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NATURAL AND APPLIED CONTROL OF INSECTS BY PROTOZOA¹

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Protozoa have been recognized as important factors in the natural regulation of the densities of certain insects. However, they have been given little consideration as applied microbial agents because entomophilic species cause chronic or debilitating infections in a narrow range of hosts. From a control standpoint, and ultimately from an economic standpoint, they could not compete with the more rapid, broad spectrum action of chemical insecticides or the more virulent pathogens such as bacteria, viruses, and certain fungi. Protozoa appeared useful only when low levels of pests could be tolerated, as in forests.

Gradually, interest in protozoa as potential biological control agents has been increasing (98). Primarily, this is a result of pest management concepts that emphasize conservation and augmentation of existing natural control agents. This has been demonstrated by an increase in published research reports on applied use of protozoan microbial agents, along with some excellent reviews of certain aspects of protozoa (8, 9, 77, 78, 98). The body of literature dealing with natural and applied control by protozoa now is so extensive that no single review can provide a fully comprehensive treatment of the subject. Since the review by McLaughlin (77) provided a fairly complete assessment of the subject prior to 1970 it seems appropriate that it should serve as a starting point for this review in order to demonstrate the progress over the past decade.

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A comment is necessary on the systematics of this very diverse group. In several recent schemes the Microsporida were given phylum rank as Microspora, thereby removing this group from Protozoa (36, 97). Although there are basic differences between the Protozoa and Microspora, many researchers use the traditional nomenclature of Microsporida or Microsporidia; for the purpose of continuity with prior reviews, microsporidia will be treated here as part of the Protozoa.

NATURAL CONTROL BY PROTOZOA

Ciliates

Most entomophilic ciliates are associated with dipterans, primarily mosquitoes and chironomids. Although there have been reports of fairly high incidence of ciliates in certain insects, for example 43.2%, epizootics that have controlled insect populations have not been reported (77). Moreover, because of the low pathogenicity, there seems to be little potential for using ciliates against noxious insects. For example, only two ciliates were found during a five-year survey of protozoa in insects in Poland, neither of which appeared pathogenic to their hosts (64).

Flagellates

Most entomophilic flagellates belong to the order Kinetoplastida of the superclass Mastigophora. The association with insects varies, ranging from mutualism and commensalism to apparent parasitism (8). Many are digenetic and spend part of their life cycle in an insect, most often Hemiptera or Diptera, and part in other animals or plants. The immediate and long-term effects of flagellates on insects are poorly understood and there has been no effort to use them against insects.

Several epizootics by flagellates have been reported. One was caused by *Herpetomonas swainei* in a population of the sawfly *Neodiprion swainei* in an eastern forest region of Canada (94). The flagellate was observed in 86% of the sawfly larvae collected from 10–15 year-old moderately infested jack pines. Infections occurred in all larval instars, eonymphs, and adults. The parasites were most abundant in third and fourth instar larvae, but mortality never exceeded 20% or occurred before the fourth and fifth instar; it occurred only among those that contracted the infection as first or second instar larvae. Although the amount of fat tissue in larvae was reduced, the adults exhibited no gross symptoms of infection nor lowered fecundity and there was no effect on simultaneous infections by an inclusion body virus. The flagellate could not be transmitted to the insects *Galleria mellonella*, *Archips cerasivoranus*, *Malacosoma disstria*, *Pristiphora erichsonii*, and the earthworm *Lumbricus terrestris*. *Crithidia cimbexi* shows a similar lack of

pathogenicity in the sawfly *Cimbex americana* and did not infect other insects (69).

More recently, the flagellate *Blastocrithidia caliroae* was isolated from quarantined eonymphs of the pear-slug *Caliroa cerasi* during introduction of parasites of the slug into New Zealand (67). Infections were observed at several locations in Europe where high mortalities were noted among pear slugs that contained high concentrations of flagellates. The infections were observed in all stages except the first instars and adults; most mortality occurred during the fourth, fifth, and sixth instars. Young infected larvae appeared normal, but older nymphs experienced a drying of the mucous coating that resulted in a darkening and spotty general appearance. Although infections were not observed among adult pear slugs, the organism might be transmitted vertically during reproduction.

One epizootic occurred during an outbreak of the pear slug in the upper Rhine Valley in Germany. Of the 2500 insects collected and reared to eonymphs, 53% died, and of these, 90% contained flagellates. During another epizootic in Germany, pear slugs collected for shipment suffered 42% mortality among eonymphs in August 1972, but 74% mortality in November 1972. The field population began collapsing in late 1972 and declined to endemic levels in 1973.

Also in Europe, the flagellate *Herpetomonas chatoni* was observed in several noctuid adults (*Discestra trifolii*, *Agrotis exclamationis*, and *A. segetum*) caught in light traps (65). The incidence of infection within each species varied during the three seasons of the survey, but never exceeded 8%.

Coccidia

Adelina tribolii, which infects several stored product pests and causes striking epizootics in populations of the confused flour beetle, *Tribolium confusum*, is probably the most frequently encountered coccidian in insects (77). Infection rates of 68% have been reported (64), indicating that this coccidian is an important factor in the regulation of laboratory and natural populations of *Tribolium* spp. Also, an epizootic by a species of *Adelina* in a laboratory colony of the wax moth, *Galleria mellonella*, suggested that the coccidian might be used to control wax moths in bee hives (4). Recently, the coccidian *Rasajeyna nannyla* was described from the European crane fly, *Tipula paludosa* (5). An investigation of the seasonal cycles of the coccidian revealed that the incidence of *R. nannyla* was higher in *Tipula vittata* than in *T. paludosa* at a site where part of the study area was constantly under water (6). In this habitat, which was more suitable for *T. vittata* than for *T. paludosa*, coccidian oocysts were protected from both freezing and desiccation in pools of water that were not completely frozen.

