STUDIES ON THE ECOLOGY, BEHAVIOUR AND PHYSIOLOGY OF THE INFECTIVE LARVAE OF *ANCYLOSTOMA TUBAEFORME* (Zeder 1800)

by

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A B S T R A C T

The free-living development of the cat hookworm Ancylostoma tubaeforme was studied using faecal cultures and also by culturing larvae on pure bacteria. Temperature and moisture had the greatest effect on egg and larval development but there was no significant difference in the size or lipid content of infective larvae developing under the various conditions. Using the product of the percentage of larvae that developed to the infective stage and the reciprocal of the rate of development as in index of development, it was shown that the absence of high temperatures would be the significant factor influencing the numbers of infective larvae to be found in contaminated soils.

Behavioural studies on larval locomotory activity showed that the activity regime could be divided into three phases. Activity was also greatly affected by temperature.

The energetics of locomotory activity was investigated by quantitatively monitoring lipid changes following periods of larval activity using scanning microdensitometry.

From a critical analysis of longevity experiments the mortality pattern of infective larvae under controlled conditions was established. Attempts were made to relate the longevity of larvae to their lipid content and the rate of its depletion.

The respiratory rates, esterase and acetylcholinesterase activities of infective larvae of various ages kept under different storage conditions were measured so as to establish the main physiological features associated with ageing. The possible significance of these results is

discussed in terms of the ecology of the infective larvae and their ability to infect potential hosts.

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GENERAL INTRODUCTION

The life-cycle of the cat hookworm Ancylostoma tubaeforme like that of other strongyloid nematodes consists essentially of two main phases - a parasitic phase in which the adult worms live in the intestine of the host, and a free-living phase during which the eggs passed out in the faeces of the host hatch, and the first-stage larvae feed, grow and develop to the infective third-stage larvae. The infective third-stage larva is enclosed within a 'sheath' which is formed at the second moult by the uncast cuticle of the second-stage larva. It does not feed and is dependent for its energy source on the food reserves synthesised by the bacteriophagous preinfective larval stages. The infective larva constitutes the end-point of the free-living phase and its special role in the life-cycle is to infect a potential host.

These studies are concerned mainly with an evaluation of factors which could influence the ability of the infective larvae of A. tubaeforme to infect potential hosts.

Rogers (1962) believed that the ability to infect a host was the fundamental feature which distinguished a parasite from other sorts of organisms. He therefore considered an understanding of the mechanisms and processes by which infection may be effected to be very important in the study of parasitism.

The process of infection however, is very complex and the mechanisms involved differ between the various groups of nematode parasites. The infection of the host per os differs from that where the infective larvae actively penetrate the host skin (Rogers and Sommerville

1963, 1968). Nevertheless, a successful infection has two essential components:

i) the entry of the host by the infective larvae (invasion) and

ii) the subsequent development of the invading larvae into adults.

Invasion is brought about by a series of events and processes. It is dependent on contact being made between potential hosts and infective larvae as well as on the efficiency of the larval penetration mechanisms.

The establishment of contact is largely a chance process. The probability of contact would therefore be governed by the bionomics of the host and the numbers and distribution of the infective larvae. Anderson (1974; 1975) in a mathematical treatment of the chance infection process argued that certain aspects of larval behaviour would increase the probability of contact. Thus the ability of the infective larvae to respond to physical and chemical factors which might represent the presence of a potential host would enhance the process of contact. It has been suggested, for example that the stimulation of the infective larvae of hookworms to activity by carbon dioxide (Sasa et al; 1960) would increase their chances of contact with potential hosts.

The longevity of infective larvae is an important factor ensuring contact since sometime may elapse before a potential host may present itself to the larvae. The infective larvae are known to stay alive for considerable periods but because they do not feed, it is to be expected

that the exhaustion of their food reserves would set a limit to their length of life. Results of these experiments which are critically reviewed by Rogers and Sommerville (1963) and Michel (1969) differ considerably and have not been conclusive.

Longevity and infectivity however may not involve similar physiological phenomena. It has been pointed out that although infectivity decreased with the age of the infective larvae, there was apparently no correlation between infectivity and longevity (Cornwell and Jones 1970); Oishi and Hiraoki (1973), Lengy (1973)). Rogers (1939) had recognised this and emphasised the importance of the 'physiological age' of the infective larvae in assessing their ability to infect hosts. The criteria to be used in estimating the larval physiological age still remain to be clearly defined.

Lipids constitute the main food reserves of the free-living infective larvae of nematode parasites (Goodey 1930, Giovanniola (1936), Wilson (1965)). Barrett (1968a) provided quantitative data to show that the lipid reserves were neutral lipids mainly in the form of free fatty acids and triglycerides.

Several investigators have demonstrated a decline with time in the lipid content and infectivity of larvae, and had consequently used the larval lipid level as an estimate of the 'physiological age' (Payne 1923a;1923b; Rogers (1939 ; 1940; Elliott 1959; Clark 1969). Croll and Matthews (1973) have argued that there was not a direct 'cause - and - effect' correlation between the lipid content of larvae and their physiological condition.

They believed that the ageing of the infective larvae of hookworms was related directly to decreased metabolic rates and not primarily to lipid levels.

Their results were hardly surprising even though the implications of their findings had hitherto not generally been realised. Since lipids formed the main source of energy for the infective larvae, the lipid content of larvae at any given time should reflect the sum total of their previous energy-expending processes and not necessarily their physiological condition. Larvae with much lipid may therefore not always be the most infective. Thus, the study of Croll and Matthews whilst not diminishing the importance of the lipid reserves in the physiology of the infective larvae, has suggested that it is unsatisfactory to estimate the larval physiological age using lipid levels alone, without reference to their energy-expending processes.

Locomotory activity has long been believed the major energy-expending process of infective larvae (Looss 1911; Payne 1923b), although quantitative data on the energetics of activity are lacking. Croll (1972a) obtained some correlation between activity and lipid loss and considered that there was good evidence that larval activity might be an appreciable lipid-consuming process. The lipid content of larvae, therefore may well be an indication of age as the extent of previous activity.

The studies of Matthews (1972a, 1975) and Smith (1975) suggested strongly that the infective larvae of A. tubaeforme (unlike those of Necator americanus) penetrated the host skin by mechanical means and without the use of histolytic

enzymes. For these larvae, their penetration capacity would be governed by their activity potential at the time of contact. The activity of infective larvae would therefore be an important measure of larval physiological age.

Larval activity has been little studied and even those few studies that have been made (Rogers 1939 ; Barrett 1969) have not been concerned with behavioural measurements. These investigators however, had shown that the activity rate of infective larvae decreased with their age. Recently Croll (1972b) and Croll and Al-Hadithi (1972) described the peculiar nature of the activity of infective larvae. They found that these larvae were 'normally' inactive in the absence of an external stimulus. When stimulated, they became very active but only for a limited period before becoming inactive once again. The components of the activity regime and how these may be affected during the ageing of larvae have not as yet been studied.

Very little is known of the mechanisms by which the infective larvae which gain entrance to the host by actively penetrating its skin, may be stimulated to resume development. But whatever these mechanisms may be, it is reasonable to expect that once development has been triggered off, a source of energy should be available to the larvae for the subsequent developmental activities such as morphogenesis before feeding is resumed. The level of the lipid reserves at this stage would then be an important factor influencing larval infectivity.

It does not appear however, than an appreciable amount of lipid is not required for such developmental activities. Payne (1923a), Rogers (1939) had observed that some

individuals of a population of "old" infective larvae which apparently had exhausted their lipid reserves could still infect a host. Nagahana et al. (1965) believed that the "old" infective larvae of N.americanus, once they could penetrate the host skin did not differ from young larvae in their subsequent development to adults. The work of these investigators would suggest that the ability to penetrate the host skin was the main factor governing the infectivity of such larvae.

The "biological performance" of infective larvae may be affected by physical factors in their early development. For example Ciordia et al. (1966) showed that the infectivity of infective larvae of Trichostrongylus axei and T.colubriformis was influenced by the temperature at which they were cultured. The developmental temperature has also been shown to affect the size, lipid content and activity of the infective larvae of Trichonema (Odei 1968) and the size and longevity of the infective larvae of Strongyloides ratti (Barrett 1968b).

It is not clear however, those features of the infective larvae which are directly affected by temperature to explain these physiological differences between larvae developing at the different temperatures. Studies on this subject had also shown differences in the size of infective larvae. But although the length of nematodes may influence their activity rates (Wallace 1958), Weinstein (1953) had reported that the smaller infective larvae of A.caninum he obtained from sterile cultures, gave rise to normal infections in puppies. The amount of larval lipid reserves if it is dependent on the culture temperature, would affect their longevity but quantitative data on this have not been made.

In these studies, various factors which would influence the infectivity potential of A. tubaeforme have been examined by first investigating the free-living development of this parasite. This was followed by a study of the locomotory activity of infective larvae and the changes in their behaviour and physiology with time. Finally the effect of ageing on some aspects of the metabolism of larvae was investigated in an attempt to relate metabolic changes to the larval physiological condition.

GENERAL MATERIALS AND METHODS

1) The Parasite

These studies were carried out using *Ancylostoma tubaeforme* obtained in 1970, by Dr, N.A. Croll from Dr. D.A. Denham of the London School of Hygiene and Tropical Medicine, and maintained by passage through worm-free kittens.

Kittens were routinely infected *per os*. with 250-400 infective larvae in about 10ml of milk, and kept in cages to avoid infection with other nematode parasites.

2) Culture and Isolation of infective larvae

Fresh cat faeces containing eggs of the parasite were collected within 24 hours of being voided. Larvae were cultured in slurries of faecal homogenate in inverted 9cm perspex dishes as described by Croll (1972a). Charcoal-faeces and sand-faeces cultures, made by mixing about 3 parts of the faecal slurry with 1 part of charcoal or sand, were used as stated in some of the studies.

The infective larvae were extracted by the Baermann funnel method. The apparatus consisted of a glass funnel filled with water at 30-35°C and a 90u. nylon mesh sieve. The faecal cultures containing the fully-developed infective larvae were spread out on a single layer of tissue paper placed over the sieve and introduced into the funnel. Great care was taken in doing this, and in the setting up of the apparatus so as to obtain clean extracts of larvae.

3) Cleaning and surface-sterilisation of larvae

The extracted larvae were left to settle in a conical glass centrifuge tube. The water was pipetted off and the larvae washed three times with distilled water.

The surface-sterilisation procedure was suggested by Dr. T.A. Miller (pers comm). Washed larvae were transferred to a shallow petri dish and kept at 5°C and 0.5% formalin was added when they had ceased to move. After 1 hour, the larvae were removed from this solution, washed three times in distilled water and left at room temperature. After 24 hours, they were examined and if their activity had not been adversely affected, they were then placed in storage.

4) Procedure for obtaining exsheathed infective larvae

Infective larvae were placed in 5% aqueous solution of 'Milton' (≡ 1% sodium hypochlorite; 16.5% sodium chloride) after Lapage(1933) Rogers(1939),Glaser and Stoll(1940).

Within 20 minutes most of the larvae had lost their 'sheaths'. The exsheathed larvae were washed in distilled water and examined 24 hours later to ensure that they had not been damaged by the treatment before use.

5) Storage

Emphasis was placed during storage on the sterility of the storage medium. The larvae were placed in phosphate buffer pH 7.2 to which a few drops of gentamicin sulphate (British Schering Ltd) and lincomycin hydrochloride (Upjohn Ltd), both at 20 ug/ml, had been added. These antibiotics were chosen because they have been used as routine canine hookworm vaccine preservatives and are

believed not to have toxic effects on larvae (T.A. Miller, pers comm).

The larvae were stored in a controlled environment chamber in an apparatus designed to minimise their being mechanically stimulated during handling. The features of this apparatus are illustrated in Fig 0:1.

Either of two methods was used to ensure that oxygen was available to larvae during storage. In the first method, a specially perforated cork (Fig 0:1) which allowed a free flow of air whilst keeping out contaminants, was used to seal the storage vessels.

In the second method, the oxygen requirements of the larvae for the duration of the study were calculated and then provided for. The calculations were based on the data of Barrett (1969) and Bryant (1973).
Using a QO_2 of 10ul O_2/ mg dry wt./ hour and larval numbers of 4×10^4/mg dry wt. (Barrett 1969) the oxygen consumption per larva per day was calculated as 0.006ul. Less than 1,000 infective larvae which had been counted were placed in about 2ml of the storage medium in a 20ml specimen tube, in some experiments. The overlying air space containing about 3.6mls of oxygen was considered more than adequate for the oxygen needs of larvae over a 20 week period.

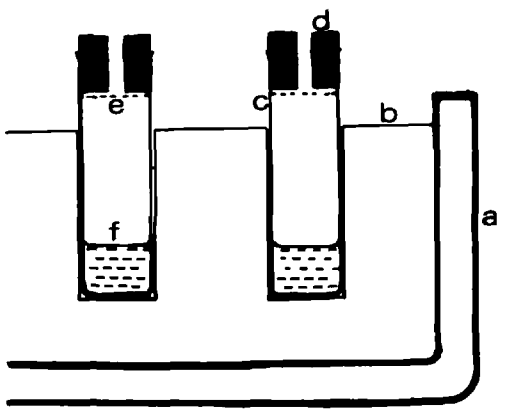
6) Lipid Staining

Infective larvae were stained for neutral lipids using a saturated solution of Oil Red O in 70% ethanol after Croll (1972a).

To eliminate variation in the depth of staining of larvae stained at different times due especially to the quality of the stain solution, a standard stain solution was used.

FIG. 0:1

THE APPARATUS USED TO STORE LARVAE.



a Metal container (wt. ≥10kg.)

b Polystyrene "packing".

c 20ml. "specimen" tube.

d Perforated cork.

e 10 nylon mesh.

f Storage medium.

A stock solution of the stain was prepared by adding 0.5 grams of the stain to 100ml of absolute ethanol at room temperature and left for 24 hours after which it was filtered using a Whatman's No.1 filter paper. When required for staining larvae, a sufficient quantity of the stock stain solution was diluted to 70% using distilled water and filtered using a Whatman's No.42 filter paper attached to a millipore filter.

7) Lipid measurement - scanning microdensitometry

The absorbance of the stained larvae mounted in glycerine jelly were measured on a Vickers M85 scanning microdensitometer at 517 m, the maximum absorption wavelength for the stain (Croll 1972a).

All measurements were made with the same spot size and area of scan.

8) Background absorbance

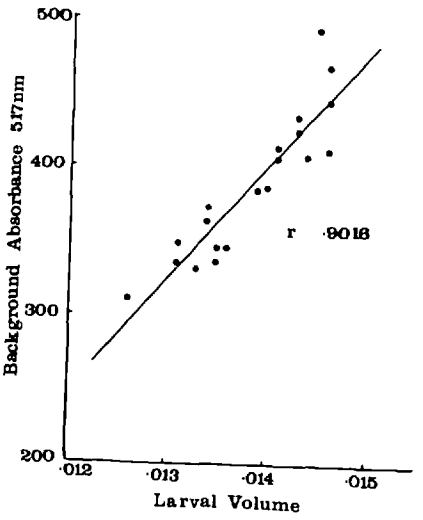
Some of the larvae from the sample to be stained for lipid were left in 70% ethanol without the stain at 60°C, for the staining period. Both the stained and unstained larvae were similarly processed for measurements.

The positions of the individual larvae on the slides were identified, and their lengths and maximum widths measured from scaled camera-lucida drawings. Each larva was then subsequently measured on the microdensitometer. The absorbance values for unstained larvae were recorded as the 'background absorbance'.

Larval volume V, was calculated from Andrassy (1956) where $V = a^2xb/1.7$, a the greatest witdth, and b the length of larva. There was a linear relationship between background absorbance and larval volume (Fig 0:2). Such background absorbance plots were made with each of the lipid measurements

FIG. 0:2

THE RELATIONSHIP BETWEEN THE BACKGROUND ABSORBANCE AND LARVAL VOLUME.



and appropriate background values obtained from them were used to correct for size on all stained larvae. This method greatly reduced, variation in the lipid values of larvae from the same culture and between sheathed and exsheathed larvae (Table 1).

TABLE 1

The lipid level of ensheathed larvae and exsheathed '0-day' old infective larvae of A.tubaeforme obtained from the same culture at 30°C (30 larvae used in each of the measurements).

	Mean larval length ± SE	Mean background absorbance at 517nm ± SE	Mean of stained worms measured at 517nm ± SE	Mean lipid level (stained worm-background absorbance) ± SE
Ensheathed larvae	582 ± 24u	391 ± 58	1093 ± 90	698 ± 39
Exsheathed larvae	567 ± 18u	257 ± 36	889 ± 64	635 ± 27

Note that measurements of the exsheathed larvae were always lower than those with sheaths.

S E C T I O N I

FACTORS IN THE DEVELOPMENT OF THE EGGS AND FREE-LIVING STAGES OF *Ancylostoma tubaeforme*

INTRODUCTION

The development of the free-living stages of nematode parasites and their distribution are governed by climatic factors of which temperature and humidity are the most important. Consequently, the effects of various factors on embryonation of the eggs and development of the larval stages of parasitic nematodes have long been a subject of study. Looss (1911) in his classic monograph on the life history of the human hookworm Ancylostoma duodenale, observed that development was best between 25-30°C, and that desiccation was injurious to the eggs and larvae. He also believed that the consistency and putrefaction of the faecal cultures affected larval development.

McCoy (1930) in a detailed study of the factors influencing egg and larval development of Ancylostoma caninum, cultured the larvae on pure bacteria on agar to eliminate the variation in consistency and putrefaction products inherent in faecal cultures. His results showed that development took place within the temperature range 15°-37°C. 30°C was considered as the optimum temperature for the development of A.caninum and pH was of little significance.

Subsequently, there have been many studies on the effect of temperature on the free-living development of zoo-parasitic nematodes. Some of these were aimed at determining the temperature range for development (Furman 1944; Gibbs and Gibbs 1959; Ciordia and Bizzell 1963; Agrawal 1966; Barrett 1968b; Pandey 1972; among others). Others were field studies designed to investigate the relationship between climate, weather and parasite epidemiology (Marquardt et al. 1959; Rose 1961, 1966; Andersen et al. 1966; Ogbourne 1972; Andersen and levine 1973).

In nearly all of these studies however, very little attempt was made to investigate the lipid content and other parameters of infective larvae developing at different temperatures.

Given a favourable temperature humidity becomes the factor determining the rate of development. Spindler (1929) reported that the distribution of human trichuriasis and ascariasis was related to humidity and showed that Trichuris eggs required more moisture for development than Ascaris eggs. Sprent (1946) considered desiccation to be the most important factor affecting the development of Bunostomum phlebotomum, a hookworm of cattle in the tropics. In spite of these early observations, comparatively little is known on the effects of humidity on the developing eggs and larvae of hookworms.

In this section, experiments to determine the conditions favourable for the free-living development of the cat hookworm Ancylostoma tubaeforme are described. Measurements of the size, activity and lipid content of the infective larvae developing under the various conditions are presented and the results discussed in terms of the ecology of the parasite.

1) Separation of the eggs from faecal matter

The technique employed to separate the eggs from faeces was a modification of that devised by McCoy (1929). Heavily infected cats showing faecal egg counts of 4000 eggs and over per gram of faeces provided material for these studies.

Fresh faeces of the cats, collected within 12 hours of being voided, were mixed with water until a fluid consistency was obtained. The mixture was strained using a copper sieve (300u pores) and left for about 2 hours to sediment. The supernatant fluid was poured off and the sediment centrifuged at 300rpm for 30 minutes, after which it was stirred into a loose homogeneous slurry. Saturated salt solution was then added, mixed thoroughly with the slurry, and centrifuged for 5 minutes. The eggs floated to the surface and were collected by immediately withdrawing the supernatant, 5mls at a time, into 500mls of distilled water. Thus, the saturated salt solution was quickly diluted a hundredfold and the eggs rarely remained in this extremely hypertonic solution much longer than 5 minutes. (see Table 2)

Furthermore, the density of the solution was lowered on dilution and the eggs sank to the bottom of the beaker. The excess fluid was poured off, and the eggs were washed twice in distilled water and concentrated in solid watch-glasses.

2) Growth of the larvae on pure cultures of bacteria

The clean eggs obtained were cultured on Escherichia coli on agar in 9cm perspex dishes after McCoy (1929). The agar medium was prepared by soaking 14 grams of nutrient agar (Oxoid Ltd) in 1000mls of distilled water for 15 minutes. The mixture was poured into 250ml Erhlenmeyer flasks stoppered with plugs of cotton wool and autoclaved for 15 minutes at 15lb/square inch (121^{o}C).

The agar was poured into 9cm dishes (about 10mls per dish), and left in a sterile chamber to set. Each dish was then inoculated with pure E.coli (obtained from the Department of Biochemistry, Imperial College and grown on agar slants) and included at 37°C. After 24 hours, batches of eggs carefully counted, were introduced into each dish and kept at the desired temperatures.

RESULTS

a) Hatchability of the eggs

After the eggs had been separated from faecal matter by salt floatation, their viability was tested using 100 eggs per test. The washed eggs in distilled water were placed in leucocyte migration cells (Sterilin) using a micropipette and kept at 26°C. The number of first-stage larvae that had hatched after 30 hours were counted and recorded as percentage viability.

Sometimes eggs were refloated 2-3 times so as to get rid of all faecal material. It became desirable therefore to check the viability of eggs obtained following various periods in saturated salt solution.

Results of experiments where eggs had been left in the salt solution for periods ranging from 3-90 minutes and their ability to hatch subsequently measured are presented in Table 2. It is evident that eggs were damaged if they were left for longer than 10 minutes in saturated salt solution.

b) The free-living development of A.tubaeforme

The use of agar cultures permitted the observation of the developing stages without removing them from their surroundings. The following brief description of the course of development at 26°C of the eggs to infective larvae has been based on several of these observations.

Eggs developed and hatched within 24 hours into small rhabditiform first-stage larvae. These were weakly active appeared to feed only occassionally and soon moulted into second-stage larvae. The second-stage larvae were more active, fed almost constantly and accumulated lipid in their intestinal cells. They grew rapidly and later moulted by the third day into the ensheathed, filariform third-stage infective larvae.

TABLE 2

Hatchability of the eggs of A.tubaeforme separated from faeces by salt floatation. (means and standard errors of the results of five experiments)

Length of time eggs were in sat. NaCl (mins)	Maximum hatch (%)	Comments
5 mins	94.75 ± 0.96	No damaged eggs identified*
10 mins	87.0 ± 4.24	No damaged eggs
15 mins	77.75 ± 4.43	Few damaged eggs
30 mins	71.33 ± 2.89	Several damaged eggs
60 mins	69.5 ± 1.73	Several damaged eggs
90 mins	61.0 ± 2.16	Several damaged eggs

* damaged eggs same as degenerated eggs (see Fig 1:1)

Embryonation and hatching constituted about 30% of the total developmental time, the first-stage larvae 15% and the second-stage larvae about 55%.

It was also observed that eggs and larvae in the same culture developed at slightly different rates. Consequently, data on development are presented as either the minimum, maximum or median.

c) The effects of temperature on development

Development of the eggs to infective larvae took place only between 15-37°C. The upper limit of temperature at which development of the eggs took place was 40°C.

