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THE HELMINTHOLOGICAL SOCIETY OF WASHINGTON

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PROCEEDINGS OF THE HELMINTHOLOGICAL SOCIETY OF WASHINGTON

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Golden Nematode Population Studies

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One of the most contradictory phases of all plant nematode experimental work is the apparent irrelation between degree of prior infestation of the soil by the pathogenic organism and the degree of crop damage. This article was prompted by such paradoxes in the results of investigations on the golden nematode of potatoes (*Heterodera rostochiensis* Wollenweber).

All workers in the field have undoubtedly carried out experiments in soil fumigation in which they obtained a greater infestation in treated plots than in controls, in other words a minus efficacy, and yet the crop production was significantly better in treated plots. The natural tendency is to consider that such results fall into error, due to either variation in prior infestation or post-treatment sampling. Farmers regularly comment, with justice, that a field showing considerable crop damage in one year may show little or no damage when planted to the same crop during a following year. They say the nematodes disappear, but this is not strictly true since crop damage due to nematodes will again occur in a future year.

Where do the difficulties lie? Experimental error is merely ignorance of one or more uncontrolled natural variants in nematode behavior, in host relationships of the organism, in the soil, or in other organisms not taken into account. These may include prior soil index, post-treatment index, seasonal effects on host-parasite relationships, and natural population laws.

INDEXING METHODS

SOIL INDEXING: The total number of golden nematode cysts per unit of soil is an obvious and simple figure to obtain. Unfortunately the cyst walls are exceedingly resistant to decay, empty cysts accumulating over a period of many years. Assuming a field were uniformly infested at a prior date, and crop practices had been the same all over the field, the number of cysts per unit of soil should be sound. Workers in all infested countries have long since realized that a total cyst count is no index to possible crop damage.

Two refinements of the total cyst index have been used. (1) Number of viable eggs and larvae per unit of soil. (2) Number of cysts containing viable eggs and larvae per unit of soil. The first procedure is undoubtedly the more exact, but sampling error is so great that such work becomes impractical. The second method has found greater favor even though imperfect.

We have standardized soil indexing, for application both before and after cropping, on the number of viable cysts per 10 gm. of dry soil. A weighed quantity of dry soil is placed in a bucket, roiled vigorously with water, and the

[1] The work reported here includes data on soil profiles acquired jointly with Dr. W. M. Mai and Mr. B. F. Lownsbery of Cornell University.

supernatant fluid poured through 25 and 50 mesh screens. This process is continued until only sand and gravel remain in the bucket. Residue (debris) on the 25 mesh screen is rinsed, then discarded. Residue (containing cysts) on the 50 mesh screen, after rinsing, is removed to a porcelain table; after excess moisture is removed it is well mixed with a spatula, weighed, and aliquot portions are taken on a weight basis. All the cysts from such a portion are dissected, and the number of viable cysts recorded.

PLANT INDEXING: (1) *Stained root technic.* Miles (1930) and Franklin (1938) fixed and stained roots, standardizing on the number of specimens per centimeter of root. The roots may be fixed in 10 percent commercial formalin before the nematodes have had time to mature, thereafter boiled in lactophenol-fuchsin (phenol 100 gm, lactic acid 100 gm, glycerin 200 gm, water 100 cc and 20 cc of 1 percent acid fuchsin), washed, and examined in lactophenol (same formula except without acid fuchsin). We do not consider the estimation of root quantity by measurement as very satifactory. Instead we have weighed roots stripped from haulm after having pressed them to uniform dryness, then cut off aliquot portions of the root mass. When such material is boiled, it is also well to examine the staining fluid because very immature specimens become dislodged in boiling. This procedure has distinct advantages if conducted early in the season, but once the bulk of the population reaches the fourth or preadult stage, the specimens become dislodged in boiling. The great disadvantage is that the procedure is laborious, requiring too much time for indexing large field plots.

(2) *Timed Count Technic.* Chitwood, Clement and Gordon (1943), Chitwood (1945), and Chitwood and Buhrer (1945) reported on the reduction of nematode infections as a result of certain chemical soil treatments using a nematode index. This index was based on the mean number of nematodes observed on the root system of living potato plants in one minute (Fig. 1). In 1945 extensive tests were made using this method of indexing. It is certainly the most rapid technic yet devised and permits the testing of large numbers of plants in so short a period that degree of population maturity is eliminated as a factor. Light infections are very seldom overlooked. Heavy infections tend to be minimized because only 200 nematodes can be counted in the time given. Defects of the technic are that it is entirely relative, gives no approximation of the actual numbers of nematodes parasitizing the plants, requires experienced personnel and is considered too subjective by most people despite the fact that two equally experienced enumerators counting adjoining plants erred less than 1 percent in their counts on 1,800 plants each.

(3) *Washed Root Technic.* For this purpose potato plants are dug after the nematodes reach adulthood but before the females turn red. As the season progresses the females tend to drop off when the roots are disturbed. Mid-June therefore is the time selected for such sampling on Long Island, New York. Plants are dug, roots gently shaken to remove excess soil, tops cut off, and stems with roots attached placed in a pint jar of 10 percent commercial formalin. This should be done as rapidly as possible, first to assure collection of a uniform proportion of the total root system, and second to cover all plants and plots to be sampled in one or two days, since the records of Chitwood and Buhrer, 1946, have shown rapid changes in the number of females emerged and dislodging from potato roots. The roots are removed from the formalin to an enamelware pan, the dirt and debris also rinsed into the pan. Water is then added and the roots vigorously torn apart by hand to dislodge females. The water in the pan is then poured through a number 25 screen (0.71 mm) and a number 200 screen (.074 mm) U. S. Standard Sieve. Fresh water is added and the process repeated

several times. The roots are removed, squeezed with the hands to remove excess moisture, then weighed. The number 25 screen is then washed vigorously to force through any nematodes caught in the debris. The material caught on the number 200 screen is washed into a 500 cc beaker, using about 200 cc of water. Using a mechanical stirrer, the beaker material is mixed well and 10 one-cc samples are withdrawn and placed in marked syracuse dishes; 5 samples are withrawn from

Fig. 1. Golden nematode females as they appear on potato roots. Photo by W. J. Mead, 1946.

the upper liquid, 5 from the deeper liquid. The volume of the remaining liquid is then measured in a graduate and recorded. The females in the 10 one-cc samples are then counted under the dissecting microscope, using reflected light (Fig. 2). In order to avoid possible confusion with other nematodes, for large quantity work it is preferable to count only tear-drop to spherical-shaped females. Old cysts, being darker, can readily be distinguished and are not counted. The number of

females per gram of root can be obtained by division of the number on the entire root system by the root weight in grams. This technic is relative since no attempt is made to account for larvae entering the root late in the season. A combination of washed root technic plus stained root technic, executed on the same root systems, collected in mid-June on Long Island, would give the most

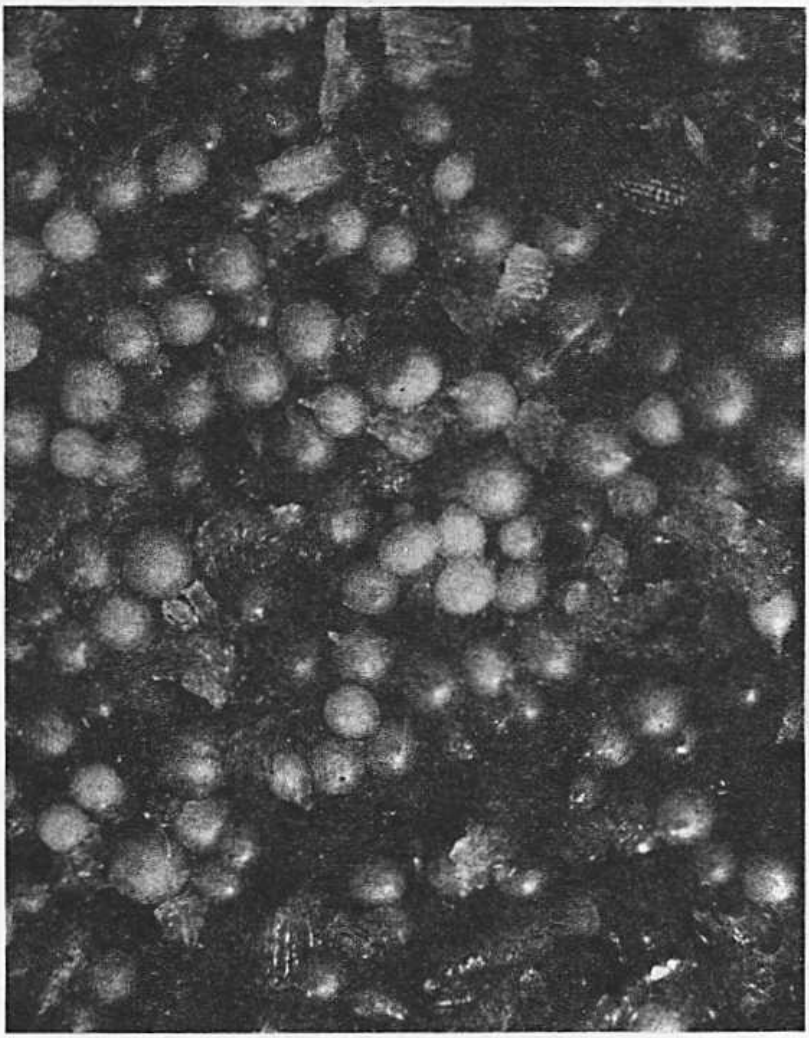

Fig. 2. Golden nematode females as they appear when washed from roots. Kilmer Photos, 1946.

accurate and absolute record of the numbers of golden nematodes actually parasitizing potato plants. Males and very young females, then, would be counted in both technics. Such a refinement, however, would reduce the value of the technic for field use.

When testing the efficacy of fumigants, using a single potato variety as indicator, the calculation of number of new females per gram of roots may be

unnecessary, but when the susceptibility of several varieties must be taken into consideration, an adjustment for quantity of root is necessary. For routine field indexing, no attempt is made to collect the entire root system. A method for adjusting numbers counted in routine indexing to the absolute numbers present may be obtained by making a special collection of the entire root system of a few representative plants.

HOST PARASITE RELATIONSHIPS

Chitwood and Buhrer (1946a and b) showed that root invasion by the golden nematode was minor during periods of weekly mean temperature 51 to 52° F., massive, when the weekly mean temperatures reached 60° F. Further investigations in constant temperature tanks showed temperatures of 53 and 56° F. too low for root invasions. Potato varieties differ as to their rate of growth at various temperatures. In general, early varieties make more rapid root growth than late varieties at temperatures between 49 and 59° F. Consequently they are better able to withstand nematode attack. The duration of mild spring weather, i.e., periods with a weekly mean of 49 to 59° F. may vary from 0 to 6 weeks, and this variance will determine the quantity of root present at the time mass nematode invasion occurs. The damage done to the host plant and the number of nematodes which will mature on it is dependent on this factor. If a half million nematode larvae attack 200 mg. of root, the damage is much greater than if the same number attacks 5 gm. of root.

Rainfall in late season is another variant affecting yields. Lack of adequate rainfall aggravates the damage caused by nematodes. Plants with an injured root system cannot survive as rigorous conditions, consequently they die early.

Crop practices undoubtedly affect the rate of nematode population increase. Killing potato vines before natural death will not permit as many nematodes to mature.

In general, we may conclude that the better the potato crop, the more nematodes it can support, and the greater the population will be built up in the soil as a menace to future crops.

POPULATION

Soil Profile: O'Brien and Prentice (1931) reported finding cysts at depths of 28 to 36 inches. On Long Island we have no record of cysts at such depths. This is probobly due to the fact that in the infested area the top soil is only 6 to 9 inches deep. Beneath this there is a plow sole of yellow clay. Plowing and cultivation is never deeper than 9 inches, 4 to 6 inches being the usual depth.

In order to obtain some information on the vertical distribution of cysts, trenches were dug, the soil scraped at the side of the trench to remove contaminant soil from other layers, and glass vials pressed or screwed into the trench wall at various depths. Each replication represents a pooled sample from 4 locations, equal quantities in the row and between rows. The mean number of cysts per gram of soil is given in Table 1. This field had been plowed but no crop planted since the previous year when potatoes had been raised.

Samples were taken from adjoining land which had been planted to rye and undisturbed for two years. These samples totalling 1184 grams were from the top one-eighth inch and showed a mean of only 12 cysts per 10 grams, and only 0.5 viable cysts per 10 grams. One to two inch layers showed up to 20 percent viable cysts.

Finally an effort was made to obtain a graphic picture of the vertical distribution of the nematode population in the presence of a growing potato plant. A single plant was selected from a group showing extreme nematode injury

(Fig. 4). This work was done June 25, 1946 at which time current females could clearly be distinguished from cysts left by previous generations of nematodes. A moat was dug around the normal spacial requirements of the plant, 32 inches cross-row and 12 inches in the row. This left a pedestal on which the plant stood. Sections of soil containing roots were dug and bagged according to diagram (Fig. 3). The soil sections were weighed, sieved according to

TABLE 1.—*Depth distribution of golden nematode cysts*

Depth in inches	Replications	Soil Weight (in grams)	Total Cysts per 10 grams	Standard Error
0	2	42	30	7.6
1	3	57	34	6.4
2	5	109	46	2.3
4	5	93	30	5.7
6	6	110	20	13.8[b]
8	6	103	1	2.2
10	9	162	0.6[a]	0.2

[a] Fault of topsoil 5 mm thick, extending into subsoil.
[b] Variation at this level extreme since some samples represented topsoil while others represented hardpan.

described procedure, the sievings weighed, and aliquot weighed parts of the sievings were indexed. The deeper samples were examined in toto to avoid error due to sampling technic of small numbers. Results are given in Table 2.