Thus, inoculum was available throughout the year. Analysis of the infection data confirmed that the incidence of infection was a function of the temperature differences between the habitats (7). Although the incidence of infection by *R. nannyla* in these crane flies reached levels of nearly 100%, there was no evidence of host density regulation.

Amoebidae

As pointed out by McLaughlin (77), *Malameba locustae*, which infects grasshoppers and some crickets, is probably the most important entomophilic amoeba from the standpoint of natural or applied control of a host. This was demonstrated in reports of a high natural incidence of *M. locustae* in the brown locust, *Locustana pardalina* (59); in one instance *M. locustae* prevented an outbreak of this locust in one area of South Africa (102).

Most recent studies of *M. locustae* examine control of the amoeba in laboratory colonies of grasshoppers (16, 17, 37, 44) or discuss the structural aspects of amoeba development as a basis for proper taxonomic assignment (19, 30, 32, 33). Infections by *M. locustae* also have been observed in silverfish (*Thysanura*) from a colony that was started with insects collected in a culture room containing desert locusts, *Schistocerca gregaria*, that were heavily infected with the amoeba (58).

Eugregarines

The eugregarines are probably the most frequently encountered protozoa in insects and the most innocuous. Many species of cephaline gregarines have been reported, particularly in the genus *Gregarina*, but few cause pathogenic effects in the host. Moderate infestations of the gregarine *Gregarina garnhami* reduced the weights of male fledglings (new adults) of *S. gregaria* without causing observable effects on molting or feeding (31). Anderson & Magnarelli (1) reported that 85% of larvae of the deerfly *Chrysops fuliginosus* were infected with the eugregarine *Cometoides pechumani* during the summer, but only 30 to 58% were infected during the winter. Less than 7% of the adults of this species were infected, as was one pupa of *Chrysops atlanticus*. In a similar study of protozoa in stored product insects in California, 4.3% of all insects of 6 species of *Trogoderma* collected at 322 locations over a four-year period were infected with a eugregarine *Pyxinia* sp. (28). Thus there is evidence that eugregarines might affect the growth of insects and can occur at high frequencies; however, critical tests have not been conducted on possible long-range effects of such infestations upon insect densities.

In an interesting case of competition, gregarines in the mosquito *Aedes aegypti* interfered with the development of dog heart worm, *Dirofilaria immitis*, for which the mosquito serves as a vector (95). The gregarine

trophozoites destroyed epithelial cells of the digestive tract and Malpighian tubules, which resulted in fewer cells available for filarial growth. Also, the Malpighian tubules of infected mosquitoes were shorter and deformed and the weights of infected pupae were significantly lower than those of uninfected pupae.

Neogregarines

Neogregarines have been reported from Lepidoptera, Coleoptera, and Orthoptera. Some species exhibit high pathogenicity and control potential (78), which undoubtedly can be attributed to one or more reproductive cycles in the life of these organisms (8). However, only a few studies have provided information on the natural occurrence and possible control of insect populations. The two neogregarines that have received the most attention are *Mattesia grandis*, described from the boll weevil, *Anthonomus grandis* (76), and *Mattesia trogodermae*, described from the khapra beetle, *Trogoderma granarium* (13). One study of the natural frequency of *M. trogodermae* showed 80% infection in seven species of beetles collected during a four-year period from 322 stored products facilities in California (28).

Microsporidia

Although the microsporidians are among the most common pathogens in insects under natural conditions, both the number of epizootic studies and field applications have been limited (98). However, this is changing rapidly and some recent studies of natural control by microsporidia provide basic information for prediction of population collapses and for applied use of the pathogens.

FOREST AND TREE CROP PESTS Early studies provided information on the host-pathogen relationship between the spruce budworm, *Choristoneura fumiferana*, and the protozoan *Nosema fumiferanae* in the pine forests of Ontario (77). Larvae that are infected early in their development from parental infections fail to mature and die. Their frass and cadavers then provide a source of spore inoculum for fourth and fifth instar nymphs that complete development but are infected in the adult stage. Adults also may contract the disease by consuming spores. Thus, increased incidence of infection usually stems from consuming food contaminated with spores, but vertical transmission maintains the microsporidium in the host population. For example, from 1955 to 1958 the incidence of disease increased progressively from 36.5 to 56.1% and the population collapse was attributed to the depressing effect of the microsporidium. Wilson (106) monitored the infection rates in *C. fumiferana* populations that had been studied some years

before when infection rates increased from 36.4 to 81.3% over a five-year period (99). Beginning about four years later and extending over a period of five years, Wilson (106) observed an increase in infection from about 36 to 69%. The results of a three-year study at another location in Ontario demonstrated similar infection patterns (104). At a point when 80 to 90% of the budworms had pupated, 66.6% of the larvae and 46.6% of the pupae were infected during the second year and 87.5% of larvae and 81.4% of pupae were infected during the third year.

The green tortrix, *Tortrix viridana*, caused widespread defoliation of two species of oaks in Germany during 1967; this threatened to deform the oaks and thus reduce their value for producing high quality veneer and acorns (22). Surveys conducted in 1968 just before applications of DDT or *Bacillus thuringiensis* established that 29% of the first instar larvae were infected with microsporidian parasites. After the treatments, 59% of the larvae in check plots were infected compared to 41% in plots treated with *B. thuringiensis* and 31% in plots treated with DDT. Infection rates among adults were 60, 58, and 42%, respectively. Although these infection rates resulted in about 75% reduction in tortrix densities, the infestation remained above the economic threshold. During the following season, 60% of the first instar larvae were infected with microsporidia. Subsequently, 80% of the third instar larvae were infected and no visible defoliation occurred. Sampling two weeks later during the fifth instar stage confirmed that the population had collapsed and that the incidence of infection had decreased to 24% in untreated plots. Also, infections decreased in plots treated with *B. thuringiensis* and DDT from 34 and 40% to 7 and 10%, respectively. These results suggested that the treatments may not have been necessary and that subsequent epizootics might be predicted by monitoring infections. *Nosema tortricis* was the most common microsporidium (43.6%) involved in the epizootic, along with *Octospora viridanae* (0.6%), *Thelohania weiseri* (0.4%), and *Plistophora* sp. (1.2%).

