Integration of Nosema locustae with chemical insecticides and entomopoxvirus for control of grasshoppers

by Gerald Louis Mussgnug

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ENTOMOLOGY
Montana State University
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Abstract:
The effects in grasshoppers of the protozoan, Nosema locustae, and formulations of N. locustae with the insecticides, malathion and carbaryl, or with an entomovirus, the grasshopper inclusion body virus (GIBV), were investigated during 1976, 1977, and 1978. A standard bioassay technique was devised for testing materials in the laboratory and mortality and incidence of infection were determined for three species of grasshoppers: Melanoplus sanguinipes, Melanoplus bivittatus, and Melanoplus differentialis. Plots 4 hectares in size were used to evaluate bait formulations against rangeland grasshoppers in Montana.

A continuous flow augering system for formulation and a modified seed spreader for application of bran bait were developed. N. locustae primarily infects the fat body of the grasshopper and results in reduced vigor and eventually death. The virulence of N locustae varied between strains, species, and subfamilies. A logarithmic relationship between the dosage of N. locustae used for initiating infection and the mortality and incidence of infection was demonstrated. Between 2.5 and 7.4 billion spores per hectare on 1.68 to 3.36 kilograms of bran were considered to be an efficient level of inoculum to apply for significant control of grasshoppers on rangeland. Applications when grasshoppers were in the third-instar of development resulted in better control than later applications. Once initiated, the infection spread through the population and caused reductions in density of 30 percent with 30 percent of the survivors showing infection within 6 weeks post-application. Infection by N_. Iocustae may increase the toxicity of malathion when fed orally to some species of grasshoppers in the laboratory but evidence of a stress phenomenon induced by the pathogen could not be demonstrated in field tests. Midseason spraying with ULV malathion to prevent oviposition might not disrupt an epizootic of N_. locustae initiated earlier in the season. Third-instar grasshoppers fed oral doses of malathion or carbaryl died within 24 hours while GIBV and N. locustae acted slower. Combinations of N. locustae with malathion, carbaryl, or GIBV were non-antagonistic and additive in action in laboratory assays. Formulations of malathion-N. locustae on bran rapidly reduced densities of grasshoppers and produced infection in the residual population. Reductions in density of 25-50 percent occurred within 3-5 days after malathion was applied as a 0.4 percent bran bait either alone or in combination with N_. locustae. Grasshopper populations treated with formulations of malathion-N. locustae displayed lower infection rates than when the pathogen was applied by itself. Slowing the spread of infection by simultaneously applying malathion with N. locustae was considered only temporary. The benefits of using an integrated approach for managing grasshopper populations on rangeland are discussed.

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FOR CONTROL OF GRASSHOPPERS

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A thesis submitted in partial fulfillment
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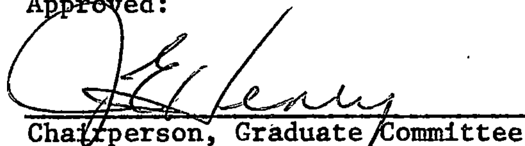
Approved:



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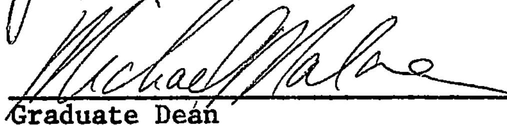

Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

May, 1980

ACKNOWLEDGEMENTS

I wish to thank Dr. J. E. Henry for his encouragement, advice, and dedication during all phases of this study and for his critical review of the manuscript. Also, I thank Dr. J. A. Onsager, Dr. N. L. Anderson, Dr. D. E. Mathre, Dr. E. L. Sharp, and Dr. C. J. Gilchrist for serving on the committee. Special thanks are due Dr. J. A. Onsager for his help in supporting and encouraging these studies, analyzing the data, and reviewing the manuscript. Sincere appreciation is extended to Dr. N. L. Anderson for his interest and for reviewing the manuscript. I wish to thank Phillip Mazuranich and Herbert Kussman for their help in the field testing of materials. I am grateful to Elaine Oma and Barbra Mullen for their assistance in the field and in the laboratory and to Vera Christie for her assistance in the preparation of this manuscript. Finally, I am especially grateful to the ranchers, Jack Galt, James Scholten, Dudley Tyler, and Newell Philbrick, for their hospitality and cooperation in allowing these experimental materials to be tested on their rangeland.

