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STUDIES ON DURATION OF LIFE OF ASPICULURIS TETRAPTERA, A NEMATODE PARASITE IN MICE, WITH OBSERVATIONS ON ITS LIFE CYCLE

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STUDIES ON DURATION OF LIFE OF *ASPICULURIS TETRAPTERA*, A NEMATODE PARASITE IN MICE, WITH OBSERVATIONS ON ITS LIFE CYCLE

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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

No subject is of deeper interest to human beings than the duration of human life. The development of the arts of science, biology, and hygiene provided basic insight into the underlying principles of the vital processes and the net effect has been to prolong the life of more and more individuals to the greatest attainable degree.

The quantitative aspect of the duration of life has been neglected by biologists because of the lack of an accumulation of an extensive, broadly-based body of quantitative data over the entire range of biological life. The general instrument for evaluating quantitative data on the duration of life is the life table. A life table is a specific conventional method of organizing and presenting the basic fundamental and essential facts on the age distribution of mortality. It has numerous ramifications and points of usefulness. The best known application of the life table, one which gives it its respectability and importance, is its contribution to the financial success of the life insurance industry. Consequently, there has been a highly sustained

interest in the accumulation of mortality records for man only. Life tables can and should be computed for other forms of life but first scientific workers must compile suitable observational data.

The first complete life tables based upon comprehensive observations on a lesser form of life were those on Drosophila melanogaster, the normal wild type and its mutant vestigial (Pearl and Parker, 1921). During the succeeding 15 years, Pearl and his co-workers continued to extend and refine the mathematical data on Drosophila using the life table approach to determine the effects of heredity, etherization, starvation, population density, and temperature on the expected average duration of life. Several other workers published partial or complete life tables for a few other species, invertebrate and vertebrate. In more recent times, the life table, or some part of it, has been used by scientific researchers as an evaluating tool to measure isolated variables affecting survival of various lesser biologic forms. In a larger sense, a rational analysis of the duration of life awaits some measure, some yardstick, of the individual's total activities, over its whole life; that is, a numerical expression for the net integrated effectiveness of the sum of all the forces acting upon an individual throughout its life. No such cleverly ingenious mathematical "law" of mortality is yet available. Meanwhile, there is a basic need for more observational data, collected for a wide variety of animal and plant species, that will follow the life history, from birth to death, of each individual in a cohort.

The motivating stimulus for this research was the idea of formulating a life table for a parasitic form of life. Ordinarily, in collecting

data for a life table, a fixed cohort of individuals of age x is followed completely through life, or any portion thereof, according to certain fixed mortality conditions. The number of deaths in each year of age is noted, as well as the number surviving each year of age, until only one survivor remains. However, for lower forms of life, time units of hours, days, weeks or months are used according to the natural life history of the organism. The idea of studying a parasite in this way introduces a few stumbling blocks to the ordinary techniques employed to gather mortality data. With the basic presumption that it might still be feasible, the following research objectives were set up:

1. To determine the essential characteristics of a suitable test organism and to work out the necessary techniques for handling both host and parasite.
2. To determine the kind of quantitative data required and to design the experiment to meet the need.
3. To determine the most suitable statistical analysis applicable to the data.
4. To make such observations on the actual development and life cycle of the parasite as are important to the comprehension of the research problem.

CHAPTER II

THE LIFE TABLE: ITS STRUCTURE, HISTORY AND SPECIAL APPLICATIONS TO LOWER ORGANISMS

Structure of the Life Table

The three essential components of a skeleton life table are l_x, the survivors at the beginning of each age interval; ${}_hd_x$, the number of deaths observed during the age interval of width h; and ${}_hq_x$, the probability of dying during the age interval h. The mathematical inter-relationships between these components are simple and direct. Among l_x survivors at age x there would be ${}_hd_x$ deaths between the ages of x and x + h, then the number of survivors at age x + h is

$$l_{x+h} = l_x - {}_hd_x.$$

The probability of dying during the h years is then

$${}_hq_x = {}_hd_x/l_x.$$

Two other functions, commonly used in vital statistics, are derived from the basic elements of the skeleton life table. The first, symbolized as ${}_hL_x$, defined as the number of "person-years" of life in the age interval h, represents the years lived by the l_x individuals from age x to x + h. If the deaths noted during the h years are assumed to occur uniformly throughout the interval, then ${}_hL_x$ derives from

$${}_hL_x = h(l_{x+h}) + h/2\ ({}_hd_x).$$

The second function, ${}^{o}e_x$, is often called the "expectation of life," for the sake of accuracy, should be interpreted as the "mean after lifetime." It represents the average number of years of life remaining for survivors at age x and is calculated from the total "person-years," T_x, thus:

$$T_x = L_x + L_{x+1} + L_{x+2} + \ldots + L_w = \sum_{x}^{w} L_x,$$

where w represents the final age in the series. Then,

$${}^{o}e_x = T_x/l_x.$$

The columns of the life table would then be as follows:

$$\underline{x \text{ to } x+h} \quad \underline{l_x} \quad \underline{{}_hd_x} \quad \underline{{}_hq_x} \quad \underline{{}_hL_x} \quad \underline{T_x} \quad \underline{{}^{o}e_x}.$$

Life Table Studies as Applied to the Comparative Mortality of Lower Organisms

Natural biological death is a rather recent evolutionary development. In Paramecium, simple fission, periodically reinforced by the rejuvenating processes of endomixis, described by Woodruff and Erdman (Pearl, 1922), and conjugation, in essence, confers a kind of immortality on the organism. With each succeeding generation, two cells are produced with no dead residue remaining. Antithetically, highly complex, multicellular organisms reproduce by the union of two germ cells. The somatic cells ultimately succumb to natural death at time intervals which vary from species to species. The germ cells, perforce, also die at the moment of somatic death but this should be considered accidental rather than biological. It would seem that the duration of life of the higher biological orders is primarily a function of genetic constitution; secondarily, an etiological effect of its biological activity and the interaction of environment on the other two factors.

This is the problem of the biology of death, interpreted and whimsically described by Pearl (1922) thus:

> It may help to visualize this problem of the determination of longevity to consider an illustrative analogy. Men behave in respect of their duration of life not unlike a lot of eight-day clocks cared for by an unsystematic person, who does not wind them all to an equal degree and is not careful about guarding them from accident. Some he winds up fully, and they run their full eight days. Others he winds only halfway, and they stop after four days. Again the clock which has been wound up for the full eight days may fall off the shelf and be brought to a stop at the third day. Or someone may throw some sand in the works when the caretaker is off his guard. So, similarly, some men behave as though they had been wound up for a full 90-year run, while others are but partially wound up and stop at 40 or 65, or some other point. Or, again, the man wound up for 80 years may, like the clock, be brought up much short of that by an accidental invasion of microbes....The essential problem is: what determines the goodness of the original winding? And what relative part do external things play in bringing the running to an end before the time which the original winding was good for?

Experimental studies on the duration of human life are not feasible, thus one must use an animal whose life span is relatively short compared with man. Pearl chose Drosophila melanogaster whose life cycle from egg to egg spans 8 to 10 days. The adult life span varies from 1 day to 90 days and parallels man in that a 90-day fly is as senile as a 90 year old man. The first life table for this organism, or any other lower organism, was published in 1921 by Pearl and Parker. The ground work was established for the technique of counting dead flies at daily intervals, beginning with the emergence of the imago. The l_x survivorship curve derived from the observed data, smoothed by fitting to a logarithmic parabolic curve, was compared with the typical l_x curve for man. The two were closely parallel except that the fly survivorship curve did not show the heavy infant mortality characteristic

of the human curve. This was attributed to the fact that only the adult stage of the fly was under study. In this first experimental mortality study the fly life span was equated to man on the basis of one day of life for the fly to one year of life for man.

