THE PATHOGENS OF HELIOTHIS PUNCTIGERA WALLENGREN

by

D.J. COOPER
R.D.A., B.Sc.(Adelaide)

Department of Entomology,

Waite Agricultural Research Institute,

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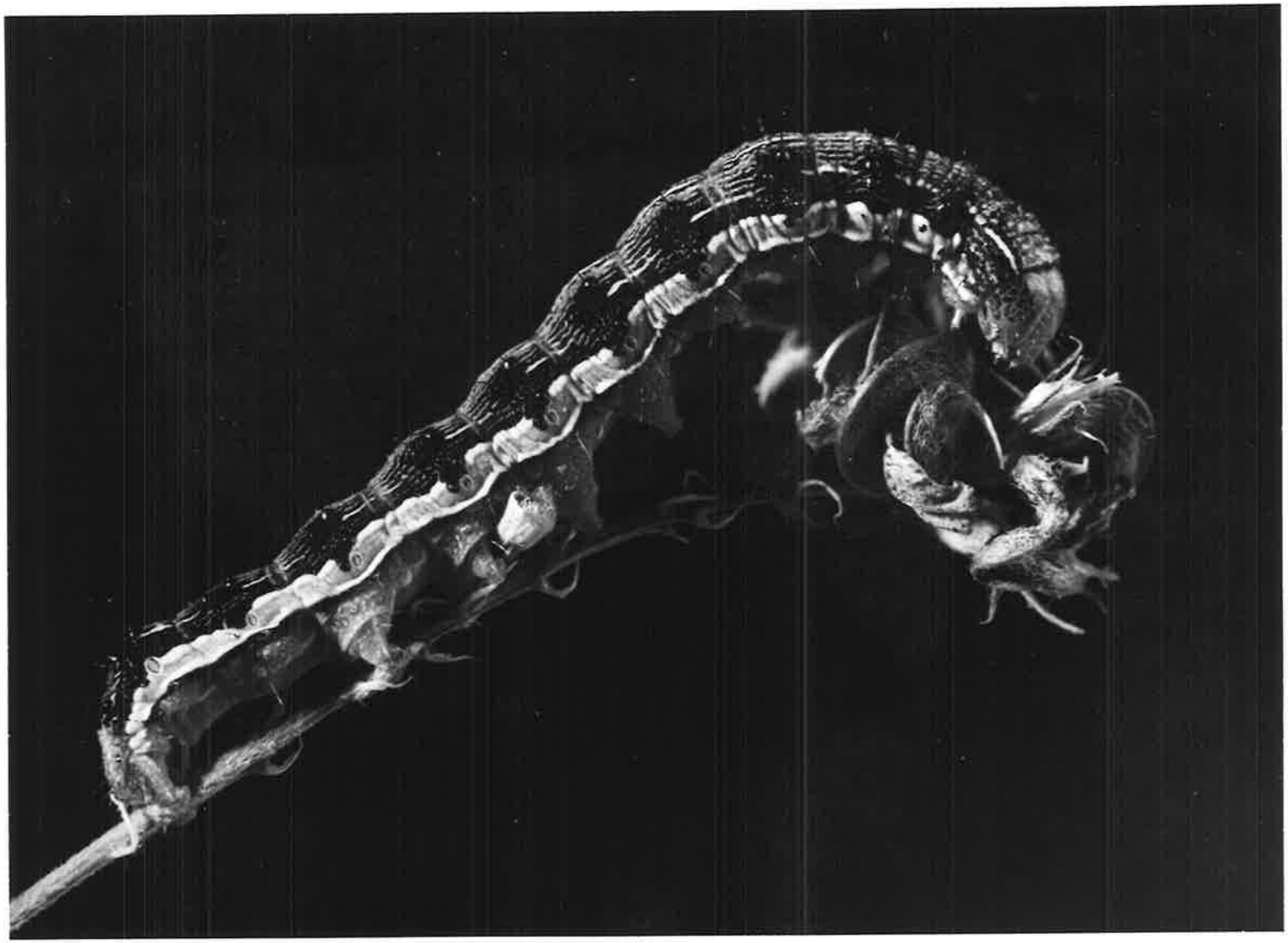
To Annie, Nicky and Patrick

Frontispiece

Sixth instar *Heliothis punctigera* larva feeding on lucerne seed pods. x6.



CONTENTS

SUMMARY

The susceptibility of Heliothis punctigera Wallengren, a pest of seed lucerne, to applied and naturally occurring pathogens was investigated. Bioassays were carried out using Bacillus thuringiensis against third and fourth instar larvae; results were sufficiently encouraging to continue the study in the field. Etiella behrii Zell., another pest of seed lucerne, was shown to be highly susceptible to B. thuringiensis. A subsequent field experiment indicated that populations of H. punctigera could be suppressed by B. thuringiensis.

A model for the prediction of target insect mortality was applied to data from the field experiment. Using the statistics of feeding rate of larvae, the initial deposit of B. thuringiensis spores on foliage and the rate of inactivation of the spores when exposed to environmental conditions, an estimate of average dose of spores was obtained. From this dose the expected mortality in the field for third instar larvae was estimated. This value was 66%, while mortality obtained in the field was 71%. The implications of the attainment of predictable field mortality as part of an integrated control programme are discussed.

A naturally occurring nuclear polyhedrosis virus is an important mortality factor in H. punctigera populations in summer and autumn. An understanding of factors leading to outbreaks of this disease might lead to eventual manipulation of epizootics. It was proposed that events such as rain or irrigation might transfer polyhedra from the soil to foliage, where larvae might ingest sufficient virus to initiate an epizootic. An immunofluorescent tracing technique was developed to detect polyhedra in conjunction with a method for washing polyhedra off lucerne foliage. This method was calibrated with respect to both recovery and infectivity of polyhedra.

The study was carried out in an irrigated lucerne stand for 2 seasons. Bioassays indicated that lethal amounts of polyhedra were present in the soil and that they were retained for long periods. Heavy overhead irrigation appeared to play no part in transferring polyhedra to foliage; however, a sudden shower of rain on one occasion coincided with a sharp increase in leaf counts and subsequently an epizootic developed. Hence it is proposed that whereas heavy watering probably washes off any polyhedra splashed up onto foliage, light overhead irrigation might well be used to contaminate the leaves and induce an epizootic. In addition some epizootics were probably not associated with rain or irrigation, hence it would appear that other factors are also involved.

Predatory insects were also involved in introducing virus inoculum into the host population. The pentatomid Oechalia schellenbergii (Guér-Mén.) readily feeds on virus-killed larvae and polyhedra were detected in the faeces for as long as 6 days after such a meal. Nymphs, which store food residues in the midgut until after the final moult, retained polyhedra for longer periods. Bioassays indicated that voided polyhedra were infective, although infectivity was reduced with increased time of retention. Field studies indicated that both O. schellenbergii and the predatory nabid Nabis tasmanicus Rem. fed frequently on virus-killed larvae.

The importance of rain, irrigation, predators and other factors likely to be involved in initiating epizootics are discussed.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

D.J. Cooper

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CHAPTER 1

THE CURRENT STATUS OF THE PATHOGENS OF HELIOTHIS SPECIES

1.1 Introduction

The genus Heliothis comprises several species of insects that are economically important throughout the world, and accordingly has been the subject of much study. Thus it is not surprising that a wide variety of pathogens has been found in Heliothis, and it is possible that more remain to be found. This thesis is concerned primarily with the use of pathogens (naturally occurring or applied) in regulating populations of H. punctigera Wallengren. However, instances of pathogens infecting other species of Heliothis will be mentioned in this introductory chapter, as in most cases cross-infectivity would be possible. It is not intended to describe each and every pathogen of the genus in detail, because some pathogens, e.g. bacteria and protozoa, have been recorded on only one or two occasions, and for the purposes of this study are not considered particularly important. Until recently the research emphasis in insect pathology has been largely devoted towards biological control - thus emphasis will be given to pathogens which are currently being used or have potential for use in controlling pest species of Heliothis. Emphasis will also be given to recent work in the field, including studies on newly discovered pathogens, as well as development of improved techniques for the efficient manipulation of known ones.

1.2 Baculoviruses

In a comparatively recent reclassification of invertebrate viruses (Vago et al., 1974), the genus Baculovirus was created; this genus comprises rod-shaped occluded viruses which have double-stranded DNA as the genetic

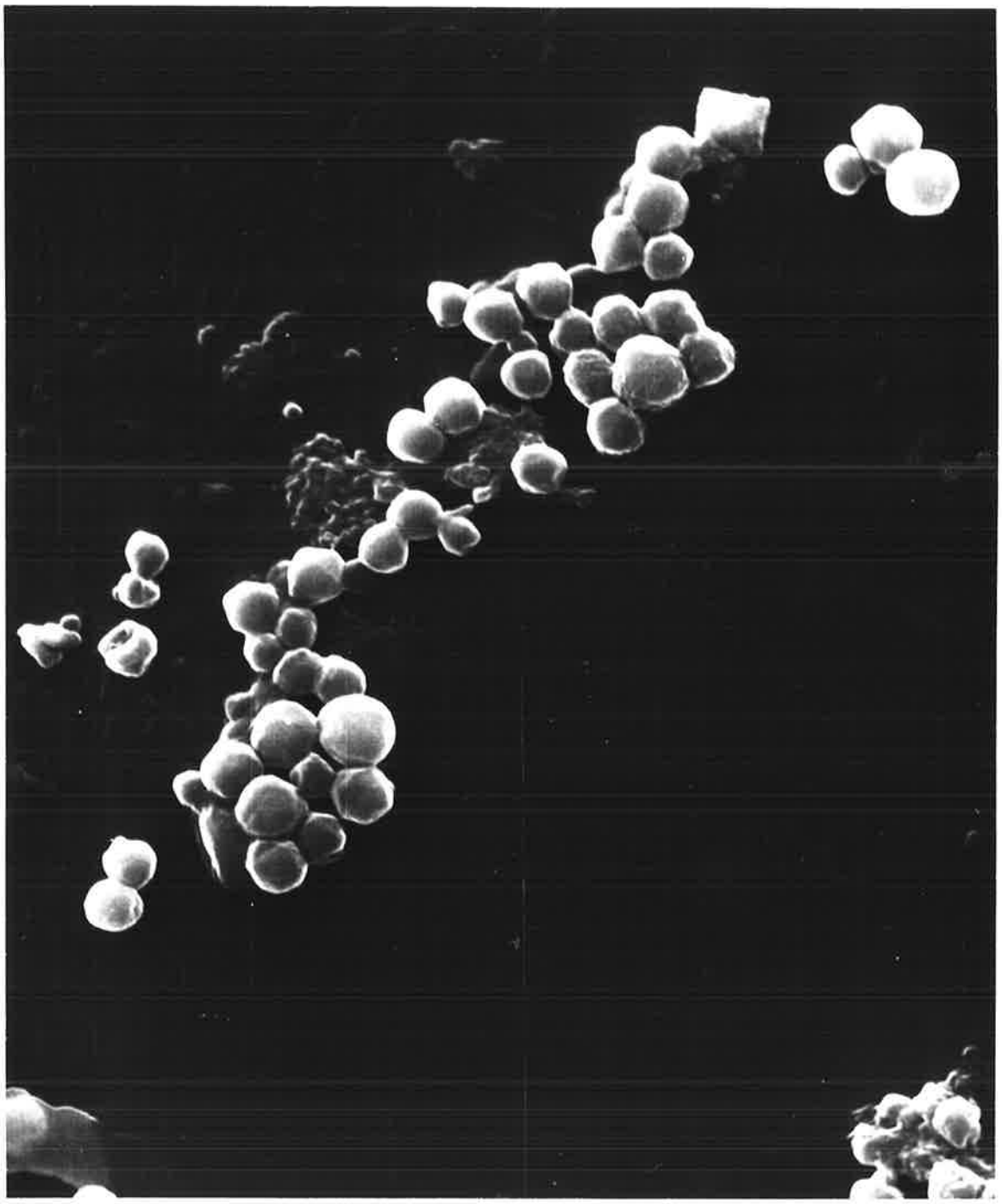
material. Thus the group is made up of the nuclear polyhedrosis viruses (NPVs) which contain many virus particles occluded in a polyhedral-shaped proteinic crystal, and the granulosis viruses (GVs) which contain 1 or, rarely, 2 virus particles occluded in a granular-shaped proteinic crystal (Wildy, 1971). Previously these viruses had been assigned to separate classifications, but comparison of the nucleic acids has demonstrated that NPVs and GVs are genetically related (Bellett, 1969). Examples of both viruses occur in Heliothis.

Nuclear polyhedrosis virus (singly embedded)

NPVs have been recorded in six species of Heliothis (Teakle, 1973). These are H. armigera (Hubn.), H. punctigera (Fig. 1.1), H. peltigera Schiff., H. phloxiphaga Grote and Robinson, H. virescens (F.) and H. zea (Boddie). It has been suggested that species from different parts of the world are susceptible to each other's virus strains (Stairs, 1971), and this has been borne out by work carried out on the NPVs of H. armigera and H. punctigera (Teakle, 1973). These two viruses could not be distinguished by serological techniques (agar gel diffusion using solubilized polyhedral protein as antigen), and in addition they showed reciprocal cross-infectivity. In another study (Ignoffo, 1965), larvae of H. virescens were shown to be significantly more susceptible ($P < 0.01$) than were larvae of H. zea to an NPV originally isolated from H. zea. The difference in susceptibility between the two species to Heliothis NPV was further substantiated in a subsequent report (Ignoffo, 1966).

Electron micrographs of H. armigera NPV were first published in 1956 (Smith and Rivers, 1956) while the structure of H. zea NPV has been described by Gregory et al. (1969), and that of H. punctigera NPV by Teakle (1973). These studies have indicated that in each case the occlusion

FIGURE 1.1 Scanning electron micrograph of purified nuclear polyhedrosis virus from Heliothis punctigera. x6000.

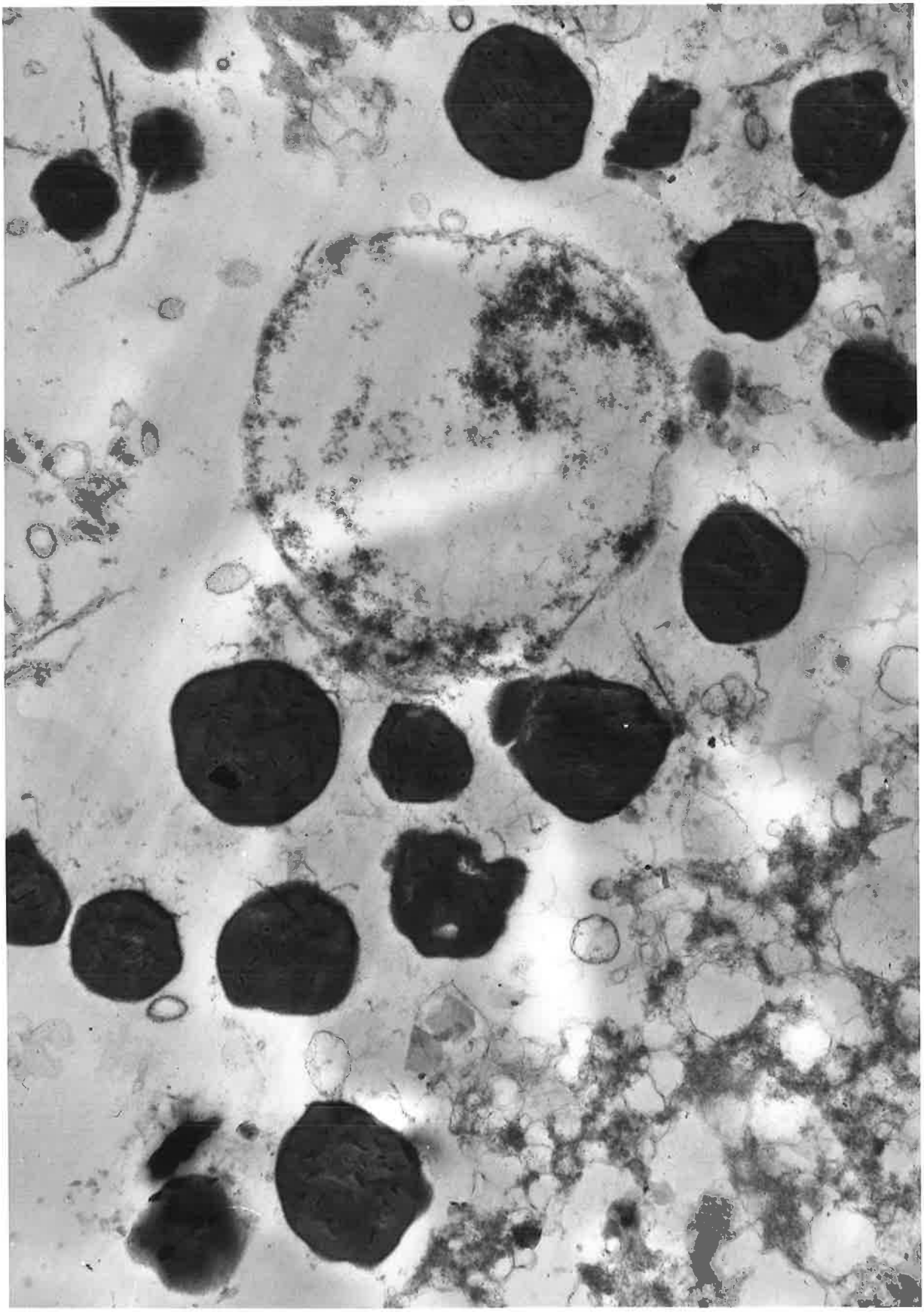


bodies contained singly embedded virions, i.e. the virions were singly eveloped within the polyhedral matrix. However, recently, a multiply embedded NPV, in which nucleocapsids occur in bundles inside a single envelope (Summers, 1975a), has been isolated from H. armigera in the U.S.S.R (see page 15).

The pathology of H. punctigera NPV has been described by Teakle (1973). The virus was observed to infect the midgut, haemocytes, fat body (Fig. 1.2), hypodermis, tracheal matrix and some cells in the muscular sheath and lining of the ventral nerve cord. The incubation period ranged from 2-14 days, depending upon the larval age at infection, with a mean time of 5.6 days.

NPVs may be quite prevalent in natural populations of Heliothis, and are often highly virulent. For reasons that are not well understood, a disease outbreak may occur leading to a spectacular collapse of a population (although in some instances, e.g. in H. zea, the virus tends to occur in nature solely as an enzootic pathogen (Burges, 1973)). Thus the high pathogenicity and specificity of Heliothis NPVs led them to be considered for use in integrated control programmes against pest species. Two species are particularly important pests in southern U.S.A., H. zea on cotton and sweetcorn, and H. virescens on cotton and tobacco. For a variety of reasons, the use of chemical insecticides to protect many crops has become unacceptable: these reasons include the well documented phenomenon of insecticide resistance, the resurgence of pest species after application of an insecticide, and the emergence of new pest species as a result of destruction of natural enemies after use of broad spectrum insecticides. Crops of cotton are particularly susceptible to attack by Heliothis species and indiscriminate use of insecticides has contributed largely to the near disaster of the cotton industries in El Salvador and Nicaragua, and to the total disaster of the cotton industry in the Matamoros-Reynosa and Tampico-

FIGURE 1.2 Section of fat tissue of third instar Heliothis punctigera larva which died from nuclear polyhedrosis, showing polyhedra and occluded virions. x25,000.



Mante areas of Mexico (Falcon, 1973). Serious problems also exist in the cotton industry in the Ord River, Western Australia, where H. armigera developed resistance to DDT.

In addition to being selective and sufficiently virulent to control the host, a pathogen must fulfil other requirements to be suitable for use in an integrated control programme (Falcon, 1973). It must be safe, easy to use, and sufficiently persistent to infect the target organism in the field. Finally, its use must be justified economically.

Field testing of Heliothis NPV

An NPV of Heliothis was first used in the field in Uganda against H. armigera (Coaker, 1958). The author used the virus in small scale tests on cotton plants, and the results were satisfactory. A dose of 4×10^9 polyhedra per plant produced approximately 80% mortality in third and fourth instar larvae. However, when similar experiments were carried out on a wider scale, none proved successful. The author attributed this to low populations and frequent movement of larvae from plant to plant.

In 1962 H. zea NPV was tried against H. zea on sweetcorn in the U.S.A. (Tanada and Reiner, 1962). NPV propagated in laboratory reared insects was applied both as a dust and a spray directly to the corn ears. It was found that the virus dust was superior, and used at a concentration of 2×10^8 polyhedra/g, was almost as effective as 5% DDT dust, the standard insecticide treatment at that time. The mean percent sound ears for the two treatments (virus and DDT) was 94.0 and 99.3 respectively.

In 1964 a study was carried out to test both the feasibility of propagating large quantities of H. zea NPV (Ignoffo, 1965) and the effectiveness of the pathogen against field populations of Heliothis on cotton, corn and grain sorghum (Ignoffo et al., 1965). Fourth instar

H. zea and H. virescens larvae were fed semi-synthetic artificial diet containing 3.6×10^6 polyhedra, and the larvae died of the virus in the final instar, yielding a 2,000- to 5,000-fold increase in the inoculum of polyhedra. It was concluded that quantities of virus large enough for field application could be propagated by this technique, and that the method could possibly be scaled up for commercial production.

When the above preparation was applied to field crops infested with Heliothis the following results were obtained: a single application of 2.46×10^{12} polyhedral inclusion bodies (PIB)/ha gave effective control of H. zea on grain sorghum. Similar results were obtained against H. zea on corn, although several applications of 1.48×10^{12} PIB/ha were necessary. H. zea and H. virescens on cotton were also effectively controlled, again by several applications of 1.48×10^{12} PIB/ha. In this study (cotton) the virus was used with methyl parathion, as it was necessary to control another insect pest, Anthonomus grandis (boll weevil). When the effects of the treatments were expressed in terms of cotton seed yield, the virus-methyl parathion compared favourably with other insecticides (Sevin-methyl parathion, Strobane-TDE and Strobane-DDT). However, when boll injury was used to evaluate effectiveness, virus-methyl parathion was significantly inferior to Sevin-methyl parathion.

Further field experiments were carried out in Kern County, California (Falcon et al., 1965), and this study was directed more towards possible integrated control than previous work. The effect of H. zea NPV, Bacillus thuringiensis and several insecticides on beneficial species was evaluated. Orius tristicolour (White), Geocorus pallens Stal, and several spiders are predators on H. zea eggs and larvae. It was found that neither pathogen influenced abundance of the two bugs, although B. thuringiensis appeared to be toxic to spiders. However, Carbaryl, Nia 10242 and Azodrin were highly toxic to all predators. Two applications of each treatment

were made, with the virus being applied at the rate of 1.48×10^{13} PIB/ha. When effectiveness in terms of damage to cotton bolls was evaluated, NPV and B. thuringiensis were superior to the insecticides, with the virus giving most effective long term control. The Nia 10242 and Azodrin treatments actually produced more damage than occurred on the untreated control plants.

Fernandez et al. (1969) tested resistant varieties of cotton along with H. zea NPV against both H. zea and H. virescens. Nectariless and glabrous species of Gossypium have been selected which reduce oviposition by the above Heliothis spp. In the experiment 30% fewer eggs were oviposited on the plants of a resistant variety, D2-321. Treatment with virus involved 17 applications of NPV over a 2 month period at the rates of 198-395 and 17-49 larval equivalents/ha (1 larval equivalent, L.E. = 6×10^{9} PIB). When yields of cotton seed were measured, it was found that the use of the virus increased the yield markedly compared to the control, both on resistant and non-resistant varieties. The resistant variety treated with virus yielded 10% more than a non-resistant variety also treated with virus. However, the number of replicates in these experiments was low. An assessment was made on the number of beneficial insects found after various treatments. Although numbers of predators were low overall, the highest number was found on one of the non-resistant cotton fields which had been treated with virus. Parasitism of eggs and larvae was also generally low throughout the experiment, but was much lower on insecticide-treated plots than on virus-treated or control plots.

Another integrated control system using Heliothis NPV was carried out in the Mississippi Delta (Allen et al., 1966). Three rates of virus (49, 124 and 247 L.E./ha) were compared with an insecticide treatment (toxaphane-DDT-methyl parathion). Predator levels on all plots were monitored throughout the trial. The Heliothis NPV-integrated control

programme compared favourably with the insecticide treatment, and in general only half as many applications of virus were necessary. The number of predators was significantly higher in the virus-treated plots, and the author stressed the importance of the timing of the first virus application of the growing season, to follow closely the peak in oviposition activity.

A synopsis of experimental work by L.A. Falcon using H. zea NPV on cotton was reported by Pinnock (1975). Briefly, trials conducted in 1969 using approximately 99 L.E./ha indicated that although larval numbers may be reduced, yields may not differ significantly from the controls. In 1971 4 treatments of approximately 99 L.E./ha did produce a 10% increase in yield, following a reduction in larval numbers of approximately 25%. Further work in 1971 indicated that differences existed in commercial preparations of the virus. Using approximately 99, 124 and 494 L.E./ha, one preparation produced consistently higher yields (6-10%) than the untreated control and another preparation produced consistently lower yields (2-8%). Larval counts did not differ between either preparation and the untreated control.

Falcon (1975) demonstrated the relative effectiveness of H. zea NPV (Viron/H) and key predators against the bollworm (H. zea) on cotton in the San Joaquin Valley in California. The systemic insecticide dimethoate (which is also used for Lygus bug control) was used to selectively kill all predators. The bollworm population then rose dramatically, compared to larval numbers in an untreated control plot. One half of the dimethoate - treated area was sprayed with an ultra-low-volume formulation of Viron/H, which had the effect of reducing the bollworm population by 50%, indicating that the NPV was approximately 50% as effective as the predators. Comparable differences in boll damage were also evident. The experiment demonstrated the strategy of controlling Lygus bug with dimethoate and then

using Viron/H to minimize subsequent resurgence of bollworm populations.

To date, Heliothis NPV has not been registered for use on any crops in Australia, although it is likely that registration will soon be granted for use of the virus against H. armigera and H. punctigera on cotton. The virus has been used in experimental work against these species on cotton, and results have varied from unsuccessful to moderately successful (Wardhaugh, pers. commun. 1977).

Problems in development

Although encouraging results have been obtained with the majority of field experiments performed so far, two major obstacles stood in the way of large-scale adoption of Heliothis NPV in integrated control (Falcon, 1973). These were, firstly, that high application rates were needed for control, and secondly, that because of low persistence in the field, frequent applications of NPV were necessary (Fernandez et al., 1969). The host crops and feeding behaviour of Heliothis species contribute largely to the first problem (Jaques, 1973). In sweetcorn, e.g., even a slight amount of damage to the cob results in that cob being unsaleable (Oatman, 1970). Thus the economic threshold in this crop is low, and compounding this fact is the incubation time of the virus, which may be 2-14 days. This situation may be contrasted with pest damage to pine forests, where quite a large amount of damage can be tolerated.

The feeding behaviour of H. zea causes particular problems in cotton. The larvae feed mainly on the inside of the cotton boll and so a high dose of the pathogen must be delivered to the larvae for the short time when they are feeding on exposed foliage. Thus high dosages are required. Various strategies have been adopted to alleviate the problem of rapid inactivation in the field - these will be discussed under "Latest

technology", page 12.

An additional problem in development is that industry is reluctant to become involved (Falcon, 1973). High costs exist in developing and testing a pathogen for registration (see below) and this cost must be borne by the prospective manufacturer. The necessary pharmacological and toxicological data required to demonstrate product safety are considerable. Unlike chemical insecticides, micro-organisms cannot be patented (Rogoff, 1973) so there is no guarantee of product protection. Thus a competitor could isolate the active agent from one company's formulation and go ahead and market a second formulation, with little outlay on research and development. A third problem is that Heliothis NPV is a relatively narrow spectrum insecticide compared to most chemicals (and to B. thuringiensis) and high volume sales sufficient to justify development costs might not eventuate. For a chemical insecticide, annual sales in the order of US$2.5 - 10 m are necessary to provide a return on capital invested. Lastly, Heliothis NPV must be propagated in live insects, which is both cumbersome and time-consuming.

Registration of Heliothis NPV

When a chemical insecticide is considered for registration, a prescribed level of tolerance, based on toxicological data, is applied (Heimpel, 1971). In the case of a microbial insecticide containing a living pathogen capable of propagating in nature, the situation is somewhat different. In the U.S.A. the Environmental Protection Agency (EPA) now requires such a control agent to be proved sufficiently safe to allow the granting of an exemption from tolerance. Before such an exemption was granted for Heliothis NPV, however, a series of tests prescribed by the Food and Drug Administration (United States) were carried out to evaluate the safety or otherwise of the virus. These tests were as

follows (Ignoffo, 1973):

1. Acute toxicity-pathogenicity

   Per os diet

   Inhalation

   Dermal

   Intraperitoneal injection

   Subcutaneous injection

2. Sensitivity-irritation

   Eye

   Skin

3. Subacute toxicity-pathogenicity

   Diet

   Inhalation

   Subcutaneous

   Teratogenicity

   Carcinogenicity

   Replication potential

   Phytotoxicty

   Invertebrate-specificity

Animals used in these tests were laboratory animals (rats, mice, rabbits and guinea pigs), and birds, fish, oysters and shrimps were used in the per os acute toxicity tests. Human subjects were used in skin sensitivity tests and human and primate cell cultures were used in replication potential tests.

The outcome of all tests was that the virus was declared completely safe for all species apart from its host, and on 8th December, 1970, the Food and Drug Administration (United States) officially granted Heliothis NPV the status of temporary exemption from a requirement of tolerance (Ignoffo, 1973). The temporary permit (which has been renewed

yearly), authorized the use of the virus on seed cotton in 19 States of the U.S.A. The permit was temporary because although the authority was satisfied with the safety data, it was not fully satisfied with efficacy; most chemical insecticides registered can claim close to 100% mortality of the target insect, but in the case of Heliothis NPV, this figure is often as low as 50%. However, it has been demonstrated (Allen et al., 1966) that the combined effect of Heliothis NPV and parasite and predator species can greatly improve the effectiveness of a treatment. The EPA is now in charge of safety testing and registration of new pesticides, and the policy now adopted is that efficacy should not be measured by the number of insects killed outright but by the product's effectiveness in an integrated control programme. The safety aspects of employing baculoviruses for insect pest control was further investigated in a joint EPA-USDA (United States Department of Agriculture) symposium (Summers et al., 1975).

More recently, however, there has been cause for some concern over the safety of baculoviruses for insect control, and doubts have been raised as to the likelihood of another baculovirus, Autographa californica NPV, also being registered as a viral insecticide. This virus would appear a suitable candidate for registration, as it has a wider host range than Heliothis NPV, which includes Heliothis species (Vail and Jay, 1973; Vail et al., 1978). However Hess et al. (1979), after infecting larvae of A. californica and Trichoplusia ni with the multiply embedded A. californica NPV (AcMNPV), observed a small icosahedral virus in the tissues of both species. The small virus occurred either alone or with AcMNPV virions in the nuclei of infected cells, and was also found occluded in polyhedra, either alone or with AcMNPV virions. Subsequent work by Morris et al. (1979) found the icosahedral virus to be a 35 nm RNA virus which possessed similarities to a mammalian calicivirus. These findings substantiate

earlier speculations (Tinsley and Melnick, 1973) that the use of viral insecticides could possibly constitute an ecological hazard and the findings may impede progress in the field until more is known about the purity or otherwise of viral preparations.

Latest technology

Application rates for virus have traditionally been expressed in terms of larval equivalents. However, this leads to quite large variations between doses, as the yield of polyhedra varies from insect to insect in the same instar, and even bigger differences exist between instars. Added to this, the number of infectious units, i.e. virions, within an inclusion body varies (Gregory et al., 1969). Use of this parameter to express dosage rates has been criticised (Pinnock, 1975). An improvement in this area would be obtained by standardizing preparations on the basis of infectivity. Whereas in some of the earlier work a somewhat arbitrary application rate was often decided upon, now a more precise dose rate can be estimated by carrying out a bioassay (Burges and Thompson, 1971). A number of insects are dosed with the virus, and usually different groups are given different dose rates. The proportion of dead insects are scored after a number of days (8-12 with Heliothis NPV) and this enables one to calculate the dose that would result in 50% mortality, which is called the "50% effective dose" or the ED_{50}. From this it is possible to make a recommendation for field rates.

The use of feeding (gustatory) stimulants has been shown to increase the effectiveness of NPV in the field. Andrews et al. (1975) used a cottonseed oil bait in conjunction with Heliothis NPV to increase mortality of H. zea and H. virescens larvae compared to control experiments using NPV without bait. Bell and Kanavel (1978) used NPV from A.

californica formulated with cottonseed flour, crude cottonseed oil and sucrose against H. virescens on cotton plants in greenhouse experiments. A mortality of 93% was obtained with the NPV-bait formulation, compared to a mortality of 33% when the plants were treated with virus in water. Ignoffo et al. (1976) developed an adjuvant which, when used with Heliothis NPV, acted as a feeding stimulant, a sunlight protectant and an evaporation retardant when tested against H. zea larvae.

An investigation reported by Falcon (1973) has indicated that virus can be disseminated by employing light traps. Adult insects attracted to a UV light source were automatically contaminated with NPV, and were then released to distribute the pathogen to other adults at mating, to eggs and to crop foliage. Non-target insects attracted to the light also disseminated the virus, but were not harmed due to the pathogen's intra-generic specificity.

Conventional application techniques have also been improved on. As the virus must be ingested to infect the target insect, adequate crop coverage is essential. It was found that improved control of Heliothis was obtained by increasing the spray volume, or by reducing the drop size. In one study (Ignoffo, 1973), virus applied to cotton at 9.9 L.E./ha with a mister blower was as effective as 10 times the dose applied with a knapsack sprayer, and doubling the volume per acre was as effective as doubling the dose. Other work (Falcon, 1973; Davidson and Pinnock, 1973) showed that by using ultra-low-volume spray equipment which delivered a droplet size of less than 50 microns, and relying on wind drift to carry the pathogen, potent deposits could be delivered to foliage at considerable distances from the source, i.e. an LD_{50} at 122 m and an LD_{10} at 305 m.

A serious shortcoming pertaining to the use of Heliothis NPV as a microbial insecticide is the rapid inactivation of the virus by sunlight (see page 81). Various UV absorbent materials have been employed to extend

the useful life of the virus (Ignoffo and Batzer, 1971; Jaques, 1973, 1977). Carbon powder incorporated in the formulation prolonged activity for approximately 6 days (Ignoffo et al., 1972). Similar effects were observed with the use of propyl gallate, and by micro-encapsulating the polyhedra in carbon and ethylcellulose. Bull et al.(1976) formulated polyhedra of Heliothis NPV into capsules, bound together with digestible, water-insoluble polymers. One preparation, incorporating titanium dioxide, and another, incorporating carbon black, provided much improved protection against both artificial ultraviolet light and sunlight, compared to commercial virus preparations. Yields of cotton from plots sprayed with these formulations did not differ significantly from the yields of plots sprayed with a standard insecticide mixture, while yields of all treated plots were significantly greater than in untreated plots.

The addition of buffers to lower the pH of cotton leaf surfaces also produced favourable results (Falcon, 1971a, 1973). Polyhedra are soluble in weak alkali (Bergold, 1963), and it seemed likely that dissolution would occur on the alkaline cotton leaf surface, exposing the virions to UV radiation which would quickly inactivate them. The addition of a proprietary line buffer, Colloidal Buffer-X$^{(R)}$ (ingredients not stated) to the formulation neutralized the leaf surface, thus extending the half-life.

All commercial production of Heliothis NPV is carried out by propagating the virus in live insects. A large proportion of the cost of the finished product must be attributed to rearing these insects. Several Heliothis cell lines exist (Ignoffo, 1973; Hink and Ignoffo, 1970) and it is envisaged that large savings in production costs could be made if NPV could be propagated in a cell line. Bombyx mori NPV has been propagated in an Antheraea eucalypti cell line, and in some strains of this line 20% of the cell population produced polyhedra (Hink, 1975). To date, however,

attempts to propagate intact Heliothis virus in cell cultures has not been entirely successful (Ignoffo et al., 1971). Infectious virus was passed 7 times through a cell line derived from the ovary of H. zea but no free virions or inclusion bodies containing virions were produced. The infectious agent in this case was probably naked DNA. Attempts are also being made to produce virus by the use of fermentation techniques (Ignoffo, 1973). Patents have been granted in South Africa for the production of insect viruses including Heliothis virus in the cells of Saccharomyces and Bacillus. Results of successful replication in these systems have not as yet been published.

H. armigera multiply embedded NPV

A multiply embedded NPV has been isolated from H. armigera. To my knowledge no reports of the biology or pathology of the virus have yet been published, although physico-chemical and serological studies have been carried out (Summers and Smith, 1978; Pritchett et al., 1979). The virus will also infect H. zea (in which it is highly virulent), H. virescens (intermediate virulence) and will also infect Spodoptera exigua, Spodoptera frugiperda and T. ni. The virus was isolated from H. armigera populations in the southern U.S.S.R. apparently near Tashkent (Hamm, pers. commun., 1979). It will be most interesting to compare the virulence of this virus with singly embedded Heliothis NPV: NPVs of T. ni occur as both singly embedded and multiply embedded forms, and the multiply embedded form is apparently more infective (Tompkins et al., 1969). This fact plus the wide host range of the virus might make H. armigera multiply embedded NPV a most suitable candidate for a biological control agent.

Granulosis virus

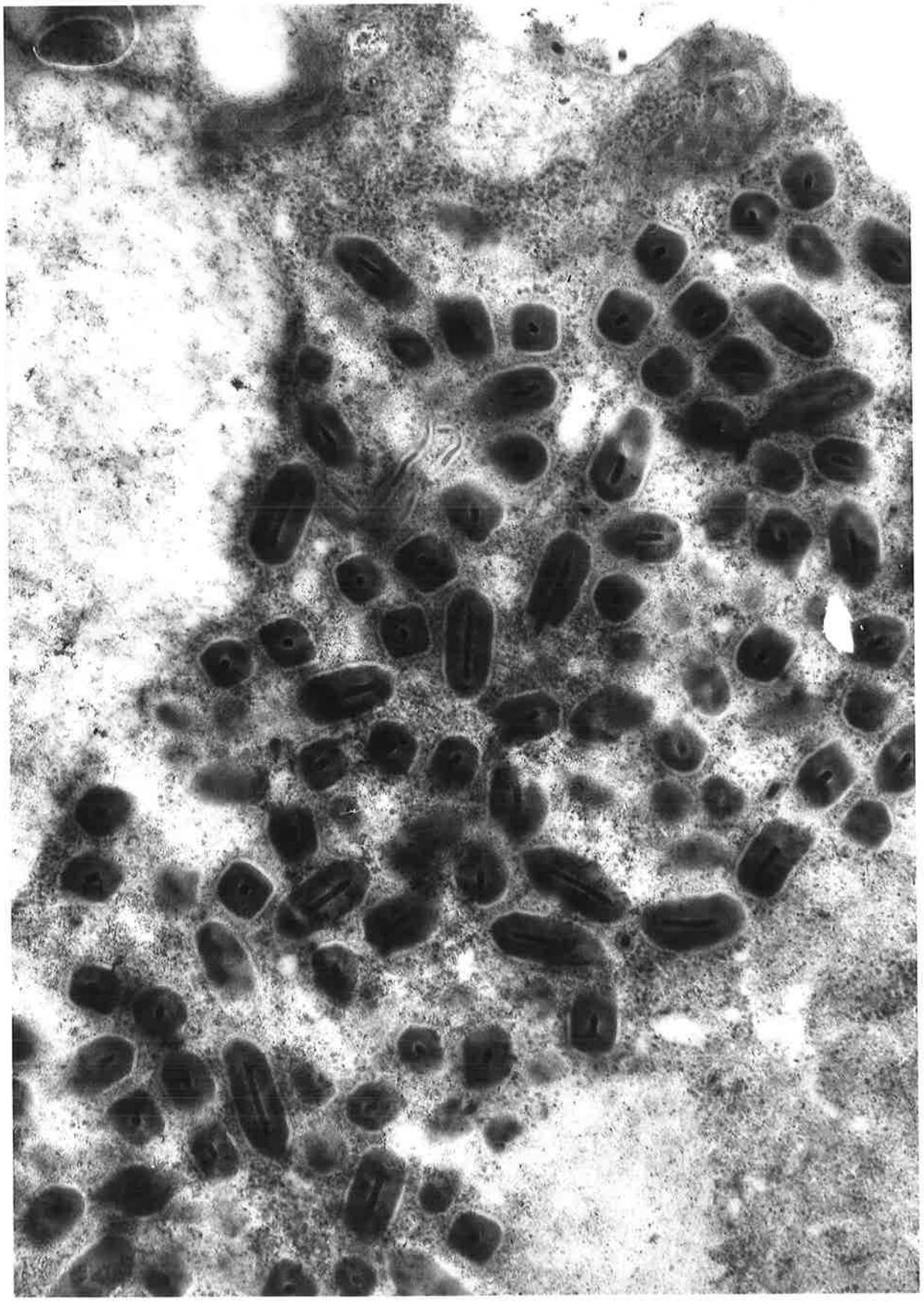
A GV has been reported in 3 species of Heliothis (Falcon et al., 1967; Gitay and Polson, 1971; Teakle, 1974). Falcon et al. recorded such a virus in H. zea, and stated that the mean capsule size was 587.2 x 252.5 nm, and that the diseased insects showed few external signs and symptoms, apart from a reduction in size. Gitay and Polson reported a GV in H. armigera in which the hypodermis was infected, and Teakle reported a GV in H. punctigera in Queensland and measured mean capsule size as 376 x 168 nm. The larvae in this case were enlarged and cream coloured and the fat body was the only organ infected. A GV has also been recorded in H. punctigera in South Australia (see page 68) and the capsule morphology and symptomatology agree closely with Teakle's description (Fig. 1.3). Thus it would appear that there exist at least two if not three GVs in Heliothis (Vaughn, 1974) - serology and cross infectivity studies have not as yet been attempted.

