A STUDY OF THE FREE-LIVING PHASE OF *DELADENUS*, NEMATODES PARASITIC IN WOODWASPS

by

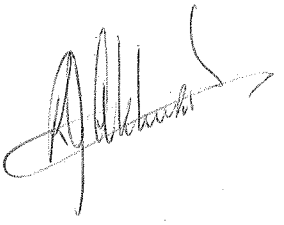
Raymond J. Akhurst, B.Sc. (Hons)

Submitted in fulfilment of the requirements for the degree of

Master of Science

UNIVERSITY OF TASMANIA
HOBART

There is no material contained in this thesis which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief this thesis contains no copy or paraphrase of material previously published or written by another person, except when due reference has been made in the text of the thesis.



CONTENTS Page

SUMMARY

1. Two populations of *D. siricidicola* and one of *D. rudyi* which had been maintained continuously in monoxenic culture for 1 year (*D. siricidicola* (S), *D. rudyi* (T)) and 6 years (*D. siricidicola* (H)) were used to study the effects of a range of environmental conditions on the egg phase of the free-living form of *Deladenus*.

2. For each population the rate of egg development increased with temperature from 5°C to 25°C.

3. The abortion rate of each population was related to temperature but the nature of the relationship varied from one population to the next. In each case, abortion rate was lowest at about 15°C.

4. There were no significant differences in the relationships of abortion rate and eclosion failure to temperature.

5. Examination of the effects of pH on the rate of egg development was inconclusive.

6. Abortion rate varied with pH from pH 2·5 to pH 8·0. Different reactions were recorded for each population. Optimum pH was about pH 5·5 for *D. siricidicola* (H) and *D. rudyi* (T), about pH 5·0 for *D. siricidicola* (S).

7. Within each population, abortion rate and eclosion failure followed a similar pattern of change with pH.

8. Considerable variation occurred between replicates in the estimation of fecundity but, on the average, *D. siricidicola* (S) were more fecund than *D. siricidicola* (H). *D. rudyi* (T) were more fecund when cultured on CMA rather than ¼ PDA.

9. The mean volume of the eggs of *D. siricidicola* (H) was significantly greater than that of *D. siricidicola* (S) which was also significantly greater than that of *D. rudyi* (T).

10. The variance of egg volume of *D. rudyi* (T) was considerably less than that of either of the *D. siricidicola* populations for which the variances were not significantly different.

11. There was no correlation between the variance of the egg volume of these populations and the time interval between hatching of the first and last eggs.

12. Cross-breeding trials conducted with seven species of *Deladenus* produced a wide range of results; from the parent female depositing only few eggs, all of which were abortive, to completely unimpeded production of the F_2 generation.

13. With only one exception, intraspecific crosses produced hybrids without reduced fecundity.

14. Interspecific crosses sometimes resulted in reduced fecundity among the parents and always among the hybrids produced.

15. The crosses, *D. rudyi* (UJ7) x *D. rudyi* (T) and *D. rudyi* (UJ7) ♂ x *D. imperialis* ♀, produced aberrant results suggesting a close relationship between *D. rudyi* and *D. imperialis*.

16. Differences between *D. siricidicola* (H) and the other two populations indicated that considerable changes can result from continuous cultures of the type used for *Deladenus*.

17. Differences between *D. siricidicola* (S) and *D. siricidicola* (H) indicate that the cultures of these populations should be regarded as different strains.

INTRODUCTION

Following the discovery of a nematode parasitising *Sirex noctilio* F. in New Zealand (Zondag, 1962), nematodes were collected by CSIRO and C.I.B.C.[1] from a wide variety of hosts, tree species, geographical regions and climates for intensive study and use in the biological control of *S. noctilio* in Australia. This insect is a potentially serious pest of *Pinus radiata* D.Don. in Australia where these trees, grown under a wide range of climatic conditions, form the basis of an industry returning almost 250 million dollars annually.

Each of the seven species of *Deladenus* collected occurs in two, remarkably dissimilar, forms which correlate with two distinct life cycles (Bedding, 1967, 1972a) (Fig. 1). In fact, if the relationship between the two forms had not been demonstrated experimentally, they would have been classified as belonging to different families; the parasitic form being placed in the insect parasitic Sphaeulariidae and the free-living form in the fungal-feeding Neotylenchidae.

The biology of these nematodes is essentially the same as that of *Deladenus siricidicola* Bedding (Bedding, 1972b). The infective form of *D. siricidicola* is an adult female which, after mating, uses its large stylet to punch a hole in the cuticle of the siricid larva to gain entry. Within a few weeks of entry, the nematode increases up to a thousand-fold in volume. It then remains quiescent in the haemocoel until the onset of host pupation when its ovary rapidly elongates, produces thousands of

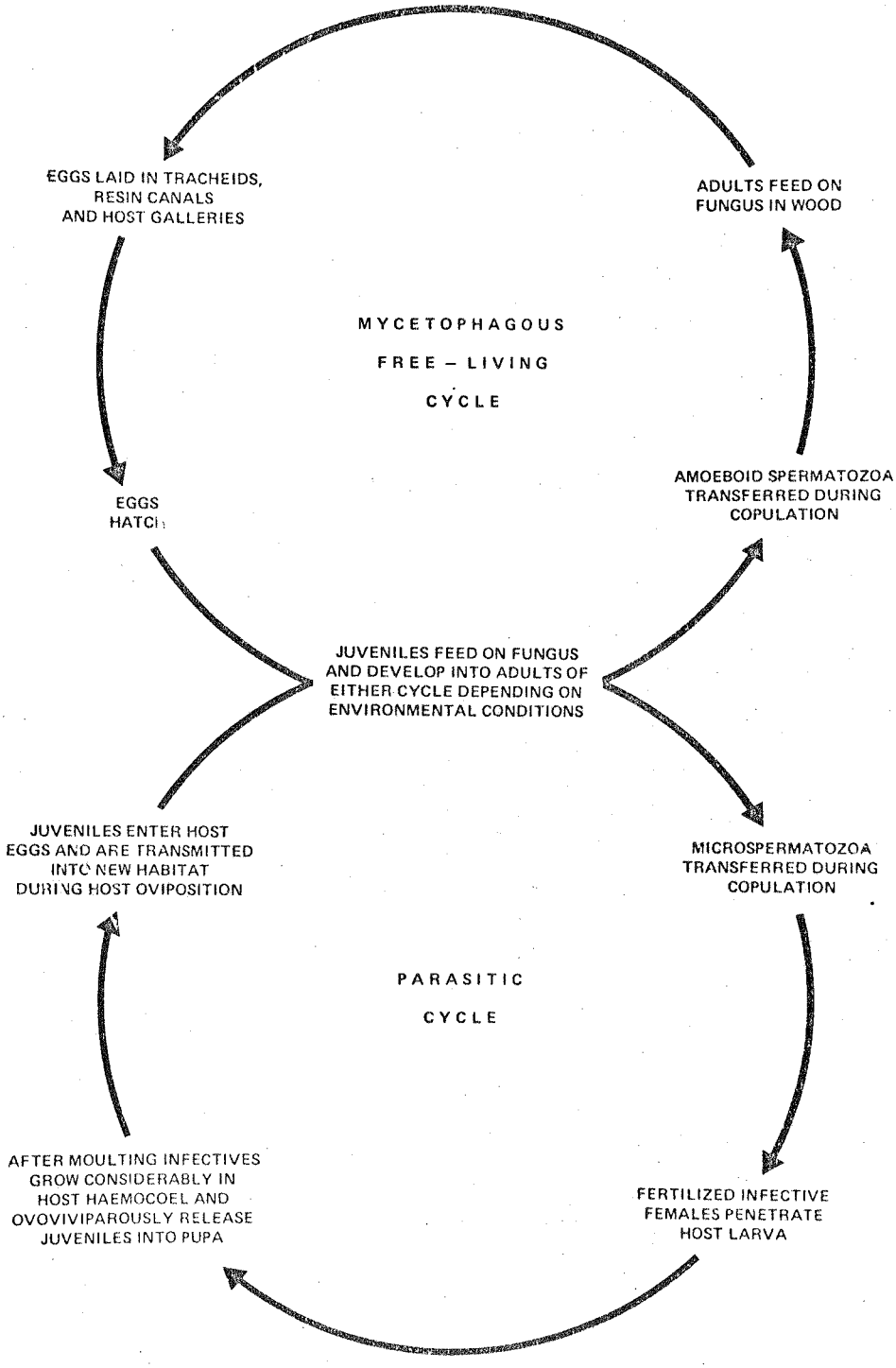
[1] Commonwealth Institute of Biological Control

eggs which soon hatch into juvenile nematodes. These pass from the parent nematode into the host's gonads. If the host is male, parasitism by *D. siricidicola* has no effect on fertility because, although the testes appear hypertrophied and devoid of sperm, the sperm are transferred to the vesiculae seminales prior to the nematode invasion of the testes. However, in the female host ovarian development is suppressed and invasion of the eggs by the juveniles results in sterility. When the female insect emerges from the tree and finds a suitable oviposition site, she deposits toxic mucus, fungal spores, and the eggs containing 50-200 juvenile nematodes which, on escaping from the eggs, feed on the fungus as it grows in the tree and develop into the free-living form which is oviparous. The free-living cycle can be repeated indefinitely or, when conditions dictate, the parasitic forms are produced and these can then infect the progeny of unparasitised *S. noctilio* attracted to the same tree.

The free-living cycles, certain features of which form the subject of this thesis, is of great importance in enabling manipulation of the nematodes for biological control (Bedding & Akhurst, 1974). It has allowed the production of hundreds of millions of nematodes for distribution in *Sirex* affected plantations and maintenance of a stock of different strains and species of *Deladenus* in culture for further evaluation and possible use.

Although the remaining six species have essentially the same life cycle as *D. siricidicola*, some have slight differences, mainly in the parasitic phase. Thus, *Deladenus imperialis* Bedding does not sterilise its natural host *Sirex imperialis* Kirby

Fig. 1 Diagram of the life history of *Deladenus siricidicola* (Bedding, 1972b).



because the juvenile nematodes do not enter the insect's eggs but lie in the ovaries and oviducts and are deposited with the viable eggs during normal oviposition. Similarly *Deladenus nevexii* Bedding does not sterilise *Sirex cyaneus* F. although 25%-75% of the eggs of *Urocerus californicus* Norton parasitised by this species and all the eggs of parasitised *Xeris* spp. are rendered inviable (Bedding, 1974). *Deladenus wilsoni* Bedding differs in being mainly a parasite of the rhyssine parasitoids of siricids where it suppresses egg development.

This thesis is an account of a study which examined the effects of a range of environmental conditions on the egg phase of the free-living form of *Deladenus*. The effects of temperature on abortion, eclosion and the duration of the egg phase were the primary subjects but led to investigation of fecundity, egg volume, and the effects of pH on abortion, eclosion and the duration of the egg phase. The effect of inter- and intraspecific hybridisation on fecundity was also examined.

A brief review of the literature relevant to this study is presented in the following paragraphs.

Temperature is often a significant factor in determining the rate of growth of a nematode population because it affects not only the rate of development but survival and transition from one phase of the life cycle to the next. Bird & Wallace (1965) compared the influence of temperature on the eggs of *Meloidogyne hapla* Chitwood and *Meloidogyne javanica* Treub by measuring, for each temperature chosen, the "percentage hatch" at 6 days. By expressing the number of eggs hatched at 6 days as a percentage of the number of eggs introduced initially, they combined

examination of the effect of temperature on both the rate of development and viability of the eggs. The effect of temperature on the rate of egg development has also been studied by determining minimum hatching time (Taylor, 1962; Crofton, 1965), median hatching time (Silverman & Campbell, 1959), mean hatching time (Fenwick, 1951) and by observing variation in the pattern of hatching (Wallace, 1966). Wallace (1971) and Banyer & Fisher (1971), studying *M. javanica* and *Heterodera avenae* Franklin respectively, examined embryonic development and eclosion separately and found that they had different temperature optima. This difference meant that, in studying the hatch responses of different populations of these nematodes at least, one must ensure that all the populations studied contain similar proportions of embryos and unhatched larvae (Wallace, 1971).

Lehman *et al.* (1971) showed that hatching of *Heterodera glycines* Ichinohe from egg masses was insensitive to pH. However, Loewenberg *et al.* (1960) found that hatching of *Meloidogyne incognita incognita* Kofoid & White was pH-sensitive and optimal at pH 6·5 while Wallace (1966) found significantly greater hatch of *M. javanica* eggs at pH 6·4 than at pH 5·8 or pH 7·6. No consideration has been given to the possibility of a relationship between pH and rate of development of nematode eggs although the data of Loewenberg *et al.* (1960) suggests this possibility. In reviewing the effect of pH on nematode eggs, Shepherd & Clark (1972) point out that the effect of pH is often inconclusive because of the osmotic effect of the buffer used.

There are at least three patterns of oviposition exhibited by nematodes (Fisher, 1969): the steady increase to a maximum rate occurring in zooparasitic nematodes (Hyman, 1951); the maintenance of a maximum rate for a period followed by a decline (*Ditylenchus dipsaci* Kühn, Yuksel, 1960; *Tylenchus emarginatus* Cobb, Gowen, 1970; *Pratylenchus penetrans* (Cobb) Chitwood and Oteifa, Mamiya, 1971); and a skewed normal distribution with the maximum rate occurring soon after the commencement of oviposition (*Aphelenchus avenae* Bastian, Fisher, 1968). Fisher (1969), Gowen (1970) and Mamiya (1971) found great variation in the fecundity of individual nematodes and that only extremely adverse conditions significantly affected this fecundity. Bedding (1972b) recorded the life span and estimated the number of eggs laid by *D. siricidicola* but gave no detail of variation between individual nematodes or the pattern of oviposition.

Crofton & Whitlock (1965b) demonstrated a relationship between the volume of an *Haemonchus contortus* Rud. egg and its rate of development. They found that "the minimum volume of eggs of a particular species determines the minimum time for hatching at any temperature, and the maximum volume will determine the theoretical maximum time required". Moreover, the minimum temperature at which hatching can occur is also related to egg volume because the larger the egg, the lower the temperature at which it will hatch (Crofton & Whitlock, 1965a).

Cross-breeding tests have been conducted with a variety of nematodes (Maupas, 1918; Augustine, 1939; Nigon & Dougherty, 1949; Sturhan, 1964; Eriksson, 1965; Potter & Fox, 1965; Poinar, 1967; Hansen *et al.*, 1968; Parrott, 1968, 1972;

Bedding, 1968). The results of such crosses have usually been interpreted as successful hybridisation or failure to hybridise. However, Potter & Fox (1965), Green & Miller (1969), and Parrott (1972) were able to produce interspecific hybrids of *Heterodera* spp., though with greatly reduced fecundity, and Eriksson (1965) found that crosses between races of *Ditylenchus dipsaci* reproduced more slowly and resulted in lower numbers of progeny than crosses between members of the same race. Although Bedding (1968) used cross-breeding tests to distinguish *D. siricidicola* and *D. wilsoni*, he gave no indication of the degree of interspecific hybridisation or of the fecundity of the intraspecific hybrids of nematodes collected from widely different sources.

MATERIALS AND METHODS

2.1 Subjects of Study.

The nematodes used in this study were taken from cultures initiated and maintained by CSIRO. Most of this study was conducted with nematodes from two populations of *D. siricidicola* and one of *D. rudyi*. The culture of one population, *D. siricidicola* (H), was initiated with one fertilised, free-living female, the original source of which was a male *Sirex juvencus* L. collected at Varbely, Sobron, Hungary. Cultures of the other two populations were initiated with hundreds of juveniles. *D. siricidicola* (S) was obtained ex testis of a male *S. noctilio* collected from Sierra Monte Planes, Llanes, Spain and *D. rudyi* (T) ex testis *S. cyaneus* from Ugurlu, Bucak, Turkey.

The Hungarian population of *D. siricidicola*[1] which had been cultured for six years was chosen for study because, of the

[1] *D. siricidicola* (H)

nematodes available at the time, it appeared to be the most likely to be effective in controlling *S. noctilio* and had been liberated experimentally in Tasmania (Bedding & Akhurst, in prep.). For comparison the Spanish population of *D. siricidicola*[1] and the Turkish population of *D. rudyi*[2] were also studied. Of all the available populations of *D. siricidicola* the Spanish population provided greatest contrast to the Hungarian: it had been in culture for only one year; the climate of its place of origin differs greatly from that of Hungary; cultures of this strain, unlike those of *D. siricidicola* (H), turn brown after about a week and produce the infective form more readily and significantly more *S. noctilio* were parasitised by *D. siricidicola* (H) under experimental conditions (Bedding & Akhurst, unpublished). Like *D. siricidicola* (S), *D. rudyi* (T) had been in culture for a year and its country of origin has a milder climate than that of Hungary. Unlike *D. siricidicola* however, *D. rudyi* cannot easily be cultured on *Amylostereum areolatum*, the symbiont of *S. noctilio*, but must be cultured on *Amylostereum chailletii* (Pers. ex Fr.) Boidin, which is symbiotic with other siricids, and rarely produces the infective form in monoxenic culture.

2.2 General Methods.

2.21 Initiation and maintenance of cultures

The free-living cycle of these *Deladenus* spp. can be kept indefinitely in monoxenic culture on the symbiotic fungi of siricids. *D. siricidicola* and *D. wilsoni* were cultured on *A. areolatum* and *D. wilsoni* and the remaining species on *A. chailletii*.

[1] *D. siricidicola* (S)

[2] *D. rudyi* (T)

These cultures were established and maintained by the methods of Bedding & Akhurst (1974).

A pure culture of *A. areolatum* was obtained from the oodial glands of an adult *S. noctilio* female by dipping the insect in ethanol, igniting and plunging it into sterile water under which it was dissected to remove the glands. The burst glands were streaked across plates of potato dextrose agar (PDA) which were then incubated at 24°C. After several days the growing fungal front was subcultured onto fresh PDA plates. A pure culture of *A. chailletii* was obtained by dissecting a *Sirex cyaneus* female in the same way.

Nematode cultures were initiated by dissecting male siricids under the same sterile conditions, removing the testes containing juvenile nematodes and placing them in the centre of fungal cultures which had grown over about one third of the plate. The nematodes migrated to the growing edge of the fungus and, after one day, subcultures (about 1 cm square) were cut from the fungal front and placed at the edge of PDA plates to establish monoxenic cultures. Subcultures were made about a week later and subsequently at fortnightly intervals just before the agar was completely covered by fungus. When subculturing, a balance between nematodes and fungus was maintained because the nematodes fed most successfully on the growing edge of the fungus; with too few nematodes the fungus covered the plate before much reproduction occurred and with too many, fungal growth was excessively stunted. The rate of reproduction improved noticeably over the first few months of culturing.

Nematode cultures on *A. areolatum* were continually sub-

cultured onto PDA but those on *A. chailletii* were maintained on corn meal agar (CMA) until approximately a week before use because *A. chailletii* attacked and killed a large proportion of eggs and some nematodes, when cultured on PDA. However, nematodes cultured on CMA were smaller than those from PDA, their rate of oviposition was lower and the number per culture plate was often less so that collection of a large number of eggs from CMA cultures was much more difficult. However, when they were sub-cultured from CMA to PDA, juvenile nematodes matured into large adults with a high rate of oviposition of viable eggs so facilitating the collection of large numbers of eggs for experimentation.

All cultures were incubated at 24°C and the plates stored in polythene envelopes. Besides reducing the risk of mites invading and contaminating the cultures, use of the polythene envelopes induced most of the nematodes to remain on the surface of the agar whence they could be harvested easily.

2.22 Selection of cultures

Nematodes used in experiments were harvested only from cultures where the fungus covered 20-80% of the plate. This avoided any undesirable end effects of this type of culturing (viz. too little fungus on a newly established culture and too high a level of metabolic wastes in an old culture).

2.23 Collection of eggs

For most experiments newly deposited eggs were required and, except when required for measurement, eggs were collected under sterile conditions in a laminar flow cabinet using

instruments which had been sterilised by being autoclaved, flamed or soaked in alcohol.

When small numbers of nematodes were involved or when knowledge of the exact number of eggs deposited was required, adult female nematodes were selected and transferred from the culture on a mounted hair. This caused no apparent disruption of their oviposition behaviour as they continued depositing eggs at the same rate as those left undisturbed on the culture.

For many experiments a large number of newly deposited eggs was required and it was physically impossible to obtain the required number by selecting nematodes with a mounted hair. Instead, the cultures were flooded with sterile Ringer's Solution, gently agitated and the suspension of nematodes and eggs pipetted into sterile watch glasses. Adult females were separated from the eggs, juveniles and most males by differential sedimentation, left in shallow Ringer's Solution for 4-5 hours at 24°C and then separated from the newly deposited eggs by differential sedimentation. With this technique up to 500 females could be handled and 2,000-3,000 newly deposited eggs collected without contamination.

2.24 Preparation of agar plates

For experiments on the duration of the egg phase and egg mortality, the eggs were incubated on plates of an ultra-purified agar, ionagar (IA). IA (1%) in distilled water provided a clear, hard medium which discouraged adult nematodes from burrowing and caused all eggs to be deposited on the surface. The eggs were covered by only a thin film of water and so impedance of the O_2/CO_2 exchange across the egg membranes was minimal. Because of the clarity of the agar, the eggs could be counted by

observation through the bottom of the plates so avoiding optical distortion caused by condensation on the lid or contamination of the agar as a result of removing the lid.

For most of these experiments, the IA was buffered with 10% phosphate/citrate buffer (McIlvaine's Series). These buffers, composed of 0·2 M disodium hydrogen phosphate and 0·1 M citric acid, were used over the range pH 2·5-8·0. To prevent acid hydrolysis of the agar, the buffer was autoclaved separately, cooled to 40°C and then mixed with the similarly cooled agar before being poured into sterile petri dishes.

Although PDA provided a suitable medium for culturing nematodes feeding on *A. areolatum* it allowed such vigorous fungal growth that small numbers of nematodes could not be cultured and so a diluted medium (¼ PDA) was used. This was prepared by using 1 part PDA and 3 parts Oxoid No.1 agar and was generally satisfactory for culturing small numbers of nematodes.

2.25 Incubation

During experiments the IA plates were held at 100% RH in a cooled incubator (±1°C at 15-35°C, ±2°C at 5-10°C). Maintaining humidity at 100% RH prevented desiccation of the agar and local variations in temperature due to loss of latent heat of vaporisation. The temperature was monitored by either a thermograph or maximum/minimum thermometer.

2.3 Experimental Methods.

2.31 Temperature effects

2.311 Effect on rate of egg development

The role of temperature in determining the duration of the egg phase was examined by measuring the time taken for 50% of the viable eggs to hatch (TH_{50}) for temperatures between 5°C and 25°C. TH_{50} was determined by constructing hatch/time curves for *D. rudyi* (T) and both *D. siricidicola* populations at each temperature. However, TH_{50} for any one population at a particular temperature varied considerably from one experiment to the next because of small but prolonged temperature fluctuations in the incubators and so valid comparison of the effect of temperature on the rate of egg development of the three populations could only be obtained by determining the hatch/time curve at each temperature for three populations simultaneously.

After washing nematodes from several cultures, 800-1500 newly deposited eggs were collected as described in section 2.23. They were evenly suspended in 2·5 ml Ringer's Solution by gentle agitation and then 0·5 ml aliquots were transferred by sterile pipette to each of five IA plates buffered at pH 5·5. The eggs were counted and then incubated at the test temperature and 100% RH until the day before expected TH_{50} when they were recounted. The following day they were counted (3-4 times at 5-15°C, hourly at 20°and 25°C) and, thereafter, daily until hatching ceased.

Hatch/time curves were constructed for 5°, 10°, 15°, 20°, and 25°C with percentage hatch at t_i being defined as in section 2.32.

2.312 Effect on abortion

Since abortion rate varied considerably between cultures of the same population, the effect of temperature on abortion could only be assessed satisfactorily by using eggs from the same source and since nematodes from any one culture provided too few eggs for these experiments, the eggs were collected from nematodes washed from several cultures. The eggs were pooled in 10 ml sterile Ringer's Solution, evenly suspended by gentle agitation, and divided into five equal lots (blocks). Each block was then evenly apportioned to five IA (pH 5·5) plates which were assigned to each of five groups so that each group contained five replicates with about 200 eggs on each. The five groups were incubated at different temperatures (5°, 10°, 15°, 20°, 25°C). Eggs were counted prior to incubation and again when hatching had ceased. The aborted eggs were examined and recorded as "developmental failure" (abortion before attainment of full larval form) or "eclosion failure" (fully formed larva within the egg).

This method was used for both *D. siricidicola* populations at 5°, 10°, 15°, 20°, and 25°C although in the experiment with the Hungarian population a malfunction in the 20°C incubator allowed the temperature to rise to 32°C. The effect of 20°C on *D. siricidicola* (H) was examined by using a similar method in which the eggs were incubated at 10°C and 20°C.

The effect of temperature on *D. rudyi* (T) eggs could not be assessed by this method because a certain proportion of the newly deposited eggs cannot be easily separated from the females and the number of females available was too small to yield a satisfactory number of eggs after separation. In the case of

D. rudyi, 100 females were transferred from one culture on a sterile mounted hair and distributed equally among five IA (pH 5·5) plates. This was repeated with a further two cultures. After five hours at 24°C the females were removed, the eggs counted and three plates (representing each culture) incubated at each temperature (5°, 10°, 15°, 20°, 25°C) and 100% R.H. When hatching ceased the eggs were counted and classified as in the previous method.