Another epizootic by microsporidia on populations of the green tortrix occurred in southern Poland (68). During the first two years, 30 and 26%, respectively, of the larvae on the oak trees were infected and 48.5 and 49.2%, respectively, died during rearing after removal from the trees. However, during the first year only 14% mortality was due to microsporidiosis; the remainder was due to bacterial infections. During the second year all mortality was attributed to microsporidia. During the third year the incidence of infection was about the same, 31.8%, but mortality increased to 91.1%. The population then collapsed and green tortrix larvae were difficult to locate during the two subsequent seasons. Of the 54 and 72 larvae collected during the fourth and fifth years, respectively, none were infected. The progression of the epizootic appeared to be due to increasing infection

rates among adults and subsequent transovarial transmission. For example, during the second season 12% of the adults were infected, whereas in the third season just prior to the collapse, 65% of the adults were infected. Not only did this epizootic follow the same general course as that in Germany, but the same four microsporidia were involved. However, in the latter epizootic *O. viridanae* was most prominent (30%), followed by *N. tortricis* (15%) and *T. weiseri* (10%). Only two larvae were observed with *Plistophora* sp.

A study in the Soviet Union of the natural occurrence of microsporidia in the codling moth, *Laspeyresia pomonella*, revealed that *Nosema carpocapsae* was present in each of the ten codling moth populations sampled (93). The incidence of infection in these populations varied from 1.5 to 89.3%; highest infections occurred at the end of the season in second generation fifth instar larvae, which also is the overwintering stage. Mortality was 58.9% during the overwintering stage, but this was due less to microsporidiosis than to both solitary and mixed infections by the fungus *Beauveria bassiana*. Apparently, infections by *N. carpocapsae* predisposed the overwintering larvae to the fungus. Also, the incidence of microsporidiosis was considerably higher and increased during the season in private orchards where insecticides were not used. In commercial orchards, the incidence of infection was much lower and remained constant during the entire season, indicating that the infected larvae were selectively killed by the insecticide.

ROW CROP PESTS Studies between 1954 and 1962 established that the most important factors affecting the natural host-pathogen relationships between *Nosema pyrausta* and the European corn borer, *Ostrinia nubilalis*, are (a) effective transmission and spread of the pathogen vertically from parent to offspring and horizontally between adults or larvae; (b) reductions in numbers of larvae that, because of the infections, are unable to attack a plant or develop into adults; and (c) a reduction in both vitality and fecundity exhibited by adults. Infection rates between 47 and 85% were observed in those earlier studies. (77).

Recent studies of the incidence of *N. pyrausta* in corn borer in North Dakota revealed that females from a disease-free population produced about twice as many eggs as females from populations with infected larvae (23). The infection rates varied from 12 to about 40% at five sites, and there was an inverse relationship between the average number of spores in larvae and the number of eggs produced by females at the five sites.

In Minnesota, 66% of all deformed pupae were infected, as were 54% of the adults that were reared from larvae collected from the field (115). Infections in adults caused reduced adult longevity, a lower percentage of

ovipositing females, lower individual fecundity, and a reduction in egg fertility. Females that contained more than 10 million spores produced less than half the number of eggs produced by disease-free females.

Surveys conducted in China revealed that 0 to 6% of *O. nubilalis* in low density populations were infected with *N. pyrausta*, whereas 16 to 38% were infected in high density borer populations (15); the microsporidium apparently exerts a significant amount of control on natural corn borer populations. Similarly, in France Lipa (66) found 11 of 30 corn borer larvae and pupae infected with *N. pyrausta* during September 1974. The other 19 insects were infected with the previously undescribed species *Thelohania ostrinae* (= *Vairimorpha necatrix*). Also, infections were not observed in any of about 900 corn borers collected in Poland during 1963–1976. He speculated that the pathogens were restricted to *O. nubilalis* populations in southern Europe.

A 16-year study of natural infection by *N. pyrausta* in 2 counties in Nebraska established that the microsporidium caused or at least contributed to declines in European corn borer populations (49). In peak years, the incidence of infection reached 100%. It appeared that high host densities and widespread spatial distribution of corn borers were essential to the development of epizootics by *N. pyrausta*.

The corn earworm, *Heliothis zea*, is commonly infected both in nature and in laboratory colonies by the microsporidium *Nosema heliothidis* (11). Examinations of moths collected by light traps revealed that the seasonal incidence of infection was seldom less than 10%, averaged about 30%, and increased to 84.9% among late season larvae during October. Because of a high frequency of infection in dead and moribund field-collected larvae, the microsporidium has been considered an important factor in the regulation of natural populations of *H. zea*. *N. heliothidis* appears to have a very narrow host range, since in nature it has been reported only from *H. zea* and *Heliothis virescens*. However, *Campoletis soronensis*, a hymenopteran parasitoid of *H. zea*, is also susceptible to infection (10).

N. heliothidis is particularly serious in laboratory colonies of *H. zea* when larval infection rates approach 100%. The infections cause "locking" of copulating adults, thus interfering with the reproductive process (96). By selection of adults that produced disease-free progeny, the microsporidium can be eliminated from a laboratory colony (29). The fungicide, benomyl, which previously had been used effectively against microsporidiosis in the alfalfa weevil, *Hypera postica*, (50) was not effective in eliminating *N. heliothidis* from corn earworm (12).

Vairimorpha necatrix, a dimorphic spore-forming microsporidium previously named *Nosema necatrix* and *Thelohania diazoma* (21, 72, 75, 85), has been isolated from at least 14 species of field-collected lepidopteran larvae.

However, infection in natural populations usually is infrequent and apparently is not important as a natural regulating factor of field crop pests (74).