This first step, the derivation of a life table and the description of survivorship curves for normal wild Drosophila, provided the impetus for research into the role of genetic constitution and environment, biological and external, in the determination of longevity for this species. Life tables were formulated under a variety of experimental conditions. Tables for the progeny of brother-sister matings indicated that line-bred strains varied significantly in their expectation of life (Pearl and Parker, 1922). Hybrid lines, resulting from crosses between long-lived and short-lived flies, gave evidence of a Mendelian pattern of inheritance for longevity (Pearl, Parker and Gonzalez, 1923). An attempt was made to determine whether certain chromosome factors controlled survivorship (Gonzalez, 1923). Tables derived from data collected for survivorship ability following drastic changes in environmental conditions were also published for the fruit fly. Successive etherizations did not seem to change the expectation of life or the l_x curves (Pearl and Parker, 1922a); but there was a 10 per cent increase in mean duration of life among flies reared under ventilated conditions as compared with those kept in unventilated jars (Pearl and Parker, 1922c). Pearl advanced the concept that the actual realization of the inborn potentiality of longevity was a function of the environment in which life was lived. He studied longevity under conditions of complete starvation (here food was

the internal environmental condition) for the normal wild type fly and the mutant vestigial (Pearl and Parker, 1924a). Under conditions of complete starvation, the duration of life and the form of the life curve were practically identical for both vestigials and normal flies. With full feeding, the normal wild type lived three times longer than the vestigials. The effects of different diets were also studied (Alpatov, 1930). Tables were published evaluating such diverse environmental effects as temperature (Alpatov and Pearl, 1929) and sterility of environment (Steinfeld, 1928). A study of the effect of crowding on the duration of life in Drosophila indicated that density of population "... is the most important and significant element in the biological, as distinguished from the physical, environment of organisms," (Pearl and Parker, 1922b). In general, the lowest density was not the optimum. The mean duration of life tended to increase with increasing population density up to a certain optimal point. After that, increased crowding was associated with diminished duration of life, even lower than under sub-optimal conditions. A follow-up study, involving the calculation of various constants from the life tables on population densities, showed that an initial density of 35 flies was optimum (Pearl, Miner and Parker, 1927). The death rates, $q_x/1000$, were age specific and tended to increase with age at all densities up to 100 flies per bottle. At higher densities, the highest death rates were experienced at the lowest ages; there were falling rates until middle life. Finally, death rates increased with advancing age.

Life tables for other non-human forms have also been published.

A graph of the ungraduated l_x line (Pearl and Parker, 1924) was derived from data on the survival of the saturniid moth, Telea polyphemus (Rau and Rau, 1914). Observations on the rotifer Proales decipiens (Noyes, 1922) were formulated into life tables and compared with those of man (Pearl and Doering, 1923). Quantitative data on life duration for Hydra fusca (Hase, 1909), for the roach Blatta orientalis (Rau, 1924), and for the slug Agriolimax agrestis (Szabó and Szabó, 1929) were graduated by fitting to suitable curves; complete life tables were formulated therefrom (Pearl and Miner, 1935) for comparison of both the l_x and d_x curves.

There exists a large body of literature on the application of survival data, or some life table function, to the study of the biology of lower forms of life. Of special interest are some examples of experimental survival studies on test organisms important from the public health point of view. Studies on the effects of temperature and humidity (Leeson, 1932) and saturation deficiency of the atmosphere (defined as mm. of Hg. at various humidities and temperatures) on the duration of life of fed and unfed Xenopsylla cheopis (Leeson, 1936) have been reported. These studies evolved from previous work which reported the mean survival time for unfed, newly emerged Xenopsylla cheopis at 20° C and 100 per cent humidity (Hopkins, 1935). Bulinus truncatus and Planorbus boissyi, both intermediate hosts for schistosomes in Egypt, are assumed to have a life span of 3-5 years. If they can carry a schistosome infection for life, their significance as vectors is closely associated with their respective life spans. Monthly mortality data on these two forms were collected

after infection and the "expectation of life" calculated from the life table l_x function (Barlow and Muench, 1951). Lightly infected snails tended to carry the infections for their entire lives, but heavily infected snails had a greatly reduced expectation of life. On a more modern note, the flour beetle, Tribolium confusum, had its life span prolonged by small doses of radiation (Cork, 1957). That life table studies can be useful in a number of ways has been amply demonstrated.

CHAPTER III

METHODS OF PROCEDURE

The objective of this study was to assemble quantitative data on the duration of life of an endoparasite for comparative analysis with the mortality of other organisms. The technique for gathering mortality data on lower forms of life is, under ordinary circumstances, simple and direct, albeit laborious. An initial population is selected and isolated; those dying at specified intervals are withdrawn from the cohort and the number recorded. Finally, when none or very few of the initial group are alive, the data on mortality can be analyzed by means of life tables. The important feature here is that the observations are direct and are always made on the same cohort. An endoparasite poses a different problem. It depends upon a specific host for survival and one cannot record the rate of attrition among the parasites without first sacrificing the host. Perforce, the survival potential of the parasite is artificially terminated. However, the problem was rationalized thus: if one were to start with a standardized universe of infective eggs from which random samples of fixed size were withdrawn and introduced into uniform host animals then, as the hosts were sacrificed in chronological sequence the mortality data on the parasites would also follow in sequence. Survivors could be counted at each periodic interval and the difference

in survivors between two successive intervals would equal the number dying in that interval. Having accepted this rationalization, the objectives of this problem were set down as listed in the Introduction and will be discussed herein in terms of methods and materials.

Selection of the Test Organism

The essential prerequisites of a suitable endoparasite for this study were formulated as follows:

1. It must be wholly parasitic, viz., have no free-living stages in its life cycle.
2. To make the collection of data a feasible undertaking, a form with a short life span was desirable.
3. It was essential that there be a 1:1 relationship between egg and adult. Each egg should eventually produce only one adult, otherwise the initial cohort would not bear any relationship to the total population exposed to risk over the entire life span. Thus, the test organism must not have propagative stages throughout its life history.
4. The most suitable organism would be one with a narrowly circumscribed habitat and no migration pattern. A parasite, which, during its life cycle, followed a complicated migration pattern would make the tabulation of quantitative survival data impractical.
5. The organism should be of sufficient size to make its isolation and identification both facile and accurate.
6. The specific host animal should be one which is neither too

difficult to maintain in the laboratory nor too expensive.

Excellent facilities for the care and maintenance of mice were readily available in this laboratory and the writer was experienced in the handling of these animals; therefore, this was a logical choice of host. Among the nematode parasites of the laboratory mouse is the oxyurid Aspiculuris tetraptera (Nitzch, 1821) Schulz, 1924, the mouse pinworm. It is host specific, wholly parasitic throughout its life cycle, and is transmitted by direct infection via fecal contamination (Mya, 1955) or by transmission of eggs through the air. There are no propagative stages (Philpot, 1924) nor did there seem to be evidence of tissue invasion or extensive migration (Wells, 1952). Its average life span was reported to be a little over a month and the colon is its essential habitat (Hsieh, 1952). All stages of the parasite, from egg through larval stages to the adult can be easily identified under the dissecting microscope at 30 to 45 magnifications. Thus, Aspiculuris tetraptera qualified as a suitable test organism.

To add to the interest of the problem it was considered preferable to select an organism of general medical interest. In recent years much attention has been centered on the use of piperazine citrate as a specific therapeutic for human pinworm infection. The mouse pinworm, Aspiculuris, has been used by many investigators, over a number of years, in anthelmintic studies.

Care and Maintenance of Experimental Animals

The experimental animals were required to be of similar genetic constitution, of the same approximate age and nutritional status, and

nematode-free. Nematode-free mice were not commercially available so a breeding and maintenance program was undertaken in this laboratory. The initial source of mice was the highly inbred Carworth Farms CF-1 strain. The original stock comprised 15 females and six males born on October 11, 1956.

Breeding Experimental Animals

Mice were selected as breeding stock on the basis of appearance, weight, and order of parity. The second parity and succeeding ones are considered more hardy (Worden, 1947; Farris, 1950). The housing units consisted of ordinary round, wide-mouth, one gallon jars, fitted with screw-on lids from which the metal centers had been sheared away and replaced with wide-mesh wire screening. The jars were preferred to metal cages because they could be cleaned readily with soap and water and stored easily. Wood shavings for bedding and water bottles, suspended vertically from the lids so the water tubes extended to within one inch of the level of the bedding, completed the housing unit. Bedding, jars and water bottles were changed weekly.

Young were usually separated from the mother at about 21 days of age. Those scheduled for breeding stock were kept together as a litter until they were 45-46 days old. From these, selected animals were marked for recognition with picric acid, then groups of three females and one male (brother and sisters) were set up per breeding jar (Poiley, 1952). It was found that a maximum number of pregnancies occurred if males were kept with females for a period of seven days. Thus, males were removed after seven days and kept in separate jars until the next mating.