Lack of incubating facilities prevented concurrent study of the effects of 27·5°C. The effects of this temperature were investigated using the method described above and incubating the eggs at 20°C and 27·5°C.

These experiments were analysed by two-way analysis of variance, after arcsine transformation of percentages, for the effect of temperature on abortion and eclosion failure.

2.32 pH effects

As the assessment of the effects of pH can be confused by ionic and osmotic factors, the buffer series chosen used the same ions over the whole range, had only a small concentration of any one ion in the pH region of particular interest, and was used in low concentration. McIlvaine's Series was chosen for these experiments because it can be used over the range pH 2·0-8·0 and preliminary experiments showed no significant difference between eggs hatched in 10% buffer (pH 6·5) and those hatched in tap water (pH 6·5). The buffers of this series are prepared from 0·2 M disodium hydrogen phosphate and 0·1 M citric acid and were incorporated into IA as described in section 2.24.

Since abortion rate varies considerably even between cultures of the same strain, attempts to measure the effect of any factor on abortion required the use of eggs obtained from the same cultural source(s). Nematodes from any one culture provided too few eggs for these experiments so large numbers of newly deposited eggs were obtained by washing nematodes from several culture plates and collecting the eggs as described in section 2.23. The eggs were then pooled in 5 ml sterile Ringer's Solution, evenly suspended by gentle agitation, and divided into five equal lots (blocks). In turn, each block was diluted to 3 ml, gently agitated, and distributed by sterile pipette among one of each of the treatment plates. This resulted in 80-120 eggs per plate and five replicates of each treatment. When excess liquid had been absorbed (about 20 mins.), the eggs were counted and incubated at constant temperature and 100% R.H. They were counted twice on the day when most hatching occurred and again when hatching ceased. The aborted eggs were examined and recorded as "developmental failure" (abortion before attainment of full larval form) or "eclosion failure" (fully formed larva within the egg).

This experiment was conducted twice with both *D. siricidicola* populations and *D. rudyi* (T) at 20°C and once with *D. rudyi* (T) at 25°C over the range pH 2·5-8·0.

After transformation of percentages by an arcsine transformation, the data were analysed by two-way analysis of variance for the effect of pH on:

1. rate of development (% hatch at t_i which is defined as the number of eggs hatched by t_i expressed as a percentage of the total number

of eggs hatched).

2. abortion (the number of eggs aborted expressed as a percentage of the number of eggs observed).

3. eclosion failure (% eclosion failure = $\frac{\text{number of eclosion failures}}{\text{number of eclosion failures + number of eggs hatched}}$).

2.33 Rate of oviposition and fecundity

The experiments described below were conducted to determine the age at which oviposition rate is maximum and the relationship, if any, between abortion rate and the age of the parent nematode. Although these data were gathered primarily to determine the optimal time for collecting large numbers of viable eggs, they were also used to estimate fecundity.

About 1,000 fourth juvenile stage females and approximately the same number of mature males were transferred by mounted hair from several cultures to an established culture of the appropriate fungus and incubated at 24°C. The following day adult nematodes were distributed evenly among five 4-day old fungal cultures and incubated at 24°C. This provided a stock of females of known age so that change in oviposition rate with maternal age and the reproductive span of the females could be determined. Confusion with the filial generation was avoided by transferring the adult nematodes by mounted hair to fresh fungal cultures whenever the filial larvae approached maturity (7-8 and 14-15 days).

Oviposition rate was determined at suitable intervals by randomly selecting 10 females (20 on the first day of maturity) from each culture and transferring them to IA plates where they were left to oviposit for 4-5 hours at 24°C before being removed

and discarded. The eggs left on the IA plates were counted and incubated at 24°C for 6 days when the remaining (aborted) eggs were counted.

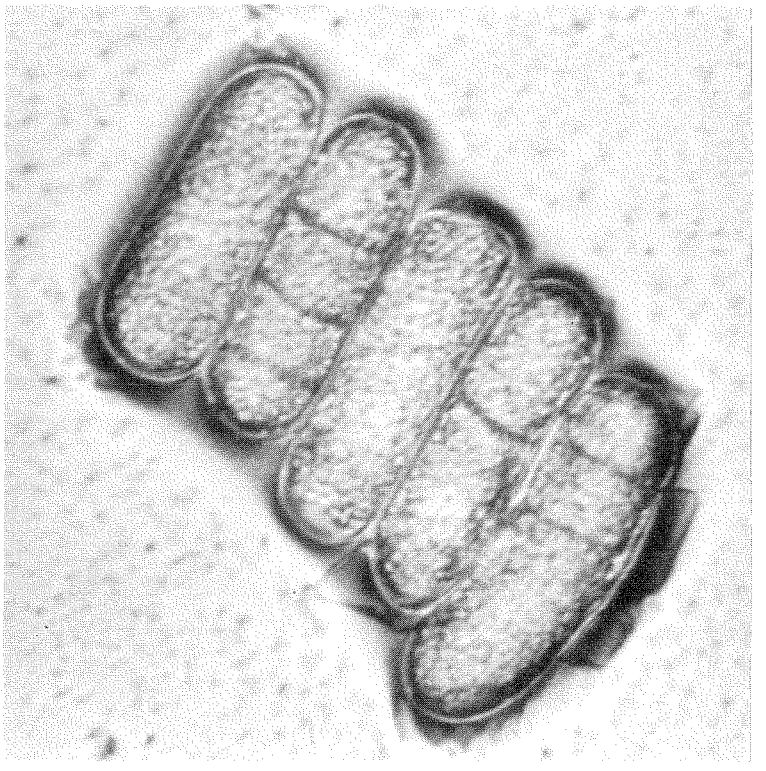
The experiment was not continued until oviposition ceased completely but was discontinued when nematode mortality was high in every replicate. Since the oviposition rate was very low when mortality was high, cessation of the experiment at this time introduced little error into the estimate of fecundity.

Stocks of *D. siricidicola* were maintained on *A. areolatum* cultured on PDA and stocks of *D. rudyi* on *A. chailletii* cultured on CMA or ¼ PDA. The newly matured *D. rudyi* females were distributed among six cultures instead of five - three on CMA, three on ¼ PDA.

Since handling this large number of nematodes was very time consuming, a simpler method involving fewer nematodes was tried. About 200 juvenile females and the same number of males were selected from culture and left overnight at 24°C on a fresh fungal culture. On each of five fungal cultures (4 days old) were placed 20 newly matured females and 20 adult males. As before they were incubated at 24°C and overlapping of generations was prevented by transferring the mature nematodes to fresh fungus as the filial generation approached maturity. Oviposition rate was determined as described above except that the nematodes were returned to their cultures after use and so each nematode was used many times, though not necessarily every time, during the course of this experiment.

The number of larvae produced in the life of the average female was estimated by measuring the area under the rate/age

Fig. 2 Eggs of *D. siricidicola*



50μ

graph. This was done by cutting out the area under the graph, weighing the paper, and, by reference to the weight of a known area, calculating the number of eggs represented. (See Appendix A).

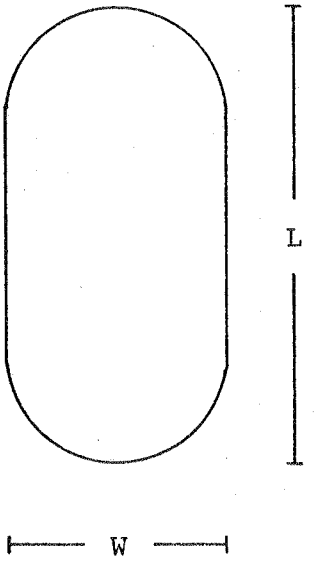
2.34 Egg size

The shape of *Deladenus* eggs (Figure 2) is approximated by a cylinder surmounted by hemispheres at either end (Figure 3) and the volume of this model can be calculated by

$$V = \frac{\pi w^2}{12} (3L-W)$$ where W is maximum width

L is maximum length

Fig. 3 Model used to estimate the volume of *Deladenus* eggs.



For each population, 100 eggs washed from each of three cultures were measured after partial separation of eggs and nematodes by differential sedimentation (complete separation was unnecessary and could have resulted in a biased sample of eggs).

The eggs were mounted in Ringer's Solution (deformation being prevented by use of glass fibre to support the coverslip) and measured at a magnification of x1250.

For each population the data from the three cultures were pooled and the differences in volume analysed by a normal t-test where the variance could be considered homogeneous and by a suitably modified t-test where not.

2.35 Hybridisation

Variation in intraspecific hybridisation and the extent of interspecific hybridisation were examined by crossing nematodes from populations which had been identified by detailed morphological examination.

All crosses were conducted on cultures of fungus growing on agar plates. Intraspecific crosses with *D. siricidicola* were conducted on *A. areolatum* cultured on ¼ PDA. Crosses involving the other species were conducted on *A. chailletii* on CMA unless they were being crossed with *D. siricidicola* in which case either both fungi were cultured on the same plate (¼ PDA) or only the fungus preferred by the female was used. In most cases these methods allowed the nematodes to feed and reproduce successfully, the completion of the trial without subculture and the observation of results of the mating.

For each trial a small block (0·5 cm square) of the appropriate fungus was subcultured onto each of 15 ¼ PDA or CMA plates. Fifteen fourth juvenile stage females, whose juvenile and sexual status had been confirmed under high magnification, were taken individually from the first culture and placed on the 15 plates. The plates were then randomly assigned to three equal

groups: on each of the first group was placed a single adult male from the same culture; on each of the second, a single adult male from the other culture, and the third was retained as a control against parthenogenicity or fertilisation before maturity. This procedure was then repeated with 15 juvenile females from the second culture. The mating pairs were incubated at 24°C and their progress through the following stages observed female: maturing and ovipositing, eggs hatching, F_1 generation maturing then ovipositing, and eggs hatching. Where this last stage was achieved, a portion of agar containing nematodes and fungus was cut out, transferred to a plate of PDA (or CMA) under sterile conditions, and the culture maintained for further observation.

Where F_1 females were observed ovipositing, their fecundity was assessed by estimating the number of eggs produced and the abortion rate. Abortion rate was measured by transferring 10-40 females from each plate onto a corresponding plate of IA where they were left 4-5 hours at 24°C. The eggs deposited in this time were counted and incubated at 24°C for 6 days when the remaining (aborted) eggs were counted.

Except where the F_1 laid many eggs with low abortion rate, each trial was repeated for confirmation of the result. Trials in which only a few F_2 larvae resulted were repeated and the parent male removed when the first F_1 larvae hatched so that there could be no backcrossing.

RESULTS

3.1 Effect of Temperature.

3.11 Rate of egg development

The effect of temperature on the rate of development was examined by plotting hatch/time curves for each population at 5^{o}, 10^{o}, 15^{o}, 20^{o}, and $25^{o}C$ and using these to estimate the time taken for 50% of the viable eggs to hatch (TH_{50}). The experiment was conducted only once for each temperature because of the difficulties involved in examining the three populations simultaneously. However, preliminary experiments in which each population was examined separately produced very similar values for TH_{50}. A hatch/time curve for *D. siricidicola* (S) at $5^{o}C$ is not presented because so few of these eggs hatched at $5^{o}C$ (38 out of 1080). The data from these experiments are presented in Tables 1-6 and Figs 4-9.

At each temperature and for each population the hatch/time curve rose quickly to about 80% hatch and then flattened (Figs 4-8). TH_{50} for each population was least at $25^{o}C$ and increased exponentially as temperature declined. At these temperatures, TH_{50} was always least for *D. siricidicola* (H) and greatest for *D. siricidicola* (S).

Table 1 Duration of the egg phase at 25°C. Values quoted for % hatch are the means of 5 replicates and were calculated after arcsin transformation of percentages

Time after ovipcsition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
50	0	0	0	0	0	0
67	39.5	1.8	2.5	4.9	9.1	4.8
68	48.4	1.0	8.1	7.7	14.6	2.9
69	51.9	0.6	15.0	15.6	19.2	3.9
70	57.6	1.3	28.2	6.1	34.0	2.0
71	65.4	0.5	36.1	4.8	44.4	3.1
72	71.0	0.8	46.4	2.9	51.9	2.7
73	77.8	2.2	56.4	0.5	61.4	1.6
74	82.5	1.8	61.2	0.5	67.8	0.7
75	86.5	2.0	78.6	7.5	71.0	0.4
92	97.8	2.7	99.5	0.9	99.4	1.3
120	100	0	100	0	100	0
Number of eggs hatched	233		88		197	

Fig. 4 Hatch/time curves for 25°C. ● *D. siricidicola* (H) ○ *D. siricidicola* (S) □ *D. rudyi* (T)

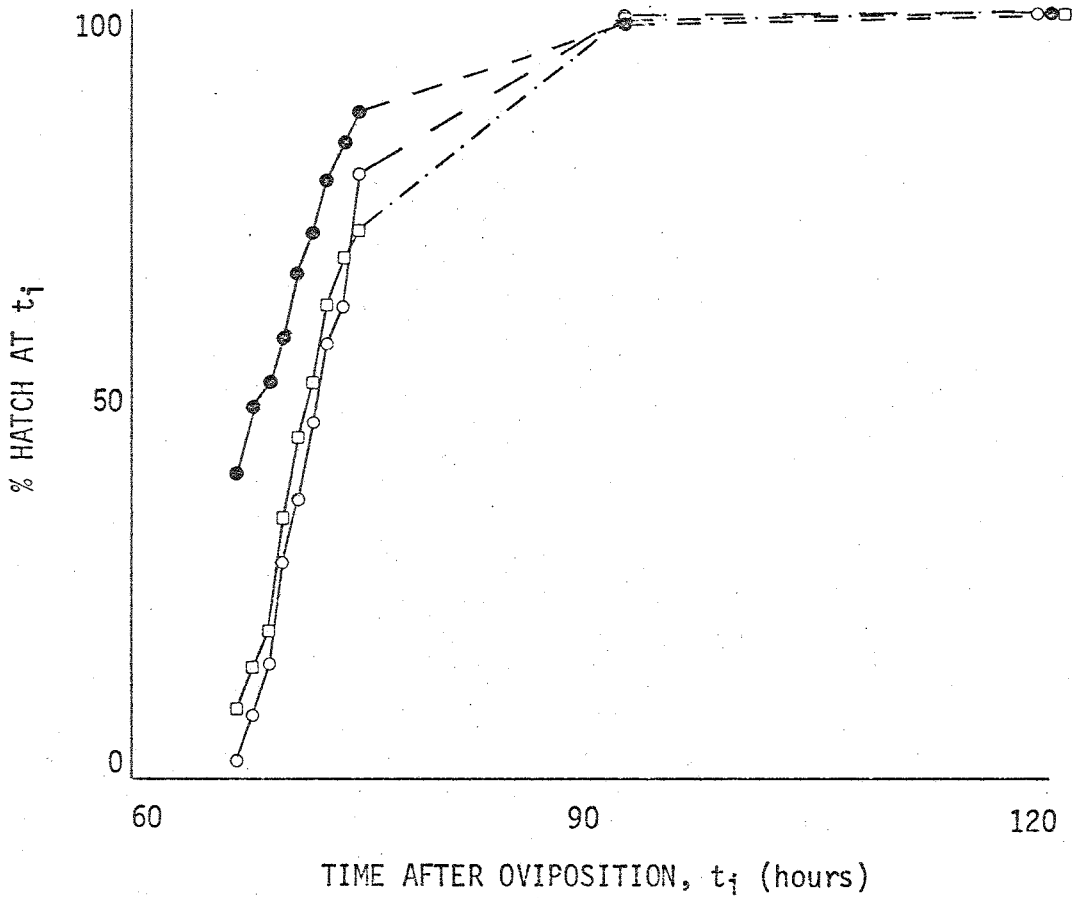


Table 2 Duration of the egg phase at 20°C. Values quoted for % hatch are the means of 5 replicates and were calculated after arcsin transformation of percentages

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
77	0	0	0	0	0	0
94	17.7	1.5	9.3	0.8	13.5	0.02
95	32.2	2.5	15.3	1.2	18.8	0.04
96	39.8	3.6	26.5	0.9	24.0	0.07
97	53.0	1.9	35.9	0.6	29.8	0.05
98	68.9	0.7	38.9	0.5	36.5	0.1
99	83.0	2.3	42.5	0.5	44.5	0.2
100	88.6	1.3	47.6	0.6	54.1	0.5
101	90.9	0.5	53.4	0.8	66.6	0.4
118	100	0	98.4	3.1	99.6	0.8
142	-	-	100	0	100	0
Number of eggs hatched	429		290		354	

Fig. 5 Hatch/time curves for 20°C. ● *D. siricidicola* (H) ○ *D. siricidicola* (S) □ *D. rudyi* (T)

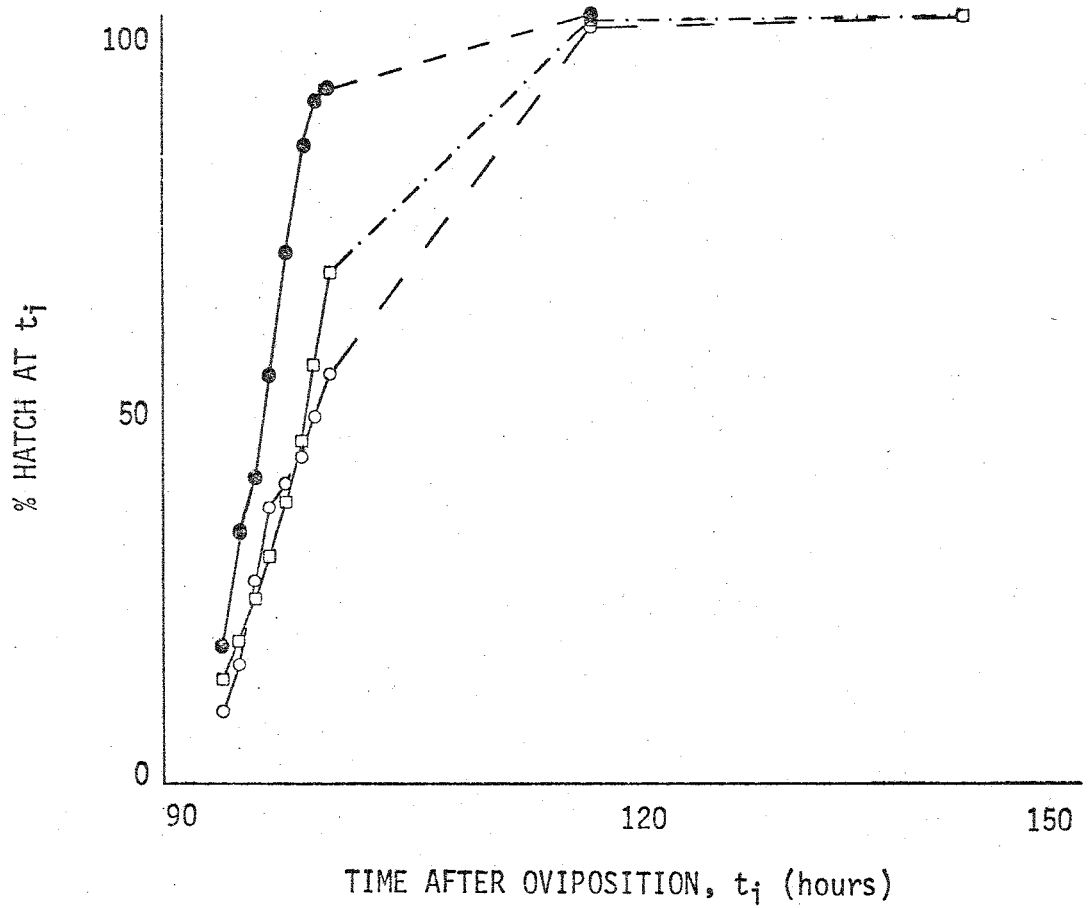


Table 3 Duration of the egg phase at 15°C. Values quoted for % hatch are the means of 5 replicates and were calculated after arcsin transformation of percentages

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
150	0	0	0	0	0	0
167	2.3	0.1	0.1	0.2	5.7	0.1
169	7.9	0.2	0.5	0.2	8.9	0.1
171	10.8	0.2	0.8	0.4	13.8	0.2
173	14.8	0.1	2.6	0.3	19.4	0.1
187	66.9	1.6	-	-	61.6	0.2
191	83.5	1.5	56.0	1.7	73.8	0.1
193	88.8	1.2	61.2	1.3	77.5	0.1
196	-	-	70.8	1.1	-	

Table 3 (continued)

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
207	98.9	0.2	94.7	0.7	92.5	0.1
229	100	0	96.8	0.3	97.9	0.1
255	-	-	99.7	0.5	99.3	0.2
264	-	-	-	-	100	0
279	-	-	99.8	0.3	-	-
293	-	-	100	0	-	-
Number of eggs hatched	983		1619		686	

Fig. 6 Hatch/time curves for 15°C. ● *D. siricidicola* (H) ○ *D. siricidicola* (S) □ *D. rudyi* (T)

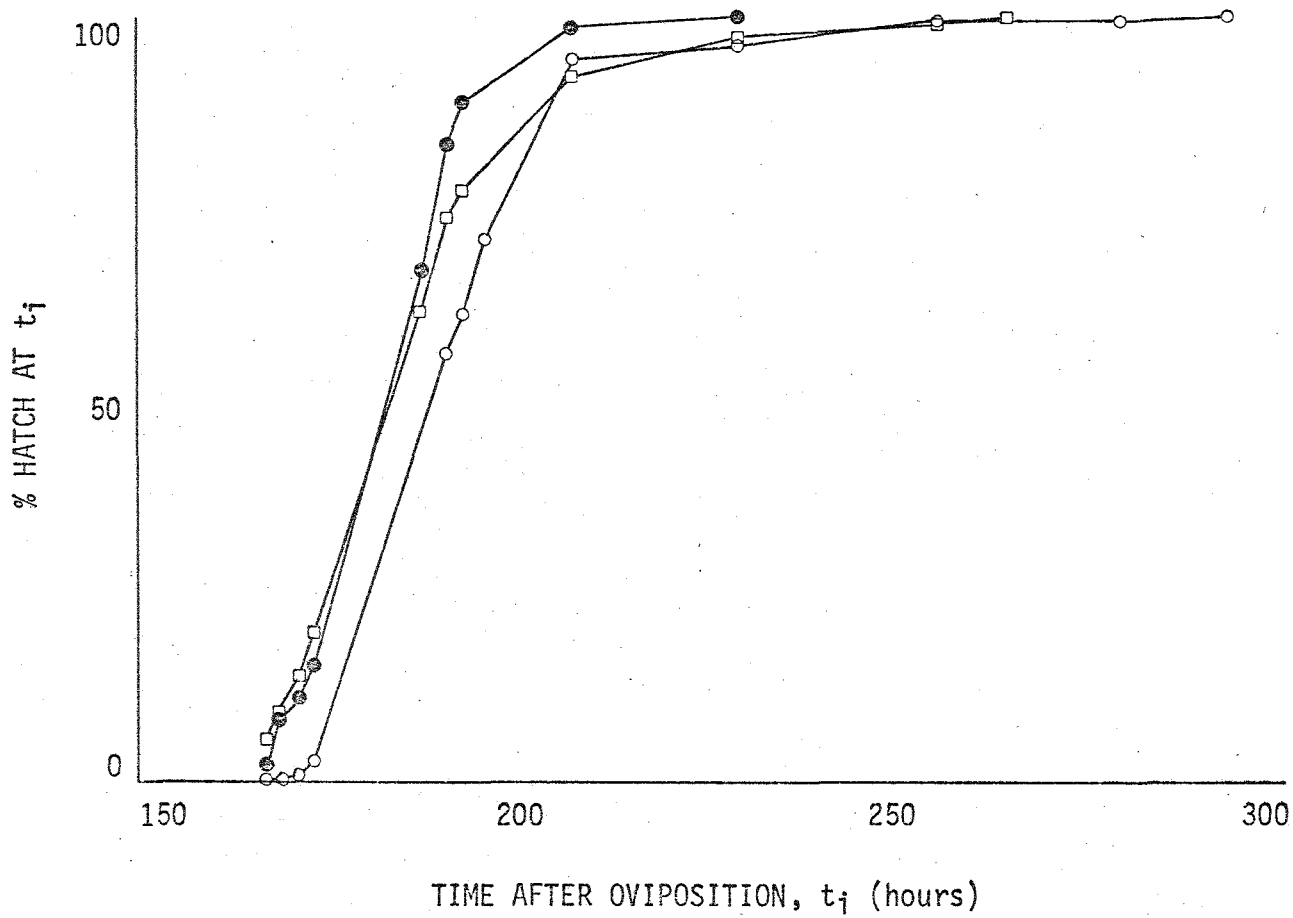


Table 4 Duration of the egg phase at 10°C. Values quoted for % hatch are the means of 5 replicates and were calculated after arcsin transformation of percentages

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
144	0	0	0	0	0	0
167	0.9	0.3	0	0	0	0
191	0.9	0.3	0	0	0	0
216	1.1	0.4	0	0	0	0
240	18.5	2.7	0.1	0.2	0.2	0.6
243	27.9	3.0	0.1	0.2	1.4	0.6
246	37.5	3.2	-	-	-	-
263	72.5	2.4	0.1	0.4	25.7	0.7
266	80.1	3.3	0.5	0.4	31.4	0.8
272	-	-	-	-	41.0	0.5
286	-	-	8.3	1.5	72.1	0.3
290	96.3	0.9	10.4	1.4	77.9	0.3

Table 4 (continued)

