FIELD EXPERIMENTS WITH THE JAPANESE BEETLE AND ITS NEMATODE PARASITE*

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Introduction

In 1929 a nematode parasite of the Japanese beetle (*Popillia japonica* Newm.) was discovered in one locality in New Jersey (1). The same year Steiner (2) placed the form among the Oxyuridæ, and described it as a new genus and species under the name of *Neoaplectana glaseri.* In 1931 (3), the senior author reported the cultivation of the parasite on an artificial medium, and the following year (4) he published some detailed studies on the subject. Among other matters, it was found that experimental infections of healthy grubs with the second-stage nemas caused a high mortality among beetles in the grub and pupal stages. The parasites infected the host by way of the mouth, developed two or three generations within the body and destroyed the grubs by feeding upon their tissues. The development of *Neoaplectana* continued within the grub cadavers until most of the tissues had been consumed. In dying and newly dead individuals all stages of nematode development were found; in cadavers that had been dead longer, the second-stage or free-living invasive form dominated. These free-living forms vacated the grub remains to seek other victims after everything had been consumed. The entire life cycle of *Neoaplectana,* corresponding to the life history within the host, was successfully cultivated upon a special artificial medium (3, 4). A generation developed every 4 or 5 days and cultivation apparently did not alter the pathogenicity of the nemas for Japanese beetles. Preliminary field experiments, on a small scale (4), indicated that the parasite could be established in a region where it did not occur naturally and, when so established, produced a high mortality.

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The field experiments initiated in 1931 on a small scale were continued. It seemed desirable to determine whether or not the introduced parasites had permanently established themselves; in other words, whether they could remain dormant through successive winters and become parasitically active again during the warm months.

Large field experiments were also planned and executed with two chief questions in mind. First, how can the nematodes be best introduced and established on a large scale in a region so that they will become an important control factor? In the solution of this problem the number of the host population in an area must be considered, as well as the actual method for introducing the parasites. Second, with all the variable factors which are encountered in the field, is it possible to obtain quantitative results which will enable an appraisal of the extent of parasitism and the consequent reduction in host population? Quantitative data, on work with insect parasites generally, are much needed at the present time. No criticism can be made of those few striking cases where a complete or nearly complete extermination of a pest has been accomplished by means of introduced parasites. In many instances, however, extermination of a noxious insect by introduced foreign parasites has not been effected. Some of these parasites established themselves and a measure of control is claimed for them, but the chief question cannot be answered from the available data. The problem of how much host reduction the parasites accomplish—*i.e.*, how effective the parasites really are, remains unsolved. In fairness to certain workers with insect parasites, it must be stated that the Japanese beetle during the grub stage lends itself very well to investigations of a quantitative nature. The larvæ live in the soil and consequently, by making a large number of standard diggings and counting the larvæ so obtained, a fairly reliable index of the population from year to year can be obtained. The effect of a parasite on such a population should yield fairly reliable results. One section of the present paper deals with quantitative aspects obtained during the course of some field work with the nematode parasite of Japanese beetle grubs.

Further Observations on Two Small Plots Inoculated with Nematodes in 1931

In the spring of 1931, as described in a previous publication (4), two localities in southern New Jersey were chosen on two separate farms about two miles apart. On each farm an experimental and a control plot, separated from each other by 150 yards, were selected. Each plot comprised 6 square feet and originally contained between four and five hundred grubs. Boards were driven one foot into the ground to enclose the grubs and to prevent their lateral migration out of the plots. During the summer the insects remain in and near the root system of plants and do not migrate vertically more than 3 or 4 inches.

On May 15, 1931, the soil within the four plots was carefully sifted to a depth of over 6 inches and all of the grubs examined and counted. They were all found to be healthy and in the second and third instars. On one farm, the grubs were equalized to 600 in each plot. On the other farm, the grubs were equalized to 450 in each plot. Since the grass was entirely uprooted and injured during this procedure, rye for food was heavily sown in the four plots.

On May 18, the soil in one plot, A, on the first farm, was treated with a culture of *Neoaplectana* and on May 22, the procedure was repeated on Plot B, on the second farm. The control plots on each farm remained untreated throughout the season. The method for preparing and applying the nematode cultures was described in the previous publication.

During the entire season of 1931, 47 parasitized grubs were found in inoculated Plot A and 64 in inoculated Plot B. No cases of parasitism were found in either of the control plots. Table I gives the number of adult beetles that emerged from each plot. Counts showed that a large number of grubs were being lost from some cause other than parasitism. It was discovered that birds were a factor, so screened cages were placed over each plot. However, in spite of the enormous losses, several points were evident. The parasite was established in the field in a region where it did not naturally occur and produced a high mortality, although the percentage of mortality from nematodes cannot be computed because of unfortunate losses from other

TABLE I
Adult Beetle Emergence Over a Period of Four Years in Experimental Plots

Year	No. of grubs in each plot	Adult Emergence				Remarks
		Experiment A		Experiment B		
		Control plot	Infected plot	Control plot	Infected plot	
1931	600 in A plots 450 in B "	50	1	175	30	Each of 6 adults collected from Infected Plot B harbored between 5–8 nemas
1932	200	128	8	158	0	Each of 4 adults collected from Infected Plot A harbored a large number of nemas
1933	200	158	0	143	0	
1934	300	146	0	180	0	

causes. The number of deaths by agents other than nematodes was approximately the same in each of the four plots.

