Microevolution in an insect-virus interaction

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Baculoviruses have been proposed as potential biological control agents, as an alternative to large scale usage of pesticides.Some strains of the stored product moth *Plodia interpunctella*, the Indian meal moth have developed resistance to malathion. Alternatives have been sought and amongst the most promising are, *Bacillus thuringiensis* and a baculovirus, *P.interpunctella* granulosis virus (PiGV).The insect-baculovirus system is the basis of this investigation.

The study of the microevolution in the insect-baculovirus interaction was centred on the two extremes of the interaction: where virus and insect evolution was most likely to occur.Selection experiments were carried out to mimic these conditions.Other possible methods of insect and virus evolution were also considered.

The insect selection experiment was designed to select for increased viral resistance in the insects by challenging them with high doses of virus over ten generations.No increase in resistance was recorded, in fact the selected insects appeared to increase in susceptibility.This is discussed in relation to insect choice for bioassays. Selection may have been unable to act on the frequency of major resistance genes to alter resistance levels in the insects, because these genes were already close to, or at a maximum, in this population.This was confirmed by retrospective comparison with two other strains of *P. interpunctella* of known resistance. Therefore it is probable that selection acted on an arrangement of background genes, and that this produced the significant developmental advancement that was observed in the selected insects but not in their non-selected counterparts.

Since granulosis viruses are often transmitted on the death of their host, it was hypothesised that passing the virus through several generations at very low dose that the virus may evolve increased virulence. Over seven generations of such selection no phenotypic increase in virulence occurred. The viruses were examined genotypically in the initial and final generations of selection using restriction endonuclease analysis.No genotypic changes were detected.An attempt was made to determine the initial level of variation in the original sample of the virus. The overall variation in the PiGV genome was determined by comparison with a limited range of PiGV isolates from the USA, again using restriction endonuclease analysis.

Less predictable routes of evolution were also explored.A particular aspect of the insect life history was observed which suggests that older females produce larger offspring.These offspring were found to be larger because they were greatly accelerated developmentally in comparison to offspring produced by younger females. The effects of male presence, female longevity, group cultures and single pair matings were investigated.It is possible that the presence of these greatly accelerated larvae may have a beneficial effect on the overall survival of the insect population when challenged by viral infection.

Traditionally it has been assumed that baculoviruses increase virulence as a method of increasing their transmissability and thus their evolutionary success. Latency of the granulosis virus may give it an alternative to this particular evolutionary route. Some evidence for the presence of viral DNA in the selected insects at certain generations was observed in the hybridization of whole granulosis virus and granulin gene specific probes to dot blots of insect DNA, thus suggesting that granulosis virus may be present in a latent form in these insects.

The implications of evolution in the insect-baculovirus system are discussed both with reference to current host-parasite coevolutionary theory and epizootiologically. In addition the commercial impact of evolution is considered with regard to the development of baculoviruses as biological control agents

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Upon completion of this project I would like to thank many people and so in true Oscar tradition....

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A man in a nearly white coat was played by D.M.Ensor.

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(Dr Begon forced to step back quickly here since he is not used to being in front. After all, he is a Spurs supporter.)

Riotous applause.

Fade to close.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 Introduction

1.1 Pesticides

The global market value of control agents used in crop protection and public health is approaching $16,000 million annually (Jutsum,1988). Despite this already heavy investment, and a projected 2 to 3% increase per year until the year 2,000 (Wood Mackenzie, 1986), the use of these synthetic agents has not been an unqualified success.Problems have arisen resulting from large scale usage of chemical control agents. The adverse effects that have been observed can be most easily categorized as (a) effects on non-target organisms (both vertebrate and invertebrate), and (b) development of resistance in target pests.

Effects of pesticides on non-target organisms, particularly vertebrates, are very well documented. Studies have been most frequently performed on birds since they are excellent indicator organisms of a widespread residue problem due to their unique mobility. In an attempt to determine the extent of persistent organochlorine residues, the flesh of starlings from 128 sites in the USA was analysed. DDT and its metabolites (<1-3ppm), and dieldrin (<0.1-0.3ppm) were found in all samples (Martin,1969). More disturbingly, data obtained from 66 bald and golden eagles found sick or dying in 18 states of the USA and Canada, showed that all contained residues of DDT, DDD, dieldrin and PCBs (polychlorinated biphenyls). Organochlorine residue levels of 133ppm were detected in the liver, and up to 73ppm in the brain (Reichel *et al*,1969). These levels were much higher than those found in starlings, indicating that the further up the food chain the residue is detected, the higher the concentration it will have reached.

A similar trend of bioconcentration exists in fish. Here, laboratory experiments

indicate that fish can build up concentrations of DDT and dieldrin at the parts per million level from parts per trillion in the water (Kenaga,1977). This assumes particular importance in areas with high agricultural run off. A survey of fish in the Great Lakes between 1965 and 1969 found residues of DDTR (DDT and degradates) and dieldrin in all fish (Reinert,1970). Fish from Lake Michigan, which drains the midwestern agricultural belt of the USA, contained 2-3 times as much of these insecticides as those from other Great Lakes, averaging 13ppm DDTR in some species (Reinert,1970).

Such studies clearly demonstrate the damage done by pesticides to non target vertebrates, but perhaps much more dramatic is the effect on non target invertebrates. Scant attention has been given to this subject even in large scale reviews of the adverse effects of pesticides (Pimentel,1971). Pesticide application disrupts the normal balance of an ecosystem, allowing previously innocuous pests to become increasingly important. For example, in the Rio Grande Valley in Texas, insecticide resistance in a major cotton pest, the boll weevil (*Anthonomus grandis*),lead to the emergence of two pests formerly of minor importance, the cotton bollworm (*Heliothis zea*) and tobacco budworm (*Heliothis virescens*).These latter two pests are now viewed as more serious than the original boll weevil, which has faded into relative obscurity. The tobacco budworm which no available pesticide will adequately control, has been brought to its present status by the use of pesticides. Such is the extent of these upsets that in intensively treated crops, it is now almost impossible to distinguish between these man-made upsets or resurgences, and the real pests (ones lacking established effective natural enemies) without long term ecological studies (Debach,1974).

The other equally dramatic adverse effect of excess pesticide usage on invertebrates is target pest resurgence. This occurs when large numbers of pests are removed from the ecosystem caused by an application of pesticide. This leaves natural

enemies of the pest with insufficient food, resulting in a subsequent drop in parasite and predator numbers. Thus any remaining pests which have survived are able to benefit from an area of plentiful food supply containining few natural enemies. The pest population can then increase with few of the normal constraints, resulting in a population explosion.An example of resurgence occurred when para-oxon was first sprayed commercially to control the cabbage aphid *Brevicoryne brassicae*. Initially the kill was extremely high. However the concurrent destruction of natural predators, resulted in the largest outbreak of cabbage aphid ever seen in England (Ripper,1956).

Several major reviews have documented the adverse effects of pesticides on insect and mite pests and their natural enemies (Ripper, 1956; Newsom,1967). Such reviews demonstrate the widespread nature of the problem, as between them they cover upsets and resurgences involving fifty or more pest species covering a wide variety of taxonomic groups.

In addition to the creation of new secondary pest problems, intensive chemical usage has lead to the rapid development of resistance in target insects. This third and final adverse effect is possibly the most damaging of all, since it results in depreciation of the pesticide's original function. A typical example of such resistance occurred in organophoshorus and organochlorine insecticide control of culicine mosquitoes in California. During World War II, DDT was discovered to be the most effective culicide, showing both high larvicidal and adulticidal activity (Mulla, 1977). By 1945, it was the major larvicide in use in California. However, by the early 1950s resistance to DDT had appeared in several culicine mosquitoes, notably *Aedes nigromaculis* and *Culex tarsalis* (Bohart and Murray,1950; Gjullin and Isaak,1957; Gjullin and Peters,1952). Substitute organophoshorus insecticides, such as malathion and ethyl parathion, were originally highly effective against both susceptible and DDT resistant mosquitoes (Isaak,1953; Lindquist,1953). But, by the early 60s, the resistant strains

had developed resistance to these (Gillies *et al,* 1967; Gjullin and Isaak,1957). In each case, heavy usage of pesticide lead to successive development of resistance: initially to DDT, then to malathion and ethyl parathion. Similar examples can be found in almost any control situation utilizing large amounts of chemical control agents.

Despite these disavantages, the agricultural value of chemical control agents cannot be underestimated, and they will undoubtedly continue to play the major role in pest control for the forseeable future. However the development of a commercial alternative has long been advocated, and at present most hopes for such an alternative lie predominantly with biological control.

1.2 Biological Control

The term biological control was originally used by Smith (1919) to describe the introduction of exotic natural insect enemies for the permanent suppression of insect pests. It has since been expanded to include virtually all pest control measures except application of chemical pesticides (Waage and Greathead,1988), but it has probably been most recently and comprehensively described by Jutsum (1988) as ' a broad spectrum of approaches ranging from the use of obligate parasites and pathogens, to facultative parasites and pathogens, to competitors to toxin producing pathogens, to toxins produced by pathogens, and finally non toxic behaviour modifying chemicals'.

The three microbial biological control agents (BCAs) which show greatest potential are bacteria, fungi and viruses (Jutsum,1988). In general, biological control agents are more selective, slower acting, and less toxicologically damaging than pesticides. They are however at present more generally difficult and expensive to produce on a large scale, and some have problems with shelf life and persistence in the

field. However problems with production costs are now being overcome with *Anticarsia gemmatalis* NPV now being produced for $2 (USA)/ Ha dose in Brazil. (P.F. Entwistle, Institute of Virology,Oxford *pers comm*). Biological contol agents are likely to be of most use as part of an integrated control programme or in specific situations, for example control of aphids and whiteflies by the fungus *Verticillium lecanii* under glass, where humidity and temperature are controlled for optimum activity (Hall and Papierok,.1982); or, in forestry, where some crop damage may be economically acceptable. Most viral pesticides are being developed for forest pest control for this reason, since higher levels of pest damage can be tolerated on trees than on smaller more valuable agricultural crops (Payne,1988). It would also require much re-education to alter the expectations of quick kill that broad spectrum pesticides bring.

At present the greatest commercial penetration of the insecticide market has been by *Bacillus thuringiensis* (*Bt*): it is the only microbial pest control agent that has been commercialized on a world wide large scale (Payne,1988). Its contribution is six times that of the cumulative total of the only other significantly represented groups of biological control agents (Table1.1). This is chiefly due to large scale use by the Canadian forestry industry where application of chemical agents has been restricted by Government, and *Bt* is sold at nearly twice the price of an acceptable insecticide, fenitrothion (Jutsum, 1988). However expansion of viral pesticide usage has taken place in developing countries, in Brazil in 1989, 40,000 Ha of soybean was sprayed with *Anticarsia gemmatalis* NPV to protect it from soybean looper attack (P.F. Entwistle, Institute of Virology,Oxford *pers comm*).

Although the total percentage contribution of biological control agents to the global pest control budget is very small, their potential for expansion into the commercial market, particularly when used as part of an integrated control programme, is very promising, especially when viewed in the light of the declining rate of chemical invention in the agrochemical industry (Jutsum,1988).

Table 1.1 Penetration of the global insecticide market by biological control agents (end-user value given in millions of U.S. dollars at 1985 value) Taken from Jutsum,1988.

1.Sales of selective chemicals/pheromones		
Selective chemicals		90
Pheromones		2
	Total	92
2. Sales of predators/ parasites and pathogens		
Bacillus thuringiensis (products for forestry, agriculture and public health)		30
Viral insecticides	ca.	1
Predators/parasites/other pathogens	ca.	3?
	Total	34

The development of any new pesticide depends on the outcome of cost-benefit analysis, and as the agrochemicals market matures it becomes increasingly more difficult to justify registration costs, making an effective BCA an attractive proposition.

1.3 Use of Viruses in Pest Control

As discussed in the previous section commercial application for insect viruses is currently very restricted. However with the advent of new molecular biological techniques, attempts are being made to genetically engineer viruses for increased effectiveness and broader host ranges.

Viruses associated with insects fall into two major categories. The first are those for which the insect acts only as a vector, carrying both plant and animal diseases without itself necessarily contracting any infection. Examples of these are Yellow Fever virus (Flaviviridae) in animals, and wound tumour virus in plants. The second major category are those viruses which are pathogenic to the insects themselves.These are obviously more useful in pest control terms.

There are, at present, eight major families of virus believed to be infecting insects (Tweeten *et al,* 1981), but a recent report of the International Committee on Taxonomy of Viruses (ICTV) proposed that up to thirteen families or sub families should be recognized (Matthews 1982). Of these eight generally accepted families, only one, the Baculoviridae, has been endorsed as a potential pest control agent (WHO,1973). This family was deemed most suitable, not only because of their prevalence [71% of all recorded insect-host associations (Entwistle and Evans, 1985)] but more specifically because as a group, they are apparently uniquely restricted to

invertebrate hosts (Stoltz and Vinson, 1979).

1.4 Baculoviruses

Baculoviruses are large rod shaped enveloped DNA-containing viruses (Payne,1988).Within the Baculoviridae, three subgroups have been recognized on the basis of structural properties (Entwistle and Evans, 1985), with a fourth proposed by ICTV (Matthews,1982)(see Table 1.2) The major division lies between occluded (Subgroups A and B), and non-occluded baculoviruses (Subgroup C). In general, members of subgroup A (nuclear polyhedrosis viruses (NPVs)) and Subgroup B (granulosis viruses (GVs)), are the of greater value as control agents due to their higher environmental stability. However, *Oryctes rhinoceros* virus has been used in a successful control program of coconut rhinoceros beetle in the South Pacific (Bedford,1980).

The occluded viruses owe their higher stability (except in UV light) to their occlusion bodies (Tweeten *et al*,1981; Consigli *et al*, 1983) . These occlusion bodies are produced late on in infection, and preserve the infectivity of the virus outside the host (Payne,1988). Occlusion bodies are composed of a protein matrix, consisting of aggregates of a single major polypeptide with a mean molecular weight of around 30 kilodaltons (Entwistle and Evans, 1985). In the case of NPVs, this protein is referred to as polyhedrin, and as granulin in GVs. The presence of an occlusion body increases the likelihood of transmission to another insect host by allowing the virus to remain viable longer in the physical environment. These are not a frequent feature in animal viruses. The occlusion body is surrounded by an envelope which was previously thought to consist primarily of carbohydrates. However recently Zuidema *et al* (1989) have also recorded the presence of phosphoprotein in association with this envelope.

Table 1.2 Classification of the Baculoviridae

Subgroup	Example	Occluded
A	Nuclear Polyhedrosis Viruses	+
B	Granulosis Viruses	+
C	*Oryctes rhinoceros* virus	-
D	'Calyx' virus	-

Note: Viruses in the proposed sub-group D are typically associated with the calyx fluid of parasitoid wasps and consist of two major types:braconid and ichneumonid (Stoltz,1982). The type virus for subgroup C is the baculovirus of the coconut rhinoceros beetle, *Oryctes rhinoceros*. (Payne *et al*,1977).

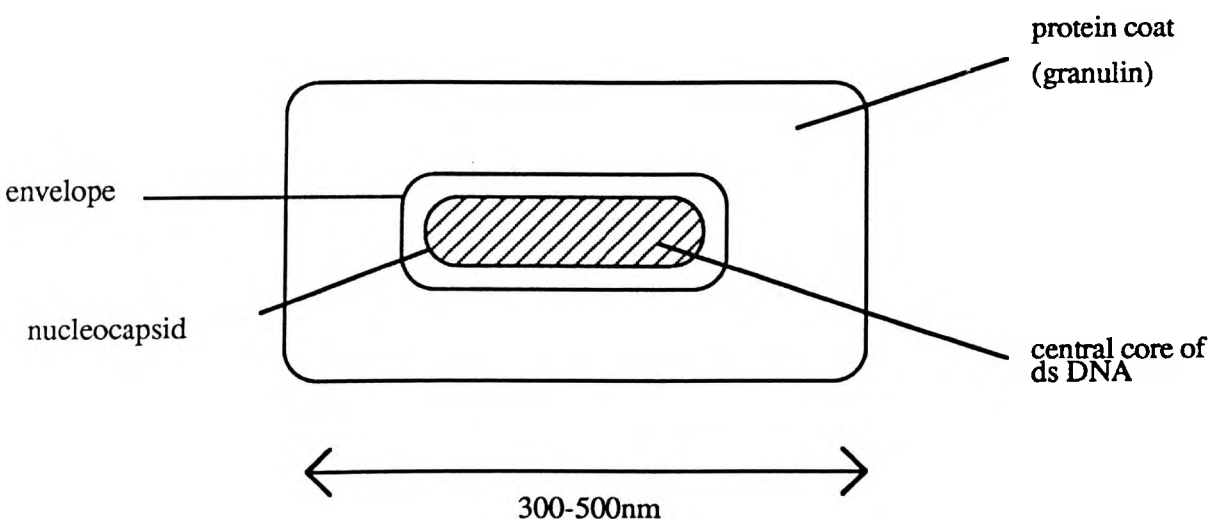
Granulosis viruses are singly occluded, having one bacilliform particle embedded in a paracrystalline lattice of granulin (Harrap and Payne, 1979; Dwyer and Granados, 1987))(see Fig 1.1). This is in direct contrast to NPVs, where each polyhedral inclusion body may contain several hundred rod-shaped particles (Smith 1976). These particles may be enclosed individually in a single envelope (singly embedded, SNPV) or collectively within one large envelope (multiply embedded, MNPV).

Occasionally, GVs have two virus particles occluded, and even more rarely produce large cuboidal inclusion bodies instead of the more usual bacilliform inclusion bodies (Arnott and Smith, 1968; Evans and Entwistle,1985).

Each virus particle or nucleocapsid contains a single molecule of circular, supercoiled, double-stranded DNA with a molecular weight of around $80x10^6$ daltons. GV DNA consists primarily of single copy sequences (Tweeten *et al*, 1981). Both subgroups multiply principally in the nucleus of the host cell. The main site of replication for GVs is in the fatbody. A diagrammatic summary of the GV infection process may be seen in Fig.1.2.

Baculovirus infection generally only develops when virus is ingested by an insect, although sub-group D viruses typically associated with calyx fluid of parasitoid wasps may be transmitted via injection to the haemocoel by external contamination of the wasp (Entwistle,1982). After ingestion,in occluded viruses, the occlusion body dissolves in the gut, liberating virus particles. These set up an initial infection in gut epithelial cells, which in Lepidoptera soon spreads to all other major tissues.

Fig. 1.1.Diagrammatic representation of the inclusion body of a granulosis virus (GV) shown in longitudinal section



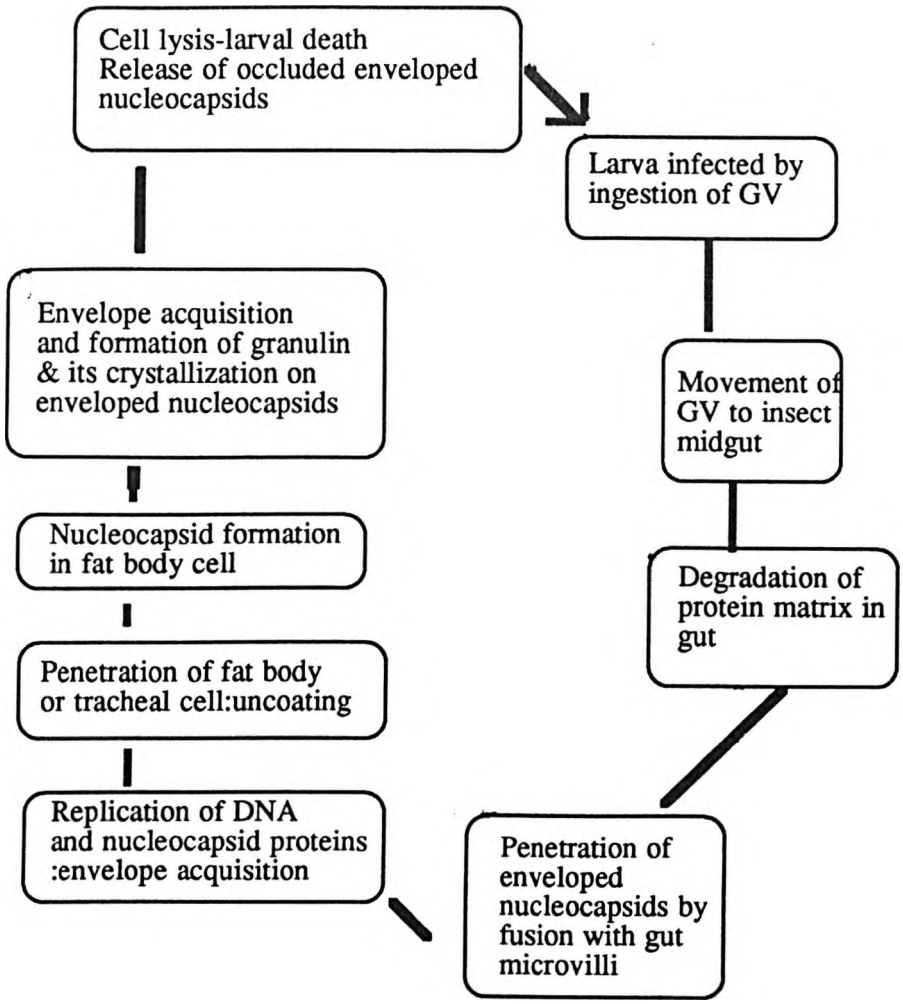


Fig 1.2 Summary of granulosis virus (GV) infection process (adapted fom Tweeten *et al* 1981)

1.5 Granulosis viruses as control agents of a stored product pest

Since granulosis virus infection was initially described by Paillot (1926), more than eighty species of lepidopterans have been reported to be susceptible to GV infections (Martignoni and Iwai, 1981). This figure includes some of the most commercially important agricultural and forest pests, such as the potato tuberworm (*Pthorimaea operculella*) and the fir budworm (*Choristoneura murinana).* Infection by GVs is limited to the order Lepidoptera and in most cases appears to be species specific (Tweeten *et al*, 1981). This makes them well suited as pest control agents, from a safety aspect.

Traditionally, when considering pest control situations, precedence is given to agricultural and forest pests, and the stored product environment is often overlooked. This oversight is unfortunate since it is an area in which biological control agents could be particularly effective.The only recommended pesticide for use on stored products is malathion (WHO, 1973). However, in common with many other pesticides, resistance has developed in many target pests, thus effectively leaving a void in the control situation, which is currently being filled by ever increasing amounts of a diminishingly effective pesticide.

Some attempt is being made to fill this void using biological control agents, both in direct and integrated control methods. One such system which has undergone successful field trials is the control of the Indian Meal Moth, *Plodia interpunctella* (Lepidoptera:Phycitidae) using a granulosis virus (McGaughey,1975).

Malathion is the principal insecticide used for controlling infestations of *P. interpunctella.* However, increasing resistance in the pesticide was causing concern and resulted in the search for a non-chemical alternative, or a method of integrated

control (Zettler *et al*,1973a; Armstrong and Soderstrom,1975; Champ and Dyte,1976; Beeman *et al* 1982; Zettler, 1982). The most viable alternative proved to be a granulosis virus.

The susceptibility of *Plodia* to infection with a GV was first recorded by Arnott and Smith (1968). Later work demonstrated the degree of pathogenicity to neonatal larvae, and suggested its possible role as a control agent (Hunter,1970; Hunter *et al*,1973).This virus has been shown to be effective in protecting dried nuts and stored grain from infestations under field conditions (Hunter and Hoffman,1973; Hunter *et al*,1973, 1977, 1979; McGaughey,1975). It also has enormous potential as an integrated control method in conjunction with malathion, since a combination of the two has been found to be more effective than either used singly (Hunter *et al*,1975 Zettler, 1974).

1.6 Biology of *Plodia interpunctella*, a stored product pest.

Plodia interpunctella is an insect of serious economic importance. Several phycitid moths have adapted with great success to the food storage environment, and whilst *P. interpunctella* seldom causes as much damage as the closely related *Ephestia* species, it has the widest distribution of all moths infesting stored products (Cox and Bell,1985).

Plodia interpunctella (Hübner) is a serious pest of stored products (Hunter *et al* , 1977). It renders stored products unfit for consumption by the action of its larvae. *Plodia interpunctella* is a cosmopolitan feeder, with all larval stages being polyphagous. It has been found on a range of products from dried fruit and nuts to cereals. It causes both primary and secondary infestations resulting in serious post

harvest losses (Hamlin *et al* , 1931; Freeman,1962; Tzanakis,1959).

The life cycle, and the effects of differing environmental conditions on the life cycle have been described many times (Tsuji,1958, 1959; Tzanakis,1959; Williams,1964; Morere and LeBerre 1967; Reyes,1969).Duration of the life cycle is temperature dependent, ranging from sixty days at 20°C, to twenty five days at 30°C (Tsuji,1963; Bell,1975). This temperature range is in line with temperature fluctuations experienced by *Plodia* in the field. In warehouses, for example, mean temperature values range from 15°C to 30.6°C , and relative humidity varies from 39% to 73%.Under such conditions the lifecycle of *Plodia* fed on raisins lasted between 27 and 305 days respectively (Hamlin *et al* ,1931). In general, larvae reared at lower temperatures (20°C) were heavier than those reared at 25°C or 30°C (Tzanakis,1959; (Tzanakis,1959; Couture and Huot,1967; Silhacek and Miller,1972)

Larvae of *Plodia interpunctella* usually go through five instars, but as many as seven have been recorded (Miles,1933; Morere and Le Berre,1967; Mossadegh,1976; Silhacek and Miller,1972), and even eight in one instance (Richards and Thompson, 1932). Temperature does not appear to have a significant effect on the number of instars (Hassan *et al*,1962). However, low temperatures, high population pressure and short photoperiod all cause the induction of diapause in the insects (Bell, 1977).The extent to which different strains diapause varies greatly, those which have been reared in laboratories for long periods of time tend to show a reduced capacity for diapause.

After a larval stage lasting on average between twelve to thirteen days, the insects progress into prepupation and pupation. Five to seven days after pupation new adults begin to emerge, with newly emerged females being on average three milligrams heavier than males (Silhacek and Miller,1972), a difference that persists throughout

adult life and is shown again as mature larvae and pupae (Couture and Huot,1967). However, this difference is not due to selective retention of water or lipid by the females (Couture and Huot,1967; Yurkiewicz,1969).

Adults survive for about a week with negligible mortality (Silhacek and Miller,1972). During this time they mate, with females laying eggs usually twenty four hours after a successful mating (Mossadegh,1976). Most viable egg production by individual moths is essentially complete four days after mating (Morere and LeBerre, 1967; Lum and Flaherty, 1969; Silhacek and Miller,1972). Eggs are laid singly or in batches up to a maximum of 218 (Mossadegh,1976). Hatching is temperature dependent: occurring 64 to 67 hours after laying at 30 +/- 5°C (Silhacek and Miller,1972); 4 to 9 days at 25 to 28°C (Richards and Thompson,1932) and 5 to 14 days at 15.5°C to 18.5°C (Hill,1928).

This species of moth is particularly suitable for laboratory experiments since they are easily reared on simple food materials under controlled conditions (Benson,1973). Thus rearing conditions in the laboratory can closely imitate conditions experienced by *Plodia* under field conditions, thus making *Plodia interpunctella* and its granulosis virus an ideal insect-virus model study system.

1.7 Microevolution in an insect virus interaction

The idea of a viral replacement for malathion as a control method for *Plodia interpunctella* has great appeal, particularly from an environmental point of view. However, the same question arises as with any insect-biological contol agent interaction: will the target pest develop increased resistance to the biological control agent ? Obviously this is a question of great importance, for if no increase is recorded,

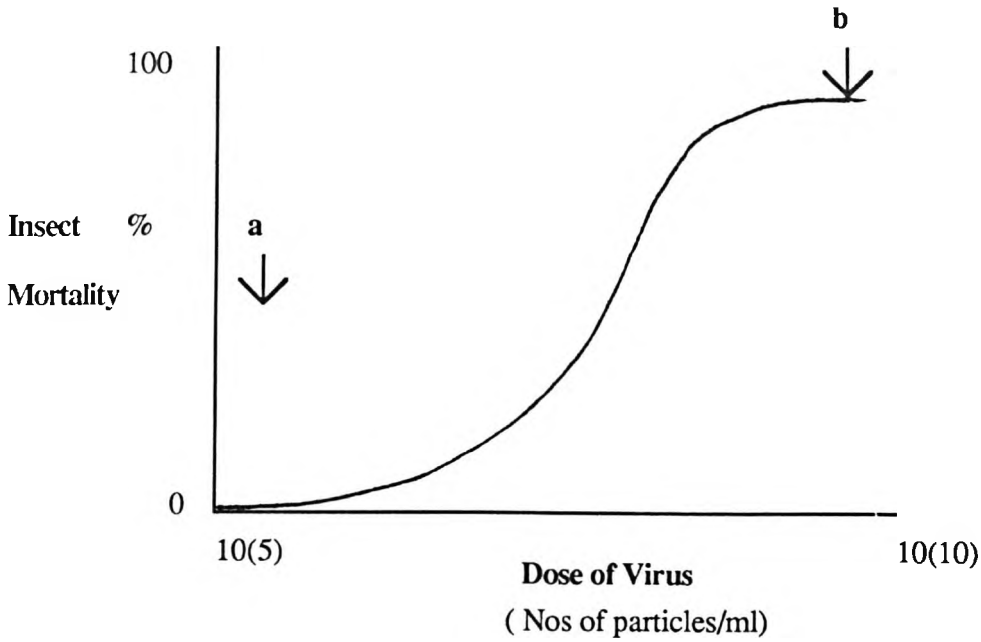
then, biological control agents may be able to overcome their perceived commercial disadvantages : slow speed of action and expense of production.

However, biological control agents do have certain advantages over pesticides with regard to resistance: unlike pesticides, they are not novel agents being introduced to the environment. Hence ecological upsets may be avoided. In addition, evolution or coevolution with the target insect may have already occurred in the generations of previous exposure. Finally, the virus may be able to keep pace with the insect by evolving to become more virulent as the insect becomes more resistant, thus avoiding any long term loss in efficiency of the virus. Pesticides being inorganic compounds are incapable of such reciprocal evolution. The topic of possible insect resistance to biological control agents, particularly viruses, has been previously well studied , and the relevant literature is reviewed in Chapter 3.

This subject of possible evolution or coevolution in the insect-virus interaction is the one that this thesis attempts to tackle.The *Plodia* -GV system has many advantages for such a study. Its primary advantage is that as a stored product pest *Plodia* is relatively easy to keep in the laboratory under conditions very similar to those that it experiences in the wild. As such laboratory studies of its evolution would not be expected to differ widely from its natural evolutionary processes in the field. In addition *P.interpunctella* and its GV are relatively well studied as insect-virus control model, unusually so for a stored product pest. In addition, the granulosis virus is a viable commercial prospect, as a biological control agent. These two factors make this system particularly useful for the type of microevolutionary study described in this thesis.

This study of the insect-virus interaction divides broadly into two parts, insect evolution and virus evolution which are best summarised by considering Fig 1.3.

Fig. 1.3 Selection Pressures leading to Evolution?



a

Low mortality

Relatively few virus particles infect hosts

Pressure on virus to become more virulent.

Increased virulence, may increase or decrease, the basic reproductive rate of the virus

VIRUS EVOLUTION

b

High mortality

Few survivors

Pressure on insects to evolve to become more resistant, and/or increase their intrinsic rate of reproduction

INSECT EVOLUTION

Thus the problem of microevolution has been addressed by considering the two extremes of the insect-virus relationship, and the selection pressures which may cause both the virus and the insect to evolve.In general terms chapters three and four deal with insect evolution, whilst chapters five and six deal with possible virus evolution

High doses of virus result in high insect mortality, leading to relatively few survivors.Continued selection pressure of this type may lead them to evolve increased resistance, and/or increase their rate of reproduction. Chapter 3 deals specifically with the response of the insects when placed under this selection pressure for several generations. Certain biotic aspects of the insect's life cycle which it is felt may have a bearing on the survival of the population in the face of viral pressure are considered in chapter 4.

At the opposite end of the curve, low doses of virus mean that relatively few virus particles are infecting the insects and mortality is correspondingly low. Since virus is transmitted via the death of the insect, then theoretically the pressure is on the virus to increase in virulence, thus increasing its chance of transmission. Such a strategy may increase or decrease the basic reproductive rate of the virus. The effect of such a selection pressure and its subsequent effect on the virulence of the virus is considered in chapter 5. A possible alternative route for evolution in which the virus could ensure survival and continued transmission is via latency. The possible occurrence of a latent form of the virus is discussed in Chapter 6.

Chapter 7 considers the results obtained in this work and reviews them in the light of current host-parasite coevolutionary theory.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter is divided into two basic areas. The first part describes general methods used in most of the experimental chapters. The second section describes molecular biology methods used mainly in Chapter 5 and 6.

2.1 Insect Rearing

2.1.1 Stock

The stock culture of *Plodia interpunctella* used in these experiments was originally supplied from a colony at Imperial College, Silwood Park,U.K. where they had been maintained as an outbred laboratory colony for 10 years.