However, the results of transfer experiments when cultures were moved from 26°C to 10°C (Table 3a) and culturing of first-stage larvae that had hatched at 26°C on E.coli at 10°C (Table 3b), showed that some of the larvae could complete their development

at 10°C.

TABLE 3

a) Transfer of faecal cultures from 26°C to 10°C.

(means of the results of five experiments)

Time at 26°C before transfer to 10°C (hrs)	Rate of development to infective larvae (minimum days)	% development to infective larvae (maximum)
24	11½	9.05
48	11½	2.97
60	-	-

b) The development of first-stage larvae hatched at 26°C and cultured at 10°C. (means of the results of five experiments)

Culture method	Number of first-stage larvae used	Number developing to infective larvae (range)	% development (mean)	Rate of development (minimum)
E.coli on agar	100	8-15	12.16	11½
E.coli in suspension in phospahte buffer pH 7.2	100	16-23	20.22	11½

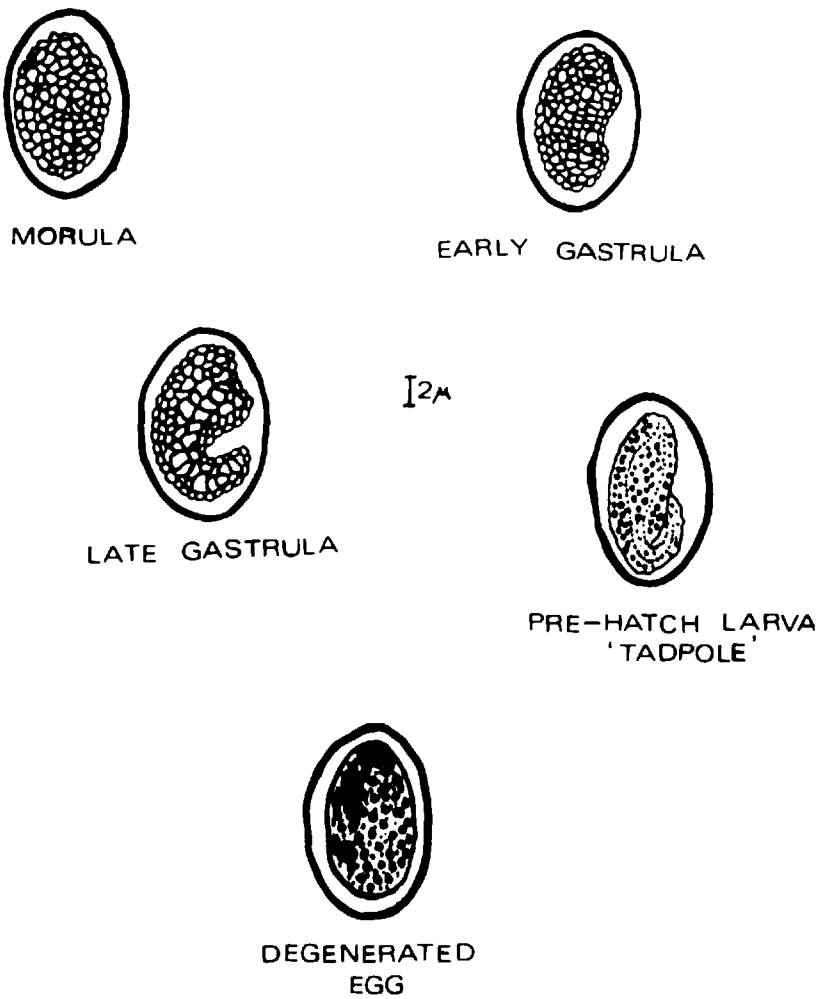
i) Embryonation and hatching

The embryonic stages were classified as morula, early gastrula, differentiating (late) gastrula, and 'tadpole' as illustrated in Fig 1:1.

20 newly hatched larvae were drawn and measured with the aid of a camera-lucida after they had been killed by gentle heat. Table 4 shows the effect of temperature on embryonation, hatching

FIG. 1:1

THE EMBRYONIC STAGES OF Ancylostoma tubaeforme.



and the length of first-stage larvae.

TABLE 4

Measurements of the length of newly-hatched larvae of A. tubaeforme grown at different temperatures. (means of the results of five experiments)

Temperature (°C)	Embryonation Minimum (hrs)	Embryonation Maximum (hrs)	Maximum hatch (%)	Length of larvae on hatching(um)
10	182	250	6.25	136 ± 41
15	80	110	90.75	211 ± 24
20	38	50	96.0	220 ± 18
26	20	24	96.75	216 ± 20
30	18	22	95.25	218 ± 22
37	12	15	93.5	216 ± 19
40	10	13	38.0	214 ± 28

Between 15-37°C, the rate of embryonation was increased by an increase in temperature. The maximum hatch remained almost unchanged over 20-37°C, and was only slightly reduced at 15°C. There was no marked differences in the size of first-stage larvae that developed within this temperature range.

At 10°C, eggs developed very slowly and 6.25% had hatched after 10 days. The hatched larvae were malformed (Fig 1:2-3). The anterior region of these larvae were enlarged, the oesophageal divisions were not distinguishable and they averaged 136um in length. They moved sluggishly, could not feed and failed to develop any further when transferred to bacterial cultures at higher (20°C and 26°C) and more favourable temperatures for development.

LARVAE DEVELOPING AT 10°C

FIG. 1:2

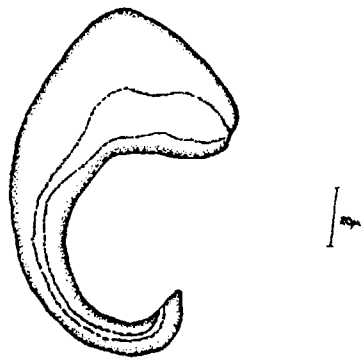
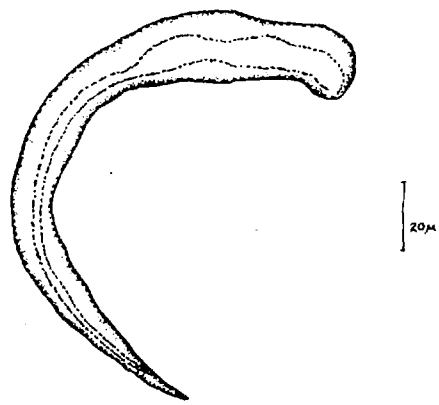


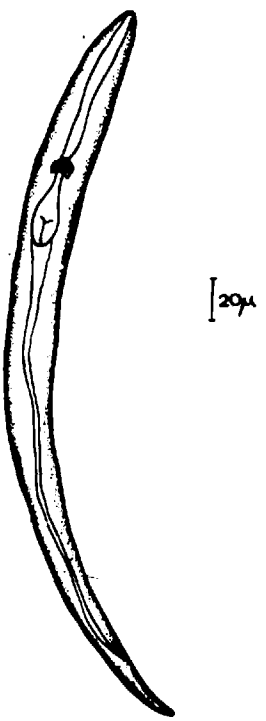
FIG. 1:3



NOTE THE MAL-FORMED OESOPHAGI.

FIG. 1:4

LARVA DEVELOPING AT 40°C



NOTE THE WELL-FORMED OESOPHAGUS.

At 40°C, many of the eggs did not develop. Those that did, however produced normal first-stage larvae with well formed oesophagi (Fig 1:4) but these died soon after hatching.

ii) the rate of development to the infective stage

The results of the effect of temperature on the mean rate of development of the eggs to infective larvae showed that the rate was greatly increased at higher temperatures. (Fig 1:5)

The relationship between the reciprocal of the rate of development and temperature was linear (Fig 1:6).

The high correlation obtained and the observation that the rate was not affected by the culture medium indicated the rate was directly dependent on temperature.

iii) the percentage development

Infective larvae developing in cultures of bacteria were counted and expressed as a percentage of the number of eggs introduced into the cultures.

For the faecal cultures, the percentage development was estimated as follows: 3 gram samples of the mixed faecal slurry to be cultured were removed for routine egg counts using the McMaster method. The number of infective larvae developing per culture was obtained after Todd et al.(1970) and expressed as a percentage of the egg count.

Cultures at 30°C and 37°C were periodically moistened with a fine spray of water to prevent their drying out. Frequently larvae failed to develop in some of the cultures and died. Such cultures smelled strongly, suggesting intense putrefactive changes and were ignored when calculating the percentage development.

Maximum development took place at 20°C, and larval development was better on E.coli cultures on agar than in faeces (Fig 1:7) It would also appear from Fig 1:7 that an increase in the

THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF

Ancylostoma tubaeforme.

FIG. 1:5

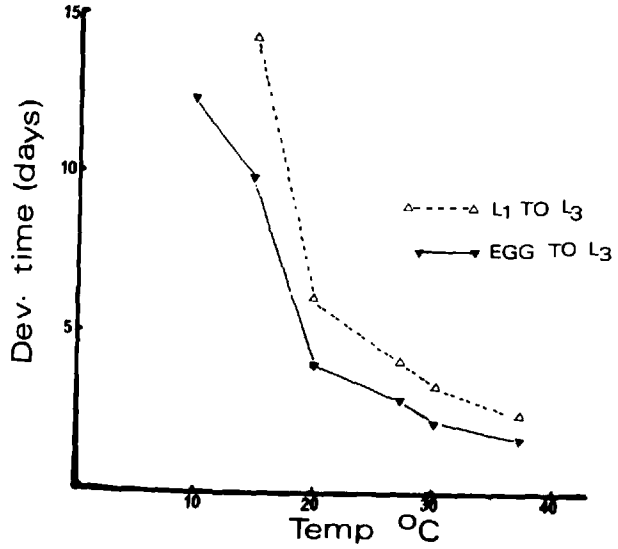


FIG. 1:6

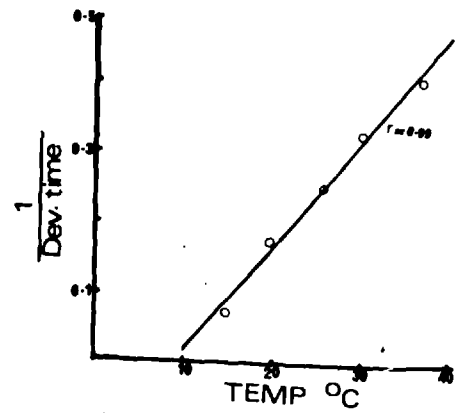
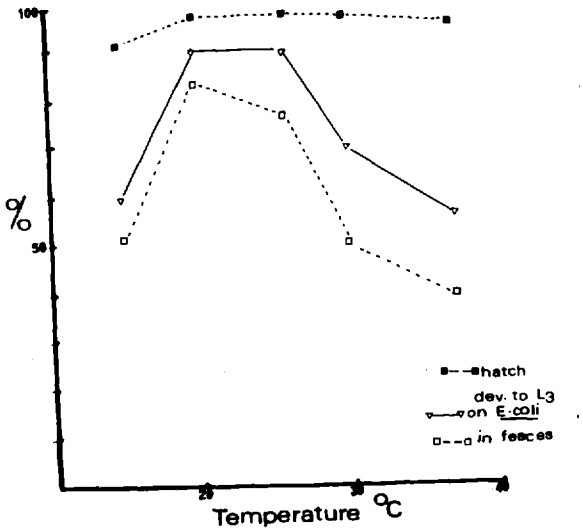


FIG. 1:7

EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF A.tubaeforme.



mortality of the preinfective larval stages accounted for lower numbers of infective larvae developing at 15°C and 30-37°C.

iv) the derivation of a development index

An index of development at a given temperature was calculated from the product of the highest percentage of larvae that developed to the infective stage and the reciprocal of the rate of development at that temperature. The latter parameter has been used because it would indicate the extent to which the apparently highly susceptible preinfective stages may be exposed to adverse factors during development as well as the turnover time for the production of larvae from eggs. The index thus calculated is therefore an estimate of the temperature at which the greatest numbers of infective larvae are to be found in contaminated soils.

A summary of the criteria used to derive the index are shown in Table 5. It is apparent that although more infective larvae developed at 20°C than at any other temperature, the calculated index of development at this temperature was lower than for 26-37°C.

TABLE 5

Summary of the criteria used to derive an index of development of A.tubaeforme at different temperatures.

Temperature °C	% development to infective larvae (on agar cultures of E.coli)	Reciprocal of the developmental time (1/D)	Developmental index (%dev x 1/D)
15	63.13	0.071	4.5
20	91.47	0.17	15.55
26	89.37	0.25	22.3
30	73.02	0.33	24.10
37	44.64	0.40	17.9

It should however be realised when interpreting these figures that high temperatures also caused a decrease in humidity, resulting in desiccation and death of the developing stages.

d) The weight of faecal matter cultured and larval development

Cultures were set up at 15°C and 30°C using 0.25, 0.5, 0.75, 1.0 and 2.0 grams of the faecal slurry per dish, after the egg counts had been determined.

Another set of cultures using similar weights of faeces and mixing them with sand as previously described were also kept at 30°C. The percentage development in these cultures were obtained as for the other faecal cultures.

Results of these experiments (Fig 1:8) showed that the development of larvae to infective stage was diminished with an increase in the weight of the cultures especially at higher temperatures. More larvae developed to the infective larvae in faeces-sand cultures than in faecal cultures alone, but charcoal-faeces cultures were poor media for development.

e) Effect of pH on development

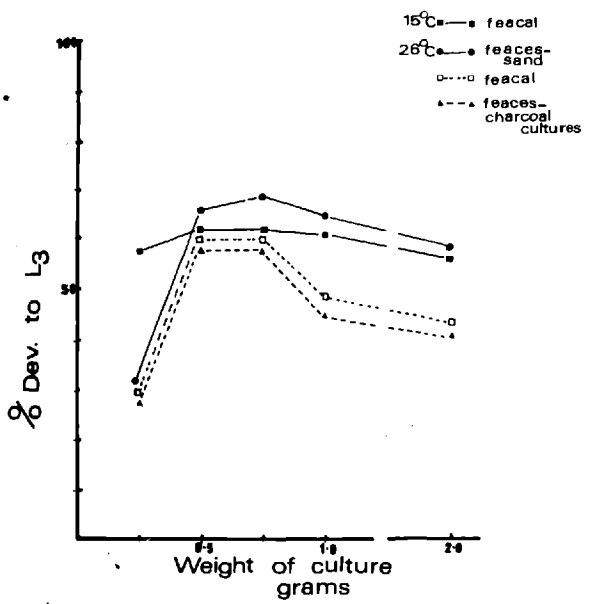
Egg and larval development was investigated over a pH range of 3.0 - 9.1 using acetate, borate, citrate, phosphate and tris buffers. Two types of acetate buffers, one made up from sodium acetate and hydrochloric acid (Ac_1), and the other from acetic acid in place of hydrochloric acid (Ac_2) were used. All buffer solutions were made up as described by Pearse (1968).

Suspensions of E.coli made by scraping the bacteria into the various buffer solutions were used to study the effect of pH on larval development. The experiments were carried out at 26°C and maximum hatch was recorded after 30 hours.

Larvae in the bacterial suspensions were observed 24 hours after hatching and the number that had developed to the second-stage

FIG. 1:8

THE RELATIONSHIP BETWEEN THE WEIGHT OF CULTURE AND DEVELOPMENT.



were counted and recorded against the percentage hatch.

Between pH 3.6 - 9.1, eggs hatched and larvae developed but the numbers that did so were reduced at the extremes of this range (Fig 1:9) Larval development was best in phosphate buffer and it was also apparent that high concentrations of certain organic acids such as citric acid and boric acid in the media adversely affected development. Eggs in Ac_1 buffer at 3.6 failed to develop when transferred after 24 hours into distilled water. Of those in Ac_2 buffer 92% hatched into viable larvae on transfer to distilled water while 2.65% of the eggs actually hatched in citrate buffer at pH 3.6.

f) Salinity and development

Eggs developed in media containing up to 5% NaCl but hatching did not occur above 1% NaCl. Most of the larvae that hatched above 0.75% NaCl failed to develop in bacterial suspensions at these concentrations and died shortly after hatching (Fig 1:10).

g) The size and lipid content of infective larvae

Temperature and type of culture medium affected the size and lipid content of the infective larvae (Table 6a and 6b).

There was considerable variation between the length and lipid content of larvae in each culture, but larvae grown on E.coli suspension in phosphate buffer were generally smaller and had less lipid than larvae from the other culture media.

There was no significant difference in the length or lipid content of the infective larvae developed below 20°-26°C. However, smaller larvae developed below 20°C and above 30°C and their lipid content were slightly reduced.

h) Developmental temperature and larval activity

Activity measurements were made at 30°C on samples of 20 infective larvae developing, at the different temperatures. The larvae were left for 24-48 hours at the test temperature before treatment.

FIG. 1:9

THE EFFECT OF pH ON THE DEVELOPMENT OF A.tubaeforme

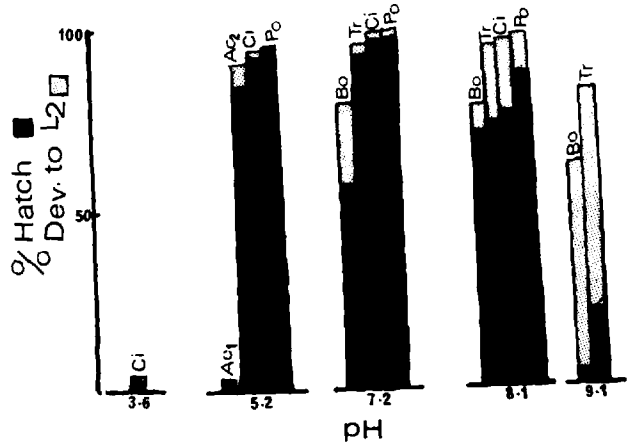


FIG. 1:10

THE EFFECT OF SALINITY ON THE DEVELOPMENT OF A.tubaeforme

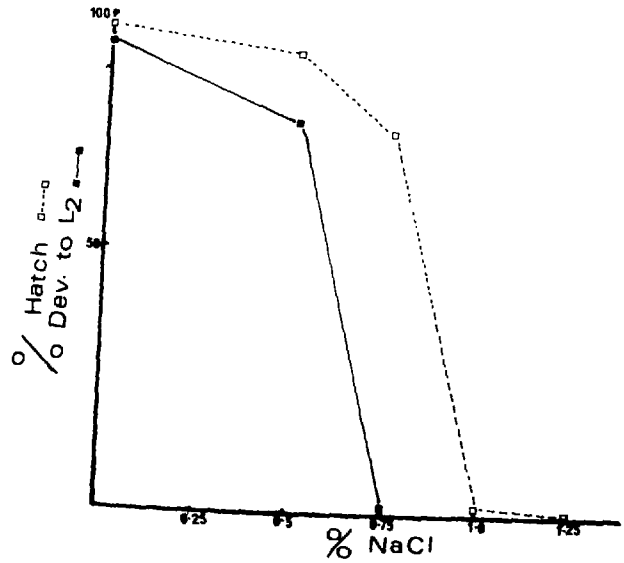


TABLE 6

a) The size and lipid content of infective larvae of A. tubaeforme grown by different methods at 20°C. (means of the results of eleven experiments)

Culture method	Larval length (um)	Larval width (um)	Ratio of length to width	Lipid content (micro-densitometer untis at 517nm)
Faeces	641 ± 28	20.04	31.99	724 ± 38
Faeces-sand	649 ± 25	20.06	32.35	730 ± 32
Faeces-charcoal	640 ± 26	20.05	31.92	721 ± 36
E.coli on agar	636 ± 20	20.04	31.74	729 ± 30
E.coli suspension in phosphate buffer (pH 7.2)	618 ± 21	20.02	30.87	689 ± 52

b) The size and lipid content of infective larvae of A. tubaeforme grown on agar cultures of E.coli at different temperatures. (means of the results of eleven experiments)

Temperature (°C)	Larval length mean (um)	Larval width mean (um)	Ratio of length to width	Lipid content (micro-densitometer units at 517um
10	617 ± 31	20.04	30.79	659 ± 39
15	628 ± 26	20.02	31.37	696 ± 32
20	638 ± 20	20.03	31.85	706 ± 30
26	640 ± 22	20.03	31.95	705 ± 28
30	636 ± 23	20.02	31.77	691 ± 37
37	629 ± 28	20.03	31.40	688 ± 36

The percentage of larvae active and the maximum undulation rate were recorded following stimulation (see p.56) of larvae.

The results are shown in Table 7. There was no difference in the activity of infective larvae that developed between 15-37°C, but those developing at 10°C had a slightly reduced activity rate.

TABLE 7

The effect of the temperature of development on subsequent activity of infective larvae. (means of the results of five experiments)

Temperature of culture (°C)	Percentage of larvae active (range)	Activity rate (waves/sec.) (maximum)
10	80 - 100	2.91 ± 0.07
15	85 - 100	2.98 ± 0.09
20	80 - 100	3.01 ± 0.06
26	90 - 100	3.00 ± 0.05
30	90 - 100	3.01 ± 0.07
37	90 - 100	2.98 ± 0.01

i) The longevity of the eggs

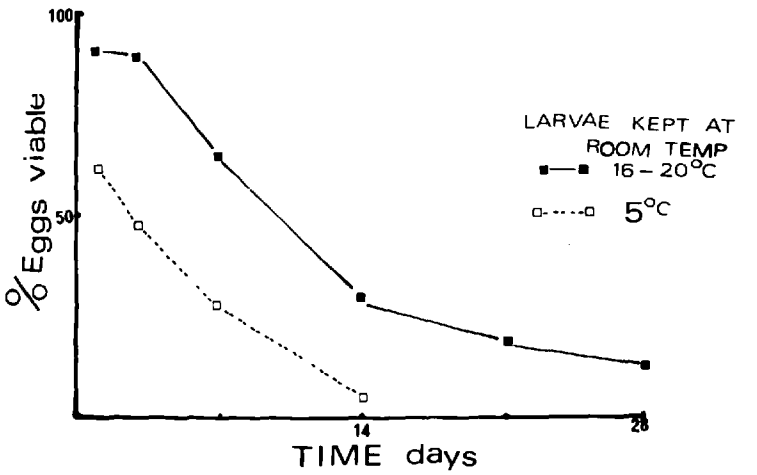
Eggs contained in slightly moist faeces were put in sealed glass tubes and kept at room temperature (16-21°C), 5°C and 15°C.

They were removed after the desired periods and the eggs were separated from faecal matter and kept in batches of 100 in distilled water at 26°C. Maximum hatch recorded after 30 hours was taken as percentage viability.

At 5°C, most of the eggs were killed within 2 weeks but at room temperature an appreciable number of eggs were found to be still viable after 4 weeks (Fig 1:11).

FIG. 1:11

THE LONGEVITY OF THE EGGS OF A.tubaeforme



None of the eggs survived after 24 hours at -15°C.

j) The effect of desiccation on the viability of eggs

Peliminary investigations on the rate of drying of water at various humidities and their effects on the eggs indicated that drying was rapid especially at low humidities and that eggs at all stages of embryonation did not survive extended periods of desiccation. Subsequent experiments were carried out at relative humidities (RH) exceeding 75%.