During 1931 after the emergence of the adults, soil was frequently sedimented in a "Baermann isolation apparatus" to see if the second-stage nemas were still active. Each test revealed many of the parasites which were always cultured to the adult stage for accurate identification. *Neoaplectana* was not recovered from similar samples of soil taken from the control plots nor from samples of soil from eleven other localities in the vicinity. Samples immediately outside of the infected areas were also frequently tested during 1931 for the presence of the parasite with negative results. Therefore no evidence on the migration of *Neoaplectana* from its place of introduction was obtained at this time. However, it will be recalled that, for the purpose of the experiments, the grubs were prevented from migrating laterally. This restraint probably also assisted in keeping the nematodes within the circumscribed area.

The small plots were studied from 1931 through 1934 (Table I). Each fall after the adult emergence a certain number of grubs were always added to the soil in each plot. In the spring two grub equalizations were made; one in April, the other during the latter part of May. In the plots inoculated with nematodes the first lots of grubs were always so reduced numerically by the parasites within 3 or 4 weeks that a second grub introduction was made. After the pupæ are fully formed, at the middle of June or later, grubs that have escaped infection or are possibly immune reach maturity.

The figures in Table I, second column, represent the numbers of grubs added each year during the last equalization. In infected Plot A no adults have emerged during the past 2 years, and in infected Plot B none have appeared since 1931. The plots were examined at intervals for parasitized grubs. During 1932 infected Plots A and B yielded 133 and 93 cases, respectively. During 1933 Plot A yielded 48 cases; Plot B was only disturbed once that year when 5 cases were collected. During 1934, 15 cases were found in Plot A and 46 in Plot B. No parasitized cases were ever found in the two controls. The data given do not present a complete record of all of the parasitized material. The observations were necessarily intermittent and during the intervals many cases undoubtedly disintegrated beyond recognition. Only diseased individuals and cadavers that revealed large numbers of the specific parasite were recorded.

The table giving the adult emergence shows losses in the control plots. Those for 1931 may be largely accounted for by birds. This cannot be true for the losses sustained during the succeeding 3 years because the plots were screened. Some trouble was encountered during 1932 and 1933 with moles and this was corrected by transferring the soil two feet in depth from each plot to wooden frames with bottoms of copper screen. The plots were now protected from birds, moles, and rodents. Nevertheless, during 1934 the two control plots showed decided losses which may possibly have been due to bacterial and other diseases.

As recorded in the table, during 1931 and 1932 all together 10 adults emerged that harbored second-stage *Neoaplectana* within their intestinal tracts. This observation presents the possibility that the adult beetles, which are vigorous fliers, may assist in the natural dispersion of the nemas.

After emergence in August and September of 1932, 1933 and 1934, samples of soil were sedimented from each of the four plots and in the soil from the inoculated ones, active second-stage *Neoaplectana* larvæ were found in abundance and cultured up to the adult stage. The same tests were repeated with the same result in the early spring before the warm weather had caused the nematodes to become parasitically active among the grubs.

From the evidence presented, therefore, there can be no doubt that *Neoaplectana* became definitely established in the inoculated areas, and caused a high mortality among the Japanese beetle grubs.

Experiments to Test the Possibility of Introducing the Nematodes by Spraying

During the early autumn of 1931, the authors selected some lawn grass which showed considerable damage by Japanese beetle grubs and staked two 15 foot square plots; one was used as a control and the other as an infection experiment. Each area was separated from the other by 15 feet. The grub population averaged ± 22 per square foot in the control and ± 29 per square foot in the other plot.*

On the day the nematodes were introduced each plot was first sprinkled with 100 gallons of water. This was considered necessary because the ground, after a prolonged drought, was exceedingly hard and dry, and it was thought that the parasites might experience difficulty in penetrating the surface. Four large pie plate cultures** with a heavy growth of second-stage nemas on

* The values of 1 square foot diggings were obtained by marking off a square foot on the surface of the ground with the handle of a grubbing hoe notched at the proper length from the end. The turf root system and the soil to a depth of 6 inches were then carefully sifted and examined for grubs. During the warm season grubs remain in and near the root systems of their food plants.

** The pie plates each contained about 60 cc. of dextrose veal infusion agar. The surface was first inoculated with a pure culture of yeast and 24 hrs. later inoculated with second-stage nemas from a Petri plate culture, after washing and sedimenting three times in water. The pie plate cultures were then incubated at room temperature (20–25° C.) for from 2 to 3 weeks. At the end of incubation the nemas had multiplied plentifully, the yeast cells had been consumed, and nearly all of the worms were in the second-stage which is the only stage that survives in the soil. (See literature citations 3 and 4.)

the agar yeast medium, previously described (3, 4), were washed off and suspended in 8 gallons of water and with sprinkling cans rapidly distributed over the experimental plot. Subsequently, although many 1 square foot diggings were made, no cases of parasitism were found. Six weeks after the treatment 4 samples each, consisting of 1 lb. of soil taken at four different points in the infected plot at levels from 1 to 6 inches deep and sedimented, did not yield any parasites. Two months later this procedure was repeated with negative results. Ordinary soil nemas were found in abundance, however.