Subsequently, those females which were obviously pregnant were isolated in separate jars about three days preceding the estimated time of arrival of the litter. The experience in this laboratory showed that greater numbers from each litter were successfully reared to weaning age if nesting material, such as shredded paper towelling, was provided. Repeat matings between the same males and females were made until each female had produced three litters. Eventually, by April, 1957, 150 to 200 mice suitable for breeding stock of oxyurid-free mice were available. Certain mice were set aside to receive anthelmintic treatment in order to establish an oxyurid-free colony. The mice not set aside for therapy were added to stock mice kept in the animal house, all of which were known to be heavily infected with Aspiculuris, and this latter colony was the constant source of parasites for the study.

Maintenance of Nematode-Free Mice

Selected mice were treated with seven daily doses of 500 mgm./kgm. body weight of "Pipizan" (Sharpe & Dohme brand of piperazine citrate) syrup (Standen, 1953). All dosages were calculated as anhydrous base. The medication was measured into a tuberculin syringe which was then capped with a 2.5 inch, 18 gauge steel needle, bent slightly and tipped with a bit of polyethylene tubing (.043 inches inside diameter by .050 inches outside diameter). The width of the plastic tubing was just sufficient to exclude the needle from the trachea, thus the needle was introduced only into the esophagus without lacerating the throat of the mouse. Fresh plastic tips could be replaced as often as was necessary. Following each treatment, the anus and perineum of each

mouse was swabbed with 95 per cent alcohol and the mice placed in freshly sterilized housing units. The exact procedure for sterilizing the units will be discussed later. The rationale behind this maneuver was to prevent reinfection of the animals from any contamination in the environment. Following the last scheduled dose one mouse from each jar was sacrificed and checked for the presence of nematodes by careful examination of the cecum and colon. If any jar yielded an infected mouse, this group of mice was discarded. If the test mouse was negative, then the remaining mice in the jar were checked by cellulose tape slides to pick up Syphacia eggs, and a modified $ZnSO_4$ fecal flotation technique (Hussey and Alger, 1951) to detect Aspiculuris eggs. Only those individual mice which were negative for both Syphacia and Aspiculuris eggs were acceptable. These nematode-free mice were transferred to an isolation room where the temperature was maintained at 70-72° F. This was the breeding and maintenance center for the nematode-free animals. The afore-mentioned breeding practices were also applied to the nematode-free mice. The isolation room had no natural lighting source. Since mice reared in complete darkness exhibit erratic and unpredictable estrus cycles (Snell, 1941), an artificial light source was provided. The room was fitted with an automatic interval timer, connected to a 100 watt light bulb. The light turned on at 6 a.m. and off at 6 p.m. giving twelve hour periods of light and dark.

Strict precautions were required to protect the animals from re-infection via contaminated food, bedding and air. Everything brought in contact with the mice was first sterilized. Housing units were changed

twice each week. Soiled jars and lids were washed in soap and water and kept in a hot air oven at 190-192° C for 16-18 hours. After cooling in the oven, the jars and lids were stored in closed boxes. Bedding, food pellets, water bottles and drinking tubes (including rubber stoppers) were autoclaved at 15 lbs. pressure for 30 minutes. Following sterilization, all equipment and materials were carefully stored to prevent contamination. The only other factor not controlled by sterilization was the air surrounding the jars. In the event some nematode-infected mice had escaped detection, circulating air currents were considered a serious means of cross-contamination between jars. To prevent this, a continuous outward flow of air from each jar was effected by directing a downward flowing air stream into the jar. Glass tubes, bent to a 90° angle, were suspended over each container with the tips extending almost to the level of the bedding. The other end was attached by rubber tubing to air valves just lightly cracked to permit a gentle flow of air. A round, filter-paper disc, slit along three-quarters of its diameter to accomodate the water and air tubes, was placed on each jar lid to prevent the settling of air and debris into the jars. The effectiveness of this technique was checked by blowing cigarette smoke across the top of the jars; the cloud of smoke drifted upward and away, but never into the jars. This procedure was introduced after all the mice for an experimental run had to be discarded when the writer proved infection was possible via inter-jar contamination.

Food pellets were sterilized because food stocks were kept in open sacks in the general animal house where the Aspiculuris-positive mice were quartered. However, in the process of sterilization, heat labile

vitamins originally in the balanced food formula were destroyed. The effect of an avitaminotic host on the survival of the parasite would be an uncontrolled variable. Thus, the heat labile vitamins were restored by coating the pellets with Pervinal syrup in amounts calculated to supply the recommended vitamin levels maintained in mouse diets by Carworth Farms.

The final check on the efficacy of all the previously described procedures was to make direct examinations on the animals periodically. Each week, one mouse from each jar of breeding stock was checked by cellulose tape and zinc sulphate techniques. If any mouse was positive for either Syphacia or Aspiculuris, the entire group housed in that jar was destroyed. Also, a randomly selected sampling of mice was sacrificed and examined for nematodes. Again, if any mouse was positive, all its companion mice were destroyed. By such rigid methods, a relatively small but nematode-free colony of mice was maintained. From this colony, young mice, about 5-6 weeks old, were used in the life table experiment.

Handling the Parasite

The protocol for the preparation of a uniform batch of infective Aspiculuris eggs was designed to preclude any confusion with Syphacia obvelata, a closely related oxyurid of the laboratory mouse. A week preceding the time scheduled for a life table experiment, five to six mice were taken from the stock of infected mice. Each mouse was killed by a sharp blow on the head; a slit was made in the abdomen, the colon and cecum excised and placed in a petri dish of distilled water filled to a depth of 3-5 mm. A small slit, a few mm. in length, was made with

a fine dissecting needle in the proximal third of the colon, just below the ileo-cecal junction. The dish was covered and allowed to remain at room temperature (21-22.5° C) for about 30 minutes. The Aspiculuris crawled out of the lumen of the segment of gut and squirmed actively in the water. To avoid clouding the water, the section of gut was removed to a petri dish with fresh water and the entire length of the colon opened. The greater part of all adult worms present were released into the water by this time. Aided by a magnifying glass, the easily visible white worms were picked up with a fine, bent needle and transferred to a dish of fresh, clean distilled water. This was done to minimize the transfer of fecal debris and to facilitate identification of the worms. Under the dissecting microscope (45 x magnification), each worm was first identified as a gravid female Aspiculuris tetraptera by the following distinctive anatomical features:

1. Three, large, inflated lips -- giving the anterior end a bluntly-rounded appearance.
2. Two prominent cervical alae with recurved margins, terminating at about the level of the esophageal bulb.
3. An anterio-ventral vulva situated about one-half of the way down from the anterior end.
4. A short, thick tail, gently tapered.
5. The uterus extending posterior to the anus, sometimes almost to the tip of the tail.

In contrast, the mature Syphacia obvelata female has less prominent lips, giving her a more narrow anterior end; the vulva is about one-third of the

way down from the anterior end; she has a long, narrow pointed tail and the uterus never extends below the level of the anus.

In order to get large numbers of infective eggs for experimental use, properly identified Aspiculuris were sorted out and those heavily laden with eggs were transferred to a fresh dish of distilled water (usually about 500 worms were gathered for each run) and incubated at room temperature for seven days. Between the third and seventh day of incubation, a random selection of worms was removed to a smaller dish half-filled with distilled water. On these, special embryonation data were collected by dissecting out the eggs, counting both embryonated and non-embryonated eggs and recording the total. The embryonated eggs were transferred to another petri dish of distilled water. These eggs were the ones used to infect the nematode-free mice. Eggs were gathered over a span of 5 days until there were enough to make a run. It was necessary to gather about 10,000 eggs for each life table experiment.

Infecting the Experimental Animals

The most demanding criterion of a life table is that the initial population under observation be counted accurately. The well-known estimation techniques for counting parasite eggs (Stoll, 1923) and protozoa (Hall et al., 1935) were found to be unsuitable. Instead a direct counting technique was devised. After all the embryonated eggs had been gathered in a dish of distilled water, they were washed several times by gently pouring off the supernate after each addition of fresh distilled water. Using fine needles (insect mounting pins), eggs were pushed to one side and counted in groups of five until there was a

small cluster containing 300 eggs. To test the accuracy of this method a series of repeat counts was made on 5 clusters, counted in this way, and each time only 300 eggs were found in each pile.

