Botswana (Roome, 1979). The moths emerge from the soil at the end of the pupal period, and after two to four days begin to mate and subsequently lay eggs (Taylor, 1982). The adult moths (Fig. 2.3) live for one to three weeks, but generally longevity is determined by the availability of nectars, pollen and dew, which are the primary food source of adult insects (Cunningham *et al.*, 1998).

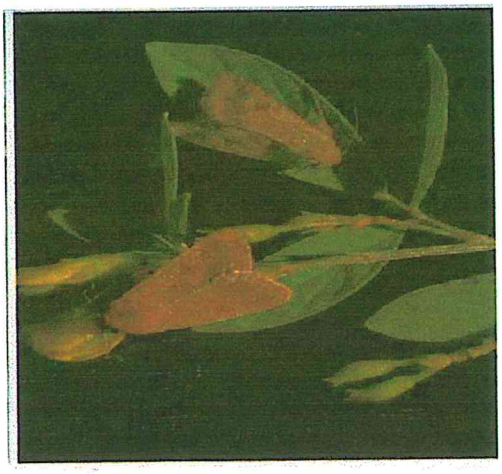

Fig. 2.3: The adult *Helicoverpa armigera*

2.1.4 **Damage and yield loss**

The destructive stage of *H. armigera* is its larval stage which feeds on plant leaves (maize, soybeans), fruits (cotton, chickpea, pigeon pea, tomatoes, soybeans), flowers (carnations, sunflowers, safflower) (King, 1994; Fitt, 1989). The larvae may completely destroy the plant part they are feeding on pigeon pea (Fig. 2.4). The levels of damage to crops vary throughout the world but generally depend on the value of crop and whether the damage is direct or indirect. In cotton, tomatoes and safflower for example, the economic threshold is very low (two to five larvae/m^2) since the larvae attack the harvested products (Cameron *et al.*, 2001). These crops also have high market value; hence any small losses of the harvested product will therefore mean loss of more money (Lawrence, 2000).

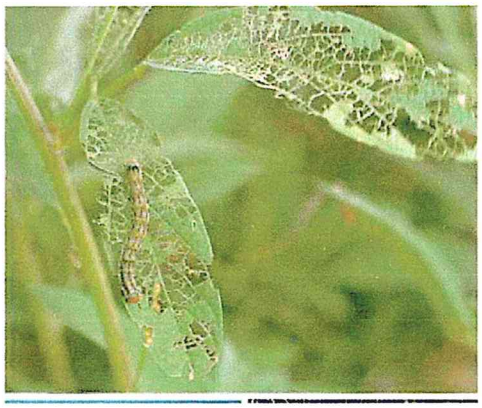

Fig. 2.4 The damage caused by *H. armigera* larvae on plant leaves

In contrast, for crops such as sunflower and sorghum, the economic threshold is very high 15 - 30 larvae/m^2 because of the indirect damage inflicted by the pest (Roome, 1975; van den Berg *et al.*, 1997). Damage to maize is considered less important since major losses are due to stem borers (Greathead and Girling, 1985). Secondary rots may also develop within the buds or fruits, which are attacked by the insect (van den Berg and Cock, 1993).

In general the economic importance of *H. armigera* as a pest relates to its preference for plant structures that are high in nitrogen, principally the reproductive structures and growing points (Greathead and Girling 1985; Firempong and Zalucki, 1990; Cameron *et al.*, 2001). In the case of crops where *H. armigera* is a primary pest, control measures need to be applied early enough when the pest population is at low densities to reduce yield loss.

2.1.5 **Control strategies for *Helicoverpa armigera***

2.1.5.1 **Chemical control**

Synthetic insecticides are often used to suppress *Helicoverpa* populations because of their availability, portability and potential for quick intervention and prevention of serious damage by larvae (King and Coleman, 1989). However, *H. armigera* rapidly develops resistance to insecticides (Gunning *et al.*, 1984; Ahmad *et al.*, 1997). Moreover, indiscriminate use of chemicals raises other concerns such as environmental safety, production efficiency and pest resurgence due to natural enemy mortality (Payne, 1988).

In Zimbabwe, Kenya, S. Africa, Australia and India, control of *H. armigera* using chemicals such as DDT, carbamates, endosulfan and pyrethroids was initially effective (Gunning *et al.*, 1984; Karel, 1985; Mathews and Tunstall, 1994; Anon, 1998b; Lawrence, 2000). Currently there are widespread reports that the pest is resistant to DDT, endosulfan, carbamates and pyrethroids (Gunning *et al.*, 1984; Hill and Waller, 1988; Denholm and Rowland, 1992).

Chemicals such as DDT, aldrin, dieldrin, mirex, endrin and heptachlor are not widely used to control pests in crops due to their deleterious effect on the environment and the ozone layer (Mathews and Tunstall, 1994). However, pyrethroids have become the most widely used chemicals for *H. armigera* control in cotton, soybeans, sorghum, tomatoes and sunflower (Gunning *et al.*, 1984). Pyrethoids proved very effective until 1983 in Australia

(Gunning *et al.*, 1984), in Eastern and Southern Africa 1980s and 1990s (Hill and Waller, 1986; Denholm and Rowland, 1992) when resistance by *H. armigera* was detected. Increased use of certain pyrethroids has also resulted in minor pests such as red spider mites gaining prominence in Zimbabwe cotton growing regions (P. Jowah, 2002 personal communication [1]). The problems associated with the use of chemicals have given an impetus to finding new control methods or improved pest monitoring to allow only need-based pesticide applications.

2.1.5.2 **Cultural control**

Cultural practices such as destruction of residues, uniform and early planting together with regular weeding have been recommended for the control of caterpillars but are seldom used to control *H. armigera* (Sithanantham *et al.*, 1997). The use of resistant varieties has been recommended as host plant resistance easily blends with cultural practices such as regular weeding. In cotton, many resistant varieties have been developed and used to control *H. armigera*. For instance, the highest mortality and lowest weight was recorded on young larvae that fed on cotton genotypes (upland cotton) with a high gossypol gland density on the ovary surface (Mohan, Raj and Kathane, 1996).

Intercrops, trap and companion crops have been tested using crops such as sorghum, maize, sunflower and beans planted with cotton and vice versa (Abate, 1998). These methods, coupled with regular weeding, have also been tested with variable success because of the polyphagous nature of the larvae (Abate, 1991).

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Other trap crops tested with considerable success are *Xemenia americana* (Ajayi, 1991). African marigold *Targetes minuta* (Dunkelblum *et al.*, 2002) and *Azadirachta indica* (neem) have been used as repellents. Use of sex pheromone traps has been recommended for scouting of adults for effective cotton spraying in Western Tanzania, Australia, USA and India (Nyambo, 1988; 1989b).

2.1.5.3 **Biological control**

Biological control involves the use of predators, parasites, and microbial organisms (King and Coleman, 1989). The importance of naturally occurring predators and parasites in regulating *Helicoverpa* populations has been recognised (Nyambo, 1989a; van den Berg *et al.*, 1997). In the absence of insecticides, natural enemies may maintain *Helicoverpa* populations below the economic threshold (King and Coleman, 1989).

An effort to preserve and increase the effectiveness of natural enemies through environmental manipulations, particularly the judicious use of pesticides, is termed biological control by conservation (Fuxa, 1987). When natural enemies populations are too low, there may be need to introduce them through propagation and release (augmentation). The potential effect of natural enemies, particularly predators and parasites on *H. armigera* has been recognised in cotton, sunflower, sorghum and soybean (Fig. 2.5) (Nyambo, 1988; van den Berg *et al.*, 1997). However, explicit guides for incorporating them into decision-making regarding action versus non-action are generally lacking (King and Coleman, 1989; van den *Berg et al.*, 1997). In any case, growers cannot be expected to adopt biological control unless they can realise meaningful monetary return compared to that realised from alternative control strategies or no control.

Fig. 2.5: The *H. armigera* predators attacking the pest under natural field conditions

There are many examples cited by different authors on the effective use of biological agents. Some of the successful records on use of parasitoids include the release of the egg parastoid, *Trichogramma* spp. (957,000 individuals/ha), *Brincklchrysa scelestes* (50,000 individuals/ha) 120 days after cotton sowing in India and USA (King and Coleman, 1989; Dhandapani *et al.*, 1992). *Microplitis croceipes* has also been used widely in USA, and it prefers to parasitise third-instar larvae (King and Coleman, 1989). All parasitised larvae were observed to move and feed less on cotton plants, which resulted in less fruit damage. Release of 15 000 adults in a 4 ha cotton field reduced *Helicoverpa* population by 76% (King and Coleman, 1989). There are attempts to use other predators such as *Chrysoperla carnae* Steph. (Chrysopidae) in China and India (Glazer, 1992) and the tachinids, *Goniophthathalmus halli.*

In Eastern and Southern Africa, several predators and parasitoids have been reported to cause significant mortality of *H. armiger*a in the fields (Greathead and Girling, 1985; Nyambo 1990; van den Berg *et al.*, 1997), but their large-scale use has not been established. Currently, there is an ongoing research at the International Centre of Insect Physiology and

Ecology (ICIPE) Nairobi, Kenya, on rearing and releasing of *Trichogramma* spp to control *H. armigera* in vegetable crops such as tomatoes (Sithanantham *et al.*, 2001).

For biological control by augmentation or importation to be feasible, efficient and cost-effective methods of rearing entomophagous arthropods must be developed. Rearing of predators and parasites necessitate the rearing of the host insects, which is expensive and often complex. Judicious use of pesticides is also vital to conserve or allow establishment of introduced natural enemies to control the pest.

2.1.5.4 **Microbial control**

Environmental concerns and problems with resistance to chemical insecticides have led to increasing interest in the use of biological agents (viruses, bacteria, fungi and nematodes) to control insect pest species (Payne, 1988). Microbial control has been a component of integrated pest management strategies for many years (Langewald and Cherry, 2000), enjoying particular success in Asia, Europe and on the American continents (Fuxa, 1987). Microbial pesticides are required to have desirable properties that can match or at least show flexibility compared to chemical pesticides in terms of different product formulations, efficacy and diverse use of application equipment in order to be accepted by the end users (Fuxa, 1987; Navon and Ascher, 2000).

They also must be registered as other pesticides before commercial use, and the registration process provides a check on their safety (Navon, Klein and Braun, 1990). Many microbes are already registered and are being used as safe and effective commercial products in that they offer safer pest control than synthetic chemical pesticides (Fuxa, 1987). The widely used microbial pesticide against *H. armigera* and other lepidopterans pest include *Bacillus thuringiensis* (Bt) (Berliner), fungi and baculoviruses. The biological control insecticide, *Bacillus thuringiensis* has been used for more than 50 years to control

lepidopteran pests (Stone and Sims, 1993). Strains of the bacteria are also used to control certain Diptera and Coleoptera. Of the five groups of entomopathogens, bacteria have the fewest species that are candidates for insect control, although they are the most widely used (Stone and Sims, 1993).

B. thuringiensis has potential of being used as a biological control agents, owing to the efficacy of the delta endo-toxins (Dulmage and Cooperators, 1981). *B. thuringiensis* are relatively easy to produce, formulate and genetically manipulate (Stone and Sims, 1993). Environmental contamination and ingestion are the two horizontal means by which bacteria are transmitted. Many of these bacteria, have wide host ranges and their persistence in the soil is good especially for *Bacillus popilae* but poor for other important species like Bt and *Bacillus sphaericus* (Fuxa, 1987). In addition to all these advantages, crops such as cotton, tobacco, and tomatoes have been genetically modified to express many types of Bt delta toxins and are commercially available for use (Gould, 1998). These engineered crops have been reported to be successful in managing *Helicoverpa* spp (Gould, 1998). The disadvantage of using bacteria as biological control agents apart from low persistence on plant leaf surface is inefficient dispersal between hosts.

Fungi are potentially the most versatile entomopathogens (Ferron, 1978). Some have toxins and potential for quick damage (Fuxa, 1987), but generally they are slow acting pathogens. Many are virulent and have wide host ranges, infecting different stages of their hosts (Ferron, 1978). There are many varieties and strains that have been identified which provide possibilities of screening for use as biological control agents. They cause natural epizootics that devastate insect populations inhabiting water, soil and aerial plant surfaces (Fuxa, 1987). Fungi are able to infect through the host's integument, making ingestion unnecessary. Their infection is not limited to chewing insects. Some aquatic fungi have the ability to search for hosts, and disperse naturally through air movements although the

effective distance and importance to epizootics of such dispersal are poorly understood (Ferron, 1978). One of the most promising approaches in using fungi as biological control agents is inoculative augmentation. This is because fungi have wide host ranges, can be raised on artificial media and produce natural epizootics (Butt *et al.*, 2001).

Species of fungi used in inoculative augmentation include *Beauveria bassiana* for *Leptinotarsa decemliniata*, *Nomuraea releyi* against lepidopteran soybeans defoliators, *Hirsutella thompsonii* against mites, *Metarrhizium anisoplae* against *Ostrinia nubilalis* and spittlebugs and are combined with other treatments against *Oryctes* (Butt, Jackson and Magan, 2001). The main disadvantage of fungi is their relative dependence on high relative humidity at several points in their life cycles (Butt *et al.*, 2001). Also, they usually take at least one week to kill or stop larvae from feeding (Butt *et al.*, 2001). Their persistence in abiotic reservoirs, especially in the soil, is known to be poor generally.

Baculoviruses, which include nucleopolyhedroviruses and granuloviruses, have also been used in the control of a wide range of pests, mainly lepidopterans including *H. armigera* for many years (Cunningham, 1998). The first attempt to use NPV as insect control agent was in 1892 in which NPV was sprayed to control *Lymantria monacha* in Germany (Cunningham, 1988). The first large-scale deliberate application of NPV as an insecticide was when NPV of alfalfa caterpillars, *Colias philodice eurytheme*, and the NPV of European pine sawfly, *Neodiprion sertifer*, in Ontario, Canada in 1950 were aerially sprayed to control these pests (Cunningham, 1988). HaNPV as a biopestide (Elcar®) has been in use against *Helicoverpa* spp. on cotton for many decades (Bell, 1982).

There is a lot of ongoing research studies and field trials on use of baculoviruses to control insect pests on crops and forests in different parts of the world (Fuxa, 1987). In Russia, USA, Europe, Australia, China and India, baculoviruses have been used to control many crop and forest pests successfully (Fuxa, 1987). Such applications are limited in Africa

with a few exceptions like use of NPV in the control of semi-loopers on soybeans in Zimbabwe and use of granulovirus (GV) to control potato tubermoth, *Phthorimaea operculella* in Egypt and Tunisia. These are also some of the success records of virus use in Africa (Taylor and Kunjeku, 1983; Kunjeku *et al.*, 1998).

In Zimbabwe, farmers multiply the NPV to control a complex of soybean semi-loopers caterpillars, *Thysanoplusia orichalsea, Chrysodeixis chalcites, Chrysodeixis acuta,* and *Ctenoplusia limberina* on soybeans (Kunjeku *et al.*, 1998). The virus is isolated naturally from the field infection and about 10 000 to 15 000 ha are treated annually (Fuxa, 1987).

Other attempts to use baculoviruses to control pests in Africa include field trials in Botswana where unpurified suspension of local HaNPV (one application of 200 larval equivalent/ha) against *H. armigera* larvae on sorghum, were found to be as effective as a standard insecticide (carbaryl at 1.1 kg/ha) in curtailing losses of sorghum (Roome, 1979).

There are several disadvantages that hinder use of baculoviruses effectively in managing pests. These include slow rate to kill, variation in efficacy from the same virus, cost of production, high specificity to host, vulnerability to ultraviolet rays (low persistence), poor application technology and some can only be produced *in vivo* (Federici, 1997). For baculoviruses to be widely accepted and adopted by many growers as biological control agents, these setbacks need to be addressed. Probably the first point to eliminate these problems is by screening isolates from different localities and selecting the most virulent strains.

The advent of recombinant DNA technology has also presented possibilities for modifying and improving baculoviruses for insect control. Consideration should be given to such parameters as host range, virulence, and enhancing resistance to inactivation of virus by ultraviolet rays when exposed to plant surfaces. An addition of foreign genes (Bt toxin or

scorpion neurotoxic venom) to baculoviruses to hasten their speed to kill insects or widen their host range is another area that is being investigated.

2.2 **Insect viruses**

There are six major groups of insect viruses, Baculoviridae, Reoviridae, Poxviridae, Picornaviridae, Iridoviridae and Parvoviridae (Murphy *et al.*, 1995). Of all these only Baculoviridae are exclusively limited to infecting arthropods, whereas the other groups affect other living organisms (Anthony *et al.*, 1999). Baculoviruses are a diverse family of entomopathogenic viruses which are pathogenic to insects particularly members of the Lepidoptera, Hymenoptera, Diptera, Coleoptera, Neuroptera, Siphonaptera, Thysanoptera and Trichoptera (Federici, 1997; Anthony *et al.*, 1999).

