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**The biology and transmission dynamics of *Echinoparyphium recurvatum* (Digenea : Echinostomatidae)**

McCarthy, A. M

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**The Biology and  
Transmission Dynamics of  
*Echinoparyphium recurvatum*  
(Digenea : Echinostomatidae)**

by

**Andrew Michael McCarthy B.Sc.(Hons.)**

**A thesis submitted for the degree of  
Doctor of Philosophy  
in The Faculty of Science of  
The University of London**

**1989**



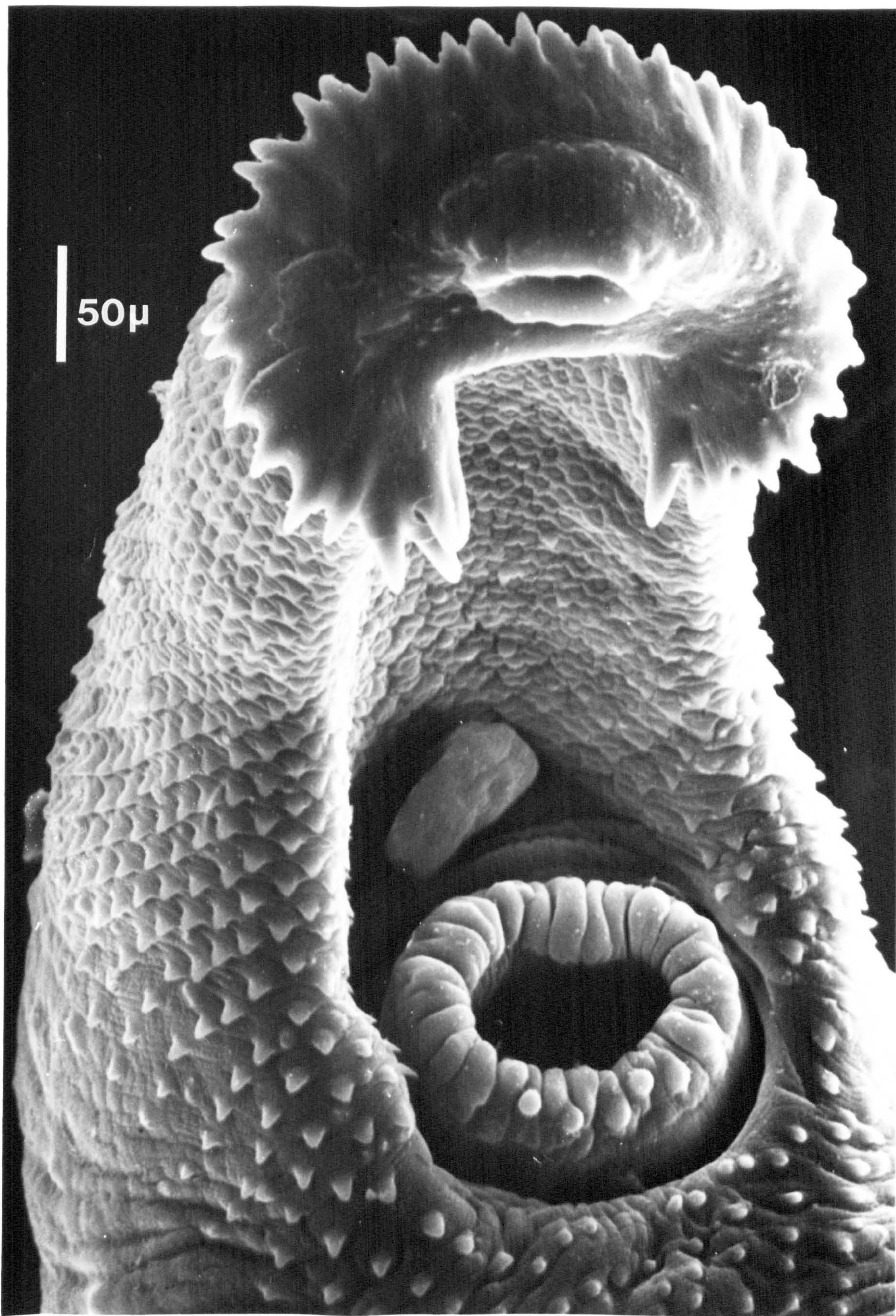
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Frontispiece (facing page) -

Scanning electron micrograph of the anterior of an adult *Echinoparyphium recurvatum* from the intestine of an experimentally infected *Anas platyrhynchos* 15 days post-infection.







## *Abstract*

Aspects of the biology and transmission dynamics of the echinostome digenean Echinoparyphium recurvatum were examined. The morphology of each life cycle stage was described. Experimental infection studies revealed that a range of gastropods and Rana temporaria tadpoles were utilizable as second intermediate hosts, although differential compatibility was observed throughout the host spectrum. Of a range of gastropods exposed to infection by E. recurvatum miracidia only the lymnaeids Lymnaea peregra and L. auricularia were utilizable as first intermediate hosts.

Hatching of E. recurvatum eggs, and the survival and infectivity of miracidia, were found to be markedly influenced by temperature. A study of the seasonal infection dynamics of E. recurvatum in a British population of the first intermediate host L. peregra revealed biannual infection peaks.

Experimental studies on the emergence of E. recurvatum cercariae from L. peregra showed emergence to be markedly photoperiodic, cercariae emerging almost exclusively during periods of light. Host-size related trends in cercarial emergence were discovered and emergence was found to be influenced by temperature. Photoperiodicity of cercarial emergence was observed from L. peregra carrying mixed infections of E. recurvatum and the plagiorchiid Plagiorchis sp. . Cercarial phototactic behaviour was found to be age-dependent.



Experimental studies on the transmission dynamics of E. recurvatum cercariae demonstrated that transmission efficiency is markedly temperature-dependent, the optimum being achieved at approximately 20°C. Cercarial transmission was influenced by second intermediate host dispersion pattern. Evidence for cercarial chemotaxis is provided.

The distribution of E. recurvatum in the intestine of Anas platyrhynchos ducklings was examined 24 hours and 15 days post-infection. Density-dependent effects on worms in the intestines of A. platyrhynchos initially infected with cyst doses of a range of sizes were observed. Metacercarial cysts 14 days old were infective to A. platyrhynchos, but 12 hour old cysts were uninfected. Infectivity of metacercarial cysts to A. platyrhynchos was found to be unaltered after a period of 16 weeks in L. peregra maintained at both 20°C and 4°C. Infectivity of cysts was independent of the species of second intermediate host utilized.

An appendix section provides experimental observations on the specificity of cercariae of the echinostome Pseudechinoparyphium echinatum toward gastropod second intermediate hosts. Patterns of cercarial transmission in host communities of different species composition were also investigated.



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# *Chapter 1*

## INTRODUCTION

*Echinoparyphium recurvatum*  
(von Linstow 1873) :  
an echinostome digenean

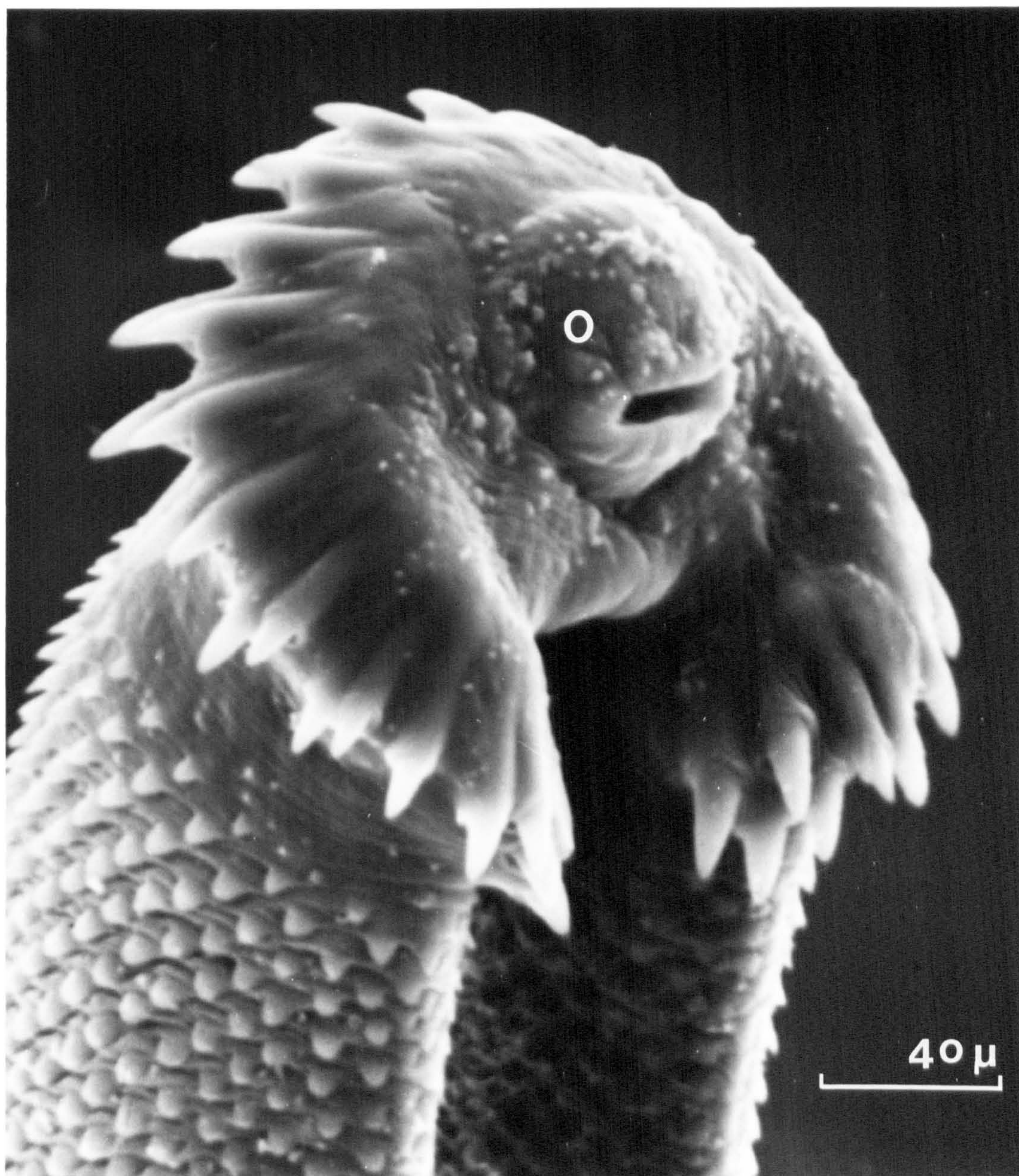


The object of the studies presented in the main body of this thesis is the digenean parasite Echinoparyphium recurvatum (von Linstow 1873). E. recurvatum is an echinostome digenean. It is a member of the Family Echinostomatidae, a group of distome digeneans of world-wide distribution, characterized by the presence of an anterior collar around the oral sucker bearing a circlet of spines, see Fig 1.1. This morphological feature is found in the cercarial, metacercarial and adult stages of the life cycle. The number and arrangement of the collar spines is of considerable taxonomic importance as a genus characteristic. For example, members of the genus Echinoparyphium are characterized by the possession of 43-45 collar spines while those of the genus Echinostoma possess 37 spines. Echinostomes of the genera Isthmiophora and Paryphostomum possess 27 spines. It is from this anterior spined collar that echinostomes receive their name (*Echino* = spine, *stoma* = opening). The echinostomes as a group are thought to be most closely related to the Families Fasciolidae, Cathaemasidae, Psilostomatidae and Philophthalmidae. Most standard classification systems place these Families together with the Echinostomatidae in the Order Echinostomida (see Erasmus 1972).

Considerable variation occurs among echinostomes with respect to life cycle patterns, although in all known forms the first intermediate host is a gastropod mollusc. Development in the first intermediate host proceeds through three generations of germinal sacs, sporocyst, mother redia and daughter redia. The second redial generation (the daughter redia) produces cercariae. Second intermediate hosts of echinostomes include a range of aquatic

FIG 1.1 Scanning electron micrograph of the anterior spined collar of the adult echinostome Echinoparyphium recurvatum ; O = oral sucker.





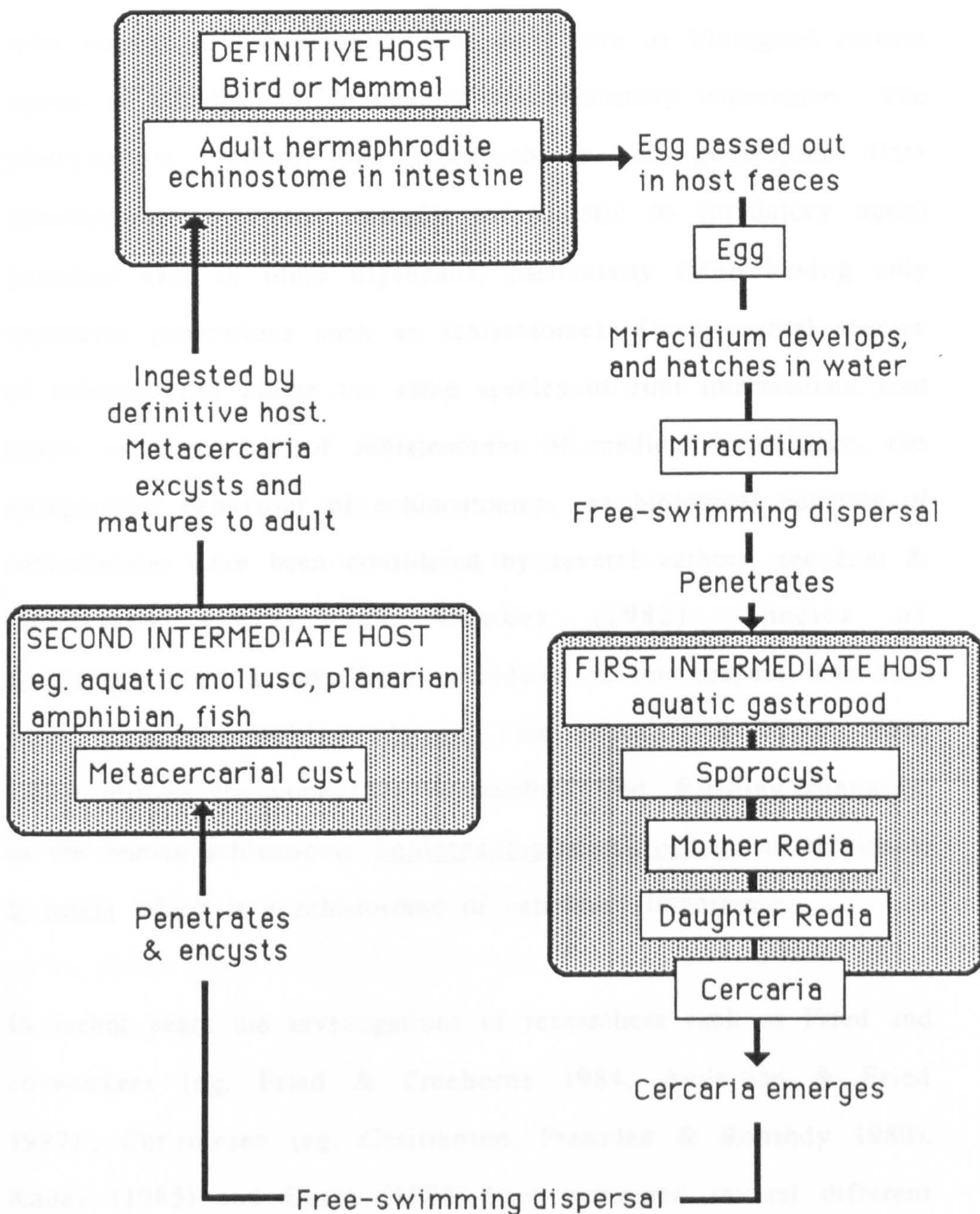


invertebrates (gastropods, bivalves, planarians) and fish. Broad specificity toward the second intermediate host is a general feature of the echinostome life cycle although certain species such as Isthmiophora melis and Paryphostomum radiatum are largely restricted to utilizing fish and amphibians (McCarthy, Jancev, Kanev & Genov 1987), (Vassilev, McCarthy & Kanev 1987). All known adult echinostomes are intestinal parasites of birds and, or, mammals, these hosts becoming infected by ingestion of metacercarial cysts in second intermediate hosts. The metacercaria excysts within the intestine of the definitive host and develops to the sexually mature adult stage. All adult echinostomes are hermaphroditic flukes. The eggs produced by adult echinostomes pass out of the definitive host in its faeces and into water. The miracidia hatch after a period of development, penetrate the first intermediate host gastropod and give rise to the mother sporocyst and the life cycle continues. The generalized life cycle of an echinostome is summarized in Fig 1.2.

As intestinal parasites of mammals certain echinostomes are potential human pathogens. However, in reality echinostomes are generally considered to be of limited medical importance to Man but, despite this, cases of intestinal echinostomiasis have been reported in the literature. For example, Donges (1962) gives a case study of duodenal ulceration caused by the echinostome I. melis in Europe. In this case infection was acquired by the ingestion of metacercarial cysts in fish. In Southeast Asia Rim (1982) and Seo, Hong, Chai & Lee (1983) have reported cases of echinostomiasis in humans caused by members of the genus Echinostoma. In these



FIG 1.2 Diagram to show the generalized life cycle pattern of an echinostome digenean





cases infections were acquired by the ingestion of raw snails or tadpoles containing encysted metacercariae.

In recent decades echinostome digeneans have been investigated with respect to the potential that they have as biological control agents of schistosomes of medical and veterinary importance. The pharyngeate rediae of echinostomes in gastropod first intermediate hosts are strongly antagonistic to (predatory upon) germinal sacs of other digeneans, particularly those having only sporocyst generations such as schistosomes. Since several species of echinostomes utilize the same species of first intermediate host snails as a number of schistosomes of medical importance, the antagonistic behaviour of echinostomes as biological controls of schistosomes have been considered by several authors, see Lim & Heyneman (1972) and Combes (1982). Species of Echinoparyphium have been considered in this respect, one such being Echinoparyphium elegans (see Mouahid & Mone' 1988) which utilizes the same first intermediate host, Bulinus truncatus, as the human schistosome Schistosoma haematobium in addition to S. bovis which is a schistosome of veterinary importance.

In recent years the investigations of researchers such as Fried and co-workers (eg. Fried & Freeborne 1984, Anderson & Fried 1987), Christensen (eg. Christensen, Fransden & Roushdy 1980), Kanev (1985) and Evans (1985) have examined several different aspects of the biology, ecology, pathology, systematics and transmission dynamics of echinostomes within the 37 collar-spined genus Echinostoma. However, members of the 43-45 collar-spined genus Echinoparyphium have received considerably



less attention, studies being confined in most cases to descriptions of the life cycles of species from various geographical locations. For example, Najarian (1954) described the life cycle of Echinoparyphium flexum from North America, Lie & Umathevy (1965) described the life cycle of Echinoparyphium dumni from Southeast Asia, and Mouahid & Mone' (1988) have recently described the life cycle of Echinoparyphium elegans from the southern Mediterranean. However, during the current decade, Evans and co-workers at King's College London have examined a number of aspects of the biology of one species of the genus Echinoparyphium in some detail, (Evans 1982, 1983b ; Evans & Gordon 1983a, 1983b ; Evans, Whitfield & Dobson 1981). This species is Echinoparyphium recurvatum (von Linstow 1873) and is the subject of the majority of the work presented in this thesis.

The adult of Echinoparyphium recurvatum was first described from material collected in Central Europe (Germany/Czechoslovakia) by von Linstow (1873). The adult worms were recovered from the intestine of naturally infected Scaup, Aythya marila. Von Linstow (1873) originally recorded the number of collar spines as 44. Luhe (1909), on re-examination of the same type material, recorded that the true number of collar spines was in fact 45. This observation has in fact been confirmed by recent examination of of the type specimens of the von Linstow collection deposited in the East Berlin Natural History Museum, DDR (Kanev 1988 - personal communication). Rasin (1933) working on material from the type locality of E. recurvatum in Czechoslovakia described the life cycle in detail with particular reference to the morphology of the larval stages. This author recorded that the

intermediate host was the freshwater pulmonate snail, Lymnaea peregra and by experimental infection studies he demonstrated that the parasite was highly specific to that species of first intermediate host. However, a variety of authors have described as "E. recurvatum" echinostomes from a range of lymnaeid, planorbid and prosobranch gastropod first intermediate hosts in a variety of geographical locations (see Table 1.1).

Second intermediate hosts of the parasite have been recorded as a wide range of freshwater molluscs (Evans, Whitfield & Dobson 1981, Evans & Gordon 1983b). In addition Vojtek (1963) has recorded metacercarial cysts of the parasite from the frogs Rana temporaria and Rana esculenta.

The only definitive hosts of E. recurvatum recorded in the natural environment have been birds, mainly anatids. A list of naturally infected bird species recorded in the literature as definitive hosts of the parasite from various geographical locations is given in Table 1.2. To this list may be added domestic bird hosts experimentally infected with the parasite which include Gallus gallus domesticus (Senger 1954, Grabda-Kazubska & Moczon 1988) and Columba livia (Diaz-Diaz 1976). Certain authors have also reported that mammals may be experimentally infected with E. recurvatum. Moravec, Barus, Rysavy & Yousif (1974) have reported that E. recurvatum metacercarial cysts from Egypt could be used to experimentally infect albino mice (Mus musculus) albino rats (Rattus norvegicus) golden hamsters (Mesocricetus auratus) and rabbits (Oryctolagus cuniculus). Senger (1954) has

**TABLE 1.1** Gastropods reported in the literature as first intermediate hosts of Echinoparyphium recurvatum in different geographical locations

Gastropod type and species	Geographical Location	Author
<b>PULMONATE</b>		
<i>Lymnaea peregra</i>	Britain	Llewellyn (1957) Probert (1966) Diaz-Diaz (1972) Evans & Gordon (1983b) Adam & Lewis (1986)
	Poland	Grabda-Kazubska & Moczon (1988)
	Czechoslovakia	Rasin (1933) Zdarska (1964)
	Britain	Adam & Lewis (1986)
<i>Lymnaea auricularia</i>	Britain	Adam & Lewis (1986)
<i>Lymnaea stagnalis</i>	Czechoslovakia	Zdarska (1964)
<i>Lymnaea palustris</i>	Czechoslovakia	Zdarska (1964)
<i>Helisoma trivolvis</i>	North America	Senger (1954)
<i>Planorbis planorbis</i>	France	Mathias (1926, 1927)
	Denmark	Wesenberg-Lund (1934)
<i>Planorbis leucostomus</i>	Czechoslovakia	Zdarska (1964)
<i>Planorbis spirorbis</i>	Czechoslovakia	Zdarska (1964)
<i>Planorbarius corneus</i>	Czechoslovakia	Zdarska et al (1987)
<i>Bulinus truncatus</i>	Africa	Moravec et al (1974)
		Bisseru (1967)
<b>PROSOBRANCH</b>		
<i>Valvata piscinalis</i>	Britain	Harper (1929)
<i>Viviparus viviparus</i>	France	Dinulesco (1939)



**TABLE 1.2 Species of bird recorded as naturally infected definitive hosts of Echinoparyphium recurvatum from different geographical locations**

Species of Bird	Common Name	Geographical Location	Reference
<i>Anas platyrhynchos</i>	Mallard	Britain North America	Beverley-Burton (1972) Hoeve & Scott (1988)
<i>Anas discors</i>	Blue-winged Teal	North America	Hoeve & Scott (1988)
<i>Anas rubripes</i>	Black Duck	North America	Hoeve & Scott (1988)
<i>Anas acuta</i>	Pintail	Europe	Dawes (1968)
<i>Aythya fuligula</i>	Tufted Duck	Britain	Beverley-Burton (1972)
<i>Aythya marila</i>	Scaup	Europe (Germany)	von Linstow 1873)
<i>Gallinula chloropus</i>	Moorhen	Britain	Beverley-Burton (1972)
<i>Cygnus olor</i>	Mute Swan	Britain	Beverley-Burton (1972)
<i>Mergus merganser</i>	Goosander	Europe	Dawes (1968)
<i>Vanellus vanellus</i>	Lapwing	Japan	Yamaguti (1971)
<i>Scolopax rusticola</i>	Woodcock	Japan	Yamaguti (1971)
<i>Circus melanoleucus</i>	Harrier	Russia (Central)	Oshmarin (in Yamaguti 1971)

also reported that in North America E. recurvatum could develop, albeit poorly, in laboratory mice.

During the current decade a number of aspects of the biology and infection dynamics of E. recurvatum have been examined by Evans and co-workers at King's College London. This research group were working with E. recurvatum material derived from Lymnaea peregra first intermediate hosts from aquatic habitats in southern England.

Evans (1983b) experimentally examined the survival of the parasite in the wildfowl definitive host Anas platyrhynchos. It was found that the maximum life span of the parasite was in the region of 30 days in this host, that 50% survival of the worm population occurred at 12.5 days and that the pre-patent period was 6 days. Evans, Whitfield & Dobson (1981) examined the distribution and occurrence of the metacercarial cysts of E. recurvatum in the molluscan community of Harting Pond, West Sussex. Seven species of mollusc were found to be functioning as second intermediate hosts of the parasite in this habitat. The bivalve Sphaerium corneum was found to be the most heavily utilized host (measured as mean number of cysts per infected specimen) followed by L. peregra. However, it was estimated that over 90% of the total number of metacercarial cysts at the sampling site were present in two host species, the bivalve Pisidium subtruncatum and the gastropod Valvata piscinalis. Evans & Gordon (1983b) examined the specificity of E. recurvatum cercariae towards a range of gastropod second intermediate hosts. They found that the gastropods Lymnaea peregra, Physa fontinalis

and Valvata piscinalis were high compatibility second intermediate hosts while Lymnaea stagnalis, Planorbis planorbis and Potamopyrgus jenkinsi were hosts of low or zero suitability. It was also discovered that the species composition of the host community was an important determinant of the pattern of cercarial transmission observed. For example it was found that in experimental infections where two host species were exposed to cercariae together, the presence of L. stagnalis, a host of low suitability, reduced the infection rate in suitable hosts L. peregra. This ability to act as a "decoy" snail was not shared however, by an alternative host of low suitability status, the planorbid Gyraulus albus.

Evans (1982) investigated the effects of the heavy metals copper and zinc on the survival and infectivity of E. recurvatum cercariae. The infection dynamics of E. recurvatum cercariae were examined experimentally by Evans & Gordon (1983a) using L. peregra as an experimental second intermediate host. Under constant temperature conditions of 18-20°C both cercarial survival and infectivity was found to be markedly age dependent. This study also showed that host size was an important determinant of cercarial transmission success, the number of cercariae establishing infection increasing linearly with increasing host size. In addition this study illustrated that establishment success increased linearly with with infective stage density up to densities equivalent to 5000 cercariae per litre. At higher cercarial densities the proportion of cercariae establishing declined progressively with increasing cercarial density.



Although the work of Evans and co-workers has examined several aspects of the biology and infection dynamics of E. recurvatum, several aspects of the ecology and transmission biology of this echinostome remain unknown. The studies described in the following chapters of this thesis were designed to examine further aspects of the biology and transmission dynamics of E. recurvatum from a source of the parasite utilizing L. peregra as first intermediate host. The studies were designed to expand upon the work of Evans et al. (1981-83) and to provide information on aspects of the biology and transmission dynamics of E. recurvatum which had not previously been examined in detail.

## Chapter 2

### The life cycle, morphology and intermediate host specificity of *Echinoparyphium recurvatum* (von Linstow 1873) (Echinostomatidae)



The life cycle, morphology and intermediate host specificity of Echinoparyphium recurvatum (von Linstow 1873) (Echinostomatidae)

## 2.1 General Introduction

The previous chapter has provided a general introduction to the parasite E. recurvatum. The work described in the following sections of this chapter describes the morphology of the life cycle stages of Echinoparyphium recurvatum, together with some aspects of the intermediate host specificity of this echinostome. The E. recurvatum worked on in this study was obtained exclusively from material utilizing the first intermediate host Lymnaea peregra at Harting Pond, West Sussex, England.

## 2.2 Completion of the life cycle and morphology of the life cycle stages

### 2.2.1 INTRODUCTION

In order to provide a description of the life cycle stages of the E. recurvatum examined in this study, the life cycle of the parasite was completed through to the sexually mature adult stage under laboratory conditions and the general morphology of the life cycle stages was examined using light microscope techniques. The length of the pre-patent period of the parasite at a temperature of 18 +/- 2°C in the first intermediate host L. peregra was also investigated.

## 2.2.2 MATERIALS AND METHODS

### The life cycle stages

Naturally infected specimens of Lymnaea peregra emitting E. recurvatum cercariae were obtained from Harting Pond, West Sussex (NGR SU 778 219) by sweep-net sampling. Snails were dissected in distilled water. Heart tissue was examined for sporocysts and the digestive glands of the snails were teased apart with fine steel needles to obtain rediae which were examined live under light coverslip pressure in distilled water. Freshly emerged cercariae from individually isolated snails were examined in both a living condition and fixed in 10% Formalin using a ZEISS RA compound microscope and OLYMPUS VANOX photomicroscope with both bright field and phase-contrast illumination.

Batches of laboratory bred, infection-free L. peregra snails in the size class 4-6mm were exposed separately to freshly emerged cercariae, maximum age 1 hour. A sample of snails was dissected 14 days post-infection and the metacercarial cysts were examined and measured under light coverslip pressure. Cysts of the same age were fed in doses of 100 cysts in gelatine capsules (EM Scope Ltd) to 3, 3 day old, infection-free Khaki Campbell (Anas platyrhynchos) ducklings. The ducklings were then maintained on a diet of non-medicated chick crumbs and water fed *ad libitum*. The hosts were killed by cervical dislocation 15 days post-infection. The intestine was removed from each host from a point at its junction with the stomach, and removed to a surgical tray containing warm (40°C) saline, 0.75% NaCl. The entire length of each intestine was then measured and the intestine was then divided into 20 sections, each being 5% of its total length. Each



section was then removed to a petri dish of saline, opened by a longitudinal cut, thoroughly searched for parasites using a stereomicroscope, and the number of parasites in each section was recorded. The number of *in utero* eggs within each worm was recorded. The intestinal caeca of each host were examined separately. The adult worms were rinsed in saline and examined in both living condition and fixed in 10% Formalin as Mayer's Paracarmine stained whole mounts. In a number of cases the head collars were severed from living worms and examined in saline under coverslip compression using phase contrast microscopy to obtain accurate collar spine counts.

Experiments involving the use of A. platyrhynchos ducklings in this study (and all others described in this thesis) were carried out under authorization of a Home Office Licence (No. ELA 24/8194), held by the author, together with Certificate A (issued under the Cruelty to Animals Act; 1876 - Experiments on Living Animals)

#### **Duration of the pre-patent period in the first intermediate host Lymnaea peregra**

The length of the prepatent period of E. recurvatum in the first intermediate host snail was investigated at a temperature of 18 +/- 2°C. Infection-free 3 day-old Khaki Campbell Mallard ducklings (A. platyrhynchos) were experimentally infected with E. recurvatum metacercarial cysts. The cysts were obtained 14 days post-infection from lab-bred L. peregra snails in the size range 3-5mm which had been exposed *en masse* to freshly emerged E. recurvatum cercariae emitted from naturally infected L. peregra first intermediate hosts. Cysts were fed in gelatine capsules in

doses of 100 per bird to infection-free 3-day old ducklings. Eggs were obtained from host faeces beginning 12 days post-infection. Eggs from the host faeces were filtered in 0.75% saline on a series of steel and nylon sieves of decreasing pore size (1mm - 150 microns) in a similar manner to that described by Mouahid and Mone' (1988) for recovering eggs of Echinoparyphium elegans from the faeces of definitive hosts. At 17 days post-infection the birds were killed and an additional supply of eggs were obtained by sieving the faecal material present in the host gut. After collection all eggs were rinsed several times in clean distilled water.

Rinsed eggs were maintained in 20ml glass vials containing clean distilled water at 18-20°C. The vials were wrapped in aluminium foil to prevent exposure of the eggs to light. Darkness inhibited hatching but not miracidial development. On day 13 of incubation at 18-20°C the eggs were exposed to a strong hatching stimulus in the form of intense light from a fibre optic cold light source.

Forty laboratory bred infection-free L. peregra snails (in the size range 4-6mm) were each exposed individually to 3 freshly hatched miracidia (maximum age 1 hour) for 24 hours in 3ml of synthetic hard water medium (HMSO 1969) at 18-20°C. The snails were then removed to individual polystyrene containers each containing 20ml of water and were maintained at a temperature of 18 +/-2°C on a diet of clean boiled lettuce fed *ad libitum*. The snails were examined daily using a stereo-microscope and the time at which each infected snail began to emit cercariae was recorded



### 2.2.3 RESULTS

#### The life cycle stages

##### Sporocyst

The sporocysts were located mainly in the heart tissue. A sporocyst from the heart tissue of a naturally infected L. peregra is shown in Fig 2.1 a. Rasin (1933) has previously reported the development of E. recurvatum sporocysts in the heart cavity of L. peregra. The sporocysts generally have the form of an elongate sac usually measuring 500 $\mu$  long x 250 $\mu$  wide under coverslip compression. In some specimens pharyngeate mother rediae could be seen developing within.

##### First generation redia (Mother Redia)

A first generation (mother) redia of E. recurvatum from L. peregra is illustrated in Fig 2.1 b. Mother rediae were found predominantly in the digestive gland tissues of L. peregra. Mature living specimens from L. peregra measured 0.75-1.5mm in length (mean=1.25mm) and 0.2-0.35mm in width (mean = 0.28mm, n=10). These rediae possess a well developed anterior collar, just posterior to which is located a protrusible birth pore. The rediae have a large pharynx leading to a short saccate gut usually filled with brown or orange material. Locomotory or ambulatory appendages, lateral projections of the redial wall, are located in the posterior third of the body. Mother rediae were found to contain 4-9 developing daughter rediae.

**FIG 2.1 Sporocyst, mother and daughter rediae of  
Echinoparyphium recurvatum**

(a) Sporocyst of E. recurvatum from the heart cavity of an infected Lymnaea peregra

DMR = Developing Mother Redia

(b) Mother redia of E. recurvatum from the digestive gland of a naturally infected L. peregra

Ph = Pharynx

c = Collar

I = Intestine

BP = Birth pore

A = Ambulatory process

DDR = Developing daughter redia

Gb = Germ balls

(c) Daughter Redia of E. recurvatum from the digestive gland of a naturally infected L. peregra.

Ph = Pharynx

c = Collar

I = Intestine

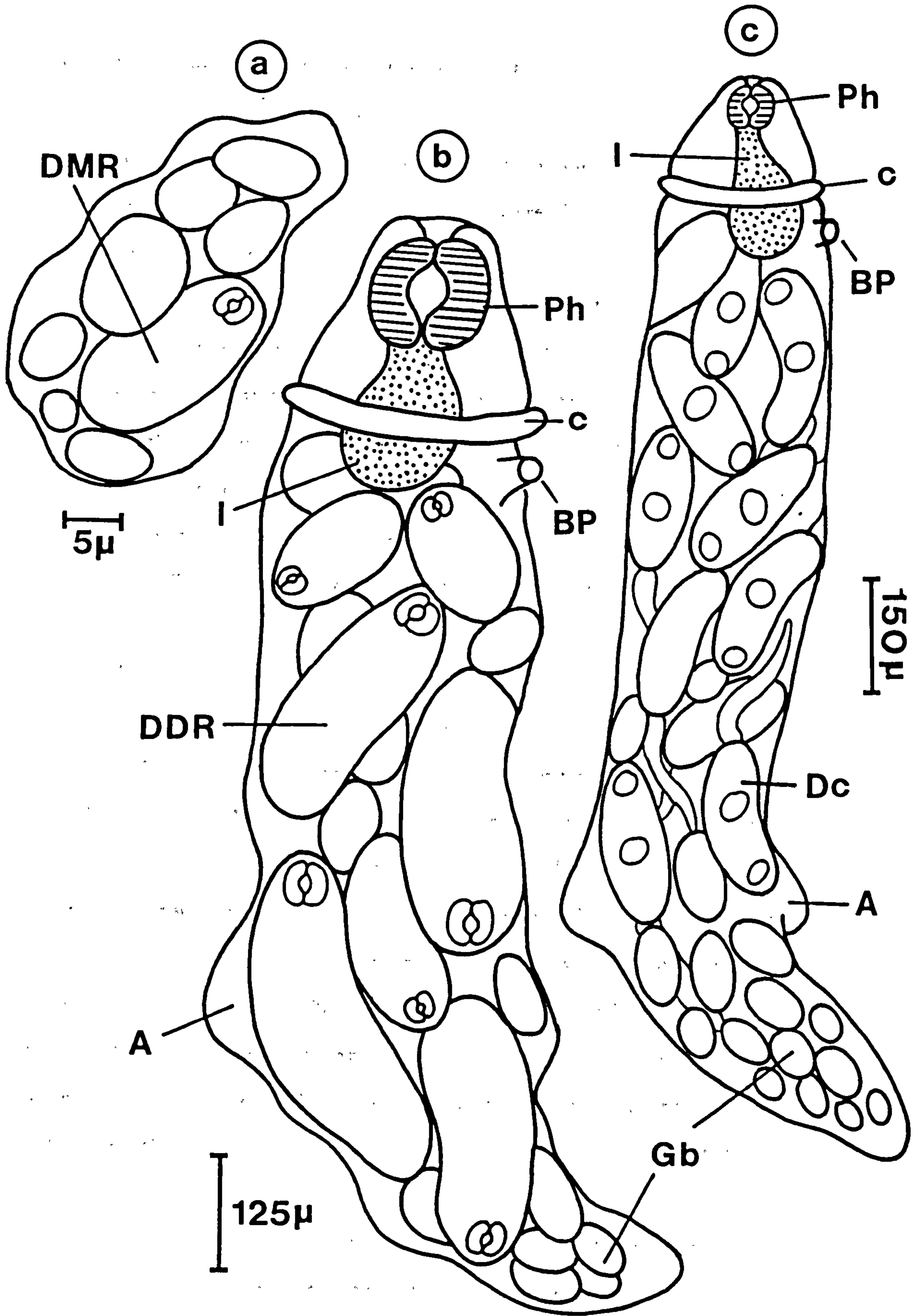
BP = Birth pore,

A = Ambulatory process

Dc = Developing cercariae.

Gb = Germ balls





### Second Generation Redia (Daughter Redia)

A mature second generation (daughter) redia of E. recurvatum from the digestive gland of L. peregra is shown in Fig 2.1 c. The digestive glands of infected L. peregra were found to carry heavy infections of these rediae. They possess a muscular anterior pharynx leading directly to a short saccate intestine containing brown or orange material. An anterior protrusible birth pore is also present. A distinct anterior collar is located between the birth pore and the pharynx and at the posterior end a pair of lateral or ambulatory appendages are present. From 6 to 12 mature cercariae were observed in these rediae together with numerous germ balls. These rediae were in the length range 1-2.5mm.

### Cercaria

The morphology of a cercaria of E. recurvatum from L. peregra is shown in Fig 2.2. The dimensions of 10% formalin-fixed cercariae are shown in Table 2.1. The cercarial body is elongate-oval and phase contrast microscopy shows the entire body surface to be covered in small posteriorly directed spines which diminish in size and number posteriorly. The anterior collar is well developed and bears 45 collar spines. The middle 37 are arranged in two tiers, those of the oral tier being slightly smaller than those of the aboral tier (see Fig 2.2b). The four spines at each corner of the collar are slightly larger and more robust in form than the rest.

The oral sucker is spherical and located at the centre of the collar. The oral sucker is smaller than the ventral sucker, (diameter ratio 1 : 1.3), which is protrusible and located slightly posterior to the middle of the body. The oral opening is located at the centre of the



**FIG 2.2 Cercaria of Echinoparyphium recurvatum**

(a) The morphology of a mature living cercaria of E. recurvatum from a naturally infected Lymnaea peregra showing cystogenous gland cell distribution on left and excretory system on right

int - Intestinal caecum

pg - Penetration gland cells

g - Cystogenous gland Cells

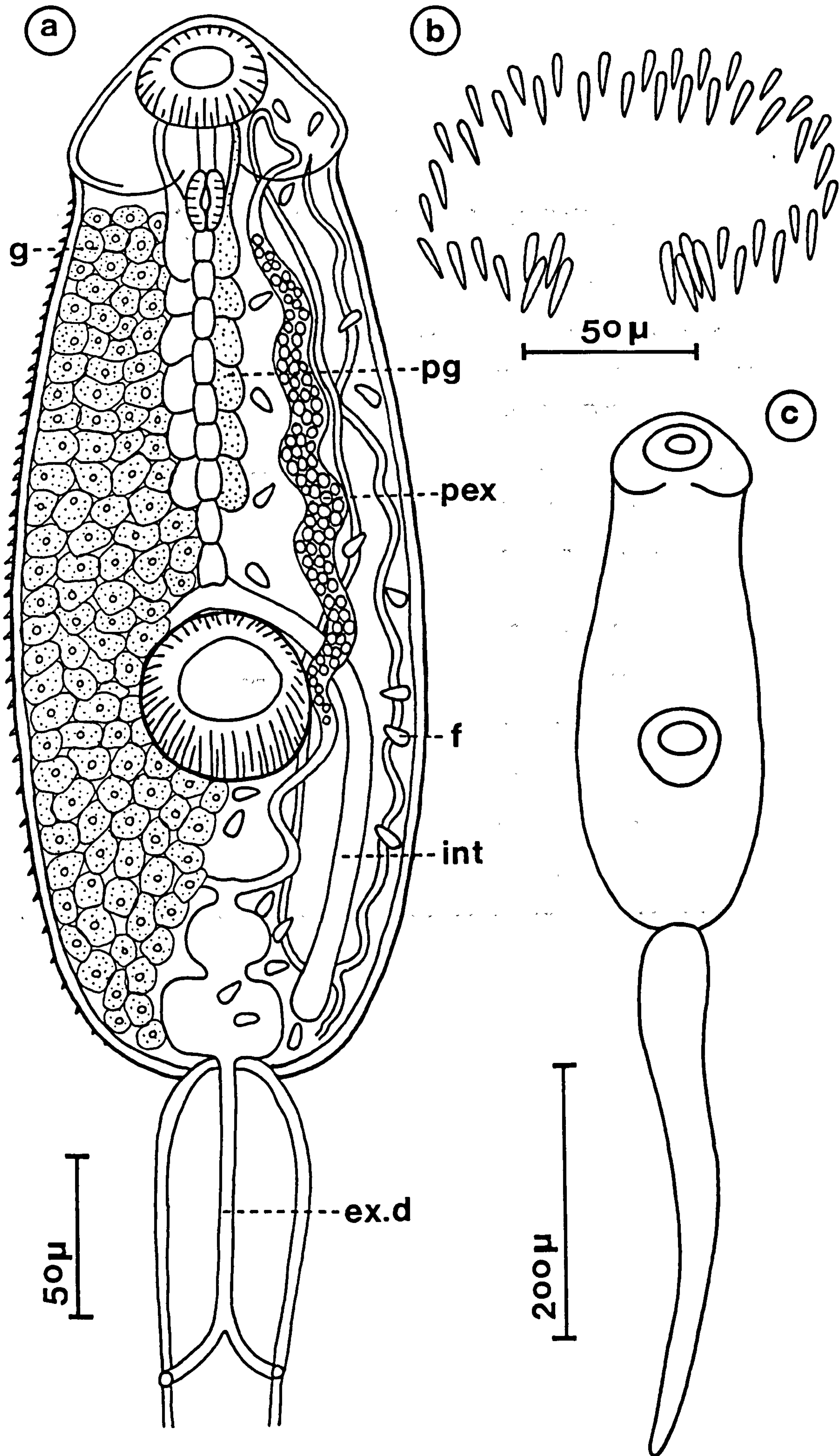
f - Flame cell

pex - Primary excretory duct

ex.d - Caudal excretory duct

(b) The arrangement of spines around the anterior collar of the cercaria (*Camera Lucida* tracing)

(c) Cercaria fixed in 10% Formalin to show relative proportions of body and tail.





**TABLE 2.1 Measurements of cercariae of Echinoparyphium recurvatum from Lymnaea peregra**

(Measurements are based on 20 freshly emitted specimens fixed in 10% Formalin).

Measurements are in  $\mu$  - range followed by mean in parentheses

Feature	Measurement
Body length	351 - 426 (381)
Body Width (mid-actetabular)	107 - 137 (121)
Oral Sucker (diam)	39 - 56 (42)
Ventral Sucker (diam)	51 - 68 (56)
Pharynx length	25 - 28 (26)
Pharynx width	20 - 23 (21)
Tail length	362 - 421 (408)
Tail width (at base)	41 - 48 (43)

oral sucker and opens into the pre-pharynx followed by the muscular pharynx and the long oesophagus. This divides just anterior to the ventral sucker and forms two long intestinal caeca which extend to the posterior end of the body. The oesophagus appears to contain a single row of cells. Six pairs of para-oesophageal gland cells located either side of the oesophagus were observed in some cercariae but they were difficult to discern, even using a variety of stains such as neutral red. The ducts from these cells appear to run anteriorly in a bundle and open around the edge of the oral sucker. Cystogenous gland cells with granular cytoplasm are distributed throughout the body from the level of the pharynx to the posterior of the body.

The excretory bladder is located at the posterior end of the body and when full is two-chambered. The excretory bladder is composed of a large basal chamber linked to a smaller spherical upper chamber. The basal chamber connects posteriorly to the caudal excretory duct which runs posteriorly into the tail. This duct bifurcates and exits via the caudal excretory pores on either side of the upper extremity of the tail. Two primary excretory ducts lead from the anterior of the upper chamber of the excretory bladder and extend in a coiled manner to the level of the ventral sucker where they dilate and are filled with spherical refractile excretory granules. The primary ducts then contract again and run dorsally to the level of the pharynx where they form anterior loops and run back on themselves passing posteriorly as narrow secondary collecting ducts containing ciliated patches. At the level of the ventral sucker these ducts divide to form the anterior and posterior collecting ducts. In the



cercariae of E. recurvatum observed in this study the flame cells appear to be arranged in groups of two. However, the exact total numbers of flame cells could not be ascertained with certainty in the specimens examined although they are numerous. Diaz-Diaz (1976) gives the flame cell formula of E. recurvatum cercariae from L. peregra as ;  $2 ( (2+2+2+2+2+2+2+2+2+2+2) + (2+2) ) = 54$ .

The cercarial tail is approximately the same length as the body, or slightly longer, (see Fig 2.2c) and is inserted sub-terminal ventrally at the posterior of the body. The tail is aspinose and phase contrast microscopy shows that it has no finfold.

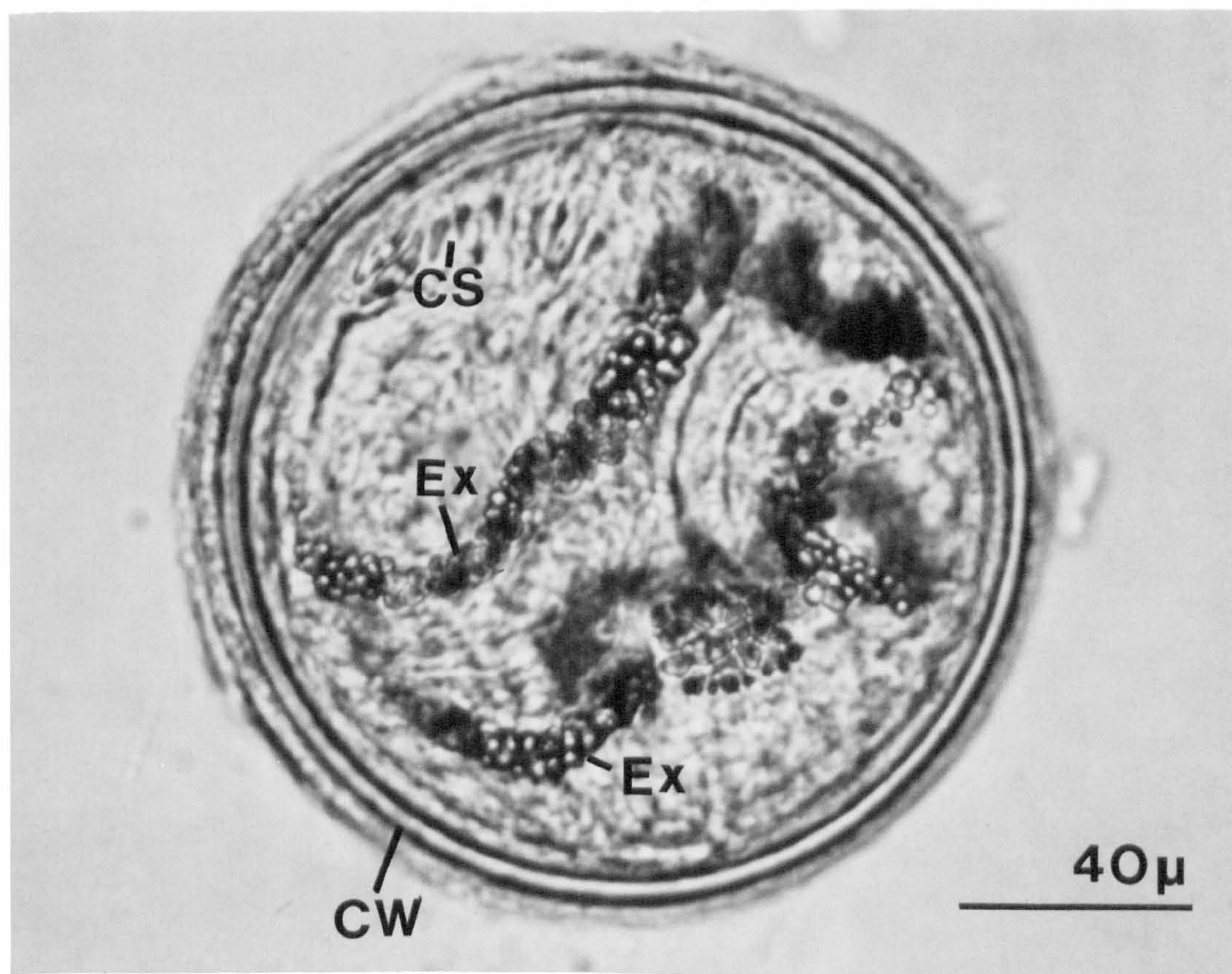
#### **Metacercarial cyst**

A 14 day old metacercarial cyst of E. recurvatum from L. peregra is shown in Fig 2.3. The diameter of cysts measured 141-153 $\mu$  (mean = 148 $\mu$  , n=20) in diameter. The cyst wall was 13-16 $\mu$ , (mean= 15 $\mu$ ), in thickness. The metacercarial cyst of E. recurvatum is spherical and contains a tightly coiled metacercaria in which the collar spines and excretory ducts containing refractile granules can be clearly seen (see Fig 2.3). Cysts were found in the kidney and digestive gland of infected L. peregra and also in the lining of the mantle and pericardial tissue. The cyst wall is transparent and consists of an outer layer, usually covered in "host reaction" material, and an inner layer. At 14 days post-infection the oral sucker, ventral sucker and pharynx of the metacercaria had slightly increased in size, and the penetration and cystogenous glands had disappeared. However, as Diaz-Diaz (1976) has



FIG 2.3 Metacercarial cyst of Echinoparyphium recurvatum (14 days old) from the kidney of a Lymnaea peregra experimentally infected with cercariae emitted from a naturally infected L. peregra first intermediate host

CS - Collar spines ; Ex - Primary excretory ducts containing spherical refractile excretory granules ; CW - Cyst Wall.





observed, the size of the cyst remains relatively unchanged during development over this period of time.

### Adult

At 15 days post-infection Echinoparyphium recurvatum adults were recovered from the intestines of all ducklings infected with cysts. These hosts yielded a mean of 18 (+/- 2.9 S.E.) adult parasites per host (13, 18 and 23). All the parasites recovered were gravid adults and contained a mean of 28 (range = 18 - 43) *in utero* eggs per worm. All the parasites were obtained from the anterior 20% of the intestine.

An adult specimen of Echinoparyphium recurvatum is shown in Fig 2.4a. The dimensions of ten 15 day-old adult specimens, fixed in 10% Formalin, stained in Mayer's Paracarmine and mounted in "Ralmount", are given in Table 2.2.

All adult E. recurvatum examined were found to possess 45 collar spines. The middle 37 spines are arranged around the collar in a double tier, the spines of the oral tier being slightly smaller than those of the aboral tier, (see Fig 2.4 b), as in the cercaria. At each corner of the anterior collar a corner group of 4 spines, each slightly larger and more robust than those of the rest of the collar, was found to exist. The anterior spine bearing collar of a 15 day old adult E. recurvatum is shown from various aspects in the following section of this chapter which examines the tegumental topography of the adult worm using SEM.

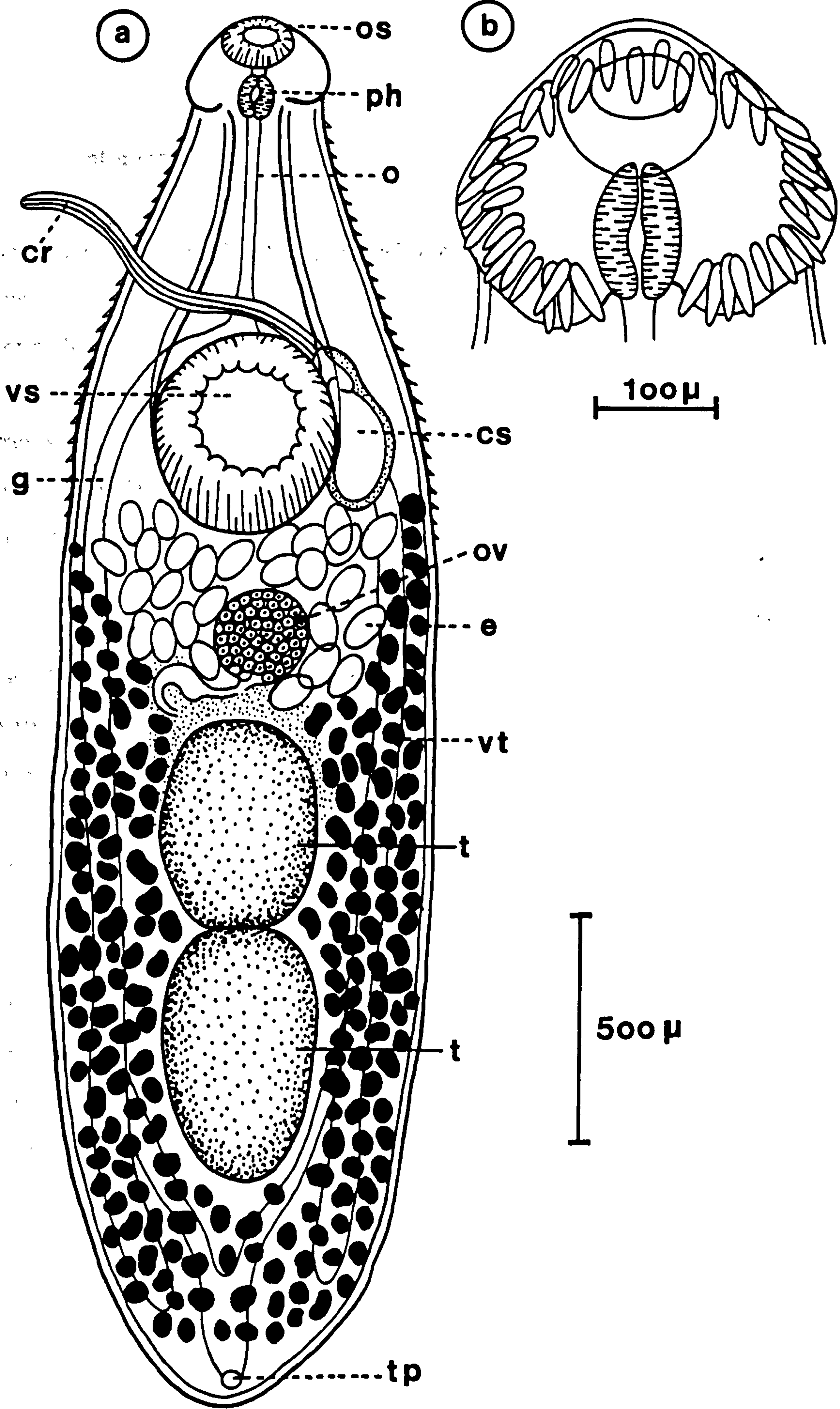
**FIG 2.4 Adult Echinoparyphium recurvatum**

**(a) A 15 day-old adult of Echinoparyphium recurvatum from the intestine of an experimentally infected duckling**

os - Oral sucker  
ph - Pharynx  
o - Oesophagus  
cr - Cirrus  
cs - Cirrus sac  
vs - Ventral sucker  
g - Gut caecum  
e - Egg  
ov - Ovary  
vt - Vitelline follicles  
t - Testes  
tp - Terminal pore

**(b) Anterior collar in detail to show the arrangement of collar spines**





**FIG 2.4 continued****(c) Cirrus sac and proximal region of cirrus**

C - Cirrus

sd - Sperm duct

pc - prostatic gland cells

cs - Cirrus sac

sv - Seminal vesicle

ve - Vasa efferentia

**(d) The female reproductive system**

Ov - Ovary

u - Uterus

e - Egg

od - Oviduct

lc - Laurer's Canal

vc - Vitelline canal

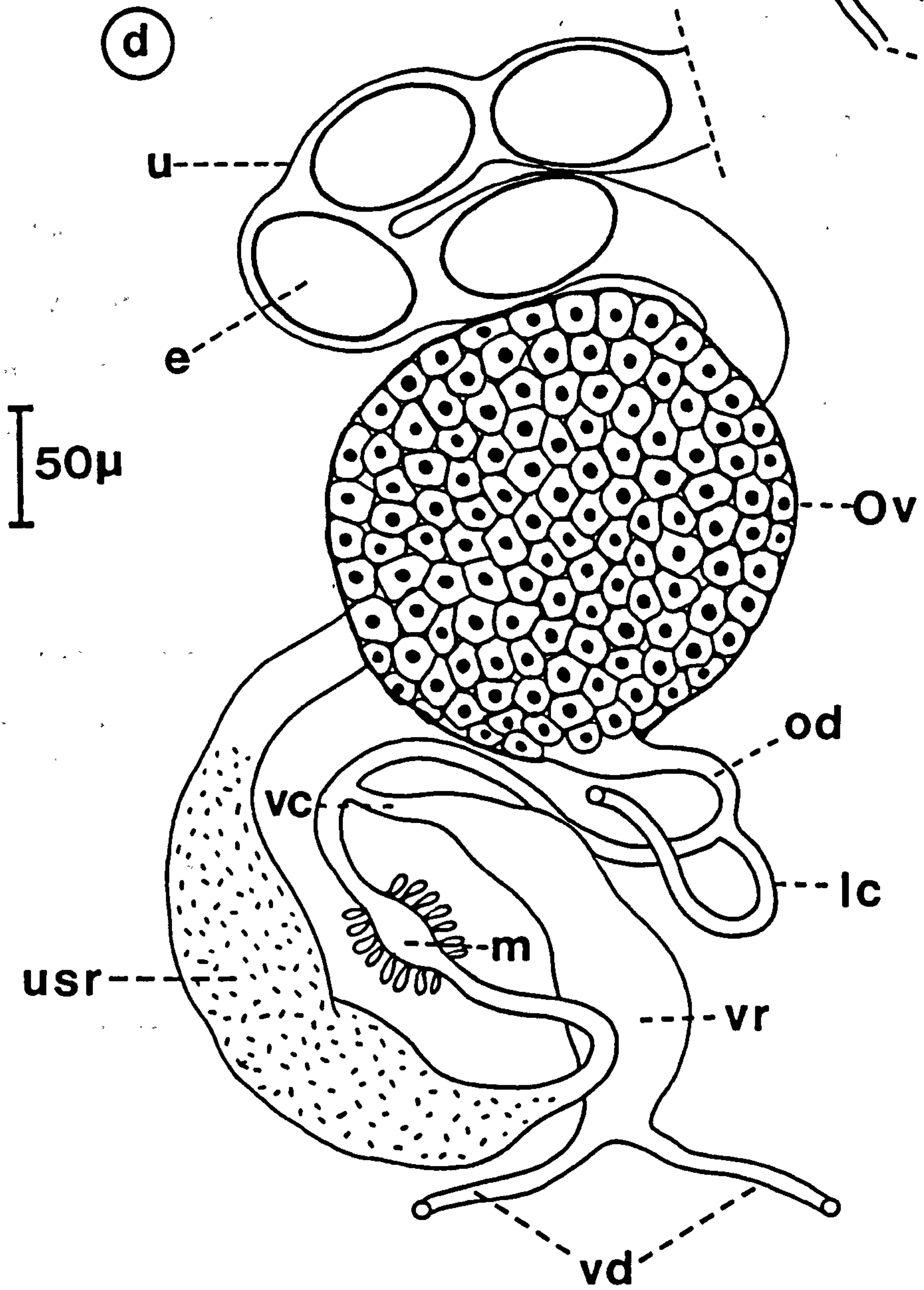
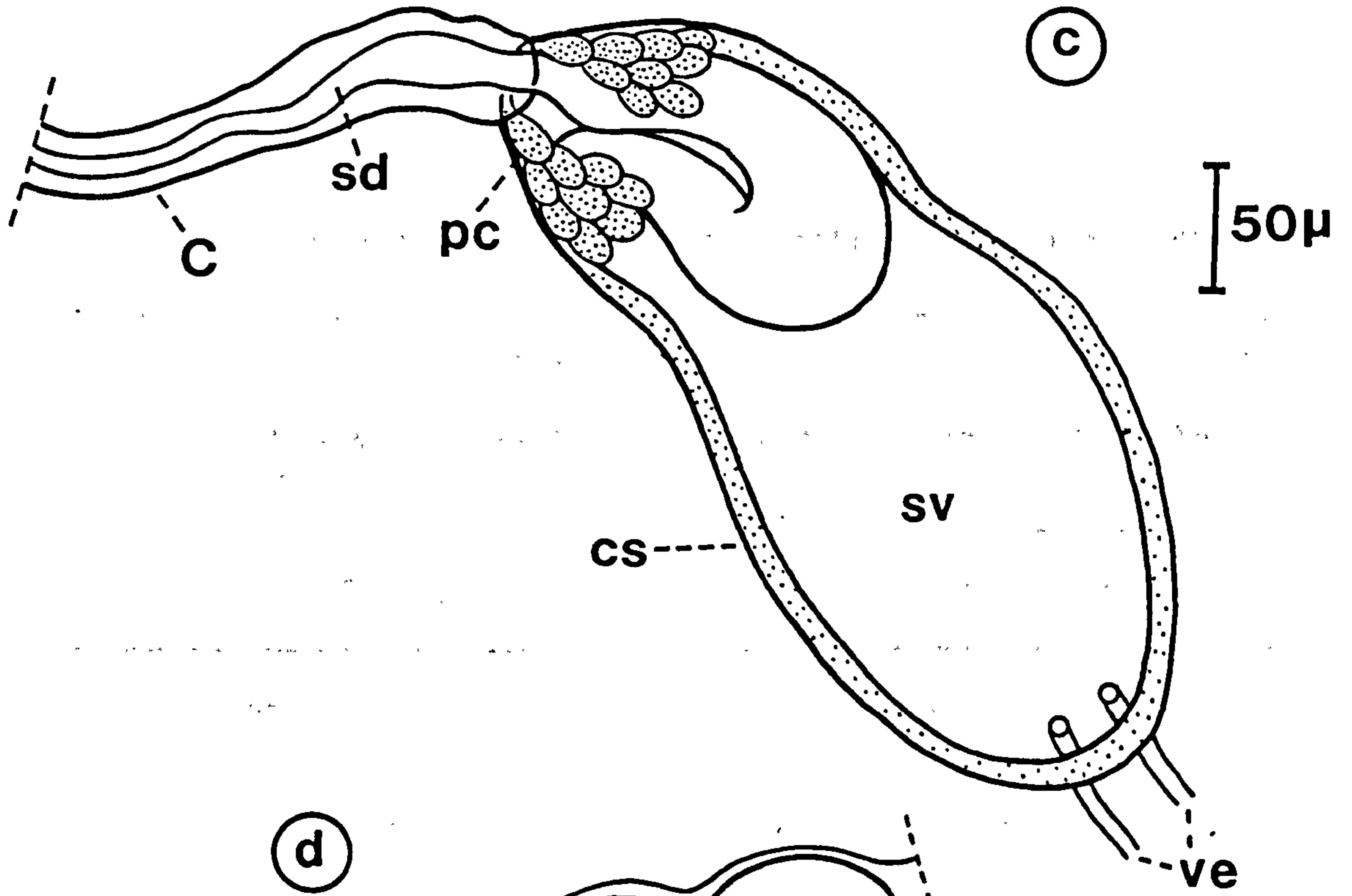
vr - Vitelline reservoir

vd - Vitelline ducts

m - Mehlis gland

usr - Uterine seminal receptacle





**TABLE 2.2 Measurements of Echinoparyphium recurvatum adults**

(Measurements are based on 10 , 15-day old , adults from experimentally infected Anas platyrhynchos. Specimens were fixed in 10% Formalin and stained in Mayer's Paracarmine).

Measurements are in  $\mu$  (except where stated) - range followed by mean in parentheses

Feature	Measurement
Body length	2.4 - 3.8 (3.1) mm
Body width	560 - 980 (800)
Oral Sucker (diam.)	115 - 127 (123)
Pharynx length	87 - 110 (102)
width	70 - 96 (83)
Ventral sucker (diam.)	253 - 410 (381)
Ovary (diam.)	178 - 208 (197)
Anterior Testis length	403 - 496 (473)
width	245 - 319 (296)
Posterior Testis length	458 - 561 (527)
width	276 - 351 (311)
No. in utero eggs	18 - 43 (28) eggs
Eggs (in utero) length	90 - 110 (96)
width	57 - 63 (61)
Collar spine lengths	
Oral	37 - 42 (39)
Aboral	41 - 44 (42)
Corner	48 - 54 (50)



The anterior region of the body is covered in rows of small spines from a point just posterior to the anterior collar to a point just posterior to the ventral sucker. The oral sucker is small, smooth and spherical and located at the centre of the anterior collar. The larger acetabulum is located in the anterior third of the body and under the light microscope appeared to have an indented rim, an observation later affirmed by SEM examination (see following section). The digestive system consists of a short pre-pharynx leading to a muscular pharynx, in turn leading to the oesophagus. The long oesophagus bifurcates just anterior to the ventral sucker to form two caeca which extend to the posterior end of the body.

The two testes are arranged *in tandem* and are located in the posterior half of the body. They are both elongate oval in outline and both have smooth margins. The anterior testis is slightly smaller than the posterior. The cirrus sac (see Fig 2.4 c) is flask-shaped and located at the lateral margin of the ventral sucker, usually on the left. The *vasa efferentia* run anteriorly from each testis and enter separately into the posterior end of the cirrus sac where they join the seminal vesicle which is looped anteriorly. The ejaculatory duct loops anteriorly and leads into the long protrusible cirrus. The latter has a single terminal pore and its surface is aspinose but ridged. Clusters of prostatic gland cells are present at the anterior of the cirrus sac.

The ovary lies in the middle of the body just anterior to the anterior testis. It is rounded in form and gives rise to the oviduct from its postero-ventral region (see Fig 2.4 d). Laurer's Canal

arises from the proximal part of the oviduct and loops on itself before opening via a small pore on to the dorsal body surface close to the posterior margin of the ovary. The oviduct extends posteriorly and receives the common vitelline duct just before the ootype. The ootype is surrounded by the Mehlis complex. The uterus extends from the ootype, usually loops back on itself, and then dilates into a large uterine seminal receptacle which was observed to be filled with sperm in living specimens. A true seminal receptacle with a duct is absent. The uterus then runs anteriorly with several coils containing the shelled eggs, which are few in number but large in size, and continues to the posterior level of the acetabulum where it forms the muscular metraterm which eventually opens at the common genital pore. The genital pore is located just anterior to the acetabulum.

The vitellaria consist of large vitelline follicles distributed laterally, extending from just posterior to the ventral sucker to the posterior end of the body. The vitelline fields unite fully behind the posterior testis and partially in front of the anterior testis. The vitellaria overlay the intestinal caeca and the excretory bladder. The vitellaria connect to two lateral vitelline ducts which run anteriorly and connect to the common vitelline reservoir just in front of the anterior testis. This reservoir is connected to the oviduct via the short common vitelline duct.

The protonephridial system of the adult consists of a tubular "Y-shaped" excretory bladder which opens terminally at the posterior extremity of the body via the excretory pore. The bladder divides into two main excretory ducts which extend to the level of the

pharynx. Although the flame cells were numerous their exact number and distribution could not be accurately ascertained.