Although Heliothis GVs may be quite virulent (Teakle, 1974), they are not worth considering as applied microbial control agents. The incubation time is particularly long, 13-19 days in H. punctigera. This is far too long for an insect which by and large spends most of its larval life feeding on the marketable part of the host crop (Jaques, 1973). However, the GV of H. punctigera is undoubtedly an important natural pathogen. The virus is almost certainly enzootic in natural populations, and was observed to infect 11% and 10% of larvae collected on lucerne in Queensland in two subsequent years (Teakle, 1974).

1.3 Iridescent viruses

Iridescent viruses have been recorded in H. armigera (Carey et al., 1978) and in H. zea (Stadelbacher et al., 1978). Carey et al. isolated a

FIGURE 1.3 Section of fat tissue from fifth instar Heliothis punctigera larva with granulosis virus infection. Note virus rods within capsules. x37,000.



130 nm iridescent virus (which they termed type 21) from a single diseased H. armigera larva from Malawi in 1974. Stadelbacher et al. isolated a 145 nm iridescent virus from field populations of H. zea in Bolivar County, Mississippi. The DNA content of this virus was 13.97 ± 1.58%. Infected larvae exhibited an iridescent lavender-blue, blue or blue-green colour. Attempts to infect test larvae per os were unsuccessful, but the virus was highly infectious when larvae were inoculated by intrahaemocoelic injection. The authors suggested that a possible route of transmission of the virus in the field was by the parasitic nematode Hexamermis, which was common in field populations of H. zea (39.4-46.7%), and was associated with 2 out of 5 larvae infected with the iridescent virus. Due to the apparent inability to infect per os, it is unlikely that this virus would prove suitable as a microbial control agent.

1.4 Cytoplasmic polyhedrosis virus

A cytoplasmic polyhedrosis virus (CPV) has been reported in Heliothis (Smith and Rivers, 1956). There have been no reports of the virus in Australia, but in the U.S.A. CPV often invades insectary cultures of Heliothis. Unlike NPV, CPV produces a more debilitative disease (Mery and Dulmage, 1975) causing such symptoms as loss of body weight, weakness, and a longer developmental cycle. Recent investigations (Sikorowski et al., 1973; Mery and Dulmage, 1975) have shown that transmission of the virus to subsequent generations is not transovarial. Surface sterilization of eggs eliminated any disease in succeeding generations.

CPVs do not have such a narrow host range as NPVs. While it is clear that Heliothis NPV is genus-specific, the same or closely related CPVs may infect several families. Thus Vaughn (1974) reports that CPVs

infecting six families of Lepidoptera are closely related serologically.

The CPV of Heliothis has never been seriously considered for use as a microbial control agent. Apart from low specificity and comparatively low virulence, the virus is unacceptable for a third reason. CPVs contain RNA as the genetic material, and the group appears to be quite closely related to some of the vertebrate RNA viruses. CPVs are structurally similar to the reoviruses of vertebrates (Summers, 1975b), and the CPV of B. mori was found to share in vitro RNA polymerase activity with a reovirus (Lewandowski et al., 1969). Registration authorities would undoubtedly consider such a virus unsafe for large scale distribution, as the chance of mutation to a mammalian pathogen would always exist (Tinsley and Melnick, 1973).

1.5 Bacillus thuringiensis

B. thuringiensis is a widely used control agent, and has a broad host range in the Lepidoptera. It has been the subject of an intense amount of research over the past 15 years, and has been registered for use in the U.S.A. on 20 crops since 1958 (Falcon, 1973). It is my intention to review only the work that has been associated with the use of B. thuringiensis on Heliothis species, and with emphasis on recent developments.

B. thuringiensis causes a rapidly fatal disease in susceptible insects when ingested. Paralysis of the midgut occurs in about 20 minutes and the insect stops feeding. For insects with voracious feeding habits, such as Heliothis, this is a distinct advantage.

Earlier work to control Heliothis species with B. thuringiensis often met with failure (Falcon, 1971b). Applications of B. thuringiensis to H. zea on corn were not always successful because of insufficient doses, and variation in potency of preparations used. More success was met in

controlling H. virescens in tobacco, where this insect is considered the major pest. Integrated control programmes were initiated (Gentry et al., 1969) and were shown to be as effective as chemical insecticides in reducing pest damage. Large quantities of B. thuringiensis are currently used in the production of insecticide-free tobacco.

Attempts to control H. zea in cotton with B. thuringiensis have met with only mediocre success. A major problem, also encountered in Heliothis NPV spray programmes, is that the feeding habits of the larvae limit the time for which they are exposed to contaminated foliage, and consequently high application rates are necessary. Thus one preparation of B. thuringiensis, Thuricide, had to be used at the rate of 45 ℓ of concentrate/ha to achieve satisfactory control (Falcon, 1971b). Use of light traps, UV protectants, and more precise timing of applications were necessary to make control more economic. Selection of strains of B. thuringiensis more pathogenic to Heliothis species was also carried out. Rogoff et al. (1969) tested several strains against H. zea and recorded var. alesti as being the most potent of the strains not producing the β-exotoxin. Dulmage (1970) isolated a new strain of B. thuringiensis serotype III (var. alesti) which produced higher levels of δ-endotoxin. The strain was called HD-1, and was found to be a new type III serotype and was named "kurstaki" serotype IIIb (de Barjac and Lemille, 1970). The insecticidal activity was found to be 15-30 times higher in the new strain, and was tested in the field on cotton against H. zea and H. virescens in the same year (McGarr et al., 1970). The new preparation, used at the rate of 1.06-1.56 x 10^{11} I.U.*/ha, gave what was in the author's opinion the first effective control

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* A primary reference standard of B. thuringiensis, E-61, is held at the Institut Pasteur, Paris, and is assigned a potency of 1000 International Units/mg. Potencies of B. thuringiensis preparations are expressed relative to this standard.

of these two species of Heliothis on cotton, and compared favourably with calcium arsenate (28.2 kg/ha). However, several applications of B. thuringiensis were necessary, and the rate used corresponded to a total of 9.0-13.5 kg/ha. Thus, even with a highly potent strain, the use of this pathogen on cotton is not yet economic.

1.6 Microsporidia

Nosema heliothidis Lutz and Splendor

N. heliothidis is a microsporidian parasite of H. zea and H. virescens (Kramer, 1959; Gaugler and Brooks, 1975). The organism has been recorded in Heliothis populations in Queensland (Teakle, 1977a), although the incidence was low. An unidentified microsporidian was recorded in H. punctigera on one occasion in South Australia in 1975 (Pinnock and Cooper, unpublished) which may have been N. heliothidis.

Infection with N. heliothidis tends to be chronic and debilitative, and does not contribute directly towards mortality. However, it is clear that N. heliothidis does reduce the reproductive potential of H. zea (Gaugler and Brooks, 1975) by reducing fecundity, longevity, and increasing the incidence of "locked" matings. Diapause intensity and duration are also reduced. Such effects no doubt serve to limit the increase of natural populations. It is unlikely that N. heliothidis would be suitable as a microbial control agent because of relatively low pathogenicity.

Vairimorpha necatrix

Another microsporidian, V. necatrix, may have potential as a control agent (Fuxa and Brooks, 1979). This organism has a relatively wide host range among Lepidoptera (including Heliothis) and produces a large number of spores in the fat tissue of an infected host. A feasibility study

carried out indicated that it would be possible to mass produce V. necatrix in H. zea larvae, using surface contamination of the pathogen on artifical diet.

1.7 Fungi

Fungal pathogens tend to be rather non-specific, and so it is not surprising that occasional instances of Heliothis species infected with entomopathogenic fungi are recorded.

Beauveria bassiana

B. bassiana has been recorded in pupae of H. punctigera in South Australia on 2 occasions (Cooper and Mew, unpublished), but no larvae have been observed infected with the fungus. Low instances of B. bassiana have also been recorded in Heliothis larvae in Queensland (Teakle, 1978). The contribution that this pathogen has to natural mortality of Heliothis is probably slight, although the mortality of less obvious stages of the host, e.g., pupae overwintering in the soil, has not been investigated to any extent.

Nomuraea rileyi

This fungus, previously Spicaria rileyi, was renamed N. rileyi by Kish et al. (1974). The fungus has not be recorded in South Australia, but epizootics of N. rileyi occur in H. armigera and H. punctigera populations on soybeans, maize and sorghum in Queensland (Teakle, 1978). The fungus has also been recorded on H. zea on cotton crops in Mississippi, where epizootics occur which on occasions cause 100% mortality (Mohamed et al., 1977). An investigation was carried out by Mohamed et al. to determine susceptibility of H. zea to N. rileyi at various temperatures.

It was found that third to fifth instar larvae were the most susceptible. Larvae were most susceptible at 20° and 25°C, with maximum mortalities of 80% and 70% respectively being obtained at these two temperatures. At 20° the LD_{50} (spores/mg body weight) were 3.0×10^4, 2.4×10^4 and 1.9×10^4 in third to fifth instar larvae respectively.

Ignoffo et al. (1978) carried out field tests using N. rileyi against H. zea on soybeans. Application rates ranging from 0.1-100 g conidia/ 0.4 ha were applied (1g = 1×10^{11} conidia). The highest mortality obtained (77%) was with the highest application rate, while the lowest application rate produced 19% mortality. The authors concluded that compared to Heliothis NPV and B. thuringiensis, N. rileyi was unsuitable as a microbial insecticide against Heliothis spp. on soybeans. They stated (in contrast to the findings of Mohamed et al. (1977)) that the fungus was not effective against older larvae.

1.8 Nematodes

Various species of entomogenous nematodes have been recorded infecting Heliothis. Poinar (1975a) recorded Hexamermis sp. infecting H. armigera, while Khan et al. (1976) recorded Chromonema heliothidis n. gen., n. sp. in H. zea. Recent work in the south east of South Australia has shown that a previously undescribed nematode is a very significant pathogen of pupating H. punctigera. Pupae were sampled from soil under a seed lucerne crop at Brecon between April and October in 1975, and 39% were found to be infected (Learmonth, unpublished). The nematode has been classified into a new family (Heterorhabditidae) and has been called Heterorhabditis bacteriphora n. gen., n. sp. (Poinar, 1975b). Poinar has also reported on the biology of the nematode. An attempt was made to determine the abundance of H. bacteriphora in the

soil at Brecon in May, 1975, by collecting 150 soil samples (approximately 50 cm^3) on a grid pattern from irrigated and dry-land seed lucerne bays (Pinnock and Mew, unpublished). Using a method similar to that described by Bedding and Akhurst (1975), Galleria mellonella larvae were used to test for presence of the nematode, and 8% of the samples contained infective larvae of H. bacteriphora. These results indicate that the nematode is probably very prevalent in soil in this area, both in irrigated and dry-land bays. Subsequent work showed that an apparently pure strain of bacteria is carried in the gut of the nematode, although the bacteria is quite distinct from that carried by Neoaplectana spp. (Poinar, 1975b). More work remains to be done on the biology of H. bacteriphora; it is not known at this stage whether the nematode infects larvae as well as pupae of H. punctigera. If larval stages are infected, H. bacteriphora could be considered as a possible microbial control agent. Tanada and Reiner (1962) tested a DD-136 strain of Neoaplectana carpocapsae to control H. zea on corn; although mortality was high, damage to corn ears was not prevented, and the treatment was considered unsuccessful.

1.9 Conclusion

Heliothis has a well documented list of pathogens; it is interesting to note that with the exception of B. thuringiensis they are all naturally occurring pathogens, and their effect in contributing to the mortality of species of Heliothis must be considerable. The NPV of Heliothis is unique in that it is the first insect virus that has received registration to be formulated as a commercial viral insecticide. It is difficult to predict if any of the other pathogens mentioned will ever be used commercially; N. rileyi and H. bacteriphora are possibly the most likely candidates.

H. punctigera is an important pest in South Australia; it is part of a pest complex on seed lucerne which lends itself well to the use of pathogens for pest control. The aim of the study to be outlined in the following six chapters is to demonstrate how two of the above-mentioned pathogens, *B. thuringiensis* and *Heliothis* NPV, may be used to regulate populations of *H. punctigera* on seed lucerne. The study will initially be involved in determining, in both laboratory and field studies, the degree of susceptibility of *H. punctigera* to *B. thuringiensis*.

ADDENDUM

Since the preparation of this thesis, a recent review of microbial control strategies of insect pests has become available (Allen *et al.*, 1978). For a synopsis of recent progress in the field, this review should be consulted.

CHAPTER 2

ASSESSMENT OF B. THURINGIENSIS AS A CONTROL AGENT OF H. PUNCTIGERA ON IRRIGATED SEED LUCERNE IN SOUTH AUSTRALIA: LABORATORY STUDIES

2.1 Introduction

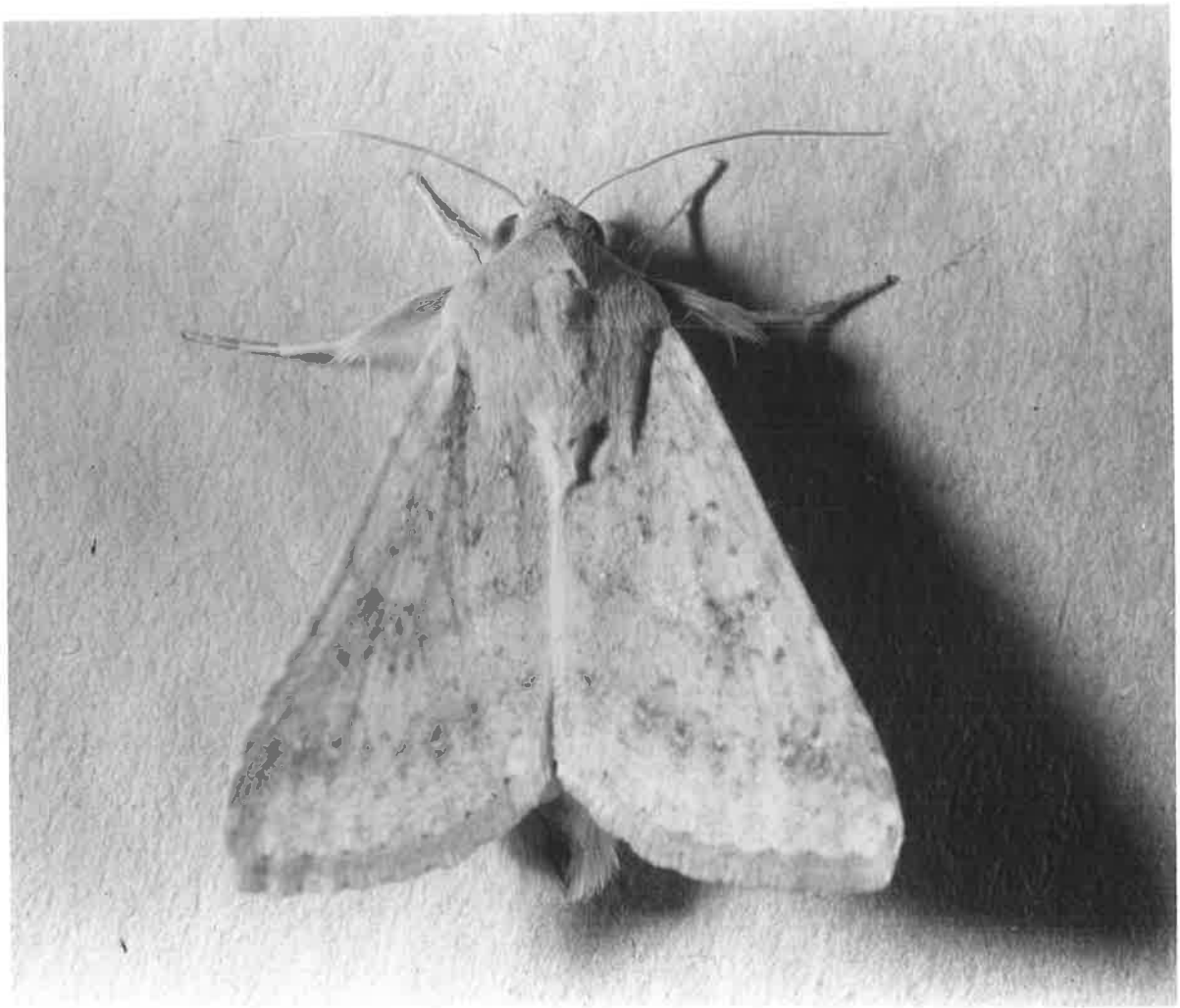
H. punctigera (Fig. 2.1) is virtually the sole representative of the Heliothis genus in South Australia. H. rubrescens (Walker) and H. armigera (Hübner) have been recorded in this state only on rare occasions (Cullen, 1969) and no other species of Heliothis has been recorded. H. punctigera is probably the most serious lepidopterous pest in South Australia. The host range includes seed lucerne, field peas, cereal crops, sweet corn and tomatoes, while in northern N.S.W. and Queensland H. punctigera and H. armigera constitute the most serious pests of cotton (Common, 1953).

H. punctigera is certainly the most serious lepidopterous pest of seed lucerne (Medicago sativa L.), and until the accidental introduction of the spotted alfalfa aphid, Therioaphis trifolii f. maculata into Australia in 1977, was the most serious pest. As mentioned in the introduction the initial section of this thesis deals with the assessment of an applied pathogen, B. thuringiensis, as a control agent of H. punctigera on seed lucerne. The rationale and likely advantages in selecting B. thuringiensis will be discussed in later sections.

Biology of H. punctigera

H. punctigera is inactive during winter and the species is usually first noticed in the spring when a large flight of adults occurs in September - October. It is not clear whether this flight of moths originates from local diapausing pupae or as a result of migration. Cullen (1969) produced circumstantial evidence in the form of light trap data that the former was the case, and this is supported by the fact that

FIGURE 2.1 *Heliothis punctigera* adult. x7.



the adults in the first spring flight were shown to have non-depleted fat reserves, making it unlikely that the moths could have taken part in a migration from any considerable distance. However, studies on larval survival failed to indicate that there would be sufficient pupae entering diapause in late summer and autumn to account for the large flight of adults in the following spring.

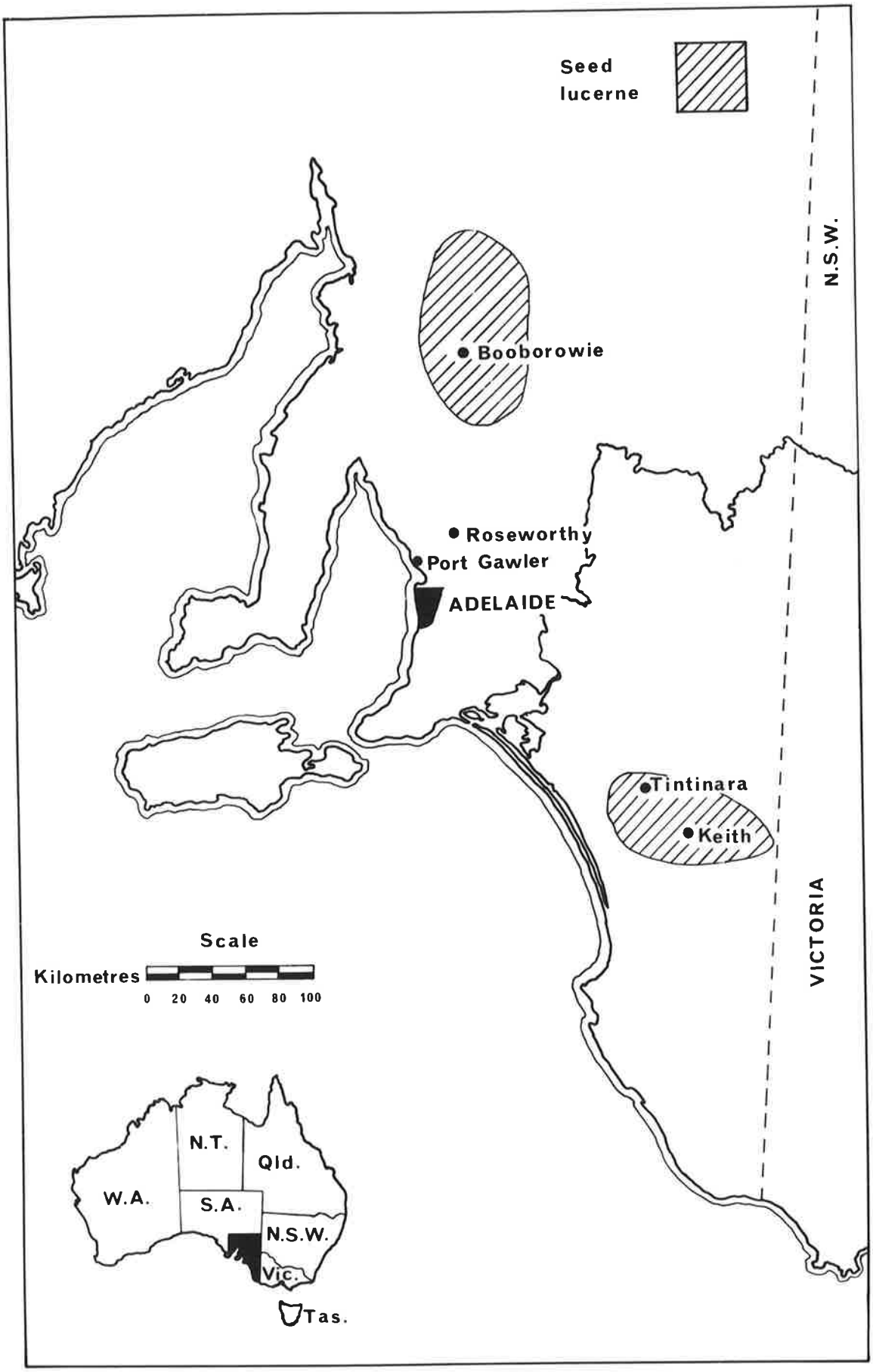
A second flight of adults occurs in early December, and there follows usually three more flights at approximately monthly intervals. In the area of study, the upper south east district of South Australia, larvae resulting from the December and subsequent flights pose the greatest threat to seed lucerne crops.

Female H. punctigera produce in excess of 1000 eggs which are oviposited on leaves and pods on the lucerne, and hatching occurs in 2-3 days. Larval development proceeds through 6 instars, which in summer takes 10-14 days, while the duration of the pupal stage, which is spent in the soil, is 12-14 days (Doull 1960a).

Seed lucerne growing in South Australia

A Mediterranean climate prevails in South Australia, typified by hot dry summers and cool but not severe winters. Most rainfall occurs during the winter months of May, June, July and August. The town of Keith, in the centre of the seed lucerne growing district in the upper south east, experiences an average rainfall of 472 mm, and annual mean maximum and minimum temperatures of 21.9^{o}C and 8.6^{o}C respectively (South Australian Year Book, 1978). The hot dry summers are well suited to lucerne seed production, and the state has a well established industry, with 11,575 ha of crop grown in 1976/77. The industry is concentrated in two main areas (Fig. 2.2), the northern area centred around Booborowie, and the slightly

FIGURE 2.2 Map of southern portion of South Australia showing principal seed lucerne growing areas.


Seed
lucerne
N.S.W.
Booborowie
Roseworthy
Port Gawler
ADELAIDE
Tintinara
Keith
VICTORIA
Scale
Kilometres
0 20 40 60 80 100
N.T.
Qld.
W.A.
S.A.
N.S.W.
Vic.
Tas.

cooler area of the upper south east, centred around Tintinara and Keith, where the growing season is slightly later. Approximately 50% of the state's lucerne seed is produced in the south eastern region (Table 2.1).

TABLE 2.1 Yields of Lucerne Seed in South Australia.

Year	Yield (kg)	Percent grown in south east (approximate)
72/3	1,175,655	50
73/4	1,282,618	50
74/5	1,824,052	44
75/6	1,564,000	50
76/7	1,436,000	50

Average yields of 100 kg/ha are obtained on dry-land lucerne, while on irrigated stands, which comprise 40% of the area sown in the south east district, yields of 672 kg/ha are common on well managed properties. Irrigation is predominantly flood irrigation, and Hunter River is the most commonly grown cultivar.

Crop management

A final hay-cut is taken off the crop in the second half of November, after which the lucerne stand is "closed up" and peak flowering occurs in the latter half of January. Conditions are ideal for pollination at this time, as there is little in the way of competing blossom for honey bees, Apis mellifera L. Preferred species such as Eucalypts, and the plants Capeweed (Cryptostomma calendula L.), Dandelion (Taraxacum officinale) and Salvation Jane (Echium lycopsis L.) have all finished flowering by late December, and so in January the bees concentrate on lucerne flowers (Doull, 1960b). A recommended practice is to introduce bee hives to the

crop at the rate of 5 hives per ha. If bees are not employed a certain amount of self-pollination occurs (Doull, 1961) but yields are often low, due no doubt to the high degree of self sterility (Cooper and Brink, 1940). The effect of native pollinating insects has never been critically measured but observations from time to time suggest that it may be of considerable importance. On two occasions I inspected high yielding crops that were grown with no bee hives on the property or in the immediate vicinity (Keith 1975 and Mundulla 1976). These observations suggest that a considerable amount of pollination may be carried out by some native insects. Harvesting is carried out 6-8 weeks after the onset of flowering.

Pest status of H. punctigera

H. punctigera larvae prefer to feed on buds and flowers of the lucerne plant, although in high infestations the upper parts of the plant may be stripped leaving only bare stems (Fig. 2.3). Newly-hatched larvae bore into the buds or flowers where they feed on the ovary, thus sterilizing the bud. After 2-3 days the larvae emerge and feed in the open. Doull (1960a) estimated that a larval density of $10/m^2$ would reduce the yield of seed by 11.2 kg/ha over the life of the larvae. This is probably a conservative estimate, and much higher larval densities are often encountered. In December 1975 I inspected a lucerne crop at Keith which was infested by an unusually high density of larvae. Very few flowers or buds were left undamaged (no control measures had been taken by the farmer), and subsequently it was found necessary to cut the crop and re-induce flowering.

The recommended control measure for H. punctigera is to spray with DDT at a rate of 140-550 g active ingredient per ha (Department of Agriculture and Fisheries, 1978). The cost of materials is low (70¢ - $2.75/ha) although larvae larger than fourth instar are not killed by this

FIGURE 2.3 a. Lucerne seed pods.

b. Feeding damage to seed lucerne caused by *Heliothis punctigera* larvae.


a
b

treatment.

Other pests of seed lucerne

1. Etiella behrii

Etiella behrii Zell. (Lepidoptera:Pyralidae (Phycitinae)) is the only other lepidopterous pest of any consequence of seed lucerne. This insect will be mentioned because it is likely that the proposed control programme using B. thuringiensis against H. punctigera may also be effective against E. behrii. The larvae of E. behrii are pod borers, thus the ripening seed-pod is the susceptible stage of the crop. Newly-hatched larvae bore into young seed pods and feed on developing seed. Towards the latter part of the larval life larvae emerge from the pods and spin a web which encloses several pods (Fig. 2.4). The larvae feed on these pods and eventually leave the web to pupate in the soil.

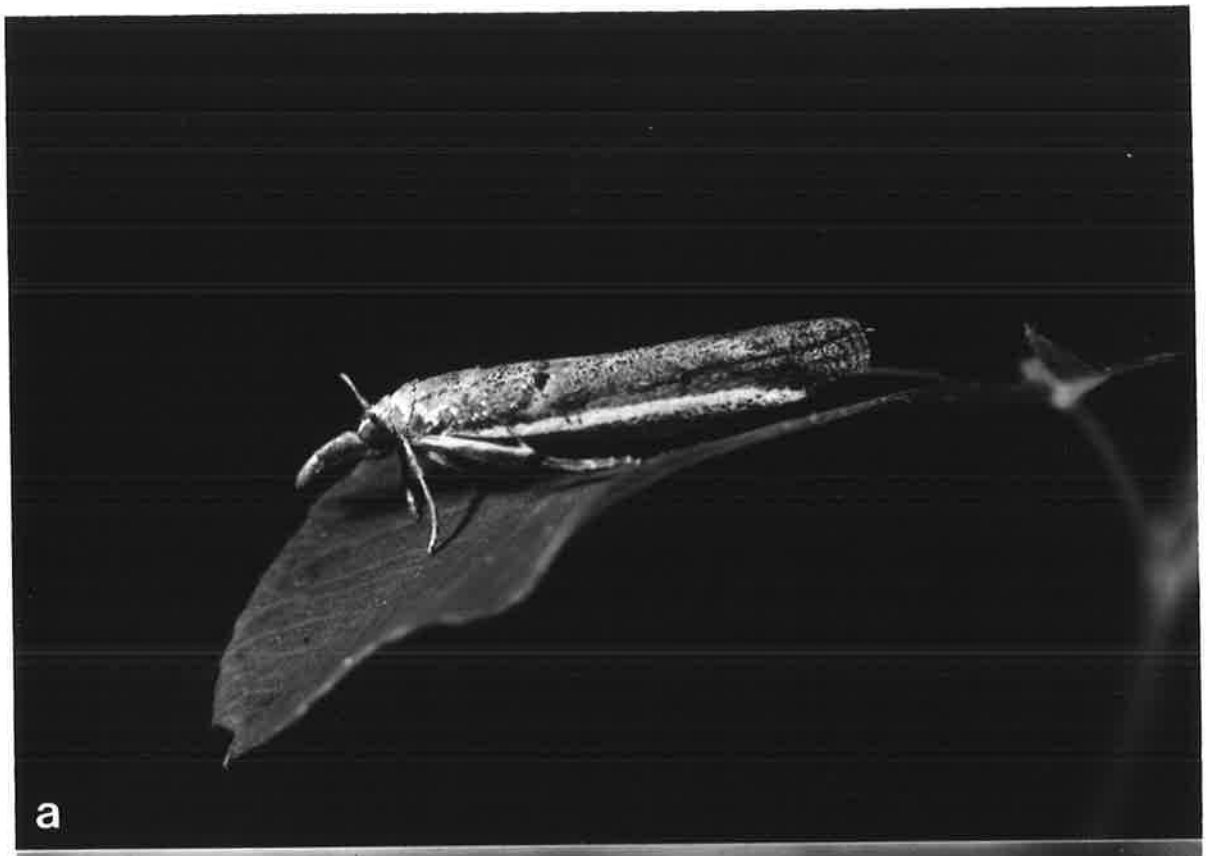
The level of damage caused by E. behrii has not been accurately assessed - it appears to be a greater problem on deep sands than on heavy soils, and possibly more damage is caused to moisture-stressed plants (Doull, 1960a). Although it would appear that only occasionally does E. behrii emerge as a pest of any consequence (Wheal, pers. commun. 1976), nevertheless farmers in the south east district routinely apply parathion at the rate of 140 g active ingredient per ha for E. behrii control. A combination of DDT/parathion for H. punctigera and E. behrii is employed, and applications are made as frequently as every 14 days during flowering and seed-setting.

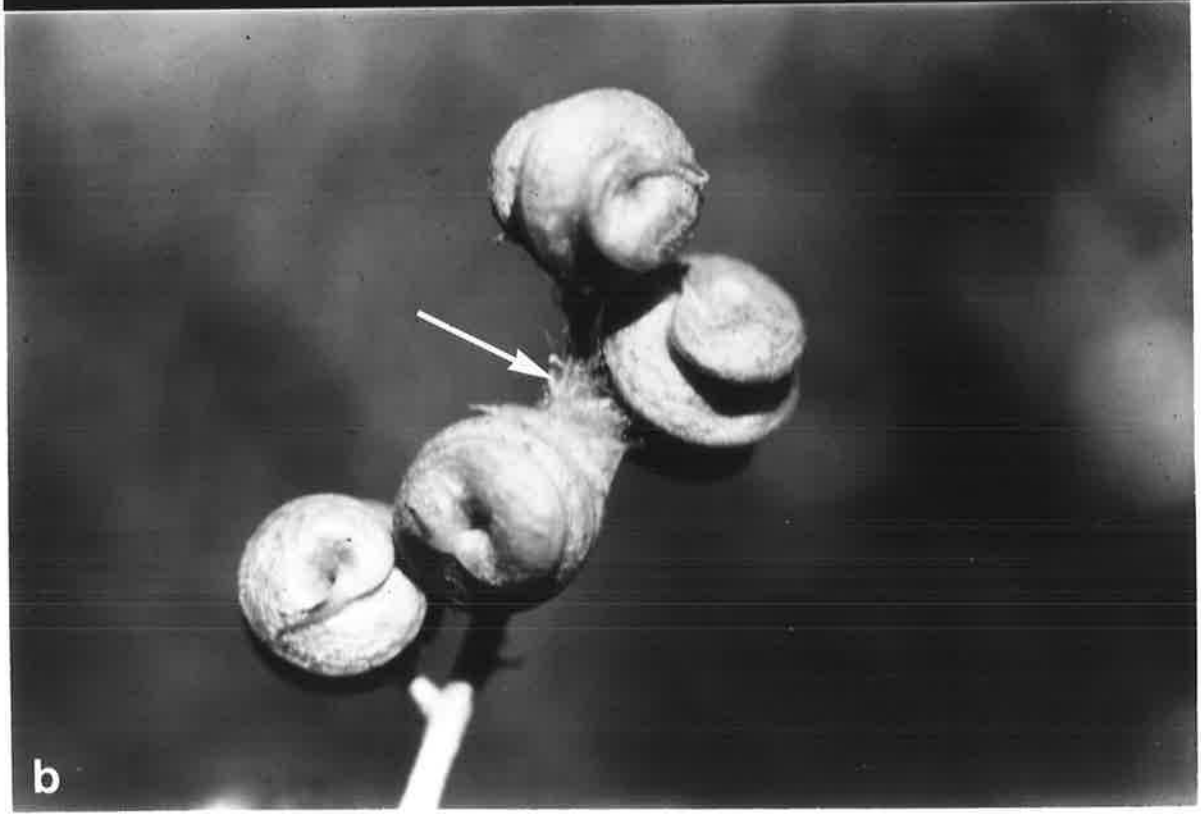
2. Therioaphis trifolii

The spotted alfalfa aphid, Therioaphis trifolii f. maculata was accidentally introduced into Australia and was first noticed in Queensland

FIGURE 2.4 a. Etiella behrii adult. x7.

b. Lucerne seed pod showing web (arrowed) spun by Etiella behrii larva. x6.


a


b

in March 1977. The population spread rapidly through eastern Australia, and by May 1977 substantial populations of the aphid were discovered in South Australia. Populations were widespread throughout the south east in the 1977/78 growing season. Prompt action by the C.S.I.R.O. and the South Australian Department of Agriculture and Fisheries have led to the importation, rearing and release of the parasitoid Trioxys complanatus. At the time of writing the wasp is believed to have become established throughout the lucerne growing areas of the state, but it is not known how effective T. complanatus will be in controlling the aphid. It is likely that eventually use will have to be made of resistant cultivars of lucerne as well as parasitoids for the industry to survive under this serious pest. It has been necessary to employ insecticides (demeton-s-methyl, dimethoate and pirimicarb) to control T. trifolii in the short term, but this has led to other problems: predators of two-spotted mite Tetranychus urticae (Koch) have been killed off by these insecticides, and consequently mites have become a major pest on some properties, and seed yields have been reduced by more than half. Use has also been made of chlorpyrifos, an insecticide claimed to control H. punctigera, E. behrii, T. trifolii and T. urticae but seed yields have been adversely affected, almost certainly due to the toxic effect of this chemical on honey bees and other pollinating insects.

3. Acryrthosiphon kondoi

Another serious aphid pest of lucerne, the blue green aphid, Acyrthosiphon kondoi Shinji has also been introduced recently into Australia. However this insect builds up to large populations in the cooler wetter months of the year and is not thought at this stage to pose a direct threat to lucerne seed production.

2.2 B. thuringiensis as a control agent

Suitability of using B. thuringiensis against Heliothis species

As mentioned in Chapter 1, B. thuringiensis has been used with mixed success against other Heliothis species. This may be due in part to the host crop involved, e.g. in cotton larvae feed inside the bolls and do not ingest sufficient quantities of spores (Parencia 1977). Feeding behaviour of larvae probably also explains the low success rate in using B. thuringiensis to control H. zea on corn (Janes and Greene, 1969). In addition a considerable range of susceptibility to B. thuringiensis occurs among different Heliothis species. Thus Dulmage (1970) while carrying out bioassays to test the pathogenicity of HD-1, a new isolate of B. thuringiensis var. kurstaki (see page 19), found that the LD_{50} for H. virescens was 0.74 $\pm$ 0.12 µg/ml of diet, while the LD_{50} for Trichoplusia ni, commonly regarded as a susceptible species (Ignoffo et al., 1968), was 5.00 $\pm$ 0.49 µg/ml of diet. On the other hand H. zea was found to have an LD_{50} of 16.0 $\pm$ 5.3 µg/ml of diet. In view of this range in susceptibility, it was considered necessary to carry out bioassays with B. thuringiensis against H. punctigera. The level of susceptibility of this species to B. thuringiensis had not been determined previously, and such information was necessary in order to be able to predict the likelihood of success in using B. thuringiensis as a control agent.

Crop suitability

Lucerne lends itself well to the use of pathogens which must be ingested by the host for insect control, as the feeding behaviour of H. punctigera on lucerne would ensure that the larvae would be likely to receive a sufficient dose of spores. Apart from the first two instars, when the larvae are feeding inside buds and flowers, the larvae feed in an

exposed position, and tend to feed towards the top of the plant, where adequate spray coverage is likely to be achieved.

Advantages in curtailing the use of chemical insecticides

Several sound reasons exist for reducing the dependence on chemical insecticides and adopting a microbial insecticide/integrated control strategy for H. punctigera control on seed lucerne. The use of DDT has been either totally banned or restricted in many countries because of residue problems (Report of the Australian Academy of Science 1972). In the U.S.A. the use of DDT was banned by the Environmental Protection Agency in June 1972 (Sherman 1977) and this ban is still in force, apart from several exemptions where no alternative form of control exists for extremely serious pest problems. In 1975 the State of Louisiana applied to the E.P.A. to have the ban on DDT lifted for the purpose of H. virescens control on cotton. This request was not granted. Although the current low rates of DDT applied to seed lucerne in South Australia do not pose a serious threat to contamination of tissues of wildlife or livestock, provided the recommended withholding periods are adhered to (Department of Agriculture and Fisheries, South Australia, 1978), it is felt that any alternative form of control is certainly worth investigating.

Resistance to DDT by H. punctigera could well be a factor to deal with in the near future. Goodyer (1976) found DDT resistance in H. armigera collected from 15 localities in New South Wales in a survey conducted in 1975. Levels of resistance ranged from 7 times to more than 150 times previous LD_{50} values. This resistance is thought to have emerged as a result of heavy use of DDT against H. armigera on cotton. No surveys of resistance to DDT by H. punctigera in South Australia have been carried out recently, but the fact that spraying must be carried out as frequently

as every 14 days at Keith might be an indication that resistance is emerging - such frequent spray applications have only been necessary over the last few seasons. Alternatively the persistent larval populations might be a consequence of constant migration of adults from surrounding bushland or alternate host crops. Use of broad spectrum insecticides such as parathion undoubtedly kills off parasites and predators of H. punctigera (see page 64) thus rapid resurgence of the pest population would occur. Seventy-five H. punctigera larvae (second and third instar) were sampled in October 1978 from a linseed crop at Roseworthy, north of Adelaide, on the same day that the crop was sprayed with DDT (700 g active ingredient per ha) and 45% of the larvae survived to pupation (N. Richardson, pers. commun. 1978). Although caution should be exercised in drawing conclusions from this observation, resistance to DDT could well explain this unexpectedly high survival rate.

