I.3 Laboratory Bioassays of *Nosema locustae*

Michael B. Hildreth, Chris W. Brey, Billy W. Fuller, and R. Nelson Foster

Introduction

The use of living insect pathogens as biocontrol agents for insects requires that the virulence (killing power) of these agents must be monitored occasionally, especially just prior to their distribution into the environment. Evaluation of an agent's virulence can be accomplished through the use of laboratory bioassays involving the target insects (raised in the laboratory) and the biocontrol agents that are to be tested.

The first biocontrol agent registered by the U.S. Environmental Protection Agency for grasshopper pests was the protozoan *Nosema locustae*. Grasshoppers acquire *N. locustae* infections by eating its spore stage. *N. locustae* infects the fat bodies of grasshoppers and is only mildly pathogenic to its host. For several years, our lab at South Dakota State University (SDSU) has been bioassaying the viability and virulence of *N. locustae* spores supplied commercially to the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (USDA, APHIS, PPQ) Grasshopper Integrated Pest Management (GHIPM) Project. Laboratory-reared third-instar *Melanoplus sanguinipes* grasshoppers were used as the target insect for these bioassays, and the grasshoppers were fed the *Nosema* spores on small disks cut from romaine lettuce. The grasshoppers were then kept in the lab for 20 days, and LD_{50} (the calculated dose of pathogen or toxin that kills half of the bioassayed grasshoppers) values were calculated based upon the percentage of grasshoppers that had died by the end of the time period.

When *Nosema* is used to control grasshoppers, spores are typically applied on rangelands with a wheat-bran bait. Lettuce bioassays can be used only to measure the viability of spores prior to the spores' addition to wheat bran. The purpose of this chapter is to describe the protocol used in our laboratory to measure the virulence of *N. locustae* spores stored in water and applied to lettuce disks, and also to describe a bioassay protocol that we've used for measuring the virulence of these spores after their addition to wheat bran. Representative results from these bioassays are reported in this chapter.

Lettuce Bioassay

Methods.—*Nosema locustae* spores used for these studies were provided by various commercial sources. All spores were stored in distilled water at –4 °F (–20 °C) until use. *Melanoplus sanguinipes* grasshoppers used in the studies were a lab-reared Canadian strain that had been maintained at SDSU for several years. These grasshoppers were reared according to the recommendations provided by Henry (1985).

The lettuce bioassay was based upon recommendations supplied by John Henry (personal communication). Spores were counted in a hemocytometer (a special slide used in hospitals to count blood cells) and applied to freshly cut lettuce disks approximately one-third inch (7 mm) in diameter disks using a 10 μL (microliter) pipettor. Six dosages of *N. locustae* spores in 10 μL distilled water (e.g., 0, 1×10^4, $10^{4.5}$, 10^5, $10^{5.5}$, 10^6 spores; $1 \times 10^{4.5}$ is equal to 3.162×10^4 or 31,620) were added to the disks (120 disks per dosage), and allowed to dry for 1 to 4 hours. Each disk was fed individually to a third-instar grasshopper that had been previously starved for 1 day in glass vials at approximately 86 °F (30 °C). To distribute the grasshoppers into vials, the insects needed to be cooled briefly from ambient 86 °F (30 °C) to approximately 39 °F (4 °C). Before adding the appropriate lettuce disks to the vials, the vials were randomly sorted and divided into the appropriate six dosage groups. Once 80 grasshoppers from each group had eaten an entire disk, they were placed in groups of 5 into 16 bioassay tubes (8 inches or 20 cm long, 2.75 inches or 7 cm in diameter) constructed of 0.08-inch (0.02-mm) sheet acetate with screened ends. Generally, grasshoppers ate an entire disk within 2 hours or did not eat it even after 12 hours. The 16 bioassay tubes were divided into 4 replicates of 4 tubes each. In the bioassay tubes, grasshoppers were fed laboratory-reared rye grass daily along with triple sulfa-coated rolled oats (Henry and Oma 1975) and maintained under continuous fluorescent illumination at approximately 86 °F (30 °C).