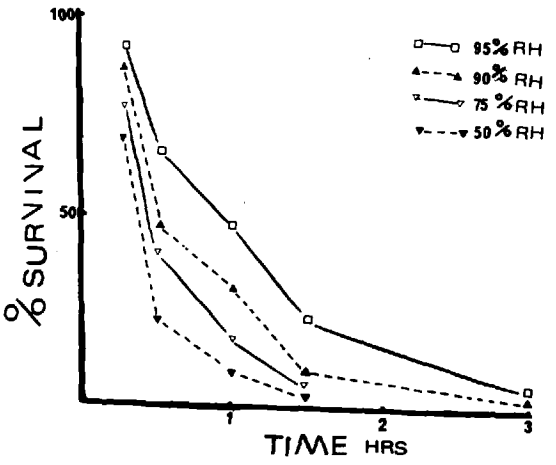
The humidity chambers were set up at 26°C in desiccators using sulphuric acid solutions as described by Solomon (1951). Clean eggs were placed in leucocyte migration cells and placed in these chambers.

A major problem of these experiments was how to minimise and keep constant the amount of water introduced into the test cells along with the eggs. In one series of experiments, 100 eggs were concentrated in a very small amount of distilled water and then transferred into the test cell in 100ul of water using a micropipette. In the other experiments, eggs were placed in the cell with as little water as possible, and excess water removed under a stereomicroscope using absorbent paper. The test cells were removed from the RH chambers at the desired intervals and the eggs covered with distilled water. The rehydrated eggs were left for 24-48 hours at 26°C and the percentage hatch recorded.

The results showed that more than 50% of the eggs did not survive desiccation after 1 hour and only very few were viable after 3 hours (Fig 1:12).

FIG. 1:12

THE EFFECT OF DESICCATION ON EGGS OF A.tubaeforme.



DISCUSSION

These observations on the factors affecting the free-living development of A.tubaeforme are consistent with those reported for other strongyloid nematodes (Belle 1959, Prasad 1959, Gupta 1961, Chhabra and Singh 1965; Okoshi and Murata 1967).

It has been shown that the eggs of A.tubaeforme were short-lived. They were committed to development once they were out of the host and were killed by long exposures to conditions which would lower or impair metabolism to such an extent as to arrest developmental process. Hookworm eggs therefore differ from eggs of Haemonchus contortus and Trichostrongylus spp which could sustain an inhibition of development for a long time under adverse conditions (Silverman and Campbell 1959).

Development was strongly influenced by temperature. Normal development of the eggs to infective larvae took place only between 15-37°C. The upper limit of temperature for development was considered to be 40°C. Eggs hatched at this temperature but larvae failed to develop and soon died. However it is possible, since 37°C was suitable for larval development, that minor fluctuations around 40°C would have resulted in some of the larvae developing to the infective stage.

An interesting observation also was that although normal egg development did not take place at 10°C, some of the larvae that hatched at temperatures higher than 10°C could complete their development at this temperature. This observation agrees with those of Gibbs and Gibbs (1959) who reported that there was a great variability in the development of the hookworm, Dochmoides stenocepohala at 7.5° and 10°C.

They observed active ovic larvae in their cultures after 21 days while some of the hatched larvae had developed to the infective stage and others were dying off and believed that a certain proportion of larvae, more vigorous that the rest could develop at these temperatures. $10^{o}C$ is therefore believed to be the minimum temperature for the development of hookworm eggs.

Previous workers (Gibbs and Gibbs 1959, Balasingham 1964, Pandey 1972) had determined the optimum temperature for development using four criteria : (1) the percentage of larvae that developed to the infective stage; (2) the rate of development; (3) the size of infective larvae; and (4) the "survival" of the infective larvae.

However, of these criteria used, only the rate of development could be directly correlated with temperature in the present studies. The percentage development and size of the infective larvae were affected by temperature as well the nature of the culture medium. McCoy (1929) had shown that not all bacteria were suitable as food for developing larvae and that larvae failed to develop on various "bacilli". Premvati (1958) believed that development required a culture of the proper consistency and Barrett (1968b) attributed differences in the size of infective larvae of Strongyloides ratti grown in different types of cultures to nutritional differences in the cultures. It is therefore not clear whether temperature has a direct effect on these parameters by controlling the feeding activity of larvae or an indirect effect through changes in the bacterial fauna of the cultures.

The "survival" (longevity) of infective larvae has been considered an end product and not a part of the free-living development, in these studies. Also results reported in

Section III have shown that survival of infective larvae involves complex physiological relationships, not comparable to those of development.

An index of development, indicating the potential for development at each temperature was calculated from the product of the percentage development to infective larvae and the reciprocal of the rate of development. Based on the development index as calculated, a temperature range of 26°-30°C has been considered as the optimum for the development of A.tubaeforme. This agrees with the view held by McCoy (1930) that 30°C should be considered as the optimum temperature for the development of A.caninum. He had argued that since the percentage of larvae that developed to the infective stage was only slightly less at 30°C than at 23°C, that 30°C was the optimum temperature because the development rate was much faster at 30°C. The use of theindex to assess development has suggested that more infective larvae would be found in contaminated soils at higher temperatures within the range for development and may partly explain the observation by (Rogers (1940a) that sheep infestation with trichostrongyles in Western Australia was highest in late summer.

McCoy (1930) using A.caninum was one of the earliest investigators to observe that eggs developed and hatched below the minimum temperature (12°C) required to complete larval development. A similar observation was reported for D.stenocephala at 5° - 7.5°C by Gibbs and Gibbs (1959). These investigators reported that the hatched larvae at these temperatures grew slowly and gradually died without reaching the infective stage.

Croll (1972b) studying the feeding activities of preinfective larvae of A.tubaeforme, thought that development had failed at 10°C, not because larvae succumbed to cold but because they could not feed effectively and starved to death. Results reported here, have shown that at 10°C embryonation was impaired and the very few eggs that had hatched produced malformed larvae which could not feed. It was also shown (Table 3b) that if viable larvae were cultured at 10°C that some of them developed to the infective stage. It would appear then that the inhibition of development at sub-minimal temperatures resulted both from loss of feeding requirements as postulated by Croll (1972b) as well as from the development of non-viable larvae.

Looss (1911) believed that intense putrefaction of the faeces especially where the host was on a high protein diet (as were the cats used in these studies), resulted in poor larval development. Nicoll (1917) had observed that mixing faeces with sand promoted larval development. Heydon (1927) showed that developing stages were highly susceptible to ammonia formed on decomposition and Shirasaka (1959) found that the development of Necator americanus was influenced by the weight of the faecal culture. It has been shown here that an increase in the weight of faecal matter cultured, resulted in decreased development of infective larvae. Since this decrease was more marked at higher temperatures it is likely that the decomposition products (which would be more pronounced at higher temperatures) would account for reduced development. However, it is important to realise that under natural conditions volatile products such as ammonia would be removed by a steady flow of air and also that faecal matter is not a homogenous medium.

Complex relationships may therefore exist between the developing stages, bacterial fauna and the physical components of faeces. Until these relationships are known, the effects of the quantity of faecal matter on larval development can only be speculative.

Very little is known on the effect of pH on development. Stoll(1923) reported that hookworm eggs were killed in very acid faeces. McCoy (1930) showed that development of A.caninum took place between pH 4.0 - 10.0. Results reported here confirm the wide pH range (3.6 - 9.1) over which development took place. Micro-organisms are known to be sensitive to the pH of their environment and Barrett (1968b) had suggested that suppression of bacterial growth in acid media such as faeces-peat cultures would affect larval development. Developing larvae have been found to utilise a great variety of bacteria as food (McCoy 1929). It is likely then that changes in egg and larval physiology rather than in the bacterial fauna, would account for reduced development observed at the extremes of the pH range. Cort et al.(1926) reported that more than 93% of hookworm eggs in faeces were killed within one month. This observation agrees with those of Takano et al.(1927) and Furuyama (1933) who found that survival was about ome month during midsummer and longer in winter.

These studies have shown that eggs of A.tubaeforme were no longer viable after 4 weeks, and were also highly susceptible to desiccation. Ellenby (1969) believed that the rate of drying was a significant factor in the ability of nematodes to survive desiccation, and that most forms were killed by rapid drying. In these studies, the rate of drying could not be accurately determined, and the eggs had responded so rapidly that it was difficult to obtain a quantitative relationship between desiccation and egg viability. The results were therefore presented only because of their significance in demonstrating the high susceptibility of hookworm eggs to drying.

Belle (1959) showed that the eggs of Bunostomum trigonocephalum, a hookworm of sheep, survived desiccation at 66% RH for 14 days at 26°C. The eggs in his experiment were in faeces and this could explain the longer survival periods he obtained. Crofton (1963) stated that faecal material had considerable temperature insulating properties and that large firm faecal droppings had a relatively small surface area to volume ratio and so tended to remain moist at the centre.

Balasingham (1964) showed that three hookworms of carnivores in North America, namely Placoconus lotoris, D.stenocephala and A.caninum had different temperature preferences for development. For example eggs of D.stenocephala survived while those of A.caninum did not. He speculated that such differences in the temperature tolerance of developing stages of these parasites would partly account for their distrinution. It may therefore be worthwhile to attempt to relate these observations on the free-living development of A.tubaeforme to its distribution.

Not much is known however, about the distribution of this parasite because for a long time, it was regarded as the 'cat strain' of A.caninum and was only recognised as a separate species following the work of Biocca (1954) and Burrows (1962). Since then, surveys in America (Lillis 1967, Power 1971), Iran (Sadighian 1970) and Belgium (Vanparijs and Thienpont 1973) have indicated that the species may have a cosmopolitan distribution. Such a widely distributed species would therefore be expected to develop over a wide temperature range as was found in these studies. The defeacating behaviour of cats which bury their faeces would also enhance development as the microenvironment in soils would be less vigorous than in the open. Furthermore, infections of cats with this parasite to provide material for these studies were found to persist for up to 9 months, and Matthews and Croll (1973) reported that the egg -laying period

for the adult females was about 30 weeks. These observations and other factors not considered in these studies, such as the biology of the host and the modes of transmission of the parasite will also influence its distribution.

S E C T I O N II

LOCOMOTORY ACTIVITY AND LIPID UTILISATION BY THE INFECTIVE LARVAE

INTRODUCTION

The locomotory activity of infective larvae is of great importance during dispersion and host entry. Matthews (1972a; 1975) believed strongly that skin penetration by the infective larvae of A.tubaeforme was not enzyme-assisted. He suggested that host entry by these larvae was a mechanical process, governed primarily by their activity potential. His results have been supported by ultrastructural studies on the oesophageal glands of the infective larvae of A.tubaeforme and Necator americanus before and after penetration of membranes (Smith 1975).

Very few studies however have been made on larval activity. Of these some were concerned with the effects of activity on lipid utilisation (Payne 1923b) and on the migratory capacity of larvae (Africa 1931). Others measured changes in the activity rates of larvae with age and attempted to relate these changes to infectivity (Rogers 1939, Barrett 1969). In nearly all of these studies behavioural activity measurements were not made and the nature of locomotory activity of infective larvae not described.

Croll (1970) found that there were differences in the activity patterns of the infective larvae of zooparasitic and phytophagous nematodes, and free-living nematodes and he related these to differences in their biology. Croll and Al-Hadithi (1972) subsequently described the activity regime of the infective larvae of A.tubaeforme. They reported that upon stimulation, these larvae became committed to a set pattern of activity.

The studies reported in this section were undertaken primarily to examine in greater detail, the nature of the activity regime of the infective larvae.

Behavioural and quantitative measurements have been made and used to describe the regime.

The effects of physical factors on larval activity are also described and quantitative data presented on the lipid utilisation by these larvae as a direct consequence of their activity.

MATERIALS AND METHODS

Infective larvae extracted after 72 hours from faecal cultures at 30°C were washed and treated as "0-day" old infective larvae. Larvae from the same culture dish were used in these studies in order to eliminate the differences in size and lipid content between infective larvae from different cultures (Croll 1972a, Matthews and Croll 1974). Limited studies were also carried out using larvae which had been artificially exsheathed using 5% aqueous 'Milton' solution as previously described.

The activity measurements were made on groups of twenty larvae selected at random from the total extract. Three such groups were measured in each treatment. The larvae were placed in 1ml M/15 phosphate buffer pH 7.2 in 'siliconed' solid watch-glasses at 30°C. When 'siliconed' watch-glasses were used there was very little dispersion of water and all larvae remained in full view and could be easily observed under the stereomicroscope. A solution if dimethyl dichlorosilane in carbon tetrachloride was used to 'silicone' the watch-glasses. The solution was evaporated at room temperature and the watch-glasses were washed twice with distilled water before use.

Larvae were left undisturbed for 1-2 hours before treatment and during this period the 'endogenous (background) activity' (Croll and Hadithi, 1972) was measured at half-hourly intervals by continuously recording the number of larvae active and the activity rate for 2 minutes.

A mechanical stimulus was provided by bubbling air through the medium using a 1ul microcapillary (Drummond Co.) attached to an air pump which had a maximum pressure capacity of 0.15 Kgf/cm2. The intensity of this stimulus was varied by altering the bubbling period.

Following stimulation, the number of larvae active, the rate

of activity, the reversal frequency and the number of larvae reversing per minute were recorded for the duration of the regime using a multi-channel event recorder. The rate of activity was taken as the number of times the head moved from one position and back through a complete cycle in unit time and was recorded in body waves per second (w/sec). The reversal frequency was taken as the number of reversals occuring in a minute (revs/min).

Activity was expressed after Croll and Matthews (1973) as a coefficient Ac calculated from

$$Ac = pa \cdot r$$

where pa is the ratio of active to total larvae per minute, and r the mean activity per minute. Total activity Ai during a response was given by

$$Ai = \sum_{i=1}^{n} pa \cdot r$$

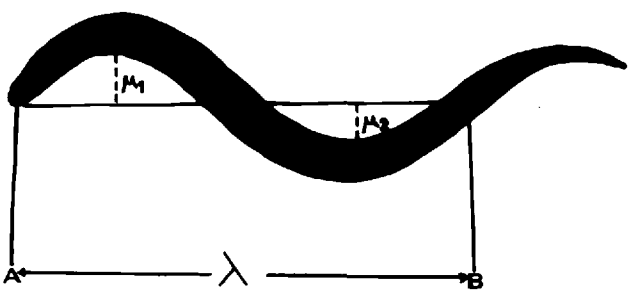
The reversal coefficient per minute was obtained from the product of the reversal frequency and the ratio of reversing to non-reversing larvae for that minute.

Wave parameters and changes in body posture during the activity regime were measured from tracings of still frames of moving larvae taken at intervals using a closed-circuit television video tape system. Wave length and amplitude were measured as in Fig 2:1 after Taylor (1951). The position of the 'kink' and the angle Q in the resting posture of larvae (Croll 1972c) were also measured.

Larval activity was measured between 5-30°C in controlled temperature rooms and at 37°C on a warm microscope stage. Phosphate and Tris buffers made up as described previously, were used to provide a pH range of 5.6 - 9.1 and larval activity measured over this range. The effect of viscosity on larval activity was investigated using aqueous Ficoll solutions to make up viscous media ranging from 1.5 - 7.15 centipoise.

FIG. 2:1

WAVE PARAMETERS OF A MOVING NEMATODE (AFTER TAYLOR 1951)



M_1 anterior wave amplitude

M_2 posterior amplitude

λ length of the wave AB

Ficoll was chosen because it provided solutions of low osmotic pressures and did not penetrate membranes.

To investigate total lipid changes associated with activity, larvae were treated in one of two ways. In the first series of experiments, groups of larvae were repeatedly stimulated as described by bubbling air for 60 seconds activity measured. 'Refractory' periods of 30 minutes were allowed between successive stimulations after activity had returned to the endogenous level.

In the second series of experiments, larval activity was measured for 1,2,4 and 8 hours in neostigmine bromide (0.1mg/ml) which had been shown to prolong activity of these larvae (Croll and Al-Hadithi, 1972). In both series of experiments unstimulated larvae in 2% piperazine and 0.5% propylene phenoxytol acted as the controls. Larval activity in the controls was also measured at half-hourly intervals by counting continuously for 2 minutes, the number of larvae active and their rate of activity.

Lipid changes were investigated by staining larvae in Oil Red O and measuring the amount of stain on a Vickers M85 scanning microdensitometer as previously described. Before each experiment a sample of the larvae ($\geq$ 25 larvae/sample) was stained for the outset lipid level, and a second sample treated for background absorbance measurements. At the end of the experiment, all the live treated and control worms were stained. The percentage lipid loss $\underline{L}$ associated with total larval activity $\sum \underline{Ai}$ was calculated from

$$\underline{L} = \frac{D - d}{O} \times 100\%$$

where $\underline{O}$ is the outset lipid level, $\underline{D}$ the difference between the outset and control lipid, and $\underline{d}$ the difference between the control and treated lipid levels.

RESULTS

a) The nature of larval locomotory activity

Undisturbed larvae under constant conditions showed very low levels of activity. Less than 10% of such larvae were active at a time and the maximum activity rate was 0.82 ± 0.3 w/sec at 30°C. The reversal coefficient remained constant at a fairly high level 1.65 ± 0.1.

When mechanically stimulated, larval activity followed a regime as described by Croll and Al-Hadithi (1972) in which activity rapidly reached a maximum within 2-3 minutes and then gradually dropped until it was again at the endogenous level after 18-19 minutes. The maximum percentage of larvae active was greater than 90% and the maximum activity rate was 3.0 ± 0.01 w/sec.

Changes in the percentage of larvae active and activity rate for the duration of the regime are summarised in Table 8. It is evident that both parameters varied between larvae and also with time. Indexing activity as total undulations per response $\sum_{i=1}^{n} pa.r$ gave consistent results and minimised this variation.

Changes in larval activity pa.r, reversal coefficient wave parameters and body postures with time (Figs 2:2 and 2:3 and Table 9) indicated that the regime could be divided into three phases:

i) phase 'a', was characterised by a high activity rate 2.9 ± 0.18 w/sec resulting in a rapid 'lashing' type movement which lasted for 15-20% of the regime. The reversal coefficient was momentarily high 2.05 ± 0.12 at the onset of activity as the inactive worms became active but very rapidly fell below 0.01.

ii) phase 'b' lasted for 50-60% of the regime.

TABLE 8

The activity response of 0-day old infective larvae of A.tubaeforme at 30°C after a 1-2 hour refractory period (results from 11 experiments)

Time (min)	% larval act mean	% larval act range	Rate of activity mean	Rate of activity range	Pa.r	Coefficient of variation
0 (Endogenous activity)	9.6	0-15	0.85	0.81-0.88	8.25	15.25
1	76.5	65-90	1.78	1.52-2.1	83.0	11.68
2	85	80-100	2.98	2.84-3.06	157.0	10.93
3	85	80-100	2.91	2.73-3.0	168.5	4.79
4	86	80-97	2.79	2.70-2.9	168.75	4.63
5	84	80-100	2.83	2.67-2.9	165.0	4.67
6	85	80-98	2.62	2.58-2.74	158.0	5.54
7	76	61-92	2.50	2.41-2.60	152.0	4.95
8	73	60-96	2.30	2.26-2.37	140.25	6.79
9	67	52-90	2.24	2.02-2.37	132.5	9.19
10	70	56-93	2.16	2.02-2.25	115.0	9.47
11	63	55-84	2.03	1.86-2.26	102.75	6.52
12	60	49-80	1.98	1.91-2.09	89.5	12.19
13	60	51-75	1.81	1.68-2.05	67.75	15.59
14	53	46-78	1.74	1.69-1.92	56.25	18.49
15	40	25-62	1.62	1.53-1.81	40.5	27.09
16	47	25-68	1.24	1.12-1.68	26.0	28.61
17	30	20-59	1.20	0.98-1.26	15.75	22.82
18	13	8-16	0.92	0.86-1.12	12.75	18.53
19	15	5-20	0.79	0.76-0.81	10.5	12.29
20	9	0-20	0.82	0.8-0.86	8.25	15.25
21	11	0-18	0.86	0.78-0.92	7.75	19.36

Duration of activity (min)

mean ± SE: 18.07 ± 0.56

C of V: 14.23

$$\sum_{i=1}^{n=21} pa.r$$

Total activity

mean: 1886 ± 47

C of V: 5.02

The activity rate decreased gradually from 2.6 to 1.7 w/sec as larvae passed into lower frequency movements. This was accompanied by an increase in the curvature of the bent region of the wave pattern at the head end (Fig 2:3, iii b). The waves were of large amplitude and short length.

iii) phase 'c', lasted for 20-30% of the regime and was characterised by a low activity rate 0.93 ± 0.52 w/sec and a high reversal coefficient (2.0). Frequently more than one wave was observed at a time, and coiling (Fig 2:3 iii c) occassionally occurred. At cessation of activity over 80% of the inactive larvae adopted the 'kinked' posture (Fig 2:3, iv c).

These three phases are a continuum and the phase boundaries are abitrary because each individual in the sample passed through them at slightly different times.

The magnitude and nature of the larval activity regime was unchanged even after 16 repeated responses and no difference in activity was observed between ensheathed and exsheathed larvae (Table 10).

b) The effect of stimulation intensity and 'refractory' period on activity.

Larval activity in response to bubbling periods of 15-120 seconds following a 1-2 hour 'refractory' (resting) period are shown in (Figs 2:4 and 2:5 and Table 11).

The 15 second bubbling resulted in reduced activity but without a change in the basic activity pattern (Fig 2:5a). The maximum percentage of larvae active was reduced (58-72%) but the maximum activity rate was unaltered (2.98 ± 0.14 w/sec).

A delayed and greatly reduced activity response followed a bubbling period of 120 seconds (Fig 2:5c). On withdrawal of the stimulus after the 120 seconds, over 90% of the larvae were inactive but had lost their 'kinks'.

FIG. 2:2

THE ACTIVITY REGIME OF "0-DAY" OLD THIRD-STAGE LARVA OF A.tubaeforme AT 30°C.