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. siricidicola* (S)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s	% hatch at t_i	s
294	-	-	15.4	1.5	-	-
311	98.8	1.5	34.9	1.6	93.3	0.1
317	-	-	44.2	1.2	94.4	0.1
360	100	0	80.2	0.9	99.0	0.8
383	-	-	90.3	0.8	99.7	0.3
407	-	-	95.7	1.4	99.9	0.2
479	-	-	99.9	0.3	100	0
503	-	-	99.9	0.3	-	-
527	-	-	100	0	-	-
Number of eggs hatched	686		523		737	

Fig. 7 Hatch/time curves for 10°C. ● *D. siricidicola* (H) ○ *D. siricidicola* (S) □ *D. rudyi* (T)

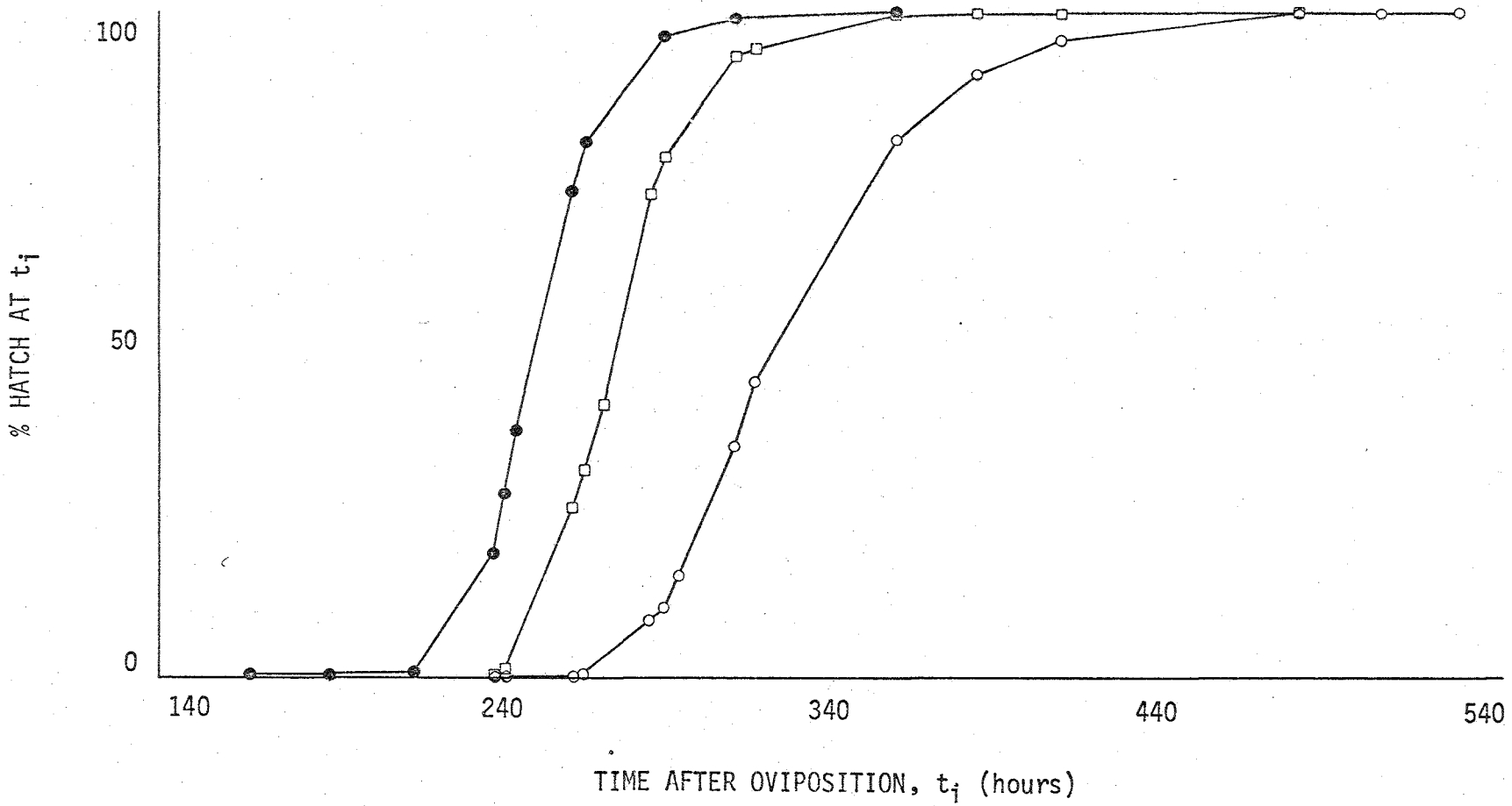


Table 5 Duration of the egg phase at 5°C. Values quoted for % hatch are the means of 5 replicates and were calculated after arcsin transformation of percentages

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s
575	0	0	0	0
598	0.9	4.5	0	0
601	1.8	5.6	-	-
628	5.6	14.2	0.2	0.7
669	34.7	12.5	2.5	2.1
671	39.8	10.4	-	-
673	43.5	9.3	-	-
676	47.8	8.0	6.1	0.4
693	60.9	3.5	11.8	0.8
696	73.1	2.3	13.5	0.8

Table 5 (continued)

Time after oviposition, t_i (hours)	*D. siricidicola* (H)		*D. rudyi* (T)	
	% hatch at t_i	s	% hatch at t_i	s
700	76.2	3.1	18.3	2.0
742	92.6	0.9	68.8	0.3
745	-	-	69.8	0.3
838	99.6	0.9	87.9	0.8
912	99.8	0.9	93.9	0.4
981	100	0	99.8	0.8
1032	-	-	100	0
Number of eggs hatched	318		252	

Fig. 8 Hatch/time curves for 5°C. ● *D. siricidicola* (H) □ *D. rudyi* (T)

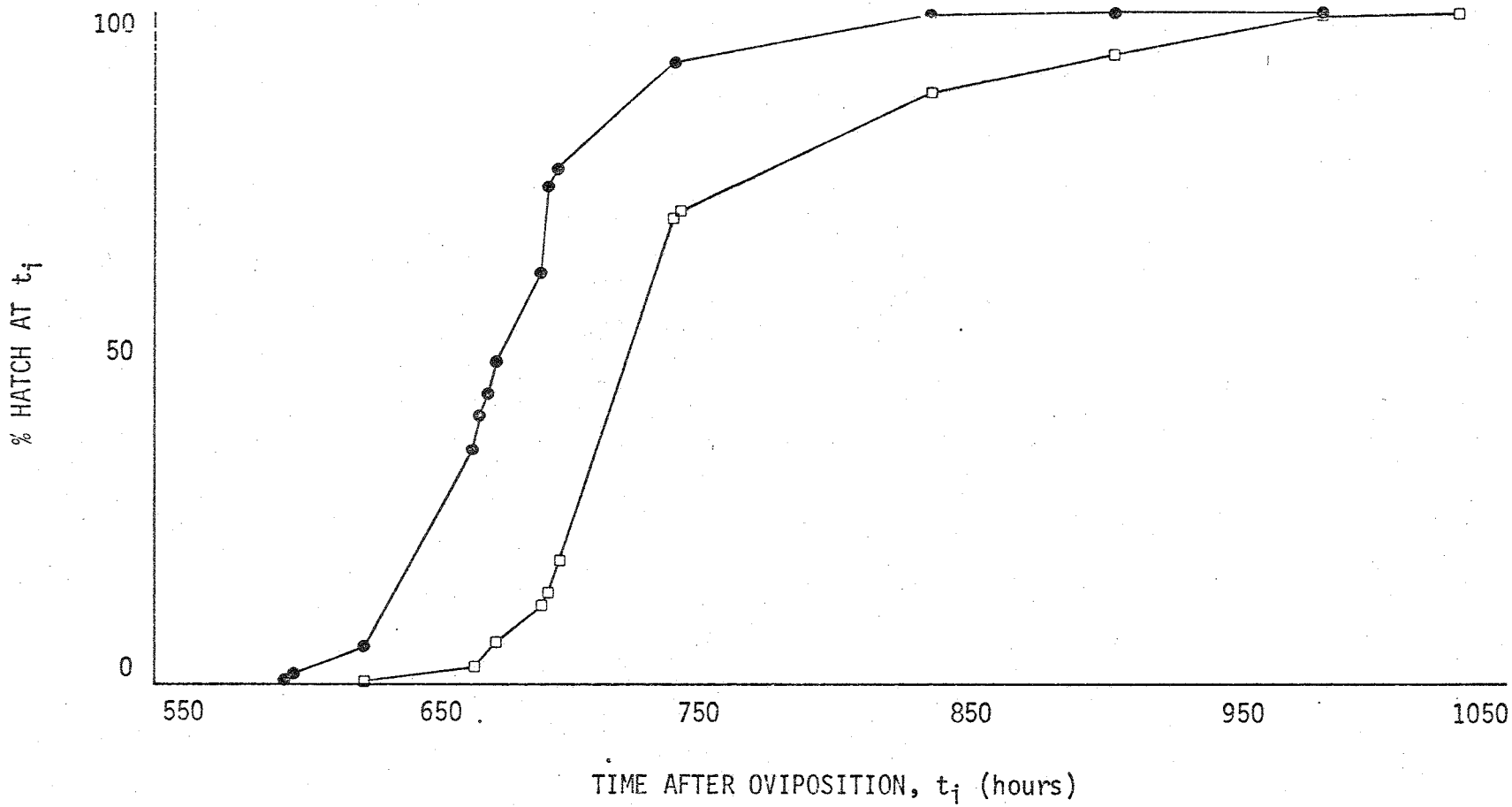
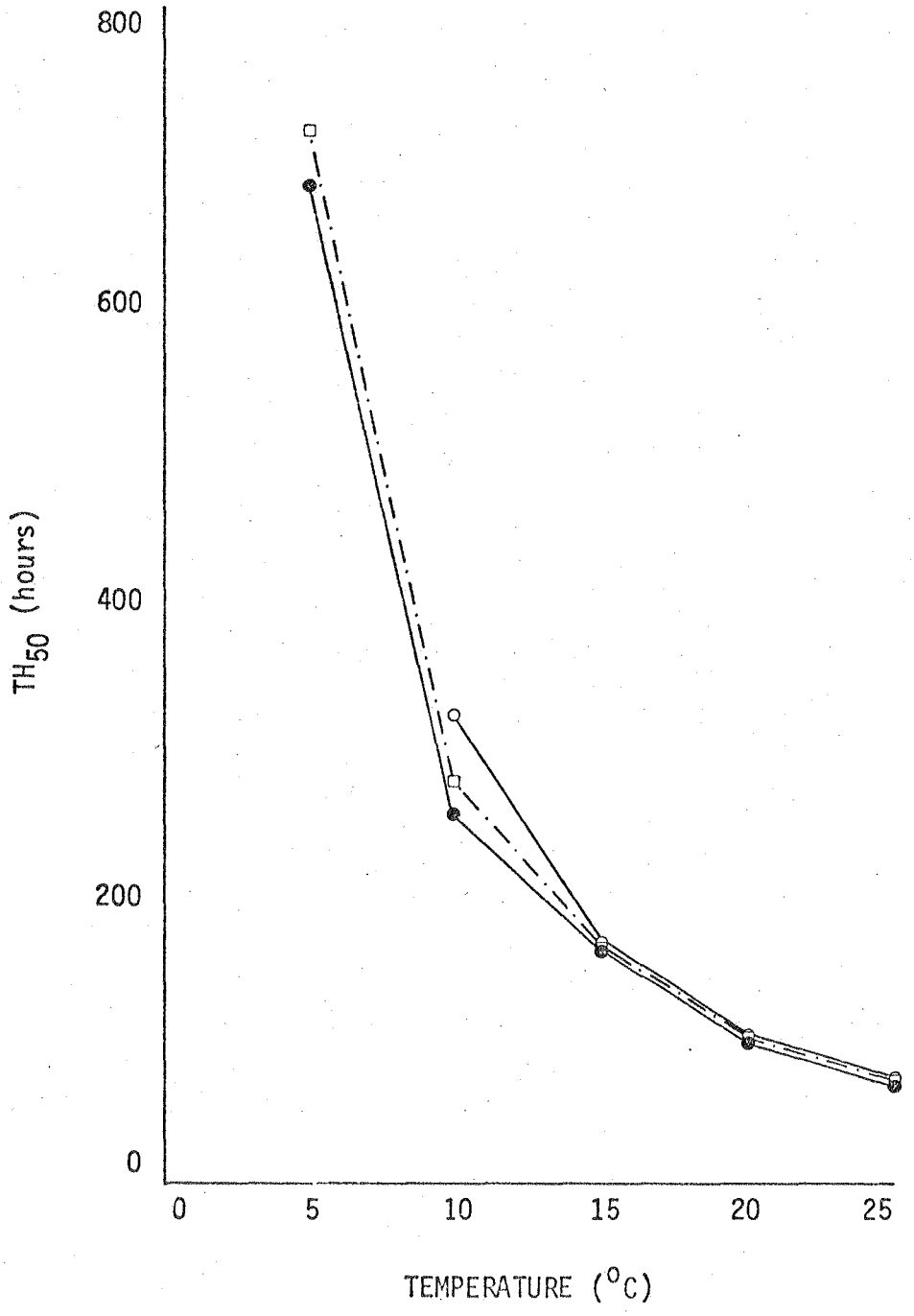


Table 6 Effect of temperature of the rate of egg development. The TH_{50} values were estimated from the hatch/time curves (Figs 4-8).

Temperature	TH_{50} (hours)		
°C	*D. siricidicola* (H)	*D. siricidicola* (S)	*D. rudyi* (T)
5	682	-	718
10	250	320	275
15	182	189	183
20	96.5	100	99
25	68.5	72.5	71.5

Fig. 9 Effect of temperature on the rate of egg development.

● *D. siricidicola* (H) ○ *D. siricidicola* (S) □ *D. rudyi* (T)



3.12 Abortion and eclosion failure

The data and analyses from these experiments are presented in Tables 7-13 and Figs 10-12. Two sets of data are presented for *D. siricidicola* (H) because an incubator malfunction subjected eggs to 32°C instead of 20°C. Eclosion failure of *D. siricidicola* (S) at 5°C was not included in the analysis because of the small number of eggs which developed to the larval form (52 from 5 replicates).

Minimum abortion occurred near 15°C for each population with that for *D. siricidicola* (S) occurring at a slightly higher temperature than for the others. Whereas the abortion/temperature curve for *D. siricidicola* (H) is symmetrical about 15°C, the curves for *D. siricidicola* (S) and *D. rudyi* (T) are J-shaped and reverse J-shaped respectively. With one exception, the results obtained were consistent with those obtained in preliminary experiments; the very high abortion rate (87.4%) recorded for *D. rudyi* (T) at 25°C was higher than expected (50-60%) and was due to the temperature being slightly in excess of 25°C for much of the experiment.

For each population, eclosion failure was lowest from 10-20°C and the optimum temperature for eclosion was about 15°C. The eclosion failure/temperature curves for *D. rudyi* (T) and *D. siricidicola* (S) are almost the same shapes as their respective abortion/temperature curves. However, the two curves differ for *D. siricidicola* (H), the eclosion failure/temperature curve being asymmetrical with greatest failure at 25°C.

It is notable that the abortion values for the two *D. siricidicola* populations are considerably greater than those

for *D. rudyi* (T) which deposited eggs directly onto agar instead of into Ringer's Solution. When the experiment was conducted with *D. siricidicola*, some females from each culture were placed on IA and their eggs incubated at 20°C; for both populations the abortion rate among these eggs (6·2% and 25·1%) was much lower than that of eggs collected by washing females from the cultures (22·7% and 48·7% respectively).

Despite the favorable conditions (eggs deposited directly onto the agar), the abortion rate at 27·5°C was very high (Table 13). However, cultures of these nematodes can be maintained at 27·5°C with ease because the abortion rate declines with time of exposure to this temperature. (After *D. siricidicola* (H) had been cultured at 27·5°C for 3 weeks, the abortion rate was 15%). Cultures of both *D. siricidicola* populations have been maintained at 30 ± 2°C although this temperature is absolutely lethal for eggs of females cultured at 24°C. Attempts to establish whether this adaptation to high temperature was physiological or genetic were thwarted by the variation in abortion rate between cultures and between samples from the same culture taken on different days. It appeared, however, that eggs deposited by some individuals were more tolerant of high temperatures than those deposited by the rest.

Table 7 Effect of temperature on abortion of *D. siricidicola* (H). The values quoted for % abortion are the means of 5 replicates of about 200 eggs each.

Temperature (°C)	1st experiment		2nd experiment	
	% abortion	s	% abortion	s
25	55.3	0.1	-	-
20	-	-	22.7	0.2
15	45.6	0.1	-	-
10	47.7	0.7	21.3	0.1
5	56.7	0.1	-	-

Table 7 (continued)

Analysis of variance data[1] (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	3	149.5068	49.8356	19.10	0.005
Blocks	4	3.0699	0.7675	0.29	ns
Error	12	31.3044	2.6087		
Total	19	183.8811			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	1	2.0885	2.0885	0.43	ns
Blocks	4	9.6222	2.4055	0.50	ns
Error	4	19.4142	4.8535		
Total	9	31.1248			

[1] Percentages were always transformed by an arcsine transformation before analysis.

Table 8 Effect of temperature on eclosion failure of *D. siricidicola* (H). Values quoted for % eclosion failure are the means of 5 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of % eclosion failure[1].

Temperature	1st experiment			2nd experiment		
(°C)	Number of eggs which completed development	% eclosion failure	s	Number of eggs which completed development	% eclosion failure	s
25	693	14.7	0.1	-	-	-
20	-	-	-	1535	0.9	0.1
15	814	1.2	0.1	-	-	-
10	960	2.5	0.4	1271	0.4	0.3
5	604	5.3	0.1	-	-	-

[1] Eclosion failure = $\frac{\text{Number of aborted eggs containing fully developed larva}}{\text{Number of aborted eggs containing fully developed larva + number of eggs hatched}}$

Table 8 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	3	148.9661	249.6554	36.18	0.005
Blocks	4	40.2871	10.0718	1.46	ns
Error	12	82.8040	6.9003		
Total	19	872.0573			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	1	8.7049	8.7049	1.38	ns
Blocks	4	16.8422	4.2106	0.67	ns
Error	4	25.1724	6.2931		
Total	9	50.7195			

Fig. 10 Effect of temperature on abortion and eclosion failure of *D. siricidicola* (H).

■ Abortion □ Eclosion failure

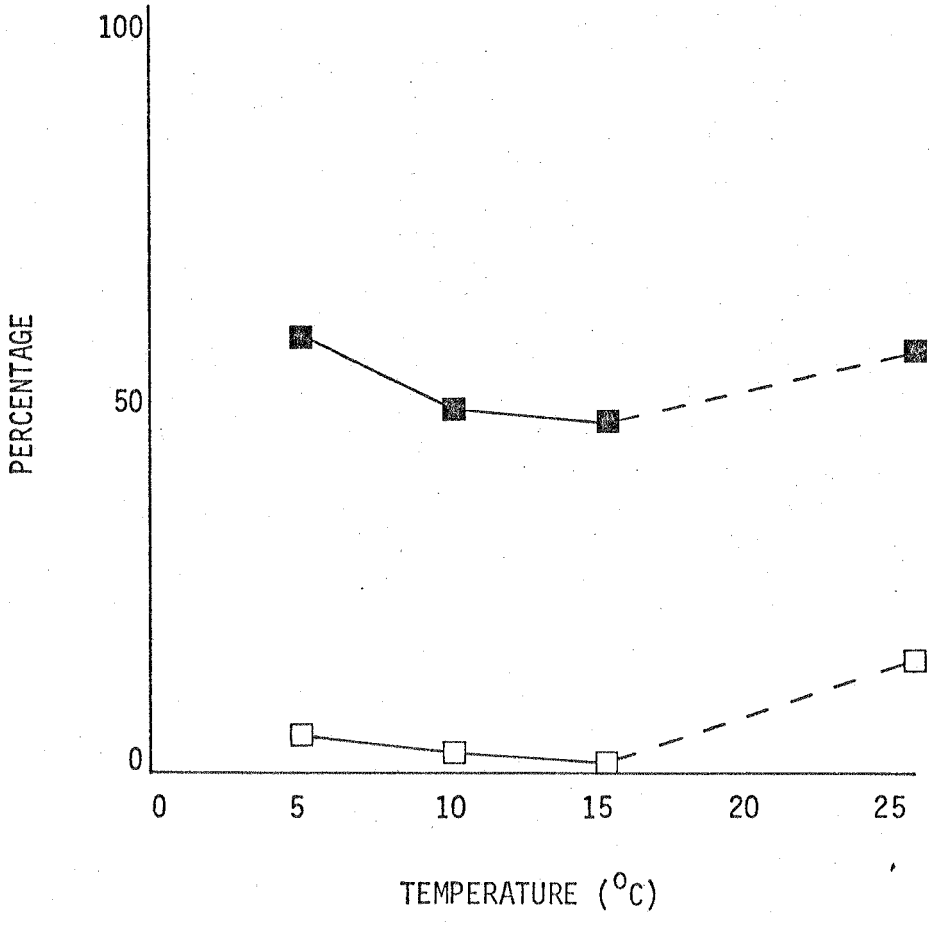


Table 9 Effect of temperature on abortion of *D. siricidicola* (S) eggs. The values quoted for % abortion are the means of 5 replicates of about 200 eggs each.

Temperature (^{o}C)	% abortion	s
25	57.1	0.2
20	48.7	0.3
15	52.7	0.1
10	61.7	0.2
5	97.0	0.1

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	4	4264.7110	1066.1778	186.15	0.005
Blocks	4	14.4227	3.6057	0.63	ns
Error	16	91.6392	5.7275		
Total	24	4370.7729			

Table 10 Effect of temperature on eclosion failure of *D. siricidicola* (S). Values quoted for % eclosion failure are the means of 5 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of % eclosion failure.

Temperature (^{o}C)	Number of eggs which completed development	% eclosion failure	s
25	496	10.9	0.4
20	658	6.4	0.1
15	551	2.6	0.3
10	452	4.4	0.4
5	52	26.0	2.0

Table 10 (continued)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Temperature	3[1]	269.8697	89.9566	7.00	0.01
Blocks	4	3.3162	0.8291	0.06	ns
Error	12	154.2634	12.8553		
Total	19	427.4493			

[1]Eclosion failure at 5°C was not included in the analysis because of the small number of eggs which completed development.

Fig. 11 Effect of temperature on abortion and eclosion failure of *D. siricidicola* (S)

▲ Abortion △ Eclosion failure

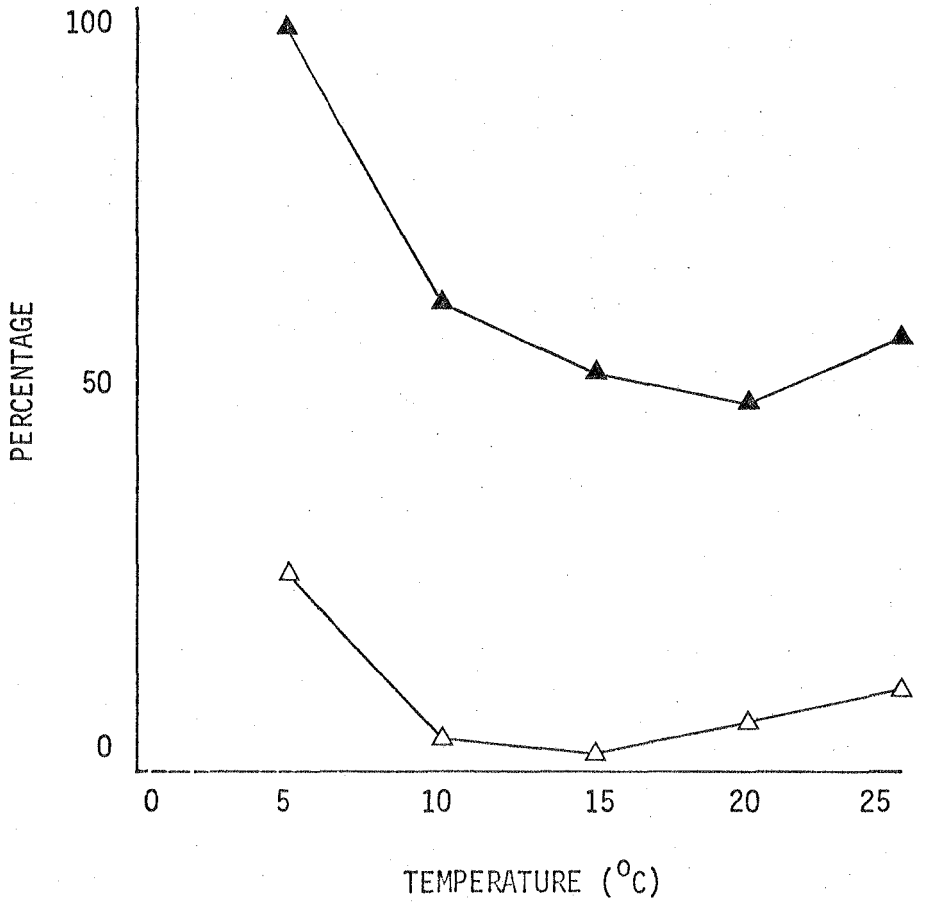


Table 11 Effect of temperature on abortion of *D. rudyi* (T) eggs. The values quoted for % abortion are the means of 3 replicates of about 120 eggs each.