Each day, we counted the number of dead grasshopper carcasses in the bioassay tubes. Grasshoppers frequently cannibalized other grasshoppers in the tubes, and portions of carcasses often were found. Therefore, we verified the number of living grasshoppers remaining in each tube to

not overlook cannibalized individuals. We calculated LD_{50} values by using the software package POLO-PC (LeOra Software, Inc., Cary, NC).

Results.—An example of the typical results obtained from the 22 bioassays conducted in our lab during the past 5 years is shown in figure I.3–1. A few of the uninfected control grasshoppers always died during the 20 days of each bioassay. For all of the 22 bioassays, generally less than 20 percent of the control grasshoppers died before the end of the bioassay. Inoculation of grasshoppers with increasing numbers of *N. locustae* spores consistently increased the mortality rate for grasshoppers infected with 1×10^6, $1 \times 10^{5.5}$, and 1×10^5 spores. Grasshoppers inoculated with 1×10^6 *Nosema* spores typically started to die sooner than the control grasshoppers within the first 8 days postinoculation (p.i.); the largest number of deaths normally occurred between days 10 and 14 p.i. By 20 days p.i., 70 to 100 percent of the grasshoppers infected with 1 million (1×10^6) spores had died among the various bioassays performed. It typically took grasshoppers inoculated with $1 \times 10^{5.5}$ spores longer to die than it did for grasshoppers infected with 1×10^6, and fewer grasshopper had died by the 20-day bioassay period (generally 40 to 90 percent). The mortality rate for grasshoppers dosed with 1×10^5 spores tended to separate gradually from the control mortality, and usually became consistently apparent only after 16 days p.i. Mortality in grasshoppers infected with the two lower concentrations (1×10^4 and $10^{4.5}$) did not consistently differ from those of the controls even at 20 days p.i. The calculated LD_{50} for the bioassay shown in figure I.3–1 was 1.19×10^5 at 20 days p.i.