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ABSTRACT

The effects in grasshoppers of the protozoan, Nosema locustae, and formulations of N. locustae with the insecticides, malathion and carbaryl, or with an entomovirus, the grasshopper inclusion body virus (GIBV), were investigated during 1976, 1977, and 1978. A standard bioassay technique was devised for testing materials in the laboratory and mortality and incidence of infection were determined for three species of grasshoppers: Melanoplus sanguinipes, Melanoplus bivittatus, and Melanoplus differentialis. Plots 4 hectares in size were used to evaluate bait formulations against rangeland grasshoppers in Montana. A continuous flow augering system for formulation and a modified seed spreader for application of bran bait were developed. N. locustae primarily infects the fat body of the grasshopper and results in reduced vigor and eventually death. The virulence of N. locustae varied between strains, species, and subfamilies. A logarithmic relationship between the dosage of N. locustae used for initiating infection and the mortality and incidence of infection was demonstrated. Between 2.5 and 7.4 billion spores per hectare on 1.68 to 3.36 kilograms of bran were considered to be an efficient level of inoculum to apply for significant control of grasshoppers on rangeland. Applications when grasshoppers were in the third-instar of development resulted in better control than later applications. Once initiated, the infection spread through the population and caused reductions in density of 30 percent with 30 percent of the survivors showing infection within 6 weeks post-application. Infection by N. locustae may increase the toxicity of malathion when fed orally to some species of grasshoppers in the laboratory but evidence of a stress phenomenon induced by the pathogen could not be demonstrated in field tests. Mid-season spraying with ULV malathion to prevent oviposition might not disrupt an epizootic of N. locustae initiated earlier in the season. Third-instar grasshoppers fed oral doses of malathion or carbaryl died within 24 hours while GIBV and N. locustae acted slower. Combinations of N. locustae with malathion, carbaryl, or GIBV were non-antagonistic and additive in action in laboratory assays. Formulations of malathion-N. locustae on bran rapidly reduced densities of grasshoppers and produced infection in the residual population. Reductions in density of 25-50 percent occurred within 3-5 days after malathion was applied as a 0.4 percent bran bait either alone or in combination with N. locustae. Grasshopper populations treated with formulations of malathion-N. locustae displayed lower infection rates than when the pathogen was applied by itself. Slowing the spread of infection by simultaneously applying malathion with N. locustae was considered only temporary. The benefits of using an integrated approach for managing grasshopper populations on rangeland are discussed.

INTRODUCTION

Grasshopper populations have historically been the most important insect pest of rangeland in the western United States. Although over 100 species are found in these areas, only about 15 percent are considered to be of economic importance since they compete with cattle for available forage or they damage cultivated crops (Hewitt 1977). Parker (1937) listed the five most important economic species as *Melanoplus mexicanus* (Saussure) (*M. sanguinipes* = *M. mexicanus*), *M. bivittatus* (Say), *M. differentialis* (Thomas), *M. femur-rubrum* (DeGeer), and *Camnula pellucida* (Scudder). Anderson and Wright (1952) showed that the amount of damage to vegetation by grasshoppers cannot be predicted by abundance only but that damage is related to the grasshopper species composition and the vegetation present. Mulkern et al. (1969) demonstrated that grasshoppers have specific food plant preferences and utilize range resources selectively.

In 1877 congress appropriated funds for the creation of the United States Entomological Commission to investigate the Rocky Mountain grasshopper, *Melanoplus spretus* (Walsh.) (Blickenstaff et al. 1974). Since that time control measures have changed considerably. Arsenical baits were used in 1936 but were replaced by the chlorinated hydrocarbons, toxaphene and chlordane, in the late forties. These were replaced later by aldrin and dieldrin in the fifties and presently malathion and carbaryl are recommended. Malathion, the chemical used most frequently, is applied in ultra low volume (ULV) formulation of

8 oz. AI/acre (585 ml/hectare) in large-scale federally sponsored programs of grasshopper control on rangelands. An assessment of the effectiveness of such programs for long-term control of grasshoppers, however, failed to demonstrate protection beyond the year of application (Blickenstaff et al. 1974).

It is generally accepted that insect population densities fluctuate both in time and space. Any attempts to evaluate applied control must therefore also include an understanding of both the organism and the environment. As proposed by Clark et al. (1967), life systems consist of a subject population and its effective environment which interact to determine its abundance and persistence. Solomon (1949) earlier emphasized the inseparable existence of the population and its environment and showed that population numbers fluctuate within limits in such a system. The life system should then serve as the basis for considering any changes in populations of grasshoppers.

L. O. Howard (1897) was the first to recognize disease as a natural regulating factor of insect densities. Steinhaus (1954) summarized the effect of disease on insect populations and pointed out that disease may be characterized according to a number of general ecological principles. In that report, as well as in a later report (Steinhaus 1957), he pointed out the potential of utilizing pathogens for practical microbial control of insect pests.

Attempts to control grasshoppers with fungi and bacteria have been reviewed by Charles (1965). Although these pathogens are capable of initiating infections, they require specific environmental conditions and have little effect on the densities of the insect populations. Protozoan and viral pathogens generally do not require specific conditions, are more host-specific, and exhibit longer controlling activity (Henry 1970). Henry (1970) has considered these pathogens, particularly Nosema locustae Canning and the grasshopper inclusion body virus (GIBV), to have the greatest potential for use in grasshopper control since they not only produce some immediate control activity but also become established and continue as a regulatory factor for prolonged periods of time.

N. locustae was first described by Canning (1953) from the African migratory locust, Locusta migratoria migratorioides (Reiche and Fairmaire). Henry (1969) reported the host range to include at least 58 species of American grasshoppers. N. locustae infects the fat bodies thus depriving the host of energy required for growth and reproduction. Canning (1962b) reported that infection by this pathogen delays the final molt to adult and decreases the flying ability of L. migratoria. Laboratory and field studies have indicated that the microsporidan is potentially useful in controlling densities of grasshoppers (Henry 1971; Henry et al. 1973; Henry and Oma 1974b). Experimental field tests have demonstrated that applications of spores

of N. locustae on bran can result in overall reductions in density of 50 percent within four weeks of application and produce infection in those surviving (Henry 1971; Henry et al. 1973).

GIBV was isolated from M. sanguinipes (Henry and Jutila 1966) and later was characterized as an entomopoxvirus (Henry et al. 1969). It replicates in the fat tissues in which inclusion bodies are formed that contain the virions and high rates of mortality result from infection. As with N. locustae, epizootics occur among grasshoppers and cause noticeable reductions in density (Henry 1970).