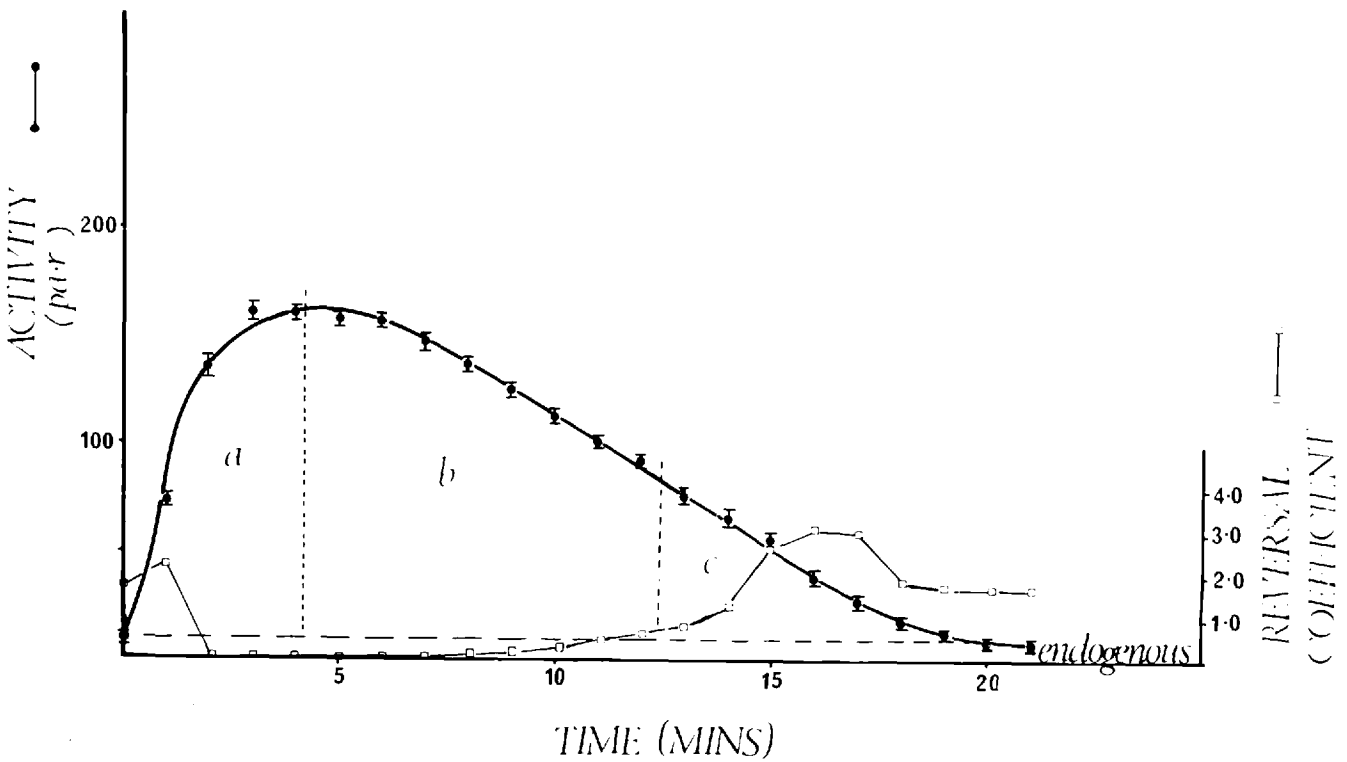
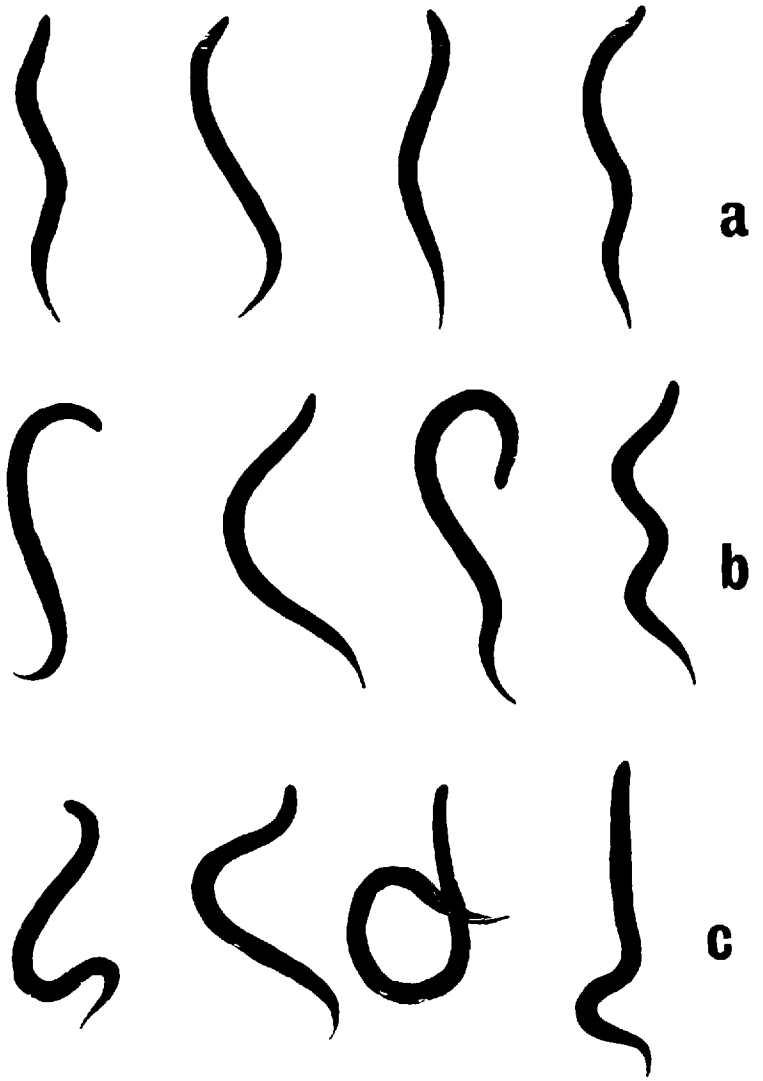


FIG.2:3

TRACINGS OF BODY POSTURES TAKEN FROM STILL FRAMES OF MOVING THIRD-STAGE LARVAE OF A.tubaeforme DURING THE ACTIVITY REGIME.



Tracings taken between

a 0-5 minutes

b 6-14 minutes

c15-21 minutes

of the activity response as the larvae passed through the "phases".

TABLE 9

Wave parameters of the '0-day' old infective larvae of A.tubaeforme during the activity regime and during activity in 0.1mg/ml neostigmine bromide (measurements on frames from video tracings of moving larvae at 30°C).

Period of the activity regime (minutes)	0 endogenous activity	1 - 5 phase a	5 - 10 phase b I	10 - 14 phase b II	15 - 21 phase c	Neostigmine bromide
Number of larvae measured	15	16	15	18	18	25
Mean larval length L	23.25	21.61	22.90	21.40	20.60	22.46
Number of waves per larval frame (range)	1.0-2.5	1.0	1.0-1.5	1.0-2.0	1.0-2.5	1.5-2.5
Mean wavelength λ	13.07	18.03	17.04	13.62	10.92	8.42
λ/L	0.56	0.83	0.74	0.64	0.53	0.38
Anterior wave amplitude u1 (mean ± SE)	3.43±2.35	2.93±1.3	5.69± 3.02	3.31±2.20	2.55±1.80	1.86± 0.92
$u1/\lambda$	0.26	0.16	0.33	0.24	0.23	0.22
Posterior wave amplitude u2 (mean ± SE)	5.6±1.08	3.14±1.5	2.68±1.82	4.62±2.03	5.2±3.21	1.58±0.73
$u2/\lambda$	0.43	0.17	0.16	0.34	0.48	0.19

TABLE 10

The activity of the ensheathed and exsheathed '0-day' old infective larvae of A.tubaeforme from the same culture at 30°C following repeated responses (means of the results of 3 experiments)

	1st Activity response			8th Activity response			16th Activity response		
	Maximum % of larvae active	Maximum rate of activity (w/sec)	duration of activity (minutes)	Maximum % of larvae active	Maximum activity rate (w/sec)	duration of activity (min.)	Maximum % of larvae active	Maximum activity rate (w/sec)	duration of activity (min.)
Ensheathed larvae	90	2.96	18.04	84	3.01	18.26	91	3.01	17.87
Exsheathed larvae	90	2.98	18.13	92	3.01	18.07	86	2.98	18.02

THE EFFECT OF THE BUBBLING PERIOD ON LARVAL ACTIVITY RESPONSE.

FIG.2:4

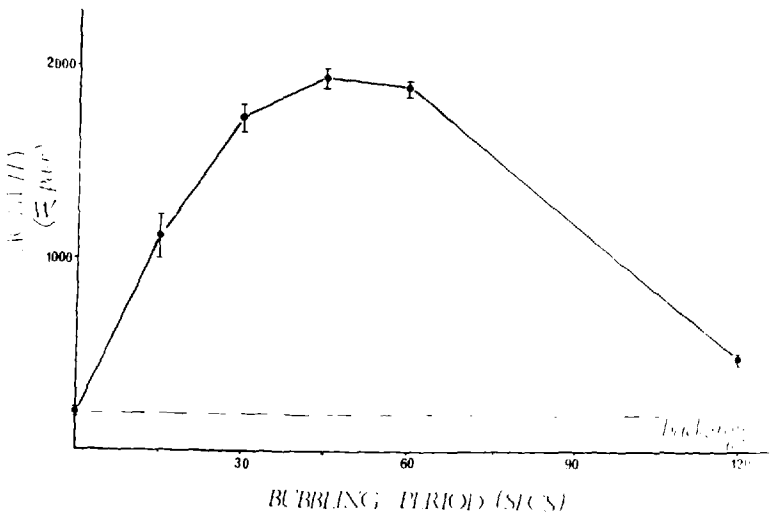


FIG. 2:5a

THE ACTIVITY RESPONSE OF LARVAE FOLLOWING A 15-SECOND BUBBLING PERIOD.

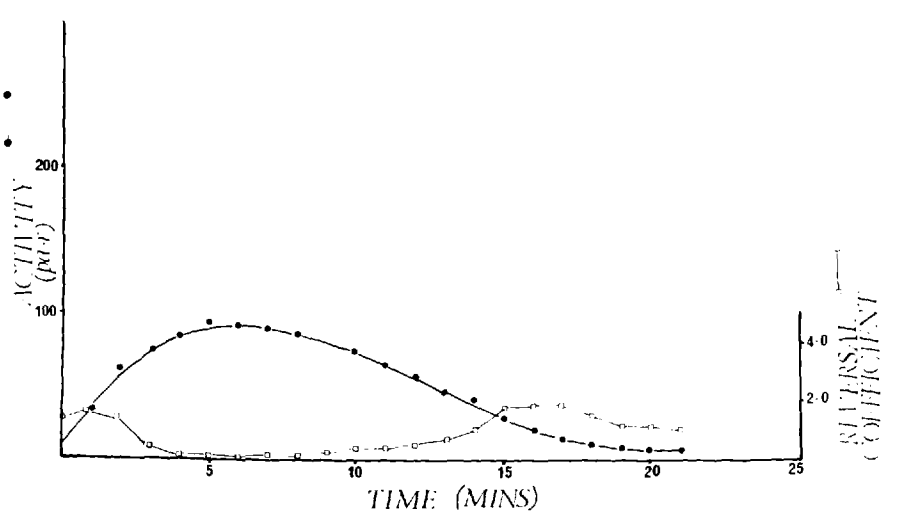


FIG. 2:5b

THE ACTIVITY RESPONSE OF LARVAE FOLLOWING 30, 45, & 60-SECOND BUBBLING PERIODS.

30 secs.

45 secs.

60 secs.

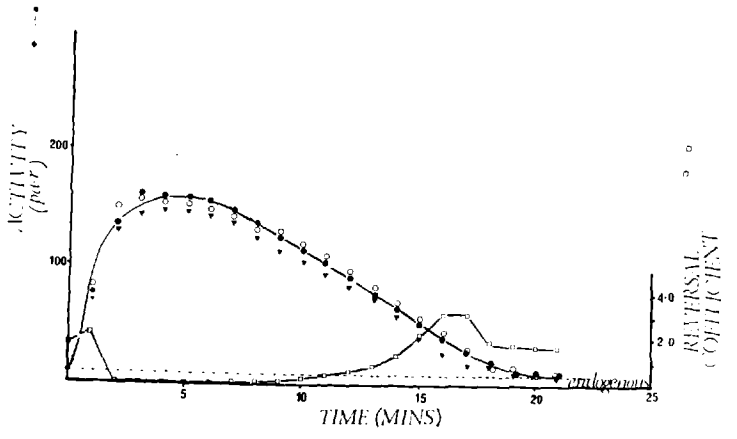


FIG. 2:5c

THE ACTIVITY RESPONSE OF LARVAE FOLLOWING A 120-SECOND BUBBLING PERIOD.

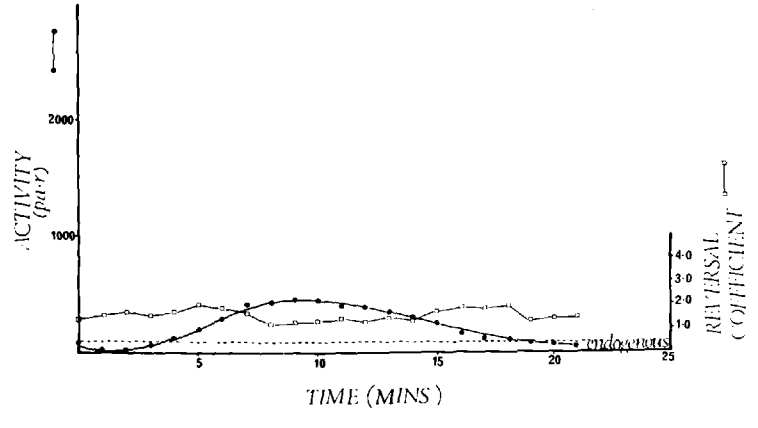


TABLE 11

Activity of the '0-day' old infective larvae of A. tubaeforme following different periods of bubbling at 30°C. (The range of the results of 5 experiments).

Bubbling period (minutes)	15	30	45	60	120
Maximum percentage of larvae active	40-65	75-100	80-100	80-100	70-95
Maximum rate of activity (waves/sec)	2.1-2.6	2.8-3.01	2.8-3.01	2.8-3.01	1.18-1.53
Time taken to attain maximum activity (minutes)	2.0-3.0	2.0-2.5	2.0-2.5	2.0-2.5	6.0-9.0
Duration of activity (minutes)	15-18	17-19	17-19	17-19	10-14

These larvae however, slowly became active but at a much reduced rate (1.5 w/sec) although the percentage of larvae active was high (80%).

Larvae did not respond to mechanical stimulation immediately after cessation of activity. The length of time during which the larvae remained unresponsive (true refractory period) varied between larvae and lasted from 10-20 minutes (Fig 2:6). There was no significant difference (at 5% level) in subsequent larval activity following refractory periods exceeding 20 minutes.

c) The 'kinked' posture

The position and angle of the 'kink' were measured from a total of 63 larvae from five experiments (at 30°C) which had adopted the kinked resting posture. The result is shown in (Fig 2:7).

The kink was mostly developed at the posterior third of the body and over 80% of the larvae adopted this posture at the cessation of activity. The posture was generally lost within 10 minutes and the larvae straightened out. The kink parameters and the percentage of larvae adopting the posture did not change following repeated responses.

At cessation of activity leading to narcosis in 2% piperazine and 0.5% propylene phenoxytol, most larvae straightened out and the percentage adopting postures similar to the typical kinked posture was less than 20%.

d) Factors affecting larval activity

i) Temperature

Larvae rapidly became immobile and straightened out when placed at 5°C, Between 10-37°C an increase in temperature resulted in an increase in larval activity (Fig 2:8a).

There was a linear increase in the activity rate with temperature (Fig 2:8a) and the percentage of larvae active following stimulation was not markedly changed between 15-30°C

but was reduced at 10°C and 37°C (Table 12).

TABLE 12

The effect of temperature and viscosity on the locomotory activity of '0-day' old infective larvae of A.tubaeforme. The viscosity measurements were at 30°C (means of the results of five experiments).

	Temperature (°C)					Viscosity (Cp)			
	10°	15°	20°	30°	37°	1.54	2.63	4.34	7.15
Maximum % of active larvae	37.5	80.1	86	88	69	79.8	82	80.2	78.1
Maximum activity rate (w/sec)	1.11	1.47	1.89	3.01	3.14	2.82	2.76	2.34	1.87
Duration of activity (minutes)	11.08	21	21	18.26	15.02	18.23	18.72	17.89	17.02

ii) pH

No significant difference was found in larval activity within the pH range 5.6 - 9.1 (Fig 2:8b).

iii) Viscosity

Fig 2:8c shows the effect of viscosity on larval activity. With an increase in viscosity of the medium, larval activity rate was reduced but the percentage of larvae active was not significantly changed (Table 12).

iv) Pharmacologically active drugs

Preliminary tests showed that piperazine and propylene phenoxytol at aqueous concentrations exceeding 5% produced an immediate narcosis of larvae, but recovery on transfer to drug-free media was very poor. In 2% piperazine and 0.5% phenoxytol, narcosis was gradual and recovery good.

FIG. 2:6

THE RELATIONSHIP BETWEEN LARVAL ACTIVITY AND THE 'REFRACTORY PERIOD'.

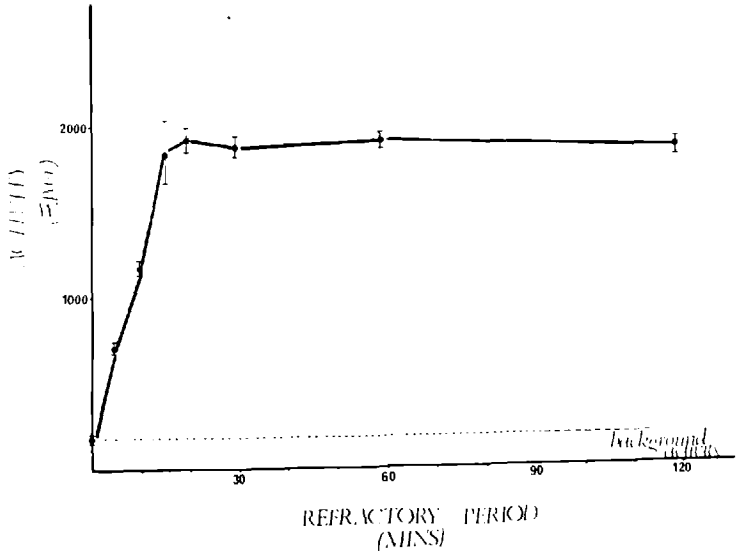


FIG.2:7

THE POSITION OF THE 'KINK' AND THE ANGLE 'Q' IN LARVAE ADOPTING THE 'KINKED' RESTING POSTURE.

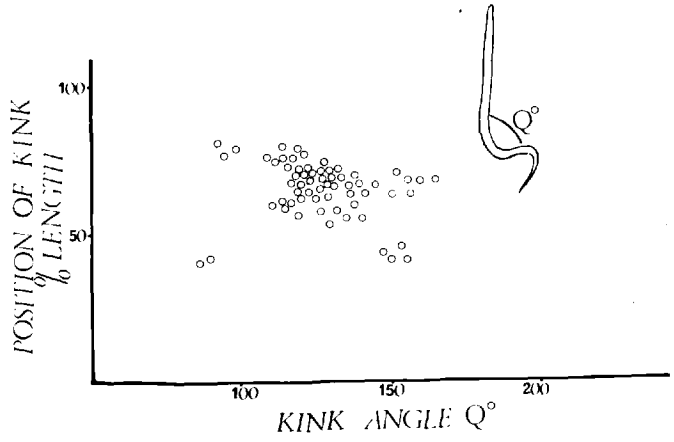


FIG. 2:8a
THE EFFECT OF TEMPERATURE ON LARVAL ACTIVITY.

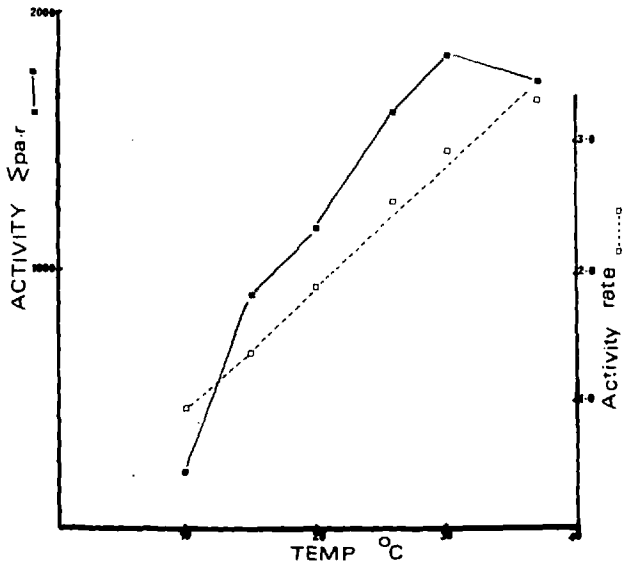


FIG. 2:8b
THE EFFECT OF pH ON LARVAL ACTIVITY.

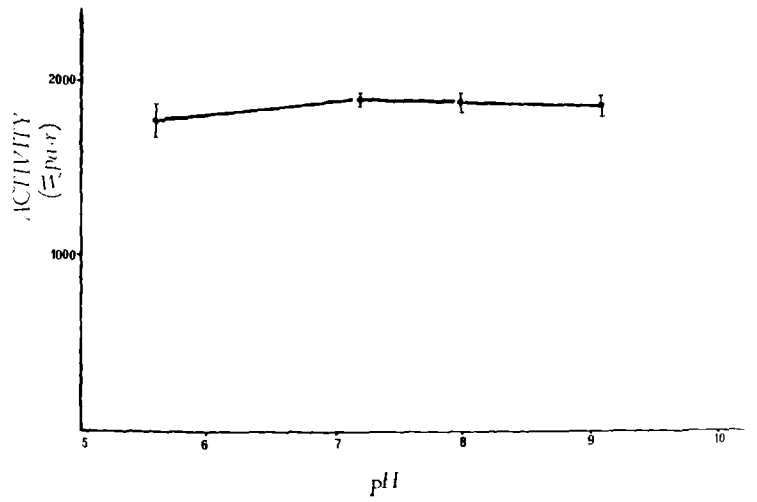


FIG. 2:8c

THE EFFECT OF VISCOSITY ON LARVAL ACTIVITY.

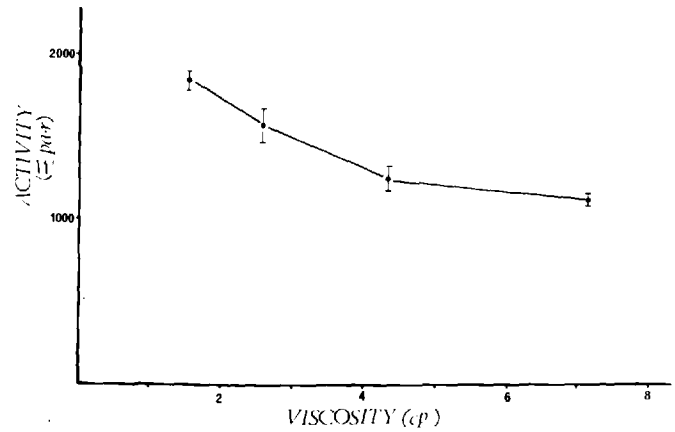
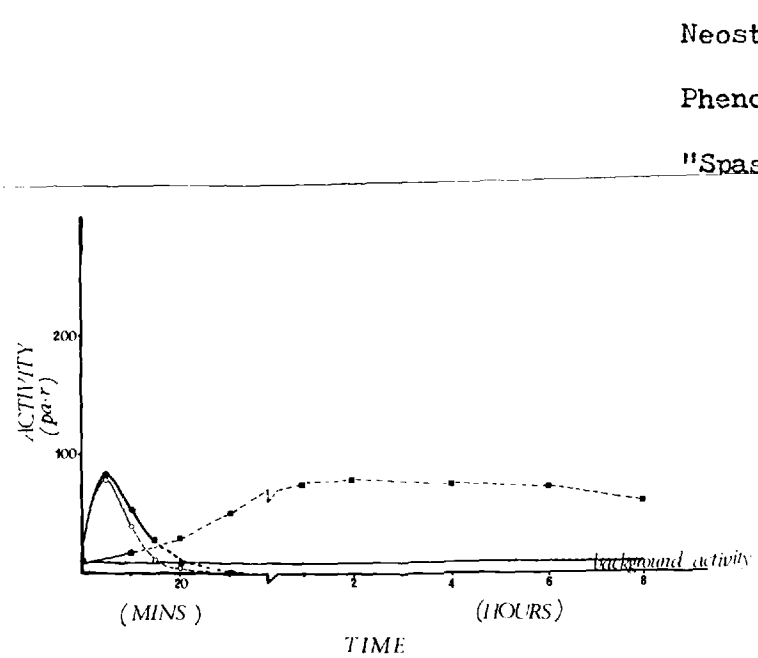


FIG.2:8d

THE EFFECT OF PHARMACOLOGICALLY-ACTIVE DRUGS ON LARVAL ACTIVITY.