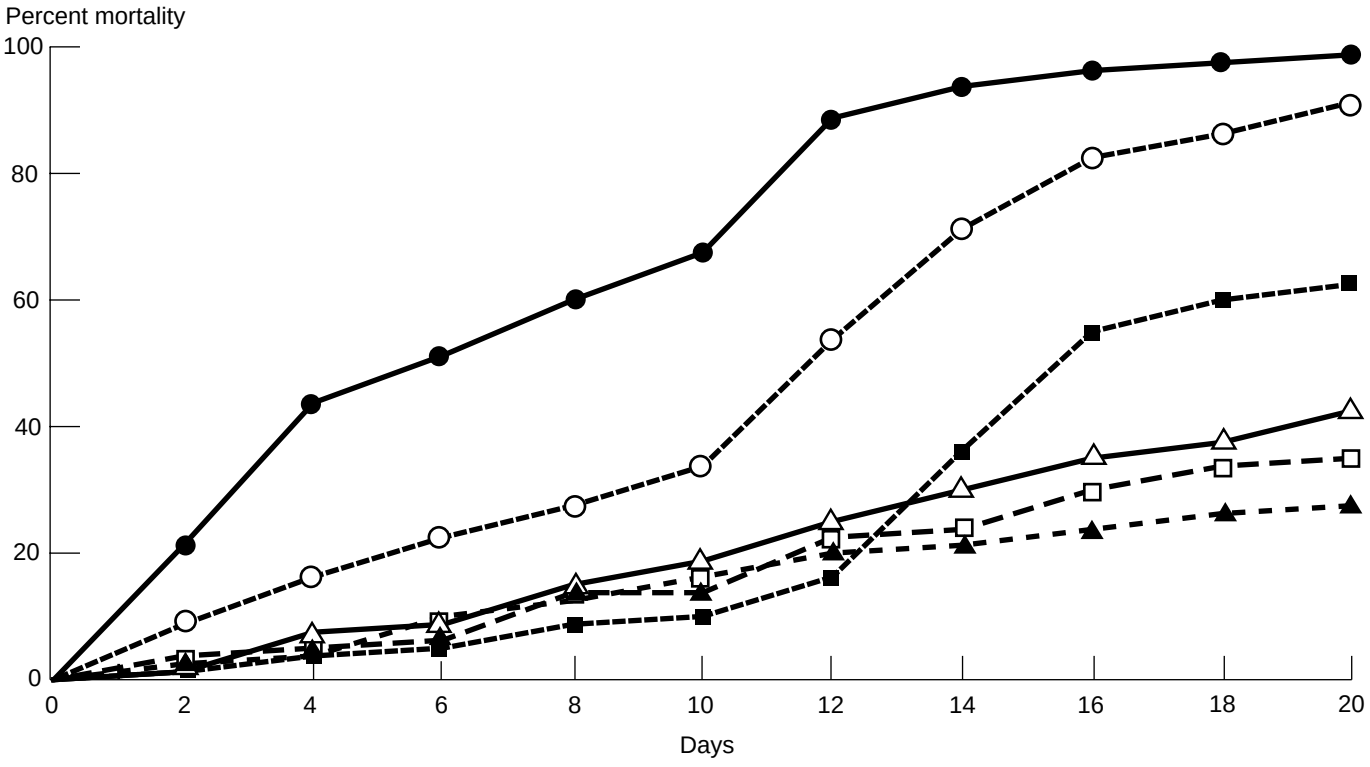

Figure I.3–1—Percent mortality of a 1990 tube bioassay involving third-instar *Melanoplus sanguinipes* grasshoppers treated with varying dosages of *Nosema locustae* spores on lettuce disks (e.g., 0, 1×10^4, $10^{4.5}$, 10^5, $10^{5.5}$, 10^6) and maintained for 20 days postinoculation at approximately 86 °F (30 °C). Solid triangle = 0 spores/grasshopper, open square = 1×10^4 spores/grasshopper, open triangle = $1 \times 10^{4.5}$ spores/grasshopper, solid square = 1×10^5 spores/grasshopper, open circle = $1 \times 10^{5.5}$ spores/grasshopper, and solid circle = 1×10^6 spores/grasshopper.

Bran Bioassay

Methods.—To calculate the theoretical quantity of spores present on average-size flakes of commercially formulated bran, we filtered several grams of the bran through a series of wire sieves with diminishing pore sizes. Most of the flakes were collected on three sieves with pore sizes of 2.36 mm (mesh 8), 2.00 mm (mesh 10), and 1.70 mm (mesh 12). From each of these sieves, 100 flakes were weighed individually. The quantity of spores on each group of flakes was calculated based upon the assumption that each pound of bran contained 1×10^9 spores.

As described in the results section, the theoretical concentration of spores on flakes of commercially formulated bran (an average 1-mg flake should contain 2.2×10^3 spores) was roughly 100 times lower than the concentration of spores easily detected in laboratory bioassays ($1 \times 10^{5.5}$ or 3.16×10^5 spores could easily be detected based upon their effect on grasshopper deaths).

Therefore, in order to bioassay spores on a single bran flake, it was necessary to formulate new bran with spores at a concentration 100 times that of commercially formulated bran (10^{11} spores/lb instead of 10^9 spores/lb). The spores were sprayed onto wheat bran while continually mixing the bran with a small cement mixer. These spores had been recently recovered from grasshoppers and bioassayed on lettuce in our lab (LD_{50} value was 3.29×10^5). In addition to the spores, the spray solution contained 0.2 percent weight to volume (w/v) hydroxymethyl cellulose in distilled water. Hydroxymethyl cellulose is thought to help the spores stick to the bran (Henry et al. 1973). An aerosol sprayer was used to spray the solution on the bran. The treated bran was then allowed to dry and was stored at 39 °F (4 °C).

Attempts were made to bioassay the 100×-treated bran using the same approach used for the lettuce bioassay. One week after formulation of the 100× bran, third-instar grasshoppers were cooled as described above and distributed individually into glass vials. The grasshoppers were starved for 24 hours, randomized, and divided into four groups. Treated bran flakes of different sizes (sieved through mesh 8, 10, or 12) were added to each appropriate vial. Untreated control flakes (sieved only through mesh size 10) were added to the tubes containing control grasshoppers. Once 80 grasshoppers from each group had consumed all bran flakes, they were placed in groups of 5 into 16 bioassay tubes and maintained as described for the lettuce bioassay.

