Some aspects of the biology of *Dactylogyrus vastator* Nybelin, 1924 (Monogenea) a gill parasite of *Cyprinus carpio* L.

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BY

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To my beloved **Father** and **Mother** whom I missed during my University Education
and
lastly to my beloved **only sister** whom I missed during this study

DECLARATION

I hereby declare that this thesis has been compiled by my self and is the result of my own investigations. It has neither been accepted, nor is being submitted for any other degrees. All the sources of information have been duly acknowledge.

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Abstract

Dactylogyrus vastator Nybelin, 1924 is a common, economically significant pathogenic monogenean parasite of European carp, *Cyprinus carpio* L. *D. vastator* attaches to the gills by means of an attachment organ, the opisthaptor, which carries two large hamuli, a connecting bar and fourteen peripheral marginal hooks.

Experiments during the present study have shown that populations of *D. vastator* on young carp reach peaks of abundance at 12°C, 19°C and 22°C within 5, 3 to 4 and 6 weeks, respectively, followed by a decline to a lower level. Parasite abundance was greatest at 19°C and lowest at 12°C. Principal component analysis was used to investigate the effects of temperature on sclerite measurements. It was shown that the basic length and internal root length of the hamuli are the major factors by which populations of the parasite reared under different temperature regimes can be discriminated. Parasites reared at 12°C were clearly separated from those reared at 14°C and 19°C.

Scanning and transmission electron microscopic studies were carried out on *D. vastator*. The outer layer of the epidermis is a syncytial structure. Circular and longitudinal muscle is found beneath this outer layer. The muscle bands are not of uniform thickness. Epidermal secreting cell bodies are located below the muscle layer and communicate with the outer layer via ducts or channels. Possible epidermal sensillae are unequally distributed over the worm's body. The parasite has four cephalic lobes each of which is provided with a cup-like opening at the border; the

unicellular cephalic gland cells empty their contents into a collecting duct. *D. vastator* shows protandous gonadial development. The female reproductive system has an oval shaped ovary, uterus, ootype, accessory glands, whereas the male reproductive system has a single lobed testis located in the posterior region of the body.

Clean sclerites prepared by using an ultrasonication technique were examined under the electron microscope. The hamuli of adult and immature dactylogyrids are divided into internal and external processes and a shaft which ends in a spike. Marginal hooks have a blade and spike. The adult and immature worms can be differentiated by the structure of the auxiliary sclerite. In mature specimens the outer and the inner surfaces of the auxiliary sclerite remain separate. The surface of the hamuli has an interlocking array of striations. The two hamuli are of unequal size in both adult and immature worms.

The parasites are not randomly distributed over the gill apparatus. There were no significant differences between gill arches but parasites aggregated in certain areas of the gills, in particular the ventral proximal secondary filaments on both sides of the hemibranch are favoured.

Smaller *D. vastator* which are found in higher numbers on fish presumably represent worms which have recently invaded the host. Larger worms are found in lower numbers. This may be due to competition or an age related mortality in which mature worms die off. *D. vastator* does not need to be attached to the host tissue to initiate egg laying. *In-vitro* oviposition was observed and described, however the egg laying rate varies with the environmental temperature. The first eggs that are

produced *in-vitro* are of a large size but as time continues the size of the eggs becomes smaller.

A severe hyperplastic tissue response was observed two weeks after the start of an experiment where fish infected with *D. vastator* were mixed with naive fish. Damage to the host gills caused by *D. vastator* was observed. Hyperplasia of gill tissue led ultimately to fusion of the secondary lamellae. Affected fish became lethargic and gulped air at the surface.

Challenge experiments were carried out to investigate whether there is an acquired immunity by carp to *D. vastator* infections. The challenged fish had a significantly lower parasite burden compared to the naive fish. The infection causes a change in the blood proteins, as was clearly shown by comparison of infected and uninfected fish, the former having very visibly separable additional bands using gel electrophoresis.

Chapter 1

Introduction

INTRODUCTION

Historically, on a global basis the fishing industry has mainly consisted of capture fisheries, the highest proportion of which have come from the marine sector. In more recent times inland fisheries have shown an increase in production. An increasing proportion of inland fish production now comes from aquaculture. There has been a tradition of extensive aquaculture in Egypt and eastern Asia for thousands of years, but in this century and especially in the last 40 years freshwater aquaculture has spread throughout the world and as technology has improved there have been dramatic increases in yields. With increasing pressure on capture fisheries because of factors such as pollution and overfishing, it may be expected that aquaculture will increase even further in importance. Cyprinids contributed some 32% of global aquaculture production in 1988 of which mirror carp alone contributed 1.1% (FAO, 1990). The ideal methods for aquaculture in tropical regions are not fully defined and poor husbandry practices often result in excessive densities of fish stocked under less than ideal conditions. This encourages high levels of parasitic infections, notably protozoans and monogeneans as confirmed by Sarig (1968, 1971). The numbers of parasites necessary to cause harm to fish varies considerably with the species and size of the host and its health status. Many parasite species are host specific to some degree and are capable of infecting one or only a limited number of host species and individual parasite species may have widely

differing effects on different host species (Roberts, 1978). Among parasites infecting fishes, the Monogenea are a group which play an important role as pathogens (Bauer, 1988). The significance of monogeneans as pathogens is related to their ectoparasitic mode of life and their direct life-cycle which allows rapid transmission between hosts potentially leading to very high population densities. The Class Monogenea is divided into two main groups, the Monopisthocotylea and Polyopisthocotylea, based on their feeding, the presence of a genito-intestinal canal and the structure of their opisthaptor. Monopisthocotyleans generally feed on mucus, and have a single, undivided opisthaptor armed with hooks. The Polyopisthocotyleans however, feed on blood, grazing on the epidermis, and have a sub-divided opisthaptor that may be either armed with several hook-sucker units or clamps or equipped with unarmed suckers. Monopisthocotylea are probably nearer to the ancestral type of Monogenea than the more specialized Polyopisthocotylea, which use their multiple attachment organs to cling to the gill lamellae of the fish host (Llewellyn, 1963).

Small monopisthocotylean monogeneans comprise 30 - 40% of the entire parasitic fauna and up to 70% of the helminth fauna of freshwater fishes (Gussev, 1988). Monogeneans are usually found in large numbers on every species of cyprinid and many other species of fishes and there can be many species and genera of parasites on a single fish species (Gussev, 1988). In most

cases monogeneans cause a dual type of injury to their hosts: through their hooks and organs of attachment they injure the tissue at the site of attachment and cause local haemorrhages and at the same time they may feed on the ruptured tissue and on the blood from the wound (Bychowsky, 1957; Uspenskaya,1962).

The importance of Dactylogyrids as parasites of fish

The genus *Dactylogyrus* is composed of about 600 nominal species, being common parasites of the external surfaces, particularly the gills, of marine, brackish and predominantly freshwater fish. Cyprinids are the main hosts for the Dactylogyrinae and Diplozooninae. Twenty five species of dactylogyrids and 2 species of *Diplozoon* occur on fish other than cyprinids (Gussev, 1973).

Golovina & Golovin (1988) showed that under certain conditions *Dactylogyrus vastator* infestation can lead to pathological changes in the blood; this pathological process is not merely local in character but affects the entire organism. Two year old carp severely affected by *Dactylogyrus extensus* showed a gradual reduction in the number of lymphocytes (from 80% to 60%) while the monocytes increased two to three times; the total number of granulocytes also increased (Golovin, 1987).

In cultured fish populations, *Dactylogyrus* spp. often causes serious outbreaks of disease. The first case of an epizootic of young common carp caused by dactylogyrosis was described in 1924. Subsequently Nybelin (1924, 1925) investigated this form of dactylogyrosis. *D. vastator* was first described by Nybelin (1924) from carp *Cyprinus carpio* L.in Sweden and because of the serious impact it has in carp culture this species has been studied extensively (Paperna, 1963b). *D. vastator* infests the gills of carp fry and fingerlings causing serious losses in many countries where carp are farmed (Nordquist, 1925; Wunder, 1926; Malevitchkaya, 1952; Ivasik, 1953). Mass mortality of *Cyprinus carpio* L. fingerlings below 13 mm in size was recorded in Israel due to an infestation of *D. vastator* (Paperna, 1963b). There were outbreaks of dactylogyrosis in the Ukraine (Malevitchkaya, 1952; Ivasik, 1953), in the Russian Federation, particularly in its central part (Lyaiman, 1948; Kanaev & Lyaiman, 1959), in Georgia and Kazakhastan (Smirnova, 1947; Agapova, 1948) and Byelo Russia (Cheshina, 1954).

MORPHOLOGY

Dactylogyrus vastator is almost cylindrical in body shape (Figure 1.1). The following description is partially based on that of Yamaguti (1940). The body is elongate, 0.8 - 1.15 mm in length, with a maximum breadth of 0.15 - 0.25mm at the level of the testis, forward of which it tapers rapidly

Cp Cephalic gland

Ey Eye spot

Ph Pharynx

Vi Vitelline follicle

In Intestine

Ac Accessory sclerite

O Ovary

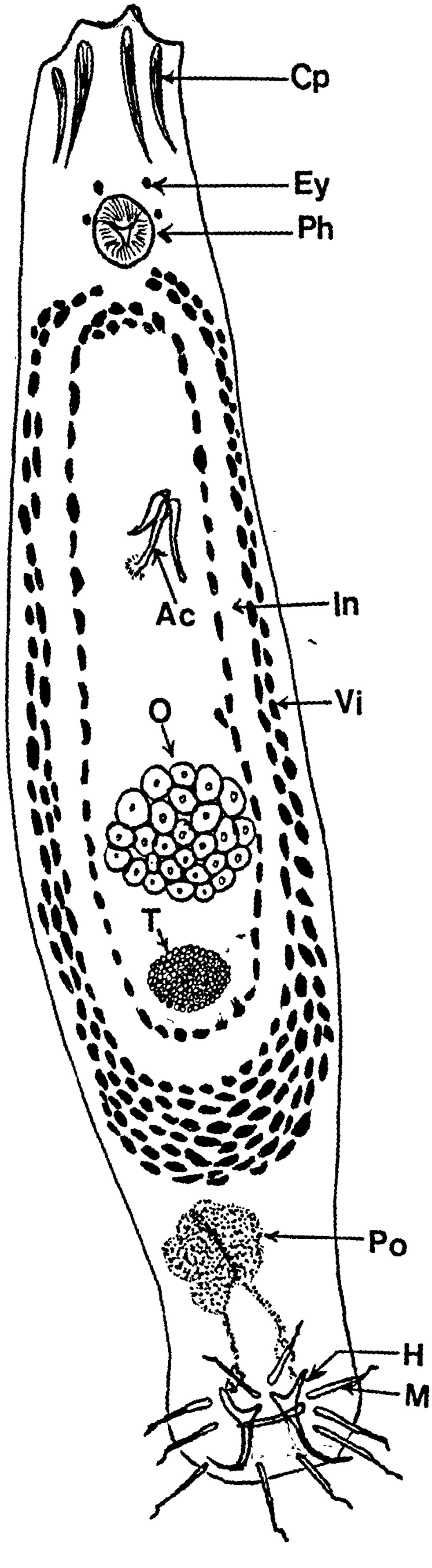
T Testis

Po Posterior gland

H Hamuli

M Marginal hook

Figure 1.1 :- The general morphology of *Dactylogyrus vastator*.


Cp
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towards the neck region. There are two pairs of very prominent head lobes. Well developed adhesive glands open on each head lobe; the secretion is discharged in the form of very fine filaments. Two pairs of eye spots are situated anteriorly to the pharynx. The caudal disk or haptor is about 120 µm in diameter, with one pair of large hooks or hamuli and seven pairs of marginal hooks. The hamuli have two truncate roots, and an external and internal root process. The hamuli are attached on each side to a connecting bar which is slightly enlarged at both ends. The copulatory organ is formed by an elongated, oval basal part, and an arched copulatory tube which is slightly funnel shaped at its base and ends in a slender fork-like branched supporting portion. The overall length of the copulatory organ is 44 - 56 µm.

The pharynx is oval and 65 - 87 x 50 - 84 µm in size. On each side of the pharynx a peripharyngeal gland is situated, opening immediately in front of the pharynx. Post-pharyngeal glands on each side of the oesophagus have an opening at the posterior end of the pharynx. The gut caeca unite with each other behind the testis. The testis is oval and 80 - 180 x 60 - 120 µm in size. The vas deferens runs forwards along the ovary on its vaginal side and after crossing the anterior end of the ovary turns toward the side opposite the vagina to curve round the caecum (Fig 1.2). The cirrus measures 54 µm long and has two prostatic reservoirs; the anterior cirrus contains granules with a greenish tinge; the posterior is elliptical to oval in shape and 12 - 33 µm in diameter, containing opalescent granules. The genital pore is placed slightly posterior to the point where the intestine bifurcates.

Ac Accessory sclerite / Acessory piece

C Cirrus

Pro Prostrate gland

U Uterus

M Mehlis' gland

O Ovary

Va Vagina

Rs Receptaculum seminis

Rvt Right vitelline duct

Lvt Left vitelline duct

Cv Common vitelline duct

T Testis

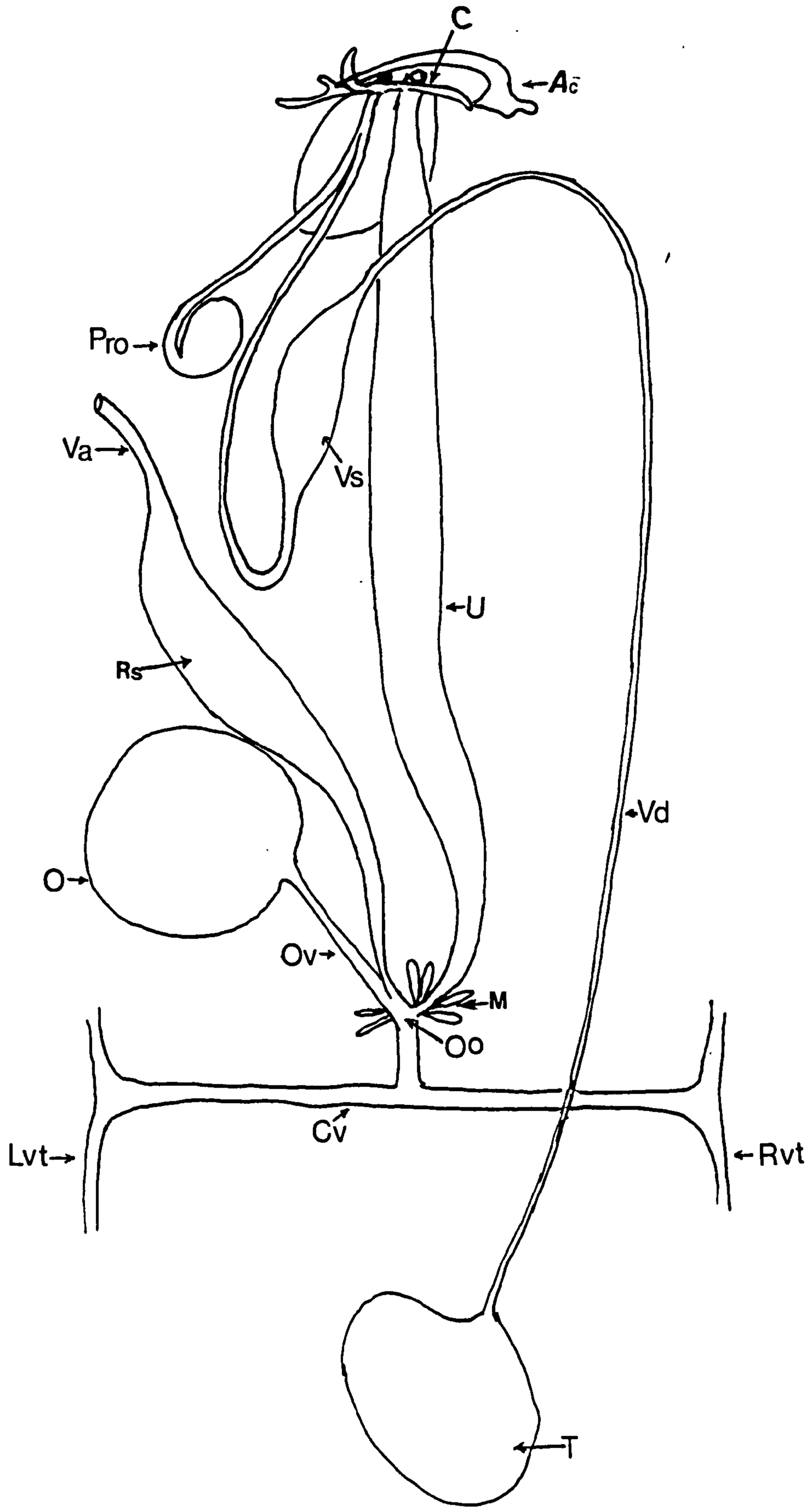
Vd Vas deferens

Vs Vesicula seminalis

Ov Ovarian canal

Oo Ootype

Fig 1.2 :- Schematic drawing of the reproductive structures of *Dactylogyrus vastator*.


C
Ac
Pro
Va
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Rs
Vd
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Ov
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Oo
Cv
Lvt
Rvt
T

The ovary is elongate, oval or elliptical, 150 - 240 x 50 - 750 µm in size with its centre a little behind the middle of the body. The ovarian canal narrows as it joins the vagina, and then widens to form a small sac (the ootype). At the anterior end where it receives the vitelline duct, it is surrounded by shell gland cells. The uterus is tubular and situated mid-ventrally. The eggs are symmetrical or asymmetrically oval, 570 - 770 x 300 - 510 µm in size with a filament 8 µm long at one end. Vitellaria extend along the caeca from the level of the posterior part of the pharynx to the anterior end of the cement glands, where they are continuous across the median line. The vagina opens on the right margin of the body at the pre-equatorial level. The vagina is lined with thick cuticle distally forming a fusiform to oval receptaculum seminis 15 - 33 µm in diameter at the point where it crosses the right caecum ventrally.

The excretory system convolutes down each side of the body before opening on the lateral margin at a point level with the cirrus; the anterior collecting vessels on both sides of the worm are united in a median line in front of the pharynx.

DISTRIBUTION

Dactylogyrus has a world wide distribution on a variety of host species. The first record of *D. vastator* came from Sweden in 1924 (Nybelin, 1924), since when it has been recorded in many countries including Britain (Kane, 1966). The species is species specific to *Cyprinus carpio* L.

Species	Host species	Country	Authority and year
D. vastator	*Cyprinus carpio*	Sweden	Nybelin, 1925
D. vastator	*C. carpio*	Czechoslovakia	Dyk, 1944
D. vastator	*C. carpio*	Israel	Paperna, 1963a
D. vastator	*C. carpio*	UK	Kane, 1966
D. vastator	*C. carpio*	India	Gussev, 1973

Host specificity

The literature suggests that monogeneans are often restricted to a single host species. For example, Hargis (1953) found a high degree of host specificity among freshwater monogeneans, whilst Llewellyn (1956) collected 18 species of gill monogenean parasites from 17 marine fish species and found that all but two parasitic species were strictly specific to their hosts. Shaharom-Harrison (1984) reported that *Cichlidogyrus sclerosus* was genus specific as it commonly infects *Sarotherodon mossambicus* and *S. spiluris*, but its presence has been recorded on other

Sarotherodon species including *S. zilli*. Some *Dactylogyrus* species are oioxenic, parasitising only one host: *D. erchardovae* and *D. sphyrna* are specific to *Rutilus rublio*; *D. alatus* and *D. minor* to *Alburnus alburnus*; *D. prostae* and *D. folkmanovae* to *Leuciscus cephalus*. Prost (1963) ranked *D. auriculatus, D. falcatus* and *D. zandti* as being species specific to *Abramis brama*. Pojmańska & Dzika (1987) reported 7 species of *Dactylogyrus* co-existing on the gills of the bream from Lake Goslawskie, Poland. Some *Dactylogyrus* species may parasitise more than one host, for example *D. vistulae* is found on *Leuciscus cephalus* and *Chondrosoma nasus*. According to Dupont & Crivelli (1988) host mucus containing free protein, glycoproteins and mucopolysaccharides, attract species specific parasites. Llewellyn (1957) suggested that strict host specificity could possibly be due to host antibodies, a relationship which would promote a high degree of host specificity at all taxonomic levels.

Infection in relation to the age of the host

Dactylogyrus vastator is a common parasite of carp fry in the first months of their life (Hanzelova & Žitňan,1983). Older fish are only occasional hosts, according to Ergens & Lom (1970) and Kollmann (1970). Hanzelova & Zitnan(1983) reported that the opisthaptoral hooks were used to attach exclusively to the tips of gill filaments of carp fry but that the gill filaments of older fish are often covered with a thick mucous layer, and this, and the relatively stronger water currents in the gill cavities were the likely reasons that *D. vastator* does not parasitise such fish.

According to Izjumova (1956), the first infestations of *D. vastator* in carp were associated with morphological differentiation of the gills in 10 - 15 day old fry. Lyaiman (1951a,b) and Kollmann (1970) agreed with this timing of first infestation.

Reproduction and population dynamics

Dactylogyrus is usually present on fish throughout the year but is generally more abundant during the spring and summer months than during the winter months. Annual cycles of *D.auriculatus* are characterized by a long period of high prevalence (78.6 - 86.9%) from February to June, a decrease from July to November (the lowest value 37.5% in November) and an increase from December through to the following spring (Dzika, 1987). The intensity exhibits a similar pattern, with a maximum in April and minimum in October to November. *D. auriculatus* reproduces most intensively in low winter temperatures to attain a maximum prevalence in March, when the monthly temperatures reach only 4 - 5°C. High summer temperatures (above 18°C) inhibit its reproduction and lead to a decrease in the number of parasites (Dzika, 1987).

The prevalence of *Dactylogyrus falcatus* increases rapidly in early spring attaining a peak (81.2%) in April and then steadily decreases to near zero in October (Dzika, 1987). Restoration of the population then begins in November. In *D. wunderi,* the rise in spring temperatures stimulates reproduction, the optimum temperature being above 18°C. *D. zandti* has a high prevalence (70 - 90%) covering

the whole warm season of the year, with an optimum temperature range above 10°C (Dzika, 1987).

Dactylogyrus extensus, initially considered as a " cold- tolerant" helminth species (Bychowsky & Nagibina, 1967) is, according to most authors, a very adaptable eurythermal species, capable of producing populations of both "warm - tolerant" and "cold - tolerant" specimens (Izjumova & Zelencov, 1969). Izjumova (1956) and Paperna (1963b) found that this parasite infests only fish smaller than 3 - 5 grams in weight, and therefore was limited to a breeding season in the spring and early summer when fish of this size were present. Bauer & Nikol'skaya (1954) showed that the optimal temperature for reproduction of *D. extensus* in the USSR was 17°C . Higher temperatures (20 to 25°C) were found to be a limiting factor for population growth for this species since egg hatching decreases when the temperature increases beyond 17°C (Paperna, 1964c). Lucky (1964a) and Zitnan (1974) have congruently reported that the invasion of carp with *D. vastator* was maximal in the warmest months of the year, i.e. from June to August. During winter the eggs laid by the worm could not develop and entered a diapause stage. When the temperature rises again in the following summer the eggs develop to give rise to infective oncomiracidia. However, a sudden drop in temperature at the end of summer stimulates its reproduction, thus causing a large rise in populaton size at a time when it is least expected (Hanzelova & Žitňan,1983).

Longevity of mature worms

Longevity of *Dactylogyrus* has not been determined directly. Incidental observations led Prost (1963) to suggest that *D. vastator* lives on the gills for about 5 days. Wunder (1949) stated that *D. vastator* lives only for 10 - 12 days, whilst Bychowsky (1957) suggested 20 - 25 days, Dogiel *et al.*(1958) 18 - 20 days and Paperna (1963b) reported 3 - 4 days. According to Izjumova (1956), the life duration of most *D. vastator* specimens was 25 - 40 days, she also suggested that some specimens pass the winter on their hosts and perish only after 6 - 7 months.

Egg production

The oviposition rate for *D. vastator* has been investigated by Lyaiman (1951a) and Bauer (1954) and the influence of temperature on *D. vastator* in all stages of development has been studied by Lyaiman (1951a), Izjumova (1953), Bauer (1954) and Bychowsky (1957) amongst others. Bauer (1954) noted a positive correlation between the rate of oviposition and temperatures up to 25°C, with a slight drop in the rate of oviposition beyond this temperature (Paperna, 1963b). Similar results were also obtained by Lyaiman (1951b). Izjumova (1953) studied the rate of oviposition for dactylogyrids *in-situ* at a temperature range of 11.5°C - 18°C. The average oviposition per 24 hour period for *D. vastator* was 4.4 eggs at 11.5°C, 4.5 eggs at 12°C and 9.3 eggs at 18°C. Paperna (1963b) observed oviposition of *D. vastator* at the temperature extremes of 12°C and 37°C, indicating that oviposition during the first 24 hours was very low: 1.5 and 2.5 eggs per parasite respectively. The number of

eggs laid was somewhat greater at 28°C than at 24°C but this difference was not significant given the great variability in oviposition rate shown by individual parasites exposed to the same temperature (Paperna, 1963b). The rate of egg development was linear with increasing temperature up to 28°C (Paperna, 1963b).

Diapause at the egg phase

Some authors (Izjumova, 1956; Paperna, 1963a) have suggested that because *D. vastator* is primarily a parasite of carp fry found only in the warmer months, the parasite produces eggs which undergo an autumn diapause on the pond bottom, hatching only with spring increase in water temperature and the consequent availability of fry.

A drop in temperature induces the appearance of "winter eggs" (Nybelin, 1925). These were larger than the "summer eggs" and fall to the bottom of the pond and remain dormant until suitable temperatures occur for their further development. Eggs laid during higher temperatures in summer/ spring develop immediately.

The egg

Dimensions of the freshly laid egg of *D. vastator* are 70 - 94 x 46 -70 µm according to Nybelin (1924). The egg resembles the eggs of other *Dactylogyrus* species in that it is irregularly oval, with one surface somewhat convex, the other rather flat. At one pole it bears a small filament, 8 -10 µm long. Nybelin (1924)

stated that the eggs of *D. vastator* remained attached to the gills of its hosts. Lyaiman (1948) and Izjumova(1956) indicated that some eggs remained on the gills while others drop off. Paperna (1963a) carried out experiments on *D. vastator in-situ* and found that all eggs laid by the attached parasite fell off the gills into the water.

When laid, the eggs are unembryonated. However, twenty four hours following oviposition a clear area is formed at one end of the egg, with darker cellular masses surrounding it. A fully developed embryo is already present within the egg after 48 hours and is easily discernible due to its movements and the appearence of 2 pairs of eyes (Shaharom-Harrison, 1986). The embryo rapidly fills the greater volume of the egg. Finally, a slit forms in the wall of the egg opposite the filament, the embryo emerges, its cilia commence beating and it swims off, rotating about its longitudinal axis. The ruptured egg empties completely or it may retain some traces of an amorphous material within it. Under optimal conditions hatching occurs 2 - 3 days following oviposition (Kearn, 1986).

Larva

The larva of monogeneans is termed the oncomiracidium, the structural features of which have been described briefly by Llewellyn (1957). The morphology of the larva of *D. vastator* was described by Bychowsky (1933). The oncomiracidium has a pyriform body shape and averages 5.58 mm in length and 1.581 mm in width with a slightly narrower middle portion. It has four eye spots each with a crystalline lens, found anterior to the pharynx. On either side of the pharynx are groups of cells which

may represent flame cells or bulbs of the excretory system. The oncomiracidium has three bands of cilia: an anterior one, a median one at the posterior region of the body proper and a posterior one on the haptor or adhesive organ in *Entobdella soleae* (see Llewellyn, 1957). The cilia are borne on epidermal cells (similar to 'epidermal plates' in the miracidia of digeneans). A single oncomiracidium of *E. soleae* yielded a total of forty-five such cells, each of which was circular in outline. Of these cells, twenty were from the anterior ciliated area of the animal, sixteen from the middle area and nine from the posterior area (Kearn, 1963). The region immediately anterior to the eyes is highly mobile in the living animal and by means of this region the oncomiracidium is capable of attaching itself to the host fish skin surface. A complex system of gland cells and ducts opens on the antero-lateral borders of the head region. Centrally situated in the body of the oncomiracidium is a relatively large 'pharynx', which is seen to consist of a number of cells around a narrow lumen. The pharynx opens directly to the exterior by means of a small mid-ventral mouth. The mouth is surrounded by a 'collar' which appears to lie within a cavity communicating with the outside. The intestine occupies the median third of the animal and takes the form of a ring (Kearn, 1963). The posterior third of the oncomiracidium consists of the haptor, the lateral edges of which are folded ventrally inwards so that the concave ventral surface of the organ is enclosed, and the cavity thus formed opens to the exterior by means of a narrow antero-posterior slit; slight coverslip pressure can cause the haptor to unfold. After hatching the larva shows a preference for the upper water layer, showing photopositive behaviour. The movement of the larva is rapid and vigorous, the body rotating about its longitudinal axis as exhibits gyrating movements upwards. The larva travels in straight lines, but may occasionally turn in circles

during its search for a host. Shaharom-Harrison (1984) observed the behaviour of the oncomiracidium of *Cichlidogyrus sclerosus* towards *Sarotherodon zillii* fry. After introduction of fish, the oncomiracidium stopped swimming, only resuming after 5 minutes, as the fish started to beat its pectoral fins, creating a water current. The oncomiracidium appeared to follow or be swept along with the inflowing water current into the mouth of the fish. Once in the water current the cilia stopped beating and the oncomiracidium was engulfed in the current flowing into the buccal cavity of the fish. Shaharom-Harrison (1984) mentioned that the longevity of an oncomiracidium is short, between 45 and 60 minutes, and those failing to find a host usually stop swimming and fall to the bottom where they remain motionless in a moribund state.

The mechanism of dactylogyrid larval infections of fish has been described by earlier workers (Paperna, 1963b; Prost, 1963). Wunder (1929) noted 3 modes of infection: a) via eggs remaining attached to the gills ; b) passive infection by entry of the larvae into the gills during respiration; and c) active adhesion of the larvae to the skin of the fish.

Nybelin (1925) observed a large number of larvae on the skin of fish and similarly Bychowsky (1933) maintained that active adhesion of the larvae to the skin is the primary method of infection. Izjumova (1956), on the other hand, detected numerous larvae on the mucous lining of the mouth cavity and only a few on the gills or skin, and therefore concluded that fish become infected primarily by the passive route and that cases of active cutaneous infection were of secondary importance and quite rare.

Paperna's (1963a) observations on larval invasion indicate that the larvae tend to concentrate around a nearby fish but do not actively attach to its skin. In all cases, the larvae were passively swept into the mouth of the fish. Active penetration of the larvae through the opercula or the mouth of the fish was not observed. In fish examined 8 hours following infection, the larvae were encountered on the gills (in fish 25 mm long); in only one instance was a single larva detected on the skin; larvae were almost entirely absent from the mucous lining of the mouth cavity. Examinations of carp fry in fish ponds revealed only one instance where a single larva was found on the fins, whereas the gills in all the fingerlings were covered with larvae (Paperna, 1963a).

The larvae are active for several hours following hatching. After 3 - 4 hours, some of the oncomiracidia sink to the bottom of the container (under experimental conditions) and feebly crawl on the bottom; some larvae however, are still swimming after 12 hours while others die after 3 - 5 hours (Shaharom-Harrison, 1984). A few larvae were found barely moving at the bottom of the container after 24 - 36 hours. Larvae are presumably infective to fish only whilst swimming and probably for not more than 4 - 5 hours (Paperna, 1963b). Bychowsky (1933) estimated that the larvae survive 10 - 12 hours at 17 - 20°C and 4 - 8 hours at 10 - 14°C. It has also been noted in some instances that larvae remained active for as long as 4 - 6 hours.

Route of infection of host

Kearn (1968) found oncomiracidia and juveniles of some dactylogyrid gill parasites on the body surface and lining of the buccal cavity of several fish hosts and suggested that the parasites then migrated to the gills. The oncomiracidia of *D. amphibothrium* invaded the ruffe, *Gymnocephalus cernua* (L.) along the respiratory current and were distributed evenly among the gills (Kearn, 1968). Subsequently the parasites must move to the more preferred site, since *D. amphibothrium* is found extensively in the dorsal segment rather than median or ventral segments of the gills where water flow is greatest and where most oncomiracidia may be expected to make first contact with the gills (Wootten, 1974). According to Paperna (1963a) the larvae of *D. vastator* may be carried passively into the mouth of the fish with the respiratory current and then swept onto the gills.

Rate of development

The rate of maturation of *D. vastator* on the fish is dependent on temperature. Once settled on the gills development to egg-laying requires 4 - 5 days at 28 - 29°C. On the second day after settlement, the developing dactylogyrids appear mature except for the lack of vitellaria. At lower temperatures (22°C), maturation was attained only on the 9th - 10th day (Paperna, 1963b).

According to Prost (1963), development to egg laying at 15°C lasts 20 days; at higher temperatures development was more rapid taking on average 10 days.

Nybelin (1925) observed maturation within 6 - 7 days whilst Izjumova (1956) reported the presence of central hooks and copulatory organs in developing *D. vastator* after 4 - 5 days, with full sexual maturity being attained in 8 days at 15 - 20°C .

The influence of salinity, oxygen concentration and NH_4 ion concentration upon reproduction of *D. vastator*

Paperna (1963b) reported that salinities higher than 1500 - 2000 mg Cl/l , inhibited the growth of *D. vastator* populations. The embryos developed within eggs of the parasite at water salinities of 255 - 4000 mg Cl/l. The larvae, however, were killed immediately upon hatching in water salinity higher than 1500 - 2000 mg Cl/l (Paperna, 1963b). The adult parasites, on the other hand tolerate moderate increases in salinity, being able to survive 3 days at a water salinity of 4000 mg Cl/l, although at 7000 mg Cl/l they die off within 24 hours.

The rate of infestation with *D. vastator* did not seem to be affected by the oxygen concentration of the water (Paperna, 1963b), although Izjumova (1956) reported an inverse correlation between oxygen concentration of the water and the rate of oviposition of *D. vastator.*

The ammonia ion concentration of the water had no effect on *D. vastator* (Paperna, 1963b) which remained viable even at a concentration lethal to the host fish (52 ppm NH_4 = 150 ppm NH_4Cl).

The *Dactylogyrus vastator* problem

Golovina & Golovin (1988) found a number of changes in blood parameters in heavily infected carp (30 individual/g and over) irrespective of their weight. There was a decrease in the haematocrit and haemoglobin content and erythrocyte count with polychromatophil normoblast numbers increasing up to 44%. Changes in leucocytes were characterized by a 2 to 3 fold decrease in lymphocyte numbers, and a marginal decrease in the reticular cell and neutrophil numbers. At the same time the number of eosinophils and monocytes increased from 0.43 to 0.82 and from 0.61 to 0.80 x 10^{-3} /µl, respectively. The authors suggested that these changes should be considered as characteristic of dactylogyrosis.

The same authors evaluated the pathogenic effect of lower infection levels on blood parameters of carp weighing 2 - 2.5g. At an infection intensity from 1 to 5 ind./ fish or less than 3 ind./g, no consistent changes were noted in the haematological indices. Similar studies conducted with two other groups of fish weighing 4 - 5 and 8 g showed that changes in blood parameters characteristic of dactylogyrosis were observed at infections exceeding 40 ind./ fish or 5 ind./g fish weight. The carp with an infection level of 3 *D. vastator* / g fish weight did not show any changes in haematological parameters (Golovina & Golovin, 1988).

Golovina & Golovin (1988) evaluated the relationship between fish weight and the level of infection with *D.vastator*. This relationship was not always simple. At

relatively low numbers of parasites per unit weight of fish the more heavily infected fish had a higher weight. At higher intensities of infection the weight of the most heavily infected fish is less than that of relatively lightly infected fish, which is indicative of a negative effect by the parasite on fish growth. The negative effect of *D.vastator* on growth of young carp was noted at an infection level exceeding 10 ind./g weight. The growth rate (Km) of young carp in rearing ponds may be decreased: at 9 parasite/g - by 40%, at 33 parasite/g - by 45%, at 56 parasite/g by 78%. At infection rates exceeding 80 parasite/g carp growth stopped, fatness was reduced significantly and at an infection rate exceeding 100 parasite/g mortality occurred (Golovina & Glovin, 1988).

The negative effect of parasites on fish was significantly dependent on rearing conditions, especially food availability and pond hydrochemical conditions. Thus with a relatively high growth rate of young carp (Km = 80 - 90% of the theoretically possible growth rate at a given water temperature) an infection of 10 parasite/g fish weight did not cause reduction of this index. Fish kept under such favourable conditions reach 3 - 5g in a short time, after which young carp become less susceptible to pathogens and the infection intensity drops abruptly (Bauer, 1959; Golovin,1987).

Under highly unfavourable rearing conditions (Km of uninfected fish < 15 - 20 % of the theoretically possible growth rate) the negative effect of *D.vastator* increases sharply and is manifested in a reduced growth rate at infection levels of less than 10 parasites/g fish weight.

Pool & Chubb (1987) found that in carp 15 to 45 mm in length infected with up to 65 *D. vastator* per fish, the parasites caused considerable damage to gill membranes and there was mortality of the heavily infected fish. However, no mortalities occurred at infection levels of 1 - 10 parasites per fish.

Infected fish show rapid opercular movements. According to Amlacher (1970) the edges of the gills become thickened and the opercula are held partly open when the infestation is severe.

In heavily infected small fingerlings (15 - 20 mm long), the gill structure was entirely destroyed and may carry only a small number of parasites. In such fingerlings, branchial tissue regeneration is very slow and consequently the gills become entirely covered with parasite induced hyperplastic tissue. Larger fingerlings (over 20mm long) were infected with a large number of parasites but histological changes in the gills were insignificant and occurred only at the edges of the gill filaments (Paperna, 1963a). In heavily stocked ponds, the growth of the fish is retarded and the gills of the larger fingerlings, 20 - 25 mm long, became entirely or almost entirely covered with epithelial hyperplasia with resulting mortalities. Fingerlings over 30mm are no longer likely to succumb to the parasites.

Histopathology

Molnàr (1972) reported that the gills of carp may show either local lesions or general lesions which account for the greater part or the entire gill damage that occurs when a fish becomes infected with *Dactylogyrus* sp. Local lesions are primarily caused by the penetration of the hamuli of the opisthaptor through the cartilage of the primary lamellae. General gill lesions include degeneration, haemorrhages, necrosis, atrophy and cell proliferation. The degree of lesions depends on the duration of infections and the number of parasites established on the gills.

Gill changes in fish infected with *D. vastator* are generally first noticed at the tips and then in interfilamental regions of the primary lamellae. Larger individual fish usually had greater parasite intensities but less gill damage (Thoney & Hargis, 1991). They concluded that as long as fish were not overcrowded and growth remained optimal, regeneration of gill tissue is sufficiently rapid to counteract the damage caused by a large number of parasites. Fast regeneration of the host epithelium has been reported by Kearn (1963, 1976). The hyperplasia which develops on the gills is an unsuitable habitat for *D. vastator* (Paperna, 1963a) and sloughing of hyperplastic tissue along with attached *D. vastator* resulted in the recovery of the fish (Paperna, 1964).

Physiological effects

Massive invasions of *Dactylogyrus* chiefly affect the respiratory epithelium. Degeneration or desquamation of the epithelium diminishes the oxygen intake, but more damage is done by extravasation of blood plasma across the damaged endothelium in young carp resulting in the osmotic imbalance of the blood. The observed increase in mucus secretion was not related to the hyperfunction or multiplication of goblet cells (Paperna, 1964) because most of the cells deteriorated along with the gill epithelium.

Immunity

Carp acquire an immunity to *D. vastator* infections (Paperna, 1964c). The histopathology of the gills shows hyperplasia, which is presumably related to the eventual loss of the parasite from the infected fish (Woo, 1992). Vladimirov (1971) suggested that antibody production was related to protection. The survival of the oncomiracidium was reduced when they were exposed to antisera from heavily infected carp. Although carp can be reinfected, they do not become resistant to subsequent parasitic challenges (Paperna, 1964).

Control

Both chemical and management methods have been used to control *Dactylogyrus* infections in aquaculture.

The chemical methods of prophylaxis and therapy of dactylogyrosis are highly varied. Fish are usually treated by adding chemicals to tanks or ponds as short-term baths.

D. vastator were completely eliminated by a single treatment with 250 ppm formalin for 35 - 40 minutes and this is widely used as an environmentally friendly method in comparison with the other available drugs (Nguenga, 1988). Two repeated applications with 5 ppm potassium permanganate at two day intervals were also used to eradicate *D. vastator* from fish (Nguenga, 1988).

Trichlorfon has been recommended and widely used for the treatment of monogeneans (Imada & Muroga, 1978; Schäperclaus, 1991a). Treatment with Naled (Kyldane) was completely successful in eliminating the *Dactylogyrus* infections. A dipping bath of sodium chloride at a dose of 50g/ l water for 3 min also proved effective against dactylogyrids (Lucký, 1984).

A 50 % solution of Dipterex (0.0 - dimethyl -1- hydroxy -2- trichloromethyl phosphate) at a concentration of 0.8ppm frees carp from parasites attached to their gills (Sarig *et al.*, 1965). A 2.5 % solution of NaCl easily killed *D.lamellatus* under

both experimental and field (natural) conditions; fry tolerated this concentration well for fifteen minutes (Molnàr, 1971c). Five organic phosphoric acid esters, such as crystalline Trichlorfon, Ditrifon 50, Filobiol E, Nuvanol and Gradona, administered as short and long term baths were effective in deparasitising carp during short-term and long-term bathing. Long-term baths of phosphoric acid esters were an effective means of controlling *D. lamellatus* adult stages (Molnàr, 1971c).

The transfer of fingerlings to growth tanks as early as possible to reduce their density has been practised, as it is the stage mostly affected or vulnerable to *D. vastator* infection. This method decreases the losses in the fish stock (Grabda & Grabda, 1966).

Aims and Objectives

Although *Dactylogyrus vastator* has long been recognised as a most important pathogen of young carp in Europe and elsewhere, there are surprisingly few recent studies on its biology, and thus many aspects remain unknown and relatively poorly described. Further studies on *D. vastator* would provide further information which might be of value in controlling the parasite.

The literature on the morphology of *D. vastator* is limited, especially at the ultrastructural level. This study investigates the structure of *Dactylogyrus vastator* with the help of scanning and transmission electron microscopy and compares the findings with other Monogenea. Particularly attention is paid to the structure and

function of the opisthaptoral hooks and comparisons made with those of *Gyrodactylus* as observed by Shinn *et al.* (1993).

There has been some controversy concerning the optimum temperature for *D. vastator* which has been reported to vary according to the geographical region. The effects of temperature on the population biology of *D. vastator* is manifestly extremely important in determining the abundance of the parasite under given conditions and this aspect has been studied under a range of temperatures. Variations in parasite numbers and overdispersion over time at different temperatures have been determined experimentally. Identification of monogenean species is often difficult and is to a large extent dependent on the shape and size of the opisthaptoral hooks and copulatory apparatus. If these structures vary in size and shape depending on the temperature then correct identification will be made much more difficult.

The rate of egg laying of monogeneans at different temperatures is important in influencing the size of the parasite populations and species are known to have optimum temperatures. The egg production of *D. vastator* at different temperatures *in-vitro* was therefore investigated. In addition the process of egg formation and oviposition in *D. vastator* was examined using light microscopy.

Carp are known to develop an immune response to *D. vastator* which is of great importance in the management of the disease in aquaculture and which may potentially form the basis for vaccine development. Experimental studies were carried out to investigate the effectiveness of this response in juvenile carp and to assess its impact on the population biology of the parasite. Gel electrophoresis was carried out

on sera from infected carp to determine any changes in blood parameters which might provide the basis for an immune response.

Dactylogyrus vastator is known to cause severe damage to the gill of young carp, but there are no detailed illustrated accounts of these effects. In this study the development over time of gill lesions caused by *D. vastator* has been studied experimentally using light microscopy.

Monogenean and other gill parasites often show a differential distribution over the parts of the gill apparatus, which may be related to water flow or the need to ensure successful mating. The distribution of *D. vastator* has not previously been studied but was examined here in juvenile carp and compared to the situation with other *Dactylogyrus* species.

Chapter 2

Temperature effects on *Dactylogyrus vastator* populations

2.1. Introduction

2.1.1. Temperature effects on *D.vastator* populations

Temperature is one of the major environmental factors which influences the population dynamics of *D. vastator*. Different species of dactylogyrids behave differently with the season and each species of *Dactylogyrus* probably has an optimal temperature favouring its population growth, which may depend on its particular geographical area or location. Most published accounts report that *D. vastator* invasion of carp is maximal in the warmest months of the year, i.e. from June to August and may drop to zero in September - October (Grabda-Kazubska *et al.*, 1966; Izjumova *et al.*, 1982; Hanzelova & Žitňan, 1983; Dzika, 1987). High water temperatures (20 - 26°C) are optimal for *D. vastator* and accelerate its development (Bychowsky, 1933; Kollmann, 1970, 1972). However, Reda (1988) reported that high summer temperatures (above 18°C) inhibit reproduction and cause a decrease in the number of *D. vastator*, whilst a temperature of about 4°C stimulates a new period of intensive reproduction. There are different reports, often contradictory, on the development of *D. vastator* during the winter season. Kollmann (1970, 1972) reported no *D. vastator* during the winter season. However, there are reports of its sporadic occurrence and even heavy invasion of wintering carp (Lucký, 1964a,b).

Changes in dactylogyrid abundance probably reflect, at least in part, the reproductive rate of the parasite. Izjumova (1956) investigated egg laying by *D. vastator* under natural conditions, and found a strict correlation between the number of eggs laid and temperature. At a temperature of 18.1°C one individual of *D. vastator* laid on average 10 eggs and at 11.5°C only 5 eggs per 24 hours. The development of larvae inside the egg is also dependent on temperature, the lower the temperature, the lower the percentage of development. The whole development of *D. extensus* from egg to sexual maturity and first oviposition lasted 9 days at 24°C to 25°C (Prost, 1963). However, at this temperature none of the eggs develop further. Bauer & Nikol'skaya (1954) stated the same process lasted 16 - 18 days at 17 to 19°C.

2.1.2. Principal Component Analysis (PCA)

Principal component analysis is a multivariate analysis using morphometric data used to separate morphologically close species on the basis of considering all the measured variables simultaneously and loading for differences between the specimens (Gibson *et al.*, 1992). Seasonal variation or local environmental changes may influence the parameters of the sclerotized portions of the opisthaptor in monogeneans (Kulemina, 1987; Mo, 1991a,b,c). Since opisthaptoral measurements are very important in the specific identification of dactylogyrids it is obviously important that any intra-specific

variation should be identified, in order to avoid possible mis-identifications. In view of this, a method to compare the measurements of sclerotised parts of the opisthaptor from worms kept at different temperatures would be useful.

Mathematical modelling applied to morphometric data, is a way to separate or discriminate one pattern of measurements from another. Bray & des Clers (1992) used principal component analysis in a series with stepwise linear discriminate analysis to show the existence of five species of Lepocreadiidae in gadiform fish using 22 measured variables. This present study was carried out to assess the morphological variation or discrimination from measured variables of *D. vastator* reared at three different water temperatures using PCA analysis.

2.2. Materials and Methods

2.2.1. Temperature effects on *D. vastator* populations

Fish used in this study (500) were brought in from Munton and Fison, Cedars, Stowmarket, and kept in a large 69 cm x 51 cm x 52 cm 160 litre aquarium for two weeks to acclimatise the fish to this new habitat at 17°C before the start of the experiments.

To study the effect of temperature on *D.vastator* populations at 12°C and 22°C, representing the lower and higher extremes of the temperature range of *D. vastator*, the following experiment was carried out; at each temperature experimental fish were reared in 6 separate 45cm x 30 cm x 26 cm 8 litre tanks maintained at 12°C and 22°C. Fish tanks were maintained at a constant temperature by an immersed electric water heater. Twenty fish of average 4.38 (± 0.975 cm) mean length and 4.19 (± 1.62 g) total weight with a mean *D. vastator* infection of 1.12 (± 1.14) (range 1 - 11 worms per fish) per fish were placed in each tank. One complete tank ie. twenty fish was sampled each week for six weeks in the 12°C experiment and one complete tank ie. twenty fish was sampled every 2 weeks in the 22°C experiment.

To study the effect of temperature on populations of *D. vastator* at 19°C two sets of experiments were performed. Preliminary studies had shown that 19°C represented an optimum temperature for *D. vastator* under aquarium conditions. Accordingly two experiments were performed at this temperature to examine in detail the population dynamics of *D. vastator* under optimal conditions.

In experiment I at 19°C, 12 tanks of 45 cm x 30 cm x 26 cm size, each with 8 litres of water, were maintained at the required temperature. Twenty fish of 4.24 (± 4.28) cm mean length and 1.04 (± 0.436) g mean weight with 7.9 (±

11.14) (range 5 - 25 worms per fish) *D. vastator* per fish were introduced to each tank. One complete tank ie. twenty fish was sampled every two weeks until the end of the 12 week experimental period.

In experiment II, two 78 cm x 58 cm x 33 cm tanks, each with 20 litres of water, were maintained at 19°C. One hundred and twenty fish of 3.65 (± 2.38) cm mean length and 1.04 (± 0.443) g mean weight with 5.2 (± 5.40) (range 2 -35 worms per fish) *D. vastator* per fish were introduced to each tank. Twenty fish from both tanks were taken at bi-weekly intervals.

In all experiments, fish necropsy was carried out and the gills from each side were removed and placed in separate plastic dishes with aquarium water of the same temperature. The number of *Dactylogyrus* worms were counted at x40 magnification on an Olympus stereomicroscope.

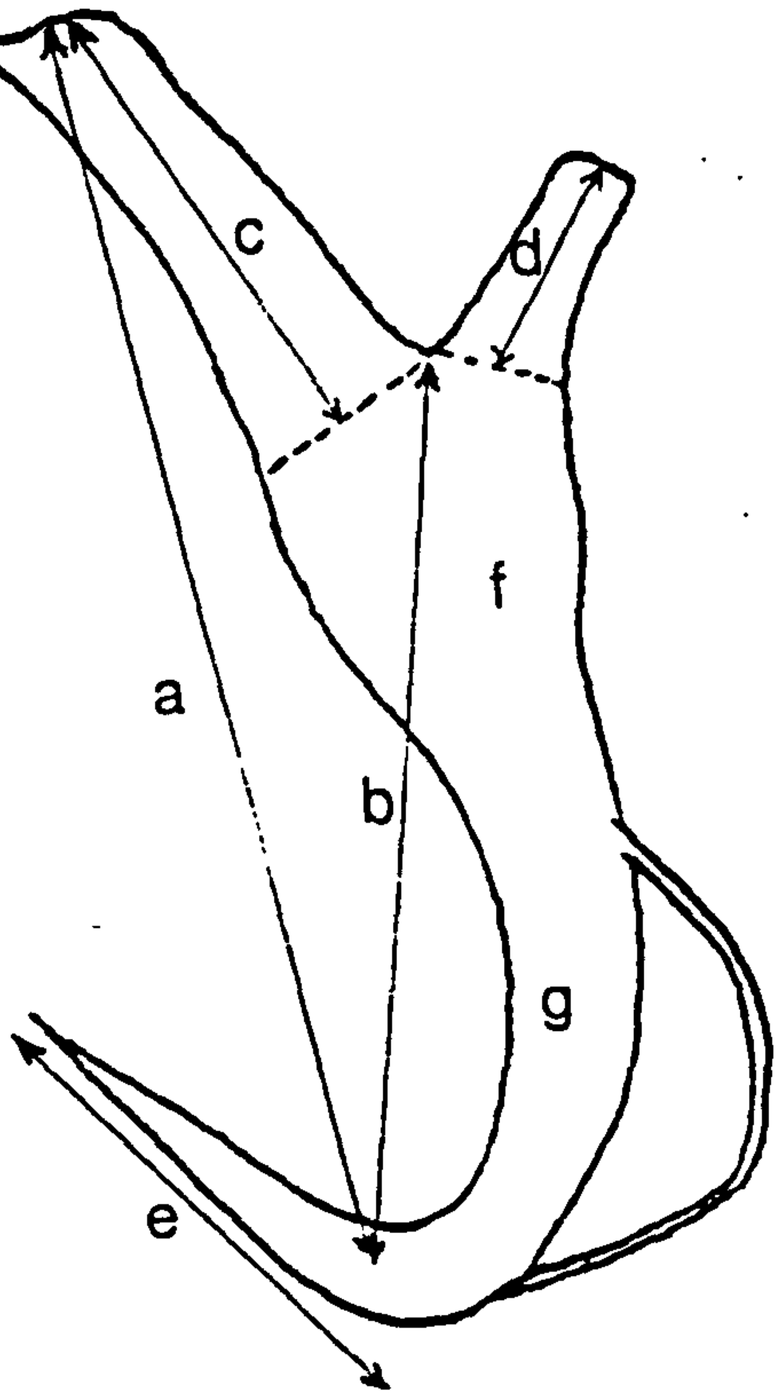
The data was interpreted using Cricket Graph (™ commercial graphics package) to show the variation of parasite number with time.

2.2.2. Principal component analysis (PCA)

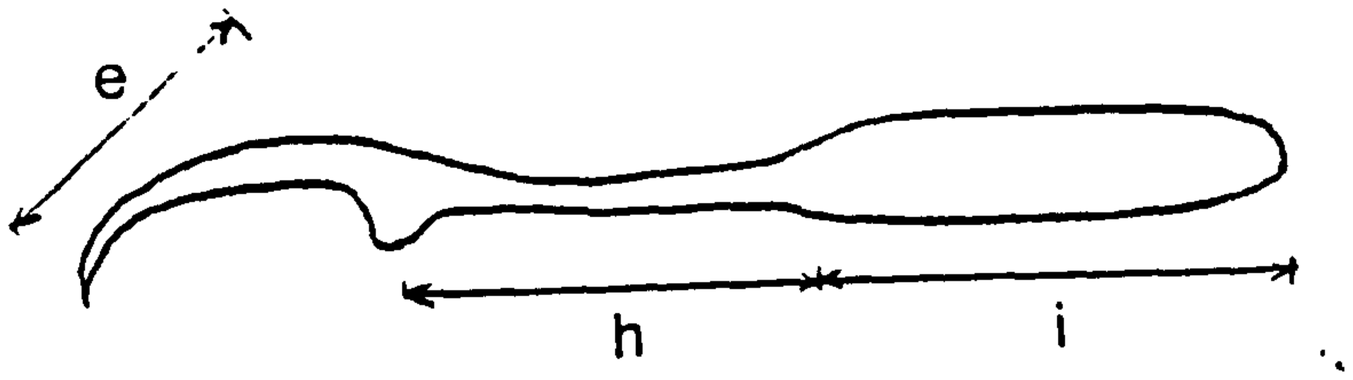
Flat preparations of *D. vastator* were prepared and mounted in ammonium picrate glycerine (Malmberg, 1970) for light microscopic studies. Ten specimens from the 12°C and 20 specimens from the 19°C experiments were selected for the analysis. In addition 10 specimens from stocked fish maintained at 14°C were also used.

Specimens were measured under x40 magnification using an Olympus BH2 binocular microscope with an eye piece graticule (100 x 0.01mm divisions). A series of measurements were taken on the opisthaptoral sclerites, these are given in detail in Figure 2.1.

All data were included in the first PCA test. Principal component analysis explains the relationship between the measured variables. Each selected axis of the PCA plot is arranged by the amount of variation which they explain (Gibson *et al.*, 1992). A cluster analysis was performed on the PCA plots to detect natural groupings within the data set. The way in which these groups are produced is by calculating some measure of dissimilarity between the specimens. Pearson's correlation can be used as the basis of dissimilarity. The cluster analysis is interactive, and is instructed to look for 2 clusters, then 3 clusters, 4,5 etc. up to 10 clusters (in this data set) (Bray & des Clers , 1992).



Hamulus



Marginal hook

a total length of hook
b length of basic portion or base
c length of outer root or external process
d length of inner root or internal process
e length of blade or spike
f expanded section of hamulus shaft
g narrow section of hamulus
h blade of marginal hook or keel
i handle of marginal hook

Fig 2.1 :- Diagram showing the details of the measurements taken from the hamulus and marginal sclerite.

The number of groupings within the data by PCA is given by the F ratio and to a lesser degree by the value of probability in the summary statistics for the number of clusters pulled out by the analysis. The clusters indicated by the analysis, were cross-referenced back to the specimens and re-examined.

2.3. Results

2.3.1. Temperature effects on *D. vastator* populations

Under all the different temperature regimes the mean number of *D. vastator* per fish increased initially, reaching a maximum (at a particular time of the experiment) and then showing a diminution to the minimum level or even to zero.

Fig 2.2a shows that at 12°C there was a continuous increase in the mean abundance of *D. vastator* up to week 5, although the mean remained low throughout. The mean number of adult *Dactylogyrus* also increased up to 5 weeks (Fig 2.2c), but the mean number of immature *Dactylogyrus* was constantly low until week 4 after which there was a sharp increase to week 5 (Fig 2.2b). Fig 2.6a shows the variation in variance to mean ratio with time at 12°C, where the ratio remained constant up to the 4th week of the experiment but after which there was a sudden increase from the 4th to the 5th week of

Fig 2.2a Variation in mean number of adult and immature *Dactylogyrus vastator* with time at 12°C.

