



Protozoan and viral pathogens of grasshoppers
by John Edward Henry

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Entomology
Montana State University
© Copyright by John Edward Henry (1969)

Abstract:

The development and pathologies of some protozoan and viral pathogens of grasshoppers are described and discussed in terms of the experimental and natural host-pathogen relationships. Included among the protozoans are: (1) *Malameba locustae*, an amoebic organism which infects tissues of the digestive tract and causes physiological changes which are reflected in the host's reproduction, (2) undetermined species of Eugregarinida which are common in grasshoppers but are considered relatively avirulent, (3) an undescribed species of Neogregarinida which from the observed stages of schizogony and sporogony probably belongs to the genus *Caulleryella*, (4) *Nosema locustae*, a species of Microsporidia that infects primarily the fat bodies of grasshoppers, (5) *Nosema acridophagus* which infects a number of host tissues in which it induces cellular aggregations described as tumors, (6) an undescribed species of *Nosema* which infects various host tissues including those of the nervous system, and (7) an undetermined number of undescribed organisms which, based on the relative size of the spore, are referred to as small spore organisms, but which exhibit developmental stages that are typical of species of Microsporidia. Included among the viruses are (1) the grasshopper inclusion body virus (GIBV) which is a DNA-containing virus that infects the fat bodies of the host and exhibits replicating forms resembling those reported during the development of pox viruses, and (2) the crystalline-array virus (CAV) which is a small RNA-containing virus that infects the pericardium, muscles and tracheal matrix of the host and is the most virulent of the reported grasshopper pathogens.

PROTOZOAN AND VIRAL PATHOGENS OF GRASSHOPPERS

by

JOHN EDWARD HENRY

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree

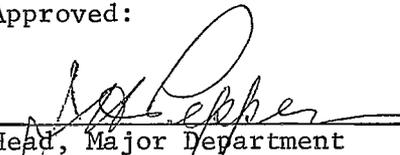
of

DOCTOR OF PHILOSOPHY

in

Entomology

Approved:


Head, Major Department


Chairman, Examining Committee


Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1969

ACKNOWLEDGMENT

I wish to thank Dr. N. L. Anderson for his help and advice while serving as chairman of the graduate committee, and Dr. J. H. Pepper, Dr. P. D. Skaar, Dr. R. H. McBee, and Dr. J. W. Jutila for serving on the committee. Also, I thank Dr. Jutila for allowing the inclusion of results from cooperative studies in this thesis. I am grateful to F. T. Cowan, Investigations Leader (retired July 1, 1967), Grasshopper Investigations, for his supporting and encouraging these studies. I thank Dr. C. C. Blickenstaff, Investigations Leader, Grasshopper Investigations, for supporting these studies. I am grateful to Mrs. Barbara McCleave, Mrs. Elaine Oma, and Mrs. Barbara Nelson for their assistance during these studies, and to Mrs. Ruth Breeden for her assistance in the preparation of this manuscript. Also, I am grateful to Dr. Dave Worley for reviewing the manuscript.

TABLE OF CONTENTS

	Page
VITA.....	ii
ACKNOWLEDGMENT.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	xiv
INTRODUCTION.....	1
MATERIALS AND METHODS.....	3
Field Collection Techniques.....	3
Rearing Techniques.....	3
Preparation of Inoculants.....	4
Experimental Inoculation.....	5
Light Microscopic Techniques.....	5
Preparation of Smears.....	6
Preparation of Sectioned Tissues for Light Microscopy.....	7
Photomicroscopy.....	8
Preparation of Tissues for Electron Microscopy.....	9
RESULTS.....	10
<u>Malameba locustae</u> (King and Taylor) (Amoebida).....	10
Host and Geographical Range of <u>M. locustae</u>	10
Description of Stages in the Life Cycle of <u>M. locustae</u>	11
Histopathology Resulting from Infections by <u>M. locustae</u> ...	16
Transmission of <u>M. locustae</u>	19
Control of <u>M. locustae</u> in Grasshoppers in Laboratory Cultures.....	20
Eugregarinida.....	22
Differentiation of Eugregarinida from Grasshoppers.....	23
Host and Geographical Ranges of Eugregarinida in Grasshoppers.....	25
Generalized Life Cycle of Eugregarinida in Grasshoppers...	26
Stages in the Life Cycle of Eugregarinida in Grasshoppers.....	28
Histopathology of Infections by Eugregarinida in Grasshoppers.....	41
Source of Infections by Eugregarinida in Grasshoppers.....	42