The next problem was to determine the best way to pick up and deliver the dosage of eggs and be able to state the exact number delivered to the mouse. A tuberculin syringe and needle were too awkward to use for picking up a small cluster of eggs. Another difficulty was encountered in trying to expel the eggs from the syringe. Despite repeated washings, too many eggs were retained within the syringe or the needle. The numbers recovered varied as much as 50 per cent from the original number picked up. To overcome this difficulty, a simple pipette was devised. A four inch length of glass tubing (3 mm. inside x 4 mm. outside) was drawn out into a fine point at one end. This end was also made to curve gently downward to match the curvature of the hard palate of the mouse. Rubber tubing, fitted with a plastic mouth piece, was mounted at the other end. Under direct observation with the dissecting microscope, the mound of eggs was drawn into the tube without sucking up surrounding eggs. The pipette was introduced into the esophagus of the mouse and its contents gently expelled with a little air pressure. The pipette was rinsed once with about 0.1 cc of water and this rinse water was also gently introduced into the mouse with a little air pressure. The exact time, to the nearest minute, was then noted because this marked the time of origin for the life table data. The now empty pipette was meticulously examined under the dissecting microscope to detect any eggs which might have adhered to the sides of the tube. If any were there, these were counted and deducted from the known number of eggs

originally picked up. If too many eggs were left behind, that mouse was discarded from the experiment. Occasionally this happened because the mouse would manage to resurge some of the eggs before the pipette could be withdrawn. Thus, for each mouse in the experiment, a known number of eggs was considered to have been successfully delivered to the host. Before using the pipette for the next animal, it was rinsed under running tap water, wiped dry and checked under the dissecting microscope to make certain no eggs were still retained inside.

The reliability of the egg-dosage-delivery technique was originally checked by following the same procedure for counting and picking up eggs, but the contents of the pipette were discharged into a small watch glass along with a single rinse. The number of eggs recovered in the dish plus the number of eggs which might have remained in the pipette were summed to see how many of the original number could be recovered. Batches of 100 eggs were counted and this test repeated ten different times; 5 batches each of 200 and 300 eggs were counted the same way. There was a variation of 0 to 3 per cent between the original number counted and the final number recovered which could be ascribed to errors in the testing technique. This was considered of sufficient accuracy to indicate the dosage.

Infected mice were then sacrificed, one each day or at whatever time interval designated, at the exact time of day noted when each received the inoculum of infected eggs. Thus, an accurate time interval was established. Mice were killed by a sharp blow on the head, the colons and cecums excised and put into petri dishes of physiological

saline to which a few drops of 1:200 dilution of neutral red had been added to give a faint pink color. The cecum was separated from the colon and put into a separate dish. The colon was cut into three equal sections and each piece placed in its own dish. All the dishes were filled with the neutral-red saline. The bits of gut were slit lengthwise with needles. After a few minutes, actively moving worms could be found in the saline and picked up, one by one, with the same kind of pipette described previously. An accurate count was kept of those worms which were picked up and transferred to saline in a small watch glass. The inner surface of the gut was then gently scraped with a bent needle and the tissue removed to a glass slide to be examined for adhering worms by the pressed slide technique. All the fecal debris and mucus scraped from the gut were minutely examined until the writer was satisfied that no more living worms could be recovered. Some worms were saved for anatomical studies and others were used for observations on molting using the technique described by Smith (1952). This will be discussed in more detail later.

CHAPTER IV

RESULTS

Life Table for Aspiculuris

An experimental life table for the parasitic nematode, Aspiculuris tetraptera, derived from raw, unfitted survival data, is shown in Table 1 (refer to pages 4 and 5 for structure of life table). Altogether, 60-70 nematode-free, white CF-1 mice, 38-42 days old, ranging in weight from 13-18 gms., were experimentally infected with embryonated Aspiculuris eggs. Not all of the mice contributed to the overall data. Some were not infected, others did not get the intended dosage of eggs or very few eggs hatched. It must be emphasized that the life table presented here is based on minimal data and is to be accepted only as a pilot life table showing a generalized survivorship trend for the test organism.

The age intervals were selected to permit the most rational assay of the data. All of the survivorship values (l_x) are based on 300 infective eggs as the original population. The most important feature of the table is the "expectation of life" column labelled $^{o}e_x$. The significant figure to note is 12.0 days, which is the mean duration of life for all the eggs starting at moment zero. With this figure established, one is prepared to order the observational data in

TABLE 1

LIFE TABLE FOR ASPICULURIS TETRAPTERA CALCULATED FROM OBSERVED SURVIVORS ISOLATED FROM THE MOUSE HOST

Age in Days	$h l_x$	$h d_x$	$h q_x$	$h L_x$	T_x	$^{o}e_x$
0-7	300	158	.5280	1547	3613	12.0
7-9	142	25	.1760	259	2066	14.6
9-13	117	12	.1025	444	1807	15.4
13-17	105	36	.3425	348	1363	12.9
17-26	69*	7	.1015	590	1015	14.7
26-30	62	0	.0000	248	425	6.9
30-36	62	59	.9530	177	177	2.9
37 +	3	3	-	-		-

*Average of l_x for 18th, 20th, 24th, and 26th day.

such a way as to make a reasonable comparison between the test organism and other lower forms of life in terms of duration of life. Tables for lower organisms analyzed by Pearl (1940) were rounded out to start with a cohort of 1000 individuals. Thus, Table 2 is a skeleton life table, based on the observed survivors of Aspiculuris, but calculated on the basis of the experimentally derived mortality rates ($_hq_x$) applied to a hypothetical stationary population of 1000 worms. Table 3 represents the distributions of survivors and deaths arranged as percentage deviations from the mean duration of life taken as zero deviation. Again the deviations were selected to permit the most rational analysis of the data with the range between +60 per cent and +80 per cent so taken because the survivors were averaged over this range in the original life table. The actual figures for $_hl_x$, $_hd_x$, and 1000 q_x were calculated by straight line interpolation between two age intervals as required.

Survivorship and Death Curves

The survivorship curve (Figure 1), death curve (Figure 2), and death rate curve (Figure 3) were plotted from data in Table 3. They are freely drawn curves shaped to fit the plotted points. The mean duration of life is taken as 100 per cent or zero deviation on the abscissal scales. The other ages (time duration) are plotted as percentage deviations on either side (plus or minus) of the mean. The ordinate scales represent the calculated survivors, deaths, or death rates per 1000 for each time unit. The rather steep initial descent of the survivorship curve (Figure 1) is not interpreted as an actual reduction in survivors, but, rather, a reflection of an intra-crypt

TABLE 2

SKELETON LIFE TABLE FOR ASPICULURIS TETRAPTERA BASED ON A HYPOTHETICAL POPULATION OF 1000 INDIVIDUALS

Age in Days	$_{h}l_{x}$	$_{h}d_{x}$	$_{h}q_{x}$
0-7	1000	528	.5280
7-9	472	83	.1760
9-13	389	40	.1025
13-17	349	120	.3425
17-26	229	23	.1015
26-30	206	0	.0000
30-36	206	196	.9530
36 +	10	-	-

TABLE 3

SURVIVORSHIP (l_x), DEATH (d_x) AND DEATH RATE (1000 q_x) DISTRIBUTIONS EXPRESSED AS PERCENTAGE DEVIATIONS FROM MEAN DURATION OF LIFE FOR ASPICULURIS TETRAPTERA

Percentage Deviations from Mean Duration of Life	Age in Days	l_x	d_x	$1000q_x$*
-100	0	1000	181	181
-80	2.4	819	181	222
-60	4.8	638	175	275
-40	7.2	463	80	172
-20	9.6	383	17	442
0	12.0	366	59	245
+20	14.4	307	72	234
+40	16.8	235	12	51
+60	19.2	223	135	606
+180	33.6	88	78	885
+200	36.0	10	10	1000
+220	36 +	0	-	-