Results from the single-flake bran bioassay study suggested that each grasshopper needed to consume additional bran before any effect could be detected. Therefore, an attempt was made to enable each grasshopper to consume a maximum quantity of treated bran before inclusion in a second bioassay. For that bioassay, 100 grasshoppers were maintained in a large screened rearing cage ($30 \times 32 \times 55$ cm) for 48 hours. The only food source during this time was 2.0 g of control or treated bran contained in a standard petri dish. After 24 hours, the uneaten bran was replaced with fresh. Weights were determined from each container of bran and compared to the weights of similar bran maintained similarly just outside the cage. At the end of the bioassay period, the grasshoppers were maintained in bioassay tubes as described for the single-flake bioassay.

Results.—The average weight for each size of Nolo Bait® bran flakes and the estimated number of spores per flake are shown in table I.3–1. The average values ranged from 1.42 mg for larger flakes sieved through mesh 8 to 0.625 mg for flakes sieved through mesh 12. If 1×10^9 spores are added to each pound of bran, then each milligram of flakes should contain 2.20×10^3 spores; therefore, the largest flake weighed in this study (2.2 mg) should contain 4.85×10^3 spores.

Figure I.3–2 illustrates the mortality rates of grasshoppers fed only one flake of 100×-treated bran from each of the various sieves. Because the average flake of bran weighed 1.05 mg, it should contain approximately 2.32×10^5 spores. After 30 days, the mortality rates from the experimental groups of grasshoppers were not significantly greater than that of the controls. In fact, fewer of the grasshoppers receiving the small flakes of experimental bran died than did the control. Unfortunately, however, the mortality rate for the control grasshoppers in this experiment was twice that of previous experiments, and may have obscured any small effects caused by *Nosema.*

Table I.3–1—Average weight in milligrams for each size of bran flakes and estimated spores per flake

Mesh size	Average weight ± standard error of the means	Weight range	Estimated spores per flake
8	1.422 ± 0.0029 mg	0.9–2.2	3.132×10^3
10	1.107 ± 0.0031 mg	0.5–2.2	2.438×10^3
12	0.625 ± 0.0030 mg	0.1–1.1	1.377×10^3