In recent years the concept of integrated pest management has become prominent as a means of maintaining insect densities below economic levels (Stern et al. 1959; Smith and Van den Bosch 1967). In its broadest sense, integrated control includes maximizing the effects of existing natural controlling factors, monitoring pests and natural enemies, and using pest suppression measures when needed (Train et al. 1972). Included in integrated control is the concept of combined use of chemicals and biological agents which according to Benz (1971) can produce interactions ranging from antagonism to synergism. Creighton and McFadden (1974) demonstrated the integrated control of two lepidopterous larvae, Trichoplusa ni (Hübner), and Pieris rapae (L.), with mixtures of the bacterium (B.t.), Bacillus thuringiensis, and insecticide. Also Jaques (1973) and Jaques and Laing (1978) have demonstrated the increased effectiveness of mixing B.t., nuclear

polyhedrosis virus (NPV), or granulosis virus (GV) with chemical insecticide for pest management of these insects. A series of reports (Morris 1972, 1975a b, 1977 a b c; and Morris et al. 1974, 1975) discussed the combined use of B.t. and various chemical insecticides against the spruce budworm, Choristoneura fumeriferana (Clem.). In addition, Hunter et al. (1975) and Luttrell et al. (1979) have shown the feasibility of combining entomoviruses with insecticides for better control of the Indian meal moth, Plodia interpunctella (Hübner), and the corn earworm, Heliothis zea (Boddie) respectively. McVay et al. (1977) suggested an additive action of simultaneous infections of B.t. and NPV in T. ni, whereas Tanada (1959) postulated a synergistic action in the use of NPV and GV against the army worm, Pseudaletia unipuncta (Haworth).

Based on his study of grasshopper pathogens, Henry (1970) suggested that an integrated approach using pathogens and insecticides might be effective for managing the densities of grasshoppers. This, together with the repeated success reported for the integrated control of lepidopterous insects during the past ten years, and the lack of similar information on which to base an integrated pest management scheme for rangeland grasshoppers, formed the basis for the present studies. The purpose of the studies was two-fold: (1) to extend the basis for using N. locustae for grasshopper control and, (2) to investigate the integrated uses of other control agents such as chemical

or virus with *N. locustae*.

METHODS AND MATERIALS

Laboratory Assays

All spores of *Nosema locustae* used as inoculum in the assays were produced in *Melanoplus bivittatus*. Eggs of *M. bivittatus* were collected in the fall or early spring from various locations in Montana and North Dakota and were brought to the laboratory for hatching. Fifth-instar nymphs were inoculated by feeding them for 3 days on romaine lettuce which had been sprayed with spores of *N. locustae* at a rate of approximately 10^7 spores/leaf. Spores were harvested 1-2 months after inoculation by homogenizing suspensions of cadavers in distilled water with a tissue grinder and then passing the homogenates through nylon organdy to remove large debris. Spores were then cleaned and concentrated by lowspeed differential centrifugation (5,000 g). Spore concentrations were quantified by direct counts with a hemocytometer and samples were stored at -10° C. The inoculum of the GIBV was produced in a non-diapause laboratory strain of *Melanoplus sanguinipes* obtained from Canada (Pickford & Randell 1969) and inclusions were recovered in a manner similar to that for *N. locustae*.

The insecticides, carbaryl (Sevin®) and malathion were diluted from technical grade stock solutions. The first material, being water based, was diluted in water and the second, being oil based, was diluted in acetone.

Three species of grasshoppers, *M. sanguinipes*, *M. bivittatus*, and *Melanoplus differentialis* were used in the tests. The non-diapause

strain of M. sanguinipes from Canada was used most extensively for the assays although field collected diapausing M. sanguinipes from Montana also were included for comparison. All M. bivittatus and M. differentialis were collected from the field in Montana and North Dakota and were of the diapause type. Grasshoppers were reared at approximately 30° C under a 12-hour light-dark regimen and were fed daily with head lettuce, wheat bran, fresh seedlings of Balbo rye, and an agar-base diet that contained a sulphonamide, Thipyrimeth®, as a prophylactic against possible amoebic infections (Henry & Oma 1975).

A standard bioassay procedure similar to that described by Henry and Oma (1974 a) was chosen for the tests (Fig. 1). All inocula were dispensed with a microinjection syringe as 5-µl drops onto 7 mm lettuce discs. The insecticides were applied to lettuce discs as 1-µl drops and combinations of pathogen and insecticide were mixed and applied in 6-µl drops. The grasshoppers were inoculated *per os* as third-instar nymphs after having been starved for 24 hours. In the tests involving the effect of N. locustae on insecticide toxicity, insecticides were administered to fifth-instar and adult grasshoppers in similar fashion. Those that failed to consume the entire disc were discarded. After inoculation, the grasshoppers were distributed in groups of 5 per rearing tube (17 cm long by 5 cm dia. constructed of sheet acetate with screened ends) (Fig. 2) and were reared as described.