The insects were kept in plastic containers, and reared on a diet that is basically the artificial diet used for rearing *Galleria melonella* containing: 400g Farex(rice based cereal-Farley Health Products, Nottingham) 100g dried brewers yeast, 180ml glycerol, 140ml honey,1g sorbic acid and 1g methyl paraben (p-hydroxybenzoic acid (methyl ester)). The open end of the container was covered with a double thickness of nylon netting and secured with two elastic bands.

The stocks were maintained at 28+/-2°C, 70+/-5% relative humidity, and 16 hour daylength. The only criterion regarding daylength was that it had to be greater than 14 hours to prevent the onset of diapause (Bell,1977).

A new generation of insects was started by adding newly emerged adults to a plastic container containing freshly prepared food. Eggs laid by these adults were allowed to hatch, and continue development until pupation and subsequent emergence of new adults. Populations were fed regularly and examined virtually on a daily basis

for any signs of disease.

2.1.2 Preparation of test insects

When larvae were required for experimental purposes, a slightly different regime was followed. Newly emerged adults were placed in a plastic container (as above). This, in turn, was placed upside down on a large plastic weigh boat (containing a small amount of food), and secured using sellotape. The adults were allowed to mate, and lay eggs for 24 hours.

The weighboat tray containing medium and eggs was then removed, and covered, using another tray as a lid. Eggs were then incubated at 28+/-2°C, until the larvae were required. The larvae were then removed from the medium using a fine camel hair paintbrush to prevent damage.

During the early parts of the selection experiment it was decided to try surface sterilising the eggs to prevent any unwanted contamination. Adults were set up to lay as above, but instead of laying eggs into food, eggs were laid onto nappy liners (Boots plc, Nottingham,U.K. which could be easily removed and sterilised.Eggs were sterilised in a Buchner funnel using the following protocol: initially eggs were soaked in 0.005% chloros then washed three times with distilled water followed by a 30 minute soak in 25% formalin solution,and a final thorough washing with water.

2.2 Development Stage

2.2.1 Age

Larvae were aged by measuring the width of the head capsule. To do this larvae were first immobilised using cold or carbon dioxide. The measurement was taken across the widest part of the head capsule (HCW), using a binocular microscope fitted with a graticule.

A selection of larvae were measured every day in order to check the complete range of head capsule widths. From this five larval instars were found to exist (see Appendix IV).This method was used to routinely size insects for bioassays.

2.2.2 Weight

Larvae were routinely weighed on a Cahn 2000 electrobalance, which gave accurate weights for the full range of instars.

2.3 Bioassay procedure

In order to quantify the effect of the granulosis virus on the insects, a bioassay procedure was employed that was originally devised by F.R.Hunter, Dept. of Microbiology, University of Reading (pers comm).

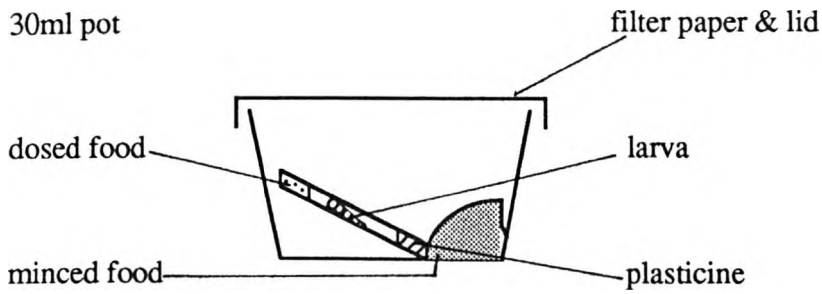
Sections of capillary tubing, 17mm in length,(1.5mm internal diameter) were sealed at one end with plasticine. A mid second instar larvae (11 days from egg laying) was then placed in the tube using a camel hair paintbrush. A small amount (3mm

length) of minced food was then used to plug the other end of the tubing, thus sealing the insect in the tube (see Fig.2. 1).The plug of food was then dosed with 1µl of virus of the appropriate dilution using a Hamilton microlitre syringe (Hamilton-Bonaduz, PO Box 26, CH-7402 Bonaduz, Switzerland).

The tube containing insect, food and virus was then placed in a 30ml plastic pot, containing enough minced food to sustain the larva once it had emerged from the tube, having eaten its way through the virus impregnated food. A 5cm circle of filter paper was then placed over the top of the tub, followed by the lid, which was slit to allow the insect suitable ventilation (see Fig.2).The pots were then incubated at 28+/- 2°C for 14 days.In each bioassay a minimum of five doses and a control were used and at least 50 larvae were tested per dose, many more with single dose mortality tests.

A slightly different method of bioassay was used for the final bioassays to determine the relative resistance level of the stock population.Larvae were placed in tubes and dosed as described above, but then instead of being placed in individual plastic tubs, the tubes were placed in Sterilin 10cm x 10cm plates containing 25 cells (2x2cm). Minimal amounts of food were placed in each cell, and the top of each cell, heat pierced to allow adequate ventilation.Strong tissue was cut to size and placed in the pierced lid of the plate. The plate was secured using two strong elastic bands and incubated at 28+/-2°C for 14 days.This method allowed more insects to be bioassayed per day.

Fig.2.1. System used in bioassaying virus.



After fourteen days the larvae were checked for the presence of viral infection. Where such an infection was present, the larva was opaque white in colour, in contrast to the normal beige colour of a healthy larva. Once the larvae had contracted the infection, they were never observed to recover. Mortality caused by handling was also recorded. No virus infection was ever observed in control larvae.

2.4 Analysis of Bioassay Results

Mortality caused by virus at different doses was analysed using probit analysis (Finney,1971). Historically probit analysis has been used almost exclusively to analyze dose-response data from the bioassay of micro-organisms pathogenic to insects, however recently an alternative method for analysis was proposed. This method is based on the exponential or independent action model (Peto, 1953; Huber and Hughes,1984), as opposed to the pharmacological or cooperative action model used in probit analysis.

Use of the exponential model was considered in this experiment. However variability between individuals was high, the majority of slope values being less than

2.0. When variability is high a probit transformation of data is more appropriate, than use of the exponential model which is suitable only if variation is small (Huber and Hughes,1984).

2.5 Extraction and purification of granulosis virus from infected insects

The following method is an adaptation based on a method of purification of granulosis virus capsules from single insects (Smith and Crook,1988).

The infected insects were homogenised in a sterile 1.5ml tissue grinder with a suitable amount of 0.1% SDS. The homogenate was spun very briefly at low speed on a bench Microcentaur centrifuge to remove the insect debris. The supernatant was removed and the pellet re-extracted to obtain maximum yield of virus by resuspending the pellet in a small volume of 0.1% SDS and recentrifuging. The resultant supernatant was added to that obtained in the initial extraction. The combined supernatants were layered onto a 50-65% (w/v) sucrose gradient in 0.1% SDS. The gradients were prepared in sterile 14ml centrifuge tubes by carefully layering equal amounts of the sucrose solutions in 5% steps of decreasing density and allowing them to equilibrate at 4°C overnight.

The homogenate was layered on top of the gradients and the gradients were centrifuged at 12500 rpm for 45 minutes at 4°C on a Sorvall OTD50B Ultracentrifuge (TST 14.1 rotor, acceleration rate=5) when the thick white capsule band was approximately halfway down the tube.The capsule band was carefully removed and transferred in another centrifuge tube which was filled with water (double distilled deionised water [ddH_20]). The suspension was thoroughly mixed by inversion, and the capsules pelleted at 15000 rpm on Sorvall Ultracentrifuge. The supernatant was

was removed quickly, and the pellet resuspended in 1ml water (ddH_20). The purified virus capsules were pelleted by centrifuging for 3 minutes at high speed on a Microcentaur centrifuge. The resulting capsule pellet was resuspended in sterile water and the process repeated. After discarding the supernatant, the capsule pellet was resuspended in a suitable volume of sterile water (ddH_20) and stored at -20°C.

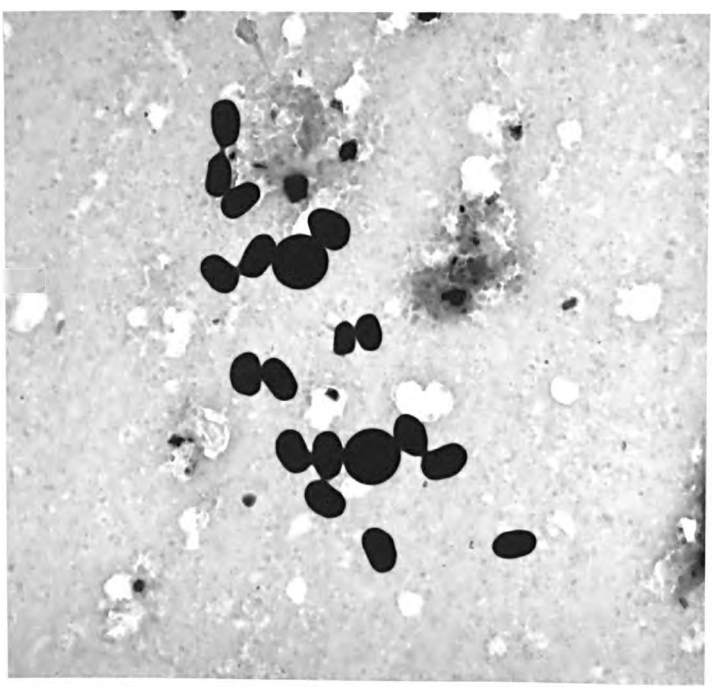
2.6 Concentration of Virus

In order to estimate the concentration of virus suspension, a method was employed which compares the proportion of unknown concentration of virus occlusion bodies, to the known concentration of Dow uniform latex spheres. The calculated ratio between the two is then used to calculate the exact concentration of the virus sample (Williams and Backus,1949).

Samples of virus and latex particle solution (1.64×10^{10} per ml, 0.481μm diameter), were sonicated separately for 2 minutes. Equal volumes of virus suspension and latex particles were then mixed and further sonicated for 1 minute.

A drop of the resulting solution was pipetted onto a carbon reinforced formvar coated copper grid (400 mesh, 3mm) (Agar Scientific Ltd., Stanstead, Essex). Excess fluid was drawn off using filter paper. Crystallised salts were then removed by washing the grid twice with distilled water. The grid was then stained using 2% phosphotungstic acid (PTA, pH 7.0), for 1.5 minutes. Excess stain was then removed by washing with distilled water.

Fig 2.2. Photograph showing latex and virus particles during counting under transmission electron microscope (magnification ~ 18,000x)



0·48μm

Particles on the grid were counted using a Zeiss transmission electron microscope with a magnification of 18,000 times . All latex and virus particles were counted on a complete sweep of the grid. This was done three times in different positions in the region of greatest diameter of the grid, covering 30-35 grid squares per sweep. The mean ratio of virus to latex particles was then calculated, and used to estimate the concentration of the virus suspension.

2.7 Materials for Molecular Biology Techniques

2.7.1 Suppliers

All chemicals were either Analar or Electran grade,and were obtained from BDH Chemicals Ltd., Liverpool, with the exception of the following:

Amersham International plc (Amersham, U.K.); ($\propto$-^{32}P)dCTP (3000Ci/mmol), Nick Translation Kit (N5000).

Anderman & Co Ltd. (Kingston upon Thames, U.K.); nitrocellulose (Scleicher and Schuell BA85, 0.45μM).

Boehringer Corporation Ltd (BCL) (Lewes,U.K.) Restriction enzymes, DNA polymerase and DNA ligase.

Difco Laboratories (East Molesey, U.K.); Bacto-agar, dehydrated yeast extract.

FMC Bioproducts (ME, USA) Seaplaque low gelling temperature agarose.

Pharmacia Fine Chemicals (Milton Keynes, U.K.) Ficoll 400.

Sigma Chemical Co. Ltd. (Poole, U.K.); bovine serum albumin (fraction V), ampicillin (sodium salt), polyvinylpyrrolidone (360 000), spermidine, trizma base.

2.7.2 Buffers

These buffers are used frequently throughout all the methods described in the following part of this chapter

a) General buffers

TE buffer	10mM Tris.HCl pH 8.0, 1mM EDTA
20xSSC	3M NaCl, 3M Na citrate
10x TBE	0.9M Tris pH 8.4, 0.9M Boric acid, 0.025M EDTA
100x Denhardts	2% Ficoll, 2% Polyvinylpyrrolidone 2% Bovine serum albumin (fraction V)

Store at -20°C

E.coli Cell Lysis Buffer (Plasmid solution I)

50mM glucose

25mM Tris-HCl, pH8.0

10mM EDTA

4mg/ml lysozyme (add immediately before use)

6x gel loading buffer

0.25% bromophenol blue

0.25% xylene cyanol

40% (w/v) sucrose in ddH2O

Store at 4°C

b) Hybridization solutions

Prehybridization buffer

6x SSC,

0.5%SDS,

5x Denhardts solution

100µg/ml denatured herring sperm DNA

(Mix 1:1 with deionised formamide)

Hybridization buffer

As above, plus:

0.01M EDTA

^{32}P denatured labelled probe

2.7.3 Bacteriological Media

a) Liquid Media

L-broth (per 500ml)

Tryptone	10g
Yeast extract	5g
NaCl	5g
Glucose	1g

b) Solid media

L-agar (per 500ml)

Tryptone	5g
Yeast extract	2.5g
NaCl	2.5g
Agar	15g/litre

2.7.4 Antibiotic

Ampicillin

A stock solution of 20mg/ml of the sodium salt of ampicillin was made up, filter sterilised and stored at -20°C. Selective media were prepared by the addition of 40 μg/ml of ampicillin. Ampicillin was only added to molten agar when cooled to 55°C or below.

2.7.5 Centrifuge rotor details

Microcentaur	MSE Microcentaur centrifuge, 1.5ml tube housing. High setting equivalent to 13,000rpm
HB-4	Sorvall RC-5B Refrigerated superspeed centrifuge
Eppendorf	Anderman Eppendorf centrifuge. Spins up to 15000rpm with 1.5ml tubes.
TST 14.1	Sorvall OTD50B Ultracentrifuge

2.8 Molecular Biology Methods

2.8.1 Extraction of Viral DNA

This extraction protocol is an adaptation of several published methods, but mainly those of Tweeten *et al*,1980 and Crook *et al*,1985.

Viral DNA was isolated from purified granulosis virus by initially disrupting the granulin matrix by adding 1M sodium carbonate pH 10.6 to a final concentration of 0.05M at 37°C and incubating for 30 to 60 minutes, until the suspension cleared. The preparation was then incubated for a further 30 minutes at 37°C with 10% SDS (sodium dodecyl sulphate)(w/v) at a final concentration of 1% in order to aid dissociation of protein.

To aid successful restriction endonuclease digestions of DNA, the DNA was frequently further purified by the addition of RNase to a final concentration of 20μg/ml and Proteinase K to a final concentration of 50-200μg/ml. The solution was incubated for 30 minutes at 37°C for the initial addition of RNase, and 30-60 minutes following the addition of proteinase K.

The solution was deproteinized by a phenol-chloroform extraction.An equal volume of phenol (equilibrated with TE pH8.0) was added to the solution containing the DNA. The two phases were mixed gently by inversion, and the two layers separated by centrifuging at low speed for a minute on a Centaur microfuge.The aqueous supernatant containing the DNA was removed carefully without disturbing the protein interface, using a wide-mouthed pipette, or using Gilson tips with the ends cut off to prevent the DNA shearing.This extraction was repeated twice with

phenol:chloroform:isoamylalcohol (25:24:1)(v/v/v)mixture, and a final chloroform :isoamyl alcohol (24:1) extraction.

The resultant DNA was dialysed extensively against large volumes (1-1.5 litres) of 0.01M Tris-hydrochloride, 0.001M EDTA (Ethylenediaminetetraacetic acid, sodium salt) pH8.0. Dialysis was carried out at 4°C, over a 24- 36 hours period, using three or four changes of buffer. The first change being 3-4 hours after dialysis was started.

Dialysis of small volumes of DNA (100µl) was carried out using the tops of 1.5ml Eppendorf tubes (Horowitz and Barnes, 1983).Dialysis membrane was stretched over the cap of the tube which containing the DNA. The top 5mm of the Eppendorf tube was removed from its base using a hot scalpel blade. This 5mm ring was then used to secure the dialysis membrane across the top of the cap, thus sealing the DNA into the cap.The DNA was then dialysed as above by placing the dialysis assembly with the top of the cap floating uppermost in slowly stirring TE buffer at 4°C.

Dialysis tubing was prepared for use by boiling in distilled deionised water for thirty minutes with two changes of water, allowed to cool and stored at 4°C. Tubing was washed thoroughly in distilled water before use. Gloves were always worn to handle tubing.

2.8.2 Viral DNA purification by isopycnic centrifugation

This method was used for purification of DNA when large volumes of virus were available (usually 4ml or above). To 4ml of the virus particles, 1ml of 10% (w/v) sodium N-lauryl sarcosinate, 10mM EDTA (sarkosyl lysis buffer) was added, and incubated for 30 minutes at 60°C. The resulting solution (2% with respect to sarkosyl)

was layered onto caesium chloride gradients (9g caesium chloride, 7.7ml TE buffer pH 8.0, 5µl Ethidium bromide (10mg/ml)), and 2-3ml of liquid paraffin was layered above this in 14ml centrifugation tubes. Supercoiled DNA was recovered in the form of a tight pink band after centrifugation at 35K for 18 hours at 15°C on a SW41 Beckman ultracentrifuge.

The band was recovered by heat piercing a hole in the base of the tube, and draining off the excess gradient then collecting the fraction containing the band. Ethidium bromide was removed from the DNA by repeated extraction with an equal volume of butan-1-ol, until DNA was colourless. It was then dialysed against TE buffer for 24 hours at 4°C, with regular changes of buffer.

2.8.3 Extraction of insect DNA

Final instar larvae were homogenized in 200µl 2xSSC using a sterile 1.5ml tissue grinder. An equal volume of sarkosyl lysis buffer was added, the suspension vortexed and left to stand for 20 minutes on ice. Then phenol-chloroform extracted as described in 2.7.1. A tenth volume of 3M sodium acetate (pH6.0) and 2 volumes of ethanol were then added and precipitated at -70°C for 1-2 hours. The precipitate was pelleted out by centrifuging for 10 minutes on an Eppendorf centrifuge, and the supernatant discarded. The pellet was washed with 1ml of 70% ethanol, by vortexing and recentrifuging as above. The supernatant was discarded and the pellet dried and resuspended in 2xSSC.

2.8.4 Estimation of DNA concentration

Where amounts of DNA were sufficient, this was estimated spectrophotometrically by using relative absorbances at 260 and 280nm and the standard double stranded DNA concentration (50μg/ml). Otherwise, DNA was run on minigels against standards of known concentration of lamda phage, and DNA concentration was estimated using a UV transilluminator to compare the intensity of fluorescence in the sample with that of the standards (Maniatis *et al*,1982)

2.8.5 Restriction enzyme digestion of DNA

All restriction enzyme reactions were carried out according to the manufacturers' specifications. Digestion was carried out for a minimum of 3 hours at 37°C, or more usually overnight at 37°C. Spermidine at a final concentration of not more than 4mM, was added to samples which proved difficult to digest.

2.8.6 Agarose Gel Electrophoresis of DNA

DNA was prepared for electrophoresis by the addition of 1/6th volume of a double dye marker (Bromophenol blue, sucrose, croesel green). In order to estimate the size of digested fragments, molecular weight markers were run concurrently with the samples on the gel. DNA molecular weight markers were prepared form wild type λDNA digested with Hind III and/or EcoRI. the size of the fragments in base pairs is given in Table 2.1. λDNA was heated to 65°C for 3 minutes to separate annealed ends.

Electrophoresis was carried out in 0.6% agarose gels in TBE buffer. Minigels (30ml, 2.5 x 5cm) were run at 40 to 80V for between 1- 3 hours. Larger gels (300ml, 20 x 25cm), were routinely run at 35V overnight. BRL horizontal gel electrophoresis

Table 2.1 Lambda DNA molecular weight markers for electrophoresis

Hind III	Hind III/Eco RI
23.130 Kbp	21.226 Kbp
9.416	5.148
6.557	4.973
4.361	4.277
2.322	3.530
2.027	2.027
.564	1.904
.125	1.584
	1.330
	.983
	.831
	.564
	.125

tanks were used, series 1025 model H4, and series model H6.

Following electrophoresis, DNA bands on the gels were visualized using a UV transilluminator (302nm, UV Products Inc., TM36). Gels were photographed using a Polaroid MP4 Land Camera with a Wratten 2A and 15 filter, and Polaroid 400ASA instant film.

2.8.7 Southern blotting: transfer of DNA to nitrocellulose

After electrophoresis, the DNA was denatured by agitating the gel in 500ml of 1.5M NaCl, 0.5M NaOH for 30 minutes. The gel was then neutralized in two changes (one rapid) of 0.5M Tris pH 7.0, 3M NaCl.Occasionally denaturation was preceded by soaking the gel in 0.15M HCl in order to hydrolyse the DNA.

Transfer of DNA to nitrocellulose was performed using essentially the method described by Southern (1975). Tissues were soaked as a block in 20xSSC and wrapped in clingfilm leaving the upper side open. On this surface was placed two pieces of Whatman 3MM Chr filter paper previously soaked in 2xSSC, the gel was in turn placed on top of the filter paper.The clingfilm was then closed over the upper surface of the tissues and around the edges of the gel to form a 'seal'.

The nitrocellulose presoaked in 2xSSC, was placed carefully on the gel, with the waterproof orientation marks correctly in position. Any trapped air bubbles were removed and if necessary the nitrocellulose reorientated. Two further pieces of presoaked 3MM paper (in 2xSSC) were placed over the nitrocellulose followed by a wad of dry tissues. The assembly was secured using a glass plate and a weight, and left to blot overnight.

Following blotting the gel was checked on the UV transilluminator to ensure that all the DNA had been transferred. The nitrocellulose filter was then bathed with gentle agitation in 2xSSC for 15 minutes, then dried on Whatman 3MMChr paper, before being baked at 80°C for 2 hours.

2.8.8. Dot Blots

Genomic DNA was denatured by boiling for 10 mins in a final sample volume of 100-400µl then rapidly snap-chilled, and used immmediately to prevent any renaturation of the sample (Anderson and Young,1985). The DNA solution was dotted onto the nitrocellulose using the dot blot apparatus (Anderman and Co. Ltd.) connected up to the suction pump. Before use the nitrocellulose was thoroughly soaked for 15 minutes in 6xSSC. DNA was dotted onto the filter, and each well in use was washed through with 300µl of 6xSSC. Upon removal from the dot blot manifold, the filter was briefly washed in 6xSSC and air dried before being baked at 80°C for 2 hours. Hybridization and prehybridization of dot blot filters were as described in 2.8.10.

2.8.9 Preparation of radioactive probes

Enzyme reactions

a) Nick Translation

Viral DNA (purified by isopycnic centrifugation) was labelled using Amersham

International's Nick Translation Kit (N 5000). 300ng DNA was labelled in a 20µl reaction mix containing 4µl buffer solution, 2µl enzyme 3µl (α-32P) d-CTP (30µCi, 9.9 pmols). The reaction was allowed to proceed for 2 hours at 15°C. The reaction was stopped by the addition of 0.5M EDTA (pH7.5) to a final concentration of 10mM.

b) Random Priming

Where DNA available was less than 100ng it was labelled using random priming (Feinberg and Vogelstein,1983). The granulin gene fragment was radiolabelled using a random priming kit supplied by Boehringer Mannheim. 70ng of DNAwas denatured by boiling for 10 minutes in 11µl water, and cooled rapidly on ice to prevent reannealing. The DNA was then added to the following reaction mix: 25µM dATP, 25µM dGTP, 25µM dTTP, 2 units of Klenow DNA polymerase, 30µCi (9.9 pmoles)(α-32P)-dCTP, and 2µl hexanucleotide primers in buffer, as specified in manufacturers details. Reaction proceeded at 37°C for one hour. Hexanucleotide primers hybridize to DNA and are extended by the Klenow enzyme, incorporating label. The reaction is terminated by heating to 65°C for 5 minutes.

Desalting DNA

Radiolabelled DNA was purified by removing the free nucleotides by desalting on a Sephadex G-50 column containing 0.02% sodium azide. The column was equilibrated with 10ml of TE buffer pH8.0. Fractions were Cerenkov counted on a scintillation counter, and the aliquots containing radiolabelled DNA were pooled to form the probe. Radiolabelled DNA routinely had specific activities greater than10^8 dpm/µg of DNA using random priming or nick translation.

2.8.10 Hybridization Conditions

In order to hybridize DNA fixed on nitrocellulose filters, filters were initially prehybridized overnight, then hybrized overnight on the following day using the method as basically described in Maniatis *et al* (1982)

The baked nitrocellulose filter was soaked in 2xSSC for two minutes, and then prehybridized overnight in 6xSSC, 50% formamide, 0.1%SDS, 1mM EDTA, 1x Denhardt's solution and 100µg/ml sheared denatured herring sperm DNA at 42°C, in a heat sealed plastic bag.

After prehybridization the buffer was replaced with hybridization buffer containing 90µl of nick translated probe (denatured by boiling for 5 minutes before addition), and then hybridized overnight at 42°C. After removing the solution containing the probe, the filter was washed briefly in 6xSSC, 0.1% SDS at room temperature, then again for an hour with two changes of buffer. Stringency of washes was varied depending on conditions required. These varied from low stringency (high salt, low temperature) to low salt, high temperature washes e.g. 0.1% SSC, 0.5%SDS at 65°C (high stringency). Individual washing conditions are cited in figure legends.

Filters were then autoradiographed at -70°C in 'X'-ograph cassettes using preflashed ' Fuji RX' film and ' Speed X' intensifying screens.

2.8.11 Dephosphorylation and ligation of plasmid DNA

5µg of plasmid vector pUC19 was digested overnight at 37°C with Bam H1. The digested plasmid was then dephosphorylated to prevent recircularization of the plasmid. Dephosphorylation was carried out by initially adding 5 units calf intestinal alkaline phosphatase (CIP) for 30 minutes at 37°C. This was repeated with a further 5 units of CIP for a 30 minute incubation at 37°C. To stop the reaction 5µl of 0.5M EDTA was added, followed by 5µl of 10% SDS incubated at 68°C for 15 minutes, in order to fully inactivate CIP and remove any excess from plasmid DNA.

The plasmid DNA was then purified using phenol-chloroform extraction and ethanol precipitation. Ethanol precipitation was carried out by adding a tenth volume of 3M sodium acetate (pH6.0) followed by 2 volumes of absolute ethanol . This was precipitated at -70°C for 1-2 hours. The precipitate was pelleted out by centrifuging for 10 minutes on an Eppendorf centrifuge, and the supernatant discarded. The pellet was washed with 1ml of 70% ethanol, by vortexing and recentrifuging as above. The supernatant was discarded and the pellet dried in a vacuum drier for 5 minutes,or at 37°C for 1-2 hours. The pellet was resuspended in TE buffer [pH8.0] to a final concentration of 50ng/µl. Resuspension was sometimes aided by placing the pellet and buffer at 37°C for 30 minutes.

Bam H1 digested viral DNA (0.25µg, 0.05µg and 0.01µg) was ligated into the vector using 0.5µl of T4 ligase, 1µl of freshly prepared ligation buffer (0.5mM Tris.Cl [pH7.4], 0.1M $MgCl_2$,0.1M DTT) and 1µl 10mM ATP. Ligation was allowed to proceed on the bench for 4 hours or overnight at 4°C

2.8.12 Preparation of competent cells

Ten ml of L-broth was innoculated with Hb101 *Escheric hia coli* cells (for details of genotype see Maniatis *et al,* 1982) from a glycerol stock and incubated overnight with gentle shaking at 37°C. To prepare competent cells, 2.5ml of the overnight culture was innoculated into a further 50ml of prewarmed L-broth, and grown at 37°C. When the culture had reached a cell density of $1x10^8$ (ideally OD600<0.4) approximately two hours twenty minutes after innoculation, it was chilled for 10 minutes on ice. The cells were pelleted, and resuspended in 25ml of ice-cold 50mM calcium chloride. This suspension was left on ice for 30 minutes, and then repelleted. Cells were gently resuspended in 2.5ml of ice-cold 50mM calcium chloride, and left on ice until required.

2.8.13 Transformation of *E.coli* with plasmid DNA

1µl of ligation mix was placed on ice. 100µl of competent cells were added with a little force. Mixture was left for 30 minutes on ice, then heat shocked for 3 minutes at 42°C. 1ml of L-broth was added and incubated at 37°C for an hour. 100µl of these cells were plated out on selective agar plates as described below.The remaining cells were pelleted in a centrifuge and the supernatant discarded. The pellet was resuspended in 100µl of fresh L-broth and plated out on selective agar plates containing 40µg/ml ampicillin, and incubated overnight at 37°C. This effectively gave two different dilutions of each transformation, 10% and 90% of all available cells having been plated out.

Transformation controls

a) negative control: competent cells only on L agar

b) positive control:1-2ng of uncut pUC19.

c) transformation control: ligation mix only to determine how many cells have been transformed.This enables the transformation efficiency of the reaction to be calculated.

2.8.15. Preparation of filters for screening of bacterial colonies

This method is based largely on that of Grunstein and Hogness(1975), and used when small numbers (100-200) of bacterial colonies are dispersed on several plates. Colonies of transformed bacteria were picked off of the selective agar plates, and placed into separate wells of a microtitre plate containing 100µl of L-amp broth. The microtitre plate was then incubated at 37°C for 3 hours.

Nitrocellulose was cut into circles in order to fit onto agar plates, then sterilised over UV light. The sterile nitrocellulose was placed onto agar plates containing ampicillin, and orientation marks placed on the filters using waterproof ink.
Transformed bacterial colonies from the microtitre plate were transferred to sterile nitrocellulose using a flame sterilised tool designed for the purpose. A master plate and two replicas of all colonies were produced. The plates were incubated overnight at 37°C.

The following day, the master plate and microtitre plate were sealed using parafilm and stored at 4°C until the results of the hybridization reaction were available.
The bacterial colonies on the replicate filters were lysed and fixed to the nitrocellulose

using the following method (Maniatis *et al* 1982).

Three trays, each containing four pieces of Whatman 3MM Chr paper were set up. The filter paper were saturated in the following solutions: 10% SDS, denaturing solution (0.5M NaOH, 1.5M NaCl) and neutralising solution (1.5M NaCL, 0.5M Tris. Cl [pH8.0]). The filters were processed as follows: the nitrocellulose was placed colony side up on the SDS impregnated 3MM paper for 3 minutes; using the side of the tray as a scraper to remove as much fluid from the underside of the filter as possible, the filter was transferred to the 3MM paper saturated with denaturing solution for 5 minutes; finally the filter was neutralised by placing in the final tray for 5 minutes. The filters were allowed to air dry at room temperature before being baked at 80°C for two hours to fix DNA permanently.

Filters were hybridized with a radio-labelled probe as described in 2.8.10.

2.8.16 Mini preparation of plasmid DNA

This alkaline lysis method for minipreparations of plasmid DNA is a modification by Ish-Horowitz (1982) of an earlier method by Birnboim and Doly (1979).

A single colony of transformed bacteria was grown to saturation in 5ml of L-broth containing 40µg/ml ampicillin. 1.5ml of the culture was pelleted, and resuspended in 100µl of ice-cold solution containing 50mM glucose, 10mM EDTA, 25mM Tris-Cl pH 8.0 and 4mg/ml lysozyme (added on day of use). Lysis was allowed to proceed for 5 minutes at room temperature, before DNA was denatured by adding a freshly prepared solution of 0.2N NaOH, 1% SDS. In order to neutralise the lysate, 150µl of ice-cold 3M potassium acetate (pH4.8) was added, after neutralisation had been allowed to proceed for 5 minutes on ice.

The flocculent precipitate containing denatured bacterial and chromosomal DNA was pelleted in the microfuge. Plasmid DNA in the supernatant was phenol-chloroform extracted and precipitated in ethanol at room temperature for 5 minutes. The pellet was washed using 70% ethanol, dried and resuspended in 20µl of TE buffer (pH8.0) and treated with 20µg/ml of RNAase. Restriction analysis of minigels followed to check for the presence of an insert.

2.8.17 Preparative gel electrophoresis of DNA from LGT agarose

In order to use a plasmid insert as a probe, the insert DNA must first be isolated using preparative gel electrophoresis techniques (Weislander,1979).

Minigels of 0.6% low gelling temperature agarose (LGT) in TBE buffer were poured and left at 4°C to ensure complete setting.The sample was prepared, loaded and run as previously described for gel electrophoresis.The resulting bands were visualized over a UV transilluminator, and the insert size checked against standard markers co-migrating on the gel. The desired band was then excised, transferred to a 1.5ml Eppendorf tube and melted at 65°C for 10 minutes. The gel was then diluted with water to a final gel concentration of 0.2% LGT, followed by the addition Tris-HCl (pH8.0) to a final concentration of 0.1M.The suspension was then extracted with an equal volume of phenol and the aqueous phase recovered. This phase was then briefly re-extracted with phenol- chloroform, and the DNA recovered by ethanol precipitation (as described previously).The pellet was resuspended in a minimum volume of TE buffer, and a 0.6% minigel run to assess the concentration of DNA.