Effect of control measures on pollinating insects

DDT/parathion spray applications on seed lucerne are routinely made early in the morning or during the late evening when honey bees are no longer foraging. This is because both chemicals, especially parathion (Barker and Waller, 1978), are toxic to bees. How soon bees return to a crop that has been sprayed has not been critically measured, but from my personal observation (Keith 1976) the period is at least 24 h. Thus it is possible that there may be a resultant reduction in seed set caused by the application of insecticides. The spores and δ-endotoxin produced by B. thuringiensis are, by and large, specific to lepidopterous larvae (Faust, 1974) and Wilson (1962) has shown that application of large quantities of B. thuringiensis to a crop has no detrimental effect on foraging bees. B. thuringiensis has in fact been used successfully inside

bee hives to protect beecomb from wax moths (Galleria mellonella and Achroia grisella (Franz, 1971). It is highly likely that other pollinating insects would be similarly unaffected by the use of B. thuringiensis.

Thus there is a good case for investigating the usefulness of B. thuringiensis as a control agent. Such a control strategy would enable naturally-occurring parasites and predators to exert their maximum effect - this argument will be developed in the next chapter. Because the control of E. behrii appears to be closely linked to that of H. punctigera, it was decided to investigate also the effectiveness of B. thuringiensis as a control agent of this insect.

2.3 Bioassay of B. thuringiensis against fourth instar H. punctigera

Introduction

To assess the potency and likely effectiveness in the field of a pathogen, it is necessary to carry out a bioassay (Burges and Thompson, 1971). The desired effect of B. thuringiensis on the target insect in the field is death, so the performance of insects in the bioassay is based on the quantal response (Meynell and Meynell, 1965; Finney, 1971), i.e. whether the insects die or survive. Daoust and Roome (1973) carried out bioassays using B. thuringiensis against the american bollworm, H. armigera, to assess the suitability of this pathogen for field use, and spores were administered to the larvae by surface application onto Bot's artificial diet. However for the purposes of this study it was highly desirable to set up the bioassay so that larvae were maintained as close as possible to the field situation, in order to minimize any factor which might bias results. Thus all larvae were reared on lucerne sprigs and in the bioassay B. thuringiensis spores were administered on lucerne leaves. The initial bioassay took the form of a preliminary small-scale experiment in order to

determine whether more detailed investigation was worthwhile.

Materials and methods

H. punctigera larvae were reared to fourth instar at 27 ± 1°C. Up to 6 pairs of adults were introduced to mating cages which were 203 x 203 mm perspex canisters (Hostess Ware(R), Michaelis Bayley); the moths laid eggs on paper towelling placed in the cages. Ten percent honey solution containing 0.5% potassium sorbate as a yeast inhibitor was provided in a cotton wick feeder as food for the adults. Eggs were surface sterilized by immersing the paper towelling for 5 min in 0.15% sodium hypochlorite, and the sodium hypochlorite was then neutralized by washing the eggs in 0.15% sterile sodium thiosulphite for a further 5 min. Lastly the eggs were washed in sterile distilled water. Newly-hatched larvae were allowed to feed on lucerne springs, and fresh sprigs were given daily until larvae attained fourth instar. Head capsule width was used as a criterion to determine instar (Kirkpatrick, 1961), and an effort was made to select larvae from mid-fourth instar, as mortality may vary in a bioassay depending upon age of the test insect within an instar (Brand et al., 1976, Burges and Thompson, 1971). In addition, larvae used in all bioassays were never more than 2 generations removed from field-caught insects.

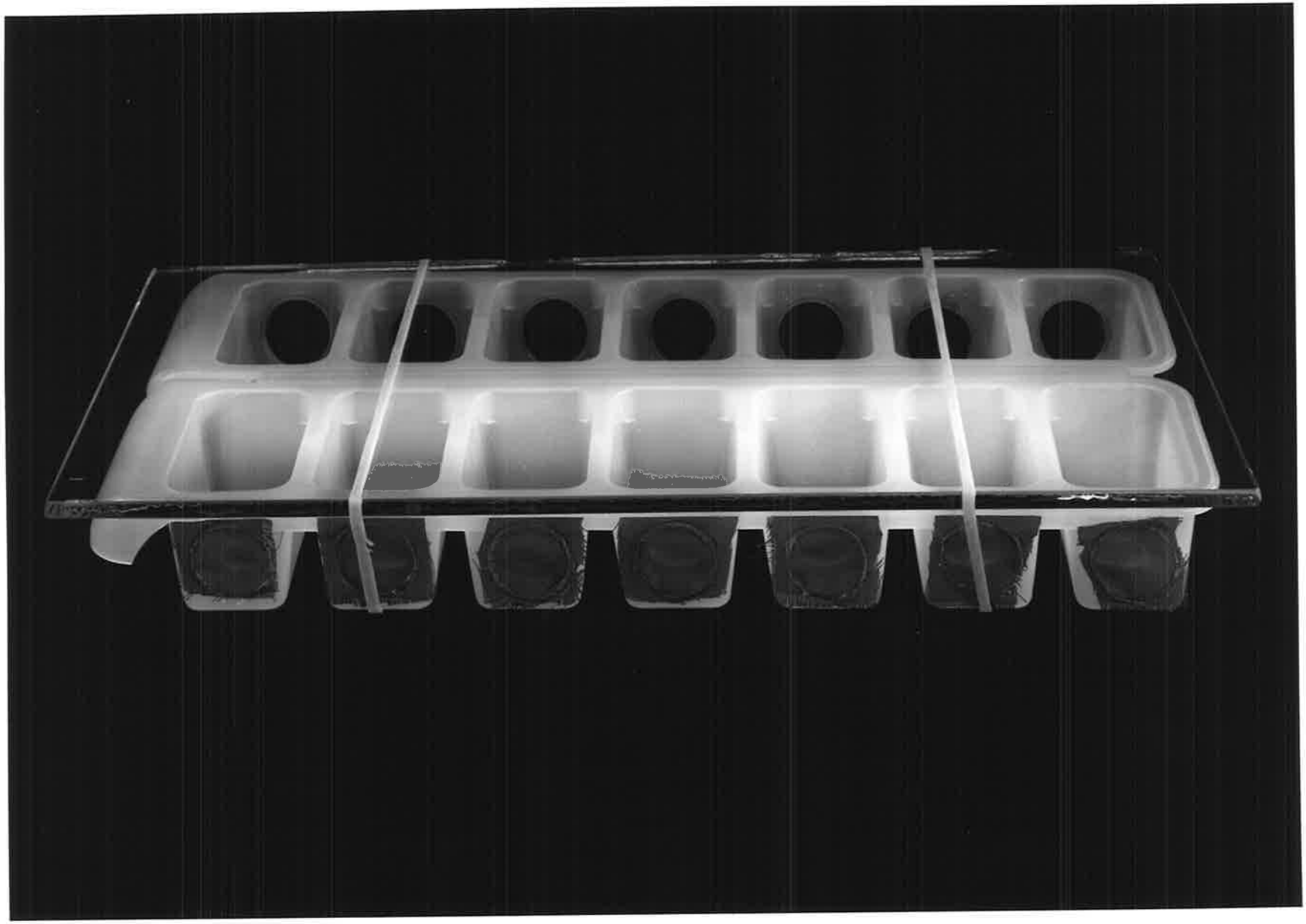
The B. thuringiensis formulation used was B. thuringiensis var. kurstaki (serotype 3b), manufactured as Thuricide(R) HPSC wettable powder (Sandoz) with a stated potency of 1.60×10^7 International Units/g; the viable spore count was estimated shortly before use to be 5.0×10^{10} viable spores/g. The formulation was administered to the larvae by allowing larvae to feed on small leaves which had been dipped into spore suspensions that had been made up in 0.1% detergent solution (Tween(R) 80, Koch-Light Laboratories Ltd.). Leaves were selected of a size (approximately 4 x 8 mm)

such that larvae could eat the entire leaf and thus ingest the administered dose within 24 h. Even coverage of the leaves was obtained by allowing the leaves to dry, after dipping and shaking, with the petiole downwards (Burges and Thompson, 1971) and by using Tween 80 in the spore suspensions. Leaves were dried at 35°C for 45 min in darkness (thus reducing any spore mortality induced by ultraviolet radiation).

The experiment was set up with 4 doses, ranging from 1.48×10^4 to 1.48×10^6 spores per leaf, and a control group in which the leaves had been dipped in 0.1% Tween 80. Twenty larvae per dose were set up. Estimates of the dose applied to the leaves were made by employing a method similar to that used by Pinnock et al. (1971). Ten leaves were dipped in a ten-fold dilution of the lowest dose used in the bioassay and, after drying, each leaf was aseptically transferred to a 150 x 20 mm test tube containing 10 ml of sterile distilled water and 15 glass beads 3 mm in diameter. Each tube was shaken vigorously for 10 min in a mechanical flask shaker, after which 7 drops from each suspension were plated onto brain heart infusion (Difco(R)) agar plates, using calibrated pasteur pipettes (Miles and Misra, 1938). The plates were incubated in darkness for approximately 18 h at 27 $\pm$ 1°C, after which colonies were counted. The mean value of the lowest dose used in the bioassay was 1.48×10^4 spores (standard error of the mean = 4.68×10^3 spores).

The bioassay was carried out with larvae caged individually in chambers of ice-cube trays (Fig. 2.5). A glass sheet prevented larvae escaping, and a piece of nylon gauze in the wall of each chamber enabled exchange of air. Individual caging of larvae was necessary because Heliothis larvae larger than third instar are cannabilistic (Cullen 1969; Ignoffo 1965). Leaves given to the larvae were kept fresh by stapling the petioles between two 1 cm disks of blotting paper which were kept moist;

FIGURE 2.5 Ice-cube tray in which Heliothis punctigera larvae were caged for bioassays.



larvae were fed fresh leaves ad lib after the first 24 h of the bioassay.

The experiment was set up (Day 0) and mortality was recorded on Day 3. Smears of dead larvae on microscope slides were stained with Gram's stain and examined under oil immersion to determine if death was associated with the presence of B. thuringiensis vegetative cells. Mortality was corrected by excluding those insects that died from causes other than due to B. thuringiensis (see Table 2.2), and Abbott's formula was used to correct for control mortality. Percent mortality was transformed to probit units, and data was analysed by the maximum likelihood method (Finney, 1971).

Results and discussion

Dose mortality data are recorded in Table 2.2.

A considerable degree of feeding inhibition was evident in the highest dose (1.48×10^6 spores per leaf) and most larvae consumed less than the entire leaf in the first 24 h of the bioassay, although all larvae in all other groups consumed the entire leaf in this period. As the larvae in the group given 1.48×10^6 spores per leaf received less than the administered dose, and considering that this dose was probably excessively high in terms of field application rates, it was considered that there was justification in excluding this last group from the estimation of the LD_{50}. Results from the probit analysis are shown in Fig. 2.6.

The LD_{90} was estimated to be 5.15×10^5 viable spores (1.59×10^6 and 1.67×10^5 - 95% confidence limits).

The LD_{50} value obtained indicates that B. thuringiensis may prove a useful control agent against H. punctigera. Daoust and Roome (1974) obtained an LD_{50} value of 6.04×10^3 spores/mm^2 of diet surface when B. thuringiensis spores were administered on artificial diet surfaces to 3 day-old

TABLE 2.2 Dose mortality data for *Bacillus thuringiensis* (Thuricide HPSC WP) assayed against fourth instar *Heliothis punctigera* (Day 3).

Dose (spores/leaf)	Total no. larvae/dose	Larvae dead from *B. thuringiensis*	Larvae dead from other causes	Test[a] mortality %	Corrected[b] mortality %
1.48×10^4	20	4	9	36.4	27.3
1.48×10^5	18	11	3	73.3	69.5
0.74×10^6	20	19	0	95.0	94.3
1.48×10^6	20	17	1	89.5	88.0
Control	20	2	4	12.5	-

[a] Test mortality = $\frac{\text{No. larvae dead due B. thuringiensis}}{\text{Total no. larvae - larvae dead from other causes}}$

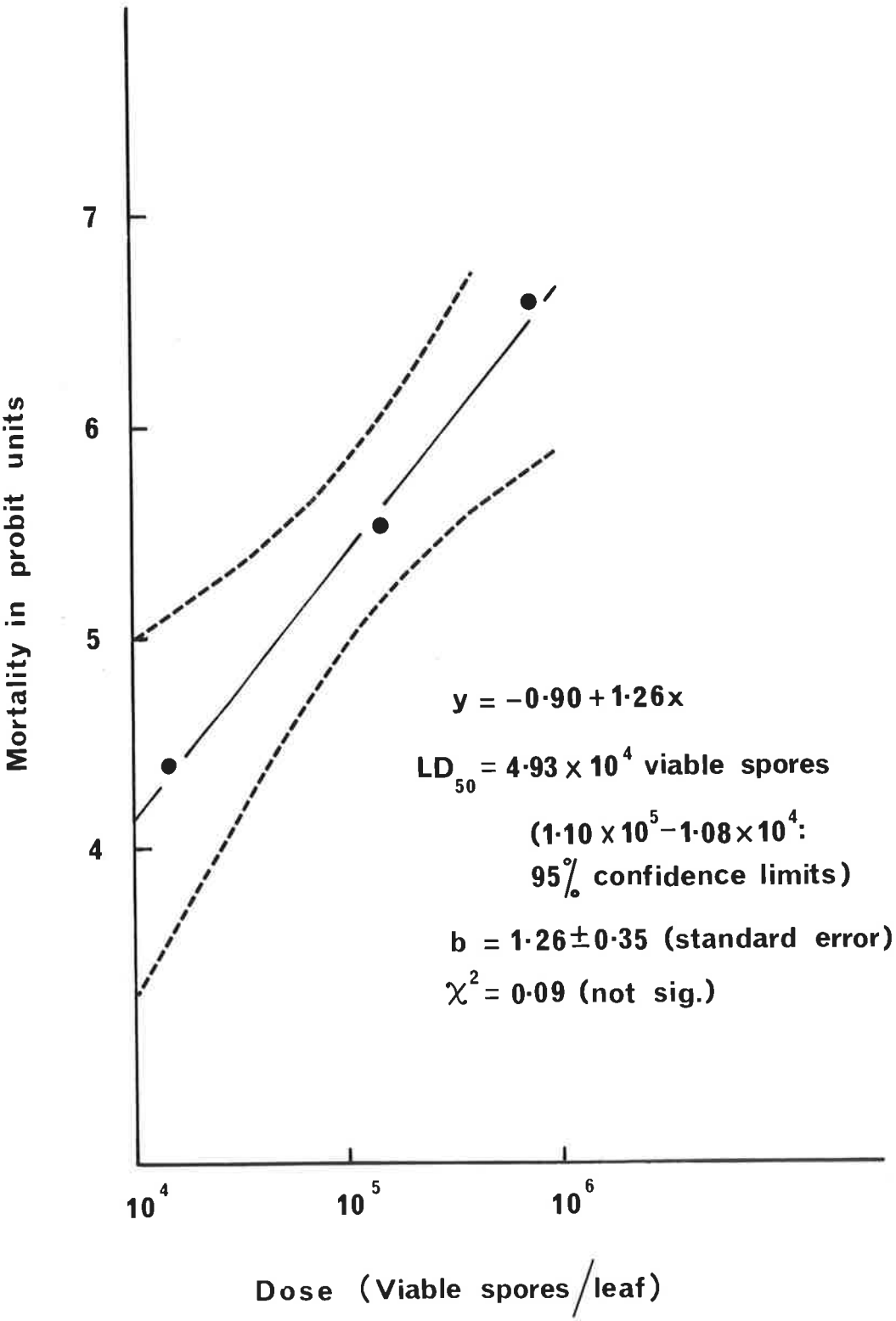
[b] Corrected mortality = Mortality corrected by Abbott's formula

$$= \frac{\text{Test mortality \% - Control mortality \%}}{100 \text{ - Control mortality \%}} \times \frac{100}{1} \%$$

H. armigera larvae. However, because much younger larvae were used, and because the experiment was set up differently, it is difficult to make a comparison. In any case it was decided that investigation of the susceptibility of younger larvae of *H. punctigera* (e.g. third instar) was warranted.

A problem encountered in both bioassays was that appreciable numbers of larvae died from a nuclear polyhedrosis virus (Teakle, 1973). Eliminating the virus from the culture proved to be difficult. Subsequently it was found that by rearing *H. punctigera* on modified Sender's diet (Griffith and Smith, 1977) containing 0.1% formalin, the incidence of the virus was reduced to an acceptably low level. Formalin in artificial diets reduces the incidence of most pathogens including

FIGURE 2.6 Bioassay of *Bacillus thuringiensis* (Thuricide HPSC WP) vs fourth instar *Heliothis punctigera* - Day 3. Dashed lines indicate 95% fiducial bands.


Mortality in probit units
7
6
5
4
10^4
10^5
10^6
Dose (Viable spores/leaf)
y = −0·90 + 1·26x
LD_{50} = 4·93 × 10^4 viable spores
(1·10 × 10^5 − 1·08 × 10^4: 95% confidence limits)
b = 1·26 ± 0·35 (standard error)
χ^2 = 0·09 (not sig.)

baculoviruses (Ignoffo and Garcia, 1968).

2.4 Bioassay of B. thuringiensis against third instar H. punctigera

Introduction

Due to the relatively low value of the slope of the curve, b, obtained in the first bioassay, it was considered necessary to increase the value of n, the number of insects per dose, in order to improve precision. In addition, to reduce the variation in the number of spores administered within each dose, aliquots of spore suspensions were pipetted onto the leaves on which the larvae were to feed.

Materials and methods

Larvae were reared as described and 42 third instar larvae per dose were selected. As previously, an effort was made to select all larvae from mid-instar. A preliminary experiment was carried out to determine the required leaf size that would be ingested within 24 h; it was found that the required size was a small leaf that had been cut to a "diamond" shape measuring 1.5 x 2.0 mm. To apply the spore suspension to each leaf, the leaf was first dipped in 0.1% Tween 80 solution, lightly blotted with tissue, and then the spore suspension also in 0.1% Tween 80 was applied in a volume of 5 µl. The leaves were then allowed to dry at 35^{o}C for 45 min in darkness. A range of 5 doses was set up, ranging from 5.69×10^{2} to 5.69×10^{5} spores per leaf, and a control group was set up in which 5 µl 0.1% Tween 80 was applied to the leaves. An estimate of the number of spores administered in each dose was made by dosing 10 extra leaves from the third-most dilute suspension, drying them, and removing the spores and counting the resultant colonies as described. The mean value of this dose was estimated to be 2.56×10^{4} spores per leaf (standard error of the mean

= 7.45 x 10^2 spores). The bioassay was set up in identical conditions as described previously, and mortality was recorded on Day 3. Dead larvae were examined for the presence of B. thuringiensis vegetative cells.

Results and discussion

Dose mortality data are recorded in Table 2.3, and data was analysed

TABLE 2.3 Dose mortality data for Bacillus thuringiensis (Thuricide HPSC WP) assayed against third instar Heliothis punctigera (Day 3).

Dose (spores/leaf)	Total no. larvae/dose	Larvae dead from B. thuringiensis	Larvae dead from other causes	Test[a] mortality %	Corrected[b] mortality %
5.69 x 10^2	36	5	14	22.7	19.3
5.69 x 10^3	40	10	22	55.6	53.7
2.56 x 10^4	40	18	12	64.3	62.7
5.69 x 10^4	41	29	8	87.9	87.4
5.69 x 10^5	42	41	1	100.0	100.0
Control	40	1	16	4.2	-

[a] Test mortality = $\frac{\text{No. larvae dead due B. thuringiensis}}{\text{Total no. larvae - larvae dead from other causes}}$

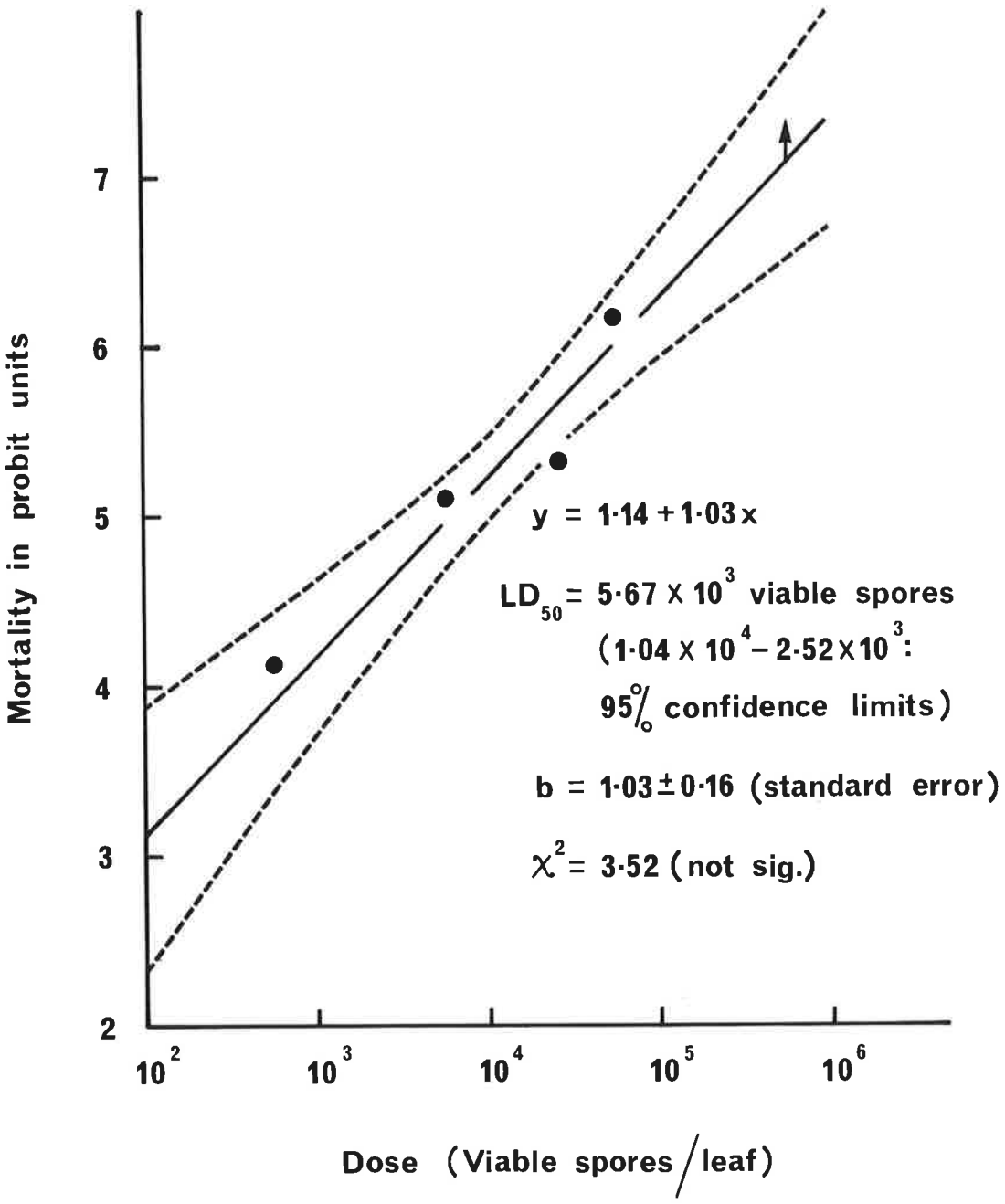
[b] Corrected mortality = Mortality corrected by Abbott's formula

$$= \frac{\text{Test mortality \% - Control mortality \%}}{100 \text{ - Control mortality \%}} \times \frac{100}{1} \%$$

by the maximum likelihood method (Fig. 2.7). The LD_{90} was estimated to be 1.00 x 10^5 viable spores (1.95 x 10^5 and 5.13 x 10^4 - 95% confidence limits).

Feeding inhibition was again evident in this bioassay - no group of

FIGURE 2.7 Bioassay of *Bacillus thuringiensis* (Thuricide HPSC WP) vs third instar *Heliothis punctigera* - Day 3. Dashed lines indicate 95% fiducial bands. Arrow indicates dose which resulted in 100% mortality.


Mortality in probit units
7
6
5
4
3
2
10^2
10^3
10^4
10^5
10^6
Dose (Viable spores/leaf)
y = 1·14 + 1·03x
LD_{50} = 5·67 X 10^3 viable spores
(1·04 X 10^4 – 2·52 X 10^3:
95% confidence limits)
b = 1·03 ± 0·16 (standard error)
χ^2 = 3·52 (not sig.)

insects completely consumed the administered dose in the first 24 h of the experiment. A similar procedure was adopted as was adopted by Brand et al., 1976, when faced with this difficulty, in that administered dose rather than ingested dose was incorporated into the analysis. At this point it became clear that feeding inhibition might in fact limit the usefulness of B. thuringiensis as a control agent in the field, as the trend appeared to suggest that younger larvae were more prone to feeding inhibition. Thus it was proposed to examine the response of each instar to applied B. thuringiensis in the subsequent field trial.

The results of the two bioassays are summarized in Table 2.4.

TABLE 2.4 Parameters of bioassays using Bacillus thuringiensis against third and fourth instar Heliothis punctigera.

Instar	b (standard error)	LD_{50}-viable spores (95% confidence limits)	LD_{90}-viable spores (95% confidence limits)
Third	1.03 (0.16)	5.67×10^3 (1.04×10^4 and 2.52×10^3)	1.00×10^5 (1.95×10^5 and 5.13×10^4)
Fourth	1.26 (0.35)	4.93×10^4 (1.10×10^5 and 1.08×10^4)	5.51×10^5 (1.59×10^6 and 1.67×10^5)

The decrease in LD_{50} between fourth and third instar larvae (by a factor of 8.7) is an indication that third and younger instars of H. punctigera are highly susceptible to B. thuringiensis, notwithstanding the feeding inhibition that was evident. The results suggest that H. punctigera is possibly more susceptible to B. thuringiensis than H. armigera (Daoust and Roome, 1974), and at this stage is was considered that it was worthwhile testing the efficacy of B. thuringiensis against H. punctigera under field conditions.

2.5 Bioassay of B. thuringiensis against third instar E. behrii

Introduction

Testing the susceptibility of E. behrii to B. thuringiensis seemed worthwhile, for should one pathogen prove effective against both pests, the case for implementing B. thuringiensis as a control agent would be strengthened. As E. behrii belongs to the subfamily Phycitinae, it was likely that E. behrii would prove to be susceptible to B. thuringiensis, as other members of this subfamily are highly susceptible. Ephestia kuehniella (Lepidoptera:Phycitinae) is regarded as being highly susceptible and is used routinely as a test insect in the standardization of new B. thuringiensis formulations (Burges and Thompson, 1971; Burges et al., 1967).

Materials and methods

E. behrii adults were caught in a light trap at the Waite Institute in November 1977, and the method of Fatzinger (1970) was used in an attempt to start a culture. Approximately 20 moths were introduced to a 30 x 30 x 30 cm gauze wood-framed cage containing 5% sucrose solution in a cotton wick feeder and bouquets of lucerne in water. The cage was put in a room at 27 $\pm$ 1^{o}C equipped with fluorescent lamps which operated 14 h a day. However as no eggs were oviposited it was assumed no mating had occurred. It was suggested (Pinnock, pers. commun. 1977) that as some species of phycitids mate in twilight conditions, more success might be met with if the moths were held in conditions of low light intensity. A subsequent group of moths in the same cage was put into a room well lit by sunlight during the day, but which by night was faintly illuminated by a 15W lamp shielded behind fine nylon gauze. These conditions were conducive to mating, as eggs were subsequently laid in the cage. The eggs were laid

in cracks and crevices in the cage, not on the lucerne bouquets as hoped, and were extremely well hidden. Rather than try to remove eggs, newly-hatched larvae were allowed to make their way to the top of the cage; there they were removed with a fine sable hair brush and put into vials containing Shorey's artificial diet (Shorey and Hale, 1965) in which peas replaced pinto beans. Formalin was omitted from the recipe, as this may have rendered the larvae less susceptible to B. thuringiensis spores in the subsequent experiment.

Although the desirability was stressed (page 34) of rearing insects and setting up a bioassay conducted for the purposes of this study in such a manner that the insects are maintained as close as possible to the field situation, very real difficulties exist with an insect with the feeding behaviour of E. behrii. Applying spores to lucerne seed pods would undoubtedly result in a large variation in dose, which would in turn result in excess variability in the response. Thus the spores were administered to the test larvae by surface contaminating the artificial diet. This experiment would suffice as a first approximation to indicate the level of susceptibility of E. behrii to B. thuringiensis.

For the bioassay, third instar (11-12 day-old) larvae were selected. Molten Shorey's diet (minus formalin) was added to 2.5 x 4.5 cm plastic vials to a depth of 0.5 cm, and when the diet had solidified, B. thuringiensis (Thuricide HPSC WP) spore suspensions were added to the vials in a volume of 0.1 ml. Drying was carried out at 35^{o}C for 45 min in darkness. Five doses were set up, ranging in five-fold steps from 5.4 to 3360.5 spores/mm^{2}, and a control group with distilled water was also set up. Fifteen larvae were set up for each dose. A plate count on brain heart infusion agar was made on a 10-fold dilution of the lowest dose to give an estimate of the number of spores applied. The mean number of spores

in this dose was estimated to be 5.36 spores/mm^2 (standard error = 0.29, n = 7). The bioassay was held at 27 ± 1°C, and mortality was recorded on Day 4. Smears were made of dead larvae, stained with Gram's stain, and examined for B. thuringiensis vegetative cells.

Results and discussion

Dose mortality data are recorded in Table 2.5, and data was analysed by the maximum likelihood method (Finney, 1971) (Fig. 2.8). The LC_{90} was estimated to be 3.65×10^3 spores/mm^2 (1.25×10^4 and 1.07×10^3 - 95% confidence limits).

The results indicate that E. behrii is highly susceptible to B. thuringiensis - the LC_{50} of 3.11×10^2 spores/mm^2 is lower by a factor of 19.4 than the LC_{50} that Daoust and Roome found for 3 day-old H. armigera (6.03×10^3 spores/mm^2). The approximate actual dose ingested by the test insects can be estimated: larvae when placed on the artificial diet burrowed into the diet and remained in the burrows and so would only have ingested spores when biting through the surface. The diameter of the burrows ranged from 1.0 - 1.5 mm, so the maximum area of diet surface eaten would have been approximately 1.8 mm^2. Thus the LD_{50} would approximately $1.8 \times 3.11 \times 10^2 = 5.6 \times 10^2$ spores/larva, which is lower than the LD_{50} obtained for third instar H. punctigera by a factor of 10.1.

The feeding behaviour of E. behrii poses problems in delivering a sufficient dose of insecticide to the larvae. Larvae are either feeding inside the pods or are protected by the web spun around the pods. However, in attempting to use B. thuringiensis as a control agent, this difficulty is likely to be offset by the high susceptibility of E. behrii to B. thuringiensis. It might be necessary for sprays to be applied at around the time of oviposition which would result in newly-hatched larvae

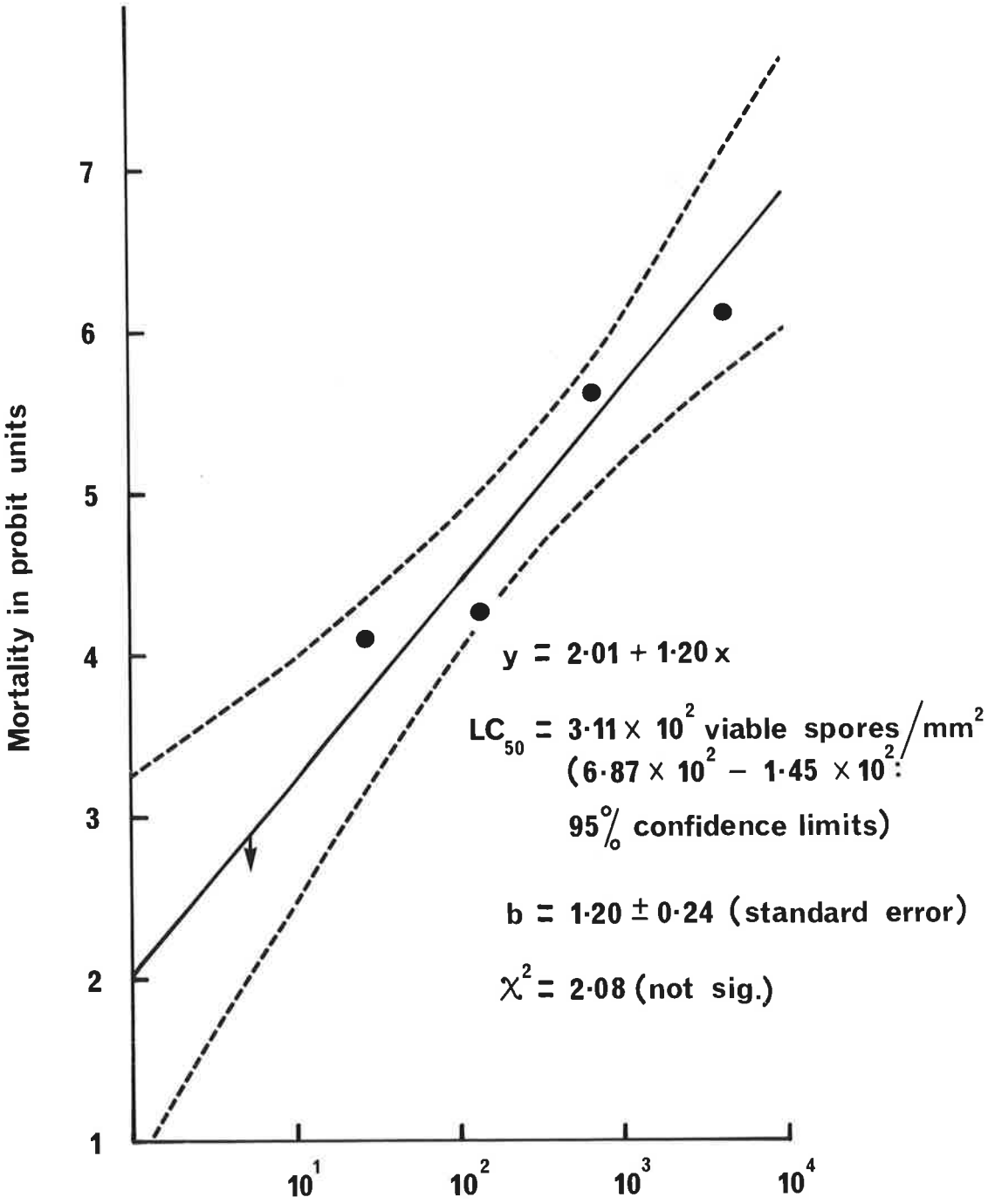
TABLE 2.5 Dose mortality data for *Bacillus thuringiensis* (Thuricide HPSC WP) assayed against third instar *Etiella behrii* (Day 4).

Dose (spores/mm^2)	Total no. larvae/dose	Larvae dead from *B. thuringiensis*	Larvae dead from other causes	Test[a] mortality %
5.4	15	0	0	0
26.8	15	2	4	18.2
134.4	15	3	2	23.1
672.1	15	11	0	73.3
3360.5	15	13	0	86.7
Control	15	0	1	0

[a] Test mortality = $\frac{\text{No. larvae dead due B. thuringiensis}}{\text{Total no. larvae - larvae dead from other causes}}$

ingesting lethal doses of spores as they bored into pods - alternatively, *B. thuringiensis* could be applied before the webs were spun, and larvae would ingest sufficient spores after emerging. At this stage, each larva would have destroyed the seeds in a single pod only, so the reduction in seed yield would not be high. It is proposed that suitably-formulated *B. thuringiensis*, protected from degradation within a cluster of pods, would have a considerable half-life (see page 59), thus timing of applications would be less critical. Unfortunately, field populations of *E. behrii* of any appreciable density were never encountered during the course of this study, and so it was not possible to carry out field experiments. The likely success of *B. thuringiensis* as a control agent of *E. behrii* as part of an integrated control programme will be discussed further (see page 65).

FIGURE 2.8 Bioassay of *Bacillus thuringiensis* (Thuricide HPSC WP) vs third instar *Etiella behrii* - Day 4. Dashed lines indicate 95% fiducial bands. Arrow indicates dose which resulted in zero mortality.


Mortality in probit units
7
6
5
4
3
2
1
10^1
10^2
10^3
10^4
y = 2·01 + 1·20 x
LC_{50} = 3·11 × 10^2 viable spores/mm^2
(6·87 × 10^2 – 1·45 × 10^2:
95% confidence limits)
b = 1·20 ± 0·24 (standard error)
χ^2 = 2·08 (not sig.)

CHAPTER 3

ASSESSMENT OF B. THURINGIENSIS AS A CONTROL AGENT OF H. PUNCTIGERA ON IRRIGATED SEED LUCERNE IN SOUTH AUSTRALIA: FIELD EXPERIMENTS

3.1 Field trial

Introduction

Demonstration in the laboratory of the susceptibility of a target insect to a pathogen does not necessarily imply that the pathogen will be successful as a control agent under field conditions. Larval feeding behaviour on a particular crop may limit the amount of pathogen ingested (see page 31) resulting in ineffective control. Pathogens, whether they be bacteria, viruses, fungi or nematodes may be subject to rapid degradation when exposed to environmental conditions (Cantwell et al., 1966; Ignoffo and Batzer, 1971; Roberts and Campbell, 1977; Gaugler and Boush, 1978). A short half-life has limited the effectiveness of many such preparations (Ignoffo et al., 1974; Jaques, 1972; David et al., 1968). Measurements of the field persistence of B. thuringiensis spores (Pinnock et al., 1971) demonstrated that a decline in spore numbers was due to a constant average force of mortality, presumably ultraviolet radiation. A subsequent study on a different host plant (Pinnock et al., 1974a) indicated that there may exist other factors which bring about a decline in spore numbers, e.g. physical removal of spores may occur from some leaf surfaces.

It is necessary to apply adequate deposits of pathogen to the host plant to ensure that the target insect receives a lethal dose. Physical properties of the foliage of different host plants may affect the amount of B. thuringiensis spores that adhere to the leaves (Pinnock et al., 1975). In addition, inclusion of spray additives (spreader-stickers) which are compatible with the preparation may be necessary to ensure adequate coverage

(Morris, 1969).

Materials and methods

The LD_{90} estimate from the bioassay against third instar larvae (1.00×10^5 viable spores) was used in selecting dose rates for the field trial. The preparation used had a stated potency of 0.0053 international units per spore. Thus in the bioassay the LD_{90} dose represented 5.3×10^2 I.U. in 5 µl, i.e. the concentration used contained 1.06×10^8 I.U./ℓ.

Commercial spraying units ("Lo-Vol") apply 100 ℓ/ha (this volume of water is sufficient to adequately wet the foliage) thus a concentration of 1.06×10^{10} I.U./ha would be applied. Thuricide has a stated potency of 1.6×10^7 I.U./g, thus would require 662.5 g/ha.

Thus it was proposed to apply B. thuringiensis at 3 rates in the trial:

A. 1121.4 g/ha

B. 560.7 g/ha

C. 112.1 g/ha

D. Control (sprayed with water plus wetting agent).

These rates were chosen as they represent a range of doses commonly used against a variety of pest species. The lowest rate is that used for highly susceptible insects e.g. Colias eurytheme (Stern et al., 1959).

The field trial was carried out in January 1976, and the site chosen was a commercial seed lucerne-growing property, "Peaton", near Keith. The crop was flood irrigated, the irrigation bays being approximately 70 m wide. The crop was in flower, and a moderate infestation of H. punctigera larvae was evident. Many moths were ovipositing in the crop just prior to the start of the trial.

The 4 treatments were laid out in a 4 x 4 latin square to accommodate variations in crop density which are often characteristic of South Australian lucerne (Doull, 1961). In this case it was probable that a north-south trend in plant growth existed, due to the northern end of the bay receiving the least irrigation water as the supply channel was at the southern end of the bay. In addition, unknown variations in larval distribution over the area had to be taken into account. A latin square design is the most suitable design where more than one source of variation exists (Clark, 1971). The plot layout was formally randomized (Federer, 1955) and was pegged out (Fig. 3.1).