GRASSHOPPERS Infections by *Nosema locustae* have been observed in at least 58 species of Orthoptera (38). Many host records occurred during a five-year study of the natural occurrence of the pathogen in southern Idaho (41). Infections varied markedly between the 52 permanent sampling sites but were most common at sites characterized by mixed vegetation that supported both consistent and moderate densities of several species of grasshoppers. The presence of three species, *Oedaleonotus enigma*, *Melanoplus bivittatus*, and *Melanopolus sanguinipes*, was important for the persistence and increase of infections by *N. locustae*. Generally, infections first appeared in *O. enigma* and *M. bivittatus* early in the season; these species produced inoculum for infection of *M. sanguinipes*, the predominant summer species, which then produced inoculum for infection of the next generation of early summer species. Although the incidence of infection among grasshoppers collected at all study sites during a season averaged only 8.11%, the incidence of infection reached 95% at one sampling late in the season.

Grasshopper densities were highest during the second season but decreased greatly during the third year and were lowest during the fifth season. Infections, however, peaked in the fourth season, followed by a sharp decline in the fifth season. The pattern depicted a lag-type host-parasite density relationship which indicated that the microsporidium might have contributed to reduced grasshopper densities. Also the study demonstrated that early seasonal infection of nymphs was necessary for developing pathogen levels that might regulate the natural levels of grasshoppers.

MOSQUITOES As pointed out by McLaughlin (77), the aquatic environment involves very different host-pathogen relationships than those that occur in the terrestrial environment. For example, transovarial transmission is most important and probably is the only means, in some cases, to maintain the pathogen and to expand an epizootic to the point where the pathogen would have a visible controlling action on host densities. However, a laboratory simulation of a natural situation that involved only transovarial transmission demonstrated that a pathogen would be eliminated from such a mosquito population (2). In that study all factors, including the number of gonotrophic cycles and total egg production by infected and uninfected females, were equal except for a single factor of 52% reduced viability of eggs from infected females. This suggests that another method of transmission has to be operative. Similarly, transovarial trans-

mission of *Nosema algerae* in populations of *Anopheles gambiae* was not important or prominent because larvae from infected eggs did not survive (14). Instead, transmission occurred by regurgitation of spores from salivary gland infections into the feeding area of other larvae; also, transovum transmission occurred because spores were present on the external surface of the eggs. Surface contamination of eggs was confirmed when infections were prevented by washing eggs from infected females. The parasite was eliminated from a mosquito colony by these hygienic techniques. Surveys for pathogens of aquatic insects in tropical regions have yielded other microsporidians that are transmitted per os (35).

Although information on the role of microsporidia in regulating natural mosquito populations is lacking, epizootics have been reported in colonized mosquitoes. For example, *Nosema algerae* was described as a serious problem in a laboratory colony of *Anopheles stephensi* (101). Also, *Nosema* sp. (probably *N. algerae*) caused epizootics among colonized mosquitoes at the University of Maryland and at Walter Reed Army Institute of Research (34). Of four species of mosquitoes colonized at the Maryland facility, *Anopheles gambiae* were most heavily infected (100%) and development to the adult stage was totally inhibited. The incidence of infection among *Anopheles albimanus* was 75%, with few becoming adults. The incidence among adults of *Anopheles stephensi* and *Anopheles quadrimaculatus* was 49 and 39%, respectively. At the Walter Reed facility colonies of *Aedes*, *Anopheles*, and *Culex* were maintained, but infections occurred only in the anophelines.

APPLIED CONTROL WITH PROTOZOA

Boll Weevil

McLaughlin (77, 78) reviewed the results of some important studies between 1965 and 1970 when baits were used to induce artificial epizootics in boll weevil (*Anthonomus grandis*) populations by the neogregarine *Mattesia grandis* and the microsporidium *Glugea gasti*. Application of the pathogen-bait formulation, which also contained feeding stimulant, resulted in 50 to 70% infection of weevils in the spring and fall populations. Such treatments suppressed populations and in one instance reduced the overwintering population by 96%, compared to the 84% natural reduction in an untreated area. However, midseason boll weevils did not respond to the baits and neither control nor crop protection could be demonstrated. No additional studies on the microbial control of the boll weevil have been conducted since the initiation of a concentrated effort to eradicate this insect.

In addition to providing some background for the use of baits, the studies of the boll weevil resulted in one of the first critical assessments of the economics of mass producing protozoans in vivo (79). In the production scheme approximately 100 larvae per plate were inoculated with about 10^6 spores, which resulted in 100% infection. The spores were harvested about 12 to 14 days later from adult weevils and were formulated with the bait at concentrations capable of producing demonstrable weevil population suppression. At this concentration, 3.3×10^5 spores per ml, the cost of production of *M. grandis* was estimated at \$50.80/acre and of *G. gasti* at about \$1.24/acre. The large difference between these costs was due to the relative efficiency of production, which in the case of *M. grandis* might be increased by using alternate hosts or by employing more efficient field application procedures, such as bait application around pheromone traps.

Stored Product Pests

Baits also have been used to introduce *Mattesia trogodermae* into populations of the stored product pest *Trogoderma glabrum* (91). It was known that males which were surface-contaminated with spores inoculated females while mating. However, the efficiency of the spore transfer was increased by releasing a natural female sex pheromone at the site where males became contaminated. A synthetic pheromone that induced males to attempt to copulate with the pheromone lure enhanced the pathogen transfer (92). During the copulation attempt, spores that entered the genital organs later were transmitted congenitally to females. Therefore, it was postulated that larvae of the subsequent generation could become infected by (a) consuming food contaminated by contact with infected adults, (b) consuming cadavers of infected adults, and (c) transovarial or transovum transmission. The results showed that male contamination at lures with the pheromone alone or pathogen alone did not increase the mortality rate beyond that among untreated beetles. Also, pathogen-pheromone treatment of high parental densities caused significantly lower F_1 and F_2 population densities, but the treatment had no effect on F_1 population densities when parental population densities were low (1/16 of high density level). Therefore, the main spore transfer pathways were larval consumption of cadavers or contaminated food; little or no transovarial or transovum transmission occurred.