Baculoviruses are named after the host species from which they were first isolated (Hunter-Fujita *et al.*, 1998). This system is unreliable for establishing the real identity of a given isolate and is not particularly helpful in providing clues about its host range and infectivity (Rovesti, Crook and Winstanley, 2000). For instance, it is now clear that there is collection of isolates that are genotypically very closely related to the *Autographa californica* MNPV. The MNPVs from *A. californica, Trichoplusia ni, Spodoptera exempta, Rachiplusia ou* and *Galleria mellonella* are considered variants of the same virus (Rovesti *et al.*, 2000).

Baculoviruses contain covalently closed, circular, double-stranded DNA genomes ranging from 88-160 kbp which are enclosed within rod-shaped enveloped nucleocaspids giving the family its name (Blissard and Rohrmann, 1990; Rohrmann, 1992). The most apparent characteristic feature of baculoviruses is the production of proteinaceous capsules, referred to as occlusion bodies (OBs) or polyhedra (Weeden, Shelton and Hoffman, 1997).

The family Baculoviridae is comprised of two genera, nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). NPVs have many virions occluded in large polyhedron shaped occlusion bodies and in GVs a single virion is occluded in the protein matrix (Murphy *et al.*, 1995; McCarthy and Di-Capua, 1979). The protein coat helps the virus to remain viable outside the host (Figueiredo *et al.*, 1999). NPVs are also subdivided by the extent of aggregation of their nucleocapsids within the envelope; some are present singly, and are called singly-enveloped nucleopolyhedroviruses (SNPVs) whereas others are found as multiples, multiple-enveloped nucleopolyhedroviruses (MNPVs) (Figueiredo *et al.*, 1999).

A novel feature of baculoviruses is the production of two types of virions. The occlusion-derived virions (ODV) type is present in polyhedra and spreads infection between insects, where as the other; the budded virions (BV) type spreads the infection between cells within insects or in cell culture (Washburn *et al.*, 1998). A major difference between these two types of virions is their envelope proteins (Rohrmann, 1992).

BV contains an envelope fusion protein that causes the merging of virion envelope and the membrane of cellular endocytic vesicles when exposed to low pH. Transmission of infection beyond the midgut is dependent on the synthesis of gp64, a protein specific to BV and lacking in ODV (Washburn *et al*, 1998). Baculoviruses have potential as biological control agents for insect pest management owing to their host specificity, efficacy, and stability (Weeden *et al.*, 1997; Volkman *et al.*, 1995; Granados and Federici, 1986).

2.2.1 **Nucleopolyhedrovirus infection cycle**

NPV infection starts when an insect larva ingests polyhedra in contaminated food, or through injury, cannibalism and possibly by transovarial transmission although evidence of this is rare (Hunter-Fujita *et al.*, 1998).

The high alkaline (pH 9.5-10.5) and possibly enzymatic activity in the midgut (Cunningham, 1988; Vail, Hall and Hoffmann, 1999) help to solubilize the ingested OBs. The dissolved OBs release the virions to begin the infection process (Granados, 1978). These virions pass through the peritrophic membrane and fuse with the microvillar membrane of the midgut epithelial cell (Vail *et al.*, 1999). The virus particles not penetrating the midgut are excreted and cause no infection. Penetration of the polyhedra directly into the haemocoel does not lead to infection because the polyhedra are not dissolved in the haemolymph.

In the midgut cells, nucleocapsids are transported into the nucleus where they uncoat as early as 1h post infection (p.i) and the virus undergoes the primary round of replication with progeny nucleocapsids observed as early as 8 h p.i (Blissard and Rohrmann, 1990). Cytopathic effects observed during this phase include enlarged nucleus and virogenic stroma within the nucleus. At 12 h p.i some progeny nucleocapsids begin to bud through the nuclear membrane. In the cytoplasm, the envelope acquired from the nuclear membrane is lost, and the nucleocapsid is subsequently transported to and buds through the plasma membrane.

In two to three days, clear areas develop within the nucleus and viral elements, the virogenic stroma, are visible under the electron microscope. About four days after infection, the first immature polyhedra can be seen. The nucleus increases in size and almost fills the entire cell. A day or two later, cells begin to break down, releasing budded virus into haemolymph and spreading the infection from tissue to tissue within the fat body, tracheal matrix, muscles haemocytes, epithelial and reproductive organs producing a secondary replication.

The frequency of formation of polyhedra varies with the virus type. Some polyhedra develop in the midgut when the larvae are infected with multiple-enveloped virus, but a few, if any, polyhedra are seen if infection is by single enveloped virus (Granados and Lawler, 1981; Blissard and Rohrmann, 1990; Vail *et al.*, 1999).

2.2.2 **Symptomatology of NPV infected larvae**

After ingestion, several days may lapse before signs and symptoms of the disease occur, depending on the insect larvae biomass (Shapiro *et al.*, 1981). Some infected larvae may be reduced in size; others may have intensified segmentation without increased turgidity. Colour and\ or behavioural changes are the first signs of infection (Granados and Lawler, 1981; Nordin, 1997).

The larvae become shiny in colour and often cease to feed. Larvae may move to different parts of the plant or light. Larvae then weaken slowly and become sluggish and flaccid, and less responsive to environmental stimuli. The haemolymph and fat body become turbid and milky because of large numbers of polyhedra. The insect hangs itself on the substrate from its hind prolegs (Fig. 2.6) and then dies (Nordin, 1997). The cadavers darken quickly after two to three days of death due to secondary infection from bacteria (Fig. 2.7) (Grzywacs, 1997).

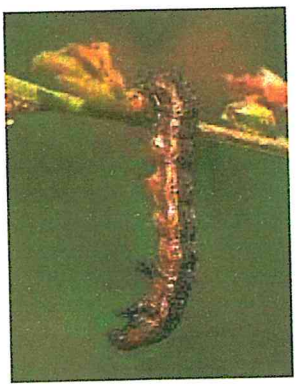


Fig. 2.6: *H.armigera* infected with NPV

Fig. 2.7: *H.armigera* larvae infected with NPV and later infected by bacteria

Body contents at this time have liquefied and the larval integument forms a sack of virus occlusions (Washburn *et al.*, 1998). Shortly thereafter, the virus oozes from the cadaver

onto foliage where it can be eaten by other larvae to repeat the infection cycle (Nordin, 1997). When a young larva is infected, the cadaver may become dehydrated and have the appearance of a small scab on the leaf. The presence of OBs in the insect tissue or cadavers is presumptive evidence of infection by this group of viruses (Hunter-Fujita *et al.*, 1998).

The incubation period from initial infection to mortality varies considerably and depends on many factors including crop type, larval age, temperature, dose, virulence of the virus isolate, and nutrition of the larval host or other sources of stress that may predispose the insect to infection (Granados and Lawler, 1981). The time of infection is inversely related to the amount of polyhedra ingested by the insect. Smaller dosages result in a longer time of death (Aizawa, 1963). Higher temperatures speed up infections, the effect of temperature increases the rate of virus production (Aizawa, 1963) and therefore the subsequent spread of virus throughout the insect body. The physiological state of the susceptible host will also affect the lethal time. Younger larvae succumb to infection faster than the older larvae (Shapiro *et al.*, 1981; Baya, 2000).

2.2.3 **Structure and Properties of NPV**

The polyhedra are an orthogonal crystalline matrix (Scharnhorst *et al.*, 1979), made up of 245 amino acids, with molecular weight of 29-kDa (Rohrmann, 1992). The polyhedra are 0.5μ to 15μ in diameter containing rod shaped virions 200 - 400 nm long and 20 - 50 nm in diameter (Rohrmann, 1992).

Polyhedra can be easily seen under the light microscope (Grzywacs, 1997). The virions consist of covalently closed, double stranded DNA genomes of 88 - 160 kbp surrounded by a protein coat made up of subunits arranged in a helical pattern (Scharnhorst *et al.*, 1979; Blissard and Rohrmann, 1990; Rohrmann, 1992). The viral DNA is

heterogeneous in size, with contour lengths ranging from 15 to 45 μm (Scharnhorst *et al.*, 1979). This structure is further enclosed in an outer polyhedron envelope, membranous in nature. The virus particles become occluded, at random, but this does not influence the regular nature of the crystalline protein matrix (Tinsley, 1979). The analysis of this protein matrix shows presence of RNA, lipid, and various trace metals, in addition to protein (Tinsley, 1979). The outer polyhedron membrane contains orthosilicate ions of iron and magnesium, which make up about 0.1% of its dry weight (Tinsley, 1979). The major function of the polyhedron is to stabilise virions in the environment, providing the viral DNA with protection from UV of sunlight (Blissard and Rohrmann, 1990). The inclusion body preserves the infectivity of the virus particles very effectively and purified polyhedra can be stored, over a wide range of temperature, either as dry powders or as aqueous suspensions.

Polyhedra are resistant to bacterial action and to a wide range of chemical treatments, but they are susceptible to treatment at high pH with solutions such as 1M sodium carbonate (Tinsley, 1979). The crystalline structure of the polyhedra is solubilized under these conditions and the virus particles can be recovered by centrifugation. The polyhedra protein can be precipitated by adjustment of the alkaline solution to its isolectric point of pH 5.8 (Tinsley, 1979). The ability of the virus to occlude and thus protect its virions and persist in the environment outside of the insect host may be a trait subject to intense selection pressure. Several other insect virus groups (entomopox viruses and cytoplasmic polyhedrosis viruses) have also evolved ability to occlude their virions (Blissard and Rohrmann, 1990).

CHAPTER THREE

3.0 REARING AND STANDARDIZATION OF THE HOST INSECT, *H. armigera* FOR BIOSSAYS

3.1 INTRODUCTION

There are two ways of building a stock culture of insects, either by requesting from an established laboratory or collecting the insect species from their natural hosts in the fields. In either case it is very important for the newly introduced insects to be quarantined so as to observe any signs of infection before taking them to the insectary for rearing. Any infected insects larvae should be removed immediately and if possible the cause of infection identified and stored for further reference as biological control agents. To limit introduction of pathogens into the insectary, an insect colony should preferably be established from eggs since eggs can be easily sterilised and thereafter incubated to hatch. Insects can be reared either on their natural hosts or on an artificial diet. The use of artificial diet is a common practice because of its efficiency and hygiene especially where mass production of insects is required (Shorey and Hale, 1965).

3.2 MATERIALS AND METHODS

The *H. armigera* colony used in the experiment was established from larvae collected the Cotton Research Institute (CRI), Kadoma, Zimbabwe and also from Hortico Fresh Produce Limited, Shamva, Zimbabwe. The insects were collected on cotton and broad-bean plants, respectively. The larvae were reared at the Department of Biological Sciences, University of Zimbabwe and the CRI. The larvae were maintained at 28 ± 2 ^{0}C and on a 12: 12 (L: D) photoperiod on an artificial diet supplied by CRI until they pupated (Shorey and Hale, 1965) (See Appendix B). The pupae were sexed, paired and allowed to emerge as

adults. The adults were first kept in mating cages for two days and later transferred to a cylindrical container of 21 cm diameter by 20 cm depth lined with black cloth along the sides and bottom on which female adults would oviposit.

The adults were initially fed on 10% sterilised honey soaked into cotton wool in a Petri dish, but the diet was later changed to 5% sterilised honey because females tended to overfeed, causing stomach distension and possibly blocking the ovipositor for egg laying. The adults were observed to oviposit two to three days post emergence. The eggs were collected and disinfected in 10% formaldehyde for five minutes to free them from any pathogen infection and then rinsed thoroughly with distilled water and left to dry. Eggs were then transferred to a clean container for hatching. Moist cotton wool wrapped in tissue paper was placed in incubation containers to prevent dehydration of eggs.

Neonates were collected daily so as to have insects from the same cohort for bioassays. Neonates were removed from the hatching containers using a fine hair artist brush. Ten larvae were placed on an artificial diet in a Petri dish. After four days, larvae were separated individually into a Petri dish to avoid cannibalism, common among all larval instars (Hardwick, 1965). During rearing and virus propagation the insectary was attended to in the morning, and the virus production units in the afternoon, to avoid introducing viral infection into the insectary.

During rearing, the life cycle of each cohort was studied. This was done by collecting data on the incubation period of laid eggs, the average development period of each instar, pupal period and the time period of moth oviposition. Twenty eggs were selected and their diameter measured on a microscope graticule. In an attempt to standardise insects to be used for bioassays, weight and head capsule width measurements were taken throughout the larval development of twenty-five insects. Based on the body weights and head capsule widths, all larval stages were established and subsequently first and second instars were selected for

bioassays. The head capsule widths, taken at the widest point, were measured on a dissecting microscope with a graticule.

3.3 **RESULTS**

The establishment and rearing of *H. armigera* under laboratory conditions proved to be very difficult at the initial stages, as the insects were either laying infertile eggs or not laying any eggs. The females were observed to emerge two days earlier than the males.

The mean diameter of twenty *H. armigera* eggs (Table. 3.1) was found to be 0.45 ± 0.034 mm. The result also indicated that the incubation period of eggs was between two to three days. The female moths started to lay eggs two days after emergence and egg laying continued till the tenth day. The eggs were laid mainly at dusk.

These eggs were typically sub-spherical or dome-shaped with the adhering surface forming the base of the dome. The eggs were uniform in shape. At an incubation temperature of 28 ^{0}C, egg colour was observed to gradually change after 14 - 36 h of deposition from yellowish white to yellowish brown, at which time a pink subequatorial band appeared at the immediate micropylar area. The band and the micropylar spot darkened to red or reddish-brown during the next 30 - 36 h after deposition, as seen under a dissecting microscope. Between two to three days after deposition the whole egg became grey as the larva matured and emerged from the chorion. Infertile eggs were white or yellowish, becoming increasingly yellow and cone-shaped as they desiccated.

Newly hatched larvae were translucent yellowish white, with faint darker longitudinal lines and brown head capsules. The thoracic and anal shields, thoracic legs, setae and their tubercle bases and spiracles were also brown to black, giving the larva a spotted appearance. These neonates were observed to partially consume the eggshell

subsequent to hatching. Immediately after hatching, larvae started to wander for about six hours before settling to feed.

The second instar was essentially similar to the first instar, but with some slight darkening of the trunk colour and some lightening of the sclerotized head capsule, thoracic and anal shields. The third instar was brown in colour. The characteristic patterning became more predominant and colouring generally got darker in later instars.

The mean head capsule widths were observed to be constant at each instar stage as shown in Table 3.2. The weight of each instar varied significantly (Table 3.3). The first, second, third, fourth and fifth instar had an average weight of 0.40 g, 4.08 g, 23.90 g, 102.30 g and 409.20 g, respectively. The relationship between weight and $\log_{10}$ of mean head capsule widths of *H. armigera* instars gave a good fit on regression line (Fig. 3.2). There was also a good fit for the relationship between age and $\log_{10}$ body weight of *H. armigera* (Fig. 3.3).

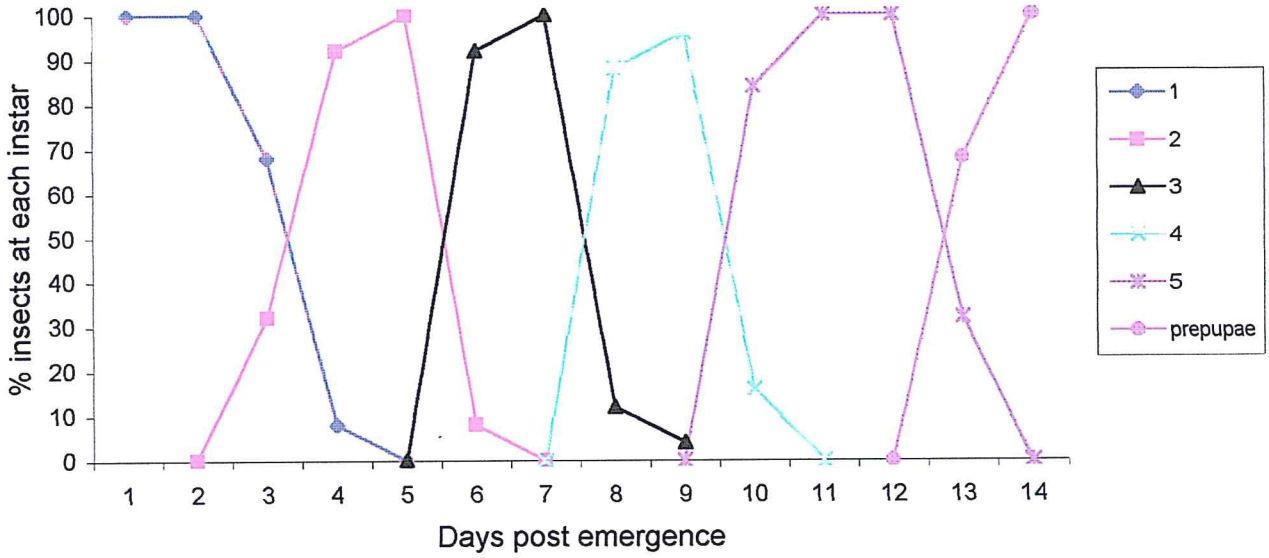
On the 15th day, upon completion of feeding, all mature larvae burrowed into the artificial diet or sand, which was placed on a tray to form prepupal cells and emergence tunnels. At the completion of burrowing the larvae became quiescent within one to two days. The prepupal period took three to five days before the pupal stage. The pupa was mahogany-brown, smooth-surfaced, rounded both interiorly and posteriorly and with two tapering parallel spines at the posterior tip. The pupal stage took about 12 - 14 days to emerge to the adult. The emergence of moths started at dusk and continued until around 0800 hours after which it virtually ceased. Feeding was found to be an essential prerequisite to mating and oviposition. The adults were observed to take a period of 10 - 18 days before dying provided they were kept on 5% honey.