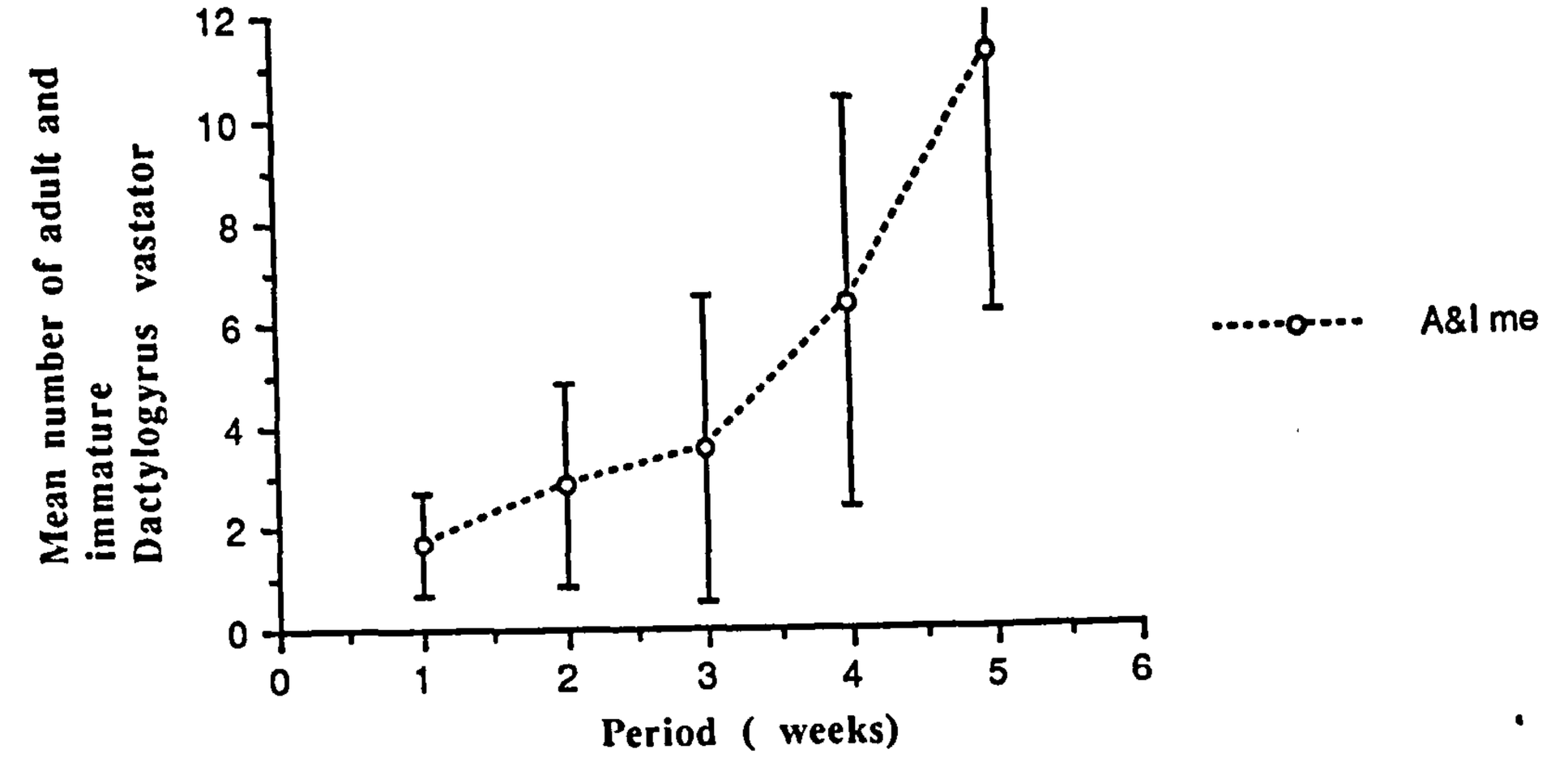


Fig 2.2b Variation in mean number of immature *Dactylogyrus vastator* with time at 12°C.

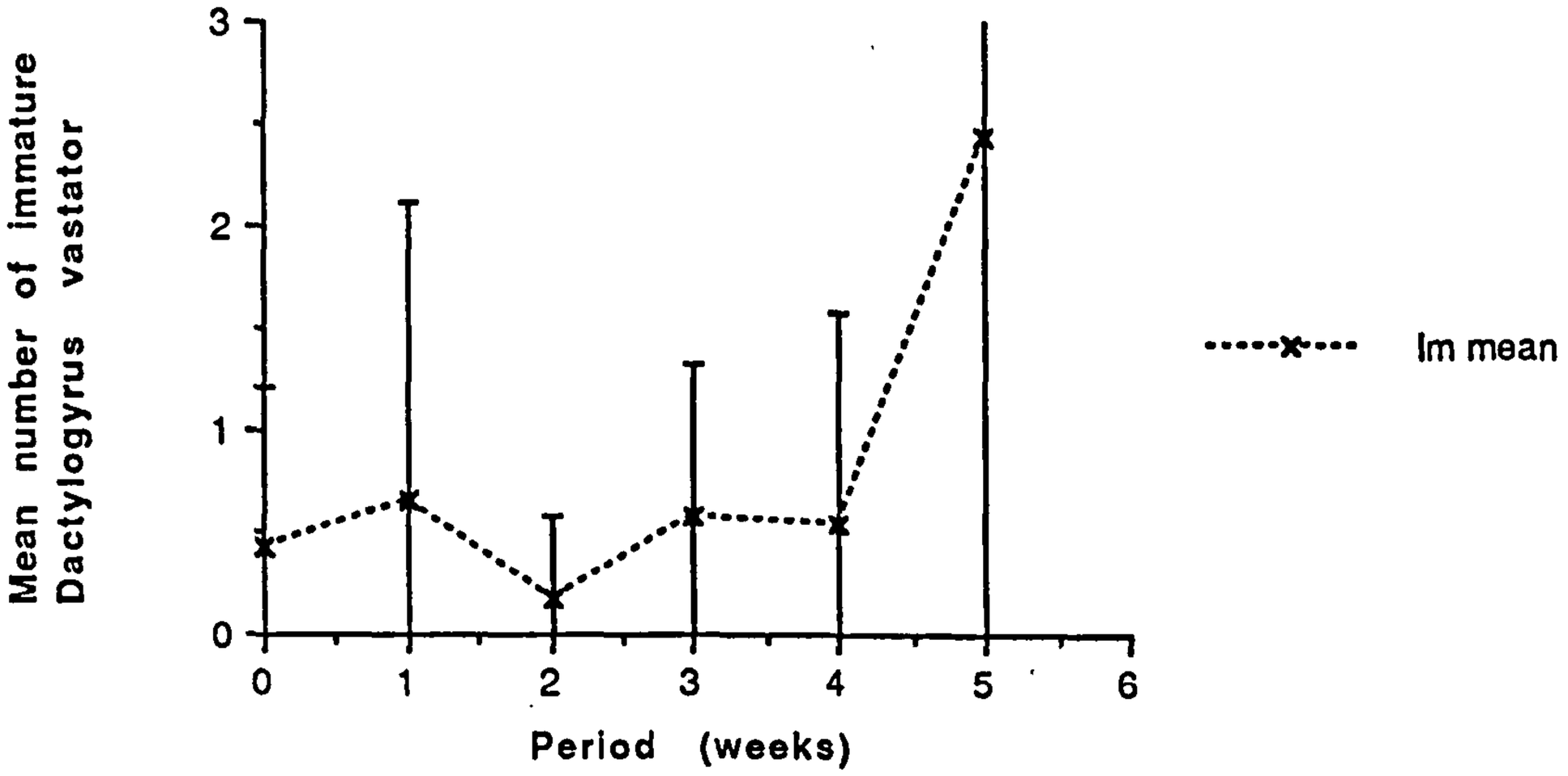
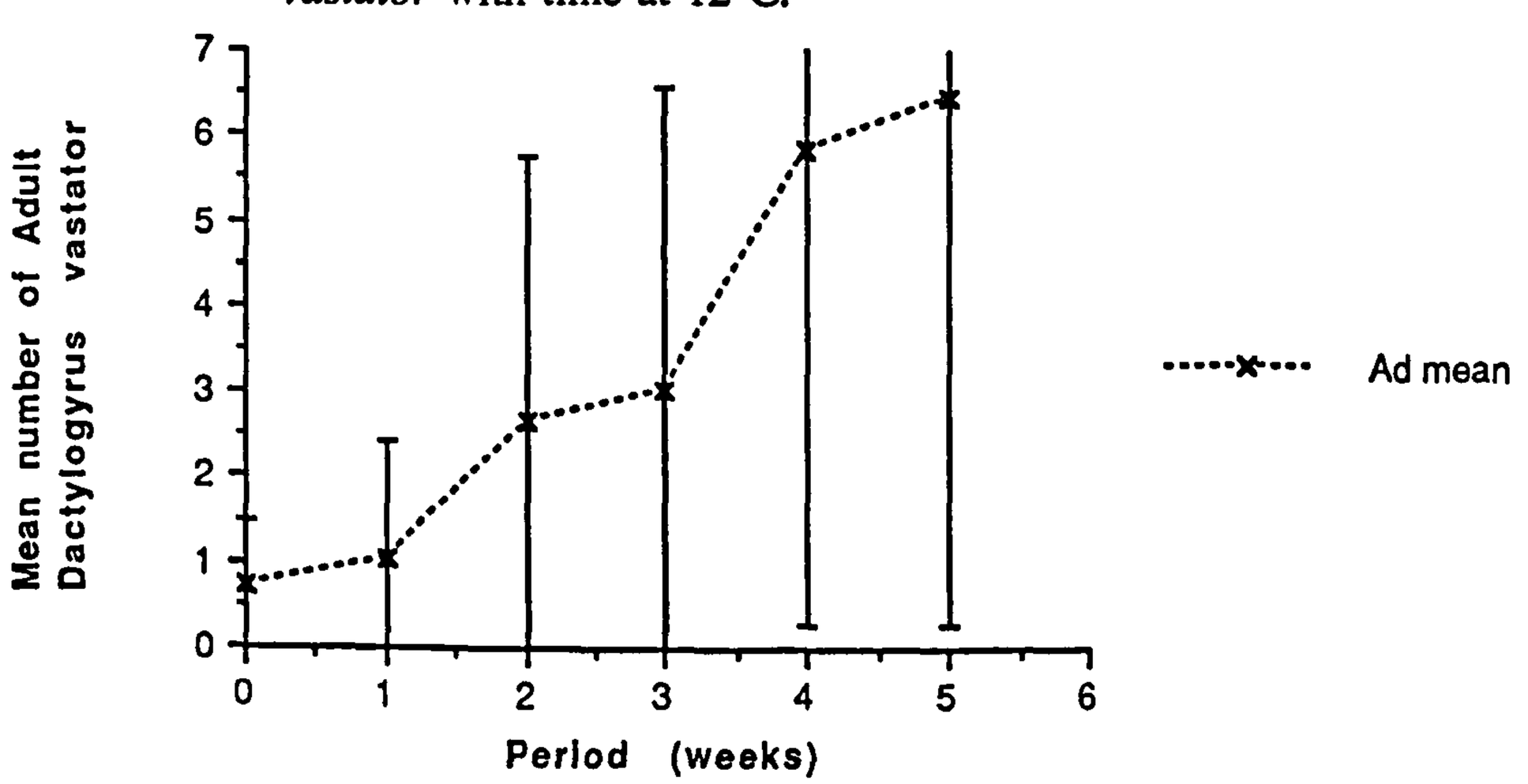


Fig 2.2c Variation in mean number of adult *Dactylogyrus vastator* with time at 12°C.



infection. In this experiment, as in those at 19°C and 22°C the range of parasite numbers between individual fish in each sample was very large.

Prevalence was lowest in the 12°C experiment, although even here the lowest level was 65%, at higher temperatures prevalence exceeded 80% in most samples. There was no association between prevalence and abundance of infection.

Fig 2.3a shows that at 19°C experiment I the mean number of immature and adult parasites remained at a low level until week 3 after which it rose dramatically to week 4 before declining equally precipitously in the following 2 weeks. After this there was a further decline to a very low level at week 10.

Fig 2.3b shows that the mean number of immature *Dactylogyrus* remains at a low level until week 2 and then rises dramatically to week 4 before decreasing sharply in the following two weeks. After this there is a continuous decline towards the minimum level at week 12. Fig 2.3c shows that at 19°C the mean number of adult *Dactylogyrus* remains low up to week 2 and then rises sharply to the maxima at week 4 followed by a slow decline in the following 4 weeks leading to the minimum level by week 12. Fig 2.6b shows a steady increase in the variance to mean ratio up to the 4th week of infection which is then followed by a rapid decline to weeks 10 and 12.

Fig 2.3a Variation in mean number of adult and immature *Dactylogyrus vastator* with time at 19°C in experiment I.

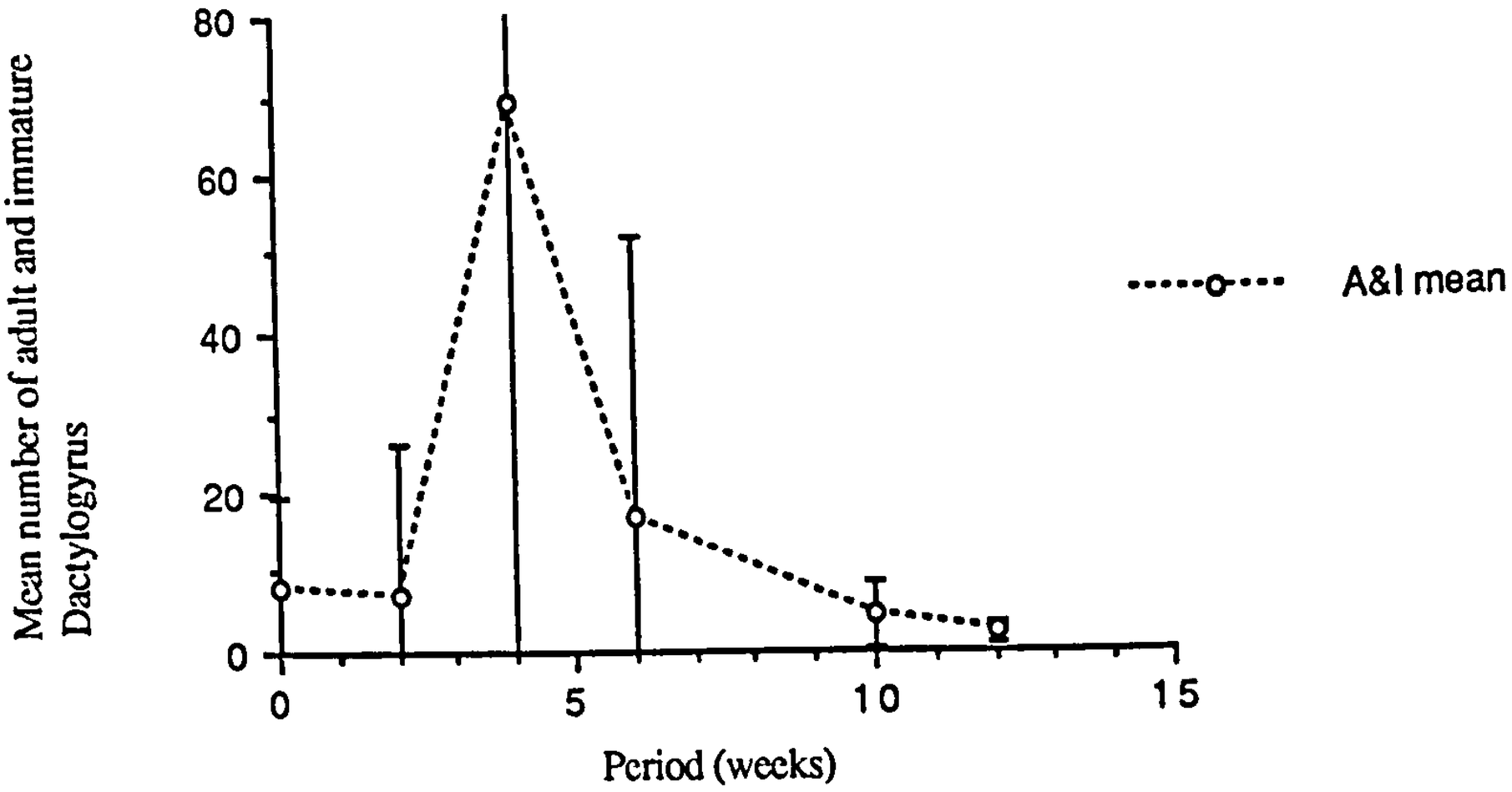


Fig 2.3b Variation in mean number of immature *Dactylogyrus vastator* with time at 19°C in experiment I.

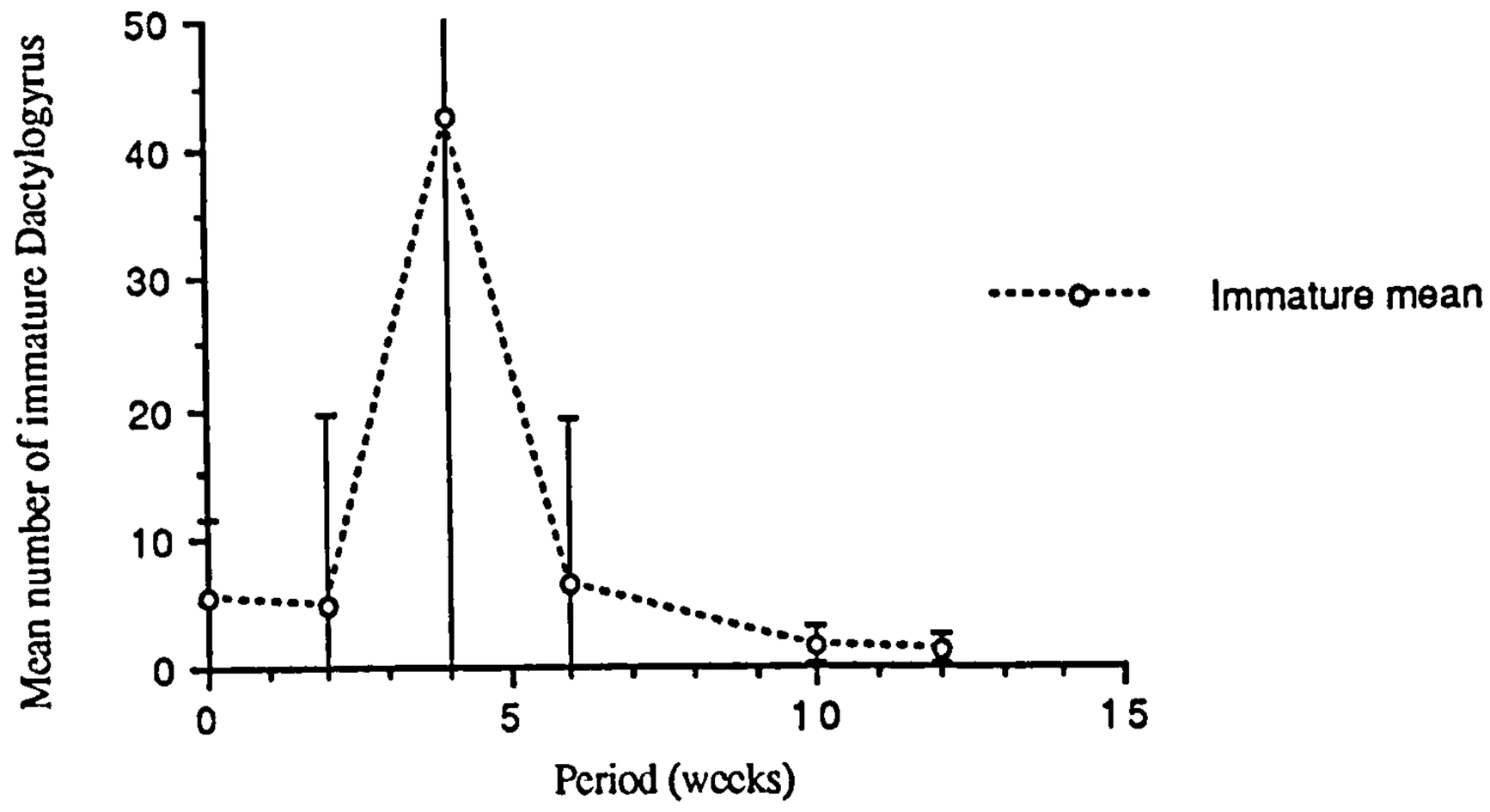
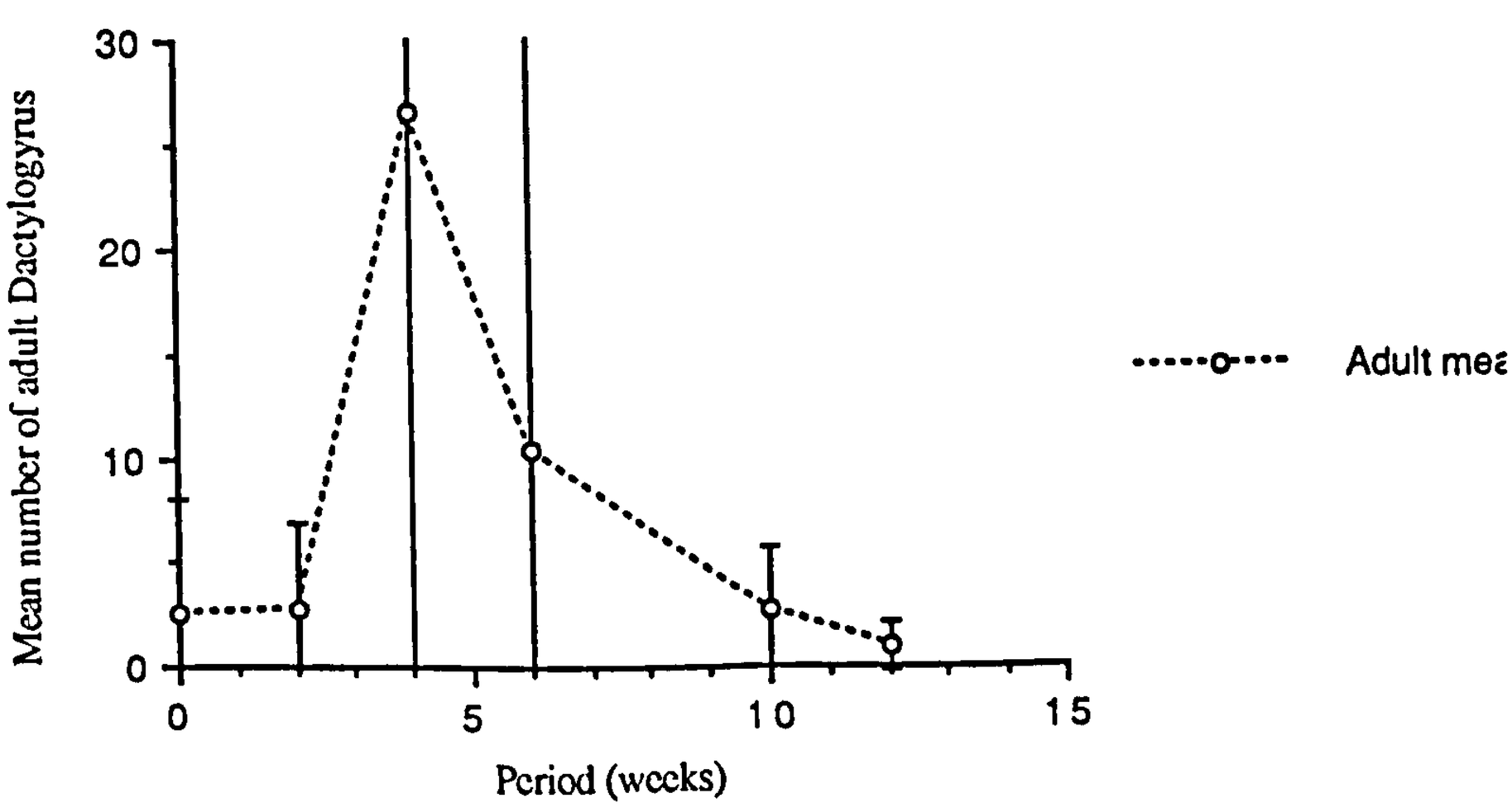


Fig 2.3c Variation in mean number of adult *Dactylogyrus vastator* with time at 19°C in experiment I.



In the second experiment at 19°C (Fig 2.4 a), parasite numbers increased very rapidly to week 2. There was then a steady decline until week 7 by which time parasite numbers were close to their original level. There was then another rise to a high level by week 12. In this experiment the mean number of adult *Dactylogyrus* (Fig 2.4 c) increased by week 1, then decreased in week 2, followed by an increase to its highest level at week 3. From week 3 there was a rapid decline to week 5 followed by a fairly constant number of parasites to week 12. Fig 2.4 b shows the variation in the mean number of immature *Dactylogyrus* with time. There was a very rapid rise to a high maximum number at week 2, followed by an equally rapid decline to week 3. Numbers then remained relatively constant to week 8 after which there was some increase to week 12. The variance to mean ratio for this experiment is shown in Fig 2.6 c. The ratio showed maximum levels at weeks 2, 11 and 12, but between these it was relatively low. Overall numbers of *D. vastator* were much higher in this experiment than in experiment I.

At 22°C parasite numbers rose slowly to week 6 but then increased very rapidly to reach a maximum level by weeks 8 and 10 followed by a drop almost to zero at week 12 (Fig 2.5 a). Parasite numbers were not so high in this experiment. Fig 2.5 b shows that there is a continuous increase in the mean number of immature *Dactylogyrus* worms to week 8 which then drops to the minima at week 12. Fig 2.5 c shows that at 22°C there is a continuous

Fig 2.4a Variation in mean number of adult and immature *Dactylogyrus vastator* with time at 19°C in experiment II.

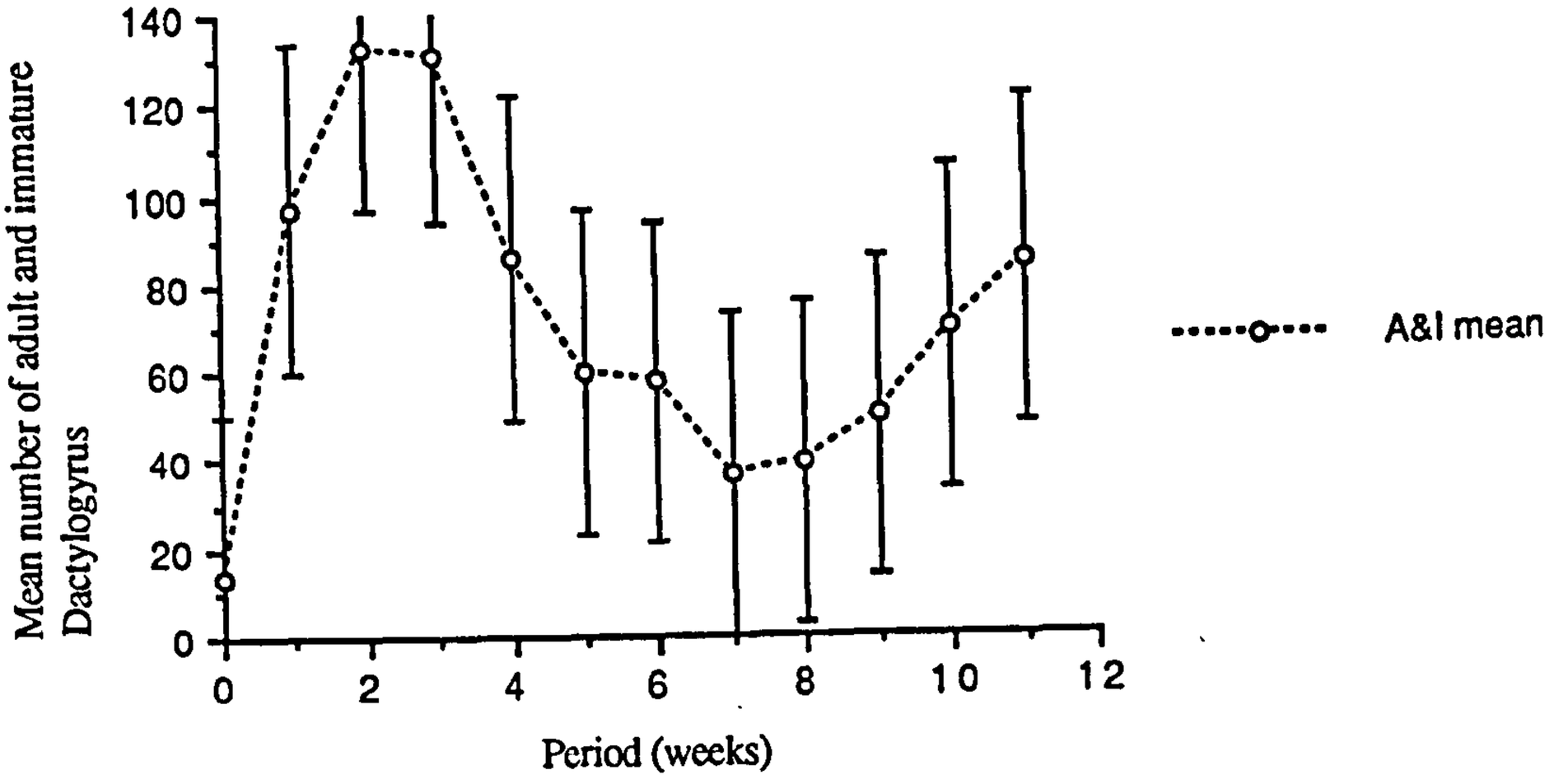


Fig 2.4b Variation in mean number of immature *Dactylogyrus vastator* with time at 19°C in experiment II.

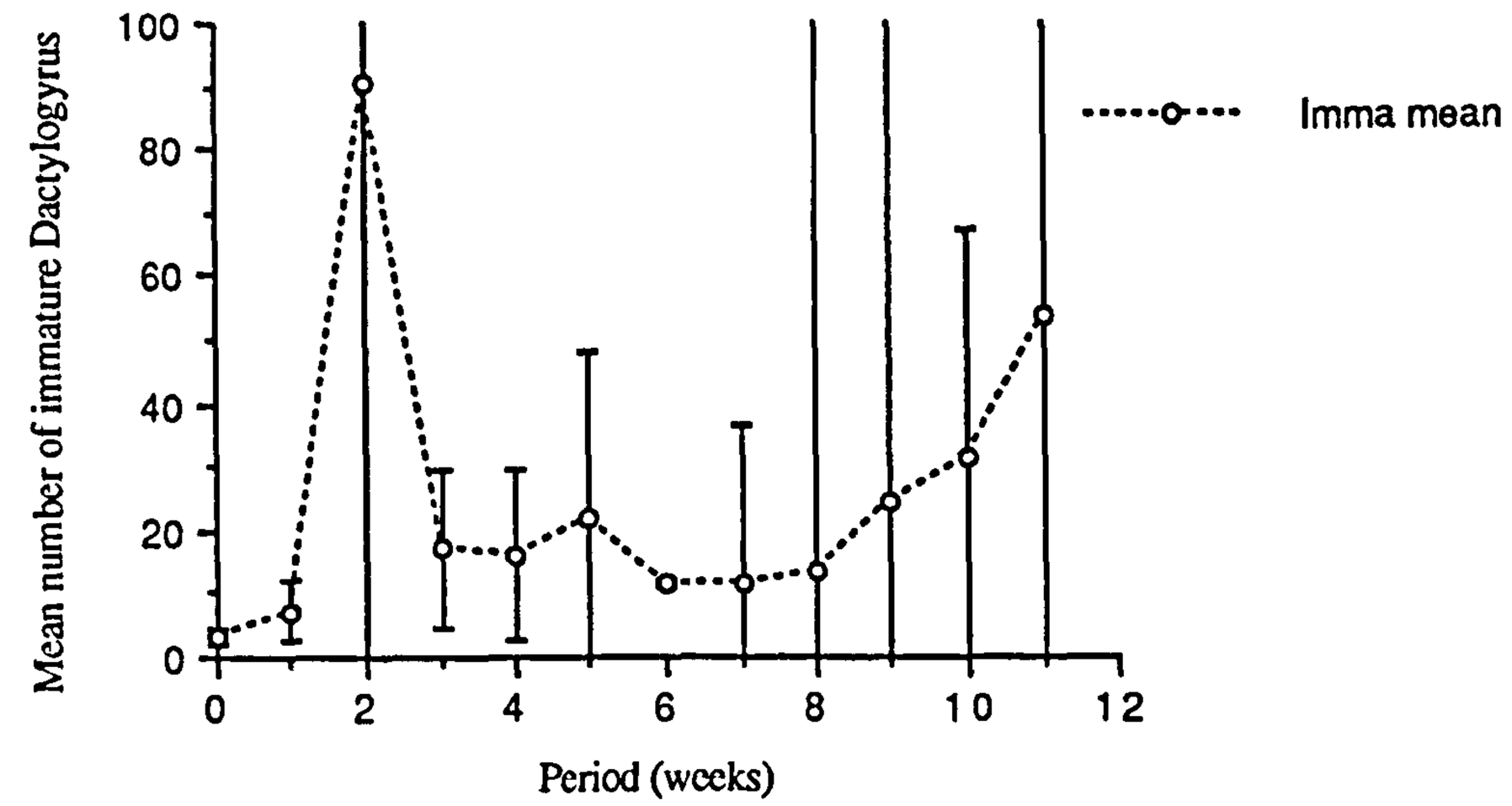


Fig 2.4c Variation in mean number of adult *Dactylogyrus vastator* with time at 19°C in experiment II.

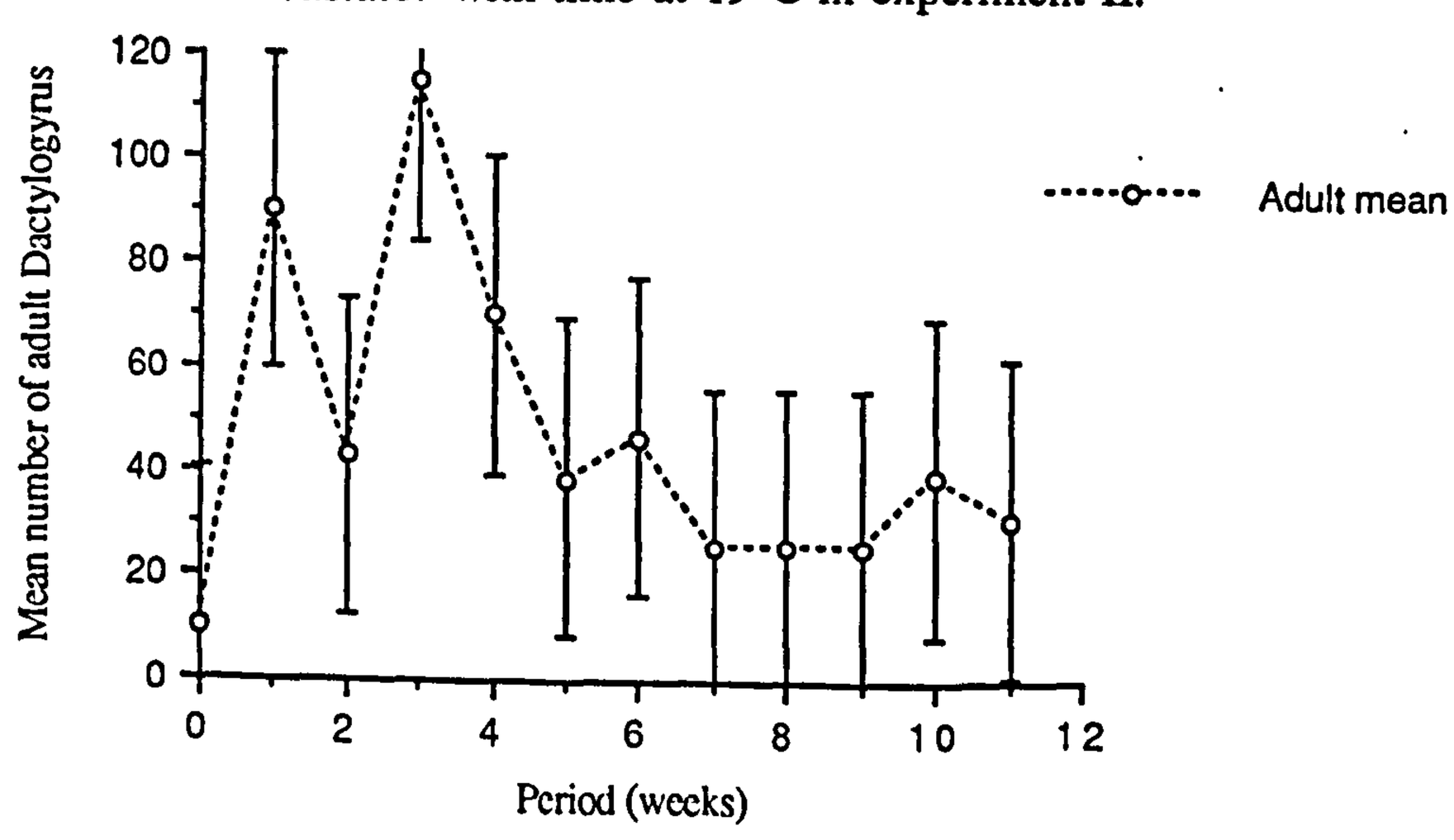


Fig 2.5a Variation of mean number of adult and immature *Dactylogyrus* with period of time at 22°C.

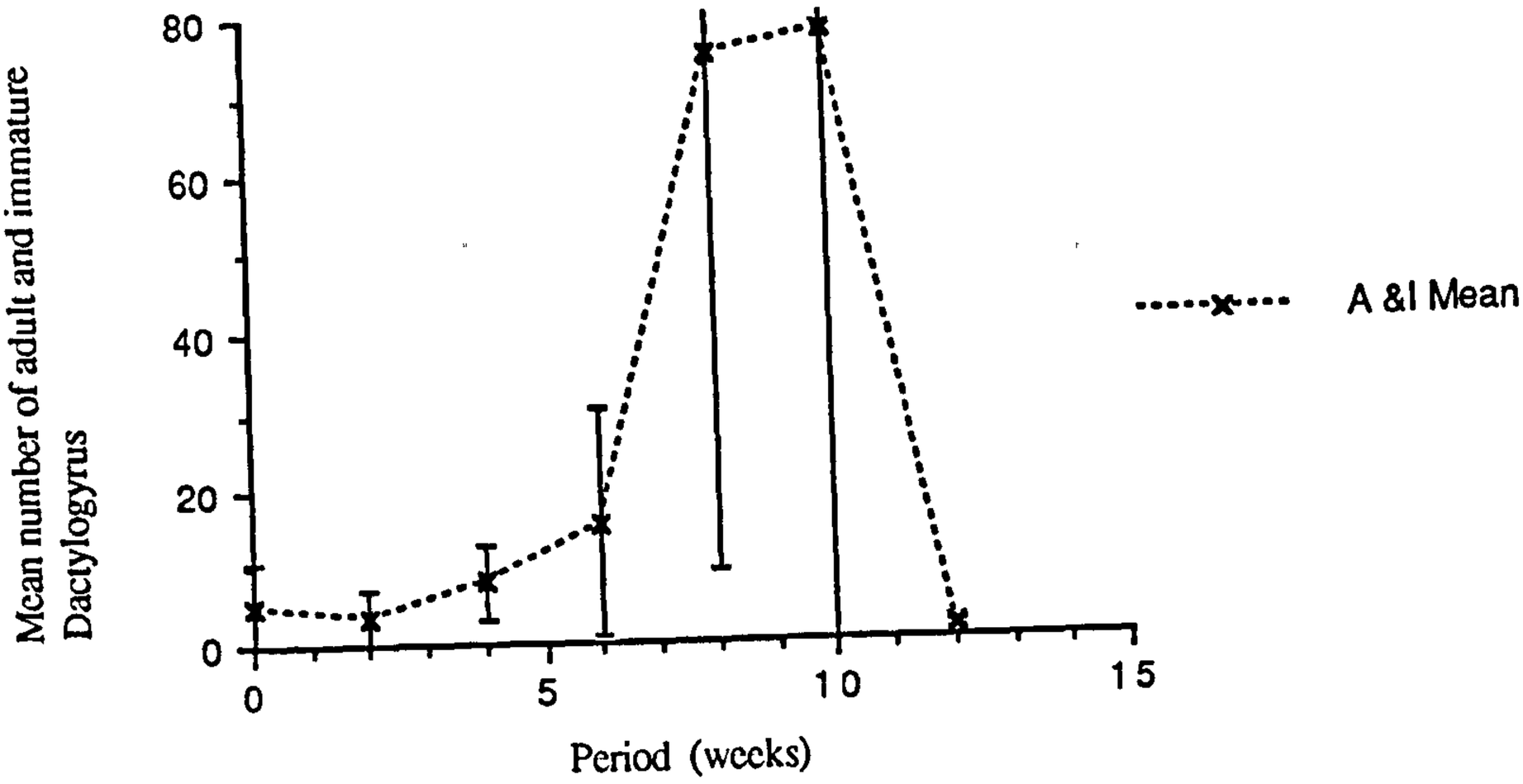


Fig 2.5b Variation of mean immature *Dactylogyrus* with period of time at 22°C.

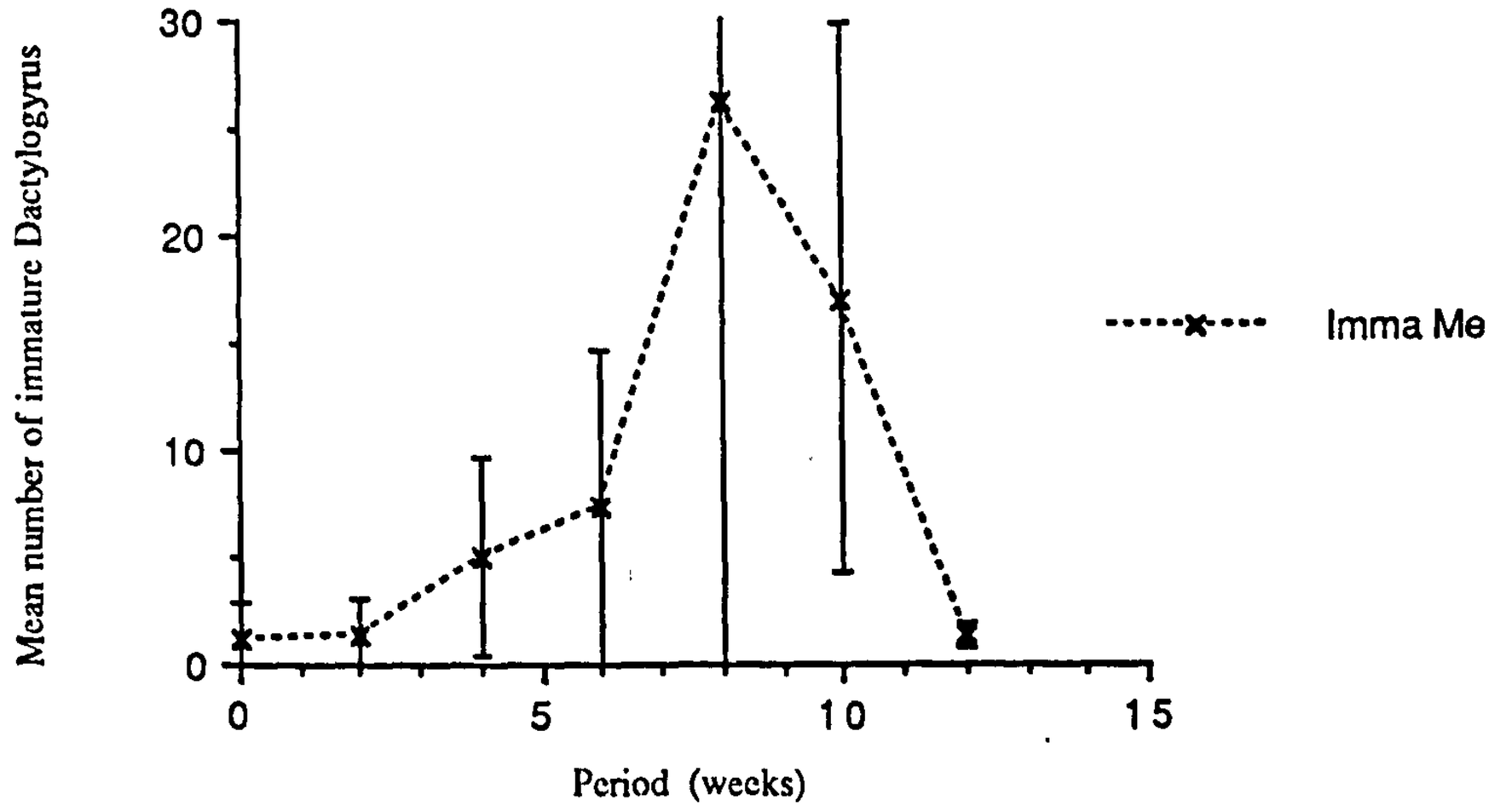
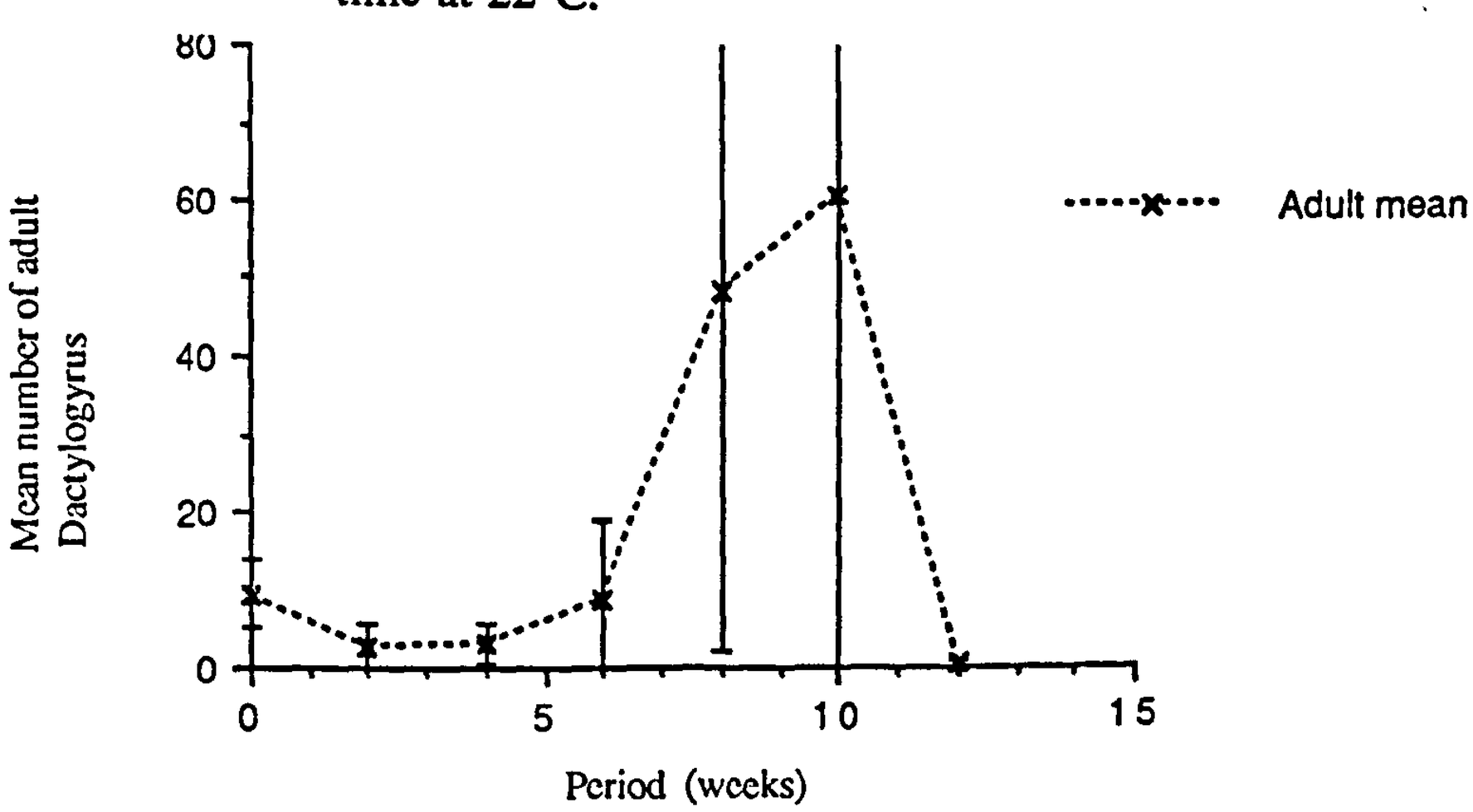


Fig 2.5c Variation of mean adult *Dactylogyrus* with period of time at 22°C.



increase in the mean number of adult worms to week 10 and the maximum value and then there is a sharp drop to zero at week 12. Fig 2.6 d shows the variation in the variance to mean ratio with time. It remained low and virtually constant until week 8, then suddenly increased at week 10 followed by a decline to a lower value at week 12.

2.3.2. Principal component analysis PCA

The first component has the longest axis, the second is the next largest and is perpendicular to the first, and the third is the next largest, in the measured value array, and arises perpendicular to the first two components. To calculate the principal component, all variables are made equal. When the components with the above variables (co-efficients) have been calculated, the total variance on the components is the same as the total variance on the original variables. The component loadings are the covariances of the original variables. If each of the loadings is squared and added up for each component then this will give the variance accounted for by each component. The eigenvector values calculated by the analysis explain how much each character contributes to each axis of the PCA and also explains how much each axis contributes to the overall variation.

Fig 2.6a The variation of variance to mean ratio with period of time at 12°C.

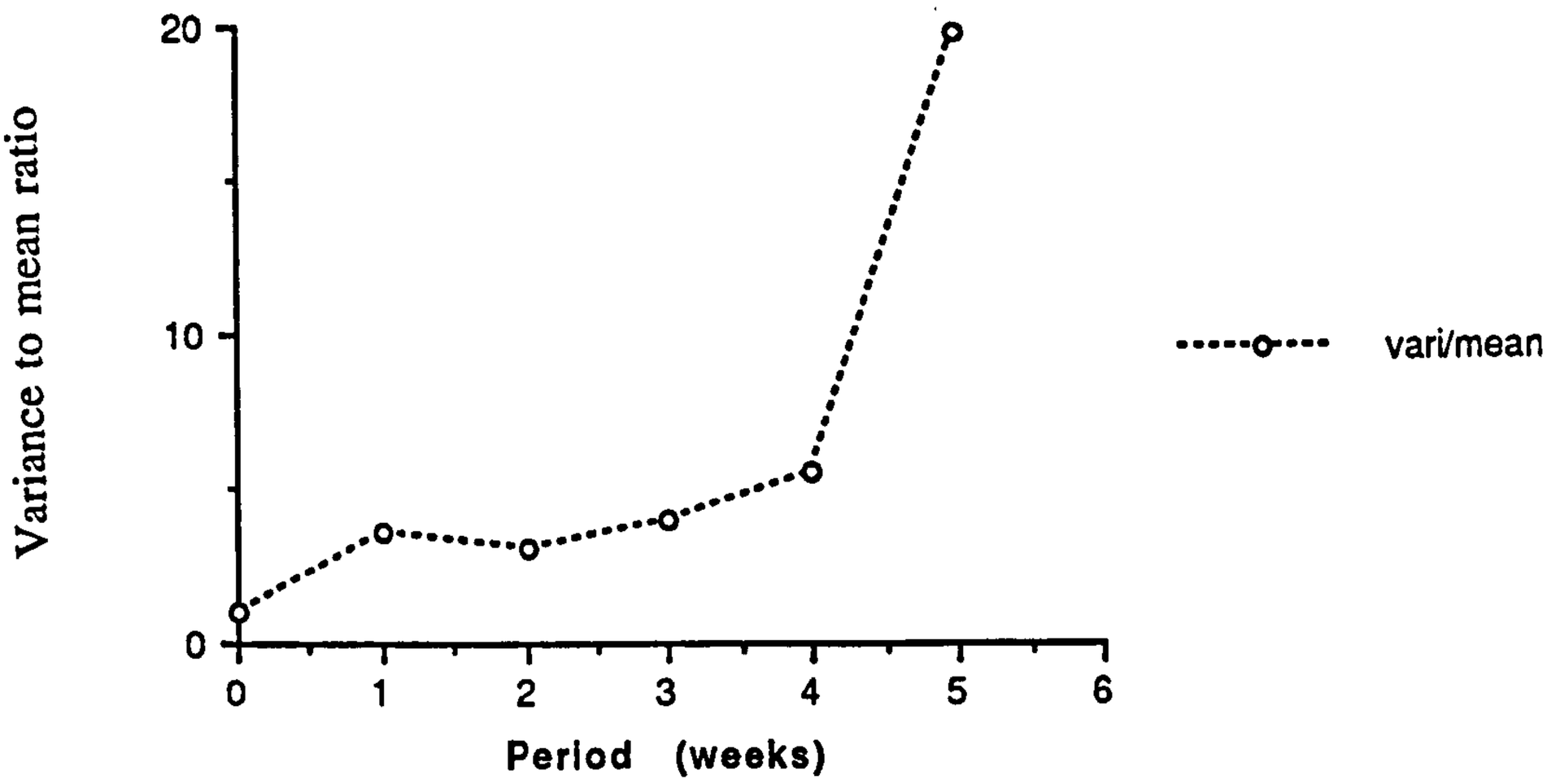


Fig 2.6b The variation of variance to mean ratio with period of time at 19°C in experiment I.

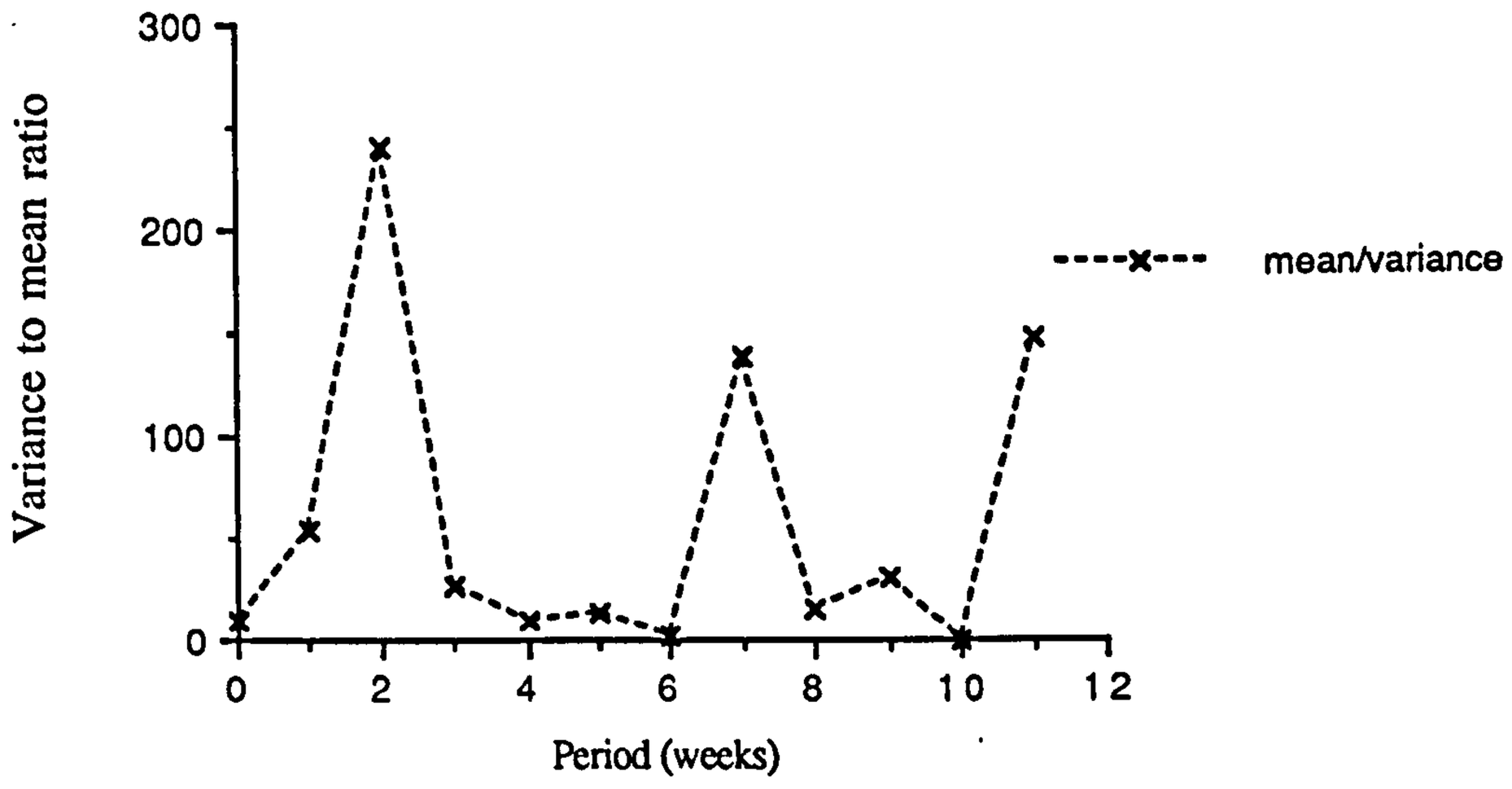


Fig 2.6c The variation of variance to mean ratio with period of time at 19°C in experiment II.