	Page
Neogregarinida.....	43
Host and Geographical Range of Neogregarinida in Grasshoppers.....	43
Stages in the Life Cycle of the Neogregarinida in Grasshoppers.....	44
Proposed Life Cycle of the Neogregarinida from Grasshoppers.....	50
Histopathology of Infections by the Neogregarinida from Grasshoppers.....	52
Microsporida.....	53
Life Cycle of <u>Nosema</u> spp. in Grasshoppers.....	54
<u>Nosema locustae</u> Canning.....	56
Host and Geographical Range of <u>N. locustae</u>	56
Stages in the Life Cycle of <u>N. locustae</u>	60
Histopathology of Infections by <u>N. locustae</u>	63
Effect of Infections by <u>N. locustae</u> on Grasshoppers.....	64
Transmission of <u>N. locustae</u>	71
<u>Nosema acridophagus</u> Henry.....	71
Host and Geographical Range of <u>N. acridophagus</u>	71
Stages in the Life Cycle of <u>N. acridophagus</u>	72
Histopathology of Infections by <u>N. acridophagus</u>	75
Gross Effects of Infections by <u>N. acridophagus</u>	82
Transmission of <u>N. acridophagus</u>	85
A <u>Nosema</u> sp.....	85
Host and Geographical Range of the <u>Nosema</u> sp.....	85
Stages in the Life Cycle of the <u>Nosema</u> sp.....	85
Histopathology of Infections by the <u>Nosema</u> sp.....	87
Gross Effects of Infections by the <u>Nosema</u> sp.....	90
Transmission of the <u>Nosema</u> sp.....	92
Comparative Susceptibility of <u>Melanoplus bivittatus</u> to <u>Nosema locustae</u> , <u>Nosema acridophagus</u> , and the <u>Nosema</u> sp... ..	92
Small Spore Diseases of Grasshoppers.....	96
The Grasshopper Inclusion Body Virus (GIBV).....	100
Host and Geographical Range of the GIBV.....	100
Morphological and Chemical Characterization of the GIBV... ..	100
Histopathology of Infections by the GIBV.....	107
Replication of the GIBV.....	108
Gross Effects of Infection by the GIBV.....	114
Observations of the Natural Occurrence of the GIBV.....	115
Effect of Host Nutrition on the Development of the GIBV... ..	116
Relationship Between the GIBV and Other Insect Viruses... ..	119
The Crystalline-array Virus.....	120
Host and Geographical Range of the CAV.....	121
Morphological and Chemical Characterization of the CAV... ..	121
Replication of the CAV.....	127
Gross Effects of Infections by the CAV on Grasshoppers... ..	130
Similarities Between the CAV and Other Viruses.....	132

	Page
DISCUSSION AND CONCLUSIONS.....	133
SUMMARY.....	144
FOOTNOTES.....	147
LITERATURE CITED.....	148

LIST OF TABLES

Table		Page
I	Average dimensions of cysts of <u>Malameba locustae</u> from <u>Melanoplus bivittatus</u> , <u>Melanoplus differentialis</u> , and <u>Melanoplus sanguinipes</u>	15
II	Species of Orthoptera that are susceptible to infections by <u>Nosema locustae</u> and the sources of the observed infections.....	58
III	Susceptibility of <u>Melanoplus bivittatus</u> to infections by <u>Nosema locustae</u>	65
IV	Effect of infections by <u>Nosema locustae</u> on the rate of development of <u>Melanoplus sanguinipes</u> as determined by the frequency of adults in the test groups.....	69
V	Effect of infections by <u>Nosema locustae</u> Canning on the fecundity of <u>Melanoplus differentialis</u>	70
VI	Susceptibility of <u>Melanoplus bivittatus</u> to infections by <u>Nosema acridophagus</u>	84
VII	Susceptibility of <u>Melanoplus bivittatus</u> to infections by the <u>Nosema</u> sp.....	91
VIII	Effect of different host diets on infection by the grasshopper inclusion body virus in <u>Melanoplus sanguinipes</u>	118
IX	Cumulative percent mortality among third instar nymphs of <u>Melanoplus bivittatus</u> and <u>M. sanguinipes</u> after <u>per os</u> inoculation with CAV.....	131

