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The path to registration of a microbial pesticide

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Summary

This paper presents a historical account of our attempt to develop a pathogenic microsporidium, *Nosema locustae* (now *Paranosema locustae*), as a microbial pesticide for reducing the frequency and scope of outbreaks of grasshoppers in the US. The objective was to provide program managers with another tool for altering grasshopper densities. As a result of our investigations, in 1980 the Environmental Protection Agency of the USA (EPA) issued the registration of a product containing *Nosema locustae* spores as the active ingredient, and in 1990 the Reregistration Eligibility Document (RED) that summarized all of the information behind the rational for using *N. locustae* and the pertinent evidence relating to the registration of this organism. Biological insecticide NOLO BAIT is used for grasshopper control all over the world, and is considered one of the most effective grasshopper baits.

Key words: Microsporidia, Protists, *Nosema locustae*, Acrididae, microbiological control, biological insecticide

Introduction

This paper presents a historical account of our attempt to develop a pathogenic protozoan, *Nosema locustae*, as a microbial pesticide for reducing the frequency and scope of outbreaks of grasshoppers in the US. The objective was to provide program managers with another tool for altering grasshopper densities. When starting this project, we had no expectation of ending with registration of a microbial pesticide. Initially I was opposed to registration because of concern that it might include regulations that would limit general use of the product. When we started some 50+ years ago, we had no knowledge that the pathogen would influence host densities in applied situations. We soon learned that we could alter host densities within several weeks after application of spores and that the pathogen would persist for at least several seasons with little or no adverse effects to the ecosystem. For many program managers and scientists, the effects were too little and too slow. Hopefully, values and priorities will change to something other than the chemical insecticide standards that were prevalent at that time.

Background

The most prominent question with this project is "Why *Nosema locustae*?" More precisely, why and

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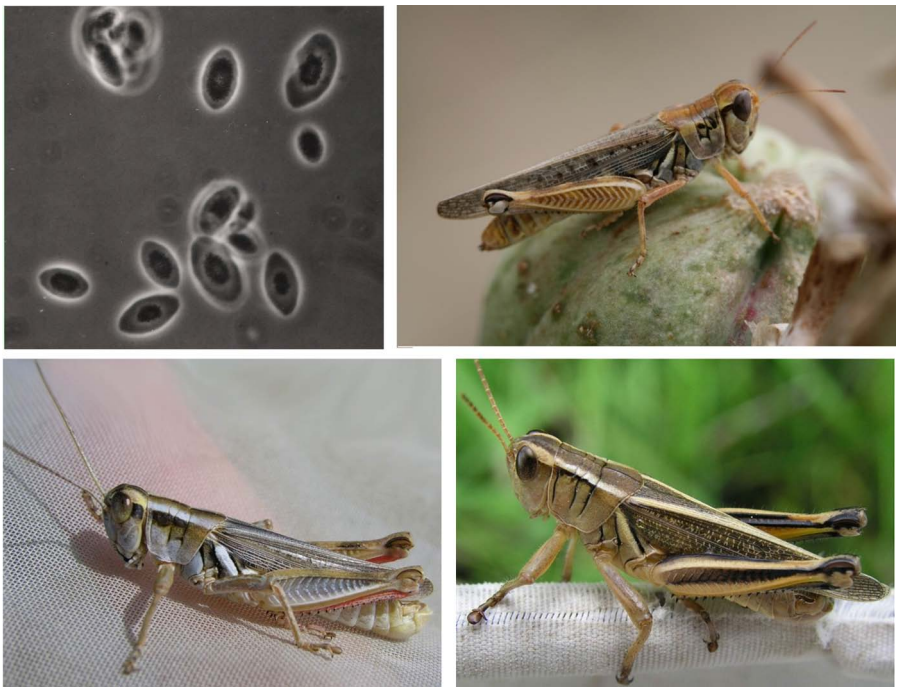