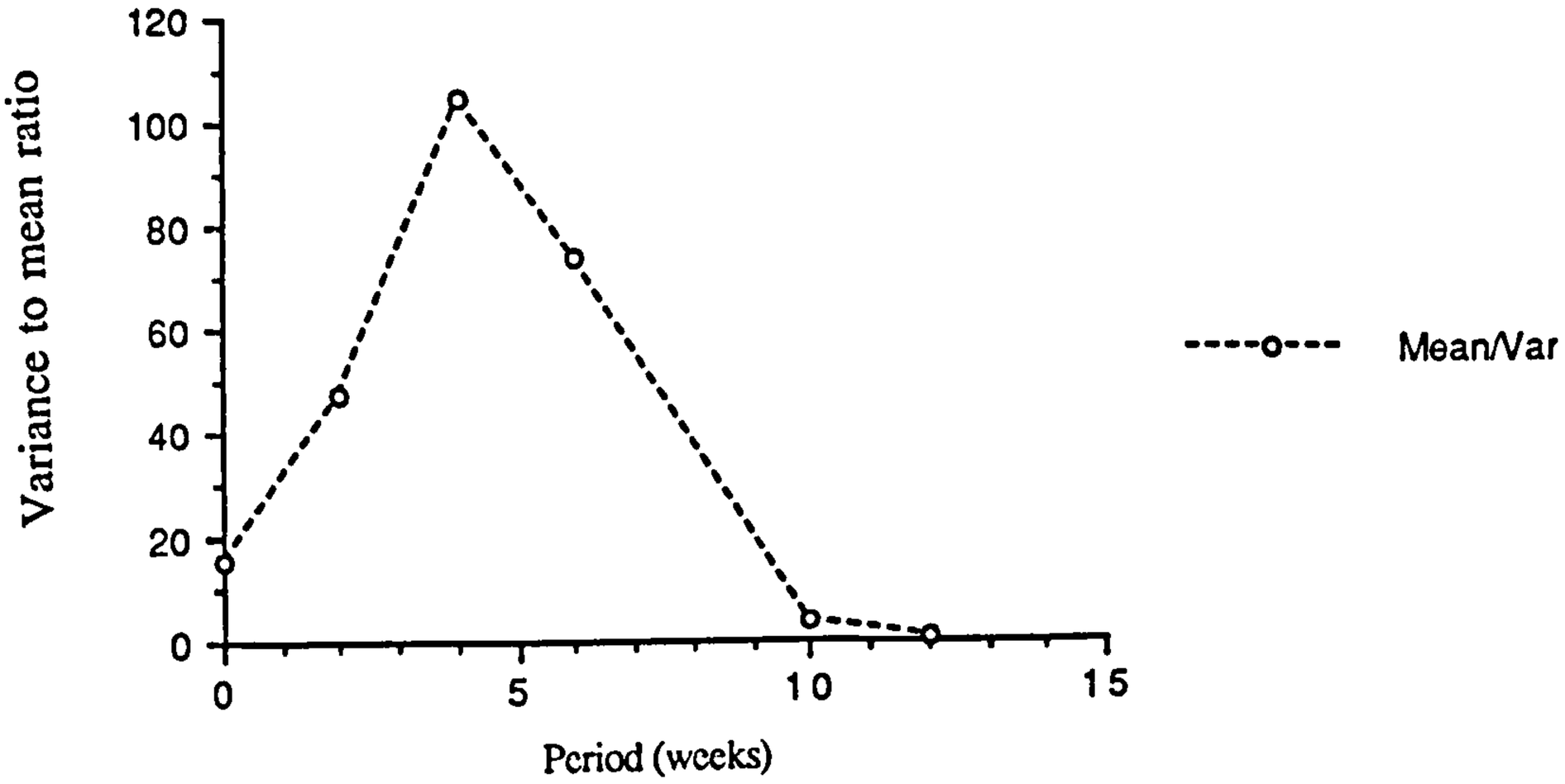
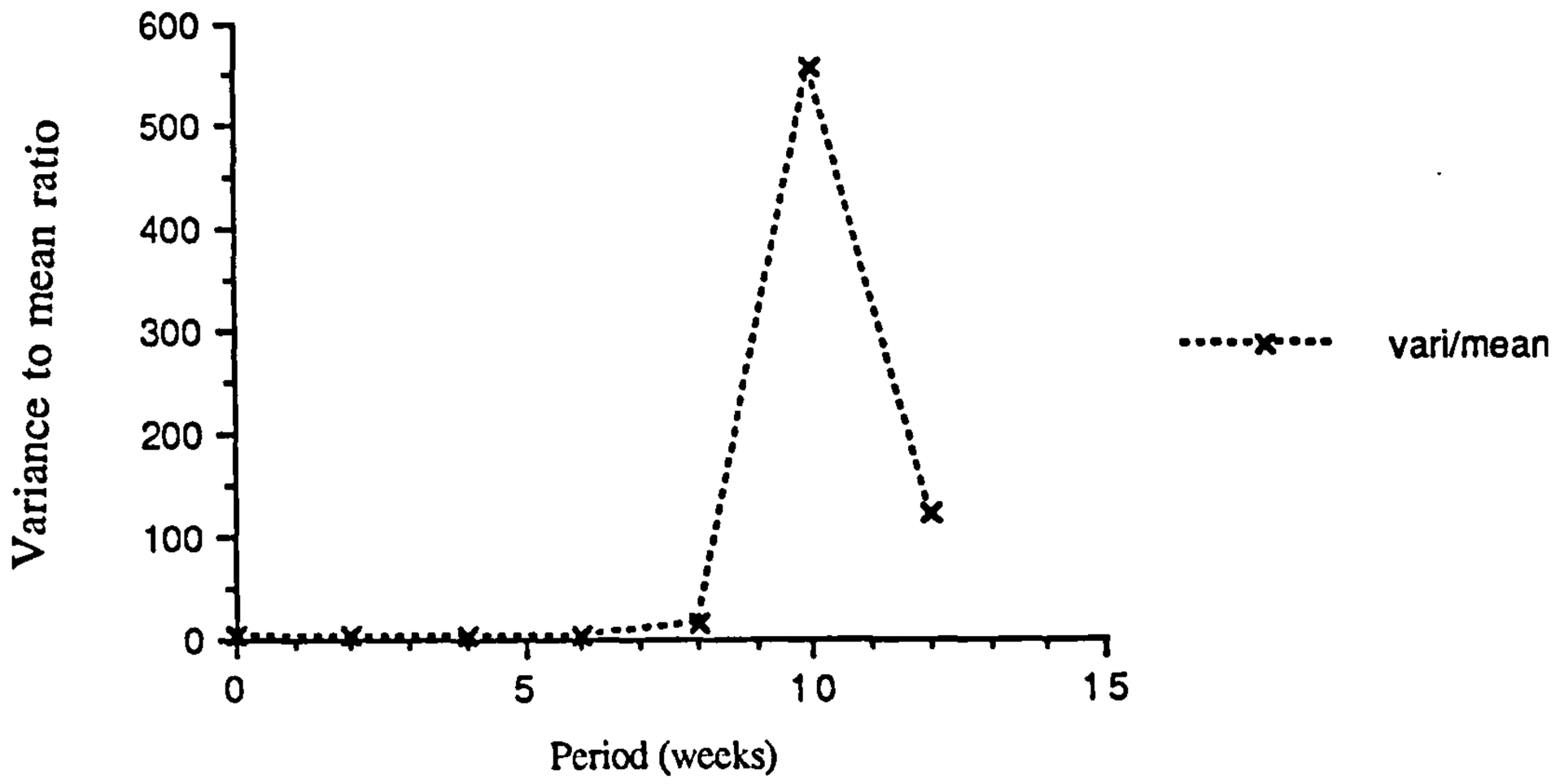


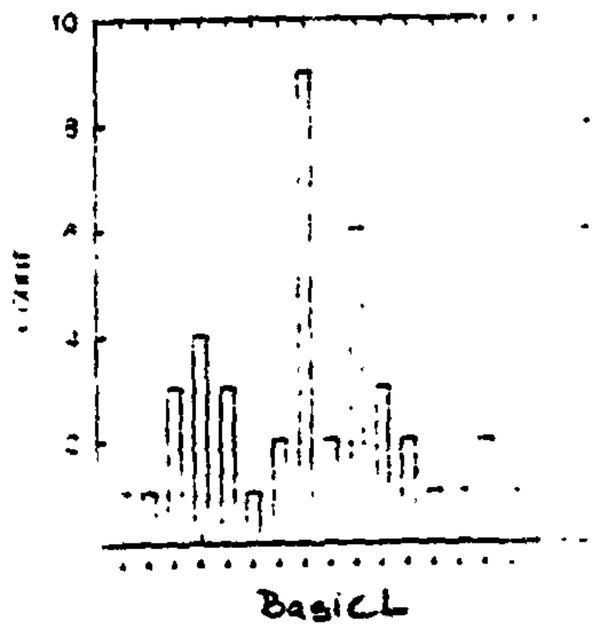
Fig 2.6d The variation of variance to mean ratio with period of time at 22°C.

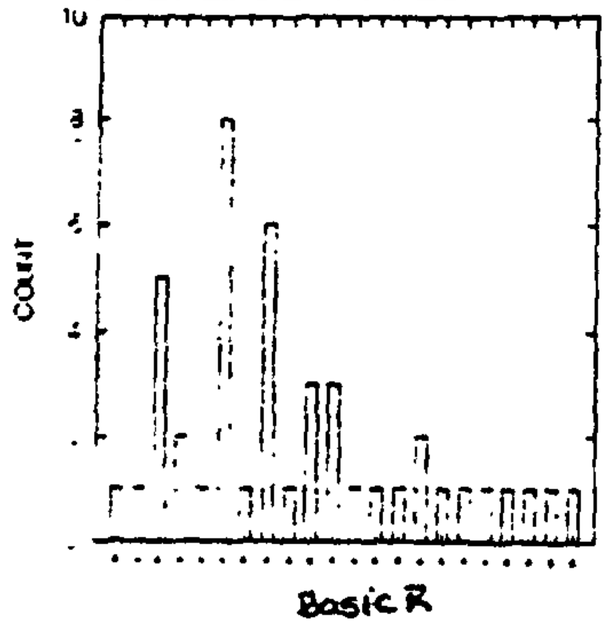


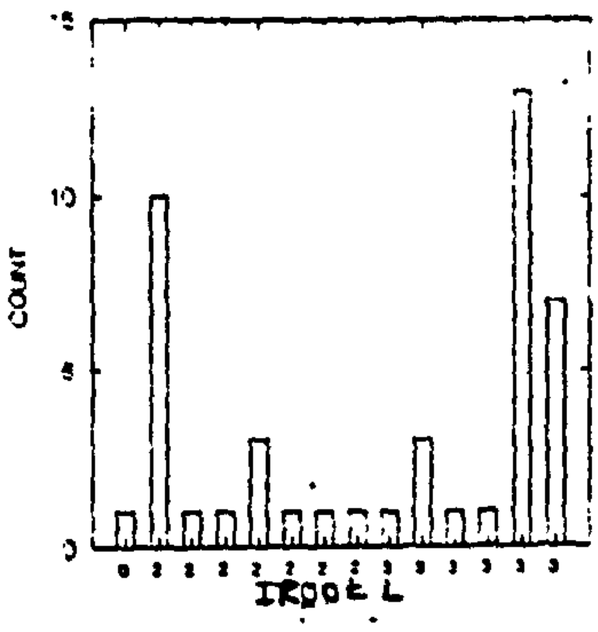
Histograms were produced for each of the measured structures to determine whether it had a normal distribution (Fig 2.7). It was found that the data did not show a normal distribution and was therefore transformed to a logarithmic value to make the histograms more symmetrical.

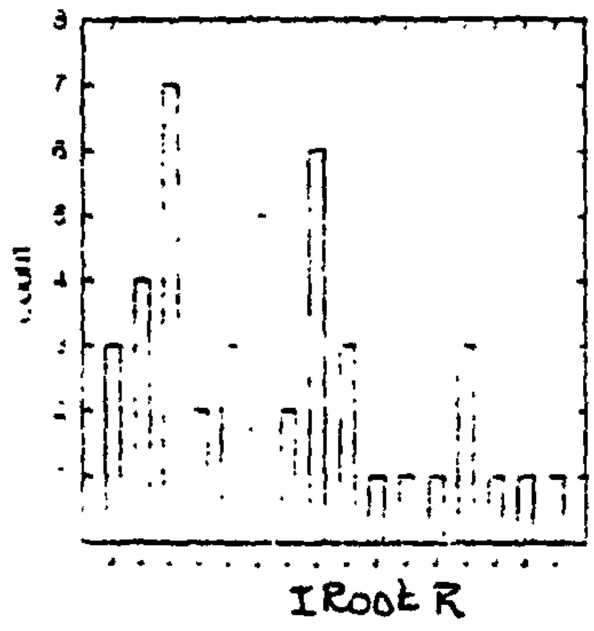
The plot of the first PCA is shown in Figure 2.8 . Table 2.1a,b gives the variance explained by the components and the percentage of the total variance explained. The first three factors here account for 55.15% of the total variance explained. The cluster analysis suggested that three natural groups existed within the specimens run in the first PCA. Two specimens deviated widely from the majority of the clustering specimen points, one in having a large internal root length of the hamuli and the other having a large total length of the hamuli. These outliers were removed prior to running the next PCA.

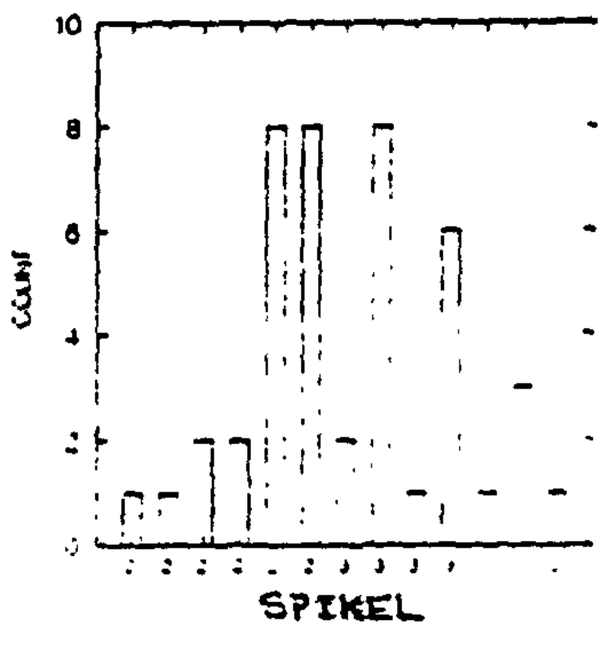
The results of the second PCA plot are shown in Fig 2.9. The component loadings for the PCA are given in Table 2.2a,b which also gives the variance explained by the components and percentage of the total variance explained. The first three factors here account for 50.72% of the total variance. The cluster analysis again suggested three natural groups within the data. The data for each cluster were then examined carefully. Factor 1, factor 2 and factor 3 were analyzed separately to ascertain the extent to which each of these factors was responsible for the clusters produced, and more importantly, which point

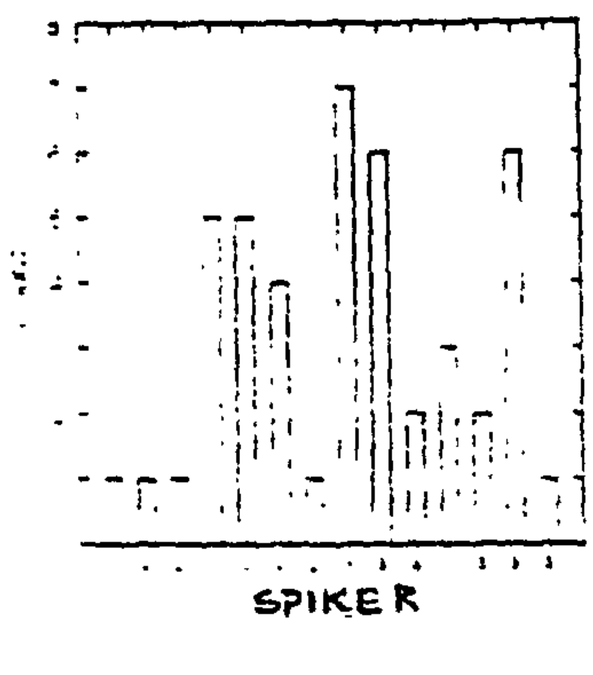


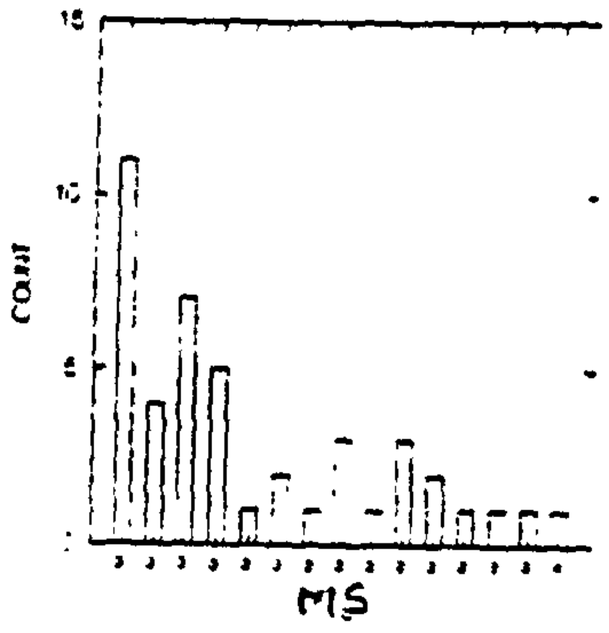


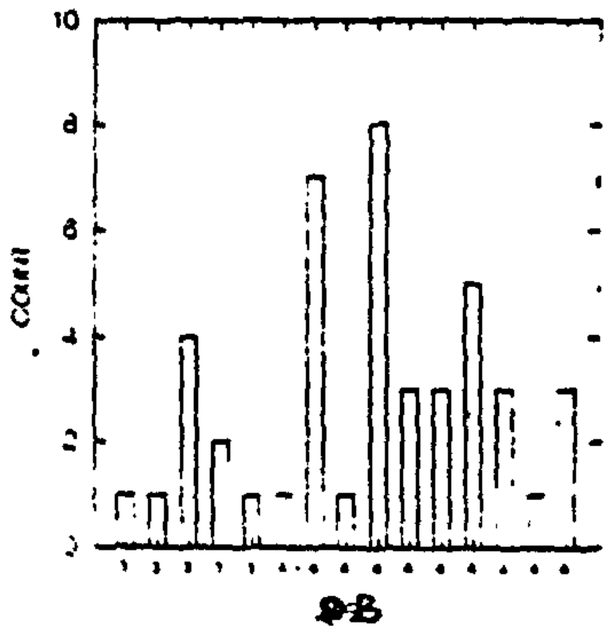


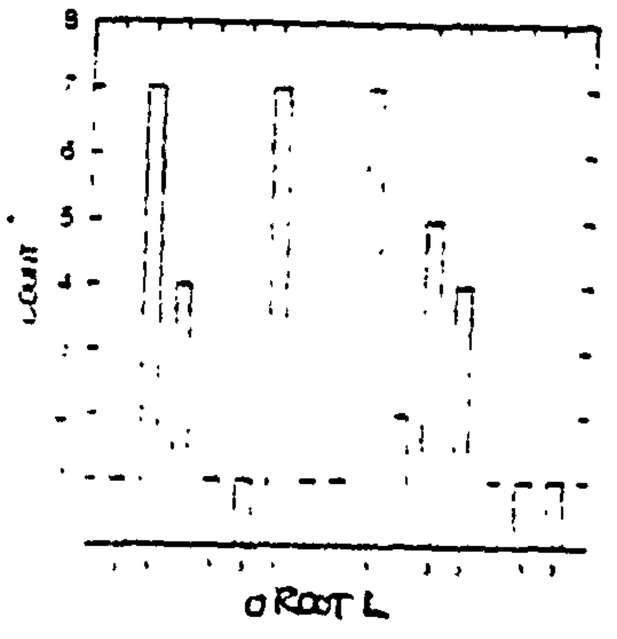












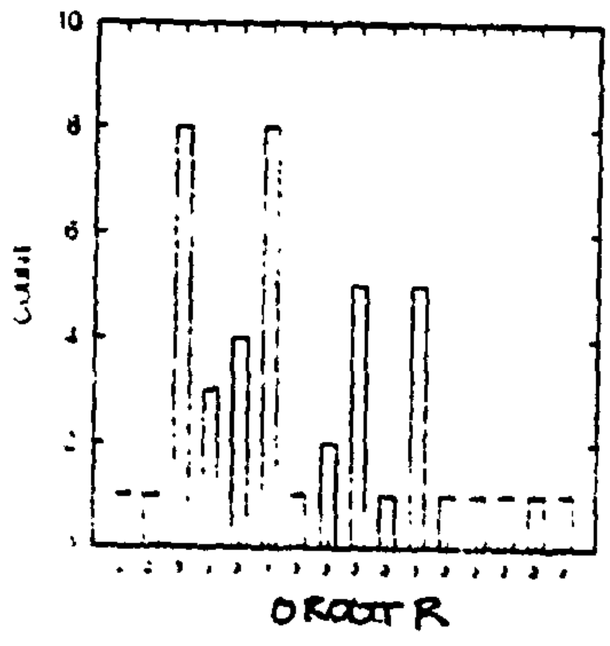


Fig 2.7 Histograms of raw data on hamulus measurements.

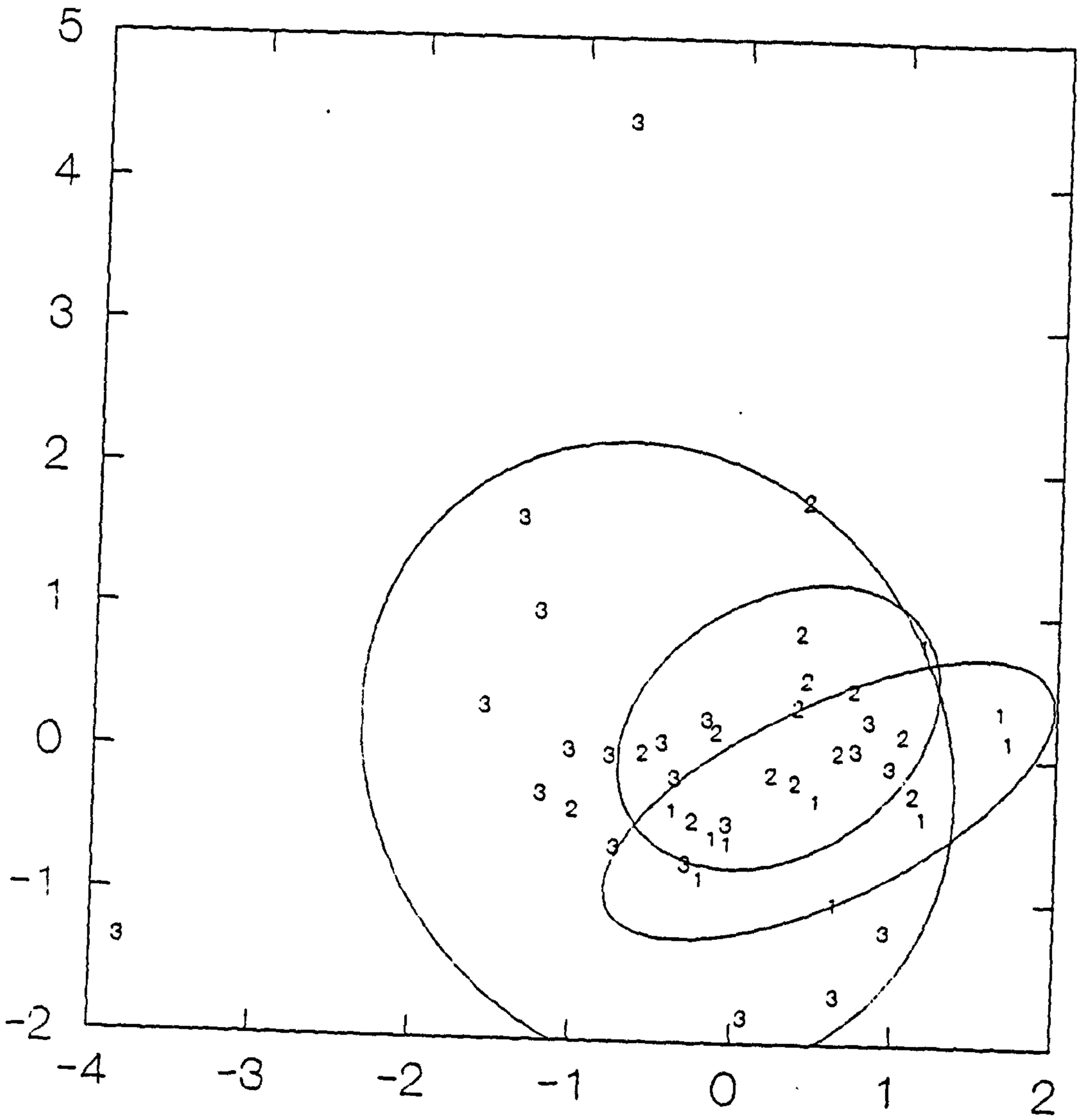


Fig 2.8 The PCA plot of logarithmic hamuli measurements for the three temperature regimes.

Table 2.1a :- Correlation matrix

	BasicL	Basic R	DB	I rootL	L length	MB	MS	OrootL	OrootR	Overall	SpikeL	SpikeR
BasicL	1.00											
BasicR	0.606	1.000										
DB	0.162	0.035	1.000									
IRootL	0.038	-0.154	-0.032	1.000								
Llength	0.282	0.386	0.064	-0.267	1.000							
MB	0.150	0.176	0.290	0.107	-0.172	1.000						
MS	0.187	-0.008	0.219	-0.020	0.135	0.236	1.000					
OrootR	0.091	0.138	-0.047	0.544	-0.226	0.378	0.128	1.000				
OrootL	0.301	0.231	0.013	0.443	-0.118	0.188	-0.063	0.421	1.000			
Overall	0.520	0.669	0.137	0.133	0.124	0.137	0.027	0.131	0.228	1.000		
Spike L	0.213	0.400	0.259	0.029	0.299	0.247	0.023	0.286	0.227	0.175	1.000	
Spike R	0.113	0.300	0.007	-0.0134	0.105	0.266	-0.114	0.235	0.196	-0.035	0.492	1.000

Table 2.1b:- Principal components analysis of the correlation between the 12 variables

Eigen values and proportion of the variance explained by the first four principal components				
Eigenvalue	3.057	2.106	1.456	1.389
Proportion	0.254	0.175	0.121	0.115
Cumulative	0.254	0.429	0.550	0.665

Coefficient of each variable on the first four principal component

Variable	PCA1	PCA2	PCA3	PCA4
Spike length right hamuli	0.148	0.008	0.375	-0.018
Outer root length of right hamuli	0.177	-0.224	-0.155	0.047
Marginal Spike length	0.053	0.022	0.139	0.549
Length of the worm	0.086	0.326	0.021	0.535
Internal root length of left hamuli	0.067	-0.356	-0.221	0.225
Basic length of right hamuli	0.253	0.192	-0.148	-0.117
Spike length of left hamuli	0.211	0.039	0.302	0.146
Overall length of left side hamuli	0.210	0.082	-0.347	-0.321
Outer root length of left hamuli	0.160	-0.322	0.046	0.273
Length of marginal blade	0.152	-0.151	0.313	-0.321
Dorsal bar length	0.089	0.050	0.252	-0.389
Basic length of left hamuli	0.223	0.132	-0.238	0.047

to point measurements contributed to the separation of the measured individuals. It was found that the length of the basal portion of the hamuli explained the most variation and was the key measurement in separating the specimens.

The variables operating in F_1, F_2, F_3 and F_4 were calculated from the component loadings. From the factor loading plots of the component loadings it can be calculated that factor 1 explains the basic length of the left hamulus acting along the x axis, factor 2 is principally explained by the spike length of the right hamulus acting principally along the y-axis and in factor 3 it is the total length of the left hamulus which acts along the z-axis.

2.4. Discussion

2.4.1. Temperature effects on *D. vastator* populations

The experiments indicate that the water temperature has a major influence on the population biology of *D. vastator*. The total abundance of *D. vastator* at 12°C increased continuously to week five, and at 19°C in experiment I increased up to the 4th week at a rapid rate, followed by a decline towards minimum values. In experiment II at 19°C the abundance increased initially at a rapid rate to week 2 and then remained at a constant level after which a decline in the abundance was observed to week 7 followed by an increase to

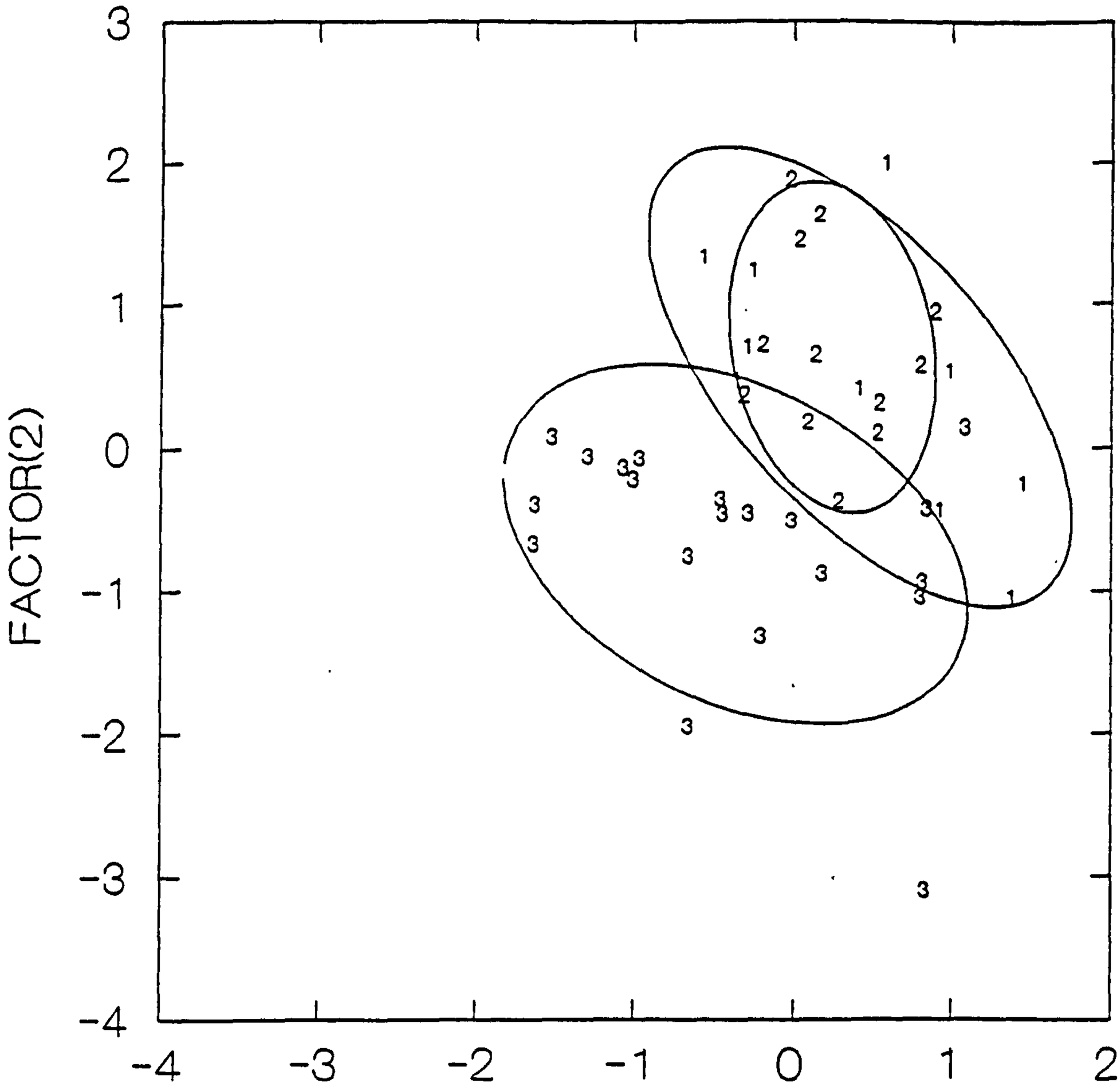


Fig 2.9 The PCA plot of logarithmic hamuli measurements for the three temperature regimes after removal of outliers.

Table 2.2a Correlation matrix

	BasicL	Basic R	DB	I rootL	L length	MB	MS	OrootL	OrootR	Overall	SpikeL	SpikeR
BasicL	1.00											
BasicR	0.511	1.000										
DB	0.183	0.068	1.000									
IRootL	0.162	-0.132	0.019	1.000								
Llength	0.150	0.249	0.054	-0.067	1.000							
MB	0.150	0.249	0.320	-0.029	-0.0103	1.000						
MS	0.173	-0.073	0.228	-0.048	0.138	0.235	1.000					
OrootR	0.119	0.040	0.043	0.288	-0.107	0.351	0.115	1.000				
OrootL	0.321	0.233	0.039	0.404	-0.085	0.146	-0.085	0.374	1.000			
Overall	0.398	0.503	0.189	0.303	-0.116	0.200	-0.027	0.047	0.232	1.000		
Spike L	0.059	0.185	0.328	0.009	0.230	0.272	-0.027	0.169	0.186	-0.120	1.000	
Spike R	-0.010	0.144	0.022	-0.150	-0.000	0.293	-0.152	0.240	0.177	-0.284	0.408	1.000

Table 2.2b Principal components analysis of the correlation between the 12 variables

Eigen values and proportion of the variance explained by the first four principal components

Eigenvalue	2.646	1.833	1.607	1.362
Proportion	0.220	0.153	0.134	0.113
Cumulative	0.220	0.373	0.507	0.620

Coefficient of each variable on the first four principal component

Variable	PCA1	PCA2	PCA3	PCA4
Spike length right hamuli	0.103	0.391	-0.132	0.238
Outer root length of right hamuli	0.231	-0.071	-0.285	0.153
Marginal Spike length	0.054	0.026	0.193	-0.540
Length of the worm	0.034	0.093	0.347	0.151
Internal root length of left hamuli	0.119	-0.233	-0.332	-0.083
Basic length of right hamuli	0.237	-0.067	0.277	0.337
Spike length of left hamuli	0.165	0.338	0.029	0.089
Overall length of left side hamuli	0.204	-0.352	0.083	0.009
Outer root length of left hamuli	0.188	0.094	-0.330	-0.149
Length of marginal blade	0.224	0.181	-0.002	-0.251
Dorsal bar length	0.159	0.099	0.172	-0.348
Basic length of left hamuli	0.245	-0.173	0.181	0.070

week 12. At 22°C *D. vastator* abundance increased dramatically to week 8 then remained more or less the same with a only slight increase to week 10 followed by a sharp decline to the minimum value. Generally in all temperature experiments there was an increase in the abundance of *D. vastator* but the length of time this took varied. At 12°C and 22°C the abundance reached a high value later than at 19°C. The results of these experiments thus suggest that the optimum of the three temperatures tested for *D. vastator* was 19°C. Temperature will have an effect on oviposition, the speed of development of the embryo, the duration of oncomiracidial life, the speed of maturation of juvenile and the longevity of matured/ adult worms. The levels of infection with *D. vastator* in the two 19°C experiments were different. In experiment I a tank was completely emptied at each sampling time, whereas in experiment II tanks containing a greater number of fish were used through out the experiment, a proportion of fish being removed on each sampling occassion. It is probable that parasite numbers built up thoughout experiment II since eggs and juvenile parasites produced from infected fish remained in the tanks between samplings and were thus available to reinfect surviving carp over the whole period of the experiment.

Each species of monogenean has an optimum temperature at which all life processes occur with maximum efficiency for the reproduction of the species (Chubb, 1977). The optimum temperature for reproduction and

propagation for *D. vastator* lies between 24 - 28°C according to Paperna (1964) and 22 - 24°C according to Bauer *et al.* (1973). The egg laying and hatching success of *D. vastator* is reduced at temperatures below 8°C and above 32°C. At 24 - 28°C, the development of the parasite from attachment of the oncomiracidium on the gills up to the beginning of egg production takes about 7 days at 17 - 20°C (Kearn, 1986).

The lowest temperature of 12°C used in these experiments did not prevent the reproduction of *D. vastator* and the pattern of infection obtained in the experiments (Fig 2.2a,b,c) was similar to that seen at higher temperatures. Nordquist (1925), Nybelin (1925) and Wunder (1929) considered that *D. vastator* was not found on fish in winter, but that new infections arose from eggs that overwintered. On the other hand, Lyaiman (1948, 1951a) showed that *C. carpio* was infected the whole year round in the USSR. The eggs of *D. vastator* can overwinter and hatch in about 100 days at 4°C (Bauer, 1959).

Paperna (1963a) postulated the existence of diapausing eggs which enabled infections to be carried over from one year to another. Such variability in the developmental potential of eggs has been confirmed by other workers. A drop in temperature induced the production of larger "winter eggs". Paperna showed some irregularity in development of *D. vastator*, with at least some eggs not developing immediately to produce larvae.

The period taken for development is 27 - 28 days at 8°C, 10 - 15 days at 12°C, 3 - 5 days at 20°C and 1 - 4 days at 24 - 28°C (Paperna, 1963a,b). Older worms lay a greater proportion of non-viable eggs up to 100 % in some cases (Kearn, 1986). It was postulated that eggs not capable of developing represented special "winter-eggs", which would only develop in the next spring. That eggs of *D. vastator* are capable of over-wintering (development stops below 5°C) has been demonstrated by Prost (1963).

The fish obtained for the experiments were less than a month old. As the fish were of the same age there should not have been any effects on the life-cycle of *D. vastator* due to the age of the host. Under natural conditions the level of infection with *D. vastator* fluctuated not only with the season of the year but also with the age of the host. Nordquist (1925) and Nybelin (1925) observed the maximum infection in *C. carpio* fry of 2 - 5 cm in length. The fishes used in these experiments were 3.26 ± 0.359 cm in standard length and thus fell within this susceptible length group. Carp fry attained a length of 2 - 5 cm in the temperate zone of USSR from the end of June to the first half of July, the time of maximum infection with *D. vastator*, following this, there was a dramatic reduction in parasite abundance. Under-developed fingerlings, weighing 3 - 5 g, were the most heavily infected (Bauer, 1959 cited from Chubb, 1979). This aspect was not studied in detail in these experiments but there was some indication that lower weight fish were more heavily infected.

Thus a fish of 3.9 cm standard length and 1.18 g weight was found to harbour 308 *D. vastator* whilst fish of the same length group but of greater weight had fewer parasites. Another fish weighing only 3.25 g but measuring 4.9 cm in standard length was found to harbour 300 *D. vastator.*

There was very great variation in numbers of *D. vastator* between individual fish in any one sample in these experiments. This must obviously reflect individual host susceptibility, which in turn may be largely due to differences in the immune status of individual fish, and / or possibly to the extent of gill hyperplasia which develops in *D. vastator* infections and eventually causes loss of parasites (Paperna, 1964).

Paperna (1963b) found *D. vastator* on carp fry from April to June. At warmer temperatures, the speed of growth of carps is also higher, thus at the end of June, the fry were in excess of 60 mm in length and were resistant to infection by *D. vastator.* However, in the experiment carried out at 19°C fish ranging from 6.0 cm to 9.0 cm in length had numbers of *D. vastator* which seemed potentially hazardous to the fish. For example, 105, 123, 185, 169 *D. vastator* were found in single fish above 60 mm in the 19°C experiment.

Paperna (1964) reported the presence of immunity in carp against *D. vastator* infections. In these experiments at different temperatures, an

increase in population numbers in the initial period at all temperatures, was followed by a decline. The initial increase in numbers is due to the reproductive ability of the parasite but the decline perhaps represents an acquired immunity. In these experiments the peak of infection mostly occurred 4 weeks after the start of the experiments, but it must be remembered that initial infections had begun 2 weeks before the start of the experiment.

Thus, Slotved & Buchmann (1993) showed that eels developed a host response against *Pseudodactylogyrus anguilli* after 4 weeks of infection. Scott (1985) reported that the guppy *Poecilia reticulata* developed a host response against *Gyrodactylus bullatarudis* 6 weeks after the initial infection with the parasite. In *Gyrodactylus colemanensis* the population rose to a peak between 28 and 42 days post-infection and by day 49 declined to a lower intensity (Cone & Cusack, 1989). Acquired immunity will vary with the age of the host and the number of discrete infestations, and will not in general last for a long period of time.

Parasitologists used the terms aggregation, clumping and over-dispersion to represent the frequent situation where most hosts are infected or lightly infected and a few hosts are very heavily infected. The latter may be important in parasite induced host mortality, which in turn regulates the size of the parasite population.

In these experiments the variance to mean ratio remained above zero indicating that the *D. vastator* population was overdispersed on the hosts and that a relatively few fish harboured the majority of the parasite population. In general the variance to mean ratio in these experiments was greatest when parasite abundance was highest.

Scott (1987) also found that in *Gyrodactylus bullatarudis* infections in guppies the parasite population became more aggregated during the increasing phase of the epidemic cycle and suggested that this was due to heterogeneity amongst the fish population and the direct reproduction of the parasite. Although *D. vastator* does not have a direct reproductive cycle on the host as does *Gyrodactylus*, host heterogeneity, ie. susceptibility to infection, may well be the main reason for the high variance to mean ratios seen in increasing parasite populations in these experiments. Scott (1987) also noted that the variance to mean ratio could be much affected by the presence of a few very heavily infected hosts and this seems to have been the case in the present study in increasing parasite populations. Scott (1987) pointed out that this made the variance to mean ratio a particularly useful indicator of parasite aggregation. The drop in variance to mean ratio in decreasing *D. vastator* populations and thus the lower degree of aggregation may reflect density dependant death of parasites, perhaps due to an immune response (see Chapter 7). Scott (1987)

suggested that a similar mechanism and perhaps also mortalities of heavily infected fish were responsible for the lower variance to mean ratios seen in falling populations of *G. bullatarudis*.

2.4.2. Principal component analysis (PCA)

Logarithmic transformation of the data was necessary as the data was not normally distributed and this makes the variance independent of the mean and the frequency distributions more symmetrical. In particular, some structures exhibited a degree of bimodality, such as the marginal spike. The length of the marginal spike represents one of the smallest structures measured in the haptoral complement, with a small range in the size of the measurements. The length of the marginal spike exhibited a high degree of variability as shown by the histogram (Fig 2.7) and therefore did not have a normal distribution. A possible reason for this is that it is a small structure (3.12 - 4.68 µm). When measuring with the light microscope, its accurate resolution is approximately 0.5 µm, the number of measurements in "score" classes is small and any errors that may happen will therefore be significant. The major result of this analysis was that in the opisthaptoral complement any structure below 10 µm in size is likely to produce a confusing picture when making a comparison of the armature at different temperatures. When the preparation is not completely flat, particularly the opisthaptor armature, this can introduce an error in the point to point

measurements. In addition some of the variation may be caused by the fixative, eg: the hamulus root is a region of unconsolidated hook material and changes in shape may occur as a result of the fixative.

The distribution of specimens within the temperature cluster were carefully analyzed. The outliers were removed to get a specific PCA plot, these structures influencing the separation of the specimens affected as a result of different temperatures on the opisthaptoral armature. It was found that the basic length of the left hamulus was the key measurement acting along factor 1 separating the specimens whilst the spike length of the right hamulus acted through factor 2 and the total length of the left hamulus acted through factor 3.

From Fig 2.8 it can be clearly seen that temperature has a marked effect on the size of the opisthaptor armature and that the hooks measured at 12°C and 14°C can be separated from those measured at 19°C. Measurements of the sclerotised parts of monogeneans taken from populations at different temperature may therefore be significantly different and this could be significant in the identification of species or the establishment of new taxa.

Chapter 3

Electron microscopic study of *Dactylogyrus vastator*

3.1. Introduction

Electron microscope investigations on fish parasitic platyhelminths have so far relatively neglected the Monogenea and have been more extensive on the endoparasitic digeneans and cestodes (Burton, 1966a,b,c; Lumsden, 1966; Morris & Threadgold, 1967; Bråten, 1968; Charles & Orr, 1968 and Erasmus, 1973). Although historically referred to as a 'cuticle' the outer surface of the ectoparasitic platyhelminths has been shown to be a living cytoplasmic 'epidermis' (Lee, 1966) and as was pointed out by Erasmus (1973) and Lumsden (1966), constitutes a metabolically active interface with host tissues and fluids. An account of the adult epidermis of *Entobdella soleae* was given by Lyons (1970) and a preliminary account of the ultrastructure and development of larval epidermis was published by Lyons (1968). The epidermis of the opisthaptor of *Entobdella* and *Diclidophora merlangi* is infolded into crevices and channels (Lyons, 1970; Morris & Halton 1971). Lyons (1970, 1971) demonstrated a confluent cytoplasmic covering connected by processes with parenchymally situated cell units or cytons in the monopisthocotylids, *Acanthocotyle elegans*, *Entobdella soleae* and *Amphibdella flavolineata* and later in the polyopisthocotylids *Rajonochotyle emarginata* and *Plectanocotyle gurnardi*. In contrast the underlying nucleated units were not observed in adults and embryos of *Gyrodactylus* spp by Lyons (1970, 1973a). However, Kritsky & Kruidenier (1976) reported the presence of cytons in *Gyrodactylus eucaliae*.

Lyons (1973a) found that a nucleate primary epidermis of flat cells in the embryonic oncomiracidia of *E. soleae* is either replaced or develops into a secondary epidermis comprising cellular regions of synaptical nucleated ciliary cytoplasm. The latter loses its nuclei and apparently fuses with a discontinuous presumptive adult epidermis which connects to cell bodies in the parenchyma. Lyons (1970) considered that the typical covering layer of monogeneans was the syncytial, microvillous epidermis of monogeneans such as *E. soleae* and *A. elegans*. Lyons (1970) reported that microvilli are found in higher concentrations closer to adhesive regions of *Acanthocotyle* and *Gyrodactylus* and suggested that they help to spread and mix the sticky secretions of gland cells. The tegument represents an anucleate cytoplasmic extension of sub-surface nucleated portions. The epidermal cytoplasm is packed with dense membrane-bound, granular inclusions which vary in shape from bi-concave rods to oval bodies and which are distributed fairly uniformly throughout the thickness of the covering layer. El-Naggar *et al.* (1990) reported that the tegument of adult *Cichlidogyrus halli typicus* is similar in its basic structure to that of other monogeneans, being a syncytial cytoplasmic layer connected to two kinds of cytons lying beneath the tegumentary muscular layers. Secretory inclusions found in the outer layer are not homogeneously distributed (El-Naggar *et al.*, 1990).

The tegument of *Dactylogyrus* follows the same general pattern as in other monogeneans, but with some variations in the individual species (Fig 3.0). Adult *Dactylogyrus amphibothrium* has small finger-like processes or spikes projecting over the general body tegument (El-Naggar & Kearn, 1983a). The spikes are scattered singly or in groups. Such structures have not been found in *Dactylogyrus hemiamphibothrium* and immature *D. amphibothrium* (El-Naggar & Kearn, 1983a). Three kinds of membrane bound or unbound secretory inclusions of differing electron density were found in the outer tegumental layer (El-Naggar & Kearn, 1983a). Three kinds of subtegumentary cells or cytons were reported throughout the tegument in adult and immature *D. amphibothrium* whereas only one kind of subtegumentary cyton was reported in *D. hemiamphibothrium* (El-Naggar& Kearn, 1983a). El-Naggar & Kearn (1983a) showed regional specializations of the tegument in dactylogyrids. Thus, in *D. amphibothrium*, the tegument lining the anterior region of the buccal cavity has numerous tegumental sacs and spikes, which are fewer in other parts of the tegument.

Little is known about the chemical nature and possible functions of tegumental secretory inclusions in monogeneans. Lyons (1970, 1971) suggested that the dense bodies in the outer tegumental layer of *E. soleae*, *Acanthocotyle elegans* and *Amphibdella flavolineata* may be mucoproteinaceous because they gave a slight reaction with the PAS test. Lyons (1971) showed that the

tegumentary inclusions of *E. soleae* showed β-metachromasia with toluidine blue, a phenomenon said to be the property of mucoproteins and suggested that the mucous coat may be derived from the tegumentary inclusions. Kritsky & Kruidenier (1976) presented evidence to indicate that one of the two secretory inclusions in the tegument of *G. eucaliae* expels its contents at the tegumental surface.

The identification of monogeneans relies on the size and shape of the sclerotised parts of the opisthaptor, as seen through the light microscope. These structures are surrounded by tissues, and their proportions can be misinterpreted depending on how the worms are fixed, prepared, and mounted. It should also be noted at this point that many smaller monogeneans are identified by means of their marginal hooks. Shinn *et al.* (1993) developed a sonication method to separate the opisthaptoral sclerites from live worms. Mo & Appleby (1990) used the enzymatic digestion of freshly collected worms to free the sclerotised parts of the opisthaptor. In this technique, the marginal hooks are not always preserved, unlike in the sonication method. The sonication used by Shinn *et al.* (1993), was modified and applied to *D. vastator* in this study.

M Mitochondrion

Go Golgi body

Cy Cyton

N Nucleus

Fm Fibrous material

Bl Basal lamina

Bm Basal membrane

S_1 Secretory inclusion

S_2 Secretory inclusion

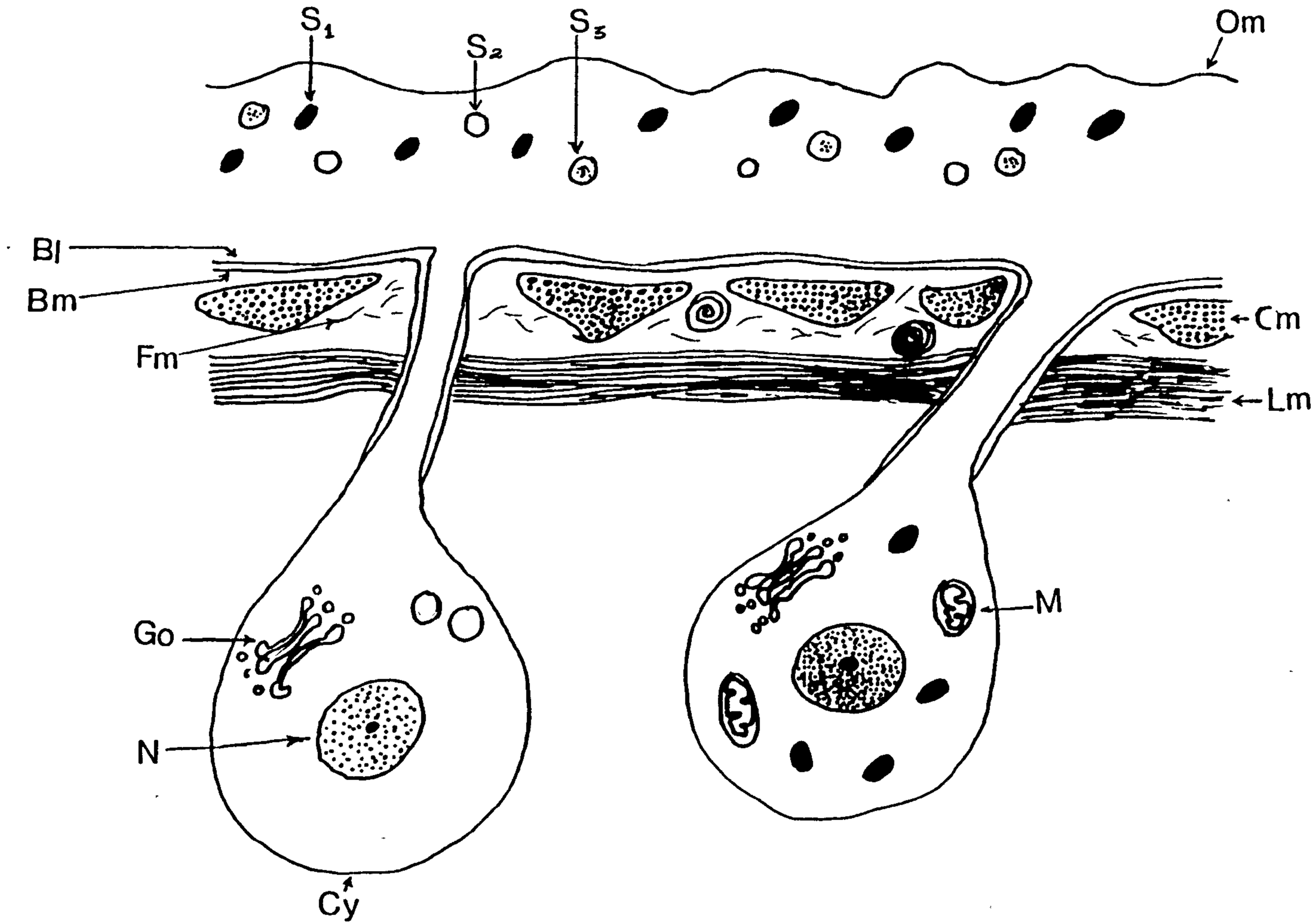
S_3 Secretory inclusion

Om Outer membrane

Cm Circular muscle fibres

Lm Longitudinal muscle fibres

Figure 3.0 :- Schematic diagram showing the ultrastructure of the body tegument of *Dactylogyrus vastator* in longitudinal section.


S1
S2
S3
Om
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Bm
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Cm
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N
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3.1.1. Sensilla

The presence of compound sense organs in turbellarian platyhelminths has long been recognized (Bullock & Horridge, 1965), although they are less common in the parasitic platyhelminths their presence is well documented (Rohde, 1965). There have also been several reports of presumed sensory 'bristles' and papillae in monogeneans (Fischthal & Allison, 1940; Wright & MacCallum, 1887), but as Baer & Euzet point out (in Grasse, 1961, p.261) 'although sensory papillae definitely exist in anterior and lateral regions of the body and on the haptor, the associated nerve endings have not been made in any particular investigations'. This was remedied to some extent by the light microscope investigation of the sense organs of the endoparasitic monogenean *Polystomoides* sp. by Rohde (1965) who used a silver stain to localize the nerve endings in the covering 'epidermis' (Lee, 1966). Harris (1983) used silverstain in scanning electron microscopic processing and observed four transverse bands of epidermal sensilla on adult *Ooegyrodactylus farlowellae*. Additional clusters occurred around the cephalic lobes, mouth, genital apertures and haptor in the same species. Two kinds of sense organs ending in highly modified cilia have been found on the head of the skin dwelling monogeneans *Gyrodactylus* spp. and *E. soleae* (larva only) and around the feeding organ of *Acanthocotyle elegans*. Cone & Burton (1981) reported that sensilla were absent from the surface of the haptor of *Benedenia* spp., while Halton (1979) reported that the

ventral surface of *Diclidophora merlangi* is provided with sensilla. Similarly, Lyons (1973b) reported numerous papillae on the ventral surface of the haptor of *D. merlangi.* These differ from the compound sense organs described from the head of adult *E. soleae* by Lyons (1969b) and which are found to terminate in cilia with the conventional 9+2 fibre structure ('fibres' = microtubules). In addition to these various compound receptors, a single sensillum consisting of a terminal cilium and nerve bulb have been found in several monogeneans and is also widespread throughout the platyhelminths (see Lyons, 1969b). A further kind of monogenean compound receptor appears to be made up of a cluster of single sensilla resembling 'tango receptors'(Lyons, 1969a). Unicellular sense organs are distributed all over the body of *C. halli typicus* (El-Naggar & Kearn, 1989). This sensilla has a superficial resemblance to the *D. amphibothrium* spikes, but structurally is quite different (El-Naggar, *et al.*, 1983a).

3.1.2. Cephalic gland

Monogeneans of the sub-order Monopisthocotylea characteristically possess several bilateral groups of unicellular glands in their cephalic and anterior trunk regions. Termed cephalic glands, they are thought to have a primary function of adhesion (Kritsky, 1978) and have been found to contain secretory cells, which expel their secretions via individual ducts either directly

at the cephalic margin or into cavities of the cephalic lobes. In some species the ducts enter the head organ prior to discharging at the surface (Kritsky, 1978). The first report of the distribution of cephalic glands was made by Wagener (1860) in gyrodactylids. Later Katheriner (1894), found bilateral groups of paired peripharyngeal, pharyngeal, and post pharyngeal glands in the dorsal half of the cephalic and anterior trunk regions in species of *Gyrodactylus*. Morris & Halton (1971) observed small bristle-bearing protuberances on the tegument lining the mouth region and covering the buccal suckers of *Diclidophora merlangi*. They have reported that the tegumental matrix beneath these regions is dense and fibrillar in appearance and they suggested that projecting bristles may have an abrasive or adhesive function (Morris & Halton, 1971).

3.1.3. Flame cells and protonephridial capillaries

The fine structure of flame cells and the protonephridial capillaries of parasitic helminths has been determined by a number of authors, although some descriptions are incomplete (Kümmel, 1958, 1959; Nieland & Weinbach, 1968; Howells, 1969; Wilson, 1969; Rohde, 1971a,b, 1972, 1973, 1980; Wilson & Webster 1974; Pan, 1980; Rohde & Georgi, 1983). Although platyhelminths have a variety of flame cells, numerous electron-microscopic studies have shown that monogeneans have flame cells of identical structure, although there

are minor differences between some species (Kümmel, 1958, 1959; Wilson, 1969; Rohde, 1971b, 1972, 1973, 1980; Wilson & Webster, 1974; Rohde & Georgi, 1983). Bruggemann(1986) and Ehlers (1986) described the morphological aspects of a flame cell by means of serial sections. Ehlers (1986) and Rohde (1986) have shown that ultrastructural studies of the structure of the flame bulb and the protonephridial capillaries is useful in establishing phylogenetic relationships between monogeneans. Kümmel (1958, 1959) described a flame cell as a single cell, consisting of a basal pericaryon with many cell processes, a 'weir apparatus' consisting of alternating internal and external ribs (rods) connected by a 'weir membrane', and a dorsal tubule. On one side of the weir apparatus and the tubule are two thick columns of cytoplasm which are connected by a desmosome. The flame bulb of *Dactylogyrus* spp. consists of a terminal cell with a nucleus which is basal and in some cases partly lateral to the basal bodies of the cilia which form the flame by their beating function (Rohde *et al.*, 1989). A thick cytoplasmic cylinder (proximal canal cell) tightly surrounds the canal where the cilia occur in the lumen. The pericaryon or terminal cell is rich in mitochondria. Protonephridial capillaries are smooth walled forming an extensive reticular system consisting of numerous interconnected cavities or ducts. Lateral flames are common, their cilia having the typical 9+2 pattern. The walls of some capillaries are rich in mitochondria.

3.1.4. Reproductive system

3.1.4.1. Male reproductive system

The process of spermiogenesis and the structure of mature spermatozoa have been studied in a number of platyhelminths, both at the light and electron microscopic levels (Gresson 1965; Davis & Roberts, 1983; Hendleberg, 1983; Ubelaker, 1983). Spermiogenesis has been studied in detail in monogeneans and digeneans (Burton, 1972; Halton & Hardcastle, 1976; Erwin & Halton, 1983). In monogeneans, each primary spermatogonium undergoes four mitotic divisions and a meiotic division to produce 64 spermatozoa. The onset of spermiogenesis is marked by the formation of a differentiation zone from which the spermatozoa develop, together with a change in the spermatid nucleus. A cytoplasmic projection appears in the differentiation zone, into which the spermatid nucleus migrates and the axonemes eventually become incorporated. Accordingly, a centriole-like body, two basal bodies and associated rootlets appear in the differentiation zone, and each basal body serves as a nucleation site for an axoneme (Maamouri, 1979). The basal bodies and the associated axoneme first appear perpendicular to the differentiation zone, and as development proceeds they rotate through 90 degrees and eventually become incorporated into the cytoplasmic extension. In the cestode, *Grillotia erinaceus*, mitochondria migrate along with the nucleus into the cytoplasmic extension and finally fuse to form a mitochondrial rod (Halton & Hardcastle, 1976; Erwin &

Halton, 1983; Erwin, 1984; Rohde & Watson, 1986a,b). Once axonemes are incorporated into the cytoplasmic extension and migration of the nucleus is completed, the spermatozoa are released from the spermatid plasmodium. In general, each spermatozoon consists of a nucleus, a single row of peripheral microtubules beneath the plasma membrane, and a single pair of axonemes of the 9+1 pattern is typical of platyhelminths. Mitochondria and acrosomes have not been observed in any of the parasitic platyhelminths (Rohde & Watson, 1986a).