These lower concentrations of the drugs were therefore chosen for use in the experiments.

Most larvae (>90%) were immediately activated on addition of the drugs but the activity rate was low (0.86 ± 0.07 w/sec). Movement became uncoordinated and "spastic" within 10-15 minutes and complete narcosis resulted after 30-45 minutes (Fig 2:8d).

The narcotised larvae recovered completely within 24 hours when transferred into drug-free media. Their activity responses when tested against those of larvae from the same culture that had been kept in phosphate buffer pH 7.2, was found not to be different.

Larval activity in neostigmine bromide (0.1mg/ml) remained unchanged over 8 hours (Fig 2:8). The percentage of larvae active was high (85 ± 5%) but the activity rate was reduced (0.98 ± 0.04 w/sec). Movement continued beyond the 8 hour study period, was uncoordinated and was often accompanied by exsheathment (32 ± 4% after 8 hours).

e) Lipid changes

Croll (1972d) pointed out that because infective larvae had low Reynold's numbers (10^{-2}) their movement would be dominated by viscous faeces rather than by inertia, and he argued that consequently the larval undulatory rate was the significant factor influencing the energetics of movement. Experiments on the effect of activity on lipid untilisation by larvae were therefore conducted at 30°C because at this temperature most larvae were active and the rate of activity was high (see Table 12).

Fig 2:9 shows camera-lucida drawings of representative larvae stained for neutral lipids after periods of larval activity. The '0-day' old infective larvae had high lipid reserves mainly in the form of large fat globules confined to the intestinal cells. In all the experiments there was no significant difference ($P=0.05$) in the lipid levels of the

FIG. 2:9

1.0 "Outset lipid"

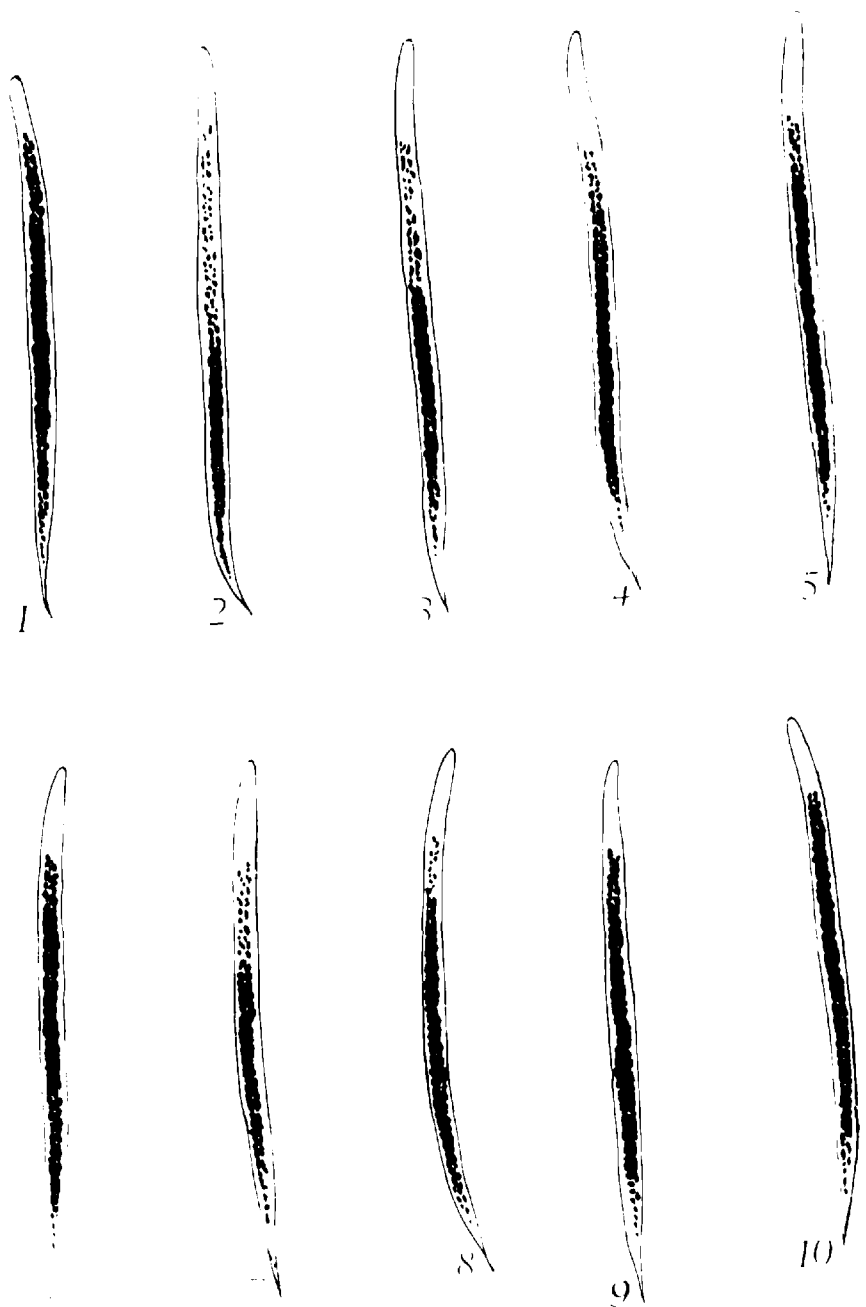
Lipid level after

2. 16 activity regimes
3. 12 " "
4. 8 " "
5. 4. " "
6. After 2 activity regimes
7. " 8 hrs. in neostigmine
8. " 4 hrs. in "
9. $Control_1$ (Unstimulated larvae)
10. $Control_2$ (Narcotised larvae)

(Larval activity per regime $\sum_{i=1}^{n=21} Pa.r = 1895 \pm 50$ unds.)

FIG. 2:9

REPRESENTATIVE THIRD-STAGE LARVAE OF A.tubaeforme STAINED FOR NEUTRAL LIPIDS AFTER PERIODS OF ACTIVITY.



control larvae and the outset lipid. Lipid loss became significant only after 4 activity regimes or 2 hours in neostigmine bromide (Table 13).

TABLE 13

The lipid loss of '0-day' old infective larvae of A.tubaeforme after several activity regimes and after periods of activity in neostigmine bromide (0.1mg/ml) (results of five experiments)

Number of activity regimes	% Lipid loss		Duration of activity in neostigmine (hours)	% Lipid loss	
	range	mean		range	mean
4	1.12-2.9	2.3	2	2.9-5.2	4.16
8	4.34-6.9	4.5	4	5.4-8.9	7.32
12	5.5-9.6	7.8	8	9.8-11.2	10.23
16	7.6-13.2	10.14			

There was a high correlation between the percentage lipid loss and extent of previous activity (Figs 2:10a and 2:10b) and more than 10% of lipid was lost after extensive activity (16 activity responses or 8 hours in neostigmine). However because the nature of larval activity in neostigmine differed markedly from movements during a normal activity regime, direct comparisons were not made between the lipid loss resulting from activity in both series of experiments.

The lipid also appeared to have been utilised in a progressive manner antero-posteriorly, and there was a reduction in the number and size of the fat globules.

THE RELATIONSHIP BETWEEN ACTIVITY AND LIPID UTILISATION.

FIG. 2 :10a

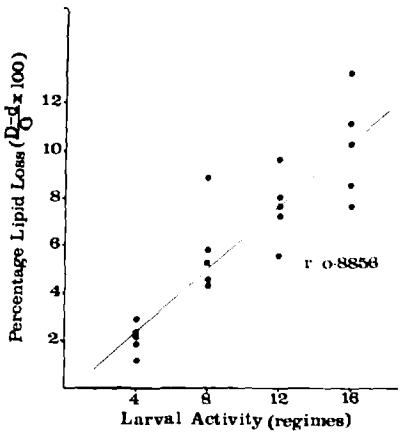
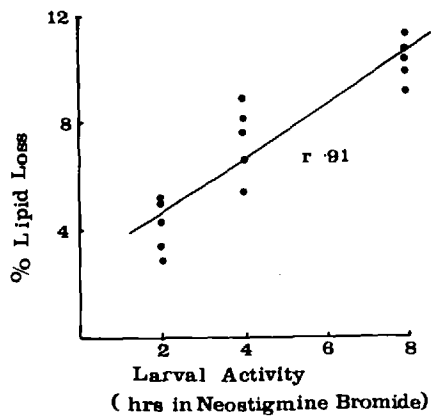


FIG. 2: 10b



DISCUSSION

The infective larvae of Ancylostoma tubaeforme showed low levels of endogenous activity and locomotory activity was greatly increased upon stimulation. Payne (1923b) and Lane (1930) pointed out that infective hookworm larvae had alternate periods of activity and "rest". Barrett (1969) observed that infective larvae of Strongyloides ratti were not continuously active and were only fully active when stimulated. Similar observations have also been reported for the infective larvae of Trichonema (Odei 1968) and Nematospiroides dubius (Bryant 1973). It would appear therefore that the activity pattern described here for the infective larvae of A. tubaeforme may be common to infective larvae of zooparasitic nematodes.

Croll (1970) has shown that the activity pattern for the preinfective larval stages of these parasites, and the infective larvae of phytophagous nematodes were similar. These larvae like the free-living nematodes Turbatrix aceti and Panagrellus redivivus showed very high levels of endogenous activity.

Croll (1972c) also postulated that nematode activity may be coordinated by two mechanisms. He regarded rapid backwardly-directed waves as being controlled by an exogenous system while slower movements and activities such as pharyngeal pumping associated with feeding were endogenously controlled. The exogenous system was believed to be entirely under nervous control and stimulated through the senses and it was dominant over the endogenous system part of which was of a myogenic origin.

On this hypothesis, the low level of endogenous activity shown by the infective larvae may be explained by the loss of tropic activities in this non-feeding stage. The activity regime (exogenous) consequently has become highly pronounced

and being under exogenous control is affected by changes in the environment. The infective larvae are therefore most active mainly under those conditions which might represent the presence of a potential host and activity soon ceased if contact had not been made.

The infective larvae do not feed and rely entirely on their lipid reserves for their energy source. Their longevity would therefore depend in part on the rate of lipid utilisation and conservation of lipid would be very important if these larvae were to stay alive and remain infective for several weeks. Payne (1923b) and Odei (1968) had suggested that the adoption of certain postures by the infective larvae might limit activity and thereby energy expenditure. It is unlikely however that the kinked posture of inactive infective larvae of A.tubaeforme had such a function. The sustainance of such a posture for long periods would involve prolonged muscular activity and the expenditure of energy. Furthermore the posture was generally lost within 10 minutes whilst many larvae were still unresponsive to stimuli, and altering the activity pattern using pharmalogical drugs greatly reduced the number of inactive larvae that adopted the posture.

The 'kink' seemed to appear as a result of the arrest of locomotion because once the kink had been formed, waves could not be transmitted to the posterior end and undulation of the body soon ceased. Croll (1972c,1975) showed that normal nematode activity consisted of an ordered combination of forward movement and reversals. Forward movement was brought about by backwardly propagated waves while reversals were by waves propagated from the posterior to the anterior end. A 'kinked' posture has been observed in the free-living nematode Caenorhabditis elegans and in many other species (Croll, pers comm).

In C.elegans, reversals characterised periods of low activity and the kink resulted from an equilibrium between forward and backward waves (Croll, 1975).

Reversal by the infective larvae of A.tubaeforme was usually of short duration and increased with a decrease in the activity rate (Fig 2:2). Periods of reduced activity accompanied by an increase in reversals always preceded cessation of activity and the adoption of the kinked posture. It thus appears in these larvae as in C.elegans, that reversals were governed by this forward component of movement and the 'kink' was simply a result of both components. The refractory period and not the kinked posture is therefore considered to be an important factor in the control of activity and conservation of lipid.

The mechanical activation threshold for larvae appeared to be minimal but it was not clear why larval activity was reduced following a bubbling period of 120 seconds. It is possible that the larvae were over stimulated and this might have led to a saturation of their 'mechanical sense', thus resulting in low activity. Smith and Croll (1975) have found a bilaterally arranged ciliary sense organ in the infective larvae of A.tubaeforme and Necator americanus, and thought it might have a proprioceptive function. However detailed studies on the physiology of this 'new' sense organ are necessary before any conclusions can be drawn as to its involvement in the repsonses of larvae to mechanical stimulation.

On the other hand it could be that the larvae had been activated when bubbling had gone on for more than 30 seconds and then inactivated because their rhythm of movement had been disrupted by mechanical forces caused by continued bubbling. Machin (1958) in an essentially mathematical treatment of the propagation of sine waves, argued that the observed wave forms on a flagellum could be explained by assuming that active

contractile elements were distributed along its length, and that these were activated by local bending. Lee (1965) considered that there was evidence for the presence of an inhibitory nerve supply to the muscles of the body wall and suggested that muscular activity in nematodes was controlled by two independent systems. Crofton (1966) argued that muscular contraction in Ascaris was of myogenic origin and could be transmitted from muscle to muscle and that the rate of the role of the nervous system was to co-ordinate and control this activity. Jarman (1970) explained the postural waves of Ascaris on the establishment of muscle-domains. He believed that the contracting cells imposed strains on adjascent cells and caused them to conform to their phase of contraction, thus maintaining a continuous wave pattern. Movement on this hypothesis, would result from a maintenance of the wave rhythm by the movement of domain boundaries or by the establishment of new domains. Reduced larval activity resulting from prolonged bubbling, may therefore not be a sensory phenomenon but a consequence of the disruption of the wave rhythm of moving larvae.

Of the various physical factors investigated, temperature had the greatest effect on larval activity. Croll and Smith (1972) suggested that temperature was the major factor stimulating penetration behaviour in these larvae. Matthews (1972b) measured the skin-surface temperature of a cat using a thermistor probe and found it to vary from 29°-33°C. It is therefore significant that total larval activity following mechanical stimulation was greatest at $30 \pm 2^{\circ}$C (Fig 2.8a). Larvae would then, on contact with the host be fully active and directed in their efforts to penetrate the skin, and it is tempting to suggest that larval activity had become adapted to enhance the invasion of potential hosts.

The activation of larvae by piperazine and phenoxytol before narcosis, is difficult to explain. It was not due to mechanical stimulation as the drugs were carefully added without agitating the medium and a similar effect was not observed with neostigmine. Propylene phenoxytol has been used as a narcotic for nematodes (Ellenby and Smith, 1964) but its mode of action is not known. Piperazine is believed to act at the neuromuscular junctions (Norton and Beer 1956, Fiakpui 1967) and may also affect the membrane potential of muscle cells (del Castillo 1969). The general similarity in the sequence of events preceding narcosis in both drugs would suggest similarities in their modes of action, and the almost instant activation of larvae indicated that there might be an initial effect of these drugs peripherally at the sense organ level.

It has generally been recognised that larval activity was a major factor in the loss of lipid by the infective larvae (Payne 1923b; Barrett 1969) but quantitative data are lacking. Clark (1969) gave quantitative results to show that lipids were the primary food reserves in the infective larvae of Ancylostoma caninum. Barrett (1968a,1969) showed that the free fatty acids were the major component of the lipid reserves of the infective larvae of S.ratti and that the amounts of the free fatty acids became greatly reduced with time. Croll (1972a), Croll and Matthews (1973) stored the infective larvae of A.tubaeforme under various conditions and found that those larvae kept under conditions where they had been very active had the least lipid. Quantitative data have been presented in this section to show that larvae which had been very active had considerably less lipid than those of a similar physiological age that had been inactive.

Larval activity remained unchanged following repeated responses. This would tend to suggest that it was not affected by the extent of previous activity nor was it determined by the lipid levels since larvae would have lost over 10% of the outset lipid by the 16th regime.

It is not known why the initial loss of lipid occurred at the anterior intestinal region. Localised loss of lipid has been reported for the phytophagous nematodes Meloidgyne hapla (Sayre 1964) and Tylenchorhynchus semipentrans (Cohn 1966), and this appeared to be associated with esterase activity (van Gundy et al. 1967). Lee (1968) found that there was more esterase activity in the walls of the anterior third of the intestine than in the posterior two thirds in the infective larvae of Nippostongylus brasiliensis. The effect of such localised esterase activity might therefore account for the initial loss of lipid from this region. Colam (1971) believed that digestion in Cyathostoma lari, was initiated by a non-specific esterase (possibly originating in the oesophageal glands). Smith and Harness (1971) described electron-dense bodies within the intestinal cells of the third-stage larvae of Haemonchus placei and Trichostrongylus colubriformis. These bodies apparently were confined to the anterior region of the intestine.

These results have shown that the anterior region of the intestine of the infective larvae of some nematode parasites differs structurally (and possibly functionally) from the posterior. If therefore, as may be the case, enzymes which function in the catobolism of neutral lipids are regionally localised, this may provide a built-in mechanism for lipid conservation. On the other hand, the localised use of lipid may be a result of complex phenomena and its explanation would require more information on lipid mobilisation by the infective

S E C T I O N III

LONGEVITY AND SURVIVAL OF THE INFECTIVE LARVAE

INTRODUCTION

The longevity of infective larvae of parasitic nematodes and their activity to survive adverse factors in their environment are of great epizootiological importance. Extensive studies have therefore been undertaken on the effects of temperature and humidity on the longevity and 'survival' of infective larvae and these were reviewed by Kates (1950), Rogers and Sommerville (1963 and Michel (1968).

However, results of these studies have differed widely. Augustine (1922) summarising previous work on the longevity of human hookworms found that it ranged from 3-18 months. His investigations showed that under field conditions these larvae lived for about 6 weeks and their longevity was affected by climatic factors especially temperature.

Spindler (1936) reported that the infective larvae of Oesphagostomum dentatum 'survived' for up to 14 months when protected by a heavy growth of grass and for 9 months when not thus protected. Shorb (1942) found that the parasitic stages of ovine nematodes Ostertagia, Trichostrongylus, Trichuris and Nematodirus 'survived' in pastures for 2-3 months and were not necessarily protected by the abundance of shade. Anantaraman (1942) believed that the infective larvae of O.radiatum were short-lived and Premvati and Lal (1961) showed that those of O.columbianum 'survived' for 50 days in summer and for 108 days in winter.

The longevity of infective larvae of Eustrongylides ignotus was much greater if they were stored under sterile conditions (van Brand and Simpson 1944). T.A. Miller (pers comm) has also found that infective larvae of the dog hookworm A.caninum stayed alive longer when kept under sterile conditions.

The divergence in the results of longevity experiments might be ascribed to two factors (Rogers and Sommerville 1963). In most of the studies due consideration was not always given to physical factors in the micro-environment like pH, availability of oxygen; light changes, which could affect larval physiology, and secondly, standard mortality evaluation procedures such as probit were not used to analyse the results.

Infective larvae of various parasites vary considerably in their ability to withstand drying conditions. Belle (1959) reported that the infective larvae of Bunostomum trigonocephalum did not survive desiccation. Prasad (1959) found that some infective larvae of Trichostrongylus retaetoformis survived 20% RH for 7 weeks at 20°-26°C. Ellenby (1968) observed that ensheathed infective larvae of Haemonchus contortus survived desiccation better than the exsheathed forms. The sheath was also found to be important in the desiccation - survival of infective larvae of Trichonema which survived better at low than at high humidities (Odei 1968).

The aims of studies reported in this section were to determine the longevity of the infective larvae of A.tubaeforme under controlled laboratory conditions, to investigate their ability to survive adverse environmental conditions and to assess the relationship between longevity and the larval lipid content. Throughout the studies the term longevity has been used to denote larval length of life under physiological conditions in which activity was not impaired (see section II) to distinguish it from survival of adverse factors.

MATERIALS AND METHODS

Eggs of A.tubaeforme were cultured at 30°C and infective larvae isolated from cultures as previously described. Larvae isolated after 72 hours were used in these experiments as 0-day old infective larvae. Larvae from the same culture were used in each experiment.

Storage of larvae was as described and the storage media were examined at weekly intervals for microbial growth by inoculating nutrient agar with 1.0ul samples. The pH of the media were also checked at the end of the experiments and those in which detectable microbial growth had occurred or which showed changes in pH exceeding 0.2 were ignored when calculating larval longevity.

The percentage of larvae alive after a desired period was obtained from 100ul aliquants of the stored larvae. Five of such aliquants were measured in each treatment. Dead larvae were easily detected because they had dark granular contents, and failed to move when touched with a mounted bristle or placed in neostigmine bromide (0.1mg/ml).

Larval activity was measured after worms had been allowed time (3-4 hours) to reach on equilibrium with the test temperature ($28 \pm 2^{\circ}C$). The percentage of larvae active when stimulated (as described in Section II) and the maximum activity rate were recorded. The lipid content was measured by the microspectrophotometric method already described and humidity chamberswere set up using sulphuric solutions in desiccators (Solomon 1951).

After periods of desiccation at 95% RH larvae were placed in distilled water and left at room temperature for 24-48 hours. Their activity and ability to penetrate an artificial membrane were subsequently measured. Penetrability was assayed at 37°C using Whatman's No.2 filter paper membranes in 'Matthews

penetration cells' (Matthews 1972b). At least 50 larvae were used in each test and the number penetrating after one hour were counted and expressed as a percentage of the number of larvae used.

RESULTS

a) Methods used to estimate larval longevity

i) the mortality pattern of infective larvae

Infective larvae isolated from a set of cultures at 30°C were subdivided into samples of 800-1,000 larvae and placed in storage under constant fluoresent illumination at 26°C. The number of dead and live larvae were counted at intervals over a period of 12 weeks.

Fig 3:1 shows the average percentage mortality (from eight experiments) of infective larvae for the 12 week period. Mortality was low initially (10%) and remained so for about 3 weeks. At 5 weeks, only 17.39% of the larvae had died but thereafter mortality increased rapidly. By the 8th week, about 50% of larvae were dead and mortality exceeded 95% after 12 weeks, resulting in a sigmoid mortality curve.

ii) Probit analysis

The probit transformation is generally used to evaluate dosage response data, such as the response of animals to therapeuticagents and insecticides (Finney 1964). The response curves obtained from such experiments are usually sigmoid in shape but may be converted to straight lines to facilitate analysis by plotting the probit of the percentage response against log dosage or log time for example.

The mortality curve of infective larvae was also found to be sigmoid (Fig 3:1 and 3:3). A computer programme developed by the Statistical Research Service, Department of Agriculture, Canada based on Finney (1964) was used to analyse the larval mortality data. Analysis showed that only data for the 5°C groups were not fitted as well as when $\log_{10}x$ was used. Log transformation was therefore not used for data at the other temperatures. The programme fits the regression line, $\underline{Y} = \underline{a} + \underline{bt}$ for $\underline{Y}$, the probit

of proportional response corrected for the natural rate (NRR) on t, the time of exposure and provides estimates of the median effective time (50% longevity or mortality or S50), 95% mortality (S95) and the standard errors of these values.