Fig. 1. Entomopox virus (dsDNA virus, Poxviridae) (upper left image) and its hosts, grasshoppers of the *Melanoplus* complex (Orthoptera, Acrididae). *M. sanguinipes* (upper right), *M. packardii* (bottom left), *M. bivittatus* (bottom right). Virus image from the collection of Carlos Lange, La Plata, Argentina; Images of acridids due to courtesy of Alexander Latchininski, University of Wyoming, Laramy, USA.

when was this organism selected for development as a biological tool against grasshoppers in the US? This is especially daunting when we realize the vast array and number of organisms that interact with grasshoppers in a very complex ecosystem. These organisms include multiple species of flies, bees, wasps, beetles, nematodes, fungi, viruses, protozoa, birds, snakes, etc. (Figs 1, 2). The selection process started in July 1961 when I reported for work on a newly created project on biological control of grasshoppers at the USDA/ARS Rangeland Insect Laboratory at Montana State University in Bozeman, MT. The first five to six years I spent getting acquainted with what was known from the literature and observing grasshoppers and their antagonist throughout the western US. Of particular interest were parasitic flies, mainly sarcophagids and tachinids, that often attacked grasshoppers in flight by placing an egg or larva on the thorax under the hind wing of the host. In a very limited study of this process, we found the grasshopper *Oedaleus enigma* often contained individuals with varied wing lengths, from wing buds, which do not fly, to full length, which fly the longest time and as a result suffer the highest frequency of parasitism. My thought was that there should be a selection for hoppers with no wings or shorter wings. However, by comparing the distribution of the varied wing lengths in several populations of *O. enigma*, there was no apparent evidence that wing lengths were related to the frequency of parasitism. I mention this observation because such interactions were encountered frequently, and a person could spend a career studying such an interaction.

During these years, I encountered two on-going epizootics caused by pathogenic microorganisms in commonly occurring species of grasshoppers. The first was caused by an entomopox virus among montane grasshoppers of the *Melanoplus* complex, possibly involving three or four closely related species, in an area of more than five acres of the Reynolds Pass between Idaho and Montana at about 7,000 feet elevation. This was the first entomopox known from grasshoppers, although we had previously isolated a probable similar or identical virus from one *M. sanguinipes* from Arizona. We visited the area three times during the season, beginning when many nymphs and adults were present, until late season when a few adults were left. At the time of the first visit, infections were observed in most adults

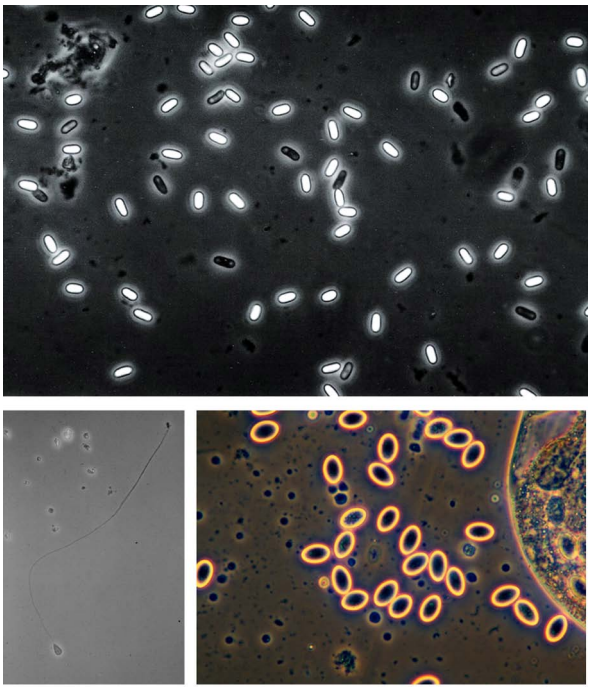

Fig. 2. Upper image, suspension of *Paranosema* (*Nosema*) *locustae* spores; bottom left image, exfilamentated *N. cuneatum* spore; bottom right image, *Malamoeba locustae* cysts. All pathogens were isolated from grasshoppers of the *Melanoplus* complex. Photographs from the collection of Carlos Lange.