These studies established the potential of using *M. trogodermae* to control various pest species in stored products. The attractiveness of *M. trogodermae* as a control agent is increased because it is specific for species of *Trogoderma*, it is not pathogenic in certain vertebrates (87), and it is fairly easy to produce in usable quantities (83).

Forest Insects

Most studies of the application of microsporidia against forest insects have been with *Nosema fumiferanae* against the spruce budworm, *Choristoneura fumiferana*. The microsporidium has been successfully introduced into populations of the spruce budworm by spraying trees with an aqueous spore suspension (110, 111). In one test 1.8×10^{11} spores per tree were applied to trees with fourth and fifth instar larvae and later to trees with sixth instar larvae. The early treatment resulted in about 2.5-fold increased infection at 11 and 25 days, posttreatment. The later treatment did not increase the incidence of infection among larvae but did increase the incidence among adults and the offspring from those adults. Also, the incidence of infection was slightly higher among insects from one tree treated with a formulation containing an ultraviolet light protectant (Sandoz adjuvant V). Samples taken the following season also indicated that the earlier treatments caused highest increases, but not significantly higher than late sprays, over the natural incidence (112). Two years after treatment the incidence of infection among insects on treated trees were still somewhat higher than on untreated trees, but in that period the incidence of infections on check trees had increased almost to treated levels (109). For example, the percentages of infection for the season of application, one year later, and two years later, were 53.0, 52.4, and 53.8, respectively, among insects from treated trees, compared to 23.0, 39.0, and 48.0, respectively, among insects from untreated trees. This indicated that the treatments advanced the infection by two to three years.

In a similar study, *N. fumiferanae* and *Pleistophora schubergi* were formulated separately in various concentrations in aqueous suspensions with 25% (vol/vol) molasses and 0.3% (wt/vol) IMC 90-001 sunlight protectant (Sandoz, Inc., Homestead, Florida) and were applied against young needle mining larvae on white spruce, *Picea glauca*, and balsam fir, *Abies balsamea* (113). The results with *N. fumiferanae* revealed that (a) single applications of 2.5×10^{10} spores per tree against second and third instar nymphs did not increase infection over the natural level; (b) later applications against fourth and fifth instar larvae, either alone or with a prior application against second and third instar larvae, significantly increased the incidence of infection; (c) single applications of doubled spore dosages, 5.0×10^{10} spores per tree, increased infections over lower spore dosages, but not significantly; and (d) the incidence of infection among insects in treated balsam fir trees was higher (72.9% at 25 days postapplication) than among insects in treated spruce trees (59.7% at 25 days postapplication). However the natural incidence of infection among insects from untreated balsam fir trees (38.8%) also was higher than among insects from untreated spruce trees (25.4%).

At 18 and 25 days postapplication of *P. schubergi* (a) 61.2 and 40.1%, respectively, of larvae on white spruce (each treated with 2.5×10^{10} spores) were infected, (b) 64.8 and 48.6%, respectively, of larvae on white spruce trees (each treated with 5.0×10^{10} spores) were infected, and (c) 96.3 and 95.2%, respectively, of larvae on balsam fir (each treated with 5×10^{10} spores) were infected. All applications were against fourth and fifth instar nymphs, and natural infections by *P. schubergi* were virtually nonexistent. These studies showed that the incidence of *N. fumiferanae* could be increased by applications against late instar larvae and that comparatively high levels of infections by *P. schubergi* could be established by such applications.

P. schubergi infects a wide variety of forest Lepidoptera and Hymenoptera (53, 103, 105) and it has been tested against second and third instar larvae of *Anisota senatoria* and first instar *Symmerista canicosta* on northern red oak trees (54). The spores were formulated as aqueous sprays with a spreader-sticker or with the spreader-sticker plus a UV protectant (Sandoz "Shade"). The UV protectant increased persistence significantly and resulted in more than eight-fold increased infectivity at four days postapplication. Tests for control of *A. senatoria* and *S. canicosta* using formulations of 2×10^8 or 2×10^7 spores per ml with only the spreader-sticker resulted in 95.8 and 72.0% infection, respectively, among *A. senatoria* and 100% infection, for both treatments, among *S. canicosta* at 14 days postapplication. Infections were not observed in insects collected from untreated trees. *P. schubergi* has been combined with *Nosema disstriae* in equal concentrations and in mixtures of 96% *P. schubergi* and 4% *N. disstriae* in aqueous sprays of 50% molasses with 0.3% IMC 90-001 UV protectant and applied at the rate of 1.8×10^{11} spores per trembling aspen, *Populus tremuloides*, against the forest tent caterpillar, *Malacosoma disstria* (114). The formulation with equal concentrations of spores caused 76.1% infection by *P. schubergi*, compared to no natural infection, but did not increase infection by *N. disstriae* over the natural incidence. The formulation with unequal spore concentrations produced similar results with a slightly higher incidence of infection (85.0%) by *P. schubergi*.

The reports of field tests with *N. fumiferanae* and *P. schubergi* showed significant increases in infection but no evidence of mortality or population control. The effect of infection on mortality and control was evident in a laboratory study in which second and fourth instar larvae naturally infected with *N. fumiferanae* were each inoculated with 5×10^4 , 5×10^6 , or 5×10^8 spores of *P. schubergi* (108). Treatment of second instar larvae increased mortality during the subsequent instars, but only the highest dosage resulted in increased mortality when fourth instar larvae were inoculated. This also was demonstrated in a study in which spruce budworm with or

without natural infection were inoculated with 2.0×10^5 spores of *N. fumiferanae* (107). Inoculation of previously uninfected larvae significantly lowered fecundity and pupal weights. Natural infections caused significantly lowered pupal weights, lowered fecundity, and lowered adult life span. Superimposed inoculation of naturally infected larvae enhanced the severity of these effects. The above studies demonstrate some potential control of forest insects by these microsporidia.

