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

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 devel-opmental 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.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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.

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 Canning, 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 Nägeli. 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 ¼ cc syringe. Precise volumes of pathogenic organisms were administered by mounting the syringe on a specially adapted micrometer caliper which, with a ¼ 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 µl 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 Haupts 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, Camnula 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.