Temperature (^{o}C)	% abortion	s
25	87.4	1.9
20	9.3	0.1
15	6.3	0.1
10	10.6	0.1
5	19.1	0.4

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
Treatments	4	6134.9240	1533.7310	94.90	0.005
Cultures	2	34.5729	17.2865	1.06	ns
Error	8	129.2975	16.1622		
Total	14	6348.7944			

Table 12 Effect of temperature on eclosion failure of *D. rudyi* (T). Values quoted for % eclosion failure are the means of 3 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of the eclosion failure.

Temperature (°C)	Number of eggs which completed development	% eclosion failure	s
25	130	61.5	7.1
20	251	1.1	0.9
15	552	0.5	0.4
10	516	1.2	0.1
5	403	4.7	1.3

Source of variation	df	Sum of Squares	Mean Squares	F	P
Treatment	4	4861.4217	1215.3554	24.08	0.005
Cultures	2	246.6978	123.3489	2.44	ns
Error	8	403.6946	50.4618		
Total	14	5511.8141			

Fig. 12 Effect of temperature on abortion and eclosion failure of *D. rudyi* (T)

● Abortion ○ Eclosion failure

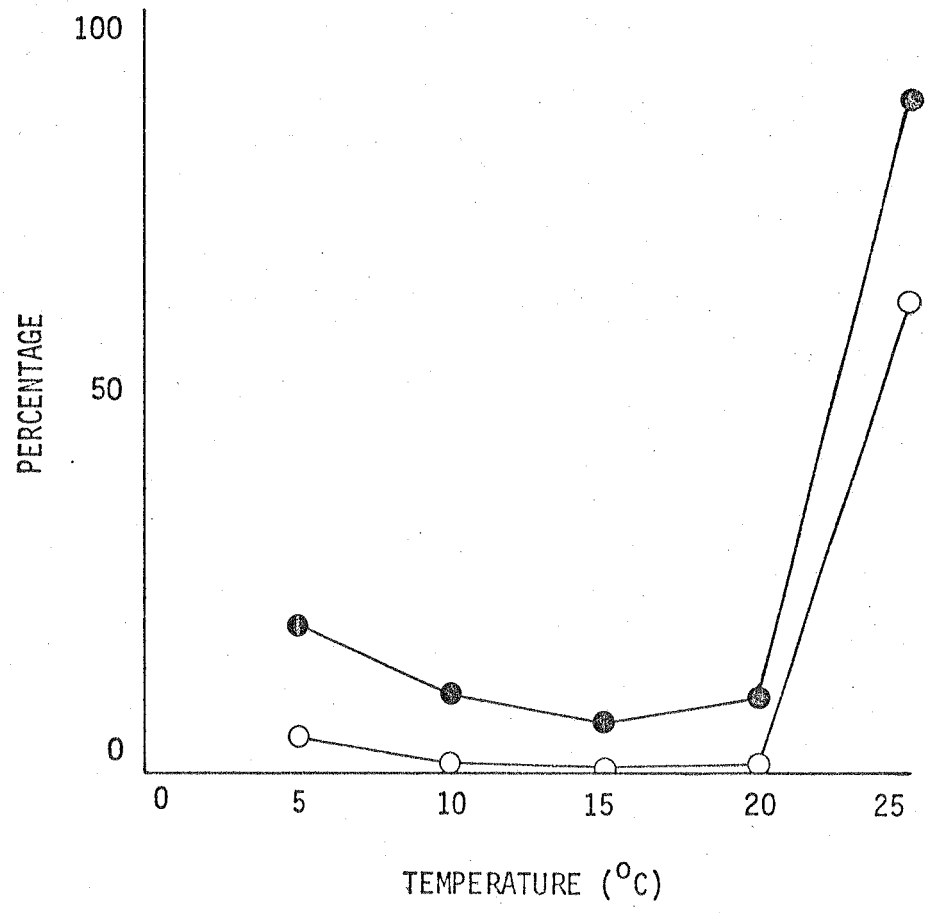


Table 13 The effect of incubation at 27·5°C on abortion. The values quoted for % abortion are the means of 3 replicates of over 100 eggs each.

	Temperature (°C)	% abortion	s	Significance level of the difference
D. siricidicola (H)	27·5	95.9	1.5	$p < 0.025$
	20	11.0	0.1	
D. siricidicola (S)	27·5	94.3	3.1	$p < 0.010$
	20	10.7	0.7	
D. rudyi (T)	27·5	97.4	0.6	$p < 0.005$
	20	9.6	0.4	

3.2 Effect of pH.

3.21 Rate of egg development

Some results of experiments to assess the effect of pH on the rate of development and the analyses of these data are presented in Tables 14-17 and Figs 13-15. Some results were omitted because of the very low number of eggs actually hatching at certain pH values (e.g. *D. siricidicola* (S) at pH 7·5-8·0).

Although in most experiments there is indication of a response to pH, aberrant results were obtained in every case and in two experiments there was no significant difference between % hatch at t_x[1] for different pH values.

[1] t_x - a time chosen so that % hatch at t_x was between 10% and 80%.

Table 14 Effect of pH on rate of development of *D. siricidicola* (H) eggs at 20°C. Values quoted for % hatch are the means of 5 replicates.

pH	1st experiment			2nd experiment		
	Number of eggs hatched	% hatch at t_x	s	Number of eggs hatched	% hatch at t_x	s
8.0	312	53.7	0.1	310	75.9	1.2
7.5	200	13.9	0.4	256	72.9	0.6
6.5	305	60.8	1.1	266	73.8	0.7
5.5	288	66.1	0.1	287	82.5	1.2
4.5	307	76.0	0.6	249	80.9	0.6
3.5	304	73.3	0.1	268	77.3	0.8
2.5	283	77.2	1.1	170	68.8	0.7

Table 14 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	5695.7588	949.2931	67.67	0.005
Blocks	4	129.6646	32.4162	2.31	ns
Error	24	336.6554	14.0273		
Total	34	6162.0789			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	306.4440	51.0740	1.82	ns
Blocks	4	77.7874	19.4469	0.69	ns
Error	24	673.6646	28.0694		
Total	34	1057.8960			

Fig. 13 Effect of pH on the rate of development of *D. siricidicola* (H) eggs.

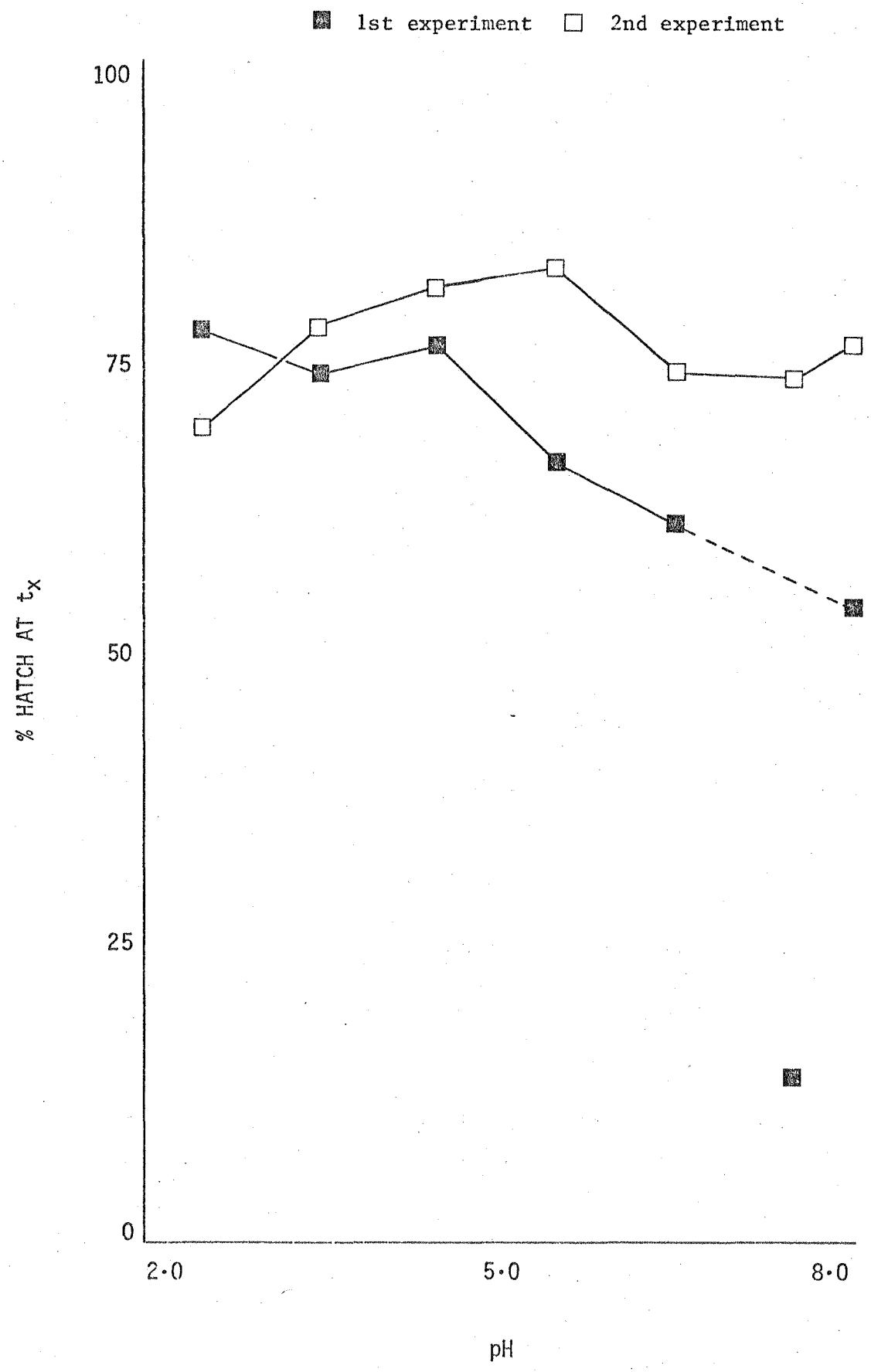


Table 15 Effect of pH on rate of development of *D. siricidicola* (S) eggs at 20°C. Values quoted for % hatch are the means of 5 replicates.

pH	1st experiment			2nd experiment		
	Number of eggs hatched	% hatch at t_x	s	Number of eggs hatched	% hatch at t_x	s
6.5	348	43.4	1.5	447	47.2	0.4
5.5	426	45.9	0.6	464	66.0	0.5
4.5	575	63.3	0.7	520	64.9	0.4
3.5	549	68.4	0.3	516	74.6	0.6
2.5	289	80.2	2.6	346	86.6	1.3

Table 15 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	4	1750.9700	437.7425	16.35	0.005
Blocks	4	317.7102	79.4276	3.00	ns
Error	16	428.3823	26.7739		
Total	24	2497.0625			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	4	1692.7984	423.1996	19.34	0.005
Blocks	4	59.2064	14.8016	0.68	ns
Error	16	349.9776	21.8736		
Total	24	2101.9824			

Fig. 14 Effect of pH on the rate of development of *D. siricidicola* (S) eggs.

▲ 1st experiment △ 2nd experiment

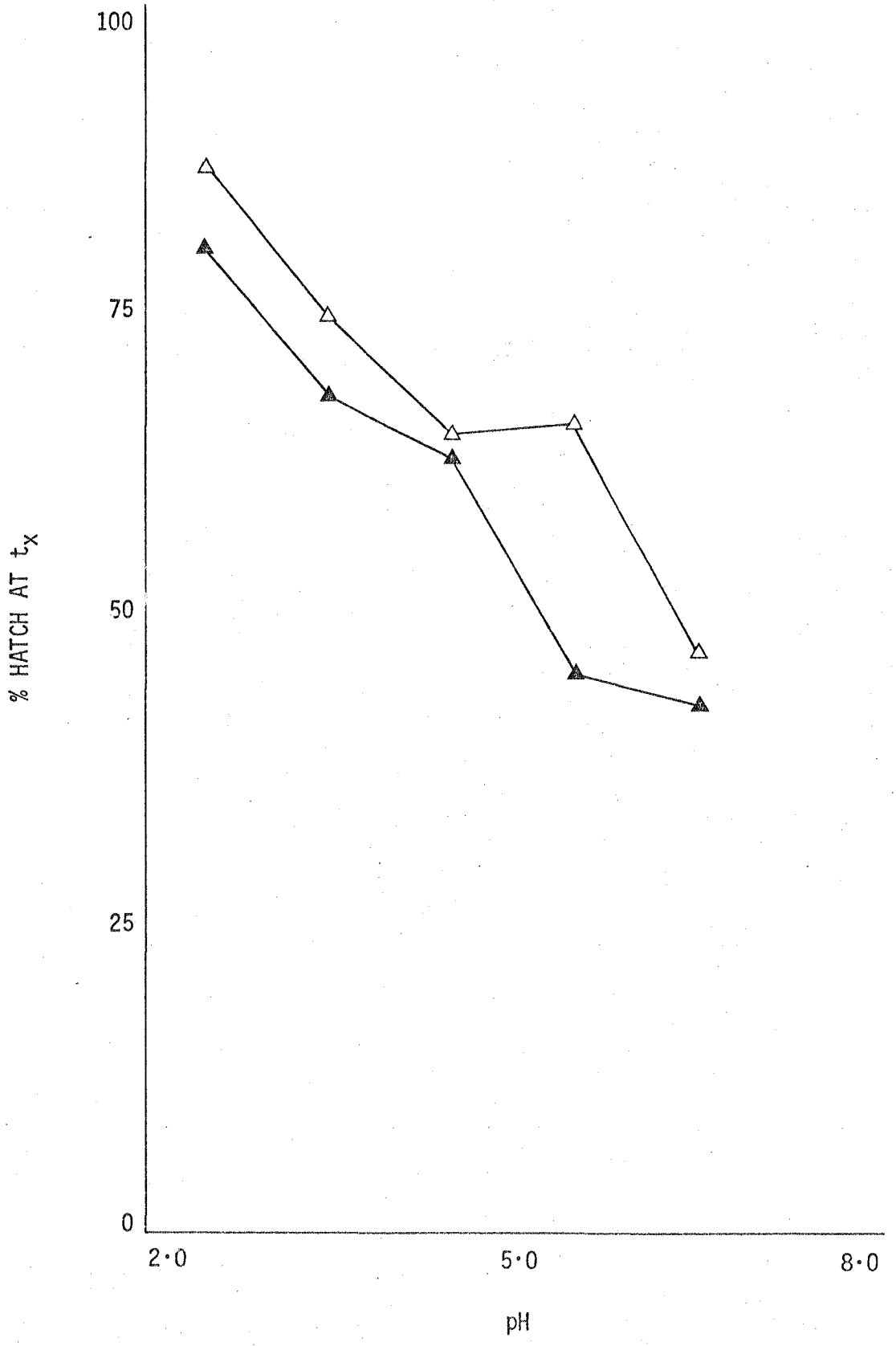


Table 16 Effect of pH on rate of development of *D. rudyi* (T) eggs at 20°C. Values quoted are the means of 5 replicates.

pH	1st experiment			2nd experiment		
	Number of eggs hatched	% hatch at t_x	s	Number of eggs hatched	% hatch at t_x	s
8.0	-	-	-	154	46.5	2.8
7.5	-	-	-	147	64.1	1.1
6.5	155	69.0	0.9	146	45.5	2.6
5.5	155	64.1	0.6	181	54.0	0.5
4.5	153	68.7	3.1	161	55.6	0.2
3.5	168	71.6	1.2	121	61.2	0.3
3.0	168	77.1	0.2	-	-	-
2.5	129	73.2	4.2	-	-	-

Table 16 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	5	194.9667	38.9933	0.86	ns
Blocks	4	448.8667	112.2167	2.47	ns
Error	20	909.5333	45.4767		
Total	29	1553.3667			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	5	474.4867	94,8973	3.24	0.05
Blocks	4	404.5754	101.1439	3.45	0.025
Error	20	586.0766	29.3038		
Total	29	1465.1387			

Table 17 Effect of pH on rate of development of *D. rudyi* (T) eggs at 25°C. Values quoted for % hatch the means of 5 replicates.

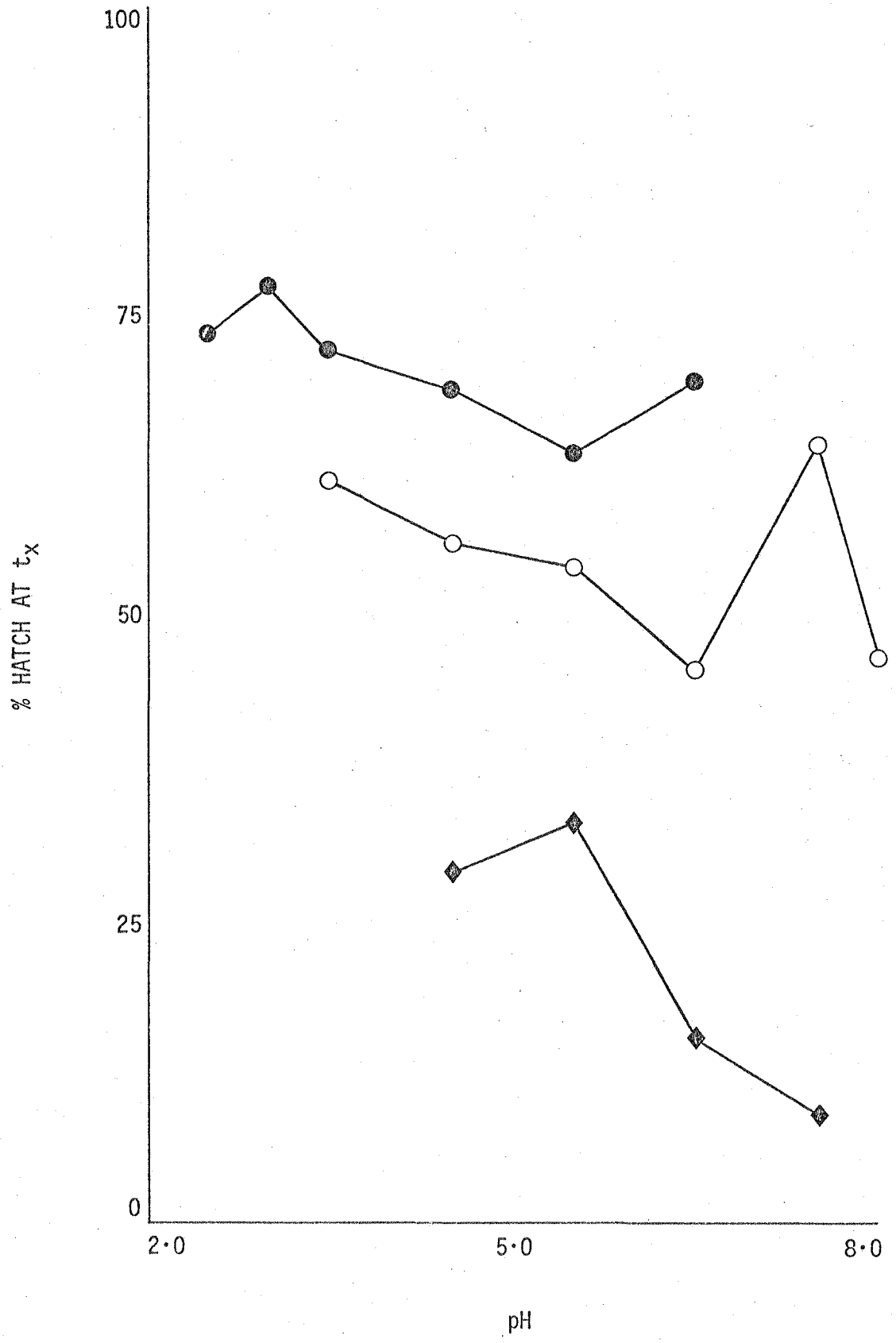
pH	Number of eggs hatched	% hatch at t_x	s
7.5	123	8.7	8.4
6.5	158	15.3	6.2
5.5	169	32.9	4.8
4.5	182	28.7	1.3

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	3	1025.8000	341.9333	3.51	0.05
Blocks	4	1406.8000	351.7000	3.61	0.05
Error	12	1169.2000	97.4333		
Total	19	3601.8000			

Fig. 15 Effect of pH on the rate of development of *D. rudyi* (T) eggs.

● 1st experiment, 20°C; ○ 2nd experiment, 20°C; ♦ 25°C.



3.22 Abortion and eclosion failure

Each experiment, except that with *D. rudyi* (T) at 25°C, was conducted twice using newly prepared buffered agar and, with one slight exception, the repeat experiment verified the results of the original. The data from these experiments and their analyses are presented in Tables 18-24 and Figs 16-18. No data on eclosion failure were collected for *D. siricidicola* (S) in the first experiment or *D. rudyi* (T) at 25°C.

Both *D. siricidicola* (S) and *D. rudyi* (T) showed a strong reaction to pH with greatest rate of abortion at pH 8·0 and 2·5 respectively. By contrast only at pH 2·5 did abortion of *D. siricidicola* (H) significantly exceed that at any other pH. The optimum pH for hatch was pH 4·5 for *D. siricidicola* (S) and pH 5·5 for *D. rudyi* (T).

The wildly aberrant result obtained for *D. siricidicola* (H) at pH 7·5 in the first experiment (Tables 18, 19) may reflect an error in preparation of the buffer or in the addition of buffer to agar and, since a similar result was not obtained in the second experiment, should probably be discarded.

The eclosion failure/pH curves for each population were similar to the respective abortion/pH curves.

Table 18 Effect of pH on abortion of *D. siricidicola* (H). The values quoted for % abortion are the means of 5 replicates of about 100 eggs each.

	1st experiment		2nd experiment	
pH	% abortion	s	% abortion	s
8.0	23.5	0.4	40.8	0.2
7.5	48.8	0.6	43.3	0.4
6.5	22.6	0.2	45.7	0.1
5.5	88.6	0.1	40.3	0.2
4.5	21.8	0.4	45.3	0.2
3.5	24.9	0.1	47.5	0.7
2.5	32.2	0.1	62.0	0.4

Table 18 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	1004.1029	167.3505	19.07	0.005
Blocks	4	20.1783	5.0446	0.57	ns
Error	24	210.6527	8.7761		
Total	34	1234.9069			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	538.5234	134.6309	11.90	0.005
Blocks	4	27.0903	6.7726	0.60	ns
Error	24	271.5537	11.3147		
Total	34	837.1674			

Table 19 Effect of pH on eclosion failure of *D. siricidicola* (H) eggs. Values quoted for % eclosion failure are the means of 5 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of % eclosion failure.