Each unit of the latin square consisted of a 12 x 12 m square (area 0.0144 ha) which was sprayed with the appropriate treatment. Within this square a 10 x 10 m square was selected, thus leaving a 1 m wide buffer zone around the perimeter to allow for spray drift. For sampling, the 10 x 10 m square was divided up into six 1.67 x 10 m transects; one transect was sampled on each day of the trial, and the sampling sequence of transects within each plot was randomized. Sampling was carried out using a sweep net. This technique, although not giving an absolute count of larval numbers, is frequently used in sampling lepidopterous larvae on lucerne (Stern et al., 1959; Tanada and Omi, 1974). It was considered that this sampling method would prove satisfactory in indicating relative differences in larval numbers, providing

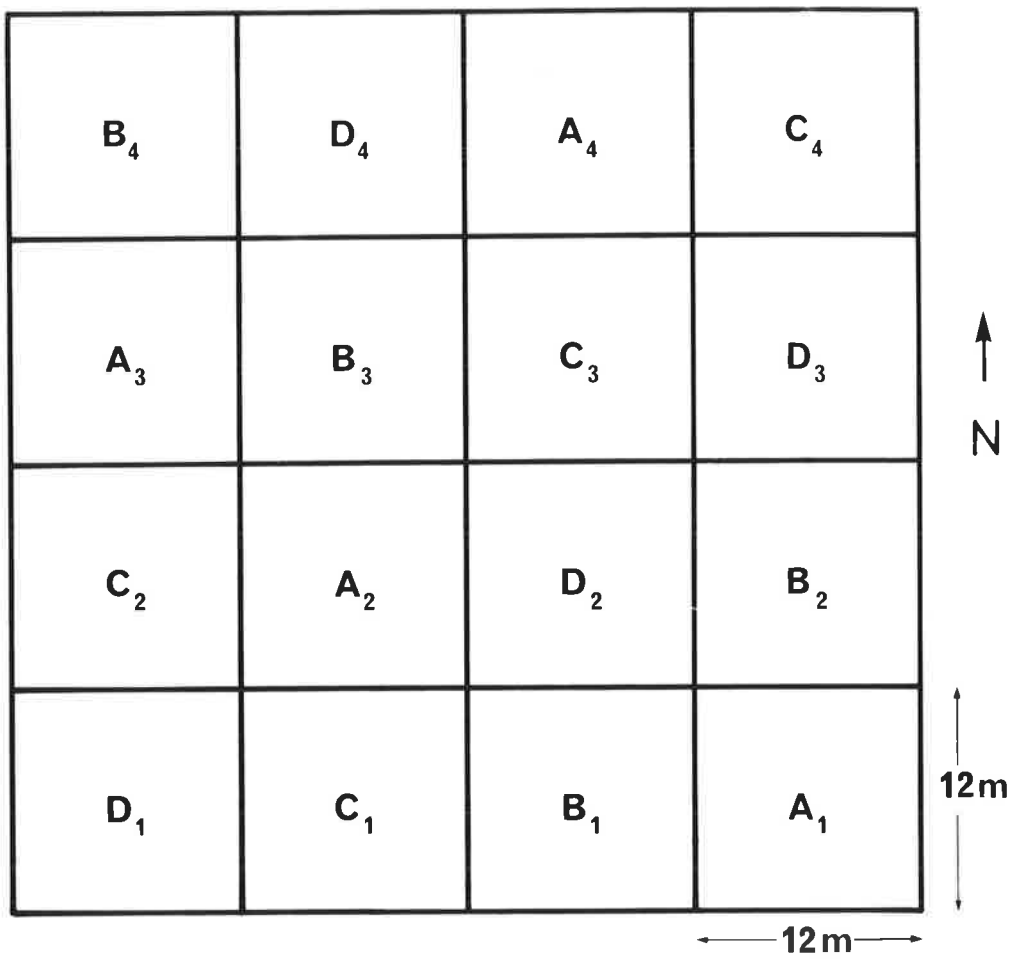
a) that a sufficiently large number of sweeps was made on each transect, and

b) that the effect of treatments being measured was sufficiently large.

A 44 cm "D"-shaped sweep net was used, each sweep being made through an arc of 180°; 17 sweeps were made per transect, i.e. 68 sweeps per treatment.

Spraying was carried out using a knapsack apparatus equipped with a

FIGURE 3.1 4 x 4 latin square design for field trial using *Bacillus thuringiensis* against *Heliothis punctigera*.



Treatments

A = 1121·4 g/ha

B = 560·7 g/ha

C = 112·1 g/ha

D = control

6-nozzle boom. "Tee-jet" 8001 (Rega) nozzles were used, and a pressure of 140 kPa was maintained during spraying. Each plot was sprayed with a volume of 12 ℓ - it was not practicable to use the calculated volume corresponding to 100 ℓ/ha (1.44 ℓ) with the equipment used. It was found necessary to use a spreader-sticker (Topwet$^{(R)}$, Schering) at a dilution of 0.1% to wet the foliage adequately - preliminary experiments indicated that this concentration of spreader-sticker had no effect on spore germination. Spraying was carried out in the early morning, when the average wind speed was 2.2 m/sec. Each plot was sprayed in a north/south and then an east/west direction. The control plots (D) were sprayed with water plus 0.1% spreader-sticker.

It was impractical to sample on the day the crop was sprayed, due to the long period of time involved in spraying. Hence plots were sampled at 10.00 a.m. on the day before spraying (called Pre-Day 0). Sampling was again carried out at 10.00 a.m. on the day after spraying and then at 24 h intervals until Day 5. The stadial composition of the larvae sampled from each plot was determined.

Results and discussion

The average maximum shade temperature over the period of the trial was 35.1^{o}C. Rain fell on 3 occasions (Days 1, 2 and more heavily on Day 5). The total larval count (all instars) on Pre-Day 0 is given in Table 3.1, and a subsequent analysis of this data is given in Table 3.2. No significant variation in larval numbers was evident over the 16 plots, i.e. no bias in the distribution of larval numbers which could affect the results of the trial was evident.

An overall increase in total larval numbers was evident in all treatments up to Day 1 (Fig. 3.2): this was almost certainly a consequence

TABLE 3.1 Total larval count (all instars) Prey-Day 0.

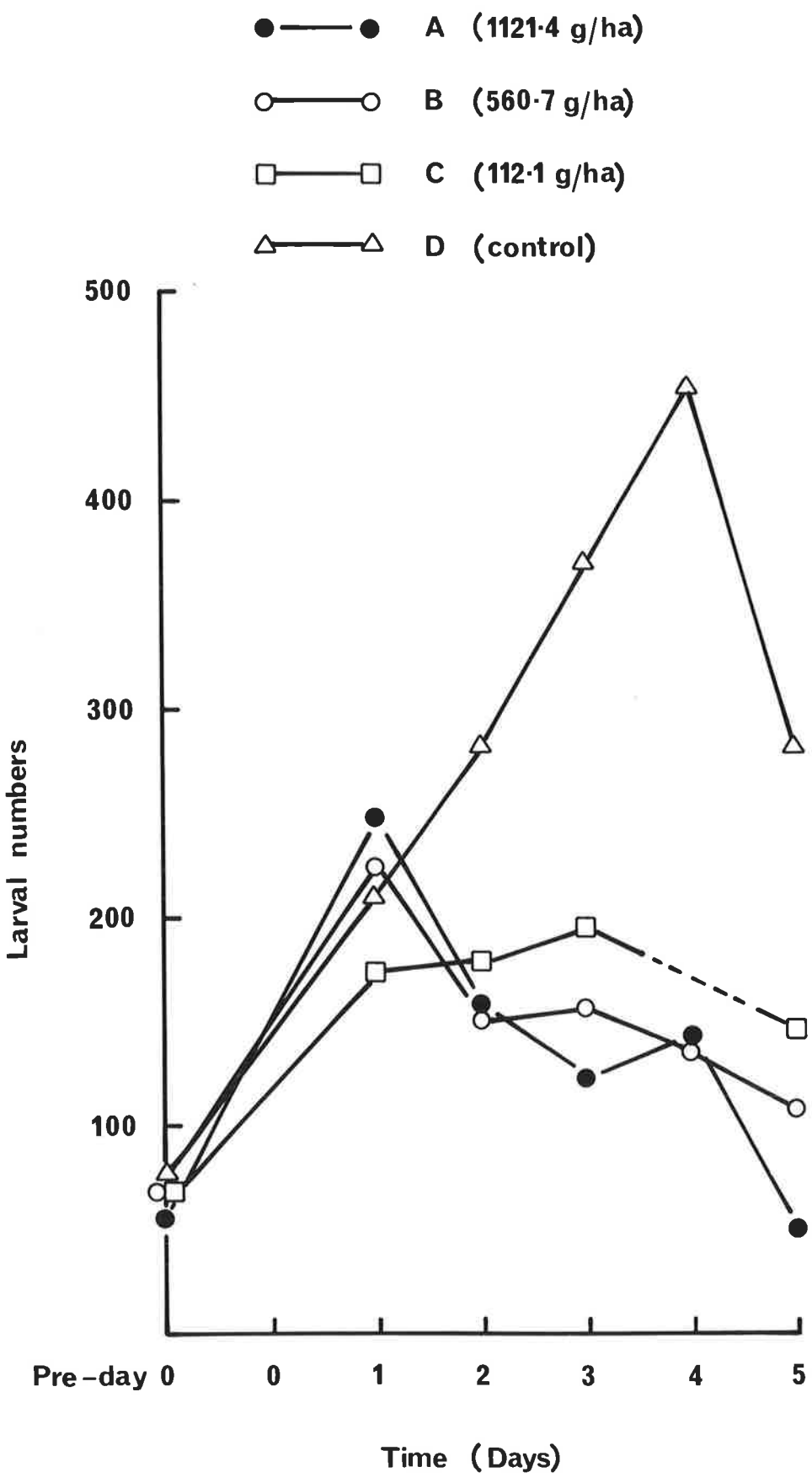
Columns	1	2	3	4
Rows	20	20	18	16
	12	21	25	23
	13	12	10	13
	22	16	17	13

TABLE 3.2 Analysis of variance. Total larval count (all instars), Prey-Day 0.

Source of variation	d.f.	s.s.	m.s.	F ratio
Treatments	3	57.69	19.23	1.20 (not sig.)
Rows	3	151.19	50.40	3.14 (not sig.)
Columns	3	3.69	1.23	0.08 (not sig.)
Error	6	96.38	16.06	
Total	15	308.94	20.60	

of second instar larvae emerging after feeding in buds and flowers prior to Day 1. This trend was continued in the control treatment (D) until Day 4, while the effect of the 3 B. thuringiensis treatments became evident after Day 1. The sudden reduction in numbers in the control (and probably also in the other treatments) between Days 4 and 5 was almost certainly due to the effect of rain which fell on Day 5. The crop was wet during sampling on Day 5, and this may have caused cessation of feeding and a net movement of larvae away from the top part of the lucerne plants. In addition the efficiency of removal of larvae from the crop when both crop and net were wet was almost certainly reduced. The count for treatment C on Day 4 is missing as a result of error (for this treatment the transects

FIGURE 3.2 Effect of Bacillus thuringiensis (Thuricide HPSC WP) on Heliothis punctigera (all instars).


A (1121·4 g/ha)
B (560·7 g/ha)
C (112·1 g/ha)
D (control)
500
400
300
200
100
Larval numbers
Pre-day 0
0
1
2
3
4
5
Time (Days)

that had been swept on the previous day were sampled again by mistake). To test the significance of the effect of B. thuringiensis, an analysis of variance was carried out on results from each day of the trial (Table 3.3). Because of the missing values on Day 4, data from the remaining 3 treatments on this day were analysed as a randomized block design (Clark, 1971).

TABLE 3.3 Treatments F ratios of analyses of variance of total larval numbers in field trial, showing levels of significance.

Day	F ratio (treatments)
1	1.49 (not sig.)
2	7.47 *
3	17.23 **
4	32.70 ***
5	69.90 ***

Although the effect of treatments on Day 5 was highly significant, there may have been some influence of weather on this result. However, on Day 4, larval numbers in treatments A and B were respectively 30.6% and 29.3% of the control, and the levels of significance of these differences are shown in Table 3.4.

TABLE 3.4 Levels of significance by which treatments A and B differed from control on Day 4.

Treatment	P
A	<0.001
B	<0.001

As mentioned in Chapter 2, it was proposed to look at the effect of B. thuringiensis on different instars. For simplicity in presenting results, the numbers of first to fourth instar larvae were summed and are plotted in Fig. 3.3, and the response of fifth and sixth instar larvae are plotted in Fig. 3.4. It is apparent that suppression of both groups of instars occurred, i.e. at the dose rates applied, younger larvae tended to ingest sufficient spores to cause mortality before feeding inhibition occurred. Fifth and sixth instar larval numbers appeared to be suppressed to a similar extent; the most likely explanation is that feeding rate was compensated by instar (Pinnock et al., 1978). Thus older larvae, although less susceptible, nevertheless received sufficient B. thuringiensis spores and endotoxin to cause mortality by virtue of their greater feeding rate. In this respect B. thuringiensis is superior to chemical control; larvae greater than fourth instar are not killed by DDT at the rates normally applied (Department of Agriculture and Fisheries, South Australia, 1978).

The B. thuringiensis formulation applied reduced the H. punctigera population significantly. The results are encouraging, but perhaps could be improved by the adoption of more refined application techniques (see page 13). Another aspect to be considered in implementing a microbial control programme is the achievement of predictable field mortality. This is desirable in an integrated control programme, as the population can be modulated to maintain sufficient numbers of parasites and predators (Pinnock et al., 1974b), yet kept below the level of economic damage. Further work remains to be done to determine such a threshold. To investigate the possibility of achieving predictable field mortality of H. punctigera, a second experiment was carried out concurrently with the field trial.

FIGURE 3.3 Effect of Bacillus thuringiensis (Thuricide HPSC WP) on Heliothis punctigera (first to fourth instars).

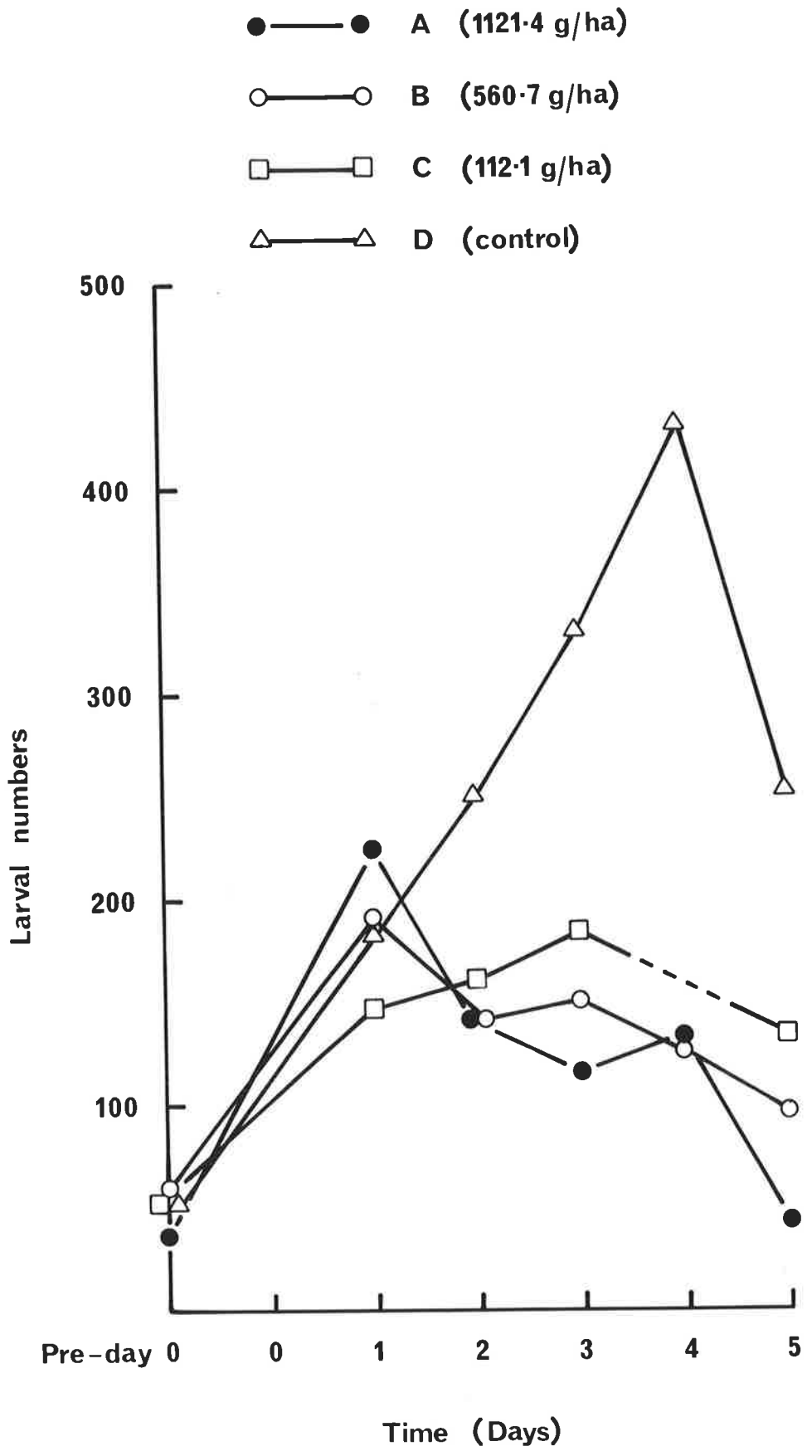
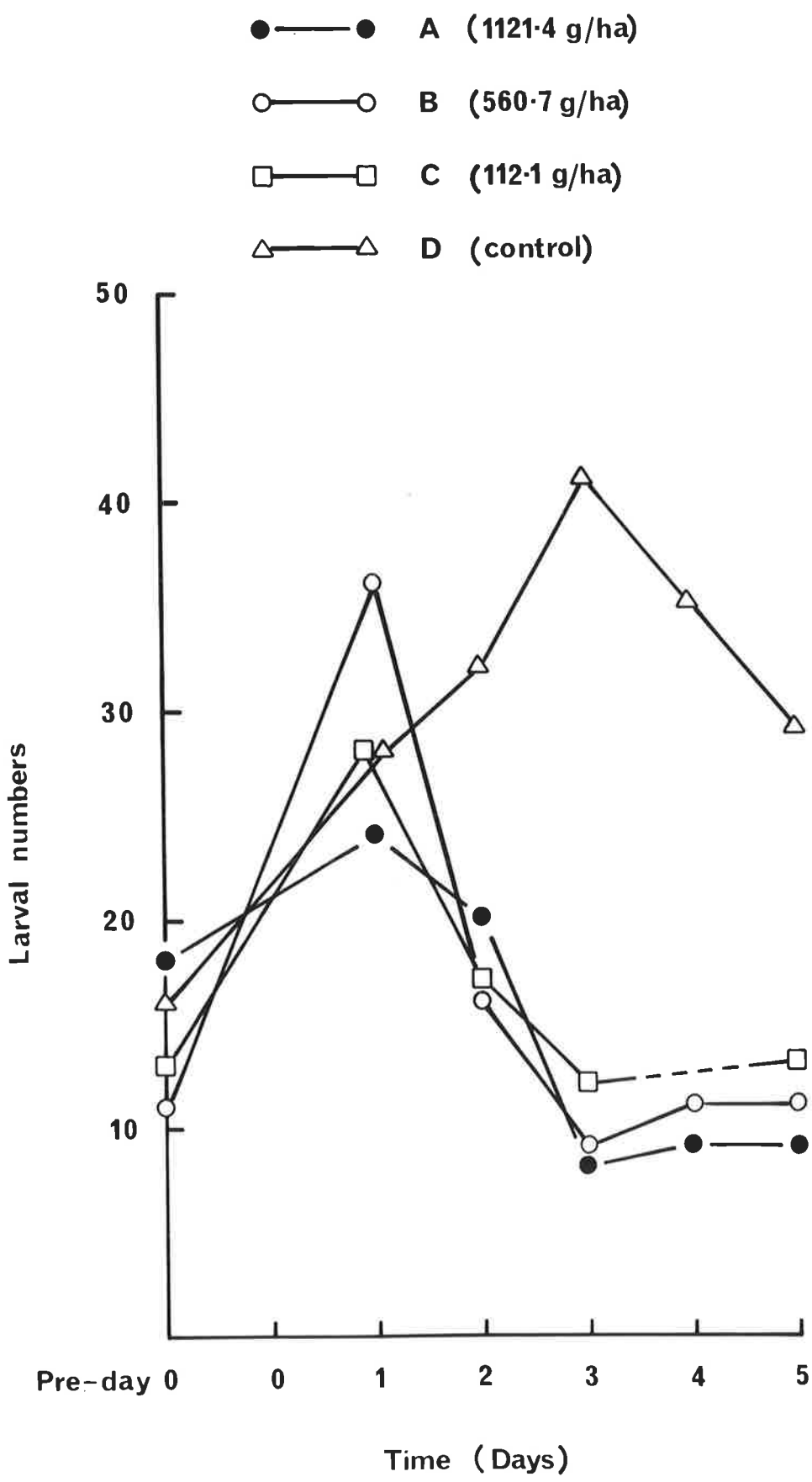

A (1121·4 g/ha)
B (560·7 g/ha)
C (112·1 g/ha)
D (control)
500
400
300
200
100
Larval numbers
Pre-day 0
0
1
2
3
4
5
Time (Days)

FIGURE 3.4 Effect of Bacillus thuringiensis (Thuricide HPSC WP) on Heliothis punctigera (fifth and sixth instars).


A (1121·4 g/ha)
B (560·7 g/ha)
C (112·1 g/ha)
D (control)
50
40
30
20
10
Larval numbers
Pre-day 0
0
1
2
3
4
5
Time (Days)

3.2 Application of a model for prediction of field mortality of H. punctigera

Introduction

The dose of B. thuringiensis ingested by the target insect population depends upon a number of factors. The initial deposit of B. thuringiensis formulation applied to the foliage, the rate at which the spores are inactivated when exposed to environmental conditions and the feeding rate of the insect in question affect the dose received by an individual. Use was made of these parameters by Brand et al. (1975) in constructing the following model for estimating average total dose, D:

$$\log_{10} D = \log_{10} r - 0.363 + a - \log_{10}(-b_e)$$

D represents the average total dose measured over the interval (O, T); r = the constant average feeding rate of the larvae and a = average log (viable spore count) at time of application (T = 0). The field persistence of B. thuringiensis spores may be measured by plotting log (viable spore count) determined from field-collected leaf samples against time. Depending on the host plant involved, a segmented linear or a linear pattern of spore decay is observed (Pinnock et al., 1974a). The slope of the early part of a segmented linear curve, b_e, or the slope of a linear curve, b, is the parameter used in the model as outlined above.

The index of effective dose is perhaps open to criticism in that it is based on viable spore count and information on the δ-endotoxin of B. thuringiensis is not incorporated into the model. However Brand et al., (1976) demonstrated that viable spore count alone is a highly significant indicator of effective dose. Subsequently a method was developed for predicting field mortality of the target insect, based both on the estimation of effective dose and also the time for which the B. thuringiensis spores were exposed on the leaf surface prior to ingestion. The following

field mortality prediction should be regarded as a simplification of the method of Brand et al. in that an estimate of effective dose alone is used to predict field mortality.

Materials and methods

An estimate of b_e was obtained by conducting a field degradation study over the first 24 h of the field trial. Twenty leaves were sampled from the plots treated with treatment B (560.7 g/ha) 30 min after spraying was completed (5 leaves were taken from each of the 4 plots which received treatment B). The leaves were handled with forceps by the petiole only, and a sterile cork borer was used to cut a 6 mm disk from each leaf. The spores were removed from the disks and the viable spore count was made as described on page 36. Leaves were sampled again after an interval of 24 h. A plot was then made of log (viable spore count) against time. Practical difficulties (in the form of supply of pre-sterilized glassware in the field) prevented the field degradation study continuing any longer than 24 h; in any case, as mentioned, the value of the slope measured over the initial (early) part of the curve, b_e, is all that is required in the estimation of effective dose. The degradation curve also provided the value of a, the value of log (viable spore count) at T_0.

The procedure for dealing with zero counts was similar to that adopted by Pinnock et al. (1974a). Zero counts were taken to represent some value between a true zero and the lowest detectable count i.e. a yield of 1 colony per plated volume. Therefore average replicate counts of zero were assigned a value of 0.1, divided by the number of replicates. Thus the difficulty of taking the logarithm of zero was avoided.

The parameter b_e may also be used to estimate π, the viable spore half-life (Pinnock et al., 1971).

Thus π (hours)

$$= \log \frac{(0.5)}{-b_e} \text{ days}$$

x 24 (hours per day)

Thus the average viable spore count at time t = twice the average viable spore count at time t + π.

The constant average feeding rate, r, of third instar larvae was estimated. Third instar larvae were selected in order to be able to make use of the bioassay using B. thuringiensis against third instar H. punctigera in predicting field mortality. Fifteen larvae were collected from the field and allowed 24 h to acclimatize to laboratory conditions, during which time they fed on lucerne sprigs. The larvae were then allowed to feed on 6 mm diameter lucerne leaf disks (28.27 mm^2) for 2 h. Six mm disks were used to maintain consistency with the leaf disks used in the field degradation study. The proportion of the disks consumed was estimated by photographing whole and partly-consumed disks using a macro lens and projecting the resulting negatives onto squared paper - counting the squares enabled an estimate of the proportion of each disk consumed to be made. The feeding rate was then expressed as the number of disks consumed per 24 h.

Results and discussion

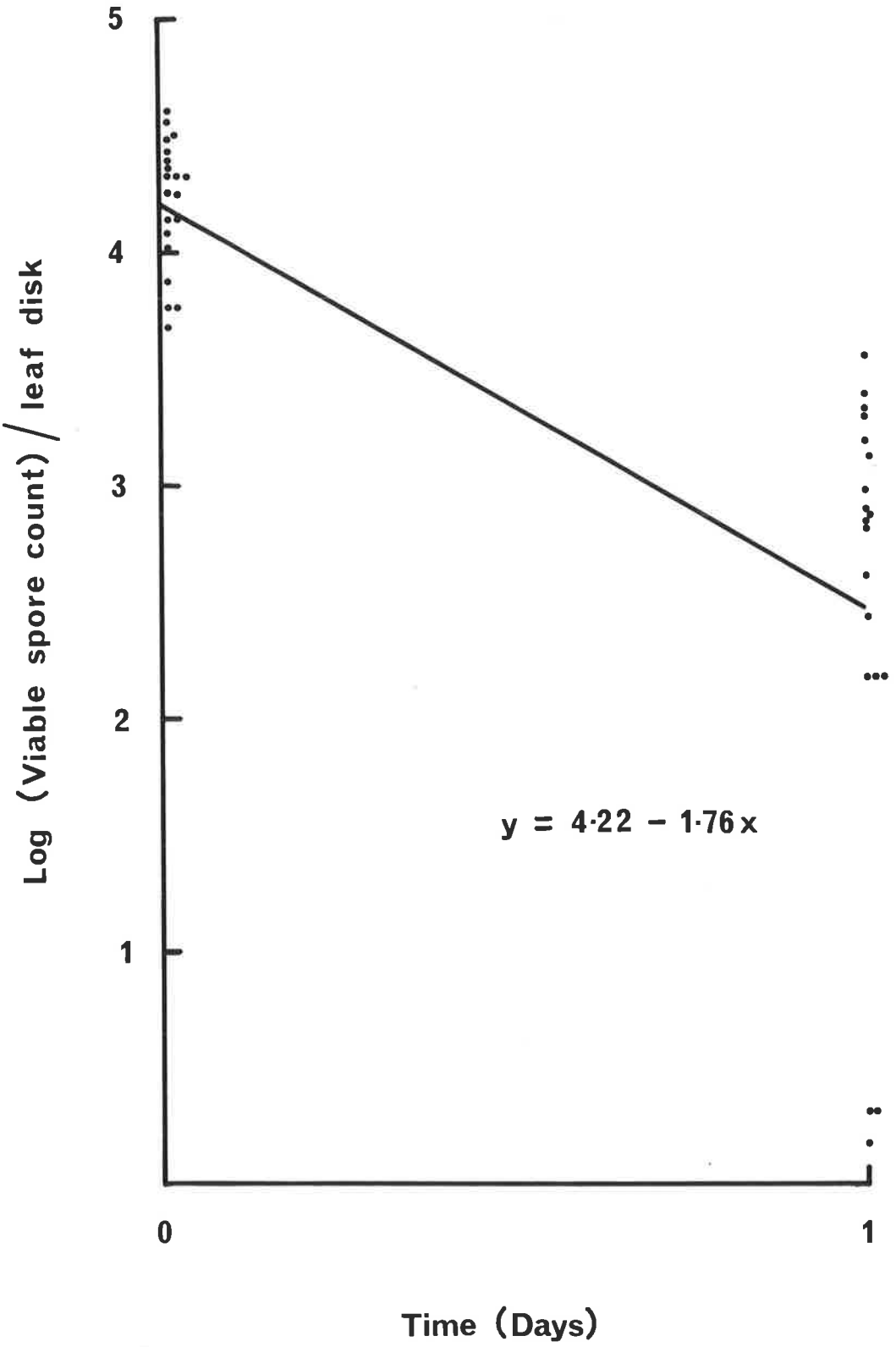
The field degradation curve (Fig. 3.5) yielded a value of $b_e = -1.76$ and a value of a = 4.22. Hence the viable spore half-life may be calculated as follows:

$$\pi = \frac{-0.3010}{-1.76}$$

$$= 0.17 \text{ days}$$

$$= 4.11 \text{ h.}$$

FIGURE 3.5 Field degradation of viable Bacillus thuringiensis (Thuricide HPSC WP) spores on lucerne leaves.


5
4
3
2
1
0
1
Log (Viable spore count) / leaf disk
y = 4·22 − 1·76x
Time (Days)

The constant average feeding rate, r, was estimated to be 3.41 leaf disks per day.

The average total dose for third instar larvae may be estimated as follows:

$$\log_{10}D = \log_{10} 3.41 - 0.363 + 4.22 - \log_{10} 1.76$$
$$= 4.14$$

$\text{Log}_{10}D$ may then be substituted into the regression equation from the third instar bioassay (Fig. 2.7) to give an estimate of predicted mortality. The limits of b of this equation (Clark, 1969) are used to compute confidence limits for predicted mortality.

$Y = 1.14 + 1.03X$ (b = 1.03 (1.17 - 0.89, 95% confidence limits)).

Thus when $X = 4.14$, $Y = 5.40$(5.98 - 4.82).

Converting probit units to % mortality (Finney, 1971) yields an expected mortality of 65.5% (83.6% - 42.9%). Observed mortality on Day 3 is calculated from both third and fourth instar larval numbers. The assumption is made that by Day 3 of the field trial, a proportion of the larvae which were third instar on Pre-Day 0 would by this time have moulted to fourth instar. Thus it is appropriate to include both third and fourth instar larvae in the count (even though the third instar count would no doubt have included some larvae that were younger than third instar on Pre-Day 0). Larval numbers on Pre-Day 0 and on Day 3 are shown in Table 3.5.

The observed mortality on Day 3 for treatment B compared to treatment D is 71.3%.

There is close agreement between observed and expected mortalities, and the results compare favourably with those of Pinnock et al. (1978). This is possibly because this study concentrated on one instar, whereas in the study of Pinnock et al., the model was applied to all instars of the

TABLE 3.5 Numbers of third and fourth instar Heliothis punctigera larvae on Pre-Day 0 and Day 3 of field trial.

Treatment	Pre-Day 0 (Third instar larvae)	Day 3 (Third and fourth instar larvae)
B	25	50
D (control)	25	174

test insect, Schizura concinna; hence it was necessary to make the assumption that feeding rate and mortality were compensated by instar. In the above study with H. punctigera, as was the case with S. concinna, the assumptions must be made that larvae feed at a constant rate and also that each larva receives the average dose.

The use of B. thuringiensis to reduce populations of H. punctigera by a predictable amount appears promising as a result of the above study. Possibly more precise agreement between observed and expected mortality might be possible if the factor concerning time for which the spores were exposed on the leaf surface prior to ingestion was also incorporated into the model. Brand et al. (1976) demonstrated that the prediction of mortality was subject to bias if the leaf exposure time factor was omitted. Further work with other instars of H. punctigera is necessary, as the response in the field of all instars must be known before this concept can be applied to a population comprising several instars. Unfortunately lack of time and the commitment to investigate other aspects of pathogens infecting H. punctigera did not permit further studies in this area.

3.3 Economics and formulation of B. thuringiensis for use against H. punctigera

Economics

B. thuringiensis formulations are currently being sold in relatively small quantities in Australia, and as all this material is imported from overseas, the cost is extremely high. The results of the field trial (Fig. 3.2) suggest that the rate used in treatment B (560.7 g/ha) or possibly (with improved application techniques), treatment C (112.1 g/ha), would achieve adequate control. The cost of Thuricide at these rates is $15.42/ha and $3.08/ha respectively. While the cost of the lower rate is comparable to many insecticides, the higher rate is very expensive. However, increased sales volumes and local formulation of B. thuringiensis would greatly reduce costs (Teakle, 1977b).

It is highly likely that considerably fewer spray applications would be necessary should B. thuringiensis be adopted as a control agent. Broad spectrum insecticides such as parathion and chlorpyrifos reduce populations of parasites and predators (see page 64), hence rapid resurgence of H. punctigera populations occur. This is almost certainly why such frequent insecticide applications are necessary. The cost of application of insecticides is often approximately equal to the cost of the material applied; hence if an expensive product such as B. thuringiensis is used less frequently than a cheaper product, it may result in no increased overall costs. In California, an integrated control programme using B. thuringiensis replaced the use of chemical insecticides for the control of S. concinna on Cercis occidentalis trees (Pinnock et al., 1974b), and the result was a net reduction in pest control expenditure (Pinnock and Brand, 1979).

More precise predictions about the overall costs of using

B. thuringiensis in the H. punctigera/seed lucerne system can only be made as a result of larger-scale field testing, which unfortunately was beyond the scope of this study. On the basis of the results so far, such investigation would appear well worthwhile. The amount of expenditure justified on pest control is related to the value of the crop; return from irrigated seed lucerne, which can vary from $500-$1800 per ha, is approaching that of many horticultural crops. Hence on irrigated lucerne especially, the use of B. thuringiensis may be justified economically. Even if the price of B. thuringiensis in Australia remains high, it is likely that its use at critical periods of seed production, e.g. at flowering, would be sound practice.

Formulation of B. thuringiensis

The value of the half-life (π = 4.11 h) obtained in the field degradation study was low - Pinnock et al. (1978) obtained a value of π = 9.1 h for Dipel$^{(R)}$, another formulation of B. thuringiensis var. kurstaki on C. occidentalis foliage. Due to the relationship of the parameter b_e with $\log_{10}D$ (page 53), any increase in the value of π should result in an increase in the effective dose. Such an increase would be obtainable by using a dust formulation. Dust formulations result in a considerable reduction in the rate of inactivation of viable spores by sunlight (Cantwell and Franklin, 1966). Pinnock et al. (1971) recorded a value of π = 22.1 days for Biotrol$^{(R)}$ Dustable BTB1832.5D, a dust formulation of B. thuringiensis. This extended value of π is thought to be due to the protective effect of dust particles adhering to the spores. Such a formulation would have obvious advantages in the conditions prevailing in summer in South Australia, where the strong sunlight and clear skies would appear to have reduced the value of π to considerably lower levels than were recorded in California.

An additional benefit in employing a dust formulation is the superior coverage obtainable compared to that obtained using water based sprays (Cantwell and Franklin, 1966). Hall (1963) reported that dust formulations of B. thuringiensis, by virtue of their excellent coverage, gave adequate control of Trichoplusia ni, whereas wettable powder formulations were not effective. Thus a dust formulation could well prove to be the most suitable formulation to use on seed lucerne. In particular, to control E. behrii, the properties of thorough coverage and extended half-life should make this type of preparation most suitable.

3.4 Integrated control

Introduction

Due to the existence of several other pests or potential pests and the need to maintain populations of pollinating insects, the control of H. punctigera on seed lucerne has become a complex problem. Control measures should not be taken against populations of H. punctigera without regard to the effect these measures might have on other species. Since the introduction of T. trifolii to this country, more caution than was previously necessary should be exercised, due to the serious nature of this new pest. Thus it is necessary to adopt a broader approach, hence the adoption of an integrated control programme might be the only satisfactory approach. Integrated control is defined (Falcon, 1973) as "a broad ecological approach to pest control that utilizes a variety of control technologies compatibly in a pest management system. To be most effective, realistic economic injury levels are used to determine the need for pest control actions. At the same time, every possible measure is taken to protect and preserve naturally occurring biotic mortality agents, such as parasites, predators, and pathogens". Thus control measures carried out

against the target insect, whether they be application of chemicals or pathogens or the release of parasites, are carried out in such a way as to be as selective as possible.

As mentioned, the economic threshold for H. punctigera has yet to be determined. As with cotton pests (van den Bosch et al., 1971), emphasis will have to be placed on the stage of development of the crop in determining this parameter. Susceptibility to damage will vary considerably between early flowering and the formation of mature seed pods. Hence the larval population that can be tolerated over this time will vary. In addition it is likely that the moisture status of the plants would affect susceptibility to damage. Thus a considerable amount of investigation is necessary to establish this important parameter.

A successful integrated control programme should operate so as to cause as little as possible ecological disruption to the system (Smith and van den Bosch, 1967). Hence maximum use should be made of biological control within the integrated control programme, i.e. parasites and predators should be allowed to exert their maximum effect. This is not possible when broad spectrum insecticides are used. However due to the specificity of B. thuringiensis, many instaces of control programmes using B. thuringiensis and naturally-occurring or applied parasites or predators have been recorded (Chepetilnikova and Fedorintchik, 1962; Falcon et al., 1968; Bullock and Dulmage, 1969; Conway, 1969; Jensen, 1969; Pinnock and Brand, 1979). To maintain sufficient stocks of parasites and predators, a residual population of the host must be maintained to prevent the phenomenon of "pest resurgence" (Stern and van den Bosch, 1959; Smith and van den Bosch, 1967). Thus it is possible to significantly reduce parasite populations as a result of B. thuringiensis application by suppressing host numbers to a low level (Hamel, 1977). Thus the aim of an integrated control

programme should be to regulate the target insect population as opposed to decimation of the population which often occurs as a result of purely chemical control. As indicated, the current study suggests that the achievement of predictable mortality of H. punctigera with B. thuringiensis is possible.

Parasites and predators

Several species of parasites and predators of H. punctigera were observed in the field (Table 3.6). Of the parasites the Tachinidae were the most common; Cullen (1969) recorded a range of 5.0-22.3% of H. punctigera larvae parasitized by Tachinidae in South Australia over 4 seasons (1965-1968). Learmonth (unpublished) commonly encountered egg parasitism by Trichogramma australicum Girault, and also recorded egg parasitism by Telenomus sp. Of the predators recorded Nabis tasmanicus Rem. was the most commonly encountered; this predator attacks eggs and first and second instar larvae (Saeed Awan, unpublished). The pentatomid Oechalia schellenbergii (Guér Mén.) was also commonly encountered until spraying for T. trifolii was commenced in late 1977. The nymphs of O. schellenbergii prey upon first, second and third instar larvae, while adults prey upon third and fourth instar larvae (Saeed Awan, unpublished). Predatory Sphex sp. wasps were frequently observed in late summer; these wasps prey upon sixth instar larvae, and bury their immobilized prey in underground chambers.

Hymenopterous parasites were also recorded from E. behrii. As mentioned previously, field populations of E. behrii were extremely low during the course of the study. The largest collection of larvae was 17, caught at Willalooka in 1976, and from these were reared the parasites recorded in Table 3.7.

TABLE 3.6 Parasites and predators of Heliothis punctigera.

Classification	Occurrence
Larval Parasites	
DIPTERA	
Tachinidae	
Chaetophthalmus sp.	Common
HYMENOPTERA	
Braconidae	Common
Ichneumonidae	
Ophion sp.	Rare
Egg Parasites	
HYMENOPTERA	
Trichogrammatidae	
Trichogramma australicum Girault	Common
Scelionidae	
Telenomus sp.	Unknown
Predators	
HETEROPTERA	
Pentatomidae	
Oechalia schellenbergii (Guér-Mén.)	Common
Cermatulus nasalis (Westw.)	Rare
Nabidae	
Nabis tasmanicus Rem.	Abundant
HYMENOPTERA	
Sphecidae	
Sphex sp.	Common
NEUROPTERA	
Hemerobiidae	
Micromus tasmaniae Walker	Abundant
Chrysopidae	
Chrysopa signata Schneider	Abundant
Chrysopa ramburi Schneider	Common
Chrysopa innotata Banks	Rare
COLEOPTERA	
Coccinellidae	
Coccinella repanda Thunb.	Abundant

TABLE 3.7 Parasites of Etiella behrii.

	Classification	Number
	HYMENOPTERA	
	Ichneumonidae	
Porizontinae (Tribe Porizontini)		1
Temelucha sp.		4
	Braconidae	
Agathis sp.		1
	Eulaphidae	
Elasmus sp.*		1

*May have been hyperparasite.

Although the sample size was small, the level of parasitism recorded suggests that for most of the time "natural" control by parasitic species keeps E. behrii populations at a low level. This finding also suggests that application of parathion for E. behrii control is probably completely unnecessary and may even be detrimental in that beneficial insects are reduced in numbers. Stern and van den Bosch (1959) state that Nabis is drastically affected by parathion; hence at least one important predator of H. punctigera is being reduced in numbers by this practice. It is highly likely that hymenopterous parasites would be similarly affected, as would pollinating insects, e.g. honey bees (see page 33).