Little work has been carried out on the ultrastructure and function of the male accessory ducts of monogeneans and digeneans, that serve to transport and store spermatozoa, and which are of taxonomic importance (Grant *et al.*, 1976; Halton & Hardcastle, 1977; Erwin, 1984).

The male accessory ducts include the vasa efferentia, vas deferens, seminal vesicle, cirrus or penis and cirrus sac, which have a cellular or syncytial lining with surface lamellae and microtriches (Mahendrasingham, 1989). In *Diclidophora merlangi* a male accessory gland, namely the prostate gland, has been identified (Halton & Hardcastle, 1977). The ducts of the prostate gland penetrate different regions of the male accessory ducts in different species and release their secretions into the lumen of the accessory ducts.

3.1.4.2. Female reproductive system

The female reproductive system includes the ovary and the accessory ducts (oviduct, vitelline duct, vagina, seminal receptacle and uterus). Oocyte development in monogeneans appears to follow the same pattern as that established for the digenetic trematodes (Gresson, 1964). Oocyte development in monogeneans has been studied in detail by Halton *et al.*, (1976).

In general, oogenesis involves a three-stage developmental sequence; oogonia, maturing primary oocytes, and secondary oocytes. The oogonia show a gonial morphology, with a large nucleus and higher nuclear/cytoplasmic ratio, a prominent nucleolus, few mitochondria, numerous unattached ribosomes and little or no granulated endoplasmic reticulum, and undergo mitosis resulting in oogonia that eventually grow into larger, primary oocytes. The maturing primary oocytes are characterized by a nuclear synaptonemal complex, an increased nucleolar volume, and Golgi complexes which are involved in the production of cortical granules. Secondary oocytes show a minimum of cellular activity and, therefore, are considered to be a resting phase. The mature secondary oocytes leave the ovary via the oviduct.

The structure and arrangement of the female accessory ducts in relation to the oogenotop have been studied in a number of parasitic platyhelminths

(Löser, 1965a,b; Ebrahimzadesh, 1966), but relatively few studies have been performed on their fine structure (Erasmus, 1973; Halton *et al.*, 1974; Grant *et al.*, 1977; Erwin, 1984; Beveridge & Smith, 1985), and none of the female accessory ducts have been studied in any monogenean species.

The vitellaria or vitelline gland is a collection of secretory cells that may be distributed as numerous follicles or as a single vitellarium. In monogeneans, the vitelline follicles are extensive and distributed in two lateral fields.

The chemical composition of vitelline cells has been examined by light microscopic histochemistry in trematodes (Smyth, 1954; Smyth & Clegg, 1959; Gupta *et al.*, 1987a,b; Shaw, 1987). Three trematodes *Fasciola hepatica, Haplometra cylindracea* and *D. merlangi* show evidence for the presence of sclerotin precursors (phenolase, phenols and basic protein) in the shell globules of the vitelline cells and in the newly formed egg shells, suggesting that egg shell of these trematodes is formed by a quinone-tanning mechanism (Smyth, 1954; Smyth & Clegg, 1959).

Ultrastructural studies of vitelline cells and vitellogenesis have been made for a number of digeneans and monogeneans (Irwin & Threadgold, 1970; Erasmus, 1973; Halton *et al.*, 1974; Grant *et al.*, 1977; Irwin & Maguire, 1979;

Erwin, 1984; Gupta *et al.*, 1987b). In general, each vitelline follicle is composed of a number of vitelline cells; all stages of vitellogenesis can be observed in a single vitelline follicle, and maturation of vitelline cells occurs towards the centre of the follicle. In the majority of digeneans, the immature vitelline cells are situated at the periphery of the follicle and exhibit gonadial morphology. The vitelline cells undergo two developmental phases, protein synthesis and the laying down of food reserves. The production of food reserves includes the synthesis of glycogen and lipid.

Vitelline glands perform two functions in that they provide nutrient for the developing embryo or larva and they participate in the formation of the egg shell.

The Mehlis' gland is a group of unicellular secretory cells surrounding the ootype. It consists of one, two or three types of cells, and their secretions generally belong to a mucous type or serous type. In most monogeneans and digeneans, the secretory substance or substances are conveyed via cytoplasmic extensions or ducts to the lumen of the ootype. In some species the ootype epithelium has been observed to be secretory. The precise function of the Mehlis' gland is obscure and it is believed it may directly or indirectly participate in the formation of the egg shell.

The oogenotop of digenetic trematodes has been reviewed by Ebrahimzadesh (1966) who suggested the existence of two types of Mehlis' gland: the F-type with two types of cell (mucous and serous), and the S-type consisting of only one type of cell (either mucous or serous); where serous cells are lacking, the epithelium of the ootype is secretory.

3.2. Materials and Methods

Carp infected with *D. vastator* were decerebrated and infected gill filaments were removed individually from separate gill arches, these were then transferred to small sized petri dishes with aquarium water. A one in 1,500 solution of phenoxyethanol was used as a parasite anaesthetic to aid dislodgement of the parasites from gills (Shinn, 1994).

3.2.1. Sonication technique

Active, live, fresh *D. vastator* were taken from freshly killed *Cyprinus carpio* gills, and collected in pointed 10 ml glass centrifuge tubes (MES) containing 3 ml distilled water. They were then sonicated in a sonic water bath (Kerry Pulsatron 125) connected to a 240 V power supply with a continuous power output of 1000W minimum, operating at a mean frequency of 40 KHz. It was found that dactylogyrids required approximately 55 minutes to release the

sclerites.

3.2.2. Post - sclerite release procedure

Following sclerite release, the resultant sonicate was centrifuged (MSE Centrifuge'S') at 6,000 rpm for 5 mins, and the supernatant decanted. The final pellet was resuspended in 3 ml distilled water and washed thoroughly. This process was repeated 4 or 5 times to get a very clean preparation without any tissue debris. The final pellet was agitated with a minimum amount of water and pipetted onto 11 mm round coverslips and allowed to air dry. The coverslips were then screened for the presence of sclerites under the light microscope (Olympus BH2 stereo microscope) using 100 times magnification. Coverslips with sclerites were sputter coated (using a Polaron Edwards 150 B sputter coater) with 15 nm gold (Gold - palladium used as a cathode in the sputter coater in an Argon gas environment) and examined under a Philips scanning field emission microscope at 12kV.

3.2.3. Scanning Electron Microscopy

Gill arches from the fish with attached worms were washed in 0.05% Rossapol (detergent) to remove surface blood and mucus and then fixed in 3% glutaraldehyde in 0.2M cacodylate buffer and left overnight at 4°C. The materials were given two washes of 1 hour each in 0.2M cacodylate buffer. The gills with worms were then post fixed in 1% osmium tetroxide for 4 hours at 4°C and then washed twice in 0.2M cacodylate buffer. They were then dehydrated through an acetone series; the specimens were left in 70% acetone for 4 hours, then transferred to 90% acetone for 4 hours, followed by absolute acetone for 2 hours. To improve the dehydration process the tissues were left overnight in 100% acetone before critical point drying. The specimens were then critical point dried in liquid CO_2 using a Bio Rad critical point dryer, mounted on aluminium stubs and sputter coated with gold-palladium in a Polaron Edwards 150 B sputter coater before examination in the scanning electron microscope.

3.2.4. Transmission Electron Microscopy

For transmission electron microscopy specimens were fixed for 4 - 6 h (or 2 h) in Karnovsky's fixative containing 1.6% glutaraldehyde and 1.3% paraformaldehyde at 4°C, washed briefly in 0.01M sodium cacodylate (pH 7.2)

at 4°C, transferred into a fresh rinse of 0.01M sodium cacodylate buffer at 4°C and then left overnight. They were then post-fixed for 1 hour in osmium tetroxide (0.5% osmic acid in cacodylate buffer) and dehydrated through graded acetone starting from 30% acetone for 10 minutes, then to 60% acetone for 10 minutes and then finally into 2 changes of 100% acetone for 15 minutes each. For impregnation the specimen was first left in Araldite CY 212 resin mix in acetone at a ratio 1:1 in a rotator for 30 minutes and then transferred to the resin mix at a ratio of 1: 3 and left for 30 minutes for the resin to penetrate into the parasite. At the end of the impregnation process the worm was left in resin mix (Araldite CY 212, 10 ml + DDSA 10 ml + BDMA 0.4 ml) alone for 1 hour in the revolving rotator. At all instances the araldite resin and DDSA hardener was maintained at 60°C before mixing with acetone. Plastic beem capsules / moulds were kept in an oven prior to embedding. First a drop of resin mix was placed into a capsule(well), followed by the worm. Then capsule was then filled by drops of resin and left in the oven at 30°C for polymerisation / hardening, for 24 hours. Ultrathin sections (5 - 7 nm) prepared using an ultramicrotome were double stained with uranyl acetate and lead citrate and viewed in a Philips transmission electron microscope operating at 80 kV.

3.3. Results

3.3.1. External appearance of the worm

Scanning electron microscopy and light microscopy reveals *D. vastator* as an elongated worm with four anterior cephalic lobes which attaches in between adjacent secondary gill lamellae by means of the posterior opisthaptor (Plate 3.1). The opisthaptor is a flattened circular organ 0.12 mm in diameter, bearing a large pair of hooks or hamuli at the centre and fourteen marginal hooks. Each hamulus has a bifurcate root, with an internal and external root process, these join to form a curved shaft which tapers to a spike. The hamuli are the main means of attachment, being deeply embedded in the gill tissue and penetrating through the primary lamella cartilage (Plate 6.9). The marginal hooks penetrate into the epidermal cells of the secondary gill lamella and serve to prevent the edges of the opisthaptor curving inwards. Each cephalic lobe is 200 - 250 μm in length and bears a 20 - 35 μm diameter cup-like opening for the duct of the cephalic glands (Plate 3.2). The opening is surrounded by a raised edge of tegument without any defined structure. Plate 3.3 shows the opening filled with what is presumably gland secretion. The external surface of the parasite has presumed epidermal sensilla varying in length from 0.28 - 1.11 μm, which are found in higher numbers at the anterior end than at the posterior end (Plate 3.4, 3.5). The sensilla appear to be randomly distributed

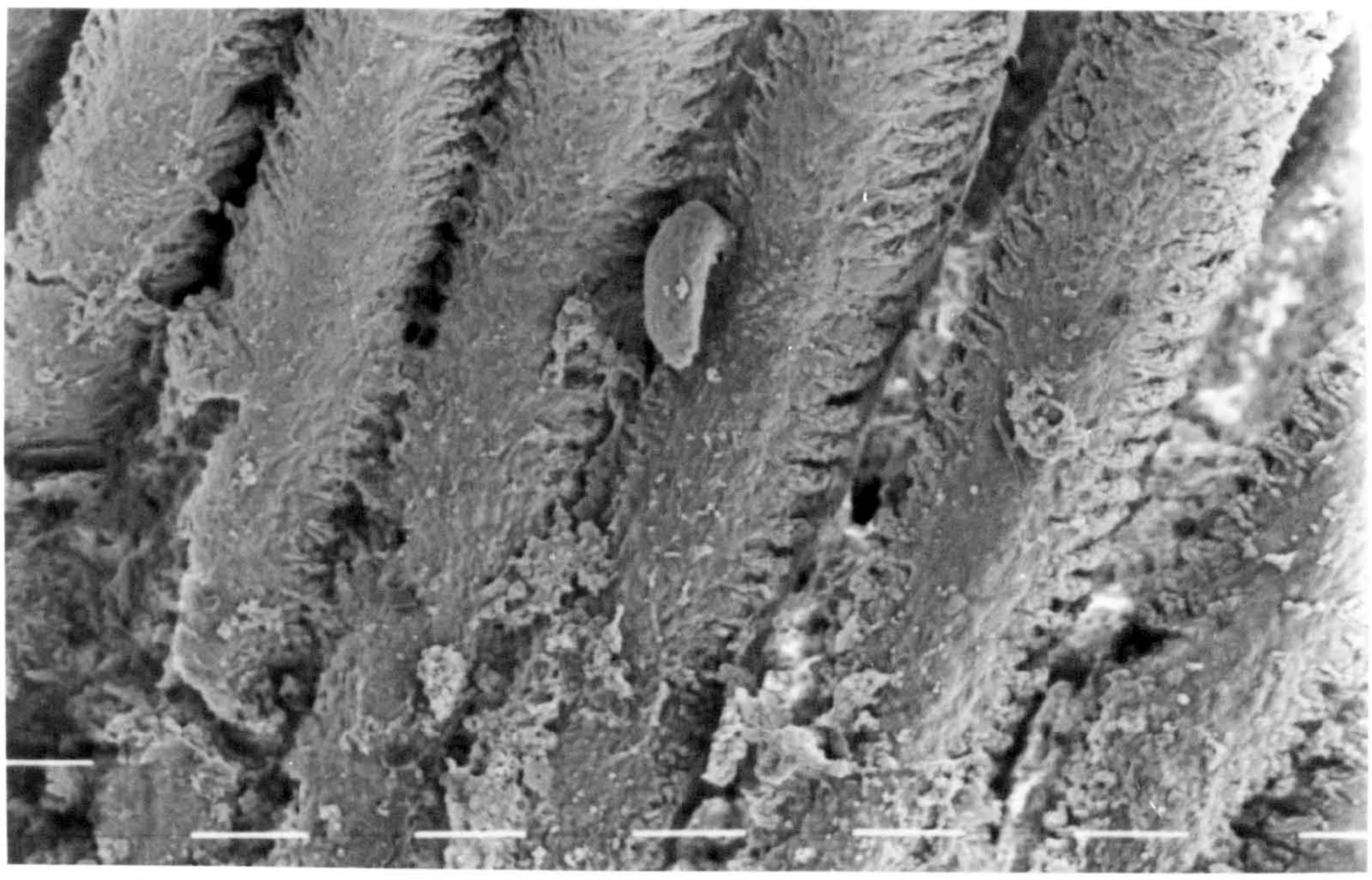


Plate 3.1 :- Scanning electron micrograph (SEM) showing an immature *Dactylogyrus vastator* attached to the gill.
Scale bar = 100μm

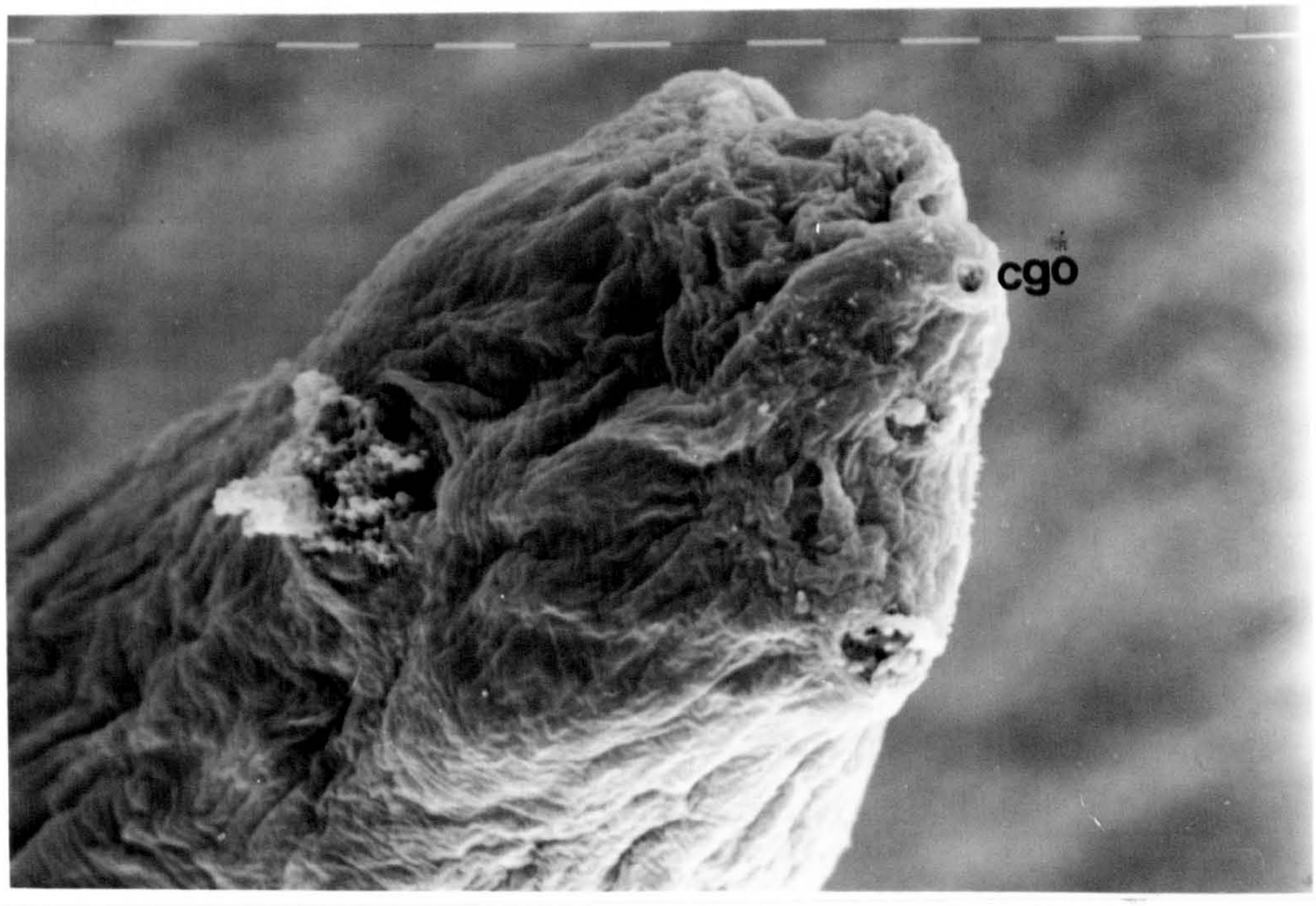


Plate 3.2 :- Scanning electron micrograph (SEM) of the anterior end of *D. vastator*: cgo = cephalic gland opening; pho = pharyngeal opening.
Scale bar = 10 μm

without any organised arrangement. On the ventral surface about 600 - 640 µm posterior from the anterior end of the parasite is the pharyngeal opening of 160 - 240 µm in diameter. During feeding the pharynx may be to some extent eversible and able to grip host tissue. Plate 3.2 shows a plug of presumably host tissue which is apparently being taken up by the worm. The genital pore is very close to the pharynx. There appears to be a flap of tissue at least partially covering the opening. Plate 3.6 shows an egg beginning to be passed out through the pore.

3.3.2. Tegument

As shown by transmission electron microscopy the structure of the tegument from different regions of the worm shows some differences. The body wall from the anterior region (Plate 3.7) has a diffuse epidermal layer, in which cell boundaries cannot be discriminated in the outer tegumental region. The thickness of the outer tegument is 2500 nm to 3000 nm and this is separated from the underlying muscular layer by a basement membrane, the hypodermal layer (Plate 3.8). Interior to this are a circular muscle layer 465 - 698 nm in thickness and a longitudinal muscle layer 233 - 465 nm thick. Plate 3.9 shows a TEM photograph of the tegument between the anterior end and the ovarian region. The thickness of the tegument is 3488 - 8139 nm, and the outer tegumental layer is not as diffused as anteriorly and cell boundaries can be

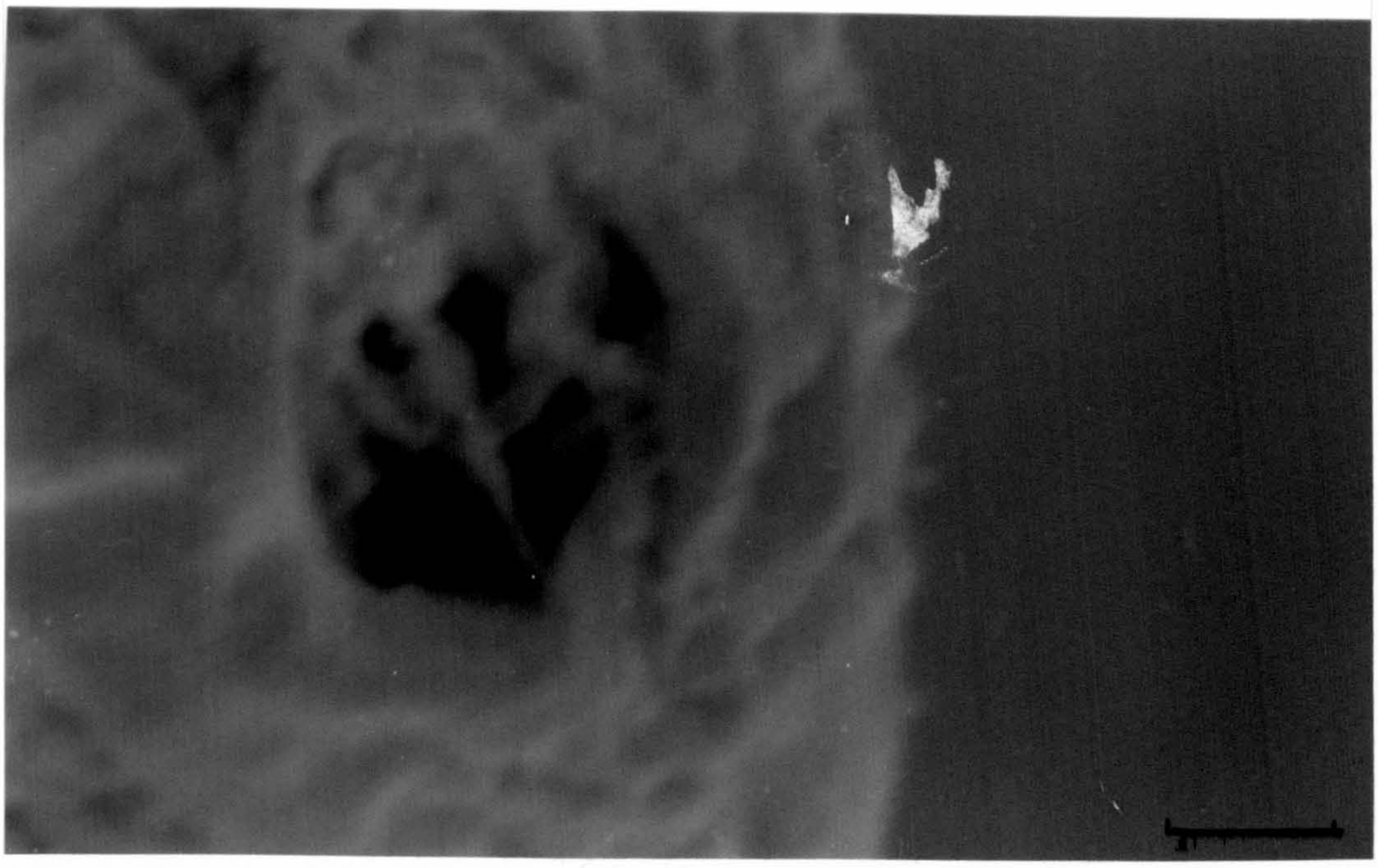


Plate 3.3 :- Scanning electron micrograph (SEM) showing the cephalic gland opening (p) at the margin of the cephalic lobe.
Scale bar = 10 μm

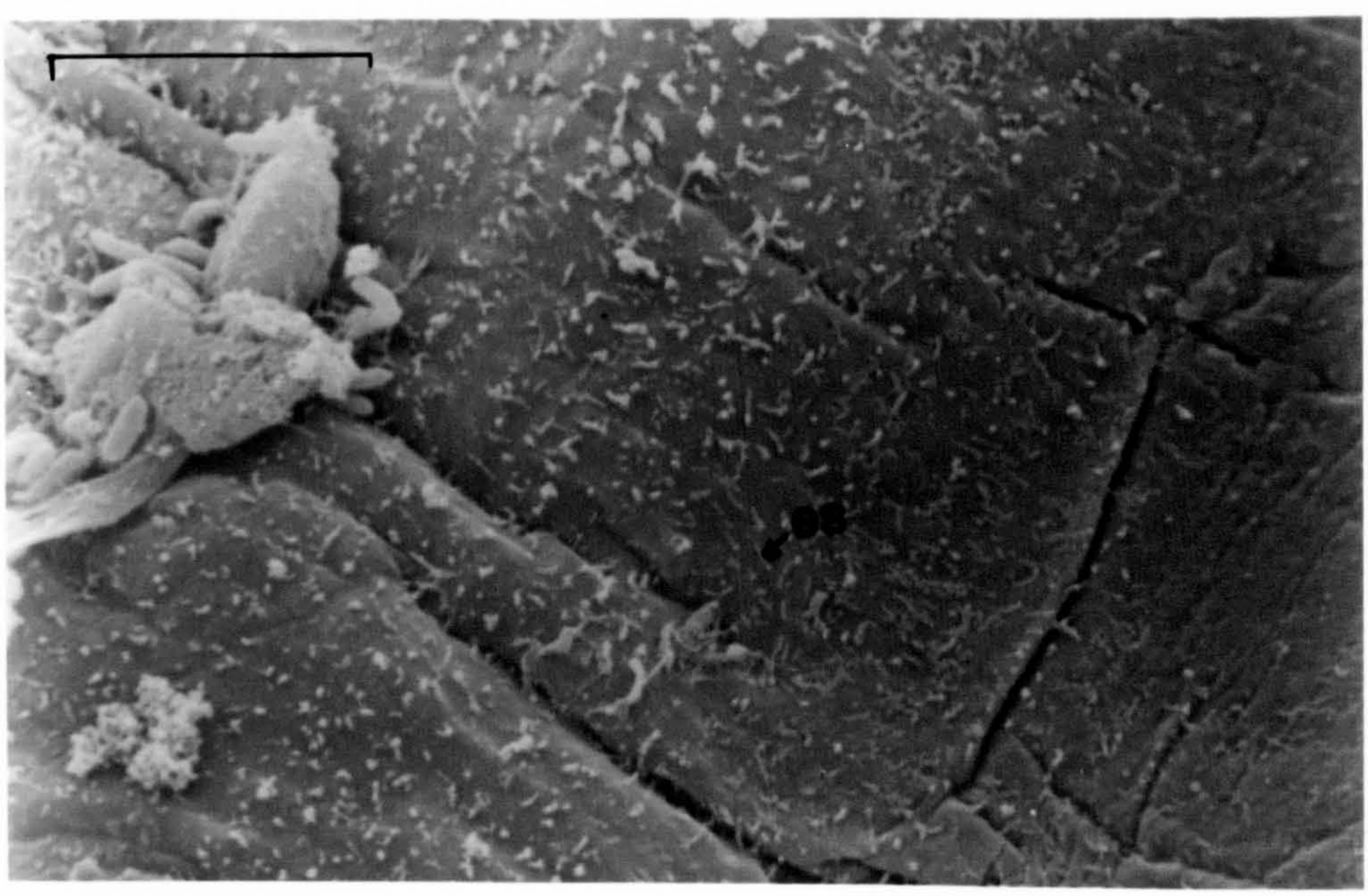


Plate 3.4 :- Scanning electron micrograph (SEM) showing the distribution of the epidermal sensilla (es) on the anterior region.
Scale bar = 10 μm

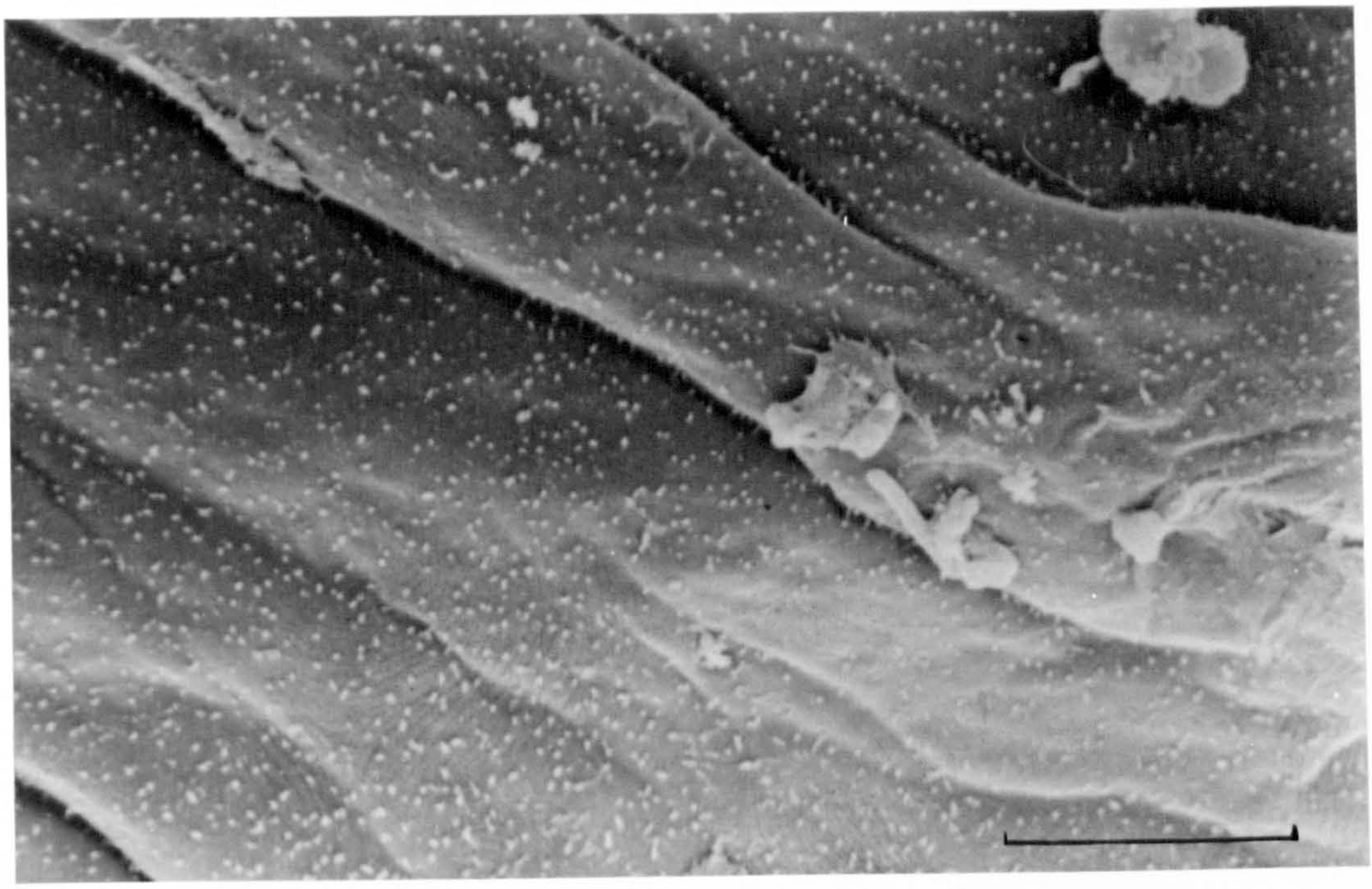


Plate 3.5 :- Scanning electron micrograph (SEM) of the posterior region of the worm showing the distribution of sensory papillae (sp).
Scale bar = 10 μm

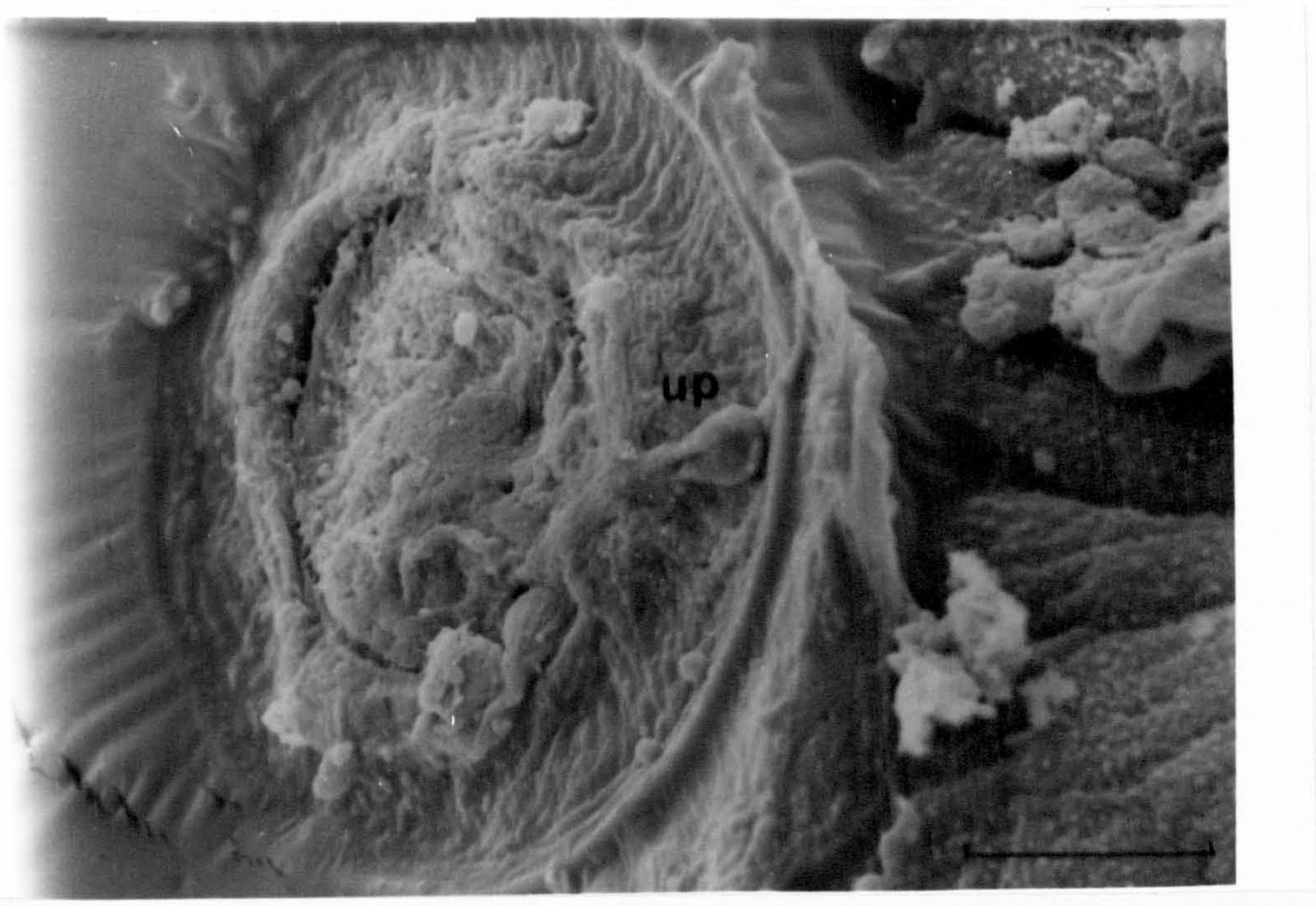


Plate 3.6 :- Scanning electron micrograph showing the egg about to exit through the uterus pore (up).
Scale bar = 10 μm

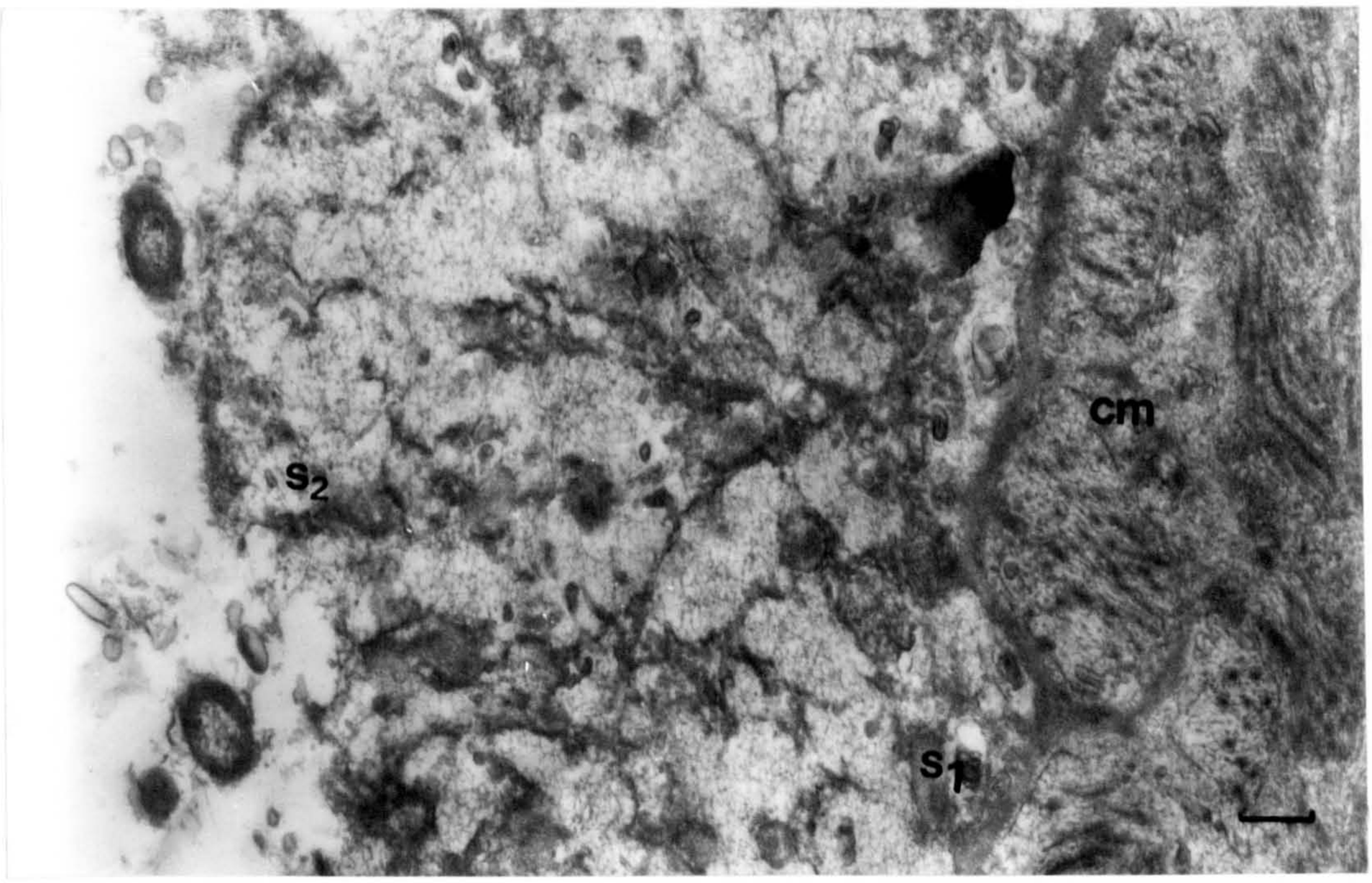


Plate 3.7 :- Transmission electron micrograph (TEM) showing the tegument from cephalic region.
S_1 = membrane bound secretory vesicle ; S_2 = electron lucent sec retory bodies; cm = circular muscle band.
Scale bar = 15 nm

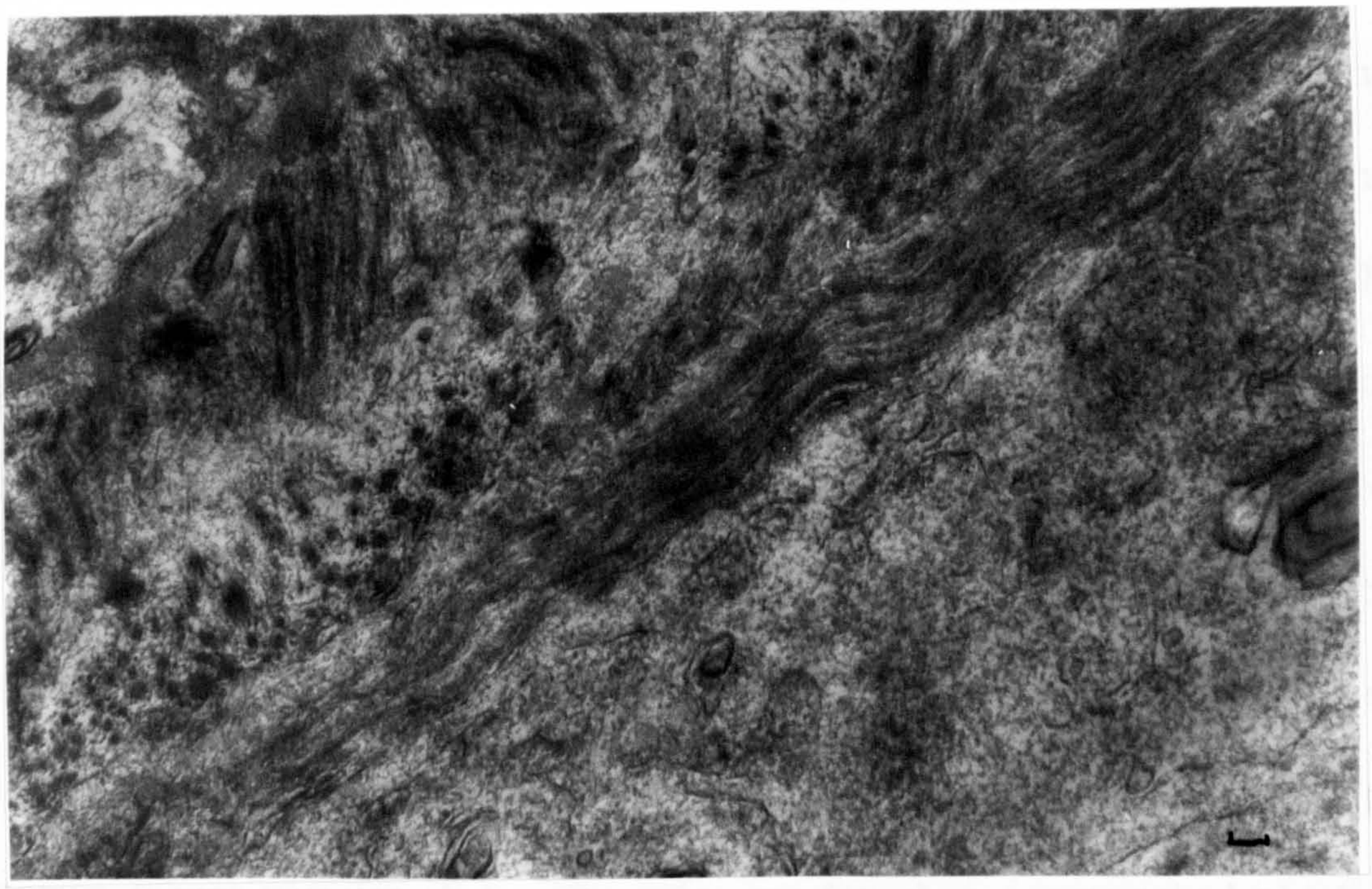


Plate 3.8 :- Transmission electron micrograph (TEM) showing hypodermal layer of the tegument.
Scale bar = 15 nm

distinguished to some extent. The outer circular muscle layer is 465 - 1628 nm in thickness and the longitudinal muscle is 698 - 1163 nm thick. Between the muscle layers is an approximately 232 nm thick layer of fibrous interstitial material. Beneath the muscular tissue are the sub-tegumentary cells (cytons) with prominent nuclei and cytoplasm containing organelles such as lysosomes, Golgi bodies and mitochondria. Within the outer syncytial layer there are three kinds of secretory inclusions. These are relatively large, oval to elongate, membrane bound vesicles containing moderately electron dense contents (S_1), roughly spherical, membrane-bound, electron lucent vesicles (S_2) and oval to spherical, membrane-bound vesicles containing moderately electron dense particles embedded in an electron-lucent ground substance (S_3). The spaces between the sub-tegumentary cells are filled with homogeneously distributed granular interstitial material. Plate 3.10 shows the structure of tegument in the ovarian region. This shows a similar overall structure but is rather thicker. The syncytial layer is 2142 - 2321 nm in thickness with the outer circular muscle layer of 143 - 249 nm and the longitudinal muscle layer 607 - 785 nm in thickness. Individual cell boundaries can be distinguished more easily compared with tegument from other sections described earlier. In the outer layer there are secretory vesicles, relatively large oval to elongate membraneous bound bodies with electron dense contents (S_1) and approximately spherical, membrane bound, electron lucent vesicles (S_2). The latter are found in comparatively higher numbers. A TEM photomicrograph of the tegument from the hamulus region

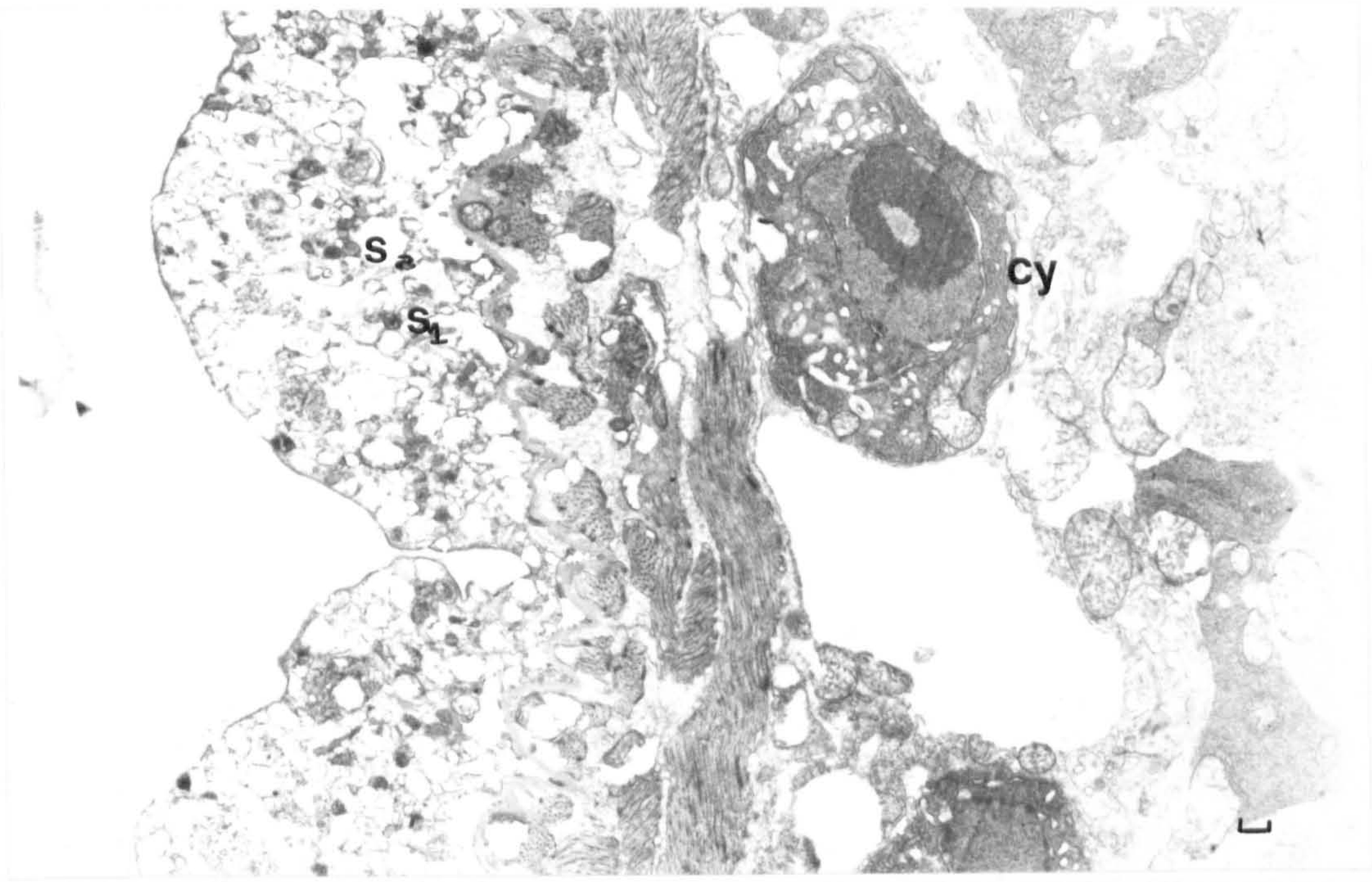


Plate 3.9 :- Transmission electron micrograph (TEM) showing the tegument between the cephalic region and the ovarian region.
S_1 = membrane bound secretory vesicle ; S_2 = electron lucent secretory bodies; cy = cyton.
Scale bar = 15 nm

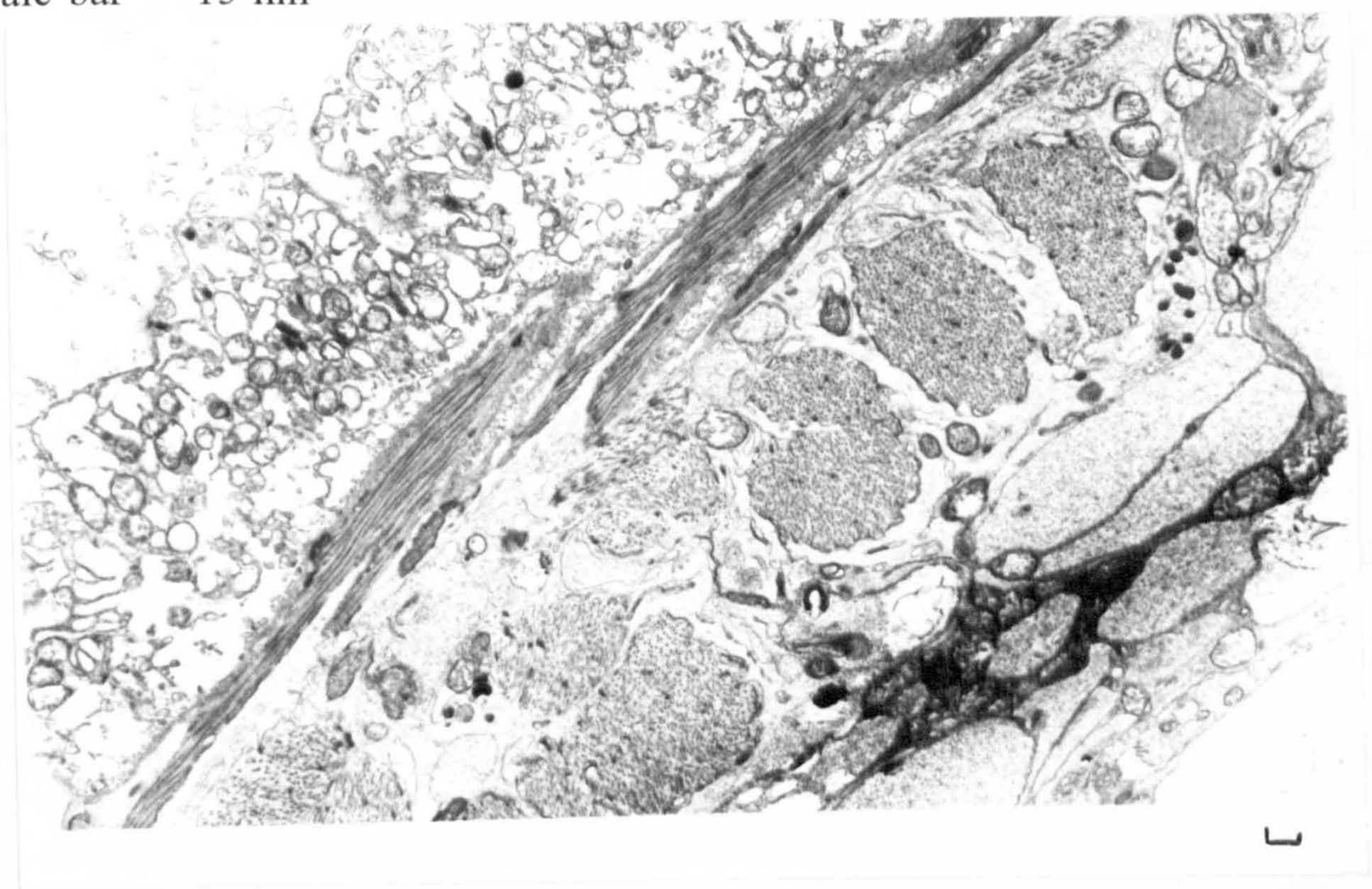


Plate 3.10 :- Transmission electron micrograph (TEM) of the body wall in the ovarian region which is slightly discrete but more muscular than in the cephalic region.
Scale bar = 15 nm

of the worm in Plate 3.11 shows the same basic structural plan described for other areas of the tegument. The tegument is 1105 - 1289 nm in thickness with the underlying outer circular muscle layer 56.63 - 158 nm and the interior longitudinal muscle layer 237 - 579 nm in thickness. In addition to the circular and longitudinal muscle bands in this particular area, is an extra diagonal muscular band of 57 - 263 nm in thickness. The underlying sub-tegument is similar to the other parts of the worm with granular interstitial material. The outer tegumental layer shows a degree of cellularity where cell boundaries can be distinguished, unlike in other areas, and it was found to contain S_1 and S_2 secretory vesicles. The S_1 spherical membraneous bound secretory vesicles are found in higher numbers. Plate 3.12 is a TEM photograph of the worm from an area very close to the hamuli. The tegumental layers are the same as the other TEM sections. The outer tegument is 818 - 1136 nm in thickness with the underlying outer circular muscular band 91 - 227 nm thick and the inner longitudinal muscle is 818 - 1045 nm thick. In this photomicrograph it is quite evident that the underlying region interior to the muscular layer has a higher concentration of concentrically arranged small membraneous bodies scattered predominantly in the outer region of the sub-tegument. An epidermal projection of length 818 - 1045 nm is shown in Plate 3.13. This projection is derived from the outer layer of the tegument and there appears to be no cellular connections entering it from the main body of the parasite. No ciliary structures were found in the projections.

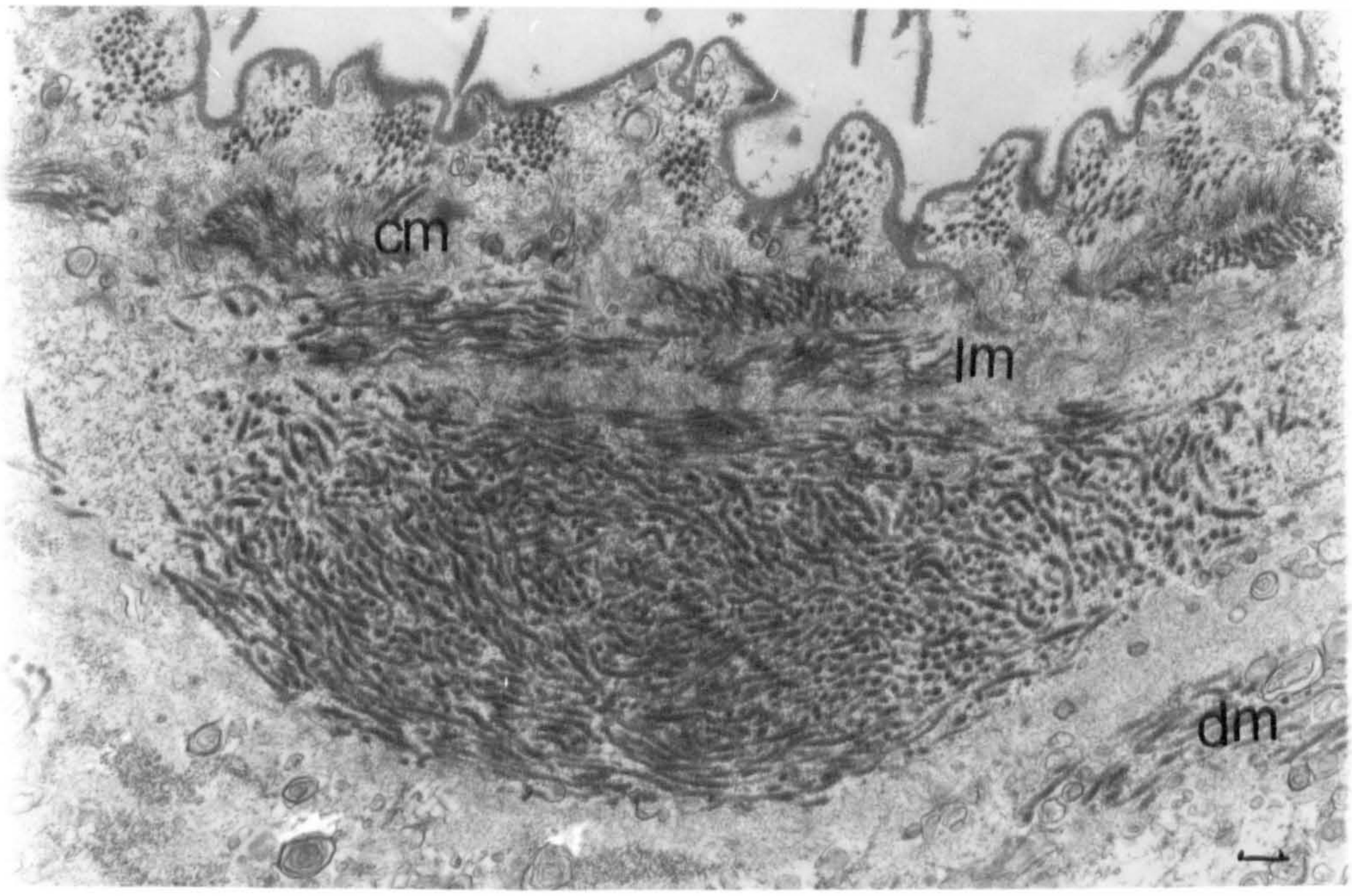


Plate 3.11 :- Transmission electron micrograph (TEM) of the body wall in the region of the hamuli showing increased muscular tissue with diagonal muscles (dm) in addition to circular (cm) and longitudinal muscles (lm).
Scale bar = 15 nm

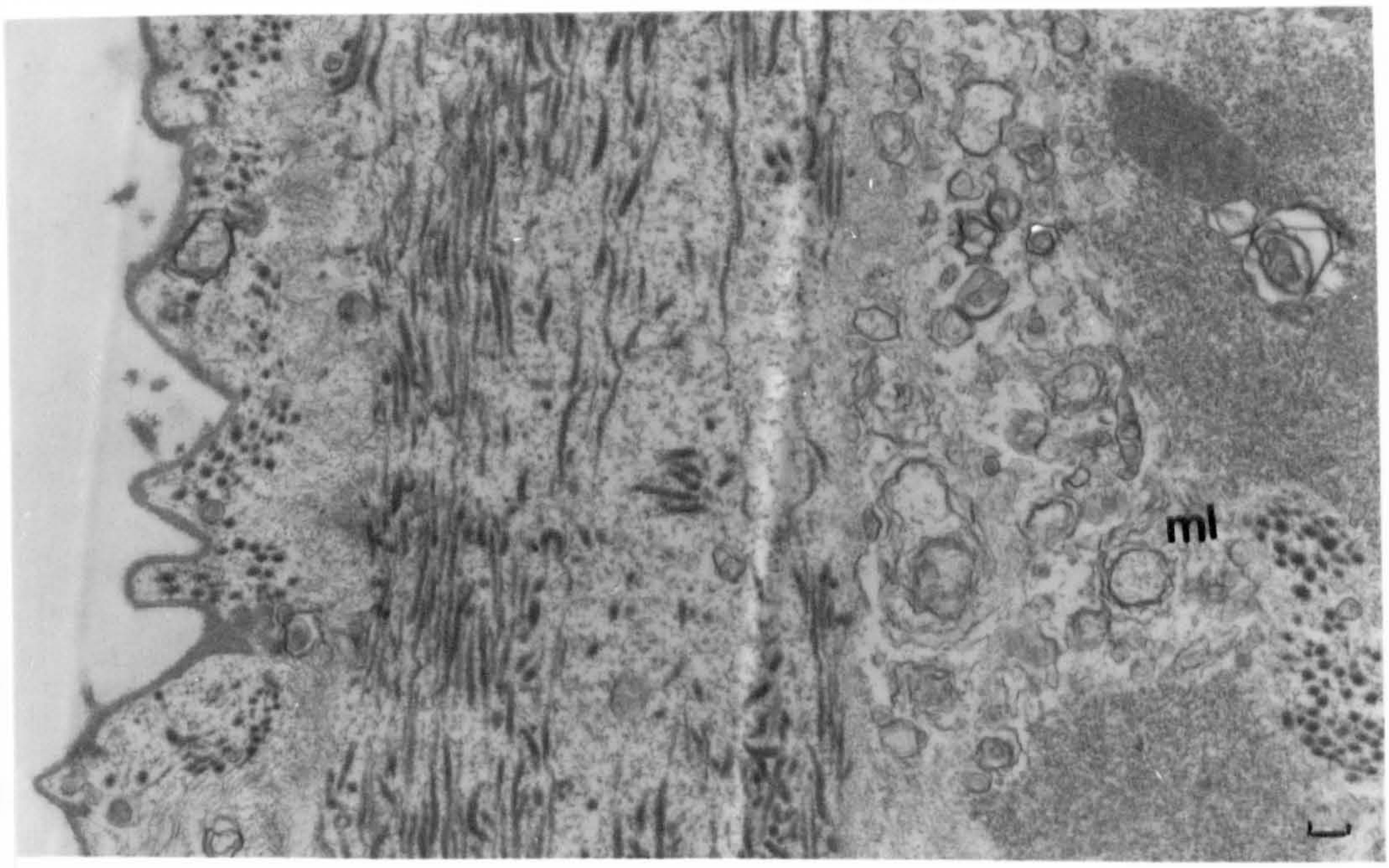


Plate 3.12 :- Transmission electron micrograph (TEM) of the body wall very close to the hamuli showing concentrically arranged membraneous layers (ml).
Scale bar = 15 nm

The tegument shows the same basic fundamental structure in all areas of the parasite. The tegument, which is bounded by apical and plasma membranes, rests on a basal lamina or hypodermis and is separated from the underlying regions by circular and longitudinal muscle blocks. Continuity between the two, however, is provided by cytoplasmic processes. The tegument is a true syncytium since compartmentalizing plasma membranes are rarely seen within the superficial layer. The outer surface of the tegument is thrown into presumed epidermal sensilla whose distribution is not uniform across the worm's body (Plate 3.4 & 3.5). Although very little endoplasmic reticulum can be seen in the outer tegument, the sub-tegumentary cells possess quite an extensive system. Mitochondria are found in the outer layer of the tegument and also interior to the muscular layer, however, they are particularly associated with the whorls of loosely arranged membraneous body. Mitochondria in the tegument are particularly concentrated in the region of the opisthaptor (Plate 3.11).