	1st experiment			2nd experiment		
pH	Number of eggs which completed development	Eclosion failure (%)	s	Number of eggs which completed development	Eclosion failure (%)	s
8.0	330	5.0	0.4	322	2.5	1.4
7.5	317	36.0	0.6	274	3.0	0.3
6.5	313	2.5	0.1	287	3.8	0.1
5.5	290	0.2	0.4	309	6.8	0.3
4.5	370	0.6	0.6	246	5.9	0.4
3.5	312	0.8	0.7	280	5.2	0.3
2.5	288	0.9	0.8	201	16.1	0.6

Table 19 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	4250.3389	708.3898	49.30	0.005
Blocks	4	114.6200	28.6550	1.99	ns
Error	24	344.864	14.3693		
Total	34	4709.8229			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	713.4560	118.9093	7.48	0.005
Blocks	4	50.0497	12.5124	0.79	ns
Error	24	381.3383	15.8891		
Total	34	1144.8440			

Fig. 16a Effect of pH on abortion of *D. siricidicola* (H) at 20°C

■ 1st experiment □ 2nd experiment

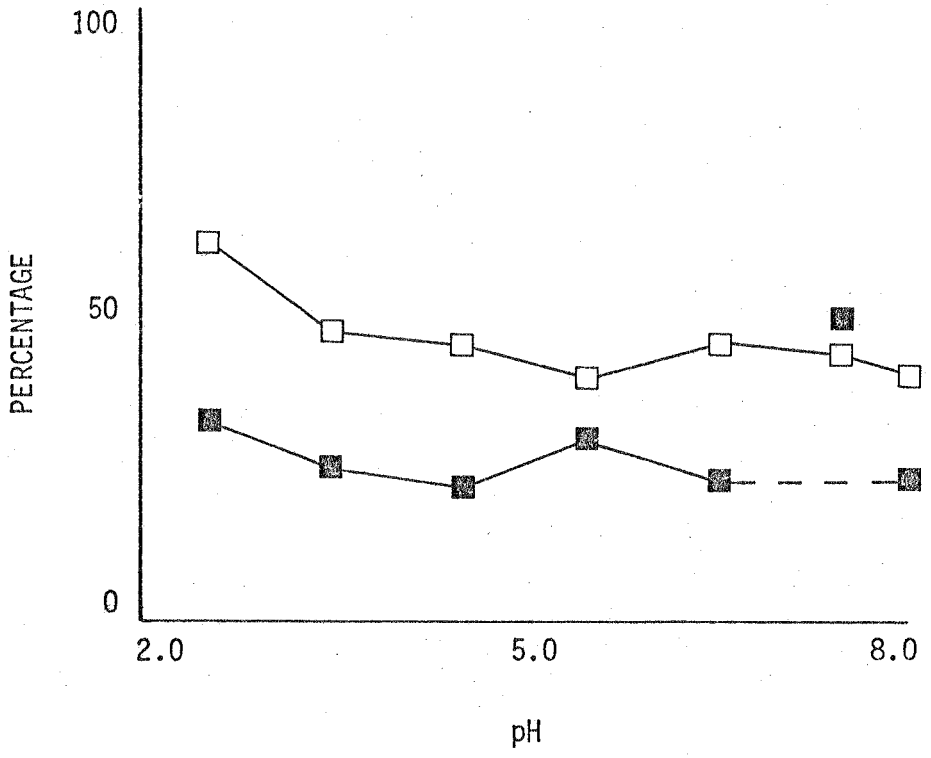


Fig. 16b Effect of pH on eclosion failure of *D. siricidicola* (H) at 20°C

■ 1st experiment □ 2nd experiment

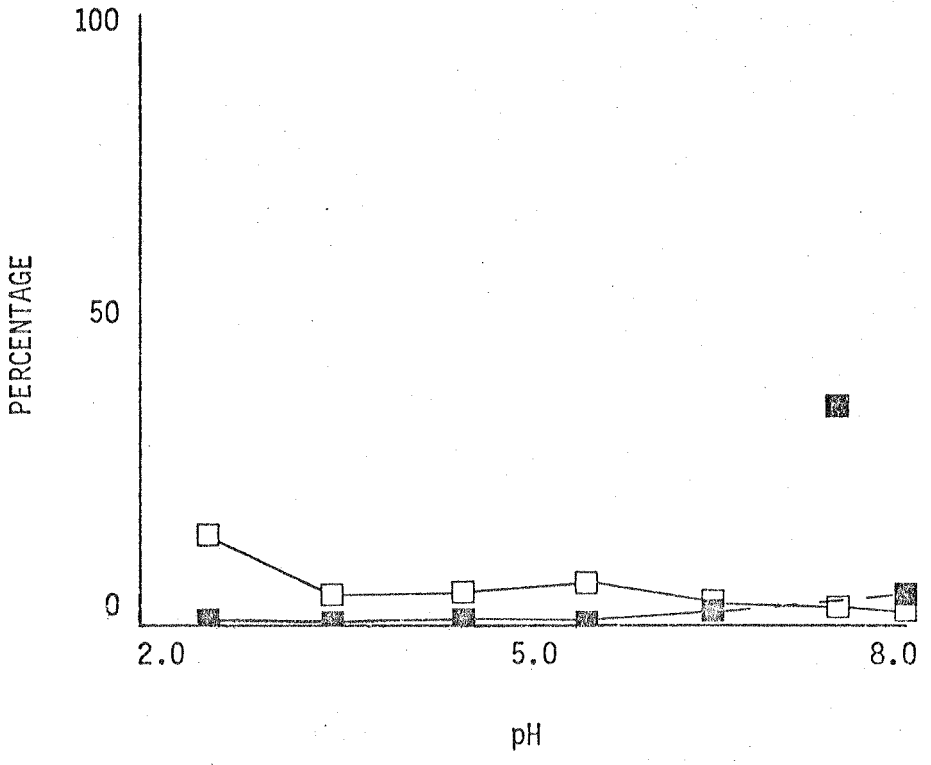


Table 20 Effect of pH on abortion of *D. siricidicola* (S). The values quoted for % abortion are the means of 5 replicates of about 200 eggs each.

pH	1st experiment		2nd experiment	
	% abortion	s	% abortion	s
8.0	99.0	1.1	92.5	0.8
7.5	97.3	1.4	96.7	0.3
6.5	65.4	0.1	48.4	0.1
5.5	53.0	0.1	47.3	0.5
4.5	40.1	0.4	39.5	0.1
3.5	42.0	0.1	45.8	0.1
2.5	67.9	0.1	64.3	0.3

Table 20 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	10,018.2897	1669.7150	106.29	0.005
Blocks	4	34.1731	8.5433	0.54	ns
Error	24	377.0189	15.7091		
Total	34	10,429.4817			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	8129.0480	1354.8413	124.73	0.005
Blocks	4	20.1617	5.0404	0.46	ns
Error	24	260.6863	10.8619		
Total	34	8409.8960			

Table 21 Effect of pH on eclosion failure of *D. siricidicola* (S) eggs ... 2nd experiment. Values quoted for % eclosion failure are the means of 5 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of % eclosion failure.

pH	Number of eggs which completed development	Eclosion failure (%)	s
8.0	347	79.4	2.7
7.5	436	93.0	0.7
6.5	512	12.4	0.4
5.5	514	9.8	0.3
4.5	555	6.2	0.1
3.5	566	8.6	0.2
2.5	464	26.8	0.7

Table 21 (continued)

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	18,021.4029	3003.5672	126.82	0.005
Blocks	4	78.5755	19.6439	0.83	ns
Error	24	568.4085	23.6837		
Total	34	18,668.3869			

Fig. 17a Effect of pH on abortion of *D. siricidicola* (S) at 20°C.
▲ 1st experiment △ 2nd experiment

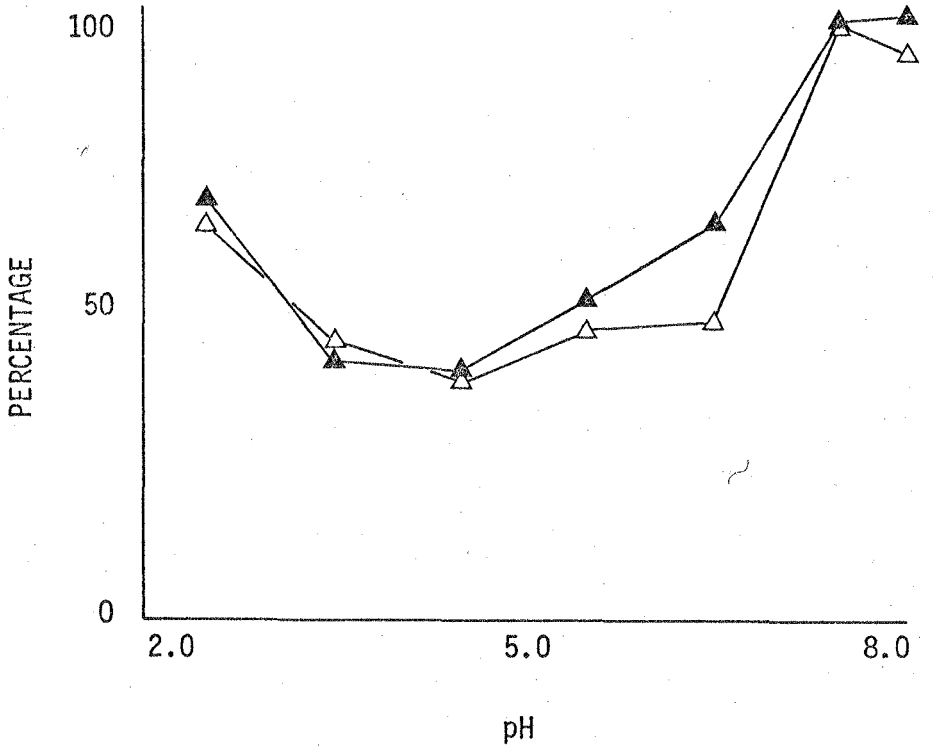


Fig. 17b Effect of pH on eclosion failure of *D. siricidicola* (S) at 20°C.

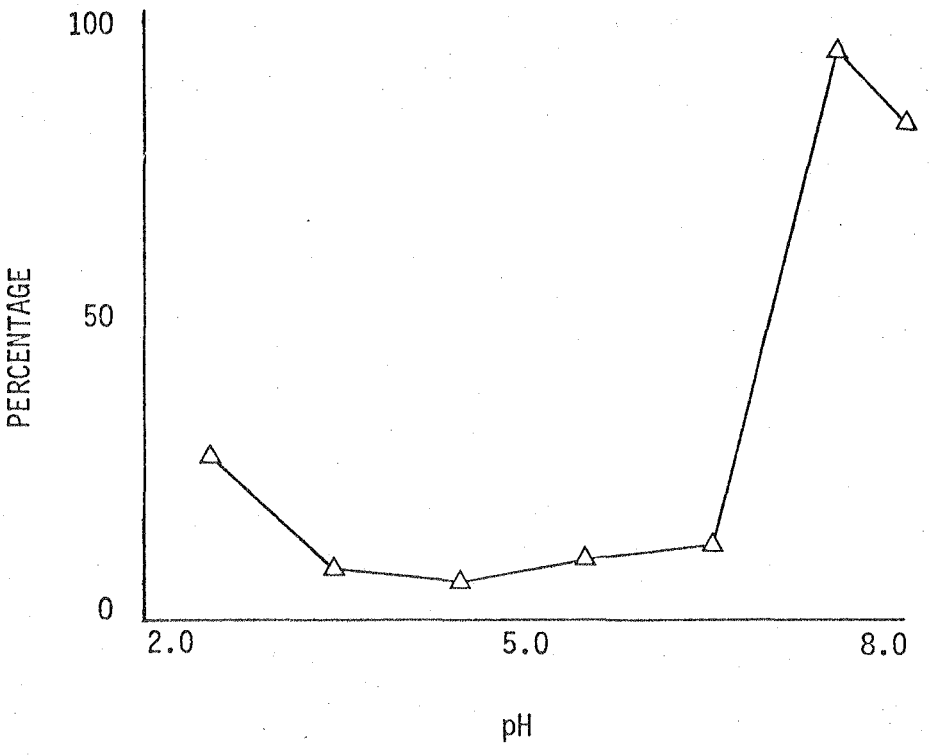


Table 22 Effect of pH on abortion of *D. rudyi* (T) at 20°C. The values quoted for % abortion are the means of 5 replicates of about 70 eggs each.

	1st experiment		2nd experiment	
pH	% abortion	s	% abortion	s
8.0	-	-	45.3	0.1
7.5	-	-	41.4	1.2
6.5	21.8	0.4	38.5	0.5
5.5	16.8	0.6	31.1	1.9
4.5	14.8	0.8	37.2	0.3
3.5	20.0	2.4	53.1	0.8
3.0	30.6	0.3	-	-
2.5	43.4	0.3	90.6	0.4

Table 22 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	5	1202.6667	240.5333	8.42	0.005
Blocks	4	62.3334	15.5834	0.55	ns
Error	20	571.6666	28.5833		
Total	29	1836.6667			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	4967.1309	827.8552	34.0	0.005
Blocks	4	105.4355	26.3589	1.08	ns
Error	24	584.3805	24.3492		
Total	34	5656.9469			

Table 23 Effect of pH on abortion of *D. rudyi* (T) at 25°C. The values quoted for % abortion are the means of about 70 eggs each.

pH	% abortion	s
8.0	83.5	0.8
7.5	71.0	1.7
6.5	54.9	3.3
5.5	45.1	2.4
4.5	52.1	2.9
3.5	95.2	1.7
2.5	98.6	2.7

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	7699.3714	1283.2286	50.74	0.005
Blocks	4	1446.6857	361.6714	14.30	0.005
Error	24	606.9143	25.2881		
Total	34	9752.9714			

Table 24 Effect of pH on eclosion failure of *D. rudyi* (T) eggs at 20°C. Values quoted for % eclosion failure are the means of 5 replicates. Only eggs which completed development (i.e. attained full larval form) were considered in the calculation of % eclosion failure.

pH	1st experiment			2nd experiment		
	Number of eggs which completed development	Eclosion failure (%)	s	Number of eggs which completed development	Eclosion failure (%)	s
8.0	-	-	-	176	12.4	0.6
7.5	-	-	-	157	6.3	0.1
6.5	188	12.9	0.4	163	2.2	2.3
5.5	163	2.4	3.6	188	3.3	1.2
4.5	162	4.2	1.7	170	3.7	1.5
3.5	176	7.5	2.9	139	11.9	0.6
3.0	202	14.7	0.9	-	-	-
2.5	180	29.1	0.6	45	41.1	2.8

Table 24 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	5	1962.4097	392.4819	6.49	0.005
Blocks	4	133.7554	33.4389	0.55	ns
Error	20	1209.2686	60.4634		
Total	29	3305.4337			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
pH	6	3891.6600	648.6100	13.32	0.005
Blocks	4	36.1218	9.0305	0.19	ns
Error	24	1168.5542	48.6898		
Total	34	5096.3360			

Fig. 18a Effect of pH on abortion of *D. rudyi* (T)
● 1st experiment, 20°C; ○ 2nd experiment, 20°C; ◆ 25°C.

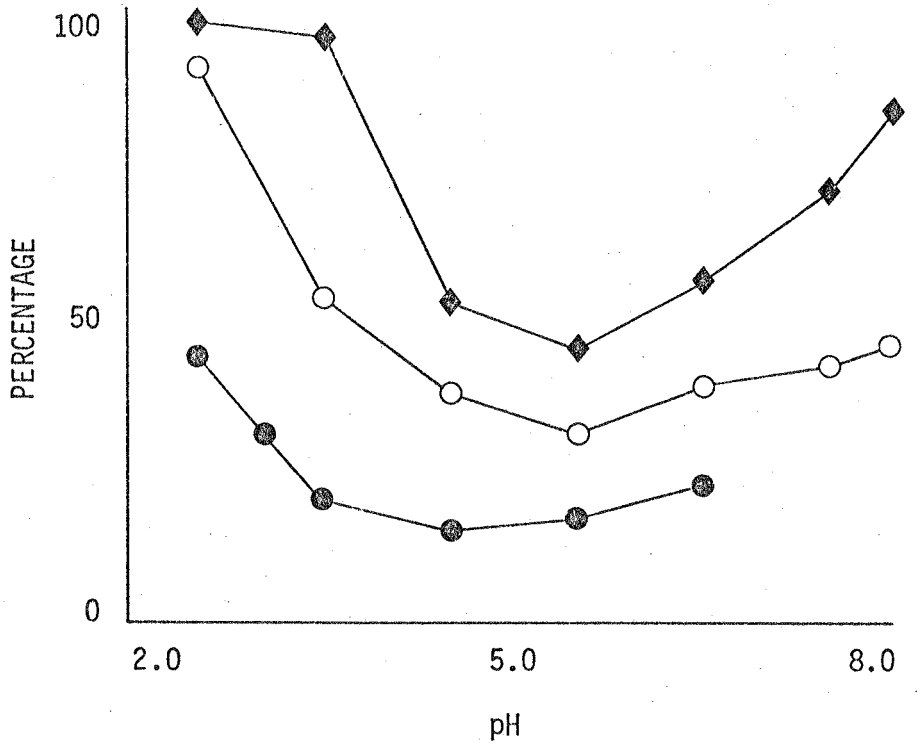
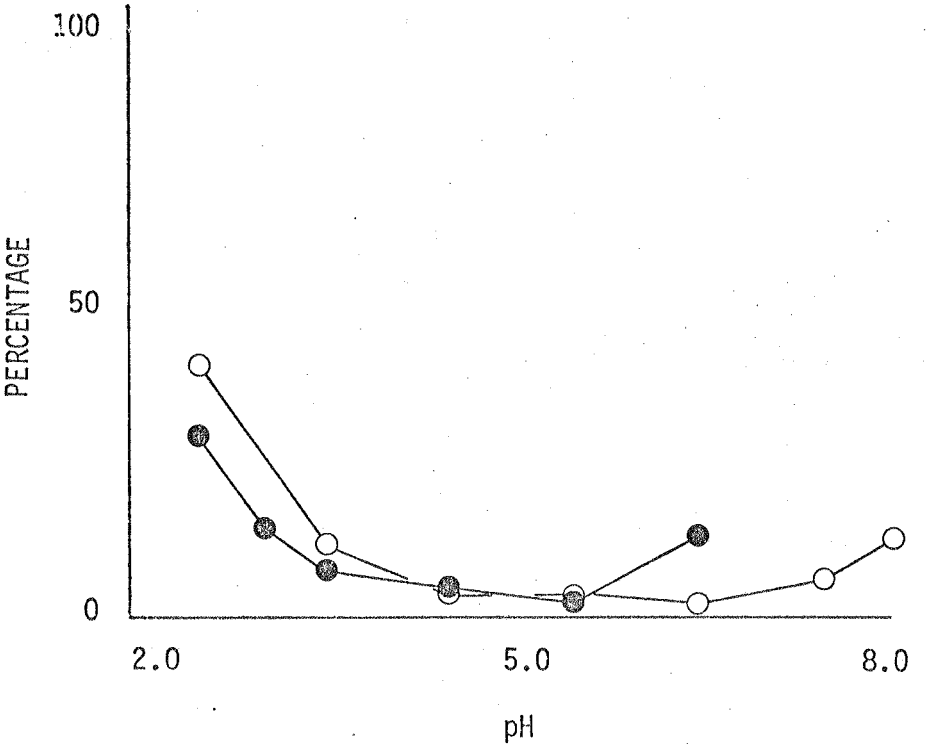


Fig. 18b Effect of pH on eclosion failure of *D. rudyi* (T)
● 1st experiment, 20°C; ○ 2nd experiment, 20°C.



3.3 Oviposition Rate and Fecundity.

In no instance was there a significant difference in egg mortality with age of the parent. It is possible that survival among the first eggs deposited was low but so few eggs were deposited on the first day (usually less than 10 during the 4-5 hours allowed) that this possibility could not be properly examined without further experimentation using much larger numbers of newly matured females.

Since there was no significant variation in mortality with the age of the parent, the rate of oviposition/age and rate of oviposition of viable eggs/age curves are, to all intents and purposes, the same shape. Since the latter curve could be used to estimate fecundity, only the data relating to viable eggs are summarised and the results of their analyses presented in the text (Tables 25-28). Data on the total number of eggs produced are presented in Appendix B.

The rate of oviposition/age curves for *D. siricidicola* (H) and *D. siricidicola* (S) (when constructed by the second method in which each nematode was used repeatedly) showed a skewed normal distribution with the maximum rate occurring early in the reproductive life of the nematodes (Figs 21, 22). When the curve for *D. siricidicola* (S) was constructed by the first method, the distribution showed almost no tailing (Figs 19, 20). The rate of oviposition/age curve for *D. rudyi* (T) cultured on CMA showed a maximum at 4 days with a minor peak at 14 days (Fig. 23). The curve for *D. rudyi* (T) cultured on ¼ PDA was less well defined and indicated maximum rate of oviposition at 10 days with possibly

a minor peak at 3 days (Fig. 23). Although the curves for *D. rudyi* (T) presented here differ from those of the *D. siricidicola* populations, some of the rate of oviposition/age curves for individual cultures of *D. siricidicola* (S) had two peaks. Since the minor peaks shown by the *D. siricidicola* (S) cultures did not coincide, the second peak was lost when the mean rate of oviposition was graphed.

The second method, used only for *D. siricidicola* (S), proved unsatisfactory. Whereas very few *D. siricidicola* (S) females died before 10 days when the first method was used, use of the second method resulted in considerable mortality after only 8 days. The second method also produced a much lower estimate of fecundity.

The reproductive life span of *D. siricidicola* (S) was only about two-thirds that of the other two populations. However, the maximum rate of oviposition attained by *D. siricidicola* (S) was greater than that attained by either of the other populations. The mean number of progeny produced by *D. siricidicola* (H) was less than that of *D. siricidicola* (S) despite the longer reproductive life and lower abortion rate of the former.

As the nematodes aged, they migrated less over the surface of the IA plate and so some estimate of variation between individuals was obtained in the latter stages of the experiments. The variation in the number of eggs deposited during the periods of observation (4-5 hours) was considerable and it was notable that even some old females deposited a large number of eggs during this period (Fig. 24). The decline in the mean rate of oviposition with increasing age appeared to be due to an increasing number of females ceasing oviposition rather than to longer intervals between periods of oviposition with few females completely ceasing oviposition.

Table 25 Fecundity of *D. siricidicola* (S) estimated by the first method described. Values quoted for oviposition rate are the means of 5 replicates of 10 females ovipositing on ionagar for either 4 or 5 hours.

Age of nematodes (days after final moult)	1st experiment Number of viable eggs[1]/♀/hr	s	2nd experiment Number of viable eggs/♀/hr	s
1	0.02	0.01	0.3	0.1
2	0.7	0.2	1.5	0.1
3	1.0	0.4	2.5	0.2
4	2.3	0.4	3.1	0.2
5	2.3	0.5	-	-
6	2.4	0.5	-	-
7	1.7	0.7	2.9	0.5
9	1.6	0.4	2.2	0.8
10	1.2	0.5	-	-
11	0.9	0.1	-	-
12	1.0	0.6	0.6	0.3
13	0	0	0	0
Estimated number of larvae produced by one female	400		550	

[1] Viable eggs are those which hatched.

Table 25 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Ages[1]	10	27.7507	2.7751	17.1302	0.005
Cultures	4	1.9500	0.4875	3.0093	0.05
Error	40	6.4782	0.1620		
Total	54	36.1789			

Analysis of variance data (2nd experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Ages[1]	6	36.3802	6.0634	37.2445	0.005
Cultures	4	0.2721	0.0680	0.4177	ns
Error	24	3.9077	0.1628		
Total	34	40.5600			

[1] The data for the 13th day were not included in the analyses.

Fig. 19 Production of viable eggs by *D. siricidicola* (S) determined by using each nematode once only. 1st experiment.

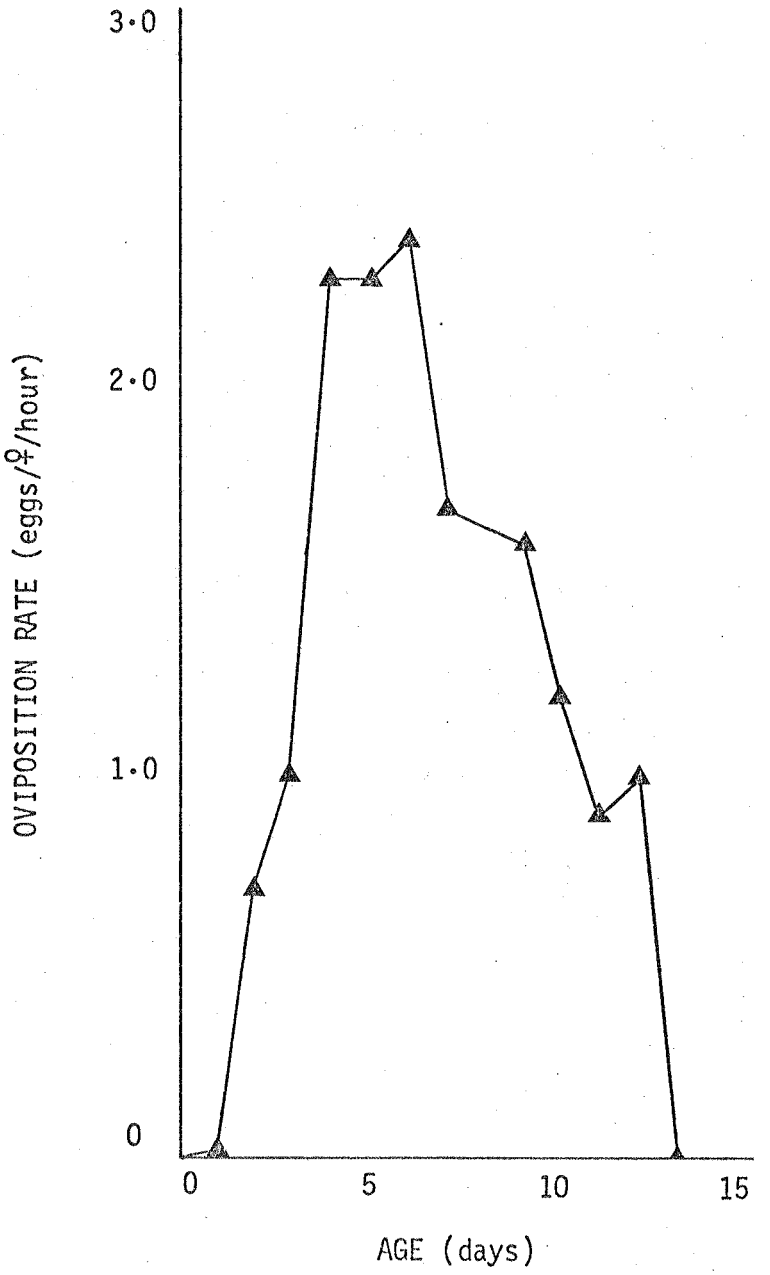


Fig. 20 Production of viable eggs by *D. siricidicola* (S) determined by using each nematode once only. 2nd experiment.