An integrated control programme is envisaged in which B. thuringiensis is used when necessary to maintain the H. punctigera population below an economic threshold, but at a level which would maintain sufficient numbers of parasites and predators. It is likely that these parasites and predators would reduce the frequency of sharp increases in H. punctigera numbers which are currently observed shortly after chemical spraying. It is possible that applications of B. thuringiensis would be necessary only after

major flights of moths, i.e. 3-4 times per season. With the information available so far, it is likely that E. behrii would emerge only rarely as a pest in such a programme, especially if dust formulations were used.

Integrated control of T. trifolii

In 1954, growers in California experienced the same problems faced now in Australia - T. trifolii was accidentally introduced and within a short time the lucerne industry was seriously threatened by the enormous populations of aphids which built up. After initial failures due to over-use of insecticides, an integrated control programme was successfully established. This programme made use of low doses of a selective insecticide (demeton) which controlled T. trifolii but which caused minimum interference with biological control (Smith and van den Bosch, 1967). Subsequent use of resistant lucerne cultivars reduced the pest status of T. trifolii even further. Hence the recommendation by the South Australian Department of Agriculture and Fisheries that a similar programme be established here would appear sound advice. Of the insecticides recommended, pirimicarb, a selective aphicide, is the least injurious to hymenopterous parasites. Farmers who are at present attempting to control T. trifolii by the use of broad spectrum insecticides (see page 30) will almost certainly encounter the problems experienced initially in California, where resistant strains of aphids quickly emerged (Smith and Hagen, 1959).

The necessity to implement integrated control to deal with T. trifolii substantially reinforces the case for setting up an integrated control programme against H. punctigera and E. behrii. Thus it would be folly to attempt to rely on selective chemical and biological control to deal with T. trifolii, and at the same time continue to use DDT and parathion

against H. punctigera and E. behrii. A juxtaposition of chemical and biological control programmes is unsatisfactory (Smith and van den Bosch, 1967). Parathion is extremely toxic to the aphid parasite Trioxys complanatus (Stern and van den Bosch, 1959) hence the effectiveness of this species would be greatly reduced. Similarly, the use of parathion would reduce numbers of native predators of T. trifolii which include species of Coccinellidae and Chrysopidae (Swincer, pers. commun. 1978).

3.5 Conclusion

The proposed integrated control programme will require a good deal more development. Detailed ecological studies on the pests involved and on the parasites and predators will have to be completed - at present, studies on the ecology of H. punctigera and on the behaviour of O. schellenbergii and N. tasmanicus are in progress at the Waite Agricultural Research Institute. Work may have to extend over several seasons before a successful programme is implemented. As mentioned, aphid-resistant cultivars will probably be necessary to further minimize the effects of T. trifolii. An approach to pest problems in seed lucerne on the broadest possible scale is required, and to succeed, such an approach will require the cooperation of specialists from several disciplines.

So far mention has been made of the use of the applied pathogen, B. thuringiensis as part of a control programme against H. punctigera and E. behrii. However, use is frequently made of naturally occurring pathogens in integrated control programmes (Falcon, 1973). In the case of pathogens which multiply in the host and are subsequently released to the environment, e.g. viruses and fungi, host density may affect the subsequent

amount of pathogen available for re-infection. Mathematical models involving such bidirectional systems may also be used to predict pest mortality (Pinnock and Brand, 1979). These models involve a greater level of complexity than do models predicting field mortality due to B. thuringiensis.

Two naturally occurring viruses have been recorded in H. punctigera in Australia (page 2, page 16). Of these the nuclear polyhedrosis virus appears to cause the highest field mortality in South Australia. In order to determine what factors lead to outbreaks of this virus, the remainder of this thesis will be concerned with the epizootiology of this disease. Such a study might prove worthwhile - firstly, from an academic viewpoint, in that it might make some contribution to the field of epizootiology; secondly, it might make possible the manipulation of the incidence of the virus to increase natural mortality of H. punctigera.

CHAPTER 4

EPIZOOTIOLOGY OF THE NUCLEAR POLYHEDROSIS VIRUS OF H. PUNCTIGERA: DEVELOPMENT OF METHODS

4.1 Introduction

Virus epizootics occur frequently in H. punctigera populations during summer and early autumn in South Australia. Cullen (1969) recorded 60-70% mortality from a virus in February and 95-100% mortality in March in larvae collected from irrigated lucerne. Cullen termed the virus a granulosis virus, but from his description of virus-killed larvae and from my own observations, the symptoms he described were almost certainly those of a nuclear polyhedrosis virus (NPV) (Teakle, 1973). Infected larvae, usually fifth or sixth instar, climb to the top of the plants and often become attached by the prolegs. The larvae turn brown and flaccid, and after death the body ruptures and releases haemolymph containing large numbers of polyhedra (Fig. 4.1). The incubation period of the virus varies from 2-14 days, depending upon larval age at infection and the dose ingested. It is thought that the virus is closely related or even identical to H. zea NPV which is marketed as a microbial insecticide (Teakle, 1978).

A granulosis virus (GV) in H. punctigera described by Teakle (1974) also occurs in South Australia. The incidence of this virus is low and no epizootics were observed. An epizootic has been recorded on only one occasion in Queensland (Teakle, 1978). GVs have been known to occur in mixed infections with NPV in Heliothis species (Teakle 1978; Whitlock, 1977) but this phenomenon was observed in a very low number of cases in H. punctigera in the course of this study.

FIGURE 4.1 Fifth instar larva of Heliothis punctigera killed by nuclear polyhedrosis virus. x10.

Initiation of epizootics

In order to be able to manipulate a naturally occurring pathogen (e.g. a virus) to effect pest control, a thorough understanding of the factors leading to disease outbreaks is necessary. Knowledge on this subject is far from complete (Tanada, 1976). In a wide range of insect hosts, the course of disease can change from an enzootic to an epizootic state, and the reason for these outbreaks is often not readily apparent. The same generalization can be applied to many viruses that infect vertebrates e.g. the causative agent of bovine ephemeral fever (St. George et al., 1977).

A once-popular explanation for the initiation of epizootics of virus diseases in insect hosts was the phenomenon of stress. Stress has been implicated as the initiator of epizootics by the activation of "latent" viruses[1], (Orlovskaya, 1967) although this view is not held so widely now (Tanada, 1976). Stress is defined as a state manifested by a syndrome or bodily changes caused by some force, condition or circumstance (i.e. by a stressor) in or on an organism or on one of its physiological or anatomical systems (Steinhaus and Martignoni, 1970). A stressor commonly associated with epizootics is crowding (although density-dependent stress phenomena are poorly understood (Surtees, 1971)). Bishop et al. (1978), investigating an NPV of the cotton looper, Anomis flava Fabr., suggested that disease outbreaks occurred when the larval population approached a certain density. They proposed a disease outbreak threshold of 11.6 larvae per metre of row. Temperature and humidity have also been implicated as predisposing factors to disease outbreaks (Wallis, 1957; Michelbacher and Smith, 1943). Cullen (1969) stated that high temperatures appeared to be

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[1] The term "latent" virus is defined by David (1978) as a virus present in an occult state in which it has lost such properties as infectivity and antigenicity, but persists in the host cell (perhaps associated with the genome) and replicates with it.

a contributing factor towards NPV epizootics in H. punctigera. However in recent investigations several systems have been described in which the onset of virus epizootics have not been associated with the above factors. Tanada and Omi (1974a) found that virus epizootics developed at low densities of Autographa californica and Spodoptera exigua. Temperature, humidity and rainfall had no obvious influence on epizootics in S. exigua and Colias eurytheme, although rainfall and temperature may have affected outbreaks of a granulosis virus in A. californica. Doane (1970) found no evidence that an NPV epizootic in Porthetria dispar was the result of high temperature, humidity or starvation. Thompson (1959) found in laboratory studies that H. zea larvae were highly resistant to H. zea NPV at a temperature of 39°C - this finding would appear to contradict Cullen's statement that NPV epizootics are initiated as a result of high temperature in H. punctigera.

Baculoviruses are perhaps unique among viruses in their ability to survive outside the host - the inclusion body affords remarkable protection against a wide variety of chemical and physical agents (Bergold, 1958; Jaques, 1977) and against microbial decomposition (Jaques and Huston, 1969). Thus disease outbreaks may be initiated as a result of an inoculum of virus being delivered (under appropriate conditions) to a susceptible host population from an external source. Although baculoviruses are rapidly degraded by sunlight (see page 81), it is widely recognised that they can remain active in soil for long periods of time (Jaques 1964, 1967a, 1969, 1970, 1974a, 1974b; Thomas et al., 1972; Tanada and Omi, 1974b; Roome and Daoust, 1976; David, 1978). Polyhedra are adsorbed onto soil particles (Hukuhara and Namura, 1971; Hukuhara, 1972) and leaching from surface layers of soil does not appear to be important. Lethal amounts of virus can be transferred to crop plants from soil by rain or irrigation (Thompson and Steinhaus, 1950; Jaques 1964, 1967a, 1974a, 1974b). Tanada

and Omi (1974b) detected virus deposits on lucerne foliage when no host larvae were present on the crop, and presumed that virus had been transferred from the soil to the crop by rain or wind. Hukuhara and Kitajima (1978) have proposed a model to depict the progress of a GV epizootic which occurs in Hyphantria cunea in Japan; according to the model the host population receives an initial inoculum of virus from the soil in the spring.

It is proposed that in the Heliothis punctigera/lucerne system under study, NPV epizootics are initiated by the above mechanism. The cropping system employed with lucerne in South Australia provides ideal conditions for such a mechanism. Lucerne crops are grown on a perennial basis, with stands of 10-15 yr being common. Hence polyhedra could be delivered to the soil from successive diseased populations, and the reservoirs of virus would not be disturbed by tillage. In addition sandy-loam soils (which occur in the area of this study) retain polyhedra longer than richer soil (Surtees, 1971). The low stature of the lucerne crop would enable maximum contamination of foliage from soil splashed up by rain or irrigation, or by wind, and the exposed feeding habits of the larvae would result in a high probability of contaminated foliage being ingested.

To test this hypothesis, a method was required for detecting virus on lucerne foliage, so that the levels of polyhedra could be monitored, in conjunction with recordings of rain or irrigation. It was proposed to carry out the study over a period of time and to determine if peaks in polyhedral counts preceded epizootics. Studies to detect virus levels on foliage have almost invariably employed bioassays (Jaques, 1967b; Bullock, 1967; Bullock et al., 1970; David et al., 1971; Ignoffo and Batzer, 1971; Young and Yearin, 1974; Tanada and Omi, 1974(b); Roome and Daoust, 1976). Bioassays have the advantage of extreme sensitivity especially if

neonate test larvae are used (Shapiro and Ignoffo, 1970). In addition, information is provided on the level of infective virus present. However, there are drawbacks with these methods. Due to the low precision of virus bioassays it is necessary to use large numbers of test larvae; hence these experiments are very labour intensive, and this may limit the scope of an investigation. Hence the proposed field monitoring experiment, involving 20 sampling stations sampled weekly over several months, required an alternate method of detecting virus on foliage. Such a method would have to entail quantitative recovery of virus, and would have to provide information on infectivity of detected virus. The study would also need to determine if substantial amounts of virus were present in the soil.

4.2 Development of an immunofluorescent detection method

Introduction

After preliminary investigation, a technique was developed for washing virus off foliage, concentrating and then detecting the polyhedra by an immunofluorescent tracing method. The immunofluorescent detection technique will be discussed first. Tracing methods of this type are well suited to detecting pathogens derived from field sources. Davidson and Pinnock (1973) used a similar method for detecting deposits of commercial *H. zea* NPV that had been applied to cotton leaves. Leaf washings would be likely to contain soil-derived debris which would make identification of polyhedra by light microscopy difficult - polyhedra and soil particles appear very similar under the light microscope (Hukuhara and Namura, 1971). Thus the immunofluorescent method could be expected to have a lower detection threshold than a method employing normal light microscopy.

Materials and methods

Purification of H. punctigera NPV

Virus-killed H. punctigera larvae were collected from the field, and stored for up to 10 weeks in water at room temperature. NPV was purified from the decomposing cadavers by a method involving sonication and repeated centrifugation in 20-50% sucrose gradients containing 0.1% sodium dodecyl sarcinate (Griffith, pers. commun. 1976).

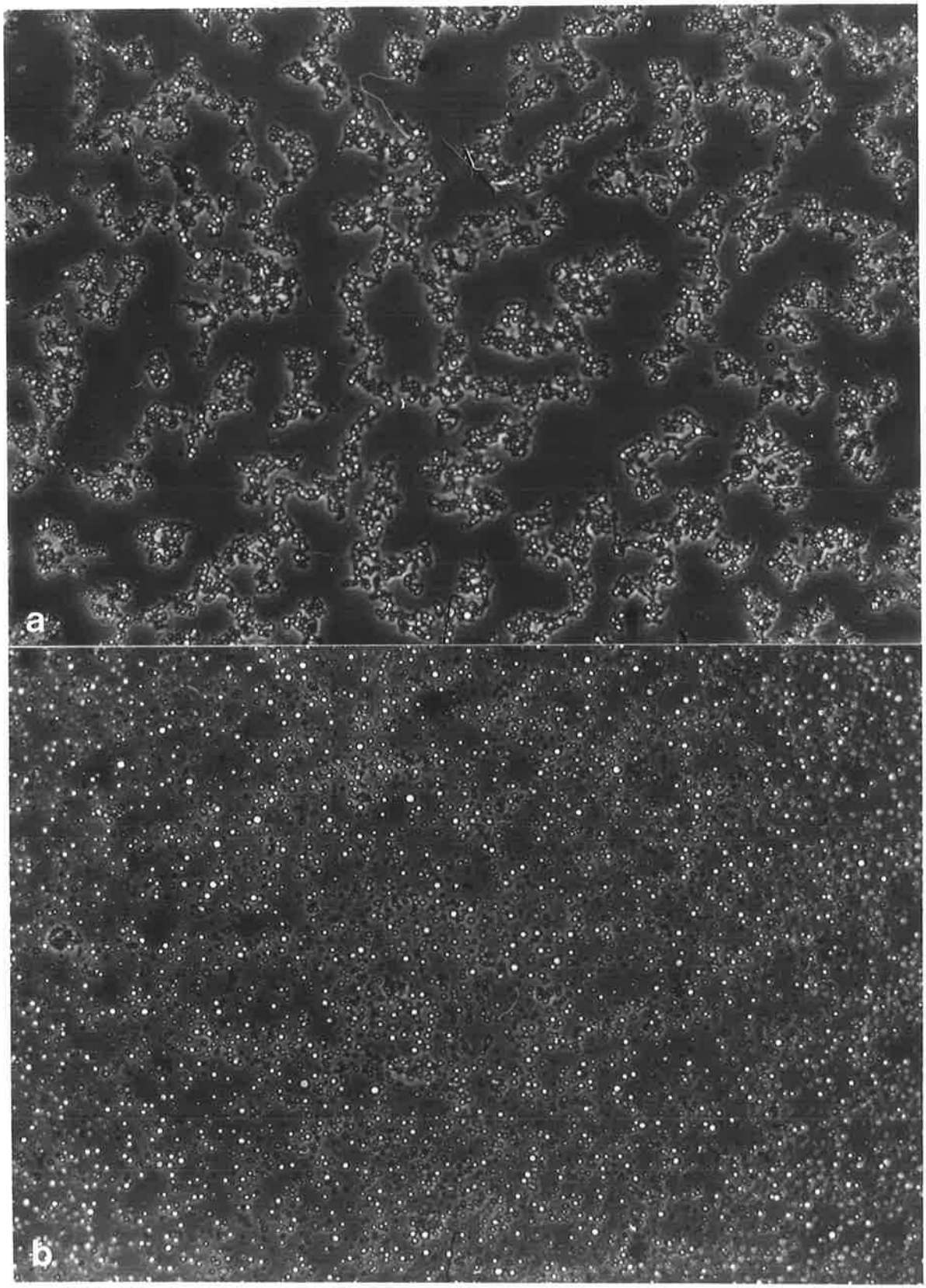
Immunization

Antiserum to intact polyhedral inclusion bodies (PIB) was produced in an adult female goat. 4.6×10^8 PIB (0.39 mg polyhedral protein) were injected subcutaneously with Freund's complete adjuvant 3 times at 10 day intervals. After an interval of 30 days 2.54×10^8 PIB (0.21 mg polyhedral protein) were injected intravenously and a similar amount was injected subcutaneously with Freund's complete adjuvant. For protein estimations, polyhedra were dissolved in dilute alkali (Bergold, 1963) and estimations were carried out by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard, and a linear relationship between optical density and protein concentration was obtained over the range 20-100 µg/ml.

An agglutination technique was used to estimate titre of antiserum (Fig. 4.2). Ten microlitre aliquots of a suspension of purified polyhedra (1.6×10^9/ml) were mixed on microscope slides with 10 µl aliquots of antiserum serially diluted 1:1 in 1/100 phosphate buffered saline (PBS). It was found that polyhedra tended to clump spontaneously in PBS presumably due to the salt concentration - however this problem was alleviated by using PBS that had been diluted 100-fold and had the pH adjusted back to pH 7.1. A wet mount preparation was made by placing a

FIGURE 4.2 a. Polyhedra of *Heliothis punctigera* nuclear polyhedrosis virus agglutinating in the presence of goat anti-NPV antiserum, titre 1/64. x700.

b. Control: polyhedra + 1/100 phosphate-buffered saline. x700.


a
b

ring of petroleum jelly around the drop of suspension and gently pressing a cover slip onto the slide. The slides were then incubated at 37°C for 1 h, and were examined by phase-contrast microscopy to determine the agglutination end-point. The goat was bled 11 days after the final injection of antigen when the titre was 1/512.

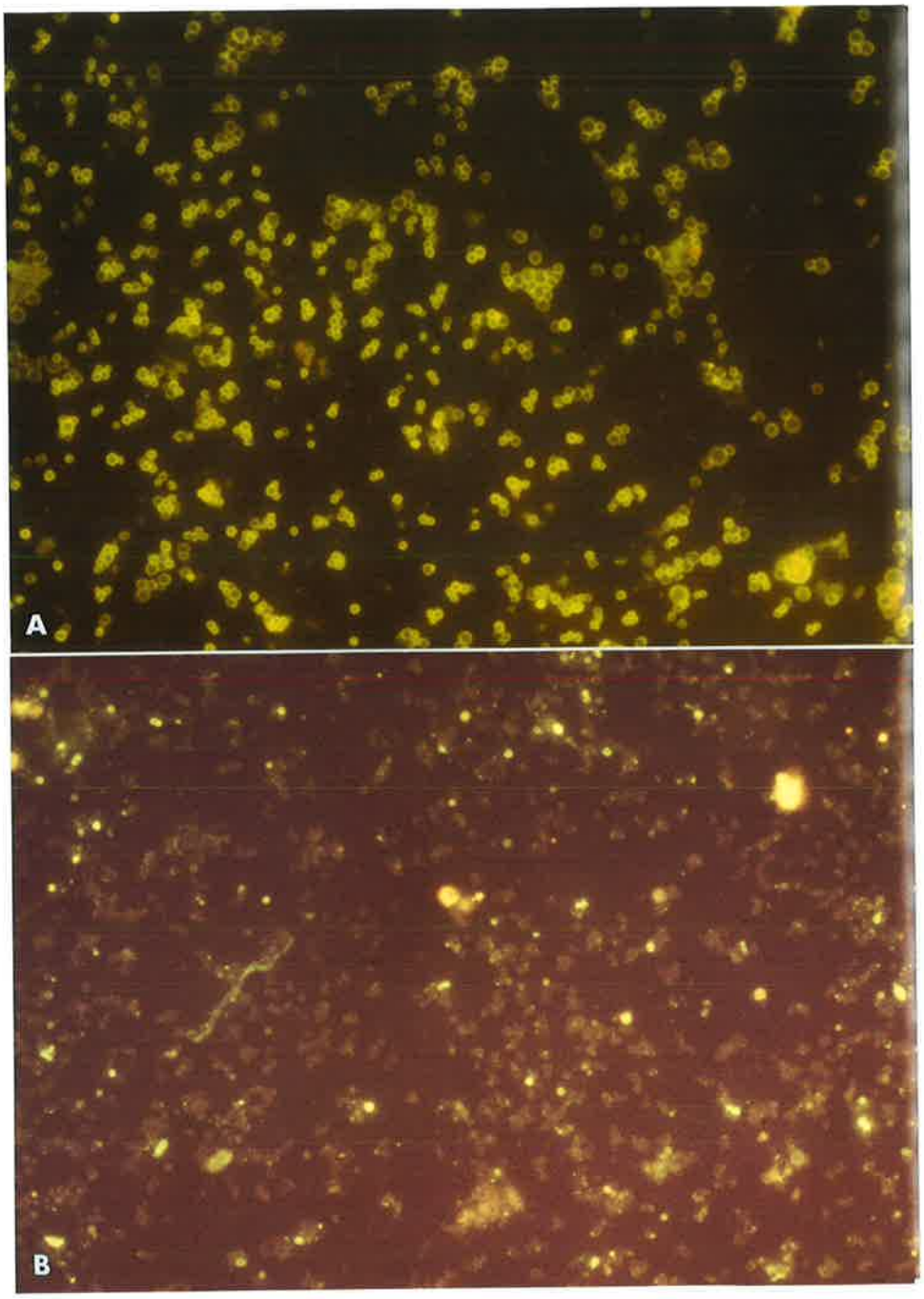
Fluorescent antibody conjugation

Antiserum was extracted, purified and conjugated with fluorescein isothiocyanate (Nairn, 1969). To remove the non-specific staining characteristic, the conjugated antiserum was absorbed with rabbit liver homogenate (prepared as described by Nairn) for 1 3/4 h at room temperature (29°C). The conjugated antiserum was then divided into small aliquots which were stored at -30°C. Checks with preparations of purified NPV and conjugated antiserum showed that polyhedra were labelled with fluorescein, while controls using conjugated non-immune serum indicated that labelling was specific. The indirect ("sandwich") staining method (Nairn, 1969) was also assessed (Fig. 4.3) using goat anti-NPV antiserum and fluorescein-labelled rabbit anti-goat immunoglobulin (Wellcome Research Laboratories, Beckenham). This technique produced marginally brighter staining than the direct method but was less specific in preparations of leaf washings; hence the direct method was routinely employed.

Staining of leaf washings on microscope slides with the conjugated antiserum was carried out as follows: to further reduce non-specific staining, the sample was first incubated with foetal calf serum (Commonwealth Serum Laboratories, Parkville) for 1 h at 37°C. The sample was then washed 3 times in sodium carbonate/bicarbonate buffer 0.5 M pH 9.1, and conjugated antiserum was applied. The sample was then

FIGURE 4.3 A. Fluorescent staining of polyhedra of Heliothis punctigera nuclear polyhedrosis virus (indirect method). Polyhedra incubated with goat anti-NPV antiserum, followed by fluorescein-labelled rabbit anti-goat immunoglobulin.

B. Control: polyhedra incubated with non-immune goat serum, followed by fluorescein-labelled rabbit anti-goat immunoglobulin.


A
B

incubated for 15 min at 37°C, washed 4 times in the same buffer and mounted in glycerol buffered to pH 9.1 (Pital and Janowitz, 1963). Slides were examined under a Zeiss fluorescence microscope, using incident illumination at 490-500 nm.

As no NPVs from other hosts were available it was not possible to investigate cross-reactivity with the prepared antiserum. However, lepidopterous polyhedral proteins are recorded as sharing common antigenic groups as determined by complement fixation and agar gel diffusion tests on solubilized polyhedral proteins (Krywienczyk and Bergold, 1960, 1961; Pritchett et al., 1979). Hence it is likely that weak cross reactivity would occur between the prepared antiserum and NPVs from other species. However, no NPVs have been recorded in 3 species of lepidoptera which are also common on lucerne in South Australia, Ectropis excursaria Guen. (Geometridae), Lampides boeticus (Linn.) (Lycaenidae) and Agrotis munda Walk. (Noctuidae). Thus it is probable that any polyhedra detected from the field would be H. punctigera NPV.

4.3 Development and calibration of method for washing polyhedra off foliage

Introduction

Davidson and Pinnock (1973) combined an immunofluorescent method for detecting H. zea NPV on cotton foliage with a method which involved stripping off polyhedra adhering to the leaves with adhesive tape. An attempt was made to use this method, but difficulties in the form of excessive background fluorescence from the adhesive tape made detection of polyhedra difficult. Hence a washing method was employed.

Little work has been carried out on quantitative washing of baculoviruses from leaves. David (1978) found Pieris brassicae GV virtually

impossible to wash off cabbage leaves; this may be partly because GV capsules are known to enter leaf stomata (Reed, 1971). Although attempts using water to wash NPVs from leaf surfaces have not been very successful (Jaques, 1977), use of dilute detergent solutions has produced more satisfactory results. Thus Heimpel et al. (1973) and Ignoffo et al. (1974) removed NPVs from leaf surfaces by washing in Tween 80(R)/salt solutions. Heimpel et al. used phosphate-buffered physiological saline - 0.25% Tween 80 to wash T. ni NPV from cabbage leaves, while Ignoffo et al. used phosphate buffered normal saline 0.025% Tween 80 to remove H. zea NPV from soybean foliage. However in both cases the methods were apparently not calibrated and no information was given as to the efficiency of removal of polyhedra.

Materials and methods

The method was calibrated by adding measured amounts of polyhedra to leaves, washing them off again, and detecting polyhedra in an aliquot of the washings. Semi-purified polyhedra were used in the experiment, as preliminary work indicated that highly purified polyhedra behaved differently in the washing process. In addition salt solutions were not employed, as this was observed to cause clumping of polyhedra (see page 73).

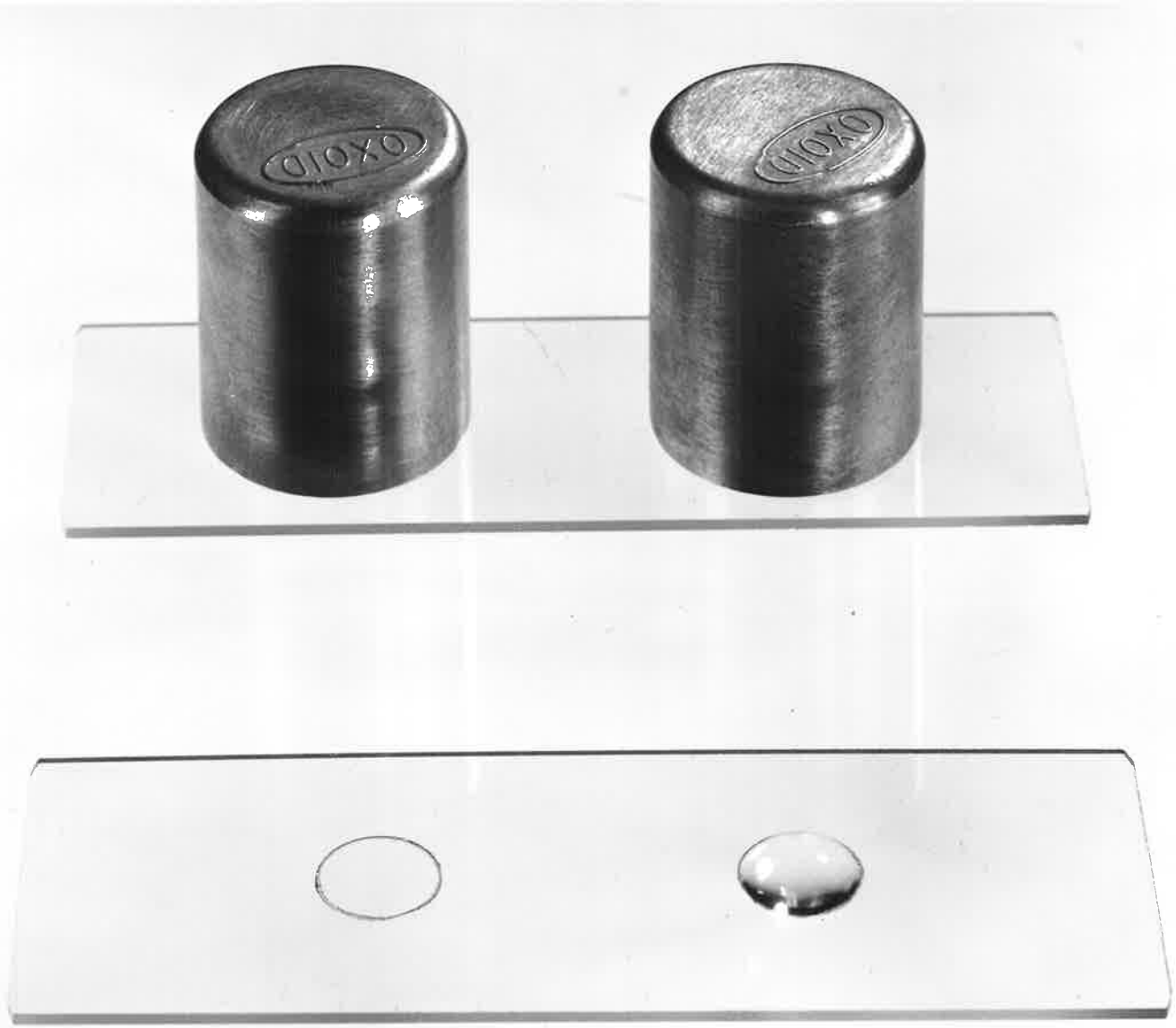
A suspension of polyhedra was counted using a haemocytometer and was serially diluted in 10-fold steps to give a final dilution of 10^{-4} of the original suspension. The dilutions ranged from 1.25×10^{7} - 1.25×10^{3} PIB/20 µl. Twenty microlitres of each suspension was applied to a fresh lucerne leaf, which was dried at 35°C for 60 min. Each leaf was placed in a 10 ml polypropylene test tube and 20 extra leaves were added to each tube (a sampling unit of 21 leaves per sampling station was adopted in the field study). In addition 21 untreated leaves were added to an extra tube

as a control. Five millilitres of 0.05% Tween 80 solution was added to each tube which was then stoppered and shaken horizontally at moderate speed for 60 min in a mechanical flask shaker. The leaves were then removed and washed for a further 5 min in 3 ml 0.05% Tween 80. The washings were then combined and centrifuged for 30 min at 38,000 x g. The volume was reduced to 0.25 ml, and the pellet was resuspended by shaking the tube on a vortex mixer. The washings were then stored at -20^{o}C.

A standardized counting technique was combined with the immunofluorescent detection method for estimating numbers of polyhedra present. Each 0.25 ml of washings was thawed at 37^{o}C and the pellets resuspended by agitation with a vortex mixer. There was still a slight tendency for polyhedra to clump or to adsorb to plant or soil debris. This was overcome by gentle ultrasonic treatment; each tube was immersed for 5 sec in chilled water in an ultrasonic bath (Branson Ultrasonic Cleaning Company, Shelton, Connecticut). A 20 µl aliquot was then applied within an 8 mm circle on a gelatin-coated microscope slide. The circle was made by coating the end of a thin-walled 8 mm diameter plastic tube with ink from a Texta$^{(R)}$ "Parcelmate" felt-tipped pen, and pressing it on to the slide for a few seconds. This produced a neat circle on the slide; thus the 20 µl of leaf washings was confined to a standard area (Fig. 4.4). The drop was covered with an aluminium Oxoid$^{(R)}$ test-tube cap so that drying occurred slowly; this prevented polyhedra concentrating at the edge of the circle, which occurred if the drop dried out too quickly. The sample was then treated with foetal calf serum and stained with conjugated antiserum as described (page 74). Counting was carried out by counting fluorescein-labelled polyhedra in 25 random microscope fields under the fluorescent microscope, using a 63 x oil immersion objective with a field diameter of 0.2 mm.

FIGURE 4.4 Foreground. Microscope slide showing 8 mm circle marked on slide and aliquot of virus suspension placed in circle.

Background. Aluminium caps placed over virus samples during drying.


DIOXO
DIOXO

Results and discussion

It was found that polyhedra were not spread to the very edges of the 8 mm circle on the slide - some repulsion occurred during drying and few polyhedra were seen in the outer 1 mm of the circle. Inside this area the polyhedra appeared to be spread evenly. The mean count per field and percent recovery are shown in Table 4.1 and log (mean polyhedra per field) vs. log (polyhedra applied to leaf) is plotted in Fig. 4.5.

TABLE 4.1 Calibration of leaf washing method-efficiency of recovery.

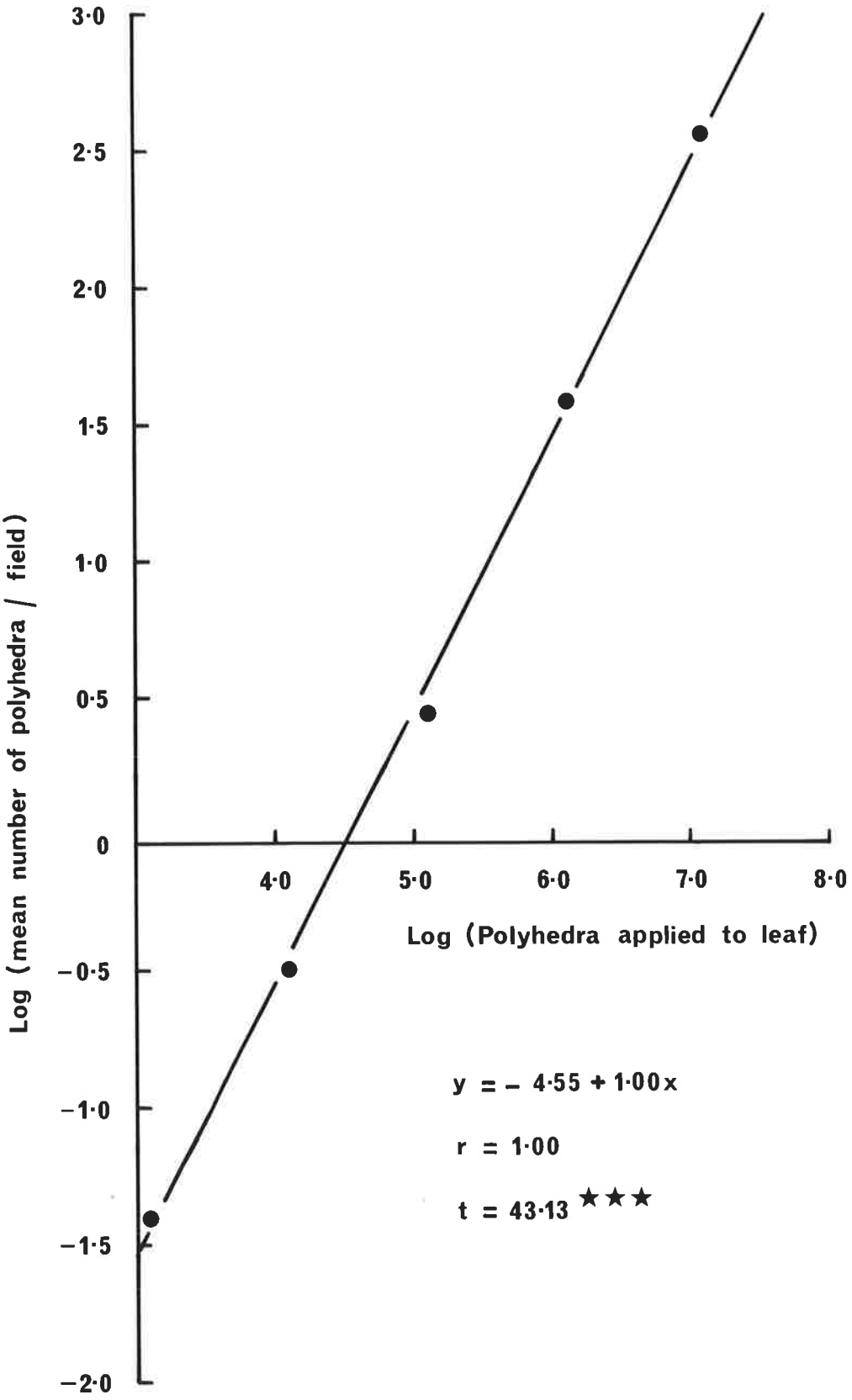
Polyhedra added per leaf	1.25×10^7	1.25×10^6	1.25×10^5	1.25×10^4	1.25×10^3	0
Mean count per field (n=25)	351.7	37.64	2.76	0.32	0.04	0
Expected[1] mean count per field	1111.11	111.11	11.11	1.11	0.11	0
Percent recovery[2]	31.65	33.88	24.85	28.81	36.02	

[1] Expected mean count per field was calculated by multiplying the expected number of polyhedra in a 20 µl aliquot by the ratio of the area of the 0.2 mm diameter microscope field and the area of the 6 mm diameter circle over which the polyhedra were spread.

[2] Mean percent recovery = 31.04, s.e. = 1.96.

The recovery of polyhedra from the leaf surfaces, although of low efficiency, nevertheless was achieved in a reproducible manner over the range of concentrations used in the experiment, i.e. b = 1.00 (Fig. 4.5). The low efficiency might possibly be due to variation in ease of removal of polyhedra, i.e. some polyhedra were adsorbed more strongly than others; alternatively, it might be due to re-adsorption of removed polyhedra to other

FIGURE 4.5 Calibration curve of method for washing *Heliothis punctigera* nuclear polyhedrosis virus off lucerne leaves and detection by immunofluorescent method.


3·0
2·5
2·0
1·5
1·0
0·5
0
−0·5
−1·0
−1·5
−2·0
4·0
5·0
6·0
7·0
8·0
Log (mean number of polyhedra / field)
Log (Polyhedra applied to leaf)
y = − 4·55 + 1·00x
r = 1·00
t = 43·13 ★★★

leaf surfaces. The method of depositing virus onto the slide may have introduced some error into the estimate - time did not permit the development of a more refined method. However an experiment carried out subsequently indicated that the error involved was similar to that involved in using a haemocytometer count (see page 115).

The detection threshold of the method was determined as follows. A count of 1 polyhedron over 25 random fields corresponds to a mean count per field of 0.04.

Log 0.04 = -1.40.

The y value -1.40 may be substituted into the regression equation $y= -4.55 + 1.00x$, which yields a value of $x = 3.15$; taking the antilog, $x = 1.41 \times 10^3$ PIB/21 leaves. The level of virus associated with a sampling unit of 21 leaves may be conveniently expressed as the amount of virus per unit area of leaf surface, i.e. PIB/mm^2. This estimate may then be compared with a threshold, based on bioassay LC_{50} values, above which a disease outbreak could reasonably be expected to occur, should susceptible larvae be present. For the purposes of this calculation, the mean total surface area of lucerne leaves is employed. This value was arrived at after taking surface area measurements on a representative sub-sample of leaves (n = 2068) sampled throughout the season as part of the subsequent field study - for description of leaf area measurement see page 91.

Thus 1.41×10^3 PIB/21 leaves corresponds to 67.14 PIB/leaf.

The mean leaf surface area = 129 mm^2, therefore the minimum virus level detectable = 0.52 PIB/mm^2. The LC_{50} for neonate H. punctigera larvae infected with H. punctigera NPV = 0.90 PIB/mm^2 (1.07-0.75-95% confidence limits) (Teakle, 1976). No data on the susceptibility of more mature larvae have been recorded; however, Daoust and Roome (1974) quoted an LC_{50} for 3 day-old H. armigera larvae infected with Heliothis NPV (local strain) of 6.4 PIB/mm^2 (7.4-5.5). The assumption must be made that

the LC_{50} for 3 day-old H. punctigera larvae would be similar to this figure. Thus the method is sufficiently sensitive to detect levels of virus large enough to initiate epizootics in neonate larvae, and well able to detect levels of virus which would initiate epizootics in more mature larvae.

The above method involving washing polyhedra from leaves has certain advantages over bioassay techniques, which usually involve direct feeding of leaves or leaf disks to test larvae. Virus levels on foliage may often be low and unevenly distributed (Young, 1975) hence leaf feeding techniques may fail to detect virus or may not give an accurate picture of virus levels. The washing method, by concentrating virus from a relatively large number of leaves, is able to cope with low levels of virus. Expressing the virus level as PIB/mm^2, although a convenient unit, does not give information on the distribution of polyhedra within a sampling unit. However, the method lends itself to handling a relatively large number of sampling units, and the between-sampling unit variation could be expected to give information on how heterogeneously the virus is distributed within the crop. In order to be able to make any prediction about the role of virus in initiating epizootics, the infectivity of this virus must be ascertained. This question will be dealt with in the following section.