TABLE 2.—*Golden nematode females and cysts in the soil occupied by a single potato plant.*

Section	Old Cysts/10 gm. soil	New Females/10 gm. soil	Total Root weight	New Females/gm. root
1	40.990	10.662	25.3	809
2 and 4	43.686	2.025	3.19	919
3 and 5	37.030	1.656	1.86	931
6	11.165	0.387	0.31	916
7 and 9	6.806	0.782	2.73	157
8 and 10	5.400	0.316	0.32	541
11	0.251	0.000	Trace	
12 and 14	0.363	0.003	"	
13 and 15	0.110	0.000	"	
16 and 19	0.074	0.004	"	
17, 18, 20, 21	0.017	0.000	"	
22 and 25	0.004	0.000	"	
23, 24, 26, 27	0.006	0.002	"	

The correlation between new female count and potato root weight in a given soil block is obvious. At the 9 inch level the roots spread out flat in a fine lace pattern, with occasional rootlets penetrating the hardpan. According to our calculations, the total number of old cysts in the soil occupied by this plant was 276,823, the new females produced totalled 31,307. Of these 254,601 old cysts and 29,854 new females were taken from the top 6 inches; only 21,555 old cysts and 1,486 new females came from the 6 to 9 inch layer. The section of soil (Block 1) occupied by the potato seed piece, though only 12 inches wide by 8 inches long by

6 inches deep, contained 20,468 of the total 31,307 new females and the bulk of the potato roots. Old cysts in this section showed only 41 viable eggs and larvae in 60 cysts dissected. Hatching in this section was nearly total. In other soil blocks the viability of old cysts was relatively higher. We believe this indicates that root secretions must be in considerable strength to cause hatching, and cysts far removed from roots may not be stimulated.

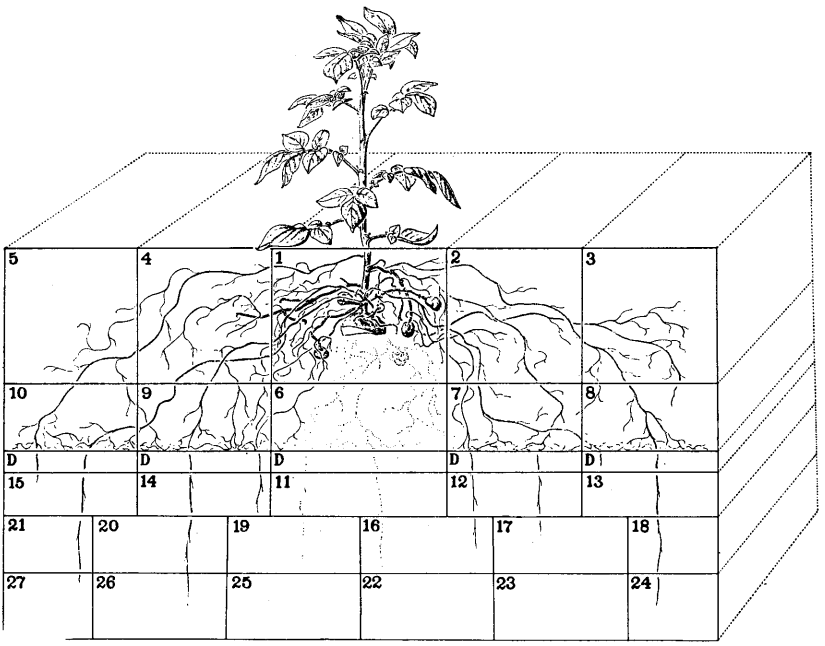

Fig. 3. Diagrammatic representation of heavily infested potato plant showing distribution of roots; soil blocks are numbered. The hardpan represented by D was discarded to prevent contamination of deeper layers.

Sex Ratio: In one series of counts, using the washed root technic, 20,270 new adult females were collected on the 60 mesh screen from one plant. Adolescent females and males were collected on the 200 mesh screen, males numbering 16,467, adolescent females 7,940. The empirical sex ratio was then 16,467 males to 28,210 females or 37 percent males. However, this presumes that there is fully as much chance of adult males remaining in intimate contact with roots as there is of females. This is probably untrue since the male regains motility while the female does not. The other extreme in calculating sex ratio would be to discard from consideration all females caught on the 60 mesh screen, counting adolescent females only. The ratio would then be 16,647 males to 7,940 females or 67 percent males. The true sex ratio probably lies between these two calculations.

In other instances we examined weighed quantities of roots by the stained root technic, counting only specimens of the third stage. The figures were 16, 16, 44, 39 and 18 males as against 21, 17, 47, 28 and 24 females or a total of 133 males to 137 females. For practical purposes it would seem reasonable to assume a sex ratio of 1: 1 or 50 percent of the larvae as potential males.

Field Records of Population Increase: Johnson and Thompson (1945) working in England found the number of viable cysts per 10 grams of soil, based on counts before and after cropping, tended to demonstrate the highest rate of in-

crease in the more lightly infested soils and the lowest in the more heavily infested soils. Crop damage was noted when the population before planting reached 4 viable cysts per 10 gm. of soil. Our field records in connection with soil fumigation experiments gave much the same results (Table 3).

TABLE 3.—*Viable cysts per 10 gm. soil before and after cropping with potatoes.*

Before Cropping	After Cropping	Rate of Increase (Decrease)
9.38	6.48	0.69 ×
3.90	5.05	1.30 ×
0.91	5.31	4.84 ×
0.11	0.83	7.54 ×

This result was striking, particularly inasmuch as the heaviest infestation resulted in a *net reduction* in viable cysts per unit of soil.

A small isolated infestation was found in a field in the fall of 1946. Soil collections were made and weighed; calculations indicated a total locus count of 2551 cysts (1624 of which were viable). It was possible to trace the history of this field and establish the source of the isolated infested spot. In 1943 this field,

Fig. 4. A—Normal Green Mountain variety potato plant, grown on chemically treated soil. Approximately 200 females were present on the root system of this plant. B—Heavily infested Green Mountain variety potato plant, growing within 50 feet of that shown in figure 4A. This is the same plant as in figure 3; the total female nematode count being approximately 31,000.

for the first time, had been operated by continuous cultivation with an adjoining, heavily-infested field, located 1000 feet away from the above-mentioned spot. At that time root examinations revealed many golden stage nematodes on those of its plants located proximal to the edge of the heavily-infested field, and occasional white females which gradually tapered off in number as the distance increased away from that field, disappearing at 400 feet. Inasmuch as these females were still white, they are certain to have invaded the roots rather late in the 1943 season.

Presumably, the locus found in 1946 (at the 1000-foot spot) had resulted from a single viable cyst which was dropped from the cultivator in 1943. If this is correct, we are justified in computing the empirical rate of increase of the nematode over the four-year period (1943–1946) as the fourth root of 2551, or 7.11 ×. A similar locus found in another field with identical circumstances showed an empirical rate of increase of 7.20 ×.

Experimental Inoculation: Carroll (1933) and Carroll and McMahon (1935) using cysts collected from roots as inoculum for 9 inch pots (approx. 5000 gm. soil) found that 5 cysts per cc or 40 per 10 gm. soil caused potato sickness in its most severe form. Rates of cyst production varied from 0.72 × at the concentration of 5 cysts per cc of planting soil to 14 × at a concentration of 0.25 cysts per cc. The maximum total cyst production amounted to 15 new cysts per cc or 60,000 per plant.

In order to gain a more exact picture of the rate of reproduction of the golden nematode, cysts were washed from soil in quantity, the sievings weighed, and aliquot portions indexed for viable cyst content. This material was then used as inoculum for 4 inch pots of clean soil, each pot containing approximately 456 grams of soil. Inoculum was varied from 1 to 12,000 viable cysts per pot. A single seed piece of Green Mountain variety potato was planted in each pot. At maturity the entire pot content was washed and the number of new females counted by aliquot dilution. Roots and tuber weights were also taken. Records were based on 5 replications except in pots inoculated with a single cyst, in which case there were 9 replications. Results are given in Table 4.

TABLE 4.—*Effect of quantity of inoculum on rate of reproduction, tuber weight and root weight.*

Inoculum in Number of Viable Cysts	Number of New Females/ Plant		Rate of Reproduction (NF/VC)[a]	Tuber wt. in grams	Root wt. in grams	Mean New Females per gram of root
	Mean	Standard Error				
0	0.0	0.0	0.0	8.5	7.2	0
1	7.5	2.4	7.5	10.9	6.8	1
10	231.6	12.0	23.2	8.1	6.5	35
20	358.6	89.6	17.9	8.1	7.0	51
40	357.4	126.0	8.9	14.3	7.9	45
80	1555.0	337.2	19.4	13.1	8.1	192
160	2598.5	238.8	16.2	10.9	6.8	382
320	3719.4	465.9	11.6	9.9	7.8	477
640	4033.2	600.0	6.3	6.9	7.0	576
1220	6008.5	879.1	4.9	6.6	8.0	751
2610	5925.2	333.9	2.3	5.6[b]	6.4	926
12000	6401.0	158.7	0.5	4.6[b]	5.2[b]	1231

[a] New females per plant divided by viable cysts used as inoculum.
[b] Significantly different from control with odds of 19 : 1.

These data show clearly that a few nematodes cause no damage. We may well suspect that a moderate quantity (40 to 80 full cysts per pot) might stimulate tuber production (in these cases the difference from controls was just short of significant). This is indeed an unexpected possibility and will require a great deal of proof. It is not totally illogical since Chitwood and Buhrer (1946) have shown that under proper weather conditions nematodes may cause root proliferation.

Marked drop in the rate of reproduction is correlated with drop in tuber weight and in the maximum inoculum (12000 full cysts per pot) there was a decline in root weight. Examination of small pieces of root earlier in the season showed necrotic areas literally teeming with first stage larvae. At plant maturity such roots no longer exist. The nematodes entering in extreme mass invasion kill the roots and themselves. This mass suicide is a major factor accounting for the drop in rate of reproduction. It also explains why a heavily infested field may have seasons of extremely poor yields followed by seasons of moderate yields. We may well conclude that nematode crop damage is chiefly due, not to the nematodes which mature on a plant, but to the excess of nematodes which fail to mature.

Projection of Data: Application of the information obtained by pot experiments to field production requires some adjustment. Certainly a potato plant growing in the field would make more growth and would produce more nematodes than such a plant in a 4 inch pot. Adding the data from various sections of the soil profile, we have seen that a heavily infected potato plant produced approximately 42 grams of root, while in 4 inch pots a heavily infected plant produced only 5.2 grams of root or one-eighth as much. This is probably the most logical comparison of nematode production in pots with production in the field. For the

TABLE 5.—*Calculated new female production with cyst concentrations of the soil before and after a crop of potatoes.*

Number of Viable Cysts per Plant		Number of Viable Cysts per 10 gm. Soil	
Before Planting	After Harvest	Before Planting	After Harvest
8	60	.00112	.008
80	1,854	.0112	.261
160	2,869	.0225	.403
320	2,859	.045	.402
640	12,440	.089	1.749
1,280	20,788	.179	2.936
2,560	29,755	.359	4.183
5,120	32,266	.719	4.536
9,760	48,068	1.372	6.757
20,880	47,402	2.949	6.663
96,000	51,208	13.479	7.197

purposes of projection of data, the inoculum and resultant new female production have been multiplied by 8, the concentration of cysts in the soil calculated on the basis of the 156 pounds of top soil normally allotted to a potato plant in the field. These figures are given in Table 5. A graph was constructed on the logarithms of the concentration of cysts in inoculum and new females produced. On the basis of this graph with our knowledge of the maximum cyst concentration in the oldest known infested Long Island field, we would conclude that the infestation was introduced in 1931 (Table 6). This is based on the introduction of a single viable cyst. Introduction could have been more recent but that would not fit with the case history of the field in question no more than an earlier introduction would fit with the case history.

The graph (Fig. 5) shows no reduction point in nematode production. This we know is not strictly true. Yet since nematode production will fluctuate above and below the point of balance, the progression is probably a fair average figure. A word of caution in the use of Table 6. It would apply only to the precise original field locus and does not represent the average cyst concentration even in the most heavily infested area of a field.

Surplus Populations: For a clear understanding of the many ramifications of population studies, we need a crude audit or accountability of the mass with which we have to deal. In 1945 J. H. Machmer, working on the most heavily infested Long Island field, estimated on untreated land 3,614,500,000 cysts per acre, of which 987,668,000 were viable. The viable eggs and larvae were estimated as 70,285,668,000 per acre. These estimates were based on total cyst counts of 200 samples and 20 cyst dissections per sample. The average planting is 15,000 potato plants per acre. This would give a mean of 4,685,711 available eggs and larvae per plant. With sexes equally divided, we would expect a mean of 2,342,855 new

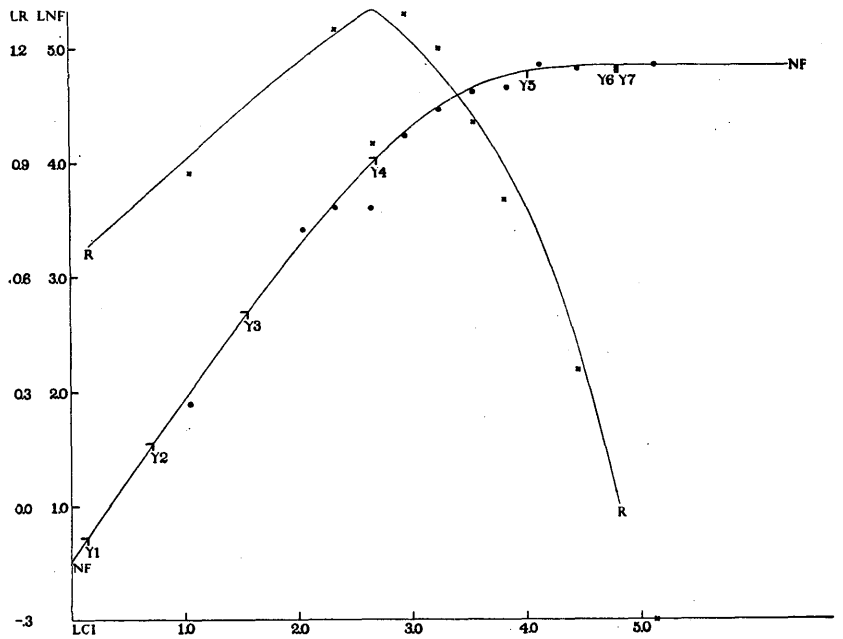

Fig. 5. Curve showing rate of reproduction and increase of golden nematode populations in soil. This is based on tables 4 and 5. NF, new females; LNF, logarithm of number of new females; R, rate of reproduction of females; LCI, logarithm of number of viable cysts in inoculum; LR, logarithm of rate of reproduction, Y 1, 2 etc., years from date of original introduction.

females per plant, if there were no natural mortality. Both in Europe and the United States the maximum recorded female production is between 30 and 60 thousand per plant. That means that not more than 1 in 40 to 1 in 80 nematodes reaches maturity in a surplus population. From the figures on experimental inoculation, it has been shown that a large part of this natural mortality is due to overcrowding, mass root invasion causing root death, reduced tuber production and reduced nematode production being correlated factors. Seasons which favor tuber production will favor nematode production which will contribute to the accumulation of a surplus population. In a year of delayed root growth such a surplus population would aggravate that condition by killing roots as they emerge. If such a year is also characterized by dry weather in late season, the damaged root system is even less able to withstand such rigorous conditions. When such an unfavorable growing season occurs, nematode production will be low and a reasonable crop might be expected in a following season.