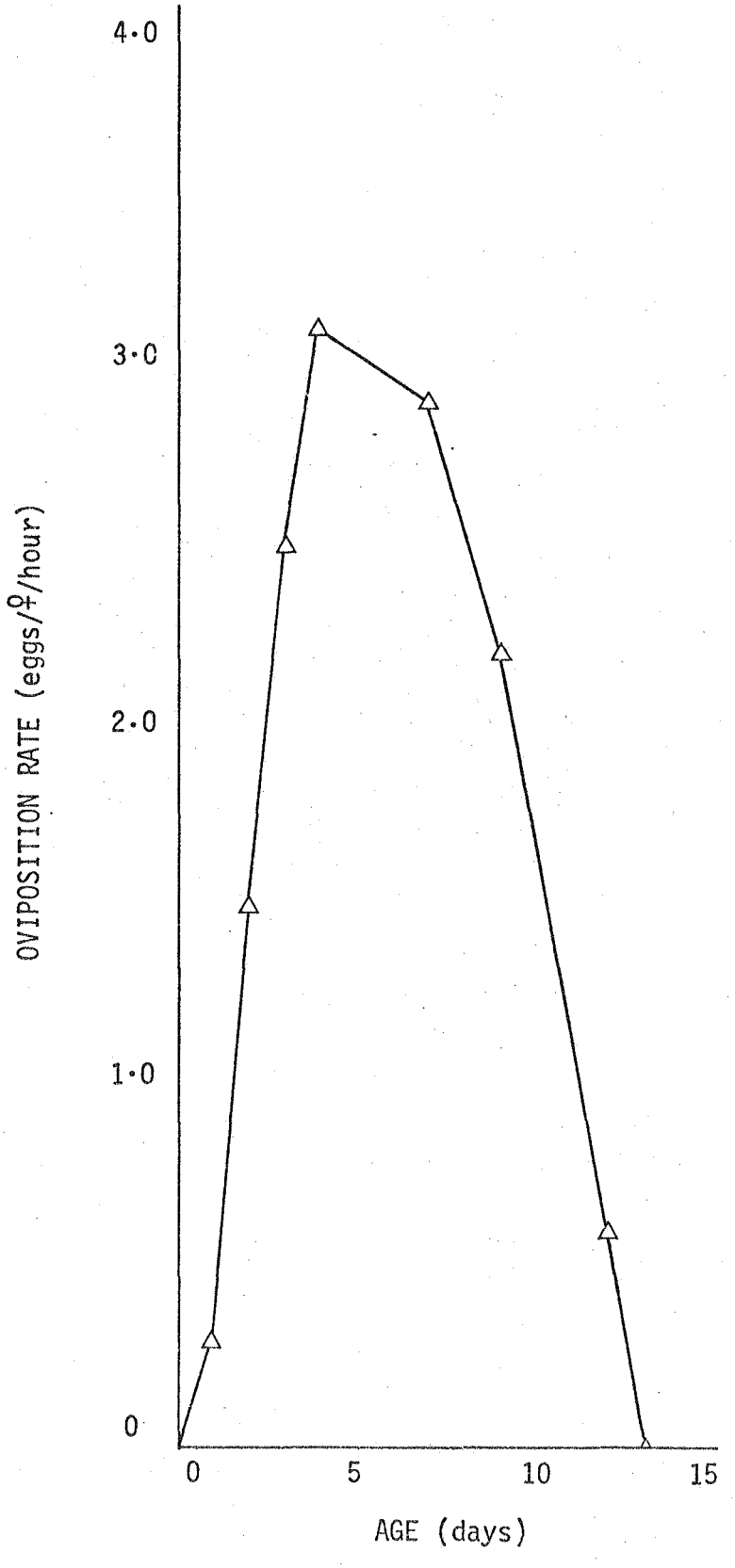


Table 26 Fecundity of *D. siricidicola* (S) estimated by the second method described. Values quoted for oviposition rate are the means of 5 replicates of 10 females ovipositing on ionagar for either 4 or 5 hours.

Age of nematodes (days after final moult)	Number of viable eggs/♀/hr	s
1	0.06	0.03
2	0.6	0.2
3	1.6	0.3
4	2.0	0.5
5	1.5	0.4
6	1.0	0.4
7	0.6	0.3
8	0.4	0.3
9	0.4	0.2
10	0.3	0.1
11	0.3	0.3
12	0.2	0.2
Estimated number of larvae produced by one female	200	

Table 26 (continued)

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
Ages	11	21.1702	1.9246	33.2401	0.005
Cultures	4	1.5645	0.3911	6.7547	0.005
Error	44	2.5476	0.0579		
Total	59	25.2823			

Fig. 21 Production of viable eggs by *D. siricidicola* (S) using each nematode repeatedly.

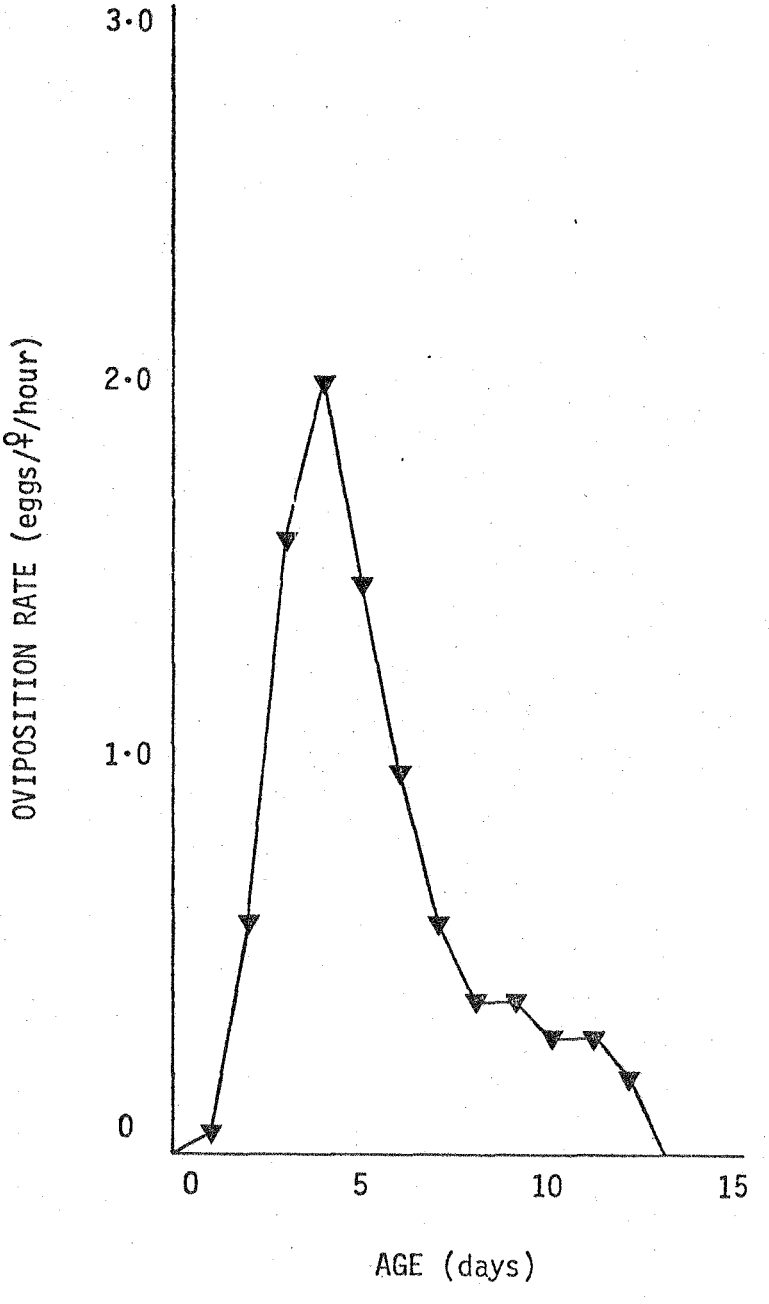


Table 27 Fecundity of *D. siricidicola* (H) estimated by the first method described. Values quoted for oviposition rate are the means of 5 replicates of 10 females ovipositing on ionagar for either 4 or 5 hours.

Age of nematodes (days after final moult)	1st experiment Number of viable eggs/♀/hr	s	2nd experiment Number of viable eggs/♀/hr	s
1	0.02	0.02	0.03	0.03
2	0.1	0.1	-	-
3	-	-	0.5	0.2
4	-	-	0.6	0.3
6	1.0	0.3	-	-
7	-	-	1.1	0.3
8	1.0	0.2	-	-
9	-	-	1.1	0.2
10	0.9	0.4	-	-
11	-	-	1.0	0.2
13	0.5	0.2	-	-
14	-	-	0.6	0.2

Table 27 (continued)

Age of nematodes (days after final moult)	1st experiment Number of viable eggs/♀/hr	s	2nd experiment Number of viable eggs/♀/hr	s
15	0.4	0.2	-	-
16	-	-	0.3	0.2
17	0.4	0.2	-	-
18	-	-	0.3	0.1
20	0.1	0.1	-	-
21	-	-	0.1	0.1
22	0.05	0.05	-	-
23	-	-	0.1	0.1
Estimated number of larvae produced by one female	250		300	

Table 27 (continued)

Analysis of variance data (1st experiment)

Source of variation	df	Sum of Squares	Mean Squares	F	P
Between ages	9	7.1962	0.7996	19.50	0.005
Between cultures	4	0.3000	0.0750	1.83	ns
Error	36	1.4762	0.0410		
Total	49	8.9724			

Analysis of variance data (2nd experiment) (1-16 days only[1])

Source of variation	df	Sum of Squares	Mean Squares	F	P
Between ages	7	4.9807	0.7115	21.56	0.005
Between cultures	4	0.6671	0.1668	5.05	0.005
Error	28	0.9233	0.0330		
Total	39	6.5711			

[1] On the 18th day one of the cultures was contaminated and had to be discarded, so for the 18th, 21st and 23rd days there were only four cultures in use.

Fig. 22 Production of viable eggs by *D. siricidicola* (H).

■ 1st experiment. □ 2nd experiment.

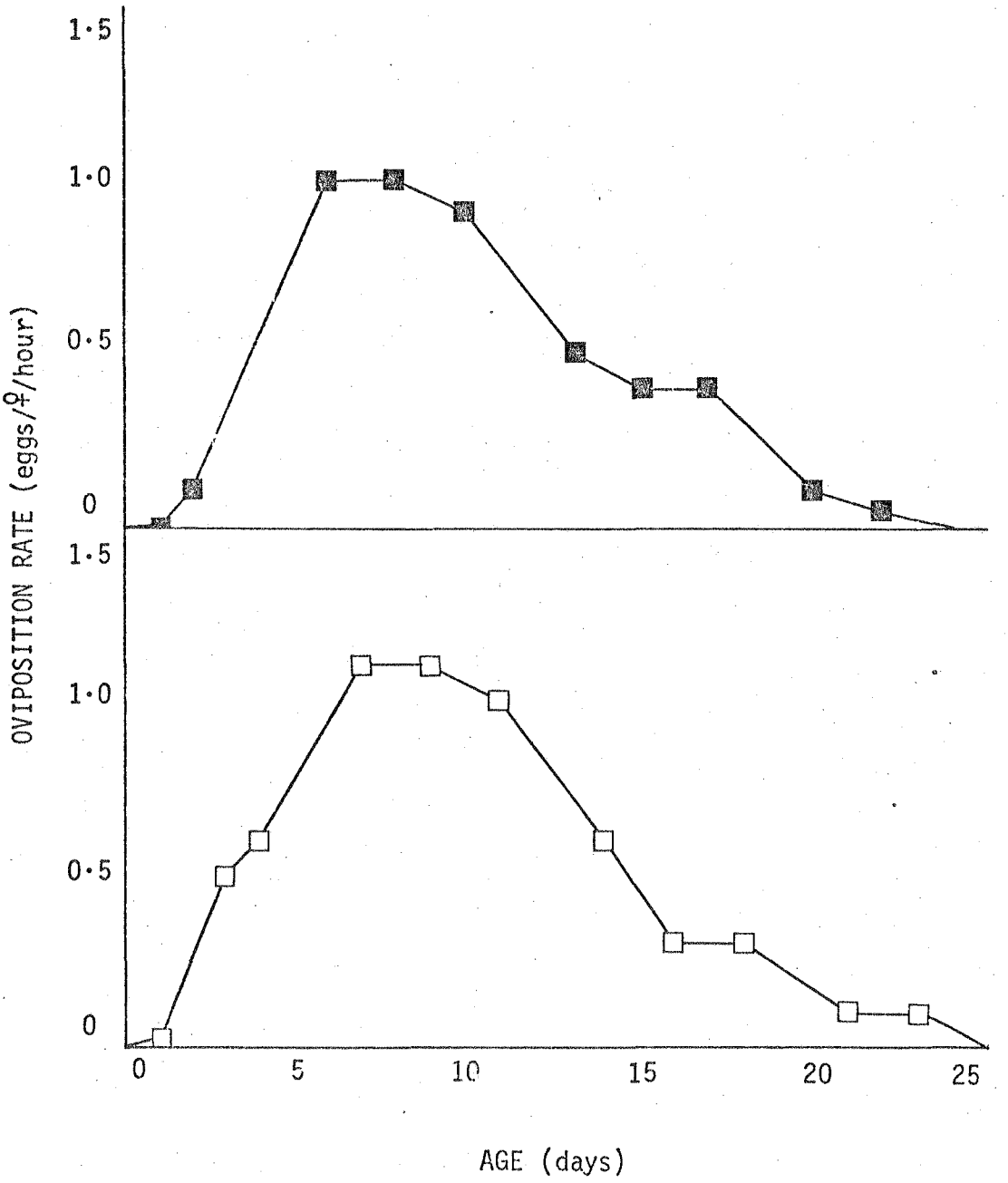


Table 28 Fecundity of *D. rudyi* (T). Values quoted for oviposition rate are the means of 3 replicates of 10 females ovipositing on ionagar for either 4 or 5 hours.

Age of nematodes (days after final moult)	CMA Number of viable eggs/♀/hr	s	¼ PDA Number of viable eggs/♀/hr	s
2	0.3	0.10	0.04	0.03
3	1.1	0.2	0.3	0.05
4	2.1	0.2	0.1	0.1
6	1.5	0.3	0.4	0.2
7	1.3	0.6	0.3	0.1
8	1.3	0.2	0.4	0.2
10	0.4	0.2	0.6	0.4
11	0.1	0.03	0.3	0.2
14	0.8	0.3	0.2	0.1
16	0.3	0.2	0.1	0.03
21	0.1	0.1	0.1	0.1
23	0	0	0.03	0.01
25	-	-	0	0
Estimated number of larvae produced by one female	350		110	

Table 28 (continued)

Analysis of variance data

Source of variation	df	Sum of Squares	Mean Squares	F	P
Between ages	11	7.1192	0.6472	13.89	0.005
Between media	1	6.0278	6.0278	129.35	0.005
Media/ages	11	6.2164	0.5651	12.13	0.005
Error	43	2.0027	0.0466		
Total	66	21.3661			

Fig. 23 Production of viable eggs by *D. rudyi* (T). ● CMA ○ ¼ PDA

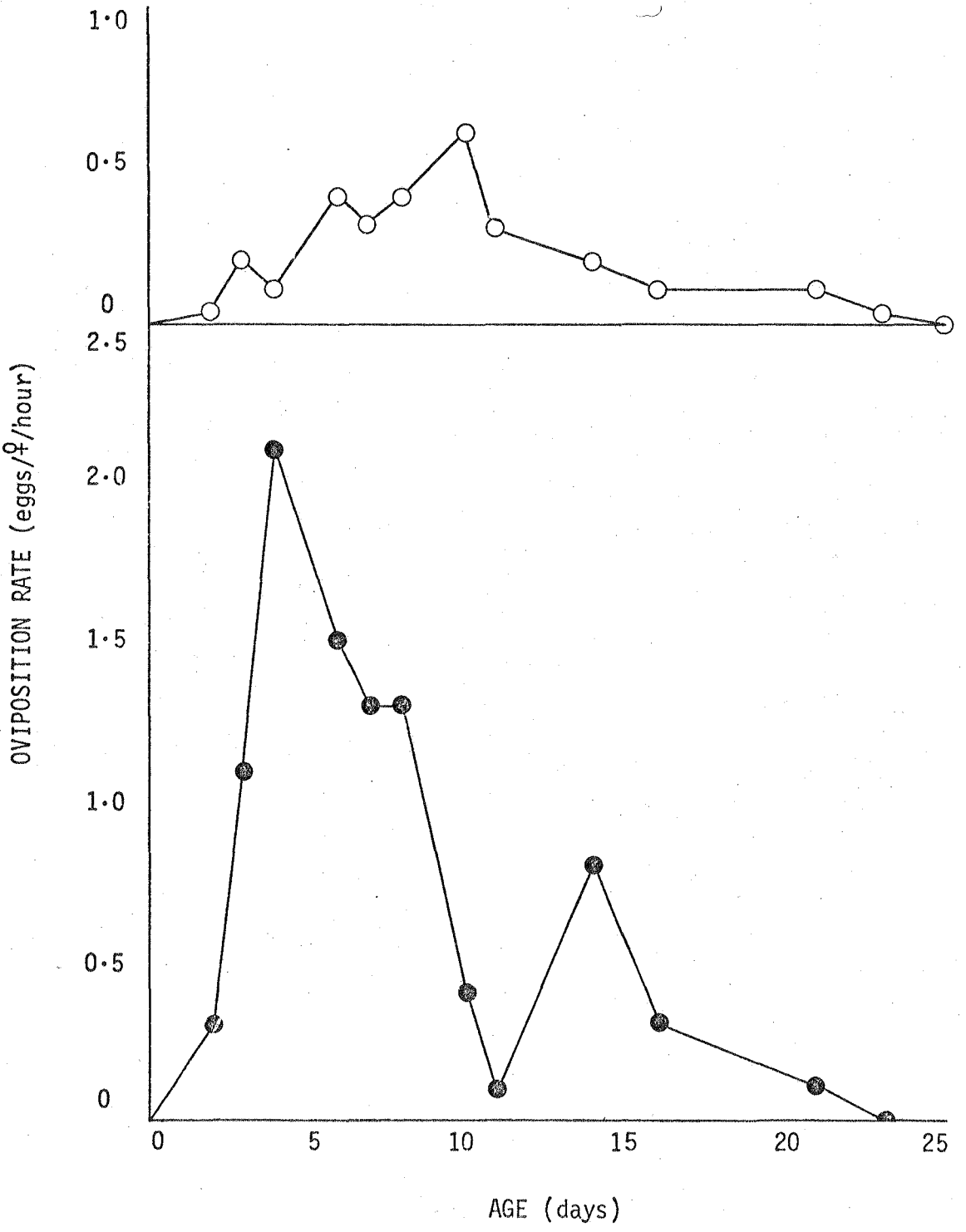
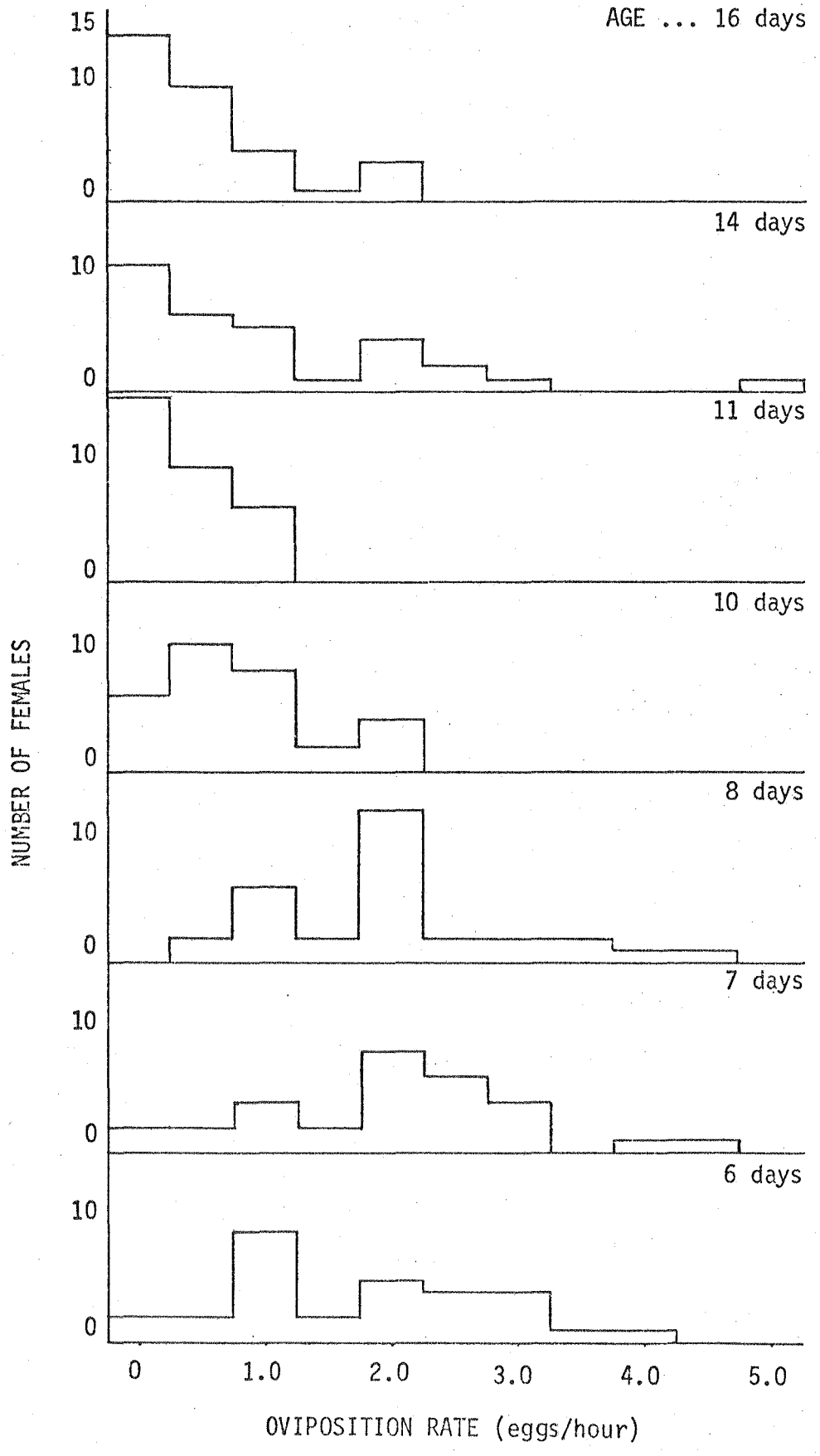


Fig. 24 Variation in oviposition rate amongst individual *D. rudyi* (T) cultured on CMA.



3.4 Egg Size.

The mean volume of eggs of each population was calculated from measurement of 300 eggs. Analysis of the data showed that the difference between each population was highly significant ($p<0.001$) with *D. siricidicola* (H) having the largest and *D. rudyi* (T) the least mean volume. There was no difference in the variance of the two *D. siricidicola* populations but the variance of *D. rudyi* (T) was considerably less than that of *D. siricidicola*. The data are summarised in Table 29 and Fig. 25.

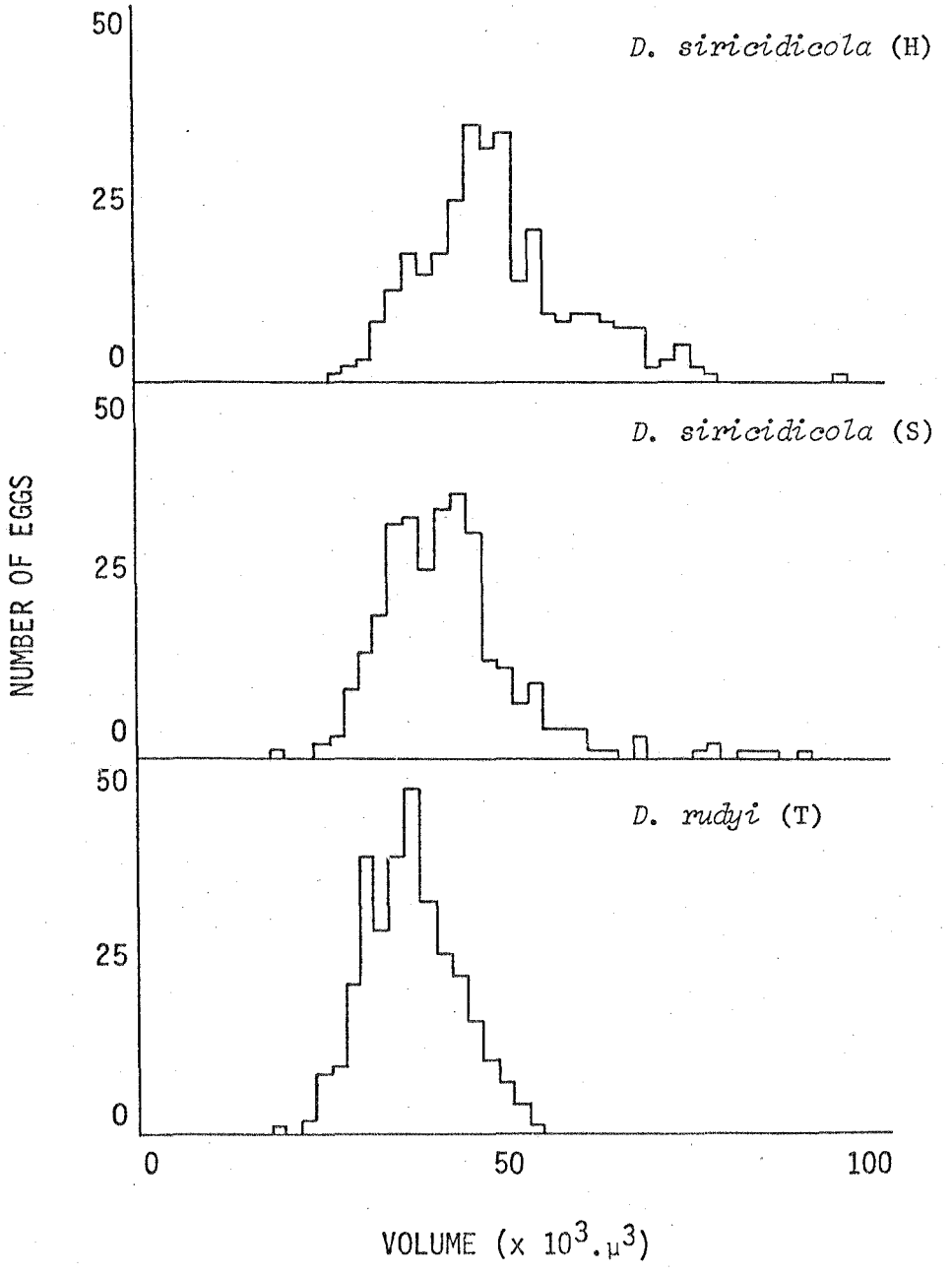
Table 29 Mean volume of eggs of *D. siricidicola* and *D. rudyi*. Values were obtained from measurements on 300 eggs of each population. Differences were analysed by t-tests.