* 1000 q_x calculated as d_x/l_x times 1000

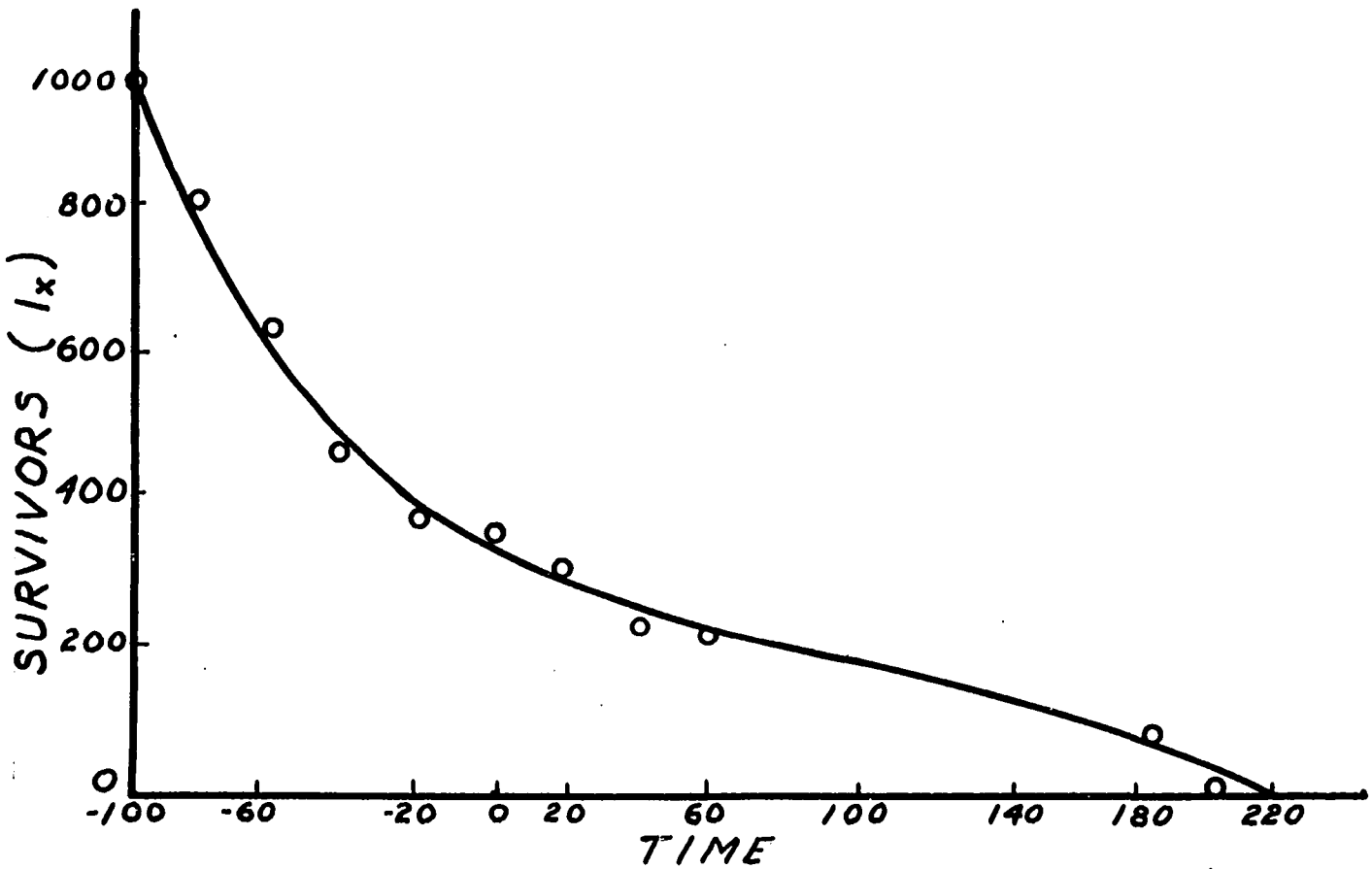

Figure 1. Survivorship Curve for Aspiculuris tetraptera - Distribution as Percentage Deviation from Mean Duration of Life Taken as Zero.

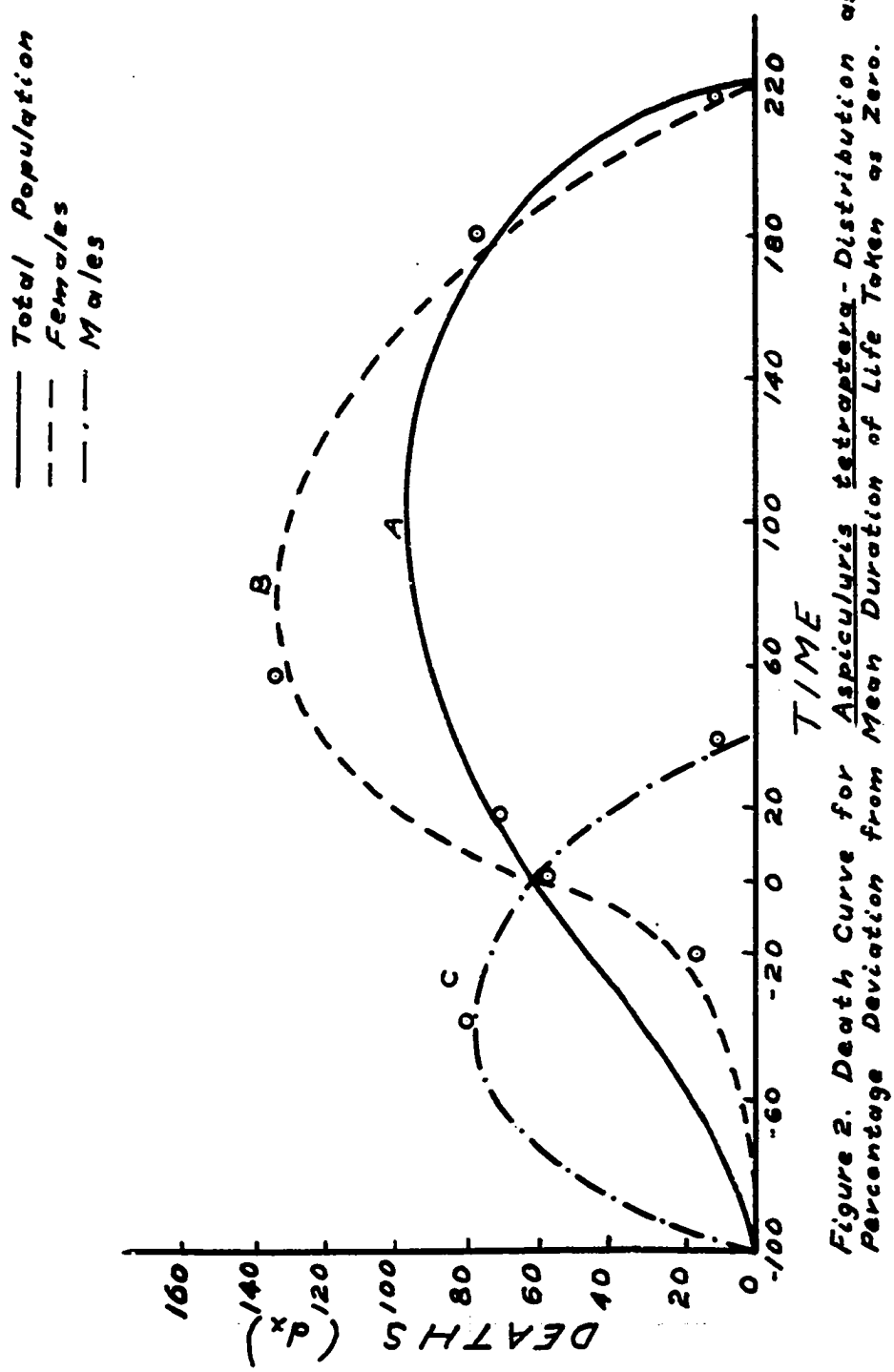

Figure 2. Death Curve for Aspiculuris tetraptera - Distribution as Percentage Deviation from Mean Duration of Life Taken as Zero.