During the early spring of 1932 the grubs in each plot averaged $\pm$ 22 per square foot, showing that no reduction in the population had occurred and no cases of parasitism were found. During the middle of June the average grub count for the control plot was $\pm$ 20 and for the inoculated plot $\pm$ 15. This slight reduction is within the bounds of experimental error and signifies nothing in so far as the treatment is concerned, because no cases of parasitism were uncovered.

The reason for the failure of this attempt at introduction is difficult to interpret. The method used was similar to the one used on the small plots which were so successful. However, the drought and condition of the soil may have prevented the nemas from penetrating. If they penetrated through cracks and crevices, they may have found conditions too dry in spite of the preliminary moistening, which probably had little effect on the soil below the surface. The grub population, and the dosage of nematodes used may also have been important factors.

In the autumn of 1931, a section of a timothy and clover pasture, showing considerable grub damage, was enclosed by a wire fence to keep out cattle. This area measured 450 feet by 50 feet and yielded an average grub count of $\pm$ 31 per square foot. The enclosed area was divided into three plots each measuring 130 feet by 30 feet. These were so spaced that each was surrounded by a so-called neutral area. A space of 20 feet existed between the plots and 10 feet between them and the fence surrounding the entire enclosure. The timothy and clover were first cut to prevent the nemas from lodging on the vegetation where they would have been rapidly desiccated. The three plots were then well watered from a clean power sprayer. One of the three remained untreated as a control. The second was treated by evenly distributing with sprinkling cans, 6 heavy pie plate cultures in doses of 1 pie plate culture to 2 gallons of water. Although most of the nemas were carefully washed off the surface of the agar, some of them stuck to the medium, so the agar was cut into small pieces and also broadcast over the surface of the ground. The ground was again gently sprinkled with the power sprayer to wash off those nemas that had lodged on the vegetation.

The third plot was treated directly with the power sprayer. This sprayer, of 300 gallons capacity, had not been used for insecticide work for an entire year. The tank, pump, pipes, hose, etc., had been thoroughly washed and treated to eliminate all traces of poison. For the inoculation the surface growth of 7 heavy pie plate cultures was washed into the tank containing 250 gallons of water. The agitator was rotated slowly to keep the worms in suspension and very little pressure was used. Samples of water taken from the end of the spray nozzle showed that the parasites issued alive at the rate of about 5 to 10 per 10 cc. of water.

Subsequently, the three plots were frequently visited and from between 10 and 20 one square-foot diggings made on each plot at every examination. No parasitized cases were found until the spring of 1932 when three typical cases were discovered in the plot treated with the sprinkling cans.

From the autumn of 1931 to the spring of 1934, the grub count dropped from $\pm$ 31 per square foot to about 12 per square foot without nematode parasitism an apparent factor. However, the grub counts in the control plot during two years dropped far below the counts in either of the infected areas until the spring of 1934 when the mean counts for all three of the plots were approximately equal; 15 for the control, 13 for the hand treated and 12 for the machine treated sections. A representative number of diggings in the neutral areas yielded approximately the same grub counts and no cases of parasitism.

The above experiment may be summarized by stating that three cases of parasitism were found in the plot treated with the sprinkling cans about 8 months after the introduction of the nematodes. Nothing was found subsequently. The parasite apparently did not become well established and this fact may be correlated with the rapid drop in grub counts due to other factors. Where grubs are numerically low, the chances for the spread of an infection become less. Other factors, as mentioned previously, such as the method of introduction, the dosage or the character of the soil, etc., might also be important. These factors must be determined, and this can only be accomplished through experimentation and observation over a long period of time. Since the parasite became established, although poorly, further studies of this locality are indicated. For instance, the host population may rise appreciably again and if this should occur, as seems likely, the parasite story might assume a different aspect.

In April, 1932, the experiment just outlined was repeated in another locality. The field used was covered with meadow grass and harbored a grub population of approximately 41 to the square foot. The three plots were handled as above with the exception that the nematode treatment followed a steady, 24-hour rain. From May, 1932, to November of the same year, the grub population gradually dropped to a mean of 3 per square foot. By September, 1933, a rise of 20 to the square foot occurred and in October one parasitized case was found in the hand-treated plot. Although 20 diggings were made in each plot at every visit and samples of soil from them were frequently sedimented, the nematodes were not again recovered. One and a half years intervened from the time of introduction until the single case recorded was found, but this poor result may have been due to the almost complete disappearance of the host due to unknown causes. Obviously, it is important to continue the study of this locality, especially as a gradual rise in population again seems to be in progress. It seemed to be impossible to obtain a population that would remain stable for a period of years.

Experiments on the Subsurface Introduction of the Nematodes

To obtain a reasonably heavy and stable population for at least a few years, the next experiment was conducted in a region invaded by the Japanese beetle during the previous year. It has been claimed that a new, heavy infestation lasts for a few years, then declines and may or may not rise slightly again. Evidence as yet does not exist that the infestation goes through a series of high peaks and low points as is the case with some other insects such as the tent-caterpillar. According to the Government entomologists engaged on the Japanese beetle project, the rise and fall of the pests seems probably to be correlated with the extent of rainfall during July and August when the soil population consists of eggs and first-instar larvæ. A deficient rainfall apparently causes a high mortality during the early stages. It is quite possible that, if several favorable years occurred in sequence, a declining population might again rise heavily. Since the main infestation in New Jersey seems to be moving slowly southward, a freshly invaded section was chosen on its southern fringe.