Row Crop Pests

Although *Nosema pyrausta* is an effective natural control of the European corn borer, *Ostrinia nubilalis* (63), the seriousness of infection by the microsporidium often depends on the interaction with additional stress factors, such as crowding or the resistance of maize plants to corn borer feeding (62, 71). For example, both leaf-feeding resistance and sheath-collar feeding resistance, which inhibit feeding by the first and second generation borers, respectively, together with heavy infections by *N. pyrausta*, decrease the number of larvae per plant and the weight of surviving larvae. Different inbred lines of leaf-feeding resistant varieties of maize did not influence the levels of infection, whereas the degree of sheath-collar feeding resistance had a direct influence on the severity of the infection. Thus, the interaction between infection and resistance was evaluated when *N. pyrausta* was applied early in the season to a large segment of the feral corn borer population (63). During the first year of the tests, treatments of 22.5×10^7 spores per plant in 25 ml of distilled water, applied to plants artificially infested with egg masses free of *N. pyrausta*, resulted in significantly fewer larvae and borer cavities per plant. Also, the average weights of larvae from treated plants were lower than those of larvae from untreated plants, and 62.1% of the larvae from treated plants were infected compared to 1.5% of the larvae from untreated plants. During the second year, applications of 24.3×10^7 spores per plant against simulated first-generation borers (artificially infested plants) failed to reduce the number of borers per plant but decreased the number of cavities per plant and caused a 7-fold increase in incidence of infection among the larvae. Also during the second year, foliar applications of 24.3×10^7 spores per plant against simulated second generation larvae reduced larval weights and the number of larvae and cavities per plant, but not significantly below those in untreated plants. However, the treatments caused 95.2 to 99.2% infection among larvae from treated plants compared to 20.8% infection among larvae from untreated plants.

In a recent test, insecticides were integrated with *N. pyrausta* in control of the corn borer (70). For 2 seasons, artificially infested corn plants were first treated with 1.2×10^7 spores; the next day they were treated with

Bacillus thuringiensis var. *kurstaki*, carbaryl, or carbofuran. In each instance larval densities were reduced following applications of *N. pyrausta* and an insecticide. Combinations of the microsporidium with *B. thuringiensis* or carbaryl were more effective than its combination with carbofuran. Generally, these mortality factors acted independently with additive effects. This study indicates a good potential for developing an integrated approach using various combinations of mortality factors, including *N. pyrausta*, for control of the European corn borer.

As with any microbial agent, the manner by which *N. pyrausta* is transmitted in the field is an important factor in persistence following application. Transovarial transmission of *N. pyrausta* to the filial generation had been established in early studies (56, 116). Recently, Lewis (60) demonstrated that infected larvae migrate to other corn plants and distribute frass contaminated with spores, which are then consumed by uninfected larvae. This form of transmission resulted in high incidence of disease, high levels of infection, and reduced larval populations.

The dimorphic microsporidian *Vairimorpha necatrix*, which is considered to be a good prospect for microbial control of numerous lepidopterous pests (74), also infects the corn borer (61). Inoculation of neonate corn borer larvae resulted in 51% mortality as first instar larvae, due primarily to septicemia, and total inhibition of reproduction. The median effective doses at which reproduction subsequently was inhibited for first, second, or third instar larvae were 1.5, 48.7, and 1385.2 spores/mm² of media surface, respectively, compared to doses of 15.3, 1055.3, and 54729 spores/mm², respectively, at which pupation was inhibited. Infection of the first, second, and third instar larvae resulted in 76%, 92%, and 96% infection, respectively, of adults. However, unlike *N. pyrausta*, *V. necatrix* is not transmitted either transovarially or transovum, or between larvae via fecal contamination. Instead it is transmitted when larvae consume infected cadavers or food contaminated with spores from cadavers.

At least 35 other species of Lepidoptera are susceptible to infection by *V. necatrix*, including such important agricultural pests as *Pseudaletia unipuncta*, *Heliothis zea*, *Heliothis virescens*, *Autographa californica*, *Trichoplusia ni*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Spodoptera ornithogalli*, *Plathypena scabra*, *Pseudoplusia includens*, and *Hyphantria cunea* (74), and it appears that *V. necatrix* is useful against the black cutworm, *Agrotis ipsilon* (51). However, not all Lepidoptera are susceptible to infection (52, 72, 75), nor are hymenopterous parasites (26) or insects of other orders (75).

In a preliminary test, spores of *V. necatrix* applied in a spray formulation at the rate of about 3.9×10^{11} spores/acre provided significant reduction of the tobacco budworm, *Heliothis virescens*, but control was not compara-

ble to that by the chemical methomyl (80). Also, the application of *V. necatrix* on late cabbage infested with *Trichoplusia ni* and *Pieris rapae* significantly reduced the populations of *T. ni* and feeding on lower leaves and provided crop protection that compared favorably with the protection by applications of *Autographa californica* virus (72). The microsporidium had no effect on *P. rapae* in laboratory tests, and thus it was combined with a granulosis virus of *P. rapae* for integrated control of both insects.

Fuxa (24) found that *V. necatrix* interacted differently with various pathogens and at varying dosages in infection of *Heliothis zea*. For example, in combination with a commercial preparation of *Bacillus thuringiensis*, mixed interactions resulted that tended to be synergistic. Combination with a commercial preparation of *H. zea* baculovirus resulted in definite antagonistic interactions. In combination with the fungus *Nomuraea rileyi* the interactions varied from synergistic at low dosages to antagonistic at high dosages. Graphic analysis of the interactions provided basically similar conclusions, but the author suggested that alteration of the bioassay inoculation system might have provided a more accurate picture of the interactions. Specifically, concentrating the inoculum on a smaller media surface (84) or better dispersion of the inoculum throughout the media might have produced less variation within the test groups.