### **Egg**

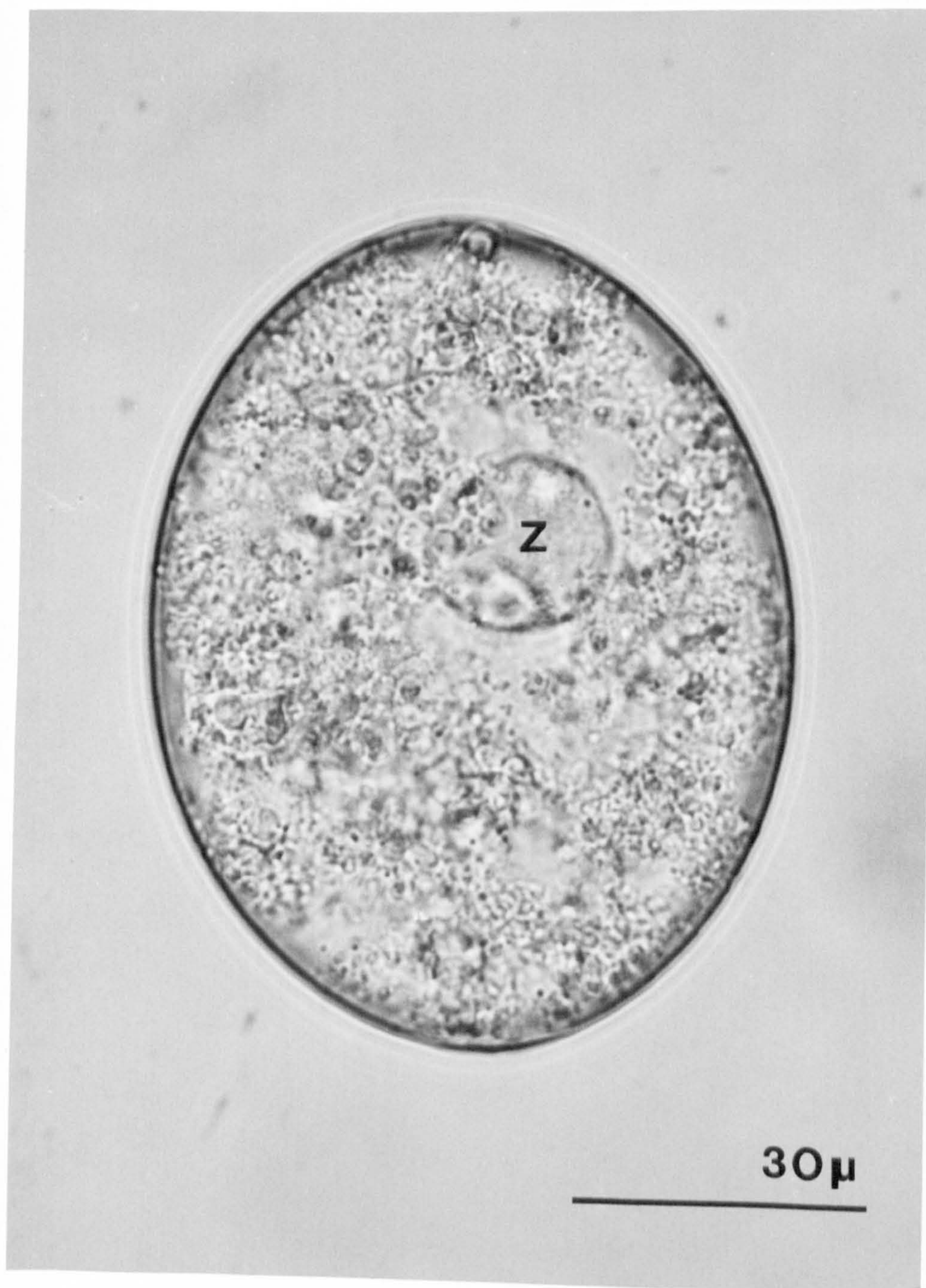
The eggs of E. recurvatum are large, golden-yellow in colour, operculate, and broadly ovoid in shape. Ten eggs from the faeces of an experimentally infected duckling were found to measure 93-115 $\mu$  long (mean= 97 $\mu$ ) x 59-65 $\mu$  wide (mean = 61 $\mu$ ). The eggs are uncleaved when laid but contain a clearly visible bi-nucleate zygote. An uncleaved egg obtained from the faeces of a duckling infected with E. recurvatum adults is shown in Fig 2.5. Eggs were found to hatch at between 11 and 13 days at 20°C, although the time to hatching is markedly temperature dependent, see Chapter 3.

### **Miracidium**

A living miracidium of E. recurvatum is illustrated in Fig 2.6 a. The miracidium is approximately 150 $\mu$  in length and possesses a pair of black pigmented eyespots with hyaline lenses. The surface of the miracidium is covered in 4 tiers of ciliated epidermal plates. A protrusible terabratorium is present at the anterior end and this is connected via a short canal to a sacculate apical gland with spherical granular contents. Two flame cells are present in the middle of the body, one positioned more anteriorly than the other. Each connects to a coiled excretory duct which exits laterally via a simple pore. The posterior of the miracidium is filled with 20-30 germinal cells.



FIG 2.5 An egg of Echinoparyphium recurvatum from the faeces of an experimentally infected Anas platyrhynchos duckling : Z = Zygote



**FIG 2.6 The miracidium of Echinoparyphium recurvatum**

**(a) - Living miracidium**

t - Terabratorium

ag - Apical gland

p - Papilla

e - Eyespot

ng - Nerve ganglion

f - Flame cell

gc - Germinal cell

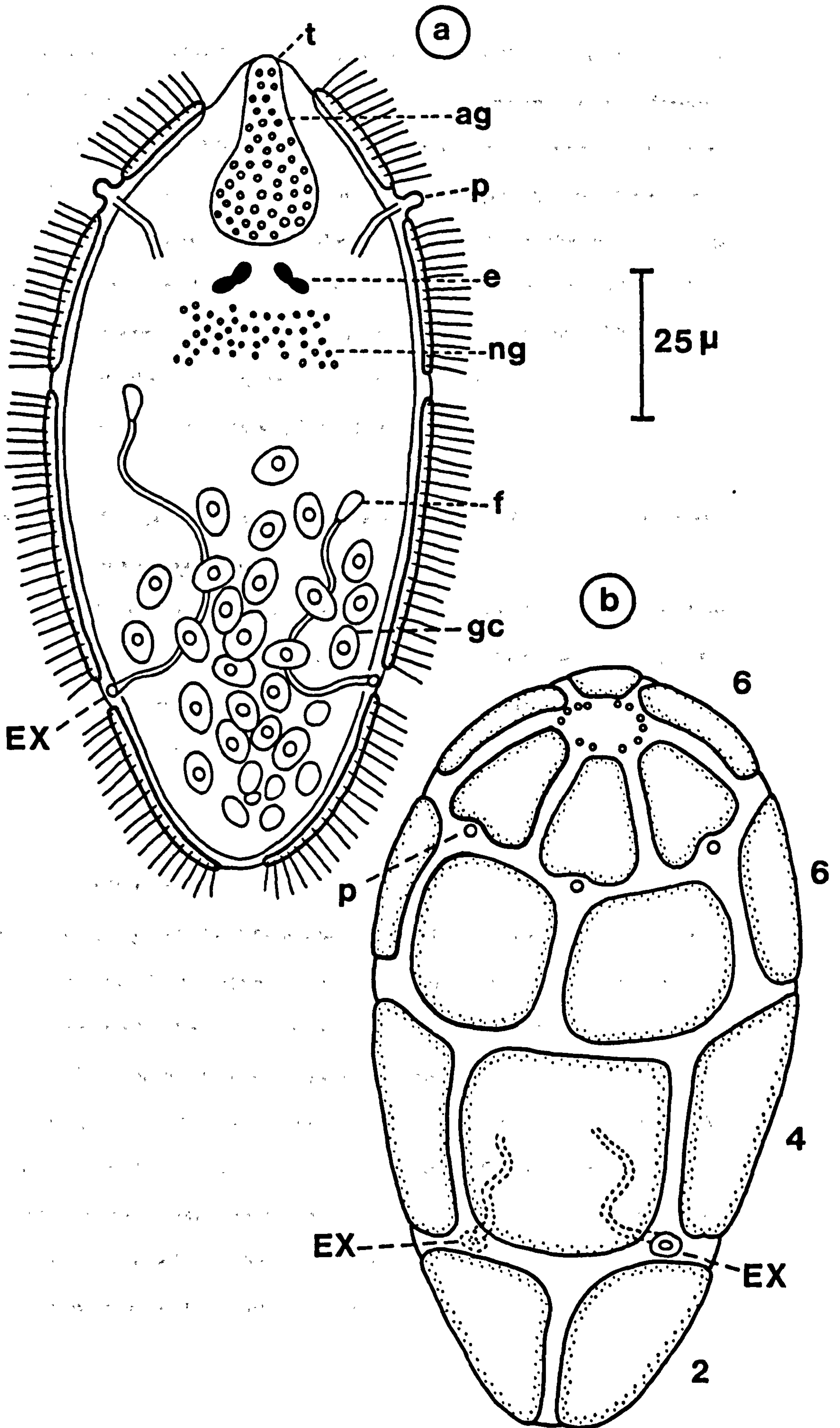
EX - Excretory pore

**(b) - Miracidium stained using silver nitrate solution to show arrangement of the epidermal plates and sensory papillae**

p = Papilla

EX = Excretory pore







Silver nitrate stained preparations of miracidia showed that the epidermal plates were arranged in 4 tiers according to the formula  $6+6+4+2=18$ , (see Fig 2.6 b). This is the classic type arrangement of epidermal plates in the genus Echinoparyphium. One large sensory papilla was found at the base of each of the plates of the first tier, and a further 12-14 smaller papillae and gland duct openings were distributed over the terabratorium. The lateral openings of the two excretory ducts can be clearly seen between the third and fourth rows of epidermal plates.

**Length of the prepatent period in the first intermediate host Lymnaea peregra.**

Of the 40 L. peregra snails exposed to infection 36 survived and of these surviving snails 24 (69%) began to shed cercariae of E. recurvatum between 28 and 41 days post-exposure. The mean time for infections to reach patency at 18-20°C was found to be 33.8 (+/- 0.62 S.E.) days.

#### 2.2.4 DISCUSSION

The morphology of the life cycle stages of E. recurvatum utilizing L. peregra as first intermediate host in the present study are consistent with the descriptions given by Diaz-Diaz (1972) and Rasin (1933) both of whom described the life cycle stages in detail from material utilizing L. peregra as first intermediate host. As such the work presented in this section of the current chapter provided a definitive identification of the parasite to be made, and therefore allowed further experimental work to be embarked upon from a firm basis of identity.

## 2.3 Scanning electron microscopy of the tegumental surfaces of adult Echinoparyphium recurvatum

### 2.3.1 INTRODUCTION

Relatively few detailed stereoscan studies have been carried out on echinostome digeneans and of these only three have provided information on the whole surface topography of adult echinostomes ; Smales & Blankespoor (1984) for Echinostoma revolutum and Isthmiophora melis , Køie (1987) for Mesorchis denticulatus and Fried & Fujino (1984) for pre-ovigerous Echinostoma revolutum . No detailed information is currently available on the topography of the tegumental surfaces of mature adult E. recurvatum. This present study therefore set out to examine using SEM, for the first time, mature adult E. recurvatum from the intestine of a definitive host. The aims of the study were twofold. The first was to provide detailed information for the first time on the tegumental morphology of adult E. recurvatum. The second was to provide a detailed description of the tegumental morphology of the adult E. recurvatum which would provide a basis for comparison of the tegumental features of adult E. recurvatum with those of echinostomes previously studied by SEM.

### 2.3.2 MATERIALS AND METHODS

E. recurvatum cercariae were obtained from naturally infected specimens of L. peregra collected at Harting Pond. Laboratory bred infection-free L. peregra snails in the size class 4-6mm were exposed individually to freshly emerged cercariae. At 14 days post-infection the metacercarial cysts from each source were fed

in doses of 100 to a group of 3, 3 day old, Khaki Campbell Ducklings. The birds were then maintained on a diet of non-medicated chick crumbs and water fed *ad libitum* and were sacrificed 15 days post-infection by cervical dislocation. Ovigerous adult worms were immediately dissected from the intestine and rinsed briefly in warm (40°C) avian saline (0.75% NaCl) followed by a single rapid wash in phosphate buffer pH 7.4. The parasites were subsequently fixed in cold (20°C) 2.5% glutaraldehyde solution buffered to pH 7.4 for 2 hours and then post-fixed for a further 2 hours in 1% osmium tetroxide buffered to pH 7.4 with 0.1 M sodium cacodylate. They were then dehydrated through a graded ethanol series to acetone and critical point dried using carbon dioxide in a Samdri-780 critical point drier. The specimens were coated with platinum in the presence of argon using an ion-sputtering apparatus and were examined using a Hitachi S-510 Scanning Electron Microscope and a JEOL 100-CX Electron Microscope with ASI4D scanning facility, at an accelerating voltage of 25 kV.

### 2.3.3 RESULTS

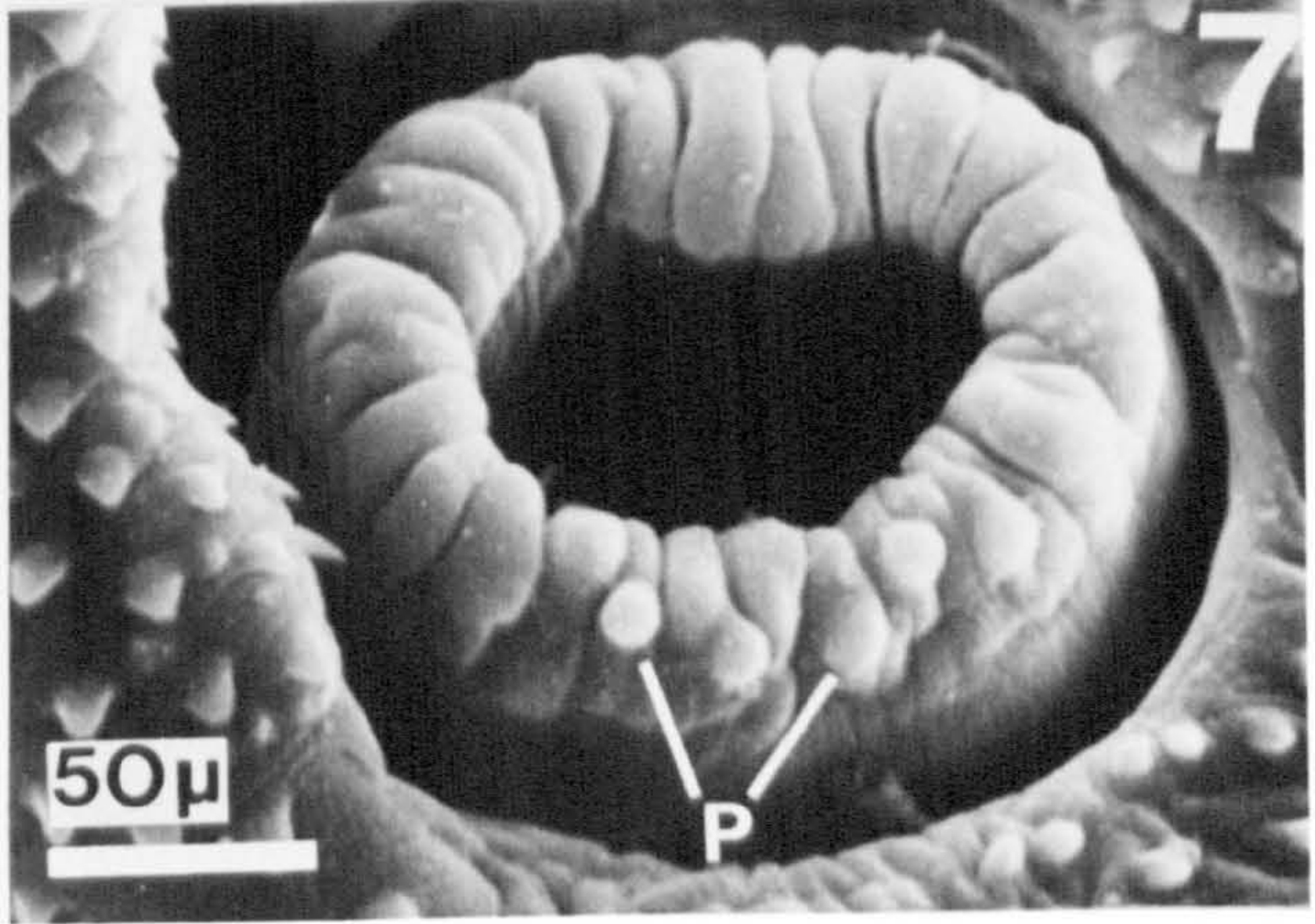
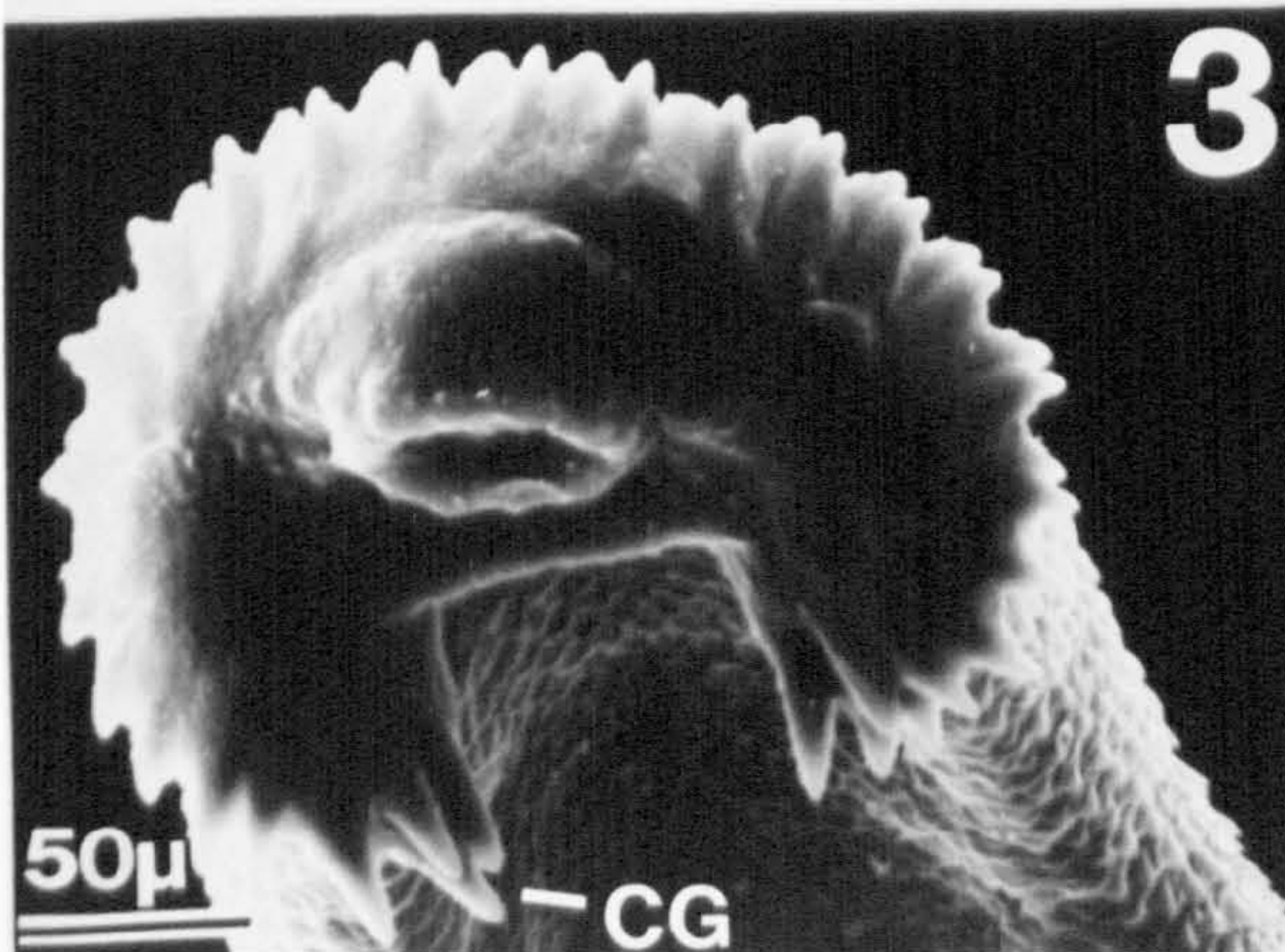
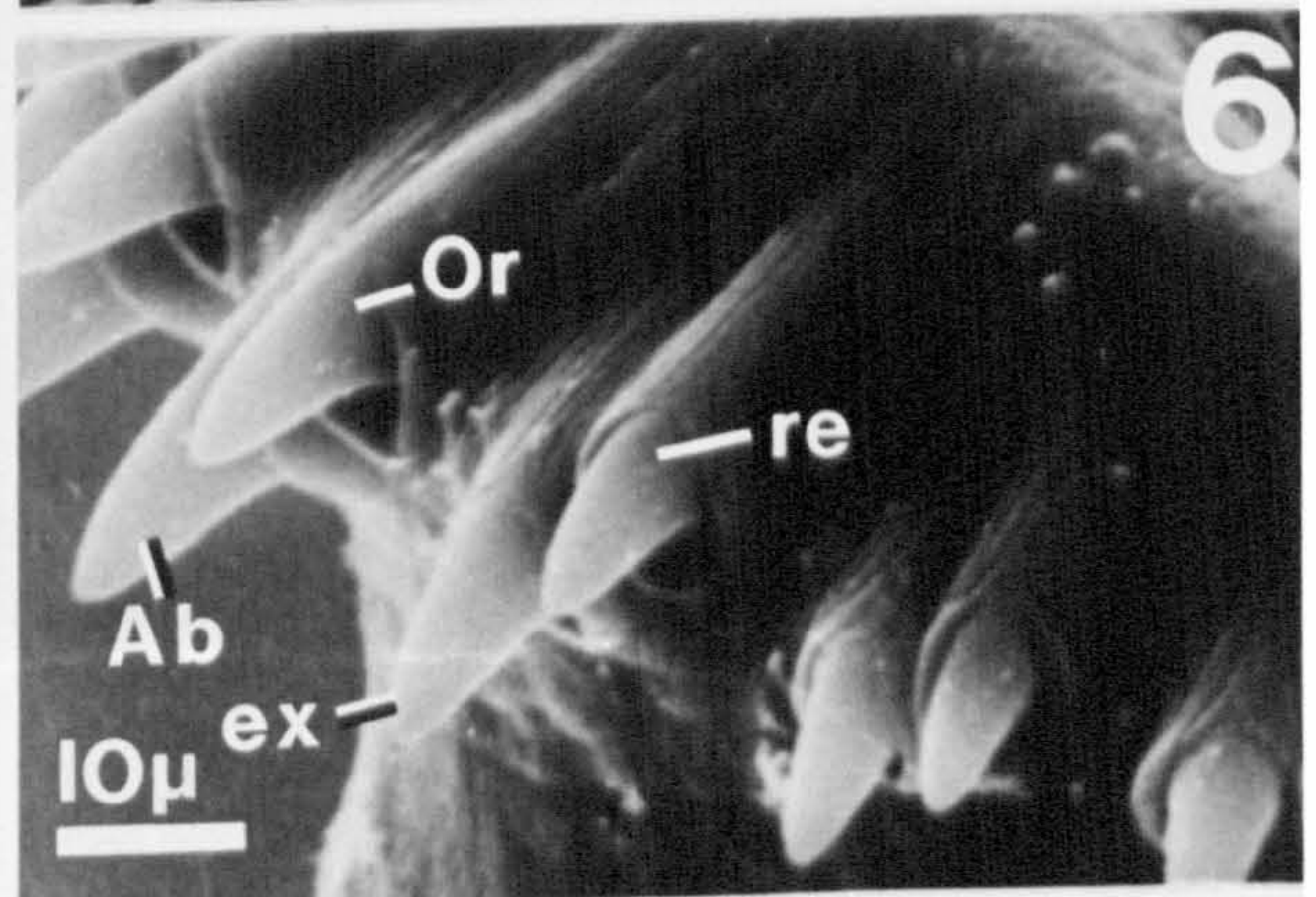
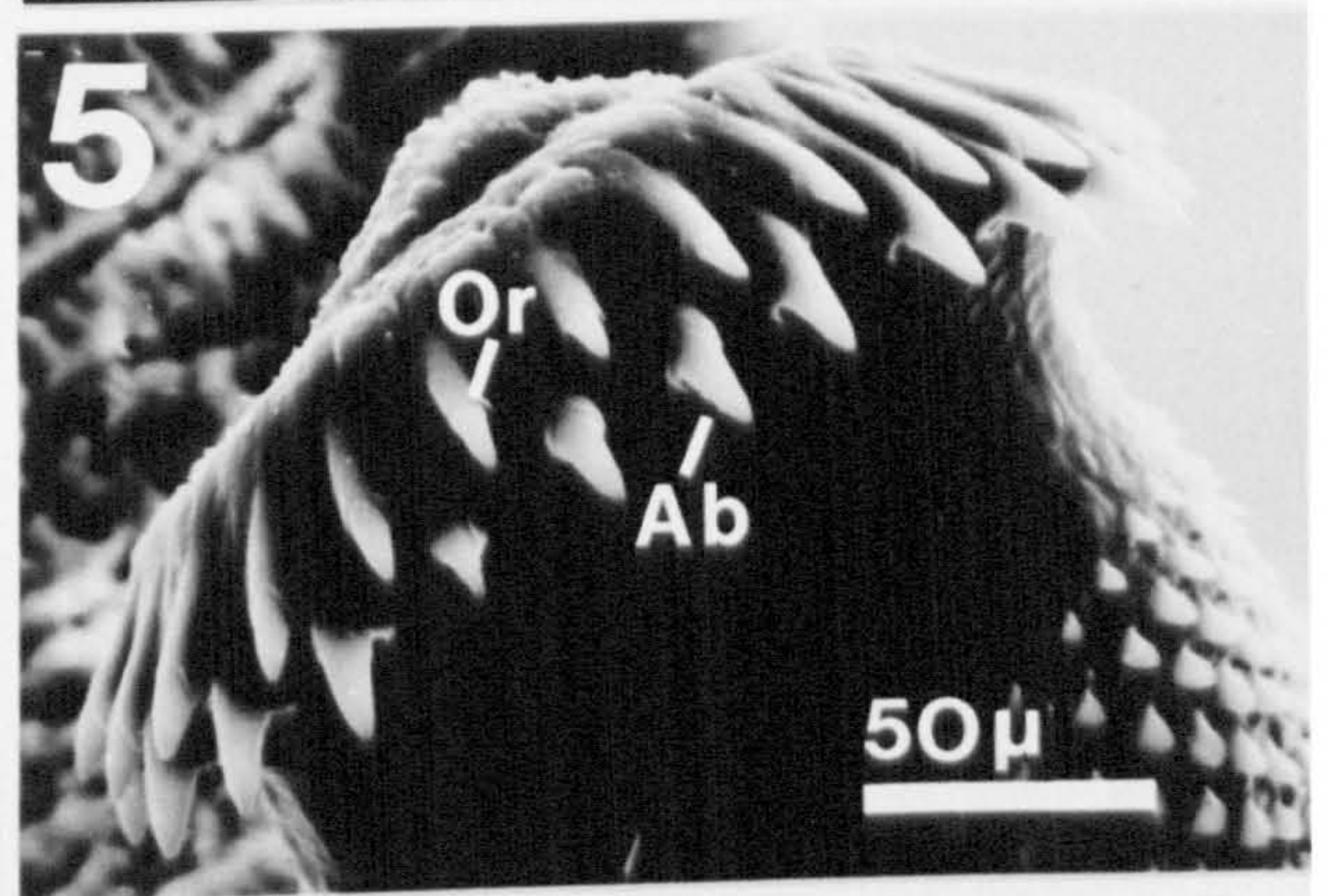
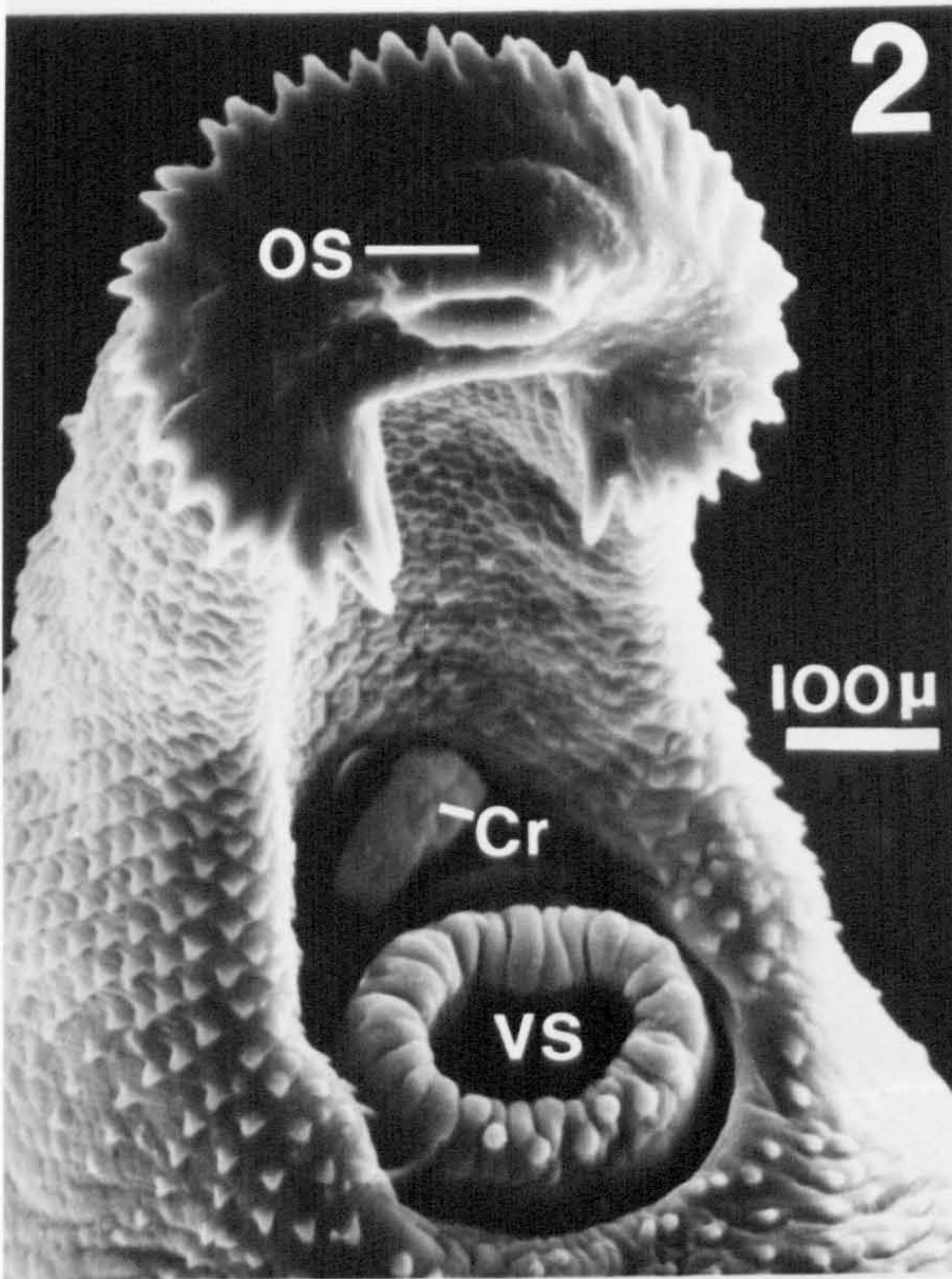
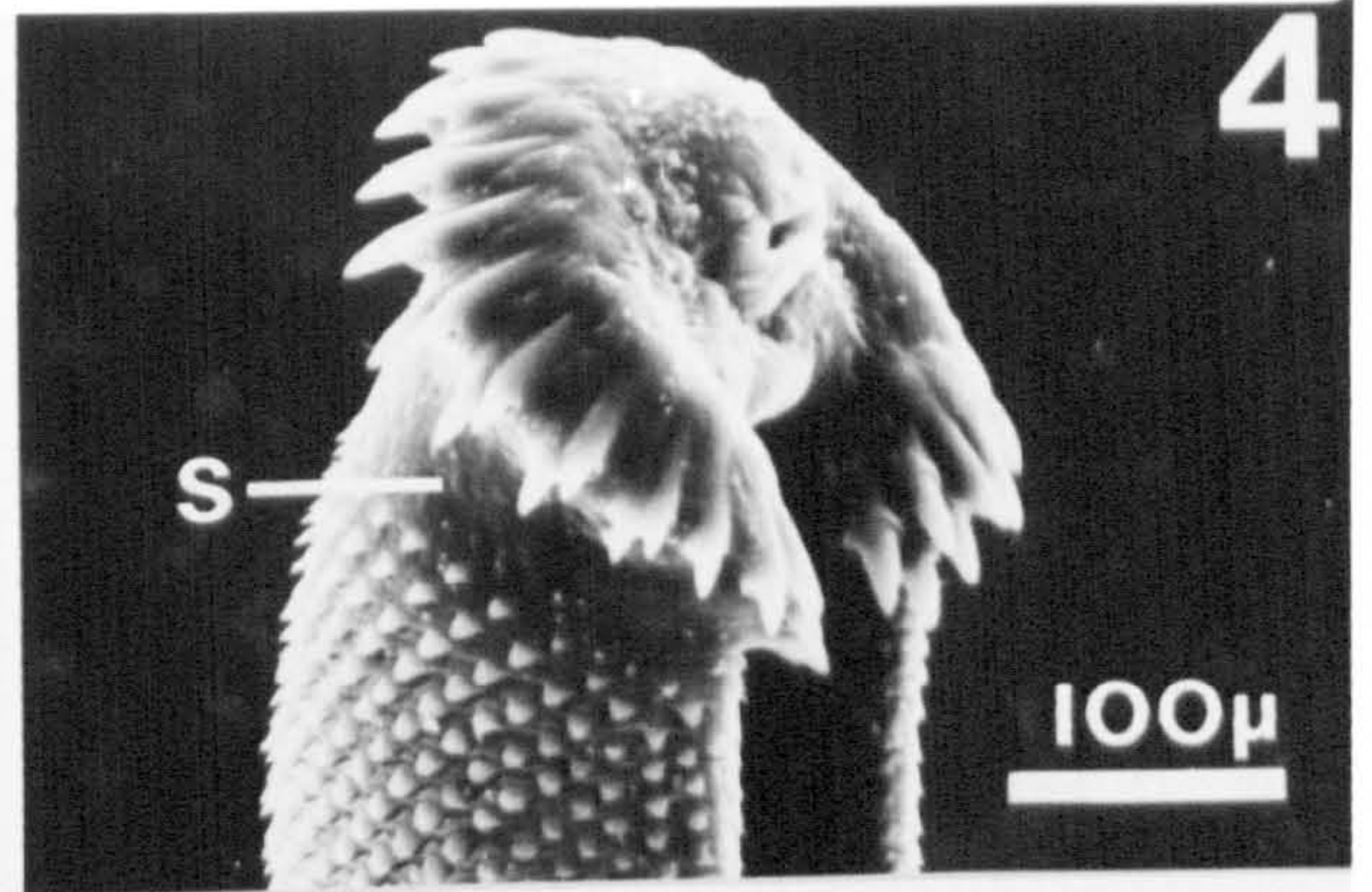
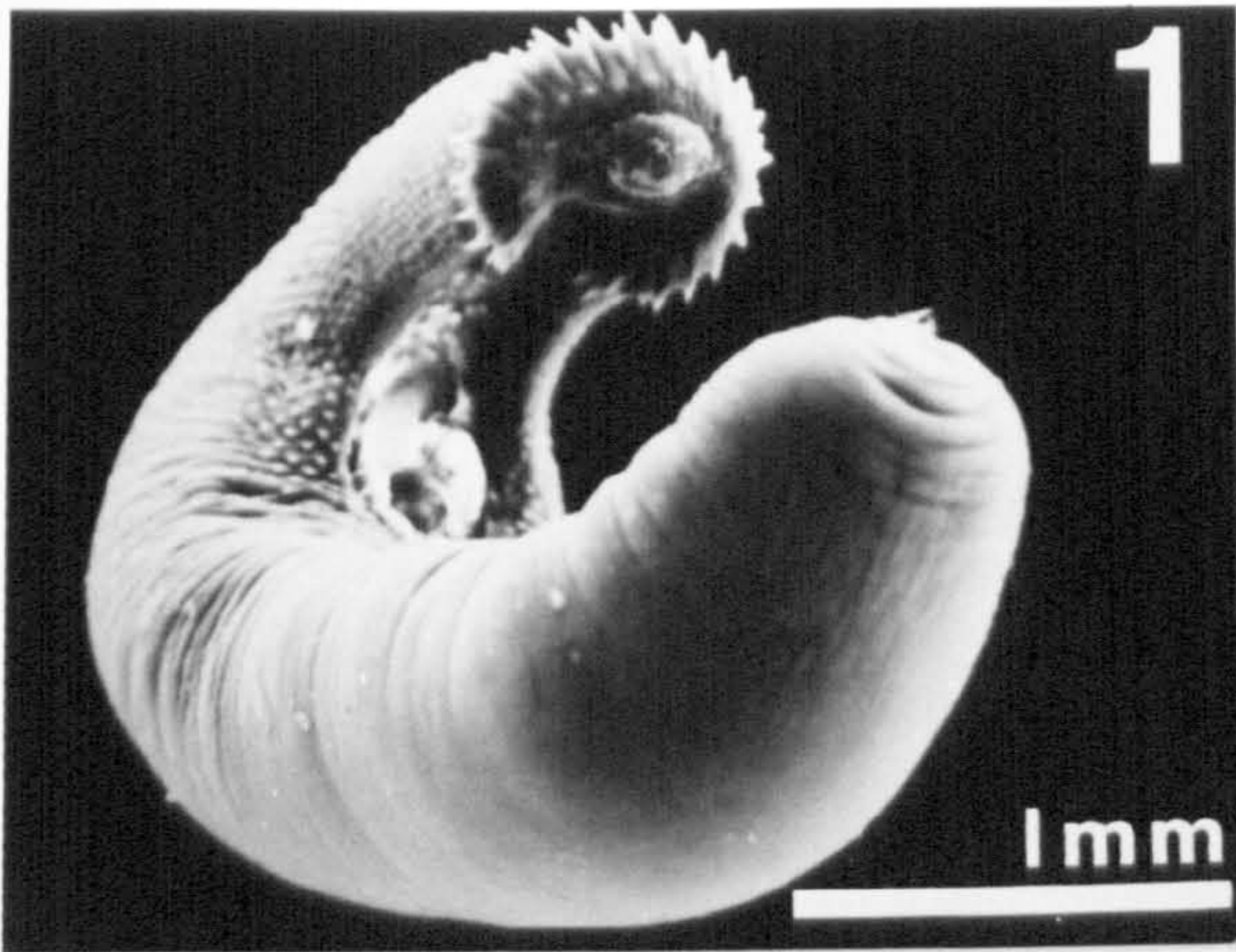
The adult *E. recurvatum* is shown in Fig 2.7 (1) lying in the classic curved position that this echinostome assumes on fixation in glutaraldehyde or formalin. The anterior third of the parasite is shown Fig 2.7 (2) from ventral aspect. The most conspicuous feature of the parasite is the anterior spine-bearing collar at the centre of which is located the simple sub-terminal oral sucker. The collar, shown *en face* in Fig 2.7 (3) and in lateral aspect in Fig 2.7 (4), is incomplete. A distinct ventral gap is present just below the oral sucker. The 45 spines of *E. recurvatum* are curved with



FIG 2.7 (1-7) Scanning electron micrographs of the tegumental surfaces of 15 day old mature adult Echinoparyphium recurvatum

- (1) Adult Echinoparyphium recurvatum 15 days old.
- (2) Anterior third of adult showing oral sucker (OS), ventral sucker (VS) and cirrus (Cr).
- (3) *En face* view of collar showing corner group of four spines (CG).
- (4) Lateral view of spined collar ; (S) indicates area of smooth tegument immediately posterior to collar.
- (5) Dorsal view of collar showing oral (Or) and aboral (Ab) tiers of spines.
- (6) Retractable collar spines showing smaller oral (Or) and larger aboral (Ab) spines accommodated in sheaths of striated collar tegument. ; (re) indicates a retracted spine and (ex) one fully extended.
- (7) Ventral sucker with indented rim and aciliate papillae (P).







**FIG 2.7 (8-16) Scanning electron micrographs of the tegumental surfaces of 15 day old mature adult Echinoparyphium recurvatum**

(8) Distal region of protrusible cirrus with ridged tegument, and sessile aciliate sensory papillae (P). Sperm (Sp) is being discharged from cirrus tip.

(9) Cirrus tip in detail showing terminal pore (Po) and filamentous sperm (Sp) being discharged.

(10. & 11) Dorsal body spines.

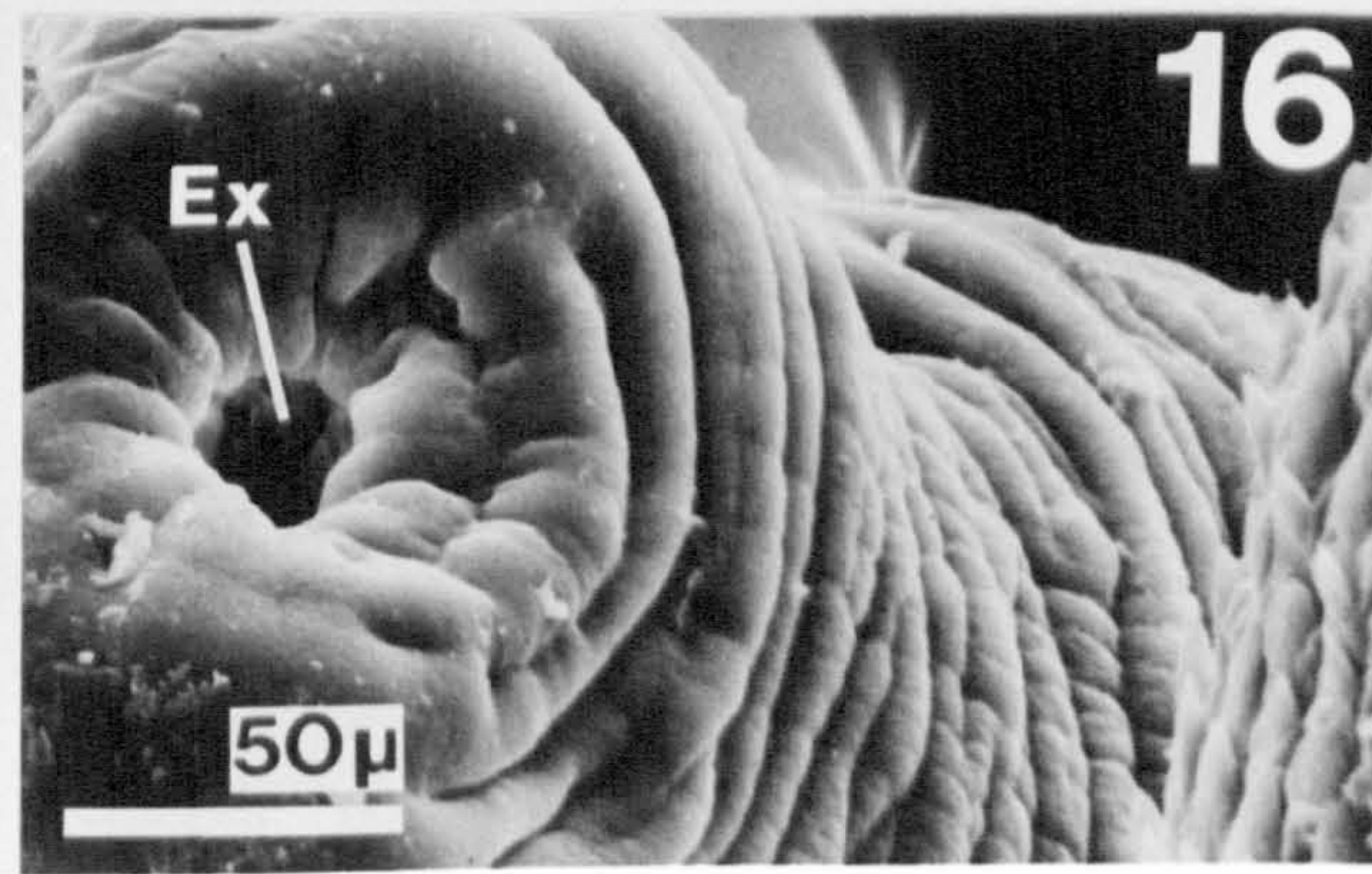
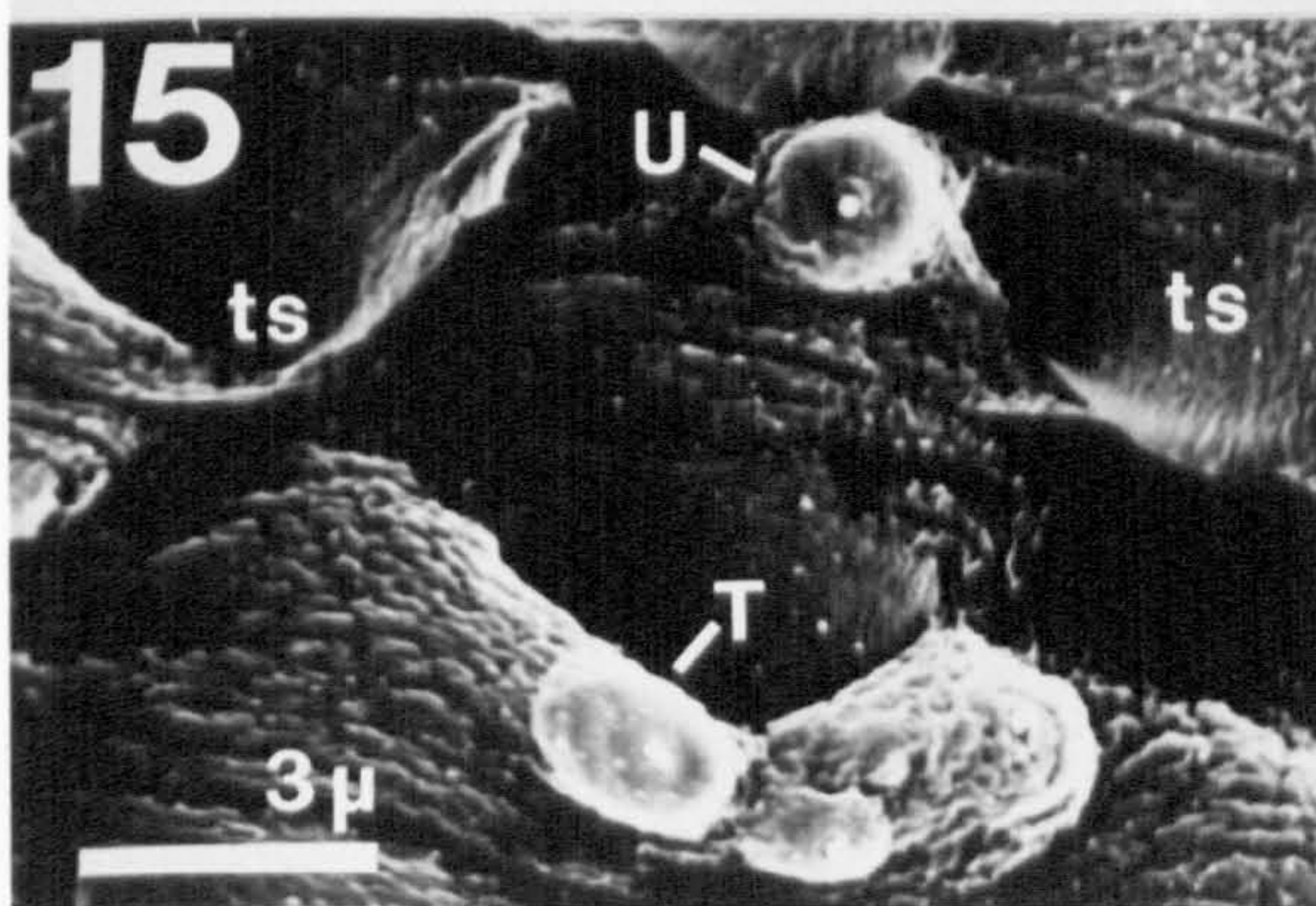
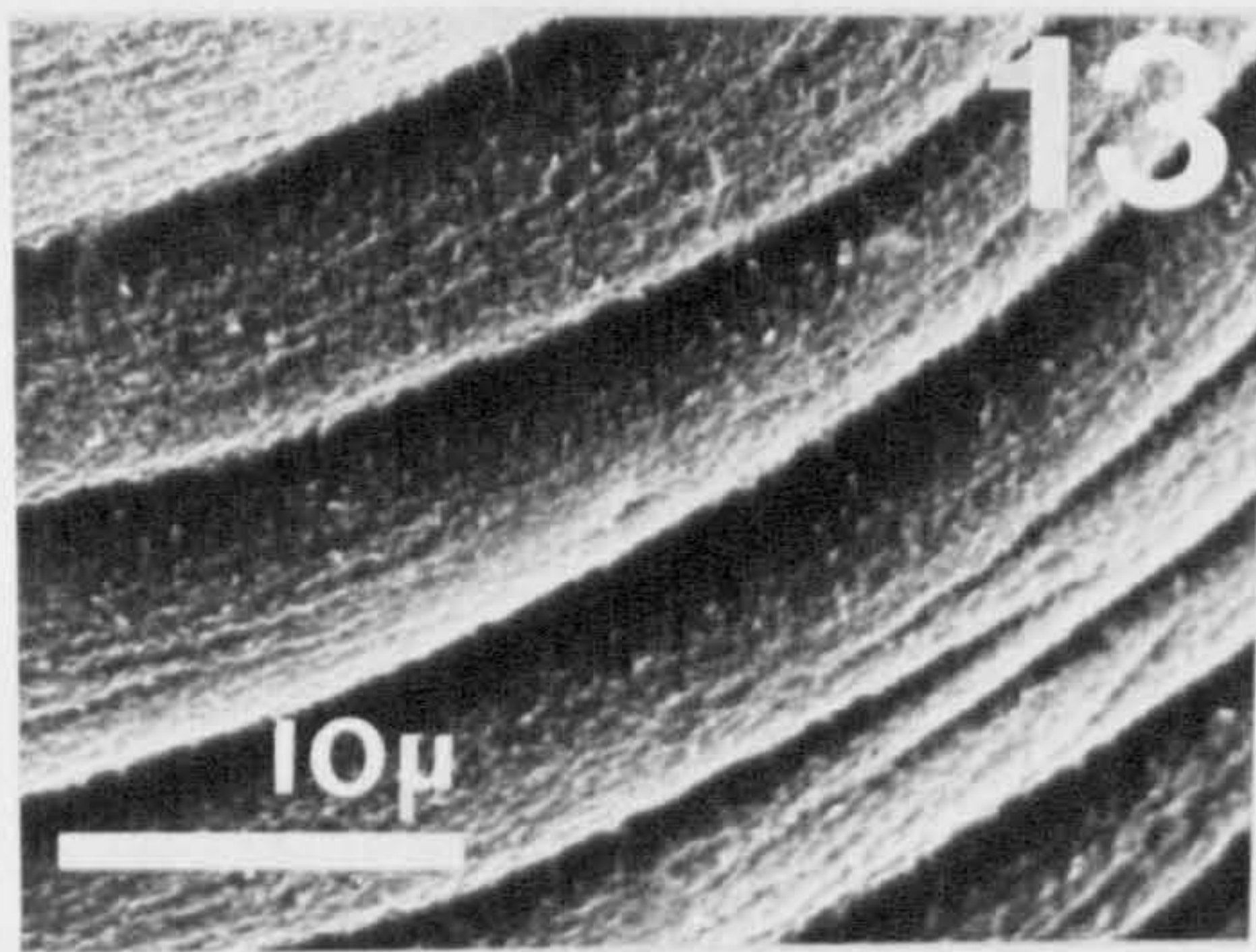
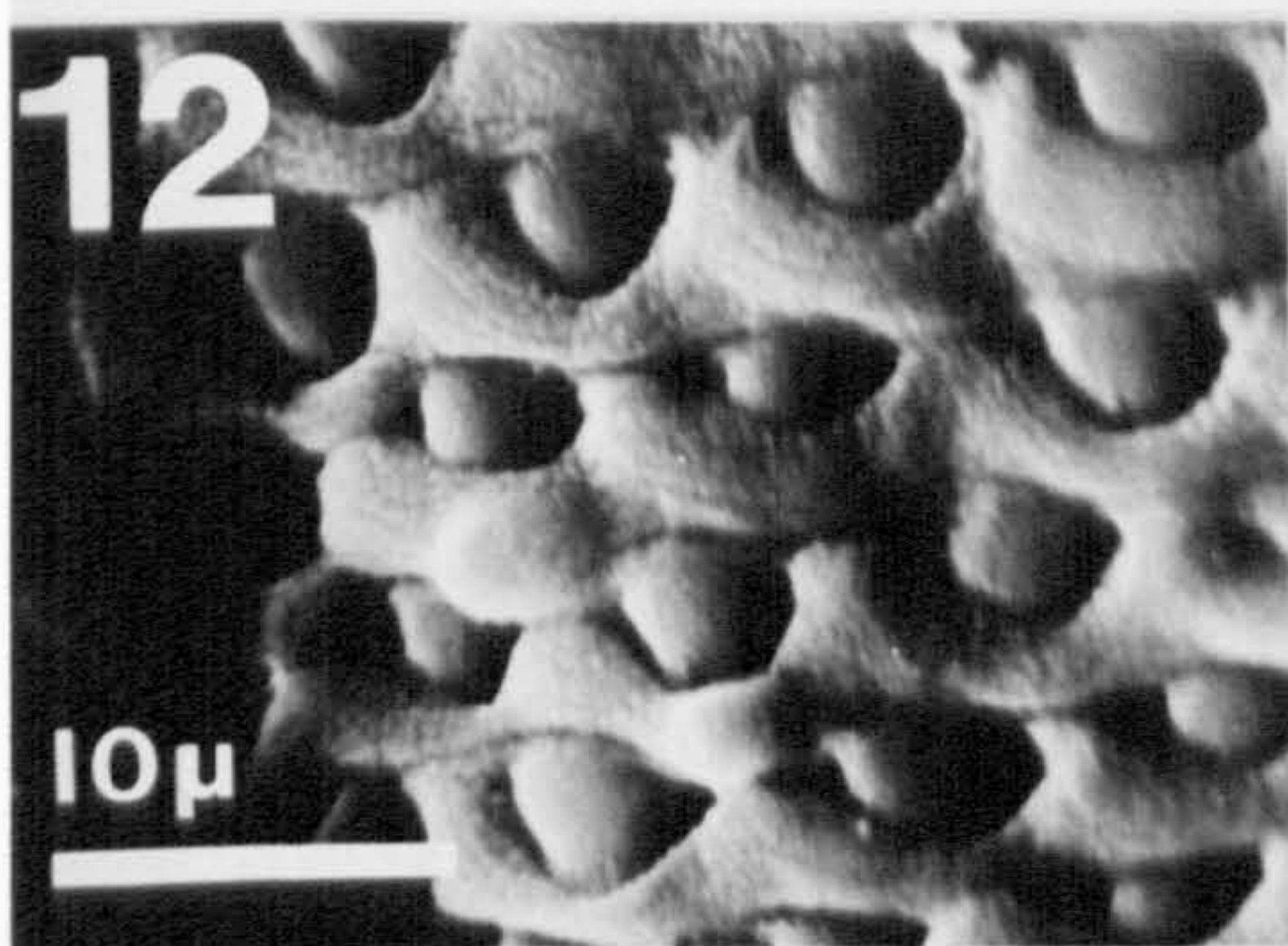
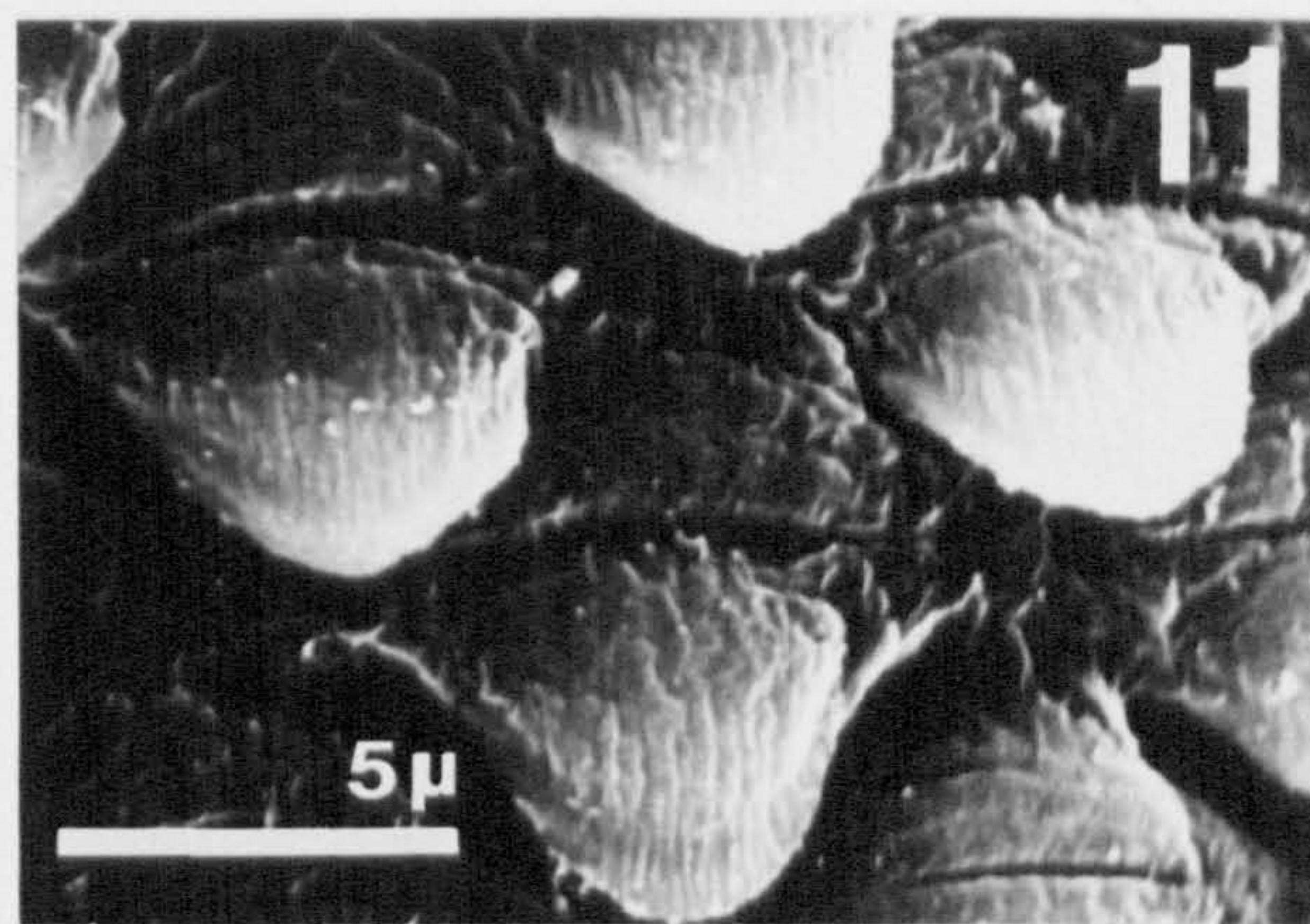
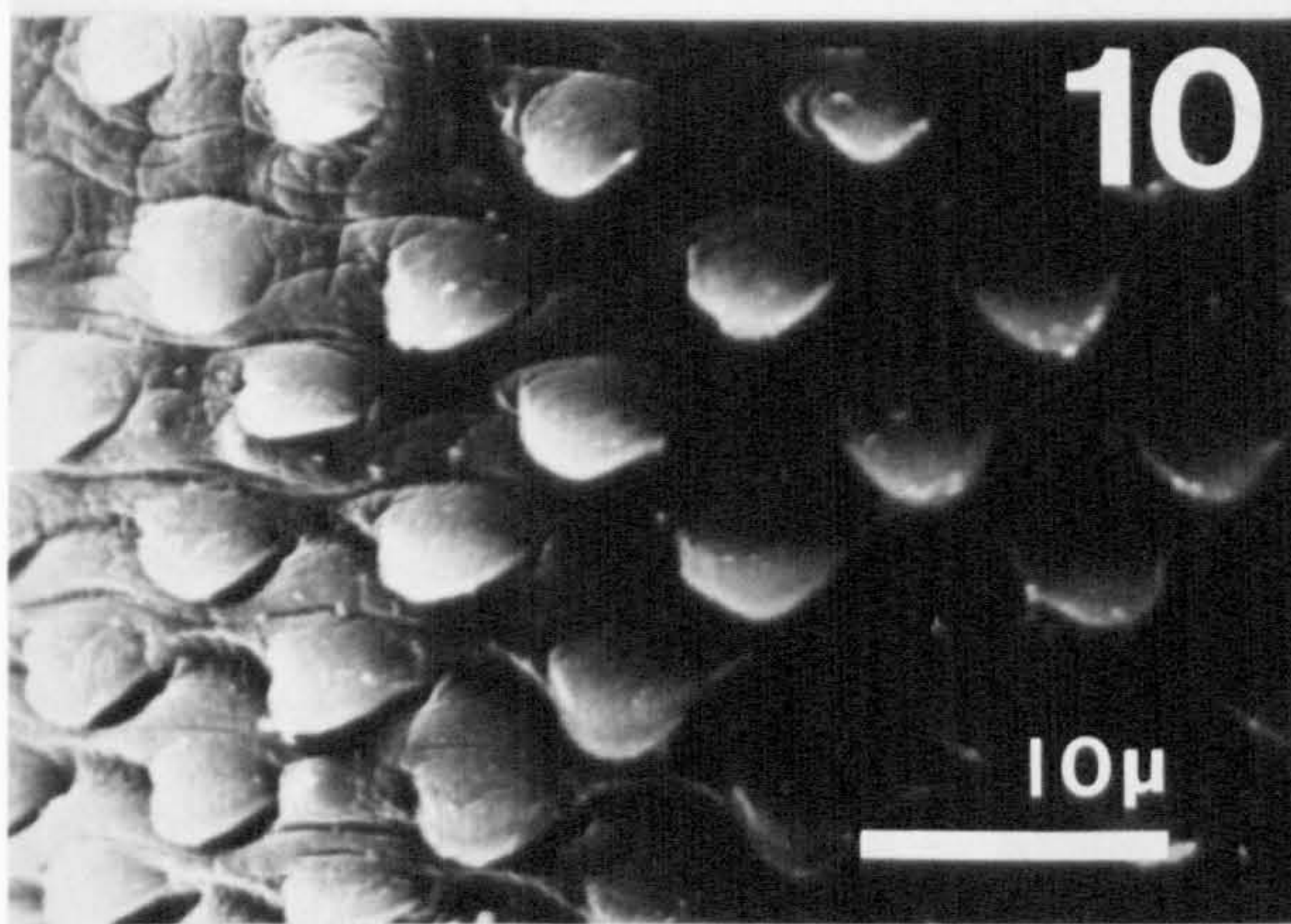
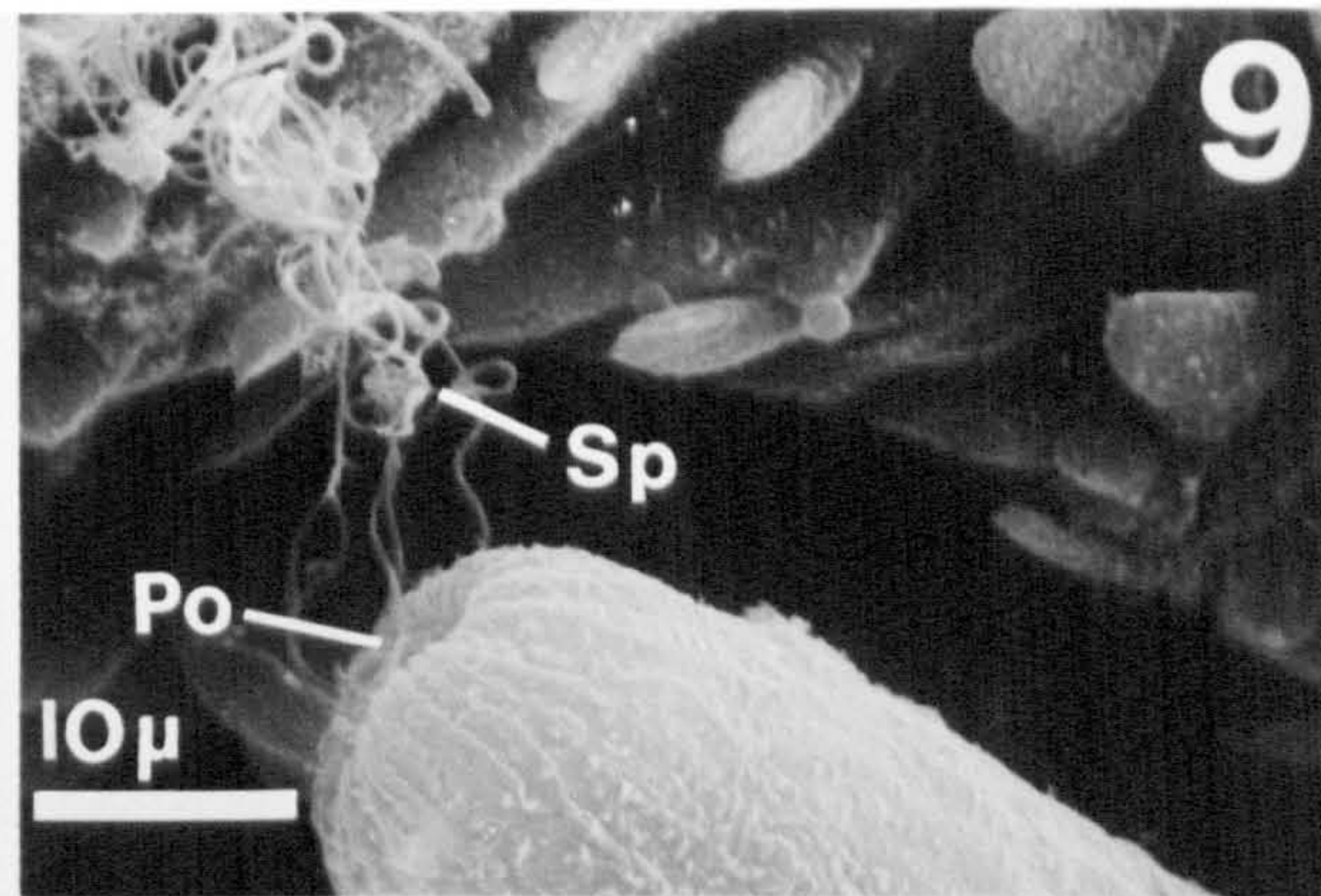
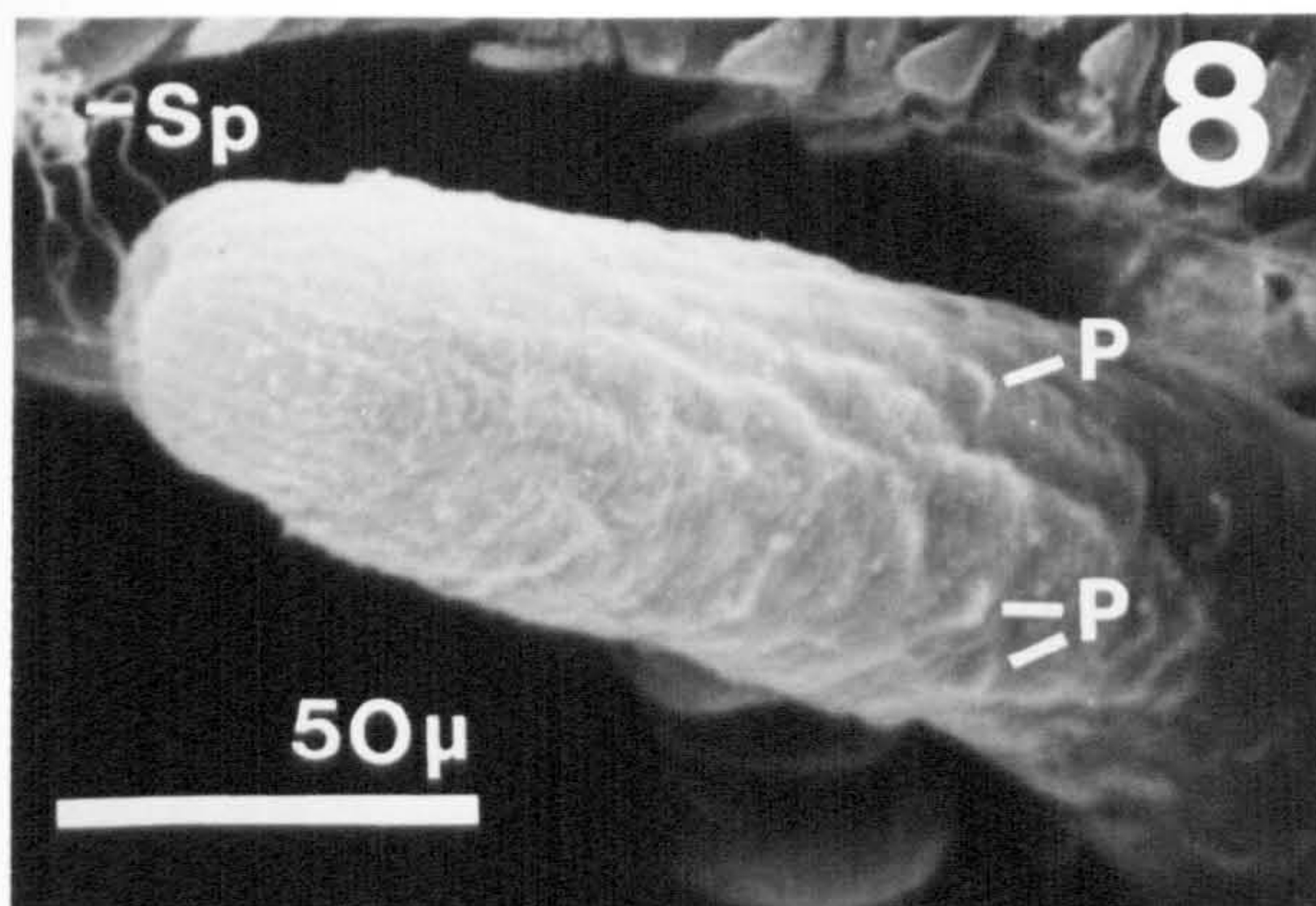
(12) Ventral body spines.