Table 3.1. The diameter of freshly laid eggs of *H. armigera*

Eggs	Diameter (mm)
1	0.49
2	0.44
3	0.46
4	0.46
5	0.46
6	0.46
7	0.44
8	0.44
9	0.37
10	0.49
11	0.44
12	0.49
13	0.49
14	0.44
15	0.46
16	0.49
17	0.46
18	0.49
19	0.41
20	0.39
Mean	0.45 ± 0.034

Table 3.2. Mean head capsule widths of *H. armigera* larval instar (n = 25)

Instar	Days	Mean head capsule width in mm (± SE)
1	1 – 3	0.24 ± 0.0
2	3 – 4	0.29 ± 0.0
3	5 – 7	0.66 ± 0.118
4	8 – 10	1.38 ± 0.035
5	11 – 14	1.90 ± 0.018



Note: The numbers in the key represent the instar stages

Fig. 3.1 Development of *H. armigea* larvae head capsule widths post emergence to prepupal stage at 28^0C

Table 3.3. Mean weights of *H. armigera* larvae (n = 25)

Age (days)	Instar stage (% instar stage in brackets)	Mean weight (mg)	Std deviation
1	I	0.3	0.000
2	I	0.5	0.000
3	I, II (68, 32)	0.64	0.000
4	I, II (8, 92)	1.56	0.000
5	II, III (4, 96)	4.08	0.000
6	II, III (8, 92)	10.06	0.001
7	III	23.9	0.002
8	III, IV (12, 88)	37.09	0.002
9	III, IV (4, 96)	102.30	0.006
10	IV, V (92, 8)	138.17	0.007
11	IV, V (16, 84)	218.21	0.015
12	V	409.20	0.026
13	V	522.92	0.017
14	V	545.30	0.024

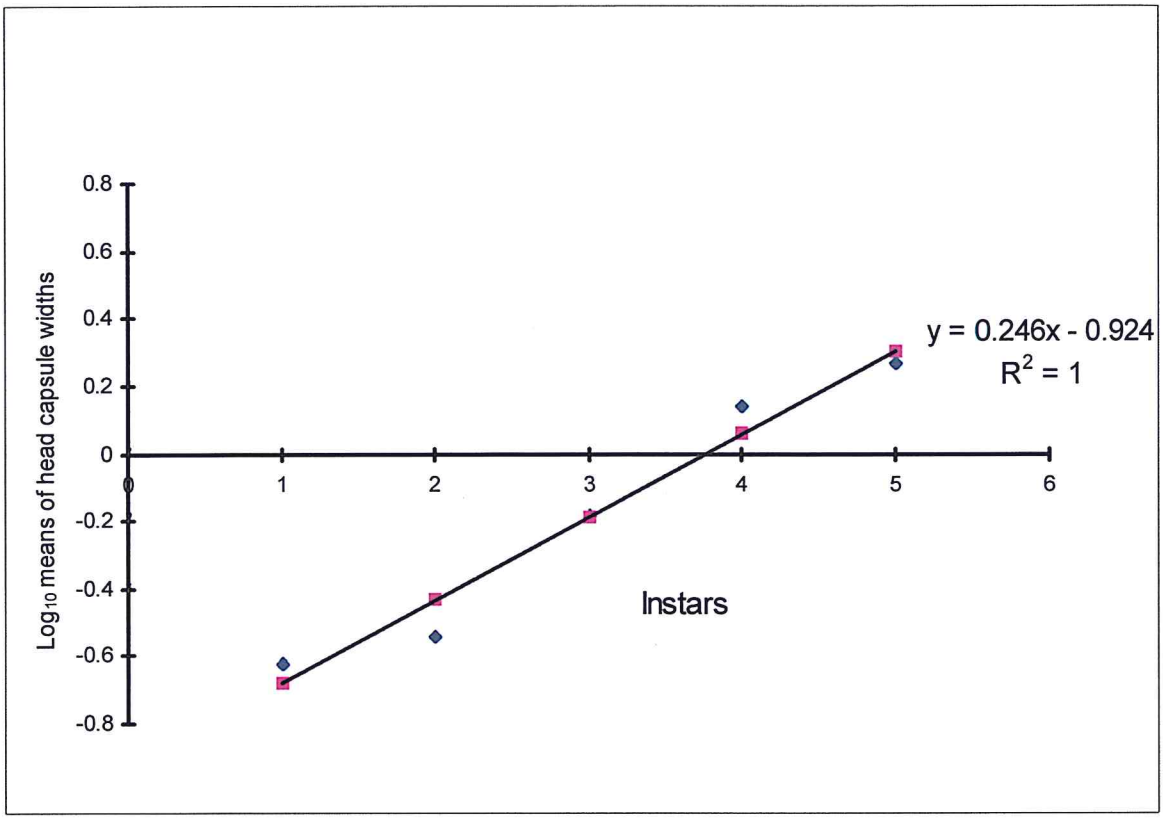


Fig. 3.2: The relationship between age and head capsule widths of *H. armigera* larval instars

Legend

- Observed Y
- Predicted Y

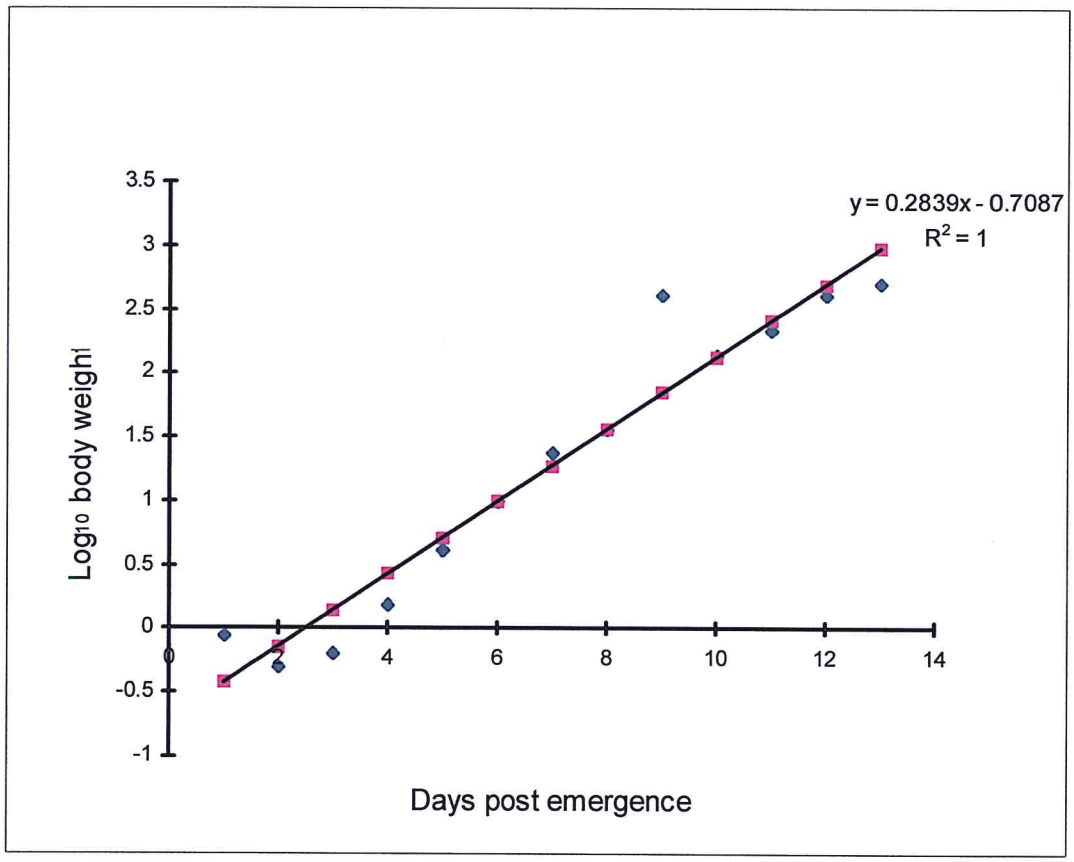


Fig. 3.3 Relationship between age and $\log_{10}$ body weight of *H. armigera* observed throughout larval development

Legend

- Observed Y
- Predicted Y

3.4 DISCUSSION

Establishing *H. armigera* cultures from the field collections initially proved very difficult, mainly because of the low mating frequency in the laboratory. The insects were either not laying eggs or laying infertile eggs. Factors that could have contributed to this mating bottleneck include differences in the rate at which the sexes reach reproductive maturity as well as the response of calling females to males. The female moths were observed to emerge on average two days earlier than males, which, added to the difference in the pre-eclosion developmental times (two days), produced a four-night gap between the sexes in reaching reproductive maturity.

Colving, Cooter and Patel (1994) observed the same phenomenon when they reared the same insect species at 26 ^{0}C. This situation in itself should not present a problem to successful mating, because *H. armigera* can live up to 10 - 23 days in the laboratory (Hardwick, 1965). However, Colving *et al.* (1994) in an experiment on receptivity showed that there is a short window of opportunity in the laboratory, immediately after females reach maturity, in which they are receptive to males.

Because of late reproductive maturity among the males, a large proportion of the females would already have become unreceptive by the time the males reach reproductive maturity and, therefore, mating would probably occur between late-maturing females and early maturing males. Mating newly emerged female moths with males emerging two or more days earlier was used to solve the problem. The insects were mated in-groups of 8 - 25, in the ratio of three females to one male.

As indicated earlier, another problem emanated from the type of diet the insects were fed on. Adult insects tended to overfeed on concentrated honey solution. This was observed to affect egg oviposition by probably blocking the ovipositor. Different dilutions of the moths diet were also tried, by feeding them on either sugar, honey or sugar and vitamin mixture

solution. Insects fed on honey and sugar plus vitamin mixture laid more eggs, which were fertile as opposed to insects fed on sugar solution alone.

Sugar solution alone was thought to lack the nutrients required for embryo formation and development, thus the infertility of the laid eggs (Nzuma, 1996). This was supplemented by adding the vitamin mixture, which is rich in ions necessary for embryo development (Nzuma, 1996). It is therefore important to feed the adults with the correct quality of diet, to establish and enhance insect cultures for rearing.

Other authors have indicated the absence of host plant materials to be one of the possible reasons for low mating among the noctuid species in the laboratory (Raina, Kingan and Mattoo 1992). Habituation by males to the females' pheromone in a confined environment has also been reported to affect mating, since *H. armigera* possess hair pencils, which are usually associated with pheromones (Raina *et al.*, 1992). Relative humidity has also been reported by some authors to affect mating success in *H. zea*. Callahan (1962) claimed that when the relative humidity was > 90% or < 35% mating of *H. zea* was poor. When *H. zea* adults are kept at 50% RH, they mated more successfully and were more fecund than when kept at 30% RH.

The incubation period of eggs was found to be between two to three days, which was also consistent with the finding of other workers (Parsons, 1940; Pearson, 1958; Hardwick, 1964; Mathews and Tunstall, 1994) who reported three to four days for the same species at 25 ± 2 ^{0}C. The mean diameter of twenty *H. armigera* eggs was found to be 0.45 ± 0.034 mm which also was consistent with the finding of other workers (Parsons, 1940; Pearson, 1958; Hardwick, 1964; Mathews and Tunstall, 1994). The egg width differs among the *Heliothis/ Helicoverpa* species (Mathews and Tunstall, 1994), with *H. zea* and *H. virescens* having diameter of 0.57 mm and 0.56 mm, respectively. Although the eggs of *H. armigera* and *H.*

punctigera are indistinguishable (Kirkpatrick, 1962) they may be separated by electrophoretic techniques (Mathews and Tunstall, 1994).

The number of larval stadia of *H. armigera* is variable between five to seven (Hardwick, 1965; Taylor, 1982; Singh, 1999; Fujisaki *et al.*, 2000), although six instars is the most common. There was evidence of overlapping instar stages (Fig. 3.1), similar results were also reported by other workers Teakle, Jensen and Giles (1985). The main cause of variation is temperature and the nutritional value of the material on which the larvae are feeding (Hardwick, 1965). In this study, the bollworm had five larval stages, similar to what Fujisaki *et al.* (2000) reported when the insects were reared on pea at 27 °C. Seven stadia have been reported to occur in S. Africa during the winter months of June and July but only six in September, October and December (Pearson, 1958). During the same study, the average larval development period was found to be 14 to 15 days. With first, second, third, fourth and fifth larval instars taking a different numbers of days, although there were some evidence of overlapping instars with age (Fig. 3.1), similar results were also reported by other workers (Teakle, Jensen and Giles, 1985).

The head capsule was observed to remain constant for each instar. A one-way analysis of variance showed very significant (99%) difference between the head capsule widths of any two instars. A plot of $\log_{10}$ head capsule widths against instar produced a straight line in accordance with Dyar's law (Richards and Davies, 1977). Although the ratio of successive progression ranged between 1.2 - 2.3 not 1.4, there was nevertheless a regular geometrical progression. The percent number of insects at a particular stage was used, as an accurate way of knowing which day has the highest number of the instar wanted for bioassay. For an example, at five days 96% of the larvae were second instar. A regression of $\log_{10}$ body weight on age showed a very strong correlation ($R^2 = 1$). Even though the body weight varied a lot in each instar (compare early fourth instar mean body weight 37.09

0.002 mg and late fourth instar 138.2 ± 0.007 mg), it was used to correlate the infectivity of the virus on the host insects since head capsule width remains constant in any instar. Larvae for bioassays were selected based on the head capsule widths and weight. The larvae used for bioassays were three and five days old, which were terminal first and second instars, respectively.

CHAPTER FOUR

4.0 PATHENOGENICITY OF THE TWO NUCLEOPOLYHEDROVIRUSES TO *H. armigera*

4.1 INTRODUCTION

Individual insects from populations differ in susceptibility as well as their rate of development even if they are from the same cohort population (Evans, 1981). The variation in insect development can be due to difference in feeding rate, possible effect of crowding at first instar stage and the duration individual insects take to moult (Twine, 1978). To minimise these variations, the physiological state of the insects was controlled by standardising the rearing conditions before and after exposure to the different virus isolates. Even with all these precautions, variations could still occur and the possible ways to reduce this further is by selecting standard insects for bioassays based on body weight and width of the head capsule (Richards and Davies, 1977).

Evans (1981) succinctly highlighted many of the problems inherent in comparative bioassays in his view of the standardisation and assay of microbial insecticides. He emphasised the need to reduce the variability of test material and suggested various criteria that could be used to increase precision of bioassays. These included the use of larvae of relatively constant weight and genetic history, supplying inoculum in such a manner as to allow test insects to ingest known dosages, and taking account of known traits of the host's behaviour, such as cannibalism. There is also need for full data presentation including method (s) used for analysis with values for LD_{50}, LT_{50}, the slope of the dosage mortality response and the associated statistical limits to allow comparisons between bioassays to be carried out by different workers. However, different approaches and techniques have been used to conduct bioassays and results are published without full data presentation and

analysis (Evans, 1981; Kunjeku, 1982). This has made it difficult to compare assays even when the same virus-host is used. Variability is mainly reflected in the methods employed to administer the virus doses (Evans, 1981).

Many authors determine lethal concentration rather than lethal dose, although dose is essential in standardisation and assay of viruses. Among these are detailed work by Ignoffo (1966a; 1966b), Vail *et al.* (1969), Whitlock (1978) and Witt and Hink (1979). The problem with using lethal concentration rather than dose is that there is no indication of the true dose ingested. However, the method can be rapid and reproducible to compare relative differences between virus samples. Some authors have also used force-feeding to administer a known dose (Paschke, Lowe and Giese, 1968), but such methods do not consider effects of stress imposed on the insects and regurgitation may also occur, altering the dose ingested (Kunjeku, 1982). To reduce variability in the test insects as a goal towards increasing precision of bioassays, virus particles were administered through a diet plug, which provided a rapid, standardised and simple procedure of estimating the activity of the virus isolates.

4.2 MATERIALS AND METHODS

4.2.1 Propagation of NPV in insect larvae

The HaNPV isolates were obtained from the International Centre of Insect Physiology and Ecology (ICIPE) Nairobi, Kenya, Department of Biological Sciences, University of Zimbabwe (Gemstar®) and Citrus Research International, South Africa. Only virus isolates from Kenya and S. Africa were propagated in insect larvae, but in different laboratories to avoid contamination. Gemstar® was used according to recommendations given by the manufacturer (AgriSense-BCS Ltd- United Kingdom).