	D. siricidicola (H)	*D. siricidicola* (S)	*D. rudyi* (T)
Mean Volume (μ^3)	48.63	41.98	36.50
s^2	104.74	94.55	36.78

D. siricidicola (H) / *D. siricidicola* (S)	t = 8.166 ***
D. siricidicola (H) / *D. rudyi* (T)	t = 17.651 ***
D. siricidicola (S) / *D. rudyi* (T)	t = 8.286 ***

Over 98% of the eggs examined conformed to the model (Fig. 4). The remainder were pear-shaped; none of these was recorded as hatching successfully and very few showed any signs of development.

Fig. 25 Size distribution of *D. siricidicola* and *D. rudyi* eggs.



3.5 Hybridisation.

More than 1,000 inter- and intraspecific single pair crosses, were conducted and these produced the wide range of results categorised in Table 30. With one exception, there was no increase in the mortality of eggs deposited by either the parental or first filial generation when the parents were members of the same morphological species. Interspecific crosses, on the other hand, frequently resulted in more than 95% mortality of the eggs deposited by the parents and, with only one exception, among those deposited by the hybrids (Table 31). The exceptions were crosses between the Turkish and a Japanese (UJ7) population of *D. rudyi* and between the latter and *D. imperialis* where the mortality of eggs deposited by the hybrids was high though less than 95% (Table 32). Although 85% of the eggs deposited by the UJ7 ♂ x *D. imperialis* ♀ hybrids aborted, a culture of these hybrids was easily maintained and the succeeding generations monitored. Over 10 generations, the mortality of the eggs of the UJ7 ♂ x *D. imperialis* ♀ hybrids decreased to 70% and that of the *D. rudyi* (Turkish) x UJ7 hybrids from 60% to 30%.

None of the nematodes used proved to be parthenogenetic. Only 20% of the control (unmated) females deposited eggs; none deposited more than 10 and all such eggs failed to develop.

Replicates often produced different results but these were invariably slight and attributable to variation in the fungal growth rate. In intraspecific crosses there appeared similar, though sometimes greater, differences between the within-culture and between-culture crosses which were due to the earlier

Table 30 Categorisation of results obtained from inter- and intraspecific cross-breeding of *Deladenus* spp.

Category of result	Eggs laid by original female	F_1 larvae	Eggs laid by F_1 females	F_2 larvae
A	Few[1]			
B	Few	Few[4]		
C	Normal[3]	Few		
D	Normal	Few	Few	Few or none
E	Normal	Few	Intermediate[2]	Few
F	Normal	Normal[6]	Intermediate	Few
G	Normal	Normal	Normal	Few
H	Normal	Normal	Normal	Intermediate[5]
I	Normal	Normal	Normal	Normal

1 <20 eggs/♀

2 20-150 eggs/♀

3 >150 eggs/♀

4 <5% eggs hatching

5 10-45% eggs hatching

6 >75% eggs hatching

Table 31 Results of inter- and intraspecific cross-breeding of *Deladenus* spp.

Female	Male						
	D. siricidicola	*D. wilsoni*	*D. rudyi*	*D. imperialis*	*D. proximus*	*D. nevexii*	*D. canii*
D. siricidicola	I	E	C	A	G	B	C
D. wilsoni	C	I	D	C	B	D	D
D. rudyi	C	C	I	C≠	B	C	C
D. imperialis	A	B	F*	I	F	E	F
D. proximus	F	B	B	D	I	F	D
D. nevexii	B	C	E	D	F	I	G
D. canii	C	B	C	F	D	F	I

≠ G When *D. rudyi* (UJ7) was used.

* H When *D. rudyi* (UJ7) was used.

commencement of oviposition (and consequently feeding) in the former creating a difference in fungal growth rate. This difference was accentuated in interspecific crosses and particularly in the cross *D. imperialis* ♂ x *D. rudyi* (T) ♀ where oviposition did not commence for several days. Differences between reciprocal crosses (i.e. species A ♂ x species B ♀ and species B ♂ x species A ♀) were also invariably due to different fungal growth rates resulting from the earlier commencement of oviposition by one group or the other.

The use of two fungi on the same plate proved unsuccessful because of their mutually antagonistic reaction which severely hampered the growth of either. However, conducting the cross on the fungus preferred by the female proved satisfactory.

Table 32 Viability of eggs deposited by F_1 hybrids of *D. rudyi* (Turkish) and UJ7 and of *D. imperialis* and UJ7.

Mating pair		Experiment 1		Experiment 2	
Male	Female	No. eggs	% hatch	No. eggs	% hatch
D. rudyi	*D. rudyi*	154	87	420	86
UJ7	UJ7	482	95	273	93
UJ7	*D. rudyi*	443	44	273	40
D. rudyi	UJ7	189	44	536	43
D. imperialis	*D. imperialis*	541	92	234	84
UJ7	UJ7	157	90	982	95
UJ7	*D. imperialis*	305	14	134	15
D. imperialis	UJ7	80	3	172	0

DISCUSSION

For each of the three populations of *Deladenus* studied, abortion rate and eclosion failure were related to temperature. The nature of the relationships, however, varied considerably from one population to the next. Abortion and eclosion failure of *D. siricidicola* (H) were only weakly affected by temperatures in the range 5°-25°C (Fig. 10) whereas for both other populations these parameters were strongly affected, albeit at opposite ends of the temperature scale (Figs 11, 12). Similar results were obtained from the experiments on the effect of pH on these parameters; in these experiments high levels of abortion and eclosion failure were recorded at the extremes for *D. siricidicola* (S) and *D. rudyi* (T) while *D. siricidicola* (H) was little affected (Figs 16, 17, 18).

D. siricidicola (H) differed from the other two populations in having been maintained continuously in monoxenic culture for a much longer period (6 years) than either of the others (1 year). It is possible that the improvement in the rate of reproduction over the first few months of monoxenic culturing (Bedding & Akhurst, 1974) coincides with selection for increased survival over a wider range of conditions. The cultures were maintained at 24°C and selection for increased survival at this temperature might be expected to change the shape of the *D. rudyi* (T) abortion/ temperature curve (Fig. 12) to one more closely resembling that of *D. siricidicola* (H) (Fig. 10). This change need not have been completed within the time of obvious improvement in reproduction since cultures in which 70% of the eggs were abortive (e.g.

D. rudyi (UJ7) / *D. imperialis*) appeared to have almost as high a reproductive rate as those in which less than 10% were abortive. Moreover, although the culture medium was pH 5·6 when prepared initially, its buffering capacity was low and so *Deladenus* in monoxenic cultures were almost certainly exposed to a range of pH conditions which could have allowed selection for increased survival throughout this range. The possibility of such selection might be explored by the re-examination of *D. rudyi* (T) after several years of continuous monoxenic culture.

Studies of the effect of pH on nematode eggs have often been contradictory because of the nature of the buffer solutions used (Shepherd & Clark, 1972). The use of a number of buffers in very low concentration (e.g. Loewenberg *et al.*, 1960) or examination of the role of inorganic ions used (e.g. Lehman, 1969) is essential for establishing the role of pH. Since neither action was taken in this study, the effects noted cannot be definitely attributed to pH. However this study was useful in establishing standard conditions for the study of the effects of temperature and revealing differences between the three populations of *Deladenus* studied.

Selecting conditions for studying the effects of temperature involved some compromises in the choice both of buffer and the method of collecting newly-deposited eggs. Though not optimal for *D. siricidicola* (S), the pH 5·5 buffer was chosen as standard for the study of temperature effects. The pH 4·5 buffer (optimal for *D. siricidicola* (S)) was not used because more care in preparation was required to prevent acid hydrolysis of the agar with this, rather than the pH 5·5 buffer and differences in rate of egg development and abortion of

D. siricidicola (S) recorded for the pH 4·5 and pH 5·5 buffers were small. For most experiments newly deposited eggs were collected by washing nematodes and eggs from culture plates, isolating the females in sterile Ringer's Solution, and 4-5 hours later isolating the newly-deposited eggs. Although the alternative method, allowing females to oviposit directly onto the agar, consistently produced a lower abortion rate, it yielded much fewer eggs. Despite the higher abortion rate resulting from use of the former method, the total number of eggs hatching was far in excess of the number hatching when collected by the latter method.

The differences between the responses of the two *D. siricidicola* populations are unlikely to be the result of choosing sub-optimal conditions for *D. siricidicola* (S). Although pH 5·5 was not optimal for successful hatching of *D. siricidicola* (S), it did not produce consistently slower development of the eggs than pH 4·5 and so the consistently higher TH_{50} values (Table 6) recorded for *D. siricidicola* (S) more than likely reflect differences between this population and *D. siricidicola* (H). It might perhaps be argued that the different shapes of the abortion rate/temperature curves (Figs 10, 11) suggest that the compounding effects of stress temperatures and the stress caused by sub-optimal pH produced the greater reaction by *D. siricidicola* (S). This is most unlikely since pH 5·5 produced only a slightly greater abortion rate than pH 4·5. Whether the differences between the two populations are due to the longer period of culture of *D. siricidicola* (H), or whether the original populations were so different that they might be considered different strains, cannot be resolved without

examination of either the wild populations or, possibly, *D. siricidicola* (S) after it had been maintained in continuous monoxenic culture for a considerable period. However, since it seems unlikely that continuous culturing at 24°C would result in selection for increased survival at 5°C, the *D. siricidicola* (H) and *D. siricidicola* (S) cultures can be considered as different strains.

Like *Panagrellus redivivus* (L.) (Lower *et al.*, 1968), *Meloidogyne hapla* and *M. javanica* (Daulton & Nusbaum, 1961), *D. siricidicola* can adapt to extreme temperatures. Cultures of *D. siricidicola* have been maintained at 30°C although eggs deposited by nematodes of this species cultured at 24°C are unable to complete development at 30°C. Adaptation to 27·5°C was quite rapid; in just 3 weeks of culturing at 27·5°C (3 generations) the abortion rate of *D. siricidicola* at 27·5°C fell from 95% to 15%. Attempts to determine whether the adaptation was genetic or physiological produced inconsistent results. Although the apparently greater tolerance of eggs deposited by some individuals indicates the validity of the genetic hypothesis, these individuals may, after all, have been those able to adapt physiologically more rapidly than the rest.

The methods used in this study to determine the fecundity of *D. siricidicola* and *D. rudyi* produced estimates of the average number of progeny of a female whereas Fisher (1968, 1969) recorded the actual number of progeny of 20 female *Aphelenchus avenae*. Although both *Deladenus* spp. and *A. avenae* were cultured on fungus growing on an agar medium, Fisher's (1968) method could not be adapted for use with *Deladenus* spp. The amount of time and effort

required by Fisher's (1968) method restricted the number of nematodes used (Fisher, 1969). Adaptation of this method would have put a maximum of 20 nematodes (instead of the 400 actually used) on each culture plate. The smaller number of nematodes would not have been enough to maintain a favorable nematode/fungus balance. The greater than normal fungal growth rate, although not preventing feeding, would have resulted in a higher abortion rate and hence, an underestimate of fecundity. The magnitude of the error would have been very difficult, if not impossible, to measure. The methods used in this study gave an overestimate of fecundity because they do not allow for a correlation between longevity and rate of reproduction which Gowen (1970) suggested for *Tylenchus emarginatus*. This overestimate was probably slight however, because little mortality occurred until late in the experiments, when oviposition rate was low, and the experiments were terminated before all the nematodes had completely ceased oviposition.

The second method used to determine the fecundity of *D. siricidicola* (S) proved inadequate. Not only was the estimate of fecundity obtained by this method far below that obtained by the first method but the pattern of oviposition differed and adult mortality occurred earlier when the second method was used. These differences are almost certainly due to the more frequent handling of individual nematodes in the second method in which the same individuals were handled almost daily.

The pattern of oviposition activity of *D. rudyi* (T) (Fig. 23) apparently differs from the patterns of both *D. siricidicola* populations (Figs 19, 20, 22) in having more than one peak. However, when oviposition rate/age curves were constructed for each replicate

in the first experiment with *D. siricidicola* (S), secondary peaks were evident. These secondary peaks were not evident in the mean rate of oviposition/age curve because they occurred at different ages in different replicates. The secondary peaks may have been evident in the mean rate of oviposition/age curve for *D. rudyi* (T) because there were only three replicates and the probability of correlation was higher. Neither the mean rate/age curve nor the curve constructed from the date of a single replicate is, of course, necessarily representative of the pattern of oviposition of individual nematodes. In fact, the data on variation in oviposition rate of individual nematodes (Fig. 24) indicates that some individuals may not attain their maximum rate of oviposition until late in life.

The variation in volume recorded for the eggs of *D. siricidicola* and *D. rudyi* was not unusual since there is often considerable variation in egg size within nematode species (Bird, 1972). It is interesting to note that the only feature the *D. siricidicola* populations had in common and in contrast to *D. rudyi* (T) was the magnitude of the variance of egg volume.

Crofton & Whitlock (1965a, b) showed that egg size determines the time taken for the egg to hatch. From this it might be argued that where the variance of egg volume is small the time interval between hatching of the first and last eggs of a sample should be less than where the variance for the sample is large. While this may be true within a population, it is not necessarily true when two species, or even two strains of the same species, are compared. Although the variance of egg volume for *D. rudyi* (T) was almost a third of that for *D. siricidicola* (H) and minimum

hatching times were similar, the duration of hatching activity for *D. rudyi* (T) bore no consistent relationship to that for *D. siricidicola* (H). Similarly, though the variance of egg volume for the two *D. siricidicola* strains were not significantly different, the duration of hatching activity for these strains did not always correspond. There were obviously other factors involved (e.g. the temperature optima of the appropriate enzymes).

Since each culture was initiated with only two nematodes for the cross-breeding experiments, the fungal growth rate and, consequently, the abortion rate was supernormal. Although this reduced the fecundity of the nematodes observed, it presented no problem in interpreting the results. All species were more or less equally affected and so comparison of the within- and between-culture crosses is valid. In these experiments fecundity was not assessed by the method described in section 2.33 but by a more subjective method. The number of eggs deposited was estimated by frequent observation of the cultures and comparison of the within- and between-culture crosses. The abortion rate was determined objectively. Again interpretation presented no serious problem since cross-breeding is far more likely to affect the abortion rate than the number of eggs produced provided insemination occurs.

Clearly defined results were obtained in almost all the cross-breeding trials; intraspecific crosses did not affect the fecundity of the progeny and interspecific crosses severely affected the fecundity of the progeny but not necessarily that of the parents. Exceptions occurred with a Japanese population (UJ7) which when crossed with either *D. imperialis* females or

D. rudyi (T) produced hybrids capable of sustaining a culture despite their low fecundity. The relationship between *D. rudyi* and *D. imperialis* is uncertain; although they may merely be very closely related species, geographical considerations do indicate the possibility of a cline.

The range of results (Table 30) could be expected because some pairs will be genotypically more compatible than others; some might copulate without fertilising and others not even copulate. However some variation, particularly between within- and between-culture intraspecific crosses and between reciprocal crosses (species A ♂ x species B ♀ and species B ♂ x species A ♀), was almost certainly due to differences in the nematode/fungus balance. This balance, important in determining the success of feeding, was affected by the number of nematodes and the time at which they commenced feeding. For cross-breeding trials the most favorable balance was achieved when the mates located each other quickly, copulated readily and deposited a large number of viable eggs.

The interspecific crosses between *Deladenus* spp. almost always produced hybrids and often in no less numbers than intraspecific crosses. In contrast, crosses between *Heterodera rostochiensis* Wollenweber pathotypes have been reported (Parrott, 1972) as having produced very few hybrids and interspecific crosses within some other nematode genera (Augustine, 1939; Nigon & Dougherty, 1949; Smart & Darling, 1963) as producing no hybrids. The many progeny resulting from interspecific crosses between the *Deladenus* spp. parasitic in woodwasps indicated a close relationship between them. It was notable that only *D. wilsoni*, usually a parasite of rhyssines rather than their siricid hosts,

did not produce a large number of hybrids with any other species.

This study has indicated that continuous monoxenic culture of the type used for *Deladenus* spp. can result in considerable changes in the response of nematodes to their environment. The number of eggs produced by, and presumably the fecundity of, *D. siricidicola* (H) has apparently declined since Bedding (1972b) described females of this population (pers. comm.) as "often laying in excess of 1,000 eggs"; the experiments described in this thesis, yielded an estimate of the mean number of eggs produced by one female as 300-350. Although the responses of *D. siricidicola* (H) to temperature and pH may not necessarily have changed during long-term continuous culture, some differences between the responses of *D. siricidicola* (H) and the other two populations are possibly due to changes resulting from continuous culture.

ACKNOWLEDGEMENTS

I would like to thank Dr. J.L. Hickman and Dr. R.A. Bedding for their advice on and criticism of both the design of the study and preparation of the manuscript. Thanks are also due to Dr. D.A. Ratkowsky for advice on statistical procedures and Mrs. E. Longmore for her assistance with some of these. Not least, I would like to thank Mrs. J. Gillie for her typing and especially her patience.

I would also like to express my gratitude for the use of CSIRO time and facilities for this study.

REFERENCES

Augustine, D.L. (1939) Experimental studies on the validity of species in the genus *Strongyloides*. III Int. Cong. Microbiol., New York, 435-437.

Banyer, R.J. & Fisher, J.M. (1971) Effect of temperature on hatching of eggs of *Heterodera avenae*. *Nematologica* 17, 519-534.

Bedding, R.A. (1967) Parasitic and free-living cycles in entomogenous nematodes of the genus *Deladenus*. *Nature, Lond.* 214, 174-175.

(1968) *Deladenus wilsoni* n.sp. and *D. siricidicola* n.sp. (Neotylenchidae), entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* 14, 515-525.

(1972a) Nematode parasitism of Siricidae and their hymenopterous parasites by *Deladenus* spp. (Neotylenchidae). Proc. 13th. int. Congr. Ent., Moscow 1968, 2, 54-55.

(1972b) Biology of *Deladenus siricidicola* (Neotylenchidae) an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* 18, 482-493.

(1974) Five new species of *Deladenus* (Neotylenchidae) entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* (in press).

Bedding, R.A. & Akhurst, R.J. (1974) Use of the nematode *Deladenus siricidicola* in the biological control of *Sirex noctilio* in Australia. *J. Aust. ent. Soc.* 13, 129-135.

Bird, A.F. (1972) The Structure of Nematodes. Academic Press, New York.

Bird, A.F. & Wallace, H.R. (1965) The influence of temperature on *Meloidogyne hapla* and *M. javanica*. *Nematologica* 11, 581-589.

Crofton, H.D. (1965) Ecology and biological plasticity of sheep nematodes. I. The effect of temperature on the hatching of some nematode parasites of sheep. *Cornell Vet.* 55, 242-250.

Crofton, H.D. & Whitlock, J.H. (1965a) Ecology and biological plasticity of sheep nematodes. IV. The biological significance of temperature x time hatching curves for eggs of sheep nematodes. *Cornell Vet.* 55, 263-274.

(1965b) Ecology and biological plasticity of sheep nematodes. V. The relationship between egg volume and hatching time. *Cornell Vet.* 55, 274-279.

Daulton, R.A.C. & Nusbaum, C.J. (1961) The effect of soil temperature on the survival of the root-knot nematodes *Meloidogyne javanica* and *M. hapla*. *Nematologica* 6, 280-294.

Eriksson, K.B. (1965) Crossing experiments with races of *Ditylenchus dipsaci* on callus tissue cultures. *Nematologica* 11, 244-248.

Fenwick, D.W. (1951) Investigations on the emergence of larvae from the cysts of potato root eelworm, *Heterodera rostochiensis*. 4. Physical conditions and their influence on larval emergence in the laboratory. *J. Helminthol.* 25, 37-48.

Fisher, J.M. (1968) Fecundity of *Aphelenchus avenae* Bastian. *Aust. J. biol. Sci.* 21, 169-171.

(1969) Investigations on fecundity of *Aphelenchus avenae*. *Nematologica* 15, 22-28.

Gowen, S.R. (1970) Observations on the fecundity and longevity of *Tylenchus emarginatus* on sitka spruce seedlings at different temperatures. *Nematologica* 16, 267-272.

Green, C.D. & Miller, L.I. (1969) *Rep. Rothamsted exp. Stn for 1968*, 153-155.

Hansen, E.L., Yarwood, E.A., Jackson, G.J. & Poinar, Jnr., G.O. (1968) Axenic culture of *Neoaplectana carpocapsae* in liquid media. *J. Parasit.* 54, 1236-1237.

Hyman, L.H. (1951) The Invertebrates. Vol. III, McGraw-Hill.

Lehman, P.S., Barker, K.R., & Huisingh, D. (1971) Effects of pH and inorganic ions on emergence of *Heterodera glycines*. *Nematologica* 17, 467-473.

Loewenberg, J.R., Sullivan, T., & Schuster, M.L. (1960) The effect of pH and minerals on the hatching and survival of *Meloidogyne incognita incognita* larvae. *Phytopathology* 50, 215-217.

Lower, W.L., Hansen, E.L. & Yarwood, E.A. (1968) Selection for adaptation to increased temperatures in free-living nematodes. *Life Sciences* 7, 139-146.

Mamiya, Y. (1971) Effect of temperature on the life cycle of *Pratylenchus penetrans* on *Cryptomeria* seedlings and observations on its reproduction. *Nematologica* 17, 82-92.

Maupas, E. (1918) Essais d'hybridisation chez les Nématodes. *Bull. biol. France Belgique* 52, 466-498.

Nigon, V. & Dougherty, E.C. (1949) Reproductive patterns and attempts at reciprocal crossing of *Rhabditis elegans* Maupas, 1900 and *Rhabditis briggsae* Dougherty and Nigon, 1949 (Nematoda : Rhabditidae). *J. exp. Zool.* 112, 485-503.

Parrott, D.M. (1968) *Rep. Rothamsted exp. Stn for 1967*, 147-148.

(1972) Mating of *Heterodera rostochiensis* pathotypes. *Ann. appl. Biol.* 71, 271-273.

Poinar, G.O., Jnr. (1967) Description and taxonomic position of the DD.136 nematode (Steinernematidae, Rhabditoidea) and its relationship to *Neoaplectana carpocapsae* Weiser. *Proc. Helminthol. Soc. Wash.* 34, 199-209.

Potter, J.W. & Fox, J.A. (1965) Hybridisation of *Heterodera schachtii* and *H. glycines*. *Phytopathology* 55, 800-801.

Shepherd, A.M. & Clark, A.J. (1972) Plant Parasitic Nematodes. Vol. II B.M. Zuckerman, W.F. Mai and R.H. Rohde (editors). Academic Press, New York. p.267-287.

Silverman, P.H. & Campbell, J.A. (1959) Studies on parasitic worms of sheep in Scotland. I. Embryonic and larval development of *Haemonchus contortus* at constant conditions. *Parasitology* 49, 23-38.

Sturhan, D. (1964) Kreuzungsversuche mit biologischen rassen des stengelälchens (*Ditylenchus dipsaci*). *Nematologica* 10, 328-334.

Taylor, D.P. (1962) Effect of temperature on hatching of *Aphelenchus avenae* eggs. *Proc. Helminthol. Soc. Wash.* 29, 52-54.

Wallace, H.R. (1966) Factors influencing the infectivity of plant parasitic nematodes. *Proc. Roy. Soc. Ser. B* 164, 592-614.

(1971) The influence of temperature on embryonic development and hatch in *Meloidogyne javanica*. *Nematologica* 17, 179-186.

Yuksel, H. (1960) Observations on the life-cycle of *Ditylenchus dipsaci* on onions. *Nematologica* 5, 289-296.

Zondag, R. (1962) A nematode disease of *Sirex noctilio* (F.). *Interim Res. Rep.*, New Zealand Forest Service, 1-6.

APPENDIX A

Calculation of the area contained by the rate of oviposition/age graph.

Calculating the area contained by a graph by counting the squares in this area of graph paper is both tedious and time consuming. The area can be more simply calculated by cutting out the area of the page contained by the graph and axes, and weighing it. By reference to the weight of a known area of the same paper, the required area can be determined.

The accuracy of determining area by the weight of paper was tested by weighing three 5x10 cm rectangles cut from each of three sheets of graph paper. Cuts were made with a sharp scalpel and only on the ruled portion of the page. The results, analysed by a one-way analysis of variance, are summarised in the following table.

Although the difference between sheets was significant, it was quite small. The greatest difference between rectangles was 0.0120g which represented an error of 15 eggs in 480. Since this difference was insignificant in comparison to the variation, in the estimates of fecundity, between replicates, this method of determining area was accepted as sufficiently accurate.

Sheet No.	Weight of 5x10 cm rectangle of graph paper		
	a	b	c
i	0.3703	0.3783	0.3736
ii	0.3677	0.3679	0.3663
iii	0.3755	0.3783	0.3759

Source of Variation	df	Sum of Squares	Mean Squares	F	P
Sheets	2	0.0001379	0.00006895	10.77	0.05
Error	6	0.0000384	0.0000064		
Total	8	0.0001763			

APPENDIX B

Total number of eggs produced by one *Deladenus* female.

The total number of eggs produced in its lifetime by one female was estimated from rate of oviposition/age graphs where rate of oviposition was calculated on the number of eggs deposited rather than the number of viable eggs as in section 3.3. The number of eggs was estimated from these graphs as described in Appendix A.