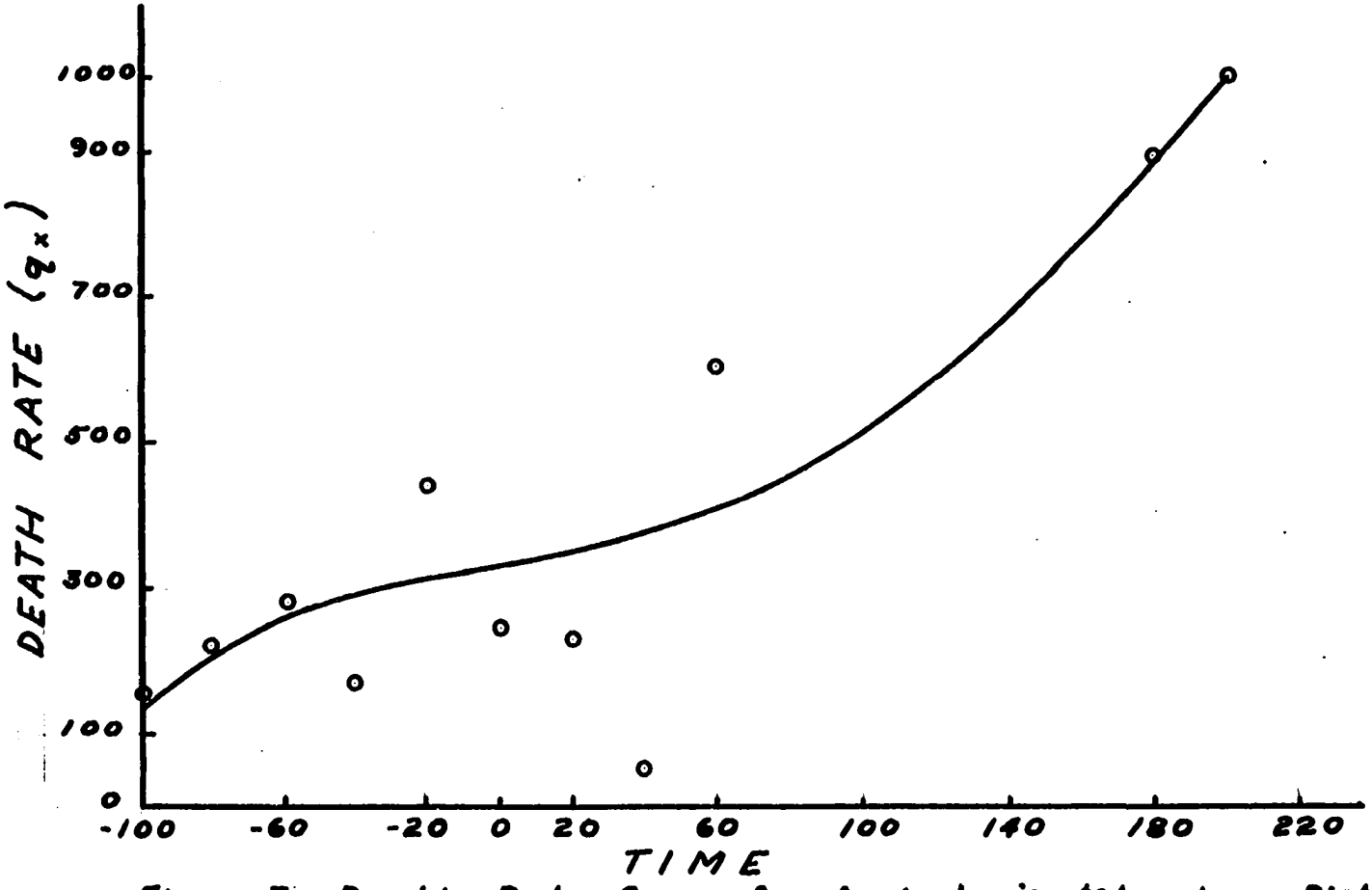

Figure 3. Death Rate Curve for Aspiculuris tetraptera -Distribution 1000 q_x per 20 Percent Deviation from Mean Duration of Life.

phase in the early part of the life cycle (to be discussed in detail later). The survivors are not truly counted until they are free in the lumen of the gut. However, the shape of the curve, in general, is a graphic representation of the overall survivorship pattern of the worm. The death rate curve (Figure 3) is not as closely fitted to the actual plotted points because there are actually two death curves. The male worms live only half the life span of the females and are no longer found after the nineteenth or twentieth day. However, the life table is computed on the basis of the total population, not each sex separately; therefore, the death curve (Figure 2, curva A) was drawn to approximate the combined deaths in the early part of the life span. Curves B and C (Figure 2) represent the theoretical female and male deaths respectively.

Observations on the Life Cycle

The eggs of Aspiculuris tetraptera are oval in shape, 84-90 μ long by 34-40 μ wide. They are not flattened on one side as are the eggs of Enterobius and Syphacia. When oviposited, the egg is in a simple morula stage (Figure 4). The egg shell has a double contoured appearance because the outer protein coat and the inner chitinous shell, together, form the external investment (Christenson, 1950). There is a third layer, the vitelline membrane. The egg shell shows faint radial striations over the entire surface. A slightly thinner area can be distinguished at the antero-ventral pole. This will be the point of emergence of the larva when it hatches. By the third day of incubation there is a well-formed embryo barely showing gut formation within the

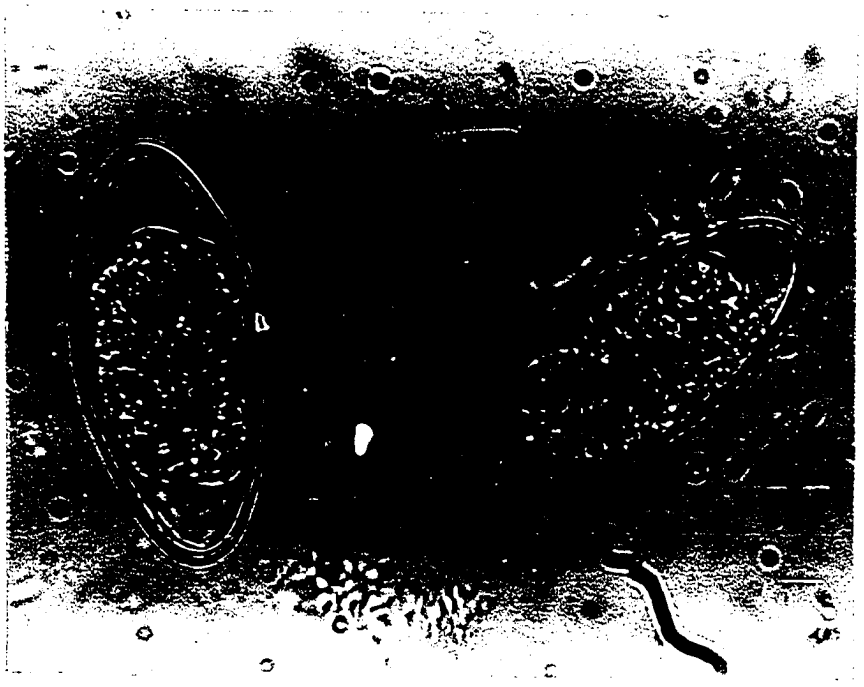

Figure 4. Eggs of Aspiculuris tetraptera as Oviposited (morula stage). x620.

egg shell. The anterior end of the worm is bluntly rounded while the tail is narrow and conical (Figure 5). Both the emergence area and the radial striations are now more readily observed. Either the head end or the tail end of the embryo may be adjacent to the emergence area (Figure 6). Larvae were seen to emerge with either head or tail end first, depending upon the position of the embryo just before hatching. After the seventh day of incubation some few larvae hatched spontaneously, but they did not actively wiggle out of the shell. Instead, the larvae seemed to float out, as if they were being pushed out by the pressure of water flowing into the opening in the shell (Figure 7).

Some of the newly hatched larvae were transferred to a small dish containing neutral red in saline. After two days there had been sufficient dye uptake to show the detailed internal anatomy of the organism (Figure 8). A very fine external cuticle was distinguishable. The mouth appeared as a simple, circular aperture; the gut was differentiated into esophagus with bulb, intestine and what appeared to be a rectum. The anterior end of the intestine showed a fine lumen and there were three distinct, dark dots on the rectum. The faint outlines of a nerve ring were also distinguished. At a mid-lateral point, a transparent ellipsoidal structure, the genital anlage, was noted. This larval stage did not show any active movements but just floated about in the water. When one and two day larvae were found in the gut of the host animal they had the same anatomical features noted above. The only motion noticed was a bending into "c" and "j" shapes.

Figure 5. Egg of Aspiculuris tetraptera Showing Emergence Area and Enclosed 3-day Embryo. x1000.