Each virus isolate was added separately to a modified artificial diet (Shorey and Hale, 1965) without formaldehyde and then fed to the terminal third larval-instar stage starved for 24 h (Grzywacs, 1997). After four to eight days post infection, larvae were harvested immediately after death before melanisation occurred thus avoiding secondary bacterial and fungal contamination. The insects were stored in a bottle and kept at 4^0C until required for purification.

4.2.2 **Purification of the HaNPV**

The infected larvae were weighed and macerated with a pestle and mortar and homogenised in an equal volume of 0.1% sodium dodecyl sulphate (SDS). The homogenate was filtered through four layers of muslin cloth into a container placed in ice to remove large debris and prevent melanisation respectively. The filtrate was added to a Sorvall 50 ml centrifuge tube and filled with 0.1% SDS. The filtrate was centrifuged at 15 000 rpm in a fixed angle S50Ti rotor in a Sorvall RC-5B superspeed centrifuge for 30 minutes.

The pellet was resuspended in 0.1% SDS and loaded onto a continuous sucrose gradient 25-60% (w/w) made up in 13.2 ml tubes. The gradient was centrifuged in an ultracentrifuge swing-out rotor (SW41) at 17 800 rpm for 30 min. The OBs bands were recovered from the gradient, diluted in 0.1% SDS and pelleted for 30 minutes at 15 000 rpm in the Sorval S50Ti rotor. The pellet was resuspended onto a continuous 45-60% sucrose gradient in 0.1% SDS (w/w). The gradient was centrifuged for 1 h at 17 800 rpm in an SW41 swing-out rotor. The OBs bands were recovered, diluted in water and centrifuged at 15 000 rpm in a fixed angle rotor for 30 min. The virus was then washed twice in water. The final pellet was resuspended in distilled water. All the above steps were carried out at 4° C as described by Scharnhort *et al.* (1977). The purity of the OBs was determined by viewing the preparation under oil immersion light microscope.

4.2.3 **Enumeration of occlusion bodies**

NPV are among the easiest viruses to quantify, as the OBs are distinctive and visible under phase contrast microscope (Grzywacs, 1997). OBs concentration was determined by counting their number in a Neubauer counting chamber using phase contrast optics (X 400) as outlined in (Hunter-Fujita *et al.*, 1998). In order to determine the viral doses to be used in the subsequent bioassays OBs were counted using a haemocytometer. The haemocytometer and coverslip were cleaned by rinsing with 70% ethanol and wiped with tissue paper. The coverslip was then placed on top of a slide so that it was covering the chamber into which the virus suspension was placed.

The coverslip was then pressed down firmly onto either side of the chamber until Newton's rings were visible. This ensured that the chamber was of the correct depth. The sample suspension was withdrawn with a micropipette; the pipette tip was placed at the junction of the coverslip and the chamber. Sufficient suspension of the virus sample was expelled to fill the chamber completely, excess suspension was drained into the channels at the side. The OBs were left for 10 minutes to settle and to reduce the degree of brownian motion of the particles. The OBs were counted in four large corner squares of the chamber, each of which comprises of 16 smaller squares. For each small square, OBs within the square, including those touching the top and left hand sides but not touching the bottom or right-hand sides were counted. Each preparation of the viruses was counted four times. The mean numbers of the OBs were then calculated and from this concentration of OBs/ml were determined and used for bioassays.

4.2.4 **Preparation and sectioning of OBs for electron microscopy**

Purified virus OBs were fixed in 2% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2) for 24 h after which it was washed and then post fixed in 1% osmium tetraoxide for further 24 hours. The pellet was dehydrated stepwise by increasing the concentration of alcohol, from 50% to 100% in 25% steps and finally transferred to acetone. The pellet was then embedded in 50:50 acetone: resin. The pellet in the resin was sectioned using a diamond knife and the section stained with uranyl acetate and viewed under an electron microscope (Joel 1220EM).

4.2.5 **Bioassay to determine LD_{50} and LT_{50}**

Bioassay was based on infecting first and second instars of *H. armigera* with known concentrations of OBs to measure quantal response (death) of the host and time taken for death to occur in relation to dosage ingested. The early instars (first and second) were of more significance because they are the target stages of control methods since the later stages (third, fourth, fifth and sixth) of the insect bore into the fruit and feed while hiding inside the plant tissues.

A series of 10-fold dilutions of the three test isolates of the virus with the titre of 0 (control), 1 x 10, 10^2, 10^3, 10^4, 10^5, and 10^6, OBs/ml were used. Forty larvae were used for each dosage in all bioassays. Concentration of OBs in each suspension was determined under oil immersion light microscope in a Neubauer chamber. Control set-up received distilled water treatment only.

A small piece (<1mg) of artificial diet (Shorey and Hale, 1965) without formaldehyde was placed into each dish and covered with 1µl of the corresponding dilution of infectious suspension. Neonate larvae of first and second instars used for bioassay were starved for 24 h before infection was done. During infection the larvae were kept at a

temperature of 27 ± 2 ^{0}C and on a damp filter paper to prevent the diet from drying. Treatments were checked after 24 h for deaths resulting from handling. At the same time Petri dishes were examined and larvae that had eaten all the infected diet were selected to continue the experiments. 1 - 2 g of fresh artificial diet with formaldehyde was added into each Petri dish at least twice a week. Larval mortality was recorded at 24 h interval and data analysed by Probit analysis (Finney, 1978).

4.2.6 **Diagnosis of mortality causes**

Larvae were examined daily and those which had died were individually smeared and stained with Giemsa stain (Appendix B). The smears were examined under oil immersion light microscope (magnification X400) for presence of OBs. Those smears having nuclei containing OBs were classed positive.

4.2.7 **Analysis**

Statistical analysis of mortality data was carried out using a Probit analysis based on SAS, a computer based programme. This computed the weighted linear regression of Probit mortality against log dose and for the calculation of LD_{50} and its 95% fiducial limits (Finney, 1978). Where control mortality occurred, the data were corrected using Abbott's formula (Finney, 1978). The time course of infection was used to calculate LT_{50} values for each instar.

4.3 **RESULTS**

4.3.1 **Purification of occlusion bodies**

Centrifugation in a horizontal position in the rotor at high speed resulted in each type of macromolecule sedimenting down the density gradient at its own rate, which was determined primarily by weight and also by density and shape. The differential centrifugation of the S. African virus isolate yielded five bands whereas the Kenyan isolate yielded three bands on a 25 - 65% (w/w) sucrose gradient (Fig. 4.1 and 4.2 respectively). Cellular debris collected at the top of the gradient and higher molecular weight aggregates pelleted at the bottom of the tube. The bands from the sucrose gradients were collected and treated as one for each virus isolate except the top most band which was discarded as it contained mainly cellular and other lighter debris.

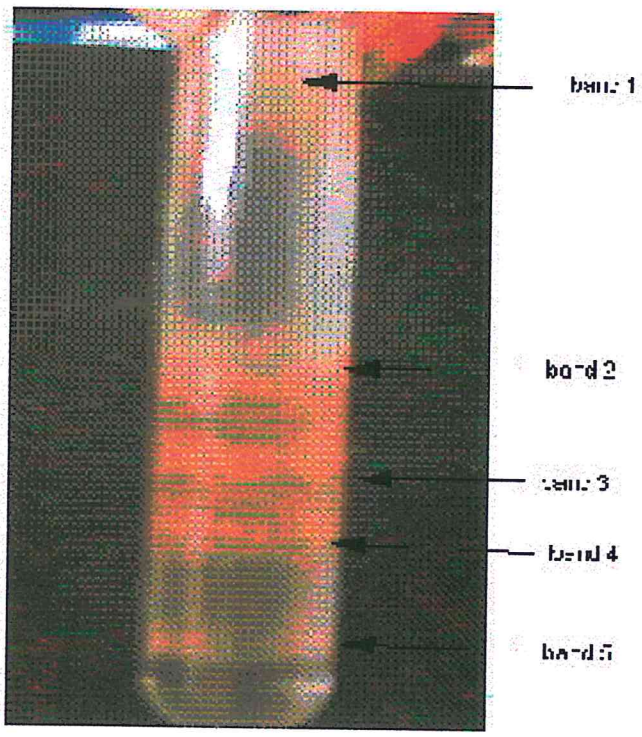


Fig. 4.1: A 25-60% sucrose density gradient of OBs from the S. African isolate of HaNPV

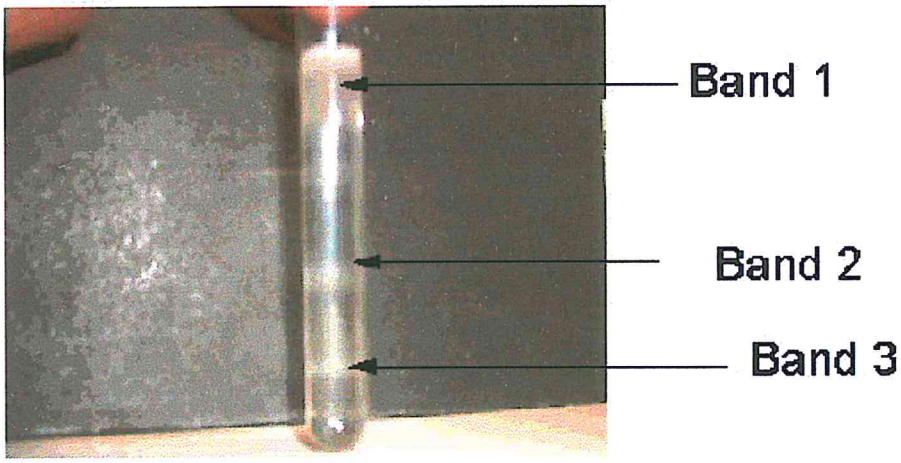


Fig.4.2. A 25-60% sucrose density gradients of OBs from the Kenyan isolate of HaNPV

4.3.2 **Virus morphology**

Fig. 4.3 and 4.4 show transmission electron micrographs of sectioned OBs of HaNPV isolates from Kenya and S. Africa respectively. A large number of scans revealed that each nucleocapsid rod (s) was singly encapsulated within the virus lipoprotein envelope for the Kenyan isolate (Fig 4.3), whereas for the S. African isolates it was multiply encapsulated in lipoprotein envelope (Fig. 4.4). The Kenyan virus isolate was assigned to the SNPV and the S. African virus isolate was assigned to the MNPV subgenera of the Baculoviridae (Murphy *et al.*, 1995). Fig. 4.5 showed purified OBs ruptured in a solution of sodium carbonate revealing the inner virus particles. A close look at the virus samples under electron microscope revealed that the virus OBs tend to aggregate (Fig. 4.6). Aggregation was also observed during counting of OBs.

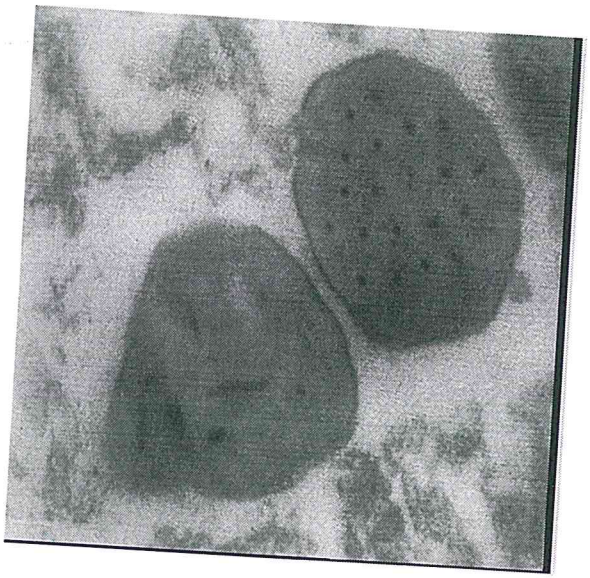


Fig. 4.3 Electron micrograph of sectioned OBs of the Kenyan HaNPV isolate (magnification x 250 000)

Fig. 4.4 Electron micrograph of sectioned OBs of the S. African HaNPV isolate (magnification x 250 000)

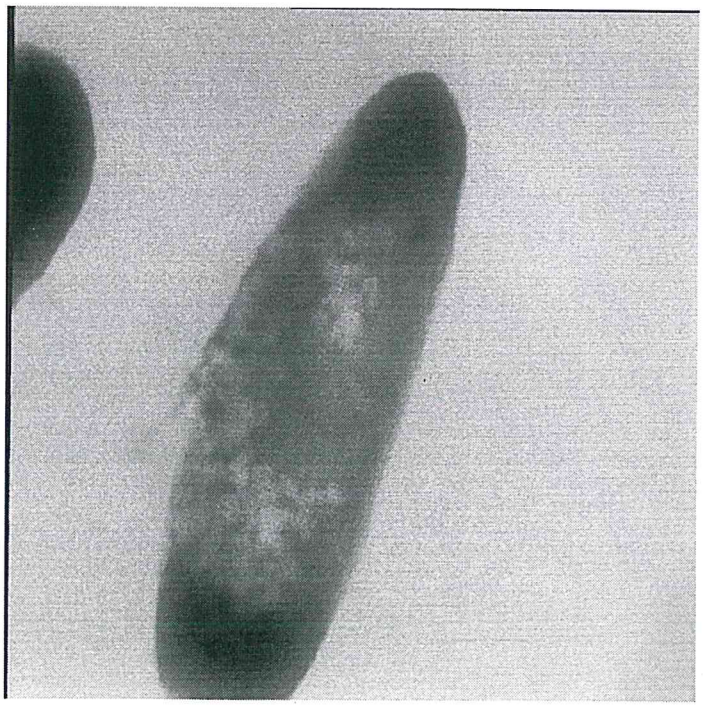


Fig. 4.5 Polyhedron lysed with sodium carbonate showing enclosed virus particles (magnification x 250 000)

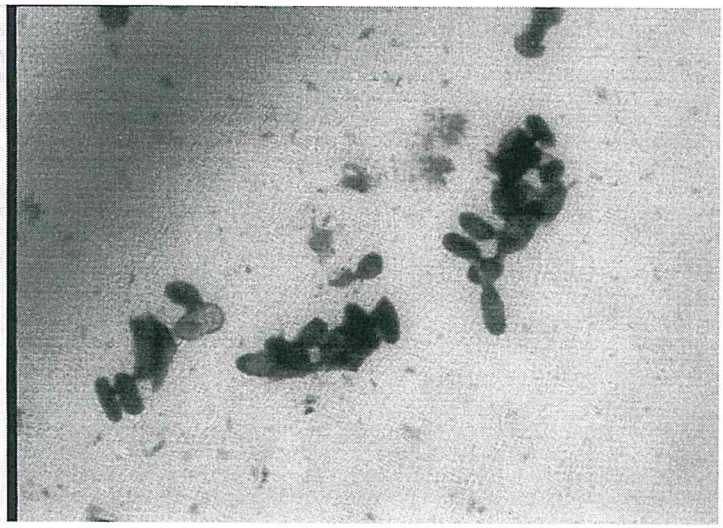


Fig. 4.6 The aggregation of the OBs (magnification x 120 000)

4.3.3 **Dosage-mortality analyses**

The LD_{50} values (with 95% confidence intervals) for the two instars in each bioassay replicate and associated statistics are given in Table 4.1. The median lethal dose (LD_{50}), expressed as a number of OBs, varies from 16 – 79 OBs for the first instar, whereas for the second instar larvae the variation ranges between 20 - 6 309 OBs. There was no significant difference in LD_{50} values between the S. African and Kenyan isolates when tested for the first instar (Fig. 4.7). The second instar assays showed significant differences among all the isolates, with the S. African isolate having much lower LD_{50} = 20 compared to the Kenyan isolate LD_{50} = 2 512 and Gemstar® LD_{50} = 6 309, indicating that the S. African isolate was more infectious than the other two isolates.

The slopes of the dose response lines, which measure variability in response, varied from 0.29 - 0.93. From the dose mortality data recorded daily, estimation was made of the median lethal time (LT_{50}) for each larval instar at a given dose (Table 4.2a and 4.2b) based on Biever and Hostetter (1971) equation. Only those larvae finally dying from NPVs infection were included in the calculations. The analysis indicated that the overall LT_{50} increases with the concentration of the dose ingested. There was a strong correlation (R^2 = 0.9) between $\log_{10}$ polyhedra and the LT_{50} values as indicated in Fig. 4.8 and Fig. 4.9 for first and second instars, respectively. Within any instar for each isolate, the LT_{50} decreased with the increase in dose that is for high dosages, insects succumbed to viral infection within a shorter time.