During the spring of 1933 a site was located on a six acre field of pastureland and an area 120 feet by 80 feet was surrounded by a barbed wire fence as a protection from cattle and pigs. The grass within the fenced area was mowed and three plots were staked off, in such wise that each measured 110 feet in length by 20 feet in breadth. (See diagram.) Each plot was separated from the other and from the fence by 5 feet. Plot A (diagram) was heavily inoculated with nematodes, Plot B served as an uninoculated control, and Plot C was lightly inoculated. The 5 feet strips that surrounded the plots were designated neutral areas. Tables II, III, IV, and V give, among other data, the average number of grubs per square foot for each plot prior to the nematode introduction, and it will be seen that the grub infestation was exceedingly heavy.

Pie plate cultures were prepared on May 9, 1933, from cultures that had been transferred on the artificial medium 7 times at intervals of from 10 days to 2 weeks. By May 23, the cultures were heavy and the nemas were practically all in the second-stage. These cultures were taken into the field and introduced on May 23 and May 29. Seventy-five holes about 3–4 inches deep and spaced approximately 5 feet apart were made on Plot A. One-half of each pie plate culture, together with the agar, was placed in each of the 75 holes. All grubs were carefully replaced, the hole watered with a sprinkling can and the soil and sod replaced. Each hole so treated was marked with a stake and the surface of

TABLE II

EXPERIMENT SHOWING ESTABLISHMENT OF INFECTION AT POINTS OF INTRODUCTION AND SPREAD AWAY FROM THESE POINTS IN PLOT A

Date of diggings	No. of dig-gings	Total No. of grubs	Average per sq. ft	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of dig-gings	Total No. of grubs	Average per sq. ft	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction						Prior to parasite introduction					
4/20/33	10	953	95.3	0	0	4/20/33	10	953	95.3	0	0
After parasite introduction at stakes						After parasite introduction between stakes					
6/12/33	17	214	12.6	6	2.80	6/12/33	7	121	17.2	0	0
9/28/33	32	1104	34.5	8	0.72	9/28/33	20	741	37.0	12	1.62
5/14/34	75	2496	33.2	64	2.56	5/14/34	75	2032	27.0	43	2.11
6/9/34	75	856	11.4	39	4.55	6/9/34	75	909	12.0	28	3.08
9/26/34	75	347	4.5	32	9.22	9/27/34	75	490	6.5	25	5.10
10/24/34	75	156	2.08	16	10.20	10/24/34	75	321	4.2	33	10.28

TABLE III

Experiment Showing Establishment of Infection at Points of Introduction and Spread Away from These Points in Plot C

Date of diggings	No. of dig-gings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of dig-gings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction						Prior to parasite introduction					
4/20/33	10	875	87.5	0	0	4/20/33	10	875	87.5	0	0
After parasite introduction at stakes						After parasite introduction between stakes					
6/12/33	10	117	11.7	6	5.13	6/12/33	10	202	20.2	0	0
9/28/33	22	580	26.3	11	1.89	10/12/33	15	397	26.4	8	2.01
5/14/34	22	460	20.9	13	2.82	5/14/34	22	473	21.5	49	10.35
6/9/34	22	141	6.4	12	8.51	6/9/34	22	196	8.9	7	3.57
9/27/34	22	129	5.8	3	2.32	9/27/34	22	121	5.5	2	1.65
10/24/34	22	50	2.2	0	0	10/24/34	22	107	4.8	1	0.93

TABLE IV

Data from Each Infected Plot Combined

Date of diggings Plot A	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings Plot C	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction						Prior to parasite introduction					
4/20/33	10	953	95.3	0	0	4/20/33	10	875	87.5	0	0
After parasite introduction						After parasite introduction					
6/12/33	24	335	13.9	6	1.79	6/12/33	20	319	16.0	6	1.88
9/28/33	52	1845	35.7	20	1.08	9/28/33	37	977	26.4	19	1.94
5/14/34	150	4528	30.2	107	2.36	10/12/33					
6/9/34	150	1765	11.8	67	3.79	5/14/34	44	933	21.2	62	6.65
9/26/34	150	837	5.6	57	6.81	6/9/34	44	337	7.7	19	5.64
9/27/34						9/27/34	44	250	5.7	5	2.00
10/24/34	150	477	3.2	49	10.27	10/24/34	44	157	3.6	1	0.64