CHAPTER 3

SELECTION FOR INSECT EVOLUTION

3.1 Introduction

The development of resistance in insects towards pest control agents, particularly pesticides, has been well documented (see Chapter 1). However, pesticides present the insects with a different problem to that encountered with biological control agents. Notably, biological control agents are not novel agents like the majority of pesticides, and unlike pesticides they are not inert inorganic entities incapable of their own evolution. Therefore, it might be expected that the insects' response to intensive exposure to large doses of biological control agents might differ from their response in a similar situation with pesticides.

Several instances of the development of resistance to microbial control agents have been recorded in insects. There are only two examples of total immunity to a viral infection, where irrespective of dose, one population of the insects is completely resistant to a pathogen to which it is normally susceptible. Both have been recorded in the silkworm *Bombyx mori* , in its response to infectious flacherie virus (Funada,1968) and to densovirus (a densonucleosis virus)(DNV)(Watanabe and Maeda,1981).This non-susceptibility is controlled in both cases by a recessive autosomal gene.

More commonly, examples of the development of increased resistance in populations of insects involve a change in the level of susceptibility, rather than total resistance. These are due to changes in the frequency of individuals showing particular responses to virus infection (Briese,1986). Most frequently, differences in the level of susceptibility are demonstrated in geographically isolated strains of an insect species (and also in direct comparisons of field and laboratory strains). Reports of resistance

microbial control agents have been comprehensively tabulated (Vigneswaren,1987). These include numerous studies involving baculoviruses.

Differences in response to peroral infection with granulosis virus amongst geographically isolated strains of insect have been recorded in *Pieris brassicae* (Rivers,1958; Sidor,1959; David and Gardiner,1960,1965,1966) and *Plodia interpunctella* (Hunter, 1972). In the latter case, a 'California' strain of insects was seven times more resistant than a 'Georgia' strain. A similar type of response has been observed in *Phthorimaea operculella* , with a laboratory strain being thirty times more resistant than a field population (Briese and Mende,1981).

Similar patterns of susceptibility have been recorded with NPVs: a designated strain B of *Spodoptera frugiperda* was five times more resistant than strain A (although both from Howard County) (Reichelderfer and Benton,1974). In addition, laboratory strains of both *Epiphyas postvittana* and *Pieris brassicae* were both more resistant than the corresponding field strains of the same insects (Sidor,1959; Geier and Briese, 1979).

Observed strain differences are a result of differences in the frequency of individuals showing a particular response to viral infection. Briese and Podgwaite (1986) described three main factors which determine the response of an individual insect: developmental, environmental, and genetic. In terms of control "procedures", genetic factors assume greater importance than either developmental or environmental factors, since the effects of the latter are mainly short term, whereas genetic changes may lead to more permanent changes in the levels of susceptibilty (Briese,1986a).

Genetic differences in resistance are due to genes arising in a population which

confer greater resistance to the virus, which then increase in frequency or even become fixed in discrete populations of the host insect, such as those known to confer resistance to chemical insecticides (Whitten and McKenzie,1982). In fact, in three of the aforementioned examples: *S.frugiperda*, *E.postvittana*, and *P.operculella* , strain difference in response to a virus has been shown to have a genetic basis (Briese,1986a).

The experiments in this chapter were carried out in an attempt to affect these genetic factors. The object of the investigation was to subject a population of insects to a consistently high selection pressure, in order to see whether after several consecutive generations of selection any change in the population had occurred , either in terms of response to the virus or in biotic terms.

Previous such selection experiments with insect viruses have experienced mixed consequences (see Table 3.1). In five out of the eight recorded cases change in response to the virus was noted. The typical range of any such change was between an eleven and a twenty-eight fold increase in resistance levels. Higher increases than this have only been achieved once, using a granulosis virus of *P.operculella*. Here a 140 fold increase was recorded over only six generations of selection (Briese and Mende,1983). Increases in resistance in other host species were achieved in similarly short generation times: a 7-8 fold increase in *Cydia pomonella* in seven generations (Huber,1974); in a similar time span (seven generations) a 4.6 fold increase in *Spodoptera frugiperda* (Fuxa *et al*,1988); and a 11-28 fold increases in *Bombyx mori* with flacherie virus (FV) and cytoplasmic polyhedrosis virus (CPV) in six and eight generations respectively (Uzigawa and Aruga,1966; Watanabe,1967).

Table 3.1 Cases in which attempts have been made to select for increased resistance to a virus in populations of Lepidoptera (adapted from Briese & Mende,1983)

Insect species	Virus	No of gens selected	Sel^n pressure (%mortality)	Resistance factor	Author
B.mori	FV	5	Not given	11-28	a
B.mori	CPV	8	53-92%	14-28	b
H. zea	NPV	25	50-70%	No diff	c
H. armigera	NPV	22	40-80%	No diff	d
H. armigera	GV	22	15-90%	No diff	e
C. pomonella	GV	7	61-90%	7-8	f
P. operculella	GV	6	34-71%	140	g
S. frugiperda	NPV	7	80%	2	h

[a]Uzigawa& Aruga (1966), [b]Watanabe (1967),[c] Ignoffo & Allen (1972),[d] Whitlock(1977), [e] Whitlock(1977),[f] Huber (1974), [g] Briese & Mende(1983), [h] Fuxa *et al*, (1988)

In contrast to this, where no increase had occurred, testing has been carried out over relatively large numbers of generations. It may be assumed therefore that if change had not occurred within a relatively short number of generations it was unlikely to occur at all.

3.2 Materials and Methods

3.2.1 Selection experiment

Selection was carried out over ten generations using the selection schedule detailed in Table 3.2. The method of selection involved individual peroral dosage of varying numbers of larvae (between 220-400, depending on the dosage of virus used), employing the bioassay method described in Chapter 2. The larvae were exposed to a range of viral doses. Mortality due to the virus was taken to be the number of insects showing positive signs of infection fourteen days after dosing. Individuals dying from other causes were omitted from the mortality totals. Uninfected insects were allowed to pupate, and the resultant emerging moths were mated. Progeny from these matings constituted the next generation of the selected population.

Concurrent with the selection schedule, unselected control larvae (derived from the same parent stock and maintained in a large out-bred population) were subjected to the same selection pressure as the selected line of insects. Therefore, a running comparison of relative mortalities of at least one virus dosage per generation was possible.

In addition after generations 5,8,9, and 10, dosage-mortality bioassays were carried out to compare the responses to granulosis virus, of the selected larvae with those of the unselected controls.This allowed a more accurate determination than single dose comparisons of whether any shift in susceptibility levels had resulted from selection. Data were then analysed using probit transformations, in order to determine LD50 values, regression line slopes and resistance factors (Finney,1971; Dulmage,1973).

3.2.2 Monitoring biotic changes in selected population

After ten generations of selection, the two populations - unselected control and selected - were compared in biotic terms. Biotic factors most likely to be affected by selection were thought to be developmental rate and size. To monitor these factors a sample of 50 larvae from each population was taken at 7,9,11,13 and 21 day intervals respectively after oviposition. Each day's sample was taken from a previously undisturbed egg batch, laid by adults of known age. These larvae were then weighed individually using a Cahn 2000 electrobalance, and their mean weight recorded. In addition the relative composition in developmental terms of each sample population was compared at day 12, by determining the proportion of different instars present by measuring head capsule width on a Kyowa binocular microscope, and eye-piece graticule.

3.2.3 Relative resistance level of stock population

A final bioassay was carried out in an attempt to place the resistance status of the original stock (control) population in a broader context. To this end the stock were

bioassayed alongside two further strains of *Plodia interpunctella* (1/SM10/85 and PS/1 brown X/ 85)kindly provided by F.R. Hunter, University of Reading. These two strains had exhibited a stable difference in resistance levels between one another (Hunter *pers comm*).

Bioassays of these two strains and the stock strain were carried out simultaneously. Two replicates of 50 insects per dose at seven dosage levels were used. Mortality was recorded, and results analysed as before using probit analysis (see section 3.2.2).

3.3 Results

3.3.1 Resistance to virus

Over ten generations of selection no control larvae died of viral infection.

Selection pressure

It was originally intended to aim for a consistently high selection pressure (at least 70-80%), in order to maximize any possible chance of evolution occurring. However this had to be traded off against the associated risk of extinction of the selected stock. Initially, high levels of selection were attained: 81% and 92% in generations one and two respectively. However between generations three and five there was a repeated pattern of decline in virus-induced mortality after exposure to one dosage level, necessitating an increase in virus dosage from generations six to ten. This resulted in a variation of selection pressure from 25-92%.A similar problem was recorded with an experiment using a granulosis virus of *Phthorimaea operculella* (Briese and Mende,1983)

operculella (Briese and Mende,1983).

The problem of repeated decline of virus-induced mortality was to some extent solved by preparing several aliquots of the viral dilutions and storing them at -20°C. These aliquots were used only once and therefore never refrozen. This largely eliminated the problem, and from generation six onwards the average selection pressure at 5.22 log dose (virus particles/larva) was 72.8%.

The only deviation from this pattern occurred at generation eight, when in order to avoid possible extinction, a lower dose was chosen, a selection pressure resulting in only 25% mortality.

Results of selection pressure : single dose mortality

The mortalities in the selected and control larvae are presented in Table 3.2. Between generations one and five, in three of the four recorded cases mortality was higher in the selected population than in the control. The mean variation in mortality between the two populations is 7.7%.

Results of selection pressure : bioassay results

Table 3.3 records the LD50s, 95% fiducial limits and slope values for each population in generations 0,5,8,9 and10, when they were compared by full bioassay. The LD50 ratio provides a measure of any change in resistance between control and selected strain .and is defined as the resistance factor. Any increase in resistance by the

Fig 3.1 Graph showing decrease in virulence of granulosis virus over successive generations

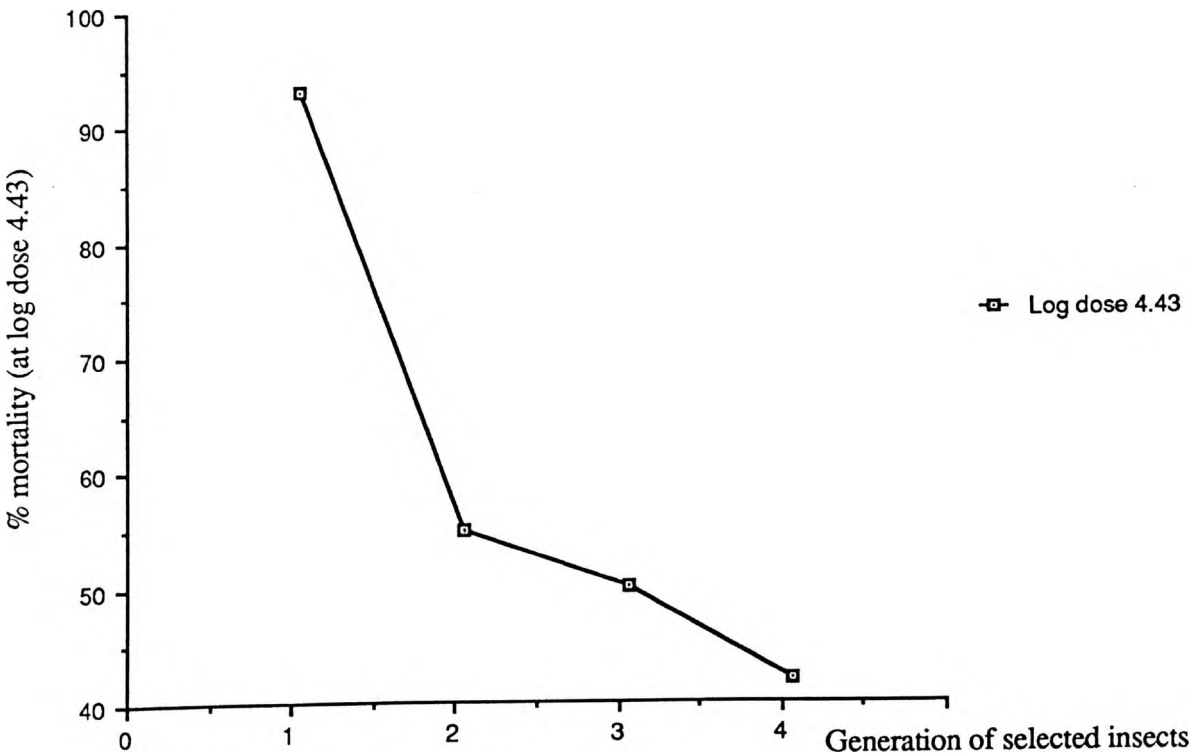


Table 3.2 Selection schedule and comparison of single dose mortalities for control and selected populations of*Plodia interpunctella* over ten generations

Generation	Log Dose	% Mortality selected	% Mortality control	Frequency of bioassay
(F_0)	4.73	81	81	Bioassay
(F_1)	4.43	92	82	-
(F_2)	4.43	57	-	-
(F_3)	4.43	49	59	-
(F_4)	4.43	41	44	-
(F_5)	5.52	74	82	Bioassay
(F_6)	5.52	88	-	-
(F_7)	4.52	25	-	-
(F_8)	5.52	74	-	Bioassay
(F_9)	5.52	91	20	Bioassay
(F_{10})	5.22	52	-	Bioassay

Dose: Nos of virus particles/larva

selected strain leads to LD50 ratio values greater than one.However, in each generation tested the value is consistently less than one, lying in the range 0.03 to 0.79. There appears to be no consistent pattern to the LD50 ratios. Therefore the selected strain can be said to have shown no increase in resistance to virus induced mortality over ten generations of selection. In fact, if anything, there appears to be a slight increase in susceptibility to the virus.

Unselected control larvae exhibited greater variation of LD50 values, 4.86 to 6.98 (log dose particles per larva), than the selected strain: 4.96 to 5.43 (log dose particles per larva). Mean LD50 values are 6.34 for control larvae and 5.11 for selected larvae. Comparison of the LD50s of the selected and control larvae at each generation based on non-overlap of 95% fiducial limits, indicated a significant difference in LD50s in each case. This method of analysis was used since a variance formula was not used to calculate the 95% fiducial limits,[the method used was one involving the roots of a quadratic equation (Finney, 1971)] and therefore statistical tests employing standard errors were not applicable (Finney,1971).Slope values for both strains covered a wide range: 0.43 to 1.47 (control) and 0.97 to 2.34 (selected). They differed significantly at generations eight and ten ($t = 4.15$, $p<0.01$; $t = 3.98$, $p<0.01$).

The value of chi squared in each of the assays was not significant (except selected generation 10) at 95% probability level indicating absence of systematic heterogeneity of response in test population of larvae, and no significant departure of the observed data from that of the calculated regression line(See Table 3.3 a).

Precision values, the ratio of the upper fiducial limit to the lower fiducial limit of the LD50 dose, were calculated for each bioassay. Ideally this value should be quite low, between 1-2. Calculated values for the selected strain ranged from 1.36-10,

Table 3.3 Comparison of dosage mortality parameters for a selected and unselected populations of *Plodia interpunctella*, after exposure of the selected strain to granulosis virus for varying numbers of generations.

Generation		Log LD50	95% Fiducial limits Lower	Upper	Slope (± s.e.) (sel/con)	LD50 ratio
1	Con:	4.33	4.17	4.48	1.47 (±0.14)	1.00
5	Con:	4.86	5.12	5.64	1.13 (±0.13)	
	Sel:	4.96	4.56	4.95	0.97 (±0.09)	0.79*
8	Con:	6.98	6.34	8.38	0.43 (±0.09)	
	Sel:	5.43	5.24	5.67	1.02 (±0.11)	0.03*
9	Con:	6.05	5.75	6.45	0.75 (±0.10)	
	Sel:	5.11	4.64	5.64	0.99 (±0.17)	0.14*
10	Con:	5.40	5.29	5.61	1.04 (± 0.13)	
		5.12	5.08	5.21	2.34 (±0.30)	0.47*

Con: Control/ Unselected Population
Sel: Selected Population

Dose: No of virus particles/larva

Table 3.3a Analysis of dosage - mortality responses of control and selected populations

Generation		LD 1¤	LD99¤	Chi-square	P
1	Con	3.68	5.90	10.254 (2df)	0.068
5	Con	2.80	6.90	15.524 (2df)	0.063
	Sel	2.36	7.10	36.454 (5df)	0.265
8	Con	1.56	12.40	4.066 (3df)	0.254
	Sel	3.14	7.71	3.817 (3df)	0.282
9	Con	2.94	9.17	0.700 (3df)	0.873
	Sel	2.75	7.47	10.951 (4df)	0.021*
10	Con	3.19	7.68	7.690 (4df)	0.093
	Sel	4.15	6.10	53.060 (4df)	0.548

* Significant level of heterogeneity

¤ Doses in log dose of number of virus particles per larva

Table3.4Precision values for dosage-mortality analysis (ratio of upper 95% confidence limits to lower 95% confidence limits).

Generation	Control	Selected
1	2.04	-
5	3.08	2.45
8	111.70	2.69
9	5.04	10.00
10	2.08	1.36

giving a mean of 3.7. Corresponding values for control strain were 2.04-111.7. The figure for the upper range is aberrant due to the low number of doses used in the generation nine bioassay. If this is omitted from the general range, the figures show a considerably tighter range: 2.04-5.04 giving an amended mean value of 3.06.

3.3.2 Biotic comparison of the selected and stock population

Table 3.5 shows the weights of larvae of different ages from control and selected populations. The larvae of the selected population are significantly larger than the control larvae at every recorded age ($p<0.001$). The selected larvae are on average 1.47 times bigger than the control larvae.

Table 3.6 shows the result of a comparison of developmental rates of 12 day old larvae from control and selected populations. All the larvae from the selected population were third instar, whereas only two thirds of the control larvae measured were third instar, the remainder being second instars. The difference in developmental rates was highly significant ($x^2= 32.7$, $p<0.001$), and the difference in weight significant and consistent with results obtained in Table 3.5 ($t = 8.76$, $p<0.001$)

3.3.3 Resistance status of the original stock population

Details of the bioassay comparison with two further insect stocks can be found in Table 3.7. The LD50 of the original stock (control) population was higher (5.40 log dose particles per larva) than that of both the other two provided insect populations, with PS/1brown/X (4.52 log dose particles per larva) showing higher LD50 than 1/SM10/85 (4.18 log dose particles per larva). Comparison of the three strains based on 95% fiducial limits demonstrated that the LD50 for the original stock population

Table 3.5 Comparison of weights of larvae of different ages from control and selected populations

	Age of larvae (days from laying)				
	7	9	11	13	21
Selected	0.016	0.048	0.050	0.134	2.94
Control	0.011	0.030	0.040	0.089	1.96
Ratio(S:C)	1.46	1.60	1.25	1.55	1.50
t	10.05*	6.25*	5.59*	6.46*	5.05*

* $p < 0.001$

NB: Weights of larvae in mg

Table 3.6 Relative rates of development of 15 day old larvae from control and selected populations

	Control	Selected
Total no weighed	150	150
No of 2nd instars	47	0
No of 3rd instars	103	150
		$X^2 = 32.7$ ($P<0.001$)
Mean weight (mg)	0.289	0.533
		$t = 8.76$ ($p < 0.001$)

Table 3.7. Comparison of dosage-mortality data of three strains of *Plodia interpunctella*

Strain	Log LD50	95% Fiducial limits Lower	Upper	Slope (± s.e.)
Stock (Con)	5.40	5.20	5.62	0.819 (±0.122)
PS/IBrown/X	4.52	4.25	4.73	0.889 (±0.109)
1/SM10/85	4.18	3.99	4.34	1.234 (±0.018)

Table 3.7a Heterogeneity analysis of dosage-mortality data from three strains of *Plodia interpunctella*

Strain	Chi-square (df)	Probability
Stock	9.532 (5df)	0.089
PS/1 Brown /X	11.879 (6df)	0.064
1/SM10/85	3.952 (6df)	0.083

was significantly higher than the LD50 of the other two strains, indicating a higher level of resistance for the stock population. Slope values of the most susceptible strain (1/SM10/85) were significantly different from both stock ($t = 103.8$, $p < 0.0001$) and PS/1Brown/X ($t = 24.6$, $p < 0.0001$).

The value of chi squared in each of the assays was not significant at 95% probability level indicating absence of systematic heterogeneity of response in test population of larvae, and no significant departure of the observed data from that of the calculated regression line.Precision values, the ratio of the upper fiducial limit to the lower fiducial limit, were calculated for each strain and ranged between 2.31 and 3.0.

3.4 Discussion

Significant differences in LD50s were recorded between the selected and stock control strains in each of the bioassays, indicating a trend towards increased susceptibility in the selected strain. In order to determine whether this is a true indication of the insect's response, or just an artefact arising from the bioassay method used, the data must be critically reviewed and subjected to detailed analysis of the factors likely to affect the selection procedure.To provide such an analysis it would be instructive to use a predetermined framework to ensure adequate coverage of all the factors involved. Georghiou and Taylor (1977) produced such a classification of the parameters influencing the selection process in insecticide resistance,and this has been used as a very general basis for this discussion of the influence of the various parameters involved in the selection for viral resistance in insects.

In the Georghiou and Taylor (1977) model,three basic categories exist:

Fig 3.2 A classification of the genetic and biological factors involved in the evolution of insecticide resistance as applied to the evolution of baculovirus resistance (adapted from Georghiou and Taylor 1977)

A. Genetic

Frequency, number and dominance of R alleles
Past selection by viruses
Extent of integration of R genome with fitness factors

B. Biological

i) Biotic
Generation turnover
Offspring per generation
Monogamy/ polygamy; parthenogenesis

ii) Behavioural
Isolation; mobility; migration
Monophagy/polyphagy
Fortuitous survival; refugia

C. Operational

i) The virus
Persistence
Relationship to earlier used viruses

ii)The application
Selection threshold
Life stage selected
Mode of application

because the model is specifically based on the development of resistance in a field situation. When applied to the bioassay-type selection regime used in this study many of the biological factors for example, become superfluous.

The majority of the factors likely to affect the reliability of the results of the selection procedures would come under the heading of 'operational'. The most important factor in directly comparative bioassays, and hence the comparison of generations in laboratory selection protocols, is the standardization of all possible parameters to minimise heterogeneity. The importance of such standardization has been reviewed in papers by Dulmage and Burgerjon, (1977) and Burges and Thom son (1971). In this experiment, test larvae were selected for uniformity of size, age and genetic background. These larvae were presented with an accurately measured dose of virus on a defined quantity of food. To minimise dependence of virus dose acquired on feeding rate and larval behaviour, complete ingestion of the food pellet, and thus the viral dose, was required before the insect was included in the bioassay results. In addition many factors are known to affect the infectivity of the virus, some of them commonly found in insect diets. Hence dietary composition was regulated, (David, Ellaby and Taylor, 1972; Matsubara and Hayashiya,1969).

Both single dose and bioassay comparisons were made of the two populations. It is interesting that the two sets of data corroborate one another: in 75% of the single dose mortalities (SDM) the control populations were more resistant than the selected, in addition the bioassay results also show that the selected population was more susceptible than the control population in all four generations of directly comparative bioassays.

When discussing resistance levels of a population, bioassays are generally recognized to be of greater value than single dose mortalities, because reliance on bioassays at one dosage level alone can be very misleading. A slope value from probit analyses of mortality data is vital when considering changing resistance levels of a population (Burges,1971). This will be considered in more depth later in the discussion. Therefore greater emphasis will be placed on the bioassay results during the course of this discussion. Detailed appraisal of the other results of probit analysis should yield a reliable evaluation of the apparent increase in susceptibility in the selected population.

An increase in resistance of a population is considered to have occurred if the resistance factor (ratio of LD50s of selected to control populations) is greater than one. In the present study the resistance factors are consistently less than one, indicating possible increased susceptibility. Values of less than one are not very unusual and in many previous studies where despite selection no increase in resistance has occurred, resistance factor values of less than one have been recorded (Ignoffo and Allen, 1972 ; Whitlock,1977). However these are usually accompanied by an equal number of resistance factor values greater than one. Such variations may be loosely attributable to fluctuations in bioassay procedure - it is generally recognized that insect bioassays are very difficult to standardise.

Obviously, then, LD50 values for the selected population were lower than those of the control population. However, changing LD50 value alone is not believed to be a reliable arbiter of increased resistance (Dyte and Blackman,1967) or of increased susceptibility. Reliable interpretation of changing resistance in a population relies equally on the slope of the probit line (Dyte and Blackman,1967). A population for example, may become more resistant (resistance factor increases) by elimination of

susceptible individuals.Such a non-uniform response across the population will mean LD99 values will remain unchanged but the slope of the resistant population will be steeper than that of the susceptible population. Then both lines will tend to the same value for LD99 whilst having significantly different LD50s (Burges,1971).By contrast, true changes in resistance involve a shift in LD99 often associated with a lowering of the slope, itself indicating increased variability within a population (Burges,1971).

Therefore to evaluate whether a true increase in susceptibility has occurred within the selected population, it should be possible to apply Burges's (1971) logic in reverse: thus for a true increase in susceptibility to have occurred, both LD50 and LD1 values should be significantly different in each case, probably accompanied by a lowering of the slope value.In fact none of the generations tested in this study show significant differences at the LD1 values, and the slope values of the selected population have increased, indicating decreased variation. Such a decrease in variation appears to have occurred in two of the selected generations (eight and ten) where the slope values are significantly higher than those of the corresponding control generations. However, its absence in generation nine, may be due to the heterogeneity recorded in the analysis of that generation's selected insects. It appears that some selection for certain types of individuals may have occurred.

Hence it is possible to state tentatively that the LD50 of the selected population has decreased, and the slope has increased. Thus the selected population has become susceptible, and the most resistant individuals of the population appear to have been removed. This is hard to explain, since the selection pressure applied to the population was designed to select for the most virally resistant individuals.

One possible way to explain it is to devalue some of the individual bioassay results.For example in the generation eight control bioassay, the slope is only 0.43, indicating that larger numbers of insects should have been dosed (approximately 200 per dose) in order to obtain accuracy comparable to a bioassay where the slope value was higher (Dulmage and Burgerjon,1977). In discussions of bioassay accuracy, slope values of two are suggested as the ideal theoretical level of precision. Unfortunately this is often not attained in practice and in fact most slope values quoted in published work are less than two. Burges (1971) found that for insect pathogens not producing known toxins, slope values lie between 0.8 and 2.1.Lower slope values indicate a higher level of variation amongst the individuals; levels of heterogeneity however were generally very low as reflected by the overwhelming number of non-significant chi-squares in the probit analyses. In fact only one generation of selected insects recorded a significant chi-square (selected generation 9).

In summary, whilst levels of accuracy in this experiment are not as high as they might ideally be, they lie well within the range regularly attained by other workers. It seems therefore that there may be a trend towards increased susceptibility. However it is important to remember that there are no actual significant differences between the two populations at either LD50 or LD1 level.

It may be possible to explain this by considering the other factors in the Georghiou and Taylor (1977) classification. In general, factors in the second, 'biological' category, are largely inappropriate to a laboratory experiment. For example, behavioural factors found to actually retard the evolution of resistance - such as immigration, refugia and mobility - when modelled by Georghiou and Taylor (1977) were inapplicable to this type of laboratory selection experiment since larvae are individually confined. Similarly biotic factors contributing to population size are

irrelevant since standard numbers of insects are dosed on each occasion.

The factors most likely to have bearing on this experiment are 'genetic'.In at least four species of insects, resistance to baculoviruses has been shown to have a genetic basis. These changes in susceptibility to virus may be controlled by complex genetic mechanisms, as in *Epiphyas postvittana* and *Spodoptera frugiperda* (Briese *et al*, 1980; Reichelderfer and Benton 1974;), or in some cases, by single autosomal genes, as in the case of *Phthorimaea operculella* (Briese1982). Virus resistance in *Plodia interpunctella* is reported to be controlled by a single, partially dominant autosomal gene (Vigneswaren,1987).

Where single genes have been identified they appear to control large shifts in susceptibility to virus. However, when large samples were examined LD50 values of individual populations have been found to be widely dispersed rather than grouped at far extremes (Aratake,1973; Briese and Mende,1981). This cannot be explained by a single gene mechanism. Similarly in other non-baculovirus infections, where resistance is controlled by a single gene mechanism, a marked F1 heterosis in resistance to viral infection has been observed in hybrid populations of *B.mori* to both CPV(Aruga and Watanabe,1961) and NPV(Aratake,1973). Both of these cases find a parallel in insecticide resistance studies which suggests that the genetic background to major resistance genes also affects the phenotypic expression of resistance to a virus (McKenzie *et al*,1980). Therefore the resulting variation in response to virus is due largely to some modifying effect that background genes may have on the major genes responsible for resistance, or to the way in which they may act through some other type of defence mechanism (Briese,1986).

The usual effect of selection is to act upon the frequency of the major resistance

genes, resulting in a relatively rapid change in resistance level of the selected population.This has been considered in the above introduction and further illustration of this point may be seen in the work of Ignoffo and Garcia (1979) and their experimental manipulation of a mixed population of *Heliothis zea* and *Heliothis virescens*. *H.virescens* is five times more tolerant of *Autographa* NPV than *H.zea*;; after only three consecutive generations of exposure to the virus the ratio of a mixed population of the two species changed from 1:1 to 366:1.

Selection may also act on the arrangement of background genes, in one of two ways: in isolation, or, in addition to affecting the frequency of major resistance genes.In the first case, selection will act on an arrangement of background genes in isolation if the resistance gene is absent from the population, or if for some reason resistance levels have plateaued out, leaving evolution of the background genes the only possible avenue open to the insects under selection pressure

In the present study there is a strong suggestion that resistance levels may have plateaued out. The original stock population used for the selection experiment was found, by retrospective comparison to exhibit a higher resistance level than two comparable populations of *P.interpunctella* which were known to show a stable difference in resistance levels (F.R. Hunter *pers comm*).Therefore it may be expected that if the resistance levels had plateaued out that selection would act on the arrrangement of background genes- since the frequency of the major resistance genes is probably high and perhaps at a maximum. One way in which selection could act would be to increase the size and/or the development rate of the insects, since increased size is known to be directly related to increased viral resistance (Huger,1963; Stairs, 1965; Sheppard and Stairs,1977; Payne *et al*, 1981). This did in fact happen , the developmental rate of the selected insects increased, so that on any day that the

selected insects were weighed, they were on average 1.49 times larger than the control insects. This size increase was found to be due to accelerated development. However, resistance levels in the selected population did not show the expected concomitant increase.

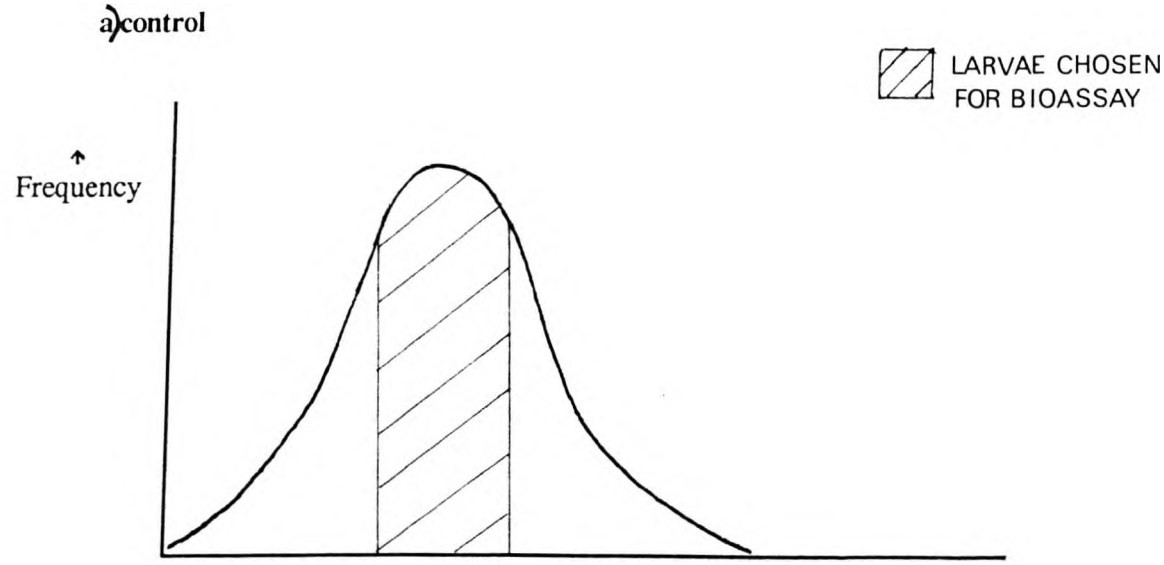
Exactly why this should have happened is unclear.However one proposal which may go some way to explaining this apparent anomaly will be outlined below.