The level at which a balance is reached will depend on the variety grown, on mean weather conditions in the locality, on planting, and on harvest dates. The viable-cyst content of the soil at which crop damage might be expected will depend on these factors. In Northern England (Yorkshire) Johnson and Thompson (1945) record severe field damage at more than 5 viable cysts per 10 gm, and between 4.25 and 5 there were patches of damage. On Long Island with the variety Green Mountain in a favorable season there was major crop reduction with a mean viable cyst index of 7.0 and minor crop injury on a portion of the same land with a viable cyst index of 5.6. Little or no crop reduction occurred in the variety Irish Cobbler under similar conditions. In a poor crop season, nematodes aggravated existing conditions so that crop injury was apparent in Cobblers at a viable cyst index of 4.0 and in Green Mountains at 0.5 per 10 grams of soil.

TABLE 6.—*Calculated accumulation of new females and old cysts in soil occupied by one potato plant at original locus, assuming the introduction of one viable cyst in a field. Based on figure 5.*

Year	No. Viable Cysts Inoculum	Rate of Reproduction (NF/VC)	Number of New Females	Cumulative Total	Year
1	1	4.8	4.8	5.8	1931
2	4.8	6.8	32.6	38.4	1932
3	32.6	13.6	443.9	482.3	1933
4	443.9	20.1	8922.4	9,404.7	1934
5	8922.4	5.9	45415	54,820	1935
6	45415	1.1	49956	104,776	1936
7	49956	1.0	49956	154,732	1937
8	49956	1.0	49956	204,688	1938
9	49956	1.0	49956	254,644	1939[a]
10	49956	1.0	49956	304,600	1940
11	49956	1.0	49956	354,556	1941[b]
12	49956	1.0	49956	404,512	1944[c]

[a] Farmer noticed crop damage.
[b] In rotation 1942 and 1943.
[c] Maximum concentration found in fall of 1944, 58 cysts per 10 gm. sample or 412,612 per plant.

Finally we will cite an example of experimental results obtained with a relatively poor soil fumigation on a surpus popuation. As based on 4 replications inoculated with indexed infested soil, 10 plants per plot, there was a 37 percent *increase* in new females *per plant* in the treated plots over that in the controls but an 18 percent *reduction* in the number of new females *per gram of root* in the treated plots. Yield was increased 21 percent in treated plots. The explanation seems obvious that the treatment killed just enough nematodes to reduce the surplus, permitting rather good root growth, thereby increasing yield and nematode production.

Nematode kill, as determined by direct examination of cysts, may differ greatly from kill, as determined by the number of new females produced on a subsequent plant. For example, a 76 percent reduction in viable cysts of an inoculum (after treatment in 1 percent ammonia for one minute at 80° F.) corresponded to only 4 percent reduction in new females produced on potato plants, using equal quantities of inoculum. Similarly a 90 percent reduction in viable cysts (after a 2 minute treatment) resulted in only 49 percent reduction of new females. It was only after treatments reached 98 percent reduction in viable cysts that the efficacy, as based on viable cyst index and new female production, became approximate.

In general, our soil fumigation studies have shown that a treatment causing a 90 percent nematode kill will slightly reduce a surplus population, accompanied by light crop injury. Treatments showing a 99 plus percent reduction in viable cysts have been accompanied by 95 percent reduction in new females the first year and 50 percent reduction in new females the second year. Such treatments permitted two crops without nematode damage.

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Experiences in Culturing *Rhabditis pellio* (Schneider, 1866) Bütschli, 1873 (Nematoda: Rhabditidae), and Related Soil Nematodes[1,2]

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I. INTRODUCTION

Our interest in free-living nematodes of the genus *Rhabditis* Dujardin, [1844], and related soil-dwelling genera[4] (families Rhabditidae and Diplogasteridae) has

[1] This work was instituted at the University of California by both authors and continued at the California Institute of Technology by the senior author.

[2] For the identification of *R. pellio* and of a number of other species in the work reported here we are greatly indebted to Dr. and Mrs. B. G. Chitwood, Division of Nematology, U. S. Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland. Dr. Victor Nigon, Travaux Pratiques de Biologie Animale, Faculté des Sciences P.C.B., Paris (V[e]), has very kindly identified the culture of *R. elegans* Maupas, 1900, used by us.

[3] John Simon Guggenheim Memorial Fellow, 1947–49, and U. S. Public Health Service Postdoctorate Fellow in Cancer Research, 1947–48.

[4] It should be noted that the taxonomy of rhabditid nematodes at the present time is still in a somewhat primitive state; and it is quite likely that the genus *Rhabditis* in particular will undergo subdivision when the species at present assigned to it are subjected to comprehensive study.

been principally conditioned by a combination of features that we feel should make them very valuable for studies on the mechanisms of differentiating growth—studies in which correlations of morphological with physiological mutations, and *vice versa*, should ultimately be possible (Dougherty and Calhoun, 1948a). In order to study the physiological genetics of these forms according to the modern approach of Beadle (1945) and his associates, it is, of course, essential to grow them on a chemically defined medium, *i.e.*, one in which the chemical identity of all food substances, or nutrilites, required for optimal growth is precisely known. The observations reported here have derived from our work toward this goal.

II. HISTORICAL SUMMARY

The culturing of both vagant (free-living) and symbiotic[5] nematodes has been rather widely studied. Certain of these investigations are of significance to the work reported here.

The culturing of vagant, soil-dwelling nematodes has been carried out for many years. One of the earliest indications of the ease with which this can be done was the observation by Dujardin (1844) that nematodes developed in very large numbers in containers in which he kept earthworms with moist soil and moss. Lieberkühn (1855) was apparently the first to note that nematode larvae encysted in earthworms developed, upon the death and subsequent putrefaction of the host, into the sexually mature organisms. In 1866 Schneider also observed that, if earthworms were killed and allowed to rot on moist soil, the decaying tissue swarmed in a few days with nematodes of the *Rhabditis* group in all stages. Perez (1866) in the same year was able to culture a species identified by him as *Rhabditis terricola* Dujardin, [1844], on an artificial medium—albumen from hens' eggs; this was thus the first cultivation of rhabditids in other than soil enrichment or rotting earthworm flesh. According to Maupas (1899, p. 568), Perez actually dealt with more than one rhabditid species.

Various other nineteenth century workers cultivated soil nematodes (Bütschli, 1873; Örley, 1886; Maupas, 1898, 1900, etc.) in the course of systematic studies, but did not describe in detail the techniques employed; they made use of various putrefying materials. A few (Conte, 1900, etc.) made minor study of soil nematode nutrition for its own sake.

A significant departure from previous techniques was introduced by Metcalf (1903), who grew a species identified by him as *Rhabditis brevispina* of de Man (1884)[6] on an agar medium containing asparagus juice. He further devised a method that he claimed would render the eggs of this species germ-free and described excellent growth of worms derived from such eggs, apparently in pure culture, on a medium resulting from the heat-sterilization of asparagus juice agar that had been partly digested by the action of bacteria and a *Fusarium* species. However, our experiences in culturing two species of *Rhabditis* in the absence of other organisms and the extensive work of Briggs (MS) (see Part III) have given strong evidence that at least one heat-labile factor is essential for the growth and

[5] In the etymologically and historically correct sense of the word—signifying parasitic, commensalistic, or mutualistic.

[6] Dr. Chitwood has examined Metcalf's figures of "*R. brevispina*" and has expressed the opinion that the latter very probably was dealing with the same form as de Man—a form that may be designated *Rhabditis brevispina* (Claus, 1862) Bütschli, 1873. (Although Bütschli was the author of this combination, the species to which he applied the name almost certainly was not the same as that originally described by Claus (1862) under the *Anguillula brevispinus* [sic]; nor is it clear that de Man (1844) dealt with Claus's species. However, since Claus's description was very meager, the most satisfactory taxonomic solution would seem to be to accept de Man as having fixed Claus's trivial name *brevispina* on an identifiable form.)

reproduction of these forms. We are of the opinion that Metcalf's medium probably contained some slow-growing microbial contaminants, which his nematodes kept so well grazed down that the presence of the former was never manifest. He did not record any specific tests for sterility except that his "agar remained uncontaminated." In any case, although we do not accept his claim of pure-culturing, the use made by him of an agar medium must be recognized as an important technical advance in the cultivation of nematodes.

Potts (1910), in extending exhaustive studies of Maupas (1899, 1900) on the reproduction of rhabditoids, observed the ease with which nematodes could be obtained from soil in great numbers by placing pieces of meat thereon and allowing these to decay. He subcultured certain species from such soil enrichments by growing them in drops of nutrient solution in watch glasses secured against desiccation by vaseline-rimmed glass covers; he made use of peptone solutions, a saturated gelatin solution, and solutions of tyrosine and leucine, but only in the first of these was growth prolific. He observed that the nematode species studied by him grew best when these solutions contained a dense bacterial growth and very poorly when the bacterial growth was slight.

Johnson (1913) applied Potts's techniques to the study of nematodes occurring as larvae in the earthworm, either encysted in the walls of the coelom or free and active in the nephridial chambers. Other workers have also used liquid media, for example Teunissen (Schuurmans Stekhoven and Teunissen, 1938), who modified the peptone medium of earlier workers by adding potato juice in various proportions. Other investigators, however, have used agar media in studying various soil-dwelling species (Hilgermann and Weissenberg, 1918; Chandler, 1924; Otter, 1933; Dotterweich, 1938; Ludwig, 1938; Stephenson, 1942; Briggs, MS; etc.). With agar media cultivation is much simplified since liquid media containing bacteria can be used only in thin layers, apparently because of the low oxygen tension produced by the bacteria, whereas, in the case of agar media, slants such as are used in routine bacteriological work can be employed and provide excellent substrate in which it is characteristic for many nematode species to tunnel extensively. Reiter (1928) used such solid media as pieces of meat, raw or cooked, and slices of potato; their opacity is, however, a serious disadvantage.

From this period the work of Zimmermann (1921) on the vinegar eel, *Turbatrix aceti* (Müller, 1783) Peters, 1927 (called by him *Anguillula oxophila*) (family Cephalobidae) merits special mention. He was able to free vinegar eel larvae from all associated microörganisms by prolonged washing in sterile water and to initiate cultures which grew through numerous generations on a variety of sterile media, the simplest of which contained peptone, NaCl, lecithin, and an alcoholic extract of yeast. This would appear to be the only acceptable claim of pure-culturing free-living nematodes until recently (Dougherty, in press).

Also worthy of mention is the success of Dotterweich (1938) in obtaining excellent cultures of nematodes by inoculating agar plates containing potato juice (among other ingredients) with samples of soil.

The most comprehensive studies on the nutrition of species of *Rhabditis* have been the work of Ludwig (1938) and the as yet unpublished experiments of Briggs (MS). The former carried out exhaustive investigations on the effect on *R. teres* [= *R. terricola* according to Chitwood (1933)] of adding various chemical substances and vegetable or fruit juices individually to a basal medium developed by Dotterweich (1938). He demonstrated that considerable variability existed in many morphological features and that some of the variations could be correlated in a consistent manner with variations in the substrate. His work is of particular importance in establishing the morphological features that are most probably of general significance in systematic work with rhabditid and related nematodes—

that is, those measurements and, particularly, indices that are most useful as reliable taxonomic criteria.

The studies of Briggs (MS) are the most intensive of investigations so far made on *Rhabditis* cultivation. She was the first to conduct a systematic survey of various substances with which enrichment cultures of soil nematodes could be made by mixing those substances with soil. She found that, in addition to potato and fresh liver, such materials as peanut butter, 10% yeast extract, and gum arabic gave excellent enrichment, and xylan, sodium lactate, soluble starch, calcium arabinate, and inulin were also useful, although giving less abundant cultures. But of much greater significance was her success in replacing the mixed bacterial flora accompanying such enrichment and subsequent transfer to agar media with single species of bacteria. She selected a single vigorous female from a crude culture on agar and with it established a single species line. This species has proved to be *Rhabditis elegans*[7] Maupas, 1900. She then devised a means of freeing the eggs of this species from associated bacteria and established two-membered cultures of her nematode with the bacterium *Pseudomonas fluorescens* Migula in Engler and Prantl, 1895. Her technique is discussed in Section IV. It thus became possible for her to conduct numerous experiments on nutritional and evironmental features in the culturing of *R. elegans*. Among other important discoveries she was able to show that it survived well on 10% yeast autolysate[8] agar in company with a number of non-spore-forming, Gram-negative, rod-shaped bacteria and with one Gram-positive bacillus, *Bacillus macerans* Schardinger, 1905, but not with most bacilli or with other Gram-positive bacteria such as streptococci and staphylococci. Her bacterial stocks were derived from a type culture collection at Stanford University, California. In this connection it is interesting to note that McCoy (1929) in culturing the free-living larval stages of the dog hookworm, *Ancylostoma caninum* (Ercolani, 1859) Hall, 1913; Lapage (1933) in culturing similar stages in species of trichostrongyles of sheep; and Chu (1936) in culturing the free-living phase of a rhabdiasid lungworm of snakes, all on single species of bacteria, found that the Gram-negative, rod-shaped bacteria were the best food for all these forms.