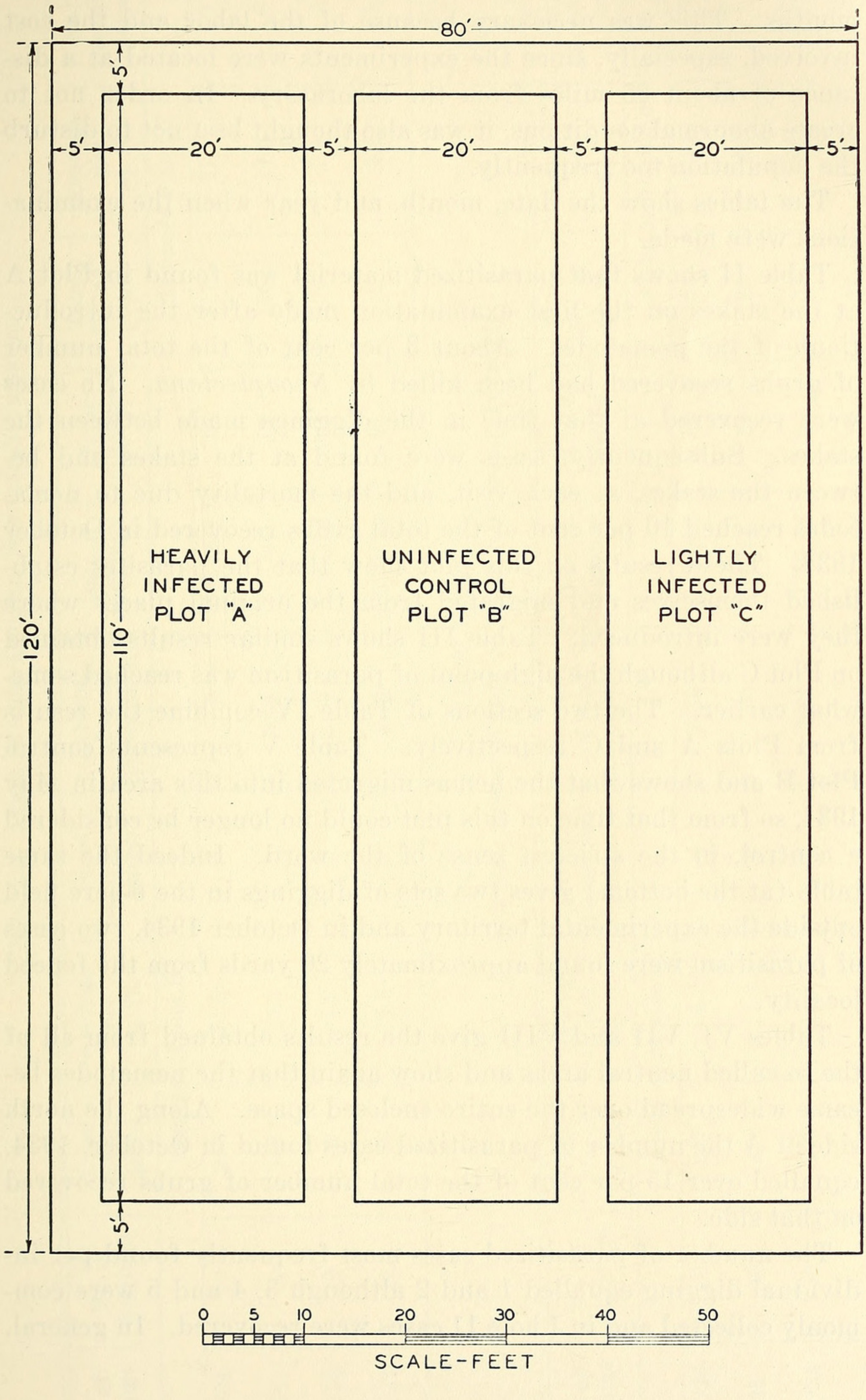
TABLE V
EXPERIMENTALLY UNINFECTED PLOT B WHICH LATER BECAME NATURALLY INFECTED

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total paratisized
Prior to parasite introduction into A and C					
4/20/33	10	787	78.7	0	0
After parasite introduction into A and C					
6/12/33	10	202	20.2	0	0
9/28/33	10	349	34.9	0	0
5/14/34	20	394	19.7	1	0.25
6/9/34	20	252	12.6	2	0.79
9/26/34	20	58	2.9	1	1.72
10/24/34	20	39	1.9	0	0
Six acre field outside experimental enclosure					
9/26/34	110	1025	9.3	0	0
10/24/34	100	719	7.19	2	0.28

the ground at each site was again watered. Plot C was similarly treated except that on May 29, 22 holes were made spaced at 18 to 20 feet from one another. Thus only 11 pie plate cultures were consumed, one-half a culture to each site.

Following the introduction, intermittent diggings were made in the three plots and later also in the neutral areas, and in the 6-acre field in which the experimental area was situated. Within the two experimental plots, diggings were made at the stakes (the points of introduction) and halfway between stakes, to obtain an idea of the rate of the migration of the parasites. Diggings in the neutral areas, the control plot and in the field outside all contributed to this rate of migration and to the final estimate of the value of the parasite. At each examination one square foot of earth was dug, the sod was shaken, the soil carefully sifted and the number of grubs and their instars recorded. A separate record was kept for each digging. A heat sterilized tin can was reserved for each hole and all diseased, dead or otherwise abnormal grubs were taken to the laboratory within 12 to 24 hours for microscopical examinations and cultural tests.

DIAGRAM OF EXPERIMENTAL PLOTS



The field examinations for grub counts and parasitized material were necessarily widely spaced in time, even during the warm months. This was necessary because of the labor and the cost involved, especially, since the experiments were located at a distance of about 85 miles from the laboratory. In order not to create abnormal conditions, it was also thought best not to disturb the population too frequently.

The tables show the date, month, and year when the examinations were made.