A critical problem in the use and application of *V. necatrix*, as with many microbials, is loss of activity of spores due to UV radiation following application. The viability of dried spores of *V. necatrix*, as well as spores of *Nosema trichoplusiae*, declined within several hours after exposure to direct sunlight on corn leaves or on artificial diet (72, 73). Mixing spores with sterile soil provides some protection against UV inactivation. Kaya (55) reported that spores of *V. necatrix* survived in the field on leaves of bean plants for more than 28 hours and that survival was better both on the undersurfaces of the leaves and when formulated with a UV protectant. In the laboratory, spores suspended in water and exposed to short and long UV wave lengths were bioassayed in the salt marsh caterpillar *Estigmene acrea*. Spores exposed to short wave lengths (254 nm) lost activity within 10 min, whereas spores exposed to long wave lengths (366 nm) remained viable after 360 min exposure. Spores were able to withstand short wave length radiation better when sprayed onto bean leaves. Kaya (54) had previously demonstrated that spores of *Pleistophora schubergi* were more sensitive to UV radiation than were spores of *V. necatrix* but that they were less sensitive when combined with a UV protectant.

Fuxa & Brooks (25) also reported that the viability of spores of *V. necatrix* declined rapidly after two days on foliage of tobacco, cotton, or soybeans. However, some spores remained viable for 10 days on soybean foliage. Spores were inactivated more rapidly on cotton foliage than on tobacco or soybean foliage. Two UV protectants, Shade® (International

Minerals and Chemical Corp., Libertyville, Illinois) combined with a stick-spreader Triton X-100 (Rohm and Hass Co., Philadelphia, Pennsylvania) and San 285 AD WP66 (Sandoz-Wander, Inc., Homestead, Florida) afforded approximately equal protection for spores.

In subsequent study, control by *V. necatrix* in spray formulations (with the UV protection of SAN 285) and on corn meal baits was compared with control provided by a commercial formulation of *B. thuringiensis* in Triton X-100 plus Shade, and the chemical insecticide methomyl (26). Although the spray formulations resulted in up to 63.2% infection of corn earworm larvae, protection to flue-cured tobacco was not evident. However, an application of spores formulated with corn meal provided protection comparable to that provided by the chemical insecticide and commercial bacterial formulations. Treatment of soybeans or sorghum with aqueous spray formulations containing 3.2×10^{13} spores/ha resulted in as much as 96% infection of larvae of *H. zea* and the green cloverworm, *Plathypena scabra*; however, again there was no evidence of crop protection, even in the presence of a significant reduction in the number of larvae of *P. scabra*. Although the chemical methomyl provided better control, resurgence of *H. zea* in methomyl-treated plots resulted in more larvae in these plots than in those treated with *V. necatrix*. Also, infections were not observed in the hymenopteran parasites *Campoletis sonorensis* and *Cardiochiles nigriceps* reared from heavily infected tobacco budworms, *H. virescens*. *C. sonorensis* has been shown to be susceptible to *Nosema heliothidis* from *H. zea* (10, 11).

In addition to a wide host range, the relative ease of mass producing spores is another reason *V. necatrix* is particularly attractive as a microbial insecticide. Inoculation of third instar *H. zea* larvae on artificial diet produced 1.7×10^{10} spores per larva at 15 days, which was a 3.3×10^6 -fold increase over the inoculation dose (27). The study also demonstrated excellent storage of spores on dry corn meal for 10 months. Survival was best when spores were stored at -15°C in water, water plus glycerin, cadavers, cadavers in glycerin, and lyophilized cadavers. *V. necatrix* is very sensitive to germicides and various other chemicals, which is critical because bacterial and fungal contaminants in spore preparations are important factors in loss of viability (57, 86). However, the antibiotics tetracycline, neomycin, and kanamycin effectively controlled bacterial and fungal contaminants without adversely affecting spore viability (86).

Grasshoppers

Three species of microsporidia, *Nosema locustae*, *Nosema acridophagus*, and *Nosema cuneatum*, appear promising as microbial agents for use against grasshoppers and possibly against certain economically important crickets. However, *N. locustae* has received the most attention because it

infects numerous Orthoptera and is easily mass-produced in certain species of grasshoppers (38, 39, 45). Field tests with small plots (4 to 40 ha) have demonstrated that applications of about 2.5×10^9 spores on 2 kg of wheat bran per ha at the time when important summer species, such as *Melanoplus sanguinipes*, are mostly third instar nymphs results in about 50% reduction within 4 weeks and 30 to 50% infection in certain predominant species (40, 42, 48). Timing is important, as early applications against young grasshoppers cause more mortality initially but less infection later, whereas later applications cause less mortality initially but more infection later. Also, application against third instars maximizes both mortality and infection four weeks later when grasshoppers become adults and are beginning to oviposit. Mortality due to infections continues during the adult stage, but more importantly reproduction is reduced significantly (20, 45).

For most field tests, spores were applied on a wheat bran carrier. However, because wheat bran is more expensive to handle and to apply than are ultra low volume (ULV) sprays, which are the common means of applying chemicals in large-scale programs for grasshopper control, a study was conducted comparing the two methods (46). Three spore concentrations in ULV sprays were compared to the standard treatment on wheat bran of 2.5×10^9 spores/ha. Both infection and mortality were higher in treatments of the wheat bran formulation than even the highest-dosage of 2.5×10^{10} spores/ha in sprays. The data indicated that at least 15-fold increase in spores would be required in ULV sprays to achieve results comparable to spores on wheat bran. Cost comparisons revealed that the expense of producing 15 times more spores for spray formulations was much higher than the added cost of handling and applying wheat bran. Applying spores on rolled barley produced results comparable to treatment by spore-wheat bran formulations; such applications may be more economical if ground application (45) is used.