LIST OF FIGURES

	Page
Fig. 1. Fresh preparation under phase contrast microscopy of cysts and trophozoites of <u>Malameba locustae</u>	14
Fig. 2. Cross section of Malpighian tubules of <u>Melanoplus sanguinipes</u> with trophozoites of <u>Malameba locustae</u> on the inner surface of the walls and cysts in the lumen of the tubules.....	14
Fig. 3. Cross section of the posterior region of the midgut of a <u>Melanoplus sanguinipes</u> showing the deterioration of part of the epithelium due to infection by <u>Malameba locustae</u>	14
Fig. 4. Fresh preparation under phase contrast microscopy of a Malpighian tubule of a <u>Melanoplus sanguinipes</u> heavily compacted with cysts of <u>Malameba locustae</u>	14
Fig. 5. Fixed and stained preparation from a <u>Melanoplus sanguinipes</u> showing the cystocytic encapsulation of a Malpighian tubule containing cysts of <u>Malameba locustae</u>	14
Fig. 6. Distributions of measurements of cysts of Eugregarinida from <u>Melanoplus bruneri</u> , <u>Melanoplus oregonensis</u> , <u>Bruneria brunnea</u> , <u>Camnula pellucida</u> , <u>Chorthippus curtippennis</u> , and <u>Neopodismopsis abdominalis</u> and <u>Melanoplus bruneri</u> alone which were collected in the Antelope Basin region of southwestern Montana.....	24
Fig. 7. Diagrammatic representation of the life cycle of Eugregarinida as determined from stages observed during studies of infections in grasshoppers.....	27
Fig. 8. Mature spores extruded from a cyst of Eugregarinida from a grasshopper.....	30
Fig. 9. Sporoblasts from a cyst of Eugregarinida from a grasshopper.....	30
Fig. 10. Fresh preparation under phase contrast microscopy of a sporozoite which was extruded from a spore of Eugregarinida from a grasshopper.....	30
Fig. 11. Young cephalont of a Eugregarinida with the epimerite segment embedded in the cytoplasm of an epithelial cell of a gastric caecum of <u>Melanoplus sanguinipes</u>	30

	Page
Fig. 12. Fresh preparation under phase contrast microscopy showing a group of cephalonts of <i>Eugregarinida</i> in tissues of a gastric caecum of a grasshopper.....	30
Fig. 13. Various sized sporonts around the satellite of an association of <i>Eugregarinida</i> from the lumen of the midgut of a grasshopper.....	34
Fig. 14. An association consisting of an anterior sporont (primita) and a posterior sporont (satellite).....	34
Fig. 15. Fresh preparation under phase contrast microscopy of the nucleus of a sporont of <i>Eugregarinida</i> showing the beaded arrangement of the karyosomes around a nucleolus.....	34
Fig. 16. Sporonts and newly formed gametocysts of <i>Eugregarinida</i> from a <u><i>Melanoplus sanguinipes</i></u>	34
Fig. 17. Newly formed sporoblasts in a sectioned preparation of a cyst of <i>Eugregarinida</i> from grasshoppers.....	39
Fig. 18. Cross section of a gastric caecum from a <u><i>Melanoplus bruneri</i></u> showing cephalonts of <i>Eugregarinida</i> attached to the epithelium.....	39
Fig. 19. Cross section of the anterior part of the midgut of a <u><i>Melanoplus bruneri</i></u> in which the lumen is nearly filled with trophozoites of <i>Eugregarinida</i>	39
Fig. 20. Fecal pellets from a <u><i>Melanoplus sanguinipes</i></u> with attached cysts of <i>Eugregarinida</i>	39
Fig. 21. Fresh preparation under phase contrast microscopy showing uninucleate, binucleate and trinucleate schizonts of the <i>Neogregarinida</i> of grasshoppers.....	46
Fig. 22. A terminal multinucleate schizont of the <i>Neogregarinida</i> of grasshoppers.....	46
Fig. 23. A vermiform trinucleate schizont of the <i>Neogregarinida</i> of grasshoppers which was observed in fresh preparations of tissues from the hemocoel of a grasshopper.....	46
Fig. 24. A granular multinuclear schizont of the <i>Neogregarinida</i> of grasshoppers observed in a smear preparation of the hemolymph of a grasshopper.....	46

	Page
Fig. 25. A group of granular gametocytes of the Neogregarinida observed in a fresh preparation of the hemolymph of a grasshopper.....	49
Fig. 26. Sectioned preparation of the fat body and pericardial tissues of a grasshopper showing a heavy concentration of gametocytes, some of which are conjugated, and host pericardial cells which show indications of deterioration.....	49
Fig. 27. Cysts of the Neogregarinida of grasshoppers which exhibit different stages of development.....	49
Fig. 28. Cysts of the Neogregarinida of grasshoppers which contain mature appearing spores.....	49
Fig. 29. Diagrammatic representation of the proposed life cycle of the Neogregarinida from grasshoppers.....	51
Fig. 30. Diagrammatic representation of the generalized life cycle of <u>Nosema</u> spp. as determined from the stages observed during studies of the life cycle of <u>Nosema acridophagus</u>	55
Fig. 31. Binucleate sporonts of <u>Nosema locustae</u> in a smear preparation.....	62
Fig. 32. Spores and sporoblasts of <u>Nosema locustae</u> in a smear preparation.....	62
Fig. 33. Spores, sporoblasts, and a megaspore of <u>Nosema locustae</u> under phase contrast microscopy.....	62
Fig. 34. Spore of <u>Nosema locustae</u> as observed during extrusion of the polar filament.....	62
Fig. 35. Section of the fat bodies and nerve cords of a nymph of <u>Melanoplus sanguinipes</u> showing the heavy accumulations of spores of <u>Nosema locustae</u>	62
Fig. 36. The rate of mortality among individually reared <u>Melanoplus sanguinipes</u> which were inoculated with 4×10^6 and 4×10^4 spores of <u>Nosema locustae</u>	67
Fig. 37. A binucleate schizont of <u>Nosema acridophagus</u>	74