3.3.3. Cephalic gland

The tegument in this region is very thin, below this there are numerous electron dense granules, possibly lipid droplets. Interior to this is a spherical shaped body limited by a membrane covered by a muscular sheath with an aggregation of ciliation in its centre. This may represent a ciliated sense organ. Unicellular gland cells are found singly and are profusely distributed near to a

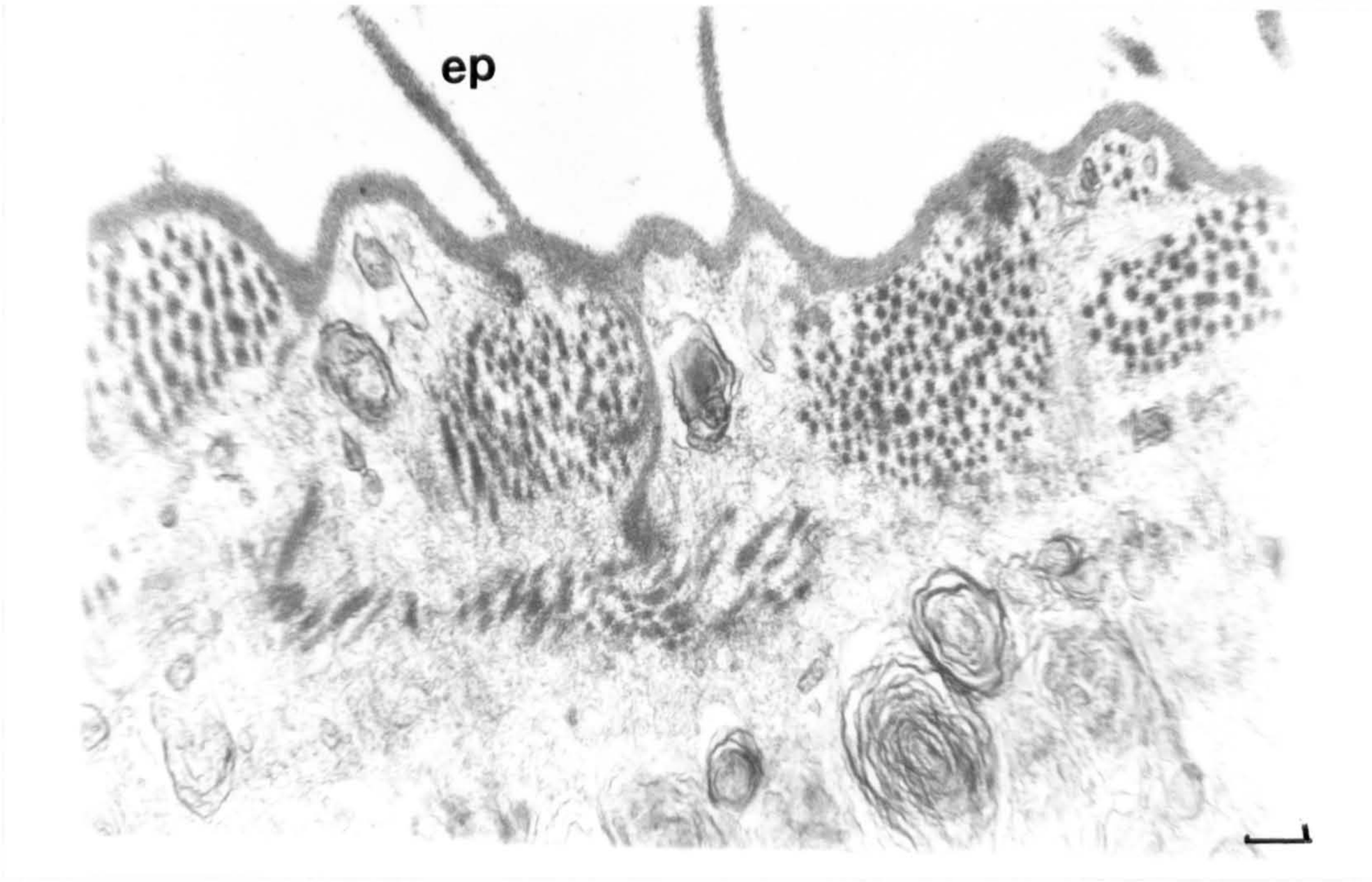


Plate 3.13 :- Transmission electron micrograph (TEM) of the body wall from the posterior region with epidermal projections (ep).
Scale bar = 15nm

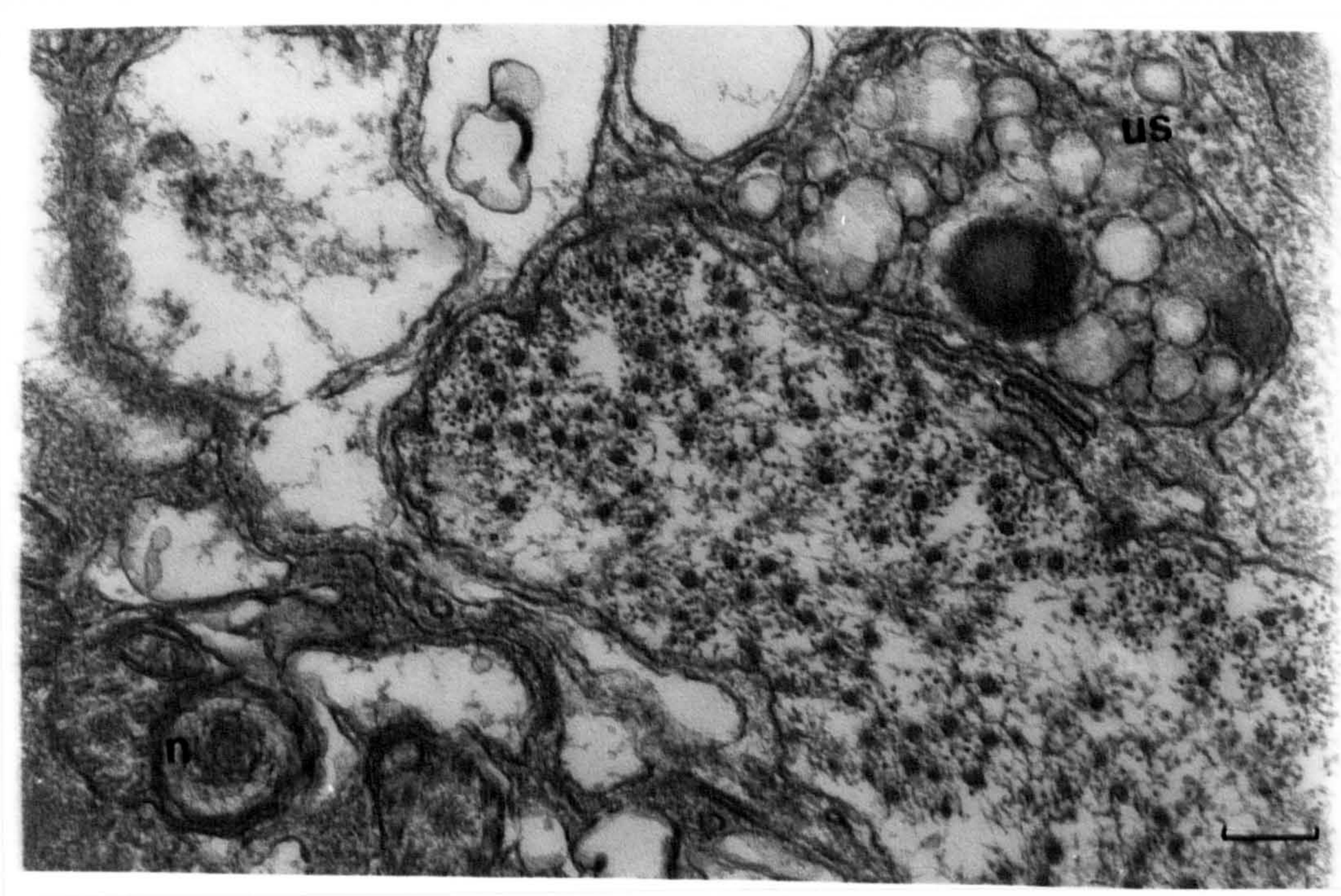


Plate 3.14 :- Transmission electron micrograph (TEM) of the cephalic area showing a unicellular secretory (us) cell with a prominent nucleus (n).
Scale bar = 15nm.

gland duct. These cells have a prominent nucleus and well developed endoplasmic reticulum and Golgi body (Plate 3.14). They also contain larger amounts of electron lucent material, presumably precursors for their secretory products. The secretion produced by the unicellular gland cells passes into a common duct approximately 1500 nm long (Plate 3.15). The duct contains large amounts of apparently secreting material and may serve as a reservoir for this material. The duct itself is bound by a membraneous border with a covering layer of muscular tissue. The duct wall opens to the exterior at the margins of the cephalic lobe via a cup-like cavity. Sections through the cephalic region show a ciliated structure lying between the sub-tegumentary cells. Plate 3.16 clearly shows seven ciliary structures lying within a pit. This is presumed to be a sensory structure.

3.3.4. Opisthaptor

The opisthaptor consists of two hamuli and fourteen marginal hooks (Plate 3.17). The shape of the hamuli enables the worm to attach to its host (Plate 3.18). Hamuli have both internal roots of 13.75 μm and external roots of 18.75 μm in length (Plate 3.19) which join to form a stiff shaft which continues to a sharp point of 9.53 μm in length. The ventral surface of the internal root and the proximal part of the shaft of the hamulus is provided with a thickened margin of 1.7 μm thickness. The auxiliary sclerite is a double

Plate 3.15 :- Transmission electron micrograph (TEM) of the cephalic area showing the duct (d) passing its contents to the exterior.
Scale bar = 15 nm.

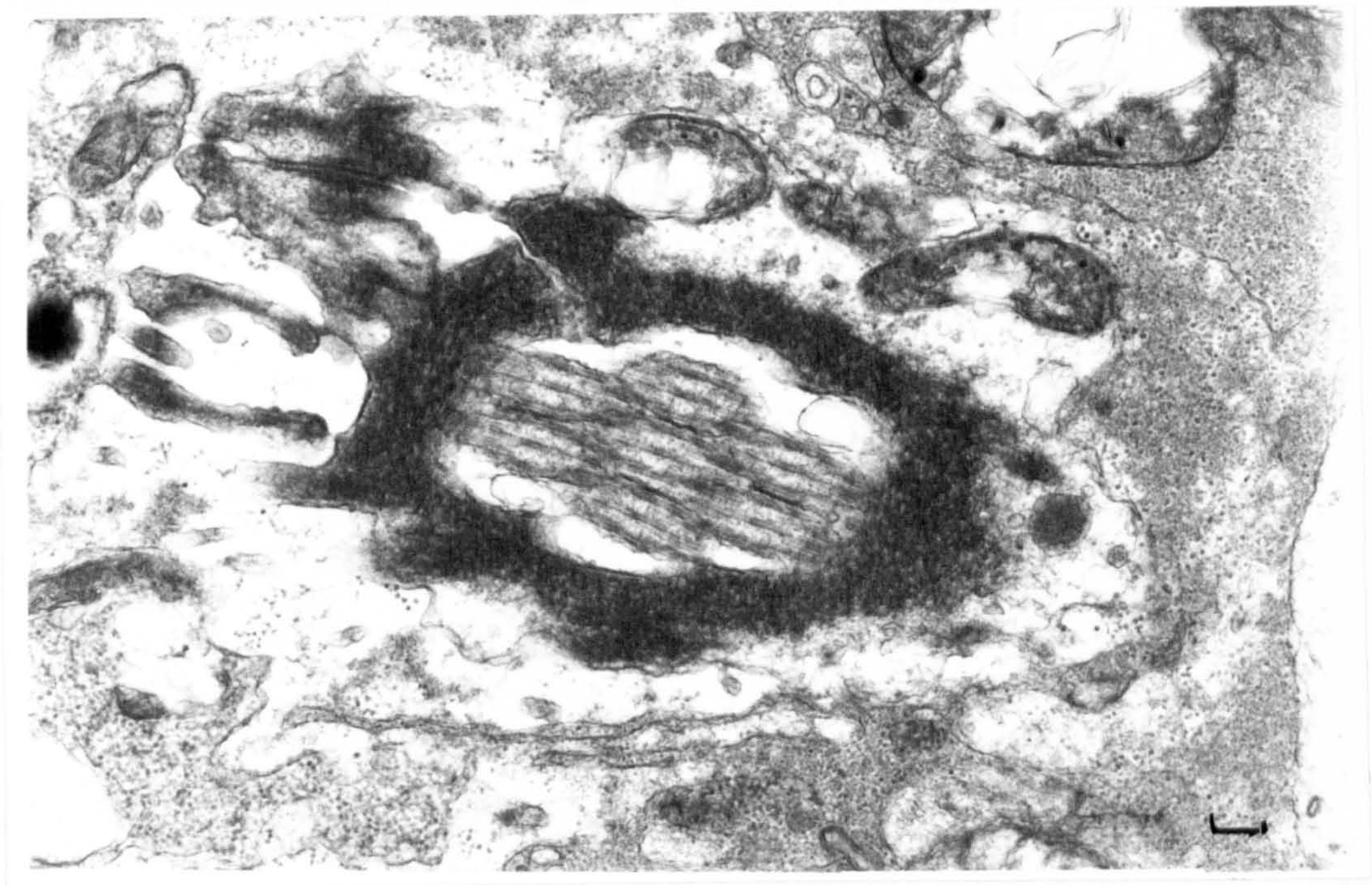


Plate 3.16 :- Transmission electron micrograph (TEM) showing a ciliated sensory pit containing seven cilia within the cephalic gland area.
Scale bar = 15 nm

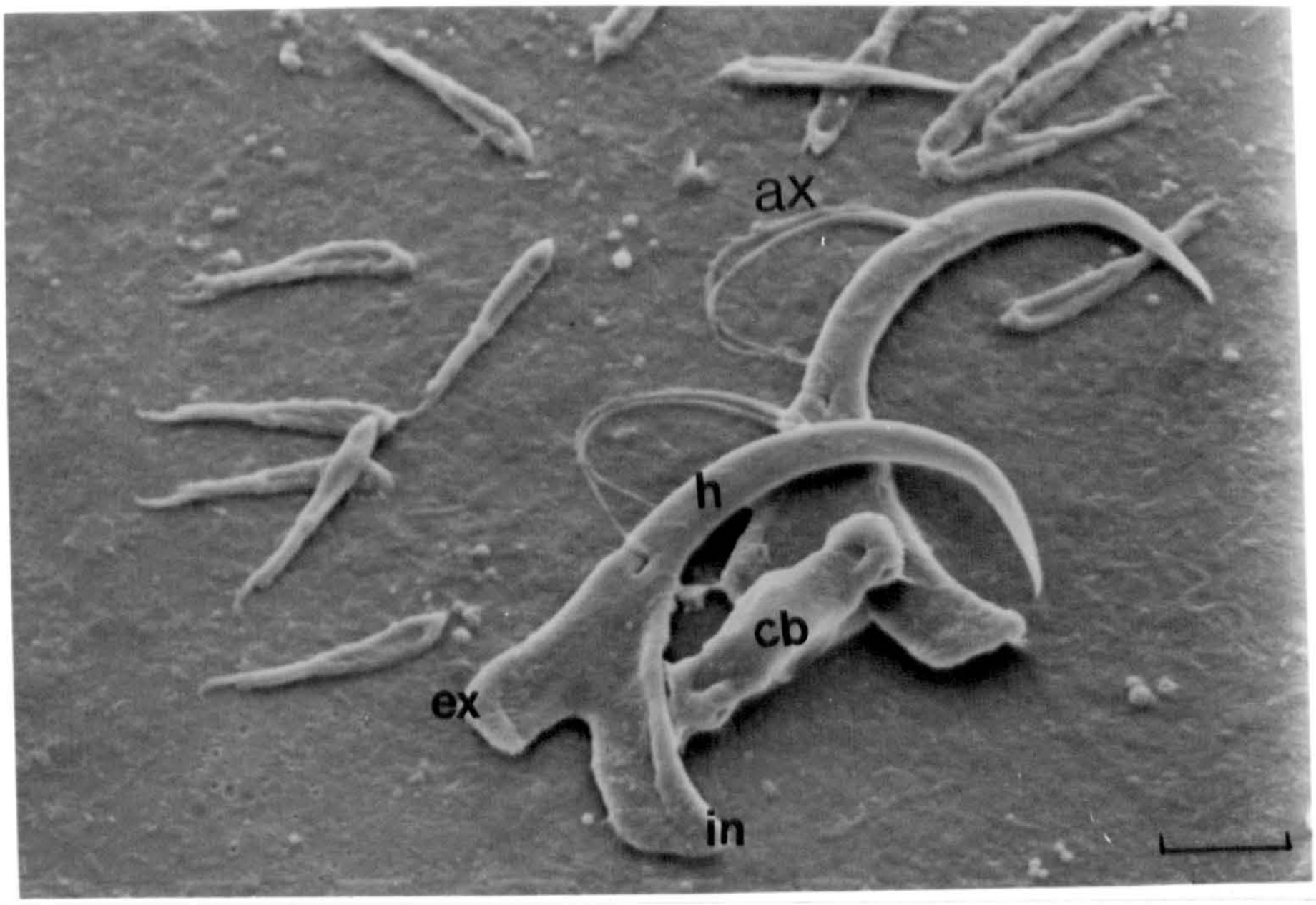


Plate 3.17 :- Scanning electron micrograph (SEM) of the hamuli and the marginals separated by the sonication technique.
ax = auxiliary sclerite; ex = external root process; h = hamuli; in = internal root process; cb = connecting bar.
Scale bar = 100 μm

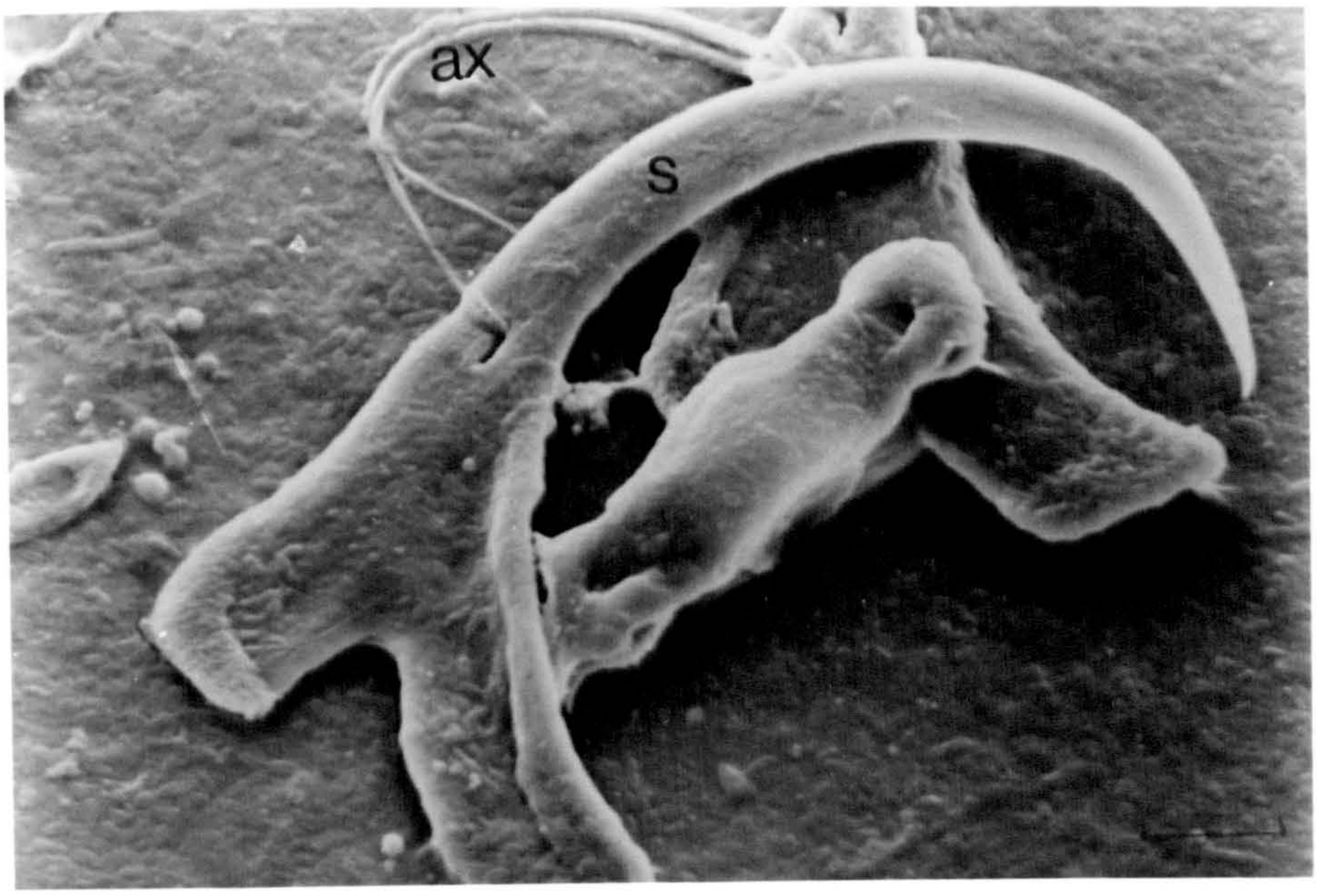


Plate 3.18 :- Scanning electron micrograph (SEM) showing the hamuli of the immature worm where the auxiliary sclerite (ax) originates at the shaft (s) as two filaments and remains separate at its distal points.
Scale bar = 10 μm

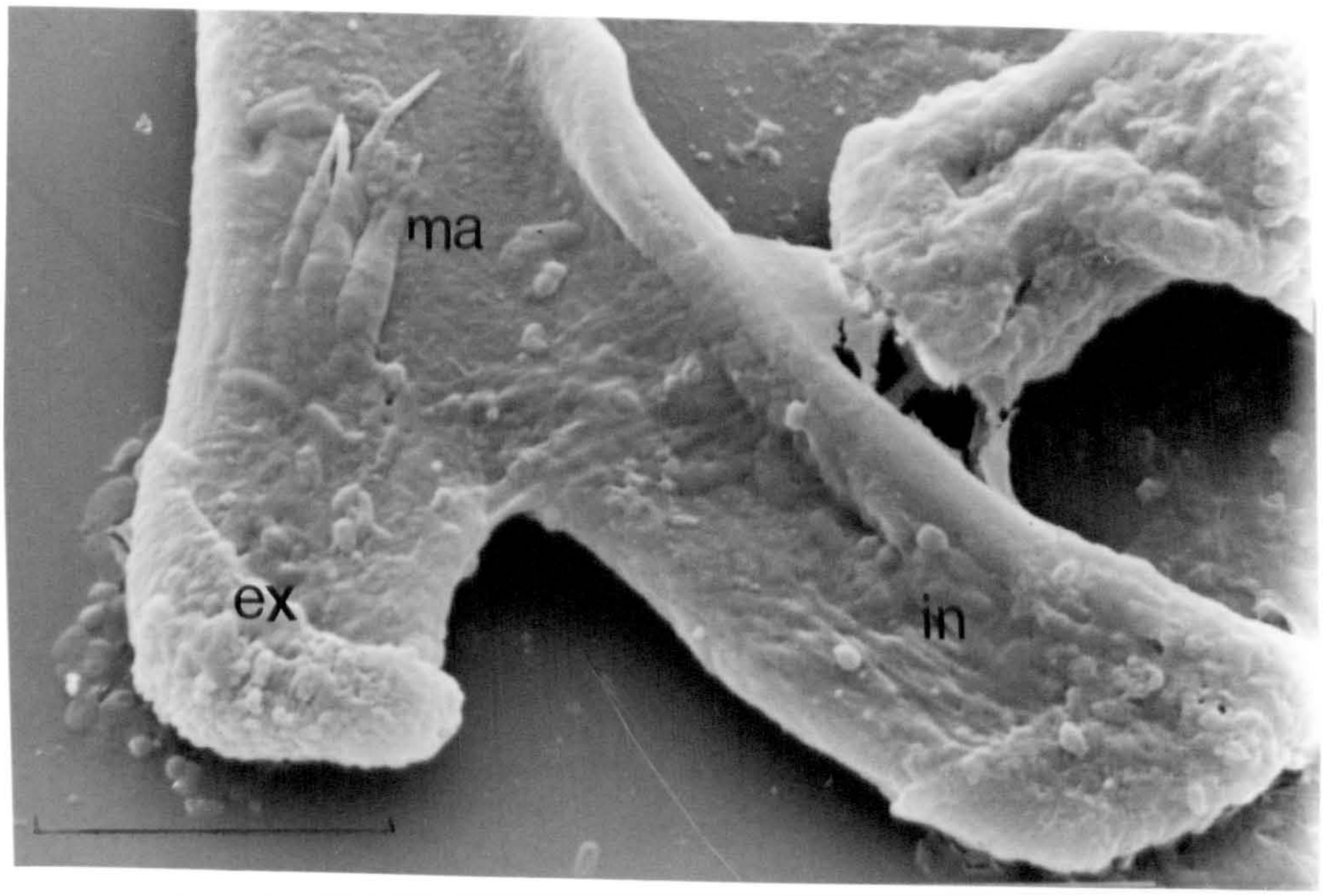


Plate 3.19 :- Scanning electron micrograph (SEM) of the basal part of the hamulus showing the external and internal root processes and their muscular attachment surfaces.
ex = external root process ; in = internal root process ; ma = muscular attachment surface.
Scale bar = 10 μm

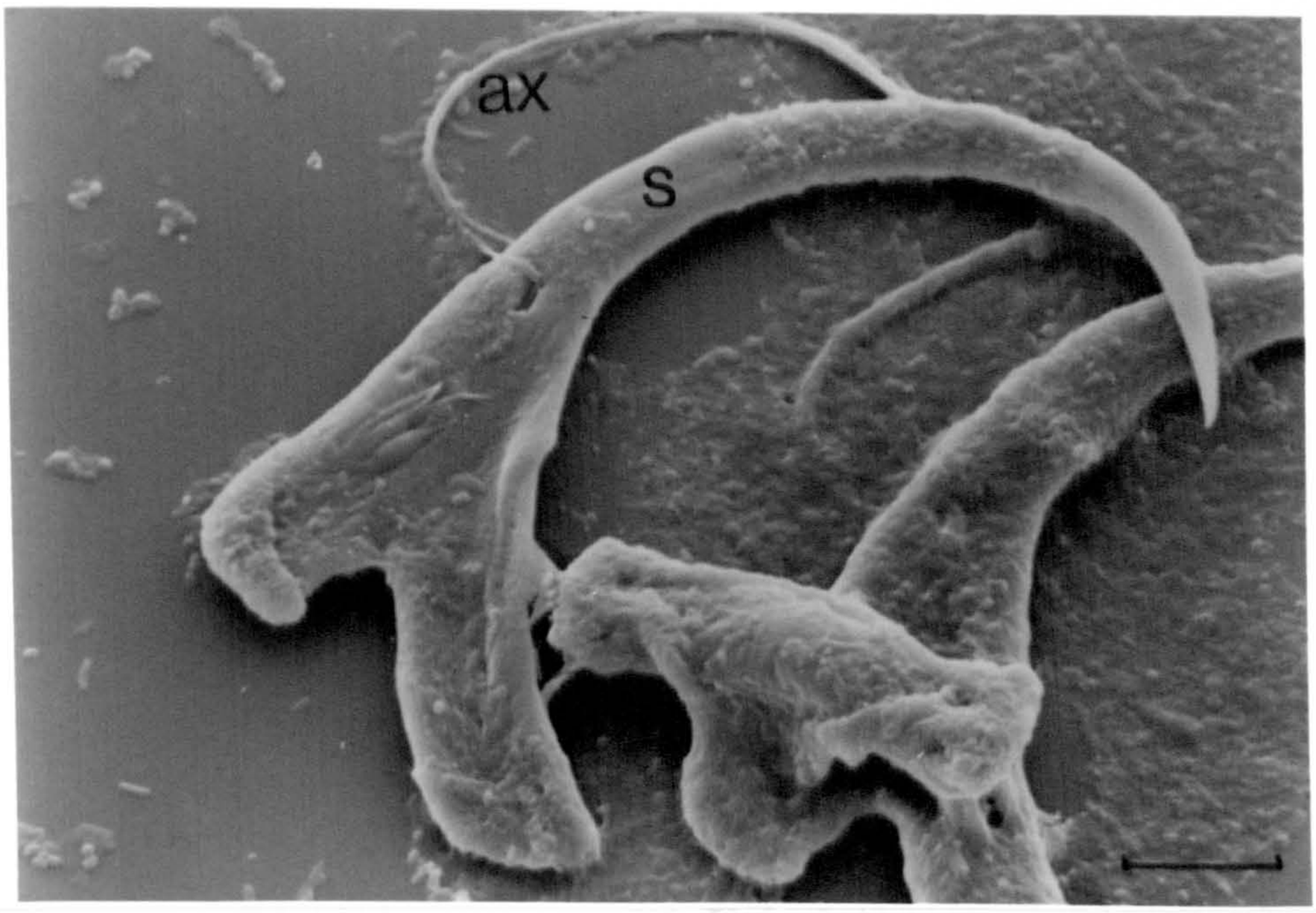


Plate 3.20 :- Scanning electron micrograph (SEM) of the hamuli of the adult worm where the auxiliary sclerite (ax) originates at the shaft (s) as two filaments and joins at the distal point.
Scale bar = 10 μm

filament (Plate 3.18) found on the dorsal surface of the shaft and point regions of each hamulus. There is variation in the attachment of the sclerite between adult (Plate 3.20) and immature worms (Plate 3.18). In the adult the auxiliary sclerite has a double origin from the outer and inner surfaces of the shaft, in association with a hole of 7.5 μm in the shaft region, but joins to terminate as one on the dorsal surface near to the angle of the shaft. In immature worms the overall structure is similar to the adult, but the two components of the sclerite remain separate throughout their length, although they attach to the same point on the dorsal surface of the hamulus.

The external and internal root processes of the hamuli are provided with attachment surfaces for muscles (Plate 3.19). The end of both the external and internal root processes are provided with a thickened cap which is 3.8 μm in thickness. The surface of the shaft and point of the hamuli are striated (Plate 3.21). Separating the two hamuli, and presumably playing an important role in maintaining their overall position within the opisthaptor, is a connecting bar (Plate 3.22). This substantial structure is attached to the flattened base of the shaft. The bar consists of three main elements, a central bar, thickened bulbous knobs at each end and a flattened triangular process ventral to the main bar. The thickened process at either end must serve as the point of attachment for the articulation of the whole connecting bar with the hamuli. The connecting bar is apparently hinged with the hamuli by ligaments (Plate 3.23).

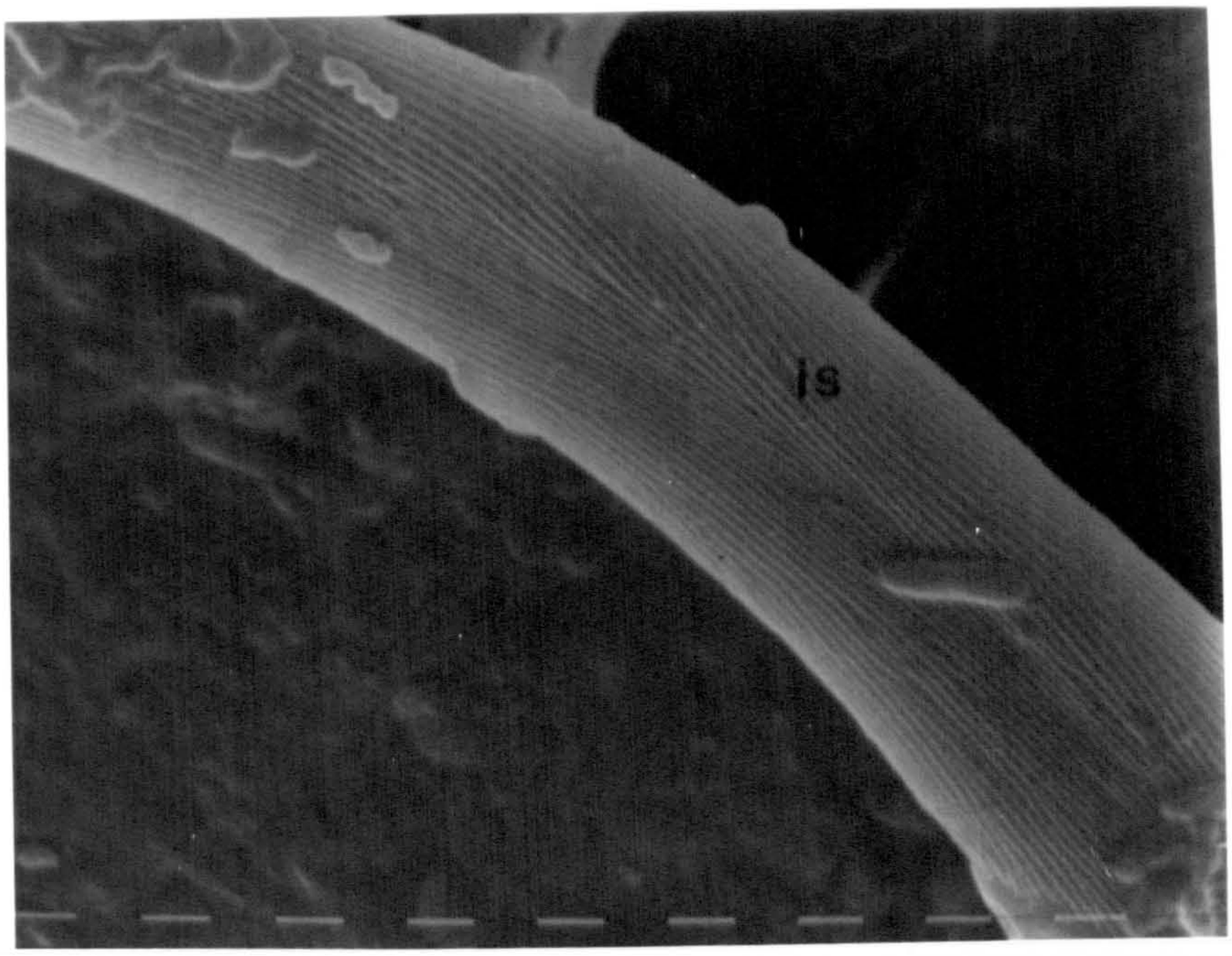


Plate 3.21 :- Scanning electron micrograph (SEM) of the surface of the hamuli showing interlocking striations (is).
Scale bar = 10 μm

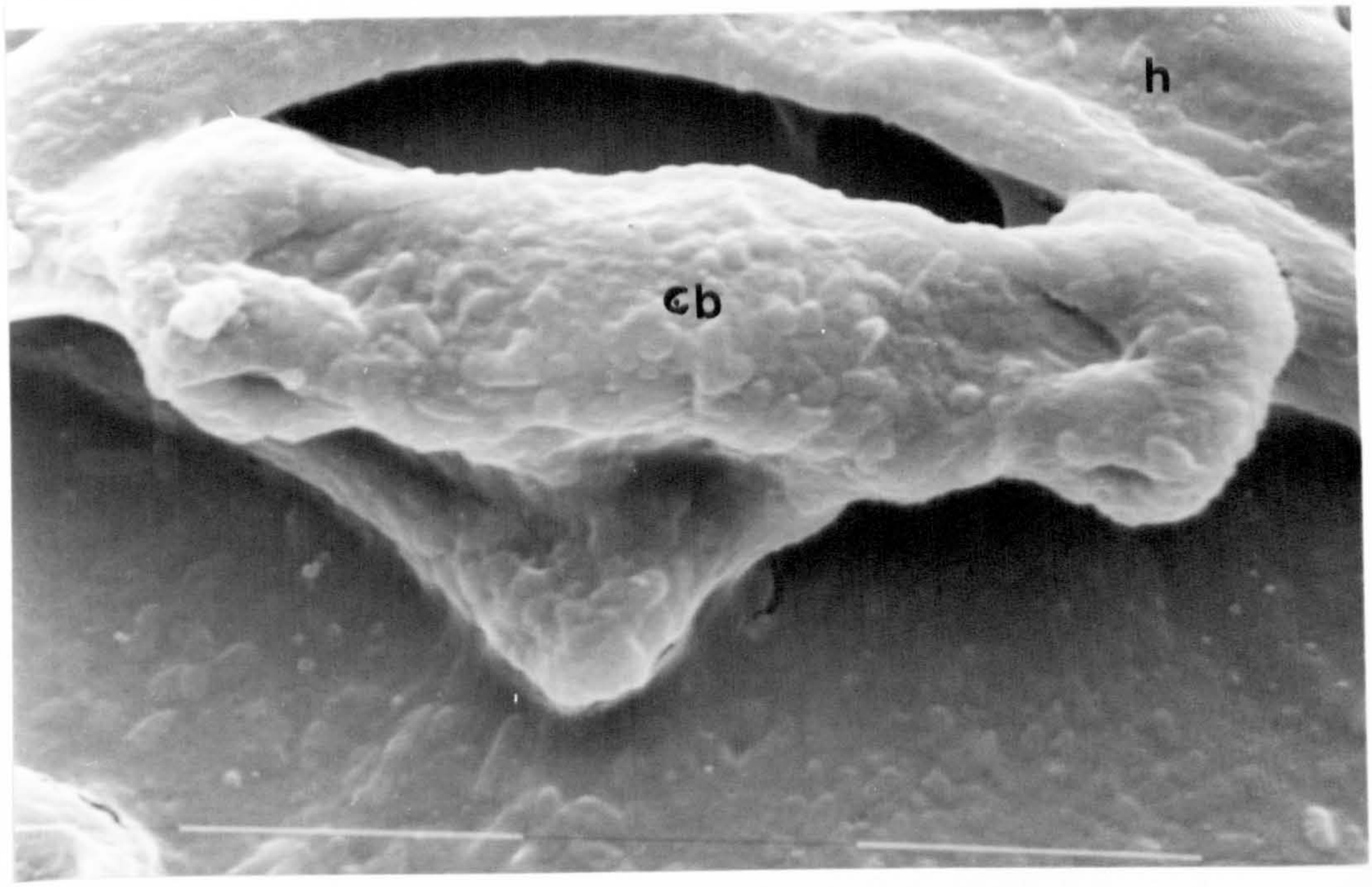


Plate 3.22 :- Scanning electron micrograph (SEM) showing the connecting bar attached to the hamuli at the point where the internal and external root processes join together.
cb = connecting bar; h = hamuli.
Scale bar = 10 μm

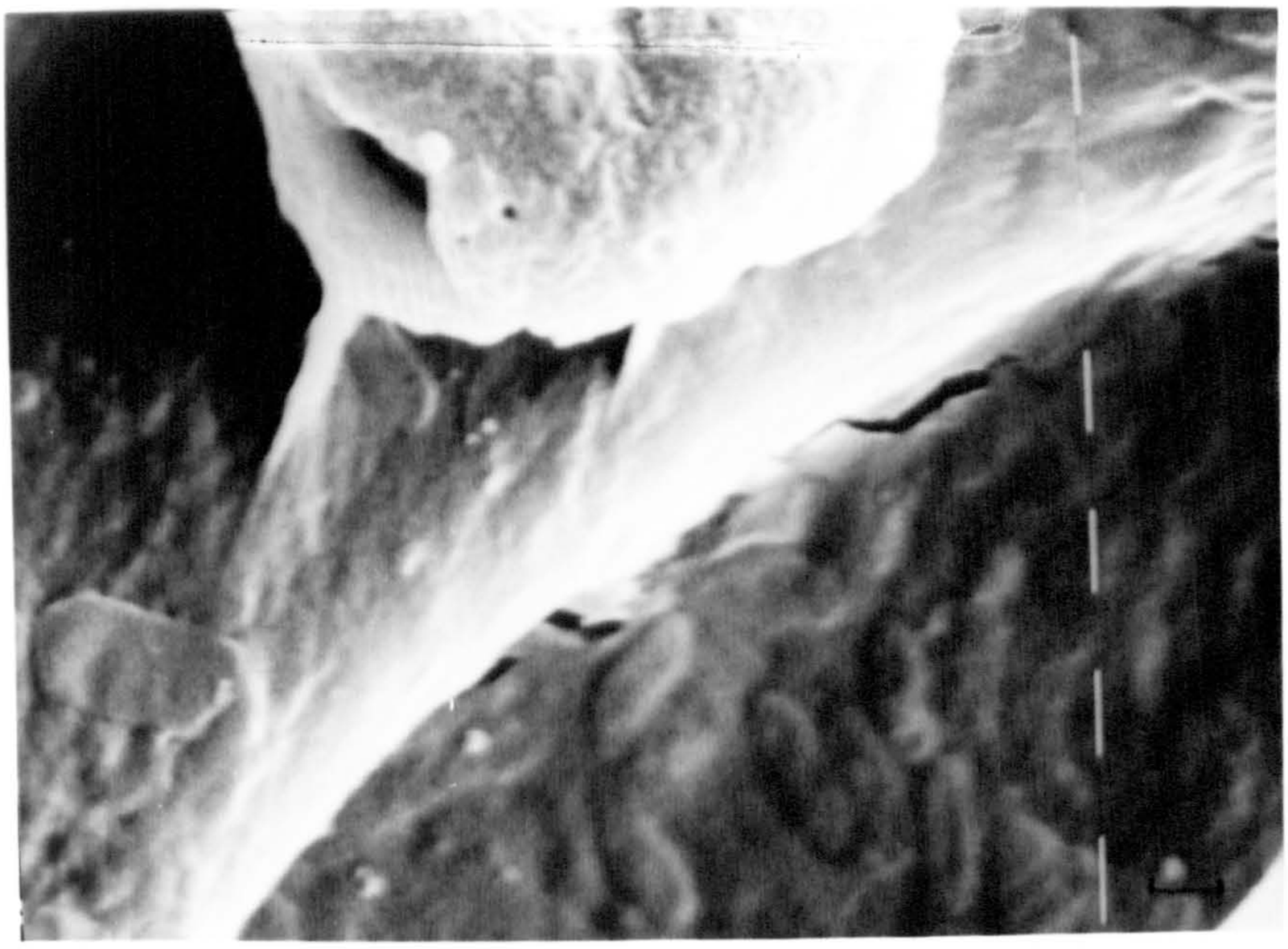


Plate 3.23 :- Scanning electron micrograph (SEM) showing the ligaments by which the connecting bar is hinged with the hamuli.
Scale bar = 10 μm

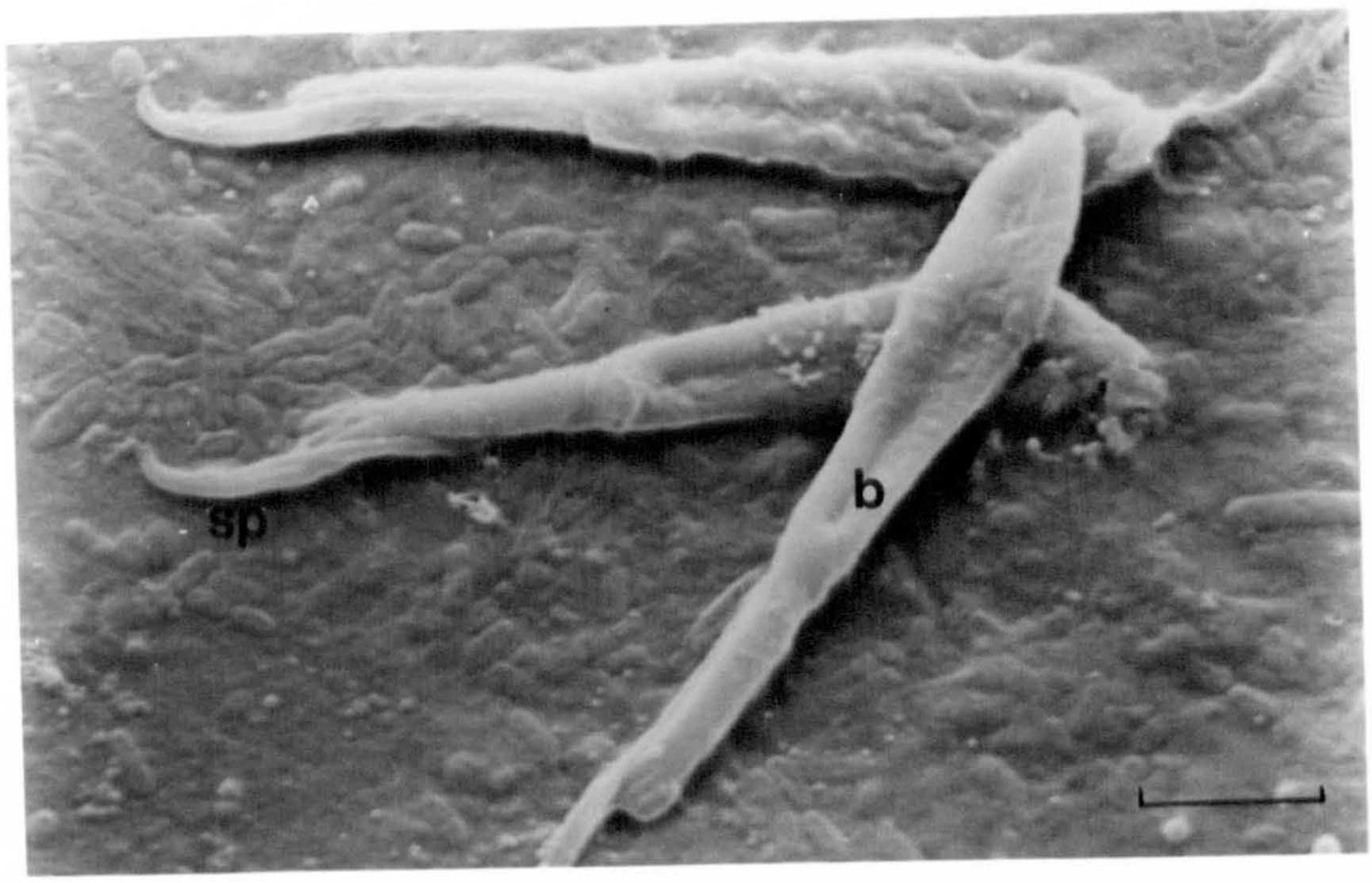


Plate 3.24 :- Scanning electron micrograph (SEM) of the marginal hooks of *D. vastator*.
b = blade ; sp = spike.
Scale bar = 10 μm

The marginal hooks consists of three elements (Plate 3.24). Proximally there is a thickened but laterally flattened handle, with a depression running along each side. Distal to the handle there is a supporting element, circular or oval in cross section which acts as a support for the blade of the marginal hook. The blade originates from the handle and is a flattened structure at this point. It runs along the dorsal surface of the supporting element before extending beyond this to become more circular or oval in cross section and curved to a point at its tip. Only the distal third of the blade is proximal to the supporting element.

3.3.5. Flame cells and protonephridial capillaries

Plate 3.25 shows the lumen of a flame bulb, it is clearly evident that the lumen is filled with the cilia forming a 'flame'. The structure of the cilia has the characteristic 9+2 pattern. The pericaryon shows a large nucleus with a prominent nucleolus and a high concentration of mitochondria (Plate 3.26).

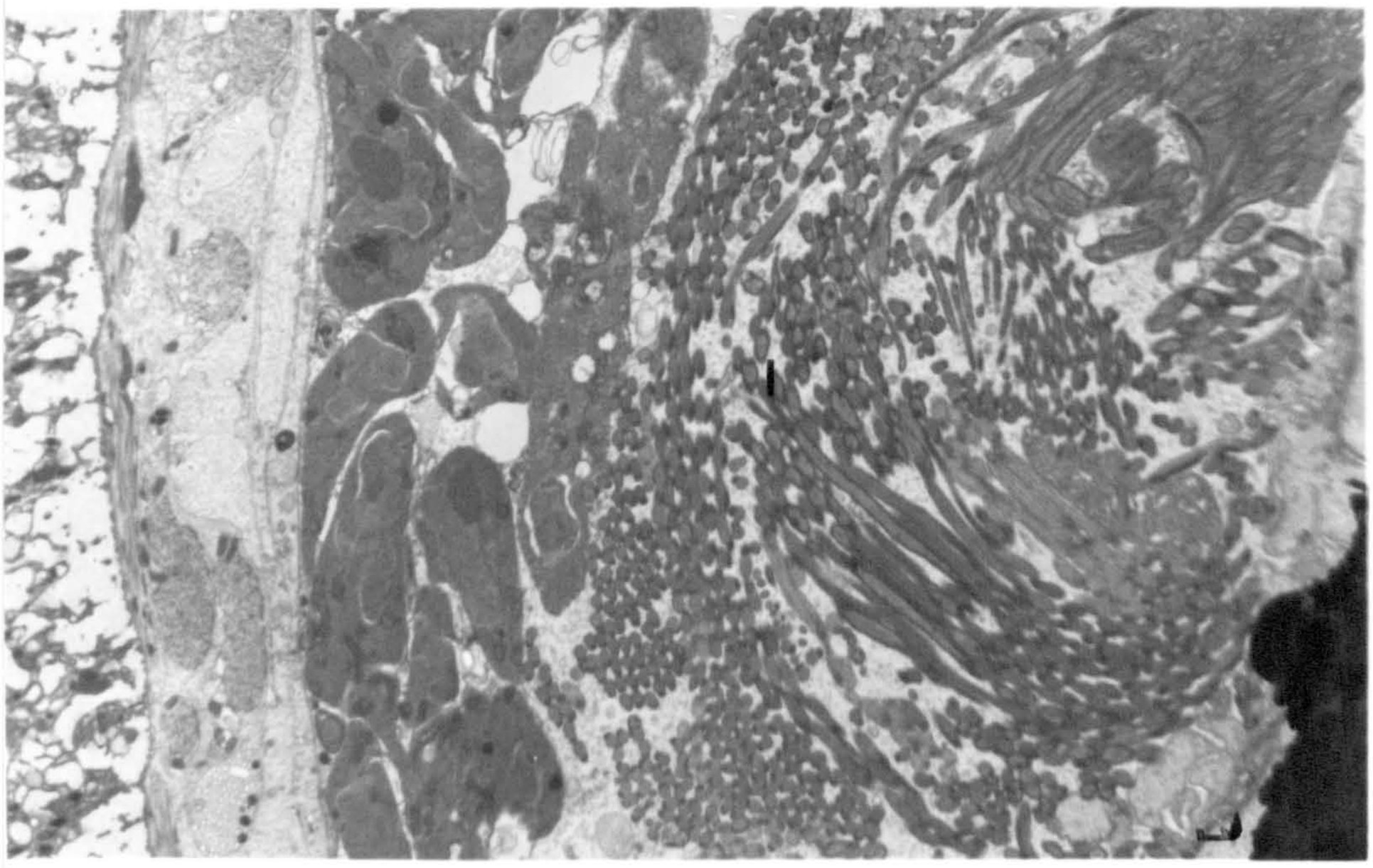


Plate 3.25 :- Transmission electron micrograph (TEM) showing the lumen (l) of the flame bulb.
Scale bar = 15 nm.

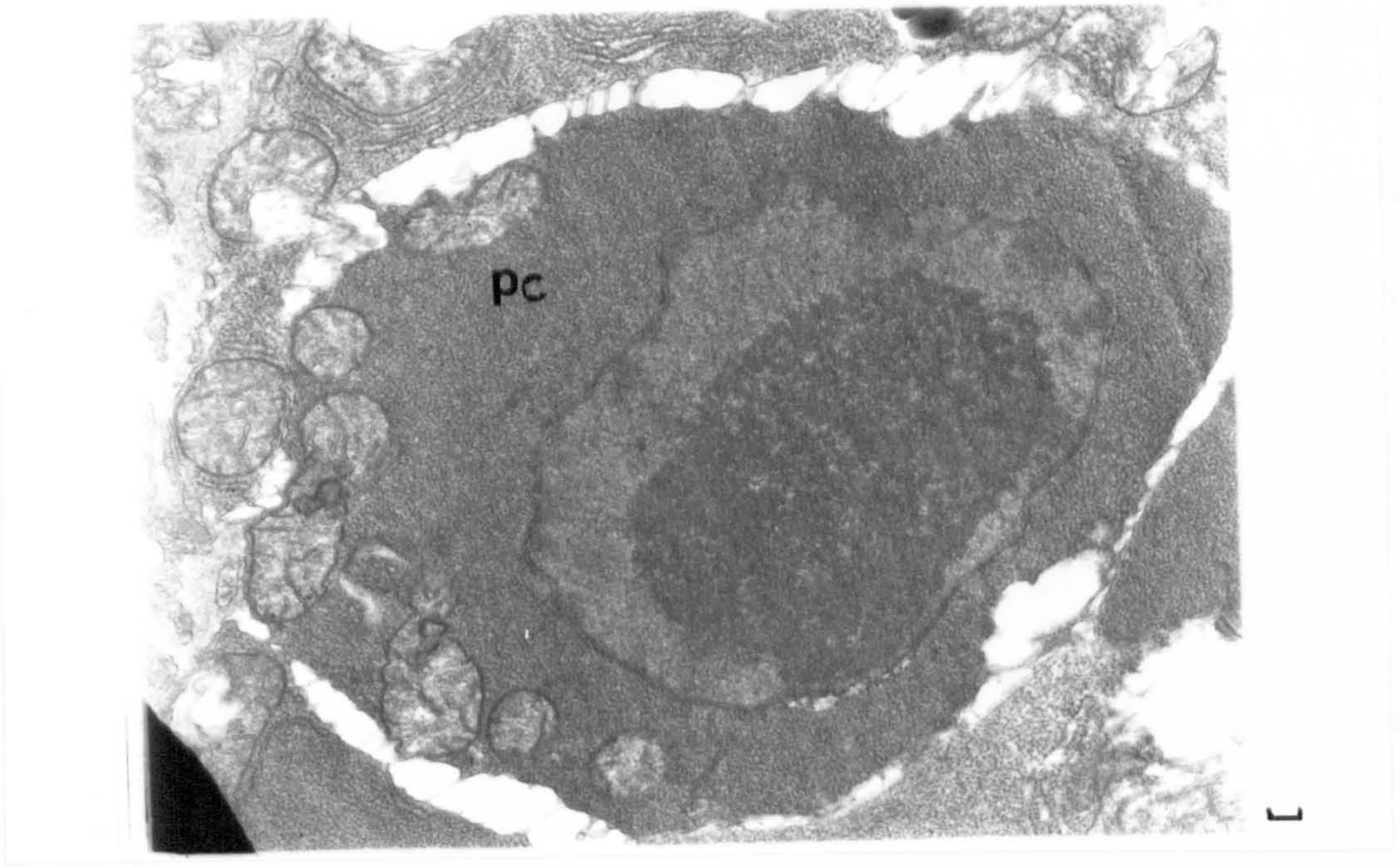


Plate 3.26 :- Transmission electron micrograph (TEM) showing the pericaryon (pc) of the flame cell.
Scale bar = 15 nm

3.3.6. Reproductive system

3.3.6.1. Male reproductive system

The testis is a lobed structure situated in the middle of the posterior third of the parasite body. It contains a mass of productive cells covered by a non-cellular germinal epithelium. Primordial germ cells in the testis give rise to the male germinative cells or spermatogonia. The spermatogonia are generally located at the periphery of the testis and appear more darkly stained than the successive stages of development. All stages of spermeiogenesis can be observed in a single testis.