S50 and S95 values obtained from probit analysis of the data presented in Fig 3:1 are shown in Table 14.

TABLE 14

Median longevity (S50) and 5% longevity (S95) of infective larvae of A.tubaeforme at 26°C obtained by probit analysis (see Fig 3:1)

Response level (≡ % mortality)	Effective dose (ED) (≡ time in weeks)	Exact 95% Confidence limit for ED	
		lower	upper
50	7.476	7.092	7.884
95	12.77	12.05	13.65

iii) Calculations on S50

Methods described by Reed and Muench (1938), Litchfield and Wilcoxon (1948) and Litchfield (1949) were also used to obtain median longevity (S50).

Litchfield method required the construction of nomographs and the use of logarithmic probability paper and was rather complicated. The method of Reed and Muench however was simple and rapid. The operations involved have been set out in Table 15 and Fig 3:2. The number of alive and dead larvae are entered in columns b and c respectively and their cumulatives in d and e. The percentage mortality was calculated from d and e and entered in column f. The cumulatives of alive and dead larvae were plotted and their point of intersection taken as median longevity.

FIG. 3: 1

THE MORTALITY PATTERN OF INFECTIVE LARVAE OF A.tubaeforme AT 26°C.

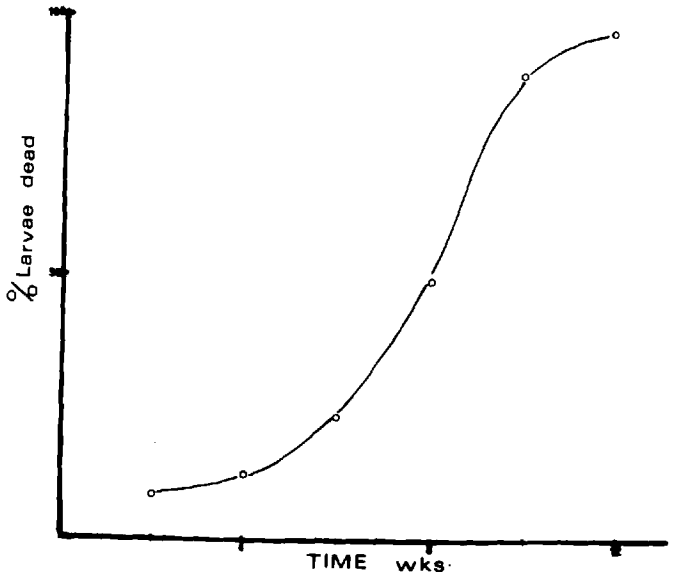


FIG. 3:2
(Explanation in the text)

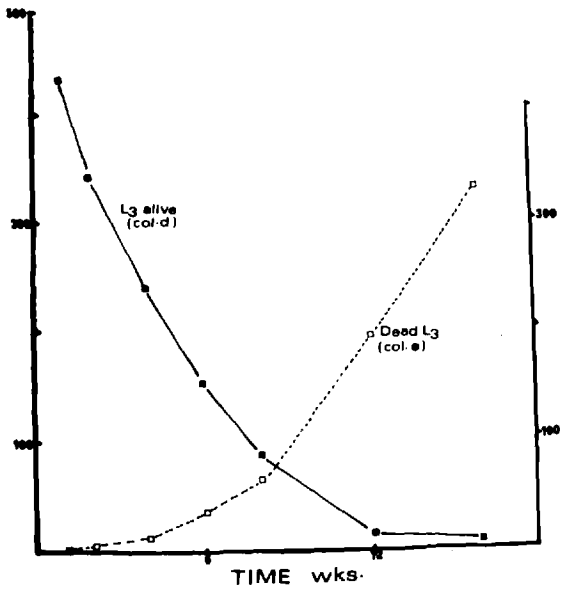


TABLE 15

(explanation in the text)

Number of weeks	Larvae alive b	Larvae dead c	Total alive d	Total dead e	Percentage mortality f
1	102	1	444	1	0
2	96	2	342	3	1
4	94	9	246	12	5
6	90	21	152	33	18
8	49	52	62	85	58
12	13	111	13	196	94
16	0	136	0	332	100

Fig 3:2 showed median longevity of larvae stored at 26°C to lie around 7.62 weeks which was not significantly different from that obtained from detailed probit transformation (Table 14). This method was therefore used in subsequent calculations.

b) the effect of the sheath on longevity

There was no marked difference in the longevity of infective larvae exsheathed using 5% aqueous 'Milton' as described, from that of ensheathed larvae from the same culture at 26°C (Table 16).

TABLE 16

The longevity of exsheathed and ensheathed infective larvae of A. tubaeforme at 26°C.

Longevity (range of results of 5 experiments)	Exsheathed larvae	Ensheathed larvae
S50 (weeks)	7.035 - 7.965	7.092 - 7.884
S95 (weeks)	12.45 - 13.61	12.90 - 13.73

c) Factors influencing longevity

I Extrinsic factors

i) temperature

To investigate the effect of storage temperature on longevity, infective larvae cultured at 30°C were kept at 5°C, 10°, 15°, 26° and 37°C. Sigmoid mortality curves were obtained for larvae at these temperatures (Fig 3:3) but larvae lived longest at 10°C (Fig 3:4). At 5°C, larvae died very rapidly - 59.6% had died after 2 weeks and all were dead within 6 weeks. Between 15-26°C, longevity of the larvae was not markedly affected, but at 37°C most of the larvae did not live beyond 8 weeks.

In another set of experiments, infective larvae cultured at 15°, 20°, 26°, 30° and 37°C were placed in storage at 26°C, after an outset lipid level had been measured from samples of larvae to be stored. The results (Fig 3:5) showed that there was no significant difference ($P = 0.01$) in the longevity of larvae cultured at different temperatures.

ii) pH

Longevity was measured between pH 2.0 - 11.8 using various buffers as indicated in Fig 3:6. Sodium acetate - acetic acid buffer, phosphate, and Tris - HCl buffers (Pearse 1968) provided pH values ranging from 2.0 - 9.1 while 0.05M Na_3PO_4 prepared from disodium hydrogen orthophosphate and sodium hydroxide produced a pH of 11.8. 0-day old infective larvae were placed in these media at 26°C, and the percentage of dead larvae estimated daily for the first week and thereafter at weekly intervals.

In very acid (pH 1.6) or very alkaline (pH = 11.8) media, all the larvae died with in 24 hours. Longevity was also reduced at pH 3.2 but remained relatively unchanged over pH 5.6 - 9.1 (Fig 3:6).

THE EFFECT OF TEMPERATURE ON THE LONGEVITY OF THIRD-STAGE LARVAE OF A.tubaeforme .

FIG. 3:3

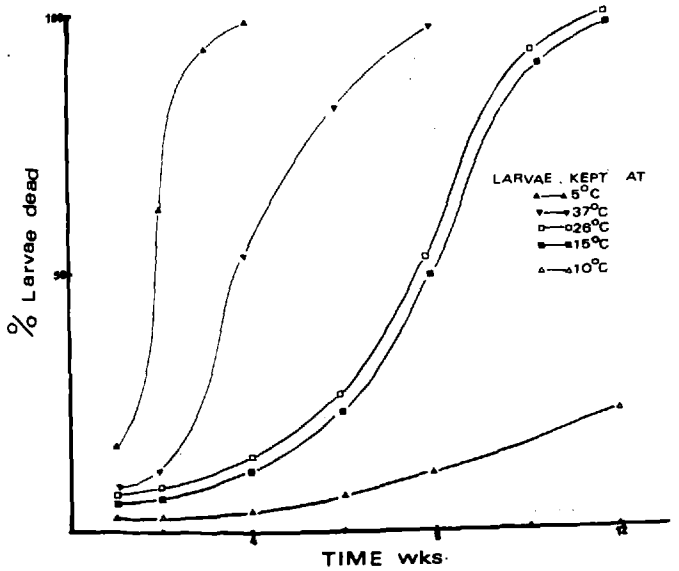


FIG. 3:4

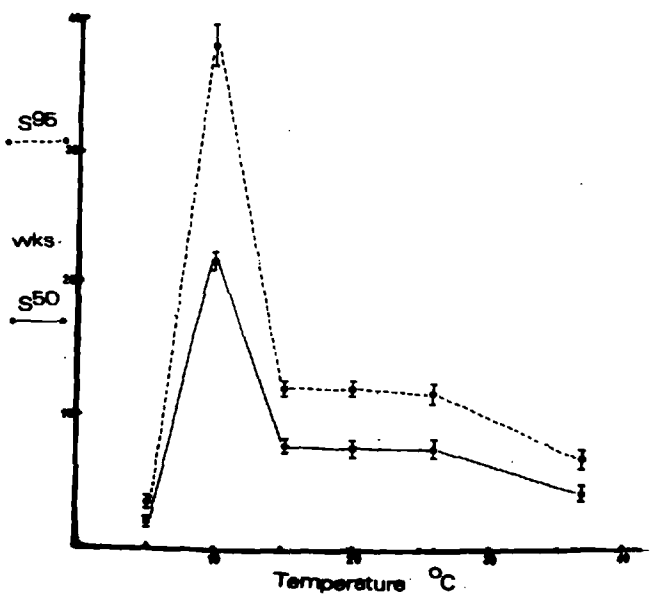


FIG. 3:5

THE LONGEVITY OF THIRD-STAGE LARVAE OF A.tubaeforme CULTURED AT DIFFERENT TEMPERATURES.

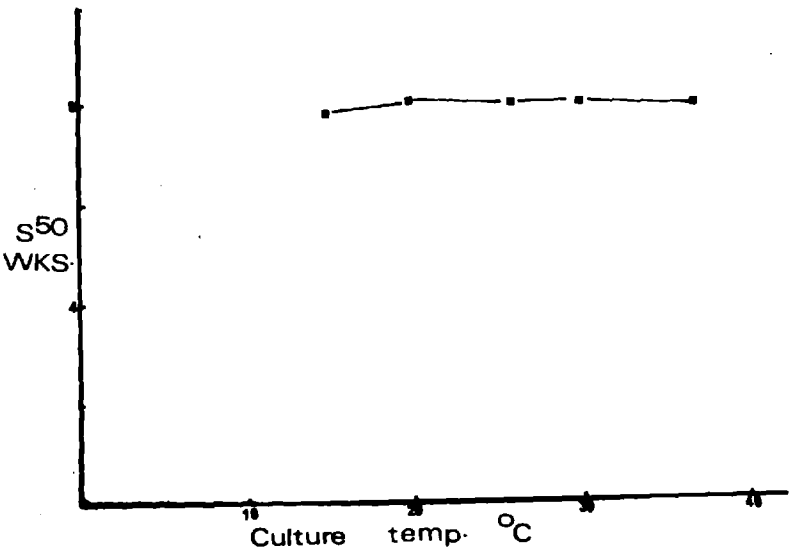
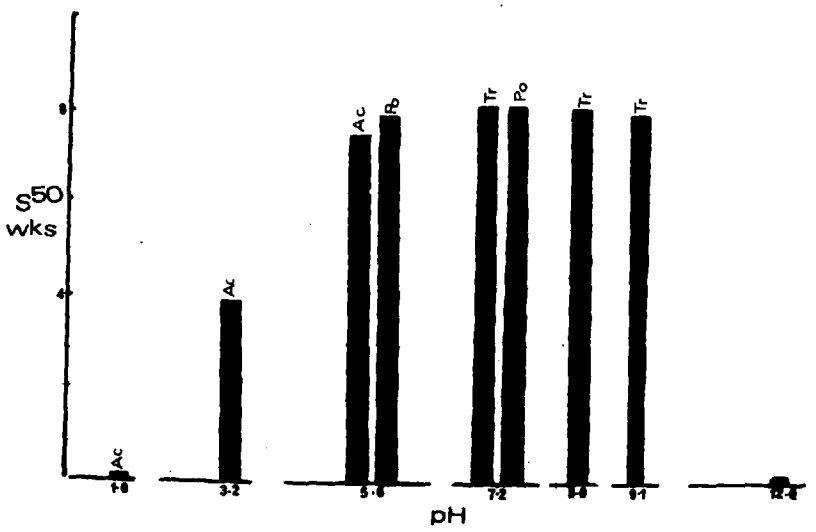


FIG. 3:6

THE EFFECT OF pH ON LARVAL ACTIVITY.



Changes in pH of the media during the course of the experiments are summarised in Table 17. Marked pH changes took place in very acid and very alkaline media, but the pH remained relatively stable between pH 5.6 - 9.1. The least changes were at pH 7.2.

TABLE 17

Changes in the pH of media used to "store" the infective larvae of A.tubaeforme at 26°C. (means of results of five experiments)

pH at the beginning	pH change After 1 week		After 8 weeks	
1.6	2.05	+0.45	-	-
3.2	3.12	-0.08	2.74	-0.46
5.6	5.6	0.00	5.48	-0.14
7.2	7.2	0.00	7.15	-0.05
8.0	8.0	0.00	7.92	-0.08
9.1	9.1	0.00	8.95	-0.15
11.8	10.17	-1.63	-	-

iii) Salinity

Saline media were prepared from the data for artificial sea water (Grimstone and Skaer 1972). Longevity was measured at 26°C in 25%, 50% and 75% dilutions as well as in 100% sea water. Larvae stored in deionised distilled water acted as the control in these experiments.

Larvae lived longest in 25% sea water and at this salinity, longevity was not significantly different from the control (Fig 3:7). Changes in size and activity of larvae took place in media exceeding 25% sea water with time. The larval activity rate in these media (compared with the control) was greatly

FIG. 3:7

THE RELATIONSHIP BETWEEN SALINITY AND LARVAL LONGEVITY.

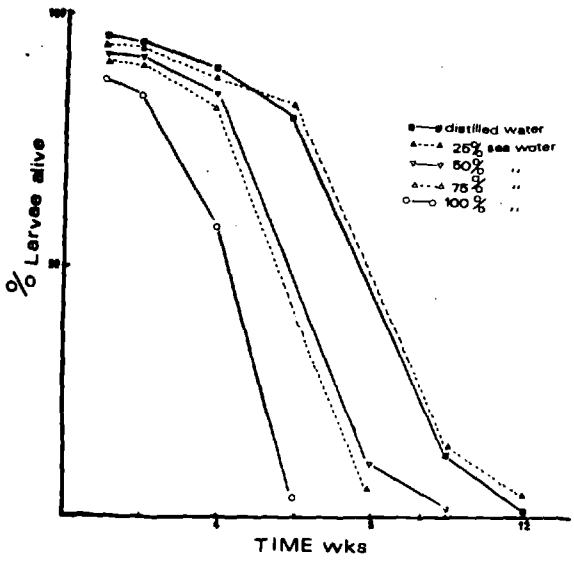
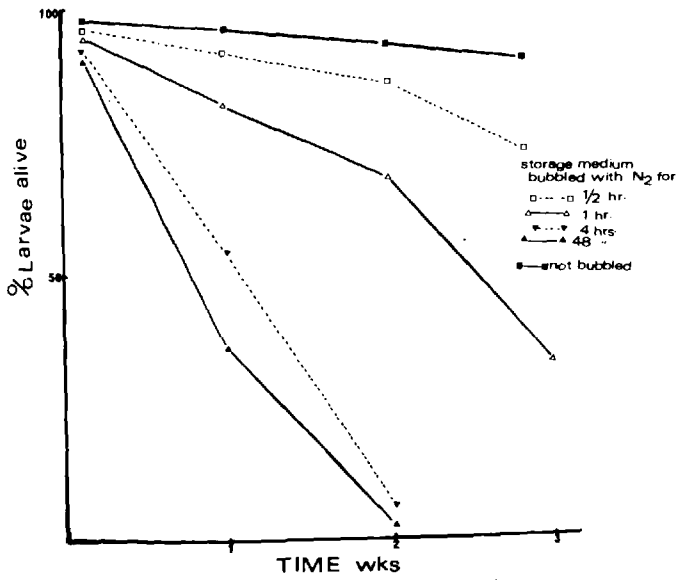


FIG. 3:8

THE RELATIONSHIP BETWEEN AERATION AND LARVAL LONGEVITY.



reduced, movement was sluggish and soon ceased altogether. Shrinkage also occurred often to such an extent that larvae were distinctly separated from their sheaths. These osmotic effects were observed at 100%, 75% and 50% sea water concentrations after 3,4 and 6 weeks respectively and most of the larvae died soon after the changes.

iv) Aeration

It was shown in Section II that larvae responded to mechanical disturbance such as was caused by bubbling air through the medium in which they were stored. In these experiments therefore microaerobic and anærobic enviroments were not maintained by continuously flushing the storage media with gaseous mixtures.

The method used to obtain anaerobic conditions was based on McCoy (1930). The storage medium (phosphate buffer pH 7.2) was flushed with commercially prepared nitrogen for 48 hours and then sealed from contact with air by a 1.5cm layer of oil and petroleum jelly. The storage vessel was sealed with a plastic stopper. To measure longevity at low oxygen tensions, the medium was flushed for 30 minutes, 1 hour and 4 hours and immediately sealed. A.tubaeforme eggs separated from faecal matter (100 eggs/chamber) and 0-day old infective larvae (900-1,000 larvae/chamber) were introduced into the microaerobic and anaerobic chambers, using sterile syringe needles. Since egg development was inhibited by very low oxygen tensions (McCoy 1930), the non-development of eggs in these media was used as an indication of their oxygen content.

Some of the larvae lived for up to 2 weeks under anaerobic conditions at 26°C but 64% of them had died after 7 days. Larvae survived microaerobic conditions for varying periods - the greater the flushing time with nitrogen, the less the longevity (Fig 3:8).

v) Light

Larvae were stored under continuous light or dark conditions at 26°C and the effect an alternating 12-hour light and 12-hour dark regimes was also investigated. Illumination was provided by cool white fluorescent lamps.

Longevity was apparently not affected by continuous light or dark conditions but was reduced by alternating light and dark periods (Table 18).

TABLE 17

The effect of light on the longevity of the infective larvae of A.tubaeforme at 26°C.

Longevity (range of results of five experiments)	Larvae under continuous illumination	Larvae under continuous darkness	Larvae under 12 hour light and 12 hour dark regimes
S50 (weeks)	7.12 - 7.85	7.05 - 7.92	7.08 - 7.80
S95 (weeks)	12.04 - 12.98	12.53 - 13.92	11.69 - 12.09

II The intrinsic factor

i) Larval 'outset' lipid

It was shown in Section I that the lipid content of infective larvae was dependent on temperature and the method of culture. In these experiments the longevity of infective larvae cultured on E.coli on agar, and on E.coli in phosphate buffer pH 7.2; in faeces, and faeces-sand cultures were also measured at 26°C after their lipid content (outset lipid) had been determined.

Fig 3:9 shows the relationship between longevity and larval 'outset' lipid level. Median longevity was similar for larvae from the various culture media but it appeared that maximum

longevity was slightly higher for larvae with more lipid.

iii) Lipid exhaustion and longevity

The time T (in weeks) at which the larval lipid reserves could no longer be detected by staining at each of the storage temperatures, was recorded. The rate of lipid utilisation by larvae at each temperature was obtained from 1/T. The rate was affected by temperature - lipid being utilised most rapidly at 37°C. There was an inverse relationship between longevity and the rate of lipid utilisation (Fig 3:10). Between 10°C - 37°C, longevity was reduced and lipid utilisation increased by an increase in temperature.

d) Longevity in faeces

Faecal cultures were set up at 26°C. After 84 hours, infective larvae were extracted from some of the cultures and placed in phosphate buffer pH 7.2 as the control. Most of the infective larvae in the cultures stored migrated out of the faecal matter onto water films and droplets bordering the cultures, and longevity was estimated by counting live and dead larvae. It was therefore very essential to keep these cultures moistened daily with a fine spray of water. These experiments were terminated after 10 weeks and the numbers of live and dead larvae counted in the cultures.

Table 19 shows the great variability in the longevity of larvae kept in faecal cultures. The mortality pattern of infective larvae stored in faeces did not appear to differ from the control but more larvae appeared to live longer in faeces than in aqueous media.

e) Desiccation-survival of infective larvae

In general techniques were similar to those described in Section I. Desiccation-survival was examined in leucocyte migration cell (Sterilin) at 26°C.

FIG. 3:9

THE RELATIONSHIP BETWEEN THE "OUTSET LIPD" AND THE LONGEVITY OF THIRD-STAGE LARVAE OF A.tubaeforme.

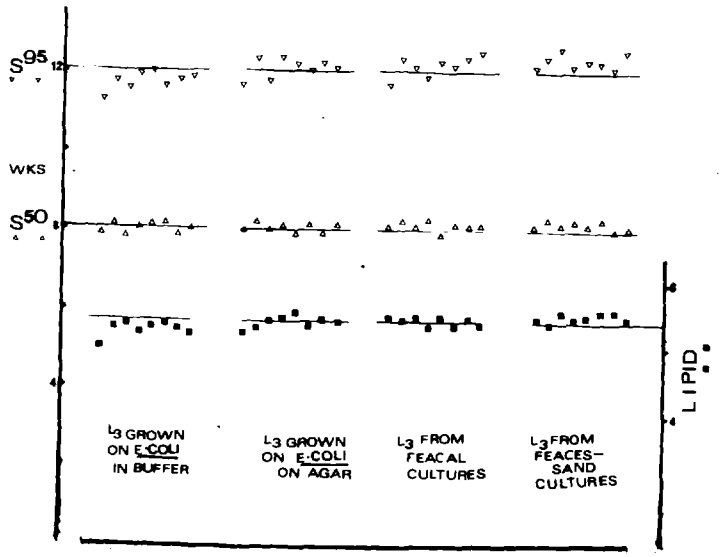


FIG.3:10

THE RELATIONSHIP BETWEEN THE RATE OF LIPID UTILISATION LARVAL LONGEVITY.

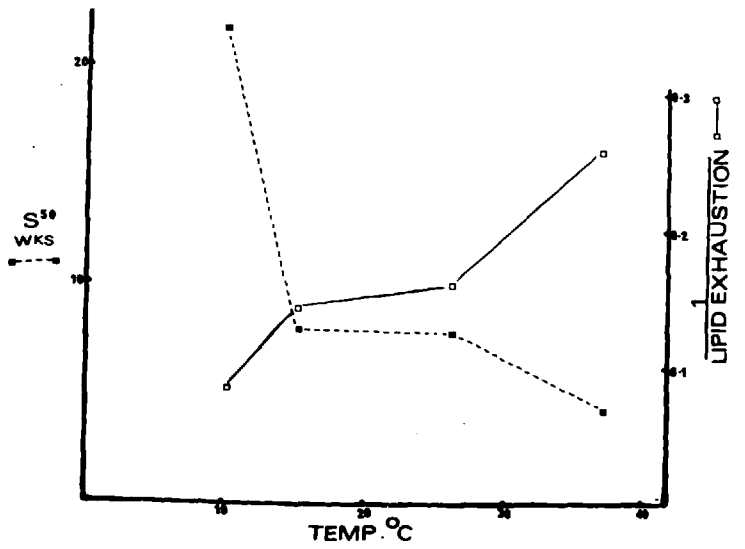


TABLE 19

Longevity of infective larvae of A. tubaeforme left in faeces and in phosphate buffer at 26°C.