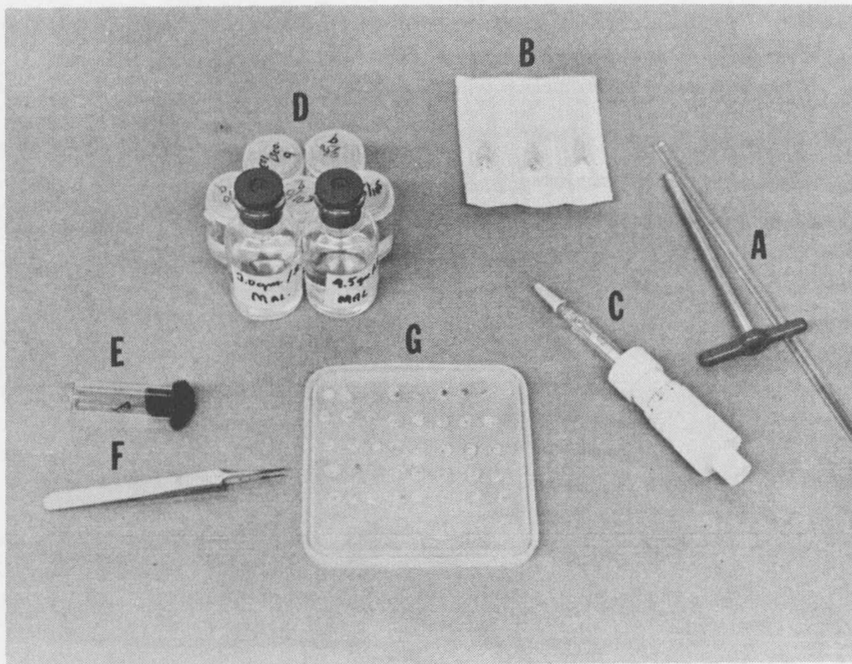

Fig. 1.--Materials utilized in inoculation of grasshoppers for standard laboratory assay. (A) cork borer for cutting lettuce discs, (B) needles, (C) syringe, (D) insecticide or pathogen, (E) third-instar grasshopper, (F) forcep, (G) treated lettuce discs.

Fig. 2.--Acetate tubes (17 cm long X 5 cm dia) used in rearing grasshoppers for standard laboratory assay.

Mortality rates were based on the number of grasshoppers that died during the post-inoculation period. Incidence of infection by the pathogen was based on the number of grasshoppers within a treatment group that exhibited either spores of N. locustae or, where applicable, inclusion bodies of GIBV. Infections were diagnosed by the presence of spores in homogenates of insects surviving 24 days post-inoculation. Samples were examined with phase microscopy at about 500 diameters magnification.

Four replicates of each treatment were included in the tests with twenty grasshoppers per replicate. Differences between treatments were assessed by an analysis of variance of arcsine square root transformations of the percentages of mortality and infeciton. Significant interaction between N. locustae and either the insecticide or the virus was interpreted as synergism or antagonism while lack of significance indicated independence or additive action. Probit analysis was used to calculate LD_{50} values.

Field Tests

Spores of Nosema locustae for dissemenation in the field were produced in the laboratory by mass inoculation of fourth and fifth-instar nymphs of Melanoplus bivittatus in cages (30 cm in dia. X 35 cm high) (Fig. 3). Grasshoppers were inoculated *en masse* by feeding them for 3 days on romaine lettuce that had been sprayed with 10^7 spores/leaf. Infected adults were collected 1-2 months after inoculation, suspended

Fig. 3.--Cage (30 cm in dia X 35 cm high) used in the mass inoculation and rearing of grasshoppers for the production of spores of *Nosema locustae* for field tests.

in distilled water, and then homogenized with a hand operated wheat mill. The homogenate was then filtered through nylon organdy to remove the large debris and spores were concentrated by low speed differential centrifugation (5,000 g). After spore concentrations were determined by direct counts with a hemocytometer, the inoculum was stored at -10° C. Malathion that was applied to bran was diluted in acetone, whereas that which was applied as ULV sprays in the field was diluted in diesel oil. In all tests the volume of spray was held constant (8.8 ml/kg and 585 ml/ha).

Formulation of bran treated with either N. locustae, malathion or combinations thereof was accomplished by spraying the bran as it moved through a continuous flow formulating system (Fig. 4). Spore suspensions were diluted so that the ratio of water to wheat bran remained constant (8.8 ml/kg bran). Hydroxymethyl cellulose (0.2% w/v) was added as a sticker in the water-spray formulations. Treated bran was generally stored for periods up to 2 weeks in burlap sacks.

The study areas were on rangeland at various locations where grass was the predominant vegetation (Fig. 5). Treated bran was applied in the field with a modified Cyclone® seed spreader (Fig. 6) that was mounted in the back of a 4-wheel drive vehicle. Applications were begun on June 28 in 1976 (when grasshoppers were mainly third-instar), on July 7 in 1977 (when grasshoppers were mainly third-instar) and on July 8 in 1978 (when grasshoppers were mainly fifth-instar). Each

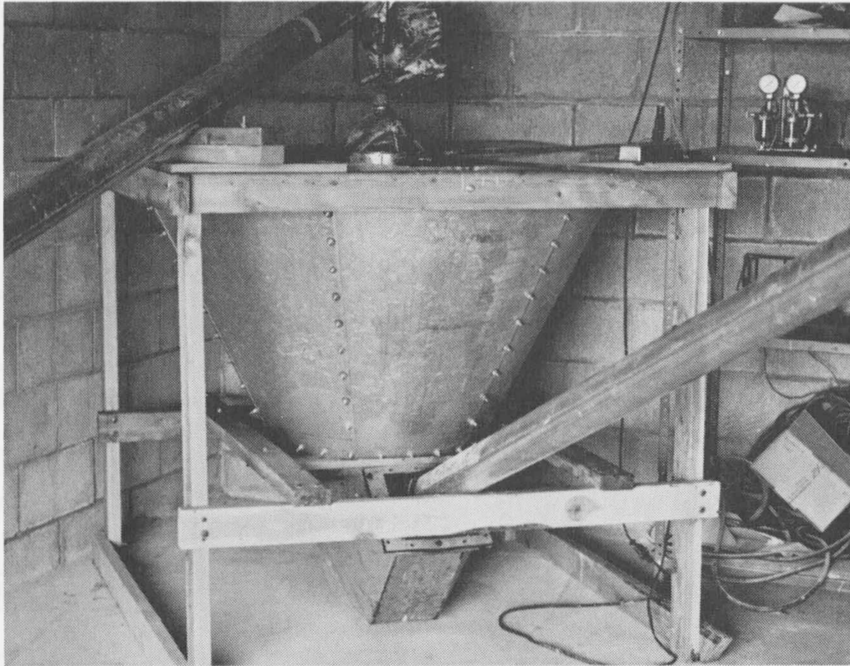

Fig. 4.--The treatment cone in which bran was sprayed with malathion, *Nosema locustae* or *N. locustae*-malathion mixtures.