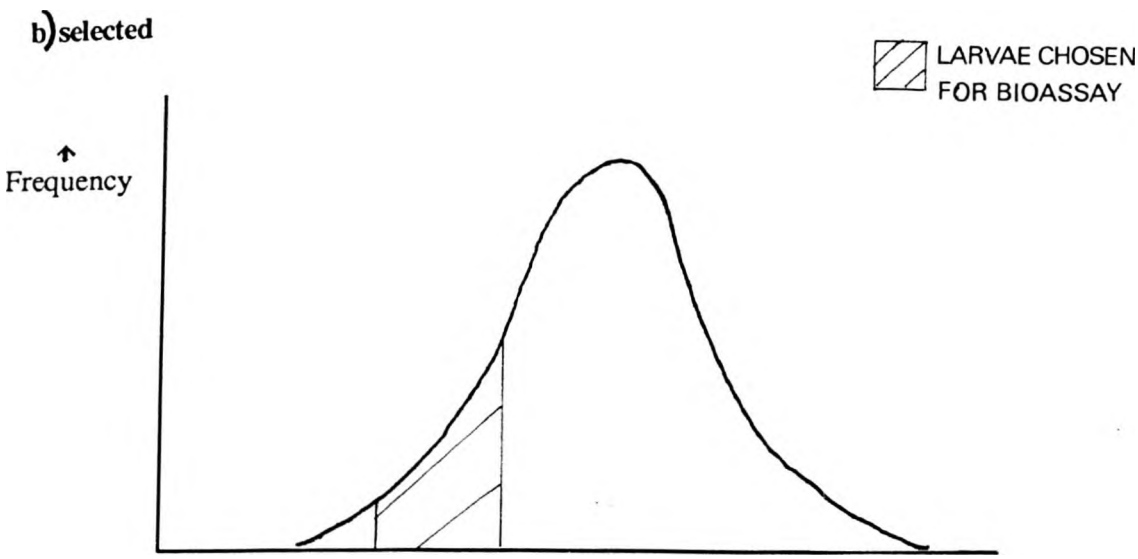
All insects, both stock and selected, used in these comparative bioassays were 11 days old. However, in order to collect sufficient numbers of larvae the adults were allowed to lay eggs for 24 hour periods.Therefore, any larvae produced by these layings would exhibit a range of sizes dependent on the exact time at which they were laid.Such a range of sizes would probably follow an approximately normal distribution,with insects both smaller and larger than the mean at either ends of the curve. In the course of a normal bioassay the insects chosen would be those centred around the mean, since previous study and knowledge of developmental rate would already have established the point in time that would provide insects of the desired size. However, by eleven days after laying the mean size of the selected insects was increased due to the increased developmental rate. Thus, in effect, the normal distribution curve will have been moved to the right.

This can be more easily demonstrated in Fig 3.3, which shows the size classes of the insects along the horizontal axis. Fig 3.3(a)shows the typical distribution of control larvae eleven days post laying, and Fig 3.3(b) shows the revised normal distribution likely to be exhibited by the selected larvae at the same point in time.

Larvae for the bioassays were chosen from within the appropriate instar by direct

Fig.3.3 Diagram to illustrate possible distribution of the range of larval weights for control and selected populations in larvae of eleven days of age





size comparison. Therefore, in choosing selected insects of the same size as control insects, it is possible that selected insects were always inadvertently chosen from the lower end of the distribution, as indicated on Fig 3.3(b). The insects from this part of the curve represent the smaller members of the selected population. They may be smaller for a number of reasons : either they may simply be younger, or they may represent the naturally less fit members of the population.If the latter is the case, then this may go some way to explaining the unexpectedly low resistance levels of the selected population, with the less fit members of the selected population showing lower resistance than fitter members of comparable size in the control population. Another possibility, is that any individuals from the left of the curve not necessarily less fit members of the selected population may be closer to moulting than their control counterparts and this may adversely affect their susceptibility.

It may be possible to go someway towards compensating for this apparent discrepancy and to obtain an empirical estimate of the LD50 for the 'mean' insects in the selected population thus allowing a revised comparison of the resistance factors.This can be done using data for weight-resistance ratios of larvae on subsequent days.Work carried out in this laboratory comparing mean weights and LD50s of larvae on two succeeding days, between mid and late second instar, indicate that larvae on the second of the two days are 1.95 times larger than those on the first day.This size increase corresponds to a 1.74 times increase in resistance (LD50) (K.B. Haji Daud *pers comm*).

Therefore, since in this experiment the mean size increase in the selected insects compared to the control insects is 1.53, the LD50 of 'average' as opposed to 'size-matched' selected individuals should be 1.53/1.95 x 1.74 =1.36 times bigger (1/1.36=0.74). This means that only RF values over 0.74 will be altered, effectively

then it alters only the generation 5 resistance factor making this just greater than unity. Such an attempt to correct for the increased weight of the selected insects has not resolved the problem of the decreased resistance of the selected insects which could be due to one of two considerations: that this calculation using weight resistance ratio is based on too limited data, or that the selected insects in the lower part of the distribution may be not only lighter and smaller than those found at the mean, but they may be significantly less fit, such that if they were 1.5 times larger they would still be less resistant than the control population.

To accurately determine whether the theory put forward here can provide an adequate explanation for the inequality of LD50s, it would be necessary to weigh and establish a number of size classes within the 24 hour egg laying period in both selected and control larvae. Each of the different size classes would then have to be bioassayed, and comparison of the mean (and therefore probably modal) classes of each population would then provide a more accurate assessment of whether any increase in resistance had occurred due to selection. Since this comparison would be based on the mean size class occurring during each 24 hour laying period, it would therefore provide a comparison of insects of equivalent 'intrinsic' fitness.Hence, resistance would be the only parameter under test. Unfortunately such an experiment is not now possible due to the loss of the selected population.

In summary, selection does seem to have occurred acting mainly on the background genes affecting size and development rather than the major resistance genes, since these appear to be already at high frequency within the population. It is uncertain whether the effects of this selection have been adequately monitored, due to the comparison criterion originally selected in this experiment: a single size at a single age. It is therefore difficult to determine whether the increase in size produced by

selection actually increased the resistance level appreciably in the selected population. Certainly, if as might be expected, there was such a size effect, then this would demonstrate that selection may act either in a relatively rapid way on major resistance genes, or in a more subtle way on the background genes producing less extreme changes in resistance.

CHAPTER 4

AN ASPECT OF LIFE HISTORY STRATEGY AS A POSSIBLE ALTERNATIVE ROUTE FOR INSECT EVOLUTION

4.1 Introduction

This chapter concentrates on differences between individuals in their response to virus, and how this may affect a population's response as a whole. The individual insects in question are those produced as a result of a relationship between maternal age and the size of offspring produced: larger offspring produced by older females. This relationship was apparently observed but not quantified in an earlier experiment. This chapter aims to investigate and quantify this relationship.

The importance of such a relationship lies in the competitive advantage such larger offspring would have over their counterparts. The advantages would be both 'ecological', in terms of fertility, ability to breed first and so on, and also in their response to virus, since an inverse relationship between larval age and/or weight and viral resistance has been well documented in many lepidopterous pest species (Huger,1963; Stairs,1965; Ignoffo,1966; Burges and Thom son,1971; Boucias and Nordin,1977; Whitlock,1977; Evans,1981). This relationship has also been demonstrated for a resistant strain of *Plodia interpunctella* (Vigneswaren,1987).
Such enhanced viral resistance may in fact only be a beneficial side effect of the particular ecological life history strategy adopted by the insect, or may be the prime evolutionary force for adopting this particular type of life history strategy.

Theoretical studies of optimal life history strategies lead to the expectation that reproductive effort (and thus reproductive output) should increase with age (Williams,1966; Gadgil and Bossert 1970; Charlesworth,1980), since with increasing maternal age, the cost of reproduction decreases because the female has no need to conserve energy for future reproduction (because of declining lifespan). However, in a number of published studies results have indicated the opposite may be true: there has been a decline in reproductive output with age. This has manifested itself by a

decline in egg size with maternal age in butterflies that lay eggs singly, *Pararge aegeria* and *Lasiommata megera* (Wiklund and Persson,1983; Wiklund and Karlsson,1984). Decline in clutch size through life has also been exhibited by the butterflies *Pieris brassicae* (David and Gardiner,1962) and *Danaus plexippus* (Zalucki,1981) with adults depositing decreasing amounts of eggs over adult life. Both egg size and clutch size have been found to decline with maternal age in the butterflies *Pieris rapae* (Gossard and Jones,1979; Jones *et al* 1982) and *Euphydryas editha* (Murphy *et al* 1983) and in the moths *Choristoneura fumiferana* (Harvey,1977) and *Tyria jacobea* (Richards and Myers 1980)

Begon and Parker (1986) produced a model explanation for the decline in reproductive output with age in terms of an adaptive maternal strategy. If a system exists in which neither egg size or clutch size is constrained, the expectation is that both egg and clutch size will decline with time as females reserves are depleted.

This chapter aims to investigate and establish whether a relationship between maternal age and larval size exists, by performing simple population age /size structure studies on both a stock population, previously unexposed to virus, and a virally selected population (see Chapter 3). In addition, an attempt will be made to assess the effect larger offspring will have on a population in response to virus.

4.2 Methods

Three types of experiment were carried out in an attempt to establish whether maternal age increases larval size. Initially, a single age comparison was performed in conditions similar to those under which the relationship was first observed. Further experiments were set up to determine whether the relationship was consistent at a

range of larval ages, whether it was dependent on the parental density, and whether there was an effect on the females of altered male availabilty.

4.2.1 Single age comparison

Four tubs each containing 25 males and 25 females (virgin insects, 24h after eclosion) were placed under experimental laying conditions (see Chapter 2 for details). Eggs were collected every 24 hours until the numbers of no surviving adults remained. This was determined by daily examination of the tubs for dead insects.

The resulting larvae from these laying trays were examined 12 days after laying (when early third instars). Initially, the total number of larvae sorted from each tray was recorded. However, this proved too costly in terms of time, so thereafter a sample consisting of the first fifty larvae sorted from the food was used.Each of these larvae was weighed individually using the Cahn electrobalance (see 2.2) and placed in a numbered Eppendorf tube. Its head capsule size was then measured using a binocular microscope and eye-piece graticule, in order to determine its developmental stage.

Therefore both mean weight and instar stage were determined for larvae produced on each succeeding day of the adult females' life. In addition, the total number of larvae produced per day was determined for one of the trays to give an indication of reproductive output.This experiment was originally carried out using only stock insects,previously unexposed to any virus. To determine any possible influence of virus on this response, the experiment was repeated twice with the virally selected population, after it had been selected for two and three generations respectively.

4.2.2 Single pair experiment

This experiment was designed to see whether the relationship recorded from 4.2.1 was also observable in single pair matings of *Plodia* , and if so with what frequency it occurred , and possibly whether the effect was entirely due to increasing maternal age.

Final instar larvae were sexed, using the presence of visible testes in the male, and placed in separate cages to develop through to adulthood.Twenty four hours after emergence, single pairs of newly emerged adults were placed in small bioassay tubs containing a minimal amount of food. After each 24 hour period, the insects were checked for deaths, and transferred to replica bioassay tubs. The vacated tubs containing food and eggs were reincubated at 28°C.

Twelve days after laying, the tubs were sorted for larvae. The number of larvae per tub, individual weights of larvae and developmental status were determined as before.Due to a low percentage of successful matings, the experiment was repeated a month later in an attempt to obtain a more complete data set.

4.2.3 Multiple age comparison.

The object of this experiment was to see how the maternal age effect varied with larval age.The experimental set up was basically the same as that in 4.2.1. Two populations of insects were used: a stock population , previously unexposed to virus, and a population of insects virally selected for ten generations. Larvae were examined on 4,6,8, and 20 days after laying. As before, mean weight and individual head capsule size were determined for each succeeding day of increasing age of the female.

4.2.4 Female longevity

Three other experiments were carried out to determine the effect of male presence on female longevity and fecundity. These involved: (i) pairing a single female with a single male for the extent of her life; (ii) placing a new male with a female every day of the female's life; and (iii) placing a new male with a female every two days for the duration of the female's life. Data for these experiments are far from complete, and they should be viewed as preliminary studies rather than a major component of the work. The intention was to monitor larval size and development while relating mating frequency to female longevity.

4.3 Results

4.3.1 Single age comparison

These results indicate that the lifespan of an adult female *Plodia interpunctella* is generally between four to six days. Larvae produced in this period show a slight increase in weight from day 1 to day 4, then a sharp increase in days 5 and 6 (see Table 4.1 and Fig 4.1). This size increase is highly significant in all three populations (Stock: $F=30.34$, $P<0.01$; Selected (3 generations): $F=10.13$, $P<0.02$; Selected (2 generations): $F=135.1$, $P<0.01$).

The increase in weight was almost exactly paralleled by the first appearance of later instars (Table 4.3). Thus, the recorded weight increase was due not to larger insects *per se*, but to an accelerated rate of development in the larvae.

Reproductive output decreased with maternal age (see Fig.4.2). This graph was produced using actual counts of larvae only, and omitting the standard samples of

Fig 4.1 Mean weight of 12 day old larvae produced by progressively older females

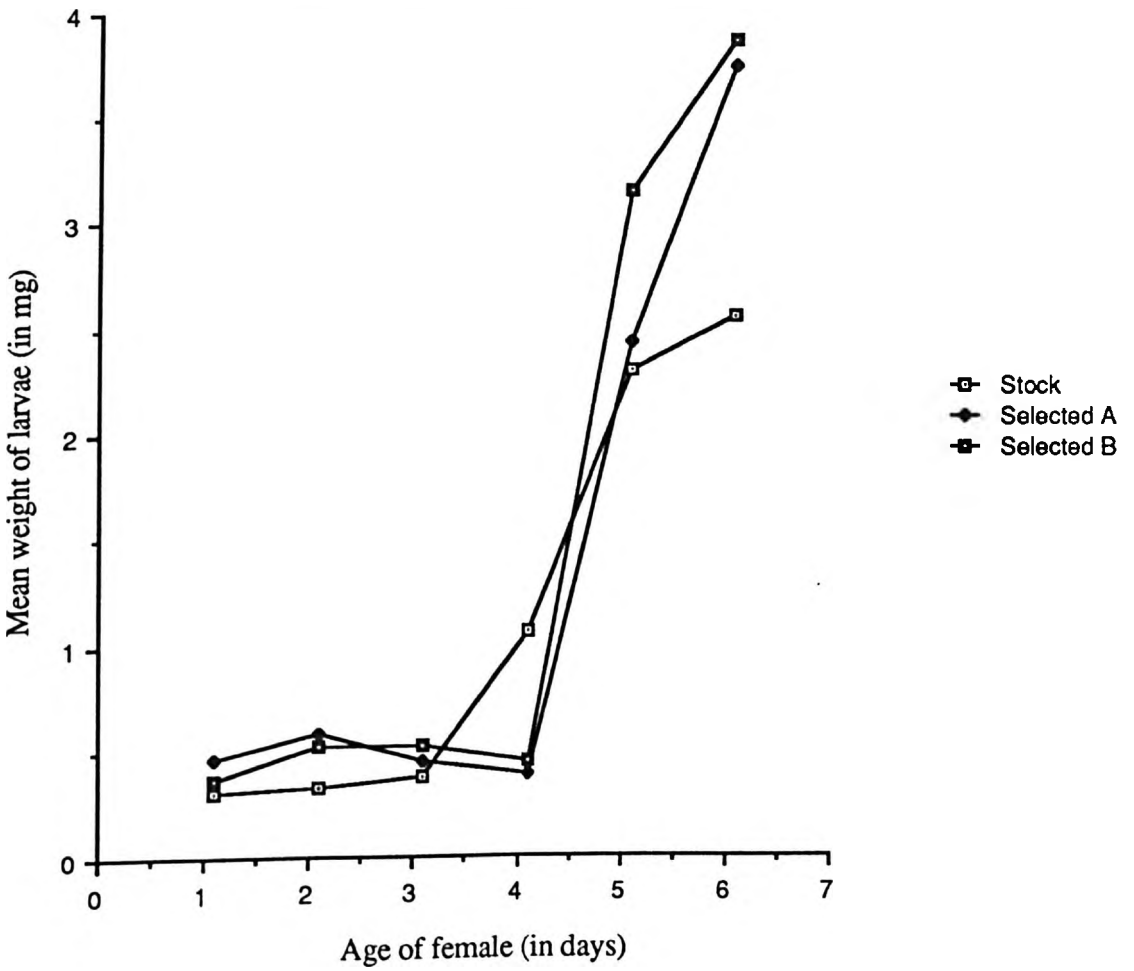


Fig 4.2 Number of larvae produced by a female of increasing age

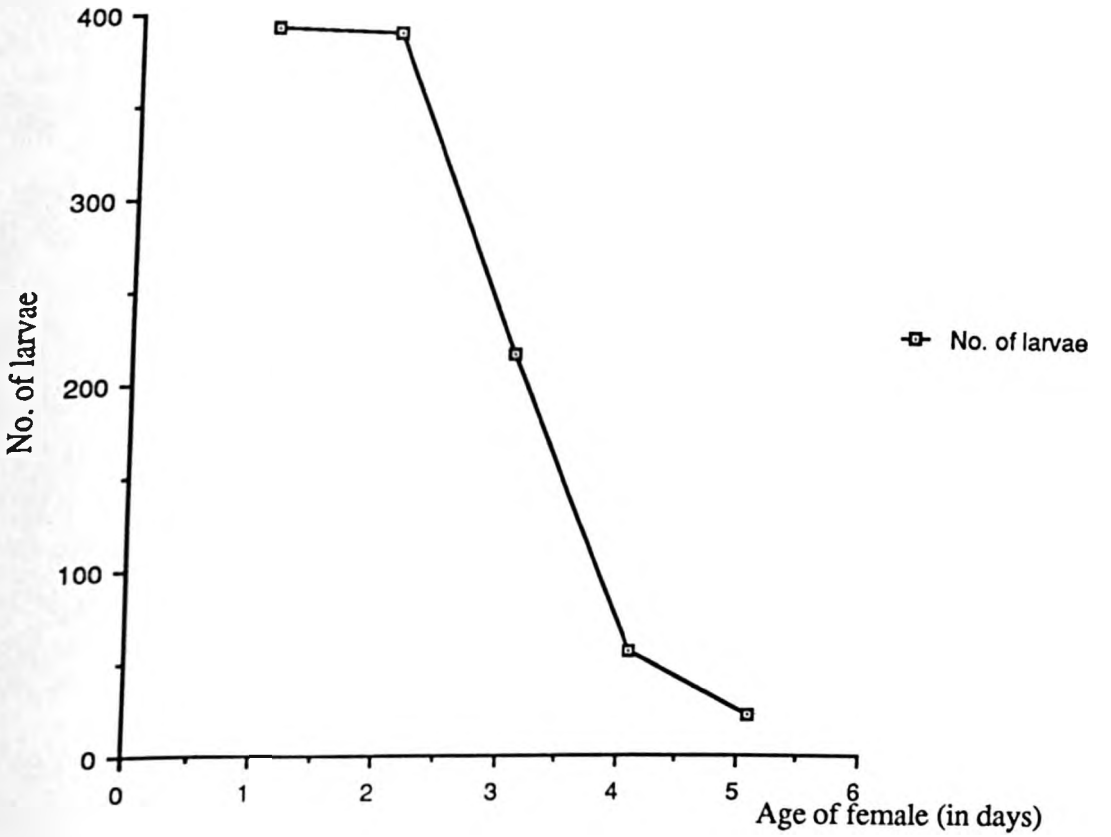


Table 4.1 Mean weight of 13 day old larvae on succeeding days of increasing maternal age (± S.E.)

Population type	Age of female (in days)					
	1	2	3	4	5	6
T_1	0.260 (±0.012)	0.272 (±0.014)	0.363 (±0.013)	1.300 (±0.104)	1.810 (±0.158)	-
T_2	0.261 (±0.010)	0.301 (±0.010)	0.360 (±0.014)	0.760 (±0.109)	2.760 (±0.191)	-
T_3	0.260 (±0.019)	0.272 (±0.008)	0.281 (±0.133)	1.000 (±0.530))	2.190 (±0.104)	2.510 (±0.195)
A_1	0.319 (±0.007)	0.451 (±0.018)	0.424 (±0.014)	0.323 (±0.05)	2.829 (±0.249)	3.570 (±0.359)
A_2	0.519 (±0.020)	0.695 (±0.014)	0.420 (±0.015)	0.368 (±0.011)	0.360 (±0.029)	3.675 (±0.173
A_3	0.388 (±0.017)	0.454 (±0.012)	0.371 (±0.012)	0.339 (±0.017)	3.972 (±0.192)	3.851 (±0.425)
B_1	0.270 (±0.008)	0.441 (±0.013)	0.501 (±0.017)	0.346 (±0.015)	2.867 (±0.320)	3.498 (±0.158)
B_2	0.343 (±0.009)	0.453 (±0.013)	0.410 (±0.017)	0.421 (±0.036)	3.373 (±0.168)	3.500 (±0.592)
B_3	0.343 (±0.018)	0.541 (±0.017)	0.513 (±0.020)	0.439 (±0.025)	3.066 (±0.360)	4.450 (±0.389)

N.B.

All larval weights in milligrams (±S.E.)
'T' denotes stock population: previously unexposed to virus
'A' denotes population selected for resistance to virus for three generations
'B'denotes population selected for resistance to virus for two generations

Table 4.2 Sample sizes of larvae used to calculate mean weights shown in Table 4.1.

Population Type	Age of females (in days)					
	1	2	3	4	5	6
T_1	805	50	50	5	59	-
T_2	388	385	211	51	17	-
T_3	50	126	50	2	6	39
A_1	50	50	50	2	12	8
A_2	25	50	50	50	3	3
A_3	50	50	50	3	5	4
B_1	50	50	50	27	9	25
B_2	50	50	50	16	14	7
B_3	50	50	50	4	13	4

Table 4.3 Proportion of different instars found in weighed samples

Population Type	Age of females (in days)					
	1	2	3	4	5	6
T1	3/2 (9)	3/2 (21)	3/2 (1)	3/4 (2)	4/3 (1)	-
T2	3/2 (5)	3/2 (11)	3/2 (1)	3/4/2*	4	-
T3	3/2 (2)	2/3 (15)	3/2 (9)	3/4 (1)	4	4
A1	3	3	3	3	4	4
A2	3	3	3	3	3	4
A3	3	3	3	3	4	4
B1	3	3	3	3	4	4
B2	3	3	3	3	4	4
B3	3	3	3	3	4	4

* Proportion of instars as follows: 38 x 3rds, 11x4ths, 1x2nd.

NB Where mixture of instars occurs, dominant instar is quoted first, secondary instar quoted second, with actual number of secondary instars per sample weighed in parentheses.

50. These data were only available for the stock population, but a similar trend, of decreasing numbers of larvae with maternal age, was observed in both virally selected populations.

The effect of viral selection on a population was apparently to delay the appearance of fourth instar larvae. Thus, fourth instar larvae appeared on day 4 in the stock population, but not until day 5 in the two virally selected populations. A comparison of larvae produced on day 2, shows that virally selected larvae are significantly heavier than stock (non exposed) larvae ($t=8.76$ $P<0.001$). Day 2 larvae were chosen for the basis of this comparison because they showed the greatest degree of standardization.

4.3.2 Single pairs experiment

This experiment was intended to demonstrate whether the effect observed in 4.3.1 was also observable in offspring from single pair matings. However, despite repeating the experiment, it was not possible to obtain a data set comparable to the one in 4.3.1. This may have been due in part to the low incidence of successful matings: 43.3% in the first instance, and 73.9% in the second. It appears from the results in Table4.1 that the age related factor responsible for the production of larger larvae becomes operational at about day 4 of the adult female life. Of all the successful matings recorded, only two produced a complete four day succession of egg laying (Tubs 7 and 14). Larvae in the former example (Tub 7) showed no rapid increase in larval size on day 4. However, neither did the the number of offspring produced on successive days show the decrease typical of the group experiment (see 4.2.1).In fact, reproductive output increased over the four day period.

Examining the other example where eggs were laid for four consecutive days (tub

Table 4.4 Mean weights of 12 day old larvae produced by a single progressively older female

Single pair number	Age of female (in days)					
	0	1	2	3	4	5
1	-	0.220	-	-	-	-
2	-	0.206	0.252	+	+	+
7	-	0.229	0.109*	0.116	0.196	-
8	0.223	0.172	0.252	-	+	+
9	-	0.078*	-	-	-	+
11	0.253	-	+	+	+	+
12	0.142	0.179	-	-	-	-
14	-	-	-	-	0.068	0.077
17	0.261	0.154	0.188	-	-	+
21	0.217	-	-	-	+	+
24	0.157	0.158	-	-	+	+
25	-	0.112	0.104	-	-	-
29	0.187	0.086	0.078	-	-	-

* one insect only
+ insect dead

Table 4.5 Sample sizes of larvae used to calculate mean values quoted in Table 4.7 (with instar size in parentheses).

Single pair number	Age of female (in days)					
	0	1	2	3	4	5
1	-	40 (2)	-	-	-	-
2	-	25 (2)	19 (2)	-	-	-
7	-	5 (2)	1 (2)	16 (2)	35 (2)	-
8	-	50 (2)	16 (2)	1 (2)	-	-
9	-	1 (4)	-	-	-	-
11	90 (2/3)	-	-	-	-	-
12	-	5 (2)	60 (2)	-	-	-
14	-	-	-	-	9 (2)	9 (2)
17	20 (2)	40 (2)	2 (2)	-	-	-
21	50 (2)	-	-	-	-	-
24	50 (2)	8 (2)	-	-	-	-
25	-	50 (2)	11 (2)	-	-	-
29	40 (2)	45 (2)	7 (2)	-	-	-

Table 4.6 Mean weight of 12 day larvae obtained from a progressively older single female (Repeat experiment)

Single pair number	Age of female (in days)					
	0	1	2	3	4	5
2	-	-	0.135	-	-	-
4	-	0.160	-	-	-	-
6	-	0.110	0.160	-	-	-
7	-	0.156	-	-	-	-
8	0.132	0.086	0.146	-	-	-
9	0.178	-	-	-	-	-
10	-	0.123	-	-	-	-
11	0.180	0.103	-	-	-	-
13	0.174	0.167	-	-	-	-
14	0.115	0.172	0.118	-	-	-
15	-	-	0.117	-	-	-
16	0.187	-	-	-	-	-
17	0.087	-	0.145	-	-	-
18	-	0.161	-	-	-	-
20	0.136	0.197	-	-	-	-
21	0.169	-	-	-	-	-
23	0.180	-	-	-	-	-

Table 4. 7. Sample sizes of larvae used to calculate mean weights in Table 4.9

Single pair number	Age of female (in days)					
	0	1	2	3	4	5
2	-	-	30	-	-	-
4	-	20	-	-	-	-
6	-	30	20	-	-	-
7	-	1	-	-	-	-
8	21	20	5	-	-	-
9	24	-	-	-	-	-
10	-	21	-	-	-	-
11	21	15	-	-	-	-
13	50	26	-	-	-	-
14	4	36	4	-	-	-
15	-	-	3	-	-	-
16	15	-	-	-	-	-
17	40	-	1	-	-	-
18	-	53	-	-	-	-
20	24	25	-	-	-	-
21	30	3	-	-	-	-
23	30	-	3	-	-	-

14), showed that they also did not exhibit the unusual size increase or increased rate of development. This would seem to indicate that the age related factor is not dependent solely on the age of the female, but also on the number of eggs produced on previous days.

The mean weight and mean numbers of offspring generally decrease over time with the exception of day 4 larvae which disrupt the general trend. Mean mating success was quite low, 56.6%, but where mating success was higher, egg laying started earlier and extended over a much shorter time period.

Where mating success was lower a higher percentage of pairs laid eggs on every day with the exception of Day 0. All larvae weighed and examined in both these experiments were second instars.There was no evidence for increased rate of development.

4.3.3 Daily experiment

This experiment was designed to see whether the pattern observed on Day12 in 4.3.1 was consistently observed on a range of days post hatch. The results obtained were less clear cut than those obtained in 3.4.1. Generally an increase in mean weight of all ages of larvae was observed with increasing female age. This was highly significant in the case of the selected population ($F= 21.82$ $p<0.001$) , and significant too in the case of the unexposed stock population ($F=3.83$, $0.025<p<0.05$). In order to calculate the ANOVAs, weights from day 2 to day 5 were used for selected, and day 2 to day 6 for the stock population because these provided the most complete data sets (see Table 4.5).

Table 4.8 Weights of selected* larvae various days post hatch produced by progressively older females (±S.E.)

No. of days post hatch	Age of female (in days)					
	1	2	3	4	5	6
4	-	0.016 (±0.0008)	0.016 (0.0005)	-	0.049 (±0.014)	-
6	-	0.041 (±0.003)	0.069 (±0.007)	0.071 (±0.047)	1.130 (±0.100)	-
8	0.470 (±0.190)	0.116 (±0.0004)	0.134 (±0.009))	0.153 (±0.038)	2.200 (±0.031)	-
20	1.922 (±0.105)	2.942 (±0.123)	3.721 (±0.370)	2.870 (±0.420))	4.370 (±0.740)	-

* Selected for ten generations with virus
All weights in mg

Table 4.9 Weights of stock larvae various days post hatch produced by progressively older females (±S.E.)

No. of days post hatch	Age of female (in days)					
	1	2	3	4	5	6
4	-	0.011 (±0.0002)	0.014 (±0.0002)	0.014 (±0.003)	0.012 (±0.0003)	0.054 (±0.014)
6	0.062 (±0.0008)	0.031 (±0.006)	0.065 (±0.0007)	0.043 (±0.0021)	0.165 (±0.060)	0.268 (±0.097)
8	0.071 (±0.001)	0.065 (±0.009)	0.131 (±0.0018)	0.263 (±0.0053)	0.392 (±0.0310)	1.235 (±0.06)
20	-	1.960 (±0.068)	1.120 (±0.153)	2.200 (±0.156)	3.110 (±0.480)	11.79 (±0.99)

All weights in mg

Table 4.10Comparison of mean larval weights from stock and selected populations various days post hatch produced by progressively older females

Population type	No. of days post hatch			
	4	6	8	20
Stock	0.011	0.031	0.065	1.960
Selected (n=10)	0.016	0.041	0.116	2.942
Ratio (Sel:Stock)	1.45	1.32	1.78	1.50

The previously observed sharp increase in weight at one particular age of the female was less pronounced in the case of the stock insects, as reflected in the significance tests above. However, the weight difference in day 13 larvae recorded between the two populations in 4.3.1 also remained fairly constant in this experiment. This comparison was made between larvae laid on day 2 of the female's life as in 4.3.1 in order to provide a consistency of approach. The ratio of the two populations varied from 1.3 to 1.7. In each case the selected insects were significantly larger ($p<0.001$)(see Table 4.6).

In the data for the selected insects one large accelerated larva was recorded on day one of the female's life, suggesting that their appearance may not be related to maternal age, however no other accelerated larvae were observed elsewhere at this stage in the female's life.

4.3.4 Female effect.

It must be stressed that results from this study are preliminary and can show only trends at most. From Table 4.7 it appears that the highest success rate in laying came when the female was presented with a new male every day: 91.7% of pairs successfully mated. The remaining two treatments, a new male every two days and only one constant male partner had approximately equal mating successes 43.3% and 50% respectively.

Female longevity obviously has great bearing on the maternal age effect observed in these studies, but results in Table 4.11 indicate that most females only lay eggs over a two day period. This correlates well with results obtained in the single pairs experiment, where only one female laid eggs over a four day period. In this study only

Table 4.11 The effect of various combinations of males on female fecundity

Experiment type	No. of days eggs laid for					Total pairs laying
	0	1	2	3	4	
One male*	17	4	6	2	1	13/30
New male/day**	1	5	4	2	-	11/12
New male/2days	5	2	3	-	-	5/10
Total	23	11	13	4	1	

Table 4.12 Effect of various combinations of males on longevity of females used in Table 4.11

Experiment Type	Length of time females survive (in days)					
	1	2	3	4	5	6
One male*	1	1	1	2	5	3
New male/day**	-	-	2	6	5	-
New male/2 days	-	1	2	4	2	1
Total	1	2	5	12	12	4

* seven pairs remained mated continuously through experiment
** one pair remained mated continuously through experiment

one pair in fifty three (1.8%) laid for a four day period.

Several pairs undergo abortive matings with the male dying whilst still in copula, and the female being unable to detach the dead male, thus preventing any further attempts at mating or egg laying.

4.4 Discussion

Two alternative theories have been proposed regarding reproductive effort in the latter stages of maternal life: reproductive effort (and thus reproductive output) may increase with age (Williams,1966; Gadgil and Bossert, 1970; Charlesworth, 1980); or, reproductive effort and therefore reproductive output such as egg and clutch size, may decrease with age as the female's reserves are depleted with age (Begon and Parker,1986).