The primary spermatogonia are situated in the periphery of the testis and divide four times by mitosis, resulting in two secondary, four tertiary, eight quartenary spermatogonia and sixteen primary spermatocytes. Cytoplasm of primary spermatogonia contains numerous unattached ribosomes, scant endoplasmic reticulum and round and oval elongate mitochondria.

Incomplete cytokinesis is found in tertiary and quaternary spermatogonia, where the cells are not separated by plasma membranes but instead are connected by narrow cytoplasmic bridges. Mitochondria are found in higher numbers closer to the outer limiting membrane. The cytophore appears less

densely stained than the rest of the cytoplasm (Plate 3.27).

The axonemes comprise 9 sets of doublet tubules arranged in a cylinder around the central unit, the so called "9+1" pattern which is typical of platyhelminths (Plate 3.28). When all the spermatozoa are released from the rosette, the residual cytoplasm displays numerous empty spaces which were previously occupied by the developing spermatozoa (Plate 3.29).

Mature spermatozoons have a broad head, a middle piece and a tail region. The head consists of a nucleus, is highly coiled and the thread-like chromatin is densely packed. Sections through the head and middle piece indicate that the axonemes are coiled helically. Conducting channels arise from the lobes and join posteriorly to form a common canal which passes the developing sperm towards the atrium (Plate 3.30).

3.3.6.2. Female reproductive system

The female reproductive system of *D. vastator* consists of the ovary, vitelline gland, Mehlis' gland and female accessory ducts: the vagina, seminal receptacle, seminal canal, oviduct, vitelline duct, ovovitelline duct and uterus.

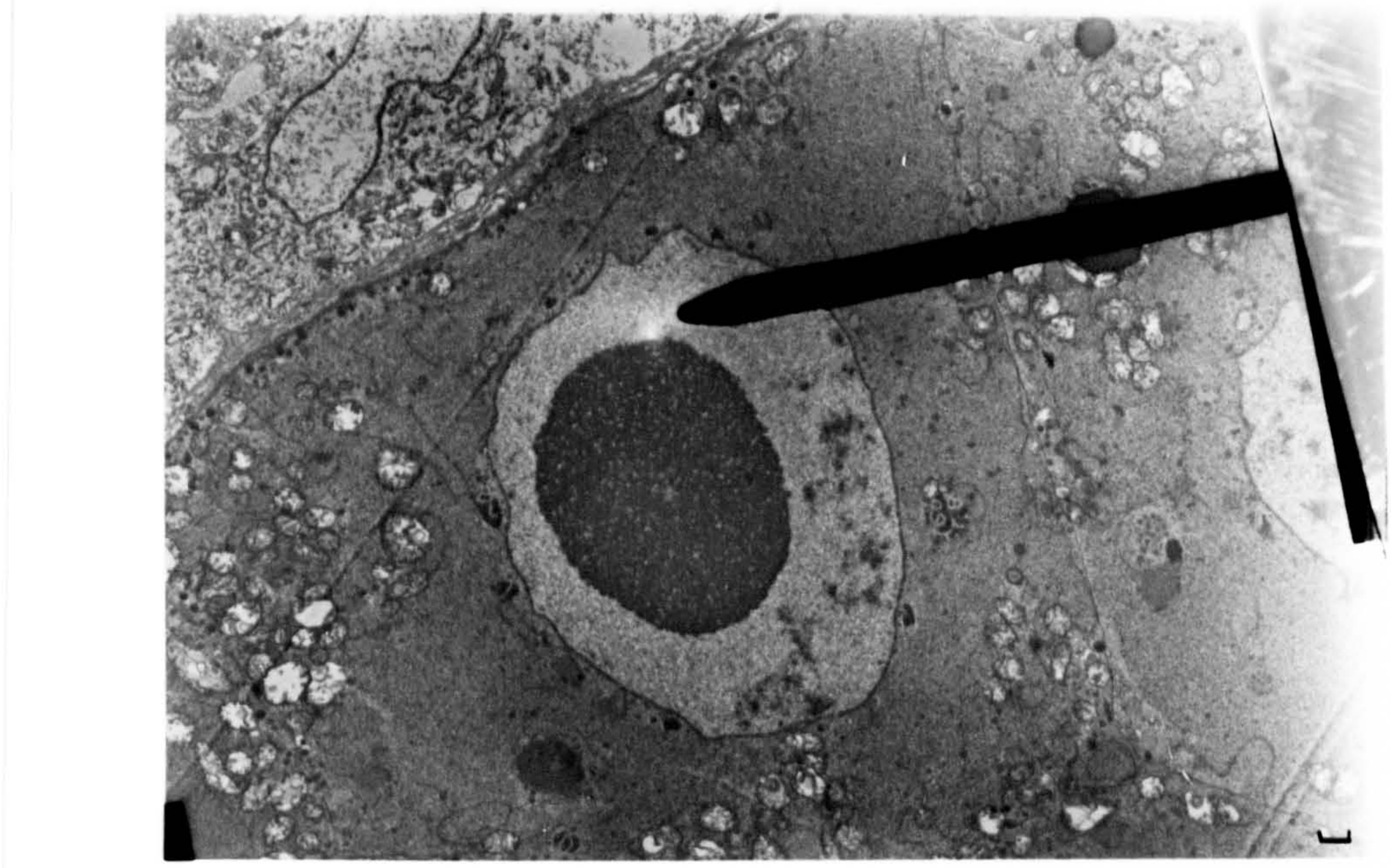


Plate 3.27 :- Transmission electron micrograph (TEM) showing the quaternary spermatogonia.
Scale bar = 15 nm

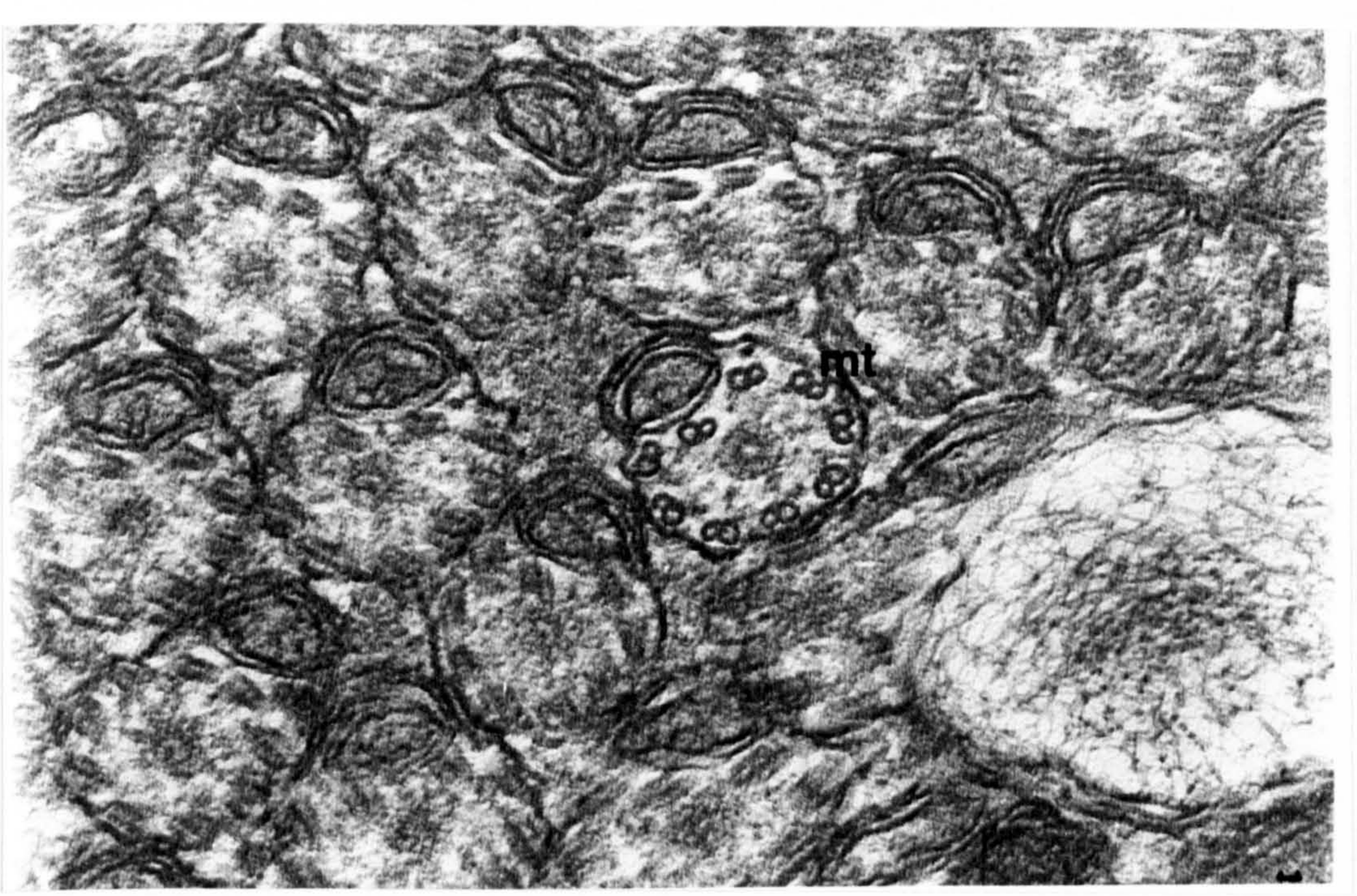


Plate 3.28 :- Transmission electron micrograph showing the structure of the tail of a mature sperm.
mt = microtubules
Scale bar = 15 nm

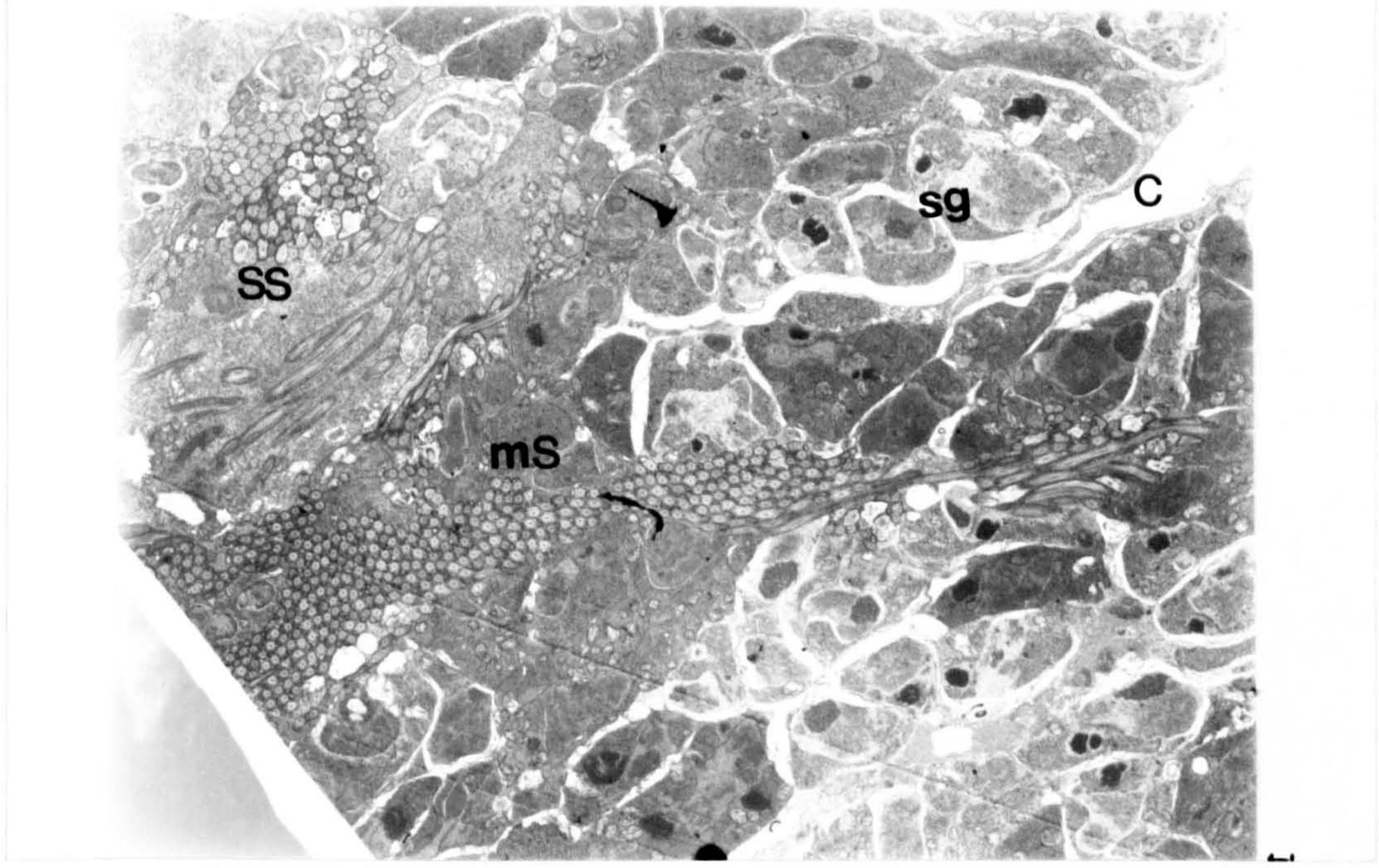


Plate 3.29 :- Transmission electron micrograph (TEM) of the testis showing the spermatogonia (sg), spermatids (ss), matured sperm (ms) and the canal (c) through which sperm passes to the anterior of the worm.
Scale bar = 300 nm

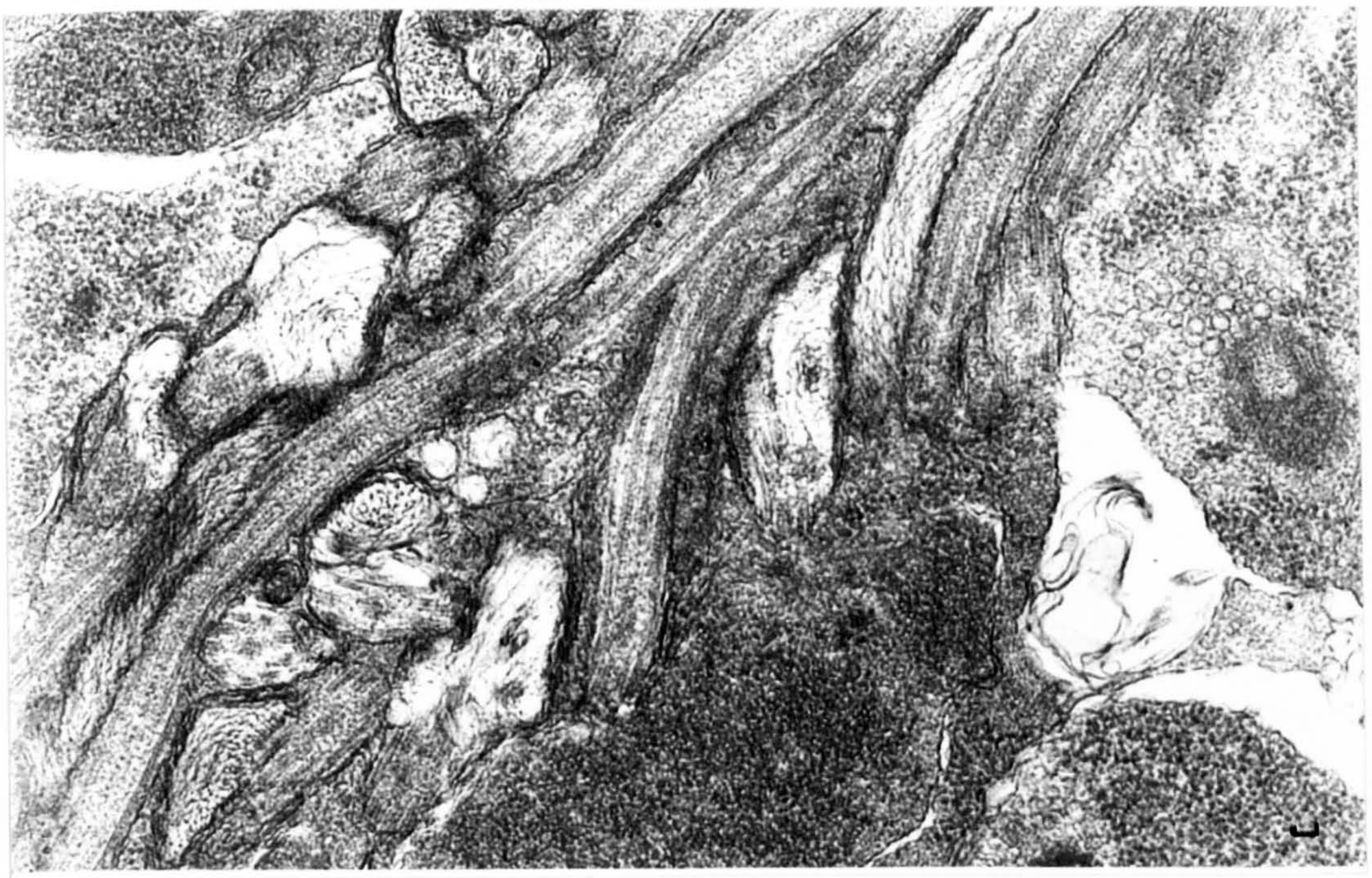


Plate 3.30 :- Transmission electron micrograph (TEM) showing the canals from separate lobes of the testis which then join to form a common canal which passes the mature sperm anteriorly towards the accessory sclerite.
Scale bar = 45 nm

A single elliptical ovary is located near the middle of the body. Three distinct stages of oogenesis can be identified in the ovary: oogonia, maturing primary oocytes and mature ooctyes. The oogonia are the smallest of the gonial cells and are situated anteriorly in the outer edges of the lobe. They undergo several mitoses before they grow and differentiate into the larger mature primary oocytes that occupy the inner edges of the lobe (Plate 3.31). The mature primary oocytes undergo further maturation as they migrate towards the posterior region of the ovary. Then these separate from one another and, as the largest of the three stages, eventually pass out of the ovary into the oviduct.

The oogonia/ early primary oocytes are round to oval in shape and form an undifferentiated mass (Plate 3.32). After a number of mitotic divisions, the oogonia grow and differentiate into larger maturing oocytes.

3.3.6.2.1. Uterus

The luminary surface of the uterine epithelium is typically pleomorphic and consists of lamellar-like folds, some of which rejoin the surface giving a looped appearance. The epithelium is a syncytium, like the external body tegument, and contains several mitochondria and rough endoplasmic reticulum in the form of parallel arrays and concentric layers (Plate 3.33). There are

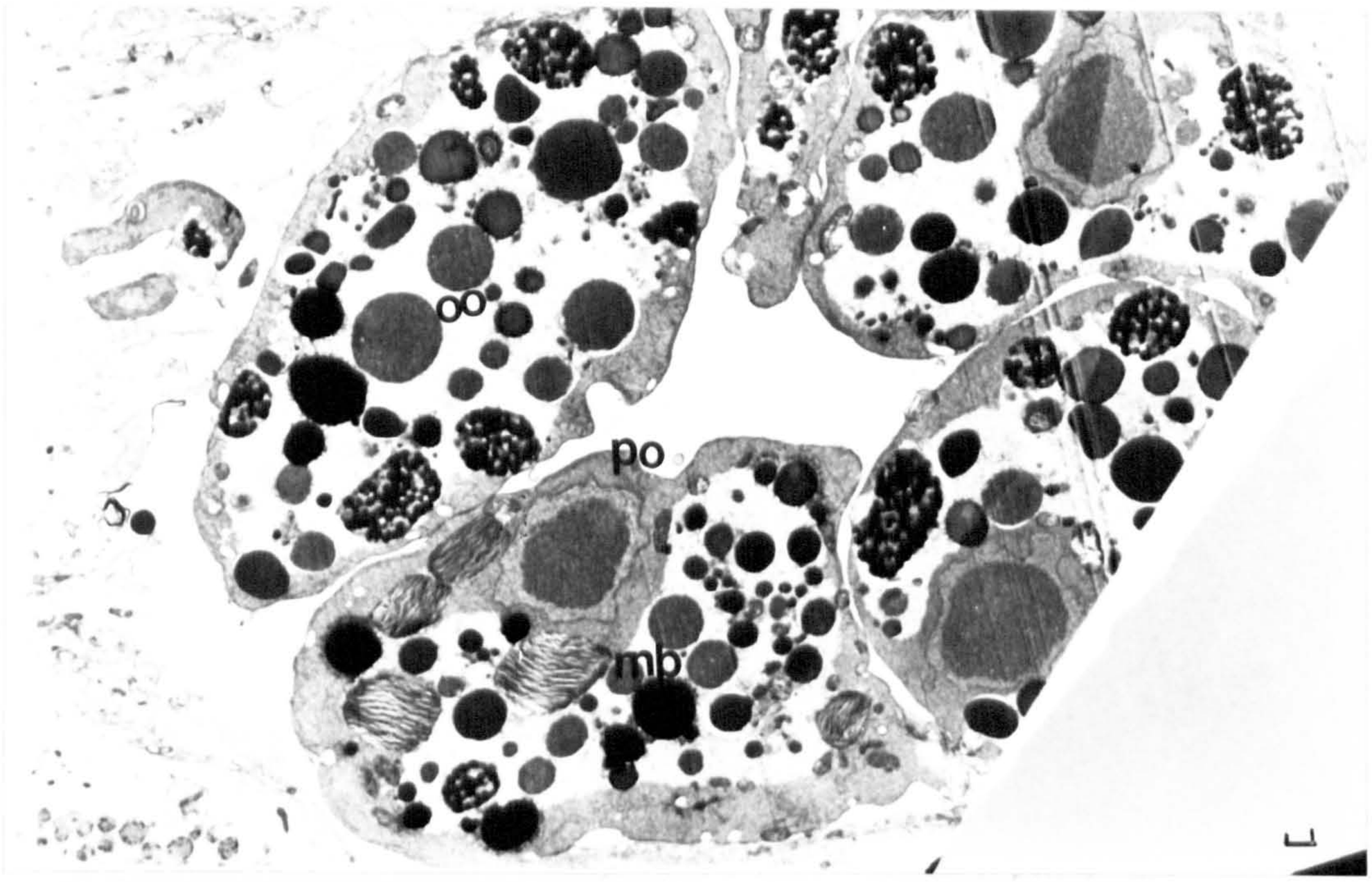


Plate 3.31 :- Transmission electron micrograph (TEM) showing the single lobe of the ovary with oogonia (oo) and primary oocyte (po) of *D. vastator*.
mb = membraneous body
Scale bar = 15 nm

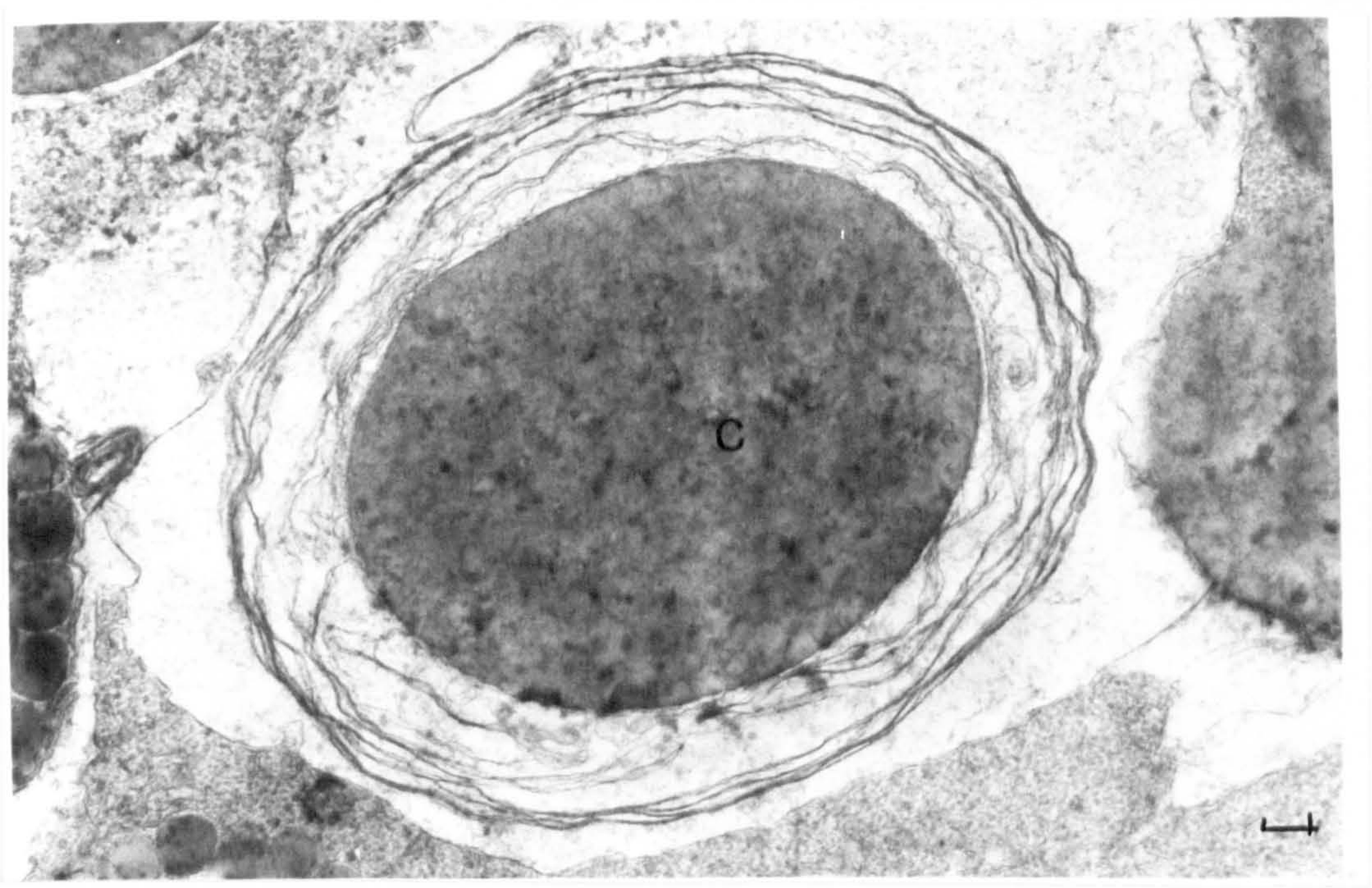


Plate 3.32 :- Transmission electron micrograph (TEM) showing the developing egg with membraneous layer (ml) and undifferentiated cytoplasmic mass (c).
Scale bar = 15 nm

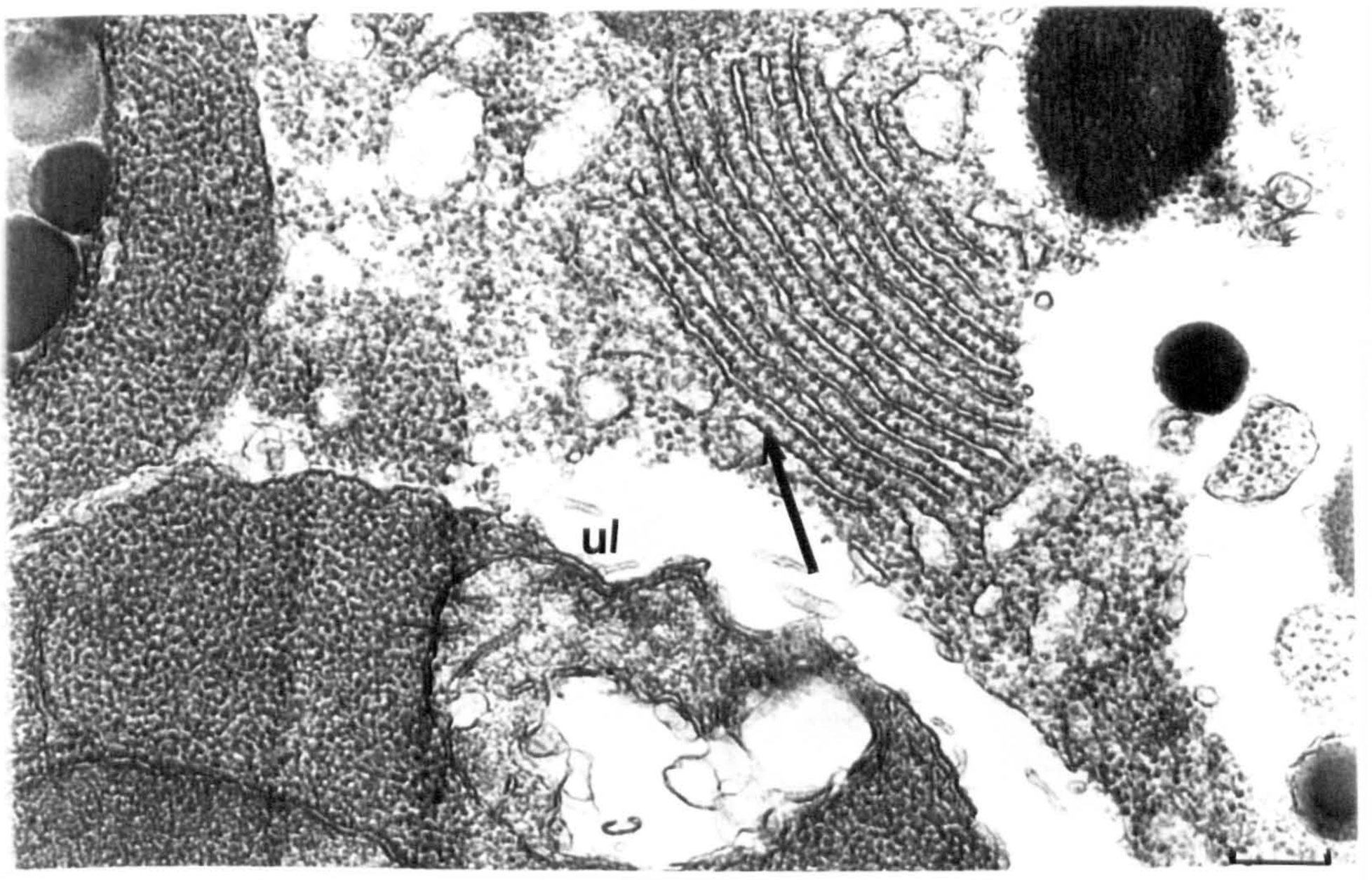


Plate 3.33 :- Transmission electron micrograph of the uterine epithelium showing granulated endoplasmic reticulum in the form of concentric layers (arrow). Uterine surface lamellae (la).
Scale bar = 50 nm.

Golgi complexes and associated electron lucent vesicles, but no conspicuous secretory activity in the uterine epithelium.

3.4. Discussion

3.4.1. Tegument

The general body tegument of adult *D. vastator* is similar in its basic structure to other monogeneans so far studied including *Cichlidogyrus halli typics* (El-Naggar, 1992), *Dactylogyrus amphibothrium* (El-Naggar & Kearn, 1983a) and *Gyrodactylus eucaliae* (Lyons, 1970). Basically the body tegument has a syncytial cytoplasmic layer connected to cytons lying beneath the tegumentary muscle layers, except in the region of the hamuli where some cell membranes can be seen. The latter has not been recorded in other monogeneans. Secretory inclusions, both membrane bound or unbound, electron dense or lucent, and spherical, oval or irregular in shape have been found in the species mentioned above, but their function is unknown. Myofibrils in the body wall of *D. vastator* resemble those of nonstriated muscles of *Gyrodactylus eucaliae* and other invertebrates, including other platyhelminths, as pointed out by Lumsden *et al.*(1968) and Lumsden & Byram (1967). The muscular layer of the tegument is not uniform in thickness in *D. vastator* and is thicker wherever muscular function is important, for example in the hamulus region.

3.4.2. Cephalic gland

Only one type of cephalic gland was observed in this study, whereas Katheriner (1894) and Wagener (1890) and many subsequent authors reported the existence of bilaterally arranged paired groups of prepharyngeal, pharyngeal and post pharyngeal glands in the cephalic region of *Gyrodactylus* sp. El - Naggar & Kearn (1983a) have reported three types of cephalic gland cells in *D. amphibothrium* and *D. hemiamphibothrium*. It is not possible to directly assign the cephalic glands found in *D. vastator* to any one of the types observed by El - Naggar & Kearn (1983a). The membraneous lining and the muscular sheath covering the cephalic gland duct presumably maintains the secretions within the duct and by contraction assists in expelling them to the exterior.

The secretory contents from the duct passes out via the cup-like cavity situated at the border of the cephalic lobe. Posterior to the duct is an aggregation of electron dense material, previously unreported but whose function and origin is unknown.

3.4.3. Opisthaptor

The opisthaptor has two large hamuli and fourteen marginals in *D. vastator*. The hamuli have two root processes and arising from the roots is a curved shaft which forms a tapering spike. The external and internal root processes of the hamuli are provided with muscular attachment surfaces which must allow the hook to move back and forth while achieving its function of anchoring the worm to the primary gill lamellar cartilage. The interlocking striations found on the hamuli shaft presumably increase the strength of attachment by providing a non-smooth surface incontact with host tissue. Interlocking striations have also been reported on the hamuli of *Gyrodactylus salaris* (Mo & Appleby, 1990). Shinn (1994) reported that in an earlier study he had found that the sides of the shaft of the hamuli were greatly thickened, giving the hook a rigid structure. Using polarising microscopy he showed that the molecules of hook material were arranged parallel to the long axis of the hook but showed a deflection at the hamulus point which the author believed added to the strength of the structure at this particular point which was subject to mechanical stress. Marginal hooks, situated in the periphery of the haptor, are anchored by muscles to their blades allowing them to move independently of the hamuli. Although the hamuli provide the main attachment mechanism, the marginal hooks probably prevent the edges of the opisthaptor moving inwards, increasing the efficiency of the worm's attachment to its host. In

contrast, in *Gyrodactylus* the marginal hooks provide the main means of attachment where as hamulus functions as a lever (Shinn, 1994). In *D. vastator* immediately prior to attachment, the worm positions itself between gill filaments such that the entire ventral surface of its opisthaptor is in contact with one filament (Gussev, 1973). The haptor then compresses allowing the hamuli to rotate outwards and flatten. The hamuli are then rapidly contracted causing the points to be drawn back together. It is essential to note that attachment to soft tissues only with the hamuli may not be durable enough. The hamuli will tend to tear the soft tissues of the gill filaments and the worms may then be easily washed off by the water current. This is avoided by penetration of the cartilage of the primary gill lamellae. The opisthaptor has additional means of attachment which disperse the forces applied to the points of attachment. Secretions produced by glands in the posterior part of the worm's body, as well as the vacuum produced between the opisthaptor and the surface of gill filament, ie. the opisthaptor acts partly as a sucker, may help in adhesion to the host (Gussev, 1973). As the worm stretches the firmer it becomes fastened to the gill filament. Gill tissue entrapped in the opisthaptor is pulled into the opisthaptor's cup under the influence of longitudinal muscles stretching from the opisthaptor into the body (Gussev, 1973).

3.4.4. Flame cells and protonephridial capillaries

The flame cell structure of *D. vastator* is similar to that found by Rohde *et al.* (1989) in *Gyrodactylus*. In *D. vastator* there is a terminal cell with a nucleus which is basal or at least lateral to the basal bodies of the cilia forming the flame found in other species.

3.4.5. Reproductive system

3.4.5.1. Male reproductive system

The fine structural organisation of the testis is protandrous in *D. vastator* and the developmental sequence of the spermatozoa agrees closely with the gametogenic pattern that has been documented for other platyhelminths (Euzet *et al.*, 1981; Davis & Roberts, 1983; Ubelaker, 1983). Four mitotic divisions of spermatogonia give rise to 16 primary spermatocytes which, through meiosis, form a group of 32 secondary spermatocytes that divide to give a rosette of 64 spermatids that transform into spermatozoa. The centriole and microtubule of the spindle are common features of monogeneans including *D. merlangi* (Halton & Hardcastle, 1976). From the TEM photomicrographs of the testis of *D. vastator*, incomplete cytokinesis was observed in quartenary spermatogonial formation where the four cells were joined together by a

cytoplasmic bridge without complete division of the cytoplasm. Transmission electron microscopic studies of *Trilocularia acanthiaevulgaris* have shown that the secondary and tertiary spermatogonia are linked clusters, and are not separated by a plasma membrane (Mahendrasingham, 1989) thus indicating that incomplete cytokinesis is taking place. Some digeneans (Gresson, 1965; Robinson & Halton, 1982), and the monogenean *D. merlangi* (Halton & Hardcastle, 1976) also exhibit incomplete cytokinesis. The quartenary spermatogonia and the successive spermatocyte and spermatid stages develop in a plasmodial rosette, with their nuclei at the periphery and their cytoplasm connected to the cytophore by cytoplasmic bridges. In *D. merlangi*, as in *D. vastator,* mitochondria are found closer to the plasma membrane. This may have an advantage for their migration into the developing spermatozoa (Halton & Hardcastle, 1976).

3.4.5.2. Female reproductive system

The elliptical ovary comprises two cell types: the predominant gametogenic cell that undergoes development and differentiation to produce mature primary oocytes, and an infrequent somatic cell, the follicle cell, whose origin and function is not known. The follicle cells are smaller than oogonia and may be round, elongated or oval in shape.

Follicle cells have been described from the ovary of a number of turbellarian and monogenean parasites. Newton (1974) observed a single accessory cell surrounding the growing oocyte of the freshwater turbellarian, *Hydrolimax gricea*, and described cytoplasmic processes that extended from the inner surface of this cell into the oocyte itself, but the cytoplasm of the two cells did not intermix. Microvilli-like structures were also seen to extend from the outer surface of the accessory cell into the adjacent parenchymal cells and, on this basis, Newton (1974) suggested that the accessory cell served as a nurse cell to transport nutrient material from the parenchyma to the growing oocyte. In *Fasciola hepatica*, the ovary is composed of germ cells and peripheral surrounding nurse cells surrounded by a capsule containing muscular tissue. The outer oogonia are in close contact with the nurse cells while the inner oocytes are loosely packed (Bjorkman & Thorsell, 1964).

Ultrastructural studies of oogonia of *D. vastator* demonstrated a prominent large nucleus and high nuclear/cytoplasmic ratio with organelles such as Golgi bodies and ribosomes. In this respect they show a similarity with the oogonia and early immature oocytes of other monogeneans such as *D. merlangi, D. paradoxum* and *Diplozoon denticulata* (Halton *et al.*, 1976). Synaptonemal complexes have also been observed in the maturing primary oocytes of monogenea (Halton *et al.*, 1976).

The membraneous bodies in the somatic cell of *D. vastator* may be involved in some way in ejection of mature oocytes into the lumen of ovarian lobule.

3.4.5.2.1. Uterus

The uterine epithelium of *D. vastator* was syncytial as recorded for *H. diminuta* and *T. acanthaevulgaris* (Mahendrasingham, 1989). Cilia-like processes as observed on the luminal surface of the uterus of *D. vastator* have also been recorded by El-Naggar & Kearn (1983a).

Chapter 4

Spatial distribution of *Dactylogyrus vastator* on the gills

4.1. Introduction

Some parasites have a greater affinity or specificity for certain sites on or in the host (Hanek & Fernando, 1978a,b). According to Llewellyn (1957), most monogeneans appear to have specific positions on the gills of their host. For example, *Gastrocotyle trachuri* is found mid-way along the primary lamellae, while *Pseudaxine trachuri* is attached nearer to the distal tips of the lamellae on the fish host, *Trachurus trachurus*. Llewellyn (1956) suggested that the site selection of polyopisthocotyleans is determined by the need to avoid the effect of the gill ventilating current. Cerfontaine (1896, 1898) was the first to record this phenomenon for *Diclidophora denticulata*, a gill parasite of *Pollachius virens*. The existence of affinities for certain regions of the gill apparatus by monogeneans has been subsequently reported by many authors, including Frankland (1955), Llewellyn (1956), Llewellyn & Owen (1960), Owen (1963) and Suydam (1971). Microhabitat distribution and interspecific relationships of gill parasites have been studied so far in fish with only a few congeneric species of parasites (Paperna, 1963a; Suydam, 1971; Mizelle, 1974; Hanek & Fernando, 1978a,b,c; Adams, 1986). Arme and Halton (1972) reported that *Diclidophora merlangi* preferred the first gill arch of its host *Merlangius merlangus*. Hanek & Fernando (1978 a,b,c) reported that there was a significant difference between the outer and innner hemibranchs of the gill arch for monogeneans such as *Actinocleidus gibbosus*, *Actinocleidus recurvatus*,

Cleidodiscus robustus, *Urocleidus acer*, *Urocleidus dispar*, *Urocleidus attennuatus*, *Urocleidus ferox* and also the glochidia of *Lampsilis radiata*. They also reported that there was no significant difference observed between the inner and outer hemibranch of the gill arch with the copepods *Achtheres ambloplitis*, *Ergasilus caeruleus* and *Ergasilus centrarchidarum*. The monogenean parasites preferred the median sector of the hemibranchs compared with the upper and lower sections of the gill arch (Hanek & Fernando, 1978 b). These authors found that the monogeneans were found in the greatest numbers on the first or outer arch than on the second, third and fourth arches in descending order. Buchmann (1988a,b, 1989) studied the microhabitats of two congeneric monogenean species on the gills of *Anguilla anguilla*. He found that *Pseudodactylogyrus bini* was most abundant on the left side of the first and second arches whilst *P. anguillae* was located on the right side of the third and fourth arches.

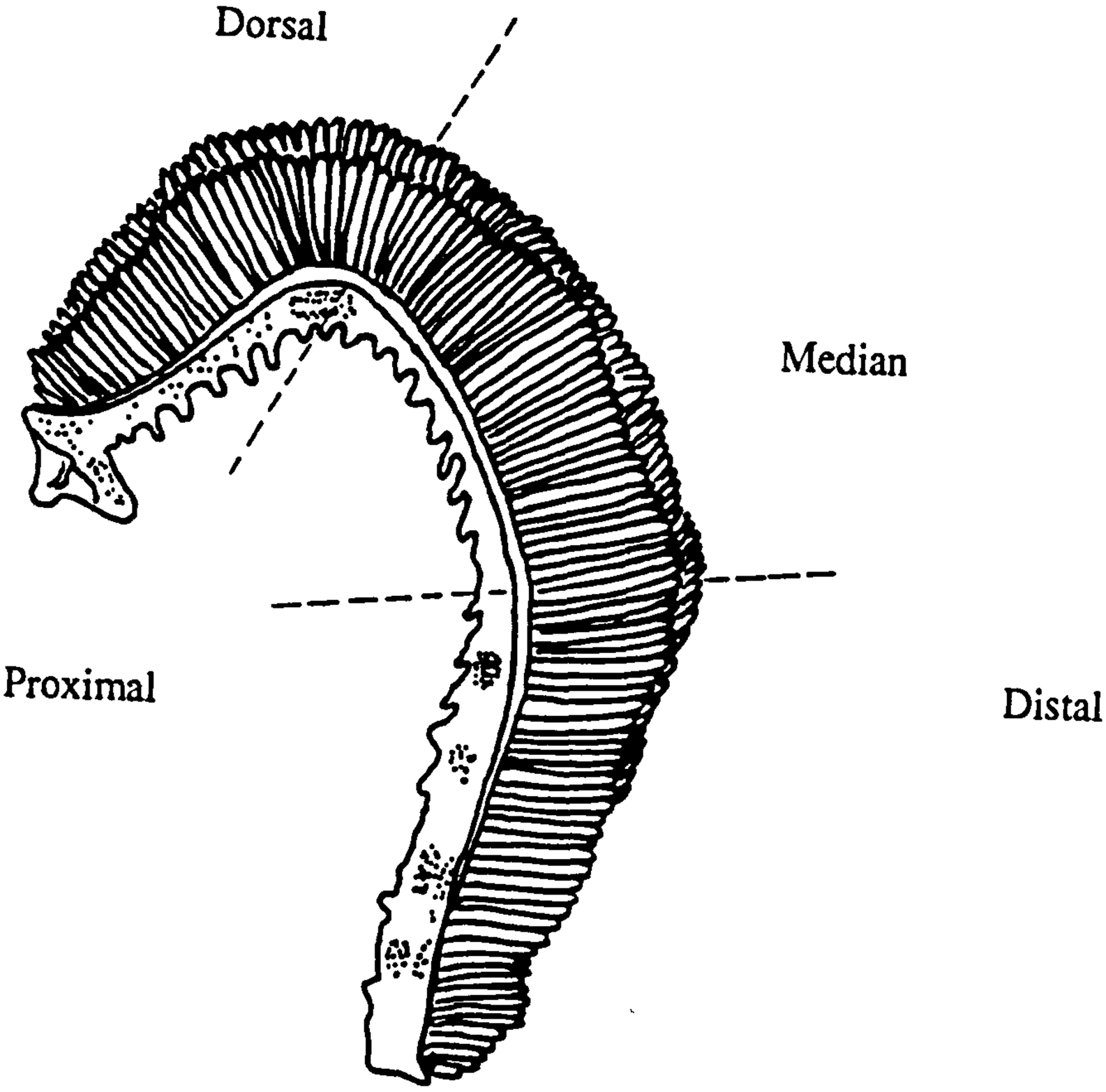
Dzika & Szymanski (1989) found that in bream, *Abramis brama* L., three species of *Dactylogyrus, D. falcatus, D. wunderi* and *D. auriculatus* mostly occurred in the same region, on the median sector of the proximal parts of the gill arches, whereas *D. zandti* mostly occurred on the extreme distal portion of the filaments. Thus, although four different species of *Dactylogyrus* coexist on the gills of one individual host, their micro-settlement sites coincide to a great degree. Koskivaara *et al.*,(1992) reported from their studies with *Dactylogyrus*

spp.on roach that there were some differences in the occurrence of the species on different gill-arches between May and June, indicating the gill arch preference and its site preference also may vary seasonally. There were species-specific differences in preference between different gill arches. These authors suggested that these results indicate that the volume of the water current over the different gill arches may not be the main reason for gill-arch preference by the parasite, as the current will not vary with the season.

Rohde (1979) evaluated the intrinsic and extrinsic factors responsible for niche restriction in Monogenea and showed that the intrinsic factors can be more important than the external factors; an important factor responsible for niche restriction in monogeneans is site selection to increase intra-specific contact and thus mating. Rohde (1976) observed that for marine gill monogeneans intraspecific relations were more important as niche-restricting factors than interspecific interactions. Fish parasite communities are likely to be isolationist in nature, mainly because of their low diversity and abundance, which make vacant niches available and competition over resources unnecessary (Kennedy, 1985; Dzika & Szymanski, 1989). On the other hand, competition and interactive site segregation have been observed to structure fish parasite communities (Buchmann, 1988c; Bates & Kennedy, 1990).

4.2. Materials and Methods

One hundred carp with a mean standard length of 2.95 ± 1.00 cm and mean weight of 1.25 ± 0.77 gm and with a mean of 24.63 (2 - 308) *D. vastator* per fish were used to study the spatial distribution of the parasite. Fish were decerebrated and were then weighed, and the standard length recorded. Gill arches were separated individually and placed in small petri dishes with water from the same aquarium from which the fish were removed. They were numbered right and left I - IV. Arch I is found nearest to the operculum, and arch IV is the innermost arch. Hemibranchs of each gill were designated as outer, ie. nearest to the operculum, and inner. Each hemibranch was divided into proximal and distal halves and three equal sections, ventral, median, and dorsal, to give a total of six areas (see Figure 4.1). The number of *D. vastator* from each gill arch, each side of each hemibranch and each area of hemibranch surface was recorded and the maturity stage of each noted. To count the number of worms, the petri dish with a hemibranch (gill) was placed on an Olympus binocular stereo microscope stage and was observed under x40 magnification. Neither the gill cartilage nor the primary lamella were separated off because this process disturbed parasite distribution. Counts were made as soon as possible after the gills were excised from the fish.



3 Proximal Dorsal	4 Distal Dorsal
2 Proximal Median	5 Distal Median
1 Proximal Ventral	6 Distal Ventral

Fig 4.1 :- Illustration of the gill arch showing its division into the six arbitrary areas which were used in this study.

Wilcoxon's signed rank test (paired samples) was performed to test the difference between the right and left sets of gill arches (Wilcoxon & Wilcox, 1964). The non parametric STP test and Dunns test were employed to test the difference in the number of parasites between the arches and between hemibranchs and hemibranch areas. Significance was noted at the 0.01, 0.05, 0.001 and 0.005 levels.

4.3. Results

4.3.1. Spatial distribution of *D. vastator*

The numbers of *D. vastator* on the different parts of the gill apparatus are given in Tables 4.1a,b. More *D. vastator* were found on the left than on the right set of gills. Comparing the four gill arches, most parasites occurred on the first arch, with decreasing numbers on arches II,III and IV in that order. The surface area of the gill arches decreases from I to IV. The ventral segment of the hemibranchs carried the greatest number of *D. vastator* and there were more parasites on the proximal rather than the distal parts of the filaments.

Table: 4.1a.

The spatial distribution of *Dactylogyrus vastator* over the gill apparatus of *Cyprinus carpio*.

Gill set	Right		Left	
No. of *D. vastator* observed	627		828	
Gill arches	1	2	3	4
No. of *D. vastator* observed	471	387	323	274
Halves of primary lamella	Proximal		Distal	
No. of *D. vastator* observed	826		629	
Segments of hemibranchs	Ventral	Median		Dorsal
No. of *D. vastator* observed	648	551		256

Table 4.1b :- The spatial distribution of *D. vastator* over the different areas of the gill apparatus of *C. carpio*

Gill Arch	Side	Mean/Std deviation	1	2	3	4	5	6
I	Right	X	0.39189	0.831168	0.164556	0.15	0.405063	0.51898
		σ	1.291706	2.91277	0.705843	0.5758	1.138138	1.421995
	Left	X	0.710526	0.71212	0.3625	0.346154	0.569620	0.708861
		σ	2.249834	2.907630	1.519858	1.696650	2.484225	3.076594
II	Right	X	0.569620	0.506329	0.151899	0.215189	0.455696	0.36708
		σ	1.374526	1.663125	0.601038	0.929169	1.517399	1.210818
	Left	X	0.423077	0.455696	0.164557	0.202531	0.263158	0.551282
		σ	1.253766	0.984462	0.649069	0.740404	1.099920	1.608802
III	Right	X	0.518987	0.35443	0.171053	0.089744	0.329114	0.265823
		σ	1.893719	1.271361	0.885259	0.432026	1.268166	1.117562
	Left	X	0.423077	0.455696	0.164557	0.202532	0.263158	0.551282
		σ	1.253765	0.984462	0.649069	0.740404	1.099920	1.608802
IV	Right	X	0.430379	0.265823	0.139535	0.102564	0.256410	0.354430
		σ	1.267782	0.811912	0.489004	0.444000	0.746175	1.166168
	Left	X	0.3875	0.481012	0.272727	0.088608	0.367089	0.506329
		σ	1.012501	1.385462	1.410826	0.364792	1.210818	2.341780

4.3.1.1. Left and right gill arches

The collected data did not show a normal distribution, therefore Wilcoxon's signed ranked test (for paired samples) was applied to compare the number of *D. vastator* between the left and right sets of gill arches. The test value was Z = 0.362538, and the tabled value for two-tailed probability was Z = 0.716947. The tabled value is higher than the test value, indicating that there is no significant difference between the number of *D. vastator* on the right and left gill arches. Therefore, data for the right and left gill arches were pooled for further analysis.

4.3.1.2. Gill arches I - IV

The data did not show a normal distribution, and thus the non - parametric STP test, and Dunn's test were used to test the number of *D. vastator* on the separate gill arches.

The summary of the STP test values are given in Table 4.2

Table 4.2 :- Summary of U values from STP test for number of *D. vastator* between gill arches.

	1	2	3	4
1				
2	295 $_{NS}$			
3	273 $_{NS}$	295 $_{NS}$		
4	289.5 $_{NS}$	320 $_{NS}$	301 $_{NS}$	

U at 0.05 = 381.43, U at 0.01 = 406.22

$_{NS}$ - non significant

The tabled value for the 0.05 level and the 0.01 level of significance are higher than the values shown in Table. Therefore, there is no significant difference in parasite numbers between gill arches.

The summary of the Dunn's test values are given in Table 4.3

Table 4.3 :- Summary of Q values from Dunn's test

	1	2	3	4
1				
2	0.762_{NS}			
3	0.166_{NS}	0.595_{NS}		
4	0.601_{NS}	1.363_{NS}	0.767_{NS}	

Tabled Q values

Q at 0.05 = 2.639, Q at 0.01 = 3.144, Q at 0.005 = 3.342, Q at 0.001 = 3.765

NS non significant

The test value for Q is higher than the tabled values at the 0.05, 0.01, 0.005 and 0.001 levels. Thus again there is no significant difference in the number of *D. vastator* between gill arches I - IV.

4.3.1.2.1. Comparison of different areas of gill arches I - IV

Dunn's test was carried out for each gill arch individually, to determine if there was any significant difference in numbers of *D. vastator* between separate areas. For gill arches I, II and III there was no apparent difference

between the areas under study. Only in gill arch IV there was a significant difference between area 1 and area 4. The test value Q for Dunn's test was 3.174. The tabled value at the 0.05 level is 2.936, and the 0.01 level is 3.403.

Table 4.4 :- Summary of Q values from Dunn's test for comparison of all areas on gill arch I - IV

	1	2	3	4	5	6
1						
2	2.178_{NS}					
3	2.683_{NS}	0.505_{NS}				
4	3.174^{*}	0.996_{NS}	0.491_{NS}			
5	1.839_{NS}	0.339_{NS}	0.843_{NS}	1.335_{NS}		
6	1.839_{NS}	0.339_{NS}	0.843_{NS}	1.335_{NS}	0.753_{NS}	

Tabled Q values

Q 0.05, 6 = 2.936 Q0.01 = 3.403 Q0.005 = 3.588 Q0.001 = 3.988

* - significant at 0.05 level

NS non significant

4.3.1.2.1.1. Comparison of gill arch areas

The data on different areas for separate gill arches was pooled. Then this was subjected to Dunn's test to elucidate any significant differences. The results are shown in Table 4.5.

Table 4.5 :- Summary of Q values from the Dunn's test for comparison of all areas

	1	2	3	4	5	6
1						
2	0.628_{NS}					
3	1.159_{NS}	0.53_{NS}				
4	4.23_{****}	3.601_{***}	3.071_{*}			
5	2.491_{NS}	1.862_{NS}	1.332_{NS}	1.739_{NS}		
6	2.879_{NS}	2.251_{NS}	1.72_{NS}	1.351_{NS}	0.388_{NS}	

Tabled Q values

Q 0.05, 6 = 2.936 Q 0.01 = 3.403 Q 0.005 = 3.588 Q 0.001 = 3.988

* - significant at 0.05 level, *** significant at 0.05, 0.01 and 0.005 level,

**** - significant at 0.05, 0.01, 0.005 and 0.001 level

NS non significant

Thus, there was a significant difference between area 4 and areas 1, 2 and 3.

4.3.1.2.1.2. Distribution of *D. vastator* on individual hemibranchs

Within individual hemibranchs *D. vastator* was found to be aggregated so that a number of parasites were often found in close proximity. Some examples of these are shown in Fig 4.2.

4.4. Discussion

This study showed no significant differences in the numbers of *D. vastator* between the gill arches of carp, although many authors have recorded such differences in other host-parasite systems. For example Wiles (1968) found that *Diplozoon paradoxum* occurred most often on gill arches I and II of *Abramis brama* and Suydam (1971) showed that the site specificity of *Diclidophora macclumi* was similar to that of *D. paradoxum.* *D. amphibothrium* prefers gill arches II and III of *G. cernua* (Wootten, 1974).