4.4 Calibration of method with respect to infectivity of polyhedra

Introduction

Polyhedra which react with the fluorescent antiserum are not necessarily infective. It is probable that two degradative processes occur when polyhedra are exposed to environmental conditions: the polyhedral surface antigens which bind the antiserum would presumably be degraded, and

the virions embedded within the polyhedral matrix are also subject to degradation. It is necessary to know to what extent these two processes are linked. That Heliothis NPV quickly loses infectivity when exposed to sunlight has been demonstrated by several authors (Bullock, 1967; Ignoffo and Batzer, 1971; Ignoffo et al., 1973, 1974; Young and Yearian, 1974; Roome and Daoust, 1976). This inactivation is attributed to the ultraviolet component of the spectrum (Bullock et al., 1970). Depending upon the host plant involved, the half-life of the virus may be less than 1 day (cotton, corn) or between 2 and 3 days (soybeans). No estimates have been made of the half-life of the polyhedral surface antigens - hence the aim of the following experiment was to expose samples of virus to sunlight, and to assay the residual virus over a period of time by both methods, i.e. by bioassay and by the immunofluorescent method.

Materials and methods

Ten microlitre aliquots of semi-purified virus containing 1.30×10^6 PIBs were applied to leaves on potted lucerne plants; 30 leaves were treated. To ensure even deposition of the suspension, horizontal leaves were selected and the suspension was spread over the surface of the leaves. The leaves were dried by placing the plants 60 cm from a hot air fan for 15 min. A sample of 5 treated leaves plus 5 untreated leaves as a control was removed from the plants, and the pots were then placed outdoors in an unshaded site at 0830 h. Samples of 5 leaves were then taken at 0.5, 1.0, 1.5, 2.0 and 2.6 days. The leaves were washed in 0.05% Tween 80 as described. To facilitate counting, aliquots were diluted 10-fold and were applied to ringed slides, stained with conjugated antiserum and counted. Only those polyhedra which fluoresced brightly were counted; a scale of intensity of fluorescence was imposed (Pital and Janowitz, 1963)

where minimal to maximal intensity of fluorescence was designated 1+ — 4+. Only those polyhedra which came within the range 3+ — 4+ were counted.

To assess infectivity of polyhedra in the leaf washings, a bioassay was carried out, using neonate H. punctigera larvae. The larvae were caged in 2 ml Auto Analyser(R) vials (Medical Plastics, Adelaide), which contained 0.45 ml of modified Sender's diet minus formalin (Griffith and Smith, 1977). Fifteen microlitres of leaf washings (diluted appropriately) were added to the diet surface and dried for 1 h at 35°C. A single larva was added to each vial and mortality was recorded after 12 days at 27 ± 1°C. Smears were made of dead larvae and were examined by phase contrast microscopy to determine if polyhedra were present. Two replicates of 30 larvae were used to determine infectivity in each sample. In addition a standard bioassay was set up, using larvae from the same batch. Five dilutions of NPV, ranging in two-fold steps from 0.23-3.6 PIB/mm^2 of diet surface were administered to vials and 30 larvae set up for each dilution. In addition an untreated control group was set up. Data was analysed by the maximum likelihood method (Finney, 1971), and a response curve (log dose vs. probit mortality) was constructed; from this curve the concentration of NPV in the unknowns was estimated. All larvae used in the above were drawn from an insectary culture reared on modified Sender's diet plus 0.1% formalin. The culture was maintained at 27 ± 1°C with a 14:10 light: dark regime; eggs were surface sterilized as described (page 35).

Results and discussion

The meteorological conditions prevailing at the time of the experiment are shown in Table 4.2.

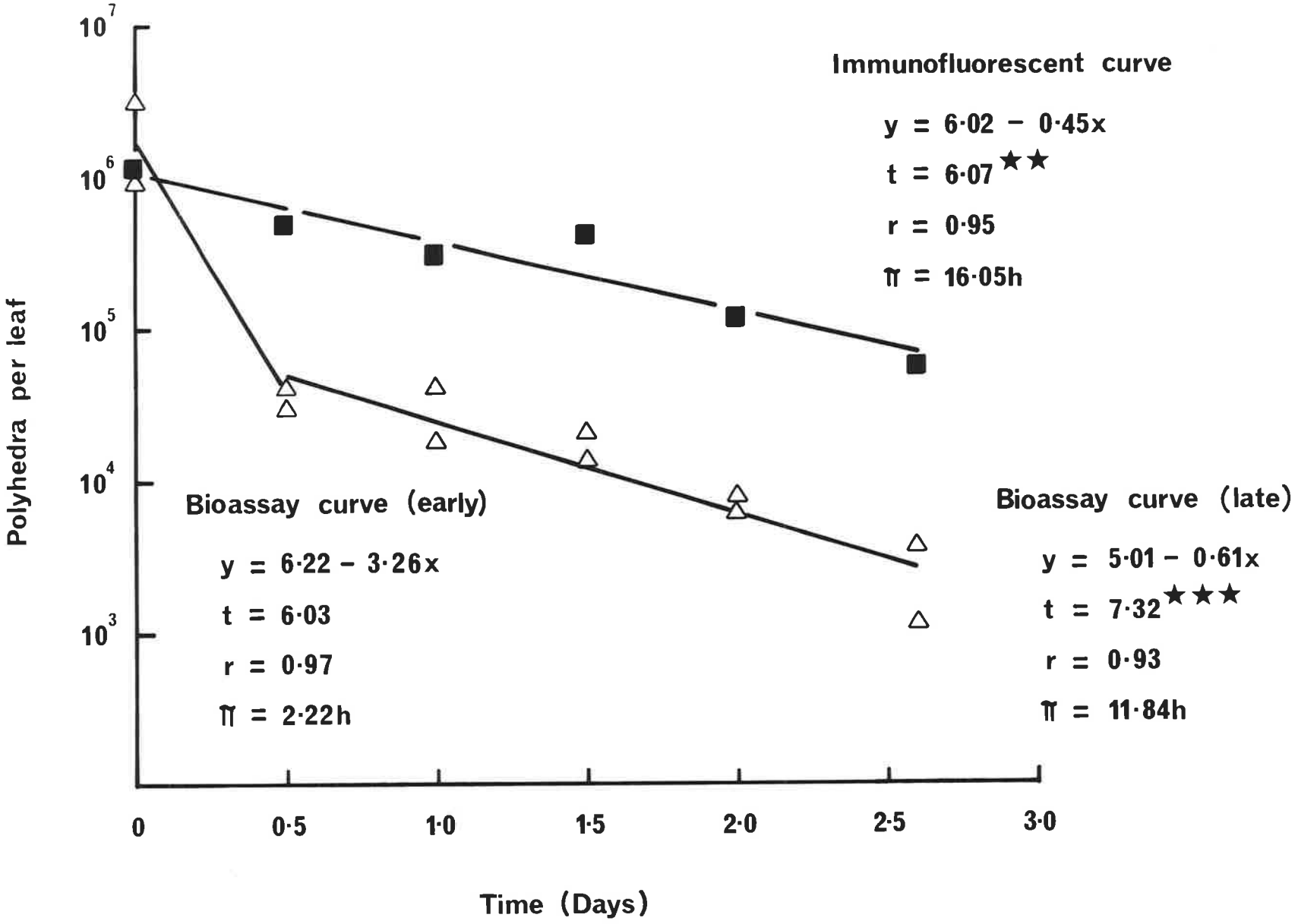
TABLE 4.2 Meteorological conditions during field degradation experiment.

Day	Air temperature max.	min.	Relative humidity (0800 h)	General
1	29	16	70%	fine, warm, no cloud.
2	31	21	58%	overcast, humid, less cloud in late afternoon.
3	29	18	49%	fine, warm, no cloud.

The results of both the immunofluorescent estimate and the bioassay estimate of polyhedral levels vs. time are shown in Fig. 4.6. No polyhedra were detected by either method from the sample of 5 untreated leaves. A substantial increase in efficiency of virus recovery compared to Fig. 4.5 was seen in the immunofluorescent curve; the value of 1.05×10^6 PIB/leaf obtained at T_0 corresponds to a washing efficiency of 80.6%. This difference is attributed to the lower number of leaves per tube present in this experiment. As mentioned previously, loss of polyhedra from the washing suspension may occur due to re-adsorption to leaf surfaces - hence in this experiment, with 5 leaves per tube instead of 21, less re-adsorption would have occurred. The half-life of the polyhedral antigens which bind the fluorescent antibodies is expressed as $\pi = 16.05$ h (see page 55 for estimation of π).

The infectivity of the polyhedra, as shown by the bioassay curve, followed a segmented response. Hence the data was analysed as two separate least squares linear regressions, a T_0-$T_{0.5}$ (early) curve and a $T_{0.5}$-$T_{2.6}$ (late) curve. At T_0 the bioassay estimate was 1.66×10^6 PIB/leaf, while

FIGURE 4.6 Field degradation of Heliothis punctigera nuclear polyhedrosis virus, assayed by the immunofluorescent counting method (—— ■ ——) and by bioassay (——△——).


Polyhedra per leaf
10^7
10^6
10^5
10^4
10^3
Immunofluorescent curve
y = 6·02 − 0·45x
t = 6·07 ★★
r = 0·95
π = 16·05h
Bioassay curve (early)
y = 6·22 − 3·26x
t = 6·03
r = 0·97
π = 2·22h
Bioassay curve (late)
y = 5·01 − 0·61x
t = 7·32 ★★★
r = 0·93
π = 11·84h
0
0·5
1·0
1·5
2·0
2·5
3·0
Time (Days)

the immunofluorescent estimate was 1.05×10^6 PIB/leaf; this discrepancy is probably a reflection of errors present in one or both methods. Thus the immunofluorescent estimate at T_0 is 63% of the bioassay estimate. In order to be able to use this calibration curve to predict infectivity of polyhedra, it was necessary to know if this relationship would remain constant. Thus two more estimates of the T_0 infectivity level were carried out (by bioassay as described). These two experiments indicated that the immunofluorescent estimate was 46.0% and 68.4% of the bioassay estimate respectively. Accordingly the mean of these three estimates, (59.1%) was subsequently used to predict the mean infectivity and the range of infectivity (page 86).

The early bioassay curve indicated a rapid degradation (π = 2.22 h) while the late curve indicated a slower rate of degradation (π = 11.84 h). Within the first 12 h infectivity was reduced by 97.7% - this represents a more rapid degradation than has previously been recorded by other authors with Heliothis NPV (Bullock, 1967; Ignoffo et al., 1974).

The infectivity data appears to adequately fit the segmented linear model and bears some resemblance to the degradation curves of B. thuringiensis spores obtained on various tree species (Pinnock et al., 1975). The reason for the differential degradation is not clear. The immunofluorescent curve suggests that in the experiment the washing process did not vary in efficiency with time, hence it is unlikely that the large reduction in infectivity recorded at $T_{0.5}$ was a result of fewer polyhedra being present in the washings. It is more likely that differential degradation of virus occurred, i.e. a small percentage of polyhedra (or perhaps a small percentage of virions within some polyhedra) are substantially more resistant to degradation. A possible explanation (if the latter were the case) is that polyhedra larger than a certain critical

size could afford more protection from ultraviolet radiation than could smaller polyhedra. A considerable size-frequency distribution of polyhedra occurs (Gregory et al., 1969; Teakle, 1973; see also Fig. 6.4) thus it is possible that the larger polyhedra could more adequately shield inner virions from ultraviolet radiation. This small proportion of non-degraded virions would account for the bioassay (late) curve (Fig. 4.6). It is possible that some force other than ultraviolet radiation may have contributed to degradation of polyhedra as both curves (except for the bioassay (early) curve) show no noticeable difference in rate of degradation between day and night. However Pinnock (pers. commun. 1978) observed a similar situation with what was presumably ultraviolet radiation-induced degradation of B. thuringiensis spores, in that there was a scarcely perceptible difference between rates of degradation that occurred during day and night.

The slopes of the immunofluorescent curve and the bioassay (late) curve are similar; b (immunofluorescent curve) = -0.45, b (bioassay - late curve) = -0.61. A comparison of the two slopes was carried out by the method of Bailey (1964) which indicated that the null hypothesis that the slopes did not differ could not be rejected ($t = 1.36$, d.f. = 12). Hence there was no significant difference between rates of degradation of the polyhedral surface antigens and the virus that remained infective after $T_{0.5}$. At $T_{0.5}$ the bioassay curve indicated a level of 5.01×10^4 PIB/leaf while the immunofluorescent curve indicated 6.17×10^5 PIB/leaf, i.e. 8.1% of the polyhedra detected by the immunofluorescent curve were infective.

On the basis of this experiment, how useful is the immunofluorescent - washing method in determining the infectivity of field-derived polyhedra? The method varies in its usefulness as a predictor depending upon whether it is known for how long the polyhedra have been exposed on the leaf surfaces. In a situation where it is not known how long the polyhedra have

been exposed, but where there has been no event (such as rain, irrigation or a recent epizootic) to indicate that the polyhedra have been on the foliage for less than 12 h, the following prediction can be made. It can be assumed that the relationship between the immunofluorescent estimate and infectivity of the polyhedra is as indicated by Fig. 4.6 after $T_{0.5}$, i.e. 8% of the polyhedra detected by the immunofluorescent method are infective.

If, however, there has been a recent event such as rain or irrigation to indicate when detected polyhedra were deposited on the foliage, a more specific prediction about infectivity can be made. For example, let us imagine that a level of 10 PIB/mm^2 was detected on leaves sampled at (for convenience) 16 h after cessation of rainfall on a crop on which previously no polyhedra were detected. The period 16 h = π for the immunofluorescent curve, so that had the leaves been sampled immediately after cessation of rain, a figure of 20 PIB/mm^2 would have been detected. At T_0 the immunofluorescent estimate is 59.1% of the bioassay estimate, hence at this time any larvae on the crop would have been exposed to 33.8 infective PIB/mm^2 (range 43.5-29.2). Comparison of this figure with known LC_{50}s would enable predictions to be made about the likelihood of an epizootic developing.

The use of the above calibration curve in predicting infectivity of detected virus is subject to the following assumptions:

a) the washing process does not vary in efficiency with time (although, as mentioned, the immunofluorescent curve suggests that this is not the case).

b) the reduction of polyhedra per leaf with time is in fact due to degradation and not to loss of polyhedra from the leaves.

c) the relationship between the two curves remains the same irrespective of:

(i) the number of polyhedra present.

(ii) the time factor involved (i.e. longer than 2.6 h).

(iii) the environmental conditions prevailing.

4.5 Detection of NPV in soil

Introduction

The purpose of this area of the study was to demonstrate that deposits of virus were present in the soil in large enough quantities to initiate epizootics (should there be some mechanism to transfer these polyhedra to a susceptible population). It was hoped initially to combine a method of desorbing polyhedra from soil with sodium pyrophosphate (Hukuhara, 1975) with the immunofluorescent detection method. However, in preliminary experiments, excessive autofluorescence from soil particles present and poor recovery of polyhedra (0.1%) indicated that this method of detection was unsatisfactory. Hence bioassays were used to determine levels of polyhedra in soil.

Materials and methods

Soil samples were passed through a 0.64 mm sieve, and the coarse fraction was discarded. The fine fraction was weighed and was then combined with an equal weight of 0.05% Tween 80 solution, and shaken vigorously on a vortex mixer. Fifteen microlitres of the resultant suspension were then applied to the surface of modified Sender's diet in Auto Analyser vials, and the bioassay was carried out as described (page 82). Two or three replicates of 30 larvae were used for each

sampling unit, and a standard bioassay (as described) was also set up. Mortality was corrected by Abbott's formula where control mortality occurred. Virus levels were expressed as PIB/g soil (fine fraction).

CHAPTER 5

EPIZOOTIOLOGY OF THE NUCLEAR POLYHEDROSIS VIRUS OF H. PUNCTIGERA: FIELD MONITORING STUDY

5.1 Design of field study

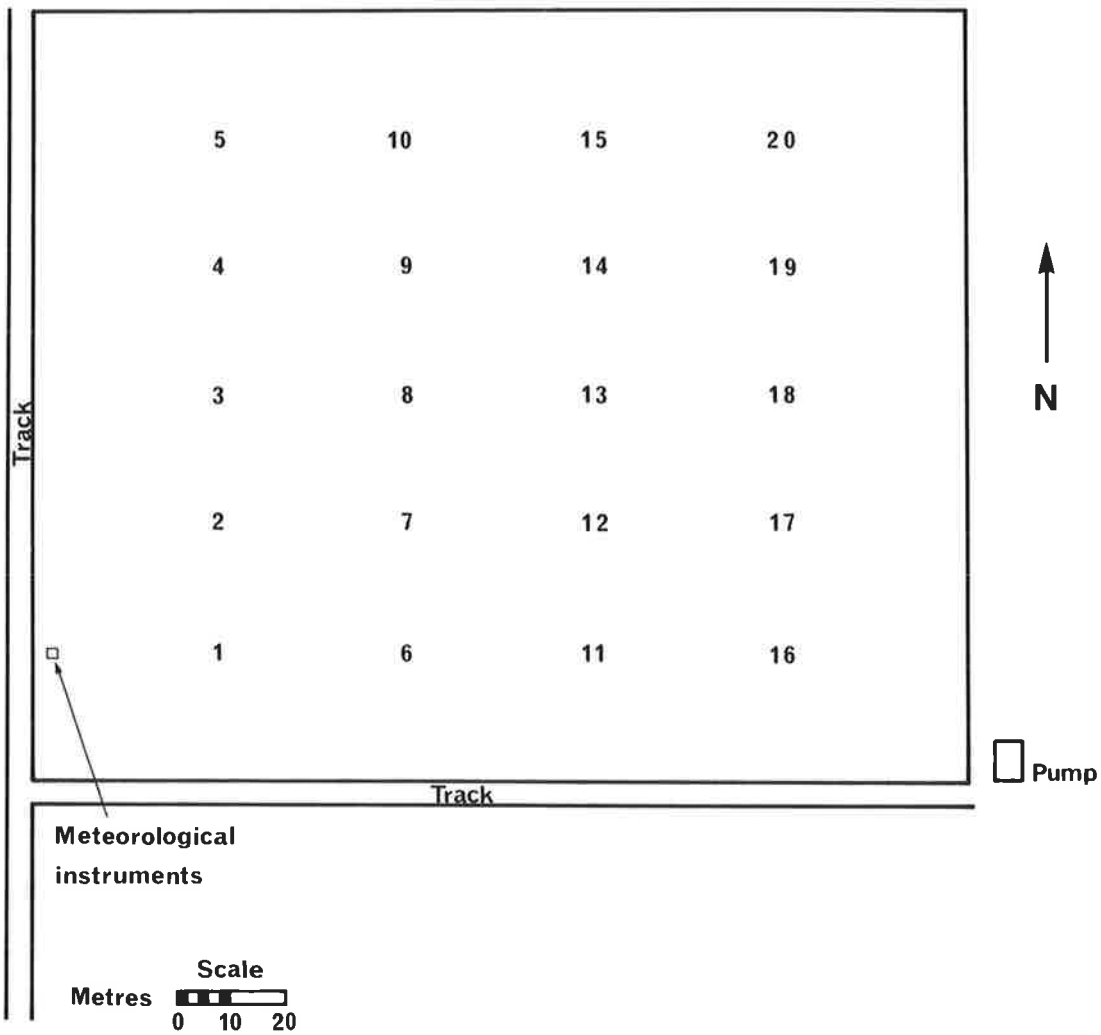
The field monitoring study was carried out for 2 seasons on a commercial lucerne hay growing property at Pt. Gawler, 50 km north of Adelaide (Fig. 2.1). Lucerne grown for hay production was an appropriate system for the study intended, as H. punctigera is not regarded as a pest of any consequence in hay production - hence no insecticides are used (although this situation changed in the 1977/78 season, when insecticides were used against T. trifolii).

The lucerne crops were permanent stands, at least 5 years old - no cultivation had been carried out during this time. The soil was a sandy loam. Irrigation was by sprinkler, using water from an artesian supply. For a hay crop, usually one heavy watering was given after the preceding cut, and this promoted enough growth until the crop was ready for cutting, which was carried out every 5 weeks at the height of summer (December to February).

Plot layout

A patchy distribution was expected for NPV on foliage and in soil. Twenty fixed sampling stations were selected (Fig. 5.1). Thus the expected large between-site variance could be excluded. Each sampling station was marked by a numbered disk, fixed to the ground by a steel spike.

FIGURE 5.1 Layout of plot for field monitoring study in irrigated lucerne crop. Numbers indicate positions of sampling stations.


5
10
15
20
4
9
14
19
3
8
13
18
2
7
12
17
1
6
11
16
Track
Track
N
Pump
Meteorological
instruments
Scale
Metres
0 10 20

First year of study

The study was first carried out over an 8 week period from 11-3-77 to 29-4-77. Weekly leaf samples were taken over this period. Conditions were somewhat atypical in the crop at this time, as no watering was carried out, due to water quotas having been exceeded. In addition, because detection methods were still being developed, this study should be regarded as a preliminary to the following season's work, i.e. the main aim of this work was to determine if virus could be detected with the sampling strategy that had been adopted. Rainfall records, in the form of weekly totals, were taken from the Two Wells meteorological station, 8 km away.

Second year of study

This was a more extensive study, from 9-12-77 to 9-5-78. Once it was realized how quickly NPV was degraded when exposed to environmental conditions (Fig. 4.6), it became apparent that more precise recording of rainfall or irrigation was required. Hence a recording rain-guage (Lambrecht, Hellmann type) was installed, which monitored amount and time of precipitation. The instrument was installed in such a position that irrigation was also recorded. A thermohygrograph was also installed to measure temperature and humidity in the crop canopy.

5.2 Leaf sampling

A sampling unit of 21 leaves (7 trifoliates) was taken at each sampling station. Although no formal randomization procedure was adopted, an effort was made to represent all portions of the crop. Sampling was normally carried out every 7 days, although mowing occasionally interrupted sampling until regrowth occurred. The leaves were stored in plastic vials

and were stored at 4°C for 2-3 days before washing. Washing and detection of NPV was carried out as described, and the standard curve (Fig. 4.5) was used to give an estimate of PIB/mm^2 of leaf area from each sampling unit. In the 1976/77 season, because no watering was carried out during the study, growth of plants was slow, hence the range of leaf sizes was small. Hence individual leaf area was not measured but the total avcrage leaf area (276 mm, standard error = 11.70, n = 25) was used to estimate PIB/mm^2.

By the 1977/78 season the sensitivity of the detection method had been established, and it become apparent that a more critical leaf area measurement was required. In addition, due to irrigation, plant growth was more rapid; hence a much larger range in leaf sizes was evident. Because of the time factor involved in individual leaf area measurement (no satisfactory electronic area measurement device was available) the following procedure was adopted. Leaves sampled from the field were matched against a series of 15 "standard" leaves of known area, which ranged in size from the smallest to the largest leaves occurring in the field. Thus each leaf sampled was classed according to the standard leaf it most closely matched in surface area. To test the reliability of this method, the total surface area of 100 leaves (10 groups of 10) was estimated by the above method, and also by direct measurement, i.e. by tracing the outline of each leaf onto 1 mm squared paper and counting the squares. A t-test indicated no significant difference between the means of the 2 sets of measurements (t = 1.33, d.f. = 9). The mean error in measurement over the 10 groups between the two methods was $\pm$ 5.9% (standard error = 1.04).

5.3 Larval sampling

Virus-killed larvae usually adhere to the foliage and either remain

stuck or, in the case of freshly-dead larvae, disintegrate when the crop is swept with a sweep net. Hence sampling by this technique is unsatisfactory. Thus when an epizootic was in progress, larvae were counted in two 1 m^2 quadrants adjacent to each station. The quadrants were within 5 m of each station, and were made in different places in successive weeks. Both healthy and virus-killed larvae were counted. Dead larvae that did not exhibit characteristic symptoms of nuclear polyhedrosis were placed in vials and subsequently smears of the cadavers were made for diagnosis.

In the 1977/78 season weekly estimates of larval populations were also made with a sweep net along 3 east-west transects, starting at stations 1, 3 and 5 respectively. Ten sweeps were made along each transect, and the stadial composition of larvae was determined.

5.4 NPV in soil

Soil samples were taken early in each season (before any epizootics developed, to determine the level of virus which remained infective from the previous season) and at the end of each season. In the first season soil was sampled on 23-11-76 and again on 29-4-77. In the second season samples were taken on 9-12-77 and on 21-4-78. Four samples were taken at each sampling station at a distance of 1 m from the station marker and equidistant from each other. Plastic vials (25 x 62 mm) were used as sampling tools, and soil was sampled to a depth of 2.5 cm. The 4 sub-samples from each station were pooled, and stored at 4^{o}C.

As supplies of neonate larvae were extremely low during the first season, bioassays were carried out on soil sampled on both dates from 1 site only, station 10. In the following season, soil sampled from 5 stations (both dates) was tested. The stations tested were chosen at

random from the 20 stations. Bioassays were carried out as described (page 82).

Results and discussion

Virus levels detected (PIB/g soil) are shown in Table 5.1. The results indicate, as other workers have found, that substantial deposits of NPV are retained in the soil, and that the virus can remain infective over the winter. The pre-season levels of virus are similar to those detected by Roome and Daoust (1976) in soil from sorghum plots which had been sprayed with H. armigera NPV. In general the post-season virus levels are substantially higher. Thus the results suggest that a build-up of virus occurs during the summer and autumn as successive generations of virus-killed larvae release polyhedra to the soil. Thus the first part of the hypothesis, that a reservoir of virus exists in the soil is confirmed.

5.5 Results and discussion, 1976/77 season (leaf samples)

Data collected during the 1976/77 season are shown in Fig. 5.2 in which virus levels detected are plotted against time. Each dot represents the level of virus detected at a sampling station. These levels comprised many values less than 1 but with 6% of the values >10 - the data is best illustrated by a logarithmic transformation of PIB/mm^2. Zero values are simply plotted at the base of the figure (the convention of adding a constant to all values before taking logarithms was not adopted as this would have increased all values in relation to known LC_{50} values). The open circles represent log (mean PIB/mm^2) for each sample. Rainfall is shown, expressed as weekly totals in millimetres - in each case a total for 7 days was summed (at the meteorological station) two days before the

TABLE 5.1 Levels of Heliothis punctigera nuclear polyhedrosis virus (PIB/g) in soil sampled during 1976/77 and 1977/78 seasons.

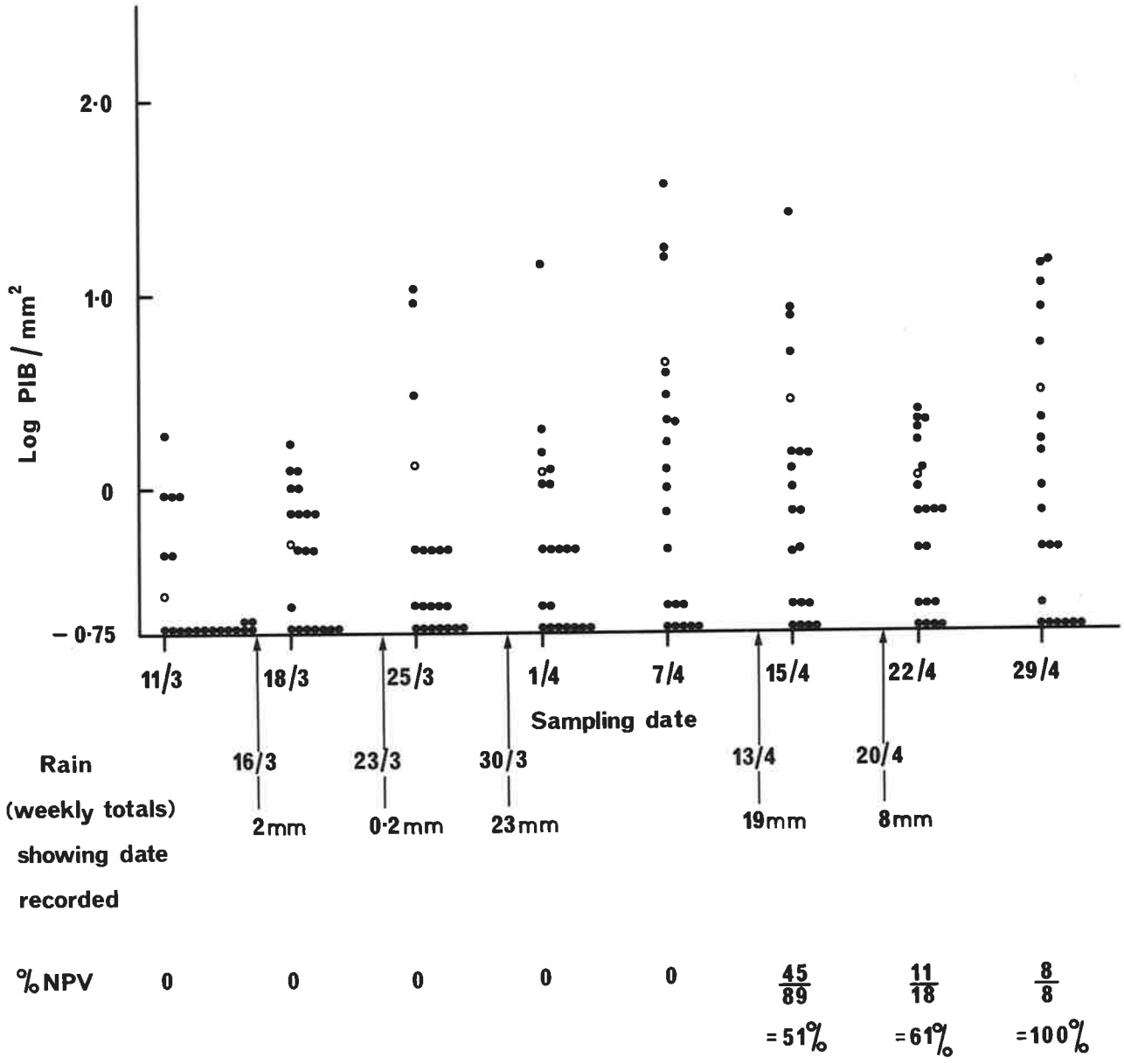
Season	Station No.	Pre-season soil sample (Date)	Post-season soil sample (Date)
1976/77		(23-11-76)	(29-4-77)
	10	2.04×10^4[1]	5.08×10^3
		(2.69×10^3)[2]	(1.21×10^3)
1977/78		(9-12-77)	(21-4-78)
	2	1.35×10^4	2.96×10^6
		(8.98×10^2)	(2.90×10^6)
	11	1.60×10^4	2.89×10^4
		(5.65×10^3)	(5.65×10^3)
	15	1.32×10^4	1.86×10^5
		(3.20×10^3)	(0)
	19	1.01×10^4	$>6.49 \times 10^6$[3]
		(5.80×10^2)	
	20	5.36×10^4	9.23×10^3
		(3.90×10^3)	(3.17×10^3)

1 Mean of 3 replicates (1976/77 season) or 2 replicates (1977/78 season).

2 Figures in parentheses = standard error of the mean.

3 100% mortality obtained in both replicates in bioassay, hence precise estimate of amount of virus present cannot be made.

FIGURE 5.2 Incidence of Heliothis punctigera nuclear polyhedrosis virus on lucerne foliage, sampled over an 8 week period, 11-3-77 - 29-4-77.


2·0
1·0
0
– 0·75
Log PIB / mm²
11/3
18/3
25/3
1/4
7/4
15/4
22/4
29/4
Sampling date
Rain
(weekly totals)
showing date
recorded
16/3
2mm
23/3
0·2mm
30/3
23mm
13/4
19mm
20/4
8mm
%NPV
0
0
0
0
0
45/89
=51%
11/18
=61%
8/8
=100%

leaf sampling date. The incidence of NPV in the population is expressed as percent diseased larvae, sampled from all stations.

Virus was detected in a large proportion (66.3%) of sampling units. There is an indication that levels of virus detected may have been influenced by rain, but for most of the sampling dates, more precise correlations are not possible due to the lack of information regarding time of rainfall. Hence the need was seen to install the recording rainguage for the following season. However, the rainfall recorded on 13-4-77 (19 mm) did in fact fall some time after 2400 h on 6-4-77; this was verified by the property manager, who observed and recorded (with a conventional rainguage) 19 mm of rain which fell in the early morning of 7-4-77. Hence assuming that there was in fact a transfer of polyhedra from soil to foliage as a result of the rain, we can calculate the expected level of infective polyhedra at the cessation of rainfall according to the immunofluorescent degradation curve, Fig. 4.6. The mean level of polyhedra at the time of sampling on 7-4-77 was 4.24 PIB/mm^2. The exact time of cessation of rainfall is not known; let us take, for the sake of argument, the figure of 8 h (0.5 π) as the interval of time which elapsed between cessation of rainfall and sampling. A convenient method to estimate the mean virus level which would have been present may be made by using the regression equation of Fig. 4.6.

$$y = 6.02 - 0.45x$$

Log 4.24 = 0.63: this may be substituted into the equation to give the x value corresponding to y = 0.63 in Fig. 4.6.

Thus $x = \frac{6.02 - 0.63}{0.45} = 11.98$ days

The time elapsing between cessation of rainfall and time of sampling, 8 h = 0.33 days. The value of y may then be calculated at 11.98 - 0.33 = 11.65 days.

$y = 6.02 - 0.45 \times 11.65 = 0.78$

Taking the antilog, $y = 5.99$ PIB/mm^2.

The under-estimation in the amount of infective virus may then be corrected for (page 84):

$$5.99 \times \frac{100}{59.1} = 10.14 \text{ PIB/mm}^2 \text{ (range = 13.02-8.76)}$$

This level of virus is approaching the LC_{70} for 3 day old H. armigera larvae (10.7 PIB/mm^2) determined by Daoust and Roome (1974). This suggests that this level of virus would be sufficient to trigger a disease outbreak in H. punctigera (should sufficient numbers of younger larvae be present). In fact an epizootic was in progress by the following week, with 51% mortality being recorded.

The mean PIB/mm^2 over all stations recorded throughout the season are shown in Table 5.2. For the first 4 weeks it is not known for how long the virus had been exposed on the leaves prior to sampling (in each case there was no indication at the time of sampling that it had rained recently). Thus the assumption must be made that only 8% of the polyhedra were infective when sampled (page 86). This level is shown in Table 5.2, and in each case is below the LC_{50} for neonate H. punctigera larvae (0.90 PIB/mm^2) (Teakle, 1976). No diseased larvae were recorded during this period. For the last 3 sampling dates of the season virus-killed larvae were present in the crop, hence it is likely that some of the polyhedra detected came from this source. Due to the protective effect of dried haemolymph from cadavers (David, 1967; Jaques, 1971, 1972a; Yendol and Hamlen, 1973), it is likely that these polyhedra would retain infectivity longer than polyhedra derived from the soil. Thus larvae feeding on the lucerne would be more likely to ingest contaminated foliage, i.e. the progress of the epizootic would be maintained if not accelerated by the large numbers of polyhedra released from virus-killed larvae. Such a

TABLE 5.2 Mean levels of Heliothis punctigera nuclear polyhedrosis virus recorded during 1976/77 season.

Sampling date	Mean PIB/mm^2 (standard error)	Log PIB/mm^2	Expected infectivity (PIB/mm^2)	Log expected infectivity
11-3-77	0.28 (0.11)	-0.55	0.02[1]	-1.65
18-3-77	0.54 (0.12)	-0.27	0.04[1]	-1.36
25-3-77	1.30 (0.66)	0.11	0.10[1]	-0.98
1-4-77	1.17 (0.68)	0.07	0.09[1]	-1.03
7-4-77	4.24 (1.96)	0.63	10.14[2]	1.01
15-4-77	2.83 (1.34)	0.45	*	
22-4-77	0.87 (0.18)	-0.06	*	
29-4-77	3.09 (1.08)	0.49	*	

[1] Expected infectivity at time of sampling = 8% of virus level detected by immunofluorescent method.

[2] Expected infectivity at cessation of rainfall (see text).

* Prediction likely to be underestimated due to presence of diseased larvae on crop (see text).

phenomenon could be expected to occur in a density-dependent fashion (see page 128). In this case because of the low larval population, the contribution made by polyhedra from this source may have been slight. The epizootic continued after 15-4-77, with 61% mortality being recorded on 22-4-77 and 100% mortality on 29-4-77. A mortality of 100% in this instance may have been due not so much to virulence of the virus as to a

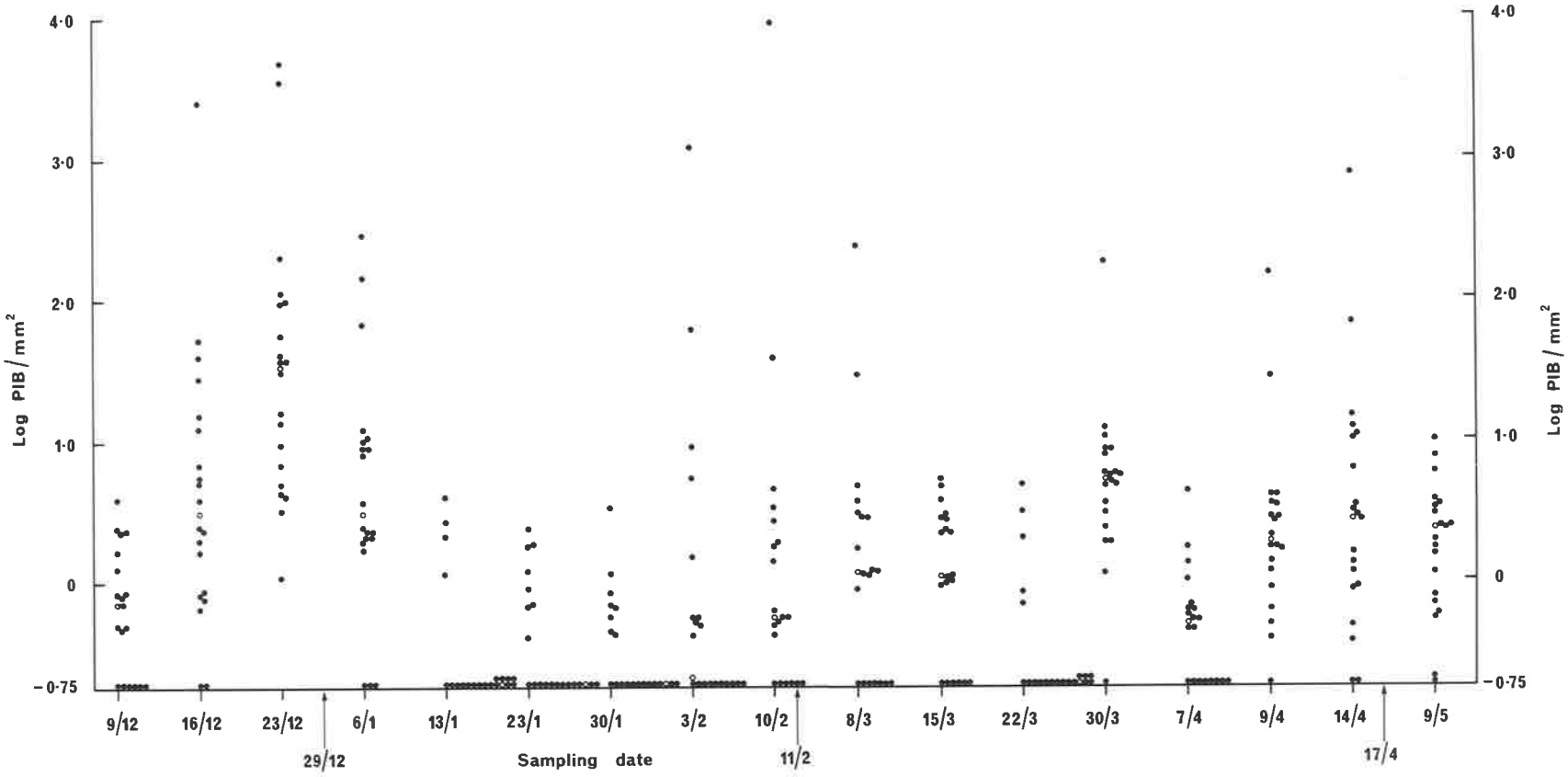
more susceptible population - because of the lateness of the season the lucerne would have slowed down in growth and may have been less nutritious to the larvae; such a reduction in food quality may have then rendered the larvae more susceptible to the virus (Tanada, 1963; Vago and Bergoin, 1968).

The first season's results indicate the usefulness of the immuno-fluorescent method in detecting virus in the field, and suggest (if somewhat tenuously) that rainfall may be a contributing factor in the initiation of epizootics. The study also made clear the need for more precise rainfall measurement, and the need to monitor healthy larval populations. In this way information concerning both levels of infective polyhedra and also the proportion of susceptible (i.e. younger) instars could be used in predicting the likelihood of epizootics occurring.