Most of the recorded evidence in the literature tends to lend more credence to the latter theory. Particularly illuminating is the work done on some species of butterflies and moths where both clutch size and egg size has been shown to decrease (Gossard and Jones,1979; Jones *et al*,1982; Murphy *et al*, 1983; Harvey 1977; Richards and Myers,1980). However there is some evidence for increased reproductive effort with increasing maternal age . In some species of locusts, correlative evidence suggests that hatchling weight increases in successive egg pods (Uvarov,1966)

Increase in offspring weight with maternal age may be due to larger larvae *per se*, or to an accelerated growth rate . An example of the latter has been demonstrated in the yellow mealworm *Tenebrio molitor*, where offspring from older mealworms have been shown to have a significantly shorter life and higher growth rate than those from young parents (Tracey,1958). In verifying this work, Ludwig *et al* (1964) reported that age related factors were not operating until the parent beetles were at least one month of age. The same workers found that the duration of the total larval stage in *T.molitor* was reduced, and the rate of larval growth greater for progeny for older mothers at all temperatures examined 20°C, 25°C and 30°C (Ludwig *et al* , 1964).

Data from the present experiments demonstrate that the larger larvae produced by

older females of *Plodia interpunctella* are due to accelerated growth as in the case of *T.molitor*. Therefore, by extrapolation, these larvae also have shorter lifespans. The age related factor responsible for producing these accelerated larvae becomes operational on day 4 of the female's life, sometimes delayed until day 5 in the case of females from virally selected populations

How then do these accelerated larvae produced at the end of an adult female's life fit in with current theories on optimal life history strategies, and more particularly theories on reproductive effort ? Results from this study indicate that in the case of *Plodia* , whilst clutch size does in fact decrease with increasing maternal age, offspring size increases.

According to theories of optimal life history strategy, when reproductive effort (and thus reproductive output) does increase with age, it is because females no longer have any need to conserve energy for future reproductive efforts (Gadgil and Bossert,1970). This seems likely to be the case in *Plodia* since adult lifespan is short, and therefore reproduction and egg laying is intensive.Viable egg production by individual moths is essentially complete within four days after mating (Lum and Flaherty,1969; Silhacek and Miller,1972).

More generally, since in life history strategy theory the two terms, reproductive effort and output are considered to be interchangeable, the soundest interpretation of the results may be obtained by calculating the total reproductive output of the female each day over the duration of her adult lifespan. The total reproductive output may be obtained by multiplying clutch size by mean larval weight for that day.However, this may only effectively be estimated for one of the samples since in only this one instance (stock insects T2) were the total number of larvae in every tray counted.Therefore this sample provides the only accurate estimate of total reproductive

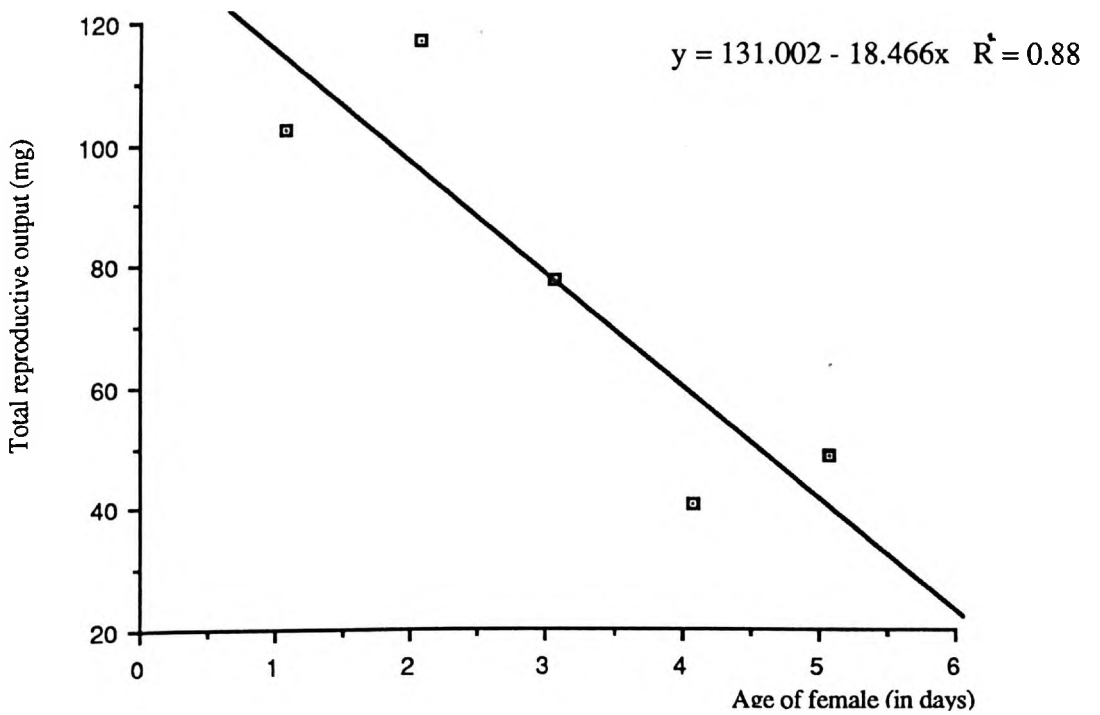
output.Total reproductive output was found to decline with increasing female age using this sample but not significantly ($F=10.219$, $0.05< p< 0.1$).

Thus the overall reproductive effort tended to decrease with age, it is also indisputable that offspring size increased with female age as recorded in *Tenebrio molitor* and some species of locusts (Tracey, 1958; Ludwig and Fiore,1960; Uvarov, 1966).The purpose and mechanisms of this size increase will be discussed in the second part of the discussion.

One possible explanation for the size increase in offspring is that in both of the life history strategies considered above, the environment is considered to be constant. However under field conditions *Plodia interpunctella* lays its eggs into an ever increasingly crowded environment.Therefore such conditions would favour the production of more developed offspring at later stages in the egg laying cycle, because without the accelerated development the larvae would be at a competitive disadvantage when compared to those produced earlier in the laying cycle.Therefore in addition it is possible that accelerated larvae may only be produced in crowded conditions, such as the mass cultures produced in the group experiments in this chapter.Interestingly ,as will be discussed later, accelerated offspring were not observed in the single pair experiments, indicating that this may be a possible explanation.

The age dependent factor appears to begin to exert its main effect on offspring produced on day 4 of the female's life. Nevertheless, before this, larval size begins to show, in the majority of cases, a progressive increase from day 1. This size differential only becomes significant on day 4, with larvae produced then being at least one instar larger than would be normally be expected: fourth instars compared with the early third instars. Therefore it would be expected that larvae produced on and after day 4 of the female's life, and possibly also those produced on day 3, would be

Fig 4.3 Total reproductive output of a female of increasing age



significantly more resistant to virus than larvae produced on any of the previous days.This pattern is demonstrated in all three populations tested, both the stock (control) population and the two virally selected populations. The only difference between the three populations is that the virally selected populations appear to be generally heavier than the stock population.This corresponds to the findings in chapter 3, that the selected populations have changed biotically.

How would the presence of these accelerated larvae in a population affect the evolutionary success of an insect population? Since third instars are reported to be on average 3000 times more resistant than second instars in a resistant population (Vigneswaren,1985).then it would require just a small percentage of accelerated larvae to enhance and possibly ensure the survival of the population in the presence of virus. Looked at in evolutionary terms, it ensures the survival of that female's offspring and maintains their presence in the population gene pool.Therefore it seems likely that the accelerated larvae form part of a strategy which provides enhanced survival of the population both under normal conditions and during viral epidemics.

The actual percentage of accelerated larvae in the group experiment is: 5.8% (n=2294) in the unexposed stock population; 5.9% (n=540) in the population selected over three generations; and 12.7% (n=569) in the population selected for over two generations. The latter two selected populations probably better reflect the idealized version of a single age population since they consist only of early thirds and accelerated fourths. However the unexposed stock population more accurately represents the actual percentage of accelerated larvae since it contains total counts of all larvae on day one rather than just standardized sample sizes of fifty in the total population number.The results also indicate that accelerated larvae are present at every larval age tested. Work done in this laboratory suggests that this pattern is consistent at the earliest stage of the lifecycle, with eggs laid on day 5 of a female's life hatching on

the day that they were laid.This is in direct comparison to eggs laid on the early days of a female's life which can take up to five days to hatch.

Attempts were made to determine whether this trend of larger offspring from older females was observable in single pair matings, and also whether there was any paternal contribution to this trend.Neither of these experiments was entirely successful, mainly due to the low numbers of larvae recorded. Since both experiments involved basically the same experimental design, it would be more constructive to discuss the experiments in tandem rather than separately.

The major problem appeared to be a lack of offspring. This may be attributable to several factors: unsuccessful matings, failure to oviposit, unsuccessful hatchings, and possible competition between larvae. Unsuccessful matings may be due to one of several things: sterility in either insect; inability of a male to mate because of a size differential; inability of the male and female to separate after mating.Sterility in insects is not a predictable factor and unless the insects have been specifically treated to render them sterile is not likely to occur in a major percentage of the population. The idea of *Plodia interpunctella* females being unable to mate with smaller males was put forward by Greenfield (1982). Courting males approach females 'head-on' or from the rear, in which case the female turns 180° to attain the head-on position (Grant and Brady, 1975). Females remain in readiness with their abdomens elevated and ovipositor extruded, the male thrusts his abdomen over his head in a copulatory strike to couple with the female.The pair remain at 180° orientation to one another at all times and this may physically prevent a small male coupling with a larger female (Greenfield, 1982).This type of rejection was recorded in 12% of cases recorded in a study on Plodia, a further 6% of matings were unsuccessful for less apparent reasons, where the males were detached (disengaged) within 15 seconds of coupling.

The third reason for an unsuccessful mating was observed during the course of these experiments where successful coupling of male and female occurred, but the male either died or was unable to separate from the female, and the pair remained linked until death. This occurred in 13% of all cases in the paternal effect experiment.

Reduction of oviposition and subsequently hatchability of eggs is known to be caused by excess carbon dioxide treatment of insects (Lum and Flaherty,1972). Carbon dioxide was only ever used to subdue insects before weighing them in the initial part of this experiment, and all these insects were subsequently decapitated to measure their head capsule.

Inter larval competition is another possibility for the low numbers of larvae recorded, although an excess of food was provided specifically to prevent any competition for that resource. Cannibalism has been recorded amongst *Plodia* larvae (C.Brown, Institute of Virology, Oxford *pers comm*), and therefore may act to decrease the number of larvae in any one pot..Therefore the reasons for the failure of this experiment are still uncertain. Adult insects in this laboratory have been observed to lay numbers of eggs concurrent with the published figures, between 40-100 eggs per day for the first few days, using a similar set-up to the one in this experiment, so possibly the problem is more related to competition.

Therefore although the trend of accelerated offspring produced by older mothers has been demonstrated unequivocally in both stock and virally selected populations, it has not been possible to determine whether there is any significant paternal contribution to the trend.Males are known to transfer proteinaceous materials within the ejaculate to females during copulation, and that the amount of this material entering unfertilized eggs increases with time after mating.This observed transfer to unfertilized eggs suggests that this is a form of paternal investment contributing nutritionally to

egg production (Thornhill,1976; Greenfield, 1982). However, neither fecundity nor the number of deposited eggs are a function of ejaculate weight. Therefore, the ejaculate may not be considered a paternal investment (Greenfield, 1982). It is possible that this ejaculate may play a part in production of larger more accelerated larvae.It is equally possible that the production of these larvae may be entirely dependent on the age of the female, which seems more likely, since this appears to be the case with *T.molitor* (Tracey,1958).However, experiments to confirm the absence of paternal investment would need to be repeated in order to verify this.

Other experiments would be needed to determine whether all females are capable of producing accelerated larvae, or whether such an ability is restricted to a small number of females, possibly constituting a separate genotype, which only lay eggs that produce accelerated larvae.

CHAPTER 5

SELECTION FOR VIRUS EVOLUTION

5.1 Introduction

Unlike the topic covered in Chapter 3 which concerned the possible evolution of increased resistance in insects to virus, the evolution of viruses with respect to their hosts has received scant attention. In general viruses are considered to evolve for one major reason: to increase its chances of transmission, and so enhance its chance of evolutionary success.

The most well-documented case of evolution of a virus resulting in increased transmissability involves the attenuation of the myxoma virus , originally introduced in the1950s in Australia and Great Britain to control rabbit populations. Myxoma virus is endemic in populations of its natural host, the South American rabbit,*Sylvilagus brasiliensias* and the Californian rabbit *Sylvilagus bachmani*, where it causes mild symptoms in a substantial proportion of the population. In the European rabbit, *Oryctolagus cuniculus* myxoma virus causes a rapidly fatal systemic disease (Fenner and Ratcliffe,1965).

In the early 1950s, myxoma virus was used as a control agent for the burgeoning rabbit populations in Australia and Great Britain. On introduction from South America, the disease was rapidly fatal, with rabbits taking less than thirteen days to die. By 1962, however, only four percent of rabbits contracted the most virulent Grade 1 form of the virus in the U.K. The majority (63.6%) of infected rabbits contracted the less virulent Grades IIIa and IIIb virus forms.This caused the survival time for those with the disease to increase to between seventeen and twenty eight days (Fenner,1965).Animals infected with a virus strain of lower grade virulence will survive longer than an animal infected with a strain of higher virulence. This is

important since myxoma virus is vectored from a live infected animal to other animals via haematophagous mosquitoes in Australia, and rabbit fleas in Great Britain. Therefore, the longer an infected animal survives, the greater is the chance of transmission of the virus. So, the evolution of an attenuated strain of myxoma virus seems to have resulted in its increased evolutionary success.

Similarly, increased transmissability has resulted in attenuation in the case of Newcastle disease, a highly virulent and contagious viral disease of poultry (Shope, 1964). Normal control measures to prevent the spread of the disease involve the slaughter of all infected stocks. This practice has led to the development of two new forms of the virus: acute and subacute. These two newer forms differ genetically from the original peracute form, and result in reduced symptoms in infected poultry (Seigmund,1950). This slows the recognition time of the disease, and hence delays the inevitable slaughter of hosts, increasing the time available for virus transmission. Such increased transmissability has given the two newer forms an evolutionary advantage over the peracute form, and lead to their becoming the dominant forms in the infected population.

Barrett (1984) has suggested that these examples demonstrate that evolution towards reduced virulence does occur in parasites, but that the process which brings this about is selection for increased transmissability, not to 'prevent the parasite eliminating the host' (Dawkins,1982).

The object of the work in this chapter is to ascertain whether given favourable conditions, granulosis virus will evolve increased virulence, and hence increased transmissability (since baculoviruses are transmitted via the death of an infected insect). Or whether such an evolutionary course - selection for increased transmissability - is limited to situations where the host and parasite meet for the first

time, as in the examples quoted above. Or indeed whether Dawkins is correct, and the virus will not evolve further virulence, thus preventing extinction of the host.

Any possible evolution has been followed on two levels: phenotypically, in terms of altered virus induced mortality; and genotypically, by examining restriction endonuclease profiles of selected virus generations.

5.2 Methods

5.2.1 Measurement of Phenotypic Change

In each of seven generations, two hundred eleven-day-old larvae were individually dosed with 1µl of varying concentrations of virus. The method used for dosing the insects was the same as that used in earlier chapters. Mortality was recorded after fourteen days, and virus extracted from the infected insects (for method see section 2.5). Virus concentration was measured using the method detailed in section 2.6.Virus collected from the first generation, was used as innoculum for the second serial generation, and so on for subsequent generations.

It was originally intended to standardize the dose of virus given in each generation but since such large numbers of particles were involved, it was thought that any error produced at the counting stage would be further magnified by a possible dilution error. Instead it was decided to use the first serial dose as a baseline for further generations.By comparing all subsequent observed mortalities with an expected mortality for each generation, (calculated using this initial mortality), an accurate representation of whether selection had resulted in any change in virulence of the virus would be attained. In order to calculate the expected mortality for each generation, it

was first essential to establish the wider relationship between dose and mortality for *Plodia interpunctella*..

Only five complete bioassays included the very low dose that was to be used in this experiment, largely because of the problem of decreased viral virulence over succeeding generations (discussed in chapter 3) leading to very low doses becoming ineffective in causing virus induced mortality. The mean slope of these five bioassays was 1.052. This relationship and the baseline mortality were used to convert the concentration of virus from each new generation to an expected mortality. To do this the following equation was used:

$$\text{Probit value}_{\text{new dose}} = \frac{\text{Log (conc new dose)} - \text{Log (conc original dose)}}{1.052} + \text{probit (orig \% mortality}$$

The expected mortalities (see Table 5.1) were then obtained by converting the probit value into a percentage using probit tables. After each generation of selection the observed mortalities were compared with the expected mortalities to determine whether selection had resulted in any change in virulence of the virus.

5.2.2 Measurement of genotypic change

A new generation of virus was produced after each passage. A sample of this was used as innoculum for the next generation, the remainder being retained for genotypic analysis. DNA was extracted from this virus using the method detailed in 2.8.1. The resulting DNA was digested using a range of restriction endonucleases found to give a

large number of bands under test digest conditions as it was hoped that this would provide the best chance of detecting any change in the virus.

Initially, major problems were encountered with DNA denaturing despite the use of proteinase. Once the DNA had apparently denatured, it was impossible to digest it despite further phenol extractions, ether extractions, an increased enzyme concentration, increased digestion time or the presence of spermidine. Unfortunately generation two virus was lost due to denaturation. The problem of DNA deterioration was never encountered using caesium chloride purification of DNA (see 2.8.2). However, due to the nature of the experiment enough virus was not available for this purification process to be used.This procedural problem was eventually overcome by eliminating any delay between the DNA extraction and its subsequent digestion with restriction endonucleases.

Samples were digested overnight at 37°C in the presence of 3mM spermidine to ensure complete digestion. The samples were run on horizontal 0.6% agarose gels for 4 hours at 35V, if a minigel, and for 18 hours at 30V if a larger gel. Bands were visualised using UV light. Since DNA concentration was very low in most cases, it was decided to Southern blot the gel in order to aid visualization of the lower bands. The blots were probed with a whole PiGV virus probe produced by nick translation.

Filters were washed at low stringency, 42°C, 6xSSC ,0.1% SDS which seemed to produce adequate results. The filters were left down for different time periods to obtain the full range of bands.

Sizes of viral fragment bands (in Kilobase pairs) were determined using the migration distances and a standard curve. The standard curve was constructed using the migration distances of Hind III and Hind III/Eco RI fragments of lamda phage

plotted against their known molecular weight. Usually, mean values from three gels are used in size estimation of bands, but practical limitations due to the nature of the experiment meant that there was only enough DNA for one gel. Microdensitometry of gels was attempted but results were found to be unacceptable, due to a lack of definition between the bands and background.

5.2.3 Determination of variation available in original virus innoculum

This was carried out to determine the level of genotypic variation present in the original isolate used in the first passage. The intention was to infect insects with a dose of virus sufficient to cause very low mortality (5%), in order to clone out any variants that exist within the viral genome Crook (1986). This required a limiting dilution of approximately 0.1 capsules per larva, so that it is likely that infected larvae receive only a single infectious virus particle. Later Smith and Crook (1988a) stated that GV capsule preparations from a high proportion of larvae infected with a dose causing 5-10% mortality were genotypically homogeneous, and that one or more constituent genotype of a mixed population may be separated out in this way. It was decided to compromise, since the lowest dose known to infect this strain of *Plodia interpunctella* was 130 virus particles per larva which caused 0.52% mortality, the larvae were infected with 536 particles per larva of the original innoculum which causes an approximately 3% mortality. Three hundred insects were individually dosed, and the virus extracted. This was counted and used at a similar low concentration to dose a further two hundred insects, causing 2.67% mortality. Virus was extracted from individual insects and restriction endonuclease digests attempted to determine whether any variation had been cloned out.

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5.2.4 Determination of variation available generally in *Plodia interpuncella* granulosis virus (PiGV)

The object of this work was to determine the available variation in the viral genome of PiGV. This would enable a comparison to be made between the virus generally and the virus otherwise used in this chapter. In order to do this, letters were written to groups known to be working on *Plodia interpunctella* requesting samples of virus. Unfortunately, many of these groups worked only on the insect and had no virus. However one group sent some virus (P.Vail, Agricultural Research Service, U.S. Department of Agriculture, Fresno, California), and the group that produced the original restriction profiles (Tweeten *et al* 1980) wrote to say that their virus sample was essentially unchanged. Thus a limited comparison of variation available in the virus was possible.

Virus was extracted from infected insects and restriction profiles of viral DNA samples were produced using methods quoted in Chapter 2 and section 5.2.2 of this chapter. These restriction profiles were compared with those obtained in 5.2.2.

5.3 Results

5.3.1 Phenotypic comparison of selected generations of virus

The difference between observed and expected mortalities is shown in Table 5.1 and also further illustrated in Fig. 5.2. These show that whilst observed mortality both exceeds and falls below the expected mortalities, it does so in no regular pattern. The actual variation involved is very small, especially when considered in terms of

Table 5.1 Results of selection pressure on virus over a period of generations showing differences in percentage recorded mortalities

Generation of virus	Log Dose of virus*	%Mortality (expected)	%Mortality (observed)	Difference (%)
0	2.73	-	3.0	-
1	2.42	1.47	7.0	+5.5
2	2.50	1.78	2.0	+0.22
3	2.43	1.48	1.50	+0.02
4	2.77	3.70	0.56	-3.14
•5	2.77	3.70	0.51	-3.19
6	2.11	0.68	0.52	-0.16
7	2.61	2.28	2.30	+0.02

* Log dose of number of virus particles per larva
• Generation 5 repeat of generation 4

Fig 5.1 Difference between observed and expected mortalities over several generations

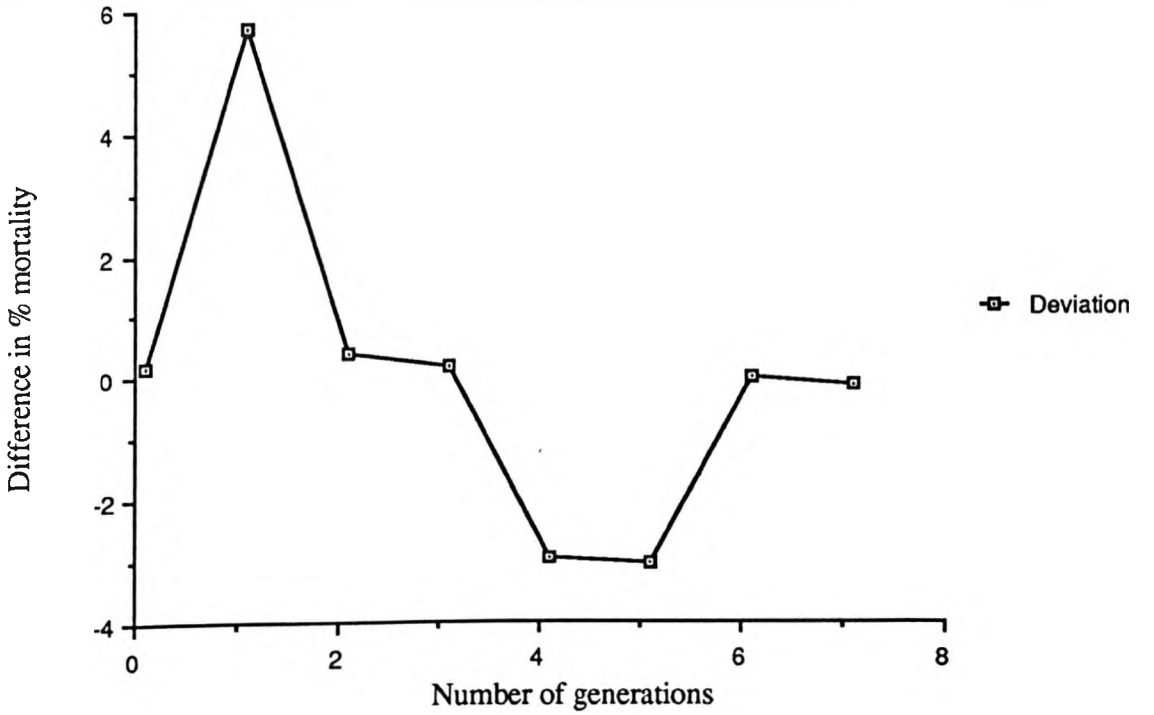


Table 5.2 Results of selection pressure on virus over a period of generations showing difference in whole numbers of larvae

Generation of virus	Log Dose of virus*	Mortality (expected)	Mortality (observed)	Difference
0	2.73	-	3	-
1	2.42	3	12	+9
2	2.50	3	4	+1
3	2.43	3	3	0
4	2.77	7	1	-6
•5	2.77	7	1	-6
6	2.11	1	1	0
7	2.61	4	4	0

* Log dose of number of virus particles per larva

• Generation 5 repeat of generation 4

differences in whole numbers of insects (Table 5.2). If we assume that the serial generations indeed represent replicate estimates of the same underlying mortality rate, then the results represent a higher level of accuracy with the technique compared to the results obtained with the bioassay. This would seem to indicate the enormous effect of storing the virus for any length of time.

Testing the differences statistically between observed and expected mortalities proved to be difficult. In this situation, a chi squared test would normally be used. However, in this case the low number of expecteds meant that chi squared was not appropriate. Analysis by Cochran (1954) concluded that no expected frequency should be less than one, and that no more than 20% of the expecteds should be less than five. This is because if values are very small, the resultant chi squared is biased in that it is larger than the theoretical chi square it is supposed to estimate, resulting in a false rejection of the null hypothesis.

For this reason, it is more instructive to look at the overall trend as illustrated in Fig 5.1. This shows that although there are differences between observed and expected mortalities, where they do occur, it is in equal and opposite directions: there is no general trend.

Therefore, no actual change in virulence seems to have occurred over the generations of selection. Those changes recorded appear to be fluctuations rather than real changes.

5.3.2 Genotypic comparison of selected generations of virus

The genotypic comparison proved more difficult to carry out than expected,

mainly due to the constraints of the selection experiment. The selection regime was deliberately severe in order to prompt possible evolution, but this meant that there were very few virus-induced mortalities. Therefore the concentration of the viral DNA obtained in some cases was very low.

This small amount of DNA available meant that only one complete set of restriction endonuclease digests was possible. As mentioned above a protocol was modified so that it would hopefully guarantee the successful digestion of the viral DNA first time, each time. However,the low concentration of DNA would mean that all the bands in a digest would not be visible, particularly those of low molecular weight. Unfortunately DNA concentrations were so low in some cases that many of the high molecular weight bands were not easily distinguishable. Difficulty in interpretation arose because the amounts of DNA used were so small that the restriction endonuclease digestion, and subsequent gel electrophoresis, were functioning at the limits of their visibility. It was hoped, originally ,that low molecular weight bands could be visualised by the use of Southern blots, but after running the gels, it was realised that Southern blots would be required in some cases to elicit the pattern of some of the higher molecular weight bands as well.

Viral DNA from the generations between the inital and final generation was extracted and digested concurrently to prevent any denaturation of DNA (as mentioned in 5.2.2). However the restriction profiles obtained from the intervening generations of selection were difficult to interpret unequivocally. It was however possible to follow general banding patterns on the autoradiographs (see Appendix VII). Whilst the Southern blot of the gels was a useful back-up technique in confirming the presence of bands seen on the photograph, and those low molecular weight bands not easily visible under UV light, it was difficult to distinguish all but the most separate of bands, in the high molecular weight region of the gel. Therefore complete information

Fig 5.2 Restriction endonuclease digest of the original sample (generation 0) of granulosis virus used in virus selection experiment.

Lanes as follows: 1 and 9 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4HindIII, 5: EcoRI,6:Pst I, 7:Asp 718, 8:Sal I.

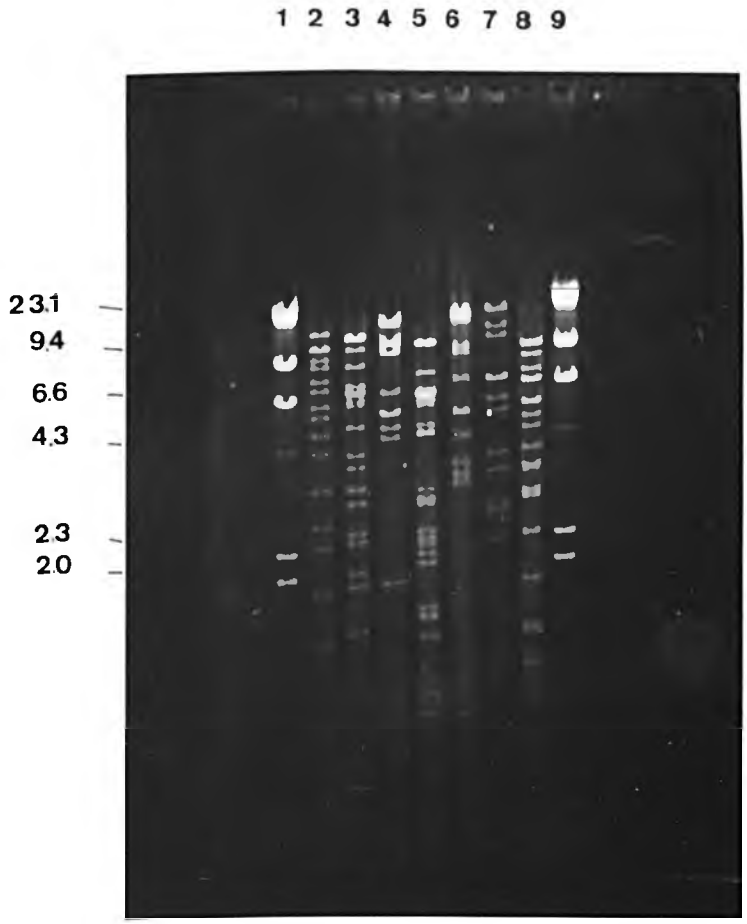
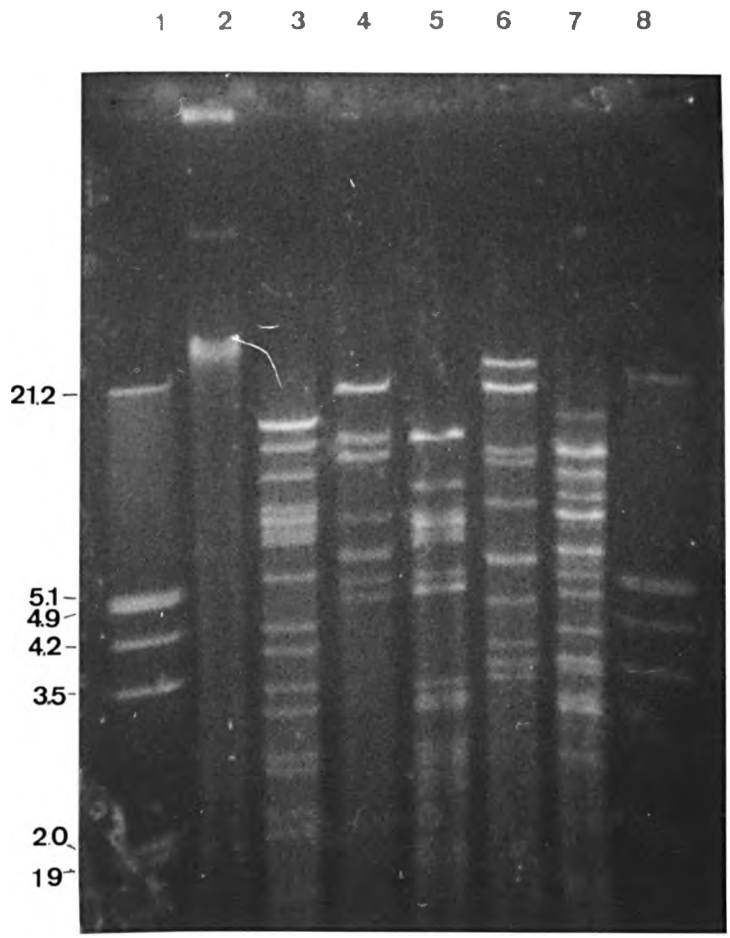

1 2 3 4 5 6 7 8 9
23.1
9.4
6.6
4.3
2.3
2.0

Fig 5.3 Restriction endonuclease digest of the final sample (generation 7) of granulosis virus used in virus selection experiment.

Lanes as follows: 1 and 8 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4:HindIII, 5: EcoRI,6:Pst I, 7:Sal I.


1
2
3
4
5
6
7
8
21.2
5.1
4.9
4.2
3.5
2.0
1.9

for many generations is not available.

These problems of clarity led to a decision to bulk up the final generation of virus. It had originally been intended to try not to bulk up the virus as had been done in other similar studies (Croizier *et al*, 1985), to be certain that any changes which may have occurred were directly detected, and not obscured by bulking up process. The final generation was bulked up, at a low level of infection (~ 6% mortality) to minimise any change.