and older nymphs but rarely in young nymphs. Dead and dying grasshoppers were collected in the higher densities of the epizootic area, all of which were infected. Apparent uninfected grasshoppers were collected out on the edges of the infested area. During the second visit virtually all grasshoppers were adults, many of which were dead or dying, all of which were infected. We estimated that within the central area of the epizootic, the death rate was approximately two to three grasshoppers per square yard during a 24 hour period. During both visits, a fair number of non-melanoplus grasshoppers were collected and examined. Light infections were occasionally observed in a single species of a spur-throat grasshopper, a group to which the melanoplus species also belong. During the last visit, only a few melanoplus hoppers were present, all of which were infected. Many non-melanoplus hoppers were collected, none of which were infected.

Some of the things we learned from those observations indicated that this virus was sufficiently virulent to cause detectable mortalities within several

Fig. 3. Habitat of grasshoppers of the *Melanoplus* complex: the site near Three Forks, Montana, where John and Carlos used to work in 1985. Photograph from collection of Carlos Lange.

weeks after infection, but that infections were limited to only a few species of grasshoppers. This seems to be the basic trade-off within virtually all host-pathogen associations; the greater the virulence expressed by a pathogen, the more restricted the pathogen is in host range, pathogen production, tissue specificity, etc. During the next 20 years of our studies, we isolated entomopox viruses (Fig. 2) from about 24 different grasshopper situations, but due to time and space constraints, only six viruses were challenged by DNA probes, which established that the challenged viruses were different. There continues to be a need for more studies of these viruses.

Finding the second natural epizootic resulted from discussions with Keith Evans, USDA/APHIS, who was responsible for grasshopper control programs in Idaho and who told me about an apparent outbreak in Camas County, Idaho in 1962. Upon visiting the area during early summer of 1963, I observed variably dense populations over an area of about 30 miles long and, in some places, 10 miles wide covering the Camas Prairie basin. Over several days, I found that numerous grasshoppers from several areas of the basin were infected with *N. locustae* (Fig. 1) and that parasitic flies also were quite active in these same areas. On the occasion of the next visit, I had decided to initiate an intense investigation of the onset and progress of this possible epizootic by establishing collection sites that would enable us to determine the grasshopper species involved and the distribution and changes in pathogen activity. I ended up with 52 sites that possibly covered variations in host species, grasshopper densities, and site selections by the hosts (Fig. 3). Sampling protocols were designed to elicit grasshopper species, development, and

movements, along with information about pathogen host relationships, namely incidence of naturally occurring infections and the severity of infections in individual grasshoppers. Previous studies had shown that all species of grasshoppers were not equally susceptible to infection.

From the samples obtained on the second visit, we learned that at least 16 species of grasshoppers were present in the area, that infections were distributed irregularly throughout the study area, ranging from no infections at a few sites to 10 sites where spore loads were observed as trace (fewer than one spore per microscopic field) to heavy (fields in which spores were touching across the field). The dominant species, *Melanoplus sanguinipes* (Fig.3), was the most widely distributed species and the most frequently encountered in the sampling process (4,368 hoppers out of a total of 5,419 hoppers examined during the entire season). However, the seasonal incidence of infection was only 3.5% among this species compared to 6.9% and 6.6% among the next two species most frequently encountered during the season.

Studies of this epizootic continued throughout four more seasons during which more than 30,000 grasshoppers were examined, of which about 6% were diagnosed as being infected. Most of the infections occurred in grasshoppers collected in three chronically infested areas where hopper densities remained higher than those throughout the study area. In these areas, the incidence of infection was around 5% early in the season when most were nymphs, but then increased to 40 to 50% toward the end of the season in late September when virtually all were adults. Numerous cadavers, some partially consumed, of adults which contained high concentrations of spores were frequently observed, and were collected in these areas during August through October. There were 12 sites from which the collected grasshoppers were never diagnosed as being infected even though some very susceptible species were dominant. Also, there were species collected throughout the area in which infections were rarely or never observed.