Table 4.1 Summary of LD_{50} values and the associated statistics for the two instars of *H. armigera* infected with the three test isolates of nucleopolyhedroviruses.

instars	Virus isolates	LD_{50}	Std mean	95% confidence limits		Slope	Std mean	Intercept	X^2
				Upper	Lower				
1st	S.Africa	20	0.451	79	3	0.73	0.142	-0.918	11.72
	Kenya	16	0.056	71	1	0.52	0.091	-0.612	9.79
	Gemstar	79	0.149	141	40	0.93	0.113	-1.755	6.55
2nd	S.Africa	20	1.426	290	2	0.61	0.184	-0.802	27.50
	Kenya	2512	0.193	6309	1047	0.48	0.053	-1.619	6.52
	Gemstar®	6309	0.369	42795	1195	0.29	0.044	-0.904	4.92

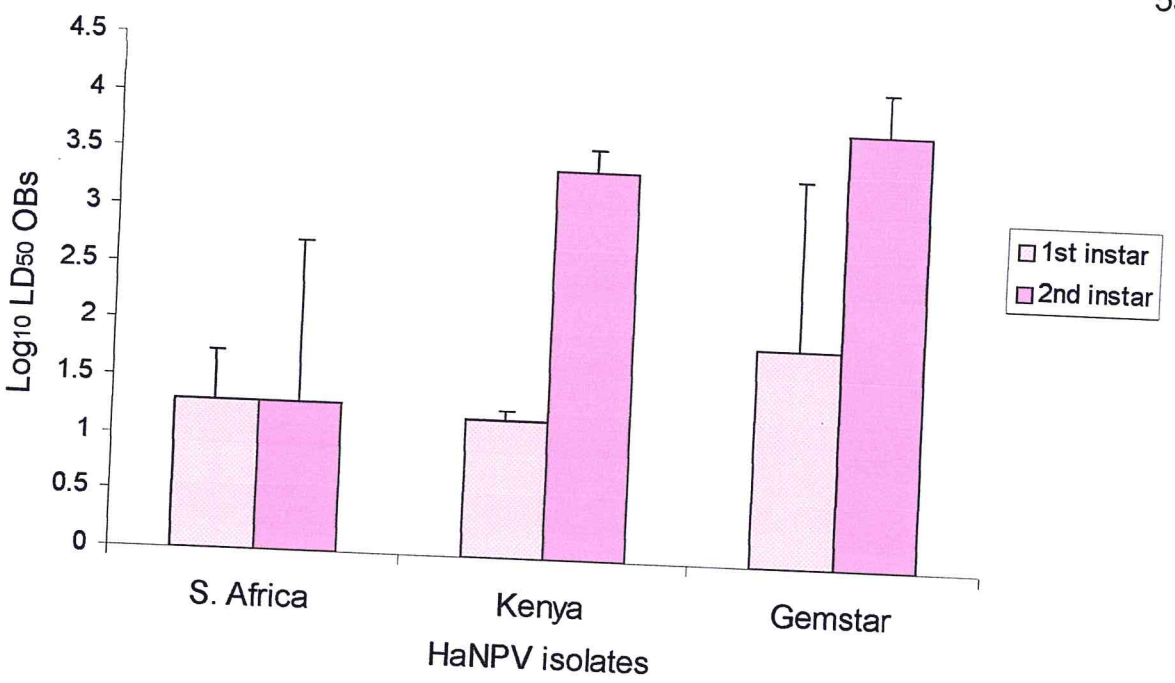


Fig. 4.7 Log-dose response of *H. armigera* infected with three HaNPV of three HaNPV isolates for first and second instars

4.3.4 **Time mortality effects**

The LT_{50} was calculated from the equation (Biever and Hostetter, 1971)

$$LT_{50} = a + e\,(c-b)/d$$

Where:

a = number of hours from initiation of the test until the reading made just before the 50% value was reached.

b = total number of larvae dead at the reading just before the 50% value was reached

c = 50% of the total number tested

d = number of hours between mortality counts

Table 4.2a: Summary of the LT_{50} for the first larval-instar of *H. armigera* in days

Log_{10} dose	1	2	3	4	5	6
S. Africa	6.4	6.0	4.4	3.7	3.5	3.0
Kenya	8.4	8.2	5.8	4.6	3.5	3.2
Gemstar	-	11.0	5.5	5.3	4.3	4.2

Table 4.2b: Summary of the LT_{50} for the second larval-instar of *H. armigera* in days

Log_{10} dose	1	2	3	4	5	6
S. Africa	6.8	6.4	5.8	4.9	4.1	3.9
Kenya	-	11.6	10.5	10.0	5.8	4.0
Gemstar	-	-	-	-	11.0	7.6

Note: (-) indicate that the dose did not yield to quantifible LT_{50} or LD_{50}

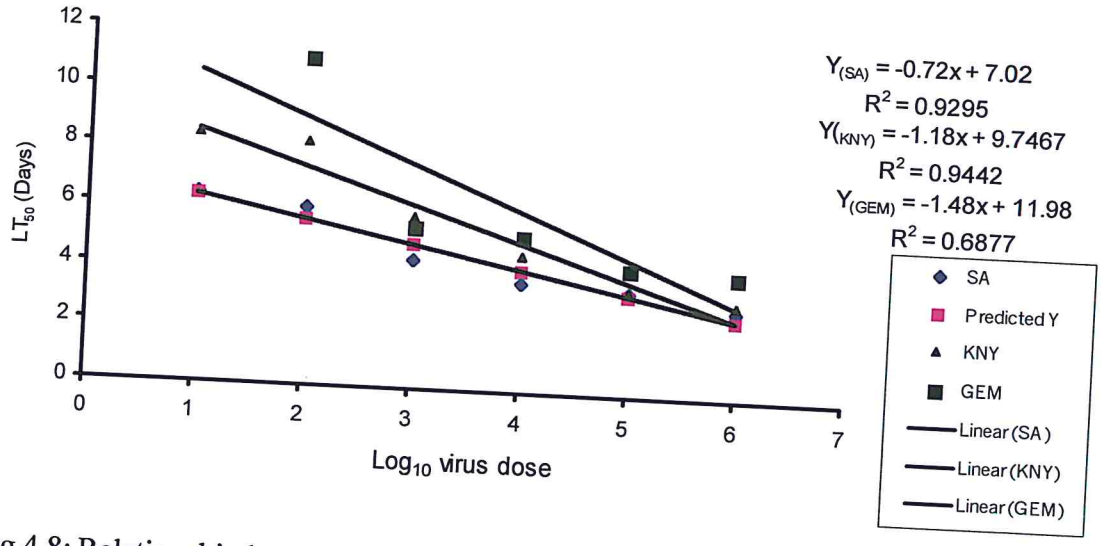


Fig 4.8: Relationship between virus dose and LT_{50} for HaNPV from Kenya, S. Africa and Gemstar® for first larval-instars

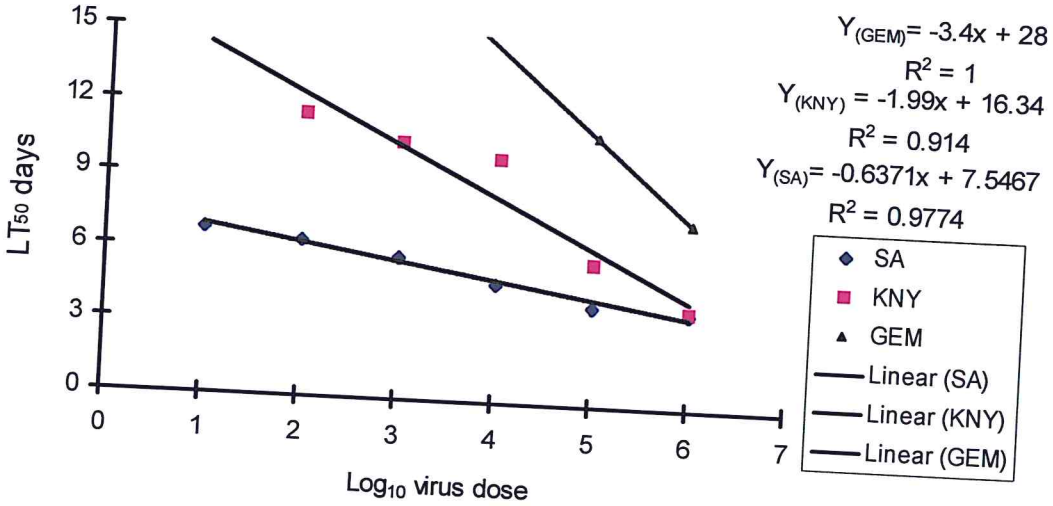


Fig 4.9: The relationship between log_{10} polyhedra and LT_{50} for *H. armigera* second larval instar

4.4 **DISCUSSION**

Centrifugation of the Kenyan and the S. African HaNPV isolates yielded three and five bands respectively instead of the expected one and two band (s) for a single and multiple nucleocapsid (s) virus envelope particle respectively. Subsequent purification of the same virus isolates showed that a narrow band of material formed in the middle of the gradient and a hazy band lower down. The formation of many bands could be a result of aggregation of OBs. Aggregates, though rare, were observed during OBs counting and electron microscope studies of OBs (Fig. 4.6).

The LD_{50} values for the first and second instars are comparable with other values found for a number of other NPVs with similar virulence against neonate larvae in homologous hosts. Teakle *et al.* (1985) reported LD_{50} values of 50 and 110 for first and second instars of *H. armigera* respectively. The value reported by Teakle *et al.* (1985) for first instar is considerably higher compared to the LD_{50} values for the S. African and the Kenyan isolates, though lower than that of Gemstar® (LD_{50} = 79). The difference could be explained by differences in bioassay techniques used, i.e. surface contamination of diet, which leads to longer virus acquisition times, especially for first instar.

Generally, LD_{50} values increase during larval development. Evans (1981) found an increase from 7 to over 900 occlusion bodies between first and second instar of *M. brassicae* larvae with the corresponding MbMNPV. Allen and Ignoffo (1969) indicated that maturation resistance decreased with larval susceptibility and were largely correlated with increase of body weight. The same trend of maturation resistance was observed in this study, first instars were more susceptible to HaNPV compared to second instar as indicated by the LD_{50} values.

It is difficult to compare the slope values obtained with those given in the literature because differences could be due to techniques rather than variation in host responses. But taking the values of the slope as they are, the slopes obtained in this study compared

favourably with those quoted in the literature of the same species (Whitlock, 1977; 1978; Teakle *et al.*, 1985). The slopes were typically low, which is expected for bioassays with micro-organisms, as it is believed micro-organisms act independently of each other to cause infection (Ridout *et al.*, 1993). Whitlock (1978) got a slope of 0.65 - 1.17 for seven days old larvae of *H. armigera* weighing between 100 - 140mg.

The values for χ^2, which tests for the linearity and homogeneity of response among the test insects, were not significantly different except for some significant heterogeneous response which occurred in second instar larvae when infected with the S. African isolate. However, Hughes *et al.* (1984) working on baculovirus infection in *H. zea* obtained slope values in excess of 2.0, showing it is possible to decrease variability by increasing precision in experimental procedures. This might be expensive in terms of time and labour, and a compromise had to be reached in this bioassay and higher variation was acceptable for quick comparative studies.

Time taken to kill the insect was a function of dose ingested as portrayed by the strong correlation between $\log_{10}$ dose and LT_{50} values (Fig. 4.8 and 4.9). The higher the dose, the shorter the LT_{50}. Teakle *et al,* (1985), obtained LT_{50} values of 3.6 - 8 days for larval age between zero to seven days old when *H. armigera* were infected with 10^3 OBs/ml at 30 ^{0}C. In this study this trend was observed but even at low doses, the LT_{50} values were comparatively low; for example, at a lower dose of 10^3 OBs/ml (S. African HaNPV), the LT_{50} was 4.4 days. The initial mortality in both larval-instars at all doses at least occurred on the fourth day.

The LD_{50} values for the three isolates on first instar are different, and the S. African isolate performed better than the two isolates in terms of both LT_{50} and LD_{50}. Whether this is due to the most obvious reason of the S. African MNPV containing more virus particles per occlusion body, is not clear. This study has shown that S. African HaNPV was extremely

infective, as can be seen from the low LD_{50} values. The insect itself seems to be very susceptible to virus infection compared to other lepidopteran species and their homologous viruses (Biever and Hostetter, 1971; Evans, 1981; Smits and Vlak, 1988).

CHAPTER FIVE

5.0 BIOCHEMICAL COMPARISONS OF TWO NUCLEOPOLYHEDROVIRUSES ISOLATES

5.1 INTRODUCTION

There were differences in biological responses of *H. armigera* larvae to infection with the two NPV isolates and Gemstar® (Chapter 4). The HaNPV isolate from S. Africa performed better in terms of dosage and lethal time of the two larval stages tested. The differences could be quantified statistically and the trends suggested that S. African isolate was more virulent than the Kenyan isolate and Gemstar®. Within these limits, dosage responses to HaNPV from Kenya and S. Africa especially for first instar were the same and it was intended to find if the two virus isolates were related by studying their polypeptides and some DNA characteristics.

Furthermore, the only knowledge available on the Kenyan and S. African HaNPV isolates was based on the signs and symptoms of the infected larvae and from microscopy study (Whitlock, 1977, 1978; Moore *et al.*, 1998; Baya, 2000). Microscopy study showed that the two isolates from Kenya and S. Africa were typically occluded viruses, with their protein lattice containing randomly arranged single virus particles (Chapter 4). It was therefore necessary to conduct biochemical studies to determine whether the two virus isolates were the same virus occurring in different locations.

REN analysis of the DNA fragments was done to test the differences on the virus isolates; this was achieved by agarose gel electrophoresis. Gel electrophoresis was useful in identification of the DNA extracted from the two viral isolates, by comparing the band patterns they yielded in electrophoresis. Agarose gel when used at concentration of 0.1% -

2.5% resolve DNA of 150 base pairs upward (Yang *et al.*, 1979). Like acrylamide, the gel percentage composition affects the rate of migration of the DNA fragments.

The S. African and Kenyan isolates structural polypeptides were compared by SDS - polyacrylamide gel electrophoresis. Acrylamide as a supporting gel was used as a media because it is chemically inert, stable, clean and reproducible (Sambrook *et al.*, 1989). Almost all-analytical electrophoresis of proteins is carried out in polyacrylamide gels under denaturing conditions. This ensures dissociation of proteins into their individual polypeptides subunits and minimises aggregation.

Proteins are amphoteric compounds; their net charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above their isoelectric points, proteins have a net negative charge and migrate towards the anode in an electrical field. Below their isoelectric points, the proteins are positively charged and migrate towards the cathode. The net charge carried by a protein is in addition independent of its size i.e. the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, both size and charge of the molecules determine the electrophoretic separation of proteins.

Nucleic acids however, remain negative at any pH used for electrophoresis and in addition carry a fixed negative charge per unit length of molecule, provided by the PO_4 group of each nucleotide of the nucleic acid. Electrophoretic separation of nucleic acids therefore is strictly according to size.

Sodium dodecyl sulphate (SDS) is an anionic detergent, which denatures proteins by "wrapping around", the polypeptide backbone and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1 (Coyne *et al.*, 1996). In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. The denatured polypeptides become "rods" of

negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size, this was done with 2- mercaptoethanol. In denaturing SDS-PAGE separations therefore, was determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. In most cases ionic detergent SDS in combination with reducing agent and heat are used to dissociate proteins.

The choice of the gel concentration is critical in that spaces in the gel matrix must be of similar dimensions to the macromolecules so that the selective sieving occurs during electrophoretic migration. Relative separation between two components increases with gel concentration because separation is size dependent (Sambrook *et al.*, 1989), the upper limit being reached when proteins cannot penetrate the gel. For general purposes, 12-13% gels are routinely used in variable ratios of acrylamide to bisacrylamide, usually 30%: 0.4% (w/v) (Coyne *et al.*, 1996). Bisacrylamide is a cross linker to increase the minimum pore size so that a maximum sieving effect is obtained. In addition, if there is no cross-link, the gel needs to be stacked so as to concentrate the proteins before they are resolved.

5.2 **MATERIALS AND METHODS**

All chemicals used were Qbiogene laboratory reagents (Qbiogene Company Limited, Rue Geiler de Kaysersberg, France) except protein markers, glycine, SDS, agarose, bisacrylamide, acrylamide, phenol, sodium carbonate, hydrochloric acid, chloroform and EcoRV (Sigma Chemical Co, USA), Silver stain Plus kit was purchased from Bio-Rad laboratory Co. Ltd.

Equipment used.

The centrifuges used were the Ultracentrifuge Beckman LC 25 high speed (100, 000 rpm), MSE superspeed 65 and eppendorf 5413 Microfuge (with speed of 15 000 rpm).

Power packs used for electrophoresis were the Shandon Vokam 500-150. Combs used for making sample slots in the gels were the Shandon types. The ultraviolet illuminator used was the UVintel from Bio-Rad laboratories.