	Page
Fig. 38. A quadrinucleate schizont of <u>Nosema acridophagus</u>	74
Fig. 39. A diplokaryon form of <u>Nosema acridophagus</u>	74
Fig. 40. A binucleate sporont of <u>Nosema acridophagus</u>	74
Fig. 41. A spindle-shaped sporoblast of <u>Nosema acridophagus</u>	74
Fig. 42. A spore-shaped sporoblast of <u>Nosema acridophagus</u>	74
Fig. 43. A spore of <u>Nosema acridophagus</u> with an extruded polar filament.....	74
Fig. 44. Schizonts and sporonts of <u>Nosema acridophagus</u> in a binucleate pericardial cell.....	77
Fig. 45. Cross section of the convoluted epithelium of a gastric caecum from <u>Melanoplus sanguinipes</u> showing initial tumor formation, as indicated by the enlarged regenerative nidi, during infection by <u>Nosema acridophagus</u>	77
Fig. 46. Cross section of the midgut of a <u>Melanoplus sanguinipes</u> showing the appearance of a tumor during initial formation and some vacuolization of the epithelial cells...	77
Fig. 47. Cross section of a gastric caecum of a <u>Melanoplus</u> <u>sanguinipes</u> which was prepared at 48 hours after inoculation with <u>Nosema acridophagus</u> showing a tumor which encompasses schizonts of the microsporidian.....	81
Fig. 48. Cross section of the midgut of a <u>Melanoplus sanguinipes</u> showing a tumor and spores and sporonts of <u>Nosema</u> <u>acridophagus</u> between layers of the basal membrane.....	81
Fig. 49. Cross section of a gastric caecum of a <u>Melanoplus</u> <u>sanguinipes</u> prepared at 72 hours after inoculation with <u>Nosema acridophagus</u> showing a large tumor with a melanotic core.....	81
Fig. 50. Cross section of the posterior region of the midgut of a <u>Schistocerca americana</u> showing tumors induced by <u>Nosema acridophagus</u> encircling the digestive tract.....	81
Fig. 51. Fresh preparation under phase contrast microscopy of the spores of <u>Nosema</u> sp. showing an extruded polar filament from a common-sized spore.....	89

	Page
Fig. 52. Fresh preparation under phase contrast microscopy showing spores of <u>Nosema</u> sp. and an extruded polar filament from a megaspore.....	89
Fig. 53. Rates of mortality among individually-reared <u>Melanoplus bivittatus</u> following inoculation with <u>Nosema locustae</u> , <u>Nosema acridophagus</u> , or <u>Nosema</u> sp.....	94
Fig. 54. Rates of mortality among group-reared <u>Melanoplus bivittatus</u> following inoculation with <u>Nosema locustae</u> , <u>Nosema acridophagus</u> , or <u>Nosema</u> sp.....	95
Fig. 55. Section of the fat bodies of a <u>Hadrotettix trifasciatus</u> showing spores, sporonts and schizonts of a small spore type organism.....	99
Fig. 56. Inclusion of the GIBV in a fresh preparation under bright light microscopy.....	102
Fig. 57. Inclusions of the GIBV in a sectioned preparation of the fat bodies of a <u>Melanoplus sanguinipes</u>	102
Fig. 58. Fresh preparation under phase microscopy of inclusions of the GIBV during initial degradation in a 0.1% solution of sodium hydroxide.....	102
Fig. 59. Fresh preparation under phase microscopy of the expanded inclusions of the GIBV containing the viral particles following treatment with a 0.1% solution of sodium hydroxide.....	102
Fig. 60. Ultra-thin section of an inclusion of the GIBV during development showing the regular dispersal of viral particles.....	105
Fig. 61. Ultra-thin section of a developing inclusion of the GIBV showing the structure of the viral particles.....	105
Fig. 62. Ultra-thin section of a fat body cell of <u>Melanoplus sanguinipes</u> showing fibrillar-granular and granular precursor masses, and presumptive viral particles "budding" off the granular masses as observed during infections by the GIBV.....	110

	Page
Fig. 63. Ultra-thin section of a fat body cell from <u>Melanoplus sanguinipes</u> showing granular precursor masses and "budding" presumptive viral particles.....	110
Fig. 64. Ultra-thin section of a fat body cell of <u>Melanoplus sanguinipes</u> showing viral particles and an initial inclusion of the GIBV.....	113
Fig. 65. Fresh preparation of the tracheal matrix of a <u>Schistocerca americana</u> containing crystals of the CAV.....	124
Fig. 66. Ultra-thin section under light microscopy showing crystals of the CAV in the muscles of <u>Schistocerca americana</u>	124
Fig. 67. Ultra-thin section of a pericardial cell of <u>Schistocerca americana</u> showing the crystalline array arrangement of viral particles in a developing crystal...	126
Fig. 68. Ultra-thin section of a pericardial cell showing developing crystals of the CAV in an electron dense replicating matrix.....	126
Fig. 69. Ultra-thin cross section of the muscles of <u>Schistocerca americana</u> containing several vesicular membrane-like structures encircling viral particles.....	129