(13.& 14) Ventral body tegument showing different degrees of folding.

(15) Single (U) and group (T) of uniciliate sensory papillae from anterior ventral tegument among body spines (ts).

(16) Posterior tip of adult showing terminal excretory pore (Ex).







bluntly rounded points and they are arranged around the collar in a double tier, (see Figs 2.7 (5) and (6)). The spines of the oral tier are slightly smaller than those of the aboral tier and this observation is supported by light microscope studies in which both sets of spines have been viewed in the same plane. A distinct "corner group" of four spines, slightly larger and more robust in form than those of the rest of the collar, is present at the edge of the collar on each side of the ventral gap, (see Fig 2.7 (3)). The latter observation verifies that of von Linstow (1873) in the original type description of this echinostome. Each collar spine is retractile and is individually accommodated in a sheath of collar tegument which shows fine longitudinal striations, (see Fig 2.7 (6)).

The ventral sucker of the mature adult lies in the anterior third of the body, (see Figs 2.7 (1) and (2)), and is shown in detail in Fig 2.7 (7). The general tegument of the sucker on both its inner and outer surfaces is smooth and aspinose. The rim of the sucker is regularly indented around its circumference. Large knob-like aciliate sensory papillae are present on the outer lip of the sucker rim in the example shown in Fig 2.7 (7), but these structures have been found at other positions around the sucker rim on other specimens examined.

The highly protrusible cirrus of E. recurvatum is located just anterior to the ventral sucker, (see Fig 2.7 (2)). It is cylindrical in general structure (Fig 2.7 (8)) and possesses a single tip at the end of which opens a simple terminal pore. The sperm of E. recurvatum may be seen in Fig 2.7 (9) exiting from the cirrus tip.



tip. The sperm are long and filamentous in appearance. The cirrus tegument is aspinose and is covered with longitudinal ridges with irregularly indented margins. Simple aciliate sensory papillae are present on the middle and distal regions of the cirrus but not on the extreme tip, (see Fig 2.7 (8)).

Rows of rounded scale-like spines ( see Figs 2.7 (10, 11 and 12)) cover both the dorsal and ventral body tegument of E. recurvatum from a point just posterior to the anterior collar to a region just posterior to the ventral sucker, (see Fig 2.7 (2)). Here the spinose tegument gives way to the smooth, folded, aspinose tegument of the posterior two thirds of the body (see Figs 2.7 (13 and 14)). The spines of the ventral anterior tegument are similar in shape to those of the dorsal surface , but the most anterior rows appear to be partly embedded in the folded tegument. The most common sensory structures found on the general body surface of the parasite are the uniciliate dome papillae shown in Fig 2.7 (15). These papillae, which occur either singly or in groups of two or three, are found most commonly over the collar and ventral tegument of the anterior regions. They are not found on the smooth tegument of the posterior of the body. The posterior of the body comes to a bluntly rounded point at the end of which is located the terminal excretory pore, (see Fig 2.7 (16)).

### 2.3.3 DISCUSSION

The tegumental features of adult E. recurvatum revealed in the present study show basic similiarity to those of other adult echinostomes previously studied by SEM, but several important

differences do exist. The overall form of the anterior spined collar is similar to that found in Echinostoma revolutum, Isthmiophora melis and Mesorchis denticulatus. However, the distinct dorsal gap in the collar of adult M. denticulatus demonstrated by Kjøie (1987) is not present in E. recurvatum. The number of collar spines is also markedly different in E. recurvatum (45) from those of E. revolutum (37), I. melis (27) and M. denticulatus (22). The shape of the collar spines in E. recurvatum also differs from those of M. denticulatus and to a lesser extent from those of E. revolutum and I. melis, see Smales & Blankspeer (1984), Fried & Fujino (1984) and Kjøie (1987), although it is likely that the spines perform similar functions in each of these species.

The function of the retractile collar spines in adult E. recurvatum, and indeed in echinostomes in general, is not known for certain. However, it is thought that they may aid in maintaining the parasite's position in the intestine of the host. E. recurvatum is known to show a marked spatial localization in the anterior quarter of the intestine of Anas platyrhynchos (see Chapter 7) and it is probable that the collar spines will prove to be an important factor in enabling worms to maintain their position in this region of the host gut against the force of peristaltic flow. The body spines may also function in this capacity. Thulin (1980) has in fact previously suggested that the adjustable spines of Aporocotyle simplex may provide increased efficiency of attachment to the host. It is conceivable that the collar spines of E. recurvatum may also function as an aid to feeding by abrading the host gut wall. McDowall & James (1988) have recently suggested a similar function for the circum-oral spines of the gut parasitic



acanthostome Timoniella imbutiforme. The spines of this fish parasite are reported to show a "digging" action. In echinostome infections villous destruction, and erosion and desquamation of the intestinal epithelium are known pathological features observed in the host gut (Huffman, Michos & Fried 1986) and it is probable that at least some degree of this damage is directly attributable to the action of the collar spines. It is interesting to note that Thorndyke & Whitfield (1987) have recently demonstrated the synthesis of a neuropeptide related to VIP, (Vasoactive Intestinal Polypeptide), in the tegument of the echinostome Echinostoma liei and this has produced another possible mechanism for the initiation of disturbance of the host mucosal structure in echinostome infections.

The distribution of tegumental spines over the general body surface of E. recurvatum seems to be similar to that in mature adult E. revolutum and I. melis extending on both dorsal and ventral surfaces from an area just posterior to the collar to just posterior to the ventral sucker. This distribution differs from that found in M. denticulatus where Kjøie (1984) observes that no spines are present on the ventral body tegument. The shape of the scale-like body spines in E. recurvatum most closely resemble those of E. revolutum, but are distinct from those of I. melis. The form of the body spines may prove to be a useful taxonomic character in distinguishing closely related species of Echinoparyphium although the status of their value in this respect remains to be investigated.

The smooth body tegument of the posterior of E. recurvatum is very similar to that observed in adult E. revolutum, I. melis and M. denticulatus. It has been suggested that the tegument of adult echinostomes has an absorptive function and it is probable that tegumental folding serves to increase the effective absorptive surface area of the adult worm. Although quantitative data is lacking for E. recurvatum, Graeber and Storch (1979) reported for E. revolutum that tegumental folding was responsible for increasing the absorptive tegumental surface areas of this parasite by factors of 1.5 (dorsal) and 3.5 (ventral).

The form of the oral and ventral suckers in E. recurvatum generally resemble those of E. revolutum and I. melis although the uniform folding of the rim of the ventral sucker seems to be unique to E. recurvatum. However, light microscope observations by Najarian (1954) have shown that the North American Echinoparyphium flexum has a ventral sucker with a similarly indented rim. In common with those of E. revolutum and I. melis the suckers of E. recurvatum lack spines and as such all three species differ markedly from 4 day old adult M. denticulatus in which large plate-like spines are present around both suckers. However, mature (10 day old) adult specimens of the latter species also lack spines on the ventral sucker while those of the oral sucker persist but become embedded in tegument.

The types of sensory papillae found on adult E. recurvatum are comparable to those found both on the adults of other echinostomes and also to those of other types of adult digeneans. The large aciliate rounded papillae found on the rim of the ventral



sucker are very similar to those found by Fried & Fujino (1984) and Smales & Blanckespoor (1984) on the ventral sucker of E. revolutum. This type of papilla is also found on the ventral sucker of M. denticulatus and is reported by Fried & Fujino (1984) to be similar to the domed papilla in Fasciola hepatica and also to the "Type C" receptor reported by Fujino, Ishi & Choi (1979) from Clonorchis sinensis. As has been suggested by Page, Nadakvukaren & Huizinga (1980) in the case of Ribierioia marini, and Hoole & Mitchell (1981) for Gorgoderina vitelliloba, these papillae may function as mechano- and stretch-receptors controlling the contact, attachment and release of the ventral sucker as the parasite moves about in the host gut. The unciliated sensory receptors found commonly on the general body tegument of E. recurvatum are of a type which have previously been reported from the body surface of E. revolutum and I. melis and M. denticulatus. As is the case in these three echinostomes these papillae appear to be especially common anteriorly in E. recurvatum.

The present study has revealed that the cirrus tegument of E. recurvatum is aspinose, and this appears to be the case in both E. revolutum and I. melis as well as in certain other non-echinostome digeneans such as R. marini (Page et al. 1980). However the form of the cirrus tegument is different from those of previously described digeneans. The basic form of the cirrus of E. recurvatum has a single tip and in this respect it differs from E. revolutum which according to Smales & Blanckespoor (1984) has a bilobed tip. The sessile aciliate papillae found on the mid-region of cirrus in E. recurvatum have been observed previously in this species (Busta, Nascincova & Kanev 1987). They do not appear to be similar to

papillae found on the cirrus surface of other digeneans although at present little scope for comparison exists as little information is available on the cirrus tegument of digeneans. Both Wittrock (1976) for Quinqueserialis quinqueserialis and Page et al. (1980) for Ribierioia marini report the absence of sensory papillae from the cirri of these trematodes. Bakke (1976) reports sensory papillae on the cirrus of Leucochloridium sp. but these structures possess short apical knobs and are clearly distinct from those found in E. recurvatum. E. revolutum has papillae restricted to the cirrus tip and I. melis has papillae distributed over the entire length of the cirrus (Smales & Blankespoor 1984) but the papillae of neither species seem to closely resemble those of E. recurvatum.

To date no other members of the genus Echinoparyphium have been examined by SEM. It therefore remains to be seen how the tegumental ultrastructure of the adult E. recurvatum of European origin demonstrated in the current study compares with that of other species of Echinoparyphium such as Echinoparyphium dunni (Lie & Umathevy 1965) from southeast Asia and Echinoparyphium elegans (Mouahid & Mone' 1988) from Sardinia and North Africa. It is hoped that the present study will provide a basis for future studies on the comparative tegumental morphology of echinostomes in the genus Echinoparyphium.



## 2.4 Experimental observations on the specificity of Echinoparyphium recurvatum cercariae toward second intermediate hosts

### 2.4.1 INTRODUCTION

An experimental study by Evans & Gordon (1983b) has examined the specificity of Echinoparyphium recurvatum cercariae from Lymnaea peregra toward a range of British second intermediate host gastropods. The latter authors obtained their infected L. peregra snails from sites in Buckinghamshire and Kent, England. The present study set out to examine separately the specificity of E. recurvatum cercariae from L. peregra snails collected at Harting Pond, West Sussex, England, toward a range of potential second intermediate host gastropods in order to provide a basis for comparison with the findings of Evans & Gordon (1983b). In addition, because other echinostome cercariae eg. Echinostoma malayanum and Echinostoma revolutum are known to be able to utilize fish, planarians and amphibians as second intermediate hosts (see Beaver 1937, Lo 1973 and Lo & Cross 1975) an additional set of experiments was carried out to examine the ability of E. recurvatum cercariae to infect these types of host.

### 2.4.2 MATERIALS AND METHODS

#### Parasite and host material

Cercariae of E. recurvatum were obtained from naturally infected specimens of L. peregra collected by sweep net sampling at Harting Pond. The following 6 species of British gastropod mollusc were exposed to infection by cercariae from each first intermediate host source ; Lymnaea peregra, Lymnaea stagnalis, Physa fontinalis,

Planorbis planorbis, Valvata piscinalis and Potamopyrgus jenkinsi. The tropical gastropod species Biomphalaria glabrata (Puerto Rican albino strain) was also exposed to infection. With the exceptions of V. piscinalis and P. jenkinsi, which unfortunately proved impossible to breed in the lab, all the snails used were laboratory bred and infection-free. L. peregra, P. fontinalis and P. planorbis were bred from parental stocks collected at Harting Pond. L. stagnalis were bred from parental stocks collected from outdoor experimental tanks at the King's College Field Centre at Rogate, Sussex. P. jenkinsi snails were obtained from Harting Pond as extensive sampling of the natural population in this habitat had revealed no infections with echinostome cysts. V. piscinalis were obtained from a natural population in a habitat on the Pevensey Levels in southern England known to be free from echinostome infections and which was utilized previously by Evans & Gordon (1983b) as a source of infection-free V. piscinalis. A sample of 50 V. piscinalis from this site was dissected at the time of the present study and this revealed no digenean infections. The identity of V. piscinalis from this source was affirmed at the British Museum (Natural History).

Non-gastropod potential second intermediate hosts were also examined for their suitability as hosts for the parasite. Three-spined sticklebacks Gasterosteus aculeatus and two species of freshwater planarian Dugesia lugubris and Polycelis nigra, were obtained from a natural habitat known to be free of echinostome infections ; a section of The Royal Military Canal near Warehorne, Kent (NGR TQ 998 322). Dissection of test samples of 30 of each species of planarian and 50 G. aculeatus confirmed their



echinostome-infection-free status. The Rana temporaria frog tadpoles used in this study were bred in the laboratory from spawn supplied commercially by XENOPUS Ltd., U.K.

#### Infections of host species with cercariae

The infectivity of cercariae to each of the potential second intermediate snail host species was examined using the following procedure. Snails of each species were exposed singly to 10 freshly emitted cercariae (maximum age 30 mins) collected from a pool of naturally infected L. peregra.

Infections were conducted in small polystyrene pots each containing 10ml of a synthetic hard water medium (HMSO 1969). As snail size is an important determinant of E. recurvatum cercarial transmission success (Evans & Gordon 1983a) all snails exposed to infection in this study were of a similar size and within the length/diameter range 3-6mm. Snails were examined 24 hours post-exposure and the total number of metacercarial cysts that had formed in each was recorded. Between 20 and 30 replicate exposures were conducted for each host species.

Non-gastropod potential hosts were also exposed to cercariae individually. The planarians D. lugubris and P. nigra were each exposed to 10 cercariae per specimen in 10ml of water. Sticklebacks and tadpoles were each exposed to 50 cercariae per specimen in 20ml of water. Between 10 and 20 replicate exposures were carried out for each species. Specimens were examined for cyst infections 24 hours post-exposure. Tadpoles and

sticklebacks were killed prior to dissection using a 2% solution of Tricaine methanesulfonate (MS 222).

### 2.4.3 RESULTS

Under conditions of single host species exposure five of the six species of freshwater gastropod exposed to infection were capable of functioning as second intermediate hosts. Only the prosobranch P. jenkinsi was unutilizable, (see Tables 2.3 and 2.4). The parameter "Transmission Success" is a convenient overall measure of infectivity of echinostome cercariae for a particular species or group of second intermediate hosts employed by Evans & Gordon (1983b) It is calculated from the cyst recovery data as follows; Transmission Success = (No. of cysts recovered /No. of cercariae to which hosts were exposed) x 100.

The six British gastropods exposed to infection in the present study could clearly be divided into two distinct groups with respect to their suitability as hosts for the parasite. The pulmonate gastropods L. peregra and P. fontinalis and the prosobranch V. piscinalis became heavily infected. Analyses of variance on cyst counts transformed by  $\text{Log}_{10}(x+1)$  indicated that the numbers of cercariae establishing as cysts in each of these three second intermediate host species were not significantly different,  $P > 0.05$  (df=2 and 72). These three second intermediate hosts therefore showed a similar high degree of compatibility with E. recurvatum cercariae. The remaining hosts, the pulmonates L. stagnalis and P. planorbis, and the prosobranch P. jenkinsi, showed a similar low degree of compatibility with cercariae of both first intermediate host origins. P. jenkinsi proved completely refractory to infection.



**TABLE 2.3 Infectivity of Echinoparyphium recurvatum cercariae from Lymnaea peregra to a range of gastropod species**

Snail species	No. of snails exposed to infection	No. cercariae per snail	Mean no. cysts recovered per snail (+/-SE)	Per cent of snails infected	Transmission Success*
<i>Lymnaea peregra</i>	30	10	8.1 (+/-0.34)	96.6	81.3
<i>Valvata piscinalis</i>	20	10	6.6 (+/-0.36)	100	66.2
<i>Physa fontinalis</i>	25	10	7.9 (+/-0.38)	100	79.3
<i>Lymnaea stagnalis</i>	27	10	0.14 (+/-0.1)	11.1	1.5
<i>Planorbis planorbis</i>	26	10	0.19 (+/-0.13)	12.5	1.9
<i>Potamopyrgus jenkinsi</i>	30	10	0	0	0
<i>Biomphalaria glabrata</i>	20	10	8.2 (+/-0.52)	100	82

\* Transmission Success = (No. cercariae / No. metacercariae) x 100

**TABLE 2.4 Infectivity of Echinoparyphium recurvatum cercariae from Lymnaea peregra to non-gastropod potential second intermediate hosts**

Potential host species	No. of specimens exposed	No. of cercariae per specimen	Mean no. (+/-SE) cysts recovered per specimen	Per cent infected	Transmission success*
<i>Rana temporaria</i> (tadpole)	20	50	7.1 (+/-1.6)	70	14.1
<i>Gasterosteus aculeatus</i>	14	50	0	0	0
<i>Dugesia lugubris</i>	10	10	0	0	0
<i>Polycelis nigra</i>	10	10	0	0	0

\* Transmission Success = (No. cercariae / No. metacercariae) x 100

The tropical snail B. glabrata proved to be a highly suitable second intermediate host for E. recurvatum. Analysis of variance on cyst counts transformed by  $\text{Log}_{10}(x+1)$  did in fact show that the number of cercariae establishing as cysts in B. glabrata exposed to infection were not significantly different from that in the high compatibility hosts L. peregra, P. fontinalis and V. piscinalis,  $P < 0.05$ ,  $df = 3$  and  $91$ .

Tadpoles of the amphibian R. temporaria were utilizable as hosts but the planarians D. lugubris and P. nigra, and the freshwater teleost fish G. aculeatus proved uninfectable, see Table 2.4. In the frog tadpoles cysts were located in the region of the kidneys.

#### 2.4.4 DISCUSSION

The results of this study indicate that E. recurvatum cercariae originating from L. peregra at Harting Pond show very similar patterns of second intermediate host utilization to those demonstrated by Evans & Gordon (1983b) for the parasite from L. peregra obtained from different locations. Cercariae showed broad specificity exhibiting the ability to utilize five of the six gastropod species exposed to infection.

The patterns of host utilization observed for cercariae throughout the range of freshwater gastropods examined agree closely with those observed by Evans & Gordon (1983b) who also found that L. peregra, V. piscinalis and P. fontinalis were highly suitable second intermediate hosts for the parasite. As the present study also



found, Evans & Gordon (1983b) reported that L. stagnalis and P. planorbis showed low degrees of compatibility with the parasite and that P. jenkinsi was completely unutilized as a second intermediate host by the parasite. In the case of the latter species P. jenkinsi failure of cercariae to infect this snail is possibly attributable to physiological incompatibility due to the fact that this snail species has only recently invaded fresh waters from brackish waters, (see Macan 1977).

The fact that the tropical snail species B. glabrata proved to be a highly suitable second intermediate host for E. recurvatum in the current study, despite the fact that this snail species is not, of course, present in the British habitat from which we obtained our parasite material, is an interesting observation. Similar findings to this have in fact been made previously with other echinostome species. Fried & Anderson (1987) for example have reported that experimental infection studies show that B. glabrata is an excellent second intermediate host for Echinostoma revolutum in North America although these authors report that the snail is not a natural host of the parasite (ie. does not occur) in this region. Similarly, the present author (see Appendix) has found that Egyptian strain Biomphalaria alexandrina is an excellent second intermediate host for the echinostome Pseudechinoparyphium echinatum, although B. alexandrina does not occur in the habitats in the Danube Valley where the author obtained his P. echinatum material, and there are no records of P. echinatum from Africa.

Variable susceptibility amongst gastropods to infection with cercariae is a well documented phenomenon in other echinostome

species. Christensen, Fransden & Roushdy (1980) demonstrated variable susceptibility amongst pulmonate snails to infection by Echinostoma liei cercariae. Species of the genus Bulinus were particularly susceptible to infection whilst Biomphalaria obtained from different geographical areas showed variable degrees of susceptibility. The present author (see Appendix) has also demonstrated under experimental conditions differential susceptibility amongst a range of second intermediate host gastropods to infection by cercariae of P. echinatum. This study found that the species Planorbarius corneus, Physa fontinalis and L. peregra were hosts of high suitability while L. stagnalis, Planorbis planorbis were hosts of low suitability and that Bithynia tentaculata and Viviparus viviparus remained uninfected.

It is interesting that the present study, and also Evans & Gordon (1983b), demonstrates that the levels of susceptibility that gastropods show to infection with echinostome cercariae are not strictly related to their phylogeny. For example, the present study and Evans & Gordon (1983b) have shown that a lymnaeid L. peregra and a prosobranch V. piscinalis are highly suitable hosts for E. recurvatum cercariae. However, a lymnaeid L. stagnalis and the prosobranchs P. jenkinsi and Bithynia tentaculata rank among the least suitable hosts for E. recurvatum cercariae. A similar situation has been demonstrated by the present author (see Appendix) who found that the planorbid Planorbarius corneus and the lymnaeid L. peregra were hosts of high suitability for P. echinatum cercariae whereas the planorbid P. corneus and the lymnaeid L. stagnalis were hosts of low suitability. In the case of these two echinostomes, groups of high suitability hosts and low



suitability second intermediate hosts may each be composed of assemblages of phylogenetically quite distantly related snail species. This particular facet of host specificity may be of ecological advantage to the parasite as with a range of high suitability hosts of a variety of gastropod groups the parasite may ensure against potentially adverse affects resulting from a local population decline occurring in any one of these host species.

The mechanisms generating differential susceptibility amongst gastropod hosts for E. recurvatum cercariae are unknown at present. However, the fact that throughout the present study no dead or moribund cercariae were found within the tissues of exposed snails suggests that incompatibility may be expressed prior to cercarial penetration. The finding of Wright (1959) that the chemical composition of mucous secretions of lymnaeid snails differed between species may be of relevance here. It may suggest that cercariae sense suitable or unsuitable snail hosts by their chemical secretions prior to penetration. Anderson & Fried (1987) have recently suggested that excretory products emitted from the nephridiopore of potential second intermediate host gastropods are probably involved in site location of echinostome cercariae. The mechanism of gastropod second intermediate host location by chemo-attraction is considered further in Chapter 6.

The present study has indicated that cercariae of E. recurvatum seem to be incapable of utilizing three-spined sticklebacks (G. aculeatus) and the planarians D. lugubris and P. nigra as second intermediate hosts. However, Lo (1973) and Lo & Cross (1973) have reported that the cercariae of the echinostome Echinostoma

revolutum are capable of utilizing both fish and freshwater planarians, in addition to snails and amphibians in this capacity. The possibility that E. recurvatum may be able to utilize other species of freshwater fish or planarians as second intermediate hosts remains to be investigated.

The present study has shown that E. recurvatum cercariae are capable of utilizing Rana temporaria tadpoles as second intermediate hosts, a finding which is confirmatory of the report of Vojtek (1963). The site of infection was also found to be the same as that reported by this author, the metacercarial cysts from all R. tempoaria tadpoles in the present study being located in the region of the kidneys. Najarian (1954) has also interestingly reported that in addition to freshwater snails cercariae of the North American species Echinoparyphium flexum are also capable of utilizing amphibian tadpoles as second intermediate hosts, the site of infection being the kidneys. This author was able to experimentally infect tadpoles of a variety of species of frog, eg. Rana pipiens (North American Leopard Frog) and Rana clamitans ; with cercariae of E. flexum. However, it was found to be impossible to infect adult specimens of the same species with cercariae, despite the fact that adult frogs were found to be infected with cysts (which were infective to experimental definitive hosts) in the natural environment. From this he concluded that infection was acquired at the tadpole stage, the cyst remaining viable as the amphibian matured.

The same is conceivably true of E. recurvatum ; indeed Vojtek (1964) reported metacercarial cysts of E. recurvatum from the



kidneys of adult Rana temporaria and Rana esculenta frogs in Czechoslovakia. An experimental study is planned by the present author to determine the suitability of other European amphibian species as second intermediate hosts for E. recurvatum. Utilization of amphibian second intermediate hosts by E. recurvatum has interesting implications with respect to extension of the range of potential definitive hosts, in that it could extend the host range from molluscivorous wildfowl to carnivorous (amphibian-eating) birds such as herons, and cormorants. However, the ability of E. recurvatum to develop to patency in such definitive hosts remains to be investigated.

## 2.5 A preliminary experimental study on the specificity of Echinoparyphium recurvatum to the first intermediate host

### 2.5.1 INTRODUCTION

Echinoparyphium recurvatum has been described in the literature as utilizing several species of first intermediate host gastropods from several different groups, see Table 1.1. In the present study it was decided to investigate experimentally whether E. recurvatum utilizing L. peregra as first intermediate host at Harting Pond had the ability to utilize other species of European freshwater gastropod as first intermediate host. In addition, it was decided to determine whether the African bulinid Bulinus truncatus, which has been reported by Moravec et al (1974) as the first intermediate host of E. recurvatum in Africa, could be

infected by miracidia of British E. recurvatum utilizing L. peregra as a first intermediate host.

## 2.5.2 MATERIALS AND METHODS

### Parasite and host material

Cercariae of E. recurvatum were obtained from large separate pools of naturally infected L. peregra collected by sweep net sampling at Harting Pond, West Sussex. Separate batches of laboratory bred, infection-free L. peregra were exposed *en masse* to cercariae from each first intermediate host source. At 14 days post-infection cysts were fed in batches of approximately 100 in gelatine capsules to a group of commercially bred, infection-free 3 day old Khaki Campbell ducklings (Anas platyrhynchos). Eggs were obtained by sieving host faeces from day 12 to day 17 of infection. The birds were killed at 17 days and additional supplies of eggs were obtained from the faecal material in the gut. Eggs obtained in this manner were given two rinses in distilled water. They were then incubated in distilled water at 20°C in darkness, in polystyrene containers wrapped in aluminium foil, for 12 days. They were then stimulated to hatch by placing them under the intense beam of a cold fibre optic light source.

In total 7 species of gastropod were exposed to infection by miracidia of E. recurvatum utilizing L. peregra as first intermediate host, these were; L. peregra, Lymnaea auricularia, Lymnaea stagnalis, Planorbis planorbis, Physa fontinalis, Valvata piscinalis and Bulinus truncatus (Egyptian strain). All snails used were laboratory bred and infection-free with the exception of V. piscinalis which were obtained from a natural population known



to be free of echinostome infections, (see previous section of this Chapter). The parental stocks of the laboratory bred L. peregra, L. stagnalis, P. planorbis and P. fontinalis used in this study were the same as those described in the previous section of this Chapter. The L. auricularia snails used were bred in the laboratory from a parental stock obtained from a section of The Royal Military Canal, near Warehorne, Kent, England (NGR TQ 998 322). All snails exposed to infection in this study were in the size range 2.5-5mm.

### **Infection of snails**

Snails were exposed individually to infection using a constant ratio of 5 miracidia per snail. Exposures were carried out in clear polystyrene plate wells (COSTAR, Cambridge U.S.A.) each containing 3 ml of synthetic hard water medium, (HMSO 1969). Snails were exposed to infection for 24 hours and were then returned to clear polystyrene tubs containing clean water. Exposed snails were maintained at 18-20°C, (22-26°C for B. truncatus) on a diet of boiled lettuce. Snails surviving 35 days post-infection were dissected and examined for active infections. The numbers of snails exposed to infection are given in Table 2.5.

### **2.5.3 RESULTS**

The results of this study are given in Table 2.5. It is apparent that miracidia derived from E. recurvatum utilizing L. peregra at Harting Pond displayed a high degree first intermediate host specificity under the conditions of this experiment and were able to develop infection only in L. peregra and the closely related lymnaeid L. auricularia. A total of 22 out of 30 L. peregra snails

**TABLE 2.5 Infectivity of Echinoparyphium recurvatum miracidia to a range of snail species**

Snail species exposed to infection	No. of snails exposed	No. of miracidia per snail	No. of snails surviving at 35 days	No. of snails infected	Per cent snails infected
<i>Lymnaea peregra</i>	30	5	30	22	73
<i>Lymnaea auricularia</i>	30	5	28	12	43
<i>Lymnaea stagnalis</i>	37	5	32	0	0
<i>Planorbis planorbis</i>	24	5	22	0	0
<i>Physa fontinalis</i>	32	5	32	0	0
<i>Valvata piscinalis</i>	31	5	27	0	0
<i>Bulinus truncatus</i>	26	5	24	0	0



surviving at 35 days (73 %) were found to be carrying patent infections of the parasite. A total of 12 out of 28 (43%) of L. auricularia snails surviving at 35 days were found to be patently infected. Snails of the species V. piscinalis, L. stagnalis, P. planorbis, P. fontinalis and B. truncatus exposed under the same conditions were uninfected.

#### 2.5.4 DISCUSSION

The results of this study have shown that of the range of gastropod species exposed to infection E. recurvatum utilizing L. peregra as first intermediate host snail at Harting Pond could utilize only the lymnaeid L. peregra and its closely related congener L. auricularia as first intermediate hosts. The parasite was unable to utilize the snail species L. stagnalis, V. piscinalis, Physa fontinalis, Planorbis planorbis or, Bulinus truncatus as first intermediate hosts; although all these species with the exception of P. fontinalis have been recorded in the literature as first intermediate hosts of the parasite. The findings of this study are very similar to those of Rasin (1933) who attempted to infect four snail species with E. recurvatum miracidia obtained from a stock of the parasite utilizing L. peregra as first intermediate host in Czechoslovakia. The snails exposed to infection were the lymnaeid Lymnaea stagnalis, the planorbids Planorbis umbilicatus (= planorbis), and Planorbarius corneus, the physid Physa fontinalis and the prosobranch Viviparus viviparus. This author obtained the same negative results from these snails, although his control groups of L. peregra exposed to miracidia became patently infected.

This finding raises the interesting possibility that E. recurvatum from L. peregra may represent a morphologically indistinct but biologically (host-specifically) distinct entity from E. recurvatum from other first intermediate hosts such as the planorbid Planorbis umbilicatus (= planorbis) (Mathias 1927) and the prosobranch Valvata piscinalis (Harper 1929). A similar suggestion has been made by Jeyarasasingham et al (1972) who suggests that these entities are probably very recently evolved, and while being distinct at the biological level of first intermediate host-specificity have changed very little from each other morphologically.

Interestingly, in a somewhat parallel situation to that found in Echinoparyphium recurvatum, Kanev & Nascincova (1988), have in recent years discovered a 37 collar-spined echinostome in Czechoslovakia utilizing the prosobranch snail Viviparus viviparus as first intermediate host. This parasite is morphologically virtually indistinguishable from Echinostoma revolutum which utilizes Lymnaea stagnalis as first intermediate host. However, cross-infection experiments have shown that both echinostomes are rigidly specific to their respective first intermediate hosts, and Kanev et al (1988) have found consistent morphological differences between the cercariae of the two with respect to numbers of para-oesophageal gland cells. Interestingly, Zdarska (1987) has recently noted a 37 collar-spined Echinostoma utilizing Planorbarius corneus as a first intermediate host, although this author records the fact that Kanev (1985) has clearly demonstrated that E. revolutum utilizing L. stagnalis as first intermediate host cannot utilize P. corneus as a first intermediate host. The findings



of Kanev (1985) have in fact prompted a revision of the nomenclature of species within the genus *Echinostoma*, (Christensen, Fried & Kanev 1989). In this taxonomically problematic echinostome genus first intermediate host specificity is thought to be of sufficient status to elevate members of the genus to formal species level, (Fried & Fujino 1987).

Experimental studies have shown that the evolution of first-intermediate host specific entities in digeneans is a relatively fast process, first intermediate host specific "strains" of *Fasciola hepatica* having been produced under experimental conditions in as little as two generations (see Boray 1966). Importantly Smyth & Halton (1983) have also discussed the frequency of host-specific strain formation in digeneans and consider it as being a natural consequence of digenean reproductive biology. This is because mechanisms such as hermaphroditic self-fertilization, and reproduction by polyembryony in first intermediate hosts, mean that large numbers of progeny may be produced from single mutations.

In the case of echinostomes there are also clear ecological driving forces for the production of first intermediate host-specific entities. Echinostome rediae in general are known to be highly predatory organisms which are known to be strongly inter- and intra-specifically competitive within the host snail's digestive gland (Combes 1982, Kjøie 1987). A high level of intra-specific competition could be expected to provide a strong selective pressure for the utilization of novel first intermediate hosts.

In the present study it was found that E. recurvatum utilizing L. peregra as a first intermediate host at Harting Pond was unable to utilize African Bulinus truncatus snails as first intermediate host. Moravec et al. (1974) reported E. recurvatum utilizing B. truncatus as sole first intermediate host in Cairo, Egypt. This would seem to suggest some degree of biological distinction between E. recurvatum in Africa and that from L. peregra in Europe. It is in fact suspected that Moravec et al. (1974) were working with a predominantly 43 collar-spined Echinoparyphium - Echinoparyphium elegans - first described by Looss (1899) from Egypt, and for which Mouhaid & Mone' (1988) have recently described the life cycle in detail beginning from 43 collar spined cercariae from B. truncatus of Sardinian origin. The fact that E. recurvatum utilizing L. peregra as a first intermediate host cannot utilize B. truncatus in the same capacity has also been found by Kanev unpublished (personal communication 1987). In this case it was found that E. recurvatum utilizing L. peregra snails in the region of the River Spree in East Germany could not utilize B. truncatus from a parental stock obtained from irrigation channels in Cairo, Egypt. In addition it was found that Echinoparyphium elegans from Cairo, Egypt could not develop in L. peregra snails of East German origin. It therefore seems that the predominantly 45 spined Echinoparyphium recurvatum utilizing L. peregra in Europe as first intermediate host may be a biologically distinct from the predominantly 43 spined Echinoparyphium elegans utilizing B. truncatus in Africa and the southern Mediterranean. However, the two entities are undoubtedly extremely closely related and their relationship to each other is made more interesting by the report of Lie & Umathevy (1965) of a form intermediate between the



two, named as Echinoparyphium durni , from Malaya. This is a predominantly 43 collar-spined Echinoparyphium which utilizes a lymnaeid snail Lymnaea rubiginosa as first intermediate host. It cannot utilize B. truncatus a first intermediate host , this having been proved by controlled experimental infection studies (Lie & Umathevy 1965).

The systematics and nomenclature of the members of the genus Echinoparyphium is at present, to say the least, controversial, and is largely dependent upon the weight which different authors afford to differences in biological and morphological differences. Because what constitutes a distinct species in this genus seems largely to be dependent on the importance that different authors place on morphological and biological differences a universal consensus is unlikely to be reached. Further studies using biochemical taxonomy techniques such those which have been applied in the case of Echinostoma (see Voltz, Richard & Pesson 1987) may provide an additional set of characteristics which could be used to decide a code of nomenclature.

Nascetti et al (1986) have in recent years stated that one of the most important contributions of multi-locus electrophoresis to taxonomy is the detection of sibling species - biological species virtually identical at the morphological level. In a detailed study (see Nascetti et al 1986) these authors used muti-locus electrophoresis to distinguish two sibling species of the nematode species complex Anisakis simplex. Multi-locus electrophoresis in the case of echinostomes can be used for examining redial and adult material (Rollinson 1988 personal communication). Detailed

multi-locus electrophoretic studies would seem therefore to be the next logical step to a fuller understanding of phylogenetic relationships of the entities within the genus Echinoparyphium. It should also be added that studies using DNA analysis may provide interesting results. DNA analysis has recently begun to be applied to interesting effect in studies on the phylogenetic inter-relationships of schistosomes (Rollinson, Walker & Simpson 1988) and also fasciolids (Blair & McManus 1988).



## ***Chapter 3***

**Hatching, survival and  
infectivity characteristics of  
*Echinoparyphium recurvatum*  
eggs and miracidia**

## Hatching, survival and infectivity characteristics of Echinoparyphium recurvatum eggs and miracidia

### 3.1 INTRODUCTION

Several previous experimental studies have provided information on aspects of the hatching, survival and infectivity of eggs and miracidia of a range of digeneans including Echinostoma liei (Christensen, Fransden & Roushdy 1980), Transversotrema patialense (Bundy 1981), Schistosoma mansoni (Anderson, Mercer, Wilson & Carter 1982), Fasciola hepatica (Smith & Grenfell 1984) and Cyathocotyle bushiensis (Menard & Scott 1987). However, no detailed information is currently available on these aspects of the biology of Echinoparyphium recurvatum.

The collective purpose of the studies described in this Chapter was to provide some basic information on the hatching, survival and infectivity of E. recurvatum eggs and miracidia under conditions of environmental temperature that could be expected to be encountered in British habitats. The studies were primarily carried out with the aim of providing an understanding of the transmission ecology of the miracidium which would aid in a more detailed analysis of a study on the seasonal infection dynamics of E. recurvatum in the first intermediate host Lymnaea peregra at Harting Pond, West Sussex in England. This seasonal study will be described in the following Chapter.



### 3.2 MATERIALS AND METHODS

Infection-free 3 day-old Khaki Campbell Mallard ducklings (Anas platyrhynchos) were experimentally infected with E. recurvatum metacercarial cysts between May and July 1986. The cysts were obtained 14 days post-infection from laboratory-bred Lymnaea peregra snails in the size range 3-8mm which had been exposed *en masse* to freshly emerged E. recurvatum cercariae emitted from naturally infected L. peregra first intermediate host snails collected at Harting Pond, West Sussex. Cysts were fed in gelatine capsules, in doses of 100 per bird, to groups of infection-free 3-day old Khaki Campbell ducklings. Eggs were obtained from host faeces beginning 12 days post-infection. Eggs from the host faeces were filtered on a series of steel and nylon sieves of decreasing pore size (1mm - 150 microns) in a similar manner to that described by Mouahid and Mone' (1988) for recovering eggs of Echinoparyphium elegans from the faeces of definitive hosts. At 17 days post-infection the birds were killed and an additional supply of eggs was obtained by sieving the faecal material present in the host gut. After collection all eggs were rinsed several times in clean distilled water.

#### Temperature-related hatching

Five separate batches of 50 eggs rinsed in dechlorinated tap water were pipetted into polystyrene culture plate wells (1 egg per well) each containing 3ml of synthetic hard water medium (HMSO 1969) at pH 7.4. The polystyrene culture plates used were supplied by COSTAR inc. (Cambridge U.S.A.), and they were equipped with covers to minimize water evaporation. The egg batches were transferred to incubators and maintained at the following

temperatures ; 4-6, 10, 15, 20, and 25°C. These water temperatures were chosen to reflect the general temperature range recorded at Harting Pond over the period October 1984 to February 1986. The water temperature range over this time period was found to be from 2 to 21.5°C (see following Chapter). The light dark regime employed in the present study was 12 hours light to 12 hours dark. The eggs were individually examined at regular intervals of time and the numbers of eggs which had hatched at each temperature were recorded. An egg was considered as hatched when it was empty (clear shell) and its operculum was open, and the miracidium could be seen in the water of the plate well.

#### **Miracidial survival**

In order to obtain supplies of freshly hatched miracidia for use in survival experiments a technique which has also been used by Menard & Scott (1987) was employed. Rinsed eggs were maintained in 20ml glass vials containing synthetic hard water medium at 20°C. The vials were wrapped in aluminium foil to prevent exposure of the eggs to light. Darkness inhibited hatching but not miracidial development. On day 13 of incubation at 20°C the eggs were exposed to a strong hatching stimulus in the form of intense light from a fibre optic cold light source.

Freshly hatched miracidia were collected within 1 hour of hatching and were pipetted individually into polystyrene plate wells containing 3ml of synthetic hard water medium. Three batches of 50 miracidia were prepared in this way and one was then placed into each of three constant temperature incubators at



15, 20 and 25°C. The miracidia in each plate were then examined at regular intervals of 1-2 hours using a stereo-microscope and the number surviving at each time was recorded. A miracidium was considered dead when it gave no response to mechanical stimulation in the form of three light contacts with a fine nylon probe.

### **Temperature- and age-related infectivity of miracidia**

#### **Temperature-related infectivity**

Miracidial infectivity was examined at three water temperatures ; 15, 20 and 25 °C. Since the results of the previous experiment on temperature-related egg hatching had shown that miracidial development and hatching did not occur at 10°C or below , examination of infectivity at these temperatures was not carried out since at these temperatures free-swimming miracidia could not be expected to be present in the natural environment.

The influence of temperature on miracidial infectivity was assessed using a method similar to that employed by Prah & James (1977). Freshly hatched miracidia (maximum age 1 hour) were obtained in the manner described above. They were pipetted into clear polystyrene containers (3 miracidia per container) each containing 20 ml of synthetic hard water medium at one of three temperatures 15, 20 or 25°C. Each container also contained a single laboratory-bred infection-free specimen of L. peregra in the size range 4-6 mm. These exposure arenas were then incubated at 15, 20 and 25°C for 24 hours. Between 33 and 41 snails were exposed to infection at each temperature. Post-exposure, all snails

were transferred to polystyrene pots containing clean synthetic hard water medium in which they were maintained at 18-20°C on a diet of boiled lettuce. At 35 days post-exposure all surviving snails were dissected and examined for active infections using a stereo-microscope .The number infected in each case was recorded.

### **Age-related infectivity**

Freshly hatched miracidia (maximum age 1 hour) were aged for various time periods up to 16 hours at a temperature of 20°C (see Table 3.5). At each desired ageing time batches of 3 miracidia were transferred to polystyrene containers each containing 10 ml of synthetic hard water medium at 20°C and a single laboratory-bred infection-free specimen of L. peregra in the size class 4-6mm. The snails were exposed to infection for 45 minutes and were then transferred to polystyrene pots containing clean hard water medium where they were maintained at 20°C on a diet of boiled lettuce for 35 days. At 35 days post-exposure surviving snails were dissected and examined for active infection using a stereo-microscope. Between 22 and 32 replicate exposures were conducted for each miracidial age investigated.

## **3.3 RESULTS**

### **Temperature-related hatching**

The effect of environmental temperature on the hatching of E. recurvatum eggs is shown in Table 3.1. Temperature had a marked effect on hatching. At 4-6°C and 10°C no hatching was observed, even though observations were continued for 40 days.



**TABLE 3.1** The influence of temperature on hatching of Echinoparyphium recurvatum eggs

Temperature (°C)	No. of eggs	Total no. of eggs hatched	% Eggs hatched	Mean hatching time (+/-SE) , (Days)
4 - 6	50	0	0	-
10	50	0	0	-
15	50	28	56	22.4 (+/-0.43)
20	50	47	94	11.3 (+/-0.2)
25	50	46	96	6.9 (+/-0.2)
Eggs stored at 4-6 then hatched at 20	50	3	6	19.7 (+/-1.7)

**TABLE 3.2** The influence of temperature on the survival parameters of Echinoparyphium recurvatum miracidia

Temperature (°C)	Maximum survival time (hours)	Time to 50% survival (hours)	Mean instantaneous death rate $\bar{\mu}$ (per miracidia per hour)
15	26	13.6	0.07
20	16	8.1	0.12
25	8	4.5	0.22

Development of eggs maintained at these temperatures had in fact been arrested at the stage prior to cleavage of the zygote ; the state in which the egg of E. recurvatum leaves the uterus of the adult worm. Over the temperature range 15-25°C increased temperature resulted in a decrease in time to hatching . Mean time to hatching was reduced from 22.4 (+/-0.2) days at 15°C to only 6.9 (+/-0.2) days at 25°C. This trend was found to be well described by a negative exponential curve of the form shown in Fig 3.1. At each temperature hatching was not simultaneous but extended over a period of several days ; 4-8 days at 25°C, 8-13 days at 20°C, and 18-26 days at 15°C.

The percentage of eggs that hatched successfully was similarly high at 20 and 25°C at 94% and 92% respectively. However, reduced hatching success was observed at 15°C with only 56% of eggs hatching successfully . Eggs maintained prior to cleavage of the germinal cell at 4-6°C for 11 weeks remained at this stage of development, showing that low temperature inhibits development. When allowed to develop and hatch at 20°C they showed a severely reduced degree of hatching success with only 6% of eggs hatching. In these eggs, hatching time was also found to be extended in comparison with eggs allowed to develop and hatch normally at 20°C. The mean hatching time was found to be 19.7 (+/-1.7) days.

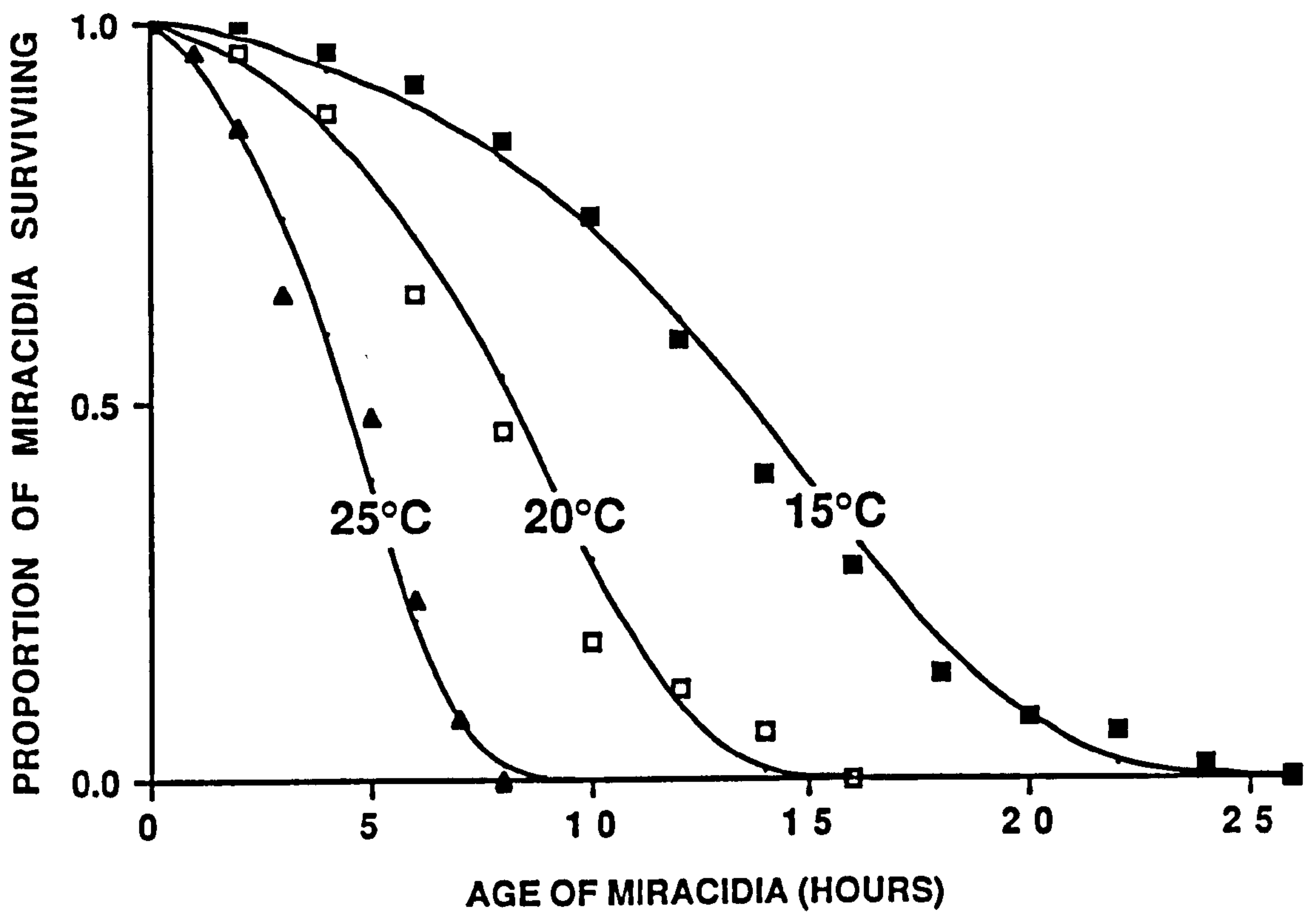
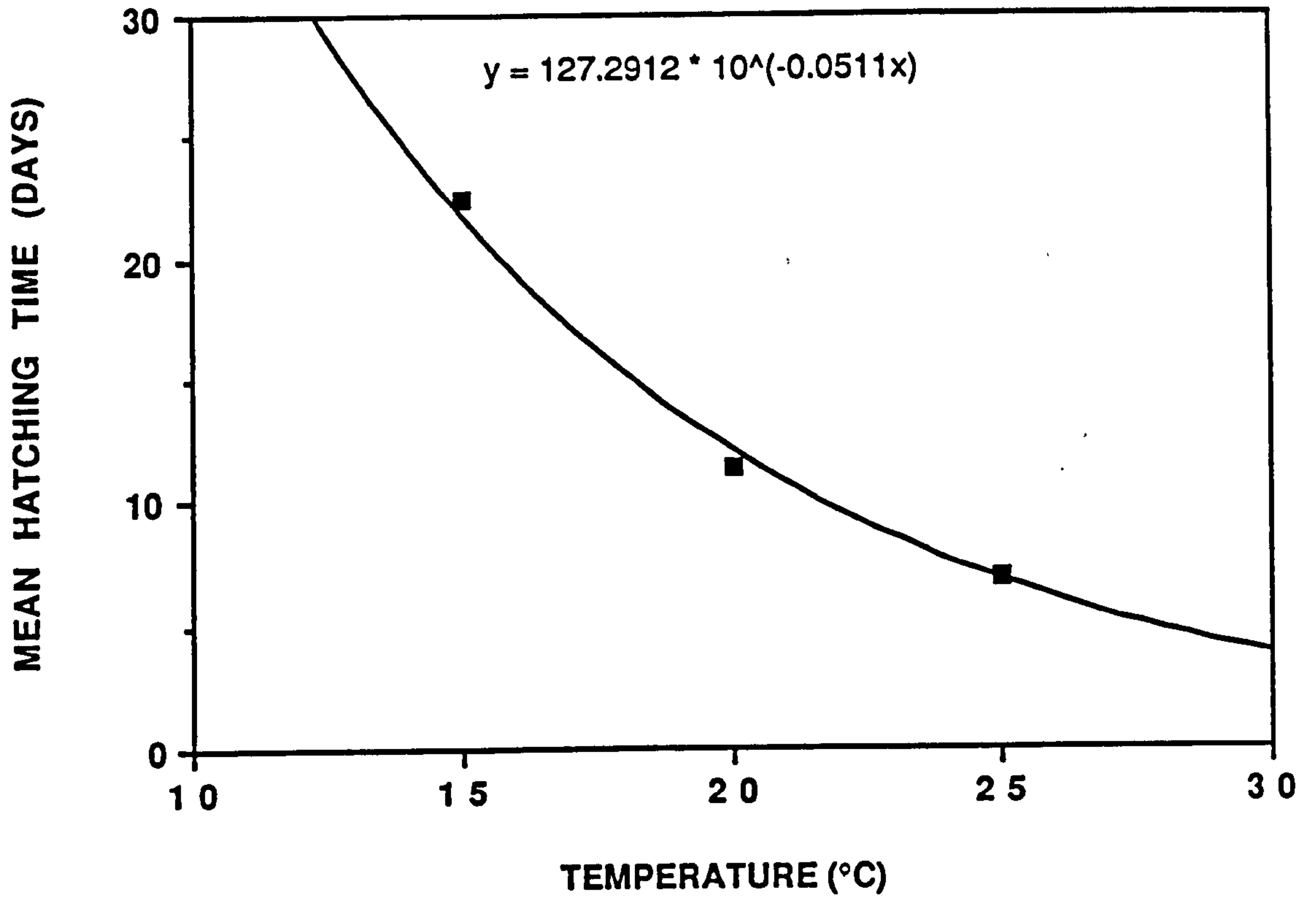
**FIG 3.1** The influence of temperature on the mean hatching time of Echinoparyphium recurvatum eggs

(At each temperature standard errors of the means were found to be smaller than the size of the points. Points indicate observed data and the solid line indicates the best fit negative exponential curve of the form described in the equation).

**FIG 3.2** The influence of temperature and age on the survival of Echinoparyphium recurvatum miracidia

(Points indicate observed proportions of miracidia surviving and the solid lines indicate the proportions predicted according to the age dependent survival model of Anderson and Whitfield (1975)).





### Miracidial survival

The survival characteristics of E. recurvatum miracidia maintained at 15, 20 and 25°C are illustrated in Fig 3.2. At each temperature the proportions of cercariae surviving at successive points in time were found to be in good agreement with those predicted by the age-dependent survival model of Anderson & Whitfield (1975) which assumes that the instantaneous death rate of the miracidia increases exponentially with time. The predicted values were generated using the purpose written BBC Micro-based programs "MUCALC" and "MUTOFIT". Copies of these programs are retained by Dr. P.J. Whitfield in The Division of Biosphere Sciences, King's College London.

The program "MUCALC" calculated the instantaneous *per capita* death rate  $\mu$ , (Anderson & Whitfield 1975) between each observation as

follows :

$$\mu (t_1+t_2/2) = (\log_n P(t_1) - \log_n P(t_2)) / t_2 - t_1$$

where  $P(t_1)$  is the proportion of miracidia alive at time  $t_1$  and  $P(t_2)$  is the proportion still alive at time  $(t_2)$ . The observed values of  $\mu$  increase with age and an exponential model of the form :

$$\mu(t) = a \exp (bt)$$

The calculated values for each successive time interval were entered into the data file of "MUTOFIT" which then predicted

values for the proportion of miracidia surviving through time according to the age-dependent survival model of Anderson & Whitfield (1975) where the predicted proportion  $P(t)$  of miracidia surviving to age  $(t)$  is given as :

$$P(t) = \exp \left[ \frac{a}{b} (1 - \exp(-bt)) \right].$$

Survival of miracidia was found to be markedly temperature-dependent, miracidial longevity decreasing significantly with increased temperature. The maximum life span of miracidia was reduced from 26 hours at 15°C to only 8 hours at 25 °C. The influence of environmental temperature on two further parameters of miracidial survival, namely the time to 50% survival and the mean instantaneous *per capita* death rate,  $\bar{\mu}$  , over the miracidial life span are shown in Table 3.2 . The values of these parameters were predicted by the Anderson & Whitfield (1975) model using the "MUTOFIT" program mentioned above.

### **Temperature- and age- related infectivity of miracidia**

#### **Temperature-related infectivity**

The infectivity of *E. recurvatum* miracidia at 15, 20 and 25°C is shown in Table 3.3. All snails exposed to infection were surviving 35 days post-exposure. The observed infectivity data was found to be adequately described by a second order polynomial curve of the form shown in Fig 3.3 which suggested that infectivity was optimal in the region of 20°C.



**TABLE 3.3** The influence of temperature on the infectivity of Echinoparyphium recurvatum miracidia to Lymnaea peregra

Exposure Temperature (°C)	No. of snails exposed to infection	No. of miracidia per snail	Exposure time (hours)	No. of snails infected	% Snails infected
15	36	3	24	9	25
20	41	3	24	26	63
25	33	3	24	10	30

**TABLE 3.4** The influence of temperature on the transmission efficiency of Echinoparyphium recurvatum miracidia to Lymnaea peregra

Temperature (°C)	Hatching success (%)	Infection success (%)	Transmission Efficiency * (T)
4 - 6	0	-	0
10	0	-	0
15	56	25	14
20	94	63	59.2
25	92	30	27.6

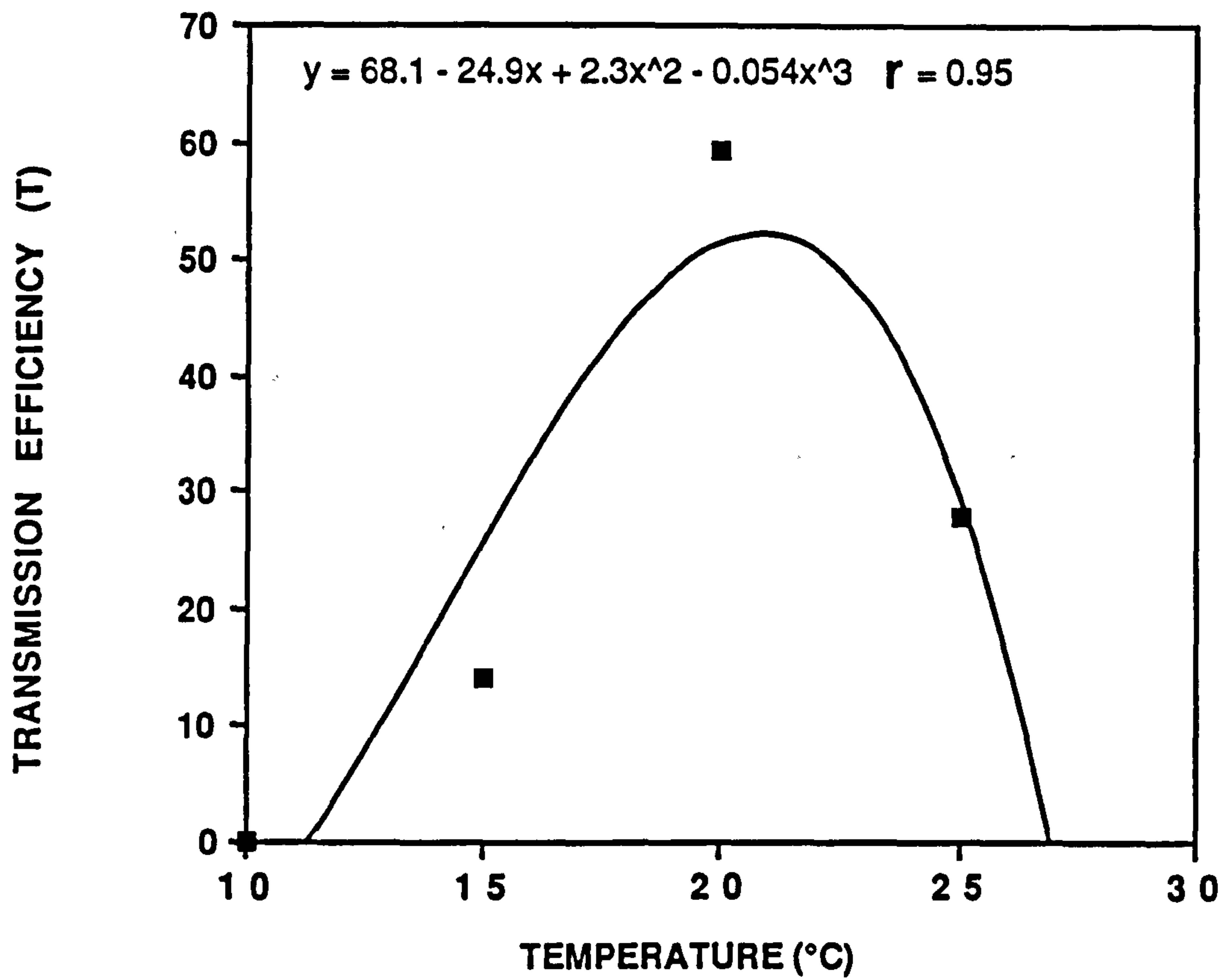
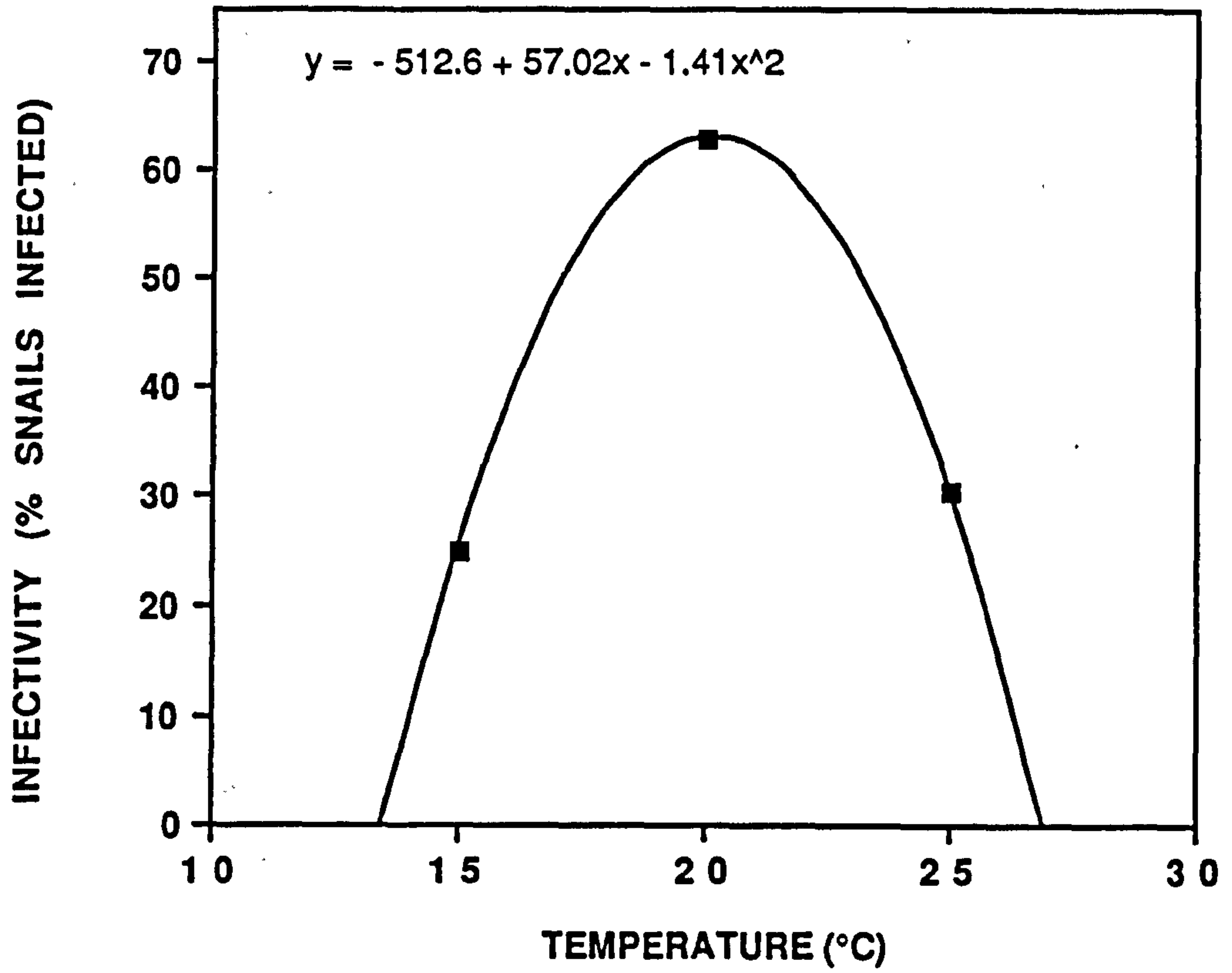
\* Transmission efficiency = (Hatching success x Infection success) / 100

**FIG 3.3** The influence of temperature on the infectivity of Echinoparyphium recurvatum miracidia to Lymnaea peregra.

(Points indicates observed data and the solid line indicates the best fit second order polynomial curve of the form described in the equation).

**FIG 3.4** The influence of temperature on the transmission efficiency of Echinoparyphium recurvatum miracidia to Lymnaea peregra.

(Points indicate observed data and the solid line indicates the best fit third order polynomial curve of the form described in the equation).





In order to provide some idea of the transmission efficiency of miracidia over the temperature range 4-25°C a parameter, T, which incorporated hatching success and infectivity data, was calculated. For each temperature T was obtained by multiplying the infectivity of miracidia (% snails infected) by the % hatching success. The product in each case was then divided by 100 to give T. At 4-6°C and 10°C infectivity in real terms was taken to be zero because at these temperatures hatching of eggs was found to be completely inhibited. The values of the transmission efficiency parameter T are given in Table 3.4. The values of T for 10-25°C are graphed in Fig 3.4 and are well described by a third order polynomial curve which suggests that transmission efficiency of E. recurvatum miracidia to L. peregra first intermediate hosts is likely to be optimal in the region of 20°C.

#### **Age-related infectivity**

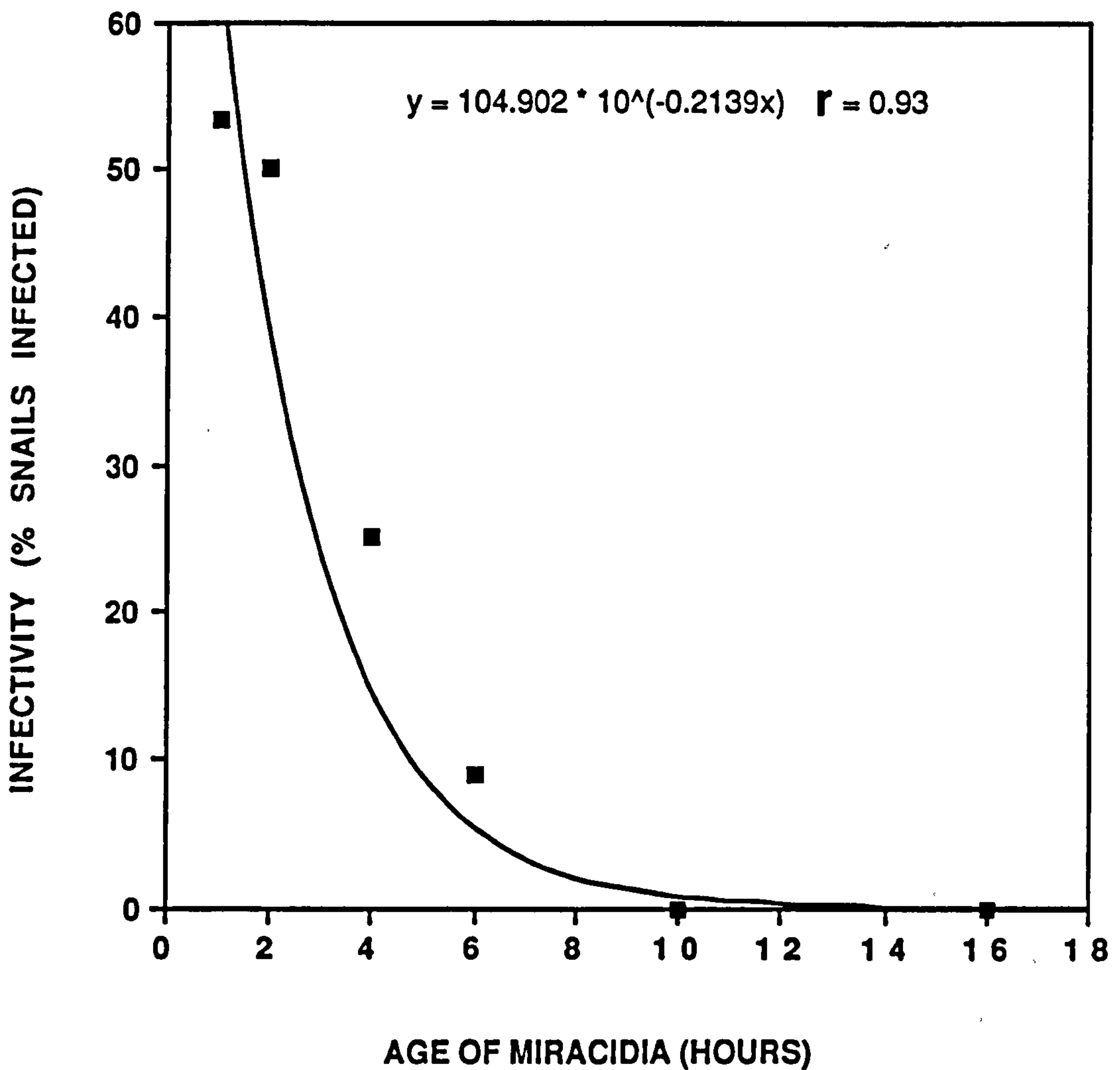
On examination 35 days post-exposure all snails used in this study were found to be alive. Infectivity of E. recurvatum miracidia was found to be markedly age-dependent at 20°C. Infectivity was found to decline with increasing miracidial age, see Table 3.5. The decline was found to be well described by a negative exponential curve of the form illustrated in Fig 3.5. The maximum infective life span of miracidia at 20°C of approximately 8 hours was found to be considerably shorter than the maximum survival time of 16 hours observed at this temperature.