In light of Briggs' work it can be understood that the interpretation of Ludwig's results is complicated by the interaction of substrate and bacterial population. In other words, it is obvious that, insofar as the various substances added to Dotterweich's basal medium favored different constitutions of the bacterial population, at least quantitatively and possibly qualitatively, it becomes impossible to distinguish between the cultural variability resulting from the substances themselves and that resulting from a changed flora. Ludwig did not, it is true, attempt to analyse his results on this basis, but in retrospect it can be perceived that such an anlysis would require that his experiments be repeated using two-membered cultures of *R. terricola* and a suitable bacterium.

Briggs also attempted to grow her nematode in pure culture, *i.e.*, in the absence of other living organisms, using various agar substrates. No autoclaved medium supported development to maturity although larvae hatched from the sterilized eggs. She has not recorded whether molting occurred in these cases. Trials were made by her with various chemical media, autoclaved bacteria, etc., as well as with yeast autolysate, fresh pressed yeast juice, unheated sterile filtrates of bacterial cultures, etc. None supported continued growth although with pressed yeast juice "partial development" occurred. Her attempt, using a sterile filtrate from a *Fusarium* culture, to reproduce with *R. elegans* Metcalf's claim (1903) for *R. brevispina* was unsuccessful. No other rhabditid had been successfully pure-

[7] See footnote 2; Miss Briggs has kindly sent us a culture of her species.
[8] 100 ml. YA, 900 ml. water, and 15 gm. agar/liter.

cultured until the recent work of the senior author (Dougherty, in press) with *R. pellio* and, as reported herein for the first time, with *R. elegans*. Thus claims of pure-culturing exist for only four species of free-living nematodes, all rhabditoids —*Rhabditis brevispina, Turbatrix aceti, R. pellio,* and *R. elegans*.

The only other nematode species recently grown for numerous generations in pure-culture are two insect parasites also of the superfamily Rhabditoidea—*Neoaplectana glaseri* Steiner, 1929, and *N. chresima* Steiner in Glaser, McCoy, and Girth, 1942 (family Steinernematidae). The former was first successfully pure-cultured by the late Dr. R. W. Glaser (1940b) and has been maintained for eight years. The latter was cultivated for some time during World War II (Glaser, McCoy, and Girth, 1942) but allowed to die out (Wm. Trager, *in litt.*). Like the vinegar eel and unlike the rhabditids (except possibly Metcalf's *R. brevispina*) the two neoaplectanas can be grown on an autoclaved medium (Glaser, McCoy, and Girth, 1942); in their case it consists of a semi-solid gel at pH 7.0, in the proportion of 20 gm. of ground beef kidney or liver, 100 ml. of water, 0.5 gm. of NaCl, and 0.5 gm. of agar. In addition to success with *Neoaplectana* the free-living larval stages of another parasitic species, the sheep wireworm *Haemonchus contortus* (Rudolphi, 1803) Cobb, 1898, have been passed free of other organisms—in a medium similar to that first used for *N. glaseri* and devised by Glaser and Stoll (1938); it contained ground-up yeast, aqueous liver extract, and pieces of sterile, unheated rabbit kidney.

Our own studies have been greatly aided by Briggs' work. The results of a year's investigations are presented herein and include our experiences applying her methods, certain advances in technique, and the working out of pure culture methods. Recently we have reported briefly on an improved procedure for preparing germ-free soil nematodes (Dougherty and Calhoun, 1948b), and one of us (Dougherty, in press) has described the first successful pure-culture technique for a species of *Rhabditis*. These subjects are treated more fully here.

III. THE PRODUCTION AND MAINTENANCE OF SOIL NEMATODE CULTURES

A. Soil Enrichment Procedures and Crude Cultures from Earthworms

The technique of placing small pieces of meat on soil and thus obtaining excellent enrichment of the nematode population has, as already been noted, been used by a number of investigators, as for example Potts (1910). It has been only Briggs (MS), however, who has made systematic trials of a number of different substances. We have successfully employed the three of these also tested by us —namely, peanut butter, inulin, and small pieces of liver—and in addition an unidentified species of mushroom.

Briggs' method, as followed by us, is to mix a selected amount of the enriching substance with 10 grams of moistened soil in a Petri plate. In a few days suitable mixtures produce a teeming nematode population. We have made almost no effort to identify the component species of the populations developing on various substrates used by us, although this is a problem well worth investigating. The only information bearing on this subject is the identity of the various species that we have isolated as single species cultures, as discussed in subsection C. Reiter (1928) and Schuurmans Stekhoven and Teunissen (1938) have observed sequences of dominating species in soil enrichments and mixed cultures. From Briggs' and our experiences it is obvious that a variety of substances can serve for enriching the soil populations of nematodes.

The soil used by us was freshly turned up, rather dry, unfertilized topsoil of the Berkeley and Pasadena campuses. Reiter (1928) gives evidence that from different sources, *i.e.*, dung, humus, etc., different dominant species can be obtained. His sources are in a sense, however, soil enrichments. It is nevertheless of in-

terest to correlate the species occurring in soil with its varying chemical and physical nature. Schuurmans Stekhoven and Teunissen (1938) have made such studies.

1. *Peanut butter crude cultures*

A 10-gram sample of soil may be mixed with 1 gm. of peanut butter and 10 ml. of water added to the mixture in a Petri plate. This is kept at room temperature in a dimly lighted cupboard. Within less than a week the surface of the culture teems with nematodes in all stages of development. Such cultures remain rich for about 2½ weeks and thereafter decline.

It has been found that, by lumping up the soil and peanut butter mixture so that an interface is established with the lid of the Petri plate, it is easier to pick out the worms from the flat culture so formed.

2. *Inulin crude cultures*

A 10 gram sample of soil may be mixed with 0.5 gm. of inulin and 10 ml. of water added to the mixture in a Petri plate. A good culture develops by the twelfth day, but not so luxuriant as in the case of peanut butter enrichment. The cultures are maximal at about two weeks.

3. *Liver crude cultures*

A few small pieces of raw liver may be placed on the surface of a soil sample in a Petri dish. In three days nematodes are seen on the bacterial scum that forms on the liver and in five days the rotting meat teems with worms. The cultures remain rich for over a month.

4. *Mushroom crude cultures*

A few pieces of mushroom may be treated in the same manner as the pieces of liver. Nematodes appear within two days and by three days are massively present. The cultures last less than two weeks.

5. *Crude cultures derived from rotting earthworms*

It has long been known that rotting earthworms are an excellent source of some of the largest soil nematodes. Furthermore, it has been demonstrated (Johnson, 1913; Hertwig, 1922) that some species regularly live in symbiosis with earthworms in the tissues and nephridia of the latter.

We have not attempted to identify the earthworm species from which cultures have been developed. Several morphological types have been represented. The worms were all obtained from the bank of a stream on the Berkeley campus of the University of California. All were washed thoroughly. Some were placed individually in Petri plates, cut into small pieces, and allowed to rot in a few millimeters' depth of water. Others were separated according to morphological type and put into Petri plates on moist sand or between moistened paper towels and maintained alive at room temperature.

The cut earthworm pieces were examined for the appearance of nematodes, also the other worms as they died and decayed. The latter died one by one over a two month period. In the case of the group placed on sand the substrate was autoclaved approximately on alternate days. The worms on paper towel were given new substrate every few days. These procedures were designed to eliminate as much as possible nematodes that might have been living facultatively in the gastrointestinal tract and were eliminated with the feces.

In most cases the decaying earthworms produced excellent crops of nematodes in three to four days. The best source was the largest type of earthworm—a red form presumably representing a single species.

B. Subculturing and Maintenance of Soil Nematodes

Agar media slanted in 6 inch test-tubes have been used for all routine subculturing of nematodes derived from soil enrichments or rotting earthworms. For

several months we regularly streaked these slants with *Pseudomonas fluorescens* before inoculating them with nemas; more recently *Escherichia coli* (Migula in Engler and Prantl, 1895) Castellani and Chalmers, 1919, has been used. Briggs used the former bacterium in her studies on two-membered cultures. We have found that agar streaked with either provides better conditions for initial subculturing and succeeding maintenance than agar on which the only bacteria are those introduced at the time of inoculating it with the worms.

The first attempts were made from the peanut butter enrichment to a mixture of 10% yeast autolysate[9] (by volume), 2% dextrose, and 2% $CaCO_3$ added to a 2–4% agar base (YDC); this was recommended by Briggs (MS) as a medium that maintains a flourishing culture for several weeks. She did not, however, use it for routine initial subculturing, and we found that it became rapidly overgrown with bacteria and that nematodes failed in all but a few cases to become established. A few transfers from inulin enrichment cultures to YDC were, however, successful. A simple nutrient agar (N)—2.3% Difco Nutrient Agar—has been found satisfactory for subculturing in many cases and entirely satisfactory for maintaining cultures over repeated transfers.

With the use of N slants streaked with *Ps. fluorescens* or *E. coli* either some time before inoculation or at the same time, crude nematode subcultures have been established from all of the enrichment mixtures. These have been maintained by transfer, at weekly to monthly intervals, of a piece of agar, richly overgrown and tunneled through with nematodes, from the old slant to a new one streaked with *Ps. fluorescens* or *E. coli*. As single species lines have been isolated, most of these crude cultures have been discarded. Over 35 successive transfers have been carried out with some of the crude stocks.

It has been interesting to note that from time to time, as judged largely by the gross appearance of the slants, different elements of the bacterial flora have dominated in our stocks. As a general rule the nematodes have kept the bacterial population considerably depressed. Occasionally densely growing spore-formers have dominated, and these can apparently be much less well controlled by the nematodes. Doubtless a good part of the bacterial flora is harvested by the feeding of the nematodes. However, in view of the fact that isolated specimens apparently cannot utilize certain bacterial species as food, it is interesting to speculate on the possibility that the soil nematodes, when present in adequate numbers, may be able to produce sufficient antibiotic substances to enable them to hold some bacterial species in check.

C. Isolation of Single Species Lines

The following rhabditid species have been established by us in single species lines by the isolation of a single gravid female or of an hermaphrodite on an N slant (numbers in parentheses refer to the number of cultures so established):

Subcultures from peanut butter enrichment:

Rhabditis dolichura (Schneider, 1866) Bütschli, 1873—(5)

Subcultures from inulin enrichment:

Diplogaster americanus Steiner, 1930—(1)

Diplogaster gracilis Bütschli, 1876—(2)

Subcultures from mushroom enrichment:

Rhabditis lambdiensis Maupas, 1919—(2)

[9] Yeast autolysate prepared as follows: 1 kg. Baker's yeast mixed well in 1 liter of water and incubated at 46–48° C for 24 hours; liquid then boiled 5–10 minutes to stop autolysis (watch for foaming); mixture filtered through a layer of kieselguhr and one filter paper in a Büchner funnel (1 cup kieselguhr per liter of autolysate); filtrate adjusted to pH 7 with 1 N NaOH and autoclaved for 1 hour; resulting liquid stored in frozen state. 10% yeast autolysate agar contains per liter 100 ml. YA, 900 ml. of water, and 15 gm. of agar.

Rhabditis, sp. nov.? [near *Rhabditis inermis* (Schneider, 1866) Örley, 1880] —(1)

Subcultures from liver enrichment:

Rhabditis lambdiensis—(1)

Rhabditis spiculigera Steiner, 1936—(1)

Subcultures from rotting earthworms:

Rhabditis pellio (Schneider, 1866) Bütschli, 1873—(15)

The females were usually isolated by placing a group of worms on a clean block of agar and picking out with a capillary pipette or microspatula those worms that crawled free of eggs and other nemas.

In addition to the species listed we have also maintained cultures of an unidentified species of the genus *Bunonema* Jägerskiöld, 1905, derived from inulin enrichment, but we have never been able to establish a line from isolated females, despite numerous attempts. We also have the culture of *Rhabditis elegans*, which was derived by Briggs from peanut butter enrichment.

We have had for the most part no success in establishing single species lines by the isolation of single gravid females directly from soil enrichment cultures. Apparently the dominating bacteria developing when single nematode females are transferred to YDC or N slants from most soil enrichment cultures used by us are inimical to the incipient nematode cultures. It is not unlikely that agar media can be developed that will favor bacteria suitable as food and thus facilitate the isolation of single species lines. Almost all of our cultures of single species, initiated in all cases by the isolation of individual gravid females, have been derived from N subcultures. The only exceptions have been the cultures of *R. lambdiensis*, *R. spiculigera*, and *Rhabditis* sp. derived from liver and mushroom enrichment cultures by the isolation of single females.

It has been very much easier to establish single species lines from crude cultures derived either from liver or mushroom enrichment of soil or from rotting earthworms than from those derived from peanut butter or inulin enrichment: Since the single species lines isolated from these different sources have been different (except for *R. lambdiensis* from both liver and mushroom enrichments), it is apparent that those favored by peanut butter or inulin are not so well supported on N plus the accompanying bacterial flora as are those favored by liver or mushroom.