		Estimated number of eggs produced by one female	
		1st experiment	2nd experiment
D. siricidicola (H)		300	350
D. siricidicola (S)	... 1st method[1]	500	770
	... 2nd method[2]	260	
D. rudyi (T)	... CMA	425	
	... ¼ PDA	150	

[1] Each nematode used once only.

[2] Each nematode used repeatedly.

APPENDIX C

USE OF THE NEMATODE *Deladenus siricidicola* IN THE BIOLOGICAL CONTROL OF *Sirex noctilio* IN AUSTRALIA

by

R.A. BEDDING and R.J. AKHURST

Commonwealth of Australia

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

USE OF THE NEMATODE *DELADENUS SIRICIDICOLA* IN THE BIOLOGICAL CONTROL OF *SIREX NOCTILIO* IN AUSTRALIA

R. A. BEDDING and R. J. AKHURST

Reprinted from the Journal of the Australian Entomological Society,

Volume 13, pp. 129-135, June 28, 1974

J. Aust. ent. Soc., 1974, **13**: 129-135

USE OF THE NEMATODE *DELADENUS SIRICIDICOLA* IN THE BIOLOGICAL CONTROL OF *SIREX NOCTILIO* IN AUSTRALIA

R. A. BEDDING and R. J. AKHURST

Division of Entomology, CSIRO, Hobart, Tasmania.

Abstract

A knowledge of the life cycles of free-living, mycetophagous nematodes has been utilized in developing methods for the monoxenic mass rearing of hundreds of millions of *Deladenus siricidicola* Bedding. This tylenchid nematode causes sterility in female *Sirex noctilio* F., a serious pest of pine trees in south-eastern Australia; it has been liberated over many of the areas affected by *Sirex* using the simple but highly effective techniques described.

Introduction

There are in Australia over $\frac{1}{2}$ million hectares of coniferous forest of which more than $\frac{3}{4}$ is the introduced pine species *Pinus radiata* D. Don; the estimated annual return from the timber harvested is now approaching 250 million dollars and the industry continues to expand rapidly.

The woodwasp *Sirex noctilio* F., a serious pest of radiata pine trees, was accidentally introduced, probably from New Zealand and was found in southern Tasmania in 1952. As a result of spectacular damage by this insect in one Tasmanian forest, where at least 40% of all trees were killed over several years, and the finding of *Sirex* in Victoria in 1961, the National Sirex Committee was set up in 1962 to examine means for eradication or control. When initial attempts at eradication proved unsuccessful, an extensive programme of "Search and Destroy" was carried out in Victoria in an effort to contain the spread of *Sirex* while research into various other aspects of control including attractants, selective breeding of resistant trees, and the introduction of insect and nematode parasites was carried out. Despite all efforts to prevent its spread, infestations of *Sirex* have spread steadily from the initial outbreak area in Melbourne over many thousands of hectares and are now only about 120 km from the vast coniferous forests of Mt. Gambier 80,000 ha). Following the discovery of nematodes infecting *S. noctilio* in New Zealand (Zondag 1962), CSIRO commenced investigations into nematode parasites of various siricids and their parasitoids from world wide sources in 1965. The life history of these nematodes was shown by Bedding (1967, 1972a, 1972b) to be extraordinary, involving profound female dimorphism associated with free-living mycetophagous and parasitic life cycles, and seven species of *Deladenus* (Neotylenchidae) found parasitizing siricids were described by Bedding (1968, in press). The present paper is concerned mainly with techniques used in the manipulation of *D. siricidicola* for the control of *Sirex* in Australia.

Biology of *Sirex*

Female *S. noctilio* drill into the wood of living pine trees to oviposit and implant toxic mucus and a symbiotic fungus (*Amylostereum areolatum* (Fr.) Boidin). The mucus and fungus kill susceptible trees (Coutts 1969) and *S. noctilio* larvae bore through, and feed on, the fungus infected wood. Adult *S. noctilio* emerge 1, 2, or 3 years after oviposition. Immature stages of *Sirex* are subject to attack from various ichneumonid, cynipid and nematode parasites and adult *Sirex* to predation by birds.

Biology of *D. siricidicola*

Parasitized *Sirex* larvae contain 1 to 100 or more adult female nematodes dispersed in the haemocoel. These nematodes vary from 5 to 25 mm in length, are cylindrical and often bright green. Their reproductive system remains undeveloped (about 0.5 mm in length) until the onset of host pupation and then expands rapidly throughout the length of the nematode, soon producing several thousand eggs,

which hatch within the parent. Juvenile nematodes escape into the haemocoel of the host pupa and migrate into its reproductive organs. In a female host, juveniles penetrate all developing eggs and tend to suppress the developing ovaries, while in a male host the testes become a solid mass of many thousands of juveniles. The female *Sirex* is sterilized, with each of its eggs containing 50 to 200 juveniles, but the male *Sirex* remains fertile because early in pupation, before juvenile nematodes penetrate the testes, most spermatozoa pass into the vesiculae seminales which are not penetrated by the nematodes.

During oviposition by parasitized female *Sirex*, eggs containing juvenile nematodes are implanted into pine trees together with symbiotic fungus. Juvenile nematodes migrate from the host's eggs, feed on the developing fungus and grow into adult free-living nematodes which lay many eggs within the tracheids around *Sirex* oviposition holes. Juveniles hatching from these eggs also feed on fungus and can develop into free-living mycetophagous adults. This free-living cycle can be repeated indefinitely.

As the tree dries out, the symbiotic fungus spreads throughout it and nematodes breed within tracheids, resin canals and even beneath the bark. In these relatively aseptic areas where fungus is sparse, juveniles develop only into mycetophagous forms, but in the vicinity of *Sirex* larvae (several *Sirex* usually attack a single tree), juvenile nematodes may develop into adult infective females which are quite unlike the mycetophagous females. After insemination, infectives penetrate *Sirex* larvae and grow as much as a thousand fold in volume within a few weeks and commence reproduction after host pupation.

Aspects of greatest importance in the use of this nematode for biological control are its ability to sterilize female *Sirex*, the free living cycle which can be utilized for maintaining cultures and mass rearing, and (Bedding, in preparation) its specificity to the *Sirex* symbiotic fungus (which restricts it to the environment of *Sirex*) and inability to affect important insect parasitoids.

Culturing techniques

Culture on agar plates

To obtain uncontaminated cultures of the symbiotic fungus *Amylostereum areolatum*, adult female *Sirex* are dipped in ethanol, ignited, and plunged into and then dissected under sterile water to remove the ooidial glands (situated internally at base of ovipositor). Under sterile conditions the burst glands are streaked on plates of potato-dextrose agar (P.D.A.) and the growing fungal front is sub-cultured after incubation at 24°C. Testes containing juvenile *D. siricidicola* similarly removed from male *Sirex* are placed in the centre of fungal cultures that have grown over about one third of the plate. After 1 day, sub-cultures (about 1 cm square) of several hundred nematodes together with fungus are cut from the fungus front and placed at the edge of P.D.A. plates to establish monoxenic cultures. At 24°C larvae mature into adults about 5 days after removal from a host insect and sub-cultures are made about 1 week later when many eggs have hatched. Further sub-cultures are made at about 2 weekly intervals just before the agar is completely covered with fungus. In sub-culturing, a balance must be maintained between nematodes and fungus since the nematodes can breed successfully only on the growing edge of the fungus; with too few nematodes the fungus covers the plate before much reproduction occurs, and with too many, fungal growth may be completely stunted. A noticeable improvement in rate of reproduction occurs during the first few months of culturing.

Use of P.D.A. produces cultures far superior to those on malt, corn meal, Czapek-Dox, nutrient, or water agars although corn meal agar is sometimes useful for storing cultures.

Cultures can be stored at 5°C in the dark for 6 to 12 months on P.D.A. or corn meal agar slopes in test tubes without sub-culturing. A 1cm square inoculum of a high density of nematodes on fungus is placed at the bottom of the tube which is closed with a tight-fitting non-absorbent cotton wool bung covered with aluminium foil. At 5°C the fungus grows and the nematodes can still feed and reproduce slowly.

Mass culture

Although P.D.A. plates each yield several thousand nematodes, this method of obtaining *D. siricidicola* in large numbers is expensive and time consuming and the nematodes are difficult to harvest. Evans (1970) developed an excellent method for mass rearing the nematode *Aphelenchus avenae* which fed on the fungus *Rhizoctonia solani* growing on autoclaved wheat in jars, but he had some contamination problems. The method used for mass rearing *D. siricidicola* is similar but utilizes Erlenmeyer flasks with cotton wool bungs and foil caps to eliminate contamination; the proportion of wheat to water is very different (1.5 cc tap water to 1 g of wheat), and the fungus used is *A. areolatum*.

Flasks of 500 ml capacity are most frequently used; 100 g wheat and 150 ml water are added to each flask and after plugging and capping they are autoclaved at 1.05 kg/cm^2 for 20 min; while still warm, the flasks are strongly agitated to separate wheat grains and aid aeration. They are inoculated from vigorous P.D.A. plate cultures of *D. siricidicola*, using sub-cultures of about 2 cm^2 of fungus and 1000 to 1500 nematodes. Incubation is at 24°C, preferably in the dark, and nematodes are ready for harvesting 4 to 6 weeks later when most of the fungus has become brown and nematodes swarm up the sides of the flask (Fig. 2).

To harvest, cold tap water (5-10°C) is added to cover the wheat and left a few minutes prior to agitation. The resulting suspension of nematodes is poured off (through a sieve to remove debris) and the nematodes allowed to settle, or centrifuged prior to washing 2 or 3 times in cold tap water. Flasks are washed out 3 or 4 times over an hour or more, but should not be left full of water for more than 5 to 10 min because of possible nematode asphyxiation. Yields vary from 3 to 10 million nematodes per 500 ml flask. Since the contents of a single flask are sufficient to inoculate about an hundred metres of timber, attempts were not made at larger scale mass rearing.

With some strains of *D. siricidicola*, infective nematodes are formed readily in such cultures so that at the usual harvest time, most nematodes are either infectives or associated males. These cannot reproduce in *Sirex* infested timber and so are of little use for inoculation. To reduce the proportion of infectives produced, flasks can be either harvested early (after 3-4 weeks) resulting in rather low yields, or infective production can be greatly reduced by blowing moist, sterile air through cultures.

Storing and transporting

Unlike many other insect parasitic nematodes, *Deladenus* has no special resistant stage adapted for prolonged survival. Generally, the nematodes are cultured as and when they are needed but can be stored for several weeks in shallow tap water with an atmosphere of almost pure oxygen at 5 to 10°C (Fig. 1).

Use of dilute formaldehyde solution, although successful for storing *Neoaplectana* spp. (Dutky *et al.* 1964), kills *Deladenus* spp. which are usually transported and stored in 150 ml Erlenmeyer flasks with up to 1 million nematodes in 20 ml tap water per flask. Each flask is flushed out with pure oxygen for several seconds before being closed by a tight fitting rubber bung.

Inoculation of timber

In order to introduce *Deladenus* into wild populations, either *Sirex* infested timber is inoculated with nematodes and distributed in *Sirex* affected areas, or trees scattered throughout affected areas are felled and inoculated on the spot. Timber of a wide range of moisture content, from freshly killed down to 50% oven dry weight can be inoculated with resulting successful establishment. Most female *Sirex* emerging from correctly inoculated timber are parasitized and oviposit on trees usually also attacked by wild unparasitized *Sirex*. The techniques used for inoculation are important; early methods of inoculating timber resulted in low, variable or no parasitism, but methods described below regularly produce over 99% parasitism.

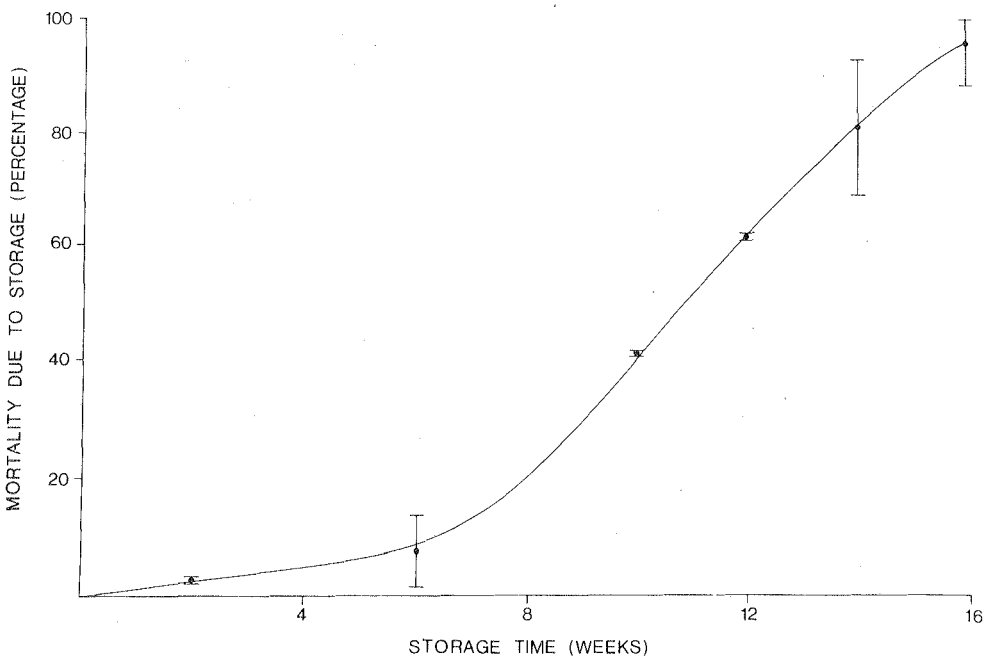


FIG. 1.—Graph of the mortality of *Deladenus siricidicola* stored at 5°C in 150 ml Erlenmeyer flasks each with 10^6 nematodes in 20 ml of tap water and an atmosphere enriched with oxygen.

Inoculation medium

Water suspensions were found to be unsatisfactory for introduction of nematodes into timber because water is rapidly absorbed into the wood leaving nematodes stranded and soon desiccated in inoculation holes, and resulting parasitism of the *Sirex* is low and variable. Most nematodes tend to remain in agar inoculum even after it dries out in inoculation holes, but gelatin based inoculum appears to be ideal.

A 12% solution of gelatin in hot tap water is cooled in a small bucket until beginning to set and is then easily made into a foam with an egg beater. A concentrated suspension of nematodes is thoroughly mixed with the foam (usually 250,000 juveniles per 100 ml gelatin) and the medium is ready for use or can be stored for several hours at 5 to 10°C. Nematode survival in the foam is high because of adequate aeration; the medium is not readily absorbed into wood, and the nematodes rapidly migrate from it into the tracheids.

Preparation of inoculation holes

Drilling holes with a wide variety of bits and speeds proved unsatisfactory. Staining in cotton blue/lactophenol (Goodey 1963) sections taken across such holes revealed that insignificant nematode penetration into the tracheids occurred because tracheal ends were damaged and blocked after drilling. Early mass inoculation of timber was made into cuts across the grain, made by a sharp 2.5 cm chisel. This gave good parasitism but required 2 operators for efficiency, used more inoculum and was more time consuming than the present method.

Mr Victor Gould of Forests Commission, Victoria, designed and manufactures a special tool for nematode inoculation (Figs. 3, 4). This consists of a wad-punch with one side cut away to release wood cores, and a heavy head welded on top. The punch is mounted in a handle and can be used like a hammer to produce inoculation holes with great rapidity. The tool is self sharpening and cuts sufficient tracheal ends cleanly enough to ensure ready access by the nematodes.

Inoculation

Inoculum (averaging 0.8 ml) is introduced into each punch hole using a 50 ml plastic syringe fitted with a 1 cm long narrow polythene tube in place of a hypodermic needle. For maximum efficiency, an operator holds the punch in one hand and syringe and a reserve of inoculum in the other (Fig. 3). After being driven in, the punch is pulled out by the head and inoculum introduced with the other hand immediately afterwards. With practice one operator can inoculate about an hundred m of timber per hour using this technique. The reserve of inoculum consisting of 200 ml in a 15 cm × 20 cm polythene bag is squeezed into the syringe via a small hole cut in one corner. During inoculation, the bag is suspended from the 2 smallest fingers while the syringe is operated by thumb and first 2 fingers.

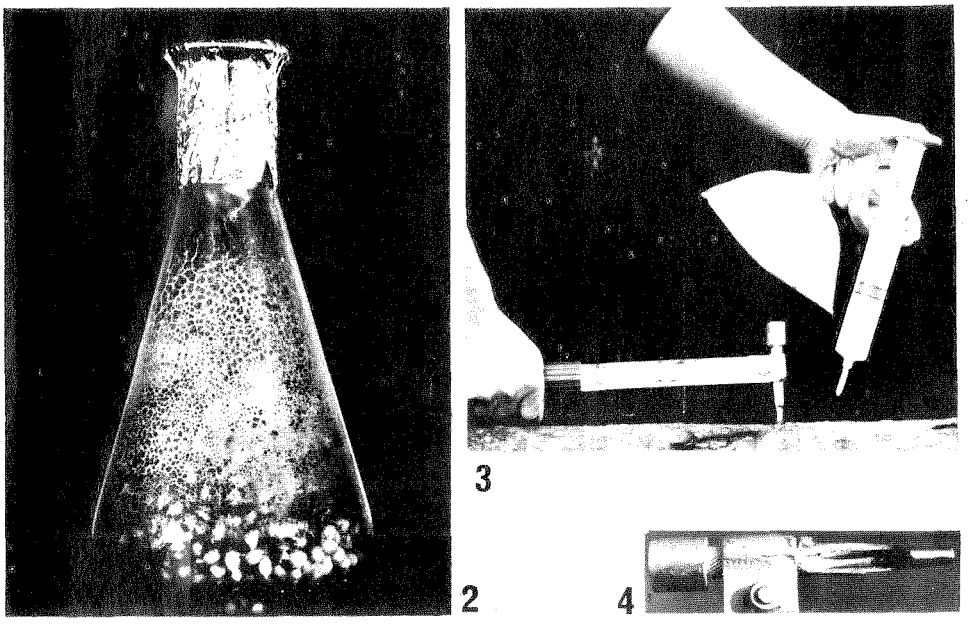


FIG. 2-4.—(2) Culture flask of *Deladenus siricidicola* ready for harvesting. (3) Inoculation of timber with *Deladenus siricidicola* by one operator using a Gould punch and syringe. (4) Head of Gould punch.

Spacing and strength of inoculations and resulting parasitism

Initially inoculations were made at 10 cm intervals on both sides of *Sirex* infested timber, but the following experiments show this density of inoculation to be unnecessarily high; the level of inoculation was in fact detrimental in that smaller parasitized *Sirex* (which fly less far and produce less nematode infected eggs) resulted, probably because of early competition for fungal food between *Sirex* larvae and nematodes (Bedding & Akhurst, in preparation).

In the following experiments (Table 1), 10 randomised, 1 m long, *Sirex* infested billets of similar source, age and moisture content were used for each treatment. The first 2 treatments were made a year later than the others.

It can be seen that excellent and almost identical results were obtained from most treatments but that 1 inoculation per m is inadequate to produce very high levels of parasitism. It is recommended that inoculations of 2000 nematodes (250,000 per 100 ml of inoculum) be made at 30 cm (1 ft) intervals along one side of *Sirex* infested logs or trees.

Field liberations

In 1971, over 1000 1 m *Sirex* infested billets were inoculated with nematodes at Hobart and sent to the Forests Commission, Victoria for distribution in areas of known *Sirex* infestation. Since then, several hundred million nematodes of 5 strains

TABLE 1

EFFECT OF INOCULATION DENSITY ON PARASITISM OF *S. NOCTILIO* BY *D. SIRICIDICOLA* IN BILLETS OF *P. RADIATA*

No. nematodes per inoculation	No. inoculations per billet	No. *Sirex* dissected	No. *Sirex* parasitized	% parasitism
1000	1	529	221	41.8
	1	844	349	41.4
	4	208	206	99.0
	16	337	335	99.4
2000	4	252	251	99.6
	16	287	280	97.6
4000	1	287	226	78.7
	2	165	162	98.2
	4 (2 rows)	335	324	96.7
	8 („)	210	209	99.5
	8 („)	368	366	99.5
	8 („)	144	143	99.3
	16 (4 rows)	275	273	99.3
	32 („)	184	183	99.5
8000	4 (2 rows)	368	366	99.5
	16 („)	144	143	99.3

have been sent to the Commission, which in a major operation, has searched for *Sirex* infested areas and cut down and inoculated selected trees over several thousand hectares of Victoria. Early results from the Commission's investigations indicate establishment of nematodes in most areas treated, with parasitized *Sirex* from original inoculations carrying nematode infection up to 19 km in a single year. Optimal levels of parasitism are not to be expected for several years.

Results from the first experimental liberation of *D. siricidicola,* made in 1970, in a 400 ha pine forest in northern Tasmania, indicate that the nematode may have great potential to control *Sirex*. An estimated 50 parasitized female *Sirex* emerged from inoculated logs at a single point in one corner of the forest and by 1972, 37% of the *Sirex* infested trees in the whole forest, and 92% in the 12 ha compartment of liberation contained nematodes (Bedding and Akhurst, in preparation). Usually over 70% of the *Sirex* emerging from a nematode infested tree are parasitized. In normal control operations, *Sirex* infested timber, inoculated with nematodes, is scattered throughout a forest, and in similar forests results comparable with those in the compartment of liberation might be expected. Forests treated in this manner are being assessed annually for development of nematode parasitism and the *Sirex* population is being monitored by ground and aerial surveys.

Discussion

This is a good example of a full study of the life history and biology of a potential biological control agent leading to developments that are of great practical importance: without knowledge of the mycetophagous phase of the life cycle of *Deladenus*, the mass manipulation of this organism for the biological control of *Sirex* would have been almost impossible. With the exception of *Neoaplectana* spp, no other nematode has been manipulated on a large scale for biological control but, since many other sphaerulariids may have comparable life cycles to *Deladenus* (Bedding 1972b), the possibility exists of similarly manipulating these.

Developments in techniques of inoculation have made it possible to produce readily large supplies of logs in which almost 100% of the *Sirex* are parasitized, an important factor when nematodes have to be released over the very long and rapidly advancing front of *Sirex* infestations. In order to obtain maximum nematode parasitism as early as possible, and hopefully to control new infestations, the greatest possible number of *Sirex* infested trees should be inoculated with nematodes; as about an hundred metres of timber can be inoculated per hour the main limiting factor is now the finding and felling of *Sirex* infested trees.

Almost inevitably *Sirex* infestations will eventually spread to all forests of *Pinus radiata*, at least in eastern Australia, but by the time this occurs, nematode and

insect parasitoids should be well distributed throughout these regions and continued inoculation of *Sirex* infested timber with nematodes and release of parasitoids should not be necessary. However, it could well become normal forestry practice to transfer *Sirex* infested timber from areas known to have high nematode parasitism to fresh outbreaks as these occur since one of the main weaknesses in nematodes as biological control agents is their inability to disperse from one host population to another.

At this stage, the techniques of liberation have been more than adequate, but it remains to be seen whether *D. siricidicola* together with insect parasitoids and changing forestry practices will be able to control *Sirex* sufficiently. In the meantime, 5 new species of *Deladenus* are being screened and selectively reared for biological control of *Sirex*, and there also exists the possibility of releasing the harmless siricid, *Xeris spectrum* (L.) as an alternative host for both nematodes and the insect parasitoids.

Acknowledgements

This study was financed by the National Sirex Committee. We would like to thank Mr G. Williams and Mr J. MacMahon for their help during these investigations, Dr F. Wilson who initiated and encouraged the early work and Mr K. L. Taylor for encouragement and constructive criticism of the manuscript. We are also grateful for much co-operation from the Forests Commission, Victoria and to Mr R. Bashford for help with photography.

References

BEDDING, R. A. (1967).—Parasitic and free-living cycles in entomogenous nematodes of the genus *Deladenus*. *Nature, Lond.* **214**: 174-175.

BEDDING, R. A. (1968).—*Deladenus wilsoni* nsp. and *D. siricidicola* nsp. (Neotylenchidae), entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* **14**: 515-525.

BEDDING, R. A. (1972a).—Nematode parasitism of Siricidae and their hymenopterous parasites by *Deladenus* spp. (Neotylenchidae). *Proc. 13th int. Congr. Ent.*, 1968, **2**: 54-55.

BEDDING, R. A. (1972b).—Biology of *Deladenus siricidicola* (Neotylenchidae) an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* **18**: 482-493.

BEDDING, R. A.—Five new species of *Deladenus* (Neotylenchidae), entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* (in press).

COUTTS, M. P. (1969).—The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* I & II *Aust. J. biol. Sci.* **22**: 915-924 & 1153-1161.

DUTKY, S. R., THOMPSON, J. V. and CANTWELL, G. E. (1964).—A technique for the mass propagation of the DD-136 nematode. *J. Insect Path.* **6**: 417-422.

EVANS, A. A. F. (1970).—Mass culture of mycetophagous nematodes. *J. Nematol.* **2**: 99-100.

GOODEY, J. BASIL (1963).—Laboratory methods for work with plant and soil nematodes. *Tech. Bull. Minist. Agric. Fish. Fd.* **2**: 72.

ZONDAG, R. (1962).—A nematode disease of *Sirex noctilio* (F.) *Interim Res. Rep.* N.Z. Forest Serv.: 1-6.

[*Manuscript received October* 16, 1973]