Table II shows that parasitized material was found in Plot A at the stakes on the first examination made after the introductioun of the nematodes. About 3 per cent of the total number of grubs recovered had been killed by *Neoaplectana*. No cases were recovered at that time in the diggings made between the stakes. Subsequently, cases were found at the stakes and between the stakes, at each visit, and the mortality due to nematodes reached 10 per cent of the total grubs recovered in October 1934. These results on this plot show that the parasites established themselves and migrated from the original places where they were introduced. Table III shows similar results obtained on Plot C although the high point of parasitism was reached somewhat earlier. The two sections of Table IV combine the results from Plots A and C respectively. Table V represents control Plot B and shows that the nemas migrated into this area in May 1934, so from that time on this plot could no longer be considered a control, in the strictest sense of the word. Indeed the same table (at the bottom) gives two sets of diggings in the 6-acre field outside the experimental territory and in October 1934, two cases of parasitism were found approximately 20 yards from the fenced locality.

Tables VI, VII and VIII give the results obtained from all of the so-called neutral areas and show again that the nematodes became widespread over the entire enclosed space. Along the north side of A the number of parasitized cases found in October, 1934, equalled over 15 per cent of the total number of grubs recovered on that side.

The number of parasitized cases most frequently found per individual digging equalled 1 and 2 although 3, 4 and 5 were commonly collected and in 1 hole 11 cases were recovered. In general,

TABLE VI

NEUTRAL AREAS WHICH BECAME NATURALLY INFECTED

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Between A and B						Between B and C					
5/24/34	10	218	21.8	0	0	5/24/34	10	217	21.7	2	0.92
6/6/34	10	152	15.2	6	3.94	6/6/34	10	106	10.6	0	0
9/26/34	10	69	6.9	2	2.89	9/26/34	10	74	7.4	1	1.35
10/24/34	10	46	4.6	2	4.34	10/24/34	10	43	4.3	1	2.32

TABLE VII

NEUTRAL AREAS WHICH BECAME NATURALLY INFECTED

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Along north side of A						Along south side of C					
5/24/34	10	247	24.7	6	2.43	5/24/34	10	124	12.4	3	2.42
6/6/34	10	118	11.8	0	0	6/6/34	10	95	9.5	0	0
9/26/34	10	109	10.9	8	7.34	9/26/34	10	38	3.8	1	2.63
10/24/34	10	51	5.1	8	15.68	10/24/34	10	28	2.8	0	0

TABLE VIII

NEUTRAL AREAS WHICH BECAME NATURALLY INFECTED

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Along east end of three plots						Along west end of three plots					
5/24/34	10	201	20.1	3	1.49	5/24/34	10	158	15.8	3	1.90
6/6/34	10	85	8.5	0	0	6/6/34	10	99	9.9	0	0
9/26/34	10	5	0.5	0	0	9/26/34	10	45	4.5	1	2.22
10/24/34	10	15	1.5	0	0	10/24/34	10	23	2.3	0	0

the number of parasitized individuals found was probably no absolute index of the mortality due to the nemas. However, the percentages parasitized, of the total number of grubs counted, is a more accurate estimate than would be possible by any other method. The data obtained during April, May and June only cover the larval time up to pupation. It is injurious to the grubs to disturb them when in the process of pupation and a record of a high mortality at this time would be misleading. Data on the extent of the adult emergence during July and early August, as were obtained from the small plots previously discussed, would have also been valuable, but it was impracticable to screen this large area. Autumn examinations were discontinued as soon as cold weather inhibited the grub and nematode activity.

Tables II to V show a general drop in the grub population from April 1933 to October 1934. Some of this downward tendency of the population may be ascribed to the parasites, as will be seen later, but it would be a misrepresentation of facts to ascribe all of it to this cause, because the same tendency was observed in the control plot and in the 6-acre area outside of the experimental enclosure before their invasion by the parasites. Frequently, during the morning hours, the surface of the ground was found riddled with bird holes and starlings, grackles, robins and others were seen feeding upon grubs. Birds, therefore, must have been a factor in this population drop. A similar claim might be made for deficient rain at certain times during the early stages of the insect. Bacterial and other diseases of the grubs were probably also factors.

Notwithstanding the difficulties enumerated and the unknown factors involved, which seem unsurmountable in any large field experiment, the data are considered significant. The degree of significance can only be determined through a statistical analysis which will be presented in the next section of this paper.

Statistical Analysis of the Data from the Previous Experiment

As indicated above, the experiment was begun April 20, 1933, by making diggings on three plots within a fenced area. The grubs found were derived from eggs laid during July and early

August, 1932. These plots were later used for separate experiments. Plot A was heavily inoculated with nematodes; B served as a control and C was lightly inoculated.

The average grubs per plot equalled:

A - 95.3; B - 78.7; C - 87.5

The first question which may be asked is whether or not it is reasonable to assume that the three plots were so chosen as to be random samples of the same general population. Working with the individual samples (10 for each plot) we find the

Variance between plots is 690 with 2 degrees of freedom
Variance within plots is 692 with 27 degrees of freedom

The ratio approximates 1.0 where 3.4 would be necessary for significance. The plots, within the errors of random sampling, may consequently be considered alike in grub population.

The nematodes were inoculated into Plot A May 23 and Plots A and C May 29. Grubs were found infected at stakes June 12, but the nematodes had not spread to "between the stakes."