In connection with our fifteen isolations of *R. pellio* from earthworms it should be noted that there has been in the past a great deal of confusion in the identification of the nematodes found in these hosts. Certain authors (Johnson, 1913; Otter, 1933) have claimed that only one form is a true earthworm symbiote —a species now known properly as *Rhabditis maupasi* Seurat, 1919, but originally described under the name of *Rhabditis pellio* [non *Pelodera pellio* Schneider, 1866] by Bütschli (1873). Paula Hertwig (1922) appears to have satisfactorily demonstrated by careful dissection that at least four species may occur encysted in earthworms—among them the original *R. pellio* of Schneider. Otter (1933), who more recently was only able to isolate one species, not *R. pellio*, but probably *R. maupasi*, overlooked Hertwig's paper; moreover, his experimental work does not appear to have been carried to a very successful point inasmuch as he was unable to get sustained subcultures of the form studied by him.

Our results, although not achieved by careful dissection, appear to support Hertwig's contention that *R. pellio* is a regular symbiote of earthworms. Our form agrees with the original description of Schneider (1866) and with the more recent figures and descriptions of Hertwig (1922), Aubertot (1928), and Reiter (1928). Moreover, Maupas (1899) repeatedly derived this species from earthworms and furthermore stated (p. 623) that only once out of many isolations was

it obtained in culture from a source other than a decaying earthworm. Despite the fact that Bütschli (1873) did not have this form, we are designating the species studied by us as *Rhabditis pellio* (Schneider, 1866) Bütschli, 1873, because he was the first to employ the combination "*Rhabditis pellio.*"

D. Production of Two-membered Cultures

Before pure-culturing could be attempted it was, of course, necessary to devise a method of eliminating at will the bacteria associated with the nematodes in agar cultures. Briggs (MS) accomplished this by treating *R. elegans* eggs for 15 minutes in a 1: 500 clorox solution, washing them three times in sterile water, and exposing them to ultraviolet light (200 μwatts/cm²) for 30 to 35 seconds. In work with *R. pellio,* however, we were unable to succeed with this method. Sterility was never achieved by us. The procedure was furthermore quite tedious especially as the eggs of *R. pellio* are sticky and the majority adhere to the walls of the capillary pipettes used for transferring them. We therefore tried various combinations of antibiotics and disinfectants. After many unsuccessful tries, a method that finally worked with *R. pellio* and more recently with *R. elegans* (a culture with mixed bacterial flora obtained from Miss Briggs) was devised. It has already been reported in abstract (Dougherty and Calhoun, 1948b) and involves the use of penicillin, streptomycin,[10] and merthiolate.

Worms in crude bacterial culture are removed from an N slant by shaking up the tube with a few ml. of water and then transferred by capillary pipette to broth containing 5,000 units of penicillin and 5,000 units of streptomycin per ml. After 24 hours small larvae are transferred to a sterile water rinse and thence to a 1: 1000 aqueous solution of merthiolate. After one hour viable larvae are rinsed once or twice in sterile water, usually containing 1,000 units of penicillin per ml. to guard against chance contamination. They may then be inoculated onto slants, each streaked with a single bacterial species.

In using this method with *R. pellio* in the original experiment, only 1 out of 3 tubes streaked with *E. coli* and inoculated with worms proved to be a two-membered culture. Gram-staining, plating out, and biochemical testing of the bacteria in the other two revealed the presence of two contaminants,[11] forms close to *Corynebacterium kutscheri* (Migula, 1900) Bergey *et al.,* 1925, and *Pseudomonas cruciviae* Gray and Thornton, 1928.

Subsequent use of this technique with other *R. pellio* cultures containing a mixed bacterial flora has never given two-membered cultures in more than 25% of the tubes set up in accordance with the procedures outlined in the preceding paragraph. On the other hand, over 75% of the sterile control tubes inoculated with the same number of worms have failed to show any bacterial growth. The only reasonable explanation for this would seem to be that *E. coli* provides an environment in which bacteria can survive that otherwise would not recover from the effects of the antibiotic and merthiolate treatment to which they have been subjected. It is obvious that some soil bacteria are exceedingly resistant to the agents used.

In the case of *R. elegans,* in contrast to our experiences with *R. pellio,* 6 out of 6 tubes streaked with *E. coli* and inoculated with treated larvae developed as two-membered cultures. However, we do not know the complete history of this culture; it may have been a secondarily contaminated two-membered culture.

This technique for rendering rhabditids temporarily germ-free was empirically

[10] One gram of streptomycin was generously donated for initiating these studies by Merck and Company, Rahway, New Jersey.

[11] These identifications were very kindly carried out by Miss Helen Jackins, Department of Bacteriology, University of California, Berkeley.

derived, and attempts to improve it by varying the lengths of the treatment periods or the concentrations of the agents used have been limited. In a few attempts to obtain germ-free *R. pellio* with only 6 to 10 hours of antibiotic treatment followed by merthiolate we have been unsuccessful. Increase of the concentration of streptomycin to 100,000 units per ml. has not improved antisepsis. The worms themselves can survive both streptomycin and penicillin concentrations of this order for several days.

From our general experiences in obtaining two-membered cultures of *R. pellio* and *E. coli* it is apparent that nematode cultures derived from rotting earthworms may be accompanied by bacterial species very resistant to antibiotic and disinfectant treatment. However, once even one two-membered culture has been successfully derived, repeated disinfection of the nematodes can be accomplished with uniform success. *E. coli* may be eliminated from association with the worms by streptomycin treatment (5,000 units/ml. in broth) for 3 hours in the case of the larvae and 4 hours in the case of the adults. Small larvae may be disinfected with a 1:1000 aqueous solution of merthiolate after one hour's treatment.

R. pellio and *R. elegans* have been found to grow well on N slants in the company of *E. coli*. They have been so maintained for over five months with over 10 transfers.

E. Pure-culturing of *Rhabditis pellio* and *R. elegans*

Aside from Metcalf's claim (1903) of pure-culturing *R. brevispina* there has been no report of the growing of rhabditids under germ-free conditions until recently (Dougherty, in press). As has already been indicated, we are inclined to the view that Metcalf's "sterile" medium actually contained slow-growing bacterial contaminants that provided heat-labile factors necessary for the growth and reproduction of his culture of *R. brevispina*. There is no evidence from Metcalf's paper that these were rigorously ruled out.

Briggs' failure (MS) with a large series of media suggested to us that an essential heat-labile factor is involved in the nutrition of *Rhabditis*. Only on pressed yeast juice did she record any growth of *R. elegans*, and this was limited.

We first tried to effect growth of *R. pellio* under otherwise sterile (*i.e., axenic*) conditions by supplementing N medium with a sterile bacterial plasmoptysate (derived from a marine bacterium, *Pseudomonas* M.D. 33.1[12]), the preparation of which was described by Taylor and van Wagtendonk (1941). It has been used successfully with the ciliates *Colpoda duodenaria* Taylor and Ferguson, 1938 (by Taylor and van Wagtendonk, 1941; Garnjobst, Tatum, and Taylor, 1943; etc.), and *Paramecium multimicronucleatum* Powers and Mitchell, 1910 (by Johnson and Tatum, 1945). We added with a capillary pipette five drops of plasmoptysate prepared in approximate accordance with the method of Taylor and van Wagtendonk (1941) to each of 19 N slants in 4 inch test tubes. To each of these was then added a gravid female *R. pellio*. The tubes were followed for almost a month, and in no case were any larvae evident on the agar surface.

Having failed with bacterial plasmoptysate and reasoning from Glaser, McCoy, and Girth's experiences (1942) with *Neoaplectana chresima*, the senior author next tried growing *R. pellio* on sterile pieces of rabbit kidney. In 5 out of 8 tubes to which 5 to 10 larvae were added, a few larvae matured to adults, but none reproduced. It was, nevertheless, evident that the raw kidney contained an important growth-promoting substance or substances lacking in autoclaved preparations.

It was finally possible to effect axenic culture (Dougherty, in press) with

[12] Very kindly supplied by Professor C. B. van Niel, Hopkins Marine Station, Stanford University, Pacific Grove, California.

the following "basal" medium: 2.5 ml. of Difco Nutrient Agar autoclaved with 0.5 ml. of homogenized liver (0.2 gm/ml of water) and overlaid, after the agar had hardened, with 0.1 ml. of sterile unheated liver extract. The last was prepared by filtering liver homogenate twice through a 5 mm. layer of kieselguhr ("Super-cel") in a Büchner funnel and then once or twice through a Seitz filter. *R. pellio* has been maintained in such sterile conditions for four months. It is now in its fifth transplant (at least 10 generations). With each transfer sterility is checked by inoculation of worms into nutrient broth and thioglycollate broth (thickened with 0.1% agar) for aërobic and anaërobic contaminants respectively. Gram stains are also occasionally made. *R. elegans* has been grown through two transfers, but is not at present being maintained in pure culture.

For routine preparation of the liver homogenate 100 grams of beef (preferably calves') liver are added to 500 ml. of distilled water and the mixture subjected to the action of a Warring blender. Liver extract is now prepared by filtering through "filter mesh" (filter paper rendered into a pulp by Warring blender action) rather than through kieselguhr.

Recently *R. pellio*, which has been transferred four times in its four months of axenic cultivation, has begun to show signs of decreased fertility. The fifth transplant is growing rather poorly. It is probable that raw liver extract is deficient in one or more essential nutrilites, and it would seem likely that the worms are suffering from an accumulated deficiency, analogous to that noted for *Neoaplectana glaseri* by Glaser (1940a) when that species was grown on a dextrose-veal agar medium with living yeast for more than six transplants. *N. glaseri* became sterile under such conditions, but Glaser was able to prevent this phenomenon by adding desiccated, bovine ovarian substance to his medium. Work is now under way to discover what supplementation of our basal medium for *R. pellio* is necessary to permit indefinitely transplantability. Obviously the same factor or factors that were deficient in the case of *N. glaseri* are not involved for *R. pellio* as a medium essentially the same as that used by us for the latter will indefinitely support axenic growth of the former.

Recently *R. pellio* has been successfully raised in liver extract alone. Larvae from the third sterile transplant were placed with 4 ml. of extract in "*Neurospora* growth tubes"—horizontal tubes open at either end and with the ends bent upwards to give the following shape:  . In these the worms can be much more easily observed than in test tubes. Starting with 50 small larvae, they passed through three transfers (6 days apart) before becoming infertile.

In axenic culture maturation of *R. pellio* has been considerably slower than in bacterial culture—5–6 days as opposed to 2–3 days. This is possibly related to the same factors that have so far prevented indefinite transplantability. Some recent cultures in the first and second sterile transplants have been exceedingly prolific, however, and, although close comparison of fertility between axenically and bacterially raised worms has not been carried out, the first impression (Dougherty, in press), that axenic cultures are even initially less prolific, has not been borne out. The addition to the sterile agar medium of a large group of vitamins and vitamin-like substances (Dougherty, in press), for the most part in the same concentrations as used in studies on *Drosophila* nutrition by Wagner and Mitchell (1948), has not materially affected growth. These vitamins included per ml.: thiamine (25 γ), riboflavin (12.5 γ), pyridoxine (12.5 γ), calcium pantothenate (12.5 γ), nicotinamide (50 γ), *p*-aminobenzoic acid (12.5 γ), inositol (100 γ), folic acid (0.05 γ), and pimelic acid (100 γ); also added were choline (125 γ) and ribosenucleic acid hydrolysate (250 γ). Whatever factor or factors it is that are required for normal rate of growth and are provided in optimal concentrations by live bacteria, but not liver extract, would not seem to be

identifiable with any of the known B vitamins. In all probability they represent as yet unknown chemical substances.

A few additional facts have been determined about the unknown factor or factors in liver extract. They are destroyed by autoclaving for 15 minutes at 15 pounds pressure. However, growth promoting properties are retained, only slightly impaired, by liver extract heated to 55° C for 30 minutes. Repeated freezing and thawing does not destroy the factors.

At present work is being carried out on the essential, heat labile factor(s) in liver extract. An assay method along the lines of that developed for *Drosophila* by Wagner and Mitchell (1948) is being sought to provide a sound quantitative measure of growth in *R. pellio*. With such a method it should be possible to attack the problem of concentrating, isolating, and identifying the heat-labile factor(s). Such factors are of general current interest in nutrition in view of the demonstration by Cooperman, Waisman, McCall, and Elvehjem (1945) of a factor (also in liver) important in the nutrition of the monkey and of the demonstration by various workers (*viz.* Johnson and Tatum, 1945) that various Protozoa require heat-labile factors.

In view of the extensive studies on the defined nutrition of the fruit fly, *Drosophila melanogaster* Meigen, 1830 (by Schultz, St. Lawrence, and Newmeyer, 1946; Wagner and Mitchell, 1948; Villee and Bissell, 1948), and of the ciliate *Tetrahymena geleii* Fergason, 1940 (by Kidder, 1947, and his associates), as well as the vast body of literature on vertebrate nutrition—studies that have demonstrated basic similarity of nutritional requirements in such diverse organisms as holozoic protozoans, arthropods, and vertebrates,—it would seem reasonable to anticipate that rhabditid nematodes will also show broad similarity—that is, in requirements for a hexose sugar, ten "essential" amino-acids, and vitamins and other growth factors—and that the main differences in dietary needs will be for a few growth-promoting substances.

IV. SUMMARY

1. The work reported here has been prompted by our interest in studying the genetics of differentiating growth of eutelous (cell-constant) organisms, specifically nematodes of the genus *Rhabditis*. Before this can be attempted, however, a chemically defined medium for their cultivation must be worked out. The present paper reports a year's work in the cultivation of various rhabitid and diplogasterid species; this work has as its immediate aim the elucidation of the minimum nutritional requirements that will support normal growth and reproduction of species of *Rhabditis*.

2. Previous studies on the culturing of soil-dwelling nematodes (particularly of the genus *Rhabitis*) are surveyed—also studies on the pure-culturing of certain symbiotic nematodes.