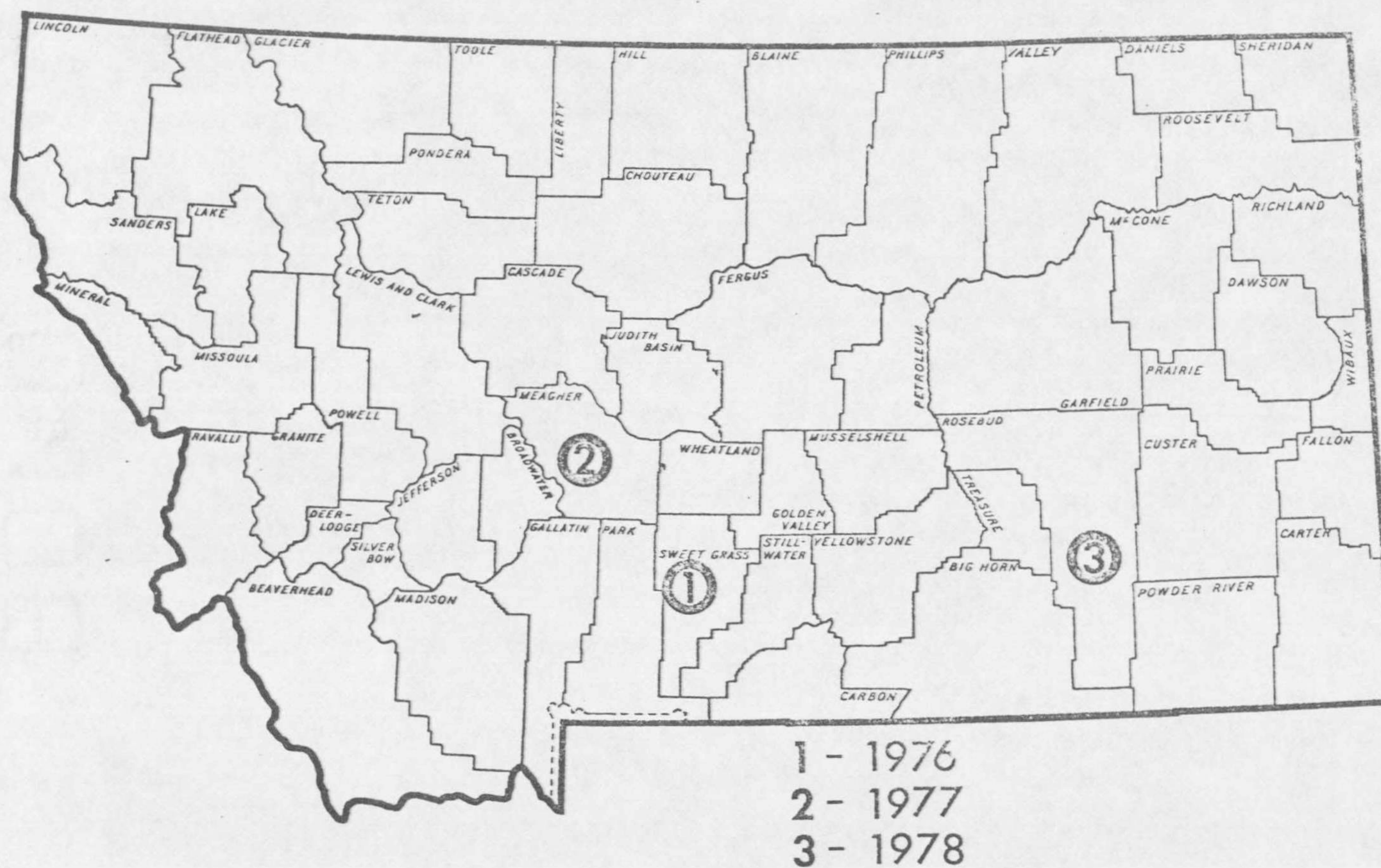

Fig. 5.--Location of field tests in Montana where formulated bran was applied to plots 4 hectares in size. (1) Big Timber, (2) White Sulphur Springs, (3) Forsyth.