	Percentage longevity (range of results of 5 experiments)	
Number of weeks	Live larvae in faecal cultures	Live larvae in phosphate buffer pH 7.2
1	86 - 100	95 - 98
2	78 - 92	90 - 98
4	70 - 85	85 - 92
6	62 - 76	76 - 89
8	45 - 58	42 - 56
12	9 - 26	0 - 13

In some of the experiments infective larvae were washed and placed in 1.0ml of 'sterile' distilled water within the test cells and transferred to humidity chambers (50% RH and 95% RH) set up using sulhpuric acid solutions (Solomon 1951). In others. infective larvae were not washed and these larvae were stored in 1.0ml of the water used to extract them from the cultures. To estimate the rate of water loss in both experiments, 1.0ml of the water but without the larvae were placed in the test cells and the time of drying recorded and used to correct for the survival times recorded for the larvae.

Sometimes all superficial water was removed under a stereo-microscope before larvae were placed in the humidity chambers. The ability of larvae artificially exsheathed (using 5% Milton as described) to survive desiccation was also measured.

Neither the ensheathed nor the exsheathed infective larvae survived extended periods of desiccation (Fig 3:11). It was also observed that the 'dirty' water in which the infective larvae were extracted took a longer time to dry out than 'clean' sterile water. A 'crusty' layer gradually formed as the dirty water dried and this slowed the rate of water loss.

f) Activity, penetration and longevity of larvae following periods of desiccation

The activity rate and penetration of larvae that recovered after periods of desiccation were measured. These larvae were subsequently stored and the maximum longevity recorded. Penetration was assayed after Matthews (1972b) using Whatman's No.2 filter paper and 50 larvae per test.

The results (Table 20) show that although larvae survived short periods of desiccation, their activity and ability to penetrate membranes was impaired and their longevity reduced.

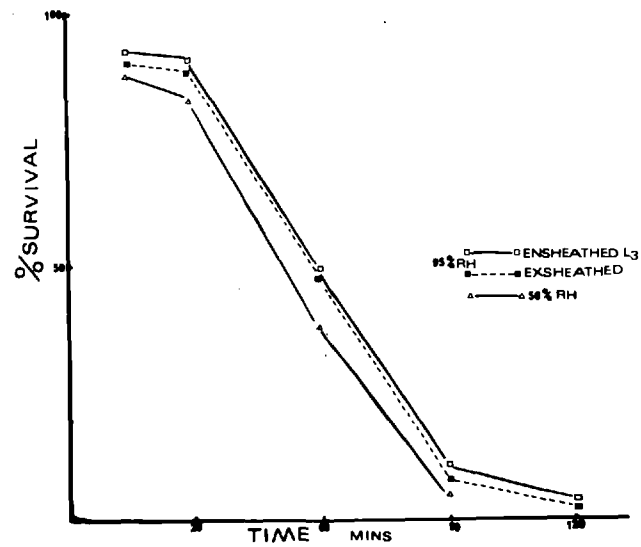
TABLE 20

The effect of desiccation-survival on the subsequent behaviour of infective larvae of A.tubaeforme. (means of the results of seven experiments.)

Period of exposure at 95% RH (mins)	% Survival	Maximum % of larvae active	Maximum activity rate (w/sec)	Penetration % of control	Longevity S50 (weeks)
15	92	85	2.98	86	7.12
30	88	86	2.41	73	6.34
60	47	52	1.74	6	3.96
90	6	-	-	-	1wk
120	1	-	-	-	-

FIG. 3:11

DESICCATION-SURVIVAL OF THIRD-STAGE LARVAE OF A.tubaeforme.



DISCUSSION

The longevity of infective larvae of Ancylostoma tubaeforme is influenced by both intrinsic and extrinsic factors. Among the latter, temperature is the most important. Larvae lived longest at 10°C - median longevity (S50) determined from experiments at this temperature exceeded 22 weeks. At higher temperatures, longevity was decreased by an increase in temperature (S50 at 15°C ≡ 7 weeks; S50 at 37°C ≡ 4 weeks), while at temperatures below that for maximum longevity, larvae died very rapidly (S50 at 5°C ≡ 2 weeks). Absence of the "sheath" did not diminish larval longevity.

These results agree in general with the findings of previous investigators who had shown the positive effect of temperature on the longevity of infective larvae. Augustine (1922) found that the loss of the "sheath" did not affect the longevity of human hookworm larvae. His observation that the longevity of the infective larvae of human hookworms in soils was about 6 weeks is supported by field studies carried out by Cort and Payne (1922), and Beaver (1953) who reported that hookworm contaminated soils became practically free of larvae after a few weeks.

Infective larvae which do not gain entry into the host by penetrating its skin such as those of trichostrongyles apparently live for a longer time (Monnig 1930; Prasad 1959; Belle 1959; Gupta 1961; Herlich and Ryan 1970). It has been pointed out (Rogers and Sommerville 1963) that most of the work on this subject is difficult to collate because standardised procedures were rarely used. For example, estimates of mortality which are dependent on the size of the sample and influenced by small chance variations, can be determined accurately only from probit analysis (Finney 1964) and the lack of use of probit had limited the value of previous studies.

In spite of such critisms, a fairly definite picture of the effect of temperature on longevity can be seen. Maximum longevity occurred at or just below the minimum temperature at which eggs of the parasite developed to the infective larvae. For example, the minimum temperature for development of T.colubriformis was 6.9°C (Levine and Andersen, 1973) while maximum longevity of the infective larvae was at 4°C (Andersen et al. 1966);and for S.ratti development did not take place below 15°C, the optimum temperature for larval 'survival' (Barrett 1968). Studies reported here show that infective larvae of A.tubaeforme lived longest at 10° - the minimum temperature for the free-living development of the parasite (see Section I).

Augustine (1922) and Barrett (1969) believed that longevity was reduced at high temperatures because larval metabolism was speeded up and consequently the lipid reserves were rapidly used up. Barrett (1968) had also suggested that the differences in longevity of infective larave grown at different temperatures could be attributed to the amount of lipid reserves accumulated at these temperatures. Longevity of the infective larvae of A.tubaeforme was affected by the "outset" lipid and more significantly by the rate at which it is utilised (Figs 3:9 and 3:10). As both parameters were also influenced by temperature, they might provide an explanation for the observed effects of temperature on longevity.

Larval metabolic rate and activity are likely to effect lipid utilisation. The rate of activity is believed by Barrett (1968) to be inversely related to longevity and Costello and Grollman (1958) had suggested that the greater the metabolic rate of infective larvae, the less their longevity. However, increase in activity with temperature is less likely to account for the observed reduction in longevity at high temperatures as larvae

stored under constant conditions that prevailed in these experiments have been shown to be ralatively inactive (see Section II). Changes in metabolic rate on the other hand are indicative of total enzyme acitivity and these changes in the case of infective larvae, would be reflected in their physiology and consequently the rate at which they utilised lipid. Increased metabolic rate at high temperatures rather than increased activity is therefore more likely to explain the reduced longevity observed at these temperatures.

Infective larvae of A.tubaeforme did not survive freezing. At these low temperatures mortality could be due not only to the metabolic rate falling below the level necessary to maintain life but also cellular damage resulting for example from the formation of intra-cellular ice crystals (von Brand 1960).

The other physical factors investigated did not greatly affect longevity. Von Brand and Simpson (1944) stated that larvae of Eustrongylides ignotus shifted solutions that were 'strongly' acid or alkaline in the direction of the pH occurring in their natural habitat. Such pH shifts do not appear to take place with infective larvae of A.tubaeforme and the pH changes reported here (Table 17) indicated that they were caused either by the decomposition of dead larvae or by instability of the buffer solutions at 26°C. The infective larvae of hookworms are free-living while those of E.ignotus exist encysted in their intermediate host (several species of fish) and these differences in their ability to 'regulate' the pH of their microenvironment might be related to differences in their natural habitats.

Light did not significantly affect larval longevity. Infective larvae are photokinetic, but when activated by light after dark adaption, the percentage of larvae active and the activity rate rose rapidly to a maximum and then gradually

subsided (Odei 1968, Croll and Al-Hadithi 1972). Changes in illumination would therefore only very slightly increase total larval activity and consequently have little (if any) effect on longevity.

In media of moderate salinity larvae lived as long as in distilled water. The ability of infective larvae to osmoregulate has been demonstrated using larvae of N.brasiliensis and Necator americanus (Weinstein 1952). Subsequent studies by Croll (1972a), Croll, Slater and Smith (1972) showed that infective larvae of A.tubaeforme were isotonic with 1.5% NaCl and that osmorequlation did not take place in hypertonic media. The inability to osmoregulate at high osmosities (50% sea water is equivalent to 1.614% NaCl (Grimstone and Skaer 1972)), and the consequent gradual impairment of metabolism would result in reduced longevity.

Infective larvae have an essentially aerobic metabolism and require an abundant supply of oxygen for an efficient utilisation of their lipid reserves. They exist under relatively high oxygen tensions (Stolzy and van Grundy 1968) and generally contain more of lipid than glycogen (Lee 1965, Saz 1969). Infective larvae of A.tubaeforme however have shown a marked tolerance to lack of oxygen (Fig 3:8). Chowdhury et al. (1958) believed that infective larvae of A.duodenale and N.americanus utilised carbohydrates when their oxygen supply became inadequate and Cooper and van Gundy (1970) found that the myceliophagous nematode Aphelenchus avenae catabolised neutral lipids at oxygen concentrations exceeding 5% and glycogen below this. It is therefore possible that non-lipid substrates such as glycogen were being utilised by larvae under microaerobic and anerobic oxygen stress and that low amounts of glycogen (Barrett 1969) limited the extent of their survival.

The ability to withstand desiccation differs considerably between infective larvae of different nematode parasites.

However, it does seem as if desiccation-survival by many actively penetrating larvae is much less than for those with a *per os* infection route. Thus infective larvae of *A.tubaeforme* like those of *A.duodenale* (Looss 1911) and *B.trigonocephalum* (Belle 1959) but unlike those of *T.colubriformis* (Andersen and Levine 1968), *T.retortaeformis* (Prasad 1959) and *H.contortus* (Ellenby 1968) did not survive extended periods of drying. This generalisation may only be partly true as Goodey (1924) had shown that infective larvae of *Oesophagostomum dentatum* could survive considerable periods of drying. For those infective larvae that survived desiccation, the 'sheath' played a significant role in slowing the rate of water loss (Ellenby 1969).

Taylor (1938) has argued that the decrease of larval populations in contaminated soils was not dependent on the "natural life-span" of infective larvae. The mortality pattern of infective larvae (see Fig 3:1) would suggest that contamination of a field by infective larvae would remain for a long time at a high level before coming to a sudden and rapid decline. However Taylor reported a high mortality rate during the first few weeks and it was only a small proportion of the larvae that remained alive for any considerable period. He also observed that those larvae which climbed on to exposed parts of grass had a much higher mortality rate than those remaining in the moister micro-climate on the lower leaves and on the stems near to the ground. Taking into consideration data on the survival of adverse factors especially desiccation, Taylor's observations would be expected. It is worthwhile to note that actively penetrating larvae (such as those of *A.tubaeforme*) generally do not climb onto vegetation but stay in close association with the soil and as the surface soil became dry, larvae migrated into the moist subsoil (Spindler 1936). Such a behavioural response on the formation of a 'crusty'

layer which slowed water loss from their microenvironment would protect these larvae from drying.

S E C T I O N IV

THE EFFECT OF AGEING ON THE PHYSIOLOGY AND METABOLISM OF INFECTIVE LARVAE.

INTRODUCTION

The primary function of infective larvae is to infect a potential host when the opportunity presents itself. They are a non-feeding stage but stay alive for long periods utilising the lipid reserves synthesised by the preinfective stages. Infectivity is lost with time at a rate which is dependent on such environmental factors as temperature, but apparently a close relationship does not exist between longevity and infectivity (Haley and Clifford 1960; Cornwell and Jones 1970; Oishi and Hiraoki 1973). A recent and perhaps the most precise study on the relationship between the infective larvae and and their ability to infect a host was that of Lengy (1973), who while examining the infectivity of larvae of S.ratti to albino rats, found that there was no correlation between their longevity and infectivity.

Changes in activity and lipid content of larvae with time have been measured and associated with infectivity (Payne 1923, Giovanniola 1936, Rogers 1939, 1940b, Clark 1969). Rogers (1940b, believed that infective larvae which had much lipid and were active would be highly 'infective'. Jaskoski (1960) showed that larvae hatching from 'old' ascarid eggs had reduced lipid reserves and that the infectivity of these eggs was related to their 'age'. More recently however, Croll and Matthews (1973) argued that the loss of infectivity was related not to lipid levels but to decreased metabolic rates and emphasised three features of the ageing of infective hookworm larvae namely - lipid; metabolic (enzymatic); and behavioural changes.

The respiratory rate of infective larvae decreases rapidly with time (Rogers 1948; Schwabe 1956; Barrett 1969; Bryant 1973), but very little is known of the changes which take place in enzyme activity as infective larvae of parasitic nematodes 'age'. In this study ageing is regarded as time-dependent changes in the infective larvae and these have been investigated for A.tubaeforme

by measuring behavioural activity, respiratory rate, and enzyme activity (non-specific esterase and acetylcholinesterase), of larvae kept at different temperatures for varying periods of time.

1) Obtaining large numbers of infective larvae

Large numbers of infective larvae (about 25,000 larvae/measurement) were required for respiration and enzyme activity studies. The procedure used to obtain infective larvae in sufficient numbers for measurements was based on Wilson and Dick (1964). Four kittens were infected per os using about 500 '0-day old' infective larvae, and their faeces collected and cultured between 5-10 weeks post infection when egg output of worms was highest (Matthews and Croll, 1973). Faeces of all the infected animals collected over the consecutive days provided sufficient material for 200-250 cultures in 9cm Petri dishes as already described. Larvae were isolated in Baermann funnels. It was found that yield of larvae was diminished with the use of large funnels. Eleven funnels not exceeding 15cm in diameter were therefore used and sieves containing the cultures were left to stand for about 5 minutes in a cold water bath (about 5°C) before they were transferred to the funnels containing distilled water at about 37°C. Extracts were removed after 12 hours and another change of distilled water at 37°C used to obtain maximal numbers of infective larvae. The larvae were washed and 'surface sterilised' before use. Total yields of larvae by this procedure from sets of cultures averaged 10^5.

Larvae obtained in this way, were of course not axenic but microbial contamination was checked by the use of the antibiotics and possibly by the absence of suitable substrates because inoculating samples of the storage medium onto nutrient agar and incubating for 48 hours at 37°C usually gave negative results.

2) Worm counts

It was of extreme importance that the numbers of infective larvae used for measurement on respiration and enzyme activity be accurately estimated. Larvae were counted by the dilution method

described by Keeling (1960). Three 50ul samples taken from a suspension of larvae in 10ml of distilled water were placed in a graduated dish (5mm squares) and counted under a low power binocular microscope. Usually it was necessary to divide 50ul samples into several subsamples in order to obtain accurate counts. If counts of the three samples varied by more than 10%, extra samples were counted until three within this range were obtained. The average count of the three 50ul samples multiplied by 200 gave the estimated number of larvae in suspension.

c) The determination of weights of larvae

The wet weight of larvae was determined using the formula of Andrassy (1956). The formula is

$$\underline{G} = \frac{a^2 \times b}{16 \times 100{,}000} \text{ ug}$$

where G is the wet weight, a the greatest body width, b the body length and 16 an empirical value determined by Andrassy.

Some attempt was also made to determine the dry weights of larvae. Suspensions of infective larvae were placed on weighed aluminium foil pans after worm counts had been made, and dried in a $CaCl_2$ desiccator at room temperature for 48 hours. After this period, they were weighed on a Beckman LM-500 microbalance observing the precautions advised by Doohan and Rainbow (1971).

RESULTS

A) Penetration and activity

The apparatus desbribed by Matthews (1972b) was used to measure penetration. About 50 infective larvae were used per test and the number that penetrated through Whatman's No.2 filter paper was counted. The maximum activity rate (w/sec) following mechanical agitation was also measured after larvae had been left for about 4 hours to attain an equilibrium with the test temperature.

Penetrability decreased very rapidly with time (Fig 4:1). By the second week, percentage penetration had fallen to less than 50% of the maximum and within 6 weeks most of the larvae, no matter at what temperature they were stored, could no longer penetrate membranes.

The percentage of larvae active and the activity rate also decreased with time (Fig 4:2). Larvae stored at $5^{o}C$ lost activity faster that those stored at the other temperatures (Fig 4:3). For larvae stored between 10^{o}-$37^{o}C$, decrease in activity occurred at somewhat similar rates but it would appear that the decrease became much more in larvae stored at higher temperatures after 4 weeks.

B) Lipid changes

The amounts of larval lipid reserves also decreased with time at a rate greatly dependent on the storage temperature (Fig 4:4). Larvae stored at $5^{o}C$ had the greatest amounts of lipid although these larvae lost activity more rapidly than those at the other temperatures (see Fig 4:3) and previous studies had shown that they also died rapidly (Figs 3:3 and 3:4 Sec. III).

Decrease in the size and numbers of the lipid globules was not localised as when larvae were subjected to intense activity (see Fig 2:9, Sec.III), but occurred evenly along the entire intestine. Dark granules appeared in larvae which had used up all

FIG.4:1

THE EFFECT OF STORAGE ON THE PENETRABILITY OF THIRD-STAGE LARVAE OF A.tubaeforme.

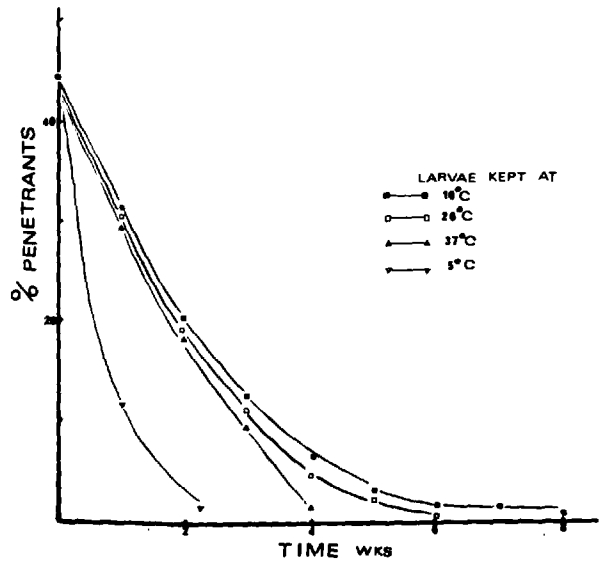


FIG. 4:2

THE EFFECT OF STORAGE ON THE ACTIVITY OF THIRD-STAGE LARVAE OF A.tubaeforme.

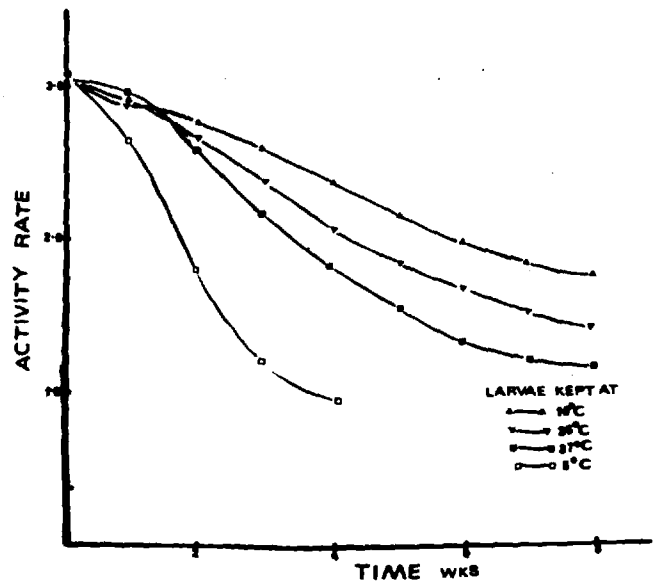


FIG.4:3

CHANGES IN THE ACTIVITY OF THIRD-STAGE LARVAE OF A.tubaeforme KEPT AT DIFFERENT TEMPERATURES.

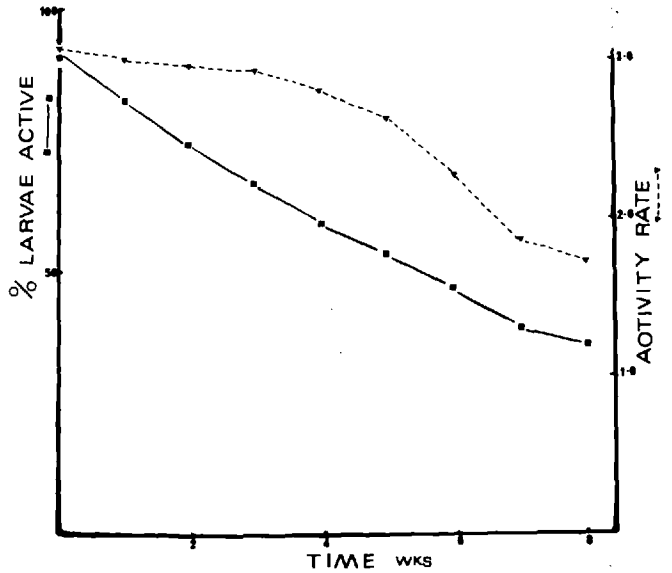
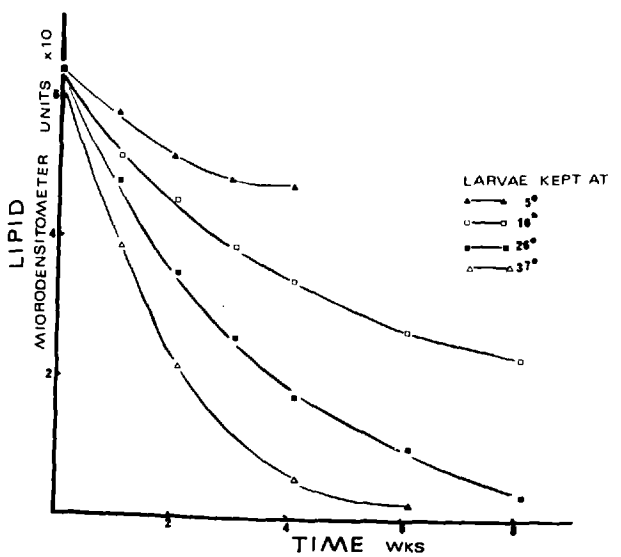


FIG.4:4

CHANGES IN THE LIPID CONTENT OF THIRD-STAGE LARVAE OF A.tubaeforme KEPT AT DIFFERENT TEMPERATURES.



their lipid. When such larvae were stained with Oil Red O and the absorption spectrum measured on the Shimadzu MPs 50L spectrophotometer, peak absorbance was found to lie beyond 600nm- well outside the peak absorbance for lipids (Fig 4:5).

c) Respiration

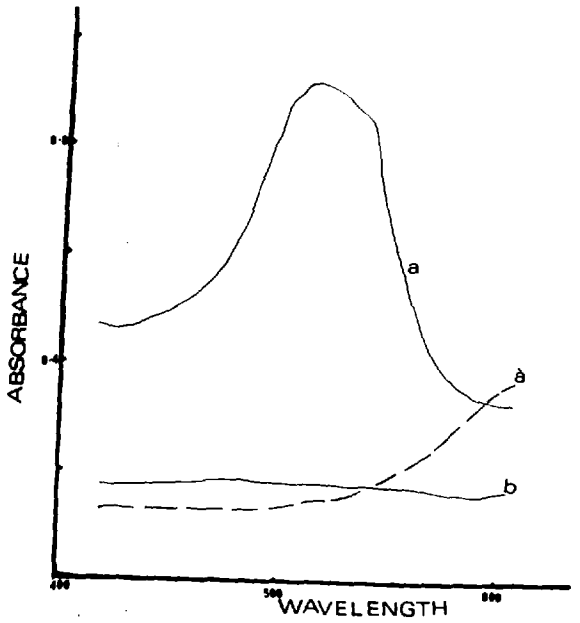
Respiratory rates (QO_2) were measured at 26 $\pm$ 0.5°C using a "Clark-type" membrane-coated oxygen electrode (Rank Bros. Ltd) in an apparatus described by Estabrook (1964). A magnetic stirrer and a small glass-encased iron rod ensured continual mixing of the raction medium thus facilitating the establishment of an equilibrium oxygen dissolved in the solution and the gas diffusing through the thin 'teflon' membrane of the electrode. The apparatus was standardised before each use with air-saturated distilled water and the oxygen content of the water calculated using appropriate data from the "Handbook of Chemistry and Physics".