3. A number of observations on cultural characteristics of the rhabditoids studied by us are made. The more important accomplishments of our work so far are:

4. Devising a method, using penicillin, streptomycin, and merthiolate for eliminating the mixed bacterial flora accompanyng cultures of soil nematodes and replacing it with a single bacterial species (*Escherichia coli*), which can in turn be eliminated at will with streptomycin;

5. Growing of *Rhabditis pellio* and *R. elegans* through several generations for the first time under axenic (germ-free) conditions in a complex medium containing Seitz-sterilized liver extract, but apparently deficient in one or more factors inasmuch as prolonged cultivation of *R. pellio* has recently led to its impaired fertility; and

6. Demonstrating that a heat-labile factor or factors in liver extract are required for growth and reproduction of *R. pellio* and *R. elegans* and that these are destroyed by autoclaving, but not by heating to 55° C for half an hour, nor by repeated freezing and thawing.

7. Work is now under way to develop an assay method for this factor or factors so that effective work on their concentration, isolation, and identification can be instituted. Efforts are also being directed toward improving the sterile medium to give indefinitely transplantable axenic growth.

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On the Nomenclature of *Trichomonas gallinae*, a Protozoan Parasite of Birds

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There is much confusion in the literature concerning the nomenclature of the flagellate found in the upper digestive tract and liver of the pigeon, the chicken,

[1] Resigned December 19, 1947.

and certain other birds. In reviewing the literature, incident to investigations of this organism, the writer was impressed by the large number of names associated with it. In an effort to determine whether these names refer to distinct species or to a single species, a study was made of their origin and possible relationship. Another purpose of this paper is to assemble this information in one report, in the hope that future workers will be spared the difficulties encountered by the writer in searching the literature for information on *Trichomonas gallinae*. Pereira and de Almeida (1943) reported the results of an extensive review of the literature, but their conclusions differ somewhat from those of the present writer, due, no doubt, to their inability to consult some of the original papers dealing with this organism.

The names which have been associated with this organism in question are listed, in order to facilitate subsequent discussion, but not necessarily to represent them all as synonyms. The names are arranged in groups in order to present a clearer picture of their evolution.

Cercomonas gallinae Rivolta, 1878
Monocercomonas gallinae (Rivolta, 1878) Neumann, 1892
Trichomonas gallinae (Rivolta, 1878) Stabler, 1938

Cercomonas hepaticum Rivolta, 1878
Monocercomonas hepatica (Rivolta, 1878) Neumann, 1892
Trichomonas hepatica (Rivolta, 1878) Nieschulz and Bos, 1936

Cercomonas columbarum Pfeiffer, 1889 (*nomen nudum*)
Monocercomonas columbarum Pfeiffer, 1889 (*nomen nudum*)
Trichomonas columbarum (Pfeiffer, 1889) Kruse, 1896

Trichomonas diversa Volkmar, 1930

Trichomonas halli Yakimov, 1934

Haemotrichomonas columbae Lanfranchi, 1917
Haemotrichomonas columbarum Rodenwaldt, 1921

Rivolta and Delprato's Infusorium, 1880
Lophomonas columbae Railliet, 1885
Trichomonas columbae (Railliet, 1885) Neumann, 1892

"*Trichomonas columbae* Rivolta" in Rátz (1910)

"*Tricomonas collumbae*" in Sousa Dias (1945)

Cercomonas gallinarum Davaine, 1875

The origin and evolution of the names will be discussed, in the order listed; then the question of synonymy will be undertaken. Unless a special reference is given, any reference to an author will be the same as that given in the list of names.

ORIGIN AND EVOLUTION

Rivolta (1878) gave the name *Cercomonas gallinae* to an organism which he described from the pigeon. Neumann (1892) placed this organism in the genus *Monocercomonas*. Stabler (1938) placed it in the genus *Trichomonas* and, in addition, pointed out that the specific name *gallinae* had priority over the other specific names. The correct citation for the name of this organism, therefore, is *Trichomonas gallinae* (Rivolta, 1878) Stabler, 1938.

In the second part of his 1878 paper, Rivolta described and named *Cercomonas hepaticum*, which he found in the liver of the pigeon. Neumann (1892) placed this organism in the genus *Monocercomonas* and Nieschulz and Bos (1936) placed it

in the genus *Trichomonas*. The latter authors changed the ending of the specific name from *hepaticum* to *hepatica*.

Pfeiffer (1889) stated that Bütschli synonymized *Cercomonas columbarum* with *Monocercomonas columbarum*. No description of the organism was given nor was any reference to a specific publication of Bütschli given. In Bütschli (1884), however, two species of *Cercomonas* were placed in the genus *Monocercomonas*, and it may have been this which caused Pfeiffer to make the above comment. A careful search of Bütschli (1878–1887) failed to reveal any mention of the specific name *columbarum*. Kruse (1896) described *Trichomonas columbarum* and although he did not refer to either *Cercomonas columbarum* or *Monocercomonas columbarum* he did mention Pfeiffer's paper. It is probable that Kruse's *Trichomonas columbarum* = *Cercomonas columbarum* Pfeiffer, 1889. The origin of the specific name *columbarum* is not clear and the possibility exists that it may be the same as *columbae*. Both specific names have the same root and both endings are of the same case; one is singular and the other plural, however. No use of the name *columbarum* was found which would have priority over *gallinae*.

"*Tricomonas collumbae*" as used by Sousa Dias (1945) is merely a change in spelling. *Tricomonas* was the original spelling of this genus but it has been amended to *Trichomonas*. The reason for spelling the specific name with a double "l" is not known, but in the original use by Railliet (1885) only one "l" was used.

The origin of the names *Trichomonas diversa* and *Trichomonas halli* is uncomplicated and no changes have been made.

Lanfranchi (1917) gave a description of an organism found in the blood of a pigeon which he called *Haemotrichomonas columbae* Martoglio, 1917. Actually, however, Lanfranchi was the first to describe and name this species, and apparently credited it to Martoglio because the latter created the genus. The corrected citation is, therefore, *H. columbae* Lanfranchi, 1917. Rodenwaldt (1921) mentioned this organism but changed the ending of the specific name to *columbarum*.

In Rivolta and Delprato (1880) there is described on page 114 an "infusorium" found in the intestine of pigeons, but it was not given a name. In addition to the description, reference was made to a figure at the end of the book. Several individuals of the organism were represented in this figure and none of them could conceivably have been a trichomonad. Although the description of the organism mentions 4 or 5 flagellae, one of the individuals figured showed 6. These flagellae leave the body of the organism at separate points and the organism looks very much like a species of *Hexamita* Dujardin, 1838 with some of the flagellae obscured. The location in which these flagellates were found in the host would tend to corroborate this belief.

To this "infusorium" described by Rivolta and Delprato (1880) on page 114, Railliet (1885) gave the name *Lophomonas columbae*. Neumann (1892) and Railliet (1893) placed it in the genus *Trichomonas*, making the combination *T. columbae*. Whether these authors failed to see the figure in Rivolta and Delprato (1880), because of its remote location from the text description, can only be surmised. Had they seen the figure, however, it seems unlikely that they would have placed this organism in the genus *Trichomonas*.

The description of "*Trichomonas columbae* Rivolta" in Rátz (1910), page 194, appears to have been taken from that of the unnamed "infusorium" described by Rivolta and Delprato (1880) on page 114. Since a search of Rivolta's papers failed to reveal any mention of the specific name *columbae*, it appears that Rátz attributed the name to Rivolta on the basis of the latter's description and not because Rivolta and Delprato actually used the name. As shown previously, Railliet (1885) was the first to give the name *columbae* to Rivolta and Delprato's "infusorium."

Rátz (1910) was in error when he assumed that the organism he found was identical with the "infusorium" described by Rivolta and Delprato (1880). The organism figured on page 196 of Rátz (1910) is definitely a trichomonad. Therefore, Rátz used the specific name *columbae* for two different organisms, one of which appears to be a species of *Hexamita* and the other a *Trichomonas*. Many authors have perpetuated Rátz's original error by using the name *Trichomonas columbae* for the *Trichomonas* found in the upper digestive tract and liver of the pigeon.

Davaine (1875) described *Cercomonas gallinarum*, a flagellate which he found in the ceca of the partridge ("perdrix").

SYNONYMY

Stabler (1938) gave *Trichomonas gallinae* as the correct name for the flagellate occurring in the upper digestive tract of pigeons and stated that *Cercomonas gallinae, C. hepaticum,* and *Trichomonas columbae* are synonyms. Later (1947), he added *T. diversa* and *T. halli* as also being synonyms of the above species. Nieschulz and Bos (1936) state that *T. hepatica, T. columbae,* and *T. columbarum* are synonymous. It is assumed that *T. columbae* as used by Stabler and Nieschulz and Bos equals "*Trichomonas columbae* Rivolta" of Rátz (1910) as used for *Trichomonas.*

"*Tricomonas collumbae*" as used by Sousa Dias (1945) is merely a misspelling of "*Trichomonas columbae* Rivolta" in Rátz (1910) as used for the *Trichomonas. Haemotrichomonas columbae* is given as a synonym of *Trichomonas gallinae* by Pereira and de Almeida (1943) and since *Haemotrichomonas columbarum* is merely an altered spelling of *H. columbae* it would likewise become a synonym of *Trichomonas gallinae.*

Trichomonas columbae (Railliet, 1885) Neumann, 1892 and "*T. columbae* Rivolta" in Rátz (1910) as described on page 194 both refer to a species of *Hexamita.* Since "*T. columbae* Rivolta" in Rátz (1910), as figured on page 196, is a trichomonad, this name is a homonym of *T. columbae* (Raillet, 1885) Neumann, 1892.

Perroncito (1901) gives "*Cercomonas gallinarum* (1875)" as a synonym of "*Cercomonas gallinae* (Rivolta)". He also states that Davaine (1875) was the first to observe it in diphtheria of the pigeon. Kruse (1896) gives *Cercomonas gallinarum* Davaine as a synonym of *Trichomonas columbarum.* He states that Davaine (1875) found *C. gallinarum* in the exudate in "Hühnerdiphtherie." It is believed that both Perroncito and Kruse have misquoted Davaine since the latter described *C. gallinarum* as occurring in the ceca of a partridge ("perdrix"). Nowhere in Davaine (1875) could any reference be found to the presence of this organism in any other host or site. The writer regards the species of trichomonads in the ceca of partridges as being distinct from the species in the mouth of pigeons. Therefore, *C. gallinarum* Davaine, 1875 cannot be considered a synonym of *T. gallinae.*

CONCLUSIONS

With the possible exception of "*Trichomonas columbae* Gedoelst, 1911" the list of synonyms of *T. gallinae* given by Pereira and de Almeida (1943) on pages 278 and 279 appears correct. The separate species of trichomonad called *T. columbae* (Rivolta *in* Railliet, 1885) and its synonyms appearing on page 284 of Pereira and de Almeida (1943) cannot be accepted as correct. As indicated earlier in the present paper, these names refer to an organism which in all probability is a species of *Hexamita.* If a species of trichomonad other than *Trichomonas gallinae* has been found to infect pigeons it will have to be renamed. The present writer has no knowledge of such an organism.

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The Duration of *Trichomonas gallinae* Infections in Individually Housed Pigeons

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A knowledge of the duration of infection of pigeons with *Trichomonas gallinae* (Rivolta, 1878) Stabler, 1938 is important in connection with the control and treatment of trichomoniasis in birds. In another paper by the author (in press), it was pointed out that subclinical infections with *T. gallinae* were rather persistent in a flock of pigeons confined in a small cement-floored house. Twenty-nine naturally infected birds were kept under observation for periods ranging from 228 to

[1] Resigned December 19, 1947.

744 days; one bird (cock number 1212) was still infected on the 744th day, whereas the others had lost their infections.

In order to ascertain the duration of the infection in individually housed birds, observations were made on 2 of the above 29 birds when so housed. These results, with observations on 7 additional birds, are reported in this paper. Presence of the parasite was determined by microscopic examination of direct smears.

Cock number 1212 was examined 10 times during a period of 670 days while he was housed with infected birds. Although the last examination was negative, 7 of the 10 had been positive. In order to determine whether or not he still harbored a low-grade infection which might become patent at some later time, he was housed individually for 61 days. During this time 10 examinations were made to determine the presence of *T. gallinae;* all of them were negative.

The mate of this cock, hen number 918, was positive on all 9 examinations made during the 670 days she was housed with infected birds. Following this, she also was housed individually for 61 days. During this period 6 examinations were made; the first 2 were positive and the next 4 were negative.

At the end of their respective periods of isolation, cock 1212 and hen 918 were reunited for 210 days. During this time each was examined 5 times and found to be negative. In addition, the pair raised 3 clutches of squabs free of the infection. These birds, therefore, represent 2 cases of a spontaneous loss of the infection.

Seven additional pigeons about 3 months old and with naturally acquired subclinical infections with *T. gallinae* were housed individually for 396 days. Each bird was examined 55 times, or approximately once a week. Three of the birds were positive on all examinations, 1 bird was positive on all but 1 examination and 1 bird was positive on all but 5 examinations. This last bird was negative on 2 consecutive examinations at one time and on 3 consecutive examinations at a later time. The sixth bird was positive on the first 21 examinations and it was negative thereafter. The seventh bird was positive on the first 30 examinations and negative thereafter. Two of the 7 birds, therefore, became free of infection with *T. gallinae* spontaneously.

SUMMARY

Four of 9 pigeons spontaneously lost their infections with *T. gallinae.* Excluding cock number 1212, which was negative at the time he was housed separately, 3 of 8 birds lost their infections while housed individually.