The speed of the respiratory current may have an influence on the settlement of the infective larval stage or of immature worms entering the gill chamber via the respiratory current. Woskobonikoff & Balabai (1936, 1937 cited in Hanek and Fernando, 1978 b) introduced the concept of a continuous gill curtain separating the buccal and opercular cavities. They also suggested

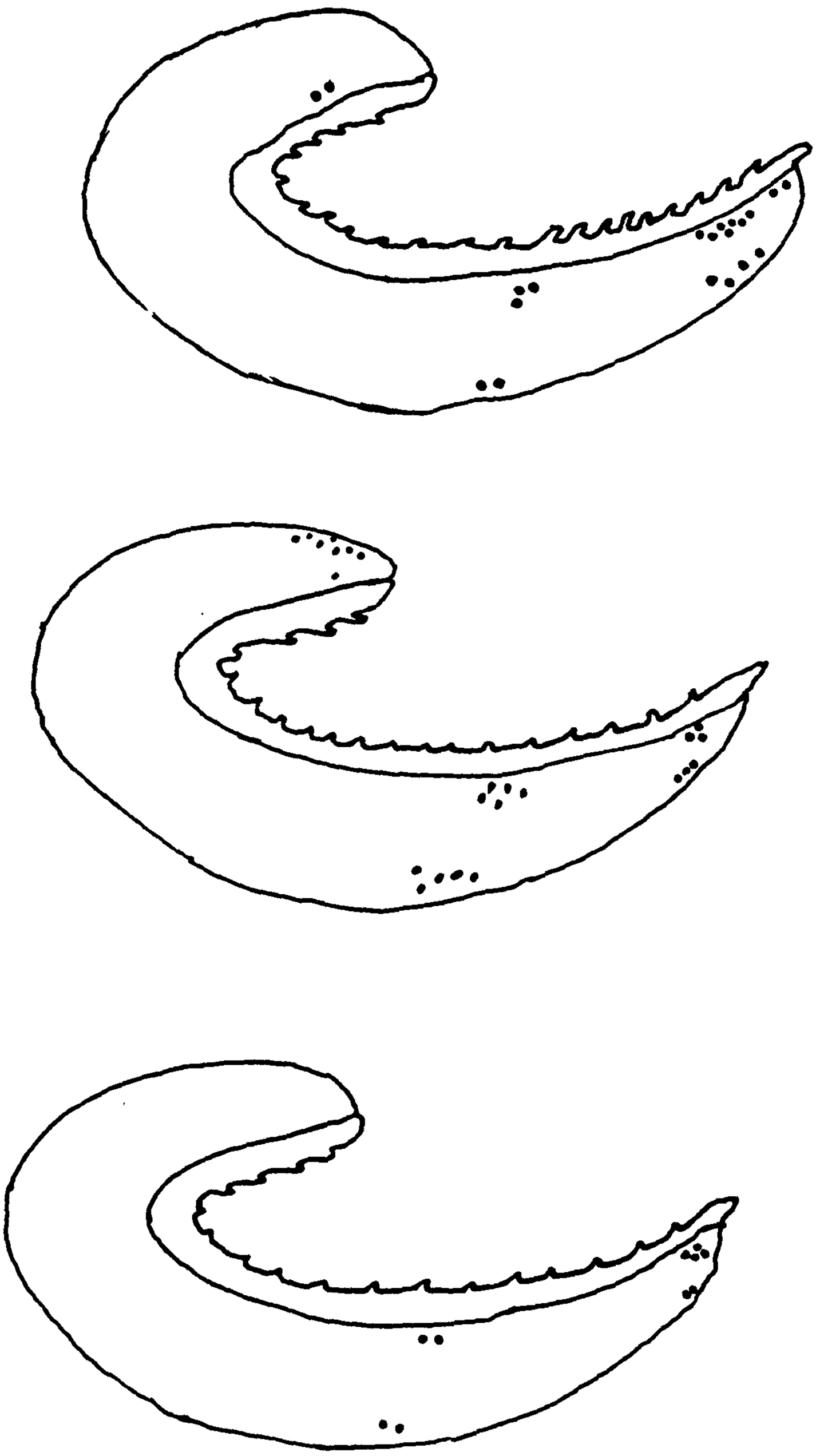


Fig 4.2 :- Showing the distribution of *Dactylogyrus vastator* on three hemibranchs

that the flow of water over the gills was essentially a continuous process. Hughes & Shelton (1958), working with cyprinid species, applied manometric methods to record the resistance of the gills to water flow over their surfaces. Differential pressure was consequently found with a gradient from the buccal to the opercular cavities. The concept of a dual pump relates primarily to water flow through the gills and has no direct anatomical basis since there is mechanical interaction through the system (Shelton, 1970). The geometry of the gills changes constantly throughout the breathing cycle (Shelton, 1970); therefore, the gill sieves are alternatively exposed to and protected from the water flow. A number of workers suggested that the gill filaments do separate during some stages in the opercular cycle (Hughes, 1961; Saunders, 1961; Pasztor & Keerekoper, 1962).

Paling (1968) used the glochidia larvae of the unionid bivalve *Anodonta cygnea* as a "marker parasites" to indicate the relative amounts of water flowing over the different parts of the gills of *Salmo trutta*. This technique relies on the fact that glochidia do not select their site of attachment on the fish gill but are taken passively through the mouth with the respiratory current and attach to the first site on the gills that they come into contact with. Thus in fish left in a homogeneous suspension of glochidia, the numbers attached to different parts of the gill apparatus of the fish after a given period of time are indicative of the relative volumes of water which have flowed over the different parts of gills.

Paling (1968) was able to demonstrate that there were indeed differences in the relative amounts of water passing over different parts of the gill apparatus of trout. The greatest volume of water passed over the middle two gill arches of brown trout, but *Discocotyle sagittata* was found in greatest numbers on the first gill arch. Thus, it appears that the parasite is selecting an area where water flow is less. Paling suggested that larval parasites invading the host via the respiratory current come into initial contact with the middle two arches, but then tend to migrate to the first arch. Wootten (1974) reported that the spatial distribution of *Dactylogyrus amphibothrium* over the gills of *Gymnocephalus cernua* was not random, parasites being aggregated on certain areas of the gill apparatus and this could be correlated with flows of water over the gills as determined by the glochidia method. There were significantly more parasites attached to the right set of gills compared to the left set and significantly greater numbers of monogeneans occurred on the middle two gill arches and on the dorsal segment of the gill compared with the median and ventral segments. *D. amphibothrium* occurred in significantly greater numbers on the distal halves of the gill filaments (Wootten, 1974).

Based on the relative areas of the gill arches it might be expected that most *D. vastator* would be found on the first gill arch of carp and this was indeed the case. The gill arches decreases in area from I to IV and the number of *D. vastator* also decreased between the arches in the same order, although

there was no significant difference in number of parasites between the arches, if parasites per unit area of gill had been analyzed it may have shown a significant difference between arches.

The fish used in this study were very small. Most authors when studying the distribution of monogeneans over the gills of the host have used bigger fish. It may be that if larger carp had been used a more restricted distribution of *D. vastator* would have been found. There may have been a size factor involved in these small carp whereby the relative flow of water over the parts of the gill was more uniform than in larger fish. Alternatively the smaller size of the gills may have simply meant that parasites had less space available to assume a restricted distribution.

Significantly less *D. vastator* are found on the distal dorsal sector of the gill compare with the proximal ventral, proximal median and proximal dorsal segment. The studies of Wootten (1974) suggested that the dorsal part of the gill arch is relatively sheltered. It may be that in carp this area is in some way less suitable as a habitat for *D. vastator*.

The biotic mechanism thought to be most responsible for niche restriction in parasites is competition (Odum, 1963; MacArthur, 1972; Ricklefs, 1980; Pianka, 1974), although other workers have doubted its importance

(Andrewartha & Brich, 1954; Andrewartha, 1970). Ktari (1971), drawing evolutionary conclusions, noted that very specific microhabitats increase the chances of successful mating in monogeneans. At the same time intra-specific phenomena like the "crowding - effect" regulate population growth and its spreading over the available niche (Rohde, 1979). Spatial niche dimensions, such as preference for certain microhabitats in the host are of special importance (Rohde, 1979). Holmes (1986) reported that continued competitive interaction between parasites in an evolutionary time frame leads to niche diversification, largely via site segregation and finally results in a narrow site specificity. Most common is "selective site segregation", in which the realized niche of a species largely coincides with its fundamental niche. Selective site segregation is a common phenomenon among parasite species occupying only one niche (Rohde, 1979). The gill microhabitat for *D. vastator* could be restricted in several ways, to certain gill arches, hemibranchs and or areas of the hemibranchs. However, *D. vastator* showed little evidence of niche restriction if all infections are aggregated, but at the individual host level there was strong evidence of restricted distribution. Rohde (1976, 1977) suggested that site selection enhances the chances of intraspecific factors and thus mating and this could well be the reason for the observed aggregations of *D. vastator* on individual gills.

Schäperclaus (1991a) reported that *D. vastator* is found on the terminal edges of the primary lamellae, but this was not found to be the case in this

study and most worms were in the proximal regions of the lamellae. Schäperclaus (1991a) discussed the location of *D. vastator* in relation to its oxygen needs. Oxygen concentration is an important environmental parameter which can affect the biology of Monogenea and it has been reported that in cases of depletion in oxygen concentration there is a fall in *D. vastator* numbers (Paperna, 1964). Schäperclaus (1991a) suggested that worms change their place of attachment on the gills in low oxygen concentrations and move towards the edge of the primary gill lamellae, where they are exposed to an oxygen rich environment. Although the immature worms during this study were seen to move from one gill lamella to another by the contractile movements of the body, there was no evidence that adults move around the gills. Indeed they seem to be very firmly attached by their hamuli which are embedded in hyperplastic tissue (see Chapter 6). Thus, only immature and presumably relatively newly invaded worms would be able to respond to changes in oxygen concentration as suggested by Schäperclaus (1991a). Dissolved oxygen levels in the tanks used in the study were high (6.2 mg l^{-1}) and it is possible that the *D. vastator* were able to obtain their oxygen requirements without moving to the distal parts of the filaments.

Chapter 5

Population and Reproductive biology

5.1. Introduction

5.1.1. Population Biology

Length frequency distribution studies of a parasite infra-population are helpful in an understanding of its structure. During a period of invasion of the host by a parasite species the proportion of smaller individuals might be expected to be greater. As these individuals mature and grow the length frequency distribution will shift in favour of larger parasites. Such a pattern will be more complicated if there is a constant influx of new parasite individuals, or waves of invasion. Thus the parasite length frequency distribution can be useful in determining the temporal characteristics of the infection of hosts by parasites, as well as providing information on the growth rates of the parasites. Length frequency distribution may also be valuable in assessing interactions between parasites at both the intra and inter-specific level in terms of competition for available resources affecting growth rate, maturation and parasite numbers.

Relatively few studies have been conducted on the length-frequency distribution of monogeneans. Kearn (1967) used length-frequency graphs to identify the population structure of *Entobdella soleae* from the dorsal and the

ventral sides of *Solea solea*. Cone & Burt (1985) were able to study the invasion periods of *Urocleidus adspectus,* as well as the spring growth of over-wintering worms, by the seasonal monitoring of length frequency distributions of parasites of different ages. In spite of some differences in individual graphs, the general picture observed by these authors was a bell-shaped / dome shaped graph.

It is known that the growth of some poikilotherms is a life-long process, despite the attainment of sexual maturity. However, Prost (1963) mentioned that in *D. extensus* the haptor, copulatory apparatus and the parasite's body attains its final size once sexual maturity is reached, but this is not so in *D. anchoratus* where sexual maturity is attained before the parasite reaches its maximum size. Kearn (1963) found continuous growth in the body size, haptor, anterior hamulus and accessory sclerite of *E. soleae*. Due to these anomalies it was therefore not generally reliable to use such measurements to separate immature from sexually mature worms. The worms with the largest sclerites may or may not be sexually mature.

In *D. vastator* there is a delay in the appearance of eggs after apparent maturation of the parasite. Although *D. vastator* has a complete set of developed reproductive organs by the third day of its life, egg laying does not begin until the fourth day at 28 - 29°C (Paperna, 1964). The time delay from

maturation to the appearance of the first egg, can be explained by the fact that the lapsed period allows for mating to occur.

Many parasite populations show either a positive or negative correlation with size of the host. An increase in parasite numbers with increasing size of the host may simply reflect an accumulation with the age and often size of the latter, or a behavioural difference. A negative correlation may also be due to behavioural changes or perhaps an immune reaction. In the Monogenea Kearn (1963) demonstrated that in *E. soleae* infections of *S. solea* parasite numbers decreased with increasing size of the host.

5.1.2. Reproductive Biology

5.1.2.1. Egg assembly

Egg production in monogeneans involves a co-ordinated sequence of events that brings a single oocyte (zygote) together with numerous vitelline cells (nurse cells) in the egg mould or ootype. The vitelline droplets located peripherally in the cytoplasm of the vitelline cells are released and coalesce around the package to form the egg shell. Shaharom-Harrison (1986) found that the proteinaceous shell undergoes a tanning process and consequently the shell becomes progressively darker, gradually changing in colour from white to yellow or brown.

5.1.2.2. The egg

Monogeneans produce eggs of different shapes and sizes and there is a remarkable diversity among the genera. The eggs of *D. vastator* are almost spherical in shape with a short filament / extension of shell material, whereas eggs of *Diplozoon* spp. and *Discocotyle* spp., *Plectanocotyle* spp, and *Diclidophora* spp. are fusiform and those of *Entobdella soleae* are tetrahedral (Kearn, 1986). The eggs of *D. vastator* are spheroidal in shape but *D. chranilovi* lays eggs that are usually tetrahedral, although Izjumova (1953) reported that oval eggs are produced (Kearn, 1986). Bychowsky (1957) proposed the terms "filament" and "little foot" to distinguish between extensions of the distal (=leading) and proximal (=trailing) edges of an egg during its passage from the ootype into the uterus. Later the same extensions were denoted as "appendages" (Kearn, 1963). Appendages are absent in *Discocotyle sagittata*, and very short in *D. vastator*.

The eggs of some monogeneans, such as *Diclidophora palmata*, also lack appendages. Ancyrocephaline monogenean tetrahedral eggs bear a long, slender, coiled appendage (Kearn, 1986). It is common for the fusiform egg of *Diclidophora merlangi* to possess needle-like appendages at both the opercular and abopercular poles. Egg appendages bear adhesive material (Kearn,1986). The *E. soleae* egg appendage carries 8 - 11 colourless droplets of adhesive

material. In *E. australis* the appendage is shorter and bears a single terminal droplet of adhesive material. *D. vastator* eggs remain separate, unlike those of *E. soleae* where eggs with sticky material on the appendages may be joined together by adhesion to form small groups (Kearn, 1963). The developed larvae of *D. vastator* emerges from the operculum of the egg, which is situated opposite to the filament or appendage. The work of Kearn (1986) on the egg of *E. soleae* indicates that the operculum is attached to the rest of the egg shell by a thin layer of opercular cement. This cement is greatly weakened by proteolytic enzymes.

Kearn (1986) described the egg formation of the capsalid monogeneans *E. soleae* and *Epibdella* (= *Benedenia*) *melleni*. The assemblage of the egg, the function of the ootype in the egg formation and egg shell formation was described by Kearn (1986). Shaharom - Harrison (1984) described the egg formation and the egg laying behaviour of *Cichlidogyrus sclerosus* and discussed its similarity to the descriptions of *Microcotyle spinicirrus* by Remley (1942). Tinsley (1983) mentioned the role of the uterus as a site where the egg shell hardens without disturbing the assembly of successive eggs in the ootype.

Several authors have attempted to measure the oviposition rate of monogeneans. However, there is a significant difference in the rate of oviposition of monogeneans *in-vivo*, compared to those removed from the gills.

This was found to be the case for *D. vastator* by Lyaiman (1951a) and Izjumova (1953) (cited in Paperna, 1963b), the detached worm's oviposition rate being higher than *in-vivo*. Prost (1963) observed that the egg size from the detached worms diminishes during the egg laying period, leading her to suggest that there is production of a larger number of smaller eggs by the dying worm contributing to an increased egg output. Therefore, the results of *in-vitro* observations must be treated with caution.

Different fecundities (oviposition rates) have been demonstrated for different monogenean species and comparatively high variation in egg laying rates can be found in the same genus at the same temperature. Thus, Paperna (1963b), Prost (1963) and Molnàr (1971a) recorded 29, 2.13 and 15 eggs per worm per day at 24°C for *D. vastator*, *D. anchoratus*, and *D. lamellatus* respectively. Kearn (1986) reported that Izjumova (1956) did not find any difference between the numbers of eggs laid by *D. vastator* during the day or during the night. However, Thurston (1968) and Macdonald & Jones (1978) found that egg laying in *Oculotrema hippopotami* and *Diplozoon homoion gracile* is affected by day and night differences, and that this difference is primarily due to the behaviour of the host fish. The difference in the egg production rate in *Diplozoon homoion gracile* is mainly dependent on the host species. Mucus from different hosts was shown to have an influence on oviposition (Macdonald & Jones, 1978). Kearn (1986) found that *E. soleae*

laid a greater number of eggs at night when the host, *S. solea*, settled on the sea bed bringing it into close contact with parasite eggs and larvae. However, Izjumova (1956) and Molnàr (1971a) did not find any difference in nocturnal and diurnal oviposition rates of *D. lamellatus*. In *Diclidophora luscae* (Macdonald, 1975) and *Oculotrema hippopotami* the egg laying rate *in-vitro* differed due to the accumulation of eggs in the ootype (Thurston, 1968).

An increase in temperature will generally lead to more eggs being produced by monogeneans. However, at the highest temperatures the egg output may fall. Paperna (1963b), Prost (1963) and Imada & Muroga (1978) found this relationship in *D. vastator*, when the temperature ranged from 20 - 25°C. Paperna (1963a,b) working in the tropical range of temperatures with *D. vastator*, found that oviposition rates at low (12°C) and high (37°C) temperatures were significantly lower compared with the rates at 24°C and 28°C. There was no pronounced/ clear difference between the oviposition rates at 24°C and 28°C.

Anderson (1981) studied the effects of salinity on the egg laying rates of the monogenean parasites of the grey mullet, *Chelon labrosus*. Young mullets up to 2 years of age live in tidal(brackish) pools and are infected exclusively by *Ergenstraema labrosi*, whereas fish aged 4 years and older live in the open sea and are infected exclusively by *Ligophorus angustus*. Fish aged between 2 and

4 years have both parasites. Anderson (1981) found evidence to suggest this change in monogenean species composition is related to salinity changes between the habitats of young and older fish. The rate of egg production in parasites separated from the host was proportional to salinity in *L. angustus* such that most eggs were laid in 100% sea water, whereas in *E. labrosi* maximum egg production occurred in 50% sea water, egg output being adversely affected by higher salinities and ceasing in 100% sea water. However, he observed that some egg production did take place in *E. labrosi* in 100% sea water. *E. labrosi* lays more eggs when detached from the host than *L. angustus* at all salinities between 0 and 80%. Anderson (1981) suggested that the higher rate of egg production by *E. labrosi* may be an adaptation to hazardous conditions in the variable environment of tidal pools compared with more stable conditions in the open sea.

5.2. Materials and Methods

5.2.1. Population studies

5.2.1.1. Gill populations

A batch of two hundred and seventy-five fish already infected with *D. vastator* were brought to the Institute aquarium from Howietown fish farm on the 23rd June 1993, of standard length between 2.0 - 7.0 cm (average 6.68 cm). Fish were left in two separate holding flow-through tanks at 19°C, one tank with 125 fish and another tank with 150 fish, and monitored for a period of 90 days. Twenty fish from each tank were sampled on days 14, 28, 35, 42, 60 and 90. Gills were removed from sacrificed fish and placed in a petri dish with aquarium water. A few drops of alcohol were added to make the worms detach from their site of attachment. The worms were removed by pasteur pipette and placed on a glass microscope slide with a drop of water. Then a flat preparation was made and fixed with ammonium-picrate glycerine (Malmberg's fixative) (Malmberg, 1970). This preparation was used to measure the length of the worm and also to determine the maturity. Immature and mature worms were differentiated by the stage of development of the ovary, testis and the copulatory apparatus. Mature worms may contain either a fully developed ovary or testis. The mature ovary has an elliptical shape and in mature worms

the copulatory tube and accessory sclerite are complete. The testis is globular in shape in mature worms. The lengths were measured directly using a calibrated eye-piece graticule (100 divisions of 10mm) fitted to an Olympus BH2 stereomicroscope.

5.2.2. Reproductive Biology

Dactylogyrus vastator collected from fish infected in the Institute aquarium were used for this study. Carp gills infected with *D. vastator* were left in a petri dish with aquarium water and viewed with an Olympus binocular stereomicroscope at x40 magnification. The behaviour of the worm was followed from production of an egg from the ovary to its expulsion to the exterior.

5.2.2.1. *In vitro* egg laying-rate

The egg-laying rate of *D. vastator in-vitro* was determined for worms at various temperatures, following their separation from the gills. A fine needle was used to separate the worm from the gills and a pasteur pipette was used to transfer the worm into another petri dish with aquarium water. Care was taken to select only active worms. Those which were inactive or apparently damaged during separation from the site of attachment were discarded. Single active

worms left in aquarium water in a petri dish were studied for the duration of their *in-vitro* life. The egg laying rate was observed at 11°C, 13°C and 19°C with 21, 6 and 9 worms, respectively. The number of eggs laid in each 30 minute period over a total time of six hours was counted. Parasites were then left for a further 6 hours and the number of eggs produced in this period again counted.

5.3. Results

5.3.1. Population structure

The length-frequencies of populations of *D. vastator* obtained from fish examined for parasites is given in Fig: 5.1 a,b,c,d at 14, 28, 35 and 42 days of sampling. At each sampling time the parasite length frequency showed a similar bell-shaped distribution. The highest frequencies occurred in the length class 1.00 - 1.50 mm at 42 days. A number of large individuals of 2.95 mm in length were also found at this time. At subsequent sampling times the majority of *D. vastator* were in the 1.00 - 1.50 mm length groups. Larger parasites were not present. Parasites of less than 1.00 mm in length were immature as judged by the size of the accessory sclerite and the structure of the ovary and testis. The proportion of these parasites ranged from 14.5 - 71.7% at each sampling interval. The length of the accessory sclerite showed a positive relationship with the length of the parasite (Fig :5.2).

Fig 5.1a :- Length frequency of D. vastator on carps after 14 days infection

Fig 5.1b :- Length frequency of D. vastator on carps after 28 days infection

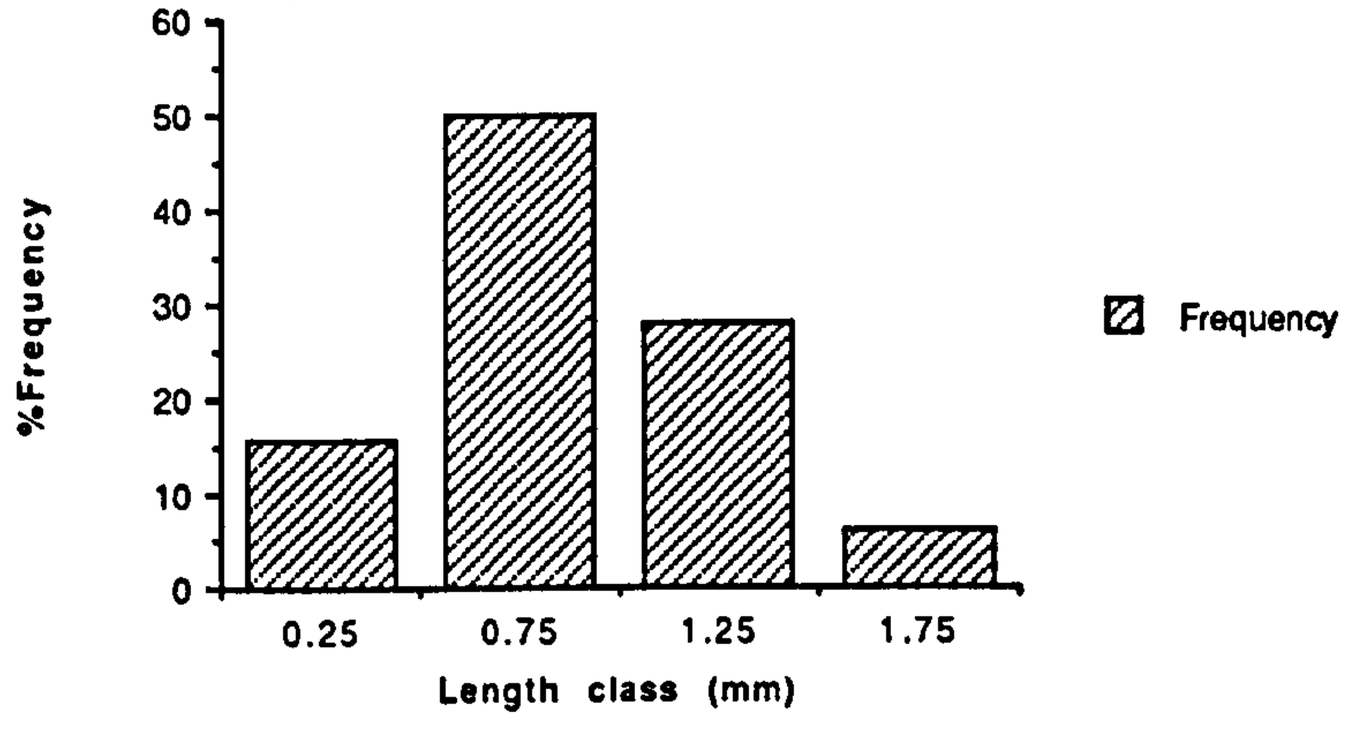


Fig 5.1c :- Length frequency of D. vastator on carps after 35days infection

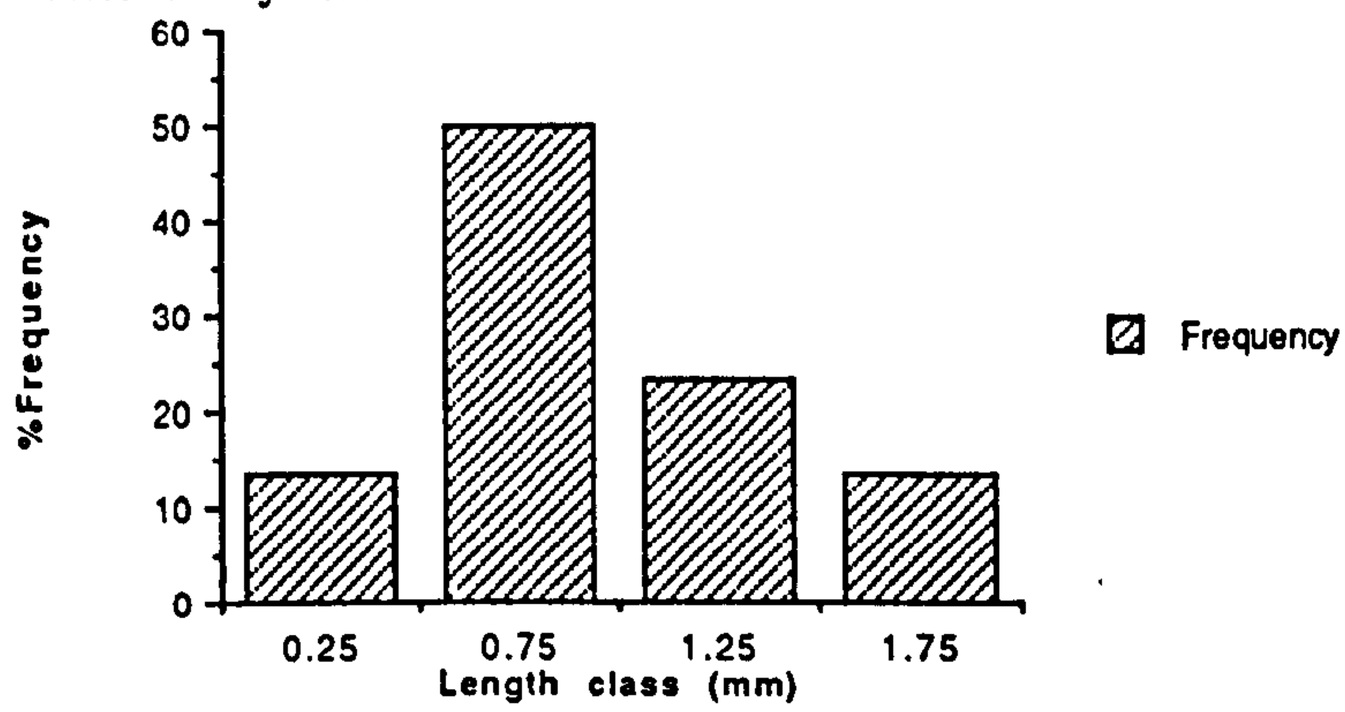


Fig 5.1d Length frequency of *D. vastator* on carps after 42 days infection.

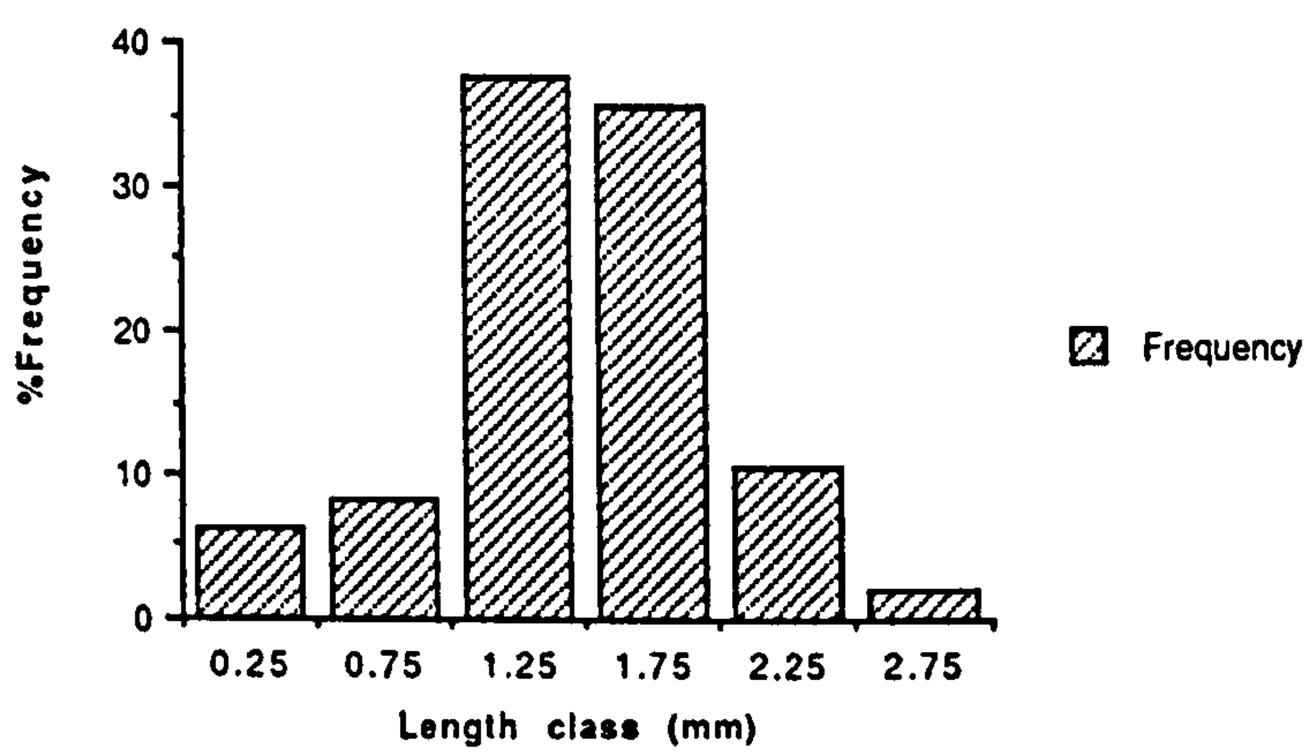
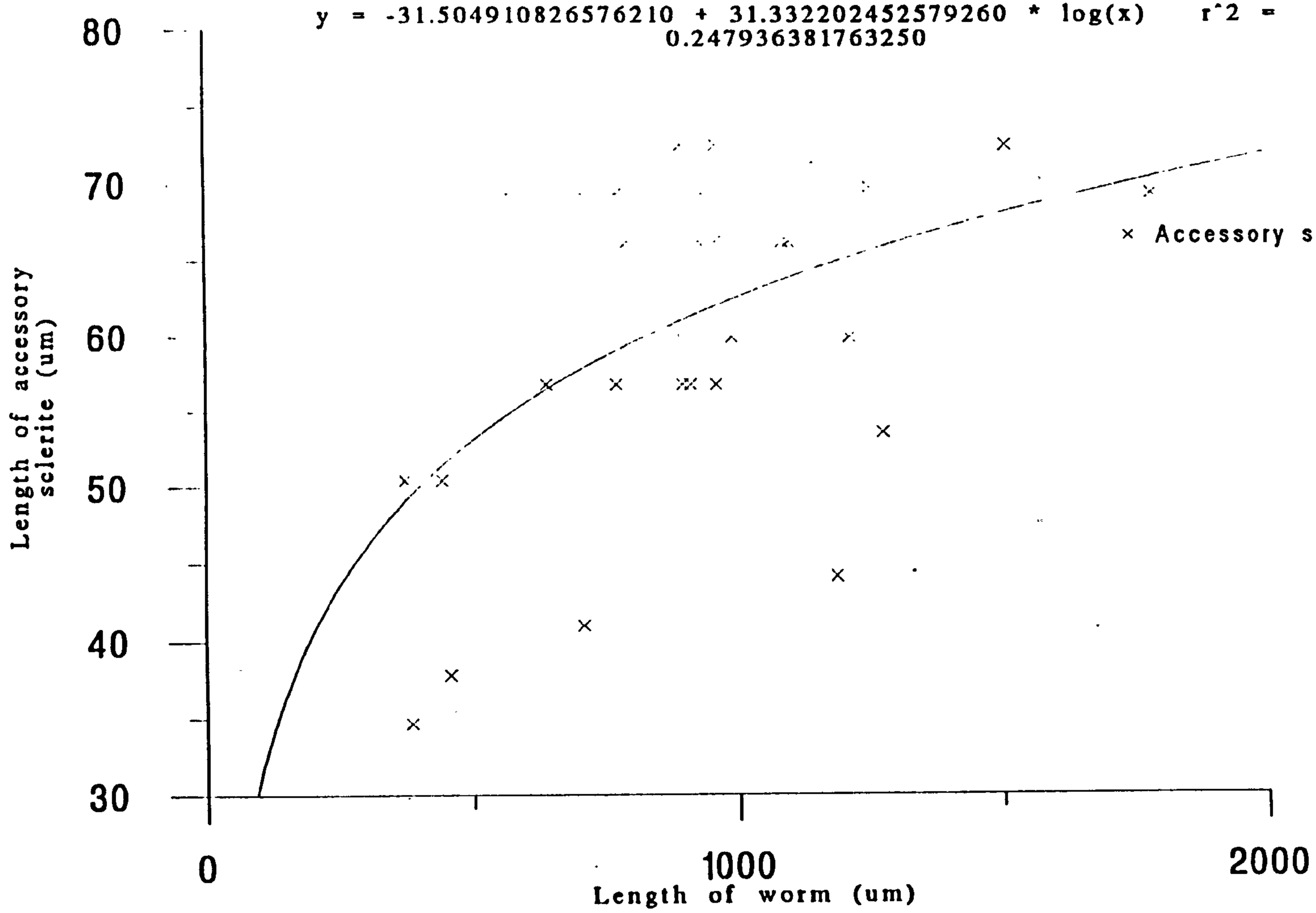


Fig:-5.2 The relationship between length of the worm and length of the accessory sclerite



5.3.2. Reproductive biology

5.3.2.1. Egg formation and oviposition behaviour

Generally *D. vastator* inhabiting the gills of carp show a searching behaviour with contraction and relaxation of body wall musculature extending and shortening the body. Occasionally immature worms under microscopic observation changed their site of attachment by looping in a leech-like fashion over the gills; this was not observed in adults.

In-vitro the egg producing adult *D. vastator* remains stationary for about 3-5 minutes before the ovum is deposited into the ovovitelline duct, where it remains for 1 - 2 minutes. The ovum then moves forward, along with the vitelline cells deposited around it, and enters into the ovovitelline duct as a rather undefined mass. After a further 4 minutes the movement stops and the egg mass is rotated at this point for about 2-3 minutes. During this time the mass acquires a shape closely resembling that of the future egg. It appears as a fairly rigid structure and yellow in colour. After a further 6 minutes the egg enters the uterus. The egg now has a well defined shape, with increased rigidity, and becomes fully tanned. During this process the worm shows alternate searching and resting behaviour. The muscular contractions and relaxations involved in the searching behaviour seem to aid in pushing the

developing egg through the reproductive tract to the exterior. The time taken to pass through the uterus is comparatively longer (5-6 minutes) than the rest of the process. On occasion an egg was observed to remain in the ootype for up to 15 minutes. During this time no further eggs entered the ootype. Once the egg has reached the genital pore, the worm shows vigorous movements including bending its anterior body against the posterior part for 2-3 minutes to push the egg out of the worm. After the expulsion of an egg, the body straightens and starts to show the typical searching behaviour.

5.3.2.2. *In-vitro* oviposition rates

Active non egg-bearing worms were selected and the numbers of eggs laid by a single worm were counted for 6 hours at 30 minute intervals, at different temperatures. Egg-laying rates at every 30 minutes at 11°C, 13°C and 19°C are given in Table : 5.1, 5.2, 5.3. The mean oviposition rates were 2.027, 1.97, 1.45 eggs $hour^{-1}$ $worm^{-1}$ respectively at 11°C, 13°C, 19°C. The time taken to lay an individual egg was calculated as 29.60 mins, 30.45 mins, 41.38 mins at 11°C, 13°C and 19°C respectively. The collected data reveals that a higher proportion of the eggs are laid during the early part of the observed period. No eggs were laid between 6 and 12 hours.

Egg laying rates of *D. vastator* at each hour No of eggs. worm^{-1} hour^{-1}																							
Hour	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	mean	std deviation
1	3	1	2	8	3	2	4	2	3	2	5	2	2	2	2	3	0	1	11	4	9	3.38	2.77
2	10	6	3	5	3	7	7	1	4	3	3	1	1	3	1	3	11	3	10	7	7	4.71	3.12
3	0	4	0	5	0	2	1	1	3	1	0	0	1	4	0	0	1	2	3	1	6	1.66	1.83
4	0	1	0	5	0	0	0	2	0	0	0	0	0	3	0	0	0	0	0	0	0	0.52	1.29
5	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.10	0.44
6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0.22
No of worms	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
Rate for whole period	2.166	2.000	2.166	4.666	3.833	1.000	5.500	2.000	2.000	1.666	1.000	1.333	0.500	0.600 0	2.000	.5000	1.000	2.000	4.000	2.000	3.666	2.027	1.31

Table 5.1 :- *In-vitro* egg laying rates of *D. vastator* for 6 hours at 11°C.

Egg laying rates of *D. vastator* at each hour No of eggs. worm^{-1} hour^{-1}								
Hour	1	2	3	4	5	6	Mean	std deviation
1	2	2	2	2	2	2	2.00	0
2	3	5	9	1	3	5	4.33	2.73
3	2	4	2	1	2	5	2.66	1.51
4	3	1	0	2	3	0	1.50	1.38
5	2	0	0	0	2	0	0.66	1.03
6	2	0	0	0	0	0	0.33	0.82
No of worms	1	1	1	1	1	1		
Rate for the whole period	2.33	2.33	2.00	2.16	1.00	2.00	1.97	0.50

Table 5.2 :- *In vitro* egg laying rates of *D. vastator* for 6 hours at 13°C.

Egg laying rates of *D. vastator* at each hour No of eggs. worm^{-1} hour^{-1}											
Hour	1	2	3	4	5	6	7	8	9	mean	std deviation
1	10	4	3	3	4	1	5	3	3	4.00	2.5
2	2	2	10	6	3	1	2	1	2	3.22	2.95
3	3	0	0	2	0	0	0	0	0	0.55	1.13
4	1	1	0	0	0	0	0	0	0	0.22	0.44
5	1	0	0	0	0	0	0	0	0	0.11	0.33
6	3	1	0	0	0	0	0	0	0	0.44	1.01
No of worms	1	1	1	1	1	1	1	1	1		
Rate for whole period	3.30	1.33	2.16	1.83	1.16	0.50	1.16	0.66	1.00	1.45	0.85

Table 5.3 :- *In-vitro* egg laying rates of *D. vastator* for 6 hours at 19°C.

5.3.2.3. Life cycle studies

5.3.2.3.1. Egg development

The eggs of *D. vastator* when laid are dark brown in colour with dense granular contents. The polar filament is clearly evident in the egg shell (Plate: 5.1a). Eggs of *D. vastator* are almost spherical in shape with a shorter polar filament or appendage at the opposite end to the operculum. In the middle region of the egg a translucent area appears within 24 hours and the dense granules align themselves in specific portions of the egg contents. The translucent area becomes transparent in the next 8 - 24 hours. This gradually enlarges and two dark eye spots appear within it during the second day of development (Plate 5.1c). Four eye spots are clearly seen 48 - 72 hours after oviposition and the developing embryo can then be recognized (Plate 5.1b). By the beginning of the fourth day the eye spots are larger and well developed, and the margins of the larval shape become clearly visible.

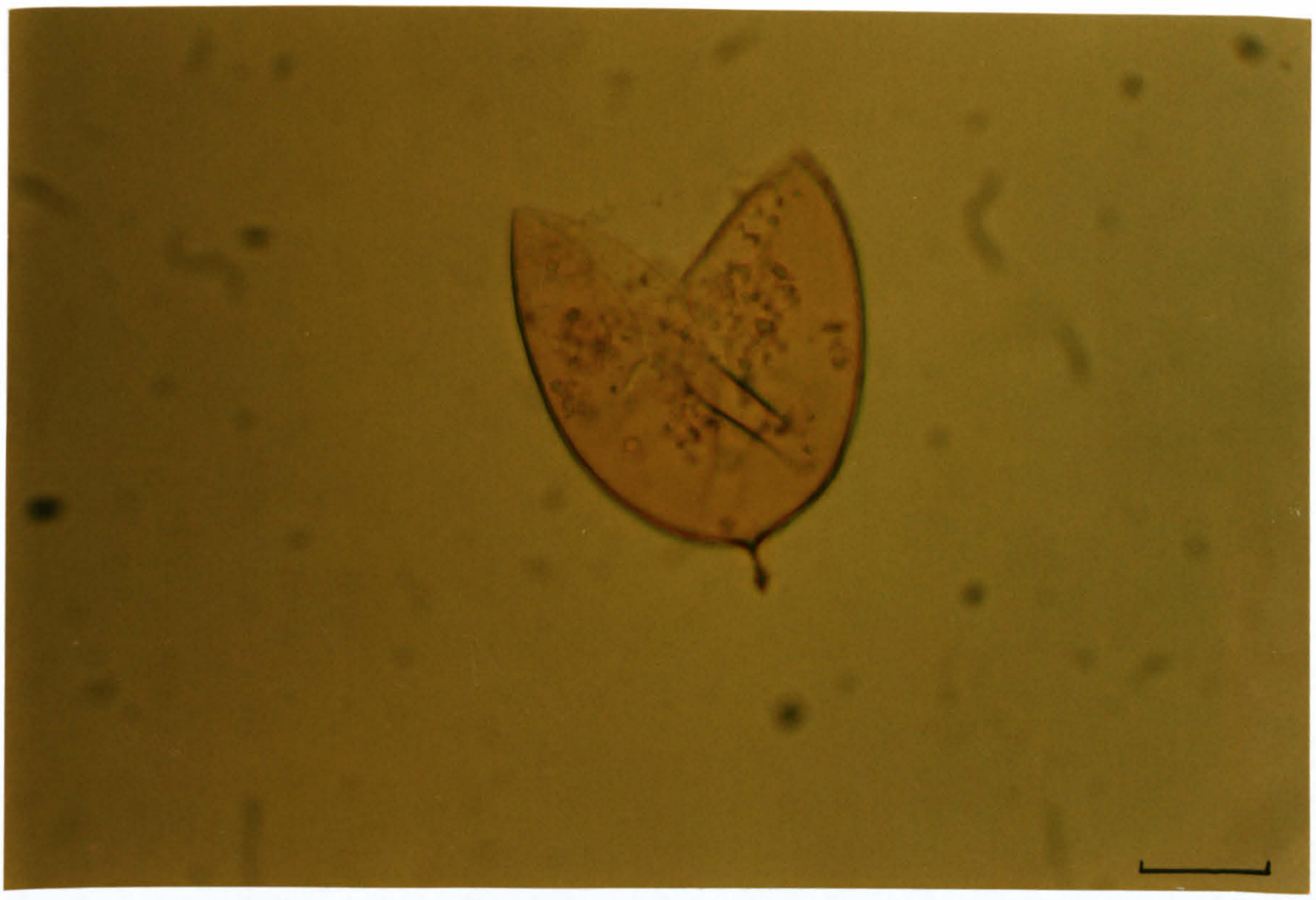


Plate 5.1a :- A light micrograph of the egg shell following the emergence of the oncomiracidium from the egg, the operculum having separated from the rest of the egg shell.
Scale bar = 10 mm

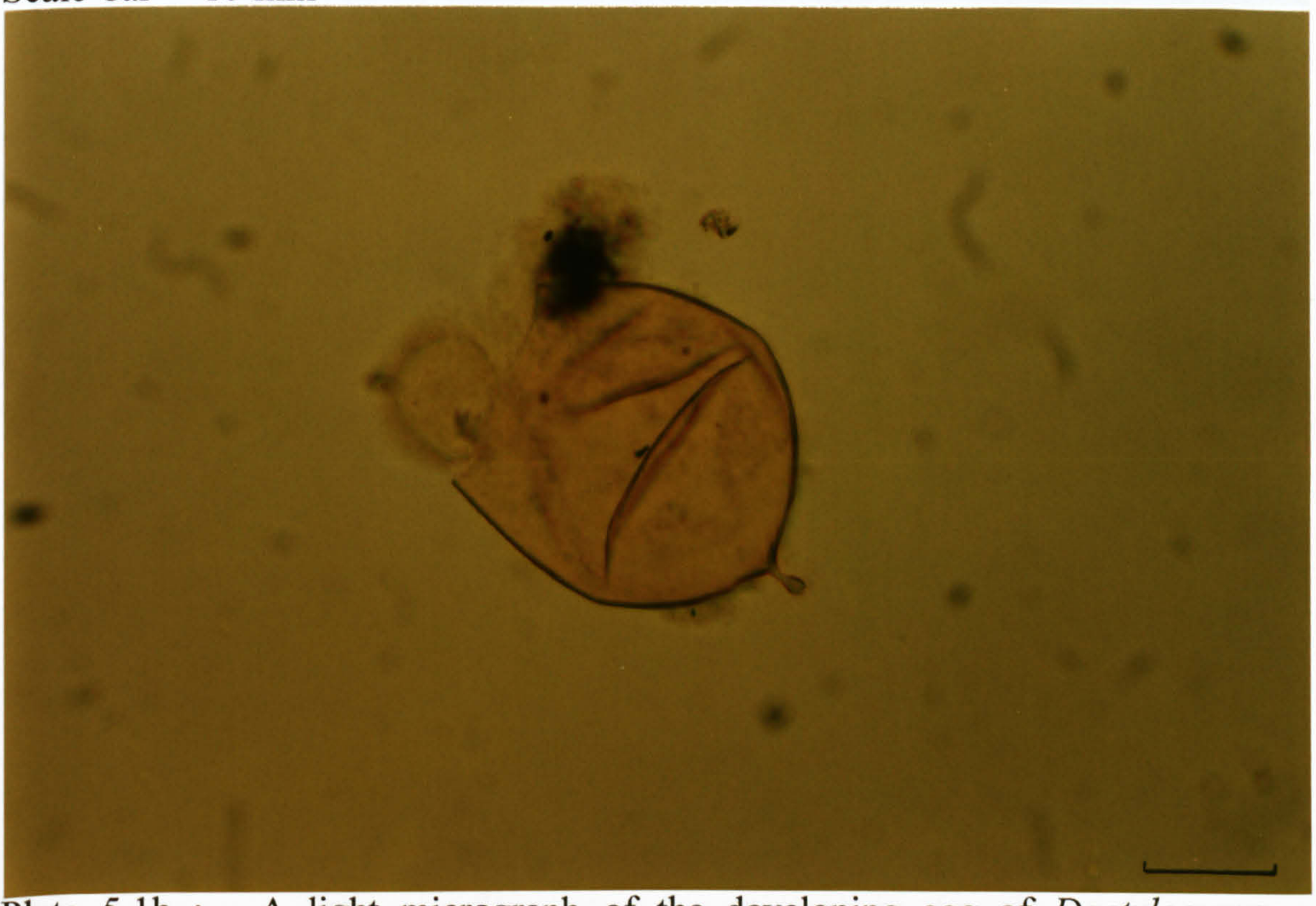


Plate 5.1b :- A light micrograph of the developing egg of *Dactylogyrus vastator* showing the oncomiracidium emerging from the egg through the operculum.
Scale bar = 10 mm

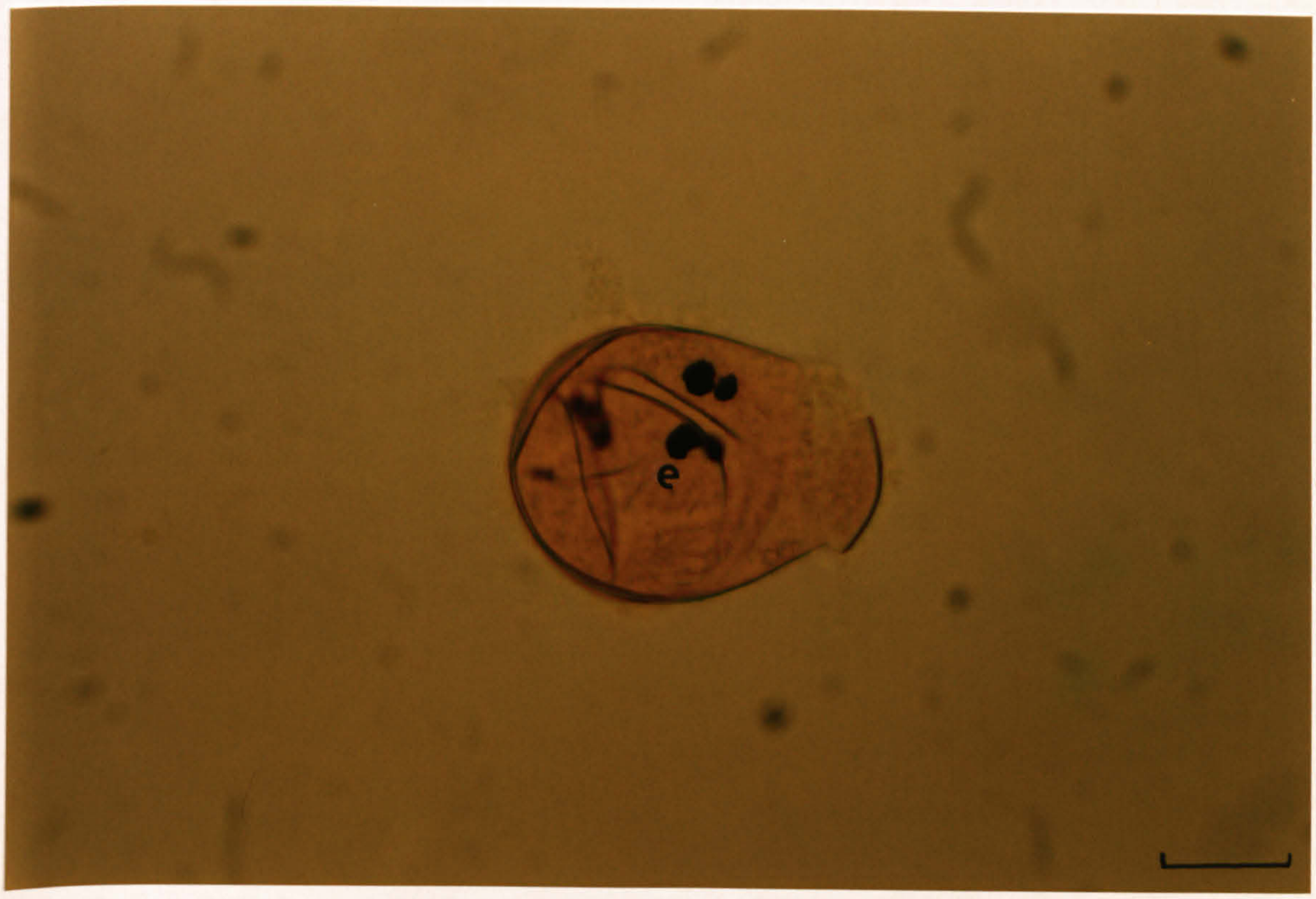


Plate 5.1c :- A light micrograph of an egg from *Dactylogyrus vastator* after 4 days development showing an undifferentiated cytoplasm with granular materials.
e = eye spots
Scale bar = 10 mm

5.4. Discussion

5.4.1. Population structure

The length frequency distribution of the parasite population was generally similar at each sampling time and showed a normal bell-shaped distribution. The proportion of immature worms remained fairly constant throughout.

D. vastator takes 10 days at 24°C to reach maturity (Paperna, 1964) and therefore those parasites of 0.75 - 1.00 mm found on carp presumably invaded the fish within the previous 7 days. Relatively few large parasites above 2.25 mm were found in these carp, indicating that most parasites die off after reaching that size. The results of this study contrast with those of Kearn (1986) who found that the smallest size group of parasites were most abundant.

The accessory sclerite showed a positive correlation with body size of *D. vastator*. This relationship held for parasites at least up to 1 mm in size. This suggests that *D. vastator* continues to increase in size throughout its life.

5.4.2. Reproductive Biology

5.4.2.1. Oviposition behaviour

Dactylogyrus vastator does not need to be attached to host tissue to initiate the egg-laying process, indeed they were found to lay eggs at a higher rate *in-vitro* than *in-vivo*. The process is very similar to that in *E. soleae* (Kearn,1985). Though the ootype is not clearly evident, the area where the ovovitelline complex is rotated can be regarded as an ootype. *E. soleae* eggs expelled from the ootype are rigid and slightly tanned whereas the eggs of *D. vastator* are fully tanned, but the whole process of tanning occurs during the passage through the uterus. The laying of fully tanned eggs is obviously an adaptation for the survival of the species in a hostile environment, as the tanned eggs are less likely to be damaged by environmental hazards. The time taken to lay individual eggs by *Enterogyrus globidiscus* was 20 - 30 minutes according to Nilakarawasam (1993), by *C. sclerosus* it was approximately 45 minutes according to Shaharom-Harrison (1984) and for *E. melleni* an average of 5 minutes as reported by Jahn & Kuhn (1932). It seems likely the time taken by each species to lay eggs varies greatly depending on its biology and the surrounding environmental conditions, particularly temperature, salinity and pH.

5.4.2.2. Oviposition rate

Estimates on the egg output of dactylogyrid monogeneans differ depending on whether such estimates were made on parasites attached to or detached from the host. A higher *in-vitro* oviposition rate for *Dactylogyrus* has been reported by Izjumova, 1953 cited in Paperna (1963a) and Lyaiman (1951a) for *D. vastator* and by Prost (1963) for *D. anchoratus*. However, Nilakarawasam (1993) reported a lower oviposition rate *in-vitro* compared to *in-vivo* in *Enterogyrus* spp. Adverse conditions found *in- vitro* may induce the worms to lay more eggs in an attempt to increase the chances of transmission to other hosts. Detached parasites, unable to replenish their resources by feeding, might be expected to produce fewer eggs than attached parasites, but Prost (1963) reported that the attached *D. anchoratus* laid fewer eggs compared to the detached parasites. The detached *D. vastator* in this study produced eggs of 72.55 x 65.38 µm during the initial *in-vitro* egg laying period and then the size of the eggs gradually diminished to 45.88 x 29.84 µm later in the period of observation. If the available reserves for egg production remain fixed throughout the life of the worm it can produce a smaller number of bigger eggs or a larger number of smaller eggs. The production of smaller eggs allows the production of greater numbers of eggs during the course of egg-laying. An interesting question is whether the viability remains the same or lower for the eggs laid later during the period of observation. Tinsley (1983) summarized

that most monogeneans deposit fewer than 100 eggs per 24 hours and may deposit fewer than 25 eggs. Paperna (1964) reported that the *in-vivo* oviposition rate varies from 7.2 to 48 eggs per hour in *D. vastator*. Following transfer of infected fish to a new experimental environment, egg laying stopped but from the fourth hour after transfer the number of eggs laid increased steadily. In this study, *D. vastator* laid 7.068 ± 4.1729 eggs a day *in-vitro*. During the initial period after separation from the gills *D. vastator* may lay eggs which have already started development. The slow down in oviposition during the period of *in-vitro* observation may be due to the final formation and expulsion of most of these eggs.

In *D. vastator* attached to the host, in general, the egg output increased with the age of the parasite over a period of 10 days (Izjumova, 1956 cited in Kearn,1986). The high fecundity of many parasites is correlated with transmission between hosts in their life cycles being associated with high mortality levels (Kino, 1984). Fecundity may be influenced by several factors. The egg output of the individual parasite is decreased with increasing population density and it is often assumed that this is due to an intraspecific competition for limited food resources (Kino, 1984). *E. soleae* continues to grow after it attains sexual maturity (Kearn, 1963), and the egg production increases with increasing size of the worm (Kearn, 1986).

5.4.2.2.1. The Ootype

Bychowsky (1957) mentioned the variety of shapes in monogenean eggs and the correspondence of the shape of the egg with the form of the ootype. Kearn (1986) noted that the egg of *E. soleae* spends a few minutes, sometimes much longer, in the ootype after the cessation of the ootype movements and before expulsion into the uterus and during this period the egg shell hardens and takes up the shape of the egg mould. The results of the present study indicate that the *D. vastator* egg spends 1 - 3 minutes in the ootype after the end of ootype movement. The actual period spent in the ootype may be due to the size of the parasite and the size of the egg. Kearn (1986) found evidence for small but significant increases in the size of the egg as *E. soleae* increased in size.

Since the process of the assembly of tanned eggs seems to be the same in all parasitic platyhelminths, monogeneans might be expected to possess similar cells associated with the ootype (Kearn, 1986). One group of cells with ducts penetrate the lining of the ootype wall, and a second group of cells with ducts converge on the ootype entrance (Goto, 1894 cited in Kearn, 1986), each of these groups of cells have been independently described as the "Mehlis gland". Goto's work revealed an apparent distinction between monopisthocotyleans, possessing glands opening at the entrance of the ootype, and polyopisthocotyleans, with the gland opening through the wall of the ootype.

Chapter 6

Gill histopathology caused by *Dactylogyrus vastator*

6.1. Introduction

Each of the four pairs of gill arches found in all teleosts is supported by a bony or cartilaginous skeleton. From each arch, diverging rows of filaments branch off and on both sides of each filament are located the plate-like lamellae where gaseous exchange occurs (Roberts, 1978). Each lamella is best regarded as a thin envelope of cells, the two surfaces of which are supported or held together by pillar cells (Ferguson, 1989). Each respiratory surface comprises a single or double layer of epithelial cells joined at their margins by interdigitations and by desmosomes and tight junctions. The inner layer or surface of cells rest on a basement membrane that passes the apposing interior faces of the lamella in grooves located within the pillar cells, thereby providing additional support for the actomyosin fibrils found within the cytoplasm of the pillar cells. The ends of pillar cells touch the basement membrane and spread laterally to form wide flanges: thus each pillar cell is shaped like a spool (Ferguson, 1989). Where the flanges touch the adjacent pillar cells, they are joined by desmosomes and tight junctions. Erythrocytes therefore percolate through the spaces created by this arrangement, except at the outer margin of the lamella, which has an endothelial lining and may be the preferred channel for blood flow.

The outer layer of the lamellar epithelium is thrown into convoluted finger-like microridges. In addition to greatly increasing the respiratory surface area, these aid in the flow and attachment of mucus. Goblet cells, osmoregulatory chloride cells and their associated "accessory" cells are present in the lamellar epithelium, particularly at the base of the lamellae.