Selection of one candidate for further development

Based on information gleaned from published accounts and from results of both laboratory and field investigations, I recognized the availability of five organisms that were possible candidates for applied use. These were *Malamoeba locustae*, *Nosema locustae, N. acridophagus, N. cuneatum*, and the entomopox virus that was involved in the natu-ral epizootic. The characteristics important to this selection were lethality, infectiousness, environmental persistence, transmissibility, mass production potential and safety to non-target organisms.

M. locustae (Fig. 2) is common in lab cultures of gras-shoppers in which it causes chronic infections that result in little or no lethality. The host range is very extensive in grasshoppers and crickets, and possibly even more insect groups. There was one report from Europe in about 1939 of a test application for control of a pest orthopteran in which no activity occurred.

N. acridophagus is quite lethal, causing mortalities following laboratory inoculations in less than 14 days, but which result in production of relatively few spores. Because of the low spore production and observed limited host range, we conducted relatively few studies that could lead to a better assessment of this microsporidan. At that time the host range appeared to include three so-called economically important species, and we had little knowledge of its persistence in nature.

N. cuneatum (Fig. 2) appeared to be somewhat lethal, but with a restricted host range and unknown geographical range. Little is known of persistence in nature and of spore production. This organism needed further study.

The entomopox virus (Fig. 2) produced significant mortalities in natural epizootics, was persistent in the field and probably in storage, but exhibited a very restricted host range. We had little information on producing sufficient quantities of inclusion bodies for field testing, although we did a small field test in which mortality and infectivity were evident.

N. locustae (Fig. 1) has been the focus of more studies than any of the organisms listed above. Lethality appeared to be dependent on age of the target and dosage of spores in the inoculum, but was particularly evident in the field toward the end of each season with relatively high numbers of spores in cadavers. The host range in 1969 included more than 65 species of grasshoppers which included most of the economically important pest species. We observed very high spore production, two to three billion spores per grasshopper, resulting from lab studies of production of spores for use in field applications.

Based on the information provided by the

investigations of natural epizootics and some preliminary tests, we selected N. locustae for further development as a microbial pesticide.

Applied studies

Many laboratory and field studies were conducted over a five to six year period to elucidate probable expectations from applied field programs. Some of the first field studies focused on the methods of applying spores with the goal of producing measurable levels of mortality and/or infection. Our initial tests were to evaluate applications of ultra-low volume (ULV) spore formulations that could be sprayed using standard chemical protocols with a buffalo turbine mounted on the back of a truck. We monitored the pattern of spray droplets with spray-drop cards placed on the ground in a number of spray swaths. Once the system was properly calibrated to deliver spore dosages of 10^5, 10^7 or 10^9, spores per acre, along with a treatment of water without spores, we applied the treatments to one acre plots with two replications of each treatment. One day later we collected grasshoppers from each plot with sweep nets from which we randomly selected 30 hoppers from each sample which were then reared separately for 30 days. Most grasshoppers were fourth instar nymphs when collected. During the post-treatment, we saw very little mortality with no correlation to treatments. None of the dead grasshoppers contained spores. At the end of the 30 day rearing, the remaining hoppers were frozen and then examined for spores. By then we had adopted a standard protocol of homogenizing a grasshopper in 5 ml of distilled water and examining a sample from the homogenate as a hanging drop on an inverted cover glass at 100× magnification. By this technique, spores were readily detected by panning across the bottom surface of the drop. Using this protocol, we found only two hoppers were infected, both from the highest dosage treatments. We concluded that this application procedure was not usable.

We then switched emphasis to the use of baits, particularly wheat bran because it had been used extensively in the past history of grasshopper control for delivering various chemical pesticides to rangelands and pastures. Based on results from using wheat bran applied at the rate of 2 lb per acre and treated with the three spore concentrations listed above, we selected a standard level of 109 spores per acre which consistently produced measurable mortality and infection that was reflected in observed changes in the densities of grasshoppers. We were aware that the results of the applications were altered with variations in species composition, age, and densities. However, we also recognized compensatory interactions which eventually achieved the anticipated regulation of grasshopper populations. For example, treating hoppers as third instars produced more mortalities than did treating fifth instars, but treating fifth instars as opposed to third instars resulted in higher numbers of infected hoppers during the late post treatment period. What was important in our estimation was the number and health of grasshoppers eventually depositing eggs. Also important was the viability of the eggs and the number and survivability of the hatchlings.