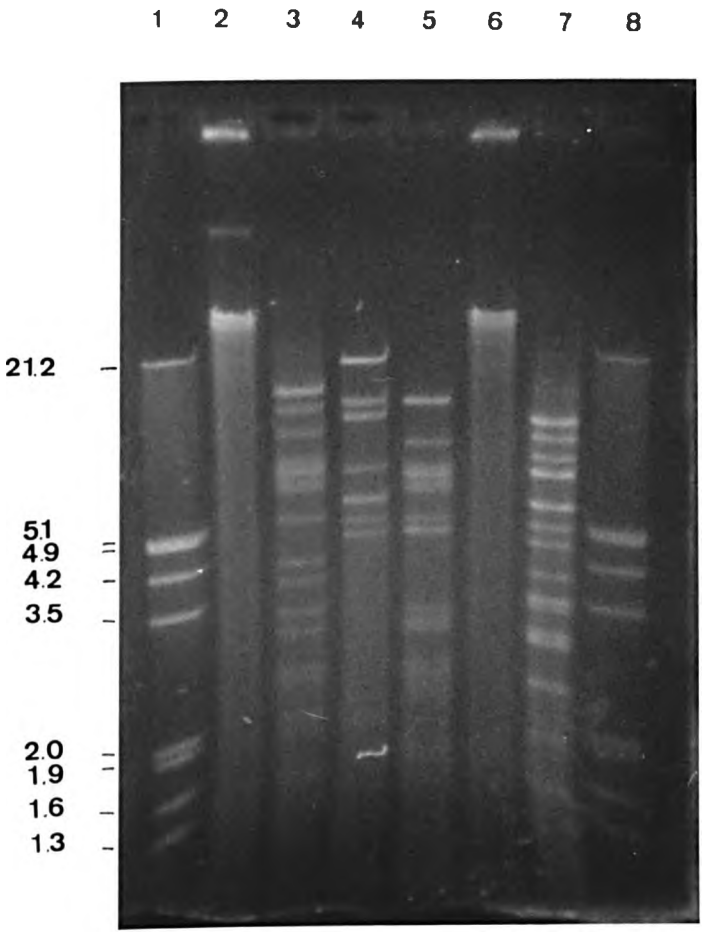
Restriction profiles of the initial innoculum and the virus extracted from the final generation (see Fig 5.3 and 5.4) were, as expected, very similar. Only two differences were noted: (i) there appeared to be an absent band at the top of the Pst I digested lane of the first generation sample, though this could have been due to a lack of clarity on the photograph of the gel. Gels were run as far as possible to ensure adequate separation of bands. The other difference (ii) appears to be the addition of a band at the top of Sal I digested final generation sample. This may be due to incomplete digestion of the sample. Some of the lower bands appear to be missing in the final generation digest, but this is thought to be due to these running off the end of the gel rather than representing any significant evolution of the virus. Molecular weights of restriction fragments obtained from the original and the final generations of the virus may be found in Tables 5.3 and 5.4 respectively.

5.3.3 Variation available in original innoculum

For the reasons discussed in 5.3.2, this was difficult to resolve. However as with the final selected generation, one of the samples of virus from a single infected insect

Fig 5.4 Digestion of bulked up sample of virus which had been cloned out in order to provide an indication of the variation available in the original innoculum.

Lanes as follows: 1 and 8 Lambda (EcoRI/HindIII); 2:XhoI, 3: BamHI, 4: Hind III, 5: EcoRI, 6: Pst I, 7: Sal I.


1
2
3
4
5
6
7
8
21.2
5.1
4.9
4.2
3.5
2.0
1.9
1.6
1.3

was retained and this sample of virus was bulked up (at low percentage mortality) and used to provide an arbitrary indication of variation (see Fig 4.5). However, when banding patterns were directly compared, there was no variation between this sample and the original innoculum. The relevant molecular weights may be found in Table 5.5. Although little emphasis may be placed on the result of this single gel, it seemed likely that any variation that was present in the genotype would have been cloned out by the end of the selection experiment.

5.3.4 Variation available in the PiGV genome

Comparisons were made between the virus used for original innoculum in this experiment, the virus supplied by P.V.Vail (PiGV-USA/V) and the published restriction profiles of PiGV (PiGV-USA/T) in Tweeten et al (1980). There proved to be very little difference between the three profiles. Tweeten *et al* (1980) noted the presence of a 12 $x10^6$ dalton submolar band, which does not occur in either the American stock of virus supplied by Vail (PiGV-USA/V), or the one used at Liverpool. Apart from this one band, the Liverpool virus and that used by Tweeten *et al* (1977) are identical. This suggests that the viruses probably originate from the same source, or that there is very little variation in the PiGV genome.

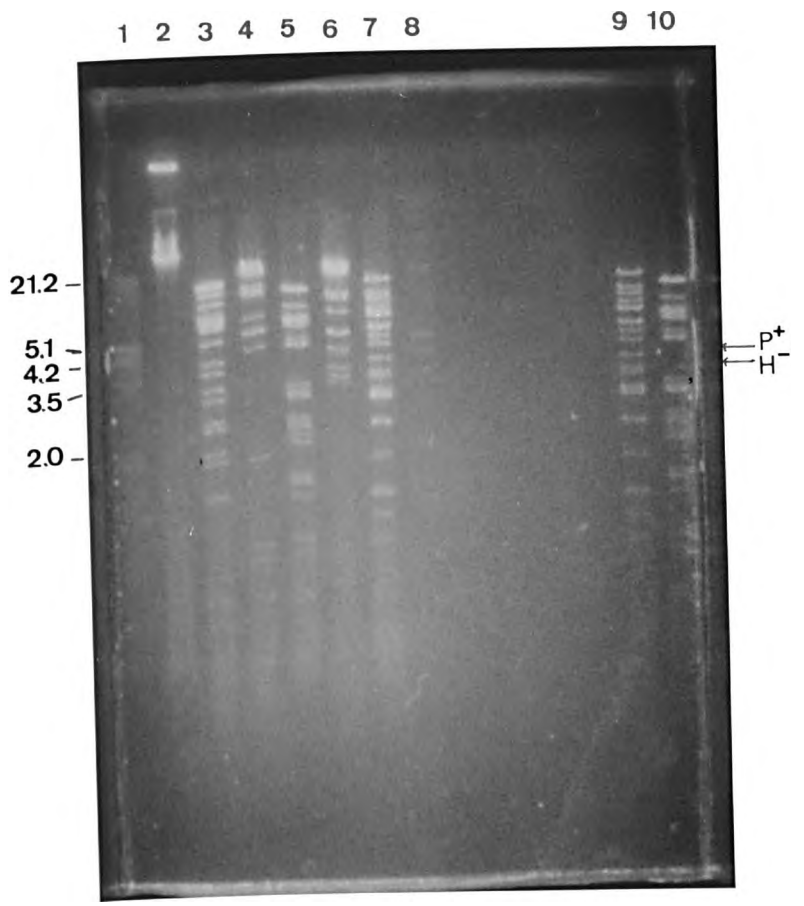
The other strain of virus (PiGV-USA/V) exhibits differences from the other two strains, with deletion of a 3.8Kb band in the Hind III digested virus, and addition of a 3.4Kb band in the Pst I digest. These differences are marked on Table 5.6. The remaining difference is the presence of a high molecular weight band (15.5Kb) at the top of the Sal I digest, similar to the band obtained in the final generation of viral selection (see Fig 5.4), possibly occurring as a result of incomplete digestion.

Fig 5.5 Restriction endonuclease digest of the USA sample of granulosis virus used in virus selection experiment (as supplied by P.Vail).

Lanes as follows: 1 and 8 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4:HindIII, 5: EcoRI,6:Pst I, 7:Sal I. Lane 9: Sal ;Lane 10 :Bam.

P^+ PST-ADDITIONAL BAND

H^- : HINDIII-DELETED BAND


1 2 3 4 5 6 7 8 9 10
21.2
5.1
4.2
3.5
2.0
P+
H−

5.4 Discussion

In summary, under selection, there has been very little change in phenotypic qualities and only minor change in the genotypic make-up of the virus. Therefore three possibilities exist: either the virus is unlikely to evolve given the current selection conditions, or the virus is in a stable equilibrium with the host so is unlikely to evolve anyway, or there was insufficient initial variation on which selection could act.

By contrast, earlier experiments that have involved the serial passaging of virus through insects have resulted in some incidence of increased virulence in the virus. Veber (1962) used a polyhedrosis virus of *Galleria mellonella* and found that over the course of twelve passages, viral induced mortality amongst the larvae increased from 20% to a stable average of 75-80%.

Smirnoff (1963) passaged a non-native nuclear polyhedrosis virus through an alternative host, resulting in a heightened infectivity of the passaged virus. An initial passage of *Trichiocampus viminalis* (poplar sawfly) NPV through the *Trichiocampus irregularis* (willow sawfly) produced very low levels of mortality (7%). Suprisingly, a second passage produced mortalities of up to 60% in the larvae. The virus attained a further slight increase in virulence (80% mortality)in a third passage, then stabilized throughout the succeeding passages.

Similarly, Stairs et al, (1981) passaged non-native *Choristoneura fumiferana* NPV (CfNPV) through *Galleria mellonella.* and *Trichoplusia ni* .Over two generations larval

Table 5.5 Cases in which changes in virus have been monitored over a period of generations

Insect	Virus	No of passages	Molecular changes?	Changes in virulence?	Author
M. brassicae	NPV	25	Yes	No	a
H. zea	NPV	20	Yes	No	b
H. virescens	NPV	25	Yes	No	b

[a] Croizier et al (1985), [b] McIntosh and Ignoffo (1986).

NB A series of earlier studies showed that the activity of a specific viral isolate may be increased >1 but <5-fold following serial passages (Veber,1962; Smirnoff,1963; Woodward and Chapman,1968).

mortality increased from 16% to 80-90% in *G.mellonella*. In *T.ni* a comparison between virus that had been passaged once and non-passaged virus, revealed that passaging had increased virulence, decreasing the lethal action time (for 100% mortality) of the virus from ten days to three days.In addition passaging the virus through both alternate hosts did not alter its infectivity for the original host . However, there was some indication after microscopic examination of infected original host larvae that the virus had been changed, since at death relatively few nuclei were found to contain polyhedra whereas normally large numbers of polyhedra could be found. Further experiments on *T.ni* using the multicapsid NPV of *Orgyia pseudotsugata* (OpNPV) demonstrated that after serial passage in the substitute host (*T.ni*), OpNPV not only maintained, but increased its virulence ten fold in its native host, after only one reverse passage in *O. pseudotsugata*.. Despite this, no observable changes occurred in the DNA profiles of five restriction endonucleases when the virus was passaged in *T.ni*. (Martignoni and Iwai, 1986)

A final available instance of increased virulence involves a non - baculovirus. It occurred as a result of serial passage of a mosquito iridescent virus. The average rate of transmission was 16%, but over a two year period (68 passages) in *Aedes taeniorhynchus*, the highest average transmission was 45% for a serial passage (Woodward and Chapman,1968). This case is slightly unusual, since although virulence increased, it did not follow a general trend of increase. Instead, it fluctuated in roughly equal and opposite directions approximately every 2 to 5 passages. It appears that unlike in the previous two examples, the increase in virulence in this case was not stable

In general, in the examples which show a stable increase in virulence, virally-induced mortality seems to have increased from a very low level (7-20%) to relatively high levels (75-80%): a 4 to 12 fold increase in virulence. As with cases of

selection for increased resistance to viruses in insects (see Chapter 3), any increase in response seems to occur fairly quickly. In both cases the initial increase of virulence seems to have occurred by the second passage: *T.irregularis* (60%) (Smirnoff, 1963)and in *G. mellonella* where mortality had both doubled to 40% (Veber,1962) and quadrupled respectively(Stairs et al,1981)

In other, more recent studies, no increase in virulence has been recorded on serial passage of virus through insects, despite fairly long term experiments. In separate studies, nuclear polyhedrosis virus was passaged through *Mamestra brassicae*, *Heliothis zea* and *Heliothis virescens* for 20 to 25 generations, with no increase in virulence. However small scale molecular changes in the virus were observed (Croizier *et al*,1985; McIntosh and Ignoffo,1986).

The conditions under which these studies were carried out were not designed to be particularly selective. As the term suggests, the viruses were passaged rather than selected over a number of generations.A major question which does arise from this literature survey is 'in which way did conditions differ between examples in which virulence increased, and those in which it did not?'

The difference seems unlikely to have been due to a lack of variation in the viruses, since molecular changes were recorded in those examples in which increased virulence did not occur. This leaves the possibilty of difference arising out of the distinction between "selection" and passaging conditions. Interpretation of the conditions is not straightforward. The most satisfactory way is to record the dose used and mortality produced. In all the cases discussed above, the virus was applied as a surface suspension to diet. In cases where stable increases of virulence were produced doses used were: 300 $x10^6$ polyhedra per cm^2, a suspension of 1 x 10^6 polyhedra per ml, and 10^3 polyhedra per mm^2 which produced initial mortalities of 7%, 20% and

16% respectively (Veber,1962; Smirnoff,1963; Stairs *et al,* 1981)). Studies carried out on mosquito iridescent virus specified only the 'use of one or two macerated infected larvae' (Woodward and Chapman,1968)

These low initial mortalities are in direct comparison to those used in studies which produced no change in virulence. Here average mortalities were between 99 and 100% in the case of *M. brassicae*, and 44% and 40.9% for *H. zea* and *H. viresecens*. The doses used in each case were: an excess of 10^5 polyhedra per mm^2 dropped to 4.5 x 10^4 polyhedra per mm^2 by the 22nd passage for *M.brassicae*, and 15 polyhedra per cm^2 for *Heliothis sp.* (Croizier *et al*,1985; McIntosh and Ignoffo,1986).

The major difference between experiments, then, appears to be the level of mortality used in the initial passage: low (< 20%) in cases of increased virulence, and higher (40-100%) in cases where no change occurred. Whether this is the only factor responsible for the difference in response is difficult to ascertain. Molecular evidence on the increasingly virulent viruses, both before and after passaging, would have nicely complemented the work done on viruses where the virulence remained unchanged. In addition it would have conferred a greater significance on those molecular changes recorded in viruses of stable virulence. As yet, despite a number of studies which have demonstrated genetic differences between different strains of the same virus (Gettig and McCarthy,1982), it has not been possible to directly relate these genetic differences to the differences in infectivity shown by the different strains.

In the experiment described in this chapter, the selection conditions used were as extreme as could be provided, assuming that changes in a measurable evolutionary

character would be required as evidence of evolution occurring. In common with the other examples quoted, such changes in this case were regarded as changes in mortality.

In this experiment, selection forces were acting at their most extreme, with very low mortalities being recorded at every generation. This included two generations where mortality was restricted to only one insect on both occasions. No increase in virulence occurred as a result of this selection pressure. It seems unlikely that increasing the number of generations would have increased the likelihood of altering the virulence, since where increased virulence has been observed to occur it has done so in a relatively short period of time. In view of the low mortality used as a selection force, it seems unlikely that selection pressure is a limiting factor in the evolution of the virus.

The other two possibilities for the lack of observed virulence are: (i) that not enough variation exists within the viral genome to permit viral evolution; or (ii) that such evolution occurs only on first contact between host and parasite. It is also possible that evolution does not occur to increase transmissability (Barrett, 1984), but instead that the host parasite relationship reaches a stable equilibrium,and that no virus evolution occurs unless in response to a similar evolution by the insect.

The amount of variation in the viral strain used in this experiment was fairly limited, but comparable to the amount available in viruses used in studies by Croizier *et al* (1985) and McIntosh and Ignoffo (1986). As mentioned above the amount of variation available in experiments where virulence increased is unknown.

limited, but comparable to the amount available in viruses used in studies by Croizier *et al* (1985) and McIntosh and Ignoffo (1986). As mentioned above the amount of variation available in experiments where virulence increased is unknown.

In a wider sense, the potential for evolution in the *Plodia interpunctella* granulosis virus genome (PiGV) does exist. Whilst studies for intra-genomic variation were carried out on only a limited range of PiGV isolates, they appear to encompass a fairly large cross section of the PiGV strains available.

A comparison of the three isolates indicated a high degree of homology between the strain used in these experiments and PiGV- USA/T. This probably indicates a similar origin for both strains. The remaining isolate showed some differences: deletion of a band from a Hind III digest, and addition of a further band to a Pst digest. This suggests that this virus is a separate genotype from the other two isolates, but also shows limited variation. It appears, then, that there are at present two available genotypes of PiGV. This corresponds quite well with the three variants available for *Cydia pomonella* GV (Crook *et al*,1985). However much larger numbers have been discovered in other baculoviruses: eight for *Artogeia rapae* GV (Smith and Crook,1988b); and over 20 for *Panolis flammea* NPV (J.C. Cory, Institute of Virology, Oxford *pers comm*)

The range of genotypes occurring are believed to arise via several routes: (a) recombination between genotypes during natural coinfection (Smith and Crook,1988); (b) recombination with foreign viruses, existing in a latent state within some insects (Roosien et al, 1986);(c) recombination with host DNA, which resulted in the production of a new phenotype in *Autographa californica* NPV (Fraser *et al* 1983) (d) rearrangement of the genome, such as sequence deletion, reiteration (Burand and Summers, 1980) or the inversion of specific regions detected in herpesvirus DNA (Hayward *et al*,1975)

(Hayward *et al*,1975). Therefore the potential for intermolecular heterogeneity is in principle enormous. It is not, however, always reflected in the number of genotypes recorded. This in turn may be a reflection of other obvious factors, the number of research groups working on the particular virus, or less obvious ones such as the specificity of the virus concerned. For example, PiGV appears to be species specific (Arnott and Smith,1968), *Panolis flammea* NPV is not. Could this be a reason for the varying degrees of intermolecular heterogeneity shown by the two viruses?

Another possibility put forward for the lack of increase in virulence in the virus, was that evolution occurs only in initial contact situations between host and parasite, as for example, with myxoma virus and European rabbits, and Newcastle disease and poultry. An increase in virulence was recorded by passaging a virus through a non native host (Smirnoff,1963; Stairs *et al*, 1981) - another possible example of an initial contact. However, Veber (1962) found that a native virus increased in virulence when passaged through its host.

In conclusion, the question must be asked-how does the work addressed in this chapter and the summary of previous work on viral evolution correspond to the theories mentioned during the course of the introduction?

Dawkins (1982) stated that viruses would not increase in virulence exponentially, that virulence would eventually plateau out in order to prevent extinction of the host. In contrast, Barrett (1984) observed that viruses evolve in order to increase transmissability, regardless of the possible extinction of the host. This was proposed with particular regard to initial contact situations.

The theory of increased transmissability seems to apply to the initial contact situation used as an example in this discussion, where virulence of the non-native NPV

increases from 7% to an average of 80% in three passages through *Trichiocampus irregularis* (Smirnoff,1963). Although some cases of 100% mortality were recorded in the third passage, mortality stabilized at the lower level (80%) after the third passage. Thus it seems that in initial contact situations, viruses do evolve increased virulence, but it is unlikely to proceed to lethal levels.

Despite earlier indications to the contrary (Veber,1962), it appears probable that once an insect- virus relationship has formed (several generations after initial contact) the result is a form of stable equilibrium. It is possible that the selection pressure required to disturb this equilibrium is a lot greater than would be required in an initial contact situation. This is probably because once it stabilizes less variation is available on either side of the relationship. Thus introducing a highly variable component may result in own subsequent evolution. The system currently in use in this laboratory lacks variation, therefore any viral evolution is only likely to be of a minor molecular type, probably by rearrangement of the genome - but it is unlikely to result in phenotypic differences.

CHAPTER 6

LATENCY AS A POSSIBLE ALTERNATIVE ROUTE OF VIRUS EVOLUTION

6.1 Introduction

The work described in this chapter is an attempt to further clarify the evolutionary position of the granulosis virus within the context of the insect-virus relationship. The previous chapter (Chapter 5) centred on a study of the evolutionary success of the virus, via changing transmissability. This chapter considers the possibility that the virus may circumvent such an expected course of evolution, by existing between epizootics in a latent state in the host.

The idea of latency in insect viruses is not new and has often been proposed as a possible explanation for periodic natural epizootics commonly recorded in Lepidoptera and sawflies. The long history of the latency concept has led to the usage of a wide variety of descriptive terms.

Latency may be defined as the ability of a virus to survive in a host, or through host generations, without causing recognizable symptoms yet remain provokable to pathological activity by certain treatments (Bergold,1958). Smith (1976) later amended this definition using latent to describe only infections. Hence a latent infection was defined as an inapparent infection which is chronic and in which a certain virus-host equilibrium is established. 'Occult virus' is the term used to describe those cases where virus particles cannot be detected, and in which the actual state of the virus cannot as yet be ascertained (Smith 1976). Later still, Entwistle and Evans(1985) termed them inapparent infections. In this chapter the term 'latent' will be used to cover both 'latent' (*sensu* Smith) and occult infections, though to add to the confusion, the term latent is often used more generally in epidemiology to describe the period in an infection cycle when the host is infected but is not itself infectious (Anderson and May, 1981)

The classic example of a latent viral infection is the mammalian herpes simplex virus where the virus remains dormant, in an unknown form, in the trigeminal nerve, until activated by a variety of physiological and physical factors resulting in an acute infection in mammals. This involves a genetic interaction between viral and host genomes (Davis *et al*,1980).

Several apparent instances of latent viral infections have been recorded in insects. Such infections are commonly activated or triggered by environmental influences (stimuli) termed "stressors" by Steinhaus (1958). Examples of such stressors include: changing physical factors, rearing conditions, certain chemicals and activation employing foreign (heterologous) viruses. On subjecting the insect to such a stimulus, a previously inapparent infection becomes an overt disease. However the mechanism of activation is as yet unresolved and it is probable that a number of components may be involved when one considers the activating role of such varied stressors as temperature, and chemicals (Entwistle and Evans,1985). There seems as yet to be no commonly accepted way of stressing out an inapparent infection. Proof will only come when it is shown that the pathogen in the host exists in a non-infective and non-replicative state and is transformed to an infective and replicative state when the insect is stressed (Tanada and Fuxa, 1987).

The aim of the work described in this chapter was to monitor the selected population for the presence of latent virus and in so doing to investigate a possible alternative evolutionary strategy in the insect virus relationship. It was decided to utilize a direct method of detection, radioactive probing of dot blots of insect DNA, in the search for possible latent virus. It was felt that with a suitably sensitive probe that dot blots would provide the most unequivocal evidence for the presence of viral DNA in the insect genome.

6.2 Materials and Methods

Samples of the insect population were collected at various stages during the selection procedure. Insect DNA was extracted and transferred to nitrocellulose paper in the form of dot blots. The dot blot was then challenged with a range of probes designed to give some degree of varying sensitivity. The insect collection protocol and some detail concerning the probes is discussed below. The remaining methods may be found in the 'general methods' in chapter 2.

6.2.1 Insects

Sampling was limited to generations 2, 5, 7 and 10. The sample from generation 2 included insects sampled in the normal way (as larvae in the generation immediately following selection); and also those which had not been exposed to virus in the immediately preceding generation, but had been selected originally then allowed to breed normally, these insects constituted a back-up population in order to prevent accidental extinction of the selected population. The eggs of both samples of generation 2 insects had been surface sterilised. In each case the sample from each generation consisted of ten fifth instars. At the end of the selection experiment a sample of the current stock population was also tested as a control. In addition, at the start of the work, large numbers of the original stock population were sampled and tested in order to check for the presence of virus.

6.2.2 Testing for the presence of latent virus

DNA was extracted from each insect sample using the method described in 2.8.3. This DNA was then transferred to nitrocellulose using a dot blot manifold and baked

at 80°C to make the transfer permanent.

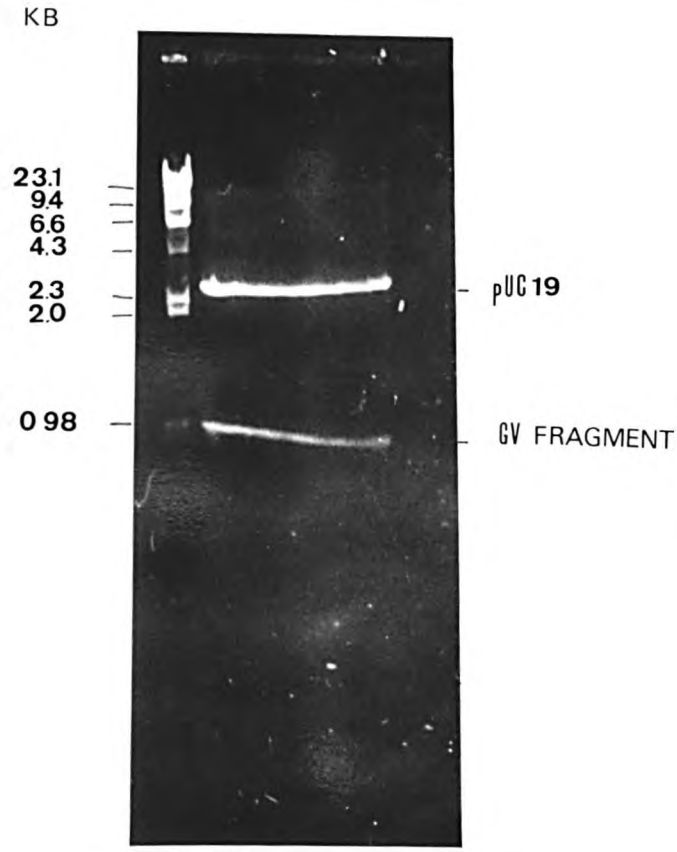
The filter was prehybridized,then hybridized under stringent conditions to the probes described in the following sections. Filters were washed with varying degrees of stringency, varying from 6xSSC, 0.1%SDS at 37°C to 0.1xSSC, 0.1%SDS at 65°C. The degree of hybridization was recorded using autoradiography. The specific washing conditions used are cited in the relevant figure legends.Calf thymus DNA and *Plodia interpunctella* granulosis virus were used as negative and positive controls respectively.Plasmid DNA (pUC 19) was used as an additional control .to see whether the granulin gene probe contained any plasmid DNA.

6.2.3. Probes used for screening insect DNA

In order to provide varying degrees of sensitivity it was originally intended to use three probes in testing for latent virus. The probes were: a probe composed of labelled whole virus (PiGV); and two probes consisting of a labelled specific fragment of the virus, the granulin gene.

This specific gene was chosen because it was known to be a highly conserved sequence of the viral genome, and also because its presence is essential to the survival of the virus outside its immediate host.A clone of the granulin gene of *Pieris brassicae* GV was kindly provided by Norman Crook of IHR, Littlehampton, Sussex. It was intended to prepare the third probe from cloned fragments of *Plodia interpunctella* granulosis virus DNA containing the granulin gene sequence. Preparation of each of these probes is described below.

Fig 6. 0 Digestion of pUC19 plasmid in LGT agarose with NdeI showing granulin gene insert


KB
23.1
9.4
6.6
4.3
2.3
2.0
0 98
pUC 19
GV FRAGMENT

6.2.3.1 Whole virus probe

Virus was extracted from infected insects using the method described in 2.8.1 and further purified using caesium chloride gradients (see 2.8.2). The purified DNA was labelled using ^{32}P and nick translation. Whole virus probe was used to test both the initial population and the selected insects.

6.2.3.2. Granulin gene probe

The granulin gene was supplied ligated into pUC19 plasmid in transformed Hb101 cells. The culture was grown up overnight in L-broth containing ampicillin. A miniprep of the plasmid was prepared and digested with Nde1. This was run on 0.6% LGT gel, and the band containing the granulin gene fragment excised (see sections 2.8.16 and 2.8.17). The fragment DNA was purified and its concentration estimated. Fragment DNA was then labelled using random priming and ^{32}P, to form a probe.

6.2.3.3 Construction of *Plodia interpunctella* granulin gene clone

A genomic library of viral DNA was constructed using pUC19. Viral DNA was digested using BamHI, which was found to give a large number of fragments after test digests with a range of enzymes. The viral DNA fragments were ligated into the plasmid, pUC19. Competent Hb101 cells were prepared, and transformed using the ligated plasmid. Transformed cells were plated on selective media, agar containing 40ug/ml of ampicillin, and incubated overnight at 37°C

The following day, the transformation efficiency was estimated by comparing the number of colonies on the control and test plates. About four hundred Amp+ colonies were selected and streaked onto nitrocellulose placed on ampicillin-containing agar plates. A master and two replicates were incubated overnight at 37°C. The resultant colonies were denatured and neutralised. The nitrocellulose filters were baked at 80°C for two hours before prehybridization. The filters were hybridized with a labelled granulin gene probe under stringent conditions. Duplicate filters were hybridized with a labelled whole virus probe under similar conditions as a control.

After screening any positive clones were picked off the master plates and grown up in 5ml of L-amp broth overnight. These were stored as glycerol stocks at -20°C. Minipreps of each potential clone were prepared, and digested to check for the presence of an insert.

Detailed methods of the procedures mentioned above can be found in the following consecutive sections of Chapter 2: sections 2.8.11-2.8.15.

6.3 Results

6.3.1 Screening the initial and selected populations of insects

Screening the initial stock population with a whole PiGV probe produced no hybridization at all with the insect DNA despite the relatively low washing stringency (42°C, 6xSSC, 0.1%SDS)(see Fig 6.1) Probing the selected insect DNA both with a whole virus probe and the granulin gene fragment from *Pieris brassicae*, produced evidence of hybridization (Figs 6.2 and 6.3).

Fig 6.2 shows that the granulin probe hybridized to the positive control and to the

Fig 6.1 Insect genomic DNA extracted from original stock(control) insects prior to the start of the selection experiment. Probed with PiGV whole virus probe. Blot washed at 42°C, 2XSSC, 0.1%SDS. Autoradiograph exposed for 3 days.

Lanes 1-4 Insect DNA from pre-selection stock insects

Lane 5 Calf thymus DNA (negative control)

Lane 6 *Plodia interpunctella* GV (positive control)

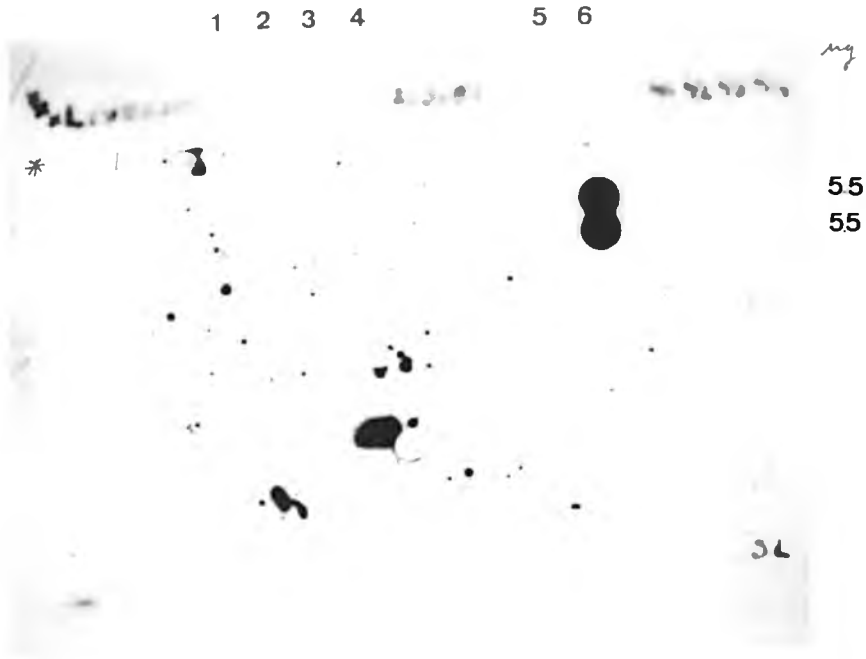

1 2 3 4 5 6
55
55

Fig 6.2 a Insect genomic DNA from selected generations: 2,5,7,and 10 probed with whole PiGVprobe. Blot washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed overnight.