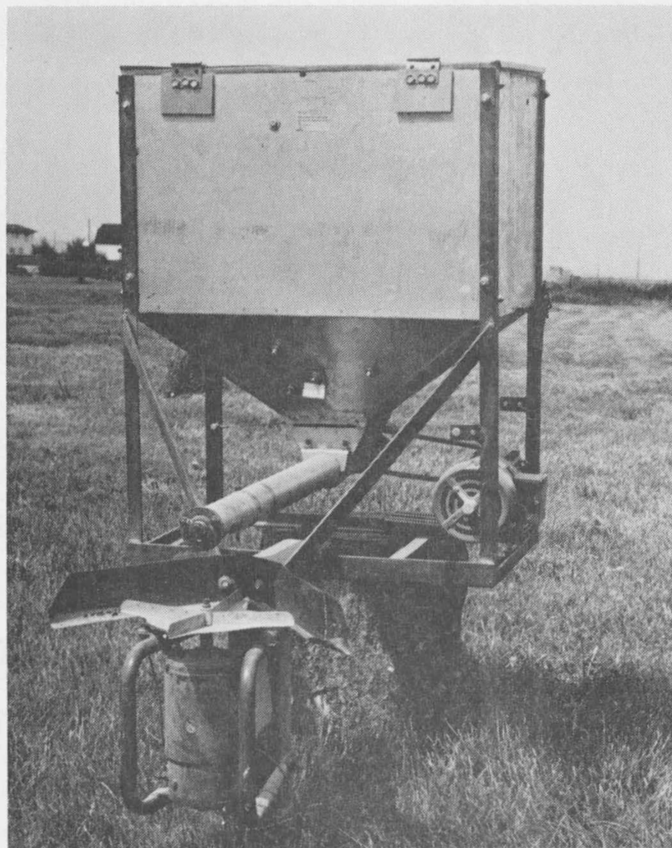

Fig. 6.--Bait spreader used in application of formulated bran for field tests.

plot was 4 hectares square and the bran was applied in 9.1 m wide swaths. In field tests in 1976 and 1978 bran was applied at the rate of 1.68 kg/ha, but in 1977 the rate was 3.36 kg/ha.

Sampling consisted of density determinations and collections of grasshoppers for species composition and incidence of infection. In some tests pre-treatment samples were taken while in other tests only post-treatment samples were made. The density of grasshoppers was determined by using 40 aluminum rings (each with an area of 0.1 m^2) distributed along a diagonal transect through the middle of the plot. Counts were made of grasshoppers within the rings the following day by approaching each ring and flushing the insects with a pointer (a 2-m long wand) (Onsager & Henry 1977). Sweep net samples of grasshoppers were obtained near the center of each plot after density determinations. The collected grasshoppers were placed in paper sacks and were frozen immediately with dry ice. Subsequently, in the laboratory the species, sex, and developmental stage of each grasshopper were recorded after which each was suspended in 5 ml of distilled water and homogenized with a tissue grinder. A sample was removed using a capillary tube and examined at 500 diameters as a hanging drop for spores of _N. locustae_. All treatments were randomized within each of 4 replications in a randomized block design. Differences between treatments were assessed by an analysis of variance of the density and of arcsine square root transformations of the incidence of infection.

RESULTS

Laboratory Assays

Nosema locustae and GIBV infection in Melanoplus sanguinipes

Grasshoppers infected with Nosema locustae exhibited reduced feeding and locomotor activity while moribund grasshoppers were almost totally incapacitated and often exhibited tetanic convulsions. Spores were evident at 16 days after *per os* inoculation of third-instar nymphs. In heavily infected grasshoppers at 24 days post-inoculation the spores of N. locustae had virtually replaced all fat tissue. The homogenate of such individuals revealed numerous spores when samples were examined under phase contrast microscopy (Fig. 7). Grasshoppers infected with GIBV exhibited a slower developmental rate and less vigor as compared to grasshoppers infected with N. locustae. Inclusion bodies were evident at 14 days after *per os* inoculation of third-instar nymphs. Fig. 8 shows the appearance of inclusion bodies of GIBV from Melanoplus sanguinipes under phase contrast microscopy. The virus was observed to infect primarily the cells of the fat body from which it spread throughout the insect. Multiple infections were evident in M. sanguinipes following dual inoculation with N. locustae and GIBV. Both pathogens were evident within 21 days after inoculation (Fig. 9).

Effect of Nosema locustae in two strains of Melanoplus sanguinipes

As shown in Table 1 those grasshoppers inoculated with spores of Nosema locustae at doses of from 10^3 and 10^6 spores per third-instar

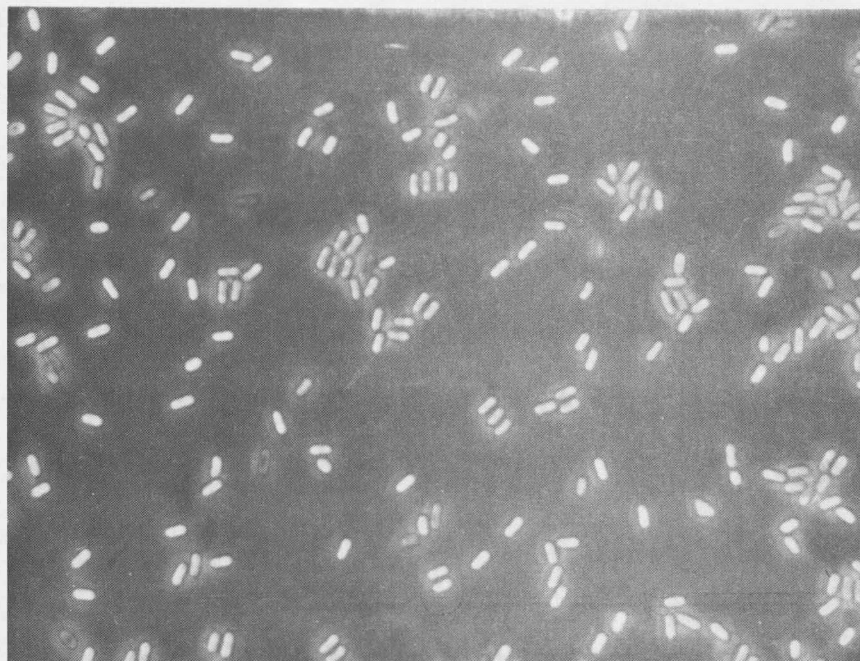

Fig. 7. Fresh preparation under phase contrast microscopy of the spores of *Nosema locustae* in *Melanoplus sanguinipes*. 800 X.