The data obtained in June show:

Heavily inoculated Plot A at stakes had 12.6 grubs
Lightly inoculated Plot C at stakes had 11.7 grubs
Untreated control Plot B had 20.2 grubs

The points in between the stakes for Plots A and C which evidently correspond to the control show respectively 17.2 and 20.2 grubs. It is evident from the numbers of grubs and the percentages of infected individuals that Plots A and C at the stakes are identical. The same may be said for the points between the stakes and the control plot.

To decide whether or not the amount by which the grub population was lower at the stakes A and C is significant; is the mean 12.3 significantly less than the mean 19.2? Working with the individual diggings, we find the

Variance between the plots is 585 with 1 degree of freedom
Variance within the plots is 60 with 47 degrees of freedom

The ratio $\frac{\text{between}}{\text{within}}$ approximates 9.7 where 4.0 would be significant. It thus appears that the nematodes had significantly reduced the grub population close to the area in which they were planted, the amount of the reduction being perhaps 40 per cent. Interestingly enough, a slightly but not significantly greater number of grubs was found parasitized in the lightly infected Plot C than in Plot A.

The September 28, 1933, diggings followed just after the laying of the eggs by the 1933 crop of adult beetles. One would expect that since the beetles came from all the surrounding territory to lay their eggs the larvæ from them would be evenly distributed over all of the plots. The evidence indicates that this is the case since there is no significant difference between the average numbers of grubs for any of the plots whether at or between the stakes.

The variance between the plots is 463 with 4 degrees of freedom
The variance within the plots is 469 with 94 degrees of freedom

The ratio approximates 1.0 which is clearly not significant.* It is of further interest that the nematodes have at this time spread over both the A and C plots.

May 14, 1934, the following spring, revealed a drop in the grub population from an average of 32 per plot to 27.6 per plot, or 12 per cent. Grubs infected with nematodes have now appeared in the untreated Plot B. Besides the diggings on this plot, examinations were made on the so-called neutral areas surrounding the plots. All but the neutral area between A and B showed invasion by the nematodes. The whole area became covered with this parasite of the Japanese beetle. The greatest drop in the numbers of beetle grubs was obtained in Plot B, from 34.9 to 19.7 per square foot. This drop would certainly not be due to the parasites, if for no other reason than that they have only just invaded this area. Temperature experiments also indicate that until the

* It might possibly be argued that the points in Plot C at stakes and between stakes do not have as many grubs as the control Plot B. Testing this difference gives:

Variance between Plots B and C is 580 with 1 degree of freedom
Variance within Plots B and C is 288 with 45 degrees of freedom

A ratio of 2.0 is obtained where 4.0 is necessary for significance. This comparison simply supports the more general one.

warmer soil temperatures of middle May, the activity of the nematodes is at a low ebb. The significant differences in the drop of the grubs in Plot B as contrasted with Plot A must therefore be attributed to other causes. The most immediately significant fact for the study of the action of the nematode parasite under natural conditions is that Plot A commences the spring, which is the beginning of the active period for the nematodes, with a considerably larger population of grubs than the other plots.

By June 9, 1934, the grubs had dropped in Plot A at the stakes from 33 to 11, a difference of 22, and between the stakes from 27 to 12, a difference of 15. In Plot C at the stakes the grubs dropped from 21 to 6, a difference of 15, and between the stakes from 22 to 9, a difference of 13. The untreated Plot B dropped from 20 to 13, a difference of 7. The drop in the grub population of the treated plots was over twice that in the so-called control plot. This control has now become invaded with parasites and therefore can no longer be regarded as an uninfected control, since part of this drop may also be due to the nematodes. The difference between the drop in the grub population of Plot A, as a whole (18.4 ± 1.08) and Plot B, the control, (7.1 ± 2.36) is 11.3 ± 2.59, and the difference between A plus C (17.4 ± 0.98) and the control (7.1 ± 2.36) is 10.3 ± 2.55. These differences are 4 times their standard errors and are consequently significant. The conclusion that there is a distinct drop in grubs due to the nematode parasite thus appears justified. The numbers of grubs found parasitized by the nematodes have increased.

The September 26, 1934, diggings represent the distribution of the larvæ from the adults of the July and early August flight. Throughout all of the plots there has been a marked decline in their numbers over the 1933 year. All of the area has now become infected with the parasites. The numbers of grubs are now approximately 5 per digging as against approximately 100 or more which were present in the fall of 1932. The question may now be asked whether this infected area has less grubs than a corresponding area in the same locality which has not yet become infected with the nematodes. One hundred and ten samplings from such an area were made, 1025 grubs were found, or 9.3 per digging. In 214 samples from Plots A, B and C 1145 grubs or 5.4 per digging were found. The differences are significant since variance between the groups is 1176, while that within the groups is 47, a ratio of 1 to 25 where a ratio of 1 to 3.8 would be significant. It would thus appear that the grubs of the Japanese beetles are distinctly less in the area parasitized by the nematodes than in the uninoculated area. This fact is supported by more data obtained during the October 24, 1934, diggings.

The General Trend of the Japanese Beetle Infestation in the Experimental Area

The general trend of the population curve of the beetle grubs is rapidly progressing downward in all of the plots. The grub population of the fall of 1932 seems to be at least 10 per cent higher than that observed on April 20, 1933, but supposing they were the same, the change would be that observed on Tables IX and X. This analysis* of these data, for the causes contributory to the reduction in the grub population, shows that the only really significant variable is the year in which the census was taken. Time, which produces the tremendous drop in numbers of grubs from the peak of 95 per square foot in 1932 to 3 per square foot in 1934, is the significant variable. Since the grubs represent eggs from the adult beetles and since these within an infested area presumably distribute themselves at random, it is likely that the character of the plot influences the number of eggs laid upon it very little,—and this is what is found,—the plot population of grubs in the fall is not significantly different from plot to plot. It is in the spring and early summer that the nematodes destroy the grubs as shown above.