Abnormalities in Development of Helminth Parasites with a Description of Several Anomalies in Cercariae of Digenetic Trematodes[1]

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A number of instances have been recorded in which there is abnormal development of helminth parasites. The cases reported have been concerned, for the most part, with cestodes, the anomalies ranging from an absence or addition of component structures of worms, i.e., reduced number of hooks in *Cysticercus cellulosae* (Huff and Bucy, 1936, Jour. Parasit. 22: 110), *Rhabdometra similis* with three suckers (Jones, 1946, Trans. Amer. Micr. Soc. 65: 357–359), cysticerci of *Taenia solium* with two scolices each (Lent, 1942, Rev. Brasil Biol. 2: 297–301), *Taenia hydatigena*

[1] The opinions contained in this paper are those of the autl or. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

with 6 suckers (Canavan, 1934, Jour. Parasit. 20: 57), etc. to partial twinning and complete duplication of strobila as found for the adult of *Taenia pisiformis* by Chandler (1930, Trans. Amer. Micr. Soc. 49: 168–173). Supernumerary suckers resulting in tri- or tetra-radiate strobilae apparently are quite common (Dobrovolny and Dobrovolny, 1935, Trans. Amer. Micr. Soc. 54: 22–27). Even greater evidence of the rather common occurrence of teratological forms among the cestodes is found in Foster's summary (1915, Jour. Parasit. 2: 5–19) in which 44 cases of poly-radiate cestodes are listed, the greater number of cases being represented by *Taenia saginata.* More recently (Clapham, 1939, Jour. Helminth. 17: 163–176) a bibliography of cestode abnormalities has been published.

Duplication of parts in larval cestodes may vary considerably within the species, as has been demonstrated in several specimens of cysticerci of *Taenia taeniaformis.* Southwell and Kirshner (1937, Ann. Trop. Med. and Parasit. 31: 37–42) and Dollfus (1938, Ann. Parasit. 16: 133–141) published descriptions of polycephalic larvae possessing, respectively, 12 and 6 segmented strobila growing from a single bladder. Another unusual condition in the strobilacercus larva of *T. taeniaformis* was reported by Kuntz (1943, Jour. Parasit. 29: 424–425) who found a specimen with two fully developed individuals tied in a compact knot and arising from one bladder.

Malformations described for adult trematodes are fewer in number, and of those reported, the majority appear to be associated with the reproductive system. In support of the latter Manter (1931, Jour. Parasit. 18: 124) has stated that monorchism is probably the most common abnormality among trematodes. He noted that monorchism as an anomaly had been described for at least nine species of trematodes representing six different families. Price (1930, Jour. Parasit. 16: 161) listed two cases of monorchism, one in *Phyllodistomum fausti* and one in *Echinostoma revolutum,* and reported the finding of a mature specimen of *Fasciola hepatica* with two apparently functional ovaries. On the other hand Hoffman (1936, Proc. Helm. Soc. 3: 62) described an adult *F. hepatica* with a dichotomously branched, bilateral ovary where normally this organ is branched but unilateral. Krull (1935, Trans, Amer. Micr. Soc. 54: 118–134) has shown that there may be a decrement in the reproductive organs of trematodes as illustrated in *Brachylaemus virginiana.* One fluke of the latter species was lacking a testis while another specimen had no ovary. In a study of *Apophallus venustus,* Cameron (1937, Canad. Jour. Res. (D) 15: 38–51) encountered several specimens with an absence of one or both testes.

Anomalous conditions thus far described in the larval stages of trematodes are much more striking than those enumerated for adults. Several cases of twinning have been observed in the miracidia of *Schistosoma mansoni.* Hoffman and Janer (1936, Proc. Helm. Soc. 3: 62) found two pairs of twinned miracidia in which the organisms were fused anteriorly for approximately one-third their length. Later, Janer (1941, Jour. Parasit. 27: 93) announced the finding of a third pair of *S. mansoni* miracidia in which the specimens again were united anteriorly and in each of them there was a normal set of four active flame cells. The paired organisms were quite active.

Abnormalities of larval development have been presented for several types of cercariae, the earliest report dates back to the work of Pelseneer (1906, Bull. Sci. France et Belgique 40: 161–186) who figured a Trichocercous cercaria, *Cercaria setifera* Müller, with a trifurcate tail, each branch possessing an apparently normal complement of bristles. Mathias (1930, Ann. Parasit. 8: 147–150) found a furcocercous cercaria (holostomid) in which the distal end of the tail was bifurcate with branch possessing a pair of furcae. Although there was no detailed study of

the excretory system the specimen was unique in that there were two caudal excretory tubes leading into the tail from a single excretory bladder. Other internal organs were normal except for the posterior half of the gut cecae which were doubled. The movements of the cercaria were similar to those of normal cercariae from the same snail. In a study of the development of the cercaria of *Alloglossidium corti*, Hussey (1941, Jour. Parasitol. 27: 92–93) dissected an abnormal, nearly mature cercaria from a sporocyst. Although there was some difference in the nearness of completion of development of the excretory system the posterior parts of the twinned cercaria was almost entirely doubled. Both tails were normal and moved actively, although the cercaria as a whole was rather sluggish when compared to normal cercariae. Gordon, Davey and Peaston (1934, Ann. Trop. Med. and Parasit. 28: 323–419) pointed out that there are aberrant forms in the cercariae of *S. haematobium*, and noted a case in which three distinct flame cells were counted in the tail instead of the usual pair.

In the course of several years study of the comparative development of the excretory system in the cercariae of digenetic trematodes (unpublished research) the author has observed several interesting monstrosities. Although such morphological freaks are not necessarily of taxonomic value it seems worth while to make note of the findings. While handling thousands of cercariae of *S. mansoni* in schistosomiasis research there has been better than average chance to find unusual specimens. The abnormalities reported herein range from a duplication of the stylet in the cercaria of *Sellacotyle mustelae* (Fig. 1) and doubling of the acetabulum in *Tamerlania bragai* (Fig. 2), to varying degrees of twinning in a cercaria of *Notocotylus urbanensis* (Fig. 3) and in three cercariae of *S. mansoni*.

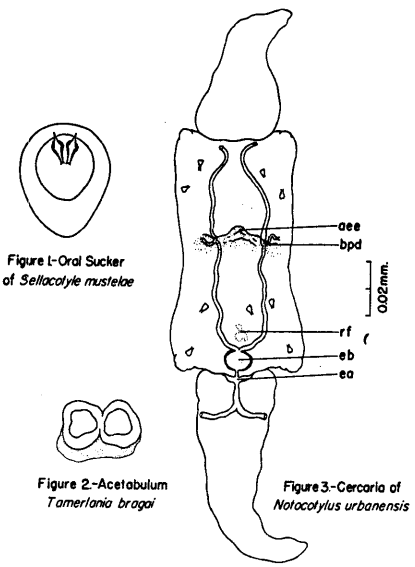

FIG. 1. Duplication of the stylet in a cercaria of *Sellacotyle mustelae*. FIG. 2. Doubling of the acetabulum in a cercaria of *Tamerlania bragai*. FIG. 3. Twinning in a cercaria of *Notocotylus urbanensis*. *aee*, anterior end of embryo; *bpd*, bifurcation of primary excretory duct; *ea*, excretory atrium; *eb*, excretory bladder; *rf*, fundament of reproductive organs.

The first abnormal schistosome cercaria found had the posterior fourth of the body doubled and each possessed a tail with a single furca (Fig. 4). There were two closely associated but normal-sized acetabula. Their tissues were connected and appeared to have arisen from a single acetabular fundament. There were two excretory bladders of normal size and an excretory atrium at the body-tail junctions but there was only a single complement of flame cells, i.e., four pairs in the body and one pair in the tail. Other organs appeared to be of average size and disposition. In a second specimen (Fig. 5) twinning had progressed much further. The posterior doubling of the body was more obvious and each part was equipped with a normal forked tail with two furcae. There were two completely developed but separated acetabula and two complete excretory systems. The remainder of the internal organs were singular except for the penetration glands

which filled the greater part of the body and appeared to be double in number. The ducts from one set of glands opened in the usual location whereas the openings of the second group appeared to be located a short distance posteriorly. A third specimen, similar to that shown in Figure 5, with two complete forked tails possessed a single complement of penetration glands, two excretory bladders and a single complement of flame cells. The acetabula were normal and separate.

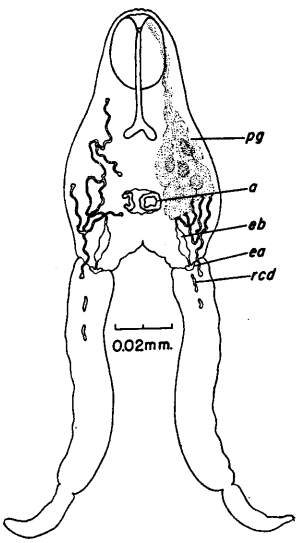

Figure 4. – Cercaria of *Schistosoma mansoni*

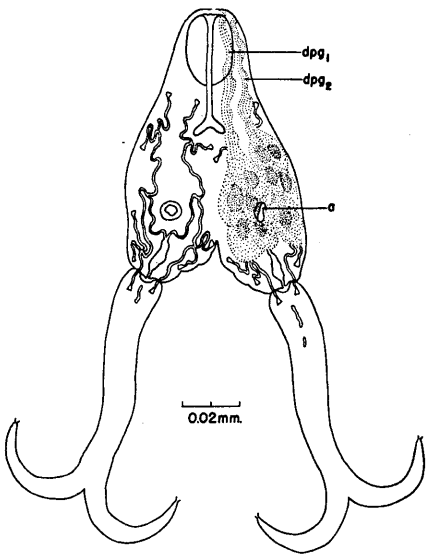

Figure 5.-Cercaria of *Schistosoma mansoni*

FIG. 4. Abnormal schistosome cercaria with the posterior part of the body doubled. *a*, acetabulum; *ea*, excretory atrium; *eb*, excretory bladder; *pg*, penetration gland; *rcd*, remnant of caudal excretory duct. FIG. 5. More complete twinning in a schistosome cercaria. *a*, acetabulum; dpg_1 and dpg_2, ducts of penetration glands, sets 1 and 2 respectively.

The three partially twinned cercariae of *S. mansoni* were taken from lots of naturally emerged cercariae. Although they were normally active their movements through the water were erratic and determind more or less by the activity of each tail. When the tail of one side showed vigorous movements the cercaria was carried through the water in one direction, then it would quickly change its course into the opposite direction as the first active tail became motionless and the other tail began to vibrate. Thus a zig-zag path was sometimes followed. On occasion both tails beat simultaneously and the cercaria was almost motionless or moved slowly in a vertical plane. The cercariae moved in normal measuring-worm fashion while they were on the bottom of observation dish.

An unusual abnormality was observed in a middle-aged cercarial embryo of *Notocotylus urbanensis* (Fig. 3) dissected from a redia. The twinned embryo was joined in the anterior fifth of the body. There was unequal growth of the two tails of the embryos as well as unequal development of the excretory systems. In one part of the embryo the primary excretory tubes had fused to form an excretory bladder in the usual manner whereas in the other embryo the primary excretory tubes remained separate. The fundaments of the oral sucker and parts of the gut usually observable at this stage of development apparently were lacking but there

was evidence of the beginning of the reproductive organs in the more advanced half of the embryo.

Such interesting anomalies, especially duplication of the greater part of an individual, as has been demonstrated for some helminths, easily lend themselves for speculation in regard to their development and genetic significance. Certainly one of the first questions to arise is that concerning the causes which are responsible for production of monstrosities. Perhaps such abnormalities in helminths (as in vertebrates) arise as a result of injury, (trauma, lack of oxygen or an excess of carbon dioxide, or radical temperature changes) at critical moments in the development of the individual. These anomalies then may arise due to the presence of physico-chemical, growth-inducing stimuli in that region of the organism which is undergoing more rapid growth. The latter could easily apply to the embryos of cercariae since it is known that in the early stages of development there is a much greater growth and differentiation of tissues in the tail region than in the anterior part of the body. It is conceivable that influential factors acting upon an embryo while in its most rapid growing stage might initiate an accelerated growth in the posterior region with subsequent formation of two tails and/or acetabula. Such factors would probably be at play even earlier to account for doubling of the excretory system. In view of what has just been postulated it seems strange, however, that only a single individual in a sporocyst or redia with many developing cercariae, would be so affected.

A Note on the Morphology of *Schistosoma japonicum*

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In examining text-books on parasitology or tropical medicine, one finds discrepancies in the descriptions of the adult *Schistosoma japonicum*. Two points not agreed upon are the location where the two intestinal crura unite to form a single intestinal caecum and the number and arrangement of testes in the male. During the past years, we have examined several hundred mature *Schistosoma japonicum* and as a result have information bearing on these two points. The material comes from rabbits and guinea-pigs experimentally infected with cercariae obtained from snails collected at Soochow, Kiangsu, China. The worms were fixed in formalin and stained either with Delafield's haematoxylin or Mayer's alum-carmine.

Intestine.—Normally, the two intestinal crura unite near the junction of the middle and posterior thirds of the body in male adults and immediately behind the ovary in female adults. The common caecum, therefore, measures about one third the body length of the male and about one half the body length of the female. However, it is not infrequent to find the length of common caecum described as occupying only one fifth or one sixth the body length (Belding; Hegner *et al*). In our material, we have found that the common caecum always occupies the posterior third of the male and posterior half of the female. During the examinations, we found some morphological peculiarities that are considered worth reporting. Figure 1, A shows a worm in which the common caecum is formed at the usual place, but after a very short course it bifurcates and its two branches immediately reunite and this process is repeated once more so the whole gives the picture of two rings in succession. Figure 1, B represents another with only one ring. Figure 1, C shows another male adult, one intestinal branch of which stops abruptly at the point where the reunion should take place; as a result, no common

caecum was formed. Figure 1, D is of a very young worm, 2 mm. long, sex still undifferentiated, with a very long oesophagus extending to the middle of the body, and a small diverticulum near the ventral sucker. The oesophagus divides into two intestinal branches, one of which runs a very short course while the other divides again near the posterior fourth of the body. This specimen is of interest because, according to Faust and Meleney (1924), adolescent worms between 0.5 and 1 mm. in length should have the two intestinal crura branched off from the oesophagus in the preacetabular region and also a common caecum formed in the posterior part of the body. Figure 1, E shows a young male with 2 diverticula at the beginning of intestinal crura. Though reproductive organs are not yet developed, the sex can readily be told by the broadness of the specimen, females of that length being much more slender.