ABSTRACT

The development and pathologies of some protozoan and viral pathogens of grasshoppers are described and discussed in terms of the experimental and natural host-pathogen relationships. Included among the protozoans are: (1) Malameba locustae, an amoebic organism which infects tissues of the digestive tract and causes physiological changes which are reflected in the host's reproduction, (2) undetermined species of Eugregarinida which are common in grasshoppers but are considered relatively avirulent, (3) an undescribed species of Neogregarinida which from the observed stages of schizogony and sporogony probably belongs to the genus Caulleeryella, (4) Nosema locustae, a species of Microsporidia that infects primarily the fat bodies of grasshoppers, (5) Nosema acridophagus which infects a number of host tissues in which it induces cellular aggregations described as tumors, (6) an undescribed species of Nosema which infects various host tissues including those of the nervous system, and (7) an undetermined number of undescribed organisms which, based on the relative size of the spore, are referred to as small spore organisms, but which exhibit developmental stages that are typical of species of Microsporidia. Included among the viruses are (1) the grasshopper inclusion body virus (GIBV) which is a DNA-containing virus that infects the fat bodies of the host and exhibits replicating forms resembling those reported during the development of pox viruses, and (2) the crystalline-array virus (CAV) which is a small RNA-containing virus that infects the pericardium, muscles and tracheal matrix of the host and is the most virulent of the reported grasshopper pathogens.

INTRODUCTION

Studies of the pathogens of insects are justified because (1) they exist; (2) they function in the dynamics of insect populations; (3) they are potential problems in the maintenance of beneficial insects in nature and the laboratory; and (4) they are potentially useful in the control of pest insects. Steinhaus (1963) defines insect pathology as a "study of whatever 'goes wrong' with an insect", and includes both infectious and noninfectious diseases, with the latter including such manifestations as genetic aberrations, nutritional deficiencies, physical and chemical injuries, etc. However, such a definition becomes unwieldy because it infers that insect pathology includes anything which is abnormal and since it is often impossible to define what is normal it is equally impossible to define what is abnormal. For this reason most studies of the pathologies of insects, including these studies of pathogens of grasshoppers, have involved descriptions of the disease manifestations resulting in the presence of some observable microorganism. Generally such studies have been directed at characterizing microorganisms, describing their effect on the host, and evaluating their potential use as agents for the microbial control of pest insects, which in this case has been species of grasshoppers.

Prior to these studies the only protozoans isolated from grasshoppers were a species of Amoebida, Malameba locustae (King and Taylor), a species of Microsporida, Nosema locustae Ganning, and from about 25 to 40 species of Eugregarinida. While these studies were in progress, Steinhaus and Marsh (1962) and Bucher (1966) reported the isolation of an undetermined species of Neogregarinida. M. locustae, N. locustae, the undescribed

neogregarine, and several species of Eugregarinida were isolated and studied during these investigations. In addition, two previously undescribed species of microsporidia were isolated. One species, Nosema acridophagus Henry, was described (Henry, 1967) while the other species has been characterized sufficiently to place it in the genus Nosema Nageli. Also, several other probable microsporidians, which have been labeled as small spore diseases for purposes of reference, were observed in grasshoppers. However, because these organisms were not experimentally transmitted, their generic characteristics were not established.

In addition to the studies of Protozoa, these investigations resulted in the isolation of the only viruses known from grasshoppers. The preliminary characterization of the grasshopper inclusion body virus (GIBV) was reported by Henry and Jutila (1966). Subsequent reports of these viruses will be published jointly with Dr. J. W. Jutila^{1/}

MATERIALS AND METHODS

The following techniques were routinely used throughout these studies. Specialized techniques will be described in the sections dealing with the organisms with which they were employed.

Field Collection Techniques

Grasshoppers usually were collected in the field with sweep nets. The collected grasshoppers were either reared individually in glass or plastic tubes, or frozen at the time of collection. The reared grasshoppers were either killed and examined after a period of post collection rearing or examined when they died. The frozen grasshoppers were examined according to the routine procedures described below.

In 1963 a study was initiated on the natural occurrence of Nosema locustae in grasshoppers in Camas County, Idaho. Fifty-two permanent sampling sites were established within the 200 square mile study area. During 1963 to 1968 each site was sampled periodically during the summer seasons. Sampling consisted of recording the time of collection, atmospheric temperatures (at about 3 feet above the surface of the ground), wind direction and speed, and the grasshopper density as determined from 40 sweeps along predetermined transects. Also up to 30 grasshoppers were collected and frozen at each sampling period from each site. In the laboratory the frozen grasshoppers were separated by species, sex and stage of development. They were then examined for the presence or absence of N. locustae or other pathogens.