**TABLE 3.5** The influence of age on the infectivity of Echinoparyphium recurvatum miracidia to Lymnaea peregra at 20°C

Age of miracidia (hours)	No. of snails exposed to infection	No. of miracidia per snail	No. of snails infected (35 days post-exposure)	% Snails infected
1	30	3	16	53
2	32	3	16	50
4	24	3	6	25
6	22	3	2	9
10	23	3	0	0
16	25	3	0	0

FIG 3.5 The influence of age on the infectivity of Echinoparyphium recurvatum miracidia to Lymnaea peregra at 20°C.

(Points indicate observed data and the solid line indicates the best fit negative exponential curve of the form described in the equation)





### 3.4 DISCUSSION

In the present study temperature was found to have a profound influence on the hatching time of E. recurvatum eggs. Over the temperature range 15-25°C the mean time to hatching of E. recurvatum eggs was found to decrease exponentially with increase in temperature. A similar exponential reduction in time to hatching with increase in temperature has also been reported for the eggs of Fasciola hepatica by Wilson, Smith & Thomas (1982). Menard & Scott (1987) have recently reported a linear decrease in hatching time with increased temperature for the eggs of Cyathocotyle bushiensis, but these authors made the comment that the relationship would probably prove to be exponential over a wider temperature range than they had investigated. Optimum hatching success of eggs of E. recurvatum was found to be reached at 20 and 25°C in the present study and this finding parallels that of Menard & Scott (1987) for C. bushiensis. Hatching success was reduced at 15°C and hatching was completely inhibited at 4-6°C and 10°C. At the two latter temperatures the observed zero hatching success can be explained by the fact that at these temperatures miracidial development was found to be completely arrested, a phenomenon which has been previously reported for other species of digenean such as Neodiplostomum intermedium (Pearson 1961).

The severely reduced hatching success of E. recurvatum eggs maintained at 4-6°C for 11 weeks before being allowed to develop and hatch at 20°C is similar to the finding made by Menard & Scott (1987) who maintained eggs of C. bushiensis at 4°C for 7 weeks prior to incubation at 20°C. This severe reduction in

hatching success of E. recurvatum eggs due to low temperature exposure could be expected to have severely detrimental effects on miracidial transmission to the first intermediate host. It does in fact suggest that the egg of E. recurvatum may be of limited significance as an overwintering stage in the life cycle of the parasite in north European habitats. This is in contrast to the metacercarial cyst, which data presented in Chapter 7 of this thesis suggest is likely to be of considerable importance as an overwintering stage in molluscan second intermediate hosts. Menard & Scott (1987) report a similar finding for the metacercarial cysts of C. bushiensis.

The survival of E. recurvatum miracidia was found to be markedly temperature-dependent in the present study. Temperature-dependency of survival is a phenomenon reported for the free-living transmission stages of a variety of digenean species including the miracidia of Schistosoma mansoni (Anderson et al 1982), and also the cercariae of, Echinostoma liei (Evans 1985) and indeed Echinoparyphium recurvatum (see Chapter 6). Miracidial death is thought to be due to the depletion of finite energy reserves within the larva. Temperature-dependency of miracidial survival is thought to be due to the fact that the rate of depletion of these finite energy reserves is greater at higher temperatures because increased demands per unit time are placed on them by increased activity (Anderson et al 1982).

The infectivity of E. recurvatum miracidia was found to be markedly age- and temperature-dependent, a phenomenon which has clearly been illustrated for the miracidia of F. hepatica by

Christensen & Nansen (1976) and for cercariae of E. liei by Evans (1985). Age-dependency of infectivity in E. recurvatum miracidia may be attributable to the fact that host location and penetration are energy-requiring processes. Therefore lower levels of infectivity exhibited with increasing miracidial age may be explained by the probability that older miracidia have depleted their finite energy reserves below that required for successful infection of a first intermediate host. As the maximum infective life span of E. recurvatum miracidia was observed to be considerably less than the maximum life span it may be assumed that the level of energy required for host penetration is likely to be much more than that required for survival alone.

Temperature-dependency of infectivity may be explained by a combination of factors. The reduced infectivity of miracidia observed at 25°C may have been mainly caused by the very high miracidial death rate experienced at this temperature. It is also possible that the host-location mechanisms in miracidia were not operating efficiently at this relatively high temperature. The seasonal water temperature at the habitat from which the stock of E. recurvatum used in this study was obtained (Harting Pond, West Sussex, England) is known not to reach much above 20°C even in the mid-summer months. Reduced infectivity at 15°C may have been caused by an inability of miracidia to locate or penetrate snails at this temperature. This could be due to the possibility that the mechanisms responsible for these functions were not, due to reduced metabolic activity, working at an optimal level at this low temperature. Similar suggestions to these were made by Evans (1985) in an attempt to explain the marked temperature-



dependency of E. liei cercarial infectivity to second intermediate host snails.

The overall findings of the present study suggest that the transmission efficiency of E. recurvatum miracidia to the first intermediate snail L. peregra may occur from or just below 15°C to just over 25°C, but that optimal transmission success (ie. optimal combination of hatching and infectivity) is likely to be reached in the region of 20°C. Below 10°C no transmission can occur due to the complete inhibition of hatching at these low temperatures. With regard to the significance of these findings to our knowledge of the transmission of the parasite in British freshwater habitats the results seem to suggest that miracidial transmission of E. recurvatum to first intermediate host L. peregra snails can occur from late Spring to late Autumn. Monthly water temperature readings taken at Harting Pond, West Sussex from October 1984 to February 1986 (see following Chapter) indicate that water temperatures tend to rise above 10°C in April and approach 15°C in May. They then remain above 15°C until October when the temperature again falls below 10°C. Water temperatures in the region of 20°C the optimal level for miracidial transmission were recorded in the mid-summer months of June, July and August. It would therefore seem that a "transmission window" for E. recurvatum miracidia at Harting Pond is likely to occur from about April to October with transmission success likely to reach its optimum level in the mid-summer months. The latter is of particular significance as it means that optimal conditions for miracidial transmission will overlap with the appearance of large numbers of juvenile L. peregra, the new first intermediate host

generation (see following Chapter). The parasite therefore seems to attain its optimal transmission potential during the period of the year when the new generation of its first intermediate host snails arrive.

Menard & Scott (1987) have in fact discovered a similar situation to that described above in the case of the miracidia of C. bushiensis. The optimal egg hatching temperatures of this digenean parasite were found to coincide closely with July and August temperatures reached in the marshes of southern Quebec, Canada. These are the two summer months in which the new generation of first intermediate host snails Bithynia tentaculata appears.

The findings reported in the present Chapter on the temperature-related transmission biology of E. recurvatum miracidia will be discussed further with respect to their relevance to the seasonal infection dynamics of the parasite in the first intermediate host L. peregra in the following Chapter.

## ***Chapter 4***

**Seasonal infection dynamics of  
*Echinoparyphium recurvatum* in the first  
intermediate host *Lymnaea peregra***



Seasonal infection dynamics of Echinoparyphium recurvatum in the first intermediate host Lymnaea peregra

#### 4.1 INTRODUCTION

The seasonal infection dynamics of digeneans in molluscan first intermediate hosts have been the subject of a number of studies in the literature to date. In Britain for example, the seasonal infection dynamics of the liver fluke Fasciola hepatica in the first intermediate host snail Lymnaea truncatula have been studied by Smith (1978). F. hepatica has been shown to have Spring and Autumn infection peaks in L. truncatula. Seasonal changes in the infection levels of digeneans in freshwater mollusc first intermediate hosts in Britain have also been observed by Pike (1968). This author reported peaks of digenean infection in first intermediate host molluscs of the Wentloog Level, in Wales, in June and August.

To date no detailed information is available on the seasonal infection dynamics of Echinoparyphium recurvatum, nor indeed any other echinostome digenean, in a population of first intermediate hosts. The present study therefore set out to examine the seasonal infection dynamics of E. recurvatum in a natural, littoral-lacustrine population of the first intermediate host snail Lymnaea peregra at Harting Pond, West Sussex in southern England. A primary aim of the study was to attempt to correlate the experimentally determined temperature-related egg hatching and miracidial transmission characteristics of E. recurvatum (see previous Chapter) with the seasonal infection dynamics of the

parasite in the first intermediate host L. peregra in the field. A similar type of study was recently attempted by Menard & Scott (1987) who experimentally examined the temperature-related egg hatching and miracidial survival characteristics of the wildfowl parasitic digenean Cyathocotyle bushiensis. These authors intended to attempt to relate their experimental findings to the seasonal infection dynamics of the parasite in a natural population of its first intermediate host gastropod Bithynia tentaculata in the marshes of southern Quebec, Canada. However, unfortunately, a seasonal study of the sporocysts in B. tentaculata proved to be virtually impossible due to the extremely low prevalence of infections. For E. recurvatum a seasonal study of active infections in L. peregra did prove possible and is described in this Chapter.

## 4.2 MATERIALS AND METHODS

### The Habitat

This study was carried out at Harting Pond, West Sussex, England (NGR SU 778 219). Harting Pond is a shallow rectangular body of inland freshwater having a surface area of approximately 2.0 hectares. The water is managed as a trout fishery and in addition to being stocked with Rainbow and Brown Trout (Salmo gairdneri and S. trutta) the pond also contains Three-spined sticklebacks (Gasterosteus aculeatus), Nine-spined sticklebacks (Pungitius pungitius). Bullheads (Cottus gobio) and Common Carp (Cyprinus carpio).

The wildfowl fauna of the pond is dominated by a resident population of Tufted Duck (Aythya fuligula). However, several

other species of waterbird have been recorded including Mallard (Anas platyrhynchos), Teal (Anas crecca), Little Grebe (Tachybaptus rufficollis), Mute Swan (Cygnus olor), Canada Goose (Branta canadensis), Coot (Fulica atra), Moorhen (Gallinula chloropus), Kingfisher (Alcedo atthis) and Grey Heron (Ardea cinerea). The molluscan fauna of the littoral regions of the pond at the time of the study was composed of the gastropods Lymnaea peregra, Physa fontinalis, Planorbis planorbis, Valvata piscinalis, Potamopyrgus jenkinsi and the bivalves Sphaerium corneum and Pisidium subtrucatum.

The littoral margins of the pond, where the present study was carried out, were of a similar substrate composition around the perimeter of the pond. The littoral substrate was composed mainly of mud, decaying leaves and other vegetation, and mollusc shells. This was overlain in places with mats of the filamentous alga Cladophora sp.. Routine chemical analysis of the pond outflow undertaken by The Southern Water Authority has indicated that the water is hard (260 ppm. hardness measured as CaCO<sub>3</sub>), well oxygenated and generally of good quality.

### Sampling Procedure

Sampling of the L. peregra population was carried out at monthly intervals over the period October 1984 to February 1986. Sampling was usually carried out on the 20th of each month. The sampling programme was similar to, and based on, that adopted by Evans (1983a) in his seasonal study on the population biology of Hymenolepis tenerrima cysticeroid infection in intermediate hosts at Harting Pond. To provide density estimates of the L.



peregra population, together with material for parasitological examination, five random quantitative samples, each removing a surface area of 132cm<sup>2</sup> of benthic littoral mud, were taken at each of 6 pre-selected sites around the perimeter of the pond. The sites were spaced at approximately equi-distant intervals around the perimeter in order to achieve some degree of spatial coverage of the habitat. The benthic samples were taken by hand using a cylindrical plastic mud-coring device. In addition to the 30 quantitative samples taken each month additional net samples of benthic mud were taken using a hand net adjacent to each of the six sites. This was to ensure that sufficient material would be available for further parasitological examination.

In the field each of the 30 mud core samples were placed into polystyrene storage tubs together with about 200ml of pond water. The samples were then returned to the laboratory. Excess mud was sieved off using a wire mesh sieve of 1mm<sup>2</sup> grid size. All the L. peregra in each sample were removed, counted and measured. The shell length of each specimen, from apex of spire to lip of aperture, was measured to the nearest millimetre. For small specimens measurement was carried out using a stereomicroscope equipped with a calibrated ocular micrometer. Larger specimens were measured using a set of malacological calipers, CAMLAB (Cambridge, U.K.).

In October 1985 it was decided to examine the relationship between the size of L. peregra and levels of active infection with E. recurvatum. In addition to the 294 snails obtained from the 30 core sample additional snails were examined in an attempt to

ensure that in the region of 30 snails were examined from each size class encountered. To provide extra snails a sweep net sample was taken parallel to each sample site. These samples were then pooled in a single large container (plastic bin) and provided a single large sample from which extra snails of the desired size classes could be obtained. In total 454 L. peregra were examined in this investigation.

### Parasitological Examination

In every month except October 1985 snails were examined for active E. recurvatum infection by crushing each snail between two glass microscope slides, or two 5cm x 5cm glass plates, depending on size. The shells of large specimens (>15mm) were removed prior to this operation. The tissues of each snail were then examined using both stereo- and compound light microscopes. Each active infection of E. recurvatum discovered was classified as being either "mature" (producing cercariae) or "immature" (not producing cercariae).

In October 1985 a sample of 454 L. peregra was examined in order to determine the relationship between host size and E. recurvatum parasitization level. All the snails were measured as above and each was then dissected in distilled water under a stereo-microscope. The digestive gland of each snail was carefully teased apart using fine stainless steel needles and the total number of rediae present in each infected snail was counted and recorded. The infection data was stored using the BBC-Micro-based database software package "Beta-Base" (CLARES MICRO SUPPLIES Ltd. U.K.).

### Temperature monitoring

On the sampling day of each month the water temperature of the pond close to the outflow was taken 30 cm below the surface of the water using a Mercury thermometer. The temperature was always taken in the midday period 12.00-14.00h.

## 4.3 RESULTS

### Water Temperature

The water temperature at Harting Pond varied between 2 and 21.5°C throughout the sampling period. The temperature for each month is shown in Fig 4.1.

### Host Population Dynamics

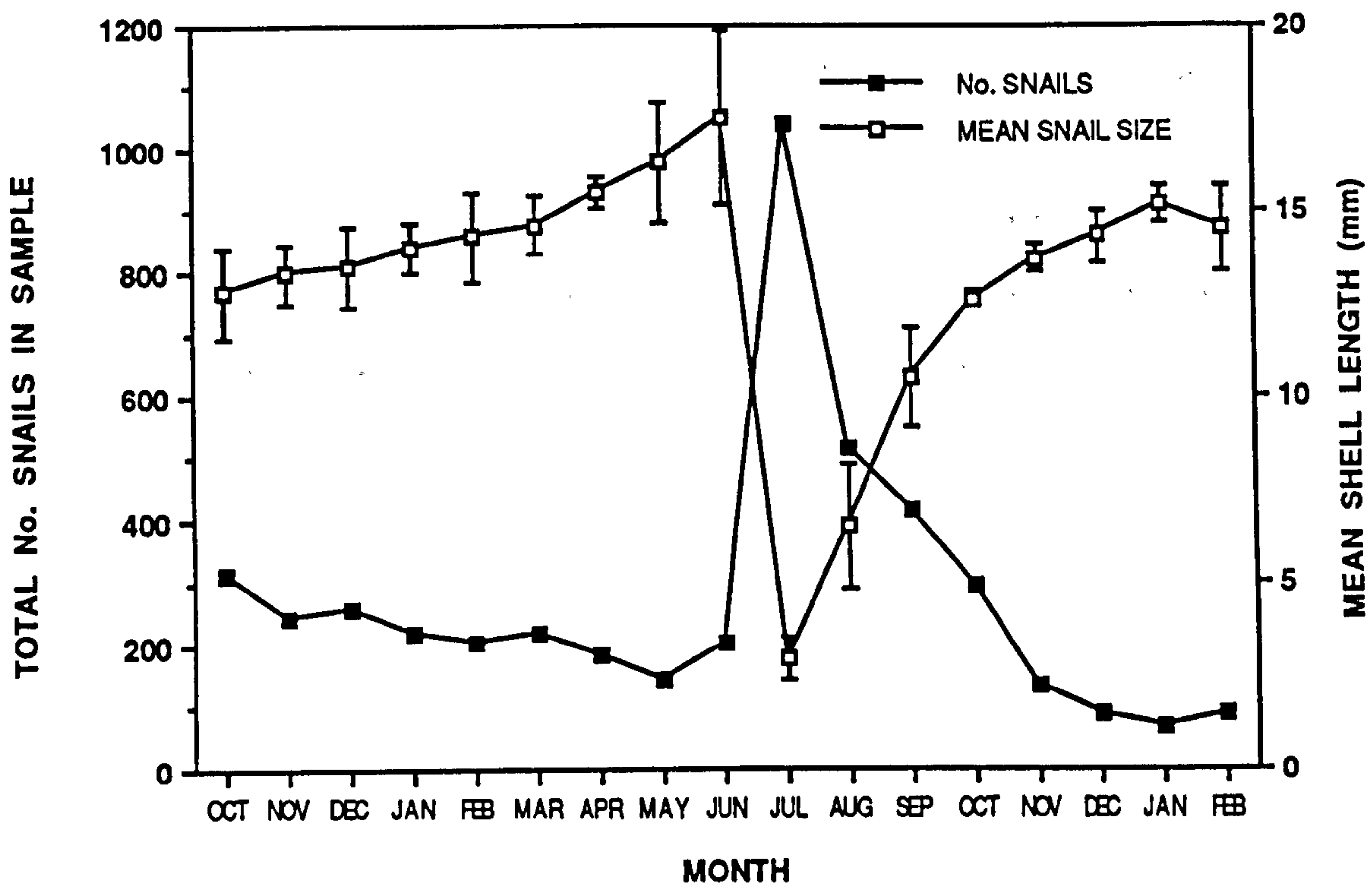
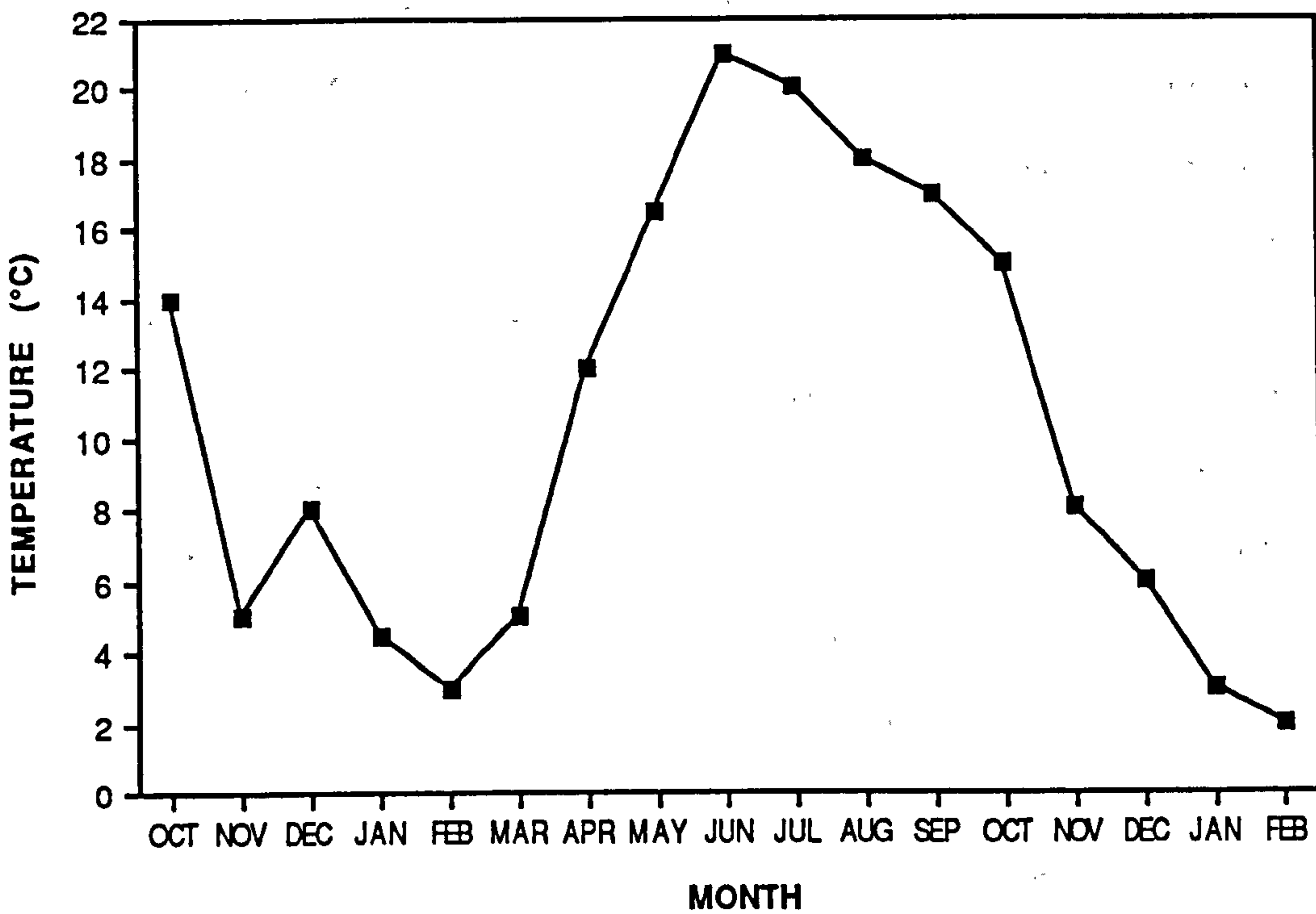
The total number of L. peregra obtained from the combined 30 core samples taken each month provided a convenient comparative monthly measure of the density of the L. peregra population at the pond throughout the sampling period. This is because the population density of snails in the field could reasonably be expected to be directly reflected in the abundance of snails in the random sample. The total number of L. peregra snails in this quantitative sample for each month is shown in Fig 4.2. The mean size (shell length) of the snails population for each month is also shown in Fig 4.2.

The L. peregra population demonstrated an annual life cycle at the pond over the period of the study, something which is well documented for British L. peregra populations, (Young 1975). From October 1984 to June 1985, a gradual decline occurred in the density of the overwintering population. However, the mean size



**FIG 4.1** The water temperature at Harting Pond from October 1984 to February 1986.

**FIG 4.2** The number and mean size of Lymnaea peregra obtained in each monthly random sample at Harting Pond over the period October 1984 to February 1986.  
(Vertical bars indicate standard errors of the means)



of snails continued to increase very slowly throughout, growth resuming at an increased rate from April onwards and reaching a peak in June 1985. L. peregra egg masses were evident in samples beginning in April. The appearance of the new generation of juvenile snails occurred in July. This was clearly indicated by the recruitment by birth of large numbers of small specimens into the population in July with a concurrent sharp drop in the mean shell size of the population, (see Fig 4.2). The July period also marked the death of the over-wintered population of parent adult snails that had reproduced in the Spring. The new juvenile population suffered severe mortality during July and August, which caused the loss of about 50% of new population. Such severe mortality among juvenile L. peregra during the period immediately after recruitment has also been clearly documented in other British populations of this pulmonate, eg. see Young (1975). In this case Young (1975) gave a mortality of about 40% of the L. peregra population in the month after hatching. At Harting Pond the mortality rate slowed between August and September and the surviving snails grew rapidly until October when the growth rate began to level off as winter approached. The population density of L. peregra in November 1985 dropped well below the level of the previous year. This decline was almost certainly explained by the fact that in mid-November the pond was drained for a period of approximately 2 weeks.



### Dynamics of Echinoparyphium recurvatum active infections

During the study the total number of L. peregra obtained in the combined 30 core random sample was found to be of adequate size to assess with an acceptable degree of accuracy the seasonal dynamics of E. recurvatum active infections in the L. peregra population. The prevalence of E. recurvatum active infections in L. peregra each month throughout the study period is shown in Fig 4.3. The sample size of snails on which the prevalence estimate is based is indicated above each column.

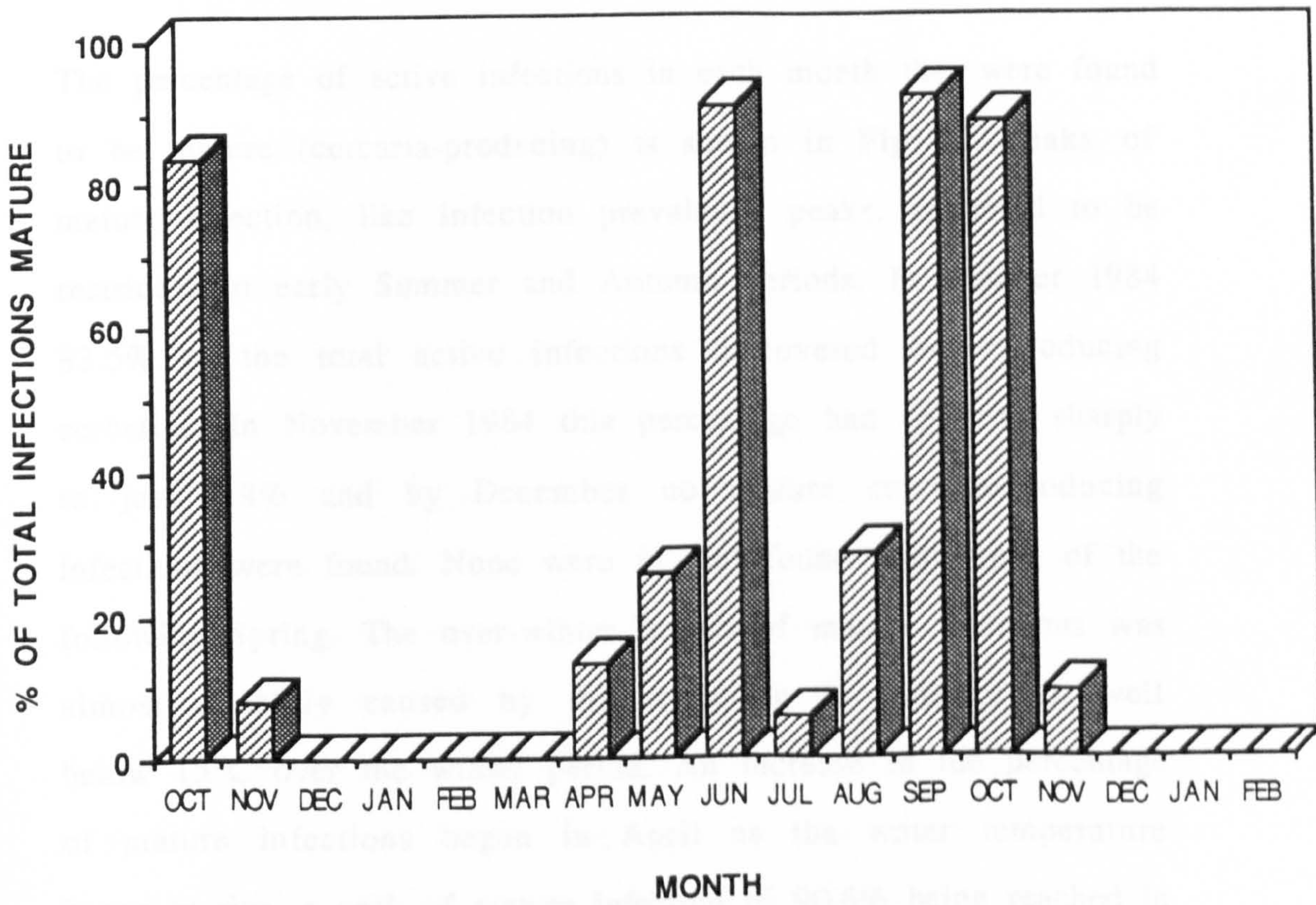
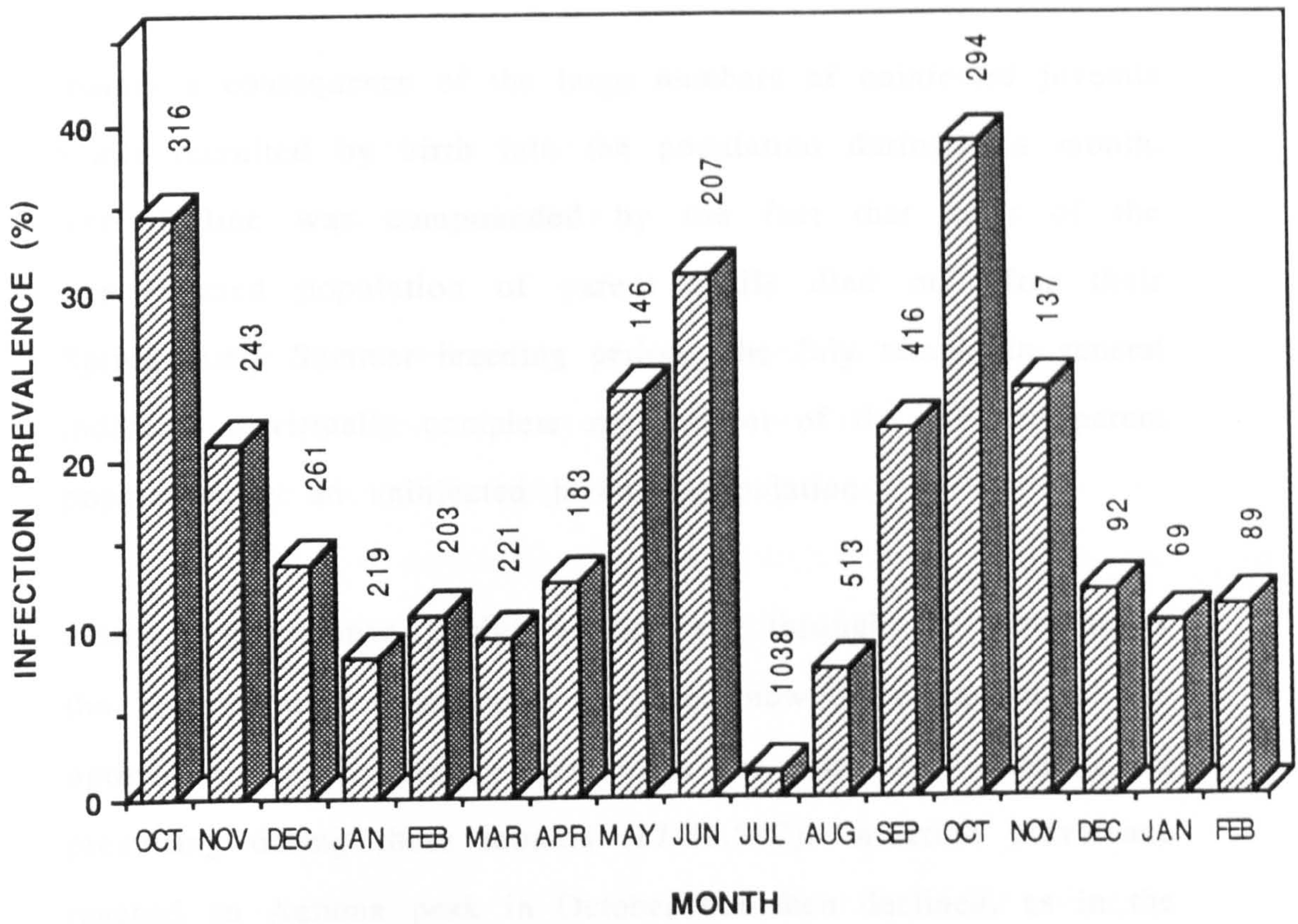
Three clear peaks of infection prevalence were observed throughout the study period these being in October 1984, June 1985 and October 1985. The prevalence of E. recurvatum active infections dropped from 34.5% in October 1984 to 13.8% in December 1984. This was caused by mortality among the L. peregra population (possibly particularly among infected snails) as it declined to its overwintering level, (see above). As the experimental results of the previous Chapter have shown that E. recurvatum miracidial transmission ceases at temperatures below 15°C it is almost certain that no new active infections were acquired during the Winter/early Spring period of November 1984 to April 1985 when the water temperature remained below 15°C. In May the infection prevalence began to increase, this being due to newly acquired infections, presumably resulting from the fact that the temperature began to rise above 15°C and miracidial transmission resumed. Infection prevalence reached a peak level of 30.9% in June after which a very sharp decline in prevalence occurred in July to its lowest level of only 1.6%. The decline was

FIG 4.3 The prevalence of Echinoparyphium recurvatum active infection in the Lymnaea peregra population at Harting Pond over the period October 1984 to February 1986.

(Numbers above the columns indicate the size of the random sample of snails on which the prevalence estimate is based)

FIG 4.4 The percentage of the total number of Echinoparyphium recurvatum active infections that were mature (cercaria-producing) in each month over the period October 1984 to February 1986.







mainly a consequence of the large numbers of uninfected juvenile snails recruited by birth into the population during this month. The decline was compounded by the fact that most of the overwintered population of parent snails died off after their Spring- early Summer breeding period. The July sample in general indicated a virtually complete replacement of the infected parent population by an uninfected juvenile population.

Infection prevalence gradually increased through the summer as the new population gradually acquired new infections under the optimal water temperature conditions for miracidial transmission prevailing during these months (17-21.5°C). Infection prevalence reached an Autumn peak in October but then declined, as in the previous year, as the L. peregra population began to decrease to its overwintering density.

The percentage of active infections in each month that were found to be mature (cercaria-producing) is shown in Fig 4.4. Peaks of mature infection, like infection prevalence peaks, appeared to be restricted to early Summer and Autumn periods. In October 1984 83.5% of the total active infections discovered were producing cercariae. In November 1984 this percentage had declined sharply to just 7.8% and by December no mature cercaria-producing infections were found. None were in fact found until April of the following Spring. The over-winter trough of mature infections was almost certainly caused by the reduction in temperature well below 15°C over the winter period. An increase in the percentage of mature infections began in April as the water temperature began to rise, a peak of mature infection of 90.6% being reached in

June. This increase to a peak was probably caused by a combination of both overwintered infections and newly acquired Spring infections becoming mature.

The percentage of mature E. recurvatum infections declined sharply in July with the appearance of the new uninfected juvenile population. The decline was also due to the death of the overwintered parent population of snails which were carrying the active infections responsible for producing the June peak. Most of the newly acquired infections over the period July- August 1987 were immature. However, by September most (92.2%) of these infections had reached maturity and were actively producing cercariae. The peak was maintained into October but a sharp decline was noted in November as cercarial production ceased in just over 90% of infected snails in response to the temperature drop below 15°C to only 8.5°C.

#### Host size-related infection trends

The October 1985 a total of 454 L. peregra were examined to see if there was any evidence for the existence of host-size-related trends in the level of E. recurvatum active infection. Prevalence of active infection was found to increase with host size, the larger size classes of L. peregra having the highest prevalence of E. recurvatum active infection, (see Fig 4.5). The number of rediae developing in each infected snail was also found to increase with host size. The size of the total redial infection load of infected snails (infection intensity) was found to increase exponentially with increase in host size as measured by maximum shell length, (see Fig 4.6).

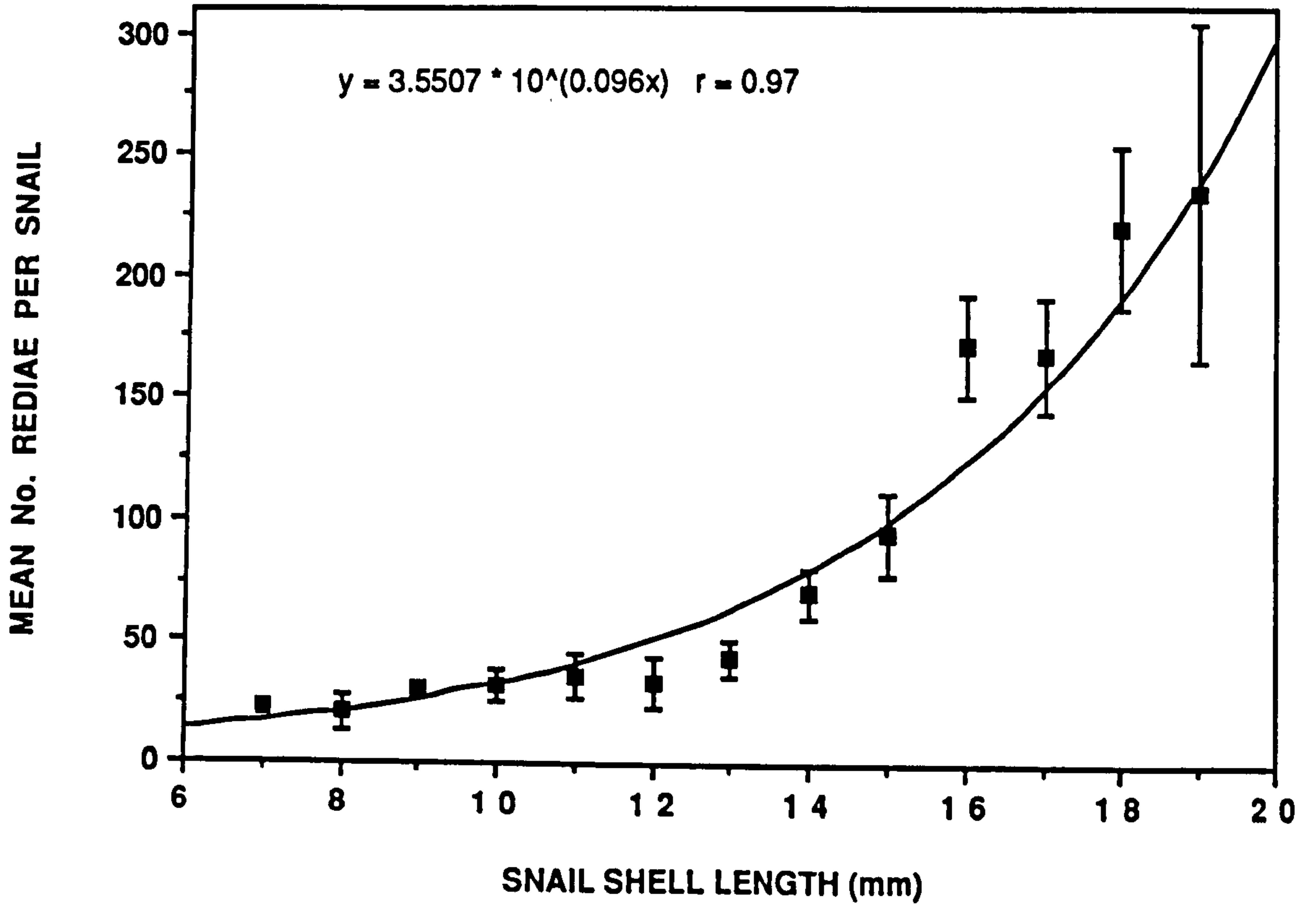
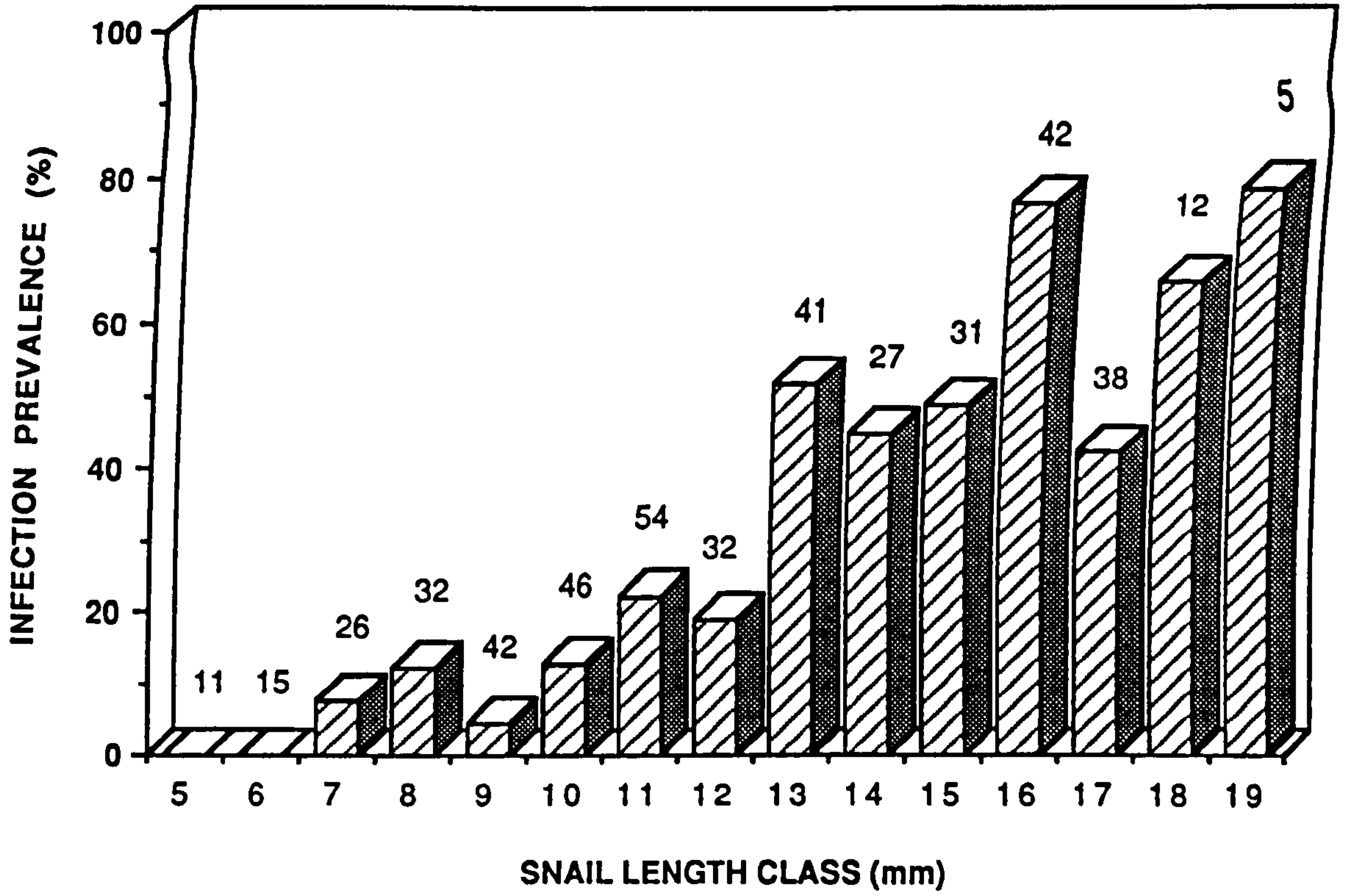
FIG 4.5 The prevalence of Echinoparyphium recurvatum active infection in each size class of Lymnaea peregra in October 1985.

(Numbers above the columns indicate the number of L. peregra on which the prevalence estimate is based)

FIG 4.6 The relationship between the size of Lymnaea peregra actively infected with Echinoparyphium recurvatum and the mean number of rediae per infected snail in October 1985.

(Vertical bars indicate standard errors of the means and the solid line indicates the best fit exponential curve of the form described in the equation)





#### 4.4 DISCUSSION

The dynamics of E. recurvatum active infections in the L. peregra population observed over the 16 month period of the present study show clear seasonality. The peaks of infection prevalence observed in early Summer and Autumn periods reveal a similar biannual infection peak pattern to that observed in previous British studies on the seasonality of digenean infections in freshwater molluscs. The general pattern of a late Spring-early Summer peak and a late Summer-Autumn peak of digenean active infections have been found previously in Britain by Rees (1932), Probert (1966), Pike (1968) and Young (1973). Spring and Autumn peaks of Fasciola hepatica infection in Lymnaea truncatula have also been recorded in Britain, (see Smith 1978). Biannual peaks of active infection prevalence in mollusc first intermediate hosts have also been recorded for digeneans in seasonal surveys carried out in geographical locations other than Britain. For example, in North America Rankin (1939) found two peaks of infection prevalence, one in December and one in June.

In the case of E. recurvatum in the present study the seasonal pattern of infection prevalence observed can be mainly explained as a combination of the temperature-related miracidial transmission characteristics of the parasite (as revealed in the previous Chapter) and the seasonal population dynamics of the first intermediate host L. peregra.

The results of the previous Chapter indicated that miracidial transmission was likely to occur only when the water temperature

reached above 15°C. The trough of active infection prevalence observed during the winter period November 1984 to April 1985 over which the water temperature varied from 3 to 12.5°C would appear to provide evidence that this is true in the natural environment. During these months the infection level dropped as actively infected snails were lost from the population, the loss not being replaced by newly acquired infections. One likely cause of the mortality of infected snails may have been that it was a natural consequence of the L. peregra population in general declining to its overwintering density. A further combinant cause may have been parasite induced host mortality operating among the infected section of the snail population. For L. peregra this could have been caused by the active infections possibly in conjunction with the large numbers of metacercarial cysts of E. recurvatum that were found within actively infected L. peregra. Parasite induced host mortality has in fact been suggested by Pike (1968) in partial explanation for the decline in the prevalence in digenean active infection levels that he found to occur after the late-Summer to Autumn infection peak in freshwater molluscs at the Wentloog level in Wales. The possibility that E. recurvatum active and metacercarial cyst infections may induce mortality in L. peregra first intermediate hosts is an interesting possibility that requires further experimental investigation.

From April to June the prevalence of active infection rose due to newly acquired infections establishing in the population. This was as a direct result of the resumption of miracidial transmission as the water temperature rose and remained above 15°C. However, the experimental findings of the previous Chapter suggest that the



miracidia responsible for producing this Spring-early Summer rise in infection prevalence had hatched mostly from eggs that had been introduced into the pond from definitive hosts in the Spring. It is unlikely that the miracidia would have originated from eggs that had overwintered in the pond as the results of the previous Chapter have shown that the egg of E. recurvatum is likely to be of little significance as an overwintering stage in the life cycle due to its low degree of low temperature tolerance. In contrast however, Chapter 7 of this thesis provides experimental evidence that the metacercarial cyst, due to its viability under prolonged conditions of low temperature, is likely to be of major importance as an overwintering stage. Interestingly, Menard & Scott (1987) came to a similar conclusion for the digenean Cyathocotyle bushiensis in which the egg has been shown to have limited overwintering potential in contrast to the metacercarial cyst. As metacercarial cysts of E. recurvatum were observed in second intermediate host snails throughout the winter it seems likely that transmission to wildfowl definitive hosts occurs throughout the winter at Harting Pond, as will egg deposition into the habitat. However, only when conditions of environmental temperature begin to become conducive to miracidial hatching and transmission, in the Spring, will new infections begin to occur in the L. peregra population, with a resultant rise in infection prevalence. This in fact was what was observed in the present study.

The experimental findings of the previous Chapter suggested that optimal hatching and transmission success of E. recurvatum miracidia would occur in the region of 20°C. This temperature approximately coincides with water temperatures at Harting Pond

during June, July and August of the present study. This was the period in the middle of which the new juvenile generation of L. peregra snails appeared. Optimal temperature conditions for miracidial transmission therefore coincided with the appearance of the new host population. This would appear to suggest a temperature-related facet of the transmission biology of E. recurvatum miracidia which serves to ensure that optimal transmission success is synchronized with the appearance of the new target host population.

During the present study miracidial transmission could have been expected to continue until October when the temperature began to fall below the 15°C level. The result of the July to October period of miracidial transmission into the new host population was evident in October 1985 when the Autumn peak in E. recurvatum active infections in L. peregra occurred. After this peak the infection prevalence again declined, this being attributable partly to the natural mortality of infected snails as the population declined to its over-wintering level and probably partly due to the stress that active infections are likely to have caused to young snails (see Pike 1968).

The seasonal pattern of occurrence of mature (cercaria-producing) infections of E. recurvatum in the present study, with peaks in the early Summer and Autumn, is a phenomenon that has been observed previously for other digeneans by Pike (1968). The low percentage of active E. recurvatum infections that were producing cercariae in the Summer period July-August can be explained by the fact that most of the infections encountered at this time were

from juvenile snails with newly acquired infections that had not had sufficient time to mature to patency. The low percentage of active infections over the winter periods was due to the cessation of cercarial production by rediae during this period of low temperature. The cessation of cercarial production in active infections over the Winter and its resumption in the Spring is a phenomenon that has been observed in other digeneans (Pike 1968) and for which environmental temperature is the primarily obvious cause. Cessation of cercarial production due to a drop in environmental temperature below a certain lower critical threshold value has been clearly demonstrated in previous studies. Ross (1930) showed that the production of E. hepatica cercariae in active infections decreased with decrease in temperature and that below 10°C cercarial production was completely inhibited. Dinnik & Dinnik (1964) reported that cercarial production in the daughter rediae of Fasciola gigantica ceased below 16°C.

The mechanism by which temperature influences cercarial production is likely to be mainly a direct one as temperature will undoubtedly have an influence on the basic metabolic and physiological activity of rediae within the host snail. However, it is interesting that the results of some previous studies are suggestive of the possibility that the resumption of cercarial production by active infections in Spring may be in part stimulated by a seasonal change in host physiological activity. Wright (1971) has tentatively suggested that production of Rhodometopa cercariae from the marine snail Turritella communis may be activated by a temperature-induced change in the



reproductive physiology of the mollusc host. Muftic (1969), in fact, claims to have isolated a hormone from the snail Australorbis glabrata which can control production of cercariae in S. mansoni sporocysts. Interestingly work by Joosse (1984) has shown that secretions of neuropeptide reproductive hormones in lymnaeid snails (L. stagnalis) are stimulated by increasing day length and are thus potentially seasonal in occurrence. The activity of the neurosecretory cells that produce the reproductive hormones in L. stagnalis appear to be most active in the Spring. The possibility that E. recurvatum rediae are stimulated in any way to resume cercarial production in Spring by a physiological change (possibly linked to host reproductive hormone release) remains an interesting subject for future experimental investigation.

Analysis of the L. peregra sample for October 1985 revealed some clear host-size-related trends in both the prevalence of E. recurvatum active infection and also the redial infection load. The increase in active infection prevalence with increase in host shell length is a relationship that has been reported previously for a number of digeneans in populations of first intermediate host molluscs. For example this relationship has been demonstrated for the E. hepatica/L. truncatula system by Smith (1984). For several digeneans host snail size is known to be a primary determinant of miracidial transmission success. Smith (1982) for example has demonstrated that the instantaneous infection rate of E. hepatica miracidia increases markedly with host size. The host size-related trend in E. recurvatum infection prevalence may therefore partially explained by the fact that larger snails present a larger

target size to miracidia and therefore acquire active infection more frequently than smaller specimens.

As shell length is generally accepted to be a function of host age in lymnaeid snails this particular size-related relationship may be explained by the fact that larger snails are older and have had a relatively longer exposure to infection in the field than smaller, younger snails. The higher incidence of active infections in larger size classes of snails may therefore primarily be a result of the fact that the older snails contained in these size classes have had a greater time in which to acquire infection. An interesting possibility that may serve to compound the observed relationship is that if E. recurvatum active infection brings about a degree of increased growth (as it does in L. truncatula snails actively infected with E. hepatica) then infected snails could be expected to enter the larger size classes at a faster rate than uninfected ones. However, this possibility remains to be experimentally tested.

In populations of L. truncatula naturally infected with E. hepatica Smith (1984) found an exponential increase in the mean redial burden per infected snail with increase in host shell length. This relationship was also observed for the E. recurvatum/L. peregra system in the present study. Mean redial burden of E. recurvatum showed a clear exponential increase with increase in shell length of infected L. peregra. The relationship is one which in general has been observed in a number of other digeneans, including the echinostomes Echinostoma revolutum (Zischke 1967), Paryphostomum segregatum (Lim & Lie 1969). The relationship between redial infection load and host size is thought to be one

mainly determined by the fact that the carrying capacity of the parasite's microenvironment is largely determined by the size of the first intermediate host snail's digestive gland. Larger snails with larger digestive glands therefore have the capacity to support larger numbers of rediae than smaller snails with correspondingly smaller digestive glands (Anderson Whitfield & Mills 1977). Under experimental conditions, studies such as that of Lim & Lie (1969) on P. segregatum in Biomphalaria glabrata have shown that the number of rediae in the digestive gland is mainly a function of the size of the host's digestive gland and is independent of the size of the initial miracidial infection dose. Thus it would seem to be a case of a parasite population expanding to the limits of its microenvironment. However, under field conditions Smith (1984) has suggested that in some cases high redial burdens in larger snails may be the consequence of multiple miracidial infections.



## *Chapter 5*

**Photoperiodic and temperature-related emergence, and phototactic behaviour of *Echinoparyphium recurvatum* cercariae**

Photoperiodic and temperature-related emergence, and phototactic behaviour of Echinoparyphium recurvatum cercariae.

## 5.1 Photoperiodic emergence of Echinoparyphium recurvatum cercariae from Lymnaea peregra

### 5.1.1 INTRODUCTION

The fact that the emergence of digenean cercariae from molluscan first intermediate hosts is photoperiodic (influenced by the light-dark regime of the external environment) has long been recognised from studies such as Cort (1922). Interest in this particular area of cercarial biology has engendered a considerable body of research reviewed by Combes & Theron (1977). Studies on the photoperiodic cercarial emergence of a range of digeneans have been carried out. These include experimental observations on the photoperiodic cercarial emergence of the cathaemasid Ribeiroia marini (Theron 1975), the transversotrematid Transversotrema patialense (Mahmoud 1983), the gorgoderid Gorgoderina vitelliloba (Mitchell & Mason 1980; Mitchell, Lees & Mason 1983), and the plagiorchiid Plagiorchis noblei (Webber, Rau & Lewis 1986). In recent years the adaptive significance of photoperiodic cercarial emergence in the transmission dynamics of schistosomes has been realized, and the work of Theron (1984) stands paramount in this respect. This author demonstrated that distinct, genetically determined, photoperiodic emergence patterns of human and murine races of Schistosoma mansoni cercariae in the French West Indies were closely linked with the diel water-visiting activity patterns of the respective target definitive hosts.

The photoperiodic emergence patterns of several schistosome species have been studied in detail in recent years, eg. S. mansoni (Theron 1984), S. haematobium (Nojima & Sato 1982), and S. bovis (Mouahid & Theron 1986). In contrast, very few studies exist on the photoperiodic emergence patterns of echinostome cercariae. However, Christensen, Fransden & Roushdy (1980) have reported that cercariae of Echinostoma liei emerge from Biomphalaria glabrata during light. In addition, Rees (1931) has reported a marked photoperiodicity of emergence of the echinostome "Cercaria Z" from Lymnaea peregra with peak emergence occurring during the daylight. No published information currently exists on the emergence pattern of Echinoparyphium recurvatum from the first intermediate host. The present study set out to examine the influence of photoperiod on cercarial emergence of E. recurvatum under experimental conditions. As part of the study it was also intended to examine the relationship between first intermediate host size and cercarial emergence.

### 5.1.2 MATERIALS AND METHODS

#### Host and parasite material

Specimens of Lymnaea peregra naturally infected with Echinoparyphium recurvatum were collected by sweep net sampling of the benthic mud at Harting Pond in September 1985.

#### Acclimatization and other general procedures

Infected snails were isolated in clear polystyrene containers each containing 15mls of a synthetic hard water medium (HMSO 1969) with a total hardness of 250 ppm and pH 7.4, hardness and pH



values identical to those of the water in Harting Pond, (Evans, Whitfield & Dobson 1981). The snails were then transferred to an experimental light-proof cabinet in which lighting and temperature were under automatic control. The cabinet was equipped with four "Daylight" cold fluorescent tubes producing a light intensity of 1600 Lux close to the snails. Temperature was maintained at a constant 18°C throughout experiments.

Prior to entering an experiment all snails were pre-conditioned to a photoperiod regime of 12 hours light : 12 hours dark (L:D 12:12) for three days during which time they were fed a diet of clean boiled lettuce *ad libitum*. Experimental light began at 08.00 hours and dark at 20.00 hours. During experiments food, boiled lettuce, was made available throughout as previous authors have reported detrimental effects of host snail starvation on cercarial emergence rates, (see Anderson Whitfield & Mills 1977).

During experimental periods, monitoring of cercarial emergence was achieved by transferring snails at the appropriate time intervals into fresh containers of water previously equilibrated to cabinet temperature. The experimental cabinet was situated in a darkroom. No appreciable change in illumination occurred during transfers in light periods. During dark periods it was necessary to use brief intervals of very low intensity photographic-safe lighting (KODAK 1A red filter) in order to carry out transfers.

Studies on the emergence of echinostome cercariae such as E. recurvatum (which are capable of penetrating and encysting in the same snail from which they have been emitted) present a

unique problem not encountered in studies such as those on schistosomes. This is because there is no easy or reliable method of determining exactly the number of cercariae which emerge during an experiment, but then subsequently penetrate and encyst in the snail being studied. This may represent a considerable potential source of error in cercarial counts, particularly if the time interval between each cercarial collection is long. This problem has recently been addressed from a technical standpoint by Jourdane at Perpignan University, France, who has experimented with set-ups designed, using water agitation, to reduce echinostome cercarial attachment and penetration rates (Jourdane 1987 personal communication). However, since this could conceivably affect snail behaviour, and since certain authors (Anderson, Nowosielski & Croll 1977) believe that there may be a host-behaviour related component in cercarial emergence, this approach was not used in the present study. Instead an interesting natural characteristic of the transmission biology of E. recurvatum was utilized to combat the problem. The results of detailed transmission studies by Evans & Gordon (1983a) and the present author in Chapter 6 of this thesis have shown that E. recurvatum cercariae do not attain maximal infectivity until about 2-3 hours after emergence. Initially infectivity is very low. This is a transmission characteristic also reported for cercariae of the echinostome Echinostoma revolutum by Lo & Cross (1975), Fried & Bennett (1979), and Anderson & Fried (1987). Therefore in the experiments of this study collection of cercariae was carried out at 1 hourly intervals in order to obtain cercariae before the majority of them became maximally infective and encysted within their first intermediate host. It was hoped that this frequency of

sampling would minimize the potential error involved. The method also had the additional advantage that cercarial emergence could be observed with a high degree of temporal resolution.

After collection, active cercariae were immobilised by the addition of approximately 2ml of 10% Formalin and were then counted exhaustively by removal under a stereo microscope, using an extruded Pasteur pipette, and their numbers recorded.

### Experiment 1

**L : D 12 : 12 (12 hours light : 12 hours dark)**

This experiment was carried out with the intention of discovering, in a general way, when cercariae of E. recurvatum emerge from L. peregra first intermediate hosts under conditions of equal periods of alternating light and darkness. Six specimens of L. peregra (in the shell length range 9.5-19.0 mm) were exposed in this experiment to an alternating light-dark regime of 12 hours light (from 08.00 to 20.00 h) to 12 hours dark (20.00 to 08.00h). Determination of the numbers of cercariae emerging per snail were made at 1 hourly intervals on days 1, 3, 5, and 7 of the experiment. Collections of cercariae began at 08.00 in the morning and continued until 08.00 the following day. Collections of cercariae were also made throughout the twelve hour dark period prior to the beginning of day 1. at 1 hourly intervals, beginning at 20.00h.



At the end of the experiment the blotted wet weight of each snail was taken using an electronic microbalance. The shell length (from tip of spire to lip of aperture) was measured using a set of adjustable calipers (CAMLAB, Cambridge U.K.). The digestive gland of each snail was dissected in distilled water and the number of mature daughter (cercaria-producing) rediae that it contained was counted and recorded.

## Experiment 2

### L : D 12 : 12 Regime reversal

In this experiment 5 L. peregra (in the size range 15.0-17.5 mm) naturally infected with E. recurvatum were exposed to a L:D 12:12 regime for three days after which the light-dark cycle was reversed and continued in reverse for a further 5 days. Cercarial monitoring was carried out every hour on days 1, 3, 4, 5, 7 and until 20.00h on day 8 of the experiment. The experiment was designed to determine whether reversal of photoperiod regime would produce a corresponding reversal in the cercarial emergence pattern.

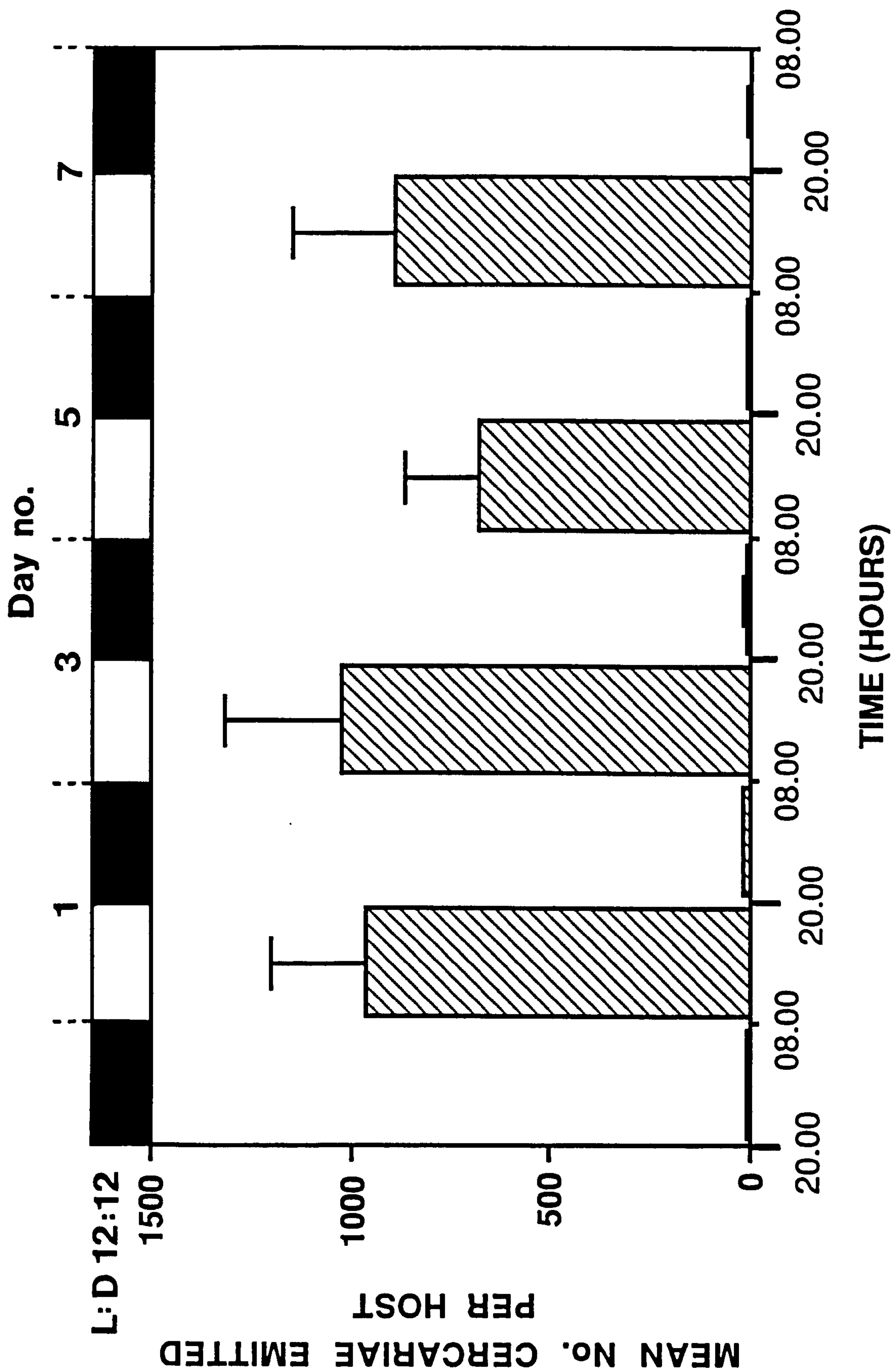
## 5.1.3 RESULTS

### Experiment 1 12hours Light : 12 hours Dark (L: D 12 :12)

The results of this experiment clearly show that E. recurvatum cercariae from L. peregra emerged almost exclusively in the light under the conditions of the experiment, (see Fig 5.1). Emergence was markedly photoperiodic. Over the 4 days of cercarial emergence monitoring 99.1% of the total number of cercariae

FIG 5.1 The mean number of Echinoparyphium recurvatum cercariae emitted per snail in each 12 hour period of Experiment 1 under photoperiod conditions of L:D 12:12.