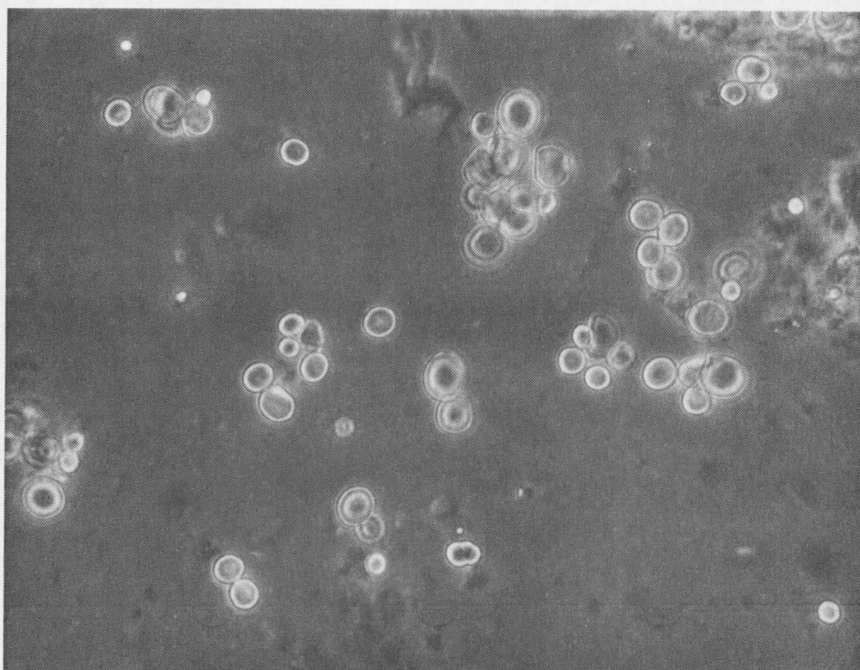

Fig. 8.--Fresh preparation under phase contrast microscopy of the inclusions of the grasshopper inclusion body virus in *Melanoplus sanguinipes*. 800 X.

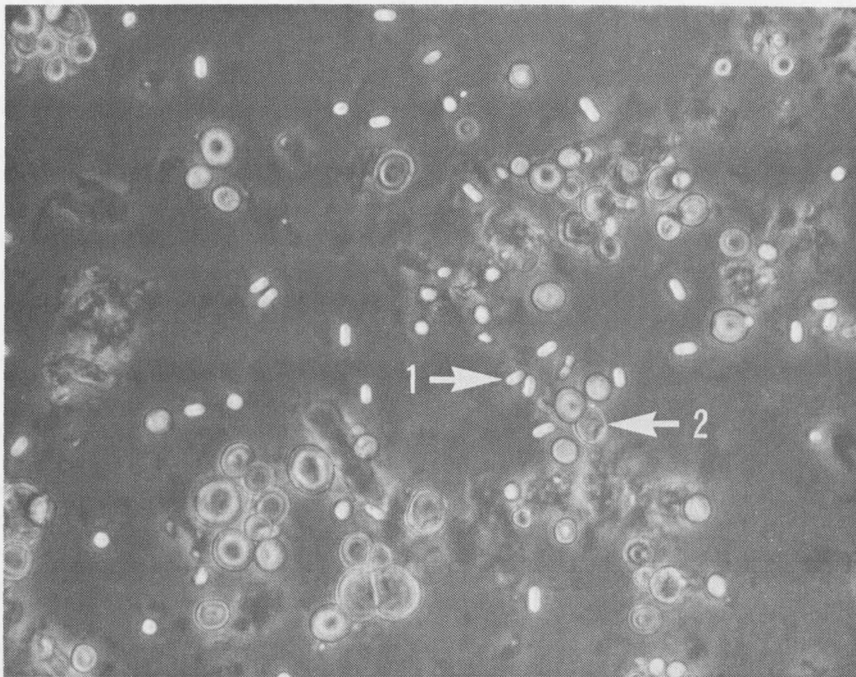

Fig. 9.--Fresh preparation under phase contrast microscopy of the spores of *Nosema locustae* (1) and the inclusions of the grasshopper inclusion body virus (2) from a multiply infected *Melanoplus sanguinipes*. 800 X.

Table 1. Percent mortality and infection at 24 days post-inoculation of third-instar diapause and non-diapause *Melanoplus sanguinipes* treated with spores of *Nosema locustae* in the laboratory.

Treatment *N. locustae* (No. spores/ grasshopper	% Mortality		% Infected Survivors	
	Non-diapause	Diapause	Non-diapause	Diapause
0	15.0 a	25.0 a	0.0 a	0.0 a
10^3	21.3 a	21.3 a	15.9 b	58.7 b
10^4	21.3 a	28.8 a	27.0 b	66.7 b
10^5	65.0 b	41.3 b	39.3 c	89.4 c
10^6	91.3 c	68.8 c	57.1 d	100.0 d

Percentages followed by the same letter indicate no significant difference between treatments at the 5% level of error.

exhibited higher rates of mortality than did the controls. Analysis of variance revealed that mortality did not differ significantly between the 10^3 and 10^4 doses, but these doses and the 10^5 and 10^6 doses were different from one another ($p \leq .05$) and varied directly with dosage. The non-diapause strain tended to be more susceptible than the field-collected strain (Fig. 10). Table 2 shows that the difference in virulence of the pathogen was significant since the 95% confidence limits of the LD_{50} values do not overlap. The LD_{50} for third-instar nymphs of the non-diapause strain was 0.9×10^5 whereas with the diapause strain the LD_{50} was 5.3×10^5 spores. However, the percentage infection among surviving grasshoppers of the non-diapause strain was significantly lower than for the diapause strain ($p \leq .05$). Regressions calculated from the data in Table 1 show the differences in infectivity of _N. locustae_ between the two strains (Fig. 11). The 10^3 and 10^4 doses were not found to produce statistically different infection rates from one another although they differed significantly from the other doses and the controls. Analysis of the dosage-infectivity data showed a linear relationship between the $\log_{10}$ of the initial pathogen dose and the subsequent percent infection.