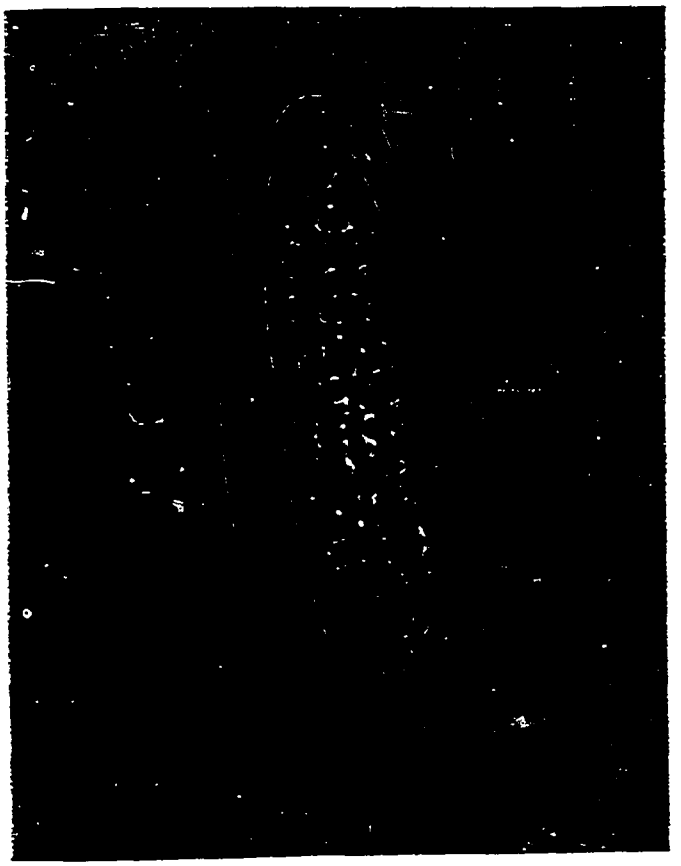

Figure 6. Egg of Aspiculuris tetraptera after Three Days of Incubation. Narrow, Conical Tail End is in Apposition to Emergence Area. x1000.

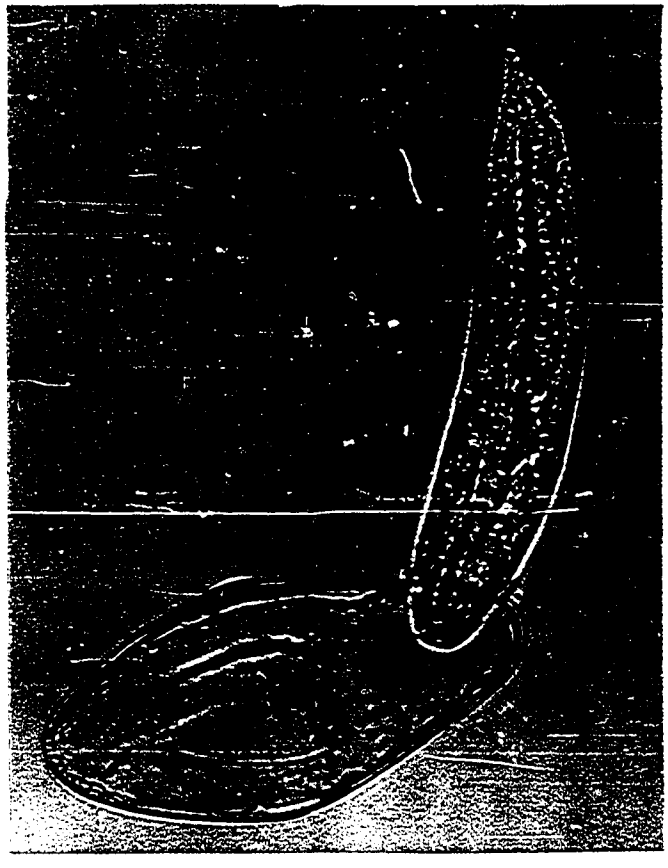

Figure 7. Larva Hatching after Seven Days of Incubation. Organism is Seen Emerging from Shell Tail First. x725.

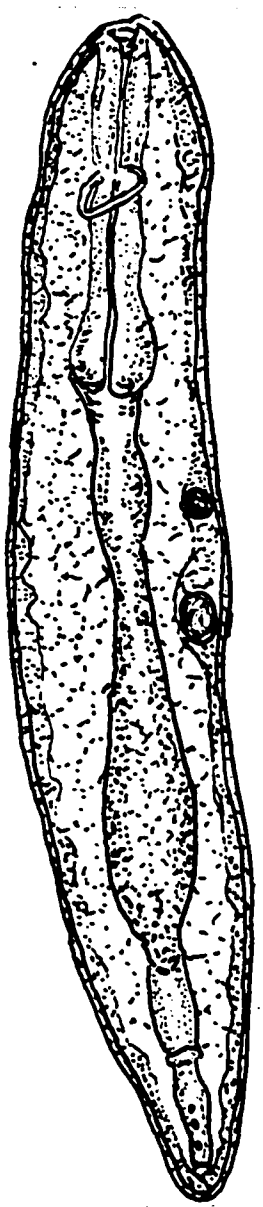

Figure 8. Newly Hatched Larva of Aspiculuris tetraptera. (Free hand drawing as observed under 430 magnifications.)

Quantitative Studies on Embryonation

The intact gravid female worms were incubated in distilled water. Quantitative embryonation studies were made on eggs dissected from the worms which had been incubated from 3 to 7 days. The data is presented in terms of the average total egg burden per worm, the average number of eggs embryonated and the average per cent embryonated (Table 4). The overall average egg burden per worm, over the five days was 105, with a range of 92-113.8. The average per cent embryonated for all the worms was 83.8 per cent. By the chi-square test of significance, there was as much variation between samples on successive days as within samples for each day. There is no demonstrated relationship between time of incubation, in vitro, of the gravid female worms and the number of eggs which develop embryos.

Molting

Chitwood and Chitwood (1950) state, "Nematodes, like arthropods, grow through the process of ecdysis. Usually, the cuticle is shed four times, the fifth stage thus formed being the adult." Molts have been observed, at some stage in the life cycle, for many species of oxyurids. However, this phenomenon has never been reported for Aspiculuris tetraptera. Attempts were made to detect molting in this species by making vaseline ring preparations in rat serum of the larvae recovered from mice at autopsy. Molting was never observed in these preparations. However, molting was observed on one occasion when a larva hatched spontaneously from the egg after seven days of incubation. The cuticle was seen trailing from the posterior end of the larva. The larva, rather

TABLE 4

AVERAGE TOTAL EGG BURDEN, NUMBER AND PER CENT EMBRYONATED EGGS PER ADULT FEMALE *ASPICULURIS TETRAPTERA* FROM THIRD TO SEVENTH DAY OF INCUBATION IN DISTILLED WATER AT 21-22.5° C

Day of Incubation	Worms in Sample	Total Eggs/Worm (Average)	Embryonated Eggs/Worm (Average)	Embryonated Eggs/Worm (Per Cent)
3	10	107.0	78.3	73.4
4	10	113.8	78.0	68.6
5	10	101.6	75.8	74.6
6	8	92.0	67.0	72.3
7	10	110.4	141.0	78.3
Overall Average		105.0	88.0	83.8

than actively wiggling out of the cuticle, seemed to float out. Some drying occurred before a photograph was taken and the cuticle was twisted about the body of the larva, but a ridge of folded cuticle is seen clearly just posterior to the anterior end (Figure 9). In an enlarged photograph of the anterior end of a seven day larva one could detect what seemed to be a new cuticle forming under the old one (Figure 10). Such observations suggest that Aspiculuris tetraptera, like other nematodes, grows through the process of ecdysis.

Intra-crypt Stage

The paradox presented by the daily recovery of increasing numbers of survivors during the first 6 days following infection was suggestive of a possible tissue penetration phase. In an attempt to demonstrate tissue penetration, three nematode-free mice were fed huge doses of embryonated eggs and sacrificed at 12, 24 and 48 hours respectively. The colon and cecum of each mouse was fixed in 10 per cent neutral formalin for 48 hours. After dehydration, clearing and imbedding in paraffin, serial histological sections were cut at 20 μ, mounted on slides and stained with hematoxylin-eosin in the usual manner. At 12 hours post infection, larvae were seen lying in the lumen of the glands of Lieberkühn (Figure 11), but there were no indications of actual tissue penetration nor any recognizable pathological changes. After 48 hours, larvae were still found in the lumen of the glands (Figure 12). This phenomenon, as far as was known, had not been reported in the literature. Later, a reference to an unpublished Master's thesis (Mya, 1955) was found in which the intra-crypt stage for Aspiculuris was mentioned.

Figure 9. Second Stage Larva Emerging from Cuticle. x825.

Figure 10. Anterior End of Seven Day Larva Showing What Appears to be New Cuticle Formation. x665.