Rearing Techniques

The following species of grasshoppers were used for most laboratory studies: Melanoplus bivittatus (Say), Melanoplus sanguinipes (Fabricius),

Melanoplus differentialis (Thomas), Schistocerca vaga vaga (Scudder), and Schistocerca americana (Drury). The three species of Melanoplus were obtained from eggs collected in the field or from eggs oviposited in the laboratory by field-collected grasshoppers. S. vaga vaga and S. americana were reared in the laboratory and the breeding cultures were held in metal cages (Mazuranich and Cowan, 1966) in a greenhouse under a 16-hour daylight regime.

The eggs of S. vaga vaga and S. americana were incubated in moistened sand at 30° C. and usually hatched 19 to 25 days after deposition. The eggs of the Melanoplus species were incubated for 2 weeks at room temperature, then 3 months at 4° C. and then until they hatched, usually about 10 days, at 30° C. The nymphs were reared in an incubator room or in growth chambers at 30° C. with continuous illumination. They were fed daily with fresh lettuce, head or Romaine, and wheat bran. In most cases the diet was supplemented with an artificial medium similar to that reported by Kreasky (1962) but which also contained wheat germ, dried lettuce powder, and Cerophyl.^{2/}

Preparation of Inoculants

Inoculants were prepared by homogenizing infected grasshoppers in distilled water using 30-ml tissue grinding vessels with teflon pestles. Inoculants for injection were prepared further by passage through double thickness cheesecloth, followed by low speed centrifugation (3700 to 4500 rpm) and then passing the supernatant through a series of millipore filters down to filters with average pore sizes of 0.80 micron or less.

Experimental Inoculations

The inoculants were administered either per os or by intrahemocoelic injection. Per os applications were made either by placing a drop of the inoculant onto the mouthparts of the grasshopper or by placing a drop onto a lettuce disc (about 5 mm in diameter) which was then fed to a grasshopper that had been starved for about 24 hours prior to treatment. Injections were made intrathoracically using a 27-gauge needle mounted on a $\frac{1}{4}$ cc syringe. Precise volumes of pathogenic organisms were administered by mounting the syringe on a specially adapted micrometer caliper which, with a $\frac{1}{4}$ cc syringe, delivered inoculants within 4% of the desired volumes. The volume was preadjusted so as to yield the desired concentration in a 5 microliter drop. In tests involving small nymphs the volumes were adjusted to deliver 2.5 μ l per drop. The concentrations of pathogens in the inoculating media were determined by direct counts using a hemacytometer.

Light Microscopic Techniques

Preliminary disease diagnosis was accomplished by examining specific organs or tissues of dissected grasshoppers for abnormalities using a dissecting microscope. Infections by gregarines were diagnosed by the detection of cysts on fecal pellets using a dissecting microscope. Preliminary characterization of the histopathology and etiological agents was based on the abnormal appearance of tissues under phase contrast microscopy. All the pathogens reported here were recognized under phase contrast microscopy at magnifications of 500 to 1250 diameters.

Routine examinations to determine only the presence or absence of

particular pathogens were conducted by homogenizing grasshoppers and examining the homogenates as hanging drops under bright light microscopy. For more critical examinations and for detection of infection by the crystalline-array virus (CAV), the homogenates were examined under a cover glass using phase contrast microscopy.

Estimates of the levels of infection (number of pathogens per grasshopper weight) for all pathogens except the CAV were conducted by weighing each grasshopper and homogenizing each in a measured amount of water. The concentrations of pathogens in the homogenates were determined by direct counts using a hemacytometer. For comparative purposes the counts were expressed as the number of organisms per milligram (mg) of grasshopper weight. Estimates of the level of infection by M. locustae also were made by counting the number of cysts per mg of feces.

Preparation of Smears

Smears of homogenates or infected tissues were prepared to aid in the characterization of the etiological agents and for detection of various developmental stages. They were fixed in either methanol, osmic tetroxide fumes (2% solution), Bouin's solution, Gilson's fluid, Schaudinn's solution or by air drying. They were stained by the Giemsa technique or with various hematoxylin solutions. The most suitable technique for most protozoans in grasshoppers was fixation of dried smears in methanol for 2 minutes and, after drying, staining according to the Giemsa technique outlined by Galligher and Kozloff (1964). Depending on the susceptibility of the specimens to this stain, the smears were left in the staining solution (4% Giemsa stock solution in buffered water) for 15 minutes to

2 hours. The Giemsa stock solution consisted of 1 gm of Giemsa stain, 66 mls of glycerol and 66 mls of methanol. For more rapid staining, but with less control over the intensity of staining, smears were flooded with the stock solution for 2 to 5 minutes and differentiated for 2 to 5 minutes by adding an equal amount of buffered water. After staining the smears were washed for 5 minutes in tap water, air dried and mounted with Histoclad.^{3/} The smears were examined using bright light microscopy at magnifications up to about 1250 diameters.