We observed reduced effectiveness of spores that had been stored on wheat bran for several weeks or longer, as well as stored frozen in water for several months. Spore viability was quite good, a year or longer, when they were stored frozen in dry cadavers. Accordingly, it was important that formulation of treatment preps was timed so as to minimize lengthy storage. Eventually, we devised equipment and protocols for applying spores to large quantities of bran that produced consistent results. The important factor was to apply spores to the bran (Fig. 4) in a manner that moisture was minimized such that the bran was essentially dry after application. Sacked wheat bran that was slightly moist often underwent a natural heating process, which inactivated spores and caused clumping of the bran. The bran clumps plugged the field delivery system and prevented uniform treatments. I think that improper handling of wheat bran during formulation and applications contributed to many of the inconsistencies reported in other studies.

Differential acceptance of wheat bran and variable susceptibility to infection by grasshoppers were recognized as inherent problems because no two species of grasshoppers respond the same to any stimulus. Accordingly, our objective was to target grasshopper species that were considered to be economically important because of their selection of particular species of plants, of their high densities in outbreak situations, and possible invasion of valuable cultivars. In selecting wheat bran as a carrier for the spores, we knew that some species would not be affected directly because many species of grasshoppers feed on a limited range of plants. However, most economically important species are generalized feeders which readily accept bran when it is available. Also, I believed that some

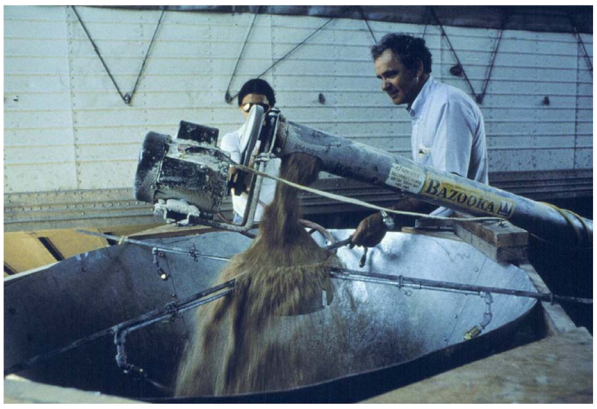

Fig. 4. John formulating *Paranosema* on wheat bran to be applied in the Pampas grasslands, about 1981. Photograph from collection of Carlos Lange.

grasshoppers needed to remain in the ecosystem such that they were available to other organisms that used grasshoppers as their prey. I am aware of studies that showed that some species of grasshoppers are very specialized in feeding on particular plants and that this feeding actually stimulates plant growth. We understood that it was a very complex ecosystem with which we were dealing.

We knew that efficient production of spores was important to the eventual applied use of this microbial. We spent much time and effort in trying to produce spores in an in-vitro cell culture system (without success) as well in a defined media, such as bacteria, also without success. Accordingly, we accepted the fact that *N. locustae* is an obligate in-vivo parasite and that spore production must be completed in live grasshoppers. For this purpose we conducted numerous test infections with various species in order to find a grasshopper that would produce the greatest spore numbers in the shortest time period. *Melanoplus bivittatus* and *M. differentialis*, both of which are relatively large hoppers that produce very large numbers of spores toward the end of the infection cycle were considered most useful for this purpose. Both species were easy to rear in the lab because they were generalized feeders, were somewhat gregarious, and could tolerate crowding in cages. Eventually we settled on the differential grasshopper because we were able to colonize it in the lab and thus we had a steady supply of fourth and fifth instar nymphs for inoculations.