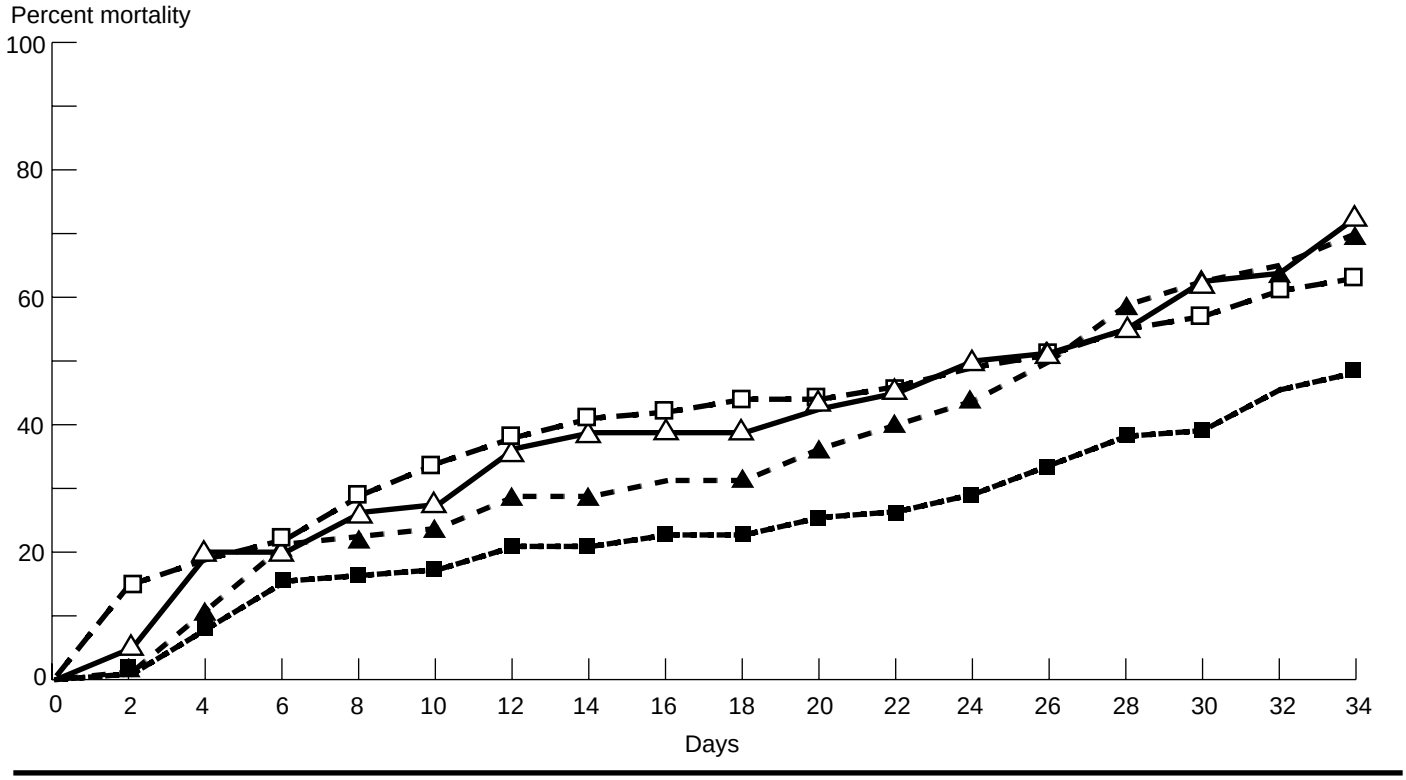

Figure I.3–2—Initial tube bioassay involving *N. locustae*-treated wheat bran flakes given individually to third-instar *M. sanguinipes* maintained for 34 days postinoculation. Solid triangle = grasshoppers given an untreated flake of bran; open triangle = grasshoppers given a treated bran flake that passed through a mesh 7 sieve but not the mesh 8 sieve; open square = flake passed through mesh 8 but not mesh 10; solid square = flake passed through mesh 10 but not mesh 12. Spores had been added to the bran at a concentration of 1×10^{11} spores per pound of bran.

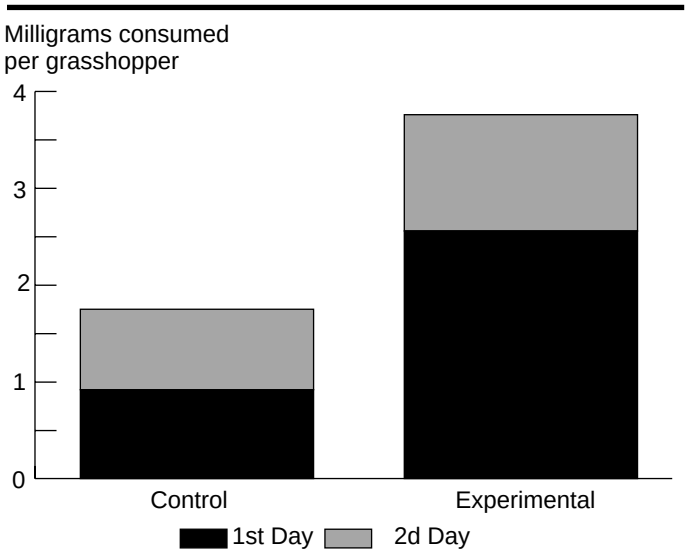

Figure I.3–3—Consumption of control and experimental *(Nosema*-treated) bran by 400 grasshoppers in each group during the first and second day of the inoculation period. Values are expressed in "grams consumed per grasshopper."