The influence of the nematodes is not widespread as yet. It seems to take them quite a while to invade adjoining fields. While, as shown above, it significantly reduces the grubs in its own area, it does not to the same extent influence the July–August flight of beetles coming from the much larger area surrounding these relatively small plots. We must at present regard other

* Since the observational data for the different classes are unequal in number the method used in this analysis is that of Yates, F., *J. Agric. Sci.*, *23*, 108, as cited by Snedecor, George W., in Analysis of Variance, 1934, Collegiate Press, Ames, Iowa, page 96.

TABLE IX

TREND OF GRUB POPULATION IN EXPERIMENTAL PLOTS

	PLOT A			PLOT B			PLOT C			TOTAL		
Fall	No. of samples	Grubs	$(\text{Grubs})^2$	No. of samples	Grubs	$(\text{Grubs})^2$	No. of samples	Grubs	$(\text{Grubs})^2$	No. of samples	Grubs	$(\text{Grubs})^2$
1932	10	935	97304	10	787	67669	10	875	83025	30	2615	247998
	Mean	95.3			78.7			87.5			261.5	
1933	52	1845	96575	10	349	14739	37	977	36171	99	3171	147485
	Mean	35.4			34.9			26.4			96.7	
1934	150	837	12203	20	58	402	44	250	2904	214	1145	15509
	Mean	5.6			2.9			5.7			14.2	
Total	212	3635	206082	40	1194	82810	91	2102	122100	343	6931	410992
	Mean	17.1			29.8			23.1			20.2	
Sum of means		136.3			116.5			119.6			372.4	

$$\text{Mean } \frac{372.4}{9} = 41.4$$

Source of variation	Degrees of freedom	Sum of squares	Variance
Total	342	270986	
Between	8	198881	24860
Within	334	72105	215.88 × .058406

$$\frac{1/10 + 1/52 + 1/150 + 1/10 + 1/10 + 1/20 + 1/10 + 1/37 + 1/44}{9} = \frac{.525652}{9} = .058406$$

Sum of squares

TABLE X
ANALYSIS OF CONTRIBUTORY CAUSES OF VARIATION IN GRUB POPULATION

Sources of variation	Degrees of freedom	Sum of squares	Variance
Between means of 9 classes	8	10755.1	
Between means of plots	2	67.3	33.6
Between means of dates	2	10560.9	5280
Interaction	4	126.9	31.7
Experimental error	334		12.6

factors such as birds, moles, and probably many as yet unidentified, which have reduced the numbers of grubs which followed the successive yearly flights. It is comforting to realize that enemies and possibly other factors (climatic) tend to hold in check such an overgrowing population. It would be of interest to know accurately what each may be and what contribution each makes to the whole control problem. In the present work all that can be said is that the nematode parasite was one such factor, and produced a distinct reduction in Japanese beetle larvae.

Summary

The small field plots inoculated with *Neoaplectana* in 1931 were studied through 1934. These plots were each periodically infested with a definite number of grubs and examined from time to time for parasitized material. The soil was frequently tested for the presence of larval nematodes in the second-stage, which is the only form capable of a free-living existence. Accurate yearly records were kept of the adult emergence from these plots. The evidence obtained showed that the parasite had permanently established itself and produced a high mortality. Large field experiments were also executed and two methods for the introduction of the nematodes were practiced. One method may be defined as surface introduction by spraying; the other subsurface introduction by burying. The spraying method, to date, has not yielded encouraging results. The subsurface method of introduction, however, yielded significant results. The nematodes became established, produced a high mortality, spread over the

entire experimental area and later to the surrounding field. No pronounced difference was noted in the results obtained between the heavily and lightly inoculated plots.

A statistical analysis of the experiment, started in the spring of 1933, showed that the plots within the errors of random sampling were alike in population. Later, during the same spring, the introduced nematodes significantly reduced the grub population close to the area in which they were planted, the amount of the reduction being perhaps 40 per cent. In the autumn of 1933, a fresh crop of beetle larvæ showed a population decline, but no significant difference was noted between the average number of grubs in any of the plots. In May 1934 a further drop in the population, not ascribed to nematodes, occurred in all of the plots. By June, however, the drop in the treated plots was over twice that in the control plot. Since by that time the control also became invaded by the parasites, part of the drop in this plot might have been due to them. The difference, therefore, between the treated and untreated areas is probably greater than indicated. The difference between one inoculated plot and the control and the difference between two inoculated plots and the control constitute differences of over 4 times their standard errors and are consequently significant. During September, 1934, a fresh crop of beetle larvæ showed a further marked decline in population. The parasites at this time were spread all over the experimental area. A test made between this parasitized area and the surrounding uninoculated field showed that the grubs had been distinctly reduced within the former.

Unfortunately it is impossible to maintain a constant, heavy population over a period of years within a given territory. Birds, moles, climate and unknown factors are undoubtedly responsible for this instability, which complicates any experimental procedure.

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