Figure 11. Longitudinal Section of Mouse Colon Showing 12 Hour Larva of Aspiculuris tetraptera Lying in the Lumen of a Gland of Lieberkühn. x515.

Figure 12. Cross Section of Gland of Lieberkühn Showing 48 Hour Larva Lying in the Lumen. x525.

This finding is of particular interest in the interpretation of the life table data.

Host-Parasite Relationship

Hsieh (1952) and Mathies (1954) suggested that at 21 days of age the mouse was most susceptible to experimental infection with Aspiculuris; while Mya (1955) found resistance to infection by suckling mice. To confirm these findings, eight nematode-free mice, four males and four females, were given 300 embryonated eggs each and were paired for mating. Young were born to all pairs. Forty-five days post infection, three of the adult pairs, with litters of 10 (9 days old), 6 (17 days old) and 6 (19 days old) mice respectively, were sacrificed. All adults showed evidence of recent reinfection with Aspiculuris. However, upon autopsy, none of the young mice were infected. The fourth adult pair and their litter of four mice were kept together until the young mice were 26 days old, at which time, all mice were sacrificed and examined for worms. The adults showed recent reinfection had taken place and all of the young had light infections with immature worms. These observations confirmed the findings of the afore-mentioned workers.

CHAPTER V

DISCUSSION

An outline of the steps in the life cycle of Aspiculuris tetraptera is presented as a prelude to the discussion of the life table. The life cycle, briefly, is as follows:

1. Eggs in the morula stage of development are laid singly by gravid females in the proximal third of the colon. They are caught up in the mucous coat surrounding the fecal pellets (Mya, 1955).
2. Following a period of incubation (3-7 days) in a moist environment at room temperature, the eggs become embryonated. This is the first stage larva.
3. Within 30 minutes to one hour after the eggs are swallowed, larvae hatch out in the cecum and lower portions of the small intestine (Hsieh, 1952). There is reason to believe that a molt occurs shortly after hatching, thus freeing the second stage larva. This stage is relatively primitive and does not seem capable of active, wriggling movements.
4. By 12 hours, some larvae are found in the lumen of the crypts of Lieberkühn. It is not clear whether the worms actively seek out the crypts or are carried there

accidentally. In view of their sluggish movements at this stage, it is possible that the latter viewpoint is the most acceptable one.

5. There are no exact data on the length of time these larvae remain in the crypts. It seems probable that they have all re-entered the lumen of the gut by the sixth day following infection. Chan (1955), who was not aware of this intra-crypt stage, suggested a migration pattern, down the colon and up again, from the first to the sixth day following infection. The writer did not confirm this. Instead, another interpretation is offered. Those larvae not in the crypts can be carried along with the fecal debris, down the length of the colon, and be expelled. The larvae hidden in the crypts at this stage can re-enter the lumen of the gut over a period of six days and maintain themselves in the upper portion of the colon for the rest of their life spans.
6. From the 8th to the 10th day, sexual differentiation occurs.
7. Males are extremely rare by the 20th day. It is suggested that the males die after fertilization (Hsieh, 1952).
8. Gravid females begin to lay eggs, after the 24th day. Egg laying continues for several days. On the average, females live about 30-35 days, with some living up to 40 days.

The detailed study of the early portion of the life cycle of Aspiculuris tetraptera was a necessary prelude to the interpretation of

the life table data. A life table should start with a known population. Here, the embryonated eggs are counted as the starting population even though the number actually hatching is not known. Hatching counts could not be made because of the intra-crypt phase. Many more eggs could have hatched than would be indicated by the daily count of living larvae over the first six days. From the first to sixth day following the initial experimental infection of the animals, increasing numbers of larvae were recovered on autopsy, ranging from about 30 on the second day to about 120 on the sixth day. This would be a biological impossibility in terms of sequential death. If larvae, not found after any time interval are considered to be dead, then they cannot be alive at the next successive time interval. The period from 0-6 days was taken with the number alive at the beginning of the interval set at 300. The number counted at the start of day seven represents the number surviving over the interval. Thus, failure to hatch, mechanical losses and natural deaths, are all distributed evenly throughout this interval. From the seventh day on the stabilized population of larvae are followed in orderly sequence.

Survivors, not deaths, are being counted from moment zero to age x and deaths are derived by subtraction. Justification in deriving 1_x^{x+h} from successive readings of 1_o^x must be established. The relationship is acceptable because the probability of survival from one age interval to another is derived thus:

$$\text{Let } P_o^x = 1_x/1_o$$

$$\text{and } P_o^{x+h} = 1_{x+h}/1_o$$

$$\text{then, } P_x^{x+h} = P_o^{x+h}/\ P_o^x = 1_{x+h}/1_x.$$

Pearl and Parker (1921) made a rough comparison between Drosophila and human life tables, equating one day of fly life to one year of human life. Later, Pearl (1922a) modified his approach to superimpose the two curves so that "at least two biologically equivalent points coincide." These were taken to be the beginning and end of the life span. Since only the imaginal life of the fruit fly was followed, Pearl took for his starting point the age when human and fly instantaneous death rates (q_x) were at a minimum. The upper end of the life span was taken at the age "at which there is left but one survivor out of 1,000 starting at age 1 day for Drosophila and 12 years for white males." Comparisons of the mortality of Proales with that of Drosophila and man were made using these reference points but with the data ordered in centile groups (Pearl and Doering, 1923). Later, Pearl (1927) devised a more trustworthy method for representing relative variability, following the criticism of Greenwood (1928). The basic point of comparison was the mean duration of life. The mean has long been accepted as the best abstract biological generalization to represent the type of species. All the observed data take a part in its determination. This generalization should be equally acceptable for comparison of life duration as for other measurable characters. The mean duration of life is a good point of comparison among species having diverse life spans because it signifies how long each species typically lives. Deviations in duration of life are then expressed as percentage deviations, rather than as absolute time units.

Curves for survivorship, death and death rates, plotted as percentage deviations from the mean duration of life, fall into three

generalized categories, the rectangular, intermediate, and diagonal. The theoretical intermediate l_x curve is a "smoothly flowing curve concave to the time axis in the first moiety of the life cycle and convex to the same axis in the last moiety...." (Pearl and Miner, 1935). The survivorship curve of Aspiculuris is considered to fall into this category, albeit it is distorted and pulled out along the abscissa. The theoretical intermediate d_x curve is a negatively skewed frequency curve representing a marked degree of scatter in interindividual variation in time of dying. The Aspiculuris d_x curves, both for each sex separately and for the total population, are considered to fall into this category.

No attempt is made to interpret the Aspiculuris curves derived from the minimal observational data. Rather, they are presented to indicate the feasibility of doing a life cycle study on an endoparasite. Since the curves are recognizable as basic theoretical curves described by Pearl (1940), the technique and the data can be considered reasonably correct. The mathematical relationships herein described are acceptable assumptions. However, one factor which was uncontrollable, viz, interanimal variations in susceptibility to infection, is worthy of mention. In general, the mice showed about a 75 per cent level of infection. About 20-25 per cent of the mice failed to get any infection or only a very small fraction of the larvae survived. If numerous repetitions of the life table experiments could have been made, this variable would have been reduced to minimal proportions. Thus, the concept of a life table study on a parasite can be considered feasible.

CHAPTER VI

CONCLUSIONS

A life table study on the endoparasite Aspiculuris tetraptera, an oxyurid nematode in the colon of mice, was undertaken to determine the feasibility of the technique. It was found that the test organism was acceptable in most respects, except that an intra-crypt phase, in the early part of the life cycle, limited the accuracy of the initial observational data. This was minimized by starting with a grouped interval of 0-6 days. There was some degree of variability introduced by inter-host variations in susceptibility to experimental infection. Sufficient repetitions of the experimental observations would tend to equalize this factor. Despite these limitations, a reasonable set of survivorship, death and death rate curves were obtained. The suggestion is made that life table studies on parasites can be useful in the evaluation of genetic and environmental factors affecting duration of life of a parasite.

Some observations were made on the life cycle of the parasite. Molting was seen shortly after hatching of the first stage larva. There was some suggestive evidence of molting at other stages in the life cycle. This is of interest because molting has not been reported for this form. No larval migration pattern was observed. However, the

intra-crypt phase, a phenomenon which has been noted by only one other writer, could explain the absence of larval forms in the upper part of the lumen of the colon during the first week after infection. There is some evidence to suggest that suckling mice, under 21 days, are resistant to natural infection with Aspiculuris.

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