Composition of buffers

1a. 10x Polyacrylamide gel electrophoresis (PAGE) buffers: -

0.5 M Tris (hydroxymethyl-aminomethane hydrochloride)

0.5 M glycine

0.1% sodium dodecyl sulphate (SDS)

1b. PAGE resolving gel buffer

1 M Tris/hydrochloric acid (HCl) pH 8.8

1c. PAGE stacking gel electrophoresis buffer

1 M Tris/HCl pH 6.8

2a. 10x Agarose gel electrophoresis buffer: -

0.4 M Tris

0.05 M sodium acetate

0.01 M ethylene diamine-tetra acetate (EDTA) pH 7.7 with HCl

3. DNA lysis buffer: -

100 mM Tris

100 mM EDTA

2% SDS

200 mM β mercaptoethanol (β merc)

54% sucrose

4. Restriction enzymes for DNA digestion buffer

HindIII extracted from *Haemophilus influenzae* 5'-A↓AGCT T-3'

10 mM TrisHCl

7.5 pH

100 mM KCl

0.1 mM EDTA

1 mM Dithiothreitol

1 mM BSA

50% Glycerol

EcoRI extracted from *Escherichia coli* 5'-G ↓AAT C-3'

10 mM KPO_4

7.4 pH

400 mM KCl

0.1 mM EDTA

5 mM 2- Mercaptoethanol

0.15% Triton x100

0.2 mg/ml BSA

50% glycerol

EcoRV extracted from *Escherichia coli* 5'GAT↓ ATC- 3'

10 mM Tris-HCl

100 mM NaCl

5 mM$MgCl_2$

1mM 2-Mercaptoethanol, pH. 8

BamHI extracted from *Bacillus amyloliquefaciens* 5' G↓GATC C-3'

20 mM TrisHCl

pH 7.5

200 mM KCl

0.2 mM EDTA

10 mM Dithiothreinol

0.1% Triton x 100, 0.2mg/ml BSA and 50% Glycerol

Protein dissociation mix

100 mM β merc

10% SDS

8% glycerol

1M Tris pH 6.8

0.2% bromophenol blue (BPB)

Deionised water

NB: The arrows indicate the point of cleavage whereas the letters indicate the base pair sequence recognised by the enzyme.

5.2.1. **Extraction of the NPV DNA**

DNA was extracted from the OBs recovered from infected *H. armigera* larvae following a modified procedure outlined by Hunter-Fujita *et al.* (1998). Viral suspension was diluted to 2 x 10^{10} OBs/ml in milli-Q water. 150 µl of the viral suspension was then dispensed into 1.5 ml eppendorf tube. 30µl of 0.5M EDTA and protease K (3 µl of a stock solution of 25 mg/ml in water) were added to the eppendorf tube with the virus sample to digest any nuclease present. The sample was then incubated for 2 h at 37 °C and thereafter 17 µl 1 M Na_2CO_3 was added to disrupt the OBs and release the virus particles. The pH was adjusted to 8.0 by adding approximately 8 µl of 1M HCl. 1 µl of RNase A (RNase A stock solution 10 mg/ml) was added to the suspension and incubated for 10 min at 37 °C. 24 µl of 10% SDS was added and incubated for 15min at 37 °C to disrupt the protein (O'Reilly, Miller and Luckow, 1992). 10% SDS was added to liberate and disrupt the virus particles so as to release the DNA.

An equal volume of (approximately 240 µl) Tris-buffered phenol (pH 8.0) was added to the DNA and the tube was mixed gently by inverting it up and down for about 30 seconds until an emulsion was formed. The mixture was then centrifuged in a microfuge at 13 000 rpm for 1 min.

The upper aqueous phase was transferred carefully into a clean eppendorf tube while avoiding sucking up the proteinaceous debris from the interface. The aqueous layer was extracted with an equal volume of a 1:1 mixture of phenol: chloroform: isoamyl alcohol (24:1) by gently inverting several times and centrifuging at 13 000 rpm for a minute. The aqueous layer was removed and transferred to another eppendorf tube and extracted with an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged as above. The final aqueous layer was transferred to dialysis chamber using forceps; the chamber was covered with a piece of dialysis membrane that had been soaking in dialysis buffer.

The aqueous layer was dialysed in three litre changes of 10 mM Tris, 1mM EDTA (TE) pH 8.0. The membrane side was placed downwards, in a beaker of dialysis buffer with magnetic stirrer bar. The DNA was dialysed for at least 24 h, with buffer changed at least three times. The dialysis chamber was removed from the buffer, blotted with tissue paper to soak up excess buffer and to prevent contamination of the DNA. A slit was made on the membrane with a surgical blade and the dialysed DNA transferred to a clean eppendorf tube. The dialysed DNA was then transferred into a clean eppendorf tube and stored at 4 °C.

5.2.2 **Restriction enzymes analyses**

5.2.2.1 **Agarose gel electrophoresis**

A 0.7% agarose solution was prepared by measuring 0.7 g of agarose which was added into a glass flask containing 100 ml 1X TAE. The solution was placed on a

microwave until agarose was completely dissolved and solution appears to be clear. Dissolved agarose was then allowed to cool to about 50 ^{0}C before pouring the gel into electrophoresis tray. The gel tray was set and the comb was then placed in position at about 1cm from one end of the tray. The comb was positioned vertically such that the teeth were about 1-2 mm above the surface of the tray. The gel solution was poured into the tray to a depth of about 5 mm. It was then allowed to solidify for about 20 minutes at room temperature. The comb was gently removed, and the tray was placed in electrophoresis chamber, and covered (just until wells were submerged) with electrophoresis buffer (the same buffer used to prepare the agarose). The samples for electrophoresis were prepared by adding 1 µl of 6x gel loading dye for every 5 µl of DNA solution and mixed well. Electrophoresis was done at 80 volts until dye markers had migrated to an appropriate distance. The gel was then stained in 0.5 µg/ml ethidium bromide until the DNA had taken up the dye and was visible under short wave UV light.

5.2.2.2 **Digestion of virus DNA with the enzymes**

Virus DNA was digested with restriction enzymes following a modified procedure by Hunter-Fujita *et al.* (1998). 10 µl viral DNA from HaNPV isolates was digested with 0.7 µl (10 units/µl) of HindIII, EcoRV, BamHI, and EcoRI and 0.7 µl incubation buffer. For HindIII, BamHI and EcoRI 0.7 µl of BSA (bovine serum albumin) was added. The contents were mixed and centrifuged in a microcentrifuge for a minute to collect the contents at the bottom of the tube. The mixtures were incubated for 3 h at 37 °C and the reaction stopped by adding 0.9 µl bromophenol blue.

The contents were then loaded onto agarose gel by placing the tip of a pipette containing DNA sample into a well expelling gently, taking care not to trap air bubbles in the well and not to contaminate the adjacent wells. Electrophoresis was carried out at 80 V until

the bromophenol blue dye had migrated three quarters of the way down the gel. Using a spatula, the gel was immersed in 0.5% ethidium bromide solution and left to stain for 1 h then photographed under ultraviolet light in UVintel machine. Restriction enzymes fragment profiles from the DNA samples were compared.

5.2.3 **SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Structural Polypeptides** (Laemli, 1970)

5.2.3.1 **Purification of polyhedrin**

Polyhedra were purified using a modification of the procedure reported by Summers and Smith (1978b). 80 µl of highly purified OBs were suspended in 0.01M Tris (pH 7.8), 0.01M EDTA and incubated for 2 h at 70^0C to inactivate the associated protease. Protease inactivated OBs were washed free of buffer by differential centrifugation. To ensure further the inactivation of the protease, the OBs were resuspended in 0.01 M Tris (pH 7.8), 0.01 M $HgCl_2$ and allowed to equilibrate at room temperature overnight. The OBs were removed from the buffer and enzyme inhibitor by pelleting, using repeated differential centrifugation and extensive washing of the pellets with water. Disruption of the OBs was achieved by adding 0.1M Na_2Co_3, 0.17M NaCl pH 10.8, for 10 min at a concentration of 5 mg of protein/ml at 4 ^{0}C. The solution was centrifuged at 30, 000 rpm (SW41) rotor for 2 h at 5 ^{0}C to remove any insoluble materials. The solution containing virus particles were then analysed by polyacrylamide gel electrophoresis.

5.2.3.2 **Polyacrylamide gel electrophoresis**

A vertical discontinuous polyacrylamide slab gel was used to compare polypeptide profiles of *H. armigera* NPV isolates from Kenya and S. Africa and to estimate the molecular

weights of the polypeptides using both high and low range standard molecular weight markers.

A 12% resolving gel was made as follows:

30%/0.4% (w/v) acrylamide: bisacrylamide (75:1)	3.5ml
1M Tris pH 8.8	3.3ml
Deionised water	1.5ml
10%SDS	88µl
15% ammonium persulphate (freshly made) (catalyst)	353µl
Tetramethylethylene diamide (TEMED) for setting	1.7µl

The resolving gel was poured into a sandwich of vertical gel plates. A thin film of water was pipetted on top of the gel prior to its setting to insure a level surface. After setting, the water was removed with a filter paper. A 3% stacking gel (for concentrating the proteins into a narrow band) was layered on the resolving gel and Shandon comb inserted.

The stacking gel was made as follows:-

30%/0.4% acrylamide:bisacrylamide (75:1)	125.0 µl
1M Tris pH 6.8	158.0 µl
Deionised water	812.0 µl
80% glycerol	83.0 µl
10% SDS	12.4 µl
1.5% ammonium persulphate	58.0 µl
TEMED	1.7 µl

An equal volume of dissociation mix was added to the virus particles and the mixture boiled in water bath for 2 min. The sample was loaded onto the discontinuous gel. Molecular weight markers (lysozyme 14 400, soybean trypsin inhibitor 21 500, bovin carbonic anhydrase 31 000 aldolase 39 000, bovine serum albumin 66 200, phosphorylase B 97 400, β Galactosidase 116 250 and myosin 200 000) were co-run with the NPV protein samples to facilitate molecular weight determinations. The gel tank contained about 500 ml of gel buffer in an upper and lower reservoir. The samples were stacked at 20 volts for 1 hour and then electrophoresed for 8 h at 60 V. The gel was stained with silver stain Plus kit (see Appendix E for components and procedures used in staining). The gel was then viewed under UV white converter illuminator.

5.3 RESULTS

5.3.1 Restriction profiles of HaNPV of the Kenyan and S. African isolates

Fig. 5.1 shows the DNA restriction endonuclease profiles of HaNPV digested with the enzymes HindIII, EcoRI, EcoRV and BamHI on a 0.7% agarose gel. All digestions showed some differences between the Kenyan and the S. African isolates. The BamHI (lane 4 -5 and lane 10 -11) and EcoRI (lane 3 and 14) fragment patterns for the Kenyan and the S. African isolates respectively illustrated the differences between these two isolates by the presence or absence of one or more fragments. For example, in lane 3, the Kenyan sample has an extra fragment of approximately 4.0kb which is not present in the profile of the S. African sample in lane 14. Both the samples had fragment patterns of sizes 9.4, 8.0, 7.0, 6.6 and 5.5 kb when digested with EcoRI (lanes 3 and 14). Both the isolates had DNA fragment profiles of size 23.1 kb when digested with BamHI. Whereas when digested with EcoRV the fragment of 23.1 kb was missing in the S. African isolate but present in the Kenyan isolate. Table 5.1 indicates the estimated DNA fragment profiles. The genomic sizes were estimated to be 107.2 and 81.5 kb for the Kenyan and S. African isolates, respectively.

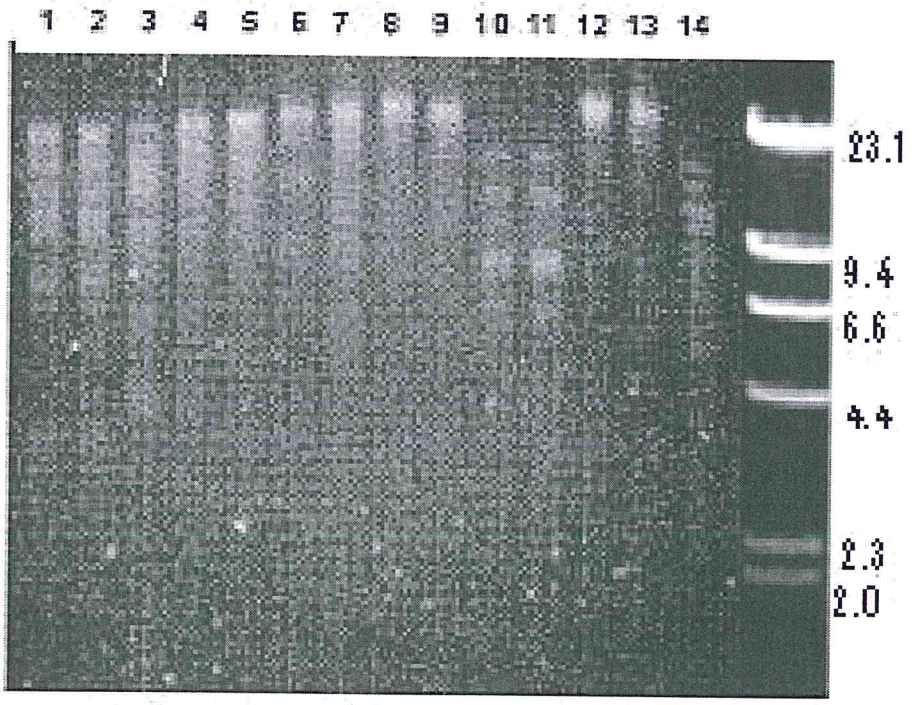


Fig. 5.1. Comparison of restriction fragments profiles of HaNPV genomic DNA from Kenya and S. Africa. 6 µl of DNA was digested with HindIII, EcoRI, BamHI and EcoRV. Lanes 1-7 are restriction profiles of the Kenyan isolate and lanes 8 -14 are restriction profiles of the S. African isolate. Next to lane 14 is λDNA/HindIII was used as a molecular weight marker. Lanes 1-2, 8-9 (HindIII), 3 and 14 (EcoRI), lanes 4 -5 and 10 -11(BamHI), lanes 6-7 and 12-13 (EcoRV).

Table 5.1. Molecular sizes (kb) of the Kenyan and the S. African HaNPV isolates DNA fragments generated by digestion with restriction enzyme EcoRI

	EcoRI	
Fragments	Kenya	S. Africa
1	21	18
2	20	15
3	18	9.4
4	9.4	9.0
5	8.0	8.0
6	7.0	7.0
7	6.6	6.6
9	5.5	5.5
10	5.0	3.0
11	4.0	
12	2.7	
Total	107.2	81.5

5.3.2 **SDS-Polyacrylamide gel electrophoresis**

SDS-PAGE structural polypeptides of HaNPV isolates from Kenya and S. Africa are shown in Fig. 5.2. Approximately one polypeptide of size 64 000 was resolved for each virus isolate. It is possible that the major proteins obscured minor proteins. The photograph showed the similarities in the migration rates of the S. African and the Kenyan isolates polypeptides. Molecular weights for the polypeptides were estimated by measuring the distance migrated by the polypeptides relative to the molecular weight markers as shown in Table 5.2.

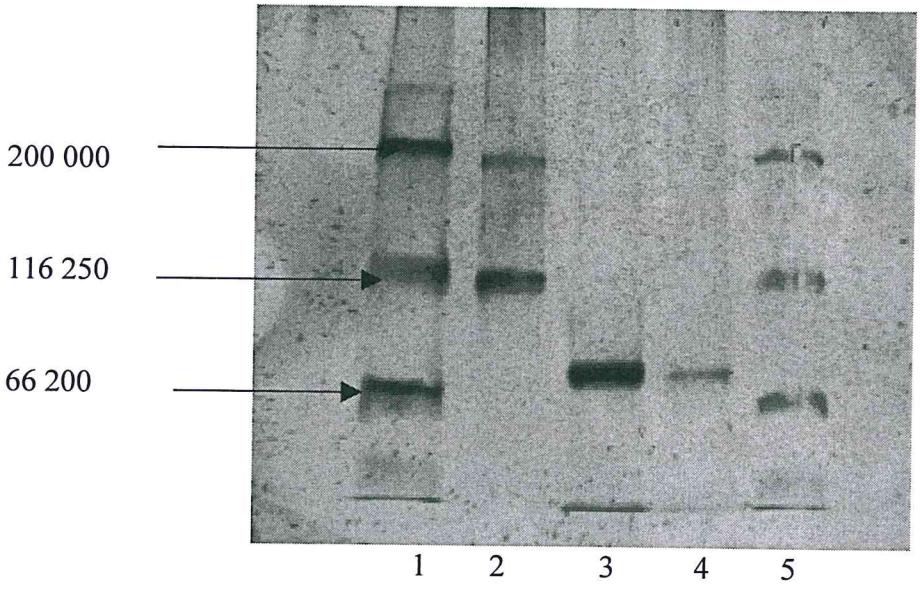


Fig. 5.2 PAGE of SDS disrupted polypeptides of HaNPV on a 12% discontinuous polyacrylamide gel. Lane 1and 2 are low and high molecular weight markers respectively. Lane 3 is the Kenyan protein sample and lane 4 is the S. African protein sample. Lane 5 is low molecular weight marker.

Table 5.2: Estimated molecular weights of the main structural polypeptides of HaNPV isolates from Kenya and S. Africa.