(Vertical bars indicate standard errors of the means)

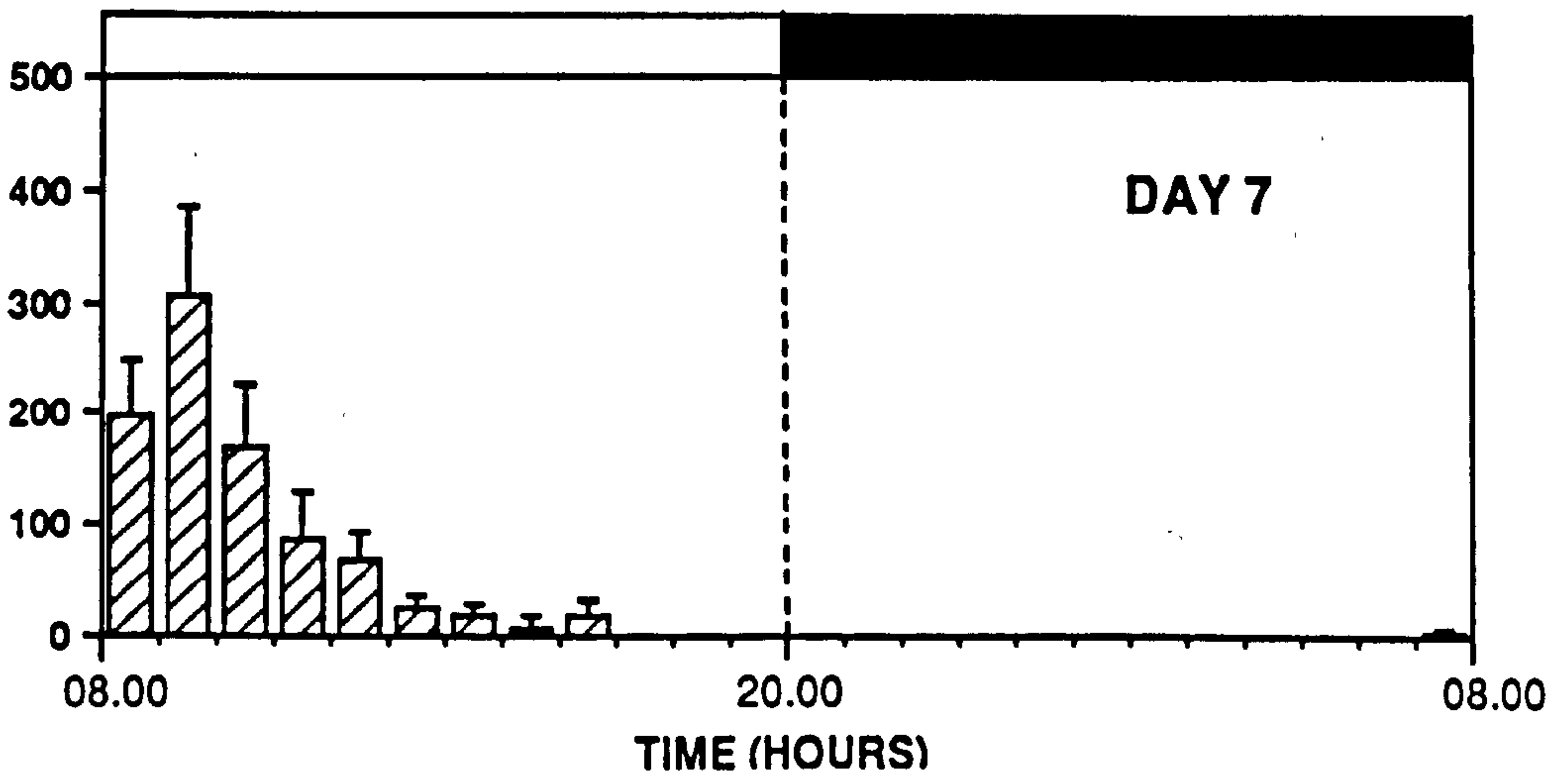
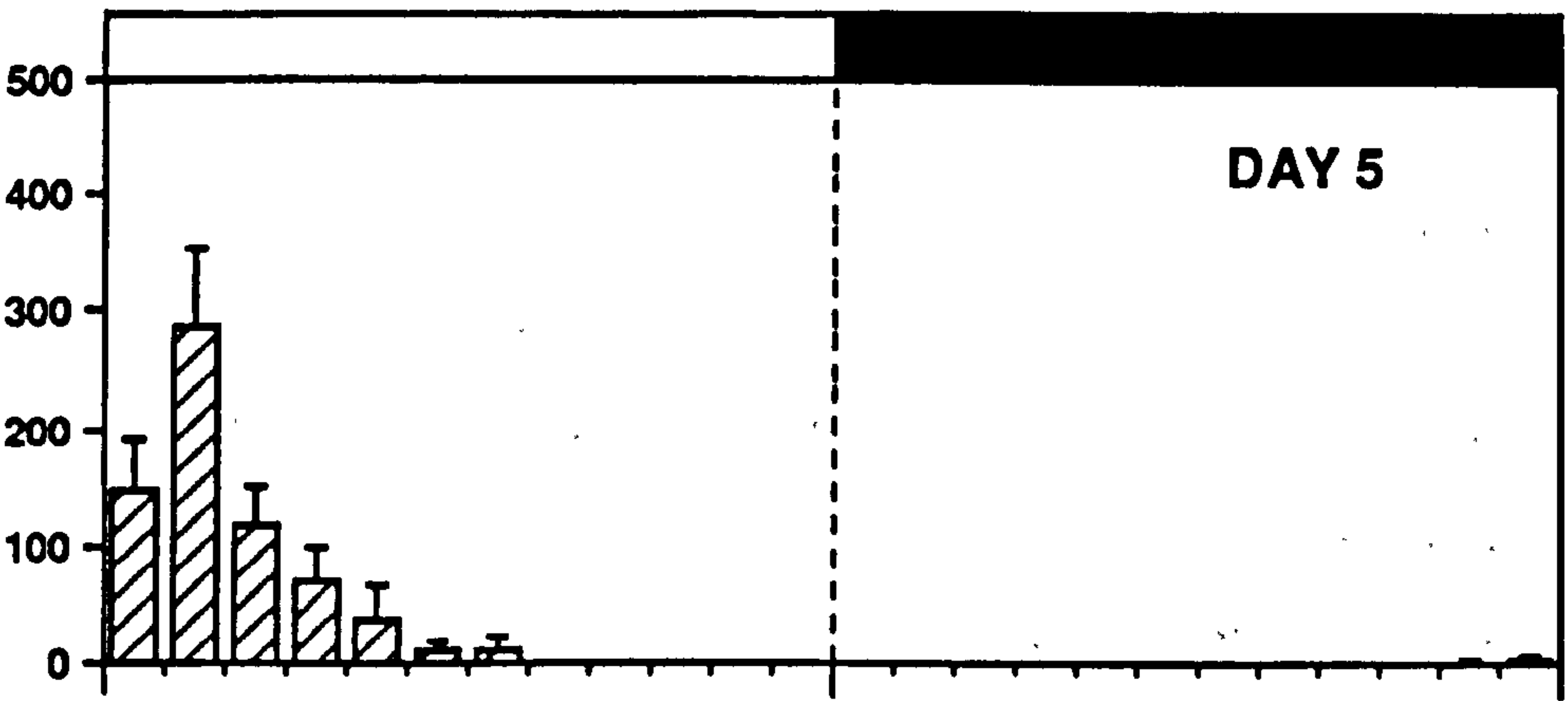
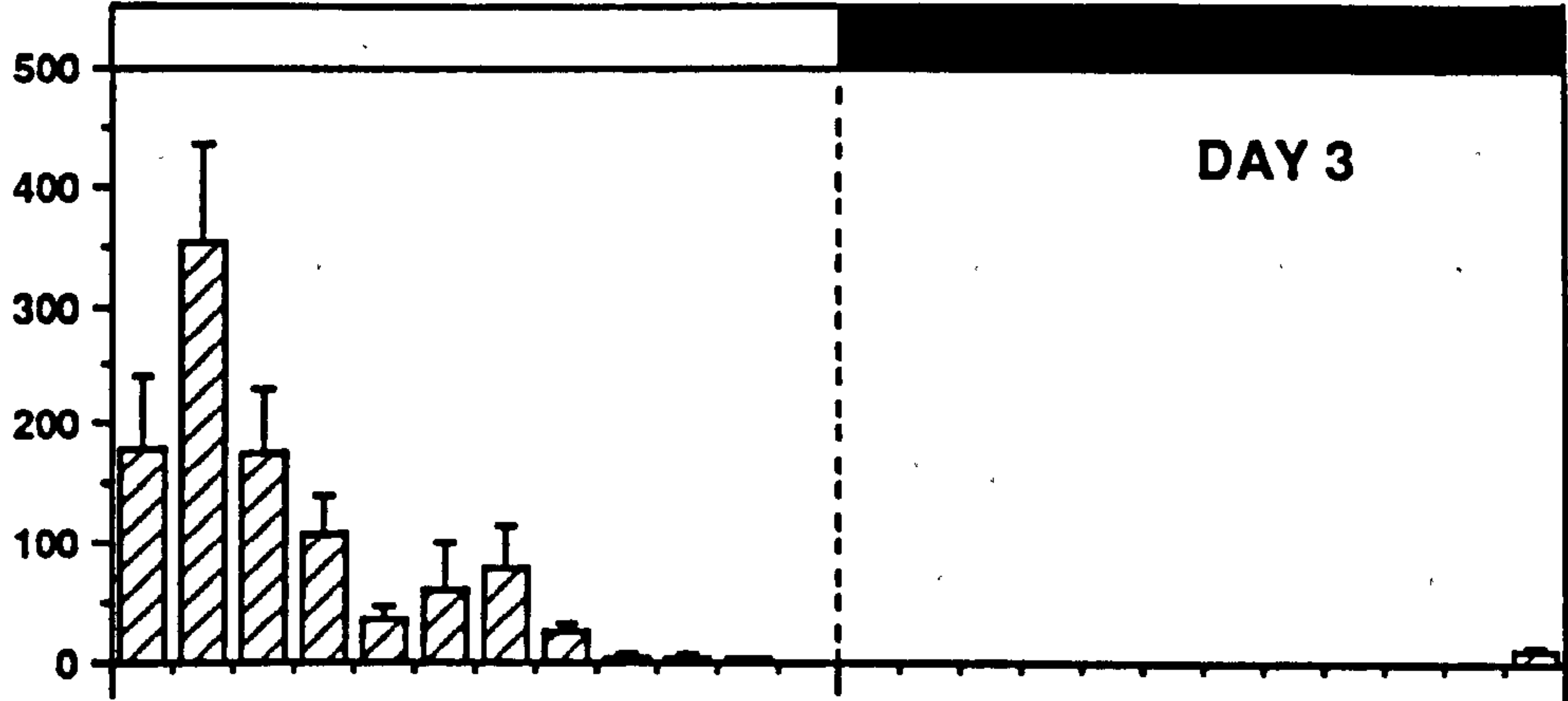
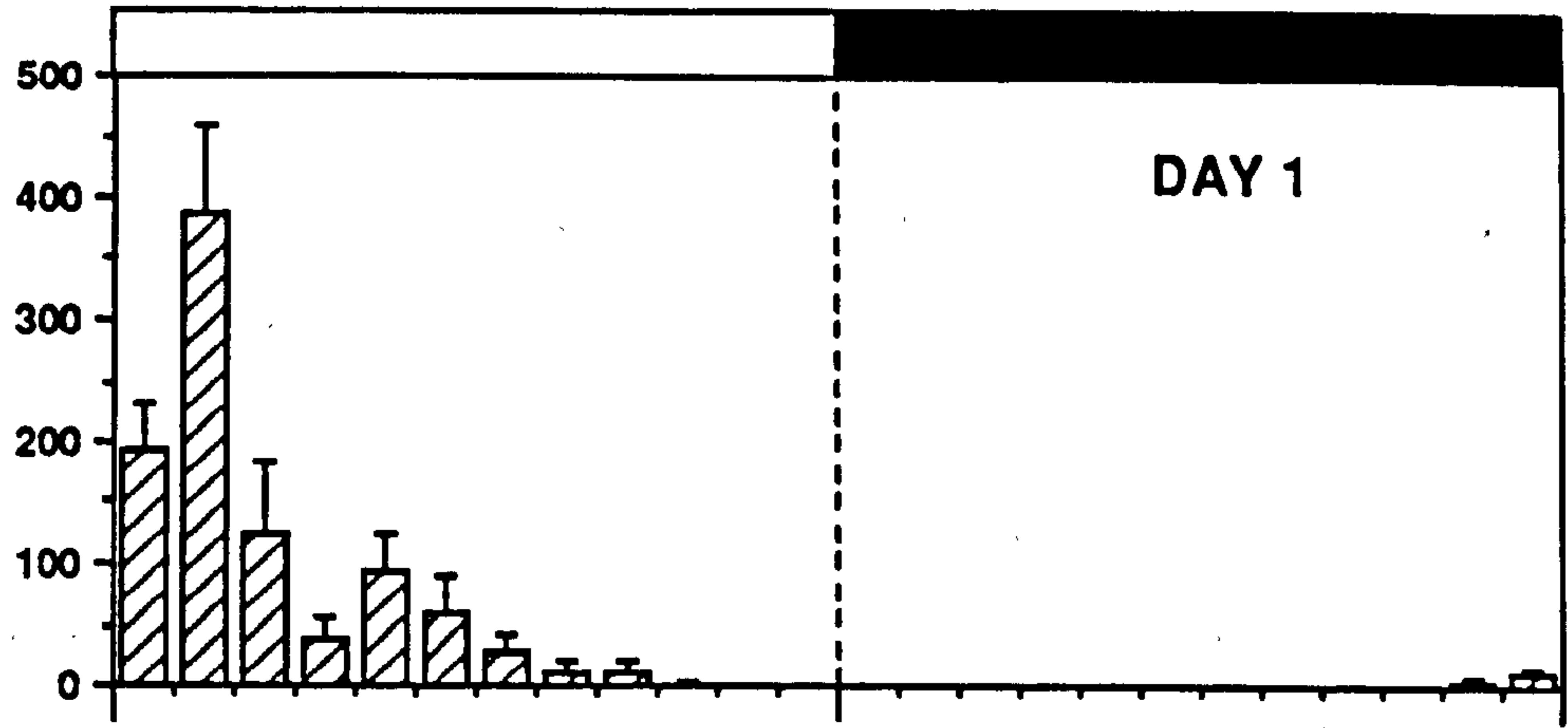




**FIG 5.2** The mean number of Echinoparyphium recurvatum cercariae emitted per snail each hour on days 1, 3, 5 and 7 of Experiment 1

(Vertical bars indicate standard errors of the means)

MEAN NUMBER OF CERCARIAE EMITTED PER HOST



emitted were emitted during periods of light. Only 0.9% were emitted during dark periods.

Examination of the numbers of cercariae emerging each hour from L. peregra over the 4 days of monitoring revealed that mean peak emergence occurred early in the light period, (see Fig 5.2). In each case mean peak cercarial emergence occurred in the second hour after the onset of experimental light. Interestingly the small amount of cercarial emergence that occurred from during dark periods was, on all 4 days, concentrated into the 1-2 hour period immediately prior to the beginning of light. This is possibly indicative of a "pre-empting" of the on-coming light period, and may suggest the existence of an endogenous component in the emergence rhythm.

During the 4 day monitoring period of this experiment the mean daily cercarial output of the six L. peregra snails varied between 1033.7 (+/- 317.8) and 687.5 (+/- 194.1). The largest number of cercariae emitted by one L. peregra in a single day (24 hour light-dark period) was 2,321 and the smallest was 56. No obvious temporal trend in mean daily cercarial output of the experimental snail population was observed. Calculation of the correlation coefficient ( $r$ ) indicated that no significant correlation existed between mean daily cercarial output per snail and time over the period of the experiment,  $P (r=0.38) > 0.05$   $df=4$ . Linear regression analysis of mean daily cercarial output ( $y$ ) against time ( $x$ ) confirmed the finding, the slope of the best fit regression line not differing significantly from zero.



The infected group of L. peregra monitored in this study was composed of individuals of a wide size (shell length and wet weight) range. There was found to be a significantly positive relationship between both shell length and wet weight and the mean numbers of cercariae produced per snail per day,  $P(r=0.95) < 0.05$   $df=4$  and  $P(r=0.94) < 0.05$   $df=4$ , respectively. The relationship in each case was found to be linear, (see Figs. 5.3 and 5.4). The number of cercariae-producing daughter rediae present in the digestive gland was found to increase with increase in host shell length and also with increase in host wet weight. The smallest snail examined 9.5mm (0.267g) was found to contain the smallest number of rediae, 38, while the largest snail examined 19mm (0.721g) was infected with the largest number of daughter rediae, 183. Calculation of the correlation coefficient ( $r$ ) using redial counts transformed as  $\text{Log}_{10}(x)$  (as recommended by Elliot 1983) revealed a significant degree of positive correlation between snail shell length and redial infection load  $P(r=0.95) < 0.05$   $df=4$ , and also between snail wet weight and redial infection load,  $P(r=0.86) < 0.05$   $df=4$ . The relationship in each case was found to be linear see Figs 5.5 and 5.6. This suggests that the host-size-related trends in mean cercarial output may be directly explained by the presence of larger numbers of daughter rediae in the digestive glands of larger snails.

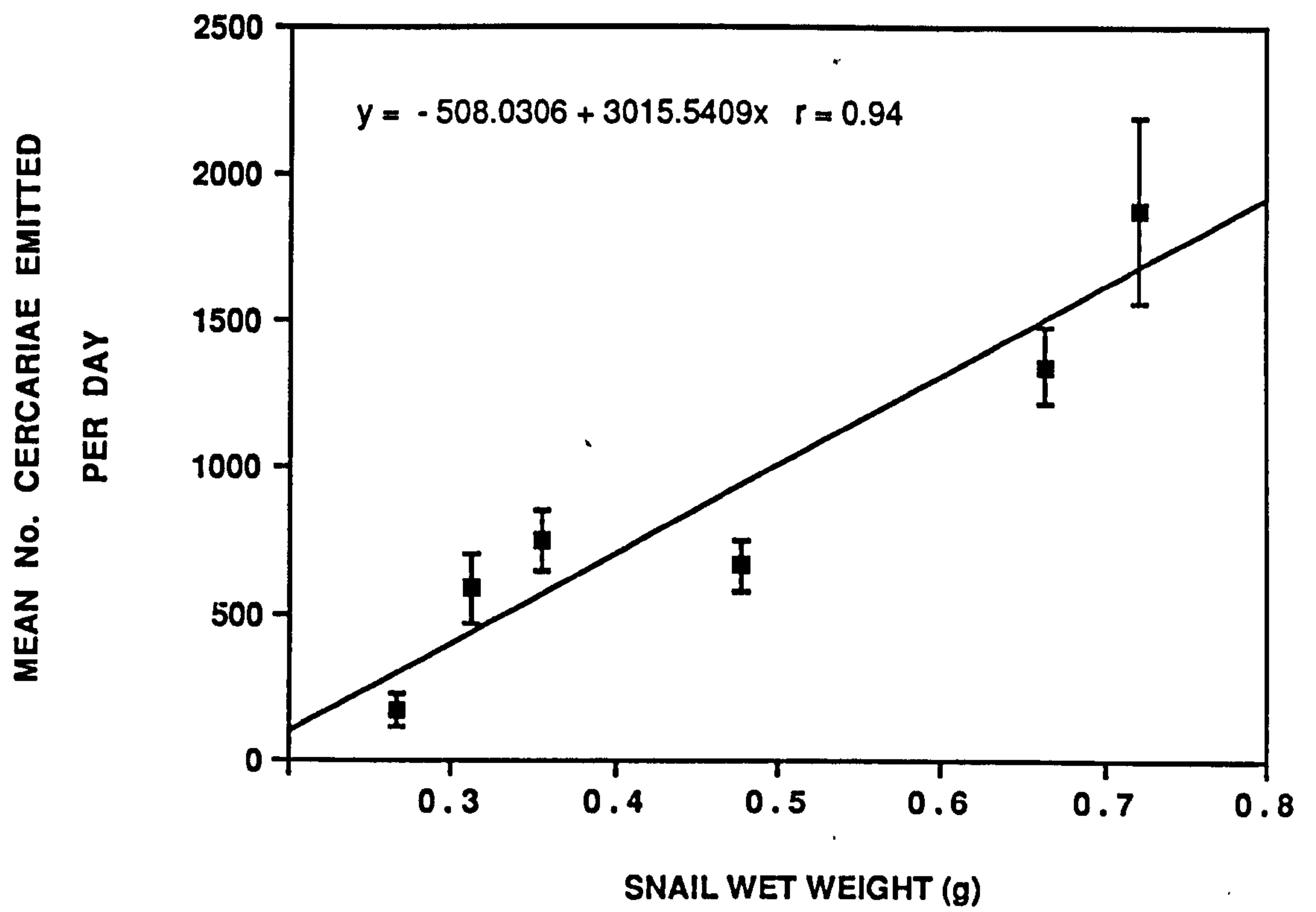
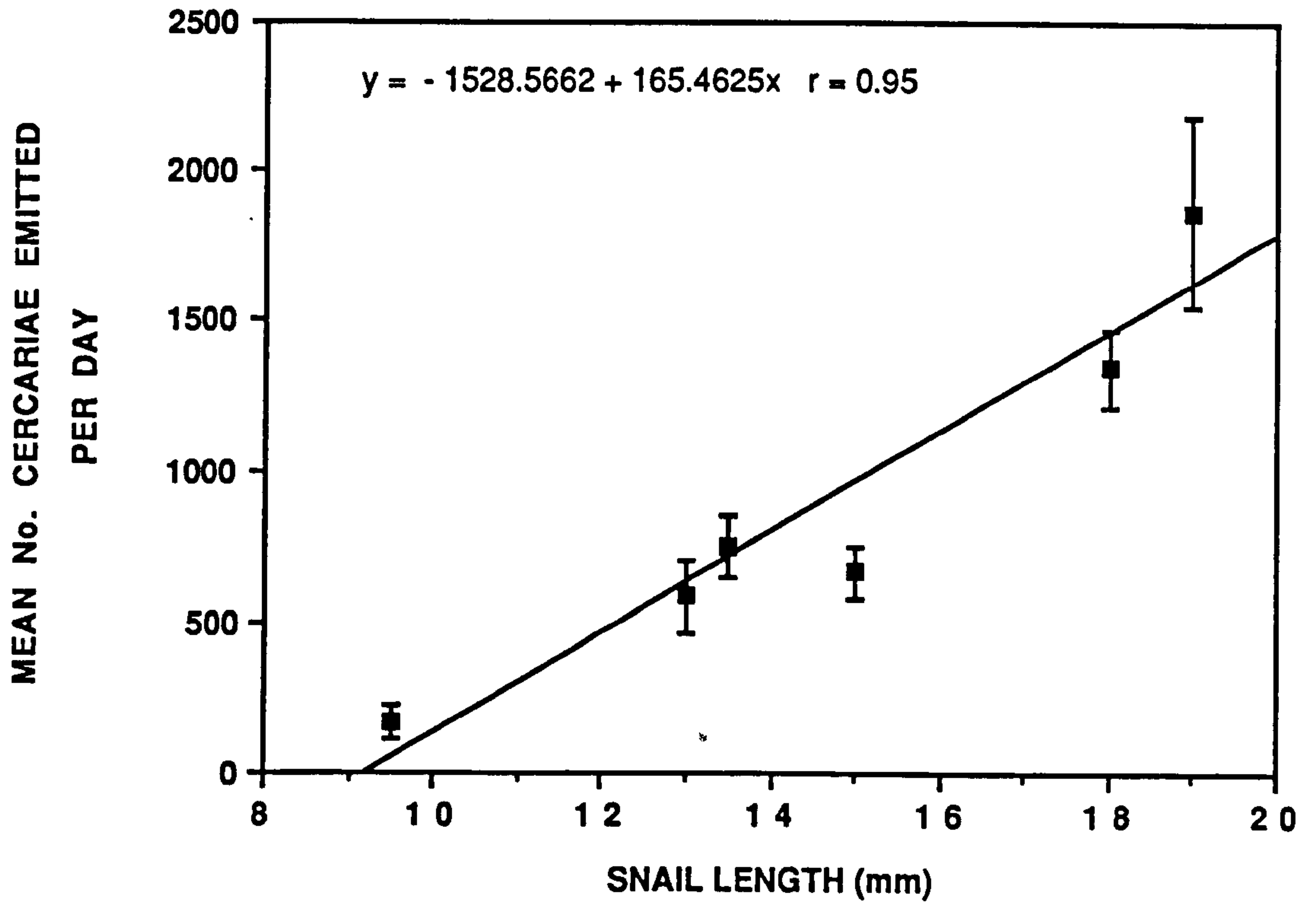
For each L. peregra snail the cercarial emission data obtained over the four days of monitoring and the redial recovery data were combined to provide an estimate of the mean number of cercariae emitted per redia per 24 hours; an estimate of mean cercarial production per daughter redia. This parameter of redial

**FIG 5.3** The relationship between host snail shell length and the mean number of Echinoparyphium recurvatum cercariae emitted per day during Experiment 1.

(Vertical bars indicate standard errors of the means and the solid line indicates the best-fit linear function of the form described in the equation)

**FIG 5.4** The relationship between host snail wet weight and the mean number of Echinoparyphium recurvatum cercariae emitted per day during Experiment 1.

(Vertical bars indicate standard errors of the means and the solid line indicates the best-fit linear function of the form described in the equation)





**FIG 5.5** The relationship between host snail shell length and the number of Echinoparyphium recurvatum daughter rediae (Log transformed) in the host digestive gland for the six snails examined in Experiment 1.

(The solid line indicates the best-fit linear function of the form described in the equation)

**FIG 5.6** The relationship between host snail wet weight and the number of Echinoparyphium recurvatum daughter rediae (Log transformed) in the host digestive gland for the six snails examined in Experiment 1.

(The solid line indicates the best-fit linear function of the form described in the equation).

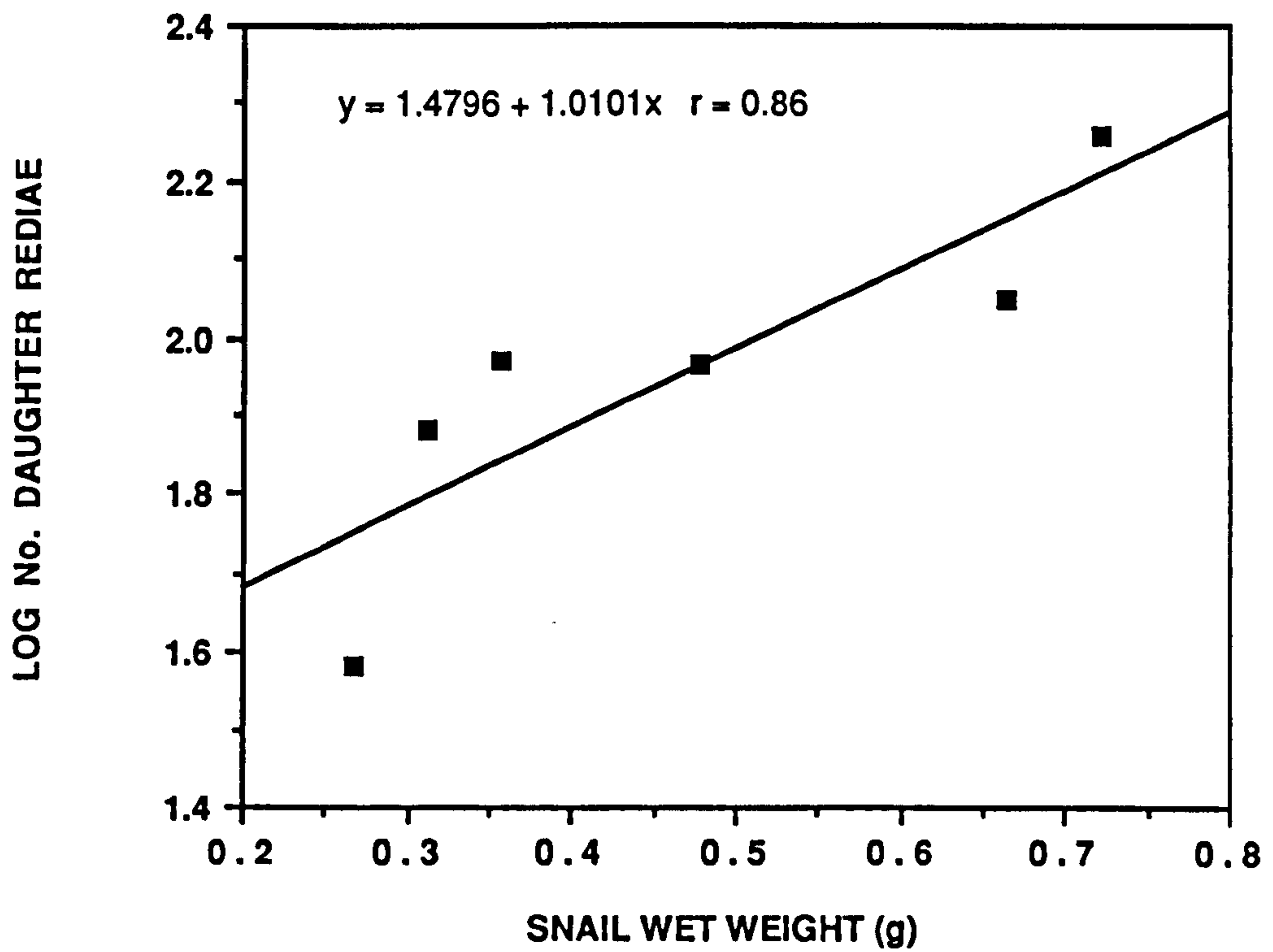
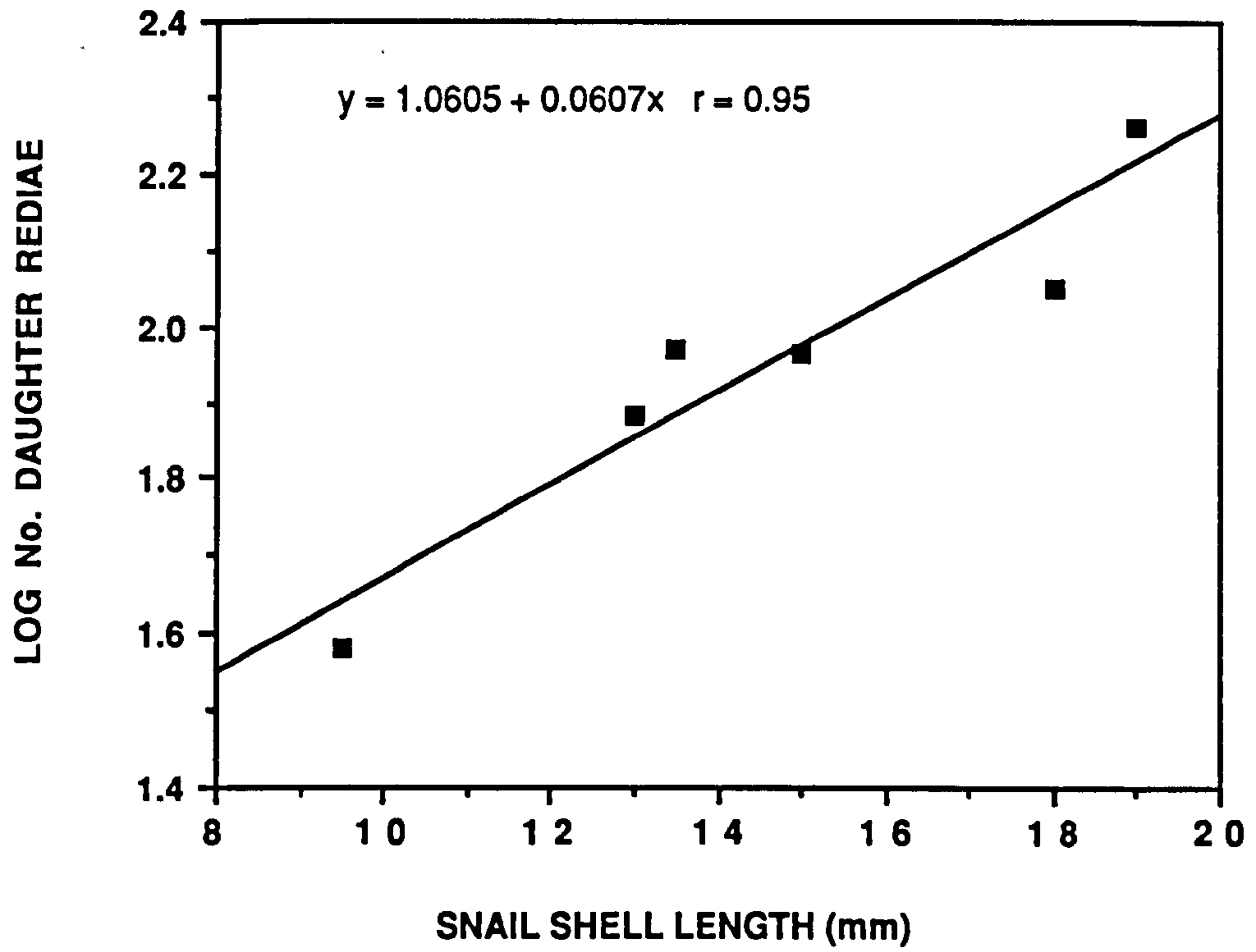
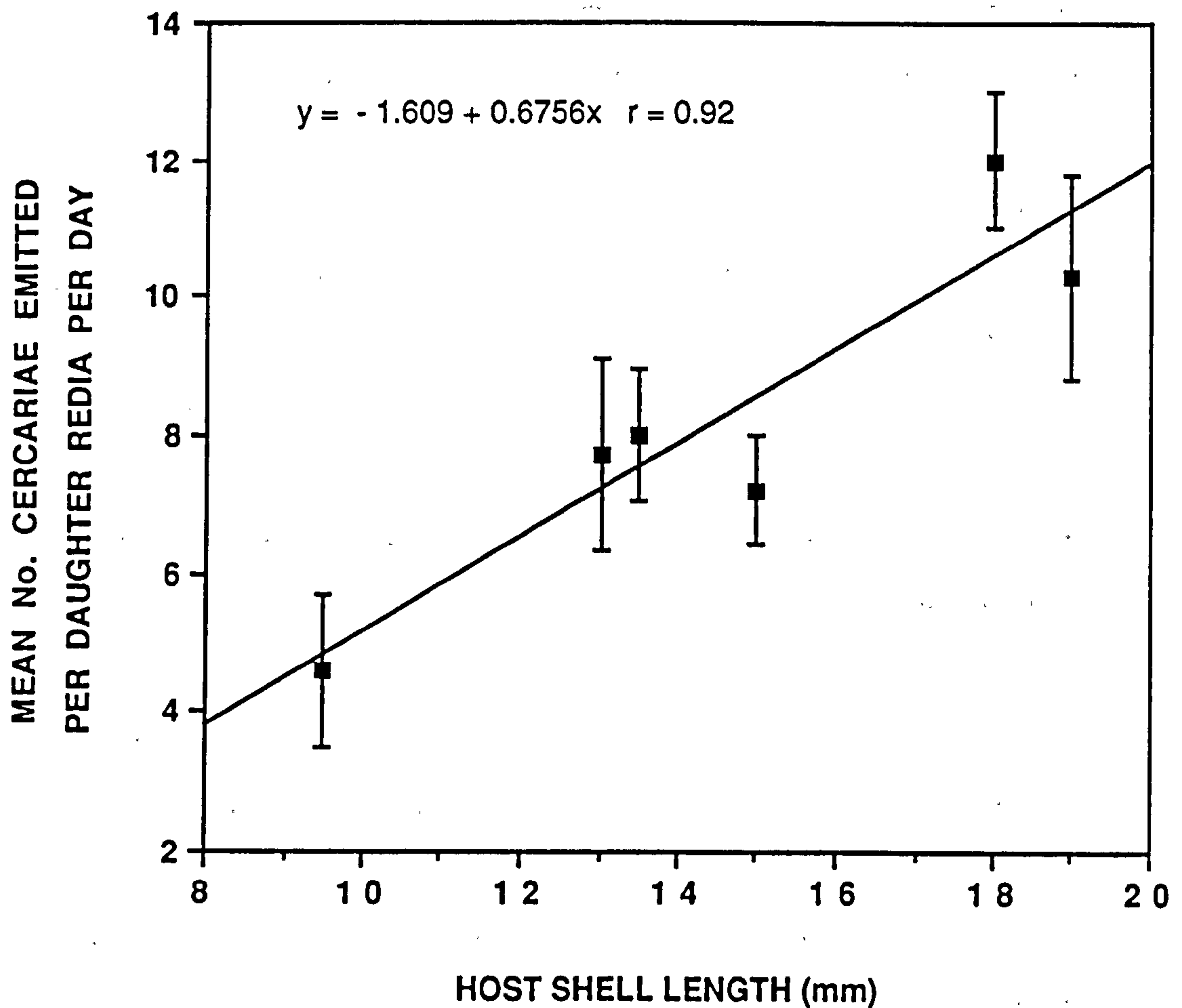


FIG 5.7 The relationship between the mean estimated number of Echinoparyphium recurvatum cercariae emitted per daughter redia per day and host snail shell length for the six snails examined in Experiment 1.

(Vertical bars indicate the standard errors of the means and the solid line represents the best-fit linear function of the form described in the equation)





productivity was also found to show a significant positive relationship with host shell length  $P(r=0.092) < 0.05$   $df=4$ . The relationship was found to be adequately described by a linear function see Fig 5.7. This relationship suggests that daughter rediae in larger L. peregra produce a larger mean number of cercariae per daughter redia than those in smaller specimens. Therefore, it seems that not only are daughter rediae more numerous in larger L. peregra, but they are also more productive in terms of cercarial output.

#### Experiment 2 (L : D 12 : 12) Regime Reversal

Reversal of the illumination regime pattern in this experiment resulted in all L. peregra snails exhibiting what in most respects constitutes a corresponding reversal of the pattern of cercarial emission as is illustrated in Fig 5.8. However, the consequence of regime reversal resulted in a complex pattern of cercarial output differing in more than one respect from the previous temporal output pattern.

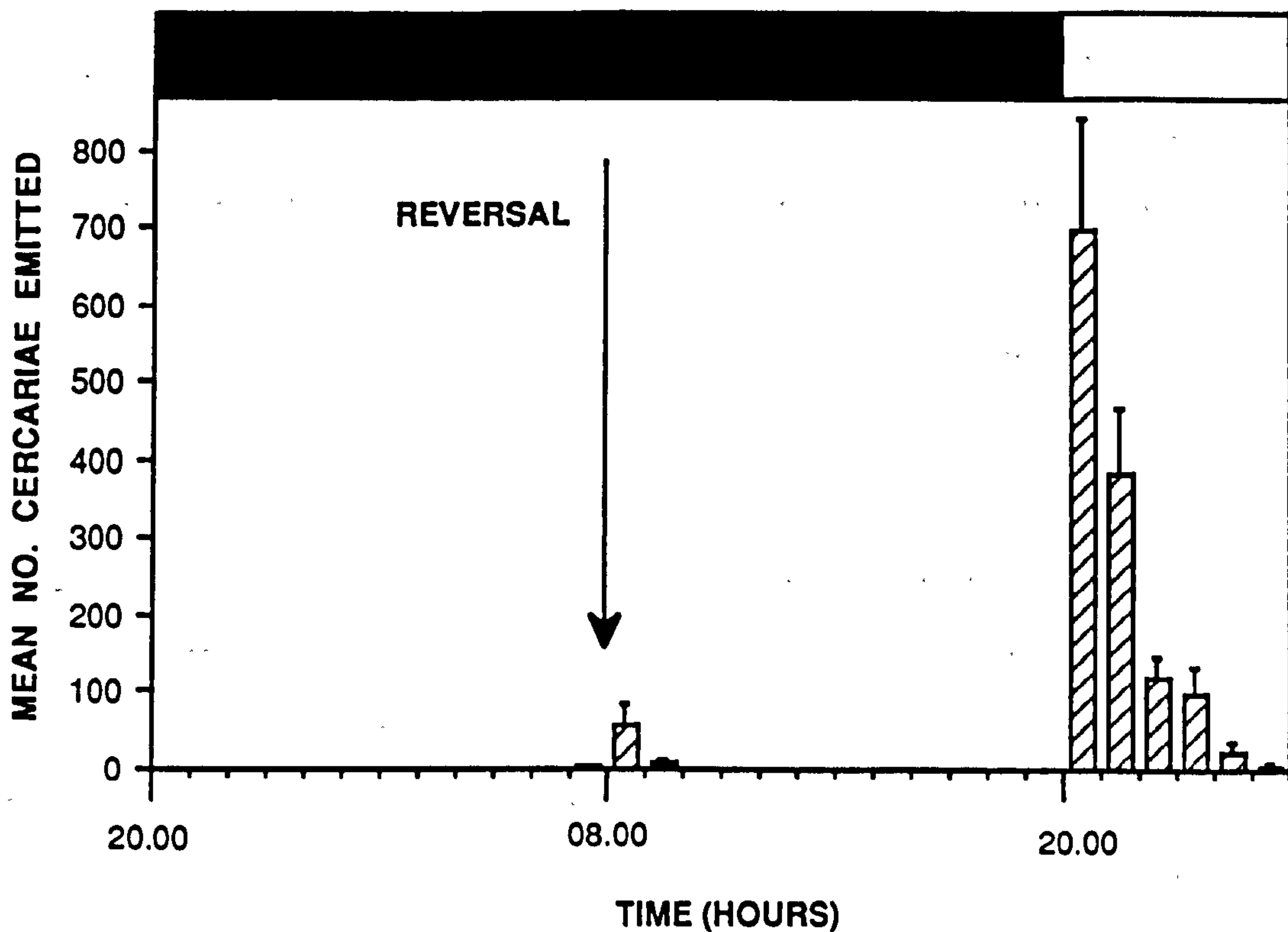
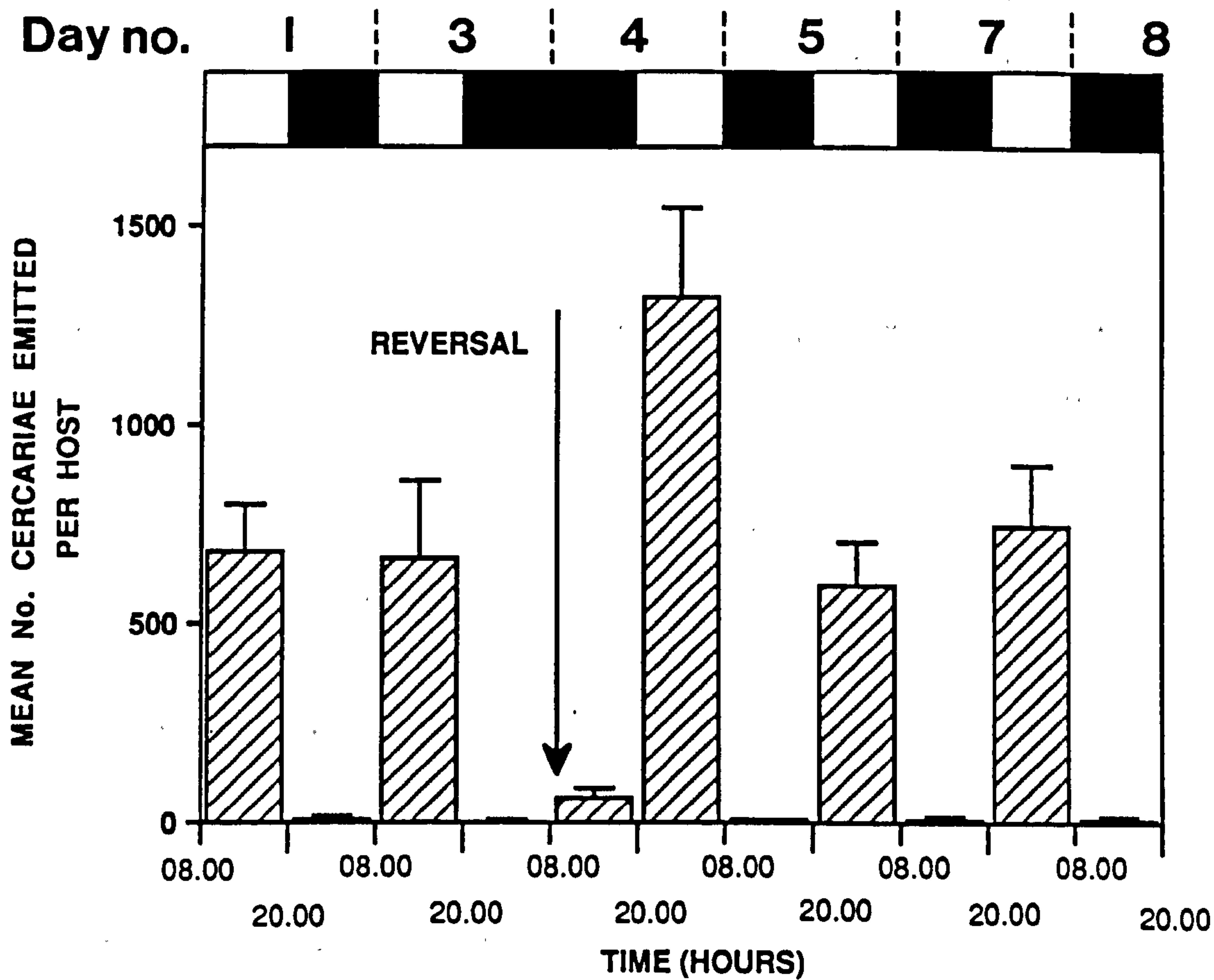
The inserted period of dark had a severely inhibitory effect on cercarial emergence. Immediately after the time when reversal itself was made a minor peak in cercarial output occurred. This coincided with the "expected" onset of light, see Figs 5.9, and is something which suggests the existence of an endogenous cercarial emergence rhythm, independent of an alternating light-dark regime. This minor emergence peak was not sustained. A much larger emergence peak, however, coincided with the initiation of the 12 hour retarded light period. The number of cercariae emitted in this light period was approximately double that of the

**FIG 5.8** The influence of photoperiod regime reversal (L:D 12:12 to D:L 12:12) on the mean number of Echinoparyphium recurvatum cercariae emitted per snail in each 12 hour monitoring period of Experiment 2.

(Vertical bars indicate standard errors of the means)

**FIG 5.9** The mean number of Echinoparyphium recurvatum cercariae emitted per snail each hour during photoperiod regime reversal in Experiment 2.

(Vertical bars indicate standard errors of the means)





pre-reversal peaks, see Fig 5.8. This finding suggests that although dark has a significantly inhibitory influence on cercarial emergence it does not inhibit the production of cercariae within the snail host.

Over the following 60 hours the cercarial output pattern from the group of snails seemed to be entirely analogous with the pre-reversal pattern, the majority of cercariae emerging in light. This indicated that the cercarial emergence pattern had adjusted completely to the new reversed regime, (see Fig 5.8).

#### 5.1.4 DISCUSSION

The results of this study clearly show that emergence of E. recurvatum cercariae from L. peregra is markedly photoperiodic. Emergence occurs almost exclusively during periods of light. Similar emergence of echinostome cercariae from first intermediate hosts have been reported as early as Cort (1922) who suggested that the phenomenon was due to positive reaction of the cercariae to light. Rees (1931) demonstrated a daylight peak in emergence of the echinostome Cercaria "Z" and Christensen, Fransden & Roushdy (1980) have more recently reported emergence of Echinostoma liei cercariae during periods of illumination.

The present study has clearly shown that cercarial emergence of E. recurvatum is markedly photoperiodic. However, at present it is not possible to state unequivocally whether cercariae are responding to a directly perceived change in light intensity or whether they are responding to a stimulus in the form of a change

in the locomotory activity or physiology of the first intermediate host. In their study Anderson, Nowosielski & Croll (1976) were able to correlate locomotive activity in infected Lymnaea stagnalis with the emergence pattern of Trichobilharzia ocellata cercariae. The ambulatory activity of the snail was suggested to be stimulatory to cercarial emission. In recent years however, a considerable body of evidence produced by Theron and co-workers at Perpignan University has accrued in support of the view that the photoperiodic patterns of cercarial emergence are primarily influenced by the parasites themselves (genetically determined) with cercariae responding directly to changes in the light intensity of the external environment (Theron 1975, 1984 ; Theron & Combes 1983 ; Mouahid & Theron 1986 ; Theron & Mone' 1986). One outstanding example of evidence that cercarial emergence is likely to be stimulated by a directly perceived change in the light intensity of the external environment is provided by Theron (1975). This author discovered that the emergence of cercariae of the cathaemasid Ribeiroia marini from Biomphalaria glabrata was normally markedly nocturnal. However, he discovered that by masking the shells of infected B. glabrata snails with aluminium foil, and thereby cutting off the light reaching the cercariae through the shell of the snail, he could induce cercarial emergence of R. marini during the day.

If the photoperiodic cercarial emergence of E. recurvatum from L. peregra is a response to a photostimulus directly perceived by the cercariae then the question of a means of photoperception must be addressed. Although ultrastructural studies have not been carried out on E. recurvatum cercariae, Fournier (1984) has reported that

echinostome cercariae do in fact possess non-pigmented rhabdomeric photo-receptors which are thought to be of a type which may intervene in the control of photoperiodic processes as monitors of day (or, night) length. The mechanisms producing the photoperiodic cercarial emergence pattern in the E. recurvatum/L. peregra system observed in the present section of this chapter will undoubtedly require much further work. As such they provide an interesting channel for future research.

In the present study positive correlation was found between the size of the first intermediate host snail and the rate of cercarial output. This is a phenomenon also reported by workers such as Betterton (see Anderson, Whitfield & Mills 1977) who found a similar host size/cercarial output trend in the case of Melanoides tuberculata snails sampled from a natural Malaysian population parasitized by Transversotrema patialense. In the present study this relationship can be directly explained by the positive relationship that was found to exist between host snail size and the number of daughter (cercaria-producing) rediae present in the digestive gland. The larger L. peregra contained larger numbers of daughter rediae and hence emitted larger numbers of cercariae per unit time than smaller snails containing smaller numbers of rediae.

Markedly positive relationships between redial infection load and host snail size have previously been reported for Fasciola hepatica in Lymnaea truncatula by Smith (1984) and the echinostome Paryphostomum segregatum in Biomphalaria glabrata by Lim & Lie (1968). The latter authors have shown the relationship to be



independent of the size of the initial miracidial infection dose. The relationship is thought to be primarily determined by the fact that the carrying capacity of the parasite's micro-environment is mainly determined by the size of the snail's digestive gland. Larger snails are therefore capable of supporting larger populations of rediae produced by asexual multiplication. As a consequence the capacity exists to produce larger numbers of cercariae.

In the present study a significantly positive relationship was also found between the mean cercarial production rate per daughter redia and the size (shell length) of the host snail. This suggests that not only are the rediae more numerous in larger snails but that they are also more productive in terms of cercarial output. This trend, to the author's knowledge, has not yet been documented for any other digenean-first intermediate host system. At present it is possible only to speculate on the probable mechanism responsible for producing this relationship. It is possible that in addition to daughter rediae being more numerous in larger snail hosts they may also be of larger size than those found in smaller hosts. In other words, expansion of the population of daughter rediae in larger snails may have proceeded on two lines ; expansion in terms of numbers of individuals and growth in terms of size of individuals. If it is assumed that larger daughter rediae have the capacity to produce larger numbers of cercariae per unit time than smaller ones, then a possible mechanism explaining the relationship under discussion is suggested. In the present study the sizes of daughter rediae within

within each snail were not measured. Therefore further studies will be required to test the relevance of the proposed mechanism.

## 5.2 The photoperiodic cercarial emergence patterns of Echinoparyphium recurvatum and Plagiorchis sp. from a mixed infection of Lymnaea peregra

### 5.2.1 INTRODUCTION

In the only previous study of this kind Theron & Mone' (1986) examined the photoperiodic cercarial emergence patterns of cercariae of Schistosoma mansoni and Ribeiroia marini in both single and mixed infections from the first intermediate host snail Biomphalaria glabrata. In single species infections both parasites showed marked photoperiodicity of cercarial emergence ; S. mansoni peaking during light and R. marini peaking during dark. In mixed infections of the same snail it was found that the cercariae of both species retained their marked, discrete, photoperiodic emergence patterns with no overlap in emergence occurring.

The very fact that the marked photoperiodic emergence patterns remained discrete in mixed infections tended to demonstrate the absence of interference at the level of mechanisms directly responsible for the emergence pattern of each species. However, S. mansoni and R. marini occupy different microhabitats in B. glabrata, the digestive gland in the case of the daughter sporocysts of S. mansoni and the genital gland in the case of the daughter rediae of R. marini. In their discussion Theron & Mone' (1986)

suggested that it would be interesting to study a host-parasite system involving digeneans with distinct diurnal and nocturnal emergence patterns but with more similar microhabitat niches within a single first intermediate host snail. It would be interesting to see if in this case the photoperiodic emergence patterns remained discrete or whether a significant degree of emergence overlap would occur. It would also be interesting to see whether evidence of any form of interference between the two parasites would be manifest.

A host-parasite system which provides the required combination exists in the case of Lymnaea peregra snails carrying mixed patent infections of Echinoparyphium recurvatum and the plagiorchiid Plagiorchis sp. The latter species is an unidentified plagiorchiid digenean which has been known to utilize L. peregra as first intermediate at Harting Pond for several years (Evans & Higgs 1982). The xiphidiocercariae of Plagiorchis sp. possess a characteristically shaped stylet, are morphologically identical to those of the "Plagiorchis elegans group", (see Bock 1984), and are known to utilize the aquatic larvae of the Megalopteran insect Sialis lutaria as a second intermediate host. Previous pilot studies (Evans & McCarthy unpublished) had indicated that the cercariae of Plagiorchis sp. exhibited a markedly nocturnal pattern of emergence from L. peregra.

The cercariae-producing stages of E. recurvatum and Plagiorchis sp., daughter rediae in the case of E. recurvatum and daughter sporocysts in Plagiorchis sp., utilize the same microhabitat niche - the digestive gland - in L. peregra. Both species show a marked



photoperiodic pattern of cercarial emergence with E. recurvatum emerging almost exclusively during light and Plagiorchis sp. nocturnally. In addition the cercariae of the two species have an obviously distinct morphology which enables them to be easily distinguished in the event of an overlap in emergence.

The discovery of specimens of L. peregra in early October 1987 at Harting Pond, West Sussex, carrying patent natural double infections of both E. recurvatum and Plagiorchis sp. allowed an examination of the photoperiodic cercarial emergence patterns of the two species within single host individuals. It also allowed their comparison with the emergence patterns observed in single species infections of these digeneans.

### 5.2.2. MATERIALS AND METHODS

Naturally infected specimens of L. peregra carrying both single and mixed patent infections of E. recurvatum and Plagiorchis sp. were obtained from Harting Pond in October 1987 by net sampling of the benthic mud of a relief channel draining the pond. Three doubly infected snails were obtained, these specimens measuring in shell length range 14 -15.5 mm. Six specimens of L. peregra, within the same size range 14 -15.5 mm, were obtained from the same net samples; three carrying single species infections of E. recurvatum and three carrying single species infections of Plagiorchis sp. . This enabled the analysis of cercarial emergence for three host-parasite combinations :- (1) E. recurvatum/L. peregra ; (2) Plagiorchis sp. /L. peregra and (3) E. recurvatum + Plagiorchis sp./L. peregra.

Infected snails were isolated in clear polystyrene containers each containing 15 ml of synthetic hard water medium (HMSO 1969), and were then transferred to the light-proof incubator described in the previous section of this chapter. Cercarial emergence from each snail was monitored every hour, over a period of 24 hours of a balanced L:D 12:12 photoperiod regime. Experimental light (1600 Lux) began at 08.00h and dark at 20.00h. The ambient temperature was maintained at a constant 18°C.

The snails were acclimatized to the experimental conditions of light/dark and temperature for a period of three days prior to the commencement of the 24 hour monitoring period. All snails were provided with an excess of clean boiled lettuce throughout the acclimatization and monitoring periods.

### 5.2.3 RESULTS

#### Emergence of E. recurvatum cercariae (single infection of L. peregra)

The emergence of E. recurvatum from L. peregra carrying single infections of this parasite is shown in Fig 5.10 (A). The emergence pattern was of a circadian type, having a single peak in 24 hours. Cercarial emergence began at the onset of experimental light at 08.00h. Mean peak cercarial emergence occurred in the second hour after the onset of light in the period 09.00-10.00h. The mean number of cercariae emerging from the three snails examined over the experimental period was 742 (+/- 84.5) cercariae per snail.

**Emergence of Plagiorchis sp. cercariae (single infection of L. peregra)**

The emergence of Plagiorchis sp. cercariae from L. peregra carrying single infections of this parasite is shown in Fig 5.10 (B) The emergence pattern was also found to be of a circadian type with a single clearly defined emergence peak in the 24 hour monitoring period. Cercarial emergence began with the onset of experimental dark at 20.00h. Mean peak cercarial emergence occurred in the second hour after the onset of dark in the period 21.00-22.00h. The mean number of cercariae emerging from the three snails examined over the experimental period was 1081.7 (+/-74.7) cercariae per snail.

**Emergence of E. recurvatum and Plagiorchis sp. cercariae (double infection of L. peregra)**

In double infections of the same snail individuals both E. recurvatum and Plagiorchis sp. cercariae were found to retain their markedly discrete respective light and dark patterns of emergence, see Fig 5.10 (C). E. recurvatum cercariae emerged exclusively during light and Plagiorchis sp. cercariae emerged exclusively during dark, with no overlap in emergence occurring.

Emergence of E. recurvatum cercariae began at the onset of light, but mean peak cercarial emergence did not occur until the third hour after the onset of light in the period 10.00-11.00h. The mean number of E. recurvatum cercariae emitted per snail over the 24 hour period was 575.3 (+/-43.4).



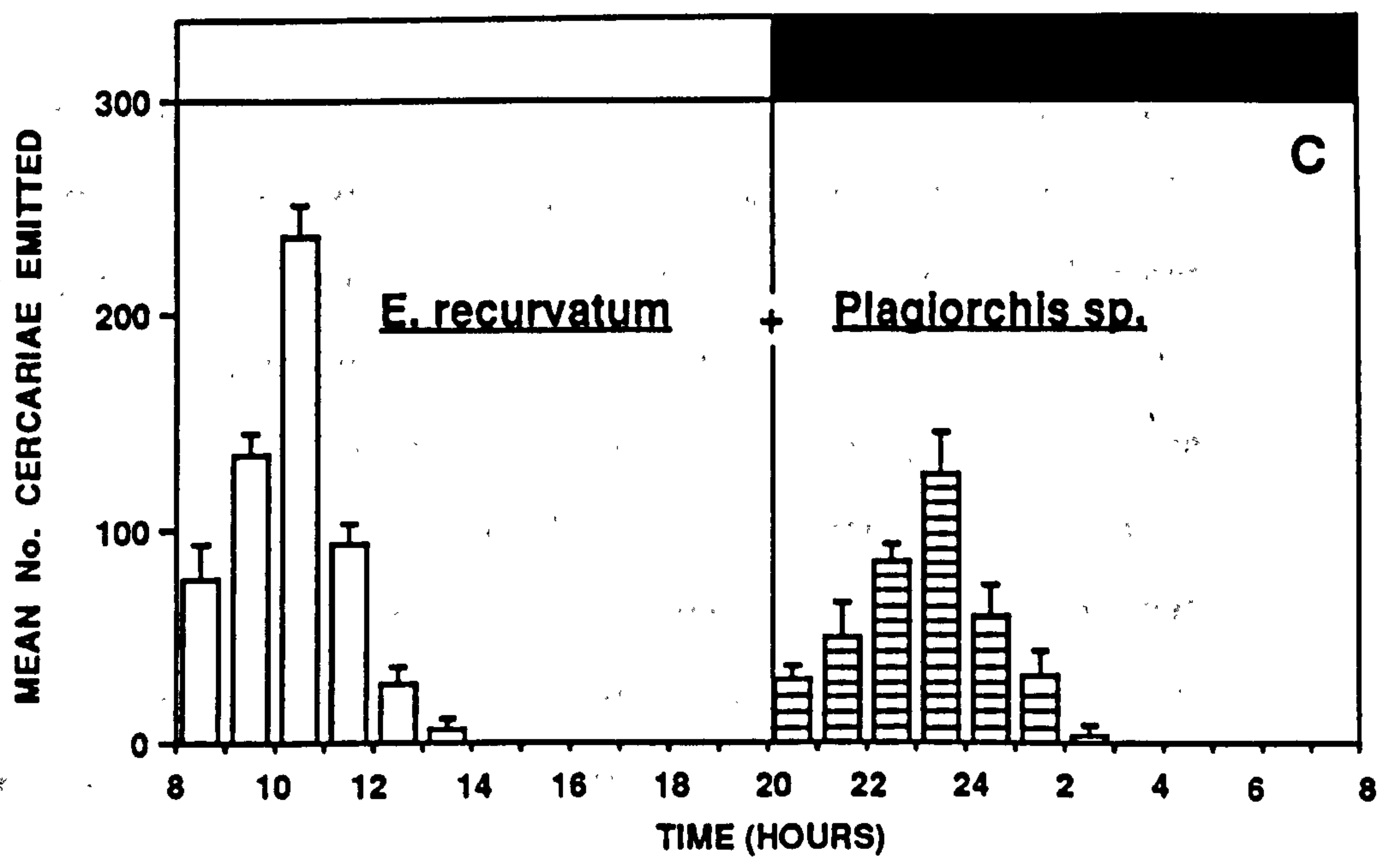
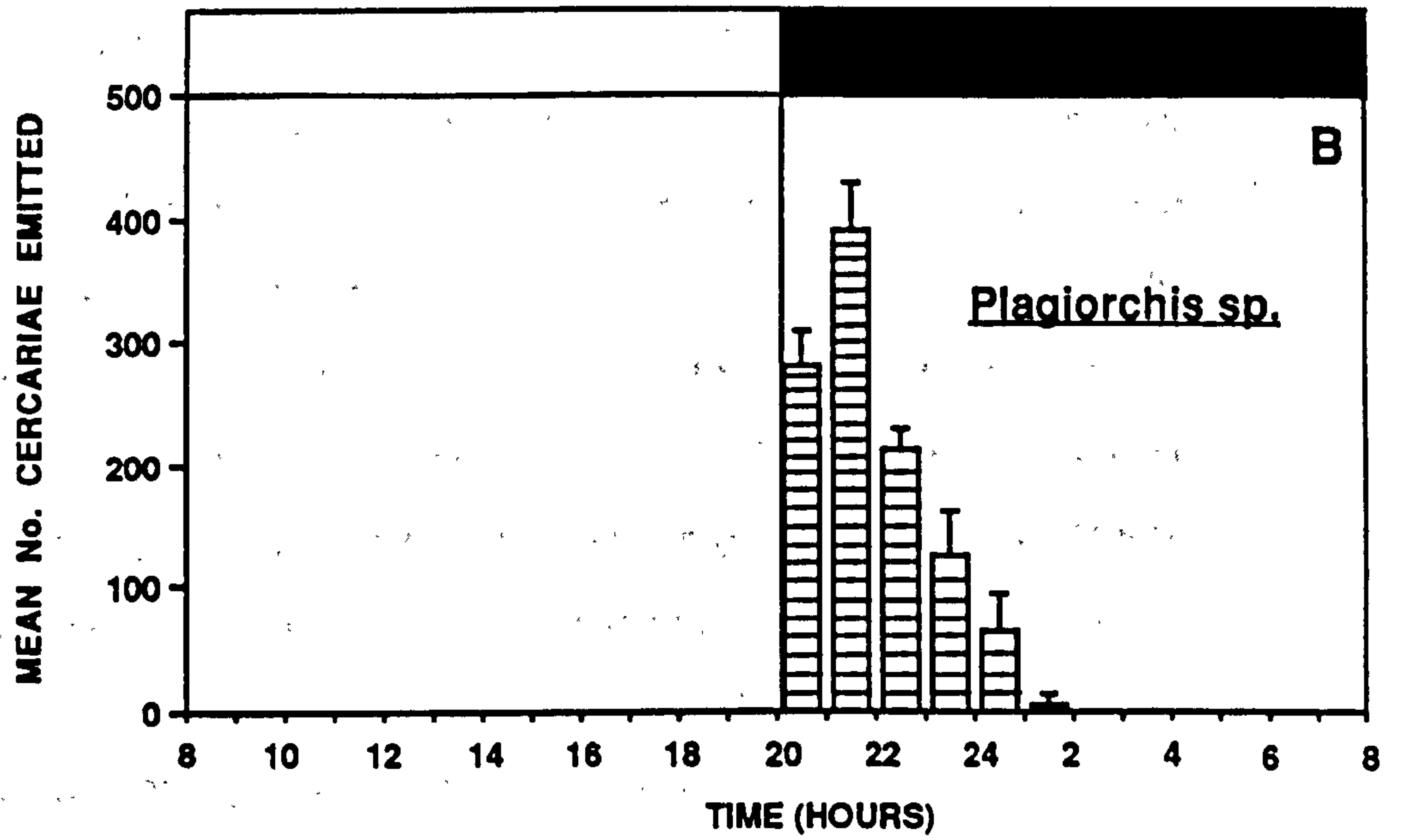
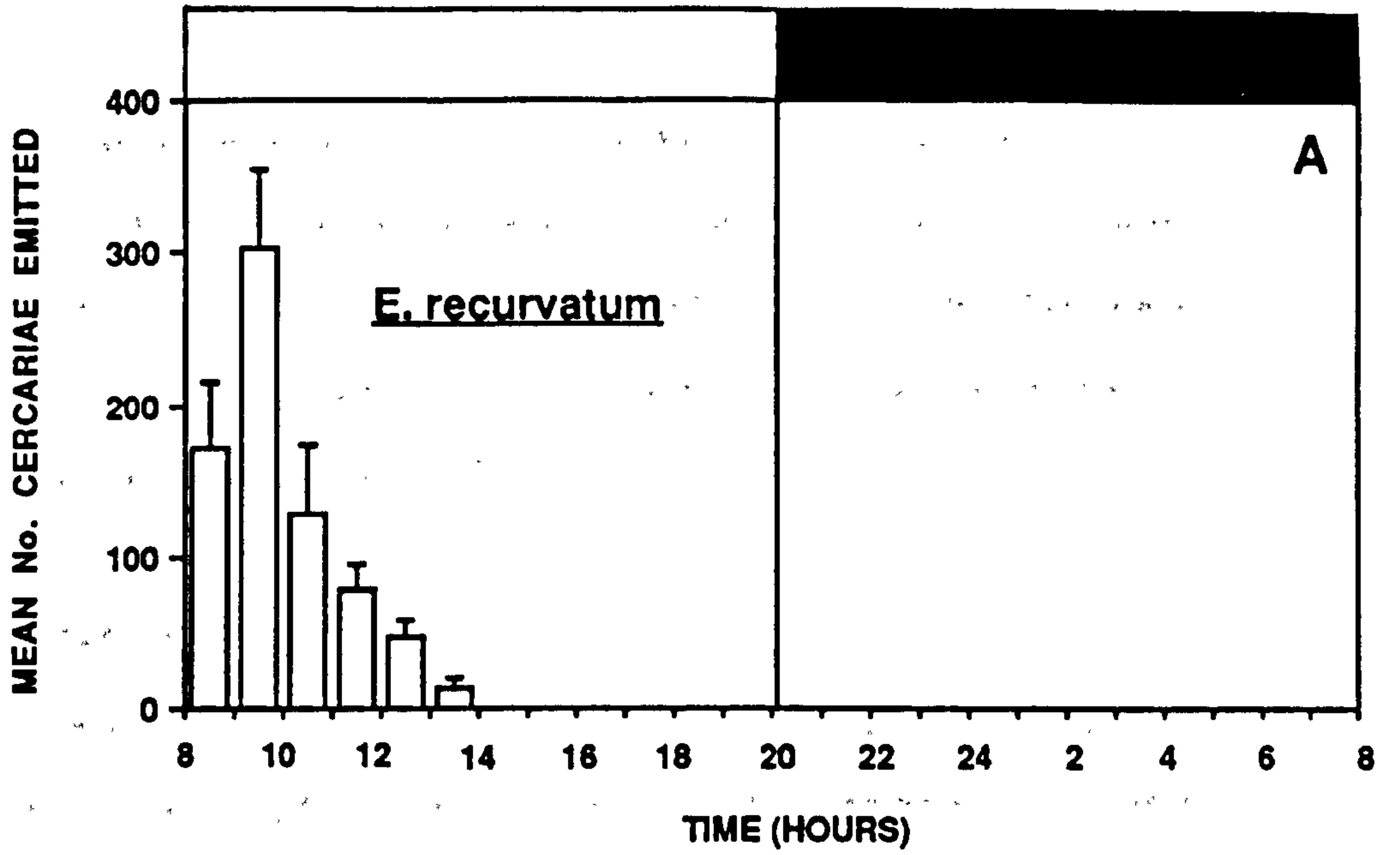
FIG 5.10 The mean number of cercariae emitted per host snail each hour over the 24 hour period of L:D 12:12 from each group of three Lymnaea peregra infected with ;

(A) Echinoparyphium recurvatum single species infection

(B) Plagiorchis sp. single species infection

(C) Echinoparyphium recurvatum + Plagiorchis sp. mixed infection.

(Vertical bars indicate standard errors of the means)



Emergence of Plagiorchis sp. cercariae began at the onset of light, but mean peak cercarial emergence did not occur until the fourth hour after the onset of dark in the period 23.00-24.00h. The mean number of Plagiorchis sp. cercariae emitted per snail over the 24 hour period was 381.6 (+/-60.5).

#### 5.2.4 DISCUSSION

In the present study in single infections of L. peregra, E. recurvatum exhibited an exclusively diurnal emergence pattern and the cercariae of Plagiorchis sp. exhibited an exclusively nocturnal pattern of emergence. For E. recurvatum this finding was the same as that made in the previous section of this Chapter. The markedly nocturnal pattern of cercarial emergence exhibited by cercariae of Plagiorchis sp. is similar to that observed for the cercariae of a variety of species of the genus Plagiorchis by several authors, eg. Plagiorchis vespertilionis parorchis (Macy 1960), Plagiorchis micracanthos (Wagenbach & Alldredge 1974), Plagiorchis neomidis (Theron 1976), and Plagiorchis noblei (Blankespoor 1977 ; Webber, Rau & Lewis 1986).

The findings of this study clearly indicate that in mixed infections of the first intermediate host L. peregra the cercariae of E. recurvatum and Plagiorchis sp. retain the markedly distinct photoperiodic patterns of emergence that they exhibit in single species infections. In mixed infections E. recurvatum cercariae began to emerge at the onset of light and emerged exclusively in light. Plagiorchis sp. cercariae began to emerge at the onset of dark and emerged exclusively in the dark. No overlap in cercarial emergence of the two species occurred. This finding is similar to



that made by Theron & Mone' (1986) who discovered that in mixed infections of the snail host B. glabrata, cercariae of S. mansoni and R. marini retained distinctly their marked respective light and dark emergence patterns without any observed overlap.

The very fact that in the present study, and also in that of Theron & Mone' (1986), markedly separate diurnal and nocturnal cercarial emergence patterns were retained by the species involved, in mixed infections, without any overlap in emergence tends to demonstrate the absence of interference at the level of the mechanisms directly responsible for stimulating cercarial emergence in each case. This is an important conclusion, one also made by Theron & Mone' (1986), as it provides us with a valuable insight to understanding what stimulates cercarial emergence. Anderson et al (1976) suggested that it was possibly the locomotory behaviour of the snail host that was primarily responsible for producing cercarial emergence of Trichobilharzia ocellata from the snail host Lymnaea stagnalis; the cercariae being pushed to suitable exit points by muscular squeezing of cercariae along haemolymph channels. It would be difficult to explain the observed cercarial emergence patterns of E. recurvatum and Plagiorchis sp. in mixed infections as being a simple product of a diel activity pattern of L. peregra. If this were the case the locomotion of the snail could reasonably be expected to stimulate an emergence of cercariae of both species at the same time. We could therefore expect that at least some degree, if not a considerable degree, of overlap in cercariae of both species emerging from the snail host would occur. The results of the present study show that this does not happen. At no time

throughout the experiment did a snail carrying a mixed infection of E. recurvatum and Plagiorchis sp. emit cercariae of both species simultaneously. It therefore seems probable that, as Theron & Mone' (1986) implied for R. marini and S. mansoni, that E. recurvatum and Plagiorchis sp. cercariae are independently responding to photo-related emergence stimuli.

Although there was no overlap in the emergence patterns of E. recurvatum and Plagiorchis sp. in the present study there was, nevertheless, some evidence suggestive of a degree of what we may term "interference" between the two in mixed infections of the same snail. In single species infections mean peak cercarial emergence of E. recurvatum occurred in the second hour after the onset of light and in single species infections of Plagiorchis sp. mean peak emergence occurred in the second hour after the onset of dark. In mixed infections mean peak emergence of E. recurvatum occurred in the third hour after the onset of light, (ie. 10.00-11.00h instead of 09.00-10.00h), and in the case of Plagiorchis sp. it occurred in the fourth hour after the onset of dark (ie. 23.00-24.00h instead of 21.00-22.00h). Therefore, in mixed infections mean peak cercarial emergence time had undergone an "en retard" time shift of 1 hour in the case of E. recurvatum and 2 hours in the case of Plagiorchis sp. One possible explanation for the observed time shift in peak cercarial emergence may be that an accumulation of E. recurvatum cercariae occurring in the digestive gland of the host could influence (slow) the emergence of Plagiorchis sp. and *vice versa*.

Some evidence of antagonism was also noted with respect to the numbers of Plagiorchis sp. cercariae emitted in mixed infections. In the case of E. recurvatum a t-test on cercarial counts (transformed  $\text{Log}_{10} (x+1)$ ) revealed no significant difference in the mean number of cercariae emitted per snail, whether the infection was E. recurvatum alone or mixed E. recurvatum + Plagiorchis sp.,  $P(t=1.49) > 0.05$ ,  $df=4$ . However, in the case of Plagiorchis sp. the same form of t-test revealed that the mean number of cercariae emitted from snails with mixed infections was significantly less than that from snails infected with Plagiorchis sp. alone  $P(t=5.3) < 0.05$ ,  $df=4$ . This suggests that in a mixed infection with the echinostome E. recurvatum, Plagiorchis sp. produces many fewer cercariae per day than when it is the only parasite in the host mollusc. A similar effect has also been noted by Theron & Mone (1986) who observed that in mixed infections with R. marini, S. mansoni produced significantly fewer cercariae than in single infections.