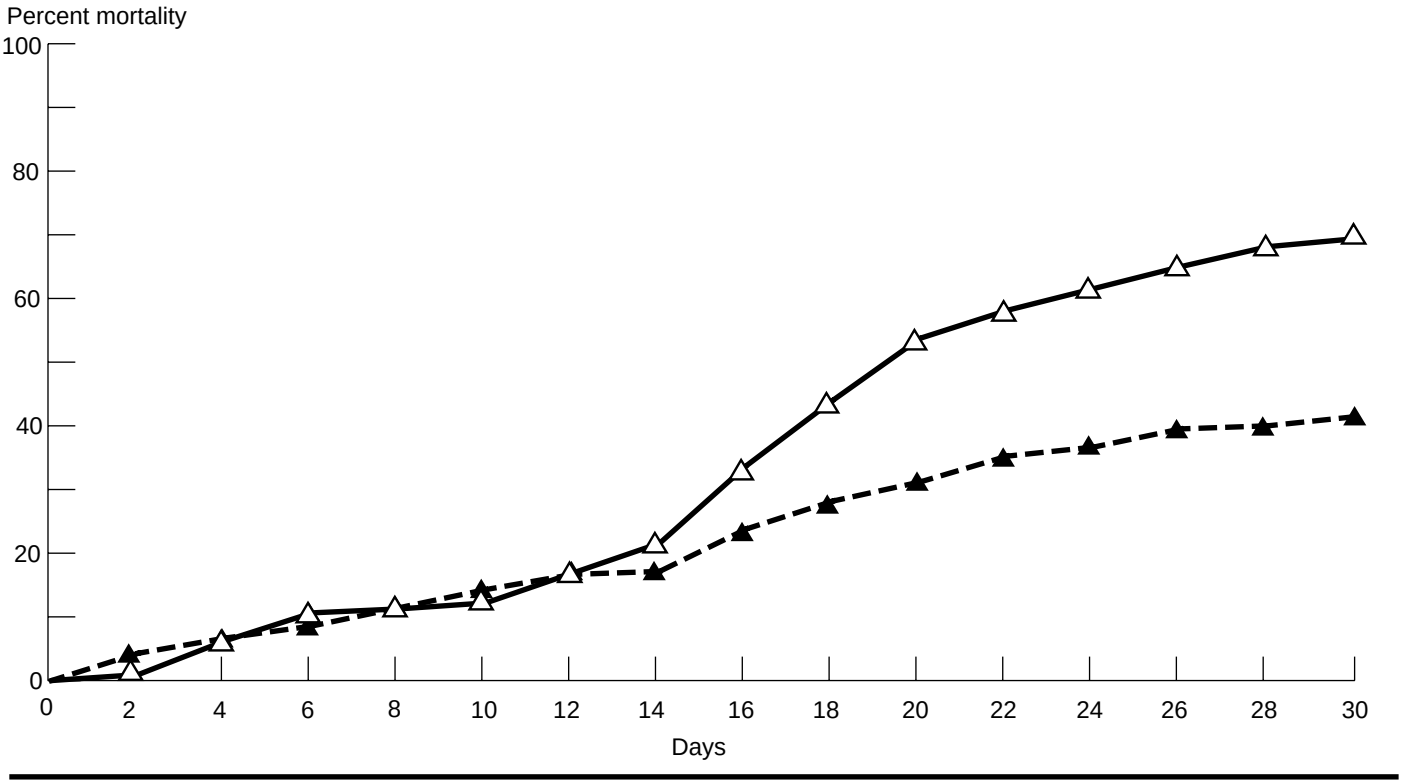

Figure I.3–4—Tube bioassay involving *N. locustae*-treated wheat bran given ad lib (from a petri dish) to third-instar *M. sanguinipes* maintained for 30 days postinoculation. Solid triangle = grasshoppers given untreated flakes of bran; open triangle = grasshoppers given 100×-strength treated bran.

Grasshoppers given as much of the 100×-treated bran as they wanted for 2 days consumed an average of 2.56 mg on the first day and 1.20 mg on the second. Therefore, each experimental grasshopper consumed an average of 3.76 mg of treated bran (roughly 6 small flakes) or 8.27×10^5 spores by the end of the second day. At the end of 2 days, control grasshoppers consumed less than half of the bran consumed by the experimental grasshoppers (fig. I.3–3). Mortality at 30 days p.i. was 75 percent higher for experimental grasshoppers than for those receiving control bran (fig. I.3–4). Mortality rates increased significantly in the experimental grasshoppers after 14 days p.i.