After inoculation, the grasshoppers were held in special rearing cages that minimized loses due to cannibalism. Insects that died prior to 30 days post inoculation were discarded. Those that died later were collected each day and frozen for eventual harvest of spores. At 42 days post inoculation, all survivors were frozen. The stored cadavers were homogenized in distilled water, after which the homogenates were filtered twice through cheese cloth and then concentrated by low speed centrifugation. Counts of harvested spores within the concentrated preps established usual per-hopper spore production of about 10^8 spores. Other checks of production showed counts of 2 to 3×10^9 spores in heavily infected females. We considered this to be a very efficient system for the production of spores.

One of our major concerns in use of *N. locustae* was persistence, not only to the following generation of hoppers, but for a number of subsequent seasons. Our previous studies of natural epizootics indicated several years persistence during which there appeared to be host population regulation. We also were aware that we could not conduct field studies in the close proximity to previous tests because infected grasshopper were frequently collected in the area in subsequent years. Fortunately, we were permitted the resources (funds, people and equipment) by our agency (ARS/USDA) to undertake a large scale test involving application of three treatments of spores (10^5, 10^7 and 10^9 per acre), an untreated check, and a standard chemical treatment of malathion at 8 oz. per acre, with four replications of each treatment to 100 acre plots. We had anticipated plot integrity possibly to three years. The area for the tests was all private land used primarily for pasture and grazing and which had not been treated for grasshoppers for at least 20 years. Protocols called for standard sampling procedures to determine host composition, densities, development and infection.

First season results followed expected trends in infections which were dose responsive in all pathogen treatments, 60 to 65% density reductions in the malathion treatments, some density reduction in pathogen treatments which were not correlated to dosages, and no density reductions or infections in untreated plots. We observed the total disappearance of flies in the malathion treatments.

During the second season we observed infections in pathogen treatments which were not dose related and all infection rates were lower than expectations as determined from previous studies. Also, fly densities in the malathion treatments were much lower than elsewhere in the study area and grasshopper densities had increased markedly in these treatments. By mid July, we determined that an extensive epizootic caused by the fungus *Entomophaga grylli* was influ-

encing the integrity of the plots. Although the results of this study were compromised by this and other unexpected factors, the study did provide useful information. The information on the reaction of fly densities together with the resurgence of grasshoppers in malathion treatments is important to managing densities of grasshoppers. This study also reinforced our knowledge that the grasshoppers were irregularly distributed in the ecosystem, resulting in high density "pockets" where they congregated and in which natural enemies were most abundant. We also observed that grasshoppers tended to move toward these hot spots.

Based on these observations, I initiated a study of treating "hot spots" in order to augment existing disease levels or to establish *N. locustae* to promote epizootics. That study had to be discontinued. I mentioned this here because I think this pathogen would be very effective in such an approach.

Registration of a microbial pesticide

Late in the 1970s I heard about some people who had mexpressed interest in commercializing *N. locustae* with the intent of selling spores. I realized this meant the need to register it for applied use, of which I was in total disagreement because I never thought of it turning into a commercial product. The EPA got involved and requested we provide fresh spores for them to inoculate mice and rats for safety tests. I examined and tested the viability of the spores from various preps by inducing polar filament extrusion prior to inoculation of the test animals. Also, I learned that the protocols for testing the pathogen were the same as those used for chemical insecticides, the only difference being that chemical analysis of the pathogen was not necessary. We examined tissues and many microscopic slides, submitted to us by the EPA, in which we never saw evidence of spores or any evidence of infection by the microsporidan. As a result of these investigations, the EPA issued the first registration of a product containing a pathogenic organism as the active ingredient on May 9, 1980. In September, 1990, the EPA issued a RED (Reregistration Eligibility Document) that summarized all of the information behind the rational for using *N. locustae* and the pertinent evidence relating to the registration of this organism (Fig. 5).

Fig. 5. Biological Insecticide NOLO BAIT™ Grashopper control.(• Safe to use around humans, plants, and animals. • Most effective grasshopper bait. • %100 Organic. • <www.amazon.com>.

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