Effect of _Nosema locustae_ on insecticide toxicity

Tables 3 and 4 show the effect of 15 days of infection by _Nosema locustae_ on the toxicity of malathion among fifth-instar non-diapause

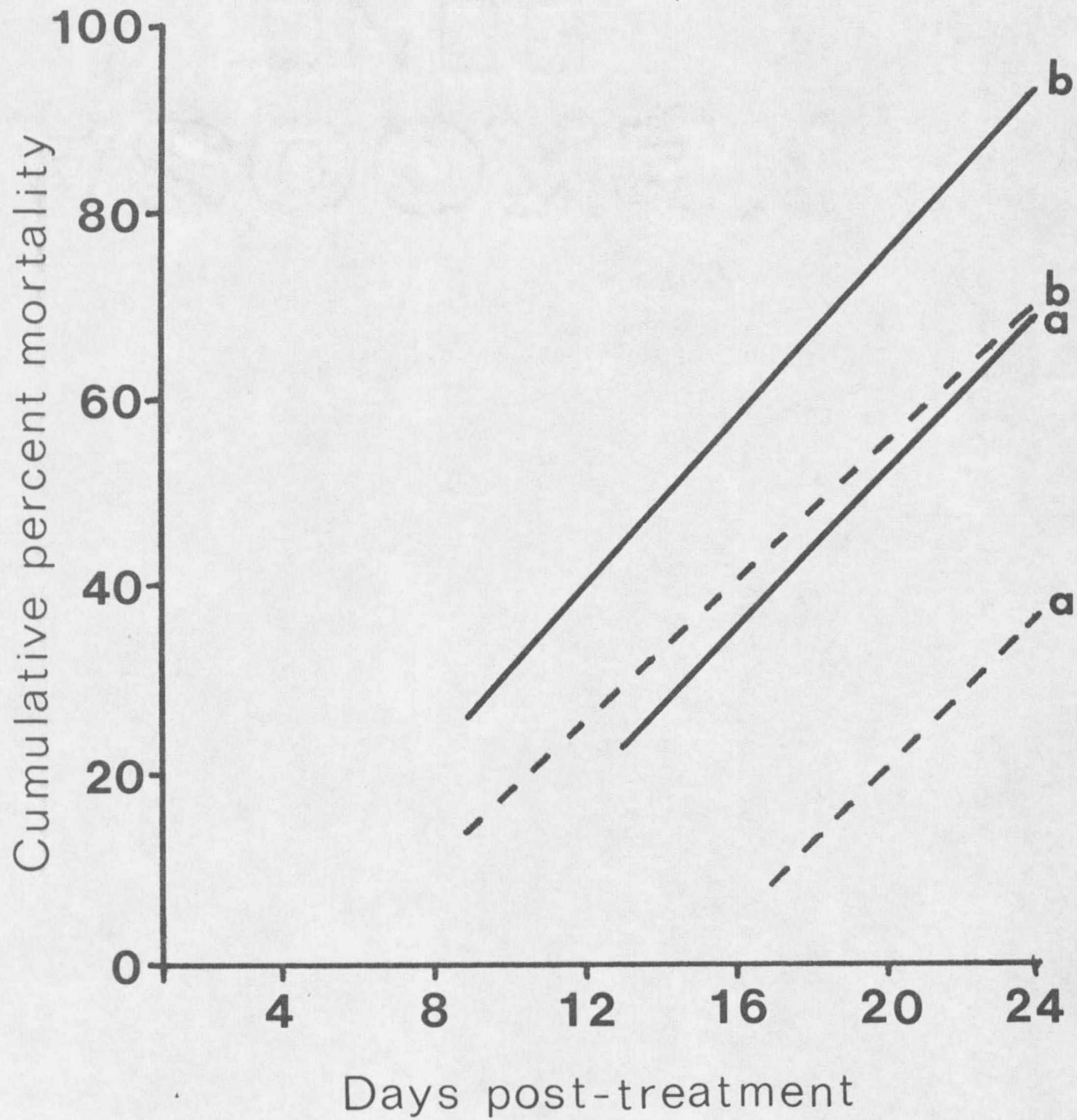

Fig. 10.--Regression of mortality vs time among a non-diapause (solid line) and a diapause (broken line) strain of Melanoplus sanguinipes after treatment with Nosema locustae at rates of 10^5 (a) and 10^6 (b) spores per third-instar grasshopper in the laboratory.

Table 2. Relative virulence of *Nosema locustae* to third-instar diapause and non-diapause *Melanoplus sanguinipes* at 24 days post-inoculation in the laboratory.

Treatment (Strain)	LD_{50} (No. of spores/grasshopper)	95% Confidence Limits
Non-diapause	0.9×10^5	$0.6 - 1.3 \times 10^5$
Diapause	5.3×10^5	$2.8 - 9.7 \times 10^5$