Conclusions from the above observation should be tempered in as much as it should be remembered that in the present study we are dealing with natural infections of E. recurvatum and Plagiorchis sp. of unknown age, and cercarial production may be influenced by age of infection. However, the observation is clearly and interestingly consistent with the well documented strongly antagonistic influence that the rediae of echinostomes (and cathaemasids such as R. marini) have on other digeneans within the same host mollusc; particularly those having only sporocyst generations. The markedly antagonistic effects of rediae of echinostomes such as Paryphostomum radiatum and Echinostoma



liei on larval schistosomes within the same host mollusc have been noted in detail by Heyneman, Lim & Jeyarsasingham (1972), Lie (1967) and Combes (1982). Kjøie (1987) has recently produced interesting SEM photographs of a cannibalistic redia of Mesorchis denticulatus. On the basis of their strongly antagonistic behaviour toward larval schistosomes, certain echinostomes have been seriously considered in recent decades as potential controls of the intramolluscan stages of human schistosomes. The fact that the rediae of E. recurvatum are predatory on cercariae of Plagiorchis sp. was affirmed in the current study by the discovery of stylets from Plagiorchis sp. cercariae in the saccate guts of some of the E. recurvatum daughter rediae recovered from the digestive glands of mixed infection snails on their dissection. The distinct probability that the rediae of E. recurvatum are directly antagonistic to the sporocysts and cercariae of Plagiorchis sp. in mixed infections provides a probable explanation for the apparently reduced cercarial output of Plagiorchis sp. in mixed infections with this echinostome.

### **5.3 The phototactic behaviour of the cercariae of Echinoparyphium recurvatum**

#### **5.3.1 INTRODUCTION**

To date no studies are available on the post-emergence phototactic behaviour of E. recurvatum cercariae and therefore the present study set out to investigate experimentally this aspect of the parasite's behavioural biology. Interesting results obtained by Evans & Gordon (1983a) and those presented in Chapter 6 of this

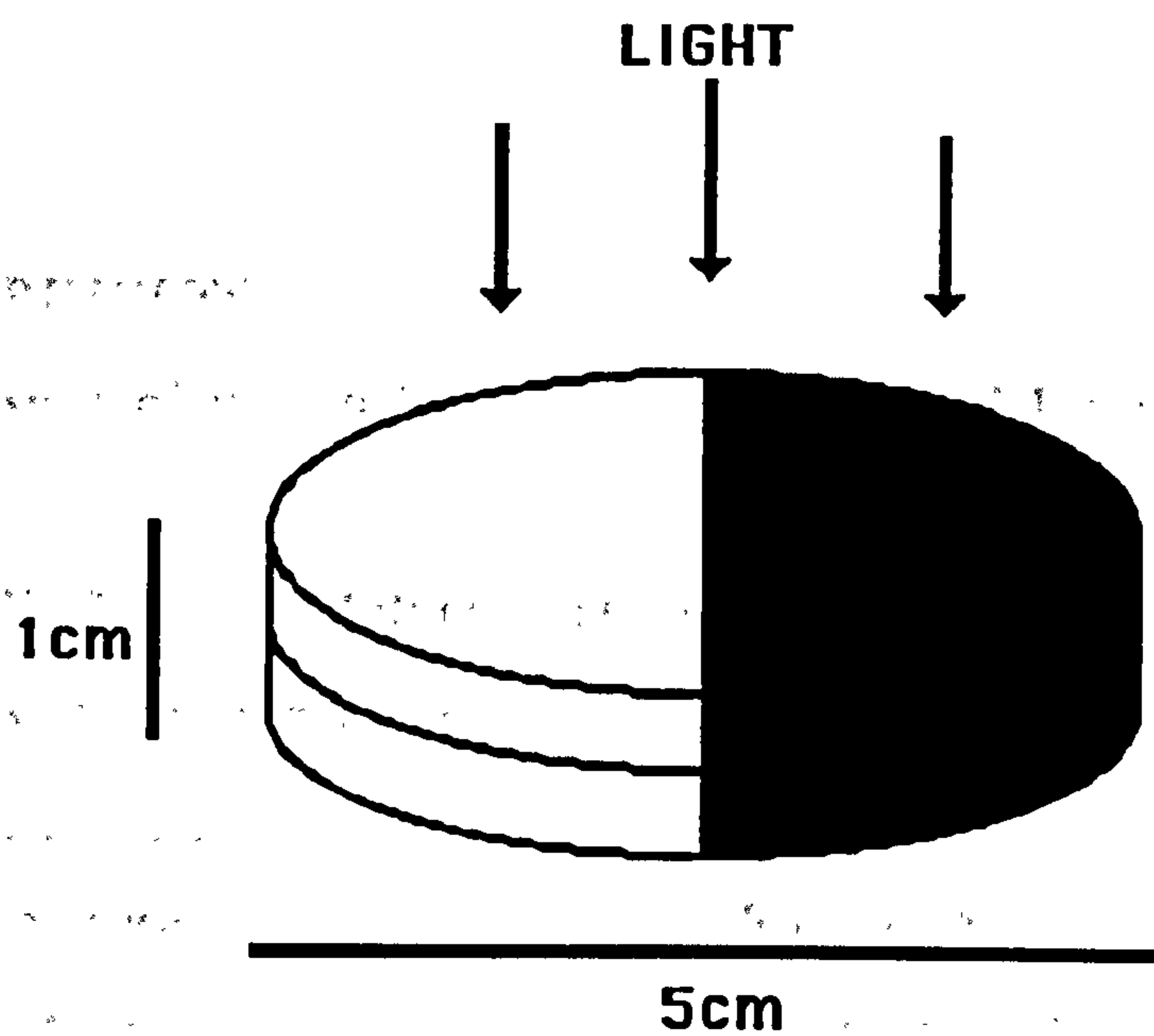
thesis, indicate that the cercariae of E. recurvatum exhibit an initial post-emergence dispersal phase of low infectivity with infectivity becoming maximal in the region of 2 hours after emergence. The phototactic response of cercariae both immediately after, and approximately 2 hours post-emergence, was examined in order to determine whether any parallel trend in cercarial phototactic behaviour would be manifest in cercariae of differing dispersal and maximal infectivity phases.

### 5.3.2 MATERIALS AND METHODS

The apparatus used to investigate the phototactic behaviour of E. recurvatum cercariae is illustrated in Fig 5.11. This simple light/dark choice chamber was constructed from a small clear polystyrene petri dish half of which was blacked out using light-proof black PVC electrical tape. The choice chamber was situated in a dark room in which the temperature was a constant 18-20°C. It was illuminated directly from above using a fibre-optic cold light source which produced a diffuse field of light of 850 Lux intensity. The choice chamber was filled with 20ml of synthetic hard water medium at a temperature of 18-20°C.

Cercariae of E. recurvatum were collected within 10 minutes of their emergence from a pool of naturally infected L. peregra obtained from Harting Pond. Twenty cercariae were introduced via a Pasteur pipette into the centre of the choice chamber and the lid was replaced. The cercariae were then allowed to distribute for a period of 20 minutes after which time the lid was removed and the numbers of cercariae on each side of the chamber were counted. The lid was then replaced and the cercariae were allowed

**FIG 5.11 The light-dark "Choice Chamber"**





to distribute for 2 hours after which the numbers of cercariae on each side of the choice chamber were again counted. The phototactic responses were thus determined for cercariae at 30 minutes post-emergence and 2.5 hours post-emergence. The entire experiment was repeated 20 times, each time beginning with freshly emitted cercariae.

### 5.3.3 RESULTS

The results of this study are shown in Table 5.1 below :

**TABLE 5.1** Phototactic responses of Echinoparyphium recurvatum cercariae of different post-emergence ages.

Post-emergence age of cercariae	Mean (+/- SE) no. of cercariae on each side of the choice chamber	
	LIGHT	DARK
30 minutes	17.1 (+/- 0.6)	2.9 (+/- 0.3)
2.5 hours	1.8 (+/- 0.5)	18.2 (+/- 0.5)

The results clearly indicate that 30 minutes after their emergence from L. peregra ; E. recurvatum cercariae show a markedly positive phototaxis. A t-test on cercarial counts transformed by  $\text{Log}_{10}(x+1)$  showed that 30 minutes post-emergence the mean number of

cercariae in the light half of the choice chamber was highly significantly greater than that on the dark side,  $P(t=9.5) < 0.01$ ,  $n=38$ . At 2.5 hours post-emergence the phototactic response of the cercariae appeared to have been reversed to a markedly negative phototaxis. A t-test on cercarial counts transformed by  $\text{Log}_{10}(x+1)$  showed that at 2.5 hours post-emergence the mean number of cercariae in the dark half of the chamber was highly significantly greater than that on the light side,  $P(t=12.8) < 0.01$   $n=38$ .

#### 5.3.4 DISCUSSION

The initial post-emergence positive phototaxis of E. recurvatum cercariae observed in the present study is similar to that observed in echinostome cercariae by Cort (1922). This author did in fact suggest that this positive reaction to light was responsible for the emergence of the cercariae from first intermediate host snails predominantly under conditions of illumination.

The results of the present study have revealed an interesting age-related change in the phototactic behaviour of the cercariae of E. recurvatum which parallels the change in infectivity of the parasite over the same period. The initial positive phototaxis of cercariae is coincident with the initial sub-maximal infectivity phase demonstrated by Evans & Gordon (1983a) and in Chapter 6 of this thesis. The marked negative phototaxis of cercariae 2.5 hours post-emergence is coincident with the maximal infectivity of cercariae that is attained at this time (see Evans & Gordon 1983a and Chapter 6). This pattern of behaviour would seem to suggest that E. recurvatum cercariae have an initially positively

low infectivity, dispersal phase followed by a negatively phototactic, maximally infective, host location and infection phase. The negatively phototactic phase could be expected to enhance the probability of contact with benthic second intermediate host snails.

Although to date no named example of a positive phototactic dispersal phase followed by a negatively phototactic infection phase has been clearly recorded for a digenean cercaria, Cable (1972) has noted that in certain cercariae, "sometimes phototaxis is initially positive and changes later, usually a short time after emerging from the first intermediate host". The phenomenon is much better documented in the case of monogenean oncomiracidia. The authors Boret (1967) and Paling (1969) have demonstrated for the oncomiracidia of Diplozoon paradoxicum and Discotyle sagittata, respectively that there exists an initial positive phototactic dispersal phase followed by a negatively phototactic host-location and infection phase. The phototactic responses of E. recurvatum cercariae observed in the present study raise the question as to how the cercariae perceive differences in light intensity. Although no ultrastructural studies have been carried out on cercariae of E. recurvatum studies by Fournier (1984) have revealed non-pigmented rhabdomeric photoreceptors in cercariae of other echinostome cercariae such as Echinostoma togoensis.



## 5.4 The influence of temperature upon the emergence of Echinoparyphium recurvatum cercariae from Lymnaea peregra

### 5.4.1 INTRODUCTION

To date very few studies have been carried out on the influence of environmental temperature on the emergence of echinostome cercariae from snail first intermediate hosts. However, Rees (1931) has commented on the inhibitory influence of low temperature on the emergence of Cercaria "Z" (a possible synonym of Echinoparyphium recurvatum) from Lymnaea peregra. Temperature is known to have an important influence on the cercarial production and emergence of a number of species of digenean including Fasciola hepatica (Kendall & McCullough 1951 ; Ross 1930), Apatemon gracilis minor (Raishte 1967) and Cercaria purpurae (Rees 1948). Temperature is also known to be an important environmental factor in determining the seasonality of cercarial production in natural systems (Pike 1968).

No detailed information is currently available on the influence of environmental temperature on the production and emergence of E. recurvatum cercariae from L. peregra. The following study was therefore carried out in order to assess the influence of temperature on E. recurvatum cercarial emergence over the broad seasonal temperature range known to occur at Harting Pond, West Sussex. The main aim of the study was to determine in a general way at what times of year cercarial emergence from infected L. peregra snails could be expected to occur in this habitat.

#### 5.4.2 MATERIALS AND METHODS

Five L. peregra snails in the shell length range 16-18.5 carrying naturally acquired patent infections of E. recurvatum were obtained from Harting Pond in June (1985). The snails were isolated individually in small clear polystyrene containers each containing 15ml of synthetic hard water medium (HMSO 1969). The snails were then transferred to a light-proof incubator where they were maintained for 3 days at a constant temperature of 20°C under a balanced L:D 12:12 photoperiod regime with light (1600 Lux) beginning at 08.00h and dark at 20.00h.

After the three day initial acclimatization period the temperature of the incubator was adjusted up to 25°C. The snails were allowed to acclimatize for one day ,(ie. 08.00-08.00h), and then 2 hourly collections of cercariae were made in the previously described manner on day 2. The temperature was then lowered to 20°C on day 3, the snails allowed to acclimatize for one day and then 2 hourly monitoring of cercarial emergence was carried out on day 4. This procedure was continued, reducing the temperature in 5°C increments until a temperature of 5°C was reached. After cercarial counts had been taken for this temperature the incubator temperature was raised to 20°C, the snails were allowed to acclimatize for 1 day and the usual 2 hourly collections of cercariae were made throughout the following day. Collections of cercariae from each snail each day were pooled before counting to provide a single cercarial count for each snail for each day. The entire experiment extended over a period of 12 days, and throughout the experimental and acclimatization periods all the

snails were provided with an excess of food in the form of clean boiled lettuce.

### 5.4.3 RESULTS

The results of this experiment are shown in Fig 5.12. Temperature clearly has a significant influence on the numbers of E. recurvatum cercariae emerging from the first intermediate host L. peregra. The greatest mean number of cercariae emitted per snail occurred at 25°C, the number decreasing significantly with decrease in temperature. Little cercarial emergence was observed at 15°C, and this result indicates that the threshold temperature for E. recurvatum cercarial emergence is likely to be in the region of 15°C, probably slightly less. At 10°C and 5°C cercarial emergence ceased completely. However, raising the temperature to 20°C from 5°C resulted in a resumption of cercarial emergence. The mean number of cercariae emitted per snail at 20°C on day 12 of the experiment was found, by a matched-pairs t-test, to be insignificantly different from that emitted at 20°C on day 4 of the experiment,  $P(t=0.67) > 0.05$ ,  $df=4$ .

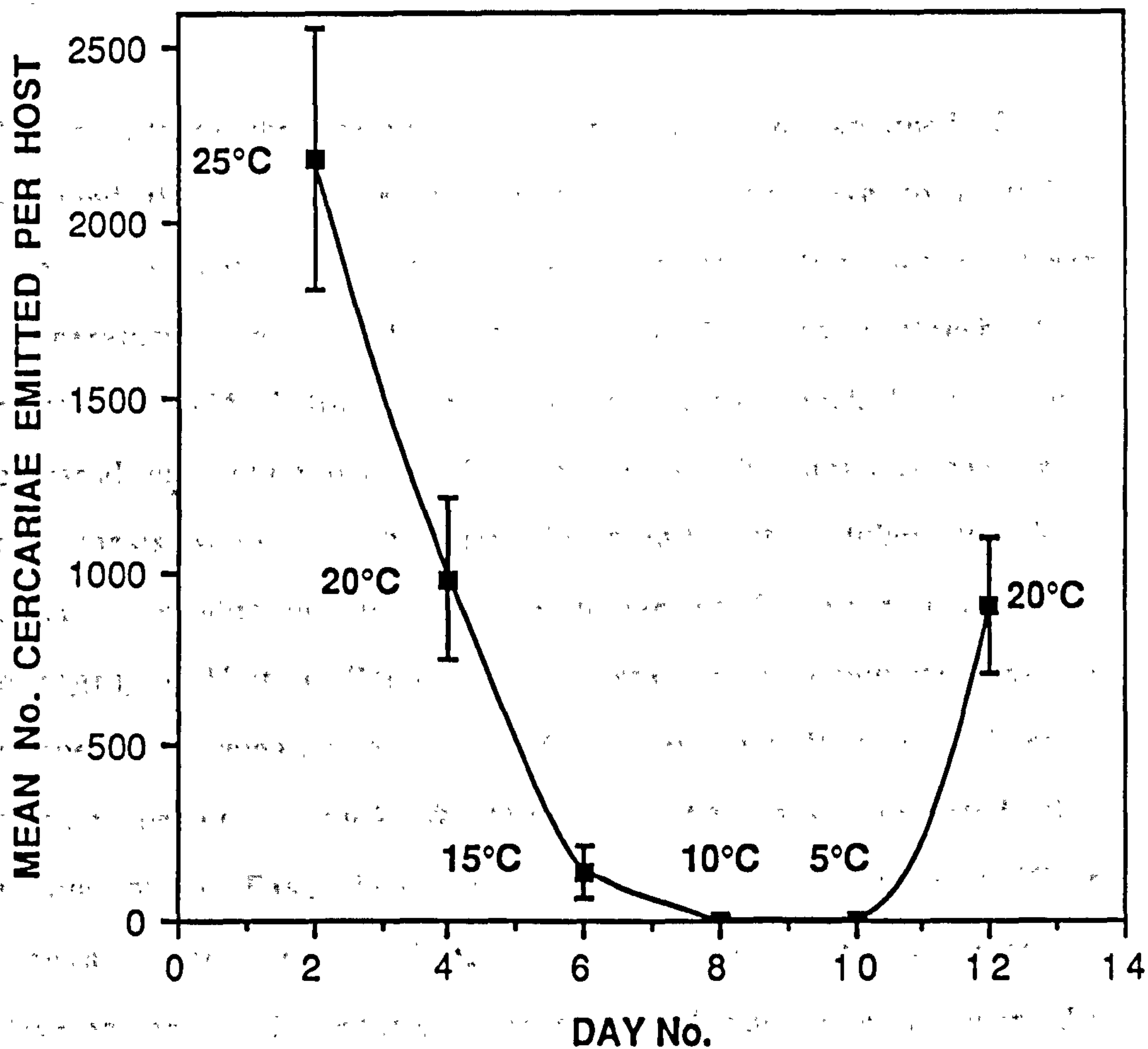
### 5.4.4 DISCUSSION

The findings presented herein clearly show that environmental temperature has a marked influence on the numbers of E. recurvatum cercariae emerging from L. peregra. The general trend of higher temperatures (up to a certain critical level) resulting in larger amounts of cercarial output is one that has been reported for several digeneans including E. hepatica (Kendall & McCullough



FIG 5.12 The mean number of Echinoparyphium recurvatum cercariae emitted per host snail over a 24 hour period at a range of different water temperatures.

(Vertical bars indicate standard errors of the means)



1951). In the present study the fact that little cercarial emergence was observed at or below 15°C is suggestive of the fact that the lower threshold level for E. recurvatum cercarial emergence is in the region of 15°C. This observation is consistent with a recent report by Adam & Lewis (1988) who state that cercarial emergence of E. recurvatum ceases below 13°C. This figure is quite high when compared to the 9°C lower threshold for cercarial emergence determined by Kendall & McCullough (1951) for E. hepatica.

The results of the present study can largely be explained if it is assumed that higher temperatures are conducive to a higher rate of cercarial production by daughter rediae while lower temperatures tend to slow this rate until a lower threshold is reached (13-15°C for E. recurvatum) below which little to zero cercarial production occurs. The findings of the previous chapter of this thesis show that between the months of October and May cercarial production by daughter rediae of E. recurvatum in L. peregra at Harting Pond, West Sussex ceases completely as the temperature remains below 15°C over this Winter and early Spring period. Dinnik & Dinnik (1964) have reported that production of Fasciola gigantica cercariae ceases below 16°C in Kenya, and Ross (1930) demonstrated that the rate of development of E. hepatica cercariae in Japan decreased markedly with a drop in temperature and ceased completely below 10°C. Low temperature is also likely to inhibit the emergence of ready-to-emerge cercariae present in the snail.

Temperature is clearly an important factor in the emergence of E. recurvatum cercariae from L. peregra. At temperatures below 15°C cercarial emergence is completely inhibited. The water temperature at Harting Pond, West Sussex, is known to vary between 2°C in February to 21.5°C in June. The daytime water temperature rises above the 15°C threshold level from about May to October. In view of the results obtained in the current study it could be expected that emergence of E. recurvatum cercariae from L. peregra could occur only between these months. This effectively means that transmission of the parasite to second intermediate hosts is likely to be restricted to the period between May, Late Spring and October (Autumn) in this habitat.



## Chapter 6

### Experimental studies on the transmission dynamics and host location biology of *Echinoparyphium recurvatum* cercariae

## Experimental studies on the transmission dynamics and host location biology of Echinoparyphium recurvatum cercariae

### 6.1 The influence of environmental temperature on the transmission of Echinoparyphium recurvatum cercariae

#### 6.1.1 INTRODUCTION

Environmental temperature is undoubtedly one of the most important of a variety of factors influencing the transmission dynamics of free-living infective stages of parasitic organisms. This climatic variable is known to have its effect on both the survival and infectivity of infective transmission stages. The quantification of the effect that it has on these factors has attracted considerable attention in the case of schistosome miracidia and cercariae (Prah & James 1977 ; Lawson & Wilson 1980 ; Anderson, Mercer, Wilson & Carter 1982). The cercariae of the African echinostome Echinostoma liei have also been studied with respect to the effect of temperature on their transmission success by Evans (1985). However, no published information is currently available on the effect of environmental temperature on the transmission dynamics of Echinoparyphium recurvatum cercariae. The present study therefore set out to provide a detailed picture of the influence of temperature on the survival, infectivity and transmission efficiency of E. recurvatum cercariae, in order to determine how well the parasite is adapted to transmission over a range of environmental temperatures.

## 6.1.2 MATERIALS AND METHODS

### Parasite and host material

Cercariae of E. recurvatum were obtained from naturally infected specimens of Lymnaea peregra collected in September 1985 from Harting Pond, West Sussex. L. peregra snails were also used as experimental second intermediate hosts in this investigation. The snails were laboratory bred from a breeding parent stock originally obtained from Harting Pond. The snails were infection-free and in the size (shell length) range 3.5-5mm ; Evans & Gordon (1983a) having shown host size to be an important factor influencing transmission success of E. recurvatum cercariae.

### Experimental Methods

#### Investigation of survival characteristics

The survival characteristics of E. recurvatum cercariae were examined at the following temperatures ; 10, 15, 20, 25 and 30°C. At each temperature survival was examined by introducing 40 freshly emerged cercariae (maximum age 30 minutes) into each of three polystyrene dishes containing 10 mls of synthetic hard water medium, (HMSO 1969). The number alive was recorded at intervals of time until all the parasites were dead. The criterion of cercarial death was the same as that used by Anderson & Whitfield (1975), Evans & Gordon (1983a) and Evans (1985), in that death was pronounced when a cercaria failed to respond to mechanical stimulation in the form of three light contacts with a fine stainless steel needle. Each experiment was carried out in a constant temperature incubator. At each temperature the dishes of water were placed in the incubator for a minimum of 2 hours to



reach the required temperature prior to the start of each experiment.

### Investigation of cercarial infectivity

Infectivity of E. recurvatum cercariae to L. peregra was investigated at the same temperatures as survival ; 10, 15, 20, 25 and 30°C. At each temperature sub-populations of cercariae collected within 30 minutes of their emergence from a pool of first intermediate hosts were aged for varying time periods at the desired temperature. After the appropriate ageing period, batches of 5 living cercariae were transferred to small polystyrene plate wells (COSTAR, Cambridge U.S.A.) each containing 3ml of synthetic hard water medium, and a single infection-free specimen of L. peregra. In each case both snails and water were placed in an incubator at least 2 hours prior to the start of an experiment. Each snail was exposed to cercariae for a period of 20 minutes and was then transferred to clean water for a period of 24 hours. Between 10 and 15 replicate exposures were carried out for each cercarial age investigated. After 24 hours each snail was crushed between two glass microscope slides and examined for metacercarial cysts using a stereo-microscope.

### Mathematical methods

Cercarial survival data obtained in this study were processed and fitted by the Anderson & Whitfield (1975) age dependent survival model using the BBC Micro Computer-based software programs "MUCALC" and "MUTOFIT" as described previously in Chapter 3. Survival data in the form of proportions of total cercariae surviving at each temperature at successive points in time were

entered into "MUCALC" which computed the instantaneous *per capita* death rate of cercariae,  $\mu$ , for each time interval. Values of this death rate with the appropriate time intervals were then entered into "MUTOFIT" which generated predicted values of the proportions of cercariae surviving at successive points in time according to the age dependent survival model of Anderson & Whitfield (1975). "MUTOFIT" also predicted the time to 50% cercarial survival ( $t_{50}$ ), and gave the mean instantaneous *per capita* death rate of cercariae,  $\bar{\mu}$ , at any given temperature by calculating this parameter as  $1/t_{50}$ .

### 6.1.3 RESULTS

The survival of *E. recurvatum* cercariae at the experimental temperatures 10-30°C is illustrated in Fig 6.1. Cercarial survival was found to be markedly age dependent. At each temperature the observed proportions of cercariae surviving through time were in close agreement with those predicted by the age dependent survival model formulated by Anderson & Whitfield (1975) from which the predicted proportion of cercariae  $P(t)$  surviving to age  $t$  is given as :

$$P(t) = \exp \left[ \frac{a}{b} (1 - \exp(bt)) \right].$$

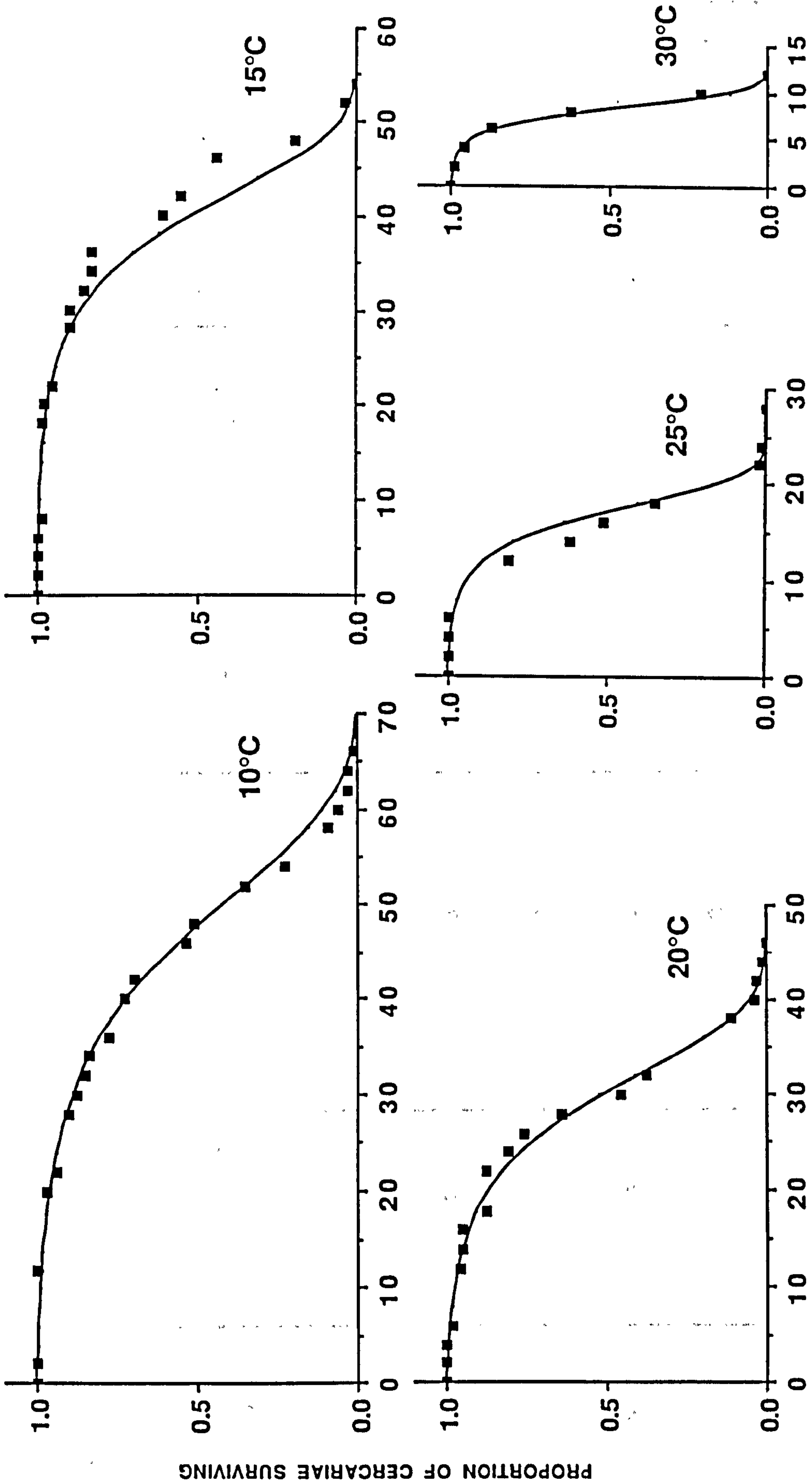
The apparent "goodness-of-fit" of the model to the observed data is due to the fact that at each temperature the instantaneous *per capita* death rate increased exponentially with cercarial age.

The most obvious temperature-related trend in cercarial survival was that longevity decreased with increase in temperature. Maximum survival time was reduced from 68 hours at 10°C to

**FIG 6.1** The influence of age and temperature on the survival of Echinoparyphium recurvatum cercariae

(Points indicate observed proportions of cercariae surviving and the solid line indicates proportions predicted according to the age dependent survival model of Anderson & Whifield (1975))





AGE OF CERCARIAE (HOURS)

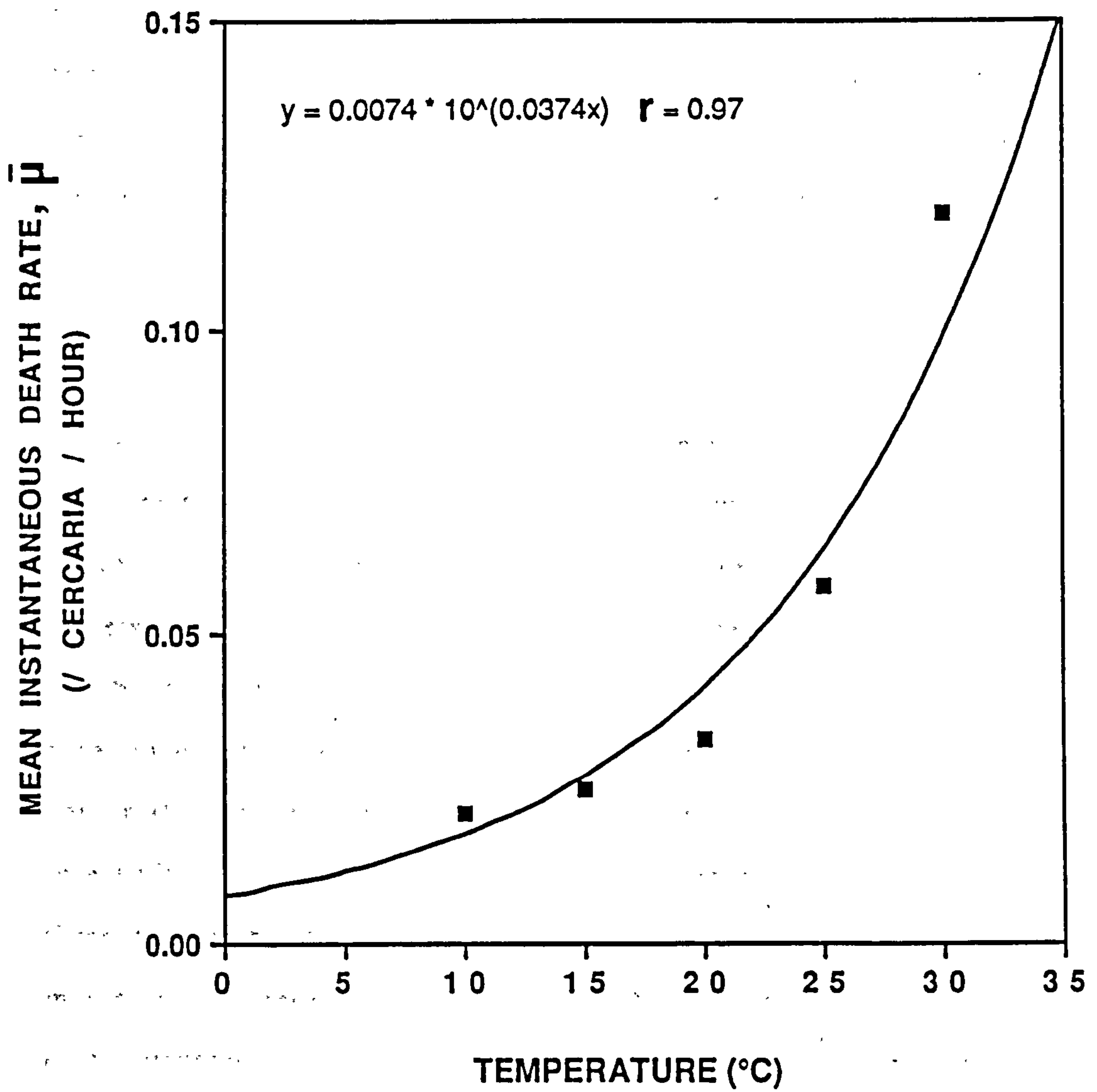
PROPORTION OF CERCARIAE SURVIVING

**TABLE 6.1 The effect of temperature on transmission characteristics of Echinoparyphium recurvatum cercariae**

Temperature (°C)	Time to 50% survival (h)	Mean instantaneous death rate, $\bar{\mu}$ (/cercaria/h)	Mean instantaneous rate of infection $\bar{\beta}$ (/cercaria/h/snail/3ml)	Transmission Efficiency $\frac{\bar{\mu}\bar{\beta}}{(H_0)}$ (1/H <sub>0</sub> )
10	47.93	0.021	0.553	0.038
15	40.4	0.025	1.005	0.025
20	30.44	0.033	1.365	0.024
25	17.25	0.058	1.623	0.036
30	8.38	0.119	0.39	0.305

FIG 6.2 The increase in mean instantaneous per capita death rate of Echinoparyphium recurvatum cercariae with increase in temperature

(Points indicate observed data and the solid line represents the best-fit exponential curve of the form indicated in the equation)





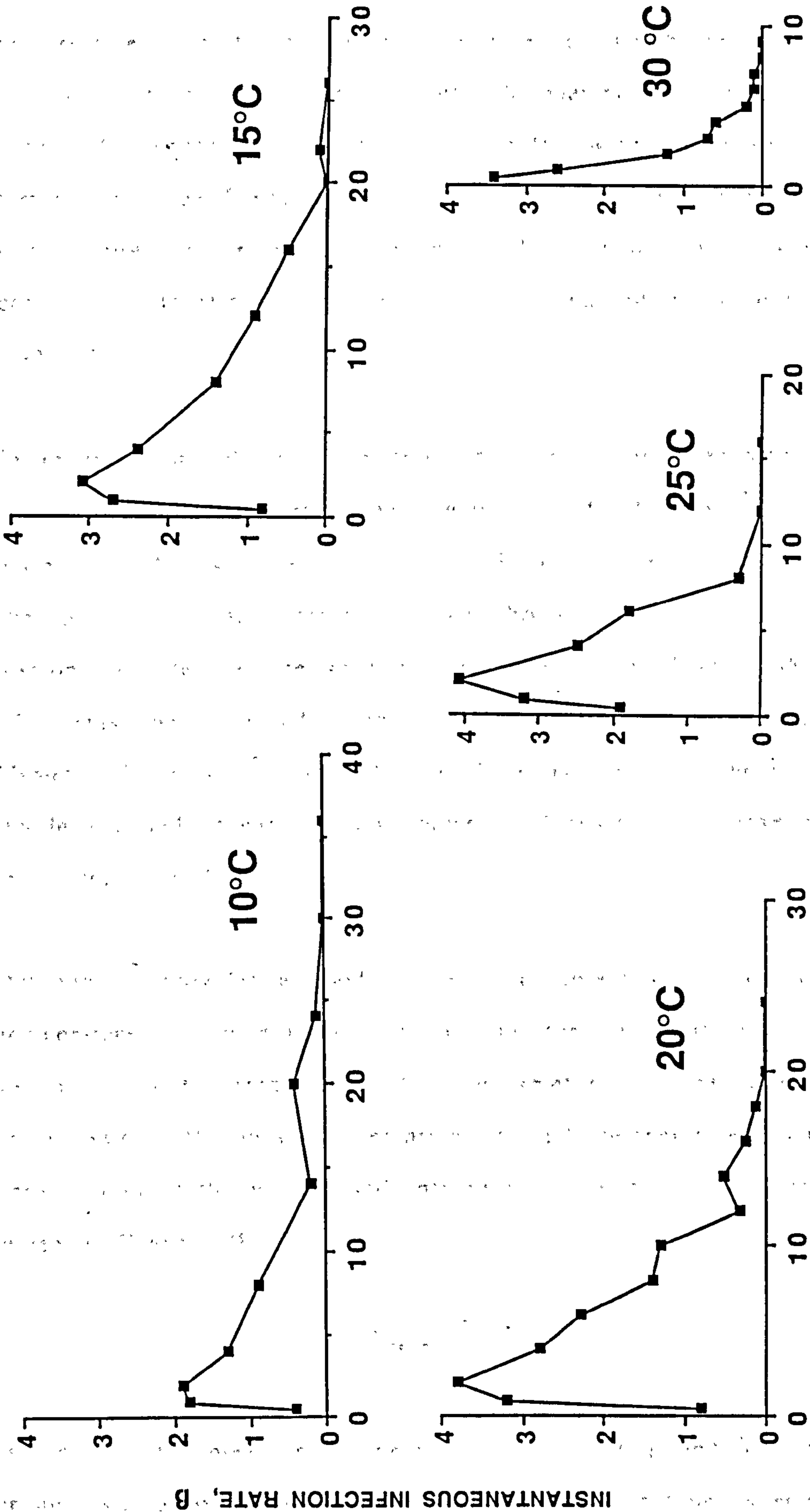
only 12 hours at 30°C, with 50% survival times being reduced from 47.93 hours at 10°C to only 8.38 hours at 30°C (see Table 6.1). A corresponding increase in the mean instantaneous *per capita* cercarial death rate,  $\bar{\mu}$ , from 10°C through 30°C was observed, the value of this survival parameter increasing exponentially with temperature, see Table 6.1 and Fig 6.2.

Infectivity of cercariae also showed marked age and temperature-related trends. In this investigation, infectivity was calculated from the metacercarial recovery data as the parameter  $\beta$ . This is the instantaneous *per capita* rate of infection formulated by Anderson et al (1982) and given by Evans (1985) as :

$$\beta = -\ln (1 - M/C_0) / t$$

where  $t$  = exposure time in hours ;  $M$  = number of metacercarial cysts recovered per snail and  $C_0$  = the number of cercariae to which each snail was exposed. This parameter according to Anderson et al (1982) may be expressed as the *per capita* rate at which the infective stage infects a host within a unit interval of time under unit host density conditions.  $\beta$  was calculated for each cercarial age at each temperature. Fig 6.3 illustrates the infectivity,  $\beta$ , through time at each experimental temperature. Over the temperature range investigated, cercarial infectivity was markedly influenced by both age and temperature. In general at each temperature infectivity was found to decline with increasing cercarial age. However, it was evident that from 10°C-25°C infectivity was initially low but rose to a peak approximately 2 hours post-emergence. The most noticeable effect of increasing

FIG 6.3 The influence of age and temperature on the infectivity of Echinoparyphium recurvatum cercariae to the second intermediate host Lymnaea peregra.



AGE OF CERCARIAE (HOURS)

INSTANTANEOUS INFECTION RATE, B



temperature was to accelerate the rate at which infectivity declined with increasing cercarial age. The maximum infective life span of cercariae decreased markedly with increase in temperature (see Table 6.1). It was also noticeable that at each temperature the maximum infective life span was always considerably shorter than the maximum life span of cercariae (see Table 6.1).

In order to provide a single summary statistic of infectivity at each temperature the mean instantaneous rate of infection,  $\bar{B}$ , was estimated. This was calculated for each temperature by dividing the area under each infection graph shown in Fig 6.3 by the appropriate infective life span as recommended by Evans (1985). The areas were obtained using of an Apple Computers "Graphics Tablet". Values of  $\bar{B}$  obtained at each temperature are shown in Table 6.1 and appear to show cercarial infectivity to be maximal in the region of 20°C.

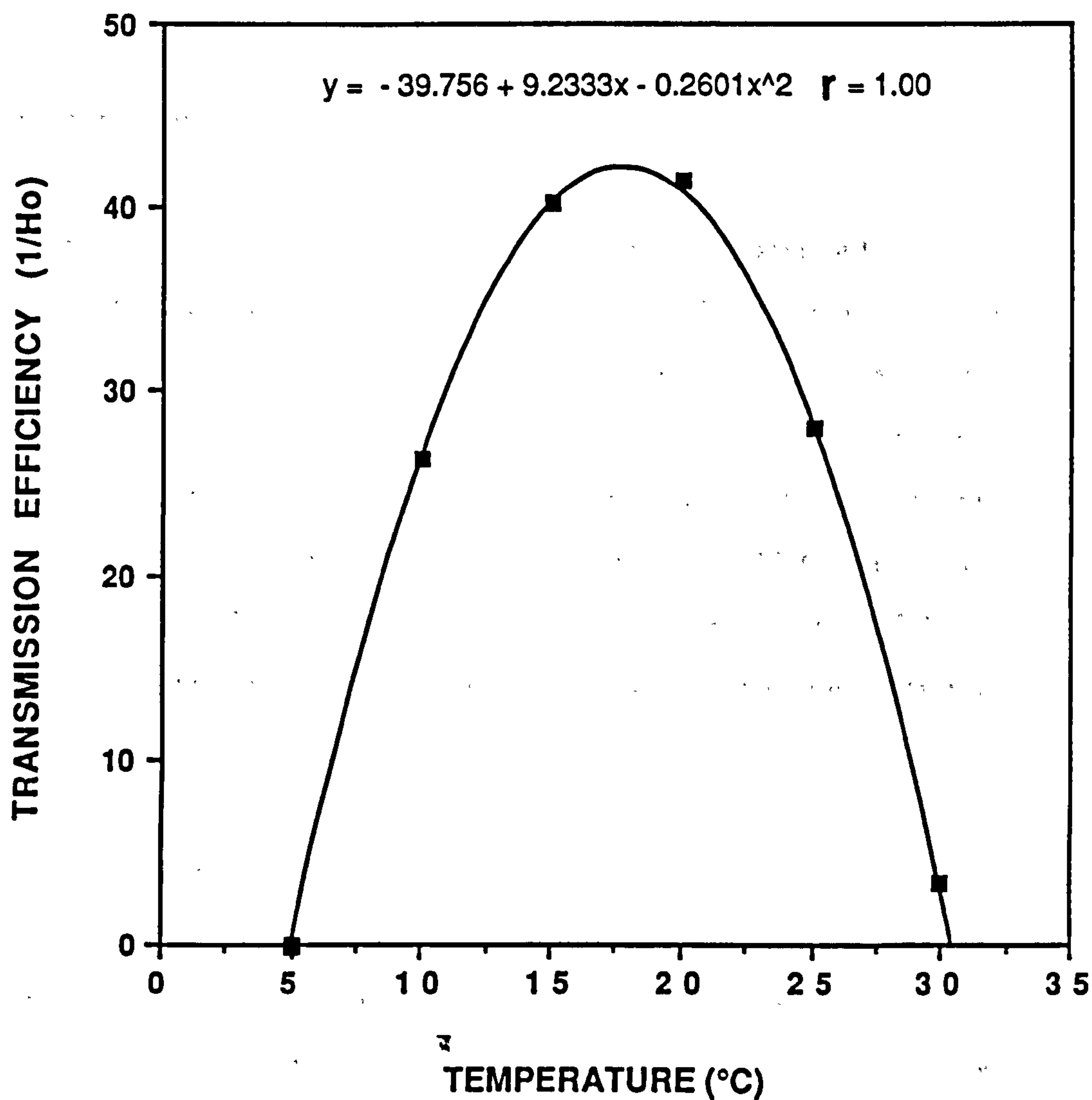
Although  $\bar{B}$  provides a good indication of infectivity at a given temperature it does not take into account the important factor of cercarial death rate. An ideal parameter of the overall transmission efficiency of cercariae at each temperature taking into account both survival and infectivity characteristics has been given by Evans (1985) as :

$$1/H_0 = \bar{B}/\bar{\mu}$$

where  $\bar{B}$  is the mean instantaneous rate of infection and  $\bar{\mu}$  is the mean instantaneous death rate per cercaria per unit time as stated

FIG 6.4 The influence of environmental temperature upon the transmission efficiency of Echinoparyphium recurvatum cercariae

(Points indicate observed data and the solid line represents the fit of a second order polynomial curve of the form indicated in the equation)



previously. Values for the transmission parameter  $1/H_0$  for each temperature are given in Table 6.1 and are presented graphically in Fig 6.4. The calculated values of the parameter were fitted by a second order polynomial curve of the form given in Fig 6.4 and suggest that optimal transmission efficiency of cercariae is attained in the region of 20°C. The parameter  $1/H_0$  was used by Evans (1985) and is a modified form of the transmission parameter  $H_0$  ( $\bar{\mu}/\bar{\beta}$ ) first formulated by May & Anderson (1978) which varies inversely with transmission efficiency. The values of  $H_0$  for E. recurvatum cercariae are included in Table 6.1 for completeness and, of course, also show transmission efficiency to be optimal around 20°C.

Although optimal transmission efficiency was attained in the region of 20°C the cercariae of E. recurvatum showed a high degree of transmission efficiency over a wide range of environmental temperature. This finding can be explained by the fact that cercarial survival and infectivity optima occurred at different temperatures. The low infection rate observed at 15°C was offset by low cercarial death rate at this temperature and at 25°C the relatively high cercarial death rate was offset by the high infection rate observed.

#### 6.1.4 DISCUSSION

The results of this experimental study clearly demonstrate that changes in environmental temperature have a significant degree of influence on the survival, infectivity and transmission efficiency of E. recurvatum cercariae, and also that cercarial survival and infectivity are markedly age dependent. Decrease in



cercarial survival time with increased temperature has previously been demonstrated for echinostome cercariae of the African species Echinostoma liei. The phenomenon is thought to be caused by higher rates of cercarial activity at higher temperatures placing increased demand per unit time on a finite energy reserve. In the case of digenean cercariae the energy reserve is thought most likely to be glycogen, (see Anderson & Whitfield 1975). In the present study both 50% and maximal survival times obtained from E. recurvatum cercariae at 20°C accord well with the values for these parameters obtained by Evans & Gordon (1983a) for this species. However, it is noticeable that at 30°C the survival times obtained for E. recurvatum were considerably lower than those obtained by Evans (1985) for Echinostoma liei cercariae, the latter presumably being better adapted to survival at relatively high water temperatures.

If it is accepted, as was suggested by Evans (1985), that increased cercarial swimming activity caused by an increase in temperature resulted in a corresponding increase in the number of contacts between cercaria and host per unit time, then it is possible to explain the increase in cercarial infectivity observed in the present study from 10°C to 25°C purely in these terms. However at 30°C a marked decrease in infectivity was observed and it is likely that at this high temperature increased swimming activity very quickly depleted the energy reserves of the cercariae so that even when a host was located the energy requiring processes of penetration migration and encystment could not be carried out successfully. Indeed, in the present study at 30°C some snails exposed to cercariae at this temperature were found to contain

cercarial bodies - cercariae that had managed to locate a host but presumably lacked the energy to encyst. An additional factor contributing to low infectivity at 30°C may be one suggested by Evans (1985) who speculated that at high temperatures the mechanisms responsible for host recognition in cercariae may be impaired.

The interesting period of initial post-emergence delayed maximal infectivity exhibited by E. recurvatum over the temperature range 10 to 25°C in the present study is a phenomenon that has been noted previously by Evans & Gordon (1983a) at 18°C. It is thought to represent a dispersal phase preventing super-infection of first intermediate host L. peregra emitting E. recurvatum cercariae. The persistence of this phenomenon throughout the temperature range 10-25°C may be testimony to its importance as a mechanism preventing super-infection and promoting dispersal.

Transmission efficiency of E. recurvatum cercariae was found to be relatively high over the temperature range 15-25°C. The relatively low degrees of transmission efficiency at 10°C and 30°C are likely to be of little real significance to transmission of the parasite in British habitats. This is because water temperatures in British habitats probably rarely, if ever, reach 30°C for extended periods of time, and (as the previous chapter has shown) emergence of E. recurvatum cercariae is completely inhibited at 10°C. Cercarial emergence of E. recurvatum cercariae has been shown to occur at temperatures between 15 and 25°C (see previous chapter). At Harting Pond, West Sussex water temperature has been observed to remain at or above 15 °C from May to mid-September, reaching

May to mid-September, reaching its peak of around 20°C in June July and August. It therefore seems that cercariae of E. recurvatum are optimally adapted to conditions of environmental temperature that they are likely to encounter in the natural environment.

It is interesting to compare the cercarial transmission efficiency of E. recurvatum cercariae observed in the present study with that of the African echinostome Echinostoma liei by Evans (1985). The latter author demonstrated that optimal transmission efficiency of E. liei cercariae occurs at 30°C, showing that this parasite is also well adapted to temperature conditions in its natural habitats. As a further step in the investigation of temperature related infection dynamics of E. recurvatum it would be interesting to compare the results of the present study on E. recurvatum from Britain with Echinoparyphium species reported by other authors from tropical habitats.

## 6.2 The influence of second intermediate host dispersion pattern on the transmission of Echinoparyphium recurvatum cercariae

### 6.2.1 INTRODUCTION

The influence of the dispersion patterns of populations of intermediate hosts on the transmission of free-swimming larvae of digeneans is at present very poorly understood. The numerous studies carried out on the transmission of miracidia of human schistosomes have delimited many host, parasite and



environment-related factors which determine the magnitude of transmission eg. Anderson et al (1982) and Prah & James (1977). However, even in this well-worked area of digenean transmission biology the role of snail host dispersion pattern on parasite transmission has received little consideration, with the exception of one study published in abstract form, (Evans 1984). This author discovered interesting trends in the levels of infection produced by Schistosoma mansoni miracidia in experimental populations of Biomphalaria glabrata arranged to produce various dispersion patterns.

At present nothing is known of the potential influence of the dispersion patterns of second intermediate snail host populations on the transmission of echinostome cercariae. The present study therefore set out to examine the levels of transmission of E. recurvatum cercariae in experimental populations of the high compatibility second intermediate host L. peregra in order to determine the effect of this potentially influential host-related factor on cercarial transmission.

## 6.2.2 MATERIALS AND METHODS

### Parasite and host material

Prior planning had indicated that a large number of L. peregra experimental second intermediate hosts would be required for this experiment, 600 in total. Existing stocks of laboratory bred L. peregra were required for important host-specificity experiments and therefore an alternative source of infection-free L. peregra was utilized. Numerous young L. peregra snails were collected by sweep-net sampling from a relief channel running parallel to

Harting Pond in July 1987. These snails were of the new generation of L. peregra that appear at this time of year at this site. Snails in the size range 4.5-5.5mm were removed from the samples taken. 1,200 snails in this size range were divided into two equal batches of 600. One batch was crushed and examined for digenean infections or the presence of Chaetogaster sp. commensals. The snails were found to be completely infection-free. Therefore it was thought reasonable to assume that this size class of L. peregra were uninfected and that it would be acceptable to use the 600 snails of the parallel sample as experimental second intermediate hosts in this investigation.

E. recurvatum cercariae in this study were obtained within 1 hour of their emergence from a pool of naturally infected L. peregra first intermediate hosts collected at Harting Pond and maintained at 20°C in synthetic hard water medium (HMSO 1969).

### Experimental methods

Transmission of cercariae was examined in experimental populations of L. peregra arranged to show three dispersion patterns. These were Ideal Regular (variance/ mean ratio = 0), Random (variance/ mean ratio = 1), and Contagious or "Clumped" (variance/ mean ratio = 10).

Populations of 100 infection-free L. peregra snails in the size range 4.5 - 5.5mm were allotted among 10 cylindrical green plastic mesh cages 30mm in diameter and 20mm high with mesh size 2.5 mm<sup>2</sup> (see Fig 6.5). The cages were anchored using a 15mm length of dacron nylon tied direct to an inert glass weight, and arranged in a

**FIG 6.5** A plastic snail cage attached to inert glass weight

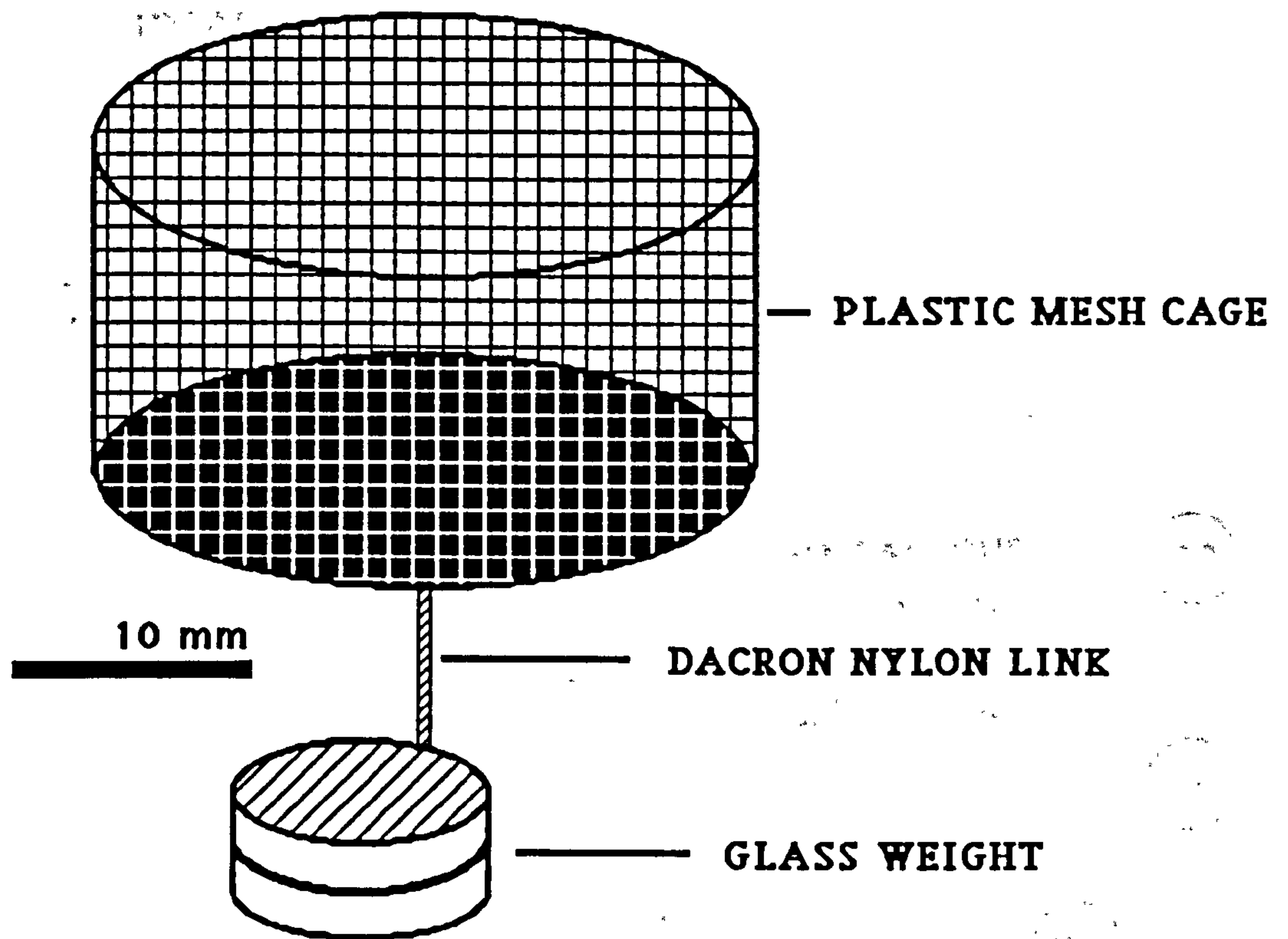
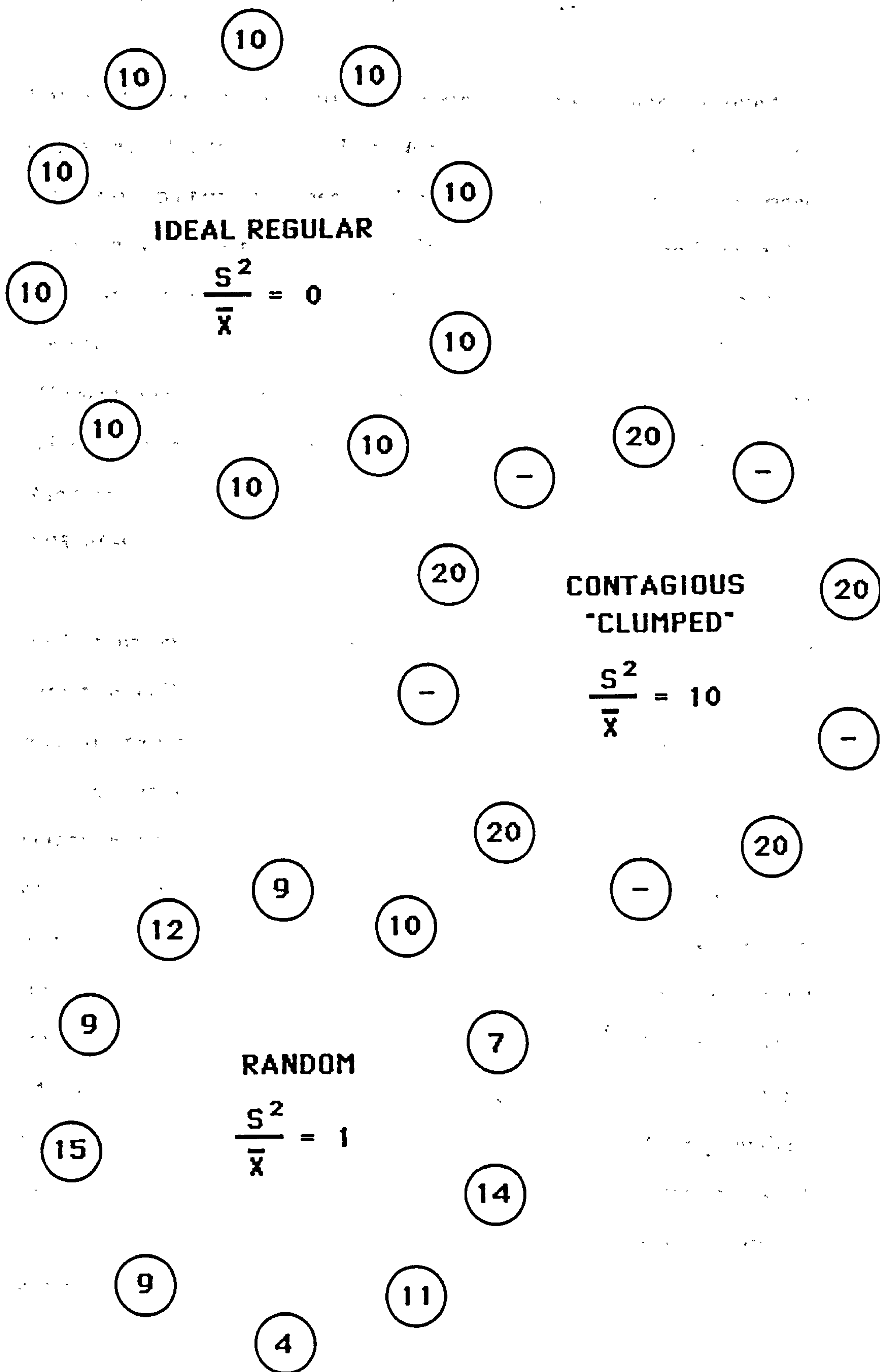




FIG 6.6 Diagram to show the distribution of *Lymnaea peregra* among the experimental cages to produce the three required host dispersion patterns



2-Dimensional circular array (diameter 25cm) in a plastic opaque-walled infection arena containing 2 litres of synthetic hard water medium (HMSO 1969) at 18-20°C.

For each required dispersion pattern the snails were allotted to cages as illustrated in Fig 6.6. The fact that the required dispersion pattern had been achieved in each case was confirmed statistically by using a 0.05 % Chi-squared test of significance for each arrangement of snails as recommended by the graphical method of Elliot (1983). In each case the Chi-squared values affirmed that the required dispersion pattern of snails had been achieved in each arrangement ; Regular P ( $\text{Chi}^2 = 0$ ) > 0.05 df=9, Random P ( $\text{Chi}^2 = 9.4$ ) > 0.05 df=9, and Contagious P ( $\text{Chi}^2 = 90$ ) > 0.05 df=9.

Each experimental population was exposed to 2,000 E. recurvatum cercariae collected within 1 hour of their emergence from a large pool of naturally infected L. peregra first intermediate hosts. This gave a cercarial density of 1 cercaria per ml and ratio of 20 cercariae per snail. The cercariae were introduced into the centre of the 2-dimensional circular array of cages, and thus at origin were equi-distant from all hosts. The snails were exposed to infection for 24 hours at 18-20°C. Post-exposure the snails were transferred in their cages to fresh clean water at 18-20°C. After 24 hours all snails were crushed individually between two glass microscope slides and the number of metacercarial cysts contained in each was counted and recorded. Two replicate populations of 100 snails were exposed for each of the host dispersion patterns examined.

### 6.2.3 RESULTS

The results displayed in Table 6.2 show the levels of transmission of E. recurvatum cercariae in experimental populations of L. peregra snails showing different patterns of dispersion. The lowest degree of cercarial transmission success was observed in the host populations exhibiting an Ideal Regular dispersion pattern while the highest was found in the host populations with Contagious ("Clumped") distribution.

The mean percentage prevalence of infection attained increased from just 57.5% (+/- 2.9%) in the ideal regular dispersed host populations to 95.5% (+/- 0.7%) in the case of the contagiously dispersed populations. Statistical t-tests on cyst counts transformed by  $\text{Log}_{10}(x+1)$  (as recommended by Elliot 1983) showed that the mean numbers of cercariae establishing as cysts in contagiously dispersed populations were significantly higher than those establishing as cysts in either the randomly dispersed populations,  $P(t=11.25) < 0.01$   $df=398$ , or the ideal regular populations,  $P(t=15.3) < 0.01$   $df=398$ . The mean number of cercariae establishing as cysts in the randomly dispersed snail populations was also found to be significantly higher than that in the regularly dispersed populations,  $P(t=2.9) < 0.01$   $df=398$ . Further, the results of the entire study taken together indicate that both the mean prevalence of infection and also the mean number of cysts recovered per snail increased significantly with the number of snails per cage, (see Table 6.3). The positive relationship between the mean number of cysts per snail and the number of snails per cage (host clumping) was found to be



**TABLE 6.2 The influence of host snail dispersion pattern on the transmission of *Echinoparyphium recurvatum* cercariae in experimental populations of *Lymnaea peregra*.**

Dispersion pattern of experimental snail populations	Variance to mean ratio (Index of snail dispersion)	No. of snails in each experimental population	No. of experimental populations examined	Mean (+/- SE) % of populations infected (Prevalence)	Mean no. (+/- SE) cysts per snail	Mean no. (+/- SE) cysts per infected snail (Intensity)	Overall Cercarial Transmission Success*
<b>IDEAL REGULAR</b>	0	100	2	57.5 (+/- 2.9)	3.3 (+/- 0.3)	5.8 (+/- 0.1)	16.7
<b>RANDOM</b>	1	100	2	67.5 (+/- 1.1)	4.9 (+/- 0.5)	7.2 (+/- 0.6)	24.4
<b>CONTAGIOUS "Clumped"</b>	10	100	2	95.5 (+/- 0.7)	11.1 (+/- 1.2)	11.6 (+/- 1.6)	55.1

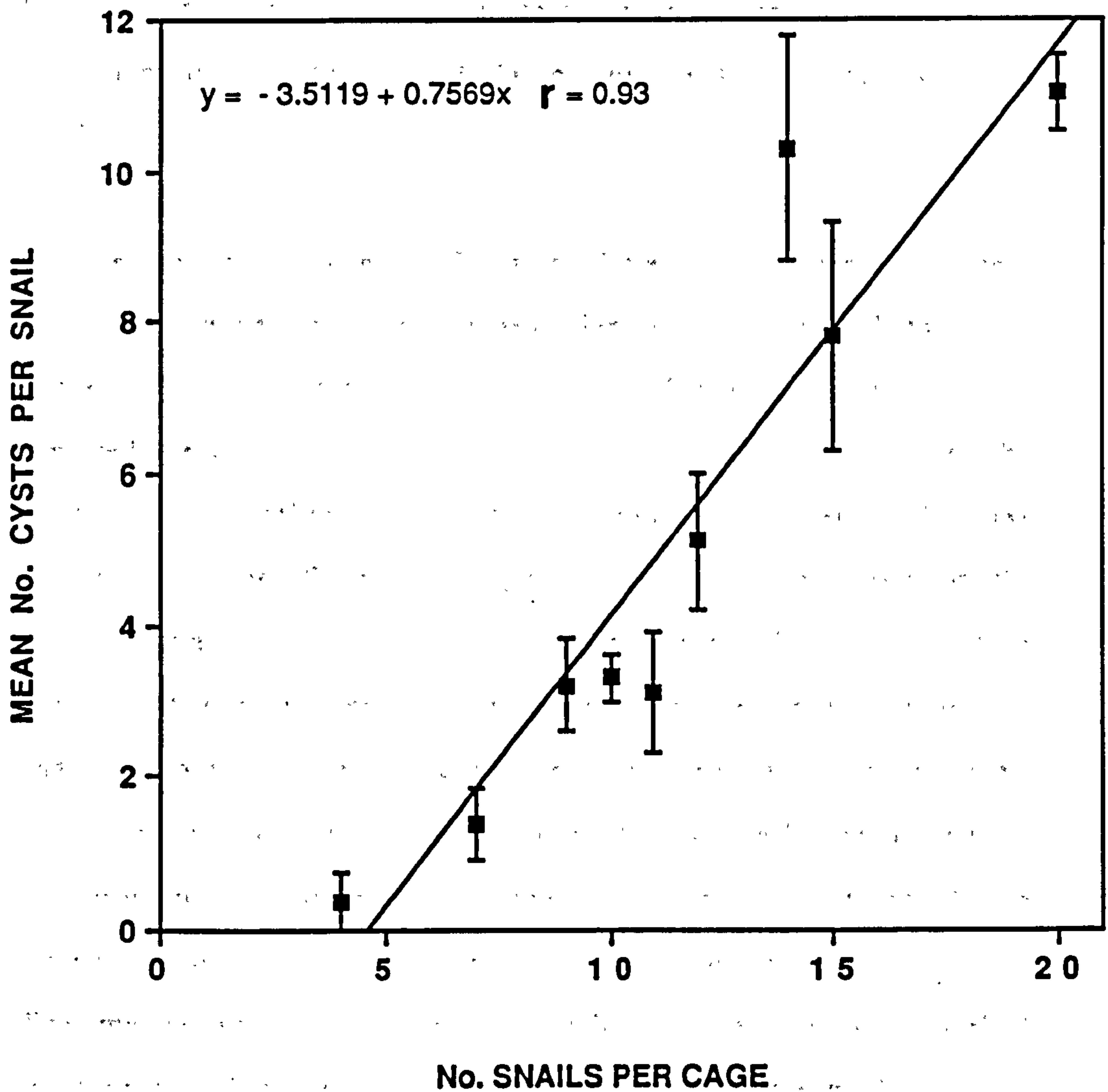
\* Overall Transmission success = (Total no. cysts established / Total no. cercariae) x 100

**TABLE 6.3** The influence of the degree of host snail contagion upon the transmission of Echinoparyphium recurvatum cercariae

Degree of snail host contagion (No. of snails per cage)	No. of snails examined	Overall prevalence of infection (% of snails infected)	Mean no. (+/- SE) cysts per snail
4	8	12.5	0.38 (+/-0.38)
7	14	50	1.4 (+/-0.46)
9	54	51.9	3.2 (+/-0.6)
10	220	57.1	3.3 (+/-0.3)
11	22	63.6	3.1 (+/-0.8)
12	24	83.3	5.1 (+/-0.9)
14	28	89.3	10.3 (+/-1.5)
15	30	76.7	7.8 (+/-1.5)
20	200	95.5	11.02 (+/-0.5)

FIG 6.7 The relationship between the degree of host contagion (no. of snails per cage) and the infection success of Echinoparyphium recurvatum cercariae (mean no. of cysts per snail).

(Points indicate observed data and the solid line indicates the best-fit linear function of the form given in the equation. Vertical lines indicate standard errors of the means.)





significant,  $P(r=0.93) < 0.05$   $df= 7$ , and linear over the range of snail densities per cage examined, see Fig 6.7.

#### 6.2.4 DISCUSSION

The findings of the present study appear to indicate that under experimental conditions cercarial transmission occurs at a higher rate in snail host populations showing a high degree of contagion relative to that which is achieved in snail populations showing low degrees of contagion. The fact that snails in cages containing larger numbers of snails were found to carry the highest parasite burdens also seems to suggest that larger clumps of snails, per unit habitat volume, attract larger numbers of cercariae than smaller clumps.