5.6 Results, analysis and discussion, 1977/78 season

The results of the 1977/78 field study are shown in Fig. 5.3. Levels of polyhedra are shown as log (PIB/mm^2) of leaf area, plotted against sampling date. On 9-12-77 only 19 values are recorded, as one sample was lost due to failure of a centrifuge tube. The open circles represent the median value of each sample (see below). Sampling was usually carried out on a weekly basis, although this was occasionally interrupted by mowing and the subsequent interval before regrowth occurred. Dates when mowing was carried out are marked by arrows. The long period between 10-2-78 and 8-3-78 was brough about by a delay in regrowth after mowing, due to pump failure. So that a sample could be taken soon after a shower of rain, the crop was sampled shortly after rain on 9-4-78, 2 days after the previous sample was taken. For the sake of convenience, all sampling dates are plotted at uniform intervals along the x axis. When an epizootic

FIGURE 5.3 Incidence of Heliothis punctigera nuclear polyhedrosis virus on lucerne foliage, sampled during 1977/78 season.



%NPV	0	82/635 = 12·9%	340/554 = 61·4%	0	0	0	0	29/299 = 9·7%	15/101 = 14·9%	0	0	0	0	8/30 = 26·7%	12/31 = 38·7%	26/76 = 34·2%	0
Rain (duration) [interval before sampling]	0	0	0	0	0	2·2mm (?) [?]	0	0	0	0	0	0	3·5mm (1·8h) [6·5h]	0	3·4mm (5·0h) [2·5h]	0	15·2mm (19·0h) [0·2h]
Irrigation	*				*	*				*		*					
Total	193	660	160	0	0	1	36	271	234	0	18	8	59	123		89	0
larval	333	504	114	0	0	2	152	536	191	0	4	5	32	105		71	0
count	381	441	96	0	0	8	222	292	126	0	7	6	48	117		26	0

occurred, a count of healthy and diseased larvae was made as described, and the incidence of NPV is expressed as percent diseased larvae, sampled from all stations.

Since the study attempted to correlate changes in levels of polyhedra with rainfall, the rain which is most likely to be of interest is that which fell shortly before the time of sampling (due to the short infectivity half-life of the virus). Thus in Fig. 5.3 rainfall which fell within 24 h of sampling is recorded; duration of the fall is shown in round brackets while the interval between cessation of rain and sampling is shown in square brackets. When rain fell as a series of showers, the interval between the end of the last shower and time of sampling is stated. The duration and precise time of the rain which fell prior to 23-1-78 was not recorded due to a clock malfunction. This rain fell some time between 1130 h, 22-1-78 and 1020 h, 23-1-78. Rainfall recorded over the entire sampling period is shown in Table 5.3.

Irrigation was carried out at somewhat irregular intervals during the study, occasioned by pump failure. On 5 occasions irrigation was carried out during the night preceding sampling; these dates are marked by an asterisk in Fig. 5.3. Irrigation was by a moveable "Sprayline" system, consisting of sprinklers mounted on a pipe which was moved over the plot in a north-south direction. Thus at the start of watering, stations 1, 6, 11 and 16 were watered. The sprinklers were run from 7 p.m. to 7 a.m. and between 70-100 mm of water was applied. Only those sampling stations adjacent to the pipe were actually watered - this is illustrated in Table 5.4.

Larval populations estimated by sweep sampling are also recorded in Fig. 5.3. Each day's sample consists of the total larval numbers caught along 3 transects which commenced at stations 1, 3 and 5 respectively.

The stadial composition of these totals is given in Appendix 1.

TABLE 5.3 Rainfall recorded during the 1977/78 field monitoring experiment.

Date	Amount (mm)	Time when rain started	Duration (h) (does not include intervals between showers)
24/25-12-78	2.15	2050	4.65
3-1-79	1.10	0445	1.75
22/23-1-79	2.20	?	?
8-2-78	0.90	1400	2.50
10/11-2-78	2.25	2300	0.92
21-2-78	1.25	0800	1.00
11-3-78	3.55	1215	2.20
25-3-78	0.20	1700	0.50
27-3-78	0.58	1100	0.17
29/30-3-78	3.50	2200	1.75
8/9-4-78	3.40	2300	5.00
9-4-78	0.88	1230	0.25
10/11-4-78	16.53	0030	28.00
25-4-78	0.28	1900	0.25
2-5-78	0.15	0700	1.00
8-5-78	15.20	1700	19.00

Larval populations were considerably higher than in the 1976/77 season, perhaps due to the more actively growing crop producing a greater number of flowers, and thus being more favourable for oviposition. The occurrence of epizootics was associated with a marked increase in extremely high levels of PIB/mm^2; polyhedra released from ruptured cadavers were probably responsible for these high values. It is likely that two populations of polyhedra are involved: polyhedra derived from the soil and polyhedra derived from cadavers (possibly small fragments of infected larval tissue adhered to the leaves causing such high values).

TABLE 5.4 Levels of Heliothis punctigera nuclear polyhedrosis virus (PIB/mm^2) detected on leaf samples taken within 24 h of irrigation. Asterisks denote sampling stations wetted by irrigation water.

Station	9-12-78	13-1-78	23-1-78	8-3-78	22-3-78
1	0.49	0	0*	2.99*	0
2	0	0	0	0	1.99
3	0.83	0	0	0	0
4	2.40	0*	0	0	0*
5	0.80*	0*	0	0.88	0*
6	1.24	0	0.90*	236.21*	0
7	2.24	2.61	0	1.13	0
8	0	0	0	1.09	0
9	3.85	0*	0	0	0.83*
10	2.28*	0*	0	4.74	0*
11	0.46	0	1.73*	2.83*	0
12	1.61	0	0	0	0
13	-[1]	3.98	0.40	1.68	0.68
14	0	0*	0.66	1.19	0*
15	0.70*	0*	0	0	4.84*
16	0	1.11	1.81*	0*	0
17	0.48	0	1.18	28.97	0
18	0	2.08	0.69	3.60	0
19	0.85	0*	0	2.80	0*
20	0*	0*	2.32	1.17	3.12*

[1] Missing value due centrifuge tube failure.

Both populations of polyhedra would probably follow skewed distributions (although there are not enough values to formally test this). Rather than calculate the mean PIB/mm^2 for each sample (unsuitable due to its sensitivity to extreme values) the median (shown in Fig. 5.3 as open circles) is used to describe central tendency - thus the weighting effect of the extremely high values is eliminated.

Analysis of variance

The differences in polyhedral levels between sampling dates may be significant or may be due to chance variation. As it is assumed that the data are not normally distributed, an appropriate statistical test to apply is the Kruskal-Wallis nonparametric analysis of variance (Siegel, 1956). This test is used to decide whether k independent samples are from different populations. No assumption can be made in the test about the data being linked (i.e. being derived from permanent stations over a period of time); no method is available which makes this assumption. However, the fact that the samples were not independent would make any differences detected by the test more reliable.

An analysis was carried out on arithmetic values of PIB/mm^2 over the entire sampling period. Results are shown in Table 5.5.

TABLE 5.5 Kruskal-Wallis one-way analysis of variance of levels of Heliothis punctigera nuclear polyhedrosis virus (PIB/mm^2) detected over entire 1977/78 sampling period.

n	Kruskal-Wallis H statistic	H corrected for ties	Probability of exceeding H
17	135.65	140.17	$P < 0.001$

We may conclude that significant variation does occur between virus levels over the period. Due to the obvious effect of epizootics in contributing to virus levels, there is justification in excluding those sampling dates when the population of what is assumed to be soil-derived polyhedra is interwoven with the population of polyhedra from cadavers. Should there be significant differences in virus levels on dates when no

diseased larvae were recorded and should any changes be associated with rain or irrigation, then this would add weight to the hypothesis. The Kruskal-Wallis test was therefore carried out on those 10 sampling dates when no epizootics were in progress, and results are shown in Table 5.6.

TABLE 5.6 Kruskal-Wallis one-way analysis of variance of levels of *Heliothis punctigera* nuclear polyhedrosis virus (PIB/mm^2) detected on sampling dates when no epizootics were in progress.

n	Kruskal-Wallis H statistic	H corrected for ties	Probability of exceeding H
10	74.14	79.09	$P < 0.001$

Similarly, we may conclude that significant changes in virus levels did occur on these sampling dates. There is now justification in determining if rain or irrigation was associated with changes in virus levels during these weeks.

Effect of rain

On three occasions, rain was recorded within 24 h preceding sampling on dates when no epizootic was in progress. On 23-1-78 the exact time and duration of the rainfall is not known - in any case the levels of virus detected on that day were at what could be regarded as a background level, i.e. the median value was zero. However, it is possible that the rain fell early on 22-1-78 and by 23-1-78, owing to the short immunofluorescent half-life (π = 16.05 h), any increased level of virus had been reduced.

Rain (3.5 mm) was recorded also on 30-3-78, 6.5 h before the leaves were sampled. On this occasion a substantial increase in virus levels was

evident: the median value, being zero on 22-3-78, increased to 5.16 PIB/mm^2 at the time of sampling. The distribution of values is clumped quite close to the median (with the exception of 2 points), in contrast to the days when epizootics were in progress, where possibly due to the presence of polyhedra from cadavers as well as from soil, the points tend to be more widely scattered. The close grouping of values suggests a somewhat more uniform application of polyhedra to the foliage, which might be expected as a result of polyhedra being splashed up from the soil.

A χ^2 analysis was used to test for the significance of the increase in virus levels between 22-3-78 and 30-3-78. Table 5.7 shows the virus levels recorded on these dates, the sign of the change in virus levels and the analysis. We may reject, at the 0.1% level, the null hypothesis

TABLE 5.7 Levels of Heliothis punctigera nuclear polyhedrosis virus (PIB/mm^2) recorded on 22-3-78 and 30-3-78, sign of change in virus levels and χ^2 analysis.

22-3-78	30-3-78	
0	5.5	+
1.99	177.82	+
0	5.76	+
0	5.76	+
0	8.57	+
0	11.92	+
0	1.11	+
0	8.45	+
0.83	7.81	+
0	2.34	+
0	4.67	+
0	3.54	+
0.68	10.42	+
0	1.86	+
4.84	1.85	-
0	0	
0	4.65	+
0	4.81	+
0	3.00	+
3.12	5.62	+

	+	-
E	9.5	9.5
O	18	1
O-E	8.5	-8.5
$(O-E)^2$	72.25	72.25

$$\chi^2 = \frac{\Sigma(O-E)^2}{E} = \frac{72.25}{9.5} + \frac{72.25}{9.5}$$

$$= 15.21$$

(χ^2 1 d.f., P = 0.001 = 10.83)

that no difference exists between these 2 samples.

The interval between cessation of rainfall and the time of sampling on 30-3-78 was 6.50 h. If we assume that the increase in polyhedral counts was caused by the rain, the level of infective virus at cessation of rainfall may be estimated from the median value 5.16 PIB/mm^2 at time of sampling. The equation of the immunofluorescent degradation curve, Fig. 4.6, may again be used:

$$y = 6.02 - 0.45x$$

Log 5.16 = 0.71. Substituting y = 0.71 into the equation gives x = 11.79 days. The time elapsing between cessation of rainfall and sampling, 6.05 h = 0.27 days.

Thus when x = 11.52, y = 0.83

Taking the antilog, y = 6.83 PIB/mm^2.

This estimate may then be corrected to indicate the median level of infective polyhedra at cessation of rainfall (page 84).

$$6.83 \times \frac{100}{59.1} = 11.56 \text{ PIB/mm}^2 \text{ (range 14.85 - 9.99).}$$

Again, comparison with the estimate of Daoust and Roome (1974) for the LC_{70} for 3 day old H. armigera (10.7 PIB/mm^2) suggests that polyhedrosis should be observed in a H. punctigera population exposed to this level of virus, should susceptible stages be present. The stadial composition data (Appendix 1) indicates that 21.6% of larvae at that date were younger than third instar. An epizootic was in progress by the following week, with 26.7% mortality being recorded.

Although an epizootic was initiated as predicted, the comparison of predicted levels of infective virus with the LC_{70} of Daoust and Roome is made with caution, and is subject to the following limitations:

a) The estimate of Daoust and Roome was made using a different (although related) species.

b) The estimate was made using larvae feeding on artificial diet to which virus had been uniformly applied, while in the field larvae are feeding on flowers, buds and leaves on which the deposition of virus is undoubtedly patchily distributed.

c) In the field the virus is rapidly degraded, while the estimate of Daoust and Roome was made under controlled conditions where presumably the degradation of virus was unimportant.

Hence to make more confident estimates of median lethal concentrations, an experiment would have to be carried out which simulated field conditions more closely. Unfortunately time did not permit such an experiment to be carried out.

The increased level of polyhedra over the background count on 9-5-78 appears to be associated with a fall of rain (15.2 mm) which ended shortly before sampling. This sample was taken only 10 minutes after the rain had stopped. However the level of virus recorded is significantly less than on 30-3-78, as shown by a χ^2 analysis carried out as in Table 5.7 ($\chi^2 = 9.8$, $P < 0.01$), although more rain fell on 9-5-78 than on 30-3-78. This is probably accounted for by the suddenness of the shower on 30-3-78, (1.75 h), compared to what must have been slow drizzle over 19 h on 9-5-78. Thus it appears that a sudden shower, with larger drop sizes, is more effective in transferring polyhedra to the foliage. This is also borne out by the data from 9-4-78, where compared to 30-3-78, a similar amount of rain fell (3.4 mm) but over a longer period (5.0 h). This sample was taken only 2.50 h after cessation of rainfall, so that the reduction in the estimate of PIB/mm^2 due to degradation would have been unimportant. Even though an epizootic was in progress, and some of the virus detected probably would have come from diseased larvae, a χ^2 analysis

indicates that the virus levels on 9-4-78 were significantly less than on 30-3-78 ($\chi^2 = 5.00$, $P < 0.05$).

Effect of irrigation

Although the data suggest that on occasions rain may transfer polyhedra to the foliage, there is little indication that this is the case with irrigation (Table 5.4). On only one occasion (16-12-77) was an epizootic preceded by irrigation, and this was not associated with any noticeable increase in polyhedral counts above background level. Hence it seems likely that the large amount of water applied during irrigation (70-100 mm) results in a washing-off effect which exceeds the rate at which polyhedra are splashed up onto the foliage.

Other factors

In the absence of rain or diseased larvae on the crop, the levels of polyhedra approached a background level, with the median equal or close to zero. However an exception to this was on 6-1-78, when high levels of virus were detected. The most plausible explanation is that factors other than rain may act to transfer polyhedra to the plants. On 6-1-78 the crop was still very short (6-10 cm) after the last mowing and undoubtedly a large residue of virus from the epizootic of 16/23-12-77 was present in the surface litter as well as soil. Thus with such short plants, one could anticipate wind movements would have more effect in swirling surface debris off the ground than in a more mature (denser) crop. Thus under appropriate conditions, wind might play an important role in transferring virus to the foliage. A light shower of rain (1.1 mm) on 3-1-78 may have made some contribution to the levels of virus detected, although any virus splashed up by this fall would have been subject to

degradation over 3 days.

On two occasions epizootics occurred which were not preceded by a high level of polyhedra being observed on the foliage (16-12-77 and 3-2-78). One explanation for this is that there was an increase in polyhedral counts either after the previous sampling date or sufficiently before so that degradation had occurred by the time the leaves were sampled. Thus the median value on 30-3-78 (5.16 PIB/mm^2) would have been reduced to 0.65 PIB/mm^2 had the sample been taken 48 h later. Hence it is possible, as mentioned, that an increase in polyhedral counts did occur after the rain that fell over the period 22/23-1-78. Such an increase, together with the low larval population, may have started a low level epizootic which was not detected on 30-1-78, and was not evident until 3-2-78. The progress of such an epizootic could be expected to be density dependent, (see Discussion, page 128) and thus would have been influenced by the increasing larval population between 23-1-78 and 3-2-78. Similarly, events prior to 9-12-77 might have been responsible for the epizootic observed on 16-12-77.

The pattern of the disease outbreak on 16-12-77 may give an indication as to the mechanism which gave rise to this epizootic. Table 5.8 shows the numbers of virus killed-larvae, total larval numbers and the percent diseased larvae at each station on 16-12-77 and 23-12-77. On 16-12-77 the presence of virus-killed larvae was recorded at all stations with the exception of station 11, i.e. it appears that by and large the initial infection of larvae occurred all over the field rather than at 1 or several localized sources. This suggests that the epizootic started as a result of contamination of foliage from virus in the soil, a process which could be expected to occur more or less evenly all over the field. However it is possible that high larval density may have been a contributing factor in this outbreak (see page 128); the distribution of larvae and the

pattern of the disease outbreak is also consistent with this mechanism. Unfortunately, numbers of diseased larvae were not sufficiently high during the other epizootics which were recorded to be able to provide much information on the pattern of these disease outbreaks.

TABLE 5.8 Incidence of virus-killed larvae and total numbers of *Heliothis punctigera* larvae during a nuclear polyhedrosis virus epizootic recorded on 16-12-77 and on 23-12-77.

Station No.	$\frac{\text{No. virus-killed larvae}}{\text{Total no. larvae}}$ = % Diseased	
	16-12-77	23-12-77
1	$^{4}/_{71}$ = 5.6%	$^{5}/_{22}$ = 22.7%
2	$^{4}/_{44}$ = 9.1%	$^{32}/_{45}$ = 71.1%
3	$^{2}/_{36}$ = 5.6%	$^{12}/_{23}$ = 52.5%
4	$^{3}/_{13}$ = 23.1%	$^{16}/_{28}$ = 57.1%
5	$^{8}/_{54}$ = 14.8%	$^{27}/_{46}$ = 58.7%
6	$^{7}/_{62}$ = 11.3%	$^{30}/_{46}$ = 65.2%
7	$^{5}/_{47}$ = 10.6%	$^{21}/_{29}$ = 72.4%
8	$^{6}/_{40}$ = 15.0%	$^{37}/_{48}$ = 77.1%
9	$^{4}/_{10}$ = 40.0%	$^{18}/_{25}$ = 72.0%
10	$^{1}/_{30}$ = 3.3%	$^{24}/_{48}$ = 50.0%
11	$^{0}/_{47}$ = 0	$^{9}/_{18}$ = 50.0%
12	$^{2}/_{25}$ = 8.0%	$^{13}/_{23}$ = 56.5%
13	$^{2}/_{12}$ = 16.7%	$^{15}/_{25}$ = 60.0%
14	$^{4}/_{19}$ = 21.1%	$^{12}/_{18}$ = 66.7%
15	$^{13}/_{38}$ = 34.2%	$^{19}/_{25}$ = 76.0%
16	$^{1}/_{13}$ = 7.7%	$^{8}/_{18}$ = 44.4%
17	$^{1}/_{19}$ = 5.3%	$^{15}/_{19}$ = 78.9%
18	$^{1}/_{12}$ = 8.3%	$^{8}/_{15}$ = 53.3%
19	$^{3}/_{10}$ = 30.0%	$^{4}/_{6}$ = 66.7%
20	$^{11}/_{33}$ = 33.3%	$^{15}/_{27}$ = 55.6%
Total	$^{82}/_{635}$ = 12.9%	$^{340}/_{554}$ = 61.4%

Surtees (1971) lists several factors which may be involved in initiating and/or propagating epizootics and those relating to transmission of pathogens are worth considering here. The study area was surrounded by other lucerne crops, which due to cultural practices were often at different stages of growth to the study plot. Larval populations were often also of different age structures, and epizootics were on occasions observed in these neighbouring plots. Hence it is possible that inocula of virus could be transmitted between adjacent areas, by abiotic factors, e.g. wind, or by biotic factors. Hamm and Young (1974) demonstrated that H. zea adults were capable of disseminating NPV. They demonstrated that virus could be transmitted between adults at mating, and subsequently progeny became infected with NPV as a result of surface contamination of eggs. They suggested that this phenomenon could occur in the field. Thus one could anticipate that if an epizootic did start as a result of this phenomenon, there would be no detectable increase in PIB/mm^2 on foliage prior to the outbreak. Predators of H. punctigera could also act as transmission agents of the virus. This is investigated in the next chapter.

Table 5.9 shows average maximum and minimum temperatures and relative humidity over the period of the 1977/78 field study. There is no evidence to support the suggestion of Cullen (1969) that high temperatures are predisposing factors to epizootics; however, on 2 occasions epizootics were preceded by a week of lower than average temperatures. It is not clear as to whether the cold weather per se contributed to the epizootics, or whether the low temperatures were simply associated with rain which occurred at about that time. Epizootics were not associated with high relative humidity.

TABLE 5.9 Meteorological data recorded during field monitoring experiment 1977/78.

Date of sample	Average temperatures (°C) during week preceding sampling date		Relative humidity (Percent of time > 80% R.H. during week preceding sampling date)
	Maximum	Minimum	
16-12	34.8	14.0	31.1
23-12	29.7	14.2	35.5
6-1	32.5	15.3	32.6
13-1	31.3	8.3	31.9
23-1	33.3	12.6	18.8
30-1	28.0	12.3	30.2
3-2	31.1	9.2	22.0
10-2	28.8	10.7	25.2
8-3	34.3	10.7	31.3
15-3	34.1	14.4	54.4
22-3	29.0	14.8	53.6
30-3	24.7	10.8	60.0
7-4	26.0	6.5	50.5
9-4	25.4	8.6	46.7
14-4	20.6	11.1	90.6
9-5	25.3	10.2	74.4

5.7 Conclusion

The field study indicated that rain, especially in the form of a quick shower, may cause a transfer of polyhedra from soil to foliage. An epizootic may then develop if larvae susceptible to this level of virus are present on the crop. However the results do not unequivocally support the hypothesis as on two occasions epizootics occurred without the detection of an increase in virus levels. Hence other mechanisms, one of which is investigated in the next chapter, may also be involved. Overhead irrigation, commonly assumed to play a similar role to rain, was not found

to be associated with the onset of epizootics. A discussion on the epizootiology of H. punctigera NPV in the light of these results, and the possibility of manipulating epizootics is made in the final chapter.

CHAPTER 6

THE ROLE OF OECHALIA SCHELLENBERGII AND NABIS TASMANICUS IN DISSEMINATING H. PUNCTIGERA NUCLEAR POLYHEDROSIS VIRUS

6.1 Introduction

As recorded in Table 3.6, Oechalia schellenbergii (Guér.-Mén.) (Pentatomidae) (Fig. 6.1) and Nabis tasmanicus Rem. (Nabidae) (Fig. 6.2) are active predators of H. punctigera. O. schellenbergii has been observed feeding on virus-killed larvae, and this observation led to the suggestion that one or both predators might be involved in disseminating the virus. Previous work has indicated that predatory insects are capable of disseminating host pathogens without becoming infected (Tanada 1963; Vago and Bergoin 1968). Predators may pick up host virus on mouth-parts and feet and subsequently contaminate foliage (Stairs, 1966) or may eliminate infective virus in faeces (Capinera and Barbosa, 1975). Smirnoff (1959) reported that Pilophorus uhleri, a hemipteran predator of Neodiprion swainei, appeared to transmit a polyhedrosis virus to healthy larvae, but no polyhedra could be detected on the proboscis or in the gut of the predator. However Franz et al. (1955), while carrying out an investigation into the epizootiology of the NPV of Neodiprion sertifer (Geoffr.), reported that the hemipteran predator Rhinocoris annulatus L. passed out infective polyhedra in faeces after feeding on virus-killed N. sertifer larvae.

Miles (1958) demonstrated that nymphs of the milkweed bug Oncopeltus fasciatus (Lygaeidae) produced no faeces but retained food residues in a distended region of the midgut until the final moult when the mass of residues was eliminated. Nymphs, however, produced a watery excreta which presumably originated entirely from the Malpighian tubes. Should a similar situation be the case with either of the predators of H. punctigera, it

FIGURE 6.1 An adult Oechalia schellenbergii feeding on a fourth instar Heliothis punctigera larva. x8.

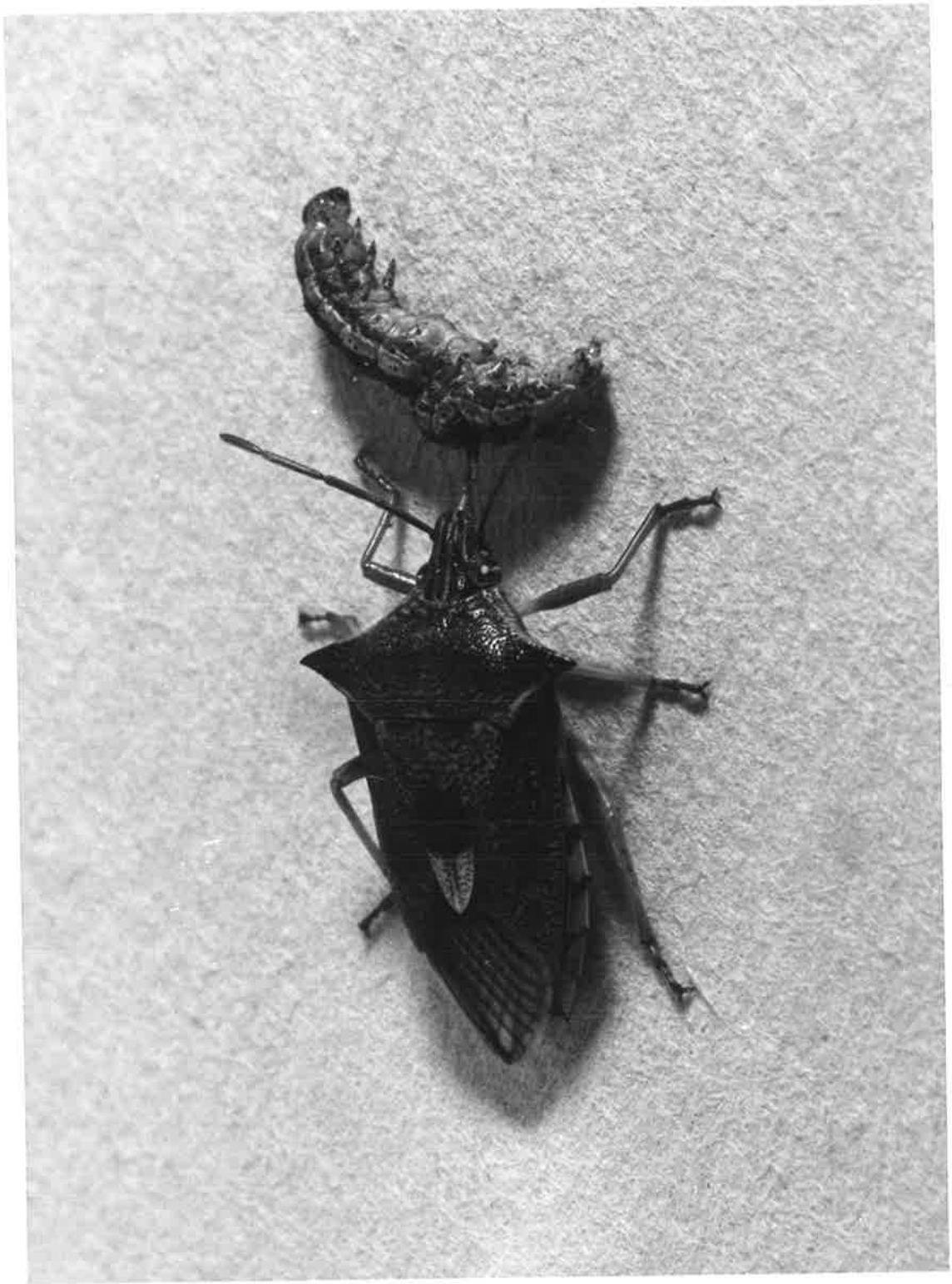


FIGURE 6.2 Nabis tasmanicus, a predator of Heliothis punctigera. x10.

gives rise to the interesting possibility of polyhedra being harboured inside the nymphs for some considerable period after feeding on virus-killed larvae.

Since many of the earlier studies were of an empirical nature, a quantitative investigation was undertaken to determine the period that infective virus is eliminated in the excreta of nymphs and adults of O. schellenbergii, after a single meal on an NPV-killed H. punctigera larva. Studies were also carried out to determine if contamination of O. schellenbergii mouthparts occurred after individuals fed on virus-killed larvae. Field-caught O. schellenbergii and N. tasmanicus were also monitored for the presence of polyhedra in excreta.

6.2 Materials and methods

Rearing of insects

O. schellenbergii nymphs and adults were reared in the laboratory in 50 mm plastic petri dishes. Nymphs were supplied daily with second or third instar laboratory-reared H. punctigera larvae, and adults were supplied with fourth instar larvae: 1-2 larvae were consumed per day. H. punctigera larvae were reared on modified Sender's diet (Griffith and Smith, 1977). For feeding experiments, first or second instar larvae were infected with NPV by administering several times the LC_{50} dose of unpurified H. punctigera NPV on artificial diet.

Scanning electron microscopy

Adult O. schellenbergii were allowed to feed in the laboratory on virus-killed H. punctigera larvae for 1 h. Mouthparts were then dissected from the adults, mounted on stubs, and were freeze-dried, using liquid freon to freeze the tissue. Specimens were then coated with gold/palladium

over carbon, and examined under an E.T.R.C. Autoscan scanning electron microscope.

Detection of NPV-immunofluorescent counting method

The immunofluorescent counting method (page 77) was used to detect polyhedra. As was the case with polyhedra in leaf washings, polyhedra in predators' faeces, especially when in low numbers, were more difficult to detect by normal light microscopy because of the presence of extraneous debris. Thus the advantage of the immunofluorescent method was a lower detection threshold, as polyhedra could be more positively identified. Although the above method was calibrated previously in conjunction with the leaf washing procedure, use of the method to detect polyhedra from suspensions also required calibration. Thus the method was calibrated using a haemocytometer count of polyhedra as a standard.

Calibration of immunofluorescent counting method

A suspension of polyhedra was counted using a haemocytometer (Neubauer-improved) and was serially diluted in 10-fold steps to give a dilution of 10^{-5} of the original suspension. Aliquots were taken from each dilution, diluted appropriately, and stained by the immunofluorescent method as described. Samples were diluted to an optimum counting density of 30-40 polyhedra per 0.2 mm microscope field, and 3 counts of 10 random fields were made for each sample. The number of polyhedra present in the 20 µl of suspension applied to within the 8 mm circle on the microscope slide (see page 78) was estimated by multiplying the mean number of polyhedra per 0.2 mm microscope field by the ratio of the area of the 6 mm diameter circle (the area over which the polyhedra were spread) and the area of the microscope field ($9.00 \times 10^2/1$). A plot was made of the number

of polyhedra in each dilution estimated by each method (immunofluorescent counting method and haemocytometer count).

Feeding experiments

Adults

Adult O. schellenbergii were allowed to feed on virus-killed fourth instar H. punctigera. Feeding was complete in 1-2 h. The bugs were then transferred to a fresh petri dish and fed healthy larvae for 10 days. Daily samples of excreta were taken by suspending the excreta in 100-200 µl 0.05% Tween 80 solution (depending on the volume produced). Samples were diluted appropriately in 0.05% Tween 80 and applied to ringed slides for assay by the immunofluorescent method, or stored at -15°C for subsequent bioassay. A control experiment was carried out by feeding an O. schellenbergii adult on healthy larvae for a 10 day period, and sampling excreta daily.

Nymphs

To determine if polyhedra were retained in the midgut until after the final moult, third and fourth instar O. schellenbergii nymphs were allowed to feed on virus-killed larvae. The nymphs were then fed on healthy larvae for the next 15 days, during which time the final moult occurred. Daily samples of excreta were taken (watery excreta from Malpighian tubes in the case of nymphs, and watery excreta plus faeces in the case of adults). Samples were assayed by the immunofluorescent method, or stored at -15°C for bioassay.

Bioassay

Infectivity of polyhedra in samples of excreta was determined by

bioassay, using neonate H. punctigera larvae (see page 82). Fifteen microlitres of excreta suspension (diluted appropriately) was added to the diet surface in each vial. A single larva was added to each vial and mortality was recorded after 12 days at 27°C ± 1°C. Smears were made of dead larvae and were examined by phase contrast microscopy to determine if polyhedra were present in the dead larvae. Usually 3 replicates of 30 larvae were used to determine infectivity in each sample. In addition a standard bioassay was set up each time as described (see page 82), using larvae from the same batch. A response curve (log dose vs probit mortality) was constructed from the standard bioassay, from which the concentration of NPV in the unknowns was estimated.

Midgut dissection

Fifth instar O. schellenbergii nymphs were dissected and the midguts examined for evidence of retention of food residues. The midgut of a nymph that had fed on virus-killed larvae during the fourth and fifth instar was triturated in 1 ml 0.05% Tween 80. Dilutions were made of the suspension for estimation of numbers of polyhedra by the immunofluorescent method.

Field studies

Field studies were carried out over a period of 11 weeks beginning in December 1977 at the irrigated lucerne property at Pt. Gawler. Weekly samples of O. schellenbergii and N. tasmanicus were made with a sweep net while walking three 150 m transects through the crop. Insects were caged in the laboratory until excreta were produced - samples were then taken, and assayed by the immunofluorescent method for the presence of polyhedra.

6.3 Results

Scanning electron microscopy

Large numbers of polyhedra were found on the labium of an adult O. schellenbergii that had fed on a virus-killed larva (Figs. 6.3 and 6.4). The polyhedra occurred in a clump adjacent to the dorsal groove on the distal end of the labium.

Calibration of immunofluorescent counting method

A highly significant regression line ($P < .001$) was obtained by plotting estimates of the concentrations of polyhedra estimated by a) haemocytometer count and b) the immunofluorescent counting method (Fig. 6.5). Over the range of concentrations used in detecting polyhedra in excreta, the mean recovery compared to the haemocytometer estimate was 95.6% (standard error of the mean = 3.7%).

Detection of polyhedra in excreta of adult O. schellenbergii

Adult O. schellenbergii fed on virus-killed larvae voided excreta containing large numbers of polyhedra. Most of the polyhedra occurred in faecal deposits, but a lower proportion occurred in watery excreta. Faeces sampled from an individual that had fed 3 days previously on a virus-killed larva contained 1.92×10^7 PIB, while watery excreta contained 2.40×10^5 PIB.

Dissection of nymph midguts

Examination of midguts dissected from nymphs revealed an identical situation with respect to retention of food residues as that described in O. fasciatus (Miles 1958). The third region of the midgut was greatly distended with accumulated food residues, while the region following it

FIGURE 6.3 Scanning electron micrograph of polyhedra adhering to labium of an adult Oechalia schellenbergii that had fed on a virus-killed Heliothis punctigera larva. Arrow indicates mass of polyhedra. x650.

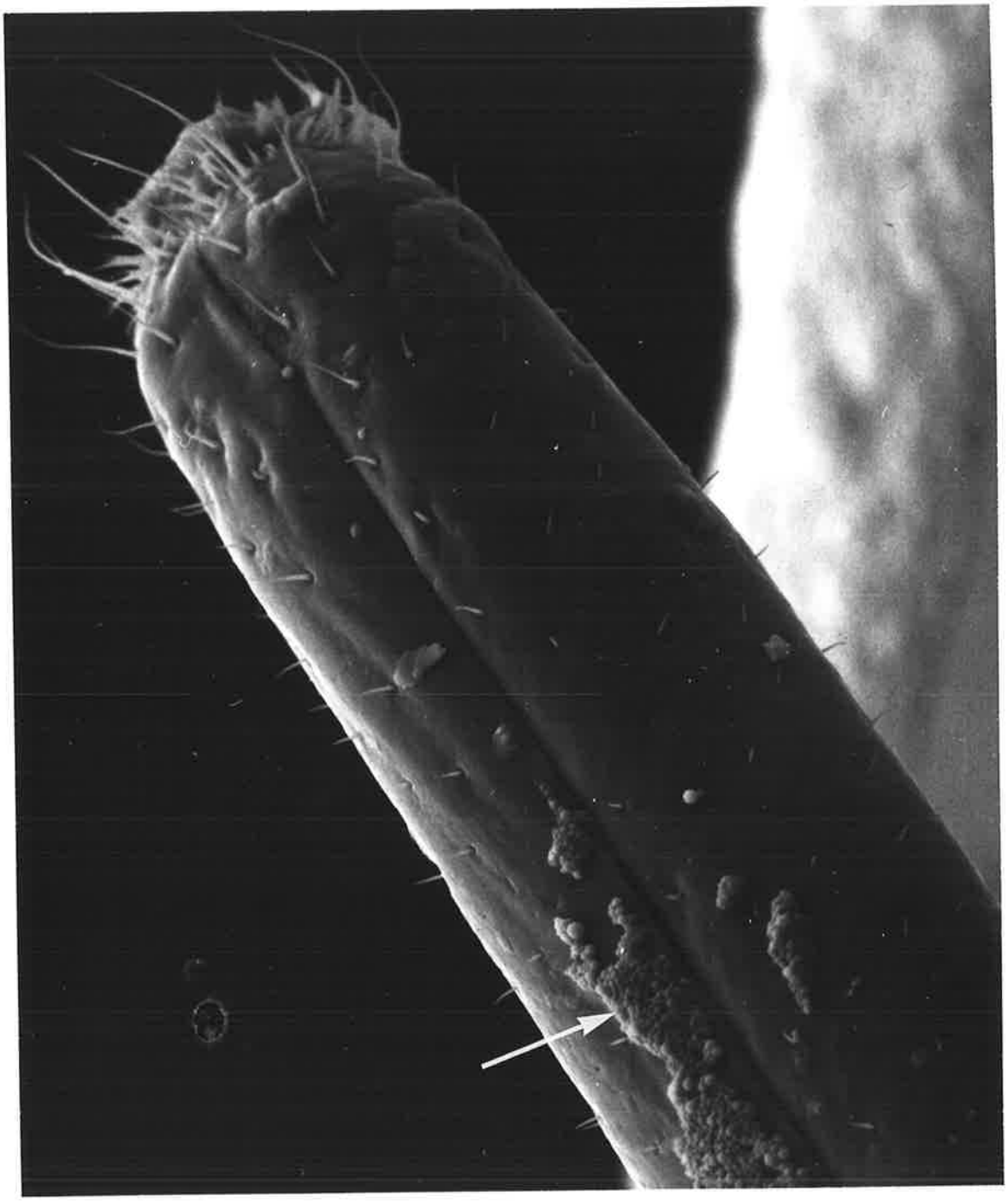


FIGURE 6.4 Scanning electron micrograph of polyhedra adhering to labium of an adult Oechalia schellenbergii that had fed on a virus-killed Heliothis punctigera larva. x2700.