Preparation of Sectioned Tissues for Light Microscopy

Tissue sections were prepared to aid in the characterization of etiological agents and histopathology. Although a number of the procedures listed by Galligher and Kozloff (1964) were used for preparation of sectioned tissues, the following procedure routinely produced the most satisfactory results with soft tissues from grasshoppers.

The tissues were fixed in Gilson's fluid for 24 hours or more, after which they were washed in 70% ethanol for at least 24 hours. They were dehydrated in 95% ethanol for 8 to 24 hours, then in absolute ethanol for 12 to 24 hours. They were then cleared in 1 to 3 parts xylene and absolute ethanol for 3 hours, followed by equal parts of xylene and absolute ethanol for 3 hours, followed by 3 to 1 parts xylene and absolute ethanol for 3 hours and then in xylene for 6 hours. The tissues were infiltrated in 3 to 1 parts xylene and melted Paraplast^{4/} for 3 to 6 hours, followed by equal parts of xylene and Paraplast for 3 to 6 hours, followed by 1 to 3 parts xylene and Paraplast for 3 to 6 hours, and then in several changes of Paraplast for 4 to 6 hours. Infiltration was conducted in an oven at

57° C. The tissues were embedded in Paraplast and cut in sections from 5 to 12 microns thick. The sections were affixed to slides using Mayers albumen or Haptas affixative and dried on a slide warmer for 4 to 12 hours. The sections were deparaffined according to the following sequence: 15 minutes in 2 changes of xylene; 2 minutes in equal parts of xylene and absolute ethanol; 2 minutes in absolute ethanol, 1 minute in 95% ethanol; 2 minutes in 70% ethanol; 1 minute in 40% ethanol, 1 minute in 15% ethanol; 2 to 3 minutes in distilled water. The sections were stained for 15 minutes in alum hematoxylin and washed in running tap water for 30 minutes. They were destained for 1 minute in each of 15%, 40%, and 70% ethanol. They were counterstained for 5 to 30 seconds in eosin, blotted, and destained for 2 minutes in 95% ethanol, 2 minutes in absolute ethanol and 5 minutes in xylene. They were mounted in Histoclad and examined at magnifications up to 1250 diameters.

Sections of tissues containing the grasshopper inclusion body virus were cut 5 microns thick and stained according to the procedures outlined by Hamm (1966). Thin sections, 0.2 to 0.5 micron thick, were prepared according to the procedures used by Harry (1965). The thin sections were prepared for studies of the small spore disease in Hadrotettix trifasciatus (Say) and some studies of gregarines.

Photomicroscopy

All photographs presented here of specimens under light microscopy were taken using a standard GFL Zeiss phase contrast microscope which was equipped with a basic body camera attachment and a Ukatron^{5/} microflash attachment. The photographic film was panatomic X (Kodak) with an ASA

rating of 32. The negatives were developed using a double bath developer (Diafine)^{6/} and Kodak fixer. The photographs were printed on hard gloss paper (Kodak medalist; F-3, F-4, or F-5).

Preparation of Tissues for Electron Microscopy

Most electron micrographs presented here were prepared according to the following procedure. Tissues were fixed in a 2.5% gluteraldehyde-potassium phosphate buffer (pH 7.0) for 15 minutes at room temperature, then postfixed in osmic tetroxide (2%) for 2 hours in a refrigerator maintained at about 4° C. They were dehydrated through a graded series of acetone followed by a final soaking in propylene oxide, after which they were embedded in Epon 6005^{7/}. After sectioning on a Reichart OM U2 Ultramicrotome, the sections were stained with aqueous uranyl acetate (2%) for 2 hours followed by Reynold's lead citrate for 5 minutes. They were examined with a Zeiss EM9 microscope. The electron micrographs were printed on hard gloss, high contrast paper (Kodabromide-5).

RESULTS

Malameba locustae (King and Taylor) (Amoebida)

Because of frequent chronic infections among laboratory-reared grasshoppers, most studies dealing with M. locustae involved the histopathology resulting from infection and the antibiotic control of the organism.