Studies on the influence of intermediate host dispersion pattern upon the transmission of digenean larvae are rare and therefore scope for comparison is very limited. However, Evans (1984) reported very similar findings to those reported herein when he studied the influence of the dispersion pattern of first intermediate host Biomphalaria glabrata snail populations on the transmission of Schistosoma mansoni miracidia. This author reported that experimental cages containing the larger numbers of snails attracted more miracidia and had higher infection rates. Furthermore, he also reported that the overall prevalence of infection which occurred in each experimental host population increased steadily as the degree of host contagion was increased. The mechanism responsible for producing the observed marked relationship between the degree of snail intermediate host contagion and the transmission success of both E. recurvatum

cercariae in the present study, and that of S. mansoni miracidia, may lie in the way in which these parasite larvae locate their target hosts. Schistosome miracidia are known to host-locate by responding to chemical conditioning of the environment by intermediate host snails. Therefore larger clumps of snails could be expected to produce a greater intensity of conditioning, a greater field of "target stimulus", and therefore would be expected to attract larger numbers of larvae. This could provide a feasible explanation for the patterns of transmission observed by Evans (1984), as was in fact suggested by him. A similar explanation would equally well explain the patterns of E. recurvatum cercarial transmission observed herein in relation to snail host contagion. However, although the explanation may seem plausible, at the moment we do not know whether E. recurvatum cercariae host-locate or respond to chemical conditioning of the environment by L. peregra snails. Reports in recent years by Motzel & Haas (1985) for Isthmiophora melis and Anderson & Fried (1987) for Echinostoma revolutum suggest that echinostome cercariae are capable of responding to chemical stimuli produced by second intermediate hosts. In order to determine whether in fact E. recurvatum do host-locate by responding to chemical conditioning of the environment, and hence whether this host-location mechanism could provide an explanation for the patterns of cercarial transmission observed in this study, a further study was carried out. This study is described the next section of this chapter.

Another factor which could have been responsible for the fact that cercarial transmission increased as snail clumping increased is one



for which some published experimental evidence already exists. Larger clumps of snails could reasonably be expected to constitute larger collective physical targets for cercariae. Evans & Gordon (1983a) have already shown in a previous detailed experimental study on E. recurvatum cercariae, using L. peregra snails as second intermediate hosts, that the target size presented by a host snail is an important determinant of cercarial transmission success. Transmission success was in fact found to increase linearly with increasing host snail size in this study.

### 6.3 Evidence for a chemotactic component of host-location in cercariae of Echinoparyphium recurvatum

#### 6.3.1 INTRODUCTION

The fact that schistosome miracidia locate intermediate host snails by responding to chemical conditioning of the environment has been known for several years (Chernin 1970 ; MacInnis 1975 ; Saladin 1979). Disko & Weber (1979) claimed to have discovered the "key" substance responsible for the chemo-location of Biomphalaria glabrata snails by Schistosoma mansoni miracidia. The substance, "Glutathione", was reported by these authors to be emitted naturally, especially from a region between the foot and edge of the shell in B. glabrata.

As was stated in the previous section of this chapter, Evans (1984) suggested that the patterns of S. mansoni miracidial transmission that he observed in populations of B. glabrata snails showing different degrees of contagion could be explained in terms of the



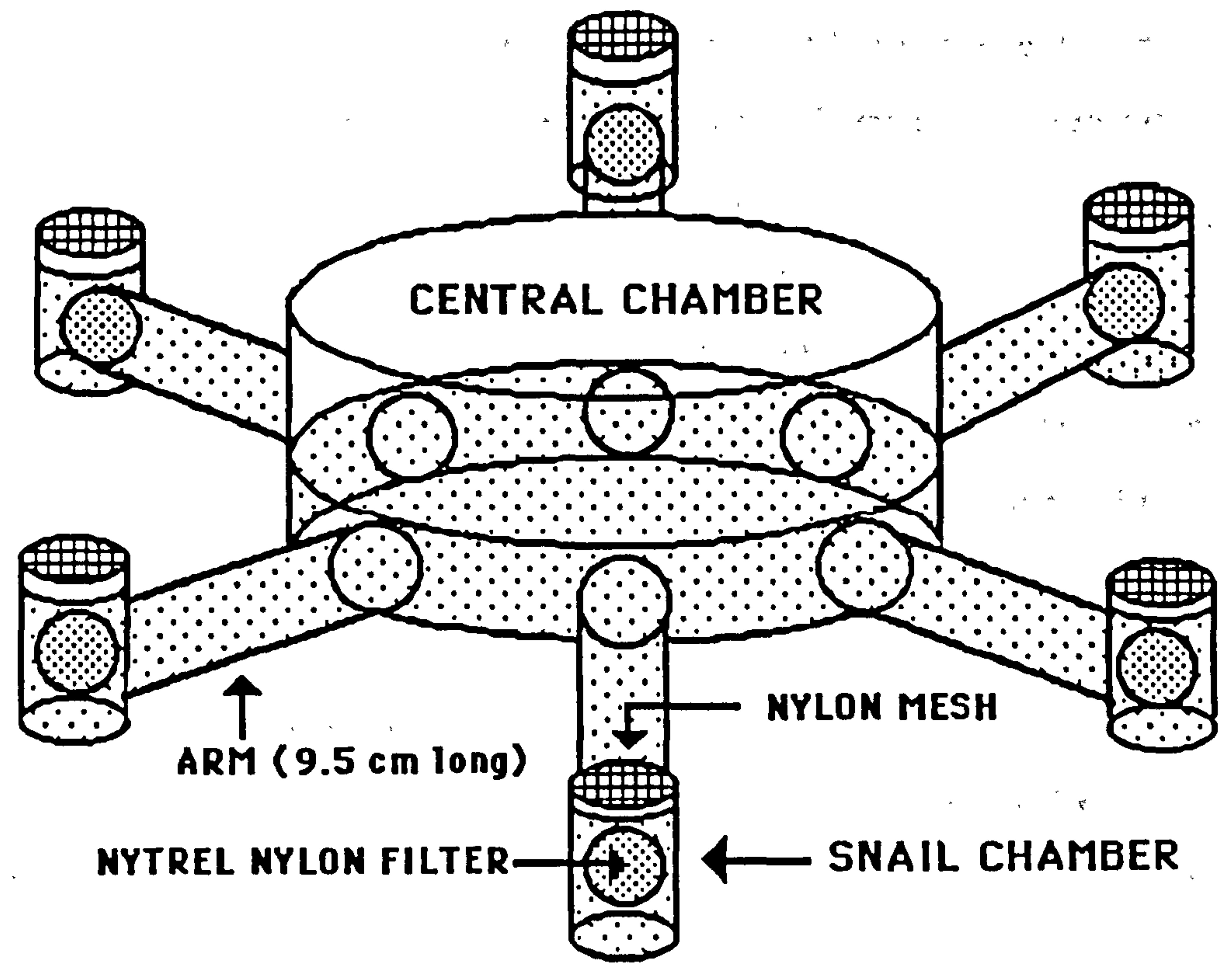
chemo-location behaviour of the miracidia. It is also possible that the patterns of E. recurvatum cercarial transmission in populations of L. peregra showing varying degrees of contagion observed in the previous section could also be explained by the existence of a chemotactic host-location mechanism, and the efficiency of this mechanism in locating the chemo-stimulus produced by larger, rather than smaller, clumps of potential host snails.

At present it is not known for certain whether E. recurvatum cercariae do host-locate by responding to chemical secretions from potential snail hosts. Evidence in the literature, although scant, suggests that echinostome cercariae can and do respond to chemical secretions from snail second intermediate hosts. A recent report by Anderson & Fried (1987) has suggested that cercariae of Echinostoma revolutum respond to renal secretions of second intermediate host snails. Interestingly a communication in abstract form by Dixon (1979) reports that cercariae of E. recurvatum show distinct responses when placed in snail (L. peregra) extract, responses which increase with increase in snail extract concentration. The present study set out to determine whether further evidence existed for a host-location mechanism in E. recurvatum cercariae based on response to chemical conditioning of the environment by potential snail hosts.

### 6.3.2 MATERIALS AND METHODS

The apparatus used in this study is illustrated in Fig 6.8. It is basically a six-armed choice chamber and is a modification on a design of a four-armed metal apparatus known as the "Chemotrometer" used by Etges & Decker (1963) to test for

FIG 6.8 The "Chemotrometer" apparatus used in this study



evidence of chemotaxis of S. mansoni miracidia among snail first intermediate hosts. The chemotrometer used in the present study was constructed from glass and clear polystyrene. The central chamber was formed by a polystyrene tub and the side arms were made of 9.5 cm lengths of 15 mm bore pyrex glass. The terminal snail chambers used were 20ml clear polystyrene pots.

In order to prevent movement of experimental snails from the terminal chambers into the side arms, and to prevent direct contact of snails with cercariae, each snail chamber was separated from its arm by a porous monofilament polyamide filter (NYTREL TI) with a 40 micron pore size. This filter was large enough to allow passage of dissolved chemicals but small enough to prevent passage of cercariae. The top of each chamber was covered using nylon mesh to prevent escape of snails. In their experiment Etges & Decker (1963) "cracked" the shells of their test snails to prevent them moving from their terminal chambers. This method was not used in the present study as it is open to the severe criticism that leaked blood and other body fluids from the damaged snails could act as chemo-attractants which under normal conditions would not be expected to be released.

Experiments were carried out by placing one group of 15 laboratory-bred Lymnaea peregra snails (5-7mm size range) into each of three of the terminal chambers. Prior to use the snails had been maintained on a diet of boiled lettuce. The groups of snails were allotted to the chambers randomly by use of random numbers electronically generated using a CASIO fx 7000G scientific calculator. Three chambers were left empty as controls.



The apparatus was filled with 280ml of synthetic hard water medium (HMSO 1969) and was allowed to stand for 12 hours to allow any substances produced by the snails to diffuse into the water. Five hundreded E. recurvatum cercariae, collected within one hour of their emergence from a pool of naturally infected L. peregra obtained from Harting Pond, were introduced into the centre of the central chamber. The experimental set-up was then allowed to stand for 6 hours at 20°C, illuminated overall by a light intensity of 1600 lux. After this time the water in each of the glass arms were sealed off with tight fitting cork bungs, and the snail chambers were sealed using snap-fit plastic lids. The water in the central chamber was then removed and the water from each individual side arm was drained into a separate container. The cercariae contained in each sample were then fixed by addition of 10% Formalin, counted exhaustively using a stereo-microscope and the number recorded. The experiment was repeated 10 times. Each time the apparatus was washed thoroughly in several changes of distilled water.

### 6.3.3 RESULTS

After a period of 12 hours a mean of 83.3% (+/- 2.6%) of cercariae were found to have entered the side arms of the apparatus. A mean number of 279.2 (+/- 10.1) cercariae per replicate experiment were found to be located in the three arms leading to the chambers containing snails with only 137.1 (+/- 12.9) cercariae per replicate being recovered from the three arms leading to the empty control chambers. A t-test on counts of cercariae transformed by  $\text{Log}_{10}(x)$  showed the difference in the means to be very highly significant,  $P (t=32.9) < 0.01$   $df=18$ . This

indicates that cercariae entering the side arms showed a marked preference for those with living snails in their terminal chamber.

#### 6.3.4 DISCUSSION

The results of this study would seem to be indicative of a significant chemotactic component in the location of second intermediate host snails by E. recurvatum cercariae. They are in fact similar to those of a study carried out by Etges and Decker (1963) which demonstrated a significant degree of chemotaxis in S. mansoni miracidia among first intermediate host snails. The results obtained herein suggest that E. recurvatum cercariae are capable of responding to chemical conditioning of the environment by host snails. Whether the cercariae are attracted along a gradient of the stimulus, or whether they are held within the field of stimulus once they have located it by random swimming is not yet known.

Cercarial response to host snail chemical stimuli has been reported previously for freshwater echinostome cercariae. Indeed, Dixon (1979) has already previously demonstrated response to L. peregra extracts by E. recurvatum. Anderson & Fried (1987) suggested that Echinostoma revolutum cercariae respond to renal secretions of second intermediate host snails emitted via the nephridiopore of the snail and that these secretions are involved in cercarial penetration and site location for encystation. Fried & King (1989) have very recently demonstrated experimentally that E. revolutum cercariae show chemo-attraction to dialysate from second intermediate host Biomphalaria glabrata snails. Several



studies by Haas and co-workers have interestingly shown that the attachment response of a range of digenetic cercariae is stimulated by water soluble chemical stimuli produced by the target host. Cercariae of Diplostomum spathaceum have been shown by Haas (1975) to respond to  $\text{CO}_2 + \text{H}_2\text{CO}_3$  produced by intermediate host fish, and Granzer & Haas (1986) have shown that S. mansoni cercariae respond to arginine secreted from target definitive hosts. Motzel & Haas (1985) have also shown that cercariae of the echinostome Isthmiophora melis respond to  $\text{CO}_2 + \text{H}_2\text{CO}_3$  and  $\text{HCO}_3^-$  produced by fish and amphibian intermediate hosts.

Studies on the chemo-location of snail intermediate hosts by schistosome miracidia have isolated several chemical components of host secretions that miracidia respond to and use to chemo-locate potential hosts. Among these are amino acids, fatty acids and glutathione, the latter being considered by Disko and Weber (1979) to be the key substance to which S. mansoni miracidia respond. It is possible that E. recurvatum cercariae also respond to these chemical components of snail secretions. It is also possible that they may respond to increased  $\text{CO}_2$  concentrations which could be expected to occur in the region of actively respiring second intermediate host snails. However, chemical analysis of snail conditioned water, followed by individual testing of cercarial response to each of its individual chemical components, will be necessary before we can begin to understand to which host chemicals E. recurvatum cercariae can respond. A recent study by Feiler & Haas (1988) has carried out a similar investigation to this



on the chemical stimuli of duck skin to cercarial attachment of Trichobilharzia ocellata cercariae.

The present study has suggested that E. recurvatum cercariae do seem to show a significant chemotactic component of snail second intermediate host-location. It therefore seems that the patterns of cercarial transmission among L. peregra experimental host populations showing different degrees of clumping observed in the previous section of this chapter may in fact be to some extent explained in terms of a chemotactic component in the host location mechanism of the cercariae. The results of the latter study indicated that larger clumps of snails attracted larger numbers of cercariae. In the light of the results of the present study it may therefore be that larger clumps of snails produce a larger field of target chemical stimulus which makes them more easily located by cercariae than smaller clumps which would not be expected to be so easily "chemically perceived" by cercariae.

The present study was conducted in the laboratory of the Department of Zoology, University of Lagos, Lagos, Nigeria. The study was carried out from 1980 to 1982. The study was carried out in the laboratory of the Department of Zoology, University of Lagos, Lagos, Nigeria. The study was carried out from 1980 to 1982.

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## Chapter 7

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### Experimental studies on

### *Echinoparyphium recurvatum*

### in the intestine of a wildfowl definitive host,

### the Mallard *Anas platyrhynchos*

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## Experimental studies on Echinoparyphium recurvatum in the intestine of a wildfowl definitive host, the Mallard Anas platyrhynchos

### 7.1 General Introduction

Our knowledge of the biology of Echinoparyphium recurvatum in the intestine of definitive hosts is limited to two previous studies, both restricted to observations on the development and survival of the parasite. In North America, Senger (1954) observed the development and survival of E. recurvatum in the fowl Gallus gallus. Evans (1983b) in Britain studied the establishment and survival of E. recurvatum in the intestine of the Mallard Anas platyrhynchos. Evans (1983b) discovered that in A. platyrhynchos gravid worms were first discovered at 6 days post-infection, maximum survival time in the intestine was 28 days and that 50% survival occurred at 12.5 days.

Virtually nothing is known of the influence that factors relating to the initial infection dose of E. recurvatum metacercarial cysts ingested by wildfowl definitive hosts have on the subsequent condition of the parasites in the intestine. This represents a considerable gap in our knowledge of the transmission of E. recurvatum to wildfowl hosts. The work presented in this chapter aimed to extend our knowledge of E. recurvatum in the intestine of A. platyrhynchos by providing some preliminary information on the potential effects on the parasite exerted by variations in the size, age, temperature treatment and second intermediate host origin of initial doses of metacercarial cysts.



Evans (1983b) observed that E. recurvatum adults show a marked spatial localization in the anterior intestine of A. platyrhynchos. As a precursor study it therefore was also decided to determine whether in fact this was the site in which parasites initially established.

## 7.2 The spatial distribution of Echinoparyphium recurvatum in the intestine of Anas platyrhynchos 24 hours and 15 days post-infection

### 7.2.1 INTRODUCTION

Several adult helminths exhibit marked spatial localization in the gut of A. platyrhynchos. For example, the digenean Apatemon gracilis minor occurs in the anterior third of the small intestine and the acanthocephalan Polymorphus minutus preferentially occupies a site in the posterior half of the small intestine (Crompton & Joyner 1980).

Evans (1983b) reported that E. recurvatum adults show a marked spatial localization in the anterior quarter of the intestine of A. platyrhynchos. The present study set out to determine whether the site of initial establishment of the parasite 24 hours post-infection was similar to that occupied by mature worms 15 days post-infection.

### 7.2.2 MATERIALS AND METHODS

Batches of laboratory bred, infection-free, Lymnaea peregra snails were exposed *en masse* in synthetic hard water medium (HMSO

1969) at 20°C to E. recurvatum cercariae collected within 1 hour of their emergence from naturally infected specimens of first intermediate host L. peregra obtained in May 1985 from Harting Pond, West Sussex. The infected snails were maintained on a diet of lettuce at a temperature of 18-20°C for 14 days. Metacercarial cysts of E. recurvatum were dissected from these snails 14 days post-infection and were fed in gelatine capsules in doses of 100 cysts per bird to two separate groups (one of three and the other of four) infection-free 3 day-old Khaki Campbell ducklings (A. platyrhynchos).

The ducklings were then maintained on a diet of non-medicated chick crumbs and water fed *ad libitum*. One group, three ducklings, was sacrificed 24 hours post-infection and the other group, four ducklings, was sacrificed 15 days post-infection by cervical dislocation. The entire intestine in each case was removed from the pylorus to the anus and placed in a surgical tray filled with warm (40°C) saline (0.75% NaCl). The entire length of each intestine was measured and then divided into 5% sections, its total length. Each section was then individually isolated in a petri dish containing fresh warm saline, opened by a longitudinal incision and searched thoroughly for parasites. The gut caeca were isolated and searched separately for parasites. All ducklings were autopsied between 12.00 and 15.00 hours. The number of worms recorded from each section of the intestine was counted and recorded.

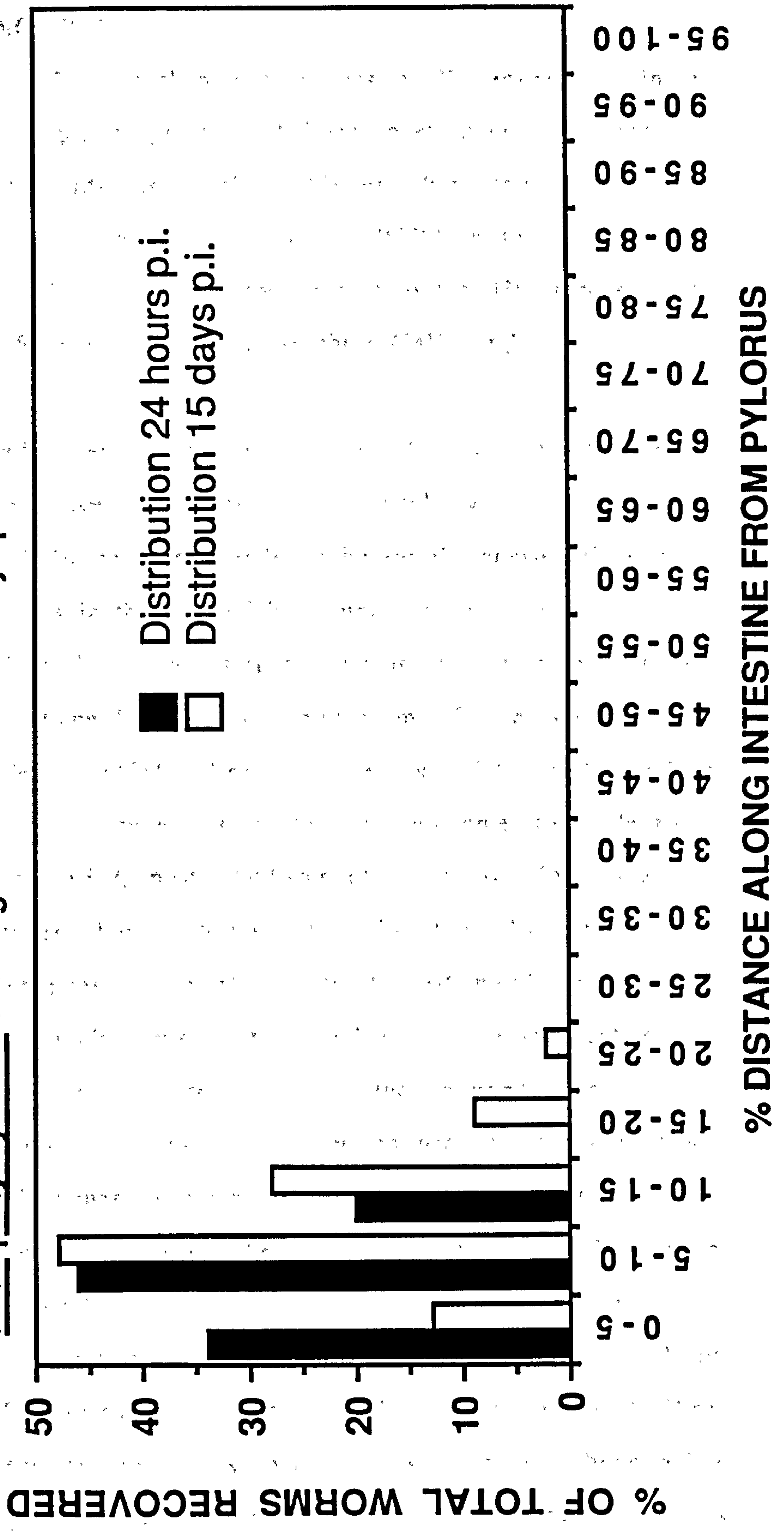
### 7.2.3 RESULTS

At 24 hours post-infection a total of 116 excysted worms were recovered from the intestines of the three ducklings infected with a total of 300 metacercarial cysts. This represents the initial establishment of parasites in the intestine and using this criterion 38.7% of the infective stages administered became established. The mean number of worms recovered per host was 38.7 (+/- 3.5, S.E.). All the parasites recovered at this stage were obtained from the anterior 15% of the intestine. Their distribution along the intestine is illustrated in Fig 7.1.

At 15 days post-infection a total of 84 adult worms were recovered from the intestines of the four ducklings infected with a total of 400 cysts. The mean number of worms recovered per host was 21.0 (+/- 1.9). This indicates that 21% of the infective stages initially administered in this experiment survived to this age. All the parasites recovered were gravid and were obtained from the anterior 25% of the intestine. Their spatial distribution in the intestine is shown in Fig 7.1. Comparison of the distribution of the initial establishment stages and that of adult worms 15 days post-infection would seem to suggest that little substantial migration of worms occurs after initial establishment in the anterior intestine of A. platyrhynchos. However, it should be noted that a definite increased posteriad spread of worms was observed at 15 days p.i. The spatial distribution of worms at 15 days p.i. extended from the pylorus to a distance 20-25% along the intestine whereas at 24 hours p.i. the distribution of establishment phase worms extended from the pylorus to a distance only 10-15% along the intestine.



**FIG 7.1 The spatial distribution of Echinoparyphium recurvatum in the intestine of Anas platyrhynchos ducklings 24 hours and 15 days p.i.**



#### 7.2.4 DISCUSSION

The initial 38.7% establishment success of E. recurvatum in the intestine of A. platyrhynchus 24 hours post-infection observed in the present study is slightly higher than the 31% initial establishment rate observed by Evans (1983b) in the same host at 12 hours p.i.. It is in fact slightly closer to the 43% recovery rate reported by Senger (1954) from the chick Gallus gallus.

The marked overlap in the spatial distributions of initial establishment stages 24 hours p.i. and adult worms 15 days p.i. seems to indicate that little substantial post-establishment migration occurs in the case of E. recurvatum in the intestine of A. platyrhynchus. It is interesting to compare this with the situation in the echinostome Echinostoma revolutum. In the domestic chick Fried & Weaver (1969), Fried & Alenick (1981) and Fried & Freeborne (1984) have shown this echinostome to undergo a substantial posteriad, post-establishment, migration down the gut. These authors have shown that in the domestic chick establishment phase E. revolutum are located mainly in the ileum while mature adult worms are located mainly in the rectum and cloaca. In the case of E. recurvatum, the observed distribution of worms 15 days p.i. would seem to be, in part at least, determined by the establishment process. A similar observation to this was made by Evans (1977) on the digenean freshwater fish parasite Asymphyiodora kubanicum which was found to excyst, establish, and remain distributed exclusively in the first intestinal limb of the Roach Rutilus rutilus. In addition, Kennedy, Broughton & Hine (1976) described the sites occupied by the acanthocephalan Pomphorhynchus laevis in the alimentary canal of a range of fishes

fishes and concluded that the observed distributions were determined primarily by the establishment process and that little substantial post-establishment migration occurred.

The marked spatial localization of E. recurvatum in the anterior intestine of A. platyrhynchos is in contrast to that of another echinostome species Echinostoma revolutum, the mature adults of which occupy a site mainly in the lower intestine, the rectum cloaca region, in domestic chicks (Fried 1984). Marked spatial localizations of echinostomes in the intestines of vertebrate definitive hosts may have potential advantages relating to the reproductive biology of the parasites. Localization of adult worms in discrete sites could be expected to promote the chances of contact and "pairing"- cross fertilization- between worms such as reported by Fried & Pallone (1984). This is important since recent findings by Balogun & Whitfield (unpublished) on Echinostoma liei have indicated that restriction of cross-fertilization opportunities in adult echinostomes eventually has severely detrimental effects on the reproductive potential of the parasites.



### 7.3 The influence of the size of the initial metacercarial cyst infection dose on the establishment, spatial distribution, size and *in utero* egg number of Echinoparyphium recurvatum in the intestine of Anas platyrhynchos

#### 7.3.1 INTRODUCTION

The influence of the population density of helminth parasites in vertebrate host guts on parasite size, spatial distribution and reproductive capacity has been studied by a number of authors. For example Jones & Tan (1971) studied the influence of population density on the size and reproductive capacity of the cestode Hymenolepis microstoma in the gut of the laboratory mouse. More recently Fried & Freeborne (1984) and Franco, Huffman & Fried (1988) have studied density dependent, "crowding", effects on worm size, *in utero* egg number and spatial distribution of Echinostoma revolutum in the intestine of domestic chicks and golden hamsters. However, to date no studies on population-density-dependent effects on E. recurvatum adults in the gut of a definitive host have been carried out. Field observations on naturally infected second intermediate host molluscs (see Evans, Whitfield and Dobson 1981) indicate that in the natural environment wildfowl definitive hosts are likely to be exposed to doses of metacercarial cyst infections of various sizes. The present study therefore set out to determine the effect of the size of the initial cyst infection dose on the initial establishment of E. recurvatum in the intestine of A. platyrhynchos. It further aimed to examine the influence of worm population density on the parasites' spatial distribution, size, and number of *in utero* eggs at

15 days post-infection in order to provide a basis for comparison of these characteristics with those obtained by Fried & Freeborne (1984) and Franco, Huffman & Fried (1988) for Echinostoma revolutum of different population densities.

### 7.3.2 MATERIALS AND METHODS

Metacercarial cysts of E. recurvatum were obtained 14 days post-infection from lab-bred L. peregra snails as described previously. Cysts were fed in gelatine capsules in doses of 20, 50, 100 and 250 cysts to separate groups each composed of either 5 or 6, 3 day-old, Khaki Campbell ducklings (A. platyrhynchos). The birds were then maintained on a diet of non-medicated chick crumbs and water fed *ad libitum*.

Three ducklings from each group were sacrificed and autopsied 24 hours post-infection. The number of worms present in the entire intestine of each duckling was counted in each case in order to provide an estimate of the initial percentage establishment. The remaining ducklings in each of the four groups were autopsied 15 days post-infection. Their intestines were removed as described in previous section and each intestine was divided into 5% sections of its total length. Each section was searched individually in a petri dish of warm (40°C) saline (0.75% NaCl) and the number of worms that it contained was recorded.

Worms recovered from the ducklings of each initial cyst dose size group were pooled in a petri dish of warm saline and then transferred, one worm per well, into wells of clear polystyrene culture plates. Each worm was allotted a number and a random



sample of 20 worms from each of the four groups was selected for measurement using random number tables.

Worms selected for measurement were fixed in Berland's Fluid. This fixative is composed of 95 parts by volume glacial acetic acid and 5 parts 40% formalin. After 30 minutes to 1 hour this fixative had the effect of relaxing and uncurling the worms. The worms were then dehydrated through an ethanol series cleared in xylene and prepared as temporary unstained whole mounts using "Ralmount". The length and maximum (mid-acetabular) width of each worm was then measured using a microscope fitted with a calibrated ocular micrometer in order to provide a first order estimate of the projected body area of each worm - length x mid-acetabular width. The number of *in utero* eggs of all worms recovered from each group of ducklings (including those fixed for measurement) were then counted and recorded. This was possible in the case of E. recurvatum since the eggs of this parasite are large and relatively few in number compared to those of other echinostomes such as Echinostoma revolutum and Echinostoma liei. The method of worm measurement used herein is that standard recommended by Fried and co-workers at Lafayette College, Pennsylvania, eg. Fried & Freeborne (1984), Hosier & Fried (1986), Franco, Huffman & Fried (1988), in their studies on infectivity, growth, distribution and crowding of Echinostoma revolutum in chick, mice and hamster intestines. This method was adopted as a previous pilot study had indicated that measurements of live worms, as recommended by Nollen (1983) for Philopthalmus gralli, would prove impractical since E. recurvatum adults have been found to curl very badly in saline or water.



### 7.3.3 RESULTS

The results shown in Table 7.1 show that the numbers of E. recurvatum established in the intestine of A. platyrhynchos 24 hours post-infection increased with increase in the size of the initial cyst infection dose. The correlation between the size of the cyst dose and the number of worms establishing 24 hours p.i. was found to be highly significant  $P(r=1.0) < 0.01$ . The relationship was found to be well described by a linear function as shown in Fig 7.2. The percentage of E. recurvatum in each initial cyst dose that became established in the intestine 24 hours p.i. remained at a relatively constant level, between 36.1% and 45%. This result suggests that no direct relationship exists between the size of the initial cyst dose and percentage worm establishment 24 hours p.i. for the range of cyst dose sizes examined herein. Initial establishment of E. recurvatum in the intestine of A. platyrhynchos therefore appears to be density independent over the cyst dose size range 20-250.

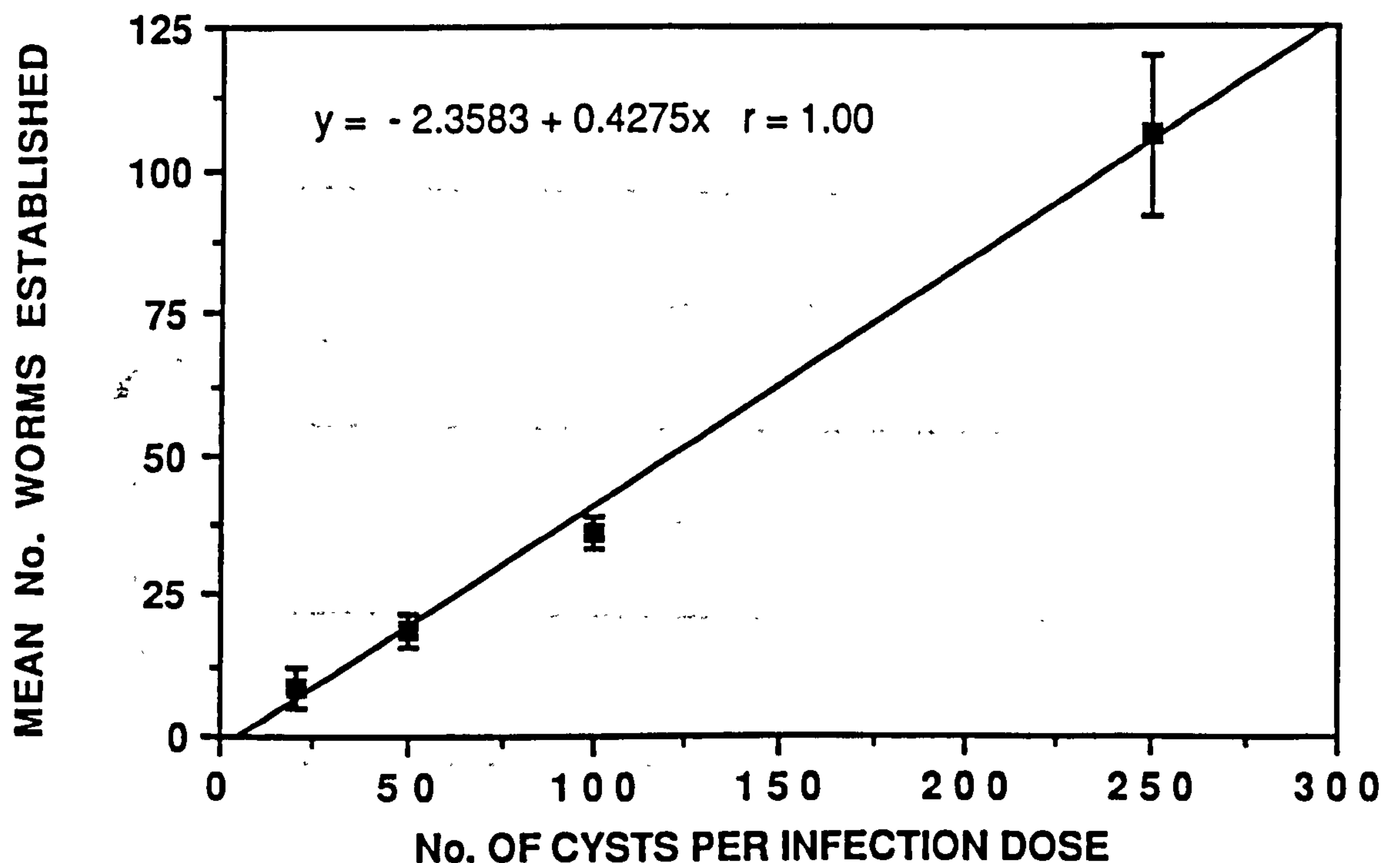
At 15 days post-infection the mean number of worms per host increased with increase in the size of the initial cyst dose, see Table 7.2. Hosts initially infected with a dose of 250 cysts contained a mean of 40.5 (+/- 2.1) worms while those infected with 20 cysts contained a mean of just 7.7 (+/- 1.5) worms per host. However, the percentage of the initial cyst dose administered surviving as adults at 15 days p.i. was found to decrease from 38.3% in hosts initially infected with just 20 cysts to only 16% in those initially infected with 250 cysts.

TABLE 7.1 The influence of the size of cyst infection dose on the initial establishment of Echinoparyphium recurvatum in the intestine of Anas platyrhynchos 24 hours post-infection

No. of cysts per infection dose	No. of hosts exposed	No. of hosts infected 24 hrs post-infection	Mean no. (+/-S.E.) worms recovered per host 24 hours post-infection	Establishment Success (% of initial cyst dose established 24 hours post-infection)
20	3	3	9 (+/-3.6)	45
50	3	3	19 (+/-2.9)	38
100	3	3	36.1 (+/-2.1)	36.1
250	3	3	106 (+/-14.3)	42.4

FIG 7.2 The relationship between size of initial cyst infection dose and the mean number of Echinoparyphium recurvatum worms establishing in the intestine of Anas platyrhynchos ducklings 24 hours post-infection

(Vertical bars indicate standard errors of the means and the solid line indicates the best-fit linear function of the form indicated in the equation)

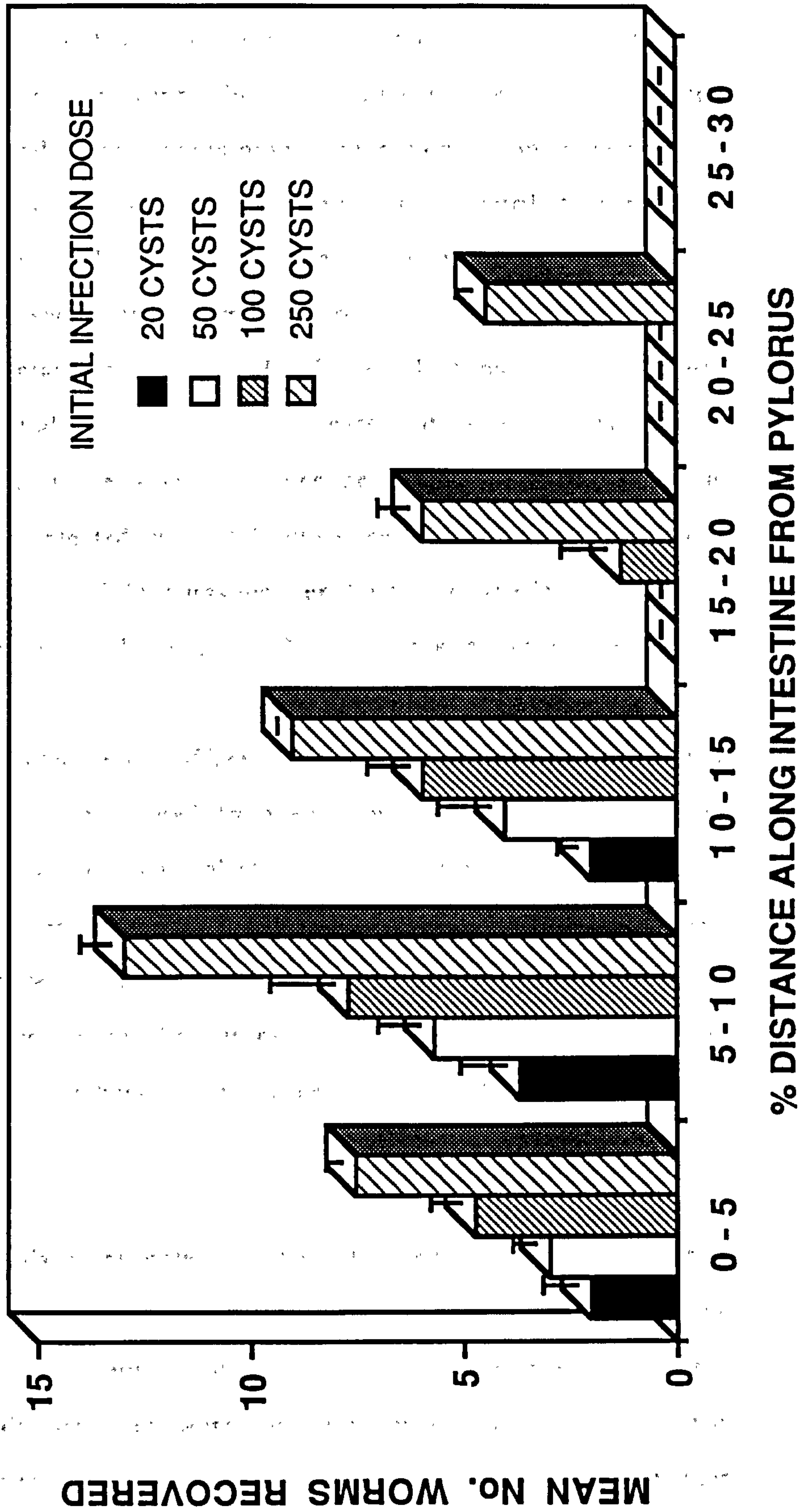


**TABLE 7.2** The influence of the size of the initial cyst infection dose on Echinoparyphium recurvatum worms in the intestine of Anas platyrhynchos ducklings 15 days post-infection.

No. of cysts in infection dose	No. of hosts exposed to infection	No. of hosts infected 15 days post-infection	Mean no. worms (+/-SE) recovered per host 15 days post-infection	% of initial cyst dose surviving as adults 15 days post-infection	Crowding Factor (mean no. (+/-SE) per infected 5% section of intestine)	Mean (+/-SE) estimated body area of worms (mm <sup>2</sup> )	% of total worms gravid	Mean no. (+/-SE) in utero eggs per worm
20	3	3	7.7 (+/-1.5)	38.3	2.5 (+/-0.52)	3.62 (+/-0.1)	100	59.3 (+/-2.1)
50	3	3	12.7 (+/-1.5)	25.3	4.2 (+/-0.7)	2.48 (+/-0.08)	100	45.9 (+/-45.9)
100	3	3	19.7 (+/-1.48)	19.7	5.9 (+/-0.7)	2.01 (+/-0.06)	98.3	32.1 (+/-1.8)
250	2	2	40.5 (+/-2.1)	16	8.1 (+/-0.97)	1.2 (+/-0.11)	57.5	7.1 (+/-1.1)



**FIG 7.3 The spatial distribution of *Echinoparyphium recurvatum* at 15 days post-infection in the intestines of ducklings infected with different sizes of cyst dose**



The spatial distributions of worms in the intestines of hosts initially infected with different sizes of cyst dose are shown in Fig 7.3. It is clear that worms in hosts initially infected with larger cyst doses, and hence containing larger population densities of worms 15 days p.i., showed extended spatial distribution relative to those worms in hosts initially infected with lower sizes of cyst dose. Fig 7.3 shows that worms in hosts initially infected with 20 cysts (and containing a mean of 7.7 (+/- 1.5) parasites per host) showed a spatial distribution which extended from the pylorus to only 10-15% distance along the intestine. In the case of worms in hosts initially infected with 250 cysts per host (and containing a mean of 40.5 (+/- 2.1) parasites per host) the spatial distribution extended from the pylorus to 20-25% distance along the intestine.

For hosts infected with different initial cyst dose sizes the mean number of worms recovered from each infected 5% section of host intestine 15 days p.i. was taken as an indication of the degree of "crowding" experienced by the parasite population in each case. Using this criterion crowding was found to be greatest in hosts initially infected with the largest cyst doses and containing the greatest mean numbers of parasites per host 15 days p.i., (see Table 7.2).

Important trends were noted in the estimated mean body size and mean number of *in utero* eggs of worms from populations showing different degrees of crowding. Both the mean estimated projected body area per worm and the mean number of *in utero* eggs per worm were found to decrease in an exponential manner with increase in worm crowding, (see Figs 7.4 and 7.5). In

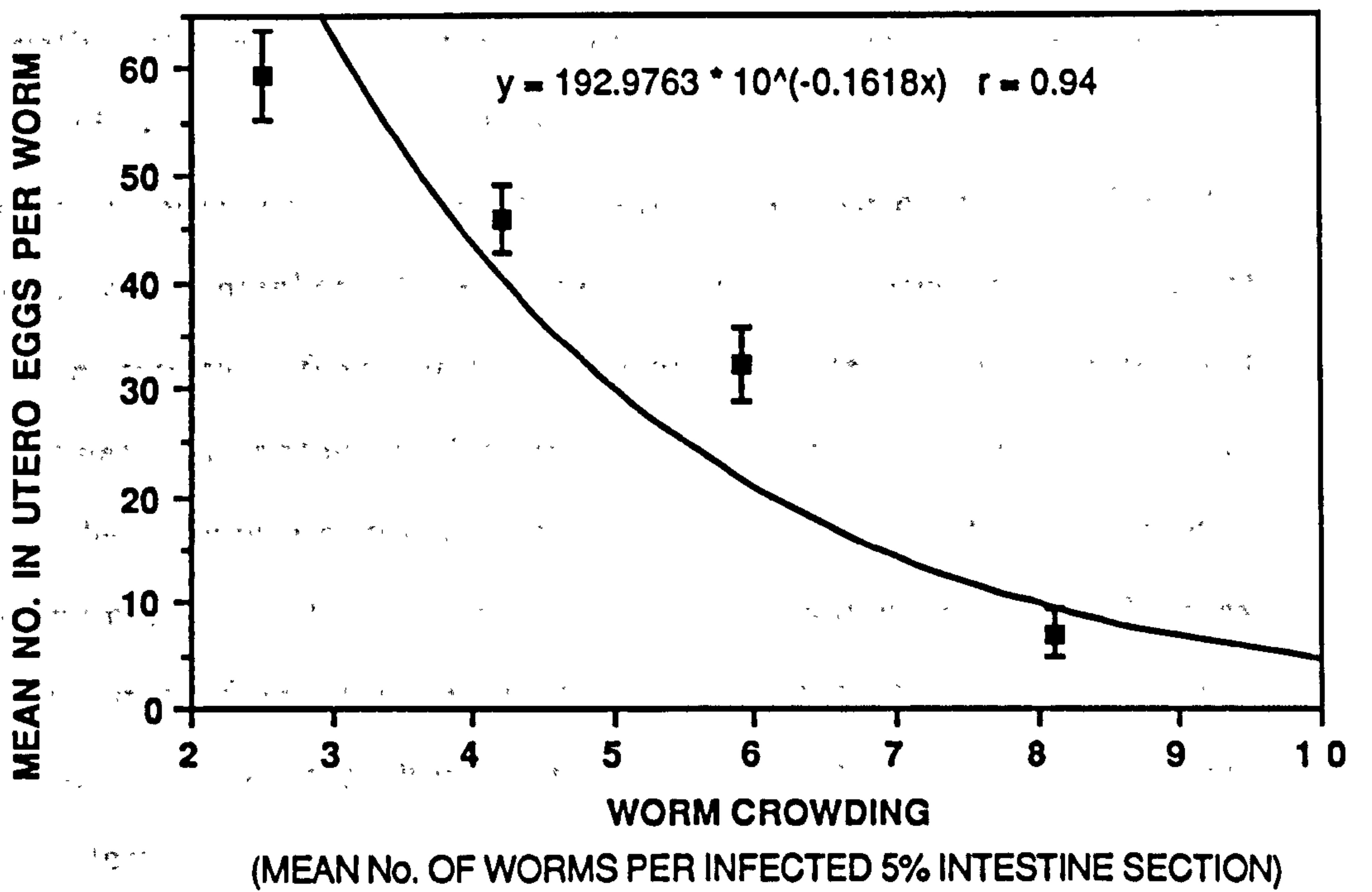
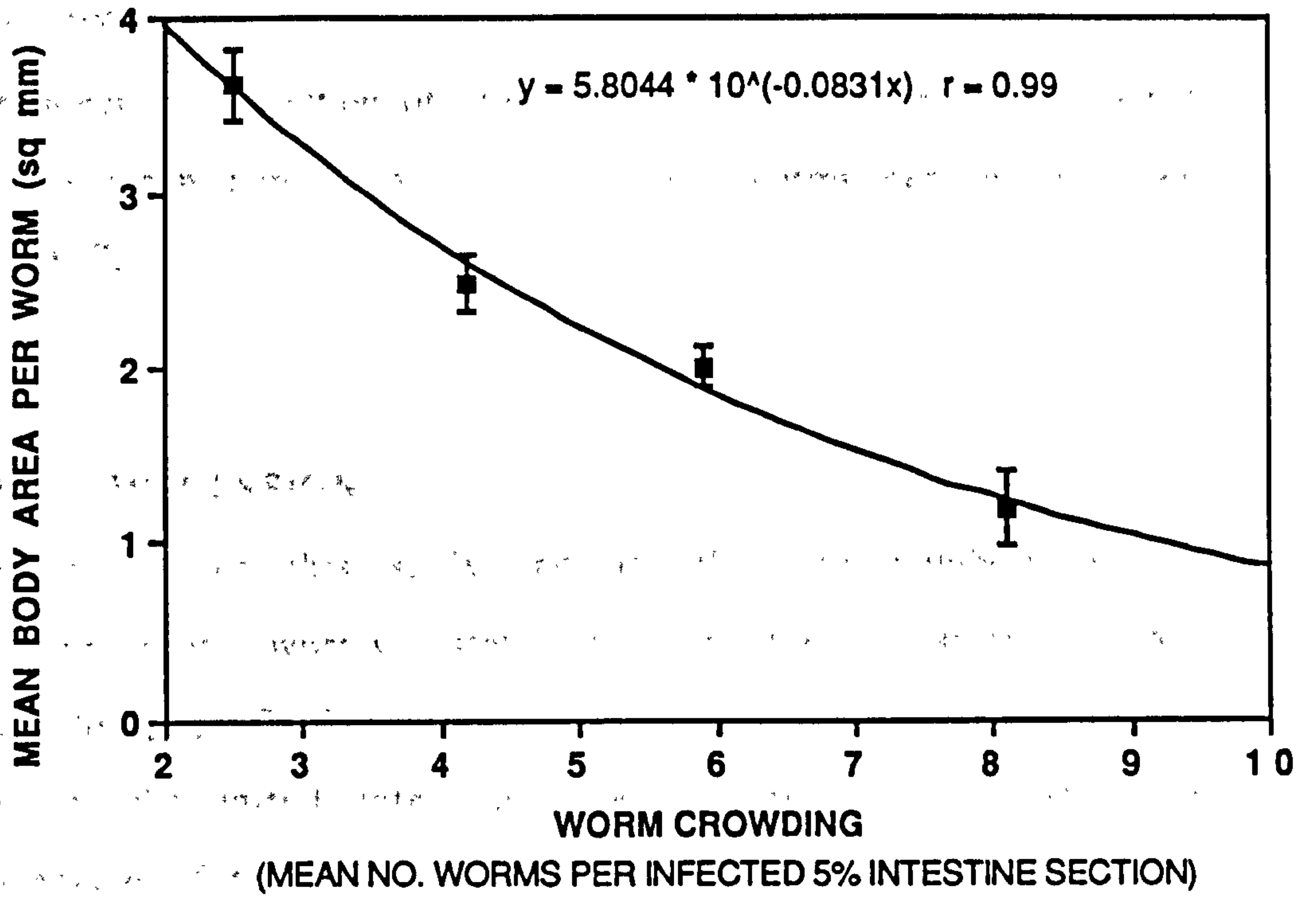
FIG 7.4 The decrease in estimated mean worm body area of Echinoparyphium recurvatum worms with increased parasite crowding in the gut of Anas platyrhynchos 15 days post-infection.

(Points indicate observed data and the solid line represents the best-fit negative exponential curve of the form indicated in the equation. Vertical bars indicate 95% confidence intervals of the means)

FIG 7.5 The decrease in mean number of in utero eggs per Echinoparyphium recurvatum worm with increase in parasite crowding in the intestine of Anas platyrhynchos 15 days post-infection

(Points indicate observed data, and the solid line represents the best-fit negative exponential curve of the form indicated in the equation. Vertical bars indicate 95% confidence intervals of the means)





addition, the percentage of gravid worms was found to decrease from 100% in hosts with a mean number of 2.5 (+/- 0.52) worms per infected 5% section of intestine to only 57.5% in hosts infected with a mean number of 8.1 (+/- 0.97) worms per section, (see Table 7.2).

#### 7.3.4 DISCUSSION

The results of this study indicate that the numbers of E. recurvatum worms establishing in the intestine of A. platyrhynchos 24 hours post-infection increases linearly with the size of the initial infection dose of metacercarial cysts. The percentage of the initial cyst dose establishing as juvenile worms in the intestine appears to be constant over the range of initial cyst dose sizes administered.

At 15 days post-infection marked differences in the population densities and degrees of worm crowding were observed in hosts initially infected with various sizes of cyst dose. In hosts initially infected with the largest size cyst dose of 250 cysts per host, high worm population density and crowding were responsible for producing a number of density-dependent or "crowding" effects of a type reported from other digenean parasites in vertebrate host intestines. Crompton & Joyner (1980) gave four characteristics of "crowded" helminth populations. These were (1) that the worms are numerous, (2) they show signs of stunted growth, (3) they show signs of reduced fecundity and (4) they occupy an extended spatial site in the host intestine. Evidence of all of these characteristic features was observed for E. recurvatum in

populations showing a relatively high degree of population density and crowding.

Detrimental effects of crowding on the body size and *in utero* egg number such as those observed in E. recurvatum worms herein have also been reported in the digeneans Zygocotyle lunata (Fried & Nelson 1978); Philopthalmus gralli (Nollen 1983) and Echinostoma revolutum (Fried & Freeborne 1984, Franco, Huffman & Fried 1988). In the case of E. revolutum Fried & Freeborne (1988) found that in the gut of the domestic chick the mean body area of E. revolutum worms and the mean number of *in utero* eggs per worm from crowded sites (> 25 worms) were significantly less than those from non-crowded sites (1-10 worms per site). In this study "site" was defined as either ileum, rectum, cloaca, caeca or bursa of Fabricius. Franco et al. (1988) also found that E. revolutum from crowded populations in the Golden Hamster also showed significantly reduced body area, and exhibited extended spatial distribution in the host gut. Mohandas & Nadakal (1978) also found that crowding reduced the body length of Echinostoma malayanum worms in host rats.

Crowding effects are generally explained by intense intraspecific competition among worms for limited spatial, nutritional or other physiological requirements. Crompton & Joyner (1980) have suggested that if stunted growth is caused by lack of space in crowded populations of the acanthocephalan Polymorphus minutus in the gut of A. platyrhynchos it is possible that the overcrowded worms may interfere with each others feeding activities and in this way be deprived of nutrients. Intraspecific competition for



limited resources is almost certain to be primarily responsible for producing the observed crowding effects. However, a contributory factor may be that large populations of worms in the gut produce an immune response by the host which adversely affects the parasites. A further contributory factor may also be that crowding causes a high concentration of worm excretory products which are possibly detrimental to worm growth and fecundity, although this clearly needs further investigation.

The present study has provided experimental information on the establishment and effects of crowding on E. recurvatum in the intestine of a wildfowl definitive host and goes some way to explaining the situation that may occur in natural systems. However, it should be noted that in this experimental study the initial infection doses of cysts were administered at a single point in time. In the natural environment wildfowl definitive hosts could reasonably be expected to acquire doses of cysts at successive points in time (trickle infection) thus producing secondary or superimposed infections. What effects a previous or existing infection of E. recurvatum will have on the establishment of an in-coming infection in the intestine of A. platyrhynchos is as yet unknown. Interestingly, Christensen, Knudsen & Andreassen (1986) have shown experimentally that in the "Echinostoma revolutum/laboratory mouse model" high levels of definitive host resistance do occur to secondary and super-imposed infections of the echinostome. Such concomitant immunity, if it exists in the "E. recurvatum/A. platyrhynchos model", could be expected to profoundly affect the establishment of worms in the intestine, and the course of existing infections. As such, it suggests a future

interesting channel of research to further extend our knowledge of E. recurvatum in wildfowl definitive hosts both under laboratory conditions and in the natural environment.

#### 7.4 The influence of second intermediate host species on the infectivity of Echinoparyphium recurvatum metacercarial cysts to Anas platyrhynchos

##### 7.4.1 INTRODUCTION

Echinoparyphium recurvatum exhibits broad specificity toward second intermediate hosts with several species of freshwater mollusc and the frog species Rana temporaria and Rana esculenta being known to function in this role (see Chapter 2). A considerable degree of variation is known to occur throughout this wide host spectrum with respect to the suitability of individual host species as second intermediate hosts for the parasite. For example, the snails Lymnaea peregra and Physa fontinalis are classed as high suitability hosts since under controlled laboratory conditions the numbers of E. recurvatum cercariae establishing as cysts in them were found to be significantly higher than those establishing in low suitability hosts such as Lymnaea stagnalis, Planorbis planorbis and Bithynia tentaculata (see Chapter 2 and Evans & Gordon 1983b). However, despite this variability in second intermediate host compatibility with the parasite nothing is known of the influence that different second intermediate hosts may have on the subsequent infectivity of E. recurvatum metacercarial cysts to definitive hosts. The present study therefore set out to examine in a general way the potential influence of various second intermediate host species on the



infectivity of E. recurvatum metacercarial cysts to the wildfowl definitive host Anas platyrhynchos using a range of six intermediate host species. These hosts were the British and European snails Lymnaea peregra, Lymnaea stagnalis, Physa fontinalis and Planorbis planorbis, the tropical freshwater planorbid snail Biomphalaria glabrata, and tadpoles of the amphibian Rana temporaria.

#### 7.4.2 MATERIALS AND METHODS

Laboratory bred, infection-free, specimens of the freshwater snails L. peregra, P. fontinalis, L. stagnalis, P. planorbis and B. glabrata (Puerto Rican strain) and laboratory bred, infection-free tadpoles of Rana temporaria, were exposed *en masse* in species groups in synthetic hard water medium (HMSO 1969) to numerous cercariae of E. recurvatum. The cercariae were collected within 30-45 minutes of their emergence from naturally infected first intermediate host L. peregra snails collected in September 1986 from Harting Pond, West Sussex. Between 20 and 40 specimens of each host were exposed to infection. All snails used were in the length/diameter size class 4-7mm.

Post-exposure, the hosts were maintained at 20°C on a diet of clean boiled lettuce. At 14 days post-exposure metacercarial cysts were obtained by dissecting hosts using fine steel needles. Batches of cysts from each second intermediate host origin were fed in doses of 50 cysts per host, in gelatine capsules, to separate groups composed of either 4, 6 or 8, 3 day old, Khaki Campbell ducklings (A. platyrhynchos). The ducklings were then maintained on a diet



of non-medicated chick crumbs and water fed *ad libitum* and were sacrificed by cervical dislocation 15 days post-infection.

The intestine of each bird was removed from pylorus to anus and was then transferred to a surgical tray containing warm (40°C) saline (0.75% NaCl). It was then divided into sections each one of which was individually searched for parasites in a separate petri dish of fresh warm saline.

Worms recovered from each intestine were counted, rinsed in clean saline, and then fixed and relaxed in Berland's Fluid. The worms were then cleared, temporarily mounted in "Ralmount" and measured using a microscope fitted with an ocular micrometer as described previously in this chapter in order to provide a first order estimate of the projected body area (length x maximum, mid-acetabular, width) of each worm. The reproductive status of each worm (ie. gravid or non-gravid) was recorded.

### 7.4.3 RESULTS

The results of this study are displayed in Table 7.3. They indicate, using the criteria of this study, that the species of second intermediate host utilized by *E. recurvatum* has no significant influence on the subsequent infection success of the parasite in the wildfowl definitive host *A. platyrhynchos*. Analyses of variance on the number of worms recovered from each host 15 days post-infection, the estimated body areas of worms transformed by  $\text{Log}_{10}(x+1)$  revealed no significant differences between the worms derived from the six different second intermediate host species origins. The results of the analyses of

**TABLE 7.3 The influence of second intermediate host species on the infectivity of Echinoparyphium recurvatum metacercarial cysts to the definitive host Anas platyrhynchos**

Second intermediate host species origin of cysts	No. of hosts exposed to infection	No. of cysts per host	No. of hosts infected 15 days post-infection	Mean no. (+/- SE) worms recovered per host 15 days post-infection	Mean (+/- SE) estimated area per worm	% of worms gravid
<i>Lymnaea peregra</i>	6	50	6	11.3 (+/- 1.2)	2.4 (+/- 0.12)	100
<i>Physa fontinalis</i>	6	50	6	10.3 (+/- 1.2)	2.32 (+/- 0.1)	100
<i>Planorbis planorbis</i>	4	50	4	11.0 (+/- 2.9)	2.25 (+/- 0.09)	100
<i>Lymnaea stagnalis</i>	4	50	4	9.5 (+/- 2.5)	2.11 (+/- 0.2)	100
<i>Biomphalaria glabrata</i>	6	50	6	12.3 (+/- 3.1)	2.26 (+/- 0.1)	100
<i>Rana temporaria</i> (frog tadpole)	8	50	8	8.5 (+/- 3.2)	2.1 (+/- 0.2)	100

variance were respectively ; worm number  $P (F=0.21) > 0.05$ ,  $df = 5$  and  $28$  ; worm size  $P (F= 0.92) > 0.05$ ,  $df = 5$  and  $348$ . All the worms recovered in this study, irrespective of second intermediate host origin, were found to be gravid, a finding which indicates that second intermediate host origin is likely to have little influence on the reproductive status of the parasite in the definitive host.

#### 7.4.4 DISCUSSION

The results of this study suggest that for the range of hosts examined the species of second intermediate host utilized by E. recurvatum is unlikely to have any significant effect on the infectivity, per metacercarial cyst, of the parasite to the definitive host A. platyrhynchos.

Experimental studies of this nature are rare and therefore the scope for comparison of results is limited. However, Christensen, Fransden & Roushdy (1980) carried out a similar study on the echinostome Echinostoma liei. These authors compared under experimental conditions the infectivity of metacercarial cysts from 14 different second intermediate host snail species to laboratory mice. Using as a criterion of infectivity the mean number of worms recovered per host expressed as a percentage of the initial cyst infection dose, Christensen et al (1980) arrived at the same conclusion for E. liei that is made for E. recurvatum in the present study ; that metacercarial infectivity to the definitive host is largely independent of the species of second intermediate host utilized.



## 7.5 The influence of age and temperature on the infectivity of Echinoparyphium recurvatum metacercarial cysts to Anas platyrhynchos

### 7.5.1 INTRODUCTION

Previous studies by Nasir (1962) Christensen ; Fransden & Roushdy (1980) have shown that for the echinostomes Echinostoma nudicaudatum and Echinostoma liei metacercarial cysts formed in the body of second intermediate host snails retain their infectivity to definitive hosts for extensive periods of time. Nasir (1962) showed that E. nudicaudatum remained infective for 14 months once encysted in a snail second intermediate host. Christensen et al (1980) found that metacercarial cysts of E. liei in the snail second intermediate host Biomphalaria glabrata remained unchanged in their infectivity to laboratory mice for at least 12 weeks from the time of their formation. These authors also showed that the infectivity of E. liei metacercarial cysts maintained at the low temperature of 4°C in filtered pond water remained unchanged for at least 18 weeks.

At present no published information is available on the influence of age or exposure to low temperature on the infectivity of metacercarial cysts of Echinoparyphium recurvatum. This represents a considerable gap in our knowledge of the transmission biology of the parasite to wildfowl definitive hosts. It means that important facets relating to the seasonal infection dynamics of the parasite, such as the significance of the metacercarial cyst as an over-wintering stage in the life cycle,

remain unknown. The present study therefore set out to examine in a general way the influence of age and exposure to low temperature on the infectivity of E. recurvatum cysts to the wildfowl definitive host A. platyrhynchos.

#### 7.5.2. MATERIALS AND METHODS

Four separate groups of 15 laboratory-bred, infection-free, L. peregra snails in the size range 4-6mm were exposed *en masse* in synthetic 'hard' water medium (HMSO 1969) to numerous E. recurvatum cercariae collected within 30-45 minutes of their emergence from naturally infected L. peregra first intermediate hosts obtained from Harting Pond West Sussex in October 1986. Post-exposure, snails were transferred in their groups to four separate polystyrene tubs each containing synthetic hard water medium at a temperature of 20°C and were maintained on a diet of clean boiled lettuce. After 14 days one group of snails was transferred to an incubator maintained at a constant 4°C, and the snails continued to be maintained on a diet of boiled lettuce.

At 24 hours post-exposure the snails of one group maintained at 20°C were dissected and the metacercarial cysts obtained were fed in doses of 50 cysts per bird in gelatine capsules to six infection-free, 3 day old, Khaki Campbell ducklings (A. platyrhynchos). This procedure was repeated using cysts from a second batch of snails maintained at 20°C for 14 days post-exposure, and again using cysts from snails maintained at 20°C for 16 weeks post-exposure. The procedure was also repeated with cysts obtained from the snail batch maintained at 4°C for 16 weeks.

In each case ducklings were killed by cervical dislocation 15 days post-infection and their entire intestines were searched for parasites in warm (40°C) , 0.75% NaCl saline. The number of worms present in the intestine of each duckling was recorded and the reproductive status of each worm (gravid or non-gravid).was noted.

### 7.5.3 RESULTS

The results of this study are shown in Table 7.4. Metacercarial cysts allowed to develop for just 24 hours post-infection of second intermediate host L. peregra were found to be completely uninfective to ducklings using the criterion of infectivity employed here. No worms were recovered from ducklings 15 days post-administration of these cysts.

In contrast, those metacercarial cysts allowed to develop for 14 days in L. peregra at 20°C, those maintained in L. peregra for 16 weeks at the same temperature, and those maintained in L. peregra for 16 weeks at 4°C all showed a very similar degree of infectivity to the definitive host. At autopsy 15 days post-infection the mean number of worms recovered from the hosts infected with metacercarial cysts from the three treatment batches were very similar. All worms recovered in each case were gravid. Analysis of variance on the number of worms recovered from each host transformed by  $\text{Log}_{10}(x+1)$  did in fact reveal no statistically significant differences between parasites originating from each of the three treatment batches ;  $P(F=0.1) > 0.05$ ,  $df= 2$  and 15.



**TABLE 7.4 The influence of age and temperature on the infectivity of Echinoparyphium recurvatum metacercarial cysts to the definitive host Anas platyrhynchos**

Treatment of cysts prior to host infection	No. of hosts exposed to infection	No. of cysts per host	No. of hosts infected 15 days post-infection	Mean no. (+/- SE) worms recovered 15 days post-infection	% of worms gravid
Allowed to develop in <i>L. peregra</i> for 24 hours at 20°C	6	50	0	0	-
Allowed to develop in <i>L. peregra</i> for 14 days at 20 °C	6	50	6	12.7 (+/- 1.5)	100
Allowed to develop in <i>L. peregra</i> for 16 weeks at 20°C	6	50	6	10.7 (+/- 2.2)	100
Allowed to develop in <i>L. peregra</i> maintained at 20°C for 14 days and then transferred to 4°C for 16 weeks	6	50	6	13.0 (+/- 3.2)	100

It would therefore seem reasonable to conclude that over the time periods examined neither age nor exposure to a temperature as low as 4°C has any significant effect on the infectivity or reproductive status of E. recurvatum metacercarial cysts to the definitive host A. platyrhynchos.

#### 7.5.4 DISCUSSION

The results of this study have shown that metacercariae allowed to develop for only 24 hours in L. peregra are uninfected to wildfowl definitive hosts. This indicates that a period of post-encystment development is necessary before infectivity to the definitive host is attained. Anderson & Fried (1987) similarly report that cysts of Echinostoma revolutum were not infective to chicks at 2 hours post-infection of the snail host but those at 4 hours were infective, thus suggesting that some maturation of the metacercaria within its cyst is a prerequisite for infectivity. Interestingly Donges (1969) has also reported that metacercariae of Echinostoma revolutum require a period of 6-8 days post-encystment before becoming infective to domestic chicks.

Metacercarial cysts of E. recurvatum allowed to develop for 14 days at 20°C in L. peregra, those allowed to develop under the same conditions for 16 weeks, and those allowed to develop for 14 days at 20°C and then maintained at 4°C for 16 weeks all showed a remarkably similar degree of infectivity to A. platyrhynchos 15 days post-infection. These findings suggest that over the time period examined the infectivity of E. recurvatum metacercarial

cysts to the definitive host is independent of age or exposure to low temperature. Both Nasir (1962) and Christensen et al (1980) have reported similar age and low temperature independence of metacercarial cyst infectivity in the echinostomes Echinostoma nudicaudatum and Echinostoma liei. However, conversely, Fashyui (1984) noted a loss of viability of Echinostoma porteri cysts after only 14 days in the second intermediate host snails Bulinus globosus and Lymnaea natalensis.

The fact that E. recurvatum metacercarial cysts can apparently retain an unaffected degree of infectivity over a period of 16 weeks at the low temperature of 4°C has profound implications in terms of the seasonal transmission dynamics of the parasite. It suggests that, unlike the egg of this parasite, the metacercarial cyst may be of considerable significance as an overwintering stage in the life cycle. Winter water temperatures at Harting Pond, West Sussex, stay at or below 10°C from November to April and reach temperatures around 4°C between late December-early January to March. With the degree of temporal and low temperature independence of cyst infectivity displayed in this study it seems likely that E. recurvatum cysts within second intermediate hosts are likely to retain their infectivity through the winter in Britain.



## *Chapter 8*

**Evidence for the existence and co-existence  
of first intermediate host specific forms of  
*Echinoparyphium recurvatum***

Evidence for the existence and co-existence of first intermediate host specific forms of Echinoparyphium recurvatum

### 8.1 INTRODUCTION

In Britain, first intermediate host gastropods of Echinoparyphium recurvatum have been recorded as the lymnaeid pulmonate Lymnaea peregra (see, for example, Diaz-Diaz 1976) and the mesogastropod prosobranch Valvata piscinalis (see Harper 1929). In Central France, Mathias (1926) recorded the planorbid pulmonate Planorbis planorbis as first intermediate host of E. recurvatum. All three of these authors experimentally completed the life cycle of the parasite to the adult stage and obtained adult worms in the intestines of experimental wildfowl hosts which they identified as 45 collar-spined adults of Echinoparyphium recurvatum von Linstow 1873.

Parasitological surveys of the aquatic gastropod fauna of a freshwater habitat, Harting Pond (Sussex, England) revealed that, of the gastropods present, only the lymnaeid pulmonate Lymnaea peregra and the mesogastropod prosobranch Valvata piscinalis were emitting morphologically indistinguishable 45 collar-spined cercariae identifiable as those of E. recurvatum. Although Planorbis planorbis snails were also present in the same habitat they have never been found to be actively infected with 45 collar-spined cercariae.