Polypeptide number	Molecular weight '00	
	S. Africa	Kenya
1	64 000	64 000

5.4 **DISCUSSION**

The use of RENs is one of the ways of determining the differences or similarities between closely related virus isolates. The major advantage of REN is that genomes can be analysed and relationships determined based on the fragment band patterns and genomic sizes. RENs recognise a particular base sequence along DNA molecules and have a specific cleavage sites. The RENs used in this study were EcoRI, EcoRV, HindIII and BamHI. These enzymes were able to recognise the viral DNA at different cleavage sites as indicated in the literature. Many enzymes were used to help in identifying any variation between the two isolates.

The two isolates shared six similar bands when digested with EcoRI (Fig. 5.1). The results from the DNA restriction profiles based on the size of the genome indicated that the isolates are different HaNPV with Kenyan isolates having 107.2 kb and S. African isolate 81.5 kb. Nevertheless, there were a number of shared DNA fragments showing that the viruses had some similarities. Based on the number of shared bands in REN profiles of the virus DNAs, HaNPV isolates from Kenya appeared to be closely related to HaNPV previously reported by Rovesti *et al.* (2000). There were only minor differences between the EcoRI and HindIII fragments of the profiles of the Kenyan isolates and the one previously reported by Rovesti *et al.* (2000). It was difficult to establish the precise relationship of the S.

African isolate from previously reported HaNPVs (Smith and Summers, 1978a; Gettig and McCarthy, 1982; Rovesti *et al.*, 2000).

As has been shown in this study, the fragmentation profiles of two different virus isolates can be identical or clearly distinct depending on the restriction endonuclease enzyme used. This validates the idea that REN analysis must include many different enzymes wherever possible to ensure that differences of the closely related genomes can be easily identified from the restriction profiles.

The presence of two distinct viruses in geographically separated populations of the same host species has been demonstrated in a few studies on the geographical variability of virus isolates, including the NPV of *Agrotis segetum* (Allaway and Payne, 1983). Most frequently however, studies of the geographical isolates of NPVs (Vlak and Gröner, 1980; Gettig and McCarthy, 1982) have shown that such isolates are often closely related strains, and that differences between these strains do not appear to be related to their site of collection (Cherry ans Summers, 1985; Vickers, Cory and Entwistle, 1991). The average molecular weights of these isolates were estimated from the summation of the restriction fragment data (Fig. 5.1). Using EcoRI-digested isolates and HindIII-lambda DNA as a molecular weight standard, the estimated sizes were 107.2 and 81.5 kb for the Kenyan and S. African isolates, respectively.

The log molecular weight of a polypeptide is inversely proportional to the distance of migration, provided the molecular weight ranges between 15 500 and 165 000 (Coyne *et al.*, 1996). Hence molecular weights of polypeptides of the Kenyan and S. African HaNPV isolates were estimated using the distance migrated, relative to molecular weight markers.

Wider bands in the structural polypeptide profiles made it difficult to determine the exact number of polypeptides resolved. Further studies on this would have involved 2-dimensional separation of the polypeptides, whereby proteins are separated first by charge

and then by size. Each protein has a charge depending on its acid-base properties, which is a reflection of ionisable groups of the polypeptide chain. Each possesses a characteristic isoelectric point, at which it remains stationary in an electric field. When polypeptides are prerun with ampholines in a pH range, a banding occurs at the isoelectric points (Coyne *et al.*, 1996). Polypeptide bands formed in electrophoresis was stained by silver stain Plus kit and viewed under UV white-converter illuminator. The density of the stain has proportionality to the amount of protein present and the peaks produced can be used to quantify the concentration of proteins (Smith and Summers, 1978b). From this study it can be deduced that the Kenyan isolate was higher in protein concentration than the S. African isolate because of the intensity of the silver stain appearance as in Fig. 5.2. The same number and size of the polypeptide bands was also an indication that the viral isolates are related.

CHAPTER SIX

6.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

Several studies have compared baculoviruses isolates obtained from different geographically separated populations of a given host species (Smith and Summers, 1978a; Vlak and Gröner, 1980; Gettig and McCarthy, 1982; Kunjeku, 1982; Allaway and Payne, 1983; Williams and Payne, 1984). Recent studies on the geographical isolates of some NPVs, including those of *H. armigera* (Figueiredo *et al.*, 1999), *Orgyia antiqua* (Richards, Speight and Cory, 1999) and *M. brassicae* (Rovesti *et al.*, 2000), have shown that such isolates are often closely related variants. In contrast, analysis of three isolates of *Agrotis segetum* NPV demonstrated the presence of two distinct viruses in geographically separate populations of the same host species (Allaway and Payne, 1983). A similar finding of two distinct viruses in geographically separate populations was found in this study, where the virus isolates from Kenya and S. Africa were found to be sigificantly different in their efficacy as indicated by the LD_{50}'s from biological studies. The morphological studies on Kenyan and S. African virus isolates also indicated that the viruses belonged to the subgenus SNPV and MNPV, respectivley.

The variations on the genomic size estimates from the restriction profiles showed that the HaNPV isolates were different viruses. Evidence of investigations of the geographical variability and the role of these genotypic differences in the biology of baculoviruses is an important area of current research. Such studies may provide insight into understanding the biology of baculoviruses and their hosts and may be useful in the development of more effective virus strains for biological control of insects. Variation in virus isolates is mainly

based on the viral potency, morphology and the arrangement of DNA fragment profiles when electrophoresed in agarose gel.

Many different methods have been applied to assess potency of NPVs. Although, infectivity of a virus can be predicted by controlling virus production processes, *in vivo* assays are the most meaningful in terms of insect response (Navon and Ascher, 2000). There are many ways of conducting bioassays. Lethal concentrations, where virus is incorporated into the diet (diet plug, leaf disk) and fed to test insects for a varying period of time, can be used. This method possesses some inherent shortcomings (Hughes and Wood, 1981). The precision of administering the doses is quite low. Larvae may ingest the virus at different times and the acquisition period can be quite long. This results in high variation within and between assays, decreasing the precision with which the median lethal dose (LD_{50}) or median lethal time (LT_{50}) can be estimated. Accordingly, both the dosage-mortality and time-mortality responses are a combination of the viral characteristics and the methodology. Despite all these shortcomings, the lethal concentration technique has been widely used because it offers a quick and cheaper alternative of assaying differences in virus isolates in terms of time and labour.

The droplet feeding bioassay generally has been used to test the virulence of NPVs using neonate larvae (Hughes and Wood, 1981; Hughes *et al.*, 1983, 1994; Huber and Hughes, 1984; Huber and Wood, 1986). It is also possible to adapt this technique even for other instars (Smits and Vlak, 1988). It seems that this technique is only best suited to noctuids, because in the case of *Leucoma salicis* and *Lymantria dispar*, the droplet feeding bioassay could only be used for neonate larvae because they do not drink (Smits and Vlak, 1988). Some authors have used force-feeding of insects to measure the lethal dose (Paschke *et al.*, 1968). This method is not ideal because of unpredictable physiological stress to the test insect.

In this study, a known dose was administered on a limited amount of medium (<1mg) that was consumed within a period of 24 h. The method was found to be inexpensive and reproducible. It is important to note that this method (diet plug method) has got also its short falls as many test insects were wasted since not all insects fed on the diet ate the whole plug and had to be discarded.

Quantitative assessment of absolute lethal dosages is more meaningful and is in fact essential as a prelude to epidemiological studies prior to field use (Kunjeku, 1982). Several baculoviruses have been registered for commercial use in USA, Europe, Asia, Australia and a similar expansion of viral usage is occurring worldwide (Fuxa, 1987; Cunningham, 1988; Hunter-Fujita *et al.*, 1998). With increased practical application of these viruses, there is a need to select strains and mutants with superior insecticidal attributes. Such selection depends largely on laboratory bioassay procedures that are sufficiently accurate and precise to discriminate between virulence of closely related viruses while being sufficiently simple in material requirements and technique to permit routine testing.

Many studies have been carried out on the virulence of the baculoviruses (Biever and Hostetter, 1971, Whitlock, 1978; Gettig and McCarthy, 1982; Williams and Payne, 1984; Hughes *et al.*, 1981, 1983, 1986; Baya, 2000). The results from these bioassays are not comparable directly, either because of different techniques employed, or because of insufficient data analysis and presentation. The present study is probably the first detailed study of HaNPV isolate from Kenya, where full analysis based on linear regression of Probit mortality against log dose, has been presented.

In cases where important statistical values such as LD_{50}s, slopes are provided in the literature, variations tend to occur possibly because of the differences in techniques used. These variations can be reduced by carefully selecting test insects based on head capsule size and weight and through rapid administration of dosage, without imposing too much

physiological stress on test insects. Within assay and between assays variation is very high and slopes of the regression lines are low. Decreasing the precision with which the median lethal dose (LD_{50}) or lethal median time (LT_{50}) can also be estimated by increasing the number of larvae required to estimate these values with a given precision.

It is generally accepted that most of the variability in dosage response is due to body weight (Whitlock, 1978; Evans, 1981; Kunjeku, 1982; Teakle *et al.*, 1986). It is important for comparative purposes that the LD_{50} values be stated for specified body weight rather than age in terms of days. For example, Whitlock (1978) stated that *H. armigera* larvae weighing 100 - 140 mg were late second and early third instars respectively, where as in the present study, those weights were typically for fourth instar. Since rate of larval development is largely dependent on the rearing conditions in terms of temperature and larval diet. The criterion of body weight is more precise rather than use of age or body size. It is therefore, necessary for careful consideration to be taken when choosing the type of technique to be used in testing the virus. The author (s) should consider giving all the details on the techniques used and present a complete data analyses for future comparison by other interested workers.

6.2 **CONCLUSIONS**

The dosage mortality studies have indicated that HaNPV isolates from S. Africa were highly infective compared to Kenyan isolate, Gemstar® and other isolates of the same hosts (Teakle *et al.*, 1985, 1986; Teakle and Bryne, 1989; Scheepens and Wyoski, 1989). The use of the S. African HaNPV isolates to control *H. armigera* is feasible as the need for alternatives to chemical insecticides gets more important, since many consumers of agricultural products require produce, which are free from both pesticides and insect damage. Field studies of the S. African virus isolates on vegetables, orchards and sorghum also

indicate that the isolates are infective and their use in initiating epidemics is practical (Roome, 1975; Moore *et al.*, 1998).

NPV have many attractive advantages including narrow specifity, adequate pathogenicity, ease of genetic manipulation, minimal residue problems, *in vivo* and *in vitro* production capability, and compatibility with intergrated pest management programmes (Ahmet *et al.*, 2001). However, NPV are not devoid of potential problems for widespread commercial use, including relatively slow speed of kill, narrow specificity, instability in the field, high production cost and short self life compared with chemical pesticides. These particular drawbacks need to be addressed by first identifying virulent virus isolates whose efficacy can be improved by incorporating gene (s) that encode invertebrate neurotoxins as done with AcNPV and *Trichoplusia ni* (Cory *et al.*, 1994).

The two virus isolates from Kenya and S. Africa were positively identified to belong to the subgenus SNPV and MNPV of the Baculoviridae, respectively, from ultrathin sectioned electron microscope study. It has been reported that the genome of baculoviruses is highly complex and occurs as circular, supercoiled, dsDNA of 88 to 160 kb (Rohrmann, 1992). The genome size of the HaNPV from Kenya and S. Africa were estimated to be 107.2 and 81.5 kb respectively, these values fall within the reported range. REN fragmentation analysis of baculovirus DNAs have proven useful in determining the genotypic relationships among various NPV isolates (Smith and Summers, 1978a; Harvey and Tanada, 1985; Anthony *et al.*, 1999; Rovesti *et al.*, 2000). Gettig and McCarthy (1982) using EcoRI for restriction enzymes fragment analysis found different genome sizes of *Heliothis* MNPV and SNPV.

Within either the *Heliothis/Helicoverpa* MNPV or SNPV genotypes, some geographical isolates have similar but not identical fragment profiles. These minor genotypic variations serve as genetic markers for the particular isolate. Genotypic similarities between

different geographical isolates from one species have also been described for MNPVs isolated from *M. brassicae* (Vlak and Gröner, 1980). In addition, the genomes from *M. brassicae* (German and Dutch isolates) and *H. armigera* were determined to have similar fragmentation profiles, and thus all representing variants (Rovesti *et al.*, 2000). The Kenyan isolate used in this study was compared with other isolates in the literature and found to be similar with the *H. armigera* isolate from Dutch and Germany reported by Rovesti *et al.* (2000). These findings suggest that baculoviruses in nature are heterogeneous populations of variant genomes.

Although little is known regarding the frequency of the genomic variants or their biological significance, their existence lends credence to the suggestion that they may serve some role in the biology of baculoviruses. In some studies on baculoviruses isolated from *Heliothis* larvae have shown that genotypic variation can be associated with differences in virulence (Gettig and McCarthy, 1982).

Comparisons of viral DNA, using methods such as RENs, could form a basis of virus classification. Many authors, including Harrap *et al.* (1977) has advocated for this type of classification rather than the usual naming viruses by host species, as it is commonly used at present. Many viruses have the ability to infect many insect species, other than their original host, as shown by infection of *Helicoverpa* species by *A. californica* NPV, *T. ni* NPV and *M. brassicae* NPV (Cibulsky *et al.*, 1977; Rovesti *et al.*, 2000). This type of classification is important because it is a prerequisite for registration of viruses for field use and in assessing possible ecological hazards. Once the virus is identified and characterized, its host range can be established.

Definitive descriptions of proteins contributing to baculovirus structure are being used to determine the relationship between virus isolates (Rohrmann, 1992). Up to 100 proteins associated with the virus structure has been reported but most of these descriptions

distinguish the proteins only by size. For the present study, it was not possible to account for protein breakdown, processing and comigration. In this regard, only one protein of size 64 000 kDa was detected on the SDS-PAGE, and was identified as capsid and envelope proteins respectively based on the molecular weight (Rohrmann, 1992).

6.3 **RECOMMENDATIONS**

There is a need to conduct laboratory assays and field trials on the potential of adjuvants (phagostimulants and optical brighteners) in enhancing the efficacy of the S. African HaNPV isolate for future adoption as a biological agent in controlling *H. armigera*, as a follow-up on this study. Characterization at the genomic level and pathogenicity of the other virus isolates obtained from different regions in Kenya (Baya, 2000) for any relatedness with the one used in this research project will also help in understanding the baculovirus diversity within the region. Hybridisation studies should also be carried out to determine whether there is restriction fragment polymorphism on these Kenyan isolates.

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APPENDICES

Appendix A: METHODS USED IN THE STUDY

A.1 Biological methods

A.1.1 Bioassay as a measure of virulence

The potency of an insect pathogen depends on a number of factors including the instar in which the infection occurs, infective dose, nutrition, temperature, degree of compatibility of the virus with its dose and the physical characteristics of the larva (Federici, 1997). A combined effect of all these factors may not be comprehensively measured at the same time, hence the use of bioassay techniques to vary at least one or two factor (s) to be measured. Bioassays are used to measure dose or time-response relationships. This is achieved by administering a known quantity of virus particles to the insect either through droplet feeding, haemocoelic injection, leaf dip or diet plug (Hughes *et al.*, 1986; Navon and Ascher, 2000). What is important is the determination of a precise dose ingested by the test insect, as it is essential for accurate LD_{50} and LC_{50} studies (Hughes and Wood, 1981; Navon and Ascher, 2000). Central to this is the ability to suspend the virus evenly throughout the diet so as to deliver precise volumes to the insect without interfering with the physiological state of the test insect (Hunter-Fujita *et al.*, 1998).

Several problems can be encountered in the process of carrying out bioassays, which emanate from both the test insects and viral pathogen. The susceptible insect is live and thus variable in its response to stimuli under different condition. This insect is used to measure the activity of a virus, itself a replicating, variable and complex entity. Therefore, bioassay must contend with variation of both virus and host. This was achieved by allowing a known number of virus particles to act on susceptible *H. armigera* larvae under controlled conditions.

The work reported here was designed to measure the virulence of isolates of HaNPV from S. Africa, Kenya and Gemstar®, a commercial preparation of NPV in terms of quantal response of dose and the time taken for death to occur in relation to dose ingested. Variation in bioassay was minimised by carefully controlling the techniques and conditions under which the bioassay was conducted. Insect populations differ in susceptibility and their variability to infection. To minimise this, the physiological state of the insect was controlled by standardising the rearing conditions before and after exposure to the viruses. The insects were reared on a synthetic diet and kept at 28 ^{0}C ± 2 individually in a Petri dishes.

A.1.2 **Biochemical methods**

A.1.2.1 **Centrifugation**

Centrifugation separates particles in solution through differential sedimentation. The principle is that while spinning, the particles are subjected to a force, the magnitude of which is a function of both angular velocity (speed of the spin) and the radius of rotation (distance between the sample containers and the centre of the rotor). Centrifugation force can be expressed as a relative gravitational force or revolution per minutes (rpm). Centrifugation is a useful tool in separating organelles, viruses, proteins, isotopes of heavy metals, and DNA. In a manner akin to gel electrophoresis; the heavier particles separate from the lighter particles along a gradient. These layers can then be aspirated or the tube cut to where the layers are. There are two broad categories of centrifugation: differential and density gradient centrifugation.