As with most protozoans, *N. locustae* is not particularly virulent and therefore is most useful as a long-term control agent of rangeland grasshoppers. The original intent was to apply *N. locustae* to areas of increasing grasshopper densities and thereby prevent or reduce the magnitude of outbreaks. However, experience has shown that because of the unpredictability of grasshopper densities from one year to the next, ranchers and other rangeland managers will not enter into control programs unless the loss of significant amounts of forage is imminent. In such instances, some immediate population suppression is necessary to reduce densities to or near tolerable economic levels. *N. locustae* alone will not accomplish this, even by applying much heavier concentrations of spores. Recent studies have established that *N. locustae* is compatible with low concentrations (10% actual ingredients) of chemical insecticides such as malathion (82), carbaryl, dime-

thoate, and carbofuran (81). Field tests have demonstrated that *N. locustae*-wheat bran formulations can be tank mixed with chemical insecticide-wheat bran formulations for simultaneous application; thus, significant short-term control is followed by added population control from *N. locustae*. This integrated approach appears to be the most effective use of *N. locustae*.

As with *V. necatrix*, one of the favorable attributes of *N. locustae* is the ease of mass production. Two species of grasshoppers, *Melanoplus bivittatus* and *Melanoplus differentialis*, appear particularly well suited for this purpose because they withstand heavy spore concentrations. Average production of 3.9×10^9 (45) spores per grasshopper has been achieved and this represented a 5×10^6 -fold increase over the number of spores used for inoculation. However, with improvements in the rearing system, average spore levels of 8.0×10^9 should be expected. Concentrations of 2.0×10^{10} spores have been recovered from heavily infected females.

Results of a series of standard toxicological tests on vertebrates and certain invertebrates (bees) that establish noninfectivity of nontarget organisms have been included with efficacy reports that are available to potential registrants [Federal Register 44(32):9609–10, Wednesday, February 14, 1979]. Recently the Environmental Protection Agency approved a request for an exemption from the requirement of a tolerance in using *N. locustae* to control rangeland grasshoppers. [Federal Register 45(94):31312–12, Tuesday, May 13, 1980].

Nosema cuneatum and *Nosema acridophagus* are considerably more virulent in grasshoppers than is *N. locustae* (43). However, possibly because of the high virulence, production of sufficient spores for field tests is difficult, and cost of spore production in grasshoppers for commercial use may be prohibitive even though fewer spores might be required for applied use because of their higher virulence. However, studies which established that *N. cuneatum* and *N. acridophagus* were infective for the corn earworm also revealed that more *N. acridophagus* can be produced per insect in the corn earworm than in grasshoppers but that both insects produced about equal concentrations of *N. cuneatum* (47). Because corn earworms are easier to rear than are grasshoppers and the rearing time (inoculation to harvest) is shorter, producing these microsporidians in corn earworms may be less expensive. Also, spores of each species from corn earworms are more virulent to grasshoppers than are spores produced in grasshoppers.

Mosquitoes

Although there has been much interest in using protozoa to control mosquitoes, few field tests have been conducted. In one test, *Plistophora* (= *Vavraia*) *culicis* was applied to small pools of water inhabited with *Culex*

pipiens fatigans (= *C. quinquefasciatus*) (88). Applications of about 6,000 to 7,000 spores per ml to the mosquito breeding pools resulted in infections ranging from 0% to about 30%. The microsporidium persisted for 24 months postinoculation at one test site and short-term population suppression was evident at several test sites; however, long-term control did not occur.

In addition to *P. culicis*, there has been considerable interest in *Nosema algerae*, the microsporidium described from a laboratory colony of *Anopheles stephensi* (101). This species exhibits a high pathogenicity for anopheline mosquitoes (3) that can interfere with the ability of mosquitoes to vector malarial organisms (90). Also, *N. algerae* can be grown in the corn earworm (100) which produces considerably higher yields of spores than is possible from mosquito larvae. *N. algerae* was infectious following injection into 11 species of insects and a crayfish (100) and following per os inoculation of a wide-range of mosquitoes, several species of muscoid flies, and a chironomid (89). In addition, one of nine species of predators of mosquitoes also was susceptible to per os inoculation (18).

In a field test against *Anopheles albimanus* in Panama, *N. algerae* was applied to plots at rates of 2.15×10^7 to 2.15×10^9 spores per m² (3). Depending on dosage, infection rates were as high as 86%, with some mortality among larvae with massive spore concentrations. Although these tests demonstrated a potential for using *N. algerae* in control of mosquitoes, subsequent tests have produced varying results because the spores of *N. algerae*, as well as other potentially useful protozoa such as *P. culicis*, do not persist long in the feeding horizon of mosquito larvae. Accordingly, further testing of these organisms will depend on the development of a formulation that will prevent the spores from sinking to the bottom of the bodies of water to which they are applied.

CONCLUDING REMARKS

Significant progress was made during the past decade on natural and applied control of insects by protozoa. Insights into the mechanisms by which protozoa regulate insect populations have been provided. More importantly, research is showing that protozoa can be used effectively and efficiently to control the densities of noxious insects.

As the body of information expands, aspects of applied use of protozoa that require more attention are becoming increasingly clear. Production of some organisms in quantities required for general use will be difficult. This might be solved by using alternative host species, but more effort on the in vitro growth of certain entomophilic protozoa is warranted. Conventional chemical insecticide formulation and application technology will not be

applicable to protozoa. Rather, the research is showing increased efficacy when protozoa are applied in baits or on particulate carriers. Development of new (and possibly specific) formulation and application techniques and equipment will be required. Because of the characteristically low virulence expressed by most protozoa, few, if any, will be used alone against insect pests. Instead, they will be integrated with other control agents that will either provide some short-term control or will predispose the insects to control by the protozoa. Microbials have generally been evaluated against standards for relatively inexpensive, broad spectrum chemical insecticides; against such standards, few microbials could compete. Now, however, with increased concern for environmental quality and increasing cost of petrochemical products (which include most chemical insecticides), our standards are changing. There has been a significant increase in integrated pest management programs in which protozoa and other microbials can and will play a role. As this course continues, and as more protozoa are brought in to operational use, we will then be better able to evaluate the true potential of these organisms.

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