1: Generation 2 (sterilised)
2: Generation 2 (sterilised and one generation removed from selection)
3: Generation 5
4: Generation 7
5: Generation 7
6: Stock (control) insects (as at generation 10)
7: Generation 10
8: Calf thymus DNA (negative control)
9:
10: *Plodia interpunctella* granulosis virus
11:
12: pUC 19

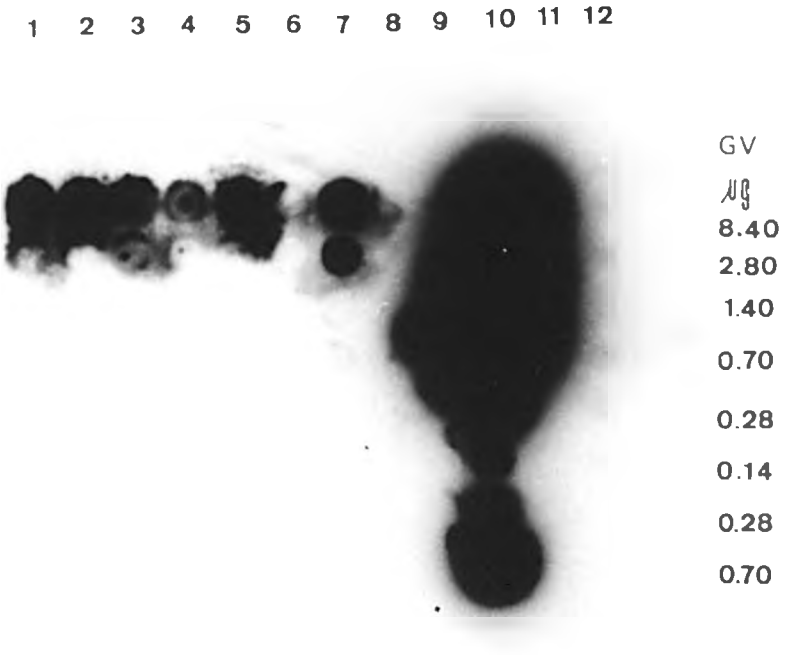

1 2 3 4 5 6 7 8 9 10 11 12
GV
μg
8.40
2.80
1.40
0.70
0.28
0.14
0.28
0.70

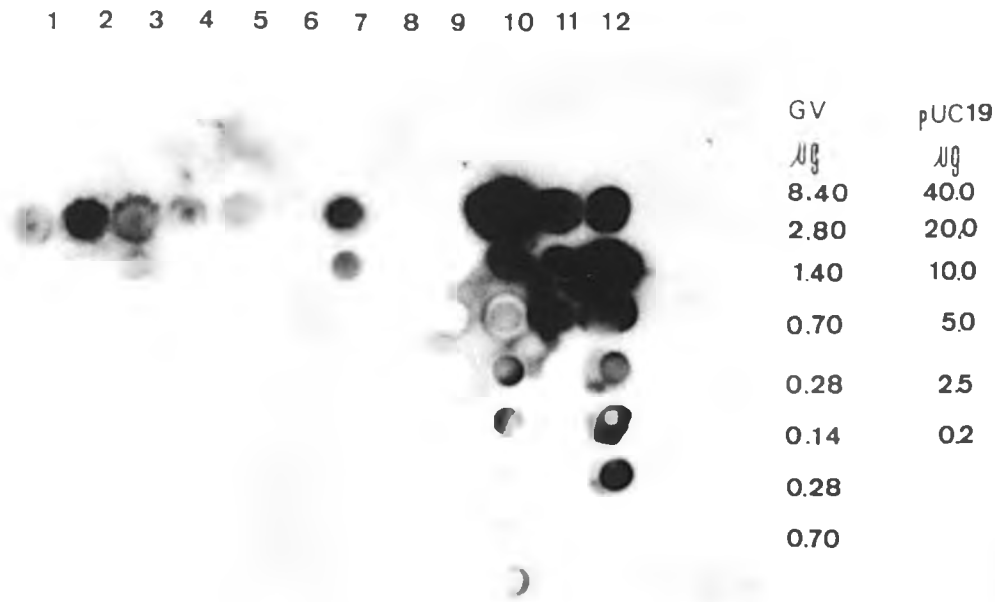
Fig 6. 2b Insect genomic DNA from selected generations: 2,5,7,and 10 probed with whole PiGVprobe. Blot washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed for 4 hours.

1: Generation 2 (sterilised)
2: Generation 2 (sterilised and one generation removed from selection)
3: Generation 5
4: Generation 7
5: Generation 7
6: Stock (control) insects (as at generation 10)
7: Generation 10
8: Calf thymus DNA (negative control)
9:
10: *Plodia interpunctella* granulosis virus
11:
12: pUC 19


1 2 3 4 5 6 7 8 9 10 11 12
GV
μg
8.40
2.80
1.40
0.70
0.28
0.14
0.28
0.70

Fig 6.3 a Insect genomic DNA from selected generations: 2,5,7,and 10 probed with granulin gene probe. Blot washed at 42°C, 6XSSC, 0.1%SDS. Autoradiograph exposed overnight.

1: Generation 2 (sterilised)
2: Generation 2 (sterilised and one generation removed from selection)
3: Generation 5
4: Generation 7
5: Generation 7
6: Stock (control) insects (as at generation 10)
7: Generation 10
8: Calf thymus DNA (negative control)
9:
10: *Plodia interpunctella* granulosis virus
11:
12: pUC 19


1 2 3 4 5 6 7 8 9 10 11 12
GV
μg
8.40
2.80
1.40
0.70
0.28
0.14
0.28
0.70
pUC19
μg
40.0
20.0
10.0
5.0
2.5
0.2

negative control. Interestingly, it also hybridized to two of the insect samples from the selected population. However only a low stringency wash was applied to this filter, so results are far from conclusive. Results do seem to suggest that some definite hybridization has occurred, the positive signal obtained from the insect samples is comparable with that of the positive virus control. The positive result from the pUC19 control is not entirely unexpected, bearing in mind that the granulin fragment was ligated into pUC19 plasmid. In addition this could possibly explain the results obtained in the next section.

Even after stringent washes (0.1XSSC,0.1%SDS at 65°C) the whole virus probe hybridized to all the insect DNA samples (Fig 6.3) and the positive control, but not to any of the negative controls. This strongly suggests the presence of viral DNA in all of the insect samples.

6.3.2 *Plodia interpunctella* granulin gene probe

The library was constructed and screened as detailed above. The results are shown in Figs 6.4 and 6.5. Figure 6.5 appears to show that the majority of colonies chosen show hybridization to the granulin gene probe, making it impossible to choose individual clones specific for granulin gene. This may have been due to hybridization to some of the plasmid still remaining with the fragment probe after excision from the LGT gel. This situation may have been rectified by adding some pUC19 to the pre-hybridization and hybridization solutions to act as a 'blocker', and prevent unwanted hybridization.

Originally however the problem was perceived as one of mislabelling, thus it was assumed that the filter with the majority hybridization was in fact the filter probed with the whole virus probe, and the one that showed only the hybridization to a few clones

Fig. 6.4a Screening of clones from a PiGV library constructed in pUC19 probed with whole *Plodia interpunctella* granulosis virus probe. Washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed overnight.

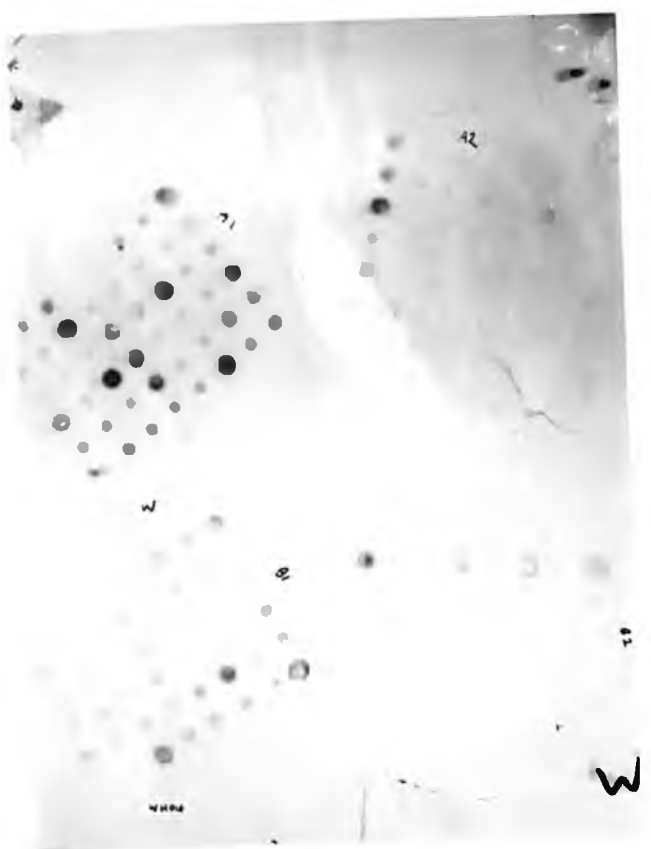


Fig. 6.4b Screening of clones from a PiGV library constructed in pUC19 probed with whole *Plodia interpunctella* granulosis virus probe. Washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed for 5 days.

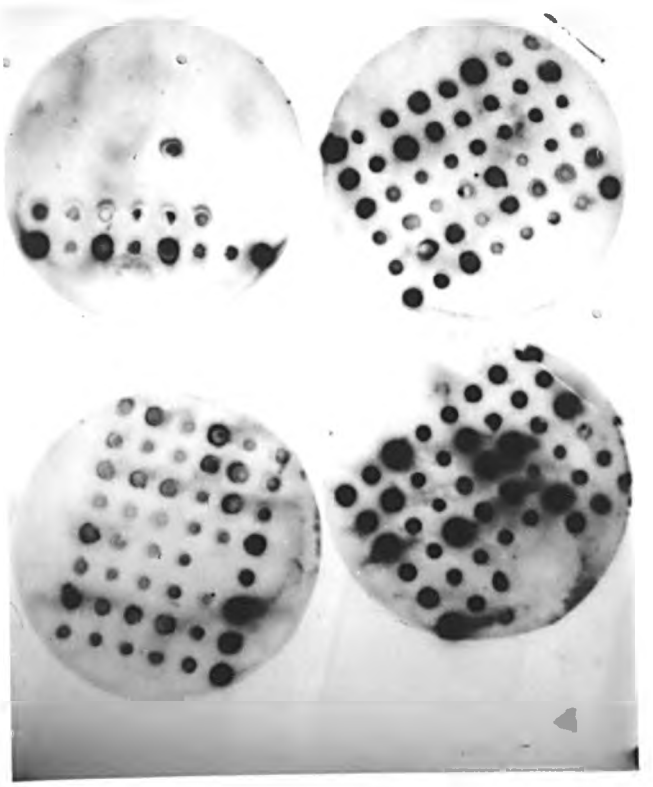


Fig. 6.5a Screening of clones from a PiGV library constructed in pUC19 probed with a granulin gene fragment of *P. brassicae* GV. Washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed overnight.

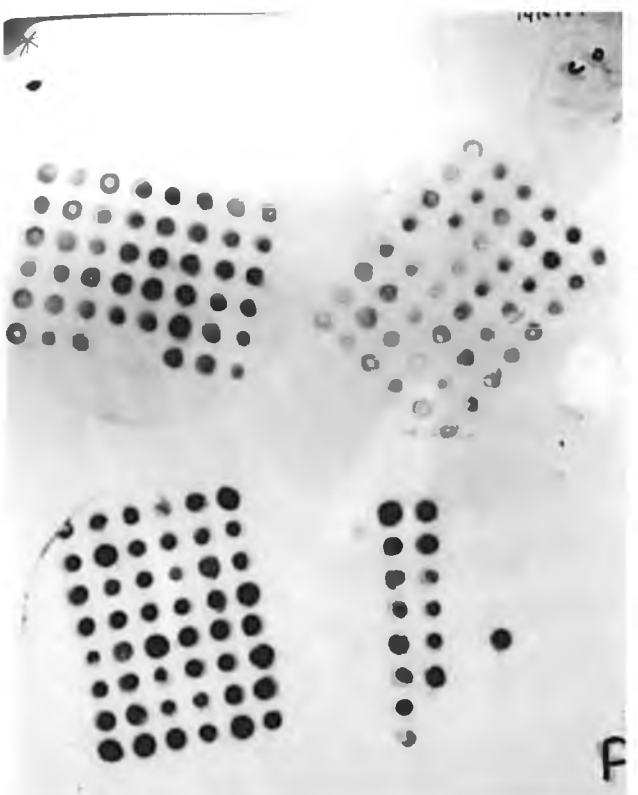
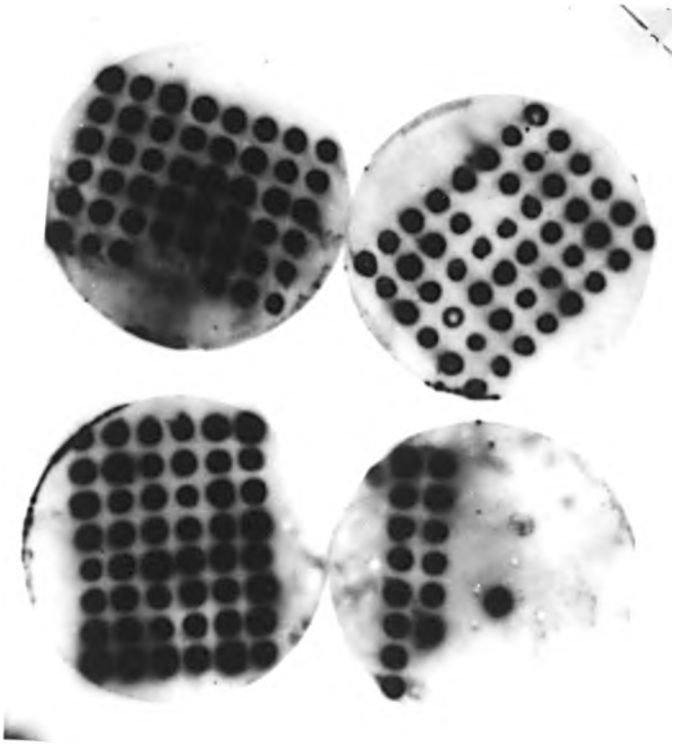


Fig. 6.5 b Screening of clones from a PiGV library constructed in pUC19 probed with a granulin gene fragment of *P. brassicae* GV. Washed at 65°C, 0.1XSSC, 0.1%SDS. Autoradiograph exposed for 5 days.



the whole virus probe, and the one that showed only the hybridization to a few clones was the filter probed with granulin gene fragment.

Since it was not possible to pick any granulin gene specific clone due to the problem mentioned above, the five clones showing the strongest hybridization to the whole virus probe were chosen.These were grown up in L-amp broth and minipreps made of each clone (see 2.8.16). Unfortunately after digestion and being run on a gel none of the chosen clones proved to have an insert.Therefore due to time constraints it was decided to just use the two readily available probes (whole virus and granulin gene fragment) to screen the selected insect DNA

6.4 Discussion

The physical evidence for inapparent or latent infections is, as mentioned in the introduction, mainly based on the conversion of an inapparent infection to an overt infection by a range of stimuli or stressors. It would be instructive to review such previous evidence at this point, in order to provide a basis for further discussion in the light of the results obtained in this chapter.

Many examples of activation using different stressors have been demonstrated, and a few will be mentioned here to give some idea of the range of occurrence. Changing physical factors, such as increased temperature and UV irradiation induce cytoplasmic and nuclear polyhedroses in the silkworm (Hukuhara, 1962) and NPV in the European pine sawfly *Neodiprion sertifer* (Krieg,1957). Lowered temperatures can also stimulate both latent cytoplasmic and nucleopolyhedroses in the silkworm (Aruga and Watanabe,1961; Himeno *et al*,1973). However, altered

temperatures do not always increase the incidence of latent infections, Steinhaus (1960) exposed different species of lepidopterous larvae to temperatures of 47°C for ten minutes and to 51°C for fifteen minutes, but recorded no increase in incidence of polyhedroses over the controls.

Altered rearing conditions, particularly increased population density and changes in food and temperature, result in the onset of a granulosis infection in *Pieris brassicae* larvae (David and Gardiner,1965), and changing the diet of *Pieris rapae* induced a similar granulosis infection (Biever and Wilkinson,1978).Overcrowding was also responsible for activating a cytoplasmic polyhedrosis virus in the alfalfa caterpillar *Colias eurytheme* (Steinhaus and Dineen,1960).

Certain chemicals were found to activate CPV in silkworms: these included sodium cyanide, sodium fluoride, arsenic acid, monoiodoacetic acid, sodium azide, ethylenediamine tetraacetic acid (EDTA), and its disodium salt (Aruga and Hukuhara,1960).However, Entwistle and Evans (1985) advise caution in the interpretation of examples involving activation of cytoplasmic polyhedrosis viruses, since CPV is " a common contaminant of insectary rearing of insects, therefore interpretation of results must be tempered by the possibility of external contamination."

By far the most impressive body of evidence has been amassed by induction of latent infections using foreign or heterologous viruses. The extent of the foreigness of the virus does not appear to affect the trigger mechanism. In general heterologous viruses used to induce infections have been other insect viruses such as cytoplasmic polyhedrosis viruses, nuclear polyhedrosis viruses, and, more rarely granulosis viruses.

For example, introducing a cytoplasmic polyhedrosis virus from the tussock moth

Dasychira pudibunda into the European sawfly *Neodiprion sertifer* produced a nucleopolyhedrosis infection in the sawfly (Krieg,1957). Similarly, feeding and injecting the granulosis virus of the European fir budworm *Choristoneura murinana* into the North American spruce budworm *Choristoneura fumiferana* produced a polyhedrosis infection in the larvae (Bergold,1951).

In an elegant extension of such earlier work, a nucleopolyhedrosis virus of the butterfly *Aglais urticae* was found to induce a host specific NPV infection in the Gypsy moth *Lymantria dispar*. Correspondingly, the reciprocal NPV of *Lymantria dispar* was also found to induce a host specific NPV infection in *Aglais urticae* (Longworth and Cunningham,1968).

Similarly, serial passage of an NPV of the velvetbean caterpillar *Anticarsia gemmatalis* in an alternative host system the soybean looper *Pseudoplusia includens*, resulted in the activation of a latent NPV native to the soybean looper between the third and fifth serial passage (Pavan *et al*,1981). In this case differences between viruses were verified by comparisons using electron microscopy and electrophoretic profiles of viral polypeptides.

More unusually, injecting concentrated suspensions of tobacco mosaic virus (TMV) into bees of the species *Bombus*, produced symptoms typical of bees infected with acute bee paralysis virus (Bailey and Gibbs,1964). This reaction was dependent on the concentration of TMV used, although whether the virus had been inactivated was not a factor.

Relatively recently such activation experiments have been taken a step further, most notably by Jurkovicova (1979), McKinley *et al* (1981) and Smith and Crook(1986), due to the wider availability of more sensitive techniques such as

restriction endonuclease digests of DNA.Jurkovicova (1979) fed larvae of the summer moth, *Adoxophyes orana* and *Mamestra brassicae* on NPV of the reciprocal species. Comparison of restriction endonuclease EcoR1 cleavage patterns of DNA isolated from virus liberated from infected larvae , and virus used as innocula showed that no cross infection had occurred. In fact results indicated that latent viruses had been activated in both insects. Previously it had been suggested that reciprocal NPVs (of both species) were pathogenic for both species. This work meant that a clear distinction could be made between cross infection and activation of latent infections thereby resulting in a clearer idea of the frequency and distribution of latent viral infections.

Using biophysical and serological techniques, McKinley *et al* (1981) were able to distinguish between the heterologous virus used to innoculate *Spodoptera littoralis* and the activated virus responsible for the subsequent infection in that species.

Smith and Crook (1986) discovered that *Pieris brassicae* harboured inert GV DNA sequences that are capable of being activated by other GVs , notably *Artogeia rapae* GV (ArGV) and *Agrotis segetum* GV . In addition they noted that in restriction endonuclease profiles of GV DNA purified from individual cadavers, a range of complex progeny genotypes could be observed. Some were identical to *Pieris brassicae* GV, others possessed bands characteristic of the innoculum virus ArGV. None however was identical. These hybrid genotypes are thought to be the result of homologous recombination between ArGV genome and latent DNA.

So, based on available evidence, it seems that latent virus does exist within certain populations of insects, and that when exposed to a factor or factors capable of activation it becomes an overt and often fatal infection. In addition, it seems probable that accidental ingestion of heterologous viruses in latently infected populations may

in fact, contribute to the variation available to the virus by homologous recombination between the genome of the heterologous virus and the viral DNA latent in the insects.

As far as it is possible to ascertain from the literature, with one exception, latent virus has remained largely undetectable by direct methods of detection,that is without subjecting the insect population to the stressors discussed above.Skuratovskaya*et al* (1985) have shown by use of the dot blot technique that *Bombyx mori* NPV DNA was integrated with cell DNA and that a similar occurrence was observed in a wild strain of *Galleria mellonella,* but not in a laboratory strain. Results obtained during the course of this work seem quite strongly to suggest the presence of viral DNA in the insects sampled at various stages from the selected population, but not in the original stock population both prior to and post selection.

Both the filters in Fig 6.2 and Fig6.3 show some degree of positive response to probing with the virus or its granulin gene fragment. High stringency hybridization conditions (42°C and 50% formamide) implied that the hybrids detected in this way were fairly well matched , consisting mainly of closely related sequences (Anderson and Young,1985). The varying stringencies of the washes used implies that more weight should be given to the positive results showing up on the filters with the highest stringency washes (0.1xSSC, 0.1% SDS, 65°C).

Therefore the hybrids formed on the filter with a whole virus probe are an obvious indication that viral DNA is present in the DNA samples from the insects. The results obtained by probing with the granulin gene fragment are interesting, in that only two of the insect samples positive for the presence of whole viral DNA have been shown to be positive for the presence of the granulin gene sequence. Such a positive result implies that the virus has been transmitted through the adult to a second generation of larvae. This host mediated vertical transmission could have occurred in one of two

ways: either the virus could exist as an external contaminant on the egg mass (transovum transmission); or, the virus may be incorporated within the egg(embryo) (transovarian transmission) (Martignoni and Milstead,1962).

In transovum transmission, the virus is generally believed to be acquired from the mother during oviposition, or from the environment. Another theoretical possibility is that virus is carried into the egg as an external passenger on the sperm (David,1978). This occurs in certain mammalian viruses (Brackett *et al*,1971). However attempts to demonstrate such a passage mechanism in *Pieris brassicae* were unsuccessful (David and Taylor,1976).

Transovum transmission is relatively easy to confirm, with many convincing examples arising from the elimination of any surface infections by the use of anti-viral agents such as formalin and sodium hypochlorite (David,1978). In *Pieris brassicae* it has been observed that some of the larvae hatching from eggs laid by females in virus infected stocks die of GV. If the eggs are surface sterilised using anti viral agents, none of the resulting larvae die, showing that the virus is either on the egg or in its superficial layers (David and Taylor,1976). Similar results have been obtained with the granulosis virus in *Zeiraphera diniana* (Schmid, 1974) and *Cydia pomonella* (Etzel and Falcon,1976). This type of adult-mediated transmission has been implicated in the build up and spread of NPV infections in both pine and spruce sawflies (Cunningham and Entwistle,1981). Conflicting results concerning trans-ovum transmission in *Plodia interpunctella*, work done in this laboratory, shows that even when the genitalia of adult moths are painted with virus prior to mating and egg laying none of the F1 generation develop viral infection. In contrast, when adult male *Plodia* were surface contaminated with GV, they transferred virus to adult females during copulation, who in turn contaminated food during oviposition and the F1 generation of larvae became

virally infected. However, feeding on contaminated corpses of adult moths was believed to be the principal route of infection (Kellen and Hoffman,1987)

Evidence for true transovarial transmission is more scarce, being subject to the same technical difficulties as the detection of true latency in viruses. There is no unequivocal evidence that any baculovirus is transmitted transovarially (David,1975). More recent reviews have also been unable to find any convincing evidence that certain of the more common insect virus groups, notably baculoviruses and CPVs undergo transovarial transmission (David,1978; Payne,1982). However germ line transmission of at least one type of insect virus has been recorded. The sigmavirus of the fruitfly, *Drosophila melanogaster* has been shown to be closely connected with cell components of the host and it is regularly transmitted to the progeny via the gametes (usually the oocytes) (Podgwaite and Mazzone,1986).

For transovarial transmission to occur the baculovirus must be present within the eggs in an occult state or as organised virions.In the former, it would be expected that the viral DNA would be intimately associated in some way with the insect genome, and would be passed to the germ cells in each successive generation. The alternate route would involve acquiring an inapparent infection at some point during the course of the the insect life cycle, and the virus invading the gonads or the germ cells.There is at present little evidence for the GVs penetrating the gonads.

The question then arises, do the positive results obtained in this work represent an example of true transovarial transmission of a latent virus, or are they an example of transovum transmission of a contaminant viral DNA?

The question is not easily resolved. Entwistle and Evans (1985) stated that in order

to resolve the problem of transovarian transmission, insect stocks must be used in which there was an "absolute certainty" that stocks were free of contamination. The original insect stocks used in this experiment were screened with a whole virus probe, before the selection experiment was started. No virus was present in the insect DNA tested. In addition, at the end of the experiment a small sample of stock larvae which had run concurrently to the selection experiment were retested. The results were again negative.Although testing of the stock population was not completely exhaustive, it represents an extensive cross section of the larvae available at the time. Obviously one can never be one hundred percent sure that a population was virus free, without causing its subsequent extinction. However the population has never produced overt signs of infection, and it has been checked on virtually a daily basis for the past three years.

Therefore it is relatively safe to assume that the original stock population was free of any viral contamination. This then leads us on to the possibility of contamination in the selected population. As mentioned before in Chapter 3, initially, for the first two generations of selection, eggs were sterilised using a sequence of formalin and sodium hypochlorite (for details see 2.1.2). Therefore insect DNA from generation one came from larvae hatched from sterile eggs but generation five and ten came from non-sterilised eggs. The practice of sterilisation was discontinued during the selection experiment, since it was thought that the risk of the potential extinction of the selected population by the sterilisation process outweighed the risk from any potential viral contamination. Viral contamination of selected insect population was viewed as unlikely, since the selected population were kept in incubators in a completely separate room from the room where virus was used for bioassays. Sterile components were used in the laying apparatus.

Thus the likelihood of contamination was small. There are two possibilities: that

all the sterilised eggs were recontaminated, or that true transovarial transmission has been demonstrated.

Positive evidence for the presence of viral DNA within insects was found by sampling what was effectively a comparatively small section of the selected population.If the virus was present as a contaminant in an infectious/active form it would be more likely to have attained greater prevalence within the selected population, and there should have been some sign of some virally infected larvae.

An interesting feature of the results is the differential hybridization of the two probes to the filter. This suggests that the virus may exist in two different forms within the insect, perhaps initially in a form lacking the granulin sequence, and a generation later as complete virus, and may suggest a possible mechanism for latency in inapparent or latent infections.Alternatively there may be no difference of form but just a difference in the copy number of the granulin gene in the selected insects which are two generations removed from selection pressure.

There is strong evolutionary significance in the acquisition of some form of latent virus, whether via transovum transmission or transovarially. Transovarial transmission would provide the virus with an alternative *modus vivendi*, since the virus would no longer have to rely upon increasing virulence as a method of effective transmission (see Chapter 5).

The ecological implications of latent virus have equally far-reaching consequences. In a recent review on the population cycles of forest Lepidoptera, Myers (1988) proposed disease susceptibility as one possible hypothesis to explain such population cycles. Baculovirus disease has often been reported in declining populations of forest Lepidoptera (Entwistle,1986).

The theory put forward by Myers (1988) is that the cumulative stress of living in high density population system increases the susceptibility of the insects to infection. Thus a high density populations of insects subjected to further stresses such as food limitations or poor weather become increasingly more susceptible to virus disease, which results in subsequent epizootic and population crash. The main foundering point for such a theory is the origin of the virus in an apparently previously virus free insect population. Retention of viral particles within the soil to provide a new focus of infection is one such possibility. The other is the existence of virus in a latent form within the population, which allows it to be transmitted amongst generations of insects (Myers,1988). This would mean that the population could appear to be virus-free for generations, increasing in number until the cumulative stresses mentioned above initiate viral infection and subsequent epizootics.

Thus latency may play a role in population control and regulation, such a theory though is dependent on the virus or pathogen being shown to exist in a non-infective, non-replicative state and is transferred to an infective and replicative form when the insect is stressed (Tanada and Fuxa,1987).

Transovum transmission has less evolutionary significance since the virus would still have to rely on virulence as its sole method of transmission. However if transovum transmission had occurred, the virus would have been introduced into the insect at the neonate stage. Since the insect then progressed to final instar with no apparent signs of infection, it is probable that the virus may have affected the insect sublethally, resulting in a possible change to the insect's biology. In turn, this may result in increased susceptibility to the virus. Thus the internal virus would have circumvented the normal viral route of infection, and yet still have contributed to the evolutionary success of the virus.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

The evolution of the insect-virus interaction has been considered in the preceding chapters primarily by studying the two extremes of the relationship where evolution would be most likely to occur given appropriate conditions. These two extremes are probably best visualised in terms of opposite ends of a bioassay curve (see Fig1.3), These two aspects of the relationship were examined by carrying out selection experiments under conditions which it was hoped would favour evolution in both insect and virus respectively. These selection experiments formed the basis of chapters three and five.

In both these selection experiments some limited measurable evolutionary change occurred. In chapter three, concerned with potential insect evolution, results showed no measurable increase in resistance occurred as a result of selection. However, the selected insects did increase their developmental rate, leading one to expect a possible recordable increase in resistance when larvae of the same age from selected and control populations were compared in bioassays.That this did not occur may be attributable to the method of choice of insects for the bioassay comparison.

Little measurable evolutionary change either phenotypic or genotypic occurred under the selection regime designed to promote virus evolution. In this work described in chapter five, no increase in virulence occurred, probably due to a lack of variation in the virus used, and possibly due to limited variation in the virus generally. After selection the virus exhibited minor genotypic changes in the shape of some alteration in the restriction endonuclease profiles.

The remaining two experimental chapters considered other aspects which may contribute to the evolutionary success of the insect and virus in the light of the

insect-virus interaction. Chapter four examined a facet of the life history strategy of the insect: older females appear to produce larvae which show greatly accelerated development in comparison to larvae of a similar age.These larger larvae may be expected to show greater resistance to virus than their larval counterparts of a similar age.Thus the production of these larvae may ultimately aid the population survival during the course of a viral epizootic

Chapter six considered the possible discovery of a latent form of the granulosis virus in selected insects. Latency is a possible aspect of the virus 'life-cycle' which may allow it to bypass the traditional route of transmission, and in doing so contribute to the continued evolutionary success of the virus .

This final chapter aims to bring together the results obtained from this experimental study of evolution, in the light of current host-parasite coevolutionary theory. In addition in a somewhat broader context it aims to examine the possible consequences of insect and/or virus evolution, both in applied terms in relation to pest control, and in terms of the normal epizootiological or population dynamics.

7.2 Theory of host-parasite coevolution

Traditionally the final outcome of any coevolution in a host-parasite system has been viewed as the attainment or establishment of a peaceful coexistence between host and parasite- a form of commensalism (Ewald, 1983). With commensalism as an evolutionary end-point, the widely held view that "successful parasites are harmless" has developed.Such ideas have been challenged most notably by Anderson and May (1981,1982,1983), in a series of papers which suggest that evolution may result in increased virulence of the parasite. Therefore, not all successful parasites are harmless.

The insect- baculovirus system is ideal advocate for such ideas since the virus is transmitted on the death of the insect, and thus parasite virulence is associated with enhanced transmission efficiency. Theoretically, increase in virulence may be unlimited since the virus is not expected to prolong the life of the host in order to increase the chance of transmission as in myxomatosis (Fenner and Ratcliffe,1965).

7.3 Evolution in an insect-baculovirus system

The extent to which a parasite weakens or kills its host is often correlated with the reproductive rate of the parasite, which should be maximised by individual selection (Futuyma,1983). Thus, in order to examine the evolutionary pressures on both host and parasite, Anderson and May posed the following question: on the basis of mathematical models of host-parasite population dynamics, how can both host and parasite maximise their respective reproductive rates ?

For the host system, the answer lies in increased virus resistance of the insect population which leads to an inevitable decrease in virus-induced mortality. However, this decrease in mortality may be at the expense of a decrease in the natural fecundity and/or the rate of growth and development of the members of the host population.

For the baculovirus the solution lies in a maximal rate of production of infective particles. The rate of release of these infective particles into an external environment is dependent on the virulence of the virus - the rate at which the virus kills its host. However the faster the virus kills its host, the smaller the yield of virus particles. Work on the gypsy moth (*Lymantria dispar* NPV has demonstrated an increase in average yield of polyhedral inclusion bodies (PIB) from 8.75×10^8 per larva from third instars to an average yield of 2.8×10^9 PIB per larva from fifth instars (Smith *et al* 1976; Shapiro,1981). Therefore to ensure maximal rate of production of virus

particles, the virulence of the virus must be high, but not so high that it kills the infected host at a small size which in turn reduces the potential number of infective particles which may be released.

Recent work on gypsy moth NPV has indicated that the virus may tend to an optimal solution, for although fourth instars produced only 80% of NPV produced by fifth instars, NPV from fourth instars showed approximately 3-fold more activity than NPV from fifth instars (Shapiro *et al*, 1986).Thus indicating that maximal production of infective virus occurs in fourth instar larvae, and that the virus in this case appears to have made a 'trade-off' between maximum virulence and maximum viral production.

Therefore, by definition, the factors which will increase the success of both host and virus respectively are diametrically opposed. It seems impossible that there should ever be a state of peaceful coexistence in this situation dubbed an "arms race" by Anderson and May. Thus there is more evolutionary antagonism than coevolution in the insect-baculovirus interaction.

Parker (1985) has attempted to extend this antagonistic relationship by considering how an increase in either component of the system will affect the other. This games theory approach considers host and virus as "players" trying to maximize their gain through certain kinds of interactions under the assumption that the other one is trying to do the same (Slatkin and Maynard Smith,1979). This approach takes into account not only the virulence or resistance levels, but also considers the population sizes. This in itself is important because population sizes of insect and baculoviruses determine the rate of encounter between the two, and thus effectively determine the level of selection pressures acting on the interaction.

Assuming that increases in virulence and resistance are not unlimited (thus preventing endless cycles of escalation) and that they therefore carry with them some cost, Parker (1985) concludes that two possibilities exist depending on whether virulence and resistance as variables are discontinuous or continuous. If the variables are discontinuous (on-off), the result will be coupled cycles of virulence and resistance. If variables are continuous then there will be an evolutionarily stable combination of the four variables, virulence, resistance and the two population sizes.