The presence of E. recurvatum active infections at Harting Pond only in the phylogenetically very distantly related pulmonate L.

peregra and prosobranch V. piscinalis , (and its absence from other species of gastropod present), suggested the possibility that two first intermediate host-specifically distinct entities of E. recurvatum may exist at this site. This final section of the current Chapter describes a preliminary study designed to determine whether E. recurvatum utilizing L. peregra and V. piscinalis at Harting Pond is a single biological entity or, whether in fact two biologically distinct, but morphologically indistinct, entities are present.

## 8.2 MATERIALS AND METHODS

### The Study Site

Harting Pond , West Sussex, England (NGR SU 778 219) is a shallow rectangular body of inland freshwater having a surface area of approximately 2 hectares. The molluscan fauna of the marginal regions of the pond at the time of the study was composed of the gastropods Lymnaea peregra, Physa fontinalis , Planorbis planorbis, Valvata piscinalis , Potamopyrgus jenkinsi and the bivalves Sphaerium corneum and Pisidium subtruncatum.

The wildfowl fauna of the pond is dominated by a resident population of Tufted Duck (Aythya fuligula). However, several other species of waterbird have been recorded including Mallard (Anas platyrhynchos), Teal (Anas crecca), Little Grebe (Tachybaptus rufficollis), Mute Swan (Cygnus olor), Canada Goose (Branta canadensis), Coot (Fulica atra), Moorhen (Gallinula chloropus), Kingfisher (Alcedo atthis) and Grey Heron (Ardea cinerea). Other relevant details of the habitat have been published



by Evans, Whitfield & Dobson (1981), and are also given in Chapter 4 of this thesis.

**Parasitological survey of the gastropod community for active infections of Echinoparyphium recurvatum**

Sampling of the gastropod community was conducted in September 1985. The sampling site chosen was a small clearly demarcated area at the eastern edge of the pond. Sampling was carried out using sweep nets. Two sweep net samples were taken, each removing material from the benthos of the margins and from the marginal vegetation. The net samples were transferred to a single large plastic container together with approximately 5 litres of pond water, and this pooled sample was then returned to the laboratory for sorting. All the gastropods in the sample were sorted into species. Each snail was then dissected individually in distilled water and thoroughly examined for active infection with E. recurvatum, initially using a stereo-microscope. Where active echinostome infections were found the identity of the parasite as E. recurvatum was confirmed by examining the collar spine number of cercariae under coverslip compression using phase contrast microscopy.

Five species of gastropod were examined for active infection with E. recurvatum; the pulmonates Lymnaea peregra, Planorbis planorbis and Physa fontinalis, and the prosobranchs Valvata piscinalis and Potamopyrgus jenkinsi. Between 97 and 724 individuals of each species were examined.

Morphological identification of Echinoparyphium recurvatum from Lymnaea peregra and Valvata piscinalis

In order to compare cercariae of E. recurvatum derived from each first intermediate host source, further sweep net samples of Lymnaea peregra and Valvata piscinalis were taken from Harting Pond. Specimens of each species were isolated in clear polystyrene pots containing a synthetic hard water medium (HMSO 1969) at 18-20°C. The snails were periodically examined for the emission of E. recurvatum cercariae. Freshly emitted cercariae from both snail species were examined under light coverslip compression both in a living condition and fixed in 10% Formalin.

Two separate batches of 10 laboratory bred, infection-free specimens of Lymnaea peregra were exposed to freshly emitted cercariae from each first intermediate host. The diameters of a sample of 20 metacercarial cysts from each first intermediate host source were measured 14 days post-exposure.

In order to obtain sexually mature adults of the parasites from each first intermediate host derived origin, further metacercarial cysts (obtained 14 days post-exposure from the two batches of experimentally infected L. peregra) were used to infect two separate groups of infection-free 3-day-old Khaki Campbell ducklings (Anas platyrhynchos). The cysts were administered orally in doses of 100 cysts per bird in gelatine capsules (EM Scope Ltd.). Three ducklings were infected with cysts derived from cercariae of L. peregra first intermediate hosts, and a separate group of three ducklings were infected with cysts derived from cercariae originating from V. piscinalis first intermediate hosts.

The birds were maintained on a diet of non-medicated chick crumbs and water fed *ad libitum*. They were sacrificed 15 days post-infection. The entire intestine of each bird was removed to a surgical tray of warm (40°C) saline (0.75% NaCl). The entire length of each intestine was then measured from pylorus to anus, and it was then divided into 20 equal sections each being 5% of its total length. Each section was then opened and thoroughly searched for parasites in a petri dish of fresh saline using a stereo-microscope. The gut caeca were searched separately. The number of worms recovered from each 5% gut section was recorded.

Ten sexually mature gravid adults obtained from each of the two groups of ducklings were fixed in 10% Formalin, dehydrated through a graded ethanol series, stained using Mayer's Paracarmine and then prepared as whole mounts using "Ralmount". They were then examined with a ZEISS RA compound microscope using bright field and phase contrast microscopy.

### Cross-infection experiment

This experiment was designed to determine whether E. recurvatum utilizing L. peregra as first intermediate host also had the ability to utilize V. piscinalis in this capacity. It was also designed to determine whether E. recurvatum utilizing V. piscinalis as first intermediate host at Harting Pond also had the ability to utilize L. peregra in this capacity. It was thereby hoped to determine whether E. recurvatum utilizing L. peregra and V. piscinalis at Harting Pond was a single biological entity, or whether in fact two separate first intermediate host specific entities existed.



Forty five collar-spined cercariae emitted from L. peregra and V. piscinalis collected at Harting Pond were used to infect two separate batches of laboratory-bred infection-free L. peregra experimental second intermediate hosts. Metacercarial cysts obtained from these snails 14 days post-exposure were used to infect two separate batches of Khaki Campbell ducklings. The cysts were fed in doses of 100 per bird. Two batches of gravid adult echinostomes, derived from each first intermediate host species, were dissected from these two groups of hosts 14-16 days post-infection. The worms were kept in separate batches and were teased apart using fine steel needles to release the large operculate eggs from their uteri. The eggs were then incubated in distilled water at 20°C in darkness, in polystyrene containers wrapped in aluminium foil, for 12 days. Miracidia were then stimulated to hatch by placing the eggs under the intense beam of a fibre optic cold light source.

This gave two batches of miracidia, one derived from the isolate of E. recurvatum utilizing L. peregra as first intermediate host, the other one derived from the isolate of E. recurvatum utilizing V. piscinalis as first intermediate host.

Two groups of infection-free specimens of L. peregra and V. piscinalis were obtained. The L. peregra were specimens bred in the laboratory from a parental stock of snails originally collected at Harting Pond. The V. piscinalis were obtained from a population on the Pevensey Levels in southern England known to be free of echinostome infections.

Thirty infection-free specimens of L. peregra and 30 of V. piscinalis (in the shell length class 2.5-4mm) were exposed to freshly hatched miracidia from the L. peregra isolate. In addition, 30 specimens each of L. peregra and V. piscinalis in the same size class were exposed to miracidia from the V. piscinalis isolate. Exposures were conducted in small polystyrene plate wells (COSTAR, Cambridge U.S.A.) each containing 3ml of a synthetic hard water medium (HMSO 1969) at a temperature of 20°C. Each snail was exposed to three miracidia for 24 hours. Post-exposure all snails were maintained at  $18 \pm 2^\circ\text{C}$  on a diet of boiled lettuce for 35 days. At 35 days post-exposure they were dissected and examined for active infection with E. recurvatum.

### 8.3 RESULTS

#### Parasitological survey of the gastropod community for active infections of Echinoparyphium recurvatum

The results of this survey are shown in Table 8.1. Although five species of gastropod mollusc were present at the sampling site only the lymnaeid pulmonate Lymnaea peregra and the prosobranch Valvata piscinalis were found to be actively infected with patent echinostome infections producing 45 collar-spined cercariae identifiable as those of Echinoparyphium recurvatum. The planorbid pulmonate Planorbis planorbis, the physid pulmonate Physa fontinalis and the prosobranch Potamopyrgus jenkinsi were all found to be negative for active E. recurvatum infection.

TABLE 8.1

The results of the survey of the gastropod community at Harting Pond for patent active infections of Echinoparyphium recurvatum

Gastropod Type	Gastropod Species	No. of specimens examined	No. infected	Infection Prevalence (%)
<b>Pulmonate</b>	<i>Lymnaea peregra</i>	352	92	26
	<i>Physa fontinalis</i>	97	0	0
	<i>Planorbis planorbis</i>	275	0	0
<b>Prosobranch</b>	<i>Valvata piscinalis</i>	536	79	15
	<i>Potamopyrgus jenkinsi</i>	724	0	0



Morphological identification of Echinoparyphium recurvatum from Lymnaea peregra and Valvata piscinalis

It was found to be impossible to distinguish morphologically between the cercariae from L. peregra and those from V. piscinalis. The general morphology of cercariae from both first intermediate host origins corresponds to that of the specimen illustrated in Fig 2.2a, (see Chapter 2). Cercariae from both hosts possessed an anterior collar of 45 spines arranged as shown in Fig 2.2b. Phase contrast microscopy revealed that none of the cercariae examined had tail finfolds. Unfortunately the exact flame cell formulae of cercariae could not be obtained, although flame cells were numerous in the body of cercariae from both first intermediate hosts. It is therefore remains unknown whether differences exist between the flame cell formulae of cercariae from L. peregra and V. piscinalis.

Measurements of Formalin-fixed cercariae showed that cercariae from each of the two first intermediate host species were of similar size. The tail length in each case was approximately the same length as the body. The body length of cercariae from L. peregra measured 351-426 $\mu$ m (mean = 381 $\mu$ m), and the mid-acetabular body breadth was 107-137 $\mu$ m (mean = 121 $\mu$ m). The tail length of these cercariae measured 362-421 $\mu$ m (mean=408 $\mu$ m) and the tail width at its base was 41-48 $\mu$ m (mean = 43 $\mu$ m). The body length of cercariae from V. piscinalis measured 324-402 $\mu$ m (mean=368 $\mu$ m), and the mid-acetabular body width was 101-128 $\mu$ m (mean =114 $\mu$ m). The tail length of these cercariae measured 326-398 $\mu$ m (mean=378 $\mu$ m) and the tail width at its base was 36-44 $\mu$ m (mean =39 $\mu$ m).

Fourteen-day-old metacercarial cysts formed by cercariae from L. peregra and V. piscinalis in experimental second intermediate host L. peregra were found to be indistinguishable from each other by morphology or size. The diameter of metacercarial cysts formed by cercariae emitted from L. peregra measured 141-153 $\mu$ m (mean=148 $\mu$ m). The thickness of the cyst wall was 13-16 $\mu$ m (mean=15 $\mu$ m). The diameter of metacercarial cysts formed by cercariae emitted from V. piscinalis measured 128-147 $\mu$ m (mean = 138 $\mu$ m) in diameter. The thickness of the cyst wall was 12-15 $\mu$ m (mean = 14 $\mu$ m).

The three A. platyrhynchos ducklings infected with cysts of cercariae derived from L. peregra first intermediate hosts yielded 13, 18 and 23 gravid adult echinostomes on dissection 15 days post-infection. All the worms were recovered from the anterior region of the small intestine, within the first 20% of the intestine posterior to the pylorus (see Fig 8.1). The three A. platyrhynchos ducklings infected with cysts of cercariae derived from V. piscinalis first intermediate hosts yielded 9, 11 and 18 gravid adult echinostomes on dissection 15 days post-infection. In the case of these three ducklings E. recurvatum worms were recovered from the posterior small intestine, and also from the rectum posterior to the ileo-caecal junction. The worms were recovered from a region 70-95% along the intestine posterior to the pylorus (see Fig 8.1). Interestingly, Harper (1929) also records that he obtained E. recurvatum adults from a posterior position (the large intestine) in the gut of A. platyrhynchos ducklings after experimentally infecting them with encysted 45 collar-spined cercariae from V. piscinalis first intermediate hosts.

FIG 8.1

The spatial distribution of Echinoparyphium recurvatum worms in the intestines of Anas platyrhynchos ducklings 15 days post-infection with metacercarial cysts formed by cercariae from Lymnaea peregra (■), and Valvata piscinalis (□).

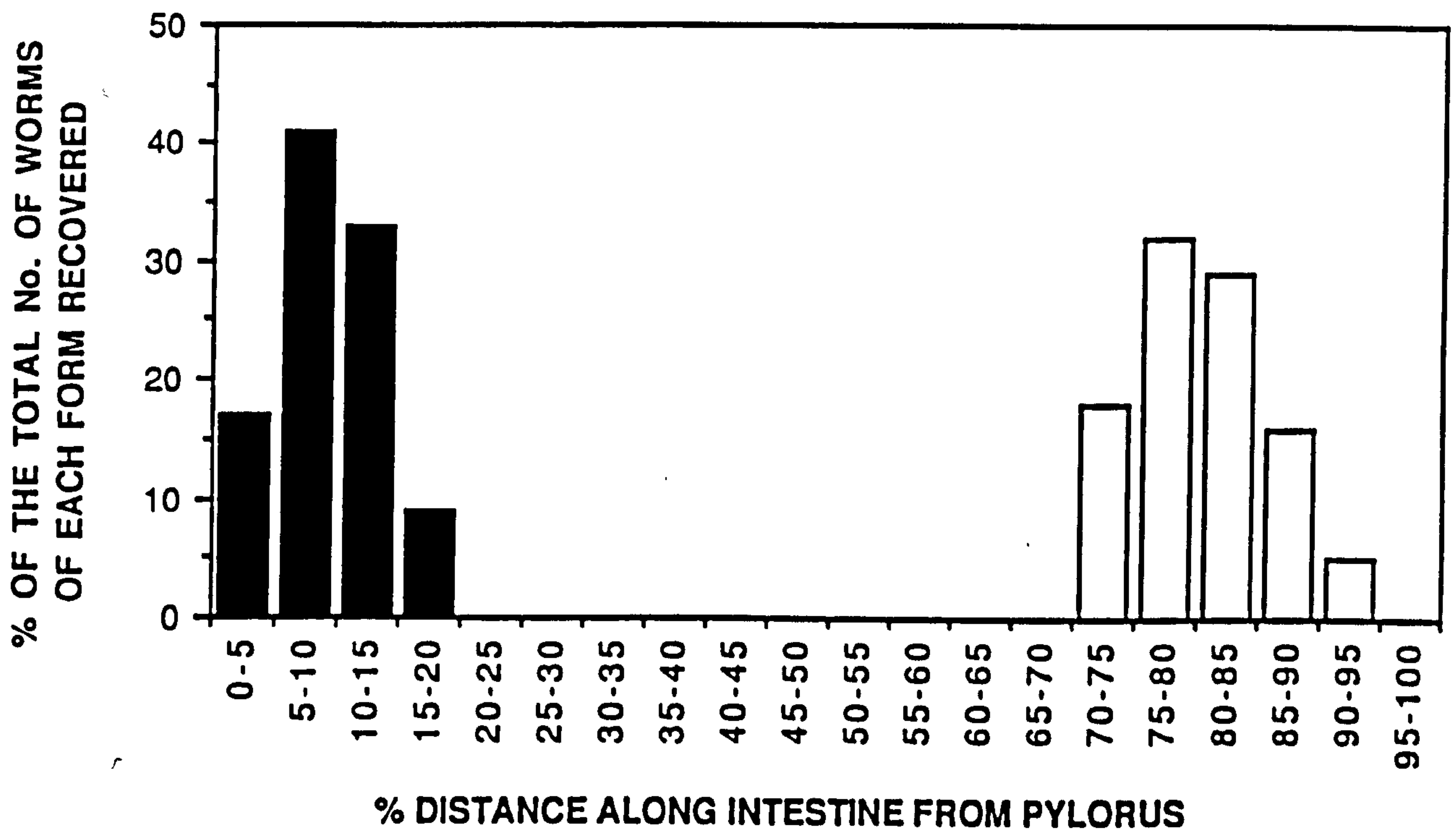




TABLE 8.2

Measurements of experimentally obtained 15 day old adults of the forms of Echinoparyphium recurvatum utilizing each of the two different species of first intermediate host gastropod ; Lymnaea peregra and Valvata piscinalis

Measurements are in  $\mu\text{m}$  (except where stated) - range followed by mean in parentheses. Measurements are based on 10 specimens of each type fixed in 10% Formalin.

Feature	Adults of the form of <u>Echinoparyphium recurvatum</u> utilizing <u>Lymnaea peregra</u> as first intermediate host	Adults of the form of <u>Echinoparyphium recurvatum</u> utilizing <u>Valvata piscinalis</u> as first intermediate host
Body length	2.4 - 3.8 (3.1) mm	2.3 - 3.6 (2.8) mm
Body width	560 - 980 (800)	524 - 977 (750)
Oral Sucker (diam.)	115 - 127 (123)	110 - 128 (114)
Pharynx length	87 - 110 (102)	92 - 105 (103)
width	70 - 96 (83)	71 - 101 (79)
Ventral sucker (diam.)	253 - 410 (381)	242 - 403 (375)
Ovary (diam.)	178 - 208 (197)	153 - 203 (186)
Anterior Testis length	403 - 496 (473)	386 - 500 (467)
width	245 - 319 (296)	232 - 314 (283)
Posterior Testis length	458 - 561 (527)	431 - 557 (504)
width	276 - 351 (311)	251 - 342 (304)
No. in utero eggs	18 - 43 (28) eggs	15 - 38 (25) eggs
Eggs (in utero) length	90 - 110 (96)	87-111 (94)
width	57 - 63 (61)	56 - 65 (64)
No. of collar spines	45	45
Collar spine lengths		
Oral	37 - 42 (39)	37-43 (38)
Aboral	41 - 44 (42)	41-44 (42)
Corner	48 - 54 (50)	46-42 (49)

It was found to be impossible to distinguish morphologically between the adult echinostomes derived experimentally from the two first intermediate host origins. The general morphology of all the worms recovered corresponded to that of the specimen illustrated in Fig 2.4 (see Chapter 2). It was found that of the two groups of 10 adult worms from each first intermediate host origin that were selected for measurement, all of them possessed an anterior collar bearing 45 spines; the spines being arranged around the collar as shown in Fig 2.4b. In each specimen 4 spines at each corner of the collar (the corner group) were found to be slightly larger than the rest. The middle 37 spines were arranged around the collar in a double tier, the spines of the oral tier being slightly smaller than those of the aboral tier. The comparative dimensions of 10 fixed, stained and mounted adult specimens derived from each first intermediate host origin are given in Table 8.2.

#### Cross-infection experiment

The results of the cross-infection experiment are as follows. Miracidia derived from E. recurvatum utilizing L. peregra as first intermediate host were infective only to L. peregra but not to V. piscinalis. At 35 days post-exposure 80% (24 out of 30) L. peregra exposed to miracidia of this isolate were actively infected with patent E. recurvatum infections. In marked contrast none of the 30 V. piscinalis exposed were infected.

It was also found that miracidia derived from E. recurvatum utilizing V. piscinalis as first intermediate host were infective to V. piscinalis but not to L. peregra. At 35 days post-exposure 63 % (19

out of 30) of V. piscinalis specimens exposed to miracidia of this isolate were actively infected with patent E. recurvatum infections. In contrast none of the 30 L. peregra exposed were infected.

These results are consistent with the hypothesis that E. recurvatum utilizing L. peregra as first intermediate host at Harting Pond represents a biologically distinct entity from that utilizing V. piscinalis as first intermediate host. The life cycles of the two entities may be taken to represent two virtually distinct transmission cycles. These two transmission cycles are summarized diagrammatically in Fig 8.2.

#### 8.4 DISCUSSION

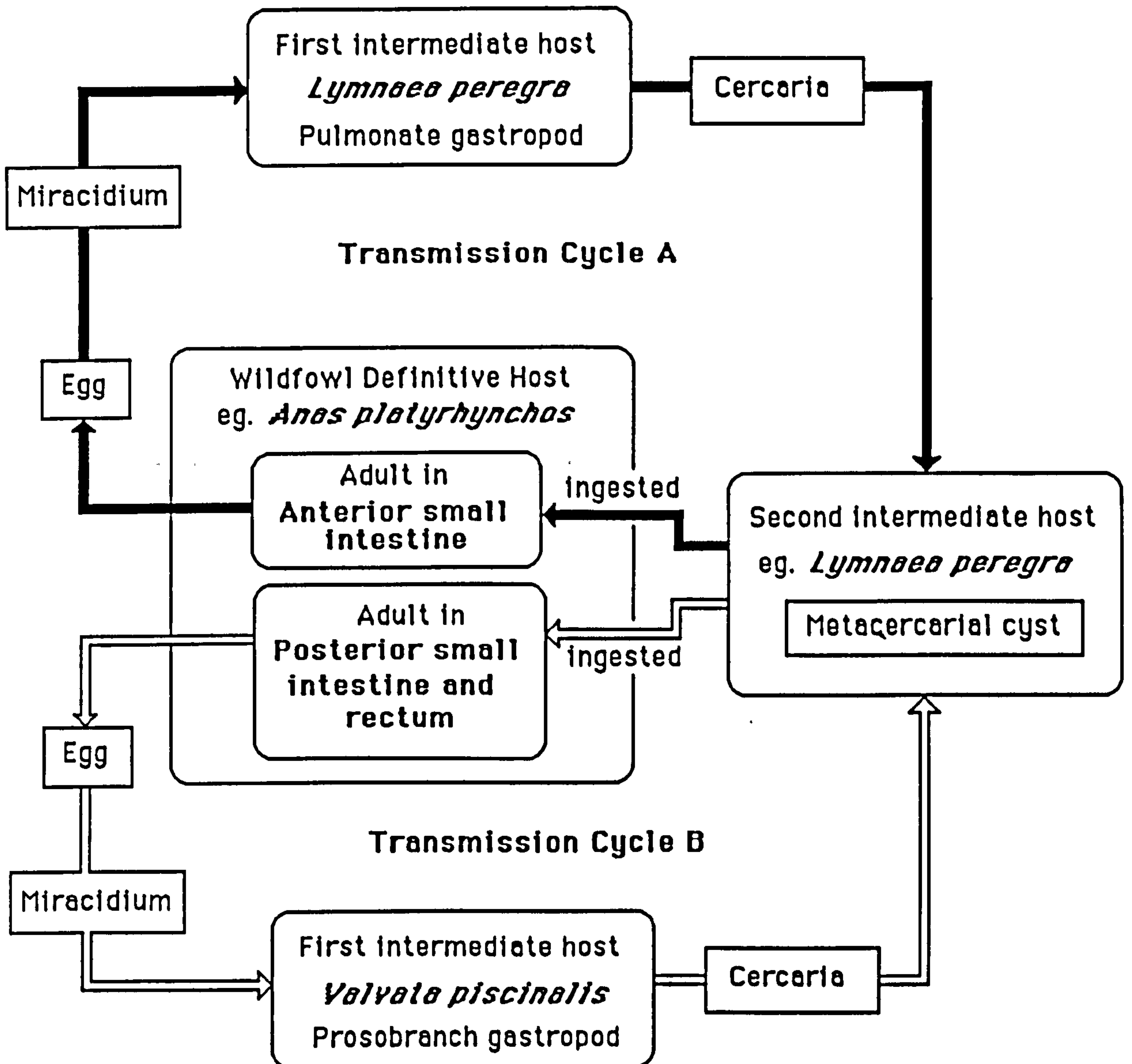
The results of this study clearly seem to indicate that at our study site in Southern England the echinostome digenean Echinoparyphium recurvatum exists as two biologically distinct, but morphologically indistinct, entities. One form utilizes the lymnaeid pulmonate gastropod Lymnaea peregra as first intermediate host, and the adult parasites of this form occur in the anterior small intestine of the wildfowl definitive host Anas platyrhynchos. The other form utilizes the prosobranch gastropod Valvata piscinalis as first intermediate host and the adults of this form occur in the large intestine of the wildfowl definitive host A. platyrhynchos. It may, in fact, be valid to consider each of these forms as sibling species within the genus Echinoparyphium.

The co-existence of these two forms at the same geographical location can, it is suggested, largely be explained by the probability that differences in their respective ecological niches



FIG 8.2

Diagram summarizing the distinct transmission cycles of the two forms of *Echinoparyphium recurvatum*. Transmission Cycle A is that of the form utilizing *Lymnaea peregra* as first intermediate host and Transmission Cycle B is that of the form utilizing *Valvata piscinalis* as first intermediate host.



are of sufficient magnitude to eliminate the occurrence of excessive competition between them. The divergence of the ecological niches of the two entities do appear to be quite substantial. Both forms utilize distinct first intermediate host species. This is particularly interesting since this is a stage of the life cycle at which the rediae of both forms could experience a marked degree of competition with each other if they were utilizing a single first intermediate host species. Echinostome rediae within first intermediate host molluscs are known to be highly antagonistic toward (predatory upon) the rediae and sporocysts of digeneans utilizing the same first intermediate host individual, (see Lim & Heyneman 1972 ; Combes 1982 ; Kjøie 1987).

In addition to the elimination of competition between the two forms at the first intermediate host stage of the life cycle, competition is also likely to be reduced in the intestine of the definitive host. Although both entities may utilize the same species of wildfowl definitive host, the adult worms appear to show a marked degree of spatial segregation within the host intestine. From the available evidence there seems to be nothing to suggest that both forms could not co-exist in the same definitive host individual. Congeneric acanthocephalans displaying similar spatial segregation have, for example, been shown to be able to co-exist in the intestine of the same individual definitive host fish. Uglem & Beck (1972) for instance reported on the co-existence of the congeners Neoechinorhynchus cristatus and N. crassus in concurrent infections of the large scale sucker fish Catostomus macrocheilus. N. crassus occupied a site in the anterior

intestine while N. cristatus occurred in the posterior intestine. Holmes (1973, 1983) has suggested that such differences in site selection by co-occurring intestinal helminths could be co-evolved, driven by selection to avoid competition. It is interesting to note that the potential ability of the two E. recurvatum forms under study herein to co-exist in the same definitive host individual is likely to be important at our study site, Harting Pond, since it is probable that both forms simultaneously utilize the same resident population of Tufted Duck (A. fuligula) as definitive hosts. However, examination of the Tufted Duck population will need to be carried out to examine this.

In summary, the life cycles of the two E. recurvatum entities studied herein may, in fact, be taken to represent two virtually distinct transmission cycles in which the two entities exhibit resource partitioning by first intermediate host, and also spatial resource partitioning in the gut of the wildfowl definitive host.

An interesting parallel to the situation reported in the current paper for Echinoparyphium is provided by Bartoli (1972). This author was working with two morphologically indistinct, but biologically distinct sibling species of the digenean genus Gymnophallus; G. nereicola and G. fossarum in the Camargue, France. Although both species are intestinal parasites of the same species of definitive host in this area, the Gull Larus argentatus, G. nereicola utilizes the bivalve Abra ovata as first intermediate host whilst G. fossarum utilizes a distinct species of bivalve Scrobicularia plana as its first intermediate host. It was demonstrated experimentally that neither species could utilize the



first intermediate host of the other. It was also shown that G. nereicola was markedly specific to a single species of second intermediate host, the polychaete annelid Nereis diversicolor. However, G. fossarum utilized marine bivalves of the genera Cardium and Tapes as second intermediate hosts. It is thought that the two different life cycle patterns represent two distinct cycles of transmission serving to reduce or, eliminate, excessive competition between the two sibling species to such an extent that both could co-exist at the same geographical locality.

In the present study differences have been demonstrated in the first intermediate host specificity of the two E. recurvatum forms, and have shown the differences in spatial distribution of the adults in the intestine of a wildowl definitive host. The next logical step in this investigation would be the examination of the two forms utilizing biochemical taxonomic techniques in order to discover whether differences between the two forms may be characterized by these means. A recent study by Renaud & Gabrion (1988) using multi-locus enzyme electrophoresis has in fact allowed the characterization of two morphologically indistinguishable sibling species of the marine fish cestode Bothrimonus occurring within the same flatfish host in the same geographical locality. DNA analysis, which has been used in studies such as those on Fascioloids described in abstract form by Blair & McManus (1988), may also be expected to provide interesting information if applied to the two forms of Echinoparyphium recurvatum described in this study.

# *Chapter 9*

## **General Overview and Discussion**

The studies presented in this thesis have examined a range of facets of the biology and transmission dynamics of the echinostome digenean Echinoparyphium recurvatum. The studies have collectively served to extend the state of our knowledge of the ecology of this parasite beyond that which existed at the time when the research programme forming the basis of the thesis was initiated. In addition to extending our knowledge of E. recurvatum several of these studies have also served to complement those of a similar nature in separate on-going research programmes carried out by other workers in both Europe and North America on members of the related echinostome genus Echinostoma.

Probably one of the most significant findings presented in this thesis relates to the very identity of Echinoparyphium recurvatum. The findings presented in Chapters 2 and 8 dealing with the first intermediate host specificity of E. recurvatum are of major importance in this respect. These studies demonstrated that E. recurvatum utilizing the pulmonate gastropod Lymnaea peregra is unable to utilize other non-lymnaeid snail species such as the planorbid Planorbis planorbis, the bulinid Bulinus truncatus and the prosobranch Valvata piscinalis, all of which have been reported in the literature as first intermediate hosts for E. recurvatum. The theory that E. recurvatum utilizing L. peregra as first intermediate host may represent a morphologically identical, but biologically distinct entity from E. recurvatum utilizing other non-lymnaeid first intermediate hosts was tested herein using E. recurvatum from the prosobranch first intermediate host V. piscinalis. Although they were found to be morphologically indistinguishable, it was demonstrated here that the two entities were completely



unable to utilize each other's first intermediate host species. Furthermore they demonstrated a marked non-overlap of spatial niche in terms of microhabitat distribution in the intestine of experimental definitive hosts. With respect to findings of this sort, Holmes (1973 ; 1983) has concluded that site segregation is usually co-evolved, driven by selection to avoid competition. [An alternative hypothesis has been advanced by Rohde (1979) who has argued that such site limitation may be the result of independent selection for greater contact for reproduction].

The present study has been valuable in that it confirms the impression that Echinoparyphium recurvatum is not a single, large, widely distributed and loosely first-intermediate-host-specific echinostome species. The suspicion that E. recurvatum may be composed of a complex of morphologically similar but biologically distinct entities (sibling species) is of course not new, but until now firm experimental evidence in support of this hypothesis was lacking. For example, Jeyerasasingham et al. (1972) suggested the distinct possibility that the name "Echinoparyphium recurvatum" in the literature might well mask a number of morphologically very similar, yet biologically distinct, sibling species.

The findings on the identity of E. recurvatum presented in this thesis are all the more interesting in the light of the parallel discoveries that have been made on the related echinostome Echinostoma revolutum. Until recently this echinostome was also regarded (purely on the basis of morphology) to be a single species with the ability to utilize a diverse range of unrelated

gastropod first intermediate hosts in various geographical regions. Due to the careful work of authors such as Kanev (1985), Fried, Scheuerman & Moore (1987) and Fried, Christensen & Kanev (1989) it is now being realized that "Echinostoma revolutum" is a complex of several morphologically similar, yet biologically distinct, entities.

Of course, in recent years the discovery of sibling species within what were once thought of as single helminth species has not been restricted to echinostome helminths. Renaud & Gabrion (1988) for example have recently characterized two sibling species in the cestode species complex Bothrimonus nylandicus. In addition, Nascetti et al. (1986) have detected the existence of two sibling species of the nematode Anisakis simplex using electrophoretic studies. MacKenzie (1981) also suspects that the trypanorhynch cestode Lacistorhynchus tenuis may be composed of a number of morphologically similar but biologically (host specifically) distinct sibling species.

With respect to further studies on the identity of E. recurvatum from different species of first intermediate host there is undoubtedly much scope for further study. Further cross-infection experiments similar to that described in Chapter 8 of the current thesis would lead towards a deeper understanding of the number of distinct biological entities in the E. recurvatum group. Perhaps the most interesting area for future study would be the biochemical characterization of E. recurvatum from different species of first intermediate host. Enzyme electrophoresis, for example, is a technique that has recently been used with excellent



effect by Renaud & Gabrion (1988) in the characterization of two sympatric sibling species in the cestode species complex Bothrimonus nylandicus. The application of DNA analysis could also be expected to yield interesting results. Rollinson, Walker & Simpson (1988) report on the usefulness of this technique in schistosome identification.

Despite the obvious appeal of biochemical characterization work, supplementary morphological work should not be entirely ignored as a potentially effective means of identifying sibling species of E. recurvatum. Scanning electron microscope studies are likely to be particularly important here. This is stressed because Fried, Irwin & Lowry (1989) have recently demonstrated that it is possible to differentiate between the adults of Echinostoma revolutum and Echinostoma liei, two very closely related echinostome species, by the characteristic shape of the body spines of each. In the present thesis adult E. recurvatum from an isolate of the parasite utilizing L. peregra as first intermediate host have been examined using SEM. Clear pictures of their body spination are thus now available. It now remains to be seen whether it is possible to distinguish morphologically between these body spines and the body spines of the form of E. recurvatum utilizing Valvata piscinalis as first intermediate host. The forms are biologically distinct, but are they also morphologically distinct in any detailed respects?

The transmission biology of the larval stages of E. recurvatum has been the subject of a major part of the work presented in this thesis. At the outset of the project our knowledge of the transmission dynamics of the cercaria was restricted to the



information provided by a single study, Evans & Gordon (1983a), and the hatching, survival and infectivity characteristics of the egg and miracidium were completely unknown. As a result of work presented here information is now available on the temperature-related transmission characteristics of both the miracidium and cercaria.

Perhaps one of the most interesting findings to have come from these studies is that both miracidia and cercariae of E. recurvatum have transmission optima in the region of 20°C. This, taken together with the intolerance of the egg to temperatures as low as 4-6°C, may suggest that the form of E. recurvatum under study here is an essentially warm water species which may be at, or at least approaching, the northern limit of its geographical range. However, the finding that the metacercarial cyst, within a second intermediate host snail is able to retain its infectivity to wildfowl definitive hosts after prolonged exposure to water temperatures as low as 4-6°C suggests that this transmission stage is likely to be able to overwinter in British aquatic habitats. It remains a possibility that different geographical forms of E. recurvatum might demonstrate distinct temperature optima with respect to transmission stage success. It would be interesting to test this hypothesis on E. recurvatum isolates from a variety of latitudinal locations.

It is interesting that Menard & Scott (1987a & b) have reported similar findings for eggs and metacercarial cysts of the digenean Cyathocotyle bushiensis to those reported here for E. recurvatum. Like the E. recurvatum under study here, Menard & Scott (1987a

& b) showed that the eggs of C. bushiensis were intolerant to exposures to temperatures as low as 4°C whereas the metacercarial cyst, within a snail second intermediate host, retained its infectivity to wildfowl definitive hosts for prolonged periods of time at a similarly low temperature. Another striking parallel with the findings of Menard & Scott (1987a) is that, like the situation in the case of C. bushiensis, the optimal temperatures for transmission of E. recurvatum miracidia prevails during the summer months, a period which overlaps with the arrival of the new generation of first intermediate host snails.

The results of the studies on the temperature-related transmission biology of the larval stages of E. recurvatum have raised a number of interesting questions. Why, for example, is the metacercarial cyst able to tolerate temperatures far lower than the egg? By what mechanism do temperatures as low as 4-6°C severely reduce the hatching success of E. recurvatum eggs? Such questions can only really be addressed by means of direct physiological and molecular investigations, for instance with respect to the temperature optima of particular enzymes which are crucial for intermediary metabolism.

In addition to information on the temperature-related transmission characteristics of the cercaria of E. recurvatum, information has been provided on other aspects of cercarial transmission. One of the most interesting facets of the age-related infectivity of E. recurvatum cercariae is the initial low infectivity phase which occurs after the cercaria has emerged from the first intermediate host snail. Evans & Gordon (1983a) first reported the



existence of this phase, and the current study has shown that it persists at temperatures up to 25°C. This uninfected phase has been described as a dispersal phase which may serve to reduce metacercarial super-infection of emitting first intermediate hosts. Of these two functions, ie. spatial dispersal of infective stages and avoidance of metacercarial super-infection, it is likely that the latter is by far the most significant in parasite fitness terms. The real spatial dispersive value of the cercaria will only be realized when experiments have been carried out to determine how far cercariae can travel from their point of emergence from the first intermediate host while still retaining sufficient energy reserves to penetrate and encyst successfully within a second intermediate host. It must remain the case, though, that an adult worm in a highly mobile avian host is a dramatically more efficient dispersal agent than any cercaria.

In addition to age-dependent changes in the infectivity of E. recurvatum cercariae, work presented in this thesis has also provided information on an age-related change in the response of cercariae to light. At 18-20°C cercariae show a photo-positive response 30 minutes post-emergence when they are relatively uninfected to the second intermediate host, but a photo-negative response at 2.5 hours post-emergence when they are maximally infective. Similar findings to this have been made on monogenean oncomiracidia, see for example Boret (1967) and Paling (1969). However, the possibility that this behavioural change is, in fact, more complex as far as monogeneans in general are concerned, has been made apparent by the observations of Kearn (1980) on the oncomiracidial behaviour of Entobdella soleae. Kearn (1980)



showed that when oncomiracidia were hatched without chemical stimulation (fish host urea) an alternating pattern of photo-positive and photo-negative vertical movements occurred, but as the oncomiracidia aged they spent an increasingly longer time in the photo-negative phase.

It should be stated here that the experiments described in the present thesis on the photo-response of E. recurvatum cercariae have given information only about this visual response in general terms. As yet it is unclear whether the nature of the response is in fact a phototaxis or photokinesis, or both. Further experiments will be necessary which examine the responses of cercariae in light gradients in order to show whether E. recurvatum cercariae do exhibit a true phototaxis, ie. a response in relation to the direction of the photostimulus, or a kinesis in which the rate of movement (or turning) is characteristically different in different light intensities.

The demonstration of a chemo-sensory component of host-location in E. recurvatum cercariae is an interesting and novel finding for this species. The existence of a chemo-sensory component in the snail host location mechanism of digenean miracidia, especially in the case of schistosomes, has been known for several years, (see short review in Smyth & Halton 1983). It has also long been known in the case of monogenean oncomiracidia that there is a chemosensory component in the location of fish hosts. Detailed experimental studies by Kearn (1967) on oncomiracidia of the monogenean Entobdella soleae have clearly demonstrated a chemo-sensory component in the fish host location mechanism of

this species. In the case of E. soleae, Kern (1967) has provided firm experimental evidence that the oncomiracidium responds to a chemical stimulus present in the mucus of the host fish, the Sole Solea solea. In addition, Kern (1974) has demonstrated that hatching of E. soleae oncomiracidia is stimulated by urea, and mucus from the skin of the Sole.

Despite the fact that a chemosensory component of host location has been recognized in a number of types of free-swimming trematode larvae only in one very recent publication, Fried & King (1989), has there been any detailed evidence that echinostome cercariae are capable of responding to chemical substances produced by snail second intermediate hosts. Fried & King (1989) have demonstrated an attraction of Echinostoma revolutum cercariae to dialysate produced by Biomphalaria glabrata second intermediate host snails. These authors have even suggested that the molecule (or molecules) to which cercariae are responding are probably below a molecular weight of 10,000, the cut-off grade of the dialysis material sac that the experimental snail hosts were retained in during the study.

Although the findings presented in this thesis for E. recurvatum and for E. revolutum by (Fried & King 1989) clearly seem to have demonstrated that echinostome cercariae are able to respond to chemical substances produced by target second intermediate host snails, it is not yet totally clear whether the response is a true chemotaxis or whether it is a chemokinesis. The question which remains to be answered is, are the cercariae capable of locating snail hosts by following a directional chemical gradient, or do they



enter a field of chemical stimulus surrounding a potential host snail (its active field) within which they then remain due to the triggering of locomotory mechanisms which serve to keep them in close proximity to the host to which they eventually attach?

Further examination of this problem would require the setting up of host chemical gradients in the laboratory and observing the response of cercariae to them. However, even if it is demonstrated that cercariae are able to locate snail hosts by following a gradient of host chemical stimulus to origin, it is doubtful if this will have any real significance when applied to the situation operating in natural habitats. In the natural environment turbulence and water currents are likely to break up gradients of host chemicals. It seems far more likely under natural conditions that E. recurvatum cercariae respond chemo-kinetically to host chemicals once they have actually entered the active chemical field of the host. Evidence that this indeed may be the case is provided by a study presented in abstract form by Dixon (1979) in which it is reported that E. recurvatum cercariae in L. peregra snail extract exhibit distinct locomotory responses (such as an increase in the rate of turning and a decrease in the degree of spatial displacement), locomotory responses which could be expected to keep them in close proximity to the potential host.

Prior to the studies on the emergence of E. recurvatum cercariae from L. peregra presented in Chapter 5 of this thesis, no detailed information was available on this aspect of the biology of this digenean. It is now known that cercarial emergence is markedly photoperiodic and occurs predominantly during the light phases of



an alternating light-dark cycle. It has also been demonstrated that darkness is inhibitory to cercarial emergence, and also that there is good evidence to suggest that an endogenous rhythm of cercarial emergence exists independently of the external stimulus of alternating photoperiod. These findings are interesting in themselves, but they also suggest an intriguing set of questions which could form the basis for future experimental investigations. For example, examination of E. recurvatum cercarial emergence under conditions of continuous light or dark could be expected to yield interesting information on the existence of an endogenous cycle of cercarial emergence, and could also be expected to allow estimations of the length of the free-running cycle period. A particularly interesting study of this nature has been carried out by Mahmoud (1983) on the emergence of Transversotrema patialense cercariae from the gastropod Melanoides tuberculata.

Another interesting question that has arisen from the preliminary studies on the photoperiodic emergence of E. recurvatum cercariae from L. peregra relates to the possible functional significance of this behaviour. In the literature to date there are several examples of where the markedly photoperiodic emergence patterns of digenean cercariae have been interpreted as behavioural strategies promoting the probability of contact between cercariae and target second intermediate hosts or, definitive hosts, see for example ; Theron (1975) on Ribeiroia marini , Theron (1976) on Plagiorchis neomidis and Theron (1984) on Schistosoma mansoni . At present it is not known if the photoperiodic emergence pattern of E. recurvatum cercariae represents a behavioural strategy with the potential of increasing

contact with, and thereby transmission success to, second intermediate hosts. Photoperiodic studies on the activity of potential second intermediate hosts will be required to discover whether there is indeed any significant overlap in the emergence pattern of E. recurvatum cercariae and the activity of these hosts.

The studies on cercarial emergence of E. recurvatum in this thesis have been of a short term nature. At present nothing is known of the dynamics of cercarial production and emergence over long time periods, and this remains as a further interesting channel for future work. In an experimental study Theron (1981) has provided information on the numbers of S. mansoni cercariae emerging each day from experimentally infected Biomphalaria glabrata for time periods in the region of 130 days post-infection. In order to examine long term temporal trends in cercarial emergence the application of time series analysis to data collected from such studies would seem to be the most logical approach. Interestingly, studies of this nature have in fact been carried out for cercariae of E. recurvatum utilizing L. peregra as first intermediate host. This has been reported in abstract form by Adam & Lewis (1989).

The work presented in this thesis has provided information on several aspects of the biology and transmission dynamics of the echinostome E. recurvatum utilizing L. peregra as first intermediate host. However, this final Chapter has shown that although several questions about this digenean have been answered, several questions have been raised as a direct consequence of the studies presented. These questions, as has been outlined in these final

paragraphs, relate to a variety of aspects including the taxonomy, host specificity, larval transmission dynamics and larval behavioural ecology E. recurvatum. Together they constitute a basis for further interesting investigations into the biology and ecology of this echinostome in which comparisons of these factors between host-adapted forms of the parasite should have a high priority.



# *Appendix*

*Pseudechinoparyphium echinatum*

(Digenea : Echinostomatidae) :

Experimental observations on cercarial  
specificity toward second intermediate hosts

The work described in this section was carried out during tenure of a visiting postgraduate research scholarship award from The British Council.

## INTRODUCTION

Pseudechinoparyphium echinatum von Siebold 1837 (Kanev & Vassilev 1986) (= Echinoparyphium aconiatum Dietz 1909) is an intestinal parasite of wildfowl occurring in Britain, Europe and Asia which utilizes the freshwater pulmonate gastropod Lymnaea stagnalis as first intermediate host. The cercariae of this 37 collar-spined echinostome are known to exhibit broad specificity towards the second intermediate host. Organisms functioning in this role in the natural environment include tadpoles and adults of the amphibian Rana ridibunda, the freshwater terrapin Emys orbicularis and freshwater gastropods; (Vassilev & Kambourov 1972, Kanev 1982). At present no quantitative information is available on the degree of compatibility of the parasite with different species of gastropod second intermediate host, although investigations carried out in Bulgarian biotopes in the Danube Valley have suggested that a high degree of preference is shown for the planorbid Planorbarius corneus and the lymnaeid Lymnaea peregra while the prosobranch Bithynia tentaculata is rarely infected.

In their study on the echinostome Echinoparyphium recurvatum Evans & Gordon (1983b) discovered close parallels between the experimentally determined order of host utilization exhibited by cercariae among gastropod second intermediate hosts and that determined by an earlier detailed field study, (Evans, Whitfield & Dobson 1981). The former authors showed that differential susceptibility of gastropods to infection by the parasite was likely to be one of the most important factors in determining the pattern of host utilization in the natural environment. The present study

set out to examine the pattern of second intermediate host snail utilization by P. echinatum with the aim of providing some information on the possible spectrum of second intermediate hosts in natural systems.

## MATERIALS AND METHODS

### Parasite and Host Material

P. echinatum cercariae were obtained from naturally infected specimens of the first intermediate host Lymnaea stagnalis. The snails were collected in June 1987 from freshwater channels close to the River Danube, 80 Km north of Mijhalovgrad, in Bulgaria. In total eleven species of freshwater gastropod were exposed to infection ; the planorbids Planorbarius corneus, Planorbis planorbis, Biomphalaria alexandrina, the bulinid Bulinus truncatus, the lymnaeids Lymnaea peregra, Lymnaea stagnalis, Lymnaea palustris and Lymnaea truncatula, the physid Physa fontinalis and the prosobranchs Viviparus viviparus and Bithynia tentaculata. All snails, with the exception of B. tentaculata obtained from a natural site known to be free from echinostome infection, were laboratory bred and infection-free. Snails used throughout this study were of similar size and in the length / diameter range 3-5mm, Evans & Gordon (1983a) having shown host size to be an important determinant of cercarial transmission success.

### Infection of Single Host Species

Cercarial infectivity towards the range of potential second intermediate hosts shown above was determined using the following method. Thirty specimens of each species were exposed



(with the exceptions of B. tentacualta, V. viviparus and B. truncatus of which ten specimens of each were used and B. alexandrina where 12 specimens were used). Snails were exposed singly to freshly emitted cercariae (maximum age 30 minutes) using a constant ratio of 20 cercariae per snail in 20 mls of dechlorinated water at pH 7 and  $23 \pm 2^{\circ}\text{C}$ . Exposures were conducted in shallow glass dishes. Snails were examined for infection 24 hours post-exposure. Examination was carried out by crushing each snail between two glass microscope slides and recording the number of metacercarial cysts formed using a stereo microscope. Cercarial transmission success was calculated as ;  
 (Total number of metacercarial cysts established / Total number of cercariae) x 100.

### Infection of Host Communities

In order to investigate patterns of cercarial transmission in host communities two experiments were carried out.

#### Experiment 1.

This experiment was designed to yield information on the pattern of cercarial transmission in a multi-species host community. Cercariae were provided with a choice of eight potential gastropod second intermediate hosts known to occur commonly in European habitats from which P. echinatum has been recorded. Ten specimens each of P. corneus, P. planorbis, L. peregra, L. stagnalis, L. palustris, L. truncatula, P. fontinalis and B. tentaculata were placed in a cylindrical aquarium containing 1.6 litres of dechlorinated water. 1600 freshly emitted cercariae were then

introduced, thus maintaining the ratio of 20 cercariae per snail, and density of one cercariae per ml, employed in single host species. exposures. Snails were examined for infection 24 hours post-exposure.

### Experiment 2.

In this experiment the degree of preference exhibited by cercariae for three species of pulmonate host P. corneus, P. fontinalis and L. peregra in mixed populations was examined. Exposures were conducted in glass dishes containing 40 mls of dechlorinated water at a host-parasite density equivalent to that employed in Experiment One. Details of the experimental design are given in Table A3.

## RESULTS

### Infections of Single Host Species

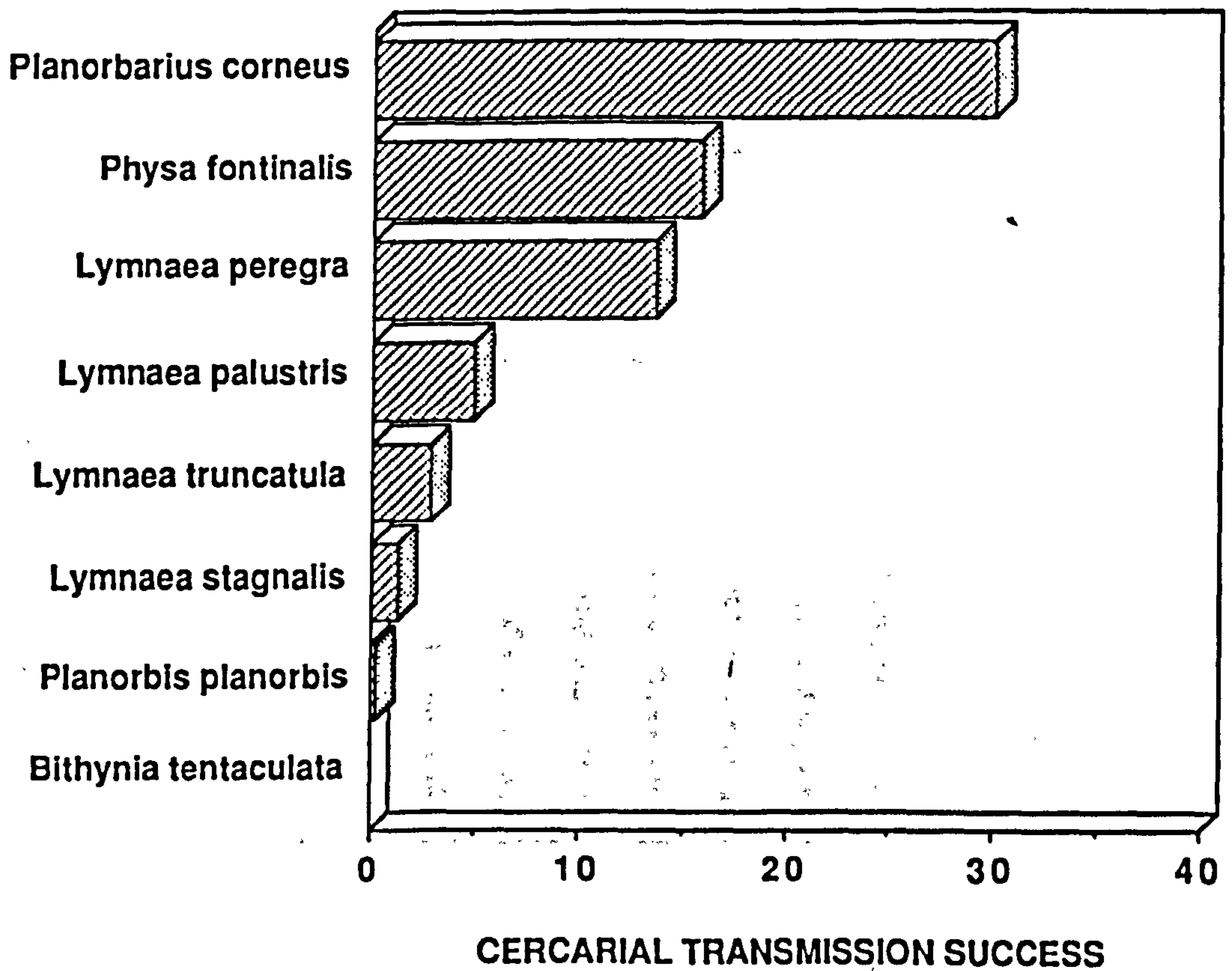
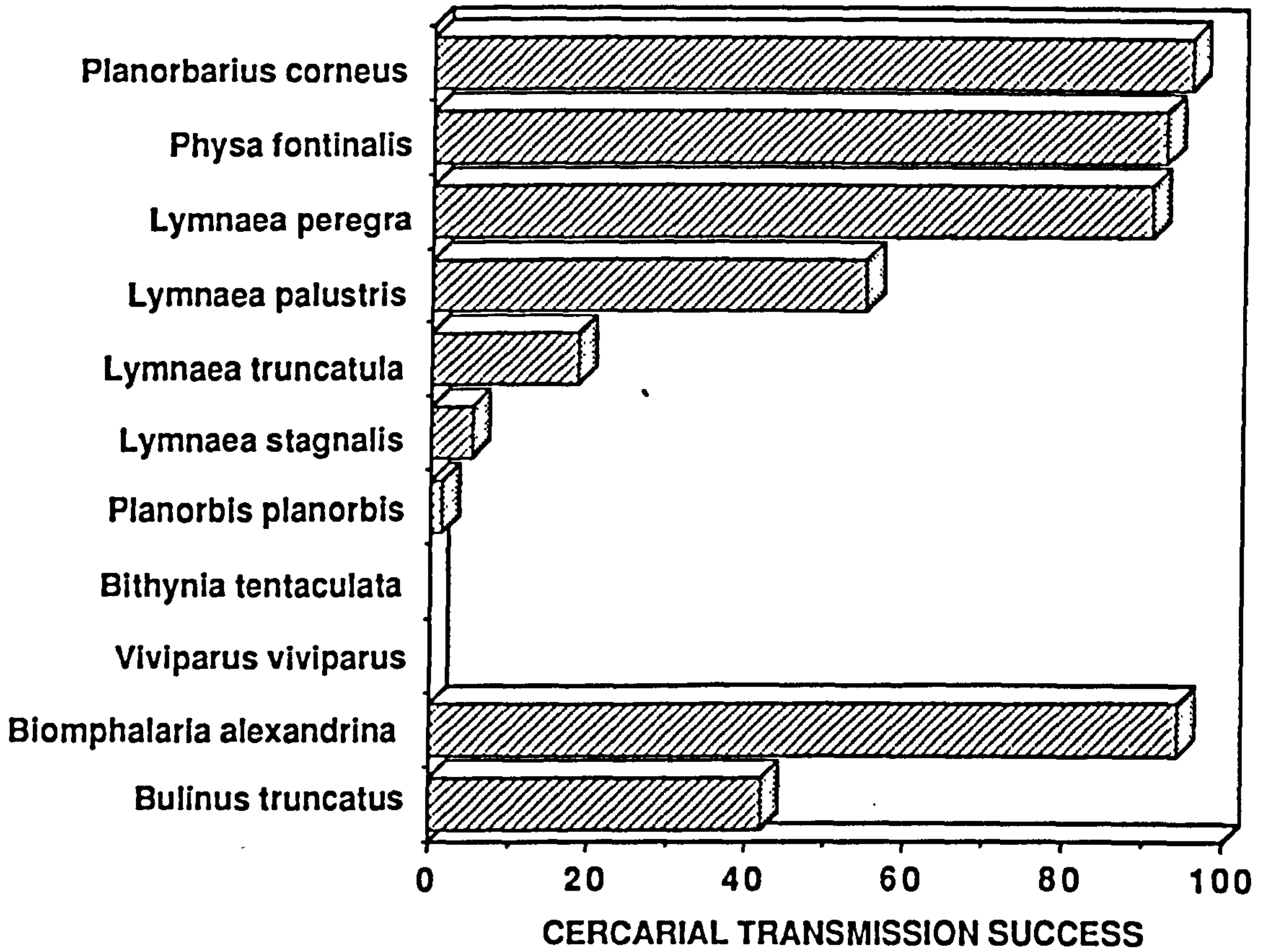
The infectivity of P. echinatum towards eleven species of potential second intermediate host, nine European and two African, is shown in Fig. A1 and Table A1. It is immediately obvious that three European pulmonates, Planorbarius corneus, Physa fontinalis and Lymnaea peregra displayed a similar degree of high compatibility with the parasite. Analysis of variance using cyst counts transformed by  $\text{Log}_{10}(x + 1)$  showed that the numbers of cercariae establishing as cysts in each of these hosts were not significantly different, ( $P(F=2.23) > 0.05$ ,  $df= 2$  and  $87$ ). The African planorbid species Biomphalaria alexandrina showed a similar high degree of compatibility whereas the African bulinid Bulinus truncatus showed intermediate compatibility comparable

FIG A1. Transmission success\* of Pseudechinoparyphium echinatum cercariae in a range of gastropods under conditions of single host species exposure.

FIG A2. Transmission success\* of Pseudechinoparyphium echinatum cercariae in an experimental multi-species host community of gastropods.

\* Transmission success = (Total number of metacercarial cysts established / Total number of cercariae) x 100





**TABLE A1** Infectivity of Pseudechinoparyphium echinatum cercariae to a range of gastropods under conditions of single host species exposure

Gastropod species	No. snails exposed to infection	No. cercariae per snail	Mean no. (+/- SE) metacercariae recovered per snail	% Snails infected
<i>Planorbis corneus</i>	30	20	19.1 (+/- 0.21)	100
<i>Physa fontinalis</i>	30	20	18.46 (+/- 0.33)	100
<i>Lymnaea peregra</i>	30	20	18.1 (+/- 0.43)	100
<i>Lymnaea palustris</i>	30	20	11.33 (+/- 1.26)	83.3
<i>Lymnaea truncatula</i>	30	20	3.7 (+/- 0.85)	46.7
<i>Lymnaea stagnalis</i>	30	20	1.03 (+/- 0.43)	20
<i>Planorbis planorbis</i>	30	20	0.3 (+/- 0.17)	10
<i>Bithynia tentaculata</i>	10	20	0	0
<i>Viviparus viviparus</i>	10	20	0	0
<i>Biomphalaria alexandrina</i>	12	20	18.92 (+/- 0.22)	100
<i>Bulinus truncatus</i>	10	20	8.4 (+/- 2.38)	70

to that of the European lymnaeid Lymnaea palustris . The low degree of compatibility of the planorbid Planorbis planorbis is similar to that shown by Evans & Gordon (1983b) for this snail species with Echinoparyphium recurvatum cercariae. The first intermediate of P. echinatum , Lymnaea stagnalis , also exhibited low compatibility with only 5% of cercariae successfully establishing as cysts. The prosobranchs Bithynia tentaculata and Viviparus viviparus proved to be completely refractory to infection.

#### Infection of Multi-Species Host Communities

The pattern of host utilization in a multi-species host community comprised of eight species of potential second intermediate host is shown in Fig A2 and Table A2. Overall transmission success of the parasite in this experimental community may be considered as high with 68.9% of cercariae establishing as cysts. This is perhaps to be expected in view of the close degree of host-parasite contact provided in this experimental system. The general order of host utilization was very similar to that determined under conditions of single host species exposures , P. corneus , P. fontinalis and L. peregra remaining the most heavily utilized hosts. However, the pattern of cercarial transmission among these high compatibility hosts differed in one important respect from that shown under individual species exposure conditions. Under the latter conditions each of these hosts showed a similar high degree of compatibility with the parasite. Under multi-species exposure analysis of variance on cyst counts transformed by  $\text{Log}_{10} (x+1)$  revealed significant differences between the numbers of cercariae establishing as cysts in each of these hosts, ( $P (F=7.05) < 0.05$  ,



**TABLE A2 Infectivity of Pseudechinoparyphium echinatum cercariae in an experimental multi-species host community of gastropods**

Gastropod species	No. snails exposed to infection	% Snails infected	Mean no. (+/- SE) metacercariae recovered per snail	% Of total cyst population in species
<i>Planorbarius corneus</i>	10	100	48.2 (+/- 11.23)	43.7
<i>Physa fontinalis</i>	10	100	25.3 (+/- 5.0)	22.9
<i>Lymnaea peregra</i>	10	100	21.9 (+/- 4.37)	19.9
<i>Lymnaea palustris</i>	10	60	7.9 (+/- 2.66)	7.2
<i>Lymnaea truncatula</i>	10	60	4.6 (+/- 1.8)	4.1
<i>Lymnaea stagnalis</i>	10	20	2.0 (+/- 1.41)	1.8
<i>Planorbis planorbis</i>	10	10	0.4 (+/- 0.4)	0.4
<i>Bithynia tentaculata</i>	10	0	0	0

**TABLE A3 Transmission of Pseudechinoparyphium echinatum cercariae in communities of high compatibility second intermediate hosts**

Community composition	No. of cercariae	No. of replicate experiments	Mean no. ( $\pm$ SE) cysts per snail		
			P. comeus	P. fontinalis	L. peregra
1 P. comeus + 1 P. fontinalis	40	20	23.05 ( $\pm$ 0.79)	11.8 ( $\pm$ 0.72)	-
1 P. comeus + 1 L. peregra	40	20	24.0 ( $\pm$ 1.18)	-	9.75 ( $\pm$ 1.0)
1 P. fontinalis + 1 L. peregra	40	20	-	16.45 ( $\pm$ 1.25)	14.25 ( $\pm$ 1.16)

df=2 and 27). Using a t-test on cyst counts transformed by  $\text{Log}_{10}(x+1)$  no significant difference was found between the number of cysts formed in P. fontinalis and L. peregra, ( $P(t=0.63) > 0.05$ , df=18), and therefore these hosts maintained the same degree of compatibility with the parasite compared to each other as they did under individual species exposures. However, a t-test on cyst counts transformed by  $\text{Log}_{10}(x+1)$  indicated that the number of cysts established in P. corneus was significantly greater than that in both P. fontinalis ( $P(t=3.5) < 0.05$ , df=18) and L. peregra ( $P(t=3.7) < 0.05$ , df=18). The transmission success of cercariae into P. corneus was in fact approximately double that of the other two high suitability hosts, see Fig A2. P. corneus contained over 40% of the total number of cysts formed in the experimental host community.

The marked preference shown by cercariae for P. corneus was confirmed by the results shown in Table A3. Again, t-tests on cyst counts transformed by  $\text{Log}_{10}(x+1)$  indicated that the number of cercariae establishing as cysts in P. corneus was significantly greater than in either P. fontinalis ( $P(t=9.1) < 0.05$ , df= 38, or L. peregra ( $P(t=8.0) < 0.05$ , df= 38), under conditions where a choice of host was made available. The results also confirm that although P. echinatum cercariae show a preference for P. corneus they do not seem to discriminate between P. fontinalis and L. peregra. Cyst counts from the latter hosts transformed by  $\text{Log}_{10}(x+1)$  were not found to be significantly different, ( $P(t=1.2) > 0.05$ , df= 38).



## DISCUSSION

The order of second intermediate host utilization by P. echinatum cercariae determined experimentally shows close parallels with that suggested by occasional field studies carried out in North Bulgarian biotopes along the River Danube in recent years which indicate that the parasite shows a preference for the pulmonates P. corneus and L. peregra but rarely encysts in prosobranchs such as B. tentaculata . It therefore seems probable that differential compatibility of the parasite with different second intermediate host snails is likely to be an important factor in determining the order of host utilization exhibited by P. echinatum cercariae in the natural environment.

The broad host specificity exhibited by P. echinatum cercariae toward second intermediate host gastropods probably confers a similar set of ecological advantages on the parasite as those suggested by Evans, Whitfield & Dobson (1981) for the cercariae of Echinoparyphium recurvatum which also exhibits broad specificity towards second intermediate hosts. It was suggested that broad specificity effectively increases the density of potential hosts in a given habitat, and may help to maintain an adaptively useful degree of genetic variability in the parasite population by periodically exposing sub-populations of the parasite to different selective environments. It may also help to protect the parasite from the deleterious effects of local population decline in any single molluscan host species. Such ecological advantages may suggest the reason why the trait of broad specificity toward second intermediate hosts has evolved in several echinostome species.

Although differential compatibility clearly operates in the case of P. echinatum cercariae among gastropod second intermediate hosts, the degree of host-parasite compatibility seems, to a certain extent, to be independent of the phyletic position of the host. For example, cercariae show significantly different degrees of compatibility with the congeneric lymnaeid species Lymnaea peregra, L. palustris, and L. stagnalis. The parasite also shows markedly different compatibility with the planorbid species P. corneus and P. planorbis. There is, however, no significant difference between its compatibility with the lymnaeid L. peregra, and the planorbid P. corneus. A similar type of finding was made by Evans & Gordon (1983b) for cercariae of the echinostome E. recurvatum. It was discovered that the prosobranch snail Valvata piscinalis and the lymnaeid pulmonate L. peregra were second intermediate hosts of similar high compatibility, whereas the prosobranch B. tentaculata and the lymnaeid L. stagnalis were both hosts of very low suitability.

The first intermediate host of P. echinatum, L. stagnalis, showed a consistently low degree of compatibility with cercariae in this study. This situation is the complete opposite of that shown by Evans & Gordon (1983b) for E. recurvatum cercariae which show a high degree of preference for the first intermediate host of this species L. peregra. The functional significance of low compatibility of P. echinatum cercariae with L. stagnalis may be that it is a mechanism, preventing metacercarial super-infection of emitting hosts and also preventing parasite pressure on the first intermediate host population in general. Low compatibility of



cercariae with the first intermediate host may therefore be likely to be of advantage to P. echinatum . Incidentally, it is interesting to note that although E. recurvatum cercariae do show a high degree of compatibility with the first intermediate L. peregra Evans & Gordon (1983a) and have demonstrated an initial post-emission period of low infectivity which may function as a mechanism preventing super-infection of emitting hosts.

The ability of the low suitability host L. stagnalis to function as a "decoy" conferring a significant degree of protection from infection by E. recurvatum cercariae on the high suitability hosts L. peregra and P. fontinalis has been shown by Evans & Gordon (1983b). The role of low suitability host decoy snails in reducing transmission levels of schistosome miracidia is also well documented, (Christensen 1980 ; Combes & Mone' 1987). In the present study we have shown that the high suitability host P. corneus effectively has the ability to confer a significant level of protection on two other high suitability hosts, L. peregra and P. fontinalis. When exposed individually to infection P. corneus , L. peregra and P. fontinalis showed an insignificantly different degree of compatibility with the parasite. However, in situations where a choice of these hosts was made available P. corneus was preferred to both of the other two high suitability hosts . It is probable that the apparently strong attractivity of P. corneus to the parasite was largely responsible for the fact that both L. peregra and P. fontinalis showed lower degrees of infection with the parasite compared to P. corneus in the community infection experiment than they did under conditions of individual host species exposure. This illustrates the point that the transmission success of P.



P. echinatum cercariae into a given second intermediate host is not a constant but a variable, an important determinant of which is the species composition of the surrounding host community.

At present it is not known how the strong attractivity of P. corneus for P. echinatum cercariae is generated. However, it is well documented that attraction to chemical stimuli produced by the host plays a role in attraction and attachment to intermediate hosts by larval digeneans, (Etges & Decker 1963 ; MacInnis 1965 ; Disko & Weber 1979 ; Haas 1974, and Motzel & Haas 1985). Interestingly, Anderson & Fried (1987) have also recently noted response by cercariae of the 37 collar-spined echinostome Echinostoma revolutum to renal secretions produced by second intermediate host snails. These authors suggest that these chemical secretions may be involved in cercarial penetration and location of site for encystation. In addition, Fried & Fujino (1987) and Zdarska et al (1987) have described a specialized multiciliate sensory receptor from the anterior of the cercaria of E. revolutum, a structure which the latter authors consider may have a chemo-sensory function. Importantly Wright (1959) has demonstrated specific differences in the chemical composition of the mucous secretions of different species of Lymnaea snails and this may prove to be a potentially important factor in the type of host selectivity demonstrated in this present study. It thus seems entirely possible that P. corneus may prove to have a higher "chemo-attractivity" to P. echinatum cercariae than either L. peregra or P. fontinalis . However, this interesting possibility clearly needs experimental investigation.

Complete incompatibility with P. echinatum in this study was limited to the prosobranchs B. tentaculata and V. viviparus. The fact that no unencysted, moribund or dead cercarial bodies were recovered from these snails, or indeed from any other of the experimental hosts used in this study, indicates that incompatibility was expressed at some stage prior to penetration. Motzel & Haas (1985) also report that second intermediate host incompatibility to cercariae of I. melis is expressed prior to penetration where incompatible hosts fail to chemically stimulate the cercarial attachment response. However, Cheng et al (1966) have reported on a host-parasite system in which incompatibility is expressed in unsuitable molluscan hosts after echinostome cercariae have penetrated.

In their field study on E. recurvatum Evans, Whitfield & Dobson (1981) found that the probable level of contribution that different host mollusc species made to the actual flow of the parasite through the natural system was dependent on a combination of factors. The two most important of these were the susceptibility of a host species to infection and the population size of that host. For example, L. peregra was found to be a high compatibility host but because its population size was relatively small its probable contribution to parasite flow into the definitive host system was assessed as minimal. A similar situation is likely to be true of P. echinatum in natural populations of gastropod second intermediate hosts. Therefore, although this present study on specificity provides information on the probable order of host utilization in the natural environment, field studies of natural host populations are clearly desirable. Such studies, should provide

data on cyst infection levels together with quantitative estimates of host population sizes. They should also ideally give information about definitive host predation levels on the various intermediate host species, although this may prove difficult to assess. It is only when such detailed field studies have been carried out that we will more fully understand which hosts are likely to contribute most to the flow of P. echinatum to its definitive hosts in natural systems.



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