Host and Geographical Range of M. locustae

King and Taylor (1936) and Taylor and King (1937) described Malameba locustae from Melanoplus differentialis, M. sanguinipes (= M. mexicanus) and M. femurrubrum femurrubrum (DeGeer) in laboratory cultures in Iowa. In unpublished reports, G. T. York (in 1951) and R. L. Newton (in 1955) reported it from laboratory-reared grasshoppers at the Grasshopper Investigations Laboratory, USDA, at Bozeman, Montana. Steinhaus and Marsh (1962) reported M. locustae from Melanoplus bivittatus, M. sanguinipes (= M. mexicanus) and Melanoplus dawsoni (Scudder) which were sent to them by J. R. Parker of the Grasshopper Investigations Laboratory at Bozeman. In the Union of South Africa, Lea (1958) and Venter (1966) reported M. locustae in nature from brown locusts, Locustana pardalina (Walker), while Prinsloo (1960) studied it in L. pardalina in laboratory cultures. Recently Henry (1968) reported that infections were common in laboratory-reared grasshoppers at the Grasshopper Investigations Laboratory (USDA), Bozeman, Montana, and that he had observed it in grasshoppers and in grasshopper feces received from several other laboratories in the United States. Dr. Peggy Ellis (personal communication, 1968) of the Anti-Locust Research Centre, London, England, reported that M. locustae was common in laboratory cultures of various species of Schistocerca, Locusta, Nomadacris, and Chortoicetes.

Taylor and King (1937) listed 37 species and subspecies of grasshoppers that were susceptible to experimental infections by M. locustae. These included 5 species in the subfamily Acridinae, 14 species or subspecies of Oedipodinae, and 18 species of Cyrtacanthacrinae. They also listed 12 species with which the results were inconclusive because of difficulty in maintaining these species in the laboratory.

During the present studies M. locustae has been diagnosed in Aeropedellus clavatus (Thomas), Melanoplus bruneri Scudder, Trachyrhachys kiowa kiowa (Thomas), Chortophaga viridifasciatus (DeGeer), and Psoloessa delicatula delicatula (Scudder) which were collected in Montana; M. sanguinipes, Melanoplus cuneatus Scudder, Aulocara elliotti (Thomas), Psoloessa delicatula, Eritettix variabilis Bruner, Hadrotettix trifasciatus (Say) and Xanthippus corallipes (Haldeman) from Arizona; and M. bivittatus from Idaho. In addition, trophozoites and cysts of M. locustae were observed in Schistocerca vaga vaga about 7 days after being placed in laboratory culture following their collection in southern California. Also in the laboratory, M. locustae has been found in Schistocerca americana, Melanoplus bivittatus, M. sanguinipes, M. differentialis, M. bruneri, Melanoplus oregonensis (Thomas), Melanoplus packardi Scudder, M. dawsoni, M. femurrubrum, Aulocara elliotti, Gamula pellucida (Scudder), Hadrotettix trifasciatus, and Chorthippus curtipennis (Harris).

Description of the Stages in the Life Cycle of M. locustae

Under phase contrast microscopy, the trophozoites of M. locustae usually appeared spherical, somewhat transparent and measured from 4 to 12 microns in diameter (Fig. 1). Characteristically, the trophozoites

contained variable numbers of small, light-refractive granules. Irregularly-shaped trophozoites were common which, as proposed by King and Taylor (1936), indicated that trophozoites moved by means of hemispherical or filiose pseudopodia. They also reported that reproduction occurred by binary fission during which each uninucleate trophozoite separated into two daughter cells. Multinucleate forms or stages representing sexual recombination have not been observed.

The cysts of M. locustae appeared ellipsoidal, measured about 12.5 microns in length by 7.8 microns in diameter in fresh preparations, possessed thick walls, and appeared refractive to light using phase contrast microscopy (Fig. 1). Most cysts contained a vacuole at one end and a variable number of light-refractive granules which appeared similar to those observed in the trophozoites. By subjecting cysts to the Feulgen reaction (Galligher and Kozloff, 1964) it was determined that the nucleus existed as an elongate band which was located along one side of the cyst. The extrusion of young trophozoites from cysts was not observed.

King and Taylor (1936) reported that cysts measured about 9.6 microns long by 5.5 microns wide, but Prinsloo (1961) reported average measurements of 12 microns long by 8 microns in diameter. These differences probably resulted from their use of different techniques and different host species. Henry (1968) observed variations in measurements of cysts from three host species. As shown in Table I, the cysts measurements were consistently larger when the cysts were measured in fresh preparations than when measured in fixed and stained preparations. In fresh preparations, the cysts from Melanoplus differentialis measured less than those from

- Fig. 1. Fresh preparation under phase contrast microscopy of cysts (C) and trophozoites (T) of Malameba locustae. 750X.
- Fig. 2. Cross section of Malpighian tubules of Melanoplus sanguinipes with trophozoites of Malameba locustae on the inner surface of the walls and cysts in the lumen of the tubules. 450X.
- Fig. 3. Cross section of the posterior region of the midgut of a Melanoplus sanguinipes showing the deterioration of part of the epithelium due to infection by Malameba locustae. 64X.
- Fig. 4. Fresh preparation under phase contrast microscopy of a Malpighian tubule of a Melanoplus sanguinipes heavily compacted with cysts of Malameba locustae. 250X.
- Fig. 5. Fixed and stained preparation from a Melanoplus sanguinipes showing the cystocytic encapsulation of a Malpighian tubule containing cysts of Malameba locustae. 330X.