For the sake of clarity the possible costs of an increase in one or other of the major variables will be outlined here. In order for insects to increase their level of resistance, energy must be diverted to costly physiological resistance mechanisms from other areas such as ecological fitness, leading to a trade-off between fitness and resistance. Thus resistant phenotypes may be at a competitive disadvantage to susceptible insects in the absence of virus (Roush and Plapp,1982)

Turning to the virus, increasing virulence or pathogenicity increases baculovirus fitness but may bear its own costs, the primary one of which is that if the virus is too pathogenic, the insect will be killed very quickly. Since larvae are most susceptible when they are very young and small, it is most likely that they will contract viral infection at this stage. If they then die relatively rapidly, relatively few infective particles will be released. Increased virulence may be expensive in other ways: it is possible that the physical configuration of the virus may change with increasing pathogenicity which may alter its transmission potential. In addition, increasing the virulence of the virus may lead to epizootics in the insect population, lowering the abundance of available insect hosts whilst concurrently lowering baculovirus fitness.

7.4 Selection experiments in the context of baculovirus-insect evolutionary theory

How do the results of selection experiments obtained in this study correspond to current theories on insect-baculovirus evolution?

In the insect selection experiment, resistance may theoretically have been expected to increase since all costs were being ignored by the experimental regime, and the larvae were being subjected to relatively high viral selection pressure. No measurable increase in resistance was recorded, but a general size increase occurred in the larvae of the selected population These selected larvae were found to exhibit accelerated growth when compared with similar aged larvae in the control population. Therefore although no increase in resistance had occurred, the selected population had undergone demographic changes. These changes may have been an alternative strategy to increase resistance in the selected population. This type of change was thought to have occurred because the resistance level of the original population before selection was already relatively high, and had possibly 'plateaued' out. Thus it is possible that the genes responsible for major resistance changes did not possess sufficient variation to be acted upon by selection. Therefore selection was only able to act on the arrangement of background genes responsible for more minor changes - a fine tuning system. As a result of selection pressure acting on these background genes, larvae with higher growth rate were favoured. These were larger at every given stage than their control counterparts and should therefore have demonstrated greater resistance to virus than control larvae. The reason that no such increase was recorded in the selected population may have been due to the choice of larvae in the bioassay process This is discussed at more length in chapter 3

In the virus selection experiment, no increase in virulence occurred. The outcome for this type of selection experiment was less clearcut. Theoretically given sufficient

variation and the correct selection pressure the virus should evolve increased pathogenicity. The most obvious explanation is that little (i.e. insufficient) variation existed in the virus stock used in the experiment. However, a limited survey of the different samples of *Plodia interpunctella* granulosis virus world-wide revealed fairly low levels of variation available within the genome. Another explanation may be advanced within the context of insect-baculovirus evolution theory discussed in the earlier part of this chapter. It is possible that the selection experiments may not differ greatly from the situation 'in the field'. In nature, insects would normally expect to encounter small amounts of virus (as in the selection experiment), and thus it is possible that the virus has evolved 'optimum' pathogenicity, and thus the selection experiment is in effect duplicating conditions in the field and will have no further effect on the virus.Therefore pathogenicity of the virus, and thus the reproductive rate may be relatively stable at intermediate levels.

7.5 'Alternative' routes of evolution?

The work in the remaining two experimental chapters (chapters four and six) suggested the occurrence of other factors likely to affect the evolution of the insect-baculovirus system either directly or indirectly.

The discovery of a small number of greatly accelerated larvae in a population lead to the hypothesis that older females produced these accelerated larvae in an attempt to give them a competitive advantage over their siblings born at an earlier stage in the female's life, or that perhaps there is a particular genotype of female which produces only small numbers of these accelerated larvae and no other eggs. It was not possible to futher explore the latter possibility since only the single pair experiments did not provide sufficient data for analysis.The impact of these greatly accelerated larvae may be to aid survival of the insect population in even very extreme virus epizootics,

because of the great discrepancy in size between these larvae and those of the same age produced by a younger female. The very accelerated larvae will be less likely to be susceptible to virus than smaller larvae of the same age. This may be an alternative route for insect evolution avoiding the potential costs of increasing resistance.

The other ' alternative route' concerns the survival and continued success of the virus. Latency would provide the virus with a method of transmission that would not necessarily rely on increasing virulence. It would obviously still play a major role in viral success, but latency would provide the necessary back-up mechanism, which would prevent viral extinction. Theoretically if a baculovirus can no longer find a host in which it can replicate and successfully reproduce, it will be unable to survive. The problem is not immediate, since the thick protein coat or granulin is able to ensure the survival of the virus in the environment for a fairly long periods of time (see Hostetter and Bell,1985; Kaupp and Sohi, 1985 for reviews). However if the virus was able to be transmitted from insect to insect in a latent form this would circumvent the problem of both transmission and continued survival, and allow the virus to be in a position to begin maximal reproduction when conditions are favourable: insect density is high and insects are stressed and therefore more susceptible to viral infections.

7.6 Possible consequences of evolution in an insect-virus system

The evolution of the insect and/or the virus has important implications on both insect epizootiology and insect population dynamics, as well as the more obvious applied pest control aspects.The theory of insect-baculovirus coevolution as discussed above suggests that the insect will tend to evolve towards increased resistance to the virus, and that the virus will tend to evolve increased virulence. What then are the consequences and likelihood of such a course of action?

With the development of baculoviruses as pest control agents the most commercially important aspect of any evolution in the insect-virus system is the possible development of viral resistance in the insect. Such resistance would decrease the efficiency of the virus as a pest control measure, and make it subject to the same strictures as the current usage of pesticides.

Earlier work (see Table 3.1) demonstrates that insects can develop resistance to virus and that this resistance is usually genetically controlled.So far this resistance has proved to be total in only two cases, both involving the response of strains of *Bombyx mori* to infectious flacherie virus and densonucleosis virus respectively (Funada,1968; Watanabe and Maeda,1981).There appears to be no record of total non-susceptibility to baculovirus infections. However many species of insects have demonstrated strain differences in response to virus suggesting that development of some degree of resistance is not uncommon.

The impact of the evolution of increased viral resistance in insects, is dependent on the stability and the extent of the resistance demonstrated by the insect population, and the fitness of the resistant individuals in the absence of the virus.

Recent work by Fuxa and Richter (1989) on the stability of resistance, has demonstrated that resistance in the fall armyworm,*Spodoptera frugiperda* to NPV (which can be quickly selected for over seven generations in the laboratory) can be lost just as quickly when the viral selection pressure is removed: within three generations the population no longer exposed to virus, showed a significant difference in resistance from the continually selected population.

Examples of reversion of resistance may be found in other biological control

agents. *Musca domestica* selected for resistance to *Bacillus thuringiensis* (Bt) for thirty generations, and then not exposed for twenty generations was significantly less than theLD50 for flies exposed to Bt for 50 generations, though it was greater than the LD50 for flies never exposed to Bt (Harvey and Howell,1965). However the resistance of *Plodia interpunctella* to Bt was stable when the selection pressure was removed (McGaughey,1985).

Fuxa (1989) has suggested that reversion of resisitance in *S.frugiperda* may be due to the fact that in the absence of the virus there is selection against the resistance gene, possibly due to reduced fecundity of the resistant females.The selective disadvantage associated with a resistance gene, whether due to an energetic burden or to the disruption of some physical process, has long been recognised in insects demonstrating resistance to insecticides.However recently work on the sheep blowfly (*Lucilia cuprina*) has shown that this selective disadvantage may be reduced or eliminated by the subsequent evolution of the blowfly (McKenzie *et al*,1982).Therefore certainly in insecticide resistance it is possible to mitigate the potential disadvantages associated with resistance,and therefore increase the stability of the resistance gene in the population. Most modern insecticides have a fairly recent association with insects therefore it is possible that such stabilizing evolution has already occurred in the insect-baculovirus interaction, and that the levels of resistance to baculovirus will never be very extreme, and therefore unlikely to be as disruptive by reducing the efficiency of baculoviruses as greatly as pesticide resistance has done.

Turning to the commercial impact of evolution in the virus, current theories of insect-baculovirus evolution suggest that the virus should tend to evolve towards increased virulence.Strains of virus showing increased virulence would be valuable from a commercial point of view. Selection experiments have as yet been unable to produce increased virulence in a virus in a native host , however increases in virulence

by passaging in non-native hosts have been recorded (Smirnoff, 1963; Stairs *et al,* 1981). Mutagenesis has been used to obtain increased virulence in a mutant of the *Autographa californica* NPV (AcMNPV) with increased virulence in *Trichoplusia ni* (Wood *et al* 1981). Chemical treatment of *Autographa californica* NPV with 3-methylcholanthrene initially decreased its virulence by two thirds, but repassaging the virus and retreating it either once or twice increased its virulence, however this increase was not stable(McClintock and Reichelderfer, 1985). Certain methods of mutagenesis and passaging viruses through non-native hosts may both eventually be used to increase the pathogenicity of virus and thus enhance its economic potential, if problems of stability are overcome.

Aside from the more commmercial, biological control aspect of the insect-baculovirus interaction, the role of evolution in the progress of natural virus epizootics should not be overlooked. Natural epizootics involve spatial and temporal components which are influenced by the ecology of both the disease and the host, and also to an extent by their respective evolution.

The disease component of an epizootic may be divided into three parts, enzoosis, sporadic epizoosis and cyclic epizoosis (Evans 1986). The appearance of these different types of infection outbreaks depends at least in part on the virulence level of the virus involved. Most examples of enzoosis occur as a result of low host population density or low virus effectiveness.Density dependent virus infection normally occurs above defined host population threshold, so that conversely, disease remains enzootic below these thresholds. For example, *Gilpinia hercyniae* NPV has an extremely low disease incidence when host density is low (Neilson and Morris,1964). Other viruses may be enzootic as a result of low pathogenicity, either intrinsically or for environmental reasons, so that they are relatively independent of host density and less likely to become epizootic

Sporadic epizootics are characteristic of those that occur in hosts that exploit unstable habitats such as agricultural crops.High pathogenicity favours the development of sporadic epizootics. Virus persistent in the soil provides the initial innoculum which may be present only locally. Rapid increases in disease incidence and efficient secondary cycling of virus are therefore essential mechanisms if virus disease is to develop epizootically before the host plant is removed(Evans,1986)

Cyclic epizootics are most frequently associated with forest Lepidoptera, and at least eighteen species in North America and Europe display population cycles with average periodicities of eight to eleven years (Myers,1988). Long-term studies studies demonstrate that insect densities show trends of three to four years of increase, one to three years of peak density, and one to three years of decline.(Myers, 1988) Collapse of forest pest outbreaks has often been associated with the appearance of baculovirus epizootics . Anderson and May (1981) have modelled the role of infectious diseases in the cyclic appearance of pest outbreaks.The major conclusion of the model, as discussed earlier in this section, was that "highly pathogenic microparasites producing very large numbers of long-lived infective stages are likely to lead to non-seasonal cyclic changes in the abundance of their invertebrate hosts and in the prevalence of infection." Therefore a highly virulent virus favours the development of cyclic epizootics. When Anderson and May's conclusion was tested on field data for *Zeiraphera diniana* which exhibits population cycles over nine to ten year period, the agreement between theory and field data was remarkably good (Evans, 1986)

Therefore in the development of two and possibly all three types of epizootic is dependent on the evolution of maximal or high virulence of the virus.The only exception to this is when the virus exists enzootically within the population.Even here, recorded examples of density independent infections, such as that seen in *Colias*

philodice eurytheme are believed to be due to poor temporal transmission or rapid inactivation of secondary innoculum that lead to the maintenance of an enzootic state rather than low pathogenicity of the virus, which has been reported to be intrinsically very high (Martignoni and Milstead, 1962; Tanada and Omi, 1974; Evans, 1986). Thus baculoviruses probably exist at an optimally high level of pathogenicity in a natural environment, with the only likely possibility of evolution of decreased virulence occurring in the virus, if resistance had to be 'traded off' against the persistence of the virus, which is the other major virally controlled factor controlling the development of an epizootic.Such a selective disadvantage such as decreased persistence being associated with increased virulence, may help to explain possibly why virulence is kept to optimally high levels rather than evolving to extremes.

Natural epizootics generally act in a delayed density-dependent manner usually requiring several host generations to develop. The spread of the virus infection amongst the host population depends not only on viral components such as pathogenicity and virus dispersal, but also on the certain characteristics of the host population, such as the resistance level. This will depend on the heterogeneity of the population in response to the virus, which in turn will depend on previous exposure to virus and the stability of any resistance genes within the population. Generally, resistance will only develop in the insect population at times when viral selection pressure is high, during viral epizootics. After the epizootic the surviving population will exhibit a high level of viral resistance, however if this is not stable or the resistant individuals are at a selective disadvantage in the absence of the virus, resistance will be lost, leaving the population vulnerable again to viral attack.Fluctuating resistance levels may help to explain the extent and scale of viral epizootics when they occur in nature. Myers (1988) has proposed that varying degrees of disease susceptibility may explain several characteristics of the cyclic population dynamics of forest Lepidoptera.

It has been assumed on a theoretical basis that the virus will tend to evolve increased virulence, this would certainly be the case if resistance is stable, but less important if the resistance is unstable, or if resistance confers a selective disadvantage, since there would be a continual supply of susceptible insects. Therefore in a natural population with unstable resistance there would be little to gain from increasing virulence, and the virus would be optimally pathogenic.If resistance in the insect population is stable, it may still tend to be selected against especially in the long time periods of relative absence of any viral stimulus experienced in cyclic populations.Comprehensive long-term fieldwork would have to be carried out on the relative resistance levels of the host population and the pathogenicity of the initial virus innoculum in order to fully evaluate the evolutionary status of both host and virus. This possibly changing status would then have to be monitored over several outbreaks, and would be a complex and time consuming task.

This review of the theory of coevolution in insect-virus interaction from natural and commercial viewpoints has arrived at no simple conclusions of their relative effects. It is likely however that generally the virus will tend to exist at an optimal level of maximal pathogenicity, but probably not at extremes of pathogenicity which may be detrimental to its future transmission. The insect population given the necessary variation will usually develop resistance in the presence of the virus however the future stability of the resistance gene in the population may depend on any biotic selective disadvantages associated with resistance.

Summary

This thesis has explored the major aspects of the evolution of an insect-baculovirus system covering both the acknowledged routes of evolution and those which are

possibly slightly more unusual. Obviously such a study can never be complete, and much work remains to be done on further aspects of the work covered particularly that covering the so-called 'alternative' routes of evolution, latency and very accelerated larvae. It would also have been interesting to see whether mean insects of the same age but different size could produce the expected increase in resistance in the selected population.

APPENDICES

Appendices

I: Dose-mortality data on selected populations

II: Dose-mortality data on control populations

III: Dose-mortality data for selection regime

IV: Insect-instar: developmental stage of larvae

V: Dose-mortality data for three strains of *P. interpunctella*

VI: Estimation of virus concentration for each generation of viral selection

VII: Autoradiographs from intervening generations of virus selection

I : Dose-mortality data for selected insects

a) Dose-mortality data for selected insects (generation 5)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	49	46	93.9
-2	$3.33x10^5$	43	32	74.4
-3	$3.33x10^4$	47	26	55.3
-3.3	$1.83x10^4$	48	14	29.1
-4	$3.33x10^3$	48	5	10.4
-4.3	$1.83x10^3$	49	1	2.04
-5	$3.33x10^2$	50	1	2.0

b) Dose-mortality data for selected insects (generation 8)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	46	39	84.8
-2	$3.33x10^5$	48	27	56.3
-3	$3.33x10^4$	49	7	14.3
-3.3	$1.83x10^4$	47	8	17.0
-4	$3.33x10^3$	49	0	0

c) **Dose-mortality data for selected insects (generation 9)**

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	47	42	89.4
-2	$3.33x10^5$	48	39	81.3
-2.3	$1.83x10^5$	49	24	49.0
-3	$3.33x10^4$	50	9	18.0
-4	3.33x103	49	3	6.1
-5	$3.33x10^2$	46	1	2.2

d) **Dose-mortality data for selected insects (generation 10)**

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	48	48	100.0
-2	$3.33x10^5$	48	42	87.5
-2.1	$2.58x10^5$	40	29	72.5
-2.3	$1.83x10^5$	118	61	51.7
-2.6	$1.08x10^5$	37	12	32.4
-3	$3.33x10^4$	43	4	6.9

II: Dose-mortality data for control (stock) insects

a) Dose-mortality data for control insects (generation 0)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$5.36x10^6$	44	44	100.0
-2	$5.36x10^5$	41	40	97.6
-2.3	$2.41x10^5$	40	38	95.0
-3	$5.36x10^4$	42	32	76.2
-4	$5.36x10^3$	48	9	18.8
-4.3	$2.41x10^3$	49	1	2.0
-5	$5.36x10^2$	42	2	4.8

b) Dose-mortality data for control insects (generation 5)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-2	$3.33x10^5$	49	40	81.6
-3.3	$1.83x10^4$	43	5	11.6
-4	$3.33x10^3$	48	3	6.3
-4.3	$1.83x10^3$	49	3	6.1

c) Dose-mortality data for control insects (generation 8)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	51	19	37.3
-2	$3.33x10^5$	49	15	30.6
-3	$3.33x10^4$	49	10	20.4
-3.3	$1.83x10^4$	49	6	12.2
-4	$3.33x10^3$	49	1	2.0

d) Dose-mortality data for control insects (generation 9)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	50	31	62.0
-2	$3.33x10^5$	45	17	37.8
-3	$3.33x10^4$	54	6	11.1
-4	$3.33x10^3$	54	2	3.7
-5	$3.33x10^2$	49	0	0

e) Dose-mortality data for control insects (generation 10)

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	50	40	80.0
-2	$3.33x10^5$	48	32	66.7
-2.1	$2.58x10^5$	46	25	54.3
-2.3	$1.83x10^5$	45	18	40.0
-2.6	$1.08x10^5$	47	12	25.5
-3	$3.33x10^4$	40	4	10.0

III: Dosage-mortality data for selection regime

Generation of insects	Log dose* (nos/larva)	Number insects tested	Number dying of virus	%Mortality
F-1	5.73	318	268	84.3
F_0	5.42	367	348	94.8
F_0	4.73	362	307	84.8
F_1	4.43	397	364	91.7
F_2	4.43	429	245	57.1
F_3	4.43	288	142	49.3
F_4	4.43	274	112	40.9
F_5	5.52	341	252	73.9
F_6	5.52	328	288	87.8
F_7	4.52	169	42	24.8
F_8	5.52	244	181	74.1
F_9	5.52	199	181	90.9
F_{10}	5.22	271	141	52.0

* Log dose of numbers of virus particles per larva

Table to illustrating method of determination of instar size of *P. interpunctella*

Instar	Head capsule width*(HCW)	HCW(mm)
1st	3.0 - 4.0	0·15 - 0·20
2nd	5.5 - 6.5	0·28 - 0·33
3rd	8.0 - 9.0	0·40 - 0·45
4th	12.0 - 14.0	0·60 - 0·70
5th	17.0 -23.0	0·85 - 1·15

* Head capsule width at x2 magnification on Kyowa binocular microscope with graticule eyepiece

V: Dose-mortality data for comparison of three strains of Plodia interpunctella

a) Dose-mortality data for original stock insects

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	50	40	80.0
-2	$3.33x10^5$	29	23	79.3
-2.1	$2.50x10^5$	45	19	42.2
-2.3	$1.83x10^5$	40	15	37.5
-2.6	$1.08x10^5$	48	17	35.4
-3	$3.33x10^4$	45	10	22.2
-4	$1.83x10^3$	13	1	7.7

b) Dose-mortality data for PS/1Brown/X strain of *Plodia interpunctella*

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	45	40	88.8
-2	$3.33x10^5$	24	21	87.5
-2.1	$2.50x10^5$	19	16	84.2
-2.3	$1.83x10^5$	50	41	82.0
-2.6	$1.08x10^5$	47	32	68.1
-3	$3.33x10^4$	25	10	40.0
-4	$3.33x10^3$	10	1	10.0
-5	$3.33x10^2$	44	1	2.3

c) Dose-mortality data for 1/SM10/85 strain of *Plodia interpunctella*

Log_{10} virus dilution	Dose per larva (nos particles)	Total number of larvae per dose	No. of larvae dying of GV infection	Mortality (%)
-1	$3.33x10^6$	37	37	100.0
-2	$3.33x10^5$	37	36	97.3
-2.1	$2.50x10^5$	40	36	90.0
-2.3	$1.83x10^5$	42	37	88.1
-2.6	$1.08x10^5$	48	41	85.4
-3	$3.33x10^4$	47	32	68.1
-4	$3.33x10^3$	32	4	12.5
-5	$3.33x10^2$	53	2	3.8

VI: Estimation of virus concentration

Generation of virus	Number of virus particles	Number of latex particles	Ratio (V:L)	Concentration of virus*
F_1	261	1044	0.25	
	107	834	0.128	
	100	911	0.11	

			Mean = 0.163	2.67×10^9
F_2	513	262	1.96	
	492	275	1.79	
	369	182	2.03	

			Mean = 1.93	3.17×10^{10}
F_3	53	31	1.71	
	80	39	2.05	
	80	68	1.18	

			Mean = 1.65	2.70×10^{10}

Generation of virus	Number of virus particles	Number of latex particles	Ratio (V:L)	Concentration of virus*
$F_{4/5}$	918	268	3.43	
	979	267	3.67	
	1073	283	3.79	

			Mean = 3.63	5.95×10^{10}
F_6	23	376	0.061	
	96	1266	0.076	
	30	298	0.100	

			Mean = 0.079	1.30×10^{9}
F_7	89	381	0.234	
	164	355	0.462	
	28	688	0.041	

			Mean = 0.246	4.03×10^{9}

* Concentration of virus in numbers of particles per ml.

Fig 5.6 Restriction endonuclease digest of the generation 2 sample of granulosis virus used in virus selection experiment

Lanes as follows: 1 and 8 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4:BamHI, 5: EcoRI,6:Pst I, 7:Sal I.

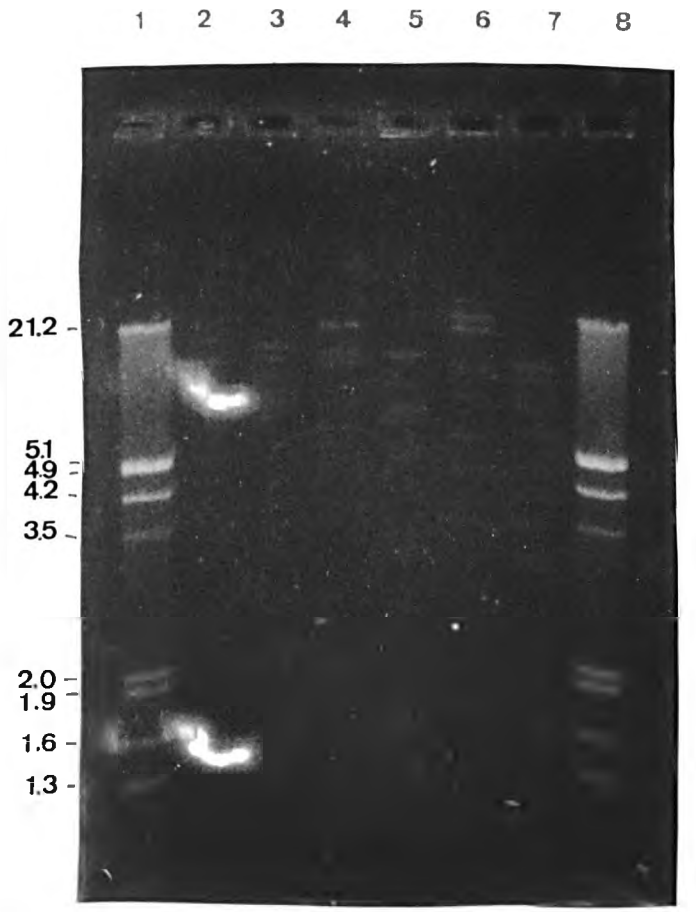

1
2
3
4
5
6
7
8
21.2
5.1
4.9
4.2
3.5
2.0
1.9
1.6
1.3

Fig 5.5 Autoradiograph of restriction endonuclease digest of the generations 2 and 5 samples of granulosis virus used in virus selection experiment.Details of hybridization in text. Autoradiographs exposed for 21 days.

Lanes as follows: 1 and 8 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4:BamHI, 5: EcoRI,6:Pst I, 7:Sal I.

2 5

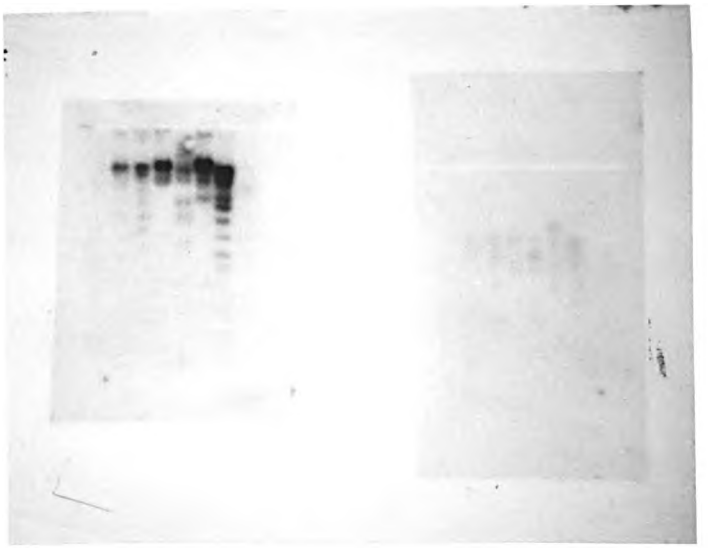


Fig 5.3 a Restriction endonuclease digest of the generation 3 sample of granulosis virus used in virus selection experiment.

Lanes as follows: 1 and 8 - Lambda (EcoR1/HindIII); 2:XhoI, 3: BamHI, 4:BamHI, 5: EcoRI,6:Pst I, 7:Sal I.

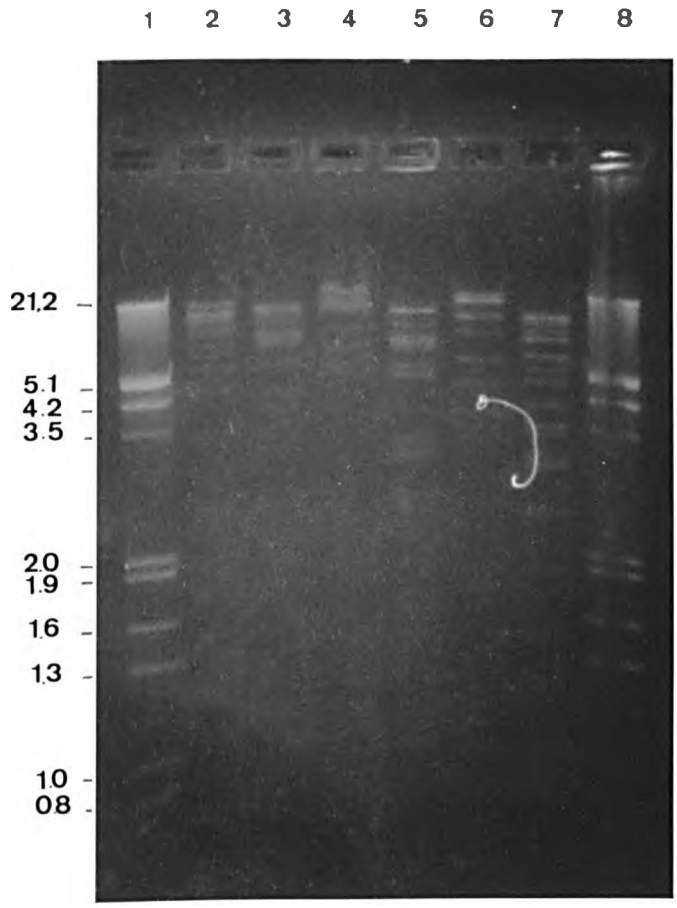

1
2
3
4
5
6
7
8
21.2
5.1
4.2
3.5
2.0
1.9
1.6
1.3
1.0
0.8

Fig 5.7 Autoradiograph of restriction endonuclease digest of generation 3 sample of granulosis virus used in selection experiment. Details of hybridization in text. Autoradiograph exposed for 3 days. (Different exposure times show different bands-results not shown here)

Lanes as follows: 1 and 8 Lambda (EcoRI/HindIII); 2:XhoI, 3: BamHI, 4: Hind III, 5: EcoRI, 6: Pst I, 7: Sal I.

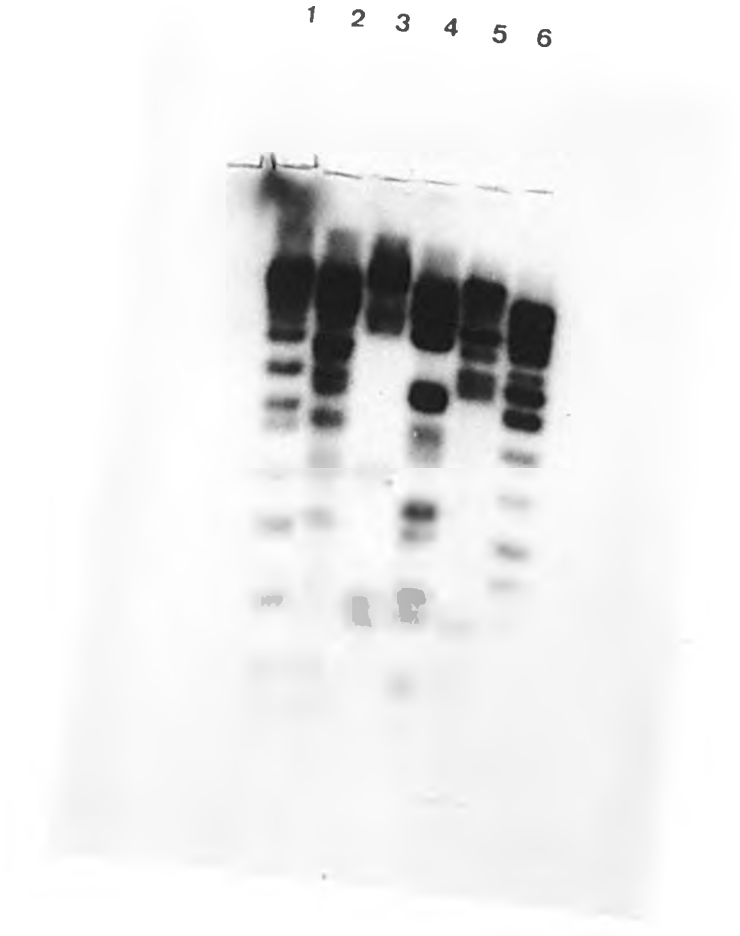
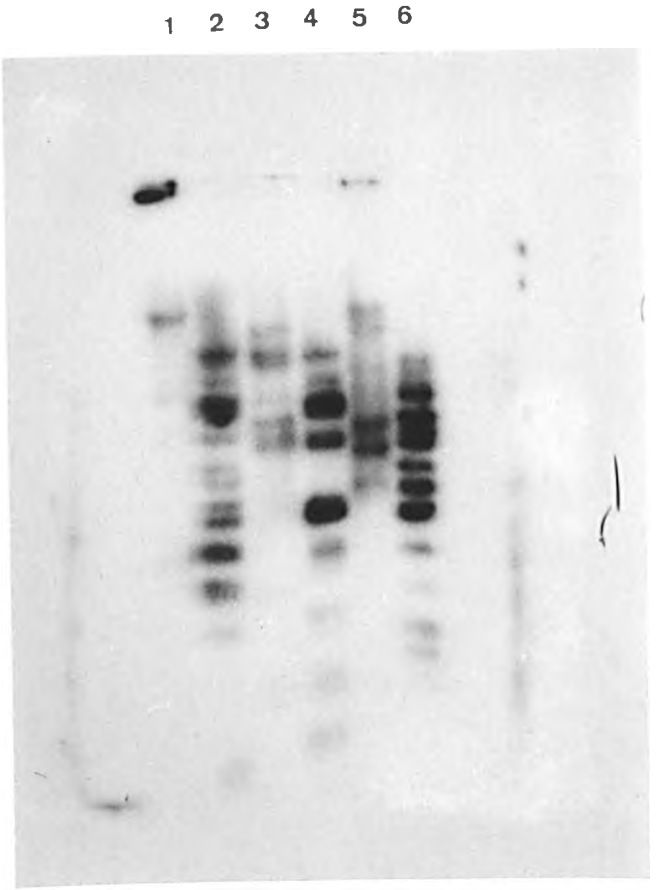

1 2 3 4 5 6

Fig 5.8 Autoradiograph of restriction endonuclease digest of generation 4 sample of granulosis virus used in selection experiment. Details of hybridization in text. Autoradiograph exposed for 3 days.(Different exposure times show different bands-results not shown here)

Lanes as follows: 1 and 8 Lambda (EcoRI/HindIII); 2:XhoI, 3: BamHI, 4: Hind III, 5: EcoRI, 6: Pst I, 7: Sal I.


1 2 3 4 5 6

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