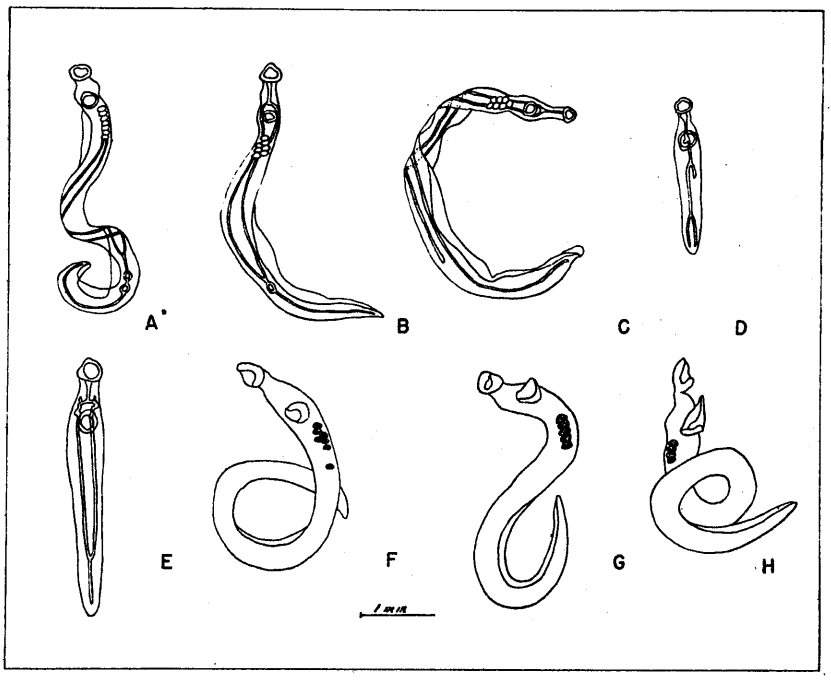

FIG. 1. *Schistosoma japonicum,* illustrating the morphological abnormalities described in the text. A to E show peculiarities of the intestine. F, G and H show variations in the number and arrangement of the testes in the male.

Testes.—In 11 texts and one monograph consulted, the authors found the following range in the number of testes reported to occur in *Schistosoma japonicum:* 5–8 (Sprehn), 6–8 (Neveu-Lemaire; Brumpt; Stitt *et al*; Manson-Bahr), 7 (Belding; Faust and Meleney; Craig and Faust; Chandler), 7–8 (Hegner *et al;* Blacklock and Southwell), 7–9 (Culbertson). Most of the time, the testes are described as arranged in a single column (Belding, Chandler, Faust and Meleney) and rarely in a cluster (Blacklock and Southwell). In our material, the number of testes was, with two exceptions, seven and the arrangement was either in a single column or clustered together variously. Perhaps some of the variation in arrangement may be explained by differences in degree of contraction of the worms. The fact that our experimental infections were made with cercariae ob-

tained from snails collected from only one place, suggests that we might be dealing with a local strain or variety. In order to have a comparison with specimens collected from other localities, we examined 15 and 92 adult male *Schistosoma japonicum* collected from Japan and Leyte respectively. The specimens were kindly furnished by Dr. L. J. Olivier of the U.S. Public Health Service, to whom the authors wish to express their indebtedness. With these specimens, the testicular region was cut off from the rest of the worm and stained in Mayer's alumcarmine. The worm fragment was then manipulated under low power so as to secure the best view, usually dorsal, of the testes. In several cases, the lateral view, which is often the only possibility in total mounts due to the ventrad curvature of the posterior portion of the male, revealed only 5 to 6 testes, but when the specimen was viewed dorsally, the presence of 7 testes became evident. There were few instances in which the number could not be clearly made out when the fragment was manipulated. Whenever a good observation was possible, the number found was always seven. In view of the frequent superimposition of the testes, when viewed from only one aspect, it is easy to understand smaller numbers reported by some authors. Reports of 8 testes could result from calling the seminal vesicle a testis. The seminal vesicle lies just in front of the anterior-most testis and when full of sperm could easily be mistaken for a testis. Faust and Meleney (1924) stated that there is a tendency toward transverse lobation in the testes. On our part, we have often observed the lobation described by them.

Among our specimens from China, we have found 3 instances in which some peculiarities about the testes were observed. Figure 1, F shows a male worm with the usual number of testes, but the last testis is separated from the others. Figures 1, G and 1, H show adult males having less than the normal number of testes. We consider them abnormal because of their rarity.

SUMMARY

As a result of the examination of several hundred adult *Schistosoma japonicum,* including 15 Japanese and 92 Philippine specimens, it is concluded that the length of the common caecum constantly occupies the posterior third of the body and the number of testes is invariably seven. Two exceptions in the number of testes and several instances in peculiar morphology of the intestine are also reported.

Report of the Brayton H. Ransom Memorial Trust Fund
December 31, 1947

STATEMENT FOR THE YEAR 1947	
ON LOAN, Jan. 1, 1947	$1400.00
BALANCE ON HAND, Jan. 1, 1947	211.92
RECEIPTS:	
Semi-annual interest on loan at $28	56.00
Interest on bank account	2.21
TOTAL RECEIPTS	$ 270.13
DISBURSEMENTS:	
Rent, safe deposit box	$ 4.20
Award to Helminthological Society of Washington	25.00
TOTAL DISBURSEMENTS	$ 29.20
BALANCE ON HAND, Dec. 31, 1947	240.93
	$ 270.13

A meeting of the Trustees was held on May 17, 1947.

ELOISE B. CRAM,
Secretary-Treasurer

A Cropworm, *Capillaria contorta*, the Cause of Death in Turkeys

EVERETT E. WEHR
U. S. Bureau of Animal Industry

During the latter part of March, 1947, the writer was requested to investigate and, if possible, to determine the cause of sickness and deaths in a small flock of Beltsville Small White laying turkeys near Manassas, Virginia. The owner of this flock lived in Manassas, while the birds were kept in an isolated place in the woods about 30 miles southwest. There were very few turkeys raised in this section of the State.

On investigation, an old shed and barn were found to serve as shelters for the birds. A small, fenced-in lot adjacent to the shed and barn was used as exercise pen and feeding grounds. The mash and small grain feed were placed in long, uncovered feeders. Corn on the cob was fed by throwing the ears on the ground and scattering them over the lot. The feeding lot was devoid of vegetation.

At the time of the writer's visit, a number of sick birds, about 25, were in a separate pen. A few birds had died and another died while he was there.

Ante-mortem examination showed the affected birds with their heads and necks drawn close to their bodies (Fig. 1). Viewed from the side, the body in the

Fig. 1. Typical appearance of turkeys infected with *Capillaria contorta*.

region of the crop protruded considerably. The birds moved only when disturbed, and then very slowly and with an unsteady gait. Occasionally a bird was seen to fall back on its hocks and assume a penguin-like posture for a brief time. Other birds were observed to extend and retract their necks more or less constantly as if attempting to relieve some congestion of the throat or experiencing difficulty in deglutition. None of the birds showed any inclination to eat. Palpation of the crop region showed that this organ was, in some cases, filled with liquid which, when expressed, was found to be very fetid.

Post-mortem examination of two of the birds showed the crop and esophagus to be very highly inflamed, with walls thickened and the mucosa covered with a thick diphtheritic membrane. Just beneath this membrane and partly or wholly embedded in the mucosal lining of the esophageal wall were numerous specimens of *Capillaria contorta*.

These parasites have been reported from the crop and esophagus of a large number of hosts, including wild and domestic turkeys, several species of wild ducks, gulls, hawks, grouse, quail, pheasants, crows and others. The adult female worm is from 2 to 3 inches long, and the male somewhat shorter. The life history is direct, and, in this respect, it differs from *Capillaria annulata* which requires an earthworm as intermediate host. The latter is frequently found in association with the species under discussion.

BOOK REVIEW

DIE TIERISCHEN PARASITEN DER HAUS- UND NUTZTIERE, SOWIE DES MENSCHEN. Dr. med. Josef Fiebiger, Wien, Austria. Urban und Schwarzenberg, 1947; pp. iii + 436 (illustrated).

The present or 4th edition of the above-mentioned book was published when the author was in his 77th year. Despite his many handicaps, such as age, lack of help, and the confusion resulting from the recent war, the author undertook the task of revising the 3rd edition, which appeared 11 years earlier.

In the preface, the author states that many erroneous statements which had appeared in the last edition were corrected, that the results of many later investigations were incorporated, and that much new material, particularly in the sections on worms and biting lice, was added. In these matters, the author was especially aided by such publications as Stempell (1938), Heidegger (1937), Schmid (1944), and Neveu-Lemaire (1938).

As in the 3rd edition, the new edition is divided into two main parts, namely, the General Section and the Special Section. The General Section covers approximately 50 pages and the contents remain much the same as those of the previous edition, except for the addition of a new chapter which he calls "Resistance, Immunity, and Praemunition." Naturally the greatest consideration was given to the Special Section and it is here that the major revision has taken place.

Had the author revised the keys and classifications of the parasite groups to conform more closely to those more recently adopted, the book would have been greatly improved. Reference is made particularly to those used for differentiating the groups of nematodes, as the reviewer is more familiar with this group of parasites than with any of the others. In retaining the old phylum "Vermes," he has chosen to remain a follower of the old school of classification. The order Acanthocephala, which some zoologists consider to be worthy of a separate phylum, has been retained in the class Nemathelminthes.

The protozoan groups Babesidae and Theileridae are treated as appendices to the Order Haemosporidia, Class Sporozoa. Along with these, and in the same category, the author also includes "Die Anaplasmen," "Die Piroplasmen," and "Die Chlamydozen." Under the Phylum Arthropoda, Class Arachnoidea, the name of the mite family has been changed from Acaridae to Sarcoptidae, and likewise the subfamily name Acarinae has been replaced by Sarcoptinae. The Suborder Tanystomata, which has been used to include the dipterous flies, known as deerflies and horseflies, is rarely, if ever, seen in the more recent classifications of the Order Diptera. According to the author, this suborder is characterized by having (1) dorso-ventrally flattened bodies, (2) short antennae and short legs, (3) long proboscis, and (4) larvae and pupae inhabiting fresh earth. He has treated it as a group intermediate between the Nematocera, those flies having long antennae, long proboscis and long legs, and the Brachycera, those flies having short antennae, short proboscis and short legs. Most students of Diptera now include the Tabanidae (horseflies and deerflies) in the Suborder Brachycera, thus eliminating this intermediate group.

The book is profusely illustrated, and keys to species, genera and families appear frequently throughout the text. Following the main body of the book, the author groups the individual parasites according to (1) their respective hosts, and (2) their location within the host.

Veterinarians, parasitologists and medical entomologists, and to some extent practitioners of human medicine, will find this book of value.

EVERETT E. WEHR

MINUTES

Two Hundred Sixty-ninth to Two Hundred Seventy-sixth Meetings

The 269th meeting was held on October 8, 1947, at the U. S. National Museum. R. Tucker Abbott, Major Elmer A. Jones, Elizabeth P. Hodges, and Dr. Dominic DeGiusti were elected to resident membership, and Dr. William L. Jellison to non-resident membership. Papers were presented by Steiner, Otto, Allen, Abbott, Chitwood, Spindler, and Price.

The 270th meeting was held on November 13, 1947, at the U. S. National Museum. Dr. B. T. Simms was elected to resident membership. Dr. Christie tendered his resignation from the editorship of the *Proceedings*, and Dr. E. G. Reinhard was elected to succeed him. Papers were presented by Schmidt, Spindler, and Kates.

The 271st meeting was held on December 12, 1947, at the School of Hygiene and Public Health of Johns Hopkins University. Dr. Foster was elected to serve as representative of the Society in the Washington Academy of Sciences. Dr. Price eulogized Dr. Christie's work as editor of the *Proceedings* and in behalf of the members presented him with a gift in token of appreciation. The following were elected: President, J. Bozicevich; Vice-president, E. G. Reinhard; Corresponding Secretary-treasurer, E. M. Buhrer; Recording Secretary, F. D. Enzie. Papers were presented by Bozicevich, Kuntz, Oliver, Chamberlain, Mix, Sadun, and Lindquist.

The 272nd meeting was held on January 14, 1948, at the U. S. National Museum. Dr. Roy W. Chamberlain and J. Anthony Morris were elected to resident membership, and Dr. Lyell F. Thomas and Dr. Norman A. Meinkoth to non-resident membership. Leon Jacobs was appointed to the Executive Committee as member-at-large for a term of two years. The price for volumes 1 and 2 of the *Proceedings* was increased from $1.00 to $3.00 (domestic) and $3.50 (foreign), and the price of the *Proceedings* to subscribers for volumes published subsequent to volume 15 was increased from $1.00 to $1.75 (domestic) and $2.00 (foreign). The report of the Executive Committee regarding the budget for 1948 was read and approved. Papers were presented by Kates, Dikmans, Spindler, and Reinhard.

The 273rd meeting was held on February 11, 1948, at the U. S. National Museum. The report of the treasurer for the year 1947 was read and approved. Papers were presented by Tarjan, Rue, McIntosh, and Wright.

The 274th meeting was held on March 17, 1948, at the National Institute of Health, Bethesda, Md. Papers were presented by Files, Cooper, Meinkoth, Chitwood, Luttermoser, and Steiner.

The 275th meeting was held on April 14, 1948, at the U. S. National Museum. Dr. James T. Culbertson was elected to resident membership, and Dr. B. S. Chauhan to non-resident membership. Papers were presented by Huff, Culbertson, and Wyckoff.

The 276th meeting was held on May 22, 1948, in conjunction with the annual picnic, at the Log Cabin of the Plant Industry Station, Beltsville, Md. Dr. Margaret A. Stirewalt and Dr. Clay Huff were elected to resident membership, and Dr. Phyllis A. Clapham and Bessie Sonnenberg to non-resident membership.

F. D. ENZIE,
Recording Secretary

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