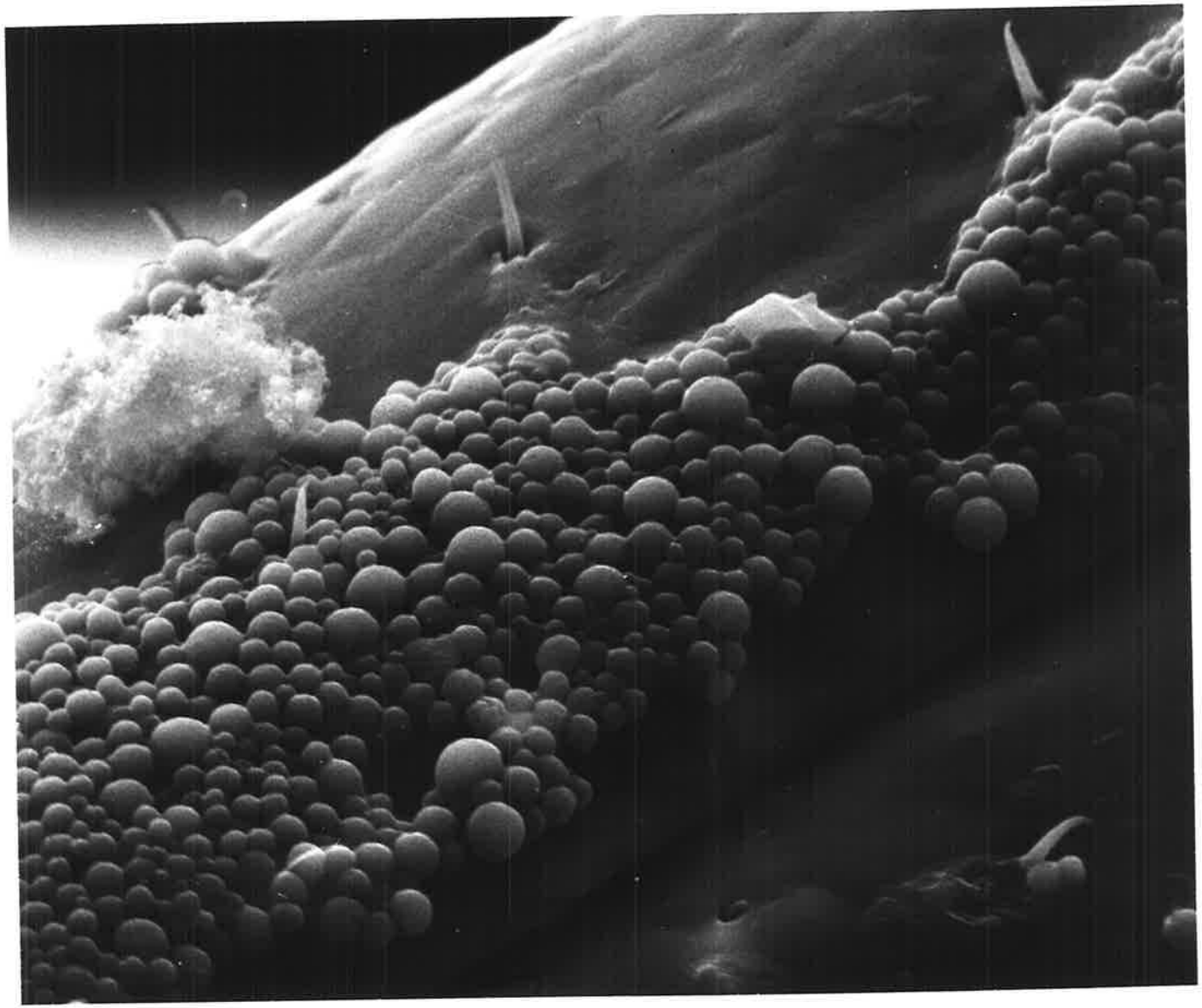
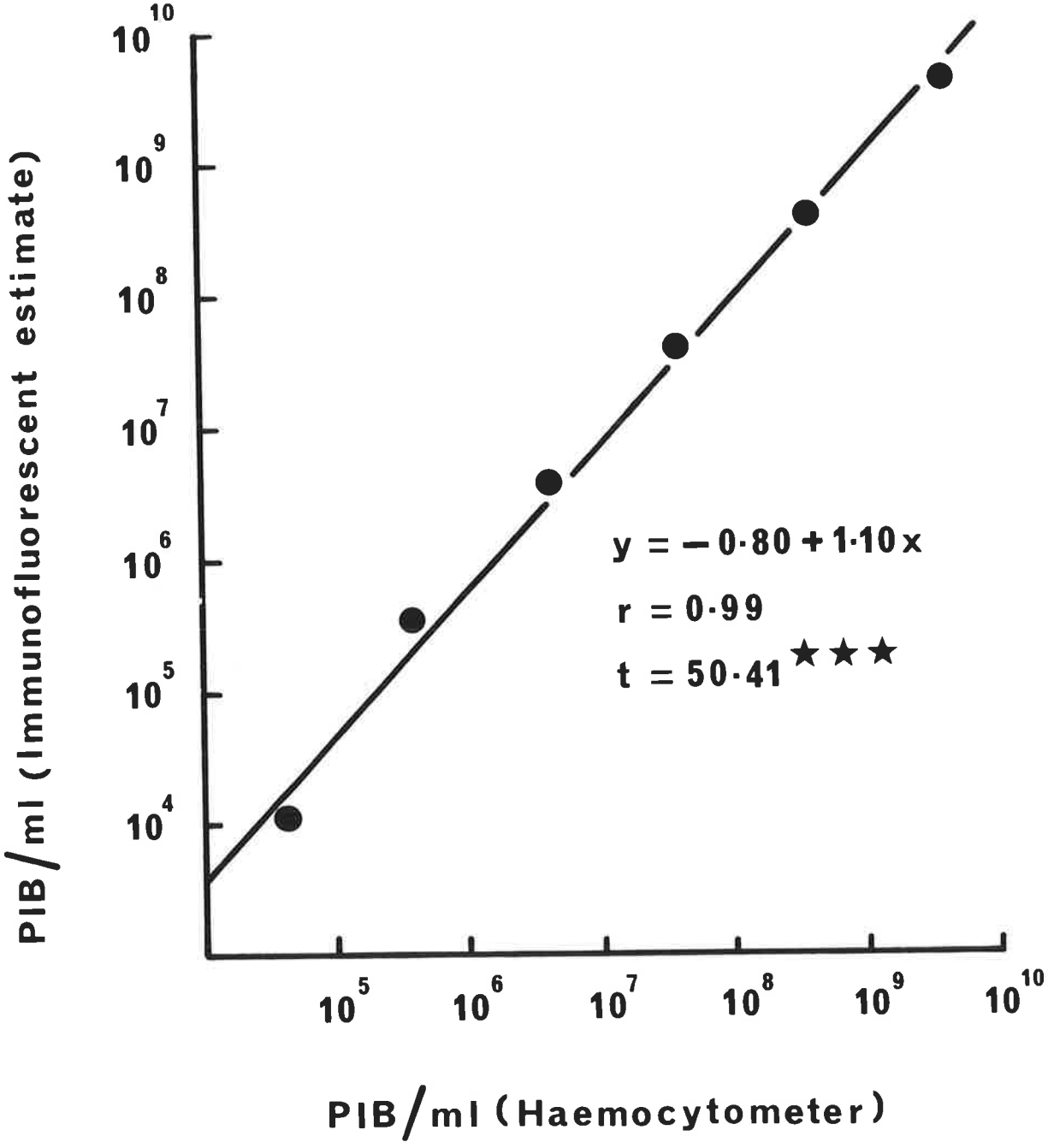


FIGURE 6.5 Estimation of numbers of polyhedra of Heliothis punctigera nuclear polyhedrosis virus in a series of dilutions by an immunofluorescent counting method compared to estimation by haemocytometer. Each point represents the mean of 3 estimates by each method.


PIB/ml (Immunofluorescent estimate)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
y = −0·80 + 1·10x
r = 0·99
t = 50·41 ★★★
10^5
10^6
10^7
10^8
10^9
10^10
PIB/ml (Haemocytometer)

was narrow and apparently closed (Fig. 6.6). This finding was consistent with the observation that nymphs produced only watery excreta presumably from the Malpighian tubes (red coloured), while faeces (brown coloured) were not produced until after the final moult. The third region of the midgut from a nymphs that had fed on two virus-killed larvae contained 6.52×10^8 PIB.

Feeding experiments

Four adults each fed on a single virus-killed larva voided excreta containing polyhedra (detected by the immunofluorescent method) within 1 or 2 days (Fig. 6.7) and for as long as between 3 and 6 days after the virus-infected meal. The control insect which fed only on healthy larvae produced no polyhedra in excreta sampled over a 10 day period. An O. schellenbergii nymph that had fed on a virus-killed larva as a fourth instar voided low numbers of polyhedra at around the time of the penultimate moult, and then large numbers of polyhedra soon after the final moult (Fig. 6.8), which occurred 8 days after the virus-infected meal. Polyhedra were not detected after Day 10.

Infectivity of polyhedra

Data are presented in Table 6.1 on the infectivity of polyhedra sampled from excreta at various times after a meal on virus-killed larvae. Insects (A) and (B) were adults. Insect (C) was a newly moulted adult that had fed on 5 virus-killed second or third instar larvae 10-15 days previously. Another adult (D), was caught in the field and faeces sampled on the following day. The mean number of infective polyhedra as indicated by bioassay is expressed as a percentage of the mean immunofluorescent estimate.

FIGURE 6.6 Midgut of fifth instar Oechalia schellenbergii nymph, showing distended third region of gut (a) and restricted region (b). x13.

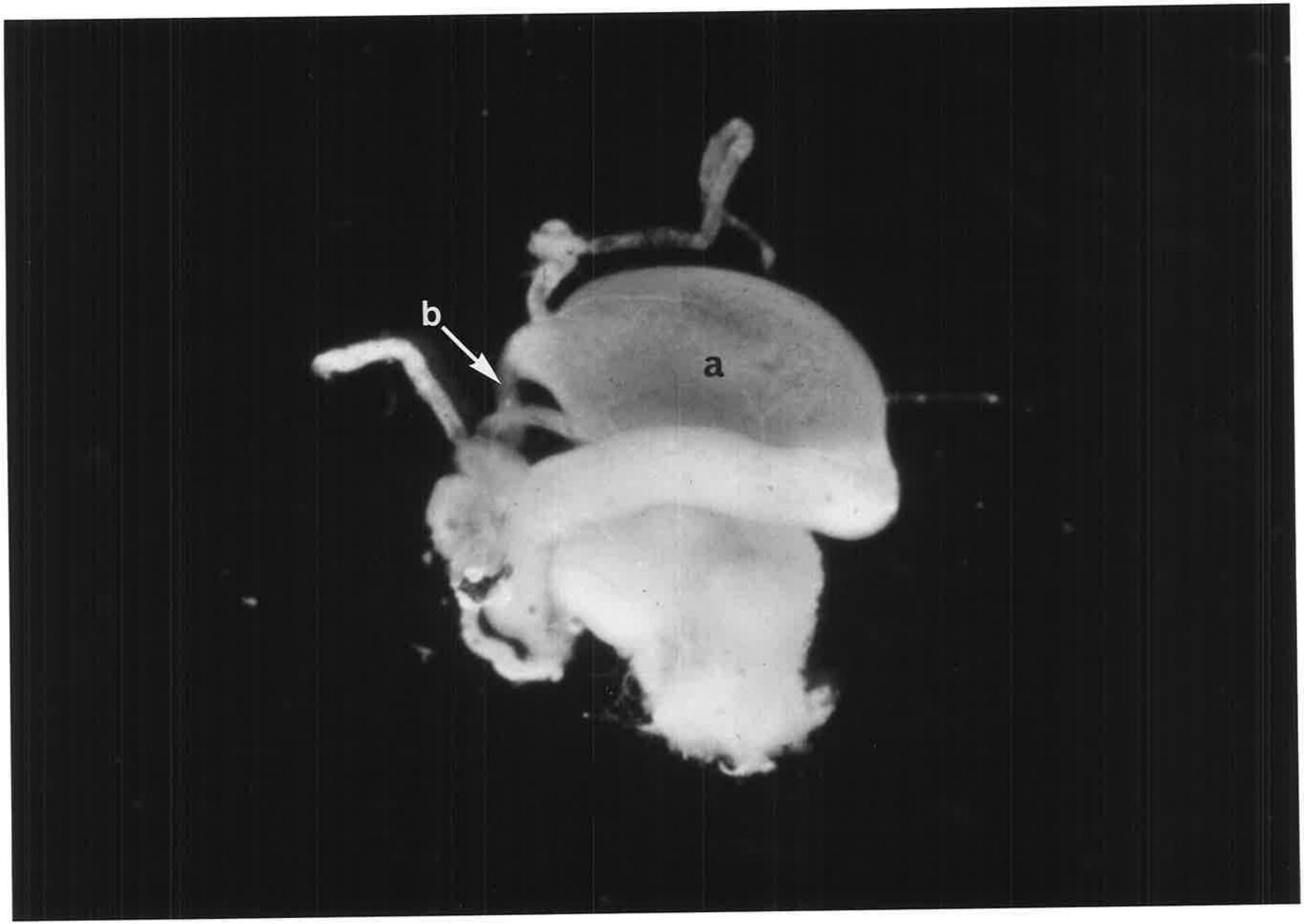

b
a

FIGURE 6.7 Levels of polyhedral inclusion bodies voided in excreta from four adult Oechalia schellenbergii fed virus-killed Heliothis punctigera larvae on Day 0 and subsequently fed healthy larvae.

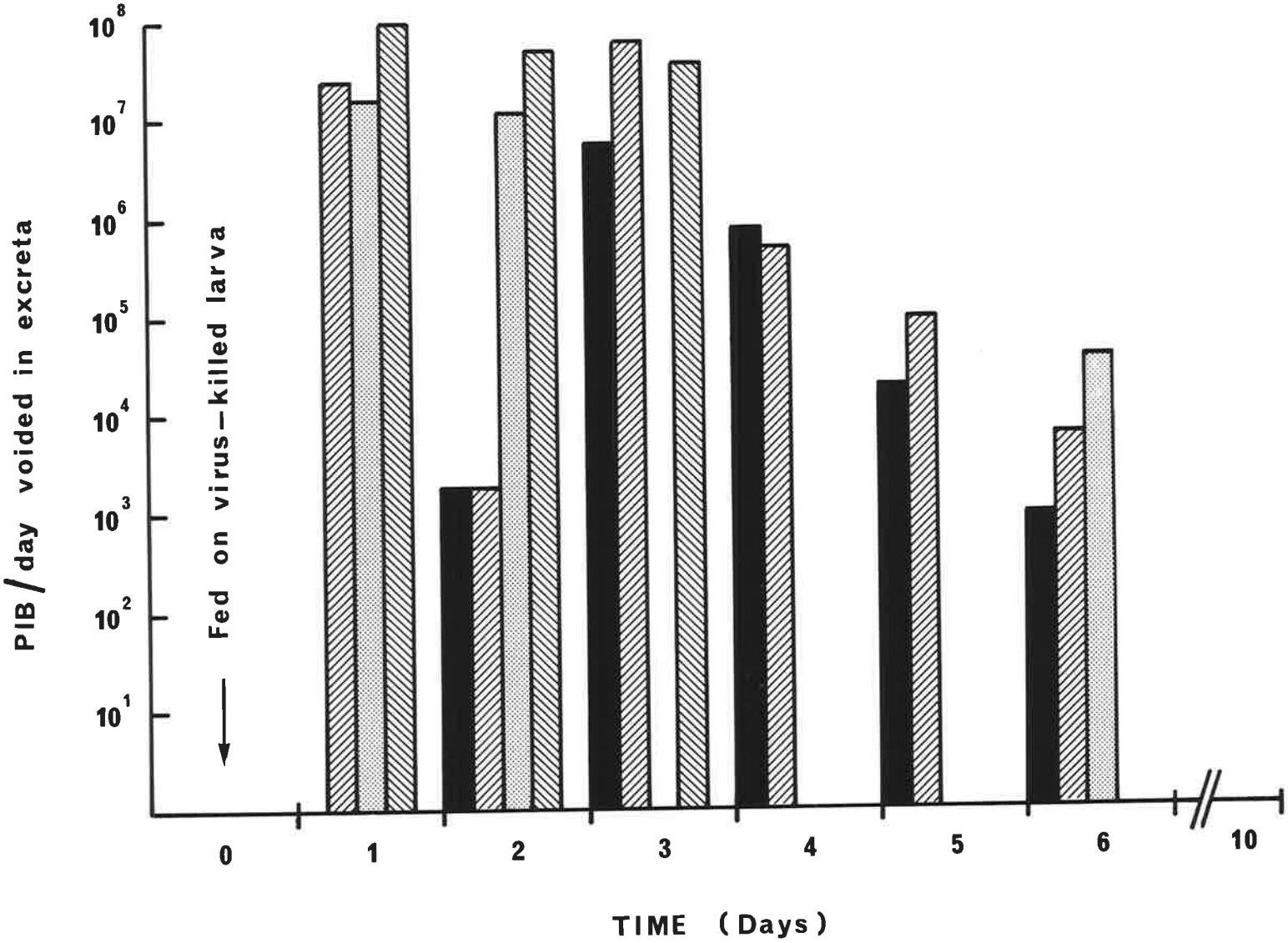

PIB / day voided in excreta
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Fed on virus-killed larva
0
1
2
3
4
5
6
10
TIME (Days)

FIGURE 6.8 Levels of polyhedral inclusion bodies voided in excreta from a single Oechalia schellenbergii which fed as a fourth instar nymph on a virus-killed Heliothis punctigera larva and subsequently fed on healthy larvae.

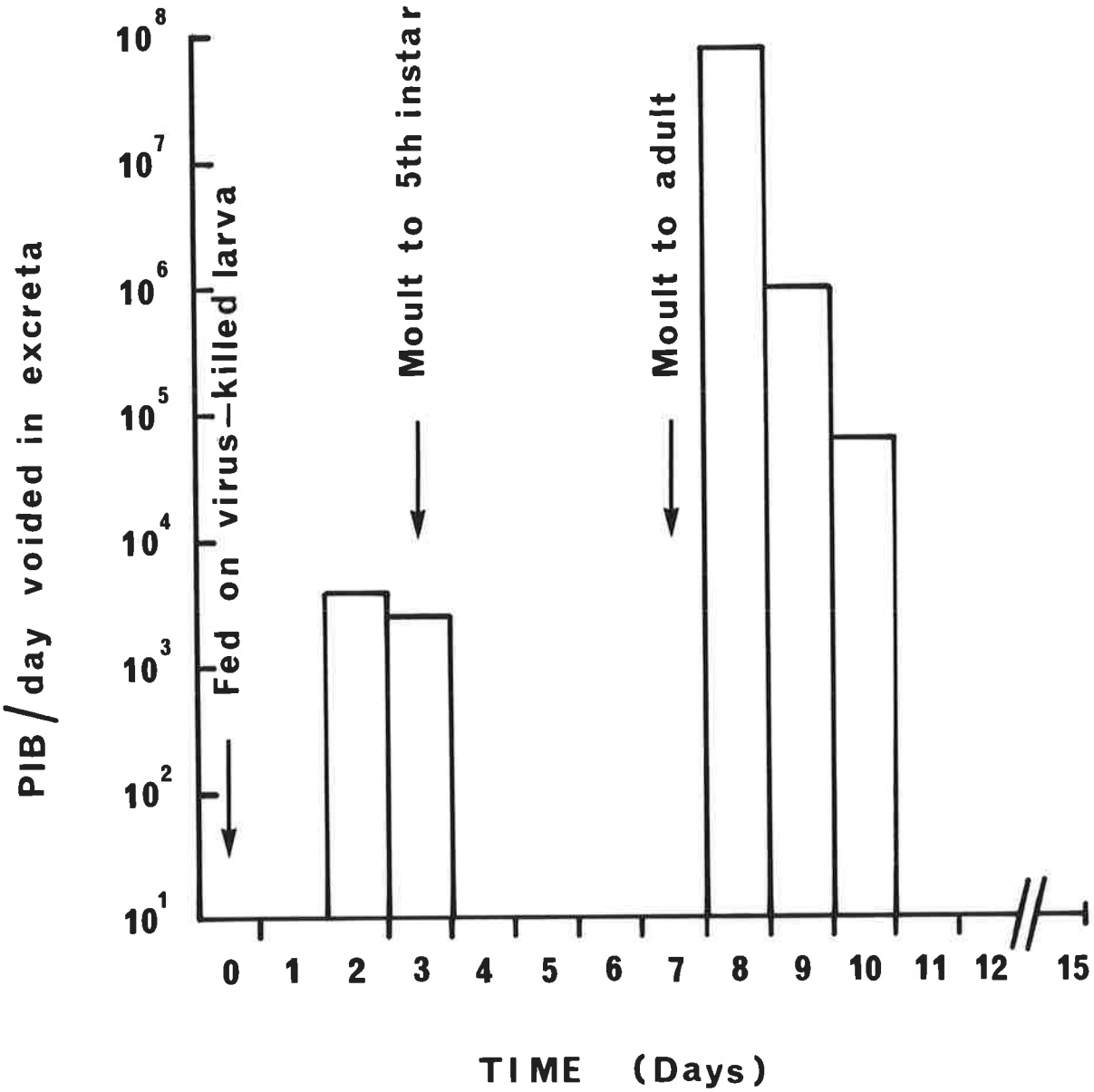

PIB / day voided in excreta
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Fed on virus-killed larva
Moult to 5th instar
Moult to adult
0 1 2 3 4 5 6 7 8 9 10 11 12 15
TIME (Days)

TABLE 6.1 Percent infectivity of nuclear polyhedrosis virus in excreta of individuals of Oechalia schellenbergii sampled at various intervals after a meal on a virus-killed Heliothis punctigera larva.

Insect	Days since meal on virus-killed larva	Mean[b] No. of PIB in excreta (bioassay estimate)	Mean[b] No. of PIB in excreta (immunofluorescent estimate)	Mean % infectivity
A	2	1.68×10^7 (8.48×10^6)[a]	1.17×10^7 (2.36×10^5)	144.0
B	4	2.27×10^5 (6.64×10^4)	8.41×10^5 (3.45×10^4)	27.0
C	10-15	5.24×10^6 (1.49×10^6)	1.26×10^8 (4.92×10^6)	4.2
D	unknown (field-caught)	2.66×10^3 [c]	8.64×10^3 (3.12×10^2)	30.8

[a] Standard error of the mean
[b] 3 replicates
[c] 1 replicate

Field studies

Although catches of both species were low compared to previous seasons (almost certainly due to the spraying of systemic insecticides against T. trifolii), a considerable proportion of each contained polyhedra in excreta. Of 22 O. schellenbergii caught (nymphs and adults), 64% contained polyhedra in excreta, and of 39 N. tasmanicus (all adults), 39% contained polyhedra in excreta.

6.4 Discussion

The feeding behaviour of individuals of O. schellenbergii when feeding on virus-killed larvae differs somewhat from the behaviour observed when

the bugs feed on healthy larvae. When feeding on healthy larvae, the labium is pressed against the cuticle of the prey insect, and the stylets penetrate into the haemolymph. However, when feeding on a virus-killed larva, the labium is inserted well into the haemolymph; this is possibly a consequence of the more fragile cuticle of the dead larva. Thus, as indicated by the SEM results, the labium becomes contaminated with polyhedra, and behaviour observed after completion of feeding suggests that dissemination of NPV by O. schellenbergii would occur. On completion of a meal, bugs would invariably clean the labium with the front tarsi, thus transferring polyhedra to the tarsi - subsequent walking would ensure contamination of foliage.

The results from Table A suggest that insect (A), which fed 2 days previously on a virus-killed larva, voided polyhedra which were all infective. The unexpectedly high estimate of percent infectivity (144%) is almost certainly a reflection of the immunofluorescent method providing an underestimate of the bioassay estimate (see page 84). Similarly, the estimates by the immunofluorescent method of the numbers of polyhedra voided by insects (B), (C) and (D) may also have been underestimated. However it is not known to what extent prolonged retention inside the insect's gut may have affected the relationship of the estimates by the 2 methods.

The data in Table 6.1 suggest that infectivity of the virus is reduced during prolonged retention inside the alimentary tract of O. schellenbergii, presumably as a result of enzymic activity. Further work is required to determine more precisely the infectivity half-life of virus inside the predator; nevertheless the results indicate that adult O. schellenbergii, after a meal on a single virus-killed larva, pass out polyhedra over the next 1-6 days, and that a proportion of these polyhedra remains infective until at least the fourth or fifth day. Thus the contents

of a dead larva, instead of contaminating foliage in one area, may be deposited over several sites, thereby increasing the likelihood of infecting healthy larvae. Thus O. schellenbergii adults, which are strong fliers, could well play an important role in disseminating virus within a host population, or between neighbouring host populations. Nymphs might play an equally important role by storing large amounts of polyhedra for a longer period and this could be important in providing inoculum of virus after deposits of NPV from decomposed cadavers have been removed from foliage by rain or irrigation. Insect (C) (Table 6.1), although storing virus as a nymph for 10-15 days, most of which was degraded, still produced a considerable quantity of infective virus (5.24×10^6 PIB). Low levels of virus may be sufficient to initiate epizootics in H. punctigera, especially when young larvae are involved (see page 132), while Allen and Ignoffo (1969) quote an LD_{50} corresponding to 9.4 PIB per larva for 3 day old H. zea. Most of the polyhedra ingested by a nymph are released at the final moult, although it appears that smaller amounts are released earlier (Days 2 and 3, Fig. 6.8). Thus it appears that some leakage occurs from the swollen midgut, or alternatively there may have been some external carry-over by polyhedra adhering to the insect's proboscis or tarsi after the virus-infected meal. Polyhedra were detected in the watery excreta of 56% of the field-caught O. schellenbergii nymphs - possibly more contained polyhedra, but it was not realized until towards the end of the investigation that nymphs retained food residues.

The high incidence of polyhedra in the excreta of field-caught insects suggests that both species feed frequently on diseased larvae. This observation, together with the results from feeding experiments, indicates that a sizeable proportion of the O. schellenbergii population preying upon a diseased H. punctigera population may be disseminating infective

polyhedra in the field. Recent investigations (Beekman, unpublished) have demonstrated that the polyhedra voided by N. tasmanicus are also infective (although unlike O. schellenbergii, it appears that the nymphs of this species do not retain food residues in the midgut). This finding suggests that N. tasmanicus may play an equally if not more important role in disseminating NPV, as this species is more abundant than O. schellenbergii. Teakle (pers. commun. 1977) observed that individuals of Cermatulus nasalis (Westw.) (Pentatomidae) also feed on virus-killed larvae of H. punctigera, hence it is likely that this predator also plays a role in disseminating NPV. The contribution that these phenomena make in maintaining and perhaps initiating epizootics in the host population can only be ascertained by more extensive laboratory and field studies.

CHAPTER 7

DISCUSSION

The study of factors leading to epizootics of H. punctigera NPV has demonstrated the feasibility of monitoring levels of polyhedra on lucerne foliage. The study has also shown that provided the level of infectivity of the polyhedra can be determined, this may be used to predict disease outbreaks. More precise predictions of expected mortality could have been made if a larger number of subsamples was made, and if sampling was carried out at more frequent intervals (desirable in view of the short infectivity half-life of the virus). In addition, as mentioned, there is a need to determine more precisely the LC_{50} values for each instar of H. punctigera on lucerne foliage.

The design of the field study is perhaps open to criticism in that no information was obtained on the within-station variance of PIB/mm^2, i.e. the study could possibly have been carried out using only 5 sampling stations and having 4 sub-samples within each station. However the advantage in the experimental layout that was adopted is that by having a larger number of stations, there was an increased likelihood of detecting the patchily-distributed virus.

There are a number of interesting questions which were raised by the results of the 1977/78 season, and which unfortunately were not investigated due to lack of time. The most obvious of these is the question of irrigation. It is assumed that the large amount of water applied during watering must have washed most polyhedra off the leaves. However if an attempt is to be made to manipulate epizootics, use of sprinkler irrigation is perhaps the best method to use to contaminate the crop, as rainfall is obviously too unreliable. That sprinkler irrigation can lead to contamination of

foliage by polyhedra derived from the soil has been demonstrated by Jaques (1967a). In this case the sprinkler was operated for 2 h. There are a number of possible variables in the application of water to the crop which could be investigated with a view to determining how closely the conditions which prevail during a sudden shower of rain can be simulated. Thus length of time the sprinklers were left operating, splashing effect at increasing distance from the sprinkler, effect of drop size and pressure of the water (do drops of water in the spray approach the terminal velocity of rain drops?) are all variables which would be well worth investigating. In addition, no information is available as to how effective is flood irrigation in transferring polyhedra to the leaves (in the south east district a large proportion of the lucerne seed crops are irrigated by flood irrigation).

The methods developed for recovering and detecting polyhedra have proved useful and could probably be used with other viruses and on other host plants. One application for which the methods would be most suitable would be in deposition studies of virus sprayed on to a crop - compared to previous work (Davidson and Pinnock, 1973) the methods described now enable a more reliable estimate to be made of the amount of virus present. Compared to methods involving bioassay, the current method has the advantage of being able to handle a large number of samples. With 3 personnel the field study could have accommodated 100 samples per week - a study on this scale would be a formidable undertaking if a bioassay technique was employed.

The results from Chapters 5 and 6 indicate 2 possible mechanisms by which epizootics may initiate. The study has provided useful information in that by investigating these mechanisms on a quantitative basis, it is now possible to say with some certainty that the host population can be exposed to lethal amounts of virus by both mechanisms. However from the

results of the investigation it is not possible to determine the relative importance of either mechanism, or to what extent other factors such as those mentioned by Surtees (1971) may be involved. Nevertheless the study (and further studies of this nature) are worth undertaking, as knowledge in this area is far from complete (Tanada, 1976). In the light of the results obtained, the initiation and progress of an epizootic of H. punctigera might proceed as follows:-

Larvae feeding on a lucerne crop are continually exposed to low levels of virus - the data in Fig. 5.3 suggest that at least some virus is present on the crop all the time, i.e. on no occasion were all samples zero. The degradation experiment (Fig. 4.6) indicates that only a small fraction of polyhedra detected would be infective, so that at times the mean level of infective virus would be less than the LC_{50} of first instar larvae. Thus at these times individuals developing polyhedrosis could be expected to occur at low frequency. Such a situation could be regarded as an enzootic phase of the disease.

This situation might alter due to an increase in the mean PIB/mm^2 on the foliage. This might occur all over the field as a result of rain, (or possibly wind), or might occur in a more localized manner due to the arrival of individuals of O. schellenbergii or N. tasmanicus (or perhaps other predators) that had been feeding on virus-killed larvae in neighbouring crops. It is possible that an inoculum of virus could also come from sources not investigated, e.g. from avian faeces (Hostetter and Biever, 1970; Gitay and Polson, 1971; Entwistle et al., 1977) or perhaps (but less likely), from alternative hosts (Surtees, 1971). Any polyhedra deposited on the foliage would be subject to inactivation, although in the case of virus in bug or avian faeces, the inactivation rate would probably be reduced due to the shielding effect of the surrounding medium.

Larvae feeding on contaminated foliage would become infected and depending on the instar and dose (Teakle, 1973) death would result in 2-14 days. Large numbers of polyhedra would be released from cadavers and this would greatly increase the levels of virus on the crop and the probability of remaining larvae becoming infected. Hukuhara and Kitajima (1978) in proposing a model depicting the progress of a GV epizootic in Hyphantria cunea describe this phase of the disease as involving a logarithmic increase in the number of diseased larvae. The progress of this phase of an epizootic and the large increase in the number of polyhedra released is well depicted in Fig. 5.3 between 16-12-77 and 23-12-77. The log median PIB/mm^2 on these two dates (0.49 and 1.53 respectively) does in fact represent a logarithmic increase in the number of polyhedra, most of which presumably came from disintegrating cadavers. The proportion of infected larvae over this period increased by a factor of 5, which suggests that by 23-12-77 the dead larvae comprised a larger proportion of mature larvae which contributed larger numbers of polyhedra. It was unfortunate that mowing on 29-12-77 prevented one more sample being made of this epizootic.

As well as the increase in the number of polyhedra, the progress of the epizootic is further enhanced by the fact that larvae almost invariably crawl to the top of the plants to die. This not only ensures that probably the major part of the plant is contaminated with virus, but also places the larvae in a position where they are most likely to be devoured by healthy larvae. Cannabilism of virus-killed larvae was frequently observed - this behaviour undoubtedly leads to a significant increase in the number of infected larvae. As mentioned, predatory bugs, when present in sufficient numbers, would also play a part in transmission of virus within the population. Thus there is often a marked increase in the number of infected larvae, which may have caused observers in the past to

believe a "spontaneous" virus outbreak had occurred, as in earlier stages the presence of the disease is not quite so obvious.

An important factor which must influence the progress of an epizootic or the probability of one being initiated is host density. When some event causes polyhedra to be deposited on leaves, the distribution of the virus will tend to be patchy (polyhedra from predators' faeces would probably be more patchily distributed that polyhedra splashed up by rain). Thus the probability of there being an encounter between host and virus will increase with increasing host density. Hence, for a given level of virus on the crop, the higher the larval density the greater will be the probability of an epizootic developing. Once an epizootic has started, the subsequent second round of infection would be expected to occur in a density-dependent manner. Thus it is possible that at very high larval densities, an epizootic may start with only background levels of polyhedra being present. Normally, in the enzootic phase, the number of individuals which die after being infected as young larvae may be too small to make subsequent infection of substantial numbers of other larvae very likely; in addition, the "yield" of polyhedra from these dead larvae is small compared to the amount of virus released from larvae which die in the fifth or sixth instar. However, at higher host densities, one could expect a larger number of larvae to be infected, even with only background levels of polyhedra on the foliage, which would then result in more polyhedra being available for a secondary cycle of infection. Thus there may exist a critical threshold of larval density above which a disease outbreak could be expected to occur with only a background level of polyhedra being present. It is possible that the epizootic which was first detected on 16-12-77 (and possibly that of 3-2-78) began due to the above mechanism. However epizootics could also be expected to occur at low host densities, provided sufficient levels of virus were present on the crop, e.g. on 7-4-78 and on 15-4-77 (Fig. 5.2).

The study has failed to indicate why the incidence of NPV is higher in irrigated (especially sprinkler irrigated) lucerne crops (Cullen, 1969; Doull, pers. commun. 1975). One explanation is that overhead irrigation does transfer polyhedra to the foliage, but only at narrowly defined zones (e.g. at the outer edge of the sprinkler arc) and in the field study these zones never coincided with the sampling stations; hence no significant increase in levels of polyhedra was detected after irrigation. An alternative (and perhaps more likely) explanation is that overhead irrigation washes cadavers and fragments of virus-infected tissue from the foliage into the soil. Here the polyhedra are far less susceptible to degradation, as opposed to being inactivated by ultraviolet radiation, or removed by mowing or grazing, if the virus remained on the leaves. Thus extensive irrigation, especially at the peak of an epizootic, would result in maximum amounts of virus being transferred to the soil. Hence in irrigated crops, epizootics may be more likely to occur as a result of larger residues of virus in the soil.

The findings of the study have in general been in agreement with the results of other investigations into the epizootiology of other insect viruses. Jaques (1964, 1967a, 1970, 1974a) found that Trichoplusia ni NPV can remain active in soil for long periods, is retained in the top layer of soil and is very resistant to leaching by rain. Jaques found that a high proportion of the virus applied to the soil was able to survive between seasons, and on one occasion 15% of the original activity remained 6 years after application. In contrast to this finding, Roome and Daoust (1976) investigating the survival of the NPV of H. armigera in Botswana, found that activity of the virus in soil in sorghum plots was reduced during winter. They stated that the likelihood of soil virus residues causing subsequent economic disease outbreaks was small. This loss in activity, which appears to be greater than occurs with H. punctigera

NPV, may have been a consequence of unfavourable (e.g. highly alkaline) soil type. Jaques (1967a, 1970) also demonstrated that application of virus to soil may be the most effective way of delivering an inoculum of virus to the host population. Virus was applied to soil and was transferred to cabbage leaves by rain or (on one occasion), by sprinkler irrigation. Thus these findings are in agreement with the findings of the current study, although the current study draws attention to the importance of the type of precipitation which might be most effective in splashing virus onto the crop. The study also confirms the findings of Tanada and Omi (1974b) and of Thompson and Steinhaus (1950) whose work suggested that lucerne foliage was contaminated by virus residues in the soil.

The rapid degradation of Heliothis NPV when exposed to sunlight (Fig. 4.6) confirms the findings of other authors (Bullock, 1967; Ignoffo and Batzer, 1971; Ignoffo et al., 1973, 1974; Young and Yearin, 1974). Roome and Daoust (1976) recorded a considerably longer half-life of H. armigera NPV on sorghum (activity was retained for as long as 30 days) - however this was accounted for by the structure of the sorghum head shielding virus contained within from the sun. Thus different half-lives of the virus obtained on different crops might be a reflection of the degree of protection afforded by different leaf surfaces although Andrews and Sikorowski (1973) concluded that the alkaline dew on cotton leaves contributed to the inactivation of Heliothis NPV deposits on the leaves.

The density-dependence of virus epizootics has been discussed by other authors (Steinhaus, 1954; Tanada, 1963; Doane, 1970; Surtees, 1971). Tanada (1963) pointed out that the relative spatial arrangement of the host may be of greater importance in the progression of epizootics than the actual number of individuals present. This appears to be the case with the gypsy moth, Porthetria dispar, where young larvae concentrate in the tops of trees and when an outbreak occurs, the inoculum load in this

zone is particularly high, hence ensuring rapid transmission of the disease (Doane, 1970). This may occur to a lesser extent in lucerne, where the majority of larvae can be observed feeding on flowers and buds at the top part of the plant, and where dead larvae are also found.

If epizootics are initiated as a result of larvae feeding on contaminated foliage, it follows (as mentioned) that provided the amount of infectious virus on the foliage is sufficiently high, epizootics could start at low as well as at high host densities. This statement is supported by Tanada and Omi (1974a) who reported that virus epizootics occurred in Spodoptera exigua and Autographa californica at low as well as high host densities. Similarly, Hall (1953) reported a high incidence of an NPV in very low host densities of A. californica. Tanada (1961) investigating the incidence of an NPV and a GV in the armyworm, Pseudaletia unipuncta, found that both viruses occurred at both low and high host densities.

Before attempts are made to use the NPV to suppress populations of H. punctigera, it is worthwhile to speculate on how the virus may have evolved to maximize both its chances of survival and also the availability of its host. The host occurs seasonally, with the population declining probably to zero from May to September each year. To accommodate this the virus is extremely well adapted (by virtue of the inclusion body) to survive over winter in the soil. Larvae of the first spring generation may then become infected by feeding on foliage contaminated with soil-derived polyhedra. A similar situation is proposed in the model of Hukuhara and Kitajima (1978).

It was assumed by early proponents of microbial control that the inclusion body would also protect the virus from degradation by the ultra-violet component of sunlight (Ignoffo and Batzer, 1971). However, it soon became apparent that this was not the case. There may, however, be

survival advantage in the short half-life of polyhedra exposed to sunlight: extremely small doses of the virus prove lethal to the host, especially to young larvae. The LC_{50} of 0.90 PIB/mm^2 for first instar H. punctigera (Teakle, 1976) indicates that probably only 1 or 2 polyhedra need be ingested by larvae to produce this level of mortality. Thus if this high virulence was combined with long persistence on the foliage, it is possible that few larvae would survive to reproduce, which would tend to limit the number of future hosts, and hence would limit future propagation of the virus. Thus the short half-life may well be a principle rate-determining step for the progress of epizootics. It is likely that the virus has evolved to ensure that 100% mortality seldom occurs (except perhaps at the end of the season - see page 97); even during a massive disease outbreak survival of some mature larvae is likely because of the degree of tolerance exhibited by these larvae (Teakle, 1973). Allen and Ignoffo (1969) reported similar tolerance to NPV by mature larvae of H. zea.

Thus it is not surprising that efforts to use baculoviruses as insecticides have often met with only moderate or variable success (Falcon, 1973). This situation has improved, however, by the incorporation of ultraviolet protectants in spray formulations (see page 13). For H. punctigera on lucerne, the concept of being able to trigger NPV epizootics, by using virus in the soil as a source of inoculum, is an attractive one. Provided an irrigation technique can be devised to transfer substantial quantities of virus onto the foliage, such a control strategy appears feasible. It would have the advantage of minimum cost, as the only inputs required would be pumping costs and irrigation water. However, alternate methods of contaminating the crop, e.g. physically blowing up soil dust on to plants, are worthy of consideration. In areas where the incidence of NPV is low, an initial spray application of virus or the release of

infected individuals would be required to build up NPV levels in the system. Frequent (possibly daily) treatments would renew levels of infective polyhedra on the crop and would offset the inactivation which would occur in sunlight. Further investigation is required to determine the most suitable larval stage to infect, and also to determine the most effective time to contaminate the foliage (probably during the middle of the day) so that maximum feeding rates can be utilized. Such a control strategy would have all the advantages of pest control by B. thuringiensis but without the cost disadvantage; thus the method might prove suitable for dryland as well as irrigated lucerne. Minimum use of insecticides would enable populations of O. schellenbergii and N. tasmanicus to assist in transmission of the virus, as well as fulfilling their role as predators of healthy larvae. The results of this study and other recent studies employing autodispersal (release of virus-infected larvae) using an NPV against H. zea on soybeans (Ignoffo et al., 1978) indicate that control methods utilizing the natural transmission of pathogens may soon occupy an important place in pest management.

APPENDIX 1

Stadial composition of *Heliothis punctigera* larvae from sweep net samples, Port Gawler, 1977/78

Sampling date	Transect	First instar	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar	Total
9-12-77	1	0	48	108	35	2	0	193
	3	40	145	108	36	3	1	333
	5	3	35	230	89	24	0	381
16-12-77	1	0	39	149	319	125	28	660
	3	0	7	86	257	135	19	504
	5	0	15	99	253	68	6	441
23-12-77	1	0	13	62	83	1	1	160
	3	0	5	48	43	16	2	114
	5	0	5	21	48	19	3	96
6-1-78	1	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
13-1-78	1	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
23-1-78	1	0	0	1	0	0	0	1
	3	0	0	1	1	0	0	2
	5	0	0	5	3	0	0	8
30-1-78	1	0	2	23	7	4	0	36
	3	0	71	58	20	2	1	152
	5	0	56	94	64	8	0	222
3-2-78	1	0	46	145	68	10	2	271
	3	0	29	257	218	26	6	536
	5	0	13	130	117	30	2	292
10-2-78	1	0	0	40	136	44	14	234
	3	0	12	69	98	7	5	191
	5	0	5	58	58	4	1	126
8-3-78	1	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
15-3-78	1	0	0	8	8	2	0	18
	3	0	0	3	1	0	0	4
	5	0	0	4	3	0	0	7
22-3-78	1	0	0	0	4	2	2	8
	3	0	0	1	1	2	1	5
	5	0	0	2	4	0	0	6
30-3-78	1	0	2	39	14	2	2	59
	3	1	13	14	3	0	1	32
	5	2	12	32	2	0	0	48

APPENDIX 1 cont'd.

Sampling Date	Transect	First instar	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar	Total
7-4-78	1	0	10	62	43	7	1	123
	3	0	19	53	27	6	0	105
	5	0	2	53	55	6	1	117
14-4-78	1	0	2	15	54	17	1	89
	3	0	7	23	32	8	1	71
	5	0	0	12	14	0	0	26
9-5-78	1	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0

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