Differential centrifugation is the most commonly used form of centrifugation. It is used to separate a suspension in two fractions, pellet and supernatant. For example, after

homogenizing cells, the cytoskeleton can be separated from the cytoplasm and buffer solution.

Density gradient centrifugation is more sophisticated than differential centrifugation. It allows separation of many or all components in a mixture and allows for measurements to be made. There are two forms of density gradient centrifugation: rate zonal and isopycnic. In rate zonal centrifugation the solutions have a density gradient. The sample has a density that is greater than all the layers in the solution. The sample is poured on top of the density gradient and spun. Over a specific length of time, the sample will separate and stratify according to weight and density. Over a prolonged period of centrifugation, the sample will ultimately settle to the bottom. However, when withdrawn at the appropriate time, the stratification can be achieved.

In isopycnic centrifugation, the solution contains a greater range of densities. The density gradient contains the whole range of densities of the particles in the sample. Sometimes it is not convenient to form the gradient before centrifugation, as in salt solutions for example. In that case one could begin with a uniform sample and the gradient solution and when placed in the centrifuge, the gradient material and the sample will redistribute according to concentration and density. The sample particles will automatically float to their isopycnic position.

The use of a centrifuge in virus purification is essentially based on separation of particles by difference in their molecular size, though density and shape also influence their sedimentation velocity. Thus virus were separated and purified from insect debris using sucrose density gradients. One important factor, which should be taken in consideration during virus purification by centrifugation, is choice of the type of rotor. There are basically two types of rotor that are frequently used; fixed angle rotor and swing out bucket rotor. To get a uniform gradient of a clean virus sample use of swing out-bucket rotor is recommended

as the OBs will form a distinct uniform layer within the gradients layer. The fixed angle rotor is mainly used when to pellet the sample within the shortest time possible.

There are different types of centrifuge used during purification, but for virus purification, centrifugation is done at 4 ^{0}C to prevent loss of virus infectivity. Centrifuges come in three general classes, low speed, up to 5 000 rpm, high-speed machines of up to about 25 000 rpm and ultracentrifuge which will turn up to 100 000 rpm. A special type of "airfuge" is in existence wherein rotor is suspended and driven by a stream of air, these can reach over 100 000 rpm, within a very short times but are limited to small sample sizes. Another form of centrifuge common in the laboratories is the microcentrifuge or microfuge. These are very simple machines that are used with 0.5-1.5 ml small disposable plastic containers (eppendorf tubes). Most of these machines are single speed and can generate between 5 000 and 15 000 rpm.

Use of lactose or acetone to recover OBs from infected insects can also be applied as a purification method (Dulmage, Correa and Martinez, 1970). Lactose or acetone purification method has the advantage that expensive centrifuge equipment is not necessary. The final product, however, has a higher level of impurities than centrifuge purification.

A.1.2.2 **Extraction of DNA**

The DNA to be manipulated must be extracted and purified from its source, either from the intact organism or from cells. The degree of purity needed is determined by the goals of the experiment. One of the common methods for extracting and purifying nucleic acids uses phenol to extract DNA (or RNA) in large or small quantities. A plethora of different phenol extraction procedures have been published, but the primary function of phenol is to remove proteins from an aqueous solution containing nucleic acids. Some of the proteins may be nucleases that could damage the DNA, while others simply could interfere

with the later manipulations. EDTA (ethylenediaminetetraacetic acid) is often added, as a chelating agent that binds Mg++ which is required for nucleases to act on the DNA.

Highly purified phenol is mixed with the sample under conditions that favour the dissociation of the proteins from the nucleic acids and the sample is then centrifuged. Centrifugation yields two phases, a lower organic phenol phase carrying the protein and the less dense aqueous phase containing the nucleic acids. Some phenol extraction protocols include chloroform, which denatures proteins, remove lipids and improves efficiency of the extractions. To reduce forming caused by chloroform, isoamyl alcohol is usually added. Extraction of DNA from organisms should be carried out as quickly as possible in ice with refrigerated buffers to minimise the activity of any nucleases present in the cell that can degrade the DNA.

Other DNA extraction methods are available (Harrap *et al.*, 1977; Vlak and Gröner, 1980; Kunjeku, 1982; Cherry and Summers, 1985). N-lauryl sarcosine lysis and caesium chloride-ethidium bromide gradients are some of the methods used to separate DNA by differences in buoyant densities. Sodium -N-lauryl sarcosinate and EDTA (ethylene – diamine-tetraacetate) are used to lyse the virus particles. Ethidium bromide an intercalating agent, binds tightly to DNA and causes the double helix to unwind. The more the ethidium bromide binds the more the density decreases because the DNA becomes longer and the buoyant densities decrease. Supercoiled DNA will be lower in the gradient than linear DNA because it binds less ethidium bromide. At high speed, the DNA moves to a position in the gradient where the density of molecules equals the density of caesium chloride and equilibrium is attained. Currently various commercial kits are also available and offer methods of rapid extraction of DNA without using toxic chemicals.

A.1.2.3 **Gel electrophoresis**

Gel electrophoresis is a method that separates macromolecules, either nucleic acids or proteins on the basis of size, electric charge, and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. Activated electrodes at either end of the gel provide the driving force. A molecule's properties determine how rapidly an electric field can move the molecule through a gelatinous medium. Many important biological molecules such as amino acids, peptides, proteins, nucleotides, and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode.

When a biological sample, such as proteins or DNA, is mixed in a buffer solution and applied to a gel, the electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules (Sambrook, Fristch and Maniatis, 1989). The frictional force of the gel material acts as a "molecular sieve," separating the molecules by size. During electrophoresis, macromolecules are forced to move through the pores when the electrical current is applied. Their rate of migration through the electric field depends on the strength of the field, size and shape of the molecules, relative hydrophobicity of the samples and on the ionic strength and temperature of the buffer in which the molecules are moving. After staining, the separated macromolecules in each lane can be seen in a series of bands spread from one end of the gel to the other. Gel electrophoresis is one of the staple tools in molecular biology and is of critical value in many aspects of genetic manipulation and study.

One use of gel electrophoresis is used in the identification of particular DNA molecules by the band patterns they yield after being cut with various restriction enzymes. Viral DNA, plasmid DNA, and particular segments of chromosomal DNA can all be

identified in this way. Another use is the isolation and purification of individual fragmen containing interesting genes, which can be recovered from the gel with full biologic activity.

There are two basic types of materials used to make gels, agarose anc polyacrylamide. Agarose is a natural colloid extracted from seaweed. It is very fragile and easily destroyed by handling. Agarose gels can be processed faster than polyacrylamide gels, but their resolution is inferior. Agarose is a linear polysaccharide (average molecular mass of about 12,000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is usually used at concentrations of between 0.5% and 3% and suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution is formed. This is poured and allowed to cool to room temperature to form a rigid gel.

A.1.2.4 **Sodium dodecyl polyacrylamide gel electrophorsis (SDS-PAGE)**

Raymond and Weintraub (1959) introduced the polyacrylamide gel electrophoresis (PAGE) technique. Polyacrylamide is the same material that is used for skin electrodes and in soft contact lenses. Polyacrylamide gel may be prepared so as to provide a wide variety of electrophoretic conditions. The pore size of the gel may be varied to produce different molecular sieving effects for separating proteins of different sizes. In this way, the percentage of polyacrylamide can be controlled in a given gel. By controlling the percentage (from 3% to 30%), precise pore sizes can be obtained, usually from 5 to 2,000 kDa (Gordon, 1973). This is the ideal range for protein polypeptide analysis. Polyacrylamide gels can be cast in a single percentage or with varying gradients. Gradient gels provide continuous decrease in pore size from the top to the bottom of the gel, resulting in thin bands. Because of this banding effect, detailed genetic and molecular analysis can be performed on gradient

polyacrylamide gels. The unparalleled resolution and flexibility possible with the polyacrylamide gel electrophoresis (PAGE) has led to its widespread use for the separation of proteins and nucleic acids. PAGE also offers more sharply defined banding than agarose gels.

Polyacrylamide gels are formed by co-polymerisation of acrylamide and bis-acrylamide (N, N'-methylene-bis-acrylamide). The reaction is vinyl addition polymerisation initiated by TEMED (tetramethylethylenediamine) and ammonium persulphate. TEMED accelerates the rate of formation of free radicals from persulphate and these in turn catalyse polymerisation. The persulphate free radicals convert acrylamide monomers to begin the polymerisation to free radicals, which react with unactivated monomers to begin polymerisation chain reaction. The elongating polymer chains are randomly cross-linked by bisacrylamide, resulting in closed loops and a complex " web" polymer with a characteristic porosity, which depends on the polymerisation conditions and monomer concentrations. Riboflavin (or riboflavin – 5'- phosphate) may also be used as a source of free radicals, sometimes in combination with ammonium persulphate (Gordon, 1973). In the presence of light and oxygen, riboflavin is converted to its *leuco* form, which is active initiating polymerisation. This is usually referred to as photochemical polymerisation.

A.1.2.5 **Restriction Enzymes Analysis**

Certain bacteria host cells produce enzymes (restriction endonucleases RENs), which can degrade foreign DNA at specific sites. These enzymes do not destroy host cell DNA because the specific covalent alteration of the host-cell DNA produced by the modification enzymes does not allow the REN to act on it. Many of these enzymes have been highly purified from bacteria and are routinely used in molecular biology. Each REN recognises and cleaves a specific sequence along the DNA molecules. These properties of RENs have

enabled them to be used in the sequencing of DNA molecules, mapping of the chromosomes, investigation of DNA replication and in genetic engineering. These enzymes are invaluable for identification, analysis and characterisation and determination of viral DNA molecules.

Although the virus type can be determined visually from the symptoms of infected larvae or by staining the OBs by Giemsa stain, accurate identification is not possible unless carried out at molecular level (Jones, 2000). REN analysis of viral DNA is one of the methods used in identification of different types of closely related organisms. REN analysis relies on the uses of specific enzymes, which recognise and cleave specific nucleotide sequences. These cut the viral DNA into fragments of different length, which can be separated through electrophoresis and produce a characteristic profile or fingerprint for each virus (Sambrook *et al.*, 1989).

A.1.2.6 **Electron microscope studies**

Electron microscopy studies offer techniques for characterisation, quantification and imaging of the baculoviruses based on their morphology and the number of nucleocapsids present in occlusion body. By using electron microscope techniques one is able to classify the baculoviruses either as MNPV or SNPV. The test samples can be examined by ultra-thin section transmission electron microscopy or by negative staining on the grid and viewing directly. The main advantage of ultrathin section microscopy has over any other types of services is that the samples can be examined at ultrastructural level. Ultrathin microscopy section provides details of the interior of the cells and tissues, including organelle structure, morphology of macromolecules, such as virus particles, and does so without causing distortion of the cells or tissues. Electron microscopy enables detection of contaminants present within the virus samples. Negative staining with glutaraldehyde for 24 h enhances the visibility of the OBs structures.

APPENDIX B: STAINING TECHNIQUES

B.1. **Simple Giemsa staining**

B.1.1 **Materials**

Microscope slides

Staining racks and jars

Giemsa fixative (absolute ethanol, 94%; formalin, 5%; acetic acid, 1%)

Phosphate buffer 0.02M

Gurr's improved R66 Giemsa, 10% in 0.02 Phosphate buffer

PH metre

B.2. **Treatment of specimen**

1. Prepare a thin smear of the virus sample

2. Air-dry the smear

3. Immerse for 1-2 min in Giemsa's fixative

4. Rinse under running tap water, 5-10 seconds

5. Stain 25-60 min in 10% Gurr's improved R66 Giemsa in 0.02 M phosphate buffer

6. Rinse off the stain in running tap water, 5-10 seconds

If over stained, stand in 0.02 M phosphate buffer until red colour on glass slide disappears

7. Gently blot with absorbent paper, dry and examine with an oil immersion objective (Total magnification of about x 1000). OBs of NPVs appear as clear, round objects, as those of GVs as very small ellipsoidal objects, also clear, while bacteria and other contaminants stain purple.

APPENDIX C: PREPARATION OF THE LARVAL DIET

1. Soak beans (400 g) in water for 2 hours and bring them to boil for 30 minutes.

2. Autoclave mealie meal (400 g) until temperature reaches 26 °C .

3. Weigh and measure out all the other ingredients shown below:

Ingredients	Quantity
95% alcohol	10 ml
Nipagin (Methyl-hydroxy benzoate)	4 g
Sodium benzoate	2 g
Yeast	14 g
Acetic acid	25 ml
Formaldehyde 10%	3 ml
Vitamin mixture	20 ml
Sodium ascorbate	25 ml

4. Mix agar (12 g) in 200 ml of water and add a small quantity of beans and blend to a fine mixture and put into a pan

5. Blend a small quantity of beans with yeast (14 g) mixed with 200 ml water

6. Blend a small quantity of beans with Nipagin (4 g) dissolved in 95% alcohol (10 ml) and add 200 ml of water

7. Blend the remaining quantity of beans with sodium benzoate (2 g) mixed in 200 ml water and pour into a pan

8. Add acetic acid, formaldehyde, vitamin mixture, sodium ascorbate in the already autoclaved mealie meal

9. Use spatula to thoroughly mix all the ingredients in the pan and then store in a cold room or refrigerator
10. Cut small piece of the preparation and give to the larvae and change as necessary

APPENDIX D: PROCEDURE FOR PREPARING *H. ARMIGERA* ADULT DIET

1. Dissolve 5 g methyl- hydroxybenzoate (Nipagin) in alcohol in a test tube

2. Combine all the ingredients indicated below in a 500 ml beaker

Ingredients	**Quantity**
Sucrose (White sugar)	50 g
Vitamin mixture	10 ml
Nipagin	1 g
95% alcohol	1 ml
Distilled water	

3. Make up volume to 500 ml with distilled water

4. Shake well until sucrose has dissolved and pour into a clean polythene wash bottle

5. Keep refrigerated

APPENDIX E: COMPOSITION OF THE SPECIAL INSECT VITAMIN MIXTURE FOR BOTH LARVAL AND ADULT DIET

Ingredients	Quantity (g)
Nicotinic acid	8
Ascorbic acid	270
Biotin	20
Calcium Pantothenate	1
Choline Chloride	50
Folic acid crystalline	250
Inositol	20
Niacinamide (nicotinic acid)	1
Pyridoxine Hydroxychloride	250
Riboflavin	500
Thiamine Hydroxychloride	250
Vitamin B12 Trituration in mannitol	2

APPENDIX F: COMPOSITION OF SILVER STAIN PLUS KIT

F.1. The silver stain Plus kit includes the following components in quantities suffiecient to stain 40 mini gels (8x10 cm) or 13 conventional gels (16x16-20 cm).

1. Fixative Enhancer Concentrate
2. Silver Complex Solution (contains NH_4NO_3 and $AgNO_3$)
3. Reduction Moderator Solution (contains tungstosilic acid)
4. Image development reagent ()contains formaldehyde
5. Development Accelerator Reagent (contains Na_2CO_3)
6. Empty 1 Litre bottle for Development Accelerator

F.2. POLYACRYLAMIDE GEL STAINING PROCEDURE

The following preparations were adequate for staining two minigels (8x10 cm), 0.75-1.mm thick.

1. Fixative step- 20min.

Reagent grade Methanol	200 ml	50% v/v
Reagent grade Acetic acid	40 ml	10% v/v
Fixative enhancer concentrate	40 ml	10% v/v
Deionised distilled water	120 ml	30% v/v

After gel electrophoresis, gel was placed in the Fixative enhancer solution for 20 min with gentle agitation.

2. Rinse step

Decant the Fixative Enhancer Solution from the staining vessel. The gel was rinsed in 400 ml deionised water for 10 minutes with gentle agitation. After 10 min, water was decanted and replaced with fresh rinse water. The gel was rinsed again for an additional 10 min.

3. Staining and Developing Step

Staining solution and preparation procedure, this was prepared within 5 min of use.

35 ml of deionised water was placed into a large Erlenmeyer flask and stirred with a Teflon coated stirring bar. The following were then added to the beaker in this order

5.0 ml Silver Complex Solution

5.0 ml Reduction Moderator Solution

5.0 ml Image Development Reagent

Immediately before use 50 ml of Development Accelerator Solution at room temperature was added quickly to the beaker. It was then swirl well for about 30 min. The content of the beaker was then added to the staining vessel. The gel was then stained with gentle agitation for 20 min. It took about 14 min before the bands started becoming visible.

4. Stop Step.

A 5% acetic acid solution was prepared to stop the staining reaction. The gel was then placed in stop solution for 15 min. After stopping the reaction the gel was rinsed in high purity water for 5 min, the gel was then viewed under UV illuminator.