Conclusions

The LD_{50} values determined through the use of lettuce bioassays described in this chapter are generally similar to values reported in other studies. For example, Mussgnug and Henry (1979) calculated the LD_{50} for *N. locustae* in their study of *M. sanguinipes* to be 1.5×10^5 spores based upon a bioassay conducted for 24 days. In lettuce bioassays conducted at SDSU, spore quantities below 1×10^5 did not exhibit mortality rates that were consistently higher than those of the controls. Because the average bran flake from commercially prepared *Nosema*-treated bran theoretically contains only 2.32×10^3 spores, each grasshopper would need to ingest 43 flakes of treated bran to become inoculated with 1.0×10^5 spores. *Melanoplus sanguinipes* grasshoppers that were given only bran flakes during a 2-day period consumed an average of approximately six flakes of bran. In field studies, it is unlikely that many wild grasshoppers ingested more than 40 flakes of *Nosema*-treated bran; therefore, other factors must have influenced the reported effectiveness of *N. locustae* in the field (Henry 1971).

By formulating bran with *N. locustae* spores at a concentration 100 times that which is generally sold commercially (10^{11} spores/lb versus 10^9 spores/lb), it was possible to measure mortality rates caused by the resultant *Nosema* infections. The results generally are consistent with those reported by Reuter et al. 1990 (unpubl.) when the 100× rate—compared to the standard rate and untreated populations only—resulted in significant mortality to one of two field-treated species tested in cages.

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Bioassays of *Nosema locustae:* An Outline of Procedures

I. Purpose of the outline is to describe two protocols to measure the virulence of *Nosema locustae* spores.

A. First Protocol: used for spores stored in water

B. Second Protocol: used for spores already adhered to bran

II. Lettuce Bioassay

A. Protocol

1. Obtain 1,000 lab-reared, third-instar *Melanoplus sanguinipes* (Canadian strain) grasshoppers.
2. Dilute spores to the following concentrations: 0, 1×10^4, $1 \times 10^{4.5}$, 1×10^5, $1 \times 10^{5.5}$, 1×10^6 per 10μL distilled water.
3. Apply 10μL of the appropriate concentration to 7-mm lettuce disks.
4. Cool grasshoppers to 39 °F (4 °C), and distribute each grasshopper into a glass vial.
5. Add disks to vials and wait until the entire disk is consumed.
6. Distribute grasshoppers into appropriate bioassay tubes.
7. Maintain grasshoppers for 20 days, daily feeding them lab-reared rye grass and sulfa-coated rolled oats.
8. Record grasshopper deaths each day.
9. Calculate the LD_{50} value based upon the total mortality after 20 days p.i.

B. Results

1. Largest number of deaths in the grasshoppers infected with 10^6 spores occurred between 10 days and 14 days p.i.
2. Calculated LD_{50} for the bioassay reported in this study was 1.19×10^5.

III. Bran Bioassay

A. Protocol

1. Formulate *Nosema locustae*-treated bran at a concentration of 1×10^{11} spores/lb (100 times higher than the concentration commercially available).
2. Prepare two large rearing cages each containing 100 lab-reared, third-instar *Melanoplus sanguinipes* (Canadian strain) grasshoppers.
3. Add 2 g of treated bran (in a petri dish) to one cage and 2 g of control bran to the other cage (add no other food source).
4. After 24 hours, replace each petri dish with petri dishes containing another 2 g of appropriate bran.
5. After another 24 hours, distribute grasshoppers into appropriate bioassay tubes, and maintain as described above for 30 days.
6. Data can be reported only as net percent mortality.

B. Results

1. Consumption of control and treated bran can be measured by comparing the weight of the leftover bran inside each cage to the weight of similar bran stored outside the cage.
2. In our first bran bioassay, on average 3.76 mg of treated bran and 1.90 mg of control bran was consumed by the grasshoppers during the 2-day infection period (theoretically 8.27×10^5 spores consumed per grasshopper).
3. Experimental grasshoppers exhibited a 75-percent increased level of mortality at 30 days p.i. compared with grasshoppers receiving control bran at rates near 2.5×10^9 per ha on 2 kg (approx. 1×10^9 spores/lb) wheat bran.