A suspension of 25,000 - 30,000 infective larvae in 2mls of air-saturated distilled water was used in each measurement. A polarising voltage of 0.6V was applied to the electrode and oxygen utilisation recorded on a chart, the rate at which the larvae utilised oxygen being given by the gradient of the recorded line. Dry weight of the larvae was calculated from worm counts as it had been previously found that there were about 38,000 infective larvae/mg dry weight.

'0-day' old infective larvae had a high QO_2 12.48 $\pm$ 0.81ulO_2/-mg dry wt./hr., at 26°C (n = 5), but this decreased very rapidly with time (Table 21). After one week and two weeks of storage at 26°C the respiratory rate had dropped by 36% and 59% respectively. There was no significant difference in the respiratory rates of larvae stored at 37°, 26° and 10°C (Fig 4:6).

FIG. 4:5

THE ABSORPTION SPECTRA OF "0-DAY"OLD AND 7-WEEK OLD THIRD-STAGE OF A.tubaeforme WHEN STAINED IN OIL RED O.



Stained "0-day" old L3

Background absorbance

Stained 7-week old L3

FIG.4:6

CHANGES IN THE RESPIRATORY RATE OF THIRD-STAGE LARVAE OF A.tubaeforme KEPT AT DIFFERENT TEMPERATURES.

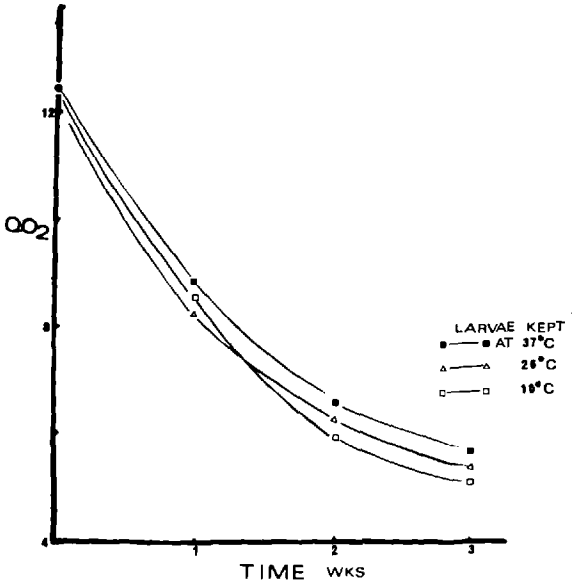


TABLE 21

The effect of ageing on the $\underline{QO_2}$ of the infective larvae of A. tubaeforme. (means ± SE of the results of five experiments)

Age of larvae in weeks	$\underline{QO_2}$ (ul02/mg dry wt 1hr)
0	12.48 ± 0.81
1	7.99 ± 0.74
2	5.17 ± 0.56
3	4.80 ± 0.51
4	3.72 ± 0.45

4) Esterase and acetylcholinesterase activities

Non-specific esterase and acetylcholinesterase activities were measured after Ellman (1961). Essentially, the method measures the rate of production of thiol as the substrate (for example acetylthiocholine iodide) is hydrolised by the enzyme (acetylcholinesterase). The thiol as it is produced reacts with 5:5 dithiobis-2-nitrobenzoate ion forming yellow anion and the rate of colour production is measured spectrophotometrically at 412nm. These rates are subsequently converted to absolute units (u moles of substrate hydrolysed/gram wet weight of tissue/hr) using the extinction coefficient of the yellow ion.

The substrates - 0.075m acetylthiocholine iodide (ATChI) or butyrylthiocholine iodide (BTChI) and the reagent 0.01M 5:5 dithiobis-2-nitrobenzoic acid (DTNB) were prepared as described by Ellman. Thiophenyl acetate (TPhA-Polysciences Inc.) used to measure non-specific esterase activity was obtained from Phase Separations Ltd, Flintshire and made up in methanol to 0.15M.

The numbers of larvae per 2ml of homogenate necessary for satisfactory readings ranged from 2.0-2.8 x 10^4. Samples of approximately 2.5 x 10^4 infective larvae were homogenised in 2mls of ice cold phosphate buffer (pH 8.0) using an MSE sonic disintegrator and assays were carried out on the 'crude' homogenates. In a typical run for acetylcholinesterase 100ul DTNB; 3.0ml buffer pH 8.0; 100ul homogenate (enzyme); and 100ul acetylthiocholine iodide were added in that order while the 'blank' consisted of DTNB, buffer and the substrate. In the esterase assay 50ul of the substrate TPhA was used. The change in absorbance at 412nm was measured at 26 $\pm$ 0.5°C with the Beckman DB (GT) spectrophotometer.

Butyrylthiocholine iodide (BTChI) was not hydrolysed by larval homogenates and ATChI hydrolysis ceased in the presence of 10^{-4} eserine (Table 22).

TABLE 22

(explanation in text)

Number of larvae	Substrate hydrolysis (absorbance/min)			
	ATChI	ATChI + eserine	BTChI	Control (no enzyme)
26,400	0.116	0.004	0.005	0.004

Non-specific esterase and acetylcholinesterase activities decreased with time, with ACHE activity decreasing at a faster rate (Table 23). Decrease in ACHE activity was gradual during the first four weeks of storage (at 26°C) but thereafter decreased more rapidly (Fig 4:7).

FIG. 4:7 THE EFFECT OF STORAGE ON CHOLINESTERASE ACTIVITY OF THIRD-STAGE LARVAE OF A.tubaeforme.

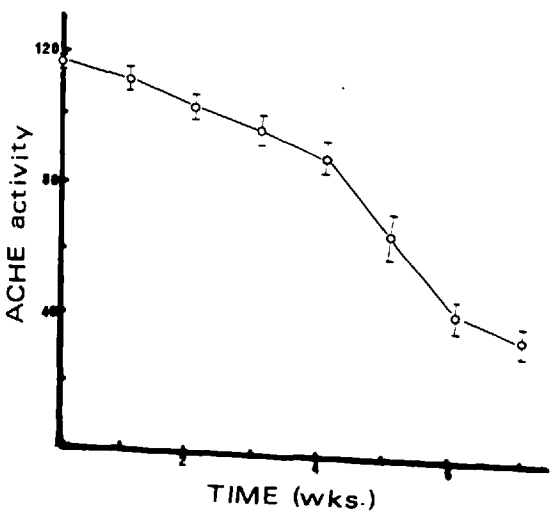


TABLE 23

Non-specific esterase and acetylcholinesterase activities of homogenates of infective larvae of A.tubaeforme 'stored' at 10°C for different periods. (means of results of nine experiments)

Age of infective larvae (in weeks)	Esterase activity	Acetylcholinesterase activity (u moles of substrate/gram wet wt. tissue / hr.)	ACHE activity as % of esterase activity
0	151.72	117.18	77
14	142.52	104.83	74
28	126.98	90.14	71
56	63.51	39.36	62

DISCUSSION

The present results support earlier observations that infectivity of the infective larvae and eggs of zooparasitic nematodes decreases with time (Payne 1923a, Rogers 1939, 1940b, Haley and Clifford 1960; Barrett 1969; Croll and Matthews 1973; Stromberg and Crites 1974). Although it is difficult to compare the present results quantitatively with those of previous studies as different methods had been used to measure 'infectivity', it is apparent that the rate of decrease depends on the storage conditions (especially temperature and differs between the various species. Thus the reported decrease in infectivity of the infective larvae of N.brasiliensis (Haley and Clifford 1960), N.americanus (Nagahana et al.1965) and possibly Anisakis (Oishi and Hiraoki 1973) where infectivity remained almost constant over a period before it gradually decreased differs from the exponential decrease observed for A.caninum(Rogers 1939a), H.contortus (Rogers 1940) S.ratti (Barrett 1969), and Dictyocaulus viviparus (Cornwell and Jones 1970). Stromberg and Crites (1974) investigating infectivity of the infective first-stage (not third-stage) larvae of Camallanus oxycephalus found that it also decreased very rapidly with time but the decrease in infectivity of Ascaridia galli where the infective agent in the embryonated egg not a larval stage presented yet a different pattern as infectivity of the infective eggs remained almost constant for a long period before coming to a sudden and rapid decline.

The infectivity pattern may be related to the host-contact and invasion mechanisms in the life-cycle. Thus the infectivity pattern of infective larvae would be expected to differ from that of infective eggs since larvae, unlike eggs, are dispersal stages and can also by their behaviour accelerate the process of contact with potential hosts. Slight differences are also to be expected between those larvae which use histolytic enzymes to penetrate

the hosts skin (eg. N.americanus - Matthews 1975) and those where penetration is largely a mechanical process (eg. A.tubaeforme - Matthews 1972b, 1975). But the best illustration of how infectivity and activity may be related to host-contact is seen in C.oxycephalus. Stromberg and Crites (1974) showed a very high correlation between the activity of the infective first-stage larvae and their ability to penetrate the copepod intermediate host (infectivity). These larvae unlike infective third-stage larvae (Croll 1970) had high levels of endogenous activity and Stromberg and Crites argued that activity of the larvae increased not only the probability of their making contact with the copepod intermediate host but also ensured that mostly young and highly infective larvae did so.

The lipid content of infective larvae of several nematode parasites has been widely used as an estimate of their 'physiological age' and consequently infectivity (Payne 1923, Cort 1925, Giovanniola 1936, Rogers 1939, 1940b, Elliott 1954, Clark 1969). The lipid content of infective larvae of A.tubaeforme decreased with time (Fig 4:4). It has also been shown that although the rate of decrease of lipid was slowed down at lower temperatures (Fig 4:4) penetrability ('infectivity') (Fig 4:1) fell rapidly so that by the 6th week when larvae stored at 10°C still contained about 40% of the outset lipid, less than 10% of them could penetrate membranes indicating that penetrability was not directly related to lipid levels.

The larval respiratory rate (QO_2) has been correlated with lipid utilisation and longevity. Costello and Grollman (1958) observed that infective larvae with a high QO_2 also had low longevity and argued thatlarval respiratory rate was inversely related to longevity. The rapid decline in the respiratory rates of infective larvae of many nematode parasites with time (Rogers 1948; Schwabe 1957; Fernando 1963; Barrett 1969; Bryant 1973)

was therefore considered by Barrett (1969) to be an adaption to conserve lipid thereby extending longevity. He calculated that if the QO_2 of S.ratti were not reduced but remained at the high level recorded for freshly-isolated ('0-day' old) infective larvae, their lipid reserves would be exhausted within 3 days ! Croll and Matthews (1973) had earlier observed that the rate of decline in behavioural activity of infective larvae of A.tubaeforme was apparently independent of lipid and believed that the mechanisms involved in this decline had 'aged' with time. They also thought that these mechanisms were either those governing the mobilisation of energy or controlling behavioural activity itself. Decrease in non-specific esterase and acetylcholinesterase activities of the infective larvae of A.tubaeforme with time have been shown in these studies. Nematodes are believed to have cholinergic nerves (Debell 1965; Lee 1965) although Castillo, Mello and Morales(1959) have suggested that in Ascaris acetylcholine acted as a modulatory (accelerating) neurohormone rather than as a transmitter of nervous impulse. Nachmansohn (1959) associated acetylcholinesterase activity with neuromuscular function. It is therefore tempting to suggest that decrease in behavioural activity (and infectivity) of infective larvae with time might be directly related to decrease in acetylcholinesterase activity. But this would be an oversimplification.

The work of Gershon and his colleagues (Gershon 1970; Erlanger and Gershon 1970, Gershon and Gershon 1970; Zeelon, Gershon and Gershon 1973) has shown that decrease in the activities of several enzymes characterised the ageing of nematodes. Van Gundy et al. (1967) using histochemical techniques also showed that non-specific esterase and acid-phosphatase, both of which function in the hydrolysis of lipids and phospholipids, had lower activity in 'aged' larvae of the phytophagous nematode Meloidogyne javanica. Mechanisms controlling behavioural activity may then involve not

either but both those enzymes controlling energy metabolism and neuromuscular function. These mechanisms may infact be closely related. For example, activity is an energy-expending process (see Section II) and those larvae which had completely exhausted their lipid would then show very little if any activity. On the other hand, the activity of infective larvae containing lipid may differ considerably and for such larvae their 'physiological age' would be reflected by their activity. To estimate the ability of a sample of infective larvae to infect a potential host ('physiological age'), 'population activity' (percentage of larvae active in the sample) and activity rate would therefore be the important factors and lipid level below a critical threshold, secondary.

GENERAL DISCUSSION

The third-stage larva forms the "bridge" between the free-living phase and the parasitic phase in the life-cycle of many parasitic nematodes. It is consequently highly specialised and adapted not only to survive two dissimilar environments (within and outside the host), but also to infect a potential host. Results obtained from this study give some indications of how this larval stage of Ancylostoma tubaeforme is suited to its biological role.

The ecology of infective larvae is governed by weather conditions, and of these moisture is likely to be of primary importance. Embryonation of the eggs and larval development took place only in aqueous media. The developing stages and infective larvae were rapidly killed on drying (see Sections I and III) in contrast to the trichostrongyles where the embryonated eggs and infective larvae could withstand considerable periods of desiccation. Dry conditions will therefore have the effect of reducing the numbers of infective larvae of this parasite to be found in contaminated soils by increasing larval mortality and also by arresting development of the eggs. However the behaviour of cats which (unlike grazing animals) tend to bury their faeces, and that of the infective larvae which do not climb onto hergage would compensate for the inability to withstand desiccation by ensuring that the free-living stages stayed in the more moist microenvironment of the soil.

Temperature is also a major factor in the ecology of infective larvae. Eggs developed to the third-stage larvae only between 15°-37°C, and using the "development index" (see Section I) it was shown that most infective larvae would be present in contaminated soils at the higher temperatures within this range. Larval longevity on the other hand was reduced at high temperatures (Fig 3:4).

However because the effect of increased the rate of development exceeded that of decreased longevity, the net effect of high temperatures (20^{o}-37^{o}C) would then be to increase the numbers of young infective larvae within the habitat.

The physiology of the infective larva is greatly influenced by the lipid reserves. As the larva does not feed it must survive on the lipid (energy) reserves synthesised by the bacteriophagous preinfective larval stages. Larval longevity was dependent not only on the level of the lipid reserves (Fig 3:9) but also on the rate at which it was utilised (Fig 3:10). Thus, the problem for these larvae would be how to avoid 'wasteful' use of their lipid reserves before they have made contact with the hosts. The subsequent development into an adult hookworm following a successful invasion is also likely to require some lipid for the development of features like the buccal capsule before feeding may be resumed.

The rate at which lipid was utilised was related to the larval metabolic rate and was influenced by temperature and larval activity. There was a rapid decline in the respiratory rate with time (Fig 4:6). The effect of such a decrease, also reported for third-stage larvae of other parasitic nematodes (Costello and Grollman 1958, Barrett 1969, Bryant 1973) would be to slow down the rate of depletion of lipid.

Quantitative data have been presented to show that larval activity resulted in considerable lipid expenditure (see Section II). This supports earlier observations by Payne (1923b) and Croll (1972a) that larval activity was a major energy-expending process of infective larvae. Under conditions of excessive activity therefore, longevity would be reduced through the rapid use of lipid. The activity regime provides a mechanism by which the infective larva maximises the chance of invasion should the

host present itself as well as ensuring that activity was not prolonged if contact with a host has not been made. A detailed analysis of the regime showed that on stimulation, the infective larva was immediately very active (phase 'a', Fig 2:2). If the activation has been caused by the presence of a potential host, due for example to mechanical disturbance in the vicinity of the larvae by the hosts foot, this rapid increase in activity might enhance the chances of successful penetration. If however larvae failed to make contact with the host, they soon passed into lower frequency movements including reversals (phase 'b', Fig 2:2), until movement was gradually arrested by the formation of the 'kink'. There was also a refractory period of 15-20 minutes following the cessation of activity during which third-stage larvae did not respond to stimuli. This restricted the number of times larvae could be activated, and together with the regime which limited the extent of activity following stimulation would conserve lipid.

Larval activity has also been considered an important indicator of the ability of infective larvae to invade potential hosts (Croll and Matthews, 1973). The rapid decrease in penetrability with time (Fig 4:1) suggested that penetration was achieved by larvae with a high activity rate. Since infective larvae of A. tubaeforme do not apparently use histolytic enzymes to penetrate the hosts skin (Matthews 1972a, 1975; Smith 1975), it seems reasonable to expect that only those larvae which could generate sufficient propulsive forces would invade. A high activity threshold would therefore be essential for a successful invasion. This threshold has not been determined in this study but was presumed to involve all the phases of the activity regime. Penetrability would be expected to be lost by those larvae where phase 'a' of the activity regime has been lost. It is also

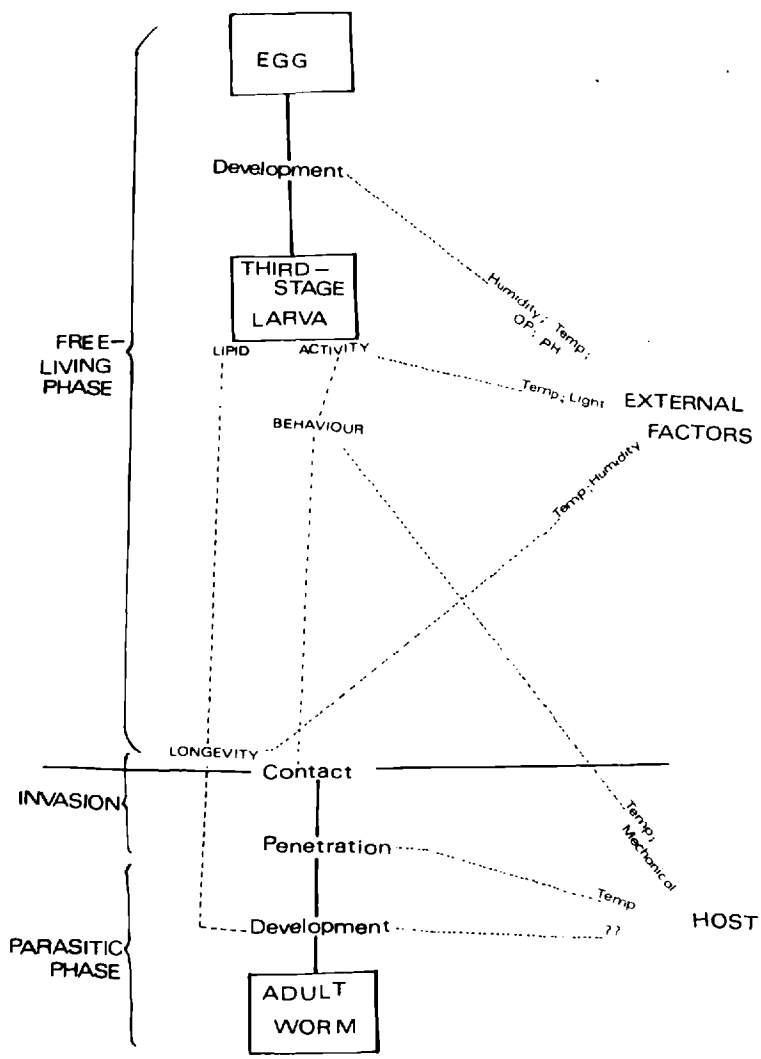
obvious that only motile larvae within a population would penetrate, so that the percentage of larvae active within the population ('population activity') would be related to invasion. Fig 4:2 showed that the population activity and activity rate both decreased with time. However either of these activity measurements alone may not completely describe the relationship between activity and penetration. It is proposed that the product of population activity and the threshold rate of activity essential to achieve penetration might prove to be a more accurate estimate of the capacity of a sample of infective larvae to invade potential hosts. An early contact with a potential host is essential for a successful invasion since penetrability is lost very rapidly (Fig 4:1). The process of contact is affected by larval behaviour. (Rogers 1962; Rogers and Sommerville 1963; Croll 1972d; Kennedy 1975). Croll and Smith (1972) believed that heat gradients occurring in and near the skin were of an order sufficient to activate larvae and ensure penetration. It may therefore be significant that the activity of infective larvae following a mechanical stimulus was highest at 30 $\pm$ 2°C (Fig 2:8a), a temperature found to be the 'surface temperature' (beneath fur, between foot-pads) of cats (Matthews 1972b).

Taking all the factors which influence the ecology, behaviour and physiology of infective larvae into consideration, a model, mainly speculative, has been made to show how they may influence infectivity (Fig 5:1). There are three main features of this model.

1. It shows the complex nature of infectivity which involves several components of which penetration of the hosts skin is one. <u>In vitro</u> measurements of parameters such as penetrability are therefore only indicative of true infectivity. It would be interesting to test whether larvae which although active were

FIG.5:1

A MODEL OF POSSIBLE FACTORS INFLUENCING THE INFECTIVITY OF THIRD-STAGE LARVAE OF Ancylostoma tubaeforme.



unable to penetrate the hosts skin, could nevertheless be infective when introduced into a potential host through some other route (eg. per os). The lack of experimental animals and other procedural matters did not allow for such an investigation to be carried out in this study.

2. It emphasises the importance of both the activity and lipid reserves of infective larvae in relation to infectivity. Since both parameters were reduced with the passage of time, they might be used jointly to estimate the physiological condition of larvae. The use of the amount of lipid reserves as an estimate of the 'physiological age' of infective larvae (Payne 1923b, Giovanniola 1936; Rogers 1939, 1940; Elliott 1954, etc) would be made more meaningful if accompanied by measurements on larval activity.

3. It recognises the importance of environmental and ecological factors. Since host-larval contact is primarily a chance process (Donald 1967; Donald and Leslie 1969; Anderson 1974) the ecology of the infective larvae in relation to the movements and behaviour of the host would affect infectivity. Unfortunately very little is known in this respect on the behaviour of cats such as, for example, how often they may visit contaminated soils.

How far this model will apply under natural conditions may therefore not be accurately assessed in the absence of data on the relevant aspects of cat behaviour.

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