The gills are the most delicate structures of the fish. Their vulnerability is considerable because of their external location and intimate contact with the environment and they may be liable to damage by any irritant materials either dissolved or suspended in the water. Under pathological conditions the cells of the secondary lamellae are often found in increasing numbers at the distal ends of the lamellae. At the base of the lamellae beneath the epithelium may be found small clumps of progenitor cells that subsequently travel up the lamellae to form epithelial cells. Filamental epithelium contains large numbers of goblet cells, especially on the leading and trailing edges and at the base of the lamellae (Roberts, 1978). Production of mucus increases when the gills are irritated by pollutants or parasites (Satchell,1984). Similarly, quantitative and qualitative variations occur with other changes in water quality such as ammonia and calcium content. Other cells found within the filamental interstitium include variable numbers of lymphocytes, macrophages, eosinophilic granular cells, neuroepithelial cells and rodlet cells. Rodlet cells are an enigma, and are found in many species of teleost and within many tissues, including intestine, renal

tubules and bulbous arteriosus. Rodlet cells have a very characteristic flask-shaped appearance with refractile eosinophilic "rods" in their cytoplasm.

6.1.1. Gill histopathology

The most detailed account of pathology due to dactylogyrosis is by Molnàr (1972) who described the effect of *D. lamellatus* on grass carp. Molnàr found that gills of carp with dactylogyrosis caused by *D. lamellatus* showed two kinds of changes: local lesions immediately surrounding the parasite, and general lesions involving either the entire gill or its greater part.

According to Molnàr (1972) acute dactylogyrosis is manifested 2 - 3 weeks after invasion of the parasite; at this time gill lesions become grossly visible. The massively infested gills are paler than normal and assume a mosaic-like appearance as if haemorrhaged. Copious mucus covers the entire lamellae. The latter appeared to be deformed and carry thick greyish bodies and processes of various sizes which form adhesions by fusion. Dactylogyrosis is characterized by proliferation of the gill epithelium originating from the cuboidal cells which grow larger and increase in number causing a thickening of lamellae. The epithelial cells between the respiratory plates enlarge, bulging above the level of the plates and eventually producing adhesions between the

lamellae.

According to Molnàr (1972) the cells of proliferative tissue have a foamy cytoplasm and a large round clear nucleus enclosing one or two lightly staining nucleoli. The cytoplasmic vacuoles are small and granular in the deeply seated cells, large and vesicular in cells of the upper layers. The cell margins are indistinct and some superficial cells necrotize and undergo cytoplasmic disintegration and karyolysis.

In moderately severe cases of dactylogyrosis the normal gill structure can be recognized at the base of the lamellae, but in the central part of the swollen lamellae degenerated cuboidal cells fill the spaces between the respiratory plates, pushing the plate epithelium upward, and the capillary net downward, deep into the proliferative tissue. At the tips of the lamellae, several layers of proliferative tissue overgrow the remains of the capillaries, thus giving rise to the respiratory plate free "lamellar processes" observed by Wunder (1929).

In severe cases, 3 - 5 or even more gill lamellae may coalesce with one another, usually at the tips, but occasionally along their entire length. The original structure is hardly perceptible within the intricate pattern of the proliferative tissue. The capillary network is pushed into the deep epithelium by proliferation. The proliferative tissue contains mononuclear cells.

Connective tissue cells encase the vessels or are found along the cartilaginous supporting structure of the gill lamellae.

In severely deformed gill lamellae cells of proliferative tissue degenerate and deform. In the softened necrotic tissue the endothelial network remains in a relatively intact state until the epithelial cells disappear. Necrosis later involves the cartilage and the arteries and affected lamellae break off leaving grossly visible gill defects. Finally parasites are expelled from their location by the proliferative tissue.

Although *D. vastator* is known as a serious pathogen there are no detailed accounts of the histopathology of infection in carp. Wunder, 1929; Uspenskaya, 1962 and Paperna, 1964 distinguished between gross lesions where the histology of the whole gill is changed with increased mucus production covering the gill and microscopic lesions where only the secondary lamellae are damaged by the presence of the parasite. The pathology caused by *D.vastator* in general resembles that caused by *D.lamellatus*. In contrast, infections of *D. extensus* caused only superficial epithelial damage (Prost, 1963).

Ancylodiscoides vistulensis has long been considered as a highly pathogenic parasite of *Silurus glanis* (Siwak, 1932) and can kill the host within a day of its infection because of its attachment all over the integument. The

gills of dead fish may be packed with parasites and coated by mucus. They have a characteristically pale colour and the whole structure of the gill is damaged such that adjacent gill filaments may fuse together (Molnàr, 1980). Molnàr reported that the larvae of *Ancylodiscoides* localize chiefly on the body surface and epithelium of the orobranchial cavity of the host, but from the third day they are exclusively found on the gill filaments. The larvae loosely attach to the epidermis and gill epithelium, by sinking their hooks and developing ventral anchors into the epithelial cells. The stratified epithelium becomes discontinuous over the skin surface invaded by the larvae. Once larvae grow enough to penetrate deep layers of the epithelium, a general gill tissue reaction develops in addition to local lesions at the site of attachment.

6. 2. 1. Materials and Methods

Carp fry used in this study were from the Munton and Fison PLC hatchery, without any previous exposure to *Dactylogyrus*. Fry were around 1 - 2 cm in standard length. These were left for two weeks in an aquarium of 78cm x 58 cm x 33 cm with 20 litres of water maintained at a constant temperature by an immersed electric water heater at 17°C to acclimatise the fish before the start of the experiment. Fish were then divided into two groups of eighty fish in one group, and transferred to 8 litre aquaria, 45cm x 30 cm x 26 cm maintained at 17°C. One set of fish was maintained as a control for the

experiment and kept under the same conditions as the infected fish. Water quality was relatively constant throughout the experiment. Dissolved oxygen remained around 6.2mg l^{-1}, pH at 6.9 ± 0.05 and total ammonia level below 0.01 mg l^{-1}.

Twenty infected carp (with an average of 15 *D. vastator* per fish) were introduced to the experimental group. After two weeks these previously infected fish were removed from the experimental tank. Samples of 10 carp from infected and control tanks were removed at 2 week intervals. The operculum was removed and the gill holobranchs were carefully dissected out using fine scissors and forceps and fixed immediately in 10% buffered formalin. The fixed gills were left in fixative for 24 hours before processing for histology. No dead fish were sampled for histological studies. Sampling of gills resulted in extensive haemorrhage over the gill tissues leading to large scale accumulation of blood cells in the interlamellar and interfilamental areas and adherence of blood cells to the filamental and lamellar epithelium. To avoid this problem the caudal peduncle was first severed to bleed the fish, subsequently the gills were removed within 2 - 3 minutes. This procedure greatly reduced the blood cell accumulation on the gill tissue and enabled clear observation of the surface epithelium and its histology, as well as parasite morphology.

6.2.2 Tissue processing and embedding

Fixed gills were cassetted, labelled and auto-processed in a histokine (HISTOKINETTE 2000). This involved passing the gills through a graded series of alcohols. The gills were first left in 50% methylated spirit for 1 hour, transferred to 80% methylated spirit for 2 hours, and then to two three hour baths in absolute spirit. They were then left in 100% alcohol for 2 hours and passed through chloroform firstly for 2 hours and then for 1 hour. Finally they were left in paraffin wax for two 2 hour periods. Tissue samples were blocked in suitably sized moulds using molten wax and were then cooled rapidly on a cold plate.

6.2.3. Sectioning

Tissue blocks were trimmed to bring the tissue close to the surface of the wax. The cut edge of the trimmed blocks were placed in decalcifier (RDC - histolab) solution. After an hour the blocks were then washed, cooled on a cold plate and 3 - 5 μm sections were cut on a Leitz-Wetzlar microtome using Reichert - Jung disposable microtome blades. Thin sections were floated in a water bath maintained at 35°C and were collected on pre-washed glass microscope slides. The slides were then marked by diamond pencil and dried at 60°C before they were stained.

6.2.4. Staining

For general observations all sections were stained with haematoxylin and eosin. Special stains such as alcian blue and periodic-acid Schiff's (PAS) were carried out when necessary to demonstrate the different components of the tissue. Procedures as outlined in *Carlton's Histological Techniques* (Drury and Wallington, 1980) were followed for the preparation of stains and staining methods. Stained sections were mounted in synthetic mounting medium (Pertex-histolab).

6.2.5. Photomicrography

Photomicrographs from the histological sections were taken on a Leitz-Orthomat automatic microscope equipped with planar lenses and a fixed camera loaded with Kodak (Gold ASA 100) film.

6. 3. Results

Macroscopically infected gills lost their natural red colour after 4 - 6 weeks, especially at the tips of the primary lamellae which became grey-white in colour, covered with mucoid secretions and were of irregular shape. In gills observed immediately after removal from the live fish the *D. vastator* were found on the proximal part of the hemibranch at the base of the secondary lamellae. At the end of the experiment infected fish were sluggish and were seen to gasp at the water surface.

In control fish at the begining and end of the experiment, the secondary lamellae showed slight proliferation at the junction with the primary lamellae. The epithelium of the secondary lamellae of the control gills was generally of single cell thickness. Swelling of cells was clearly visible, along with separation of the epithelial cells from the rest of the lamellae, in some areas.

Plate 6.1 shows the gill histology in the second week of infection by *D. vastator*. There was some degree of localised hyperplastic tissue response. There was proliferation of the basal layer of the primary lamellae resulting in hyperplastic tissue between the secondary lamellae. Secondary lamellae showed signs of adhesion and there was mucus evident between primary lamellae. At

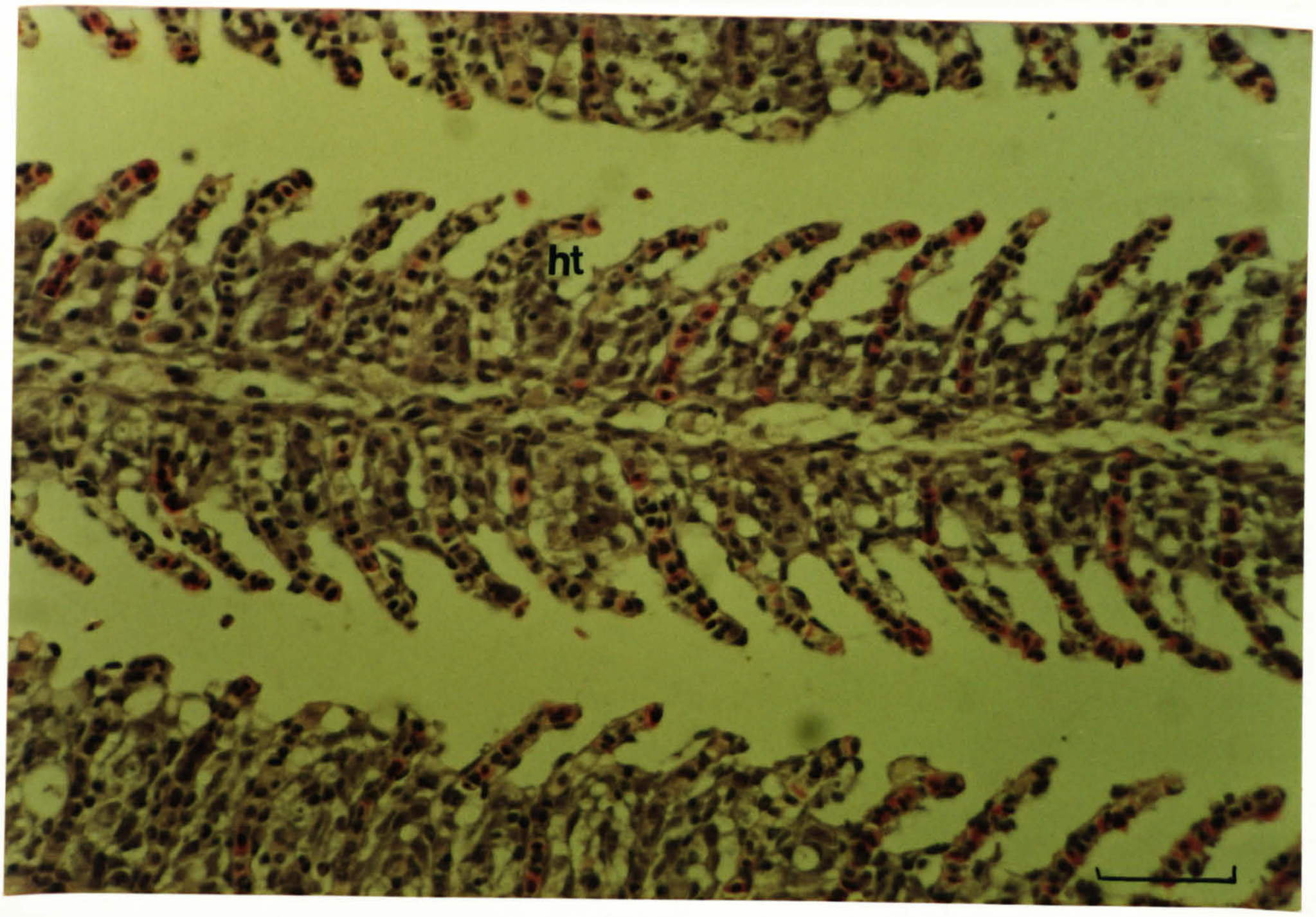


Plate 6.1 :- A light micrograph of gills two weeks after infection with *Dactylogyrus vastator*, showing the local hyperplastic tissue (ht) response and proliferation of the basal layer.
Scale bar = 0.1 mm

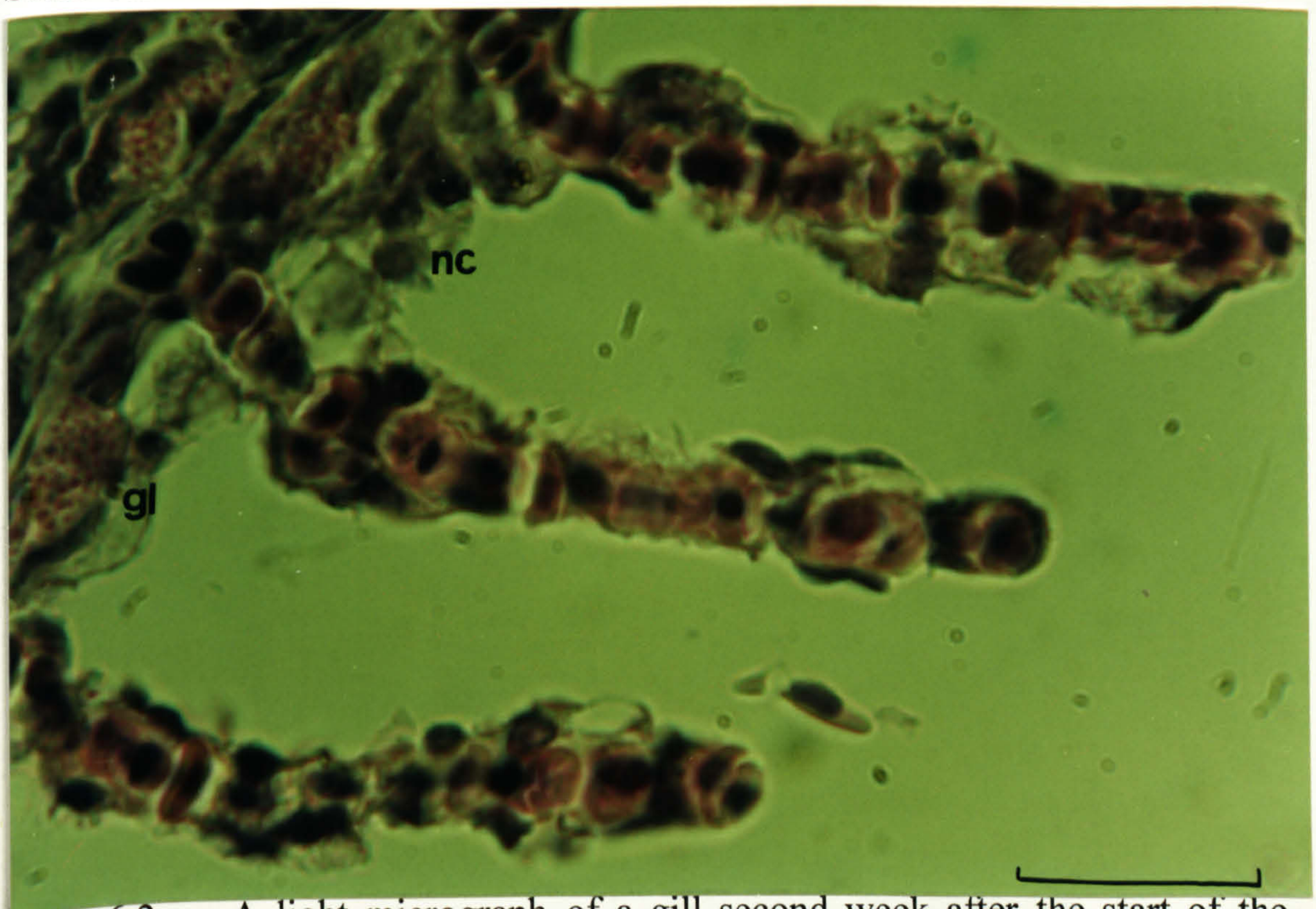


Plate 6.2 :- A light micrograph of a gill second week after the start of the experiment with *Dactylogyrus vastator,* showing the necrotic cells (nc) found in the secondary lamellae.
gl = granular leucocyte
Scale bar = 0.05 mm

higher power (Plate 6.2) some necrotic elements can be seen on the surface of the secondary lamellae. Some granular leucocytes were scattered among the secondary lamellae.

Plate 6.3 shows a section of a gill after 4 weeks infection with *D. vastator*. The degree of the pathological changes were little different from those seen in the second week of infection. The fusion of secondary lamellae was more clearly evident and there were changes in the secondary lamellar epithelial cells. The cells at the periphery lost their shape and became flattened, with a prominent nucleus at the outer edge. In some places there were necrotised cells at the basal part of the secondary lamellae. The distal end of the primary lamellae was swollen and some cutaneous elements can be seen within the mass of cells. The secondary lamellae were curved and also showed clubbing. Cavities are visible between hyperplastic cells of the secondary lamellae (Plate 6.4). At higher power (Plate 6.5) some separation of the epithelial cells from the secondary lamella can be seen. Adhesion of the filaments is clearly shown in Plate 6.6. Adjacent lamellae are joined by the epithelial cells over laid with mucus.

Plate 6.7a,b shows the gill structure of carp after 6 weeks of *D. vastator* infection. The distal end of the primary lamellae has formed a multilayered mass with cutaneous cells embedded in a mass of necrotic cells. In the

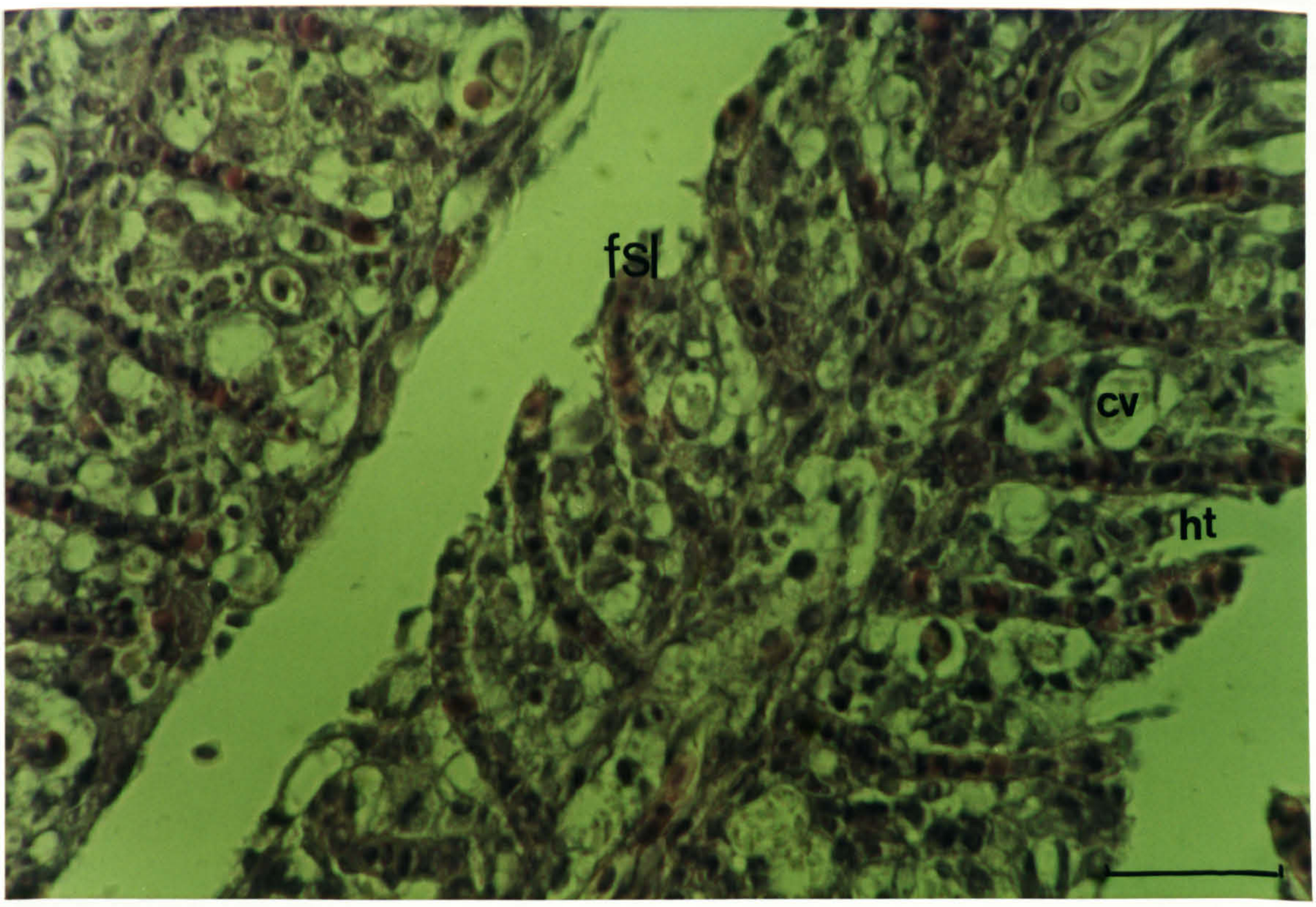


Plate 6.3 :- A light micrograph of gills four weeks after infection with *Dactylogyrus vastator* showing fusion of adjacent secondary lamellae (fsl) in addition to hyperplastic tissue (ht) response.
Scale bar = 0.1 mm

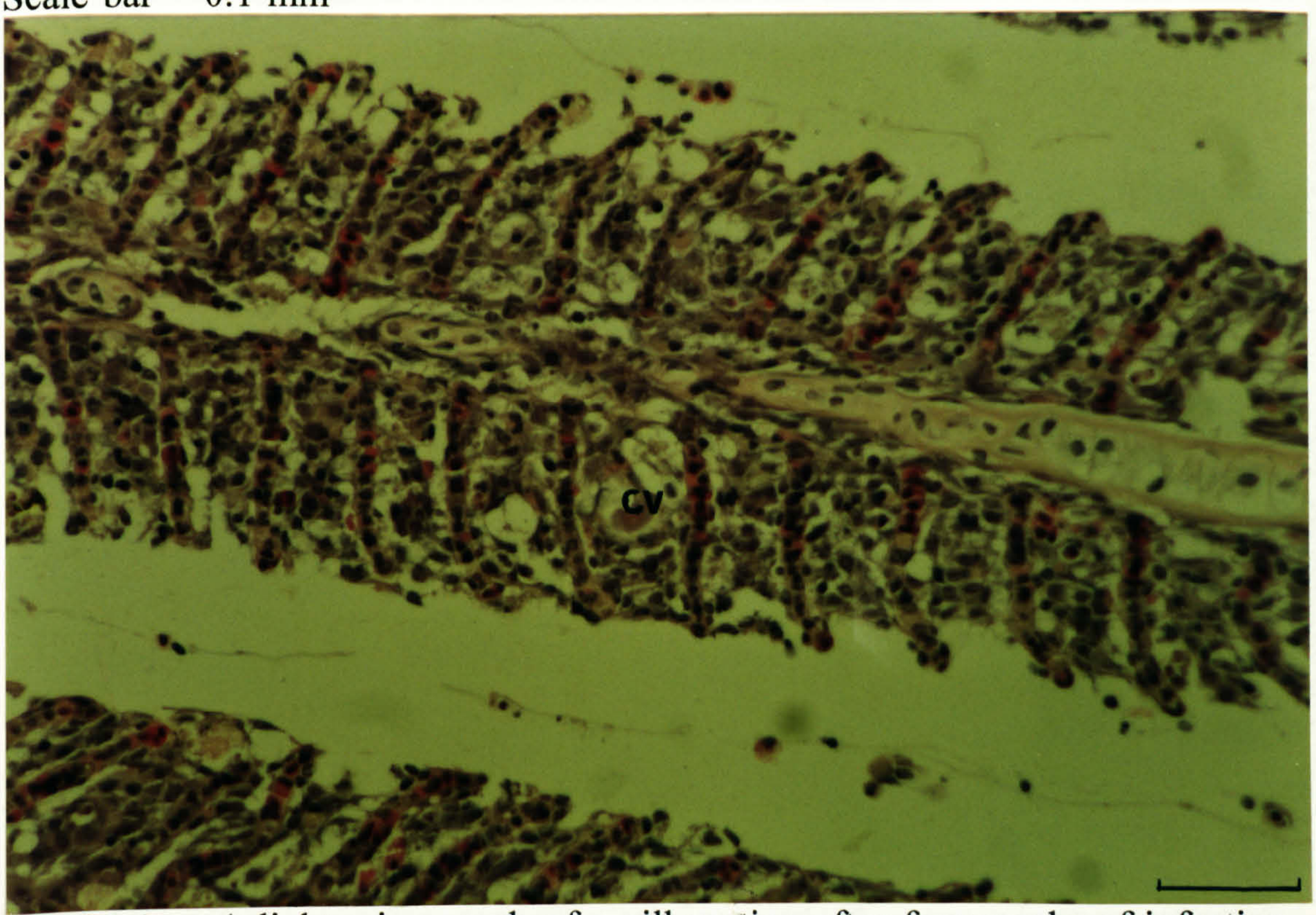


Plate 6.4 :- A light micrograph of a gill section after four weeks of infection with *Dactylogyrus vastator*.
cv = cytoplasm filled vacuoles
Scale bar = 0.1 mm

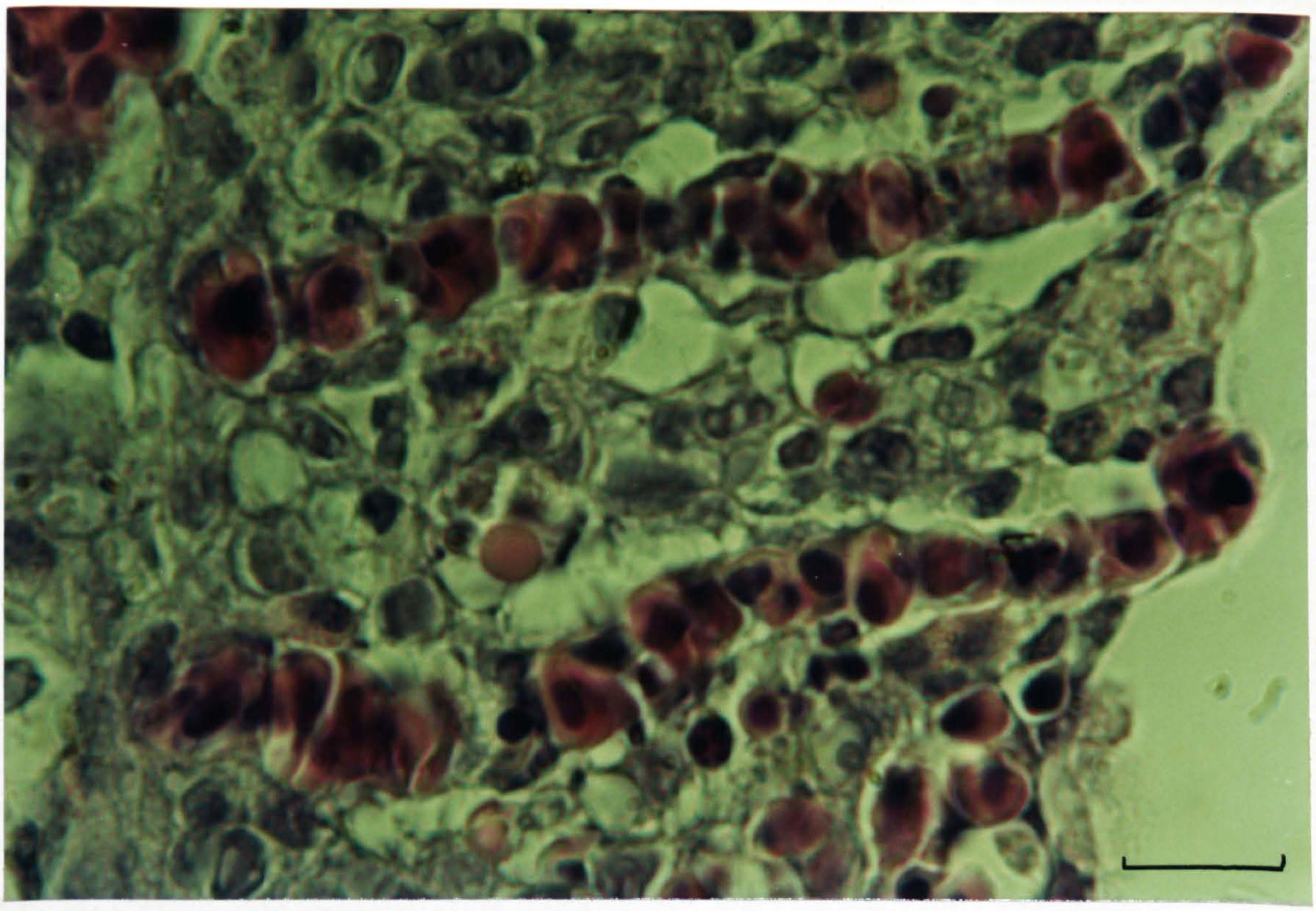


Plate 6.5 :- A light micrograph of a section of gill after four weeks of infection with *Dactylogyrus vastator* showing separation of epithelial cells from the secondary lamella.
Scale bar = 0.05 mm

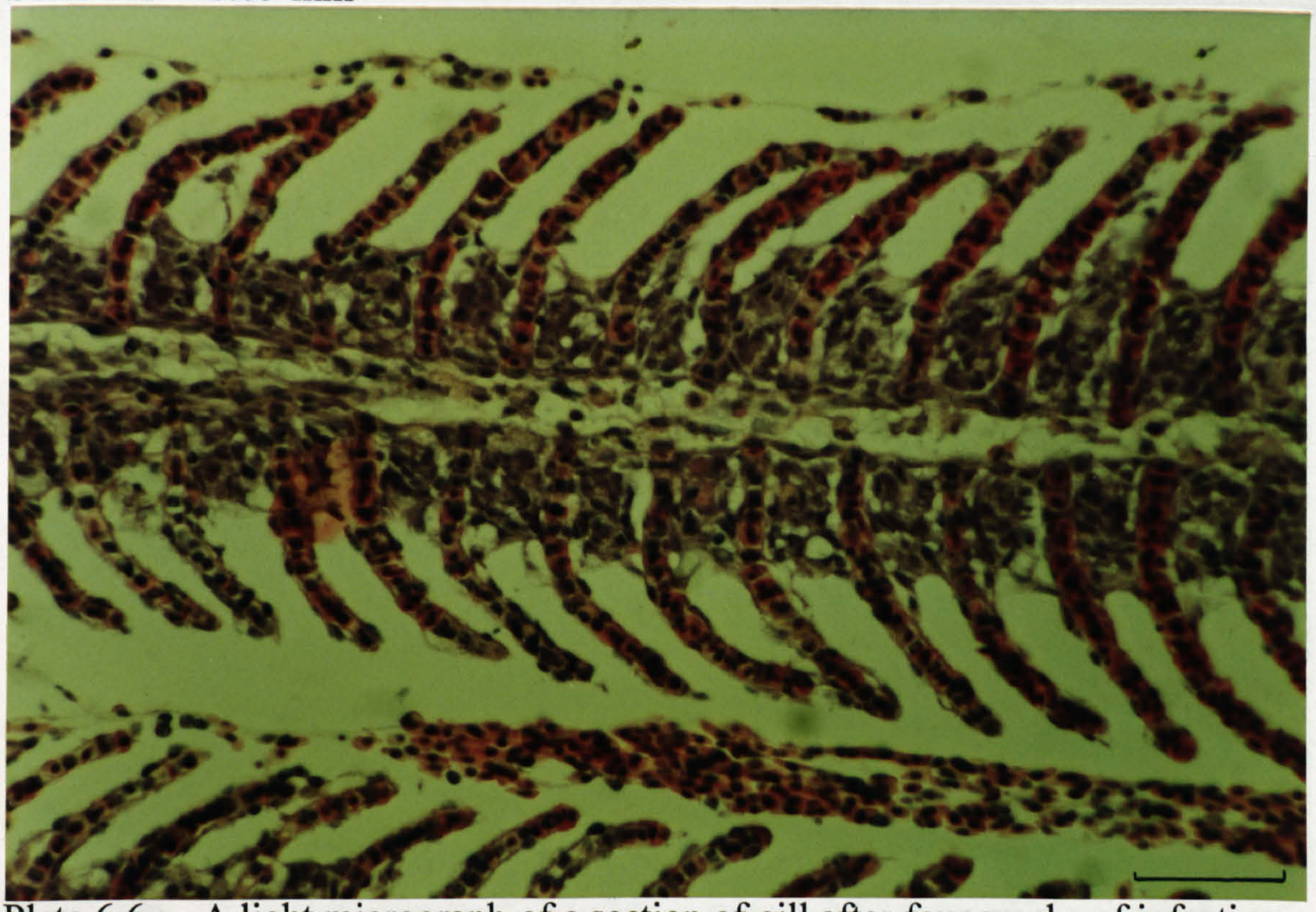


Plate 6.6 :- A light micrograph of a section of gill after four weeks of infection with *Dactylogyrus vastator* showing adhesion of adjacent secondary lamellae.
Scale bar = 0.05 mm

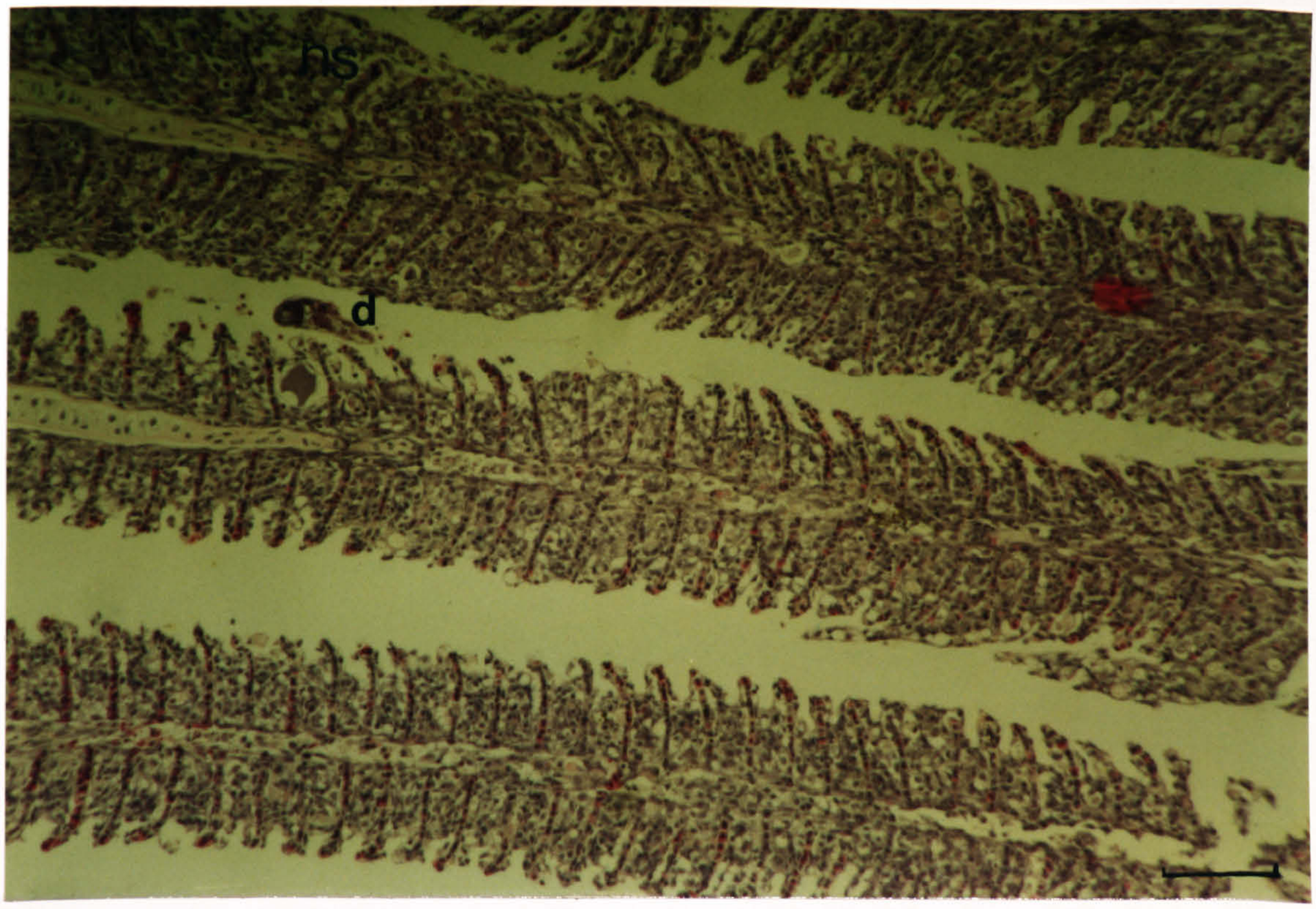


Plate 6.7a :- A light micrograph of a gill section after six weeks of infection with *Dactylogyrus vastator*.
d = *D. vastator* ; ns = mass of necrotic cells
Scale bar = 0.1 mm

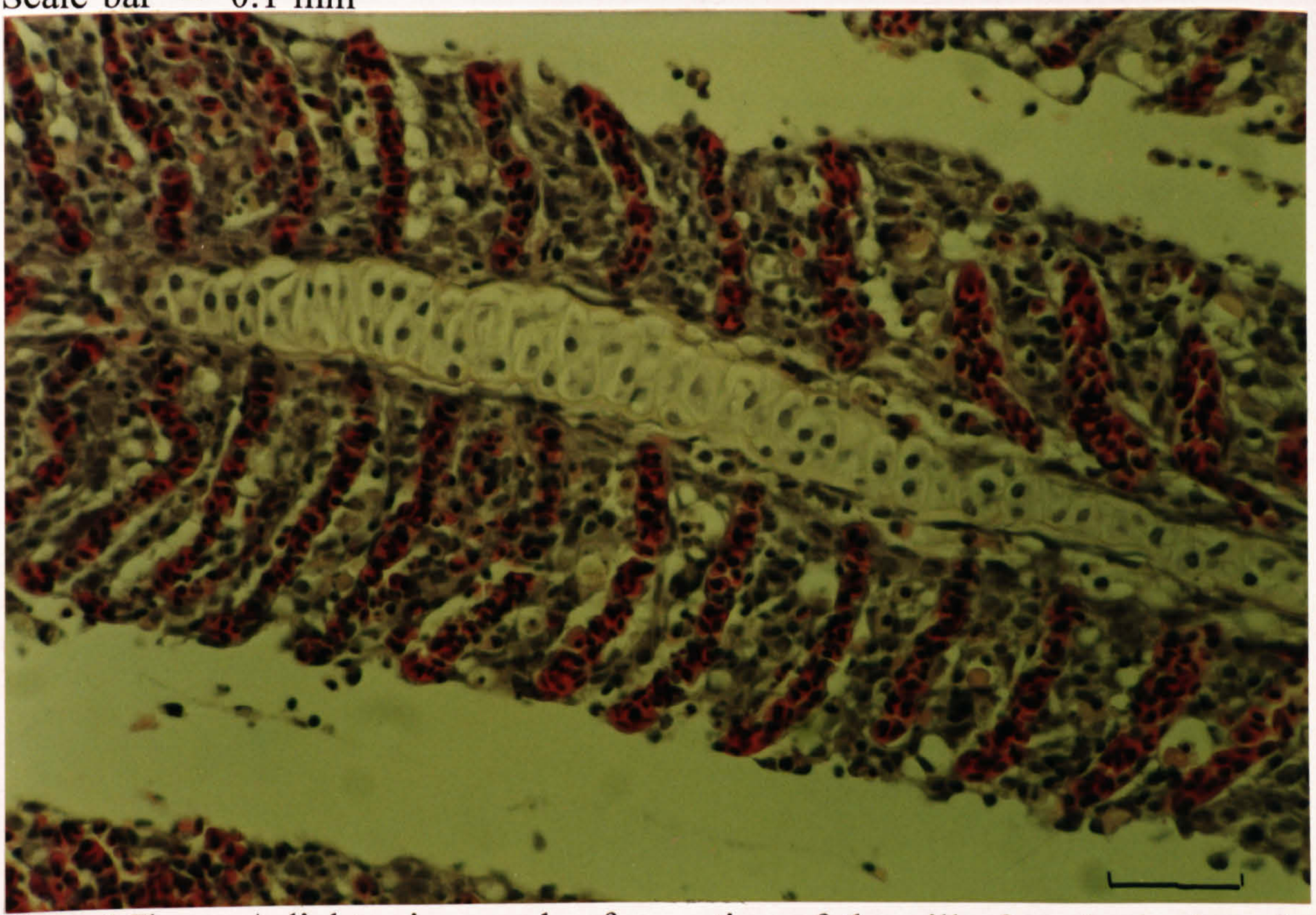


Plate 6.7b : A light micrograph of a section of the gill after six weeks of infection with *Dactylogyrus vastator* showing hyperplastic tissue response and fusion of adjacent lamellae.
Scale bar = 0.1 mm

secondary lamellae infiltration of haemocytes can be seen in the discontinuous cell array, where the continuity has been damaged by the penetration of parasite hamuli and by the cellular reaction to infestation. The secondary lamellae show a more pronounced hyperplastic tissue response and there is complete fusion of adjacent lamellae. In some areas the spaces between the epithelial cells are filled with congested blood and sloughed tissue. Oedema can be seen at the base of the secondary lamellae. Plate 6.8 shows a higher magnification of the hyperplastic tissue and granulated cells which may originate from leucocytes or other white blood cells.

Under the light microscope (Plate 6.9) the hamuli of *D. vastator* can be seen to penetrate towards the primary lamella of the gill arch during attachment to the host. At the point of attachment there is hyperplastic tissue formation surrounding the rear end of the worm (Plate 6.10).

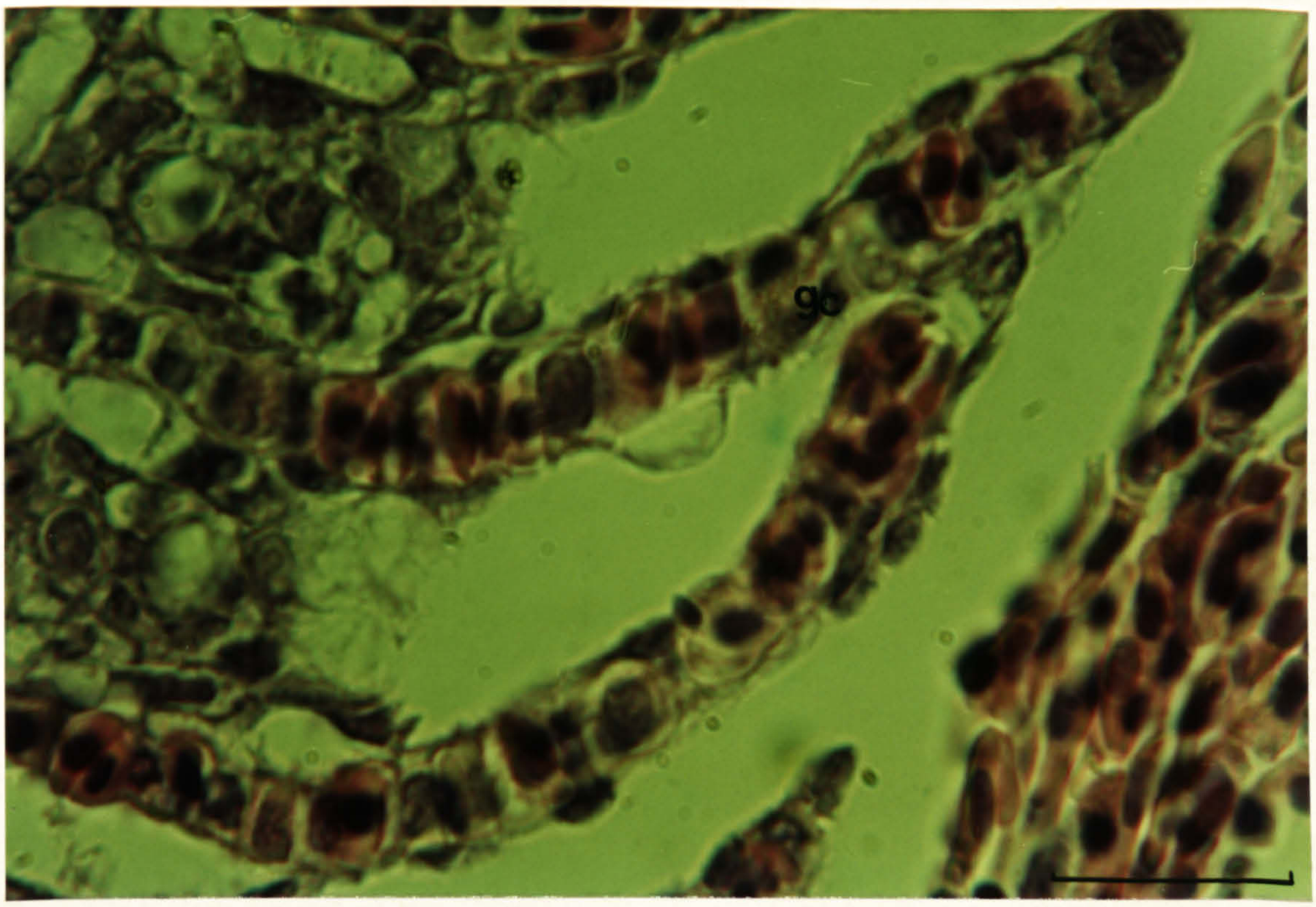


Plate 6.8 :- A light micrograph of a gill section after six weeks of infection with *Dactylogyrus vastator*.
gc = granulated cells
Scale bar = 0.1 mm

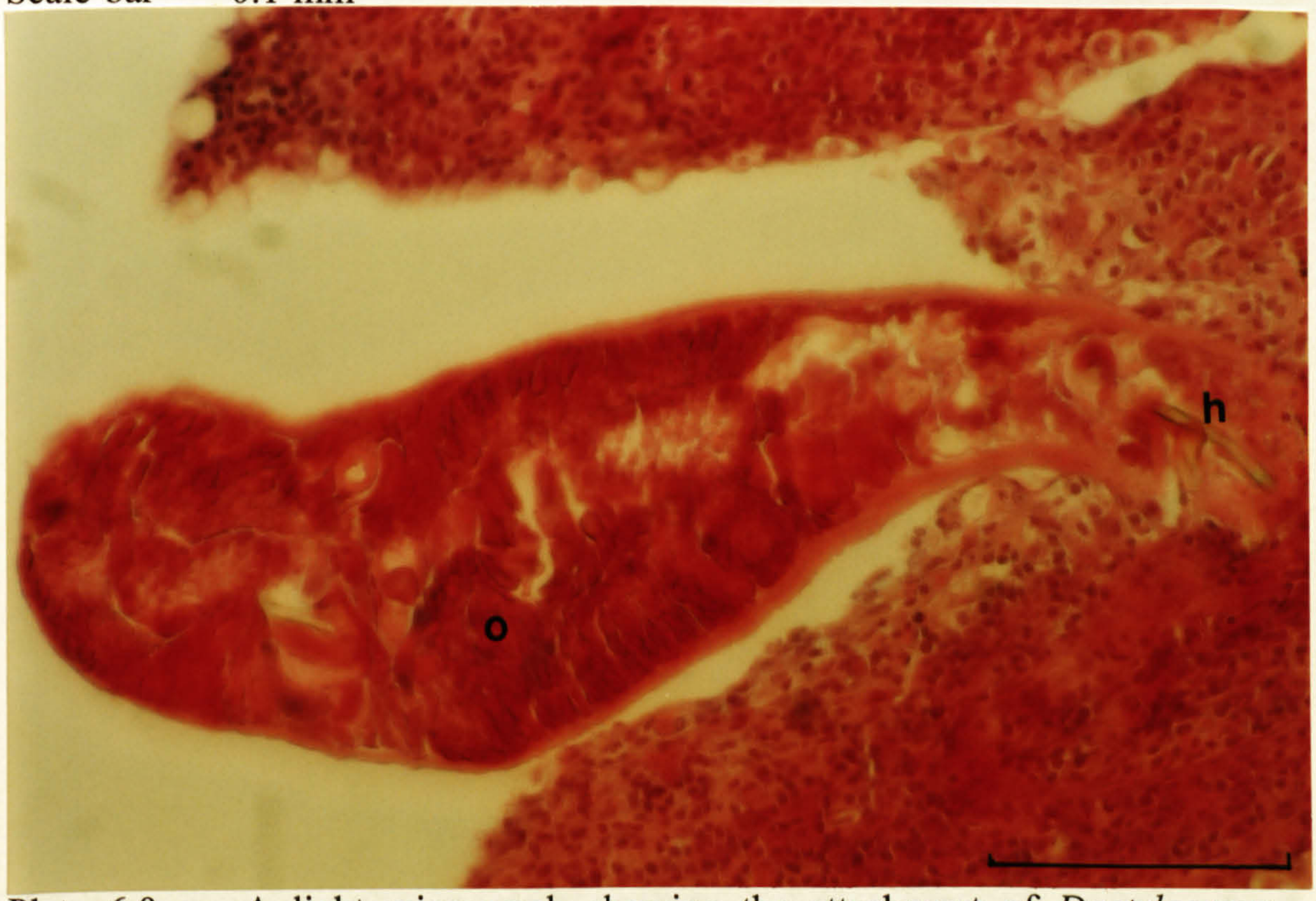


Plate 6.9 :- A light micrograph showing the attachment of *Dactylogyrus vastator* to the gill of *Cyprinus carpio*.
h = hamuli ; o = ovary
Scale bar = 0.05 mm

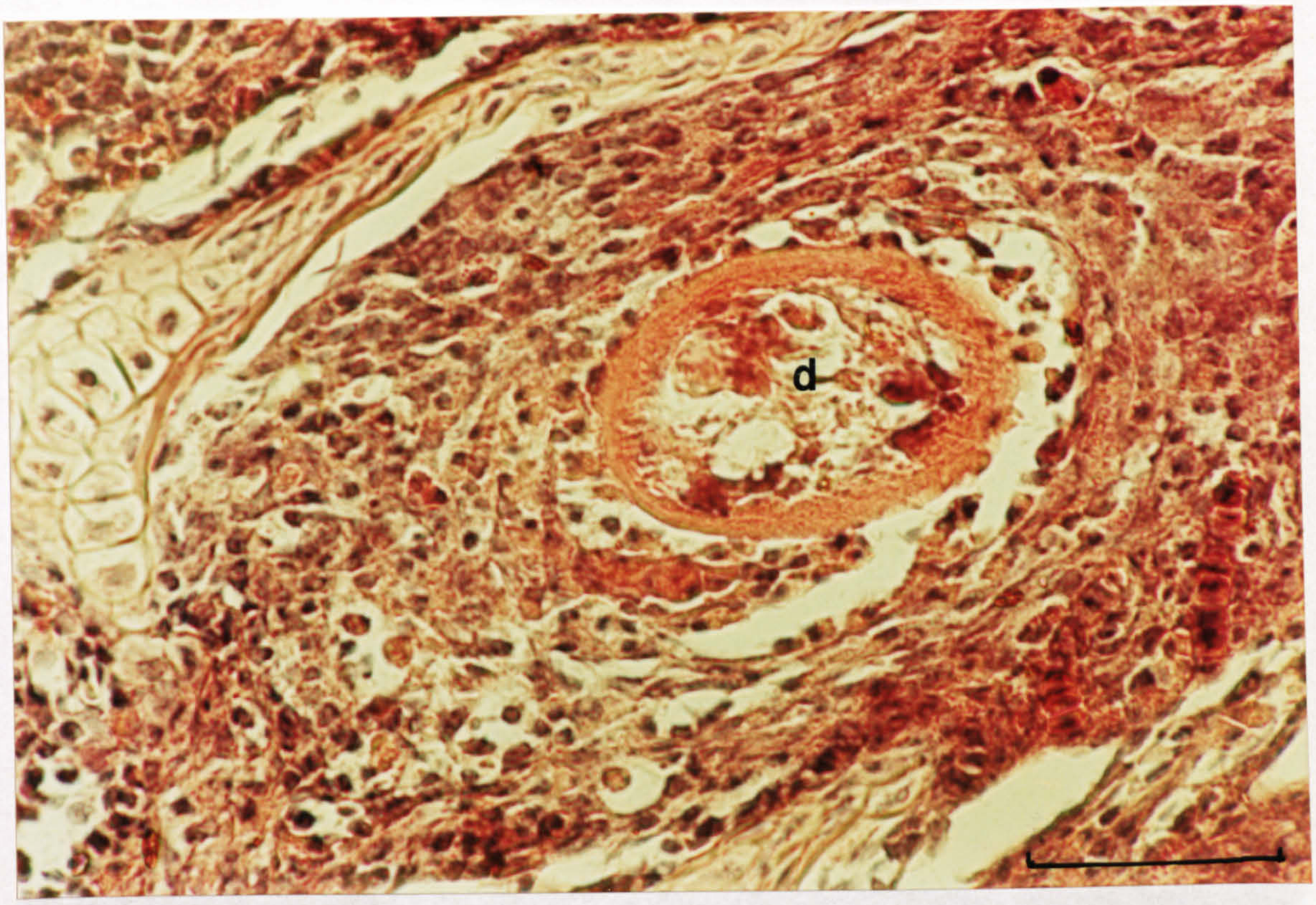


Plate 6.10 :- A light micrograph showing the posterior end of *Dactylogyrus vastator* covered with hyperplastic tissue.
d = *Dactylogyrus vastator*.
Scale bar = 0.05 mm

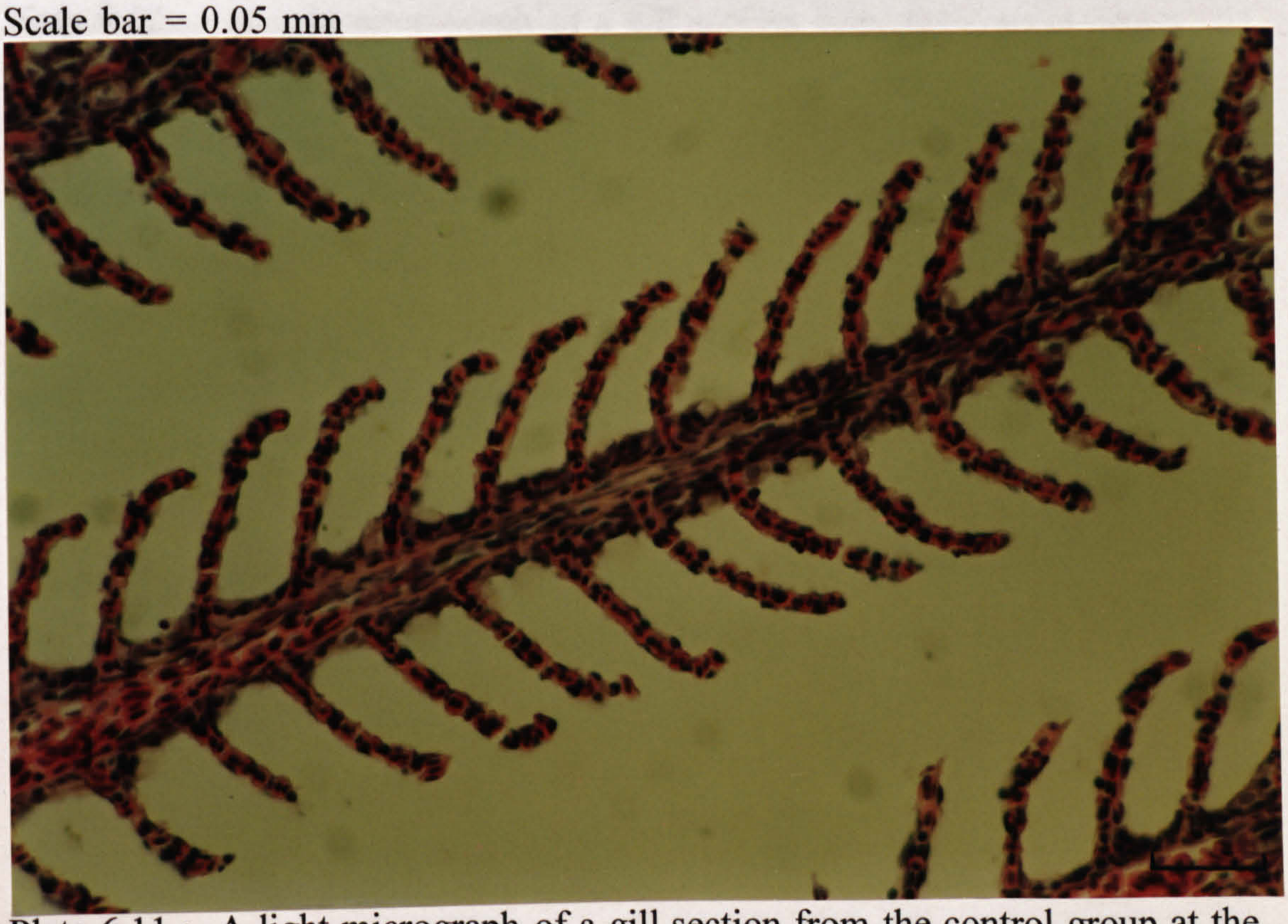


Plate 6.11 :- A light micrograph of a gill section from the control group at the start of experiment.
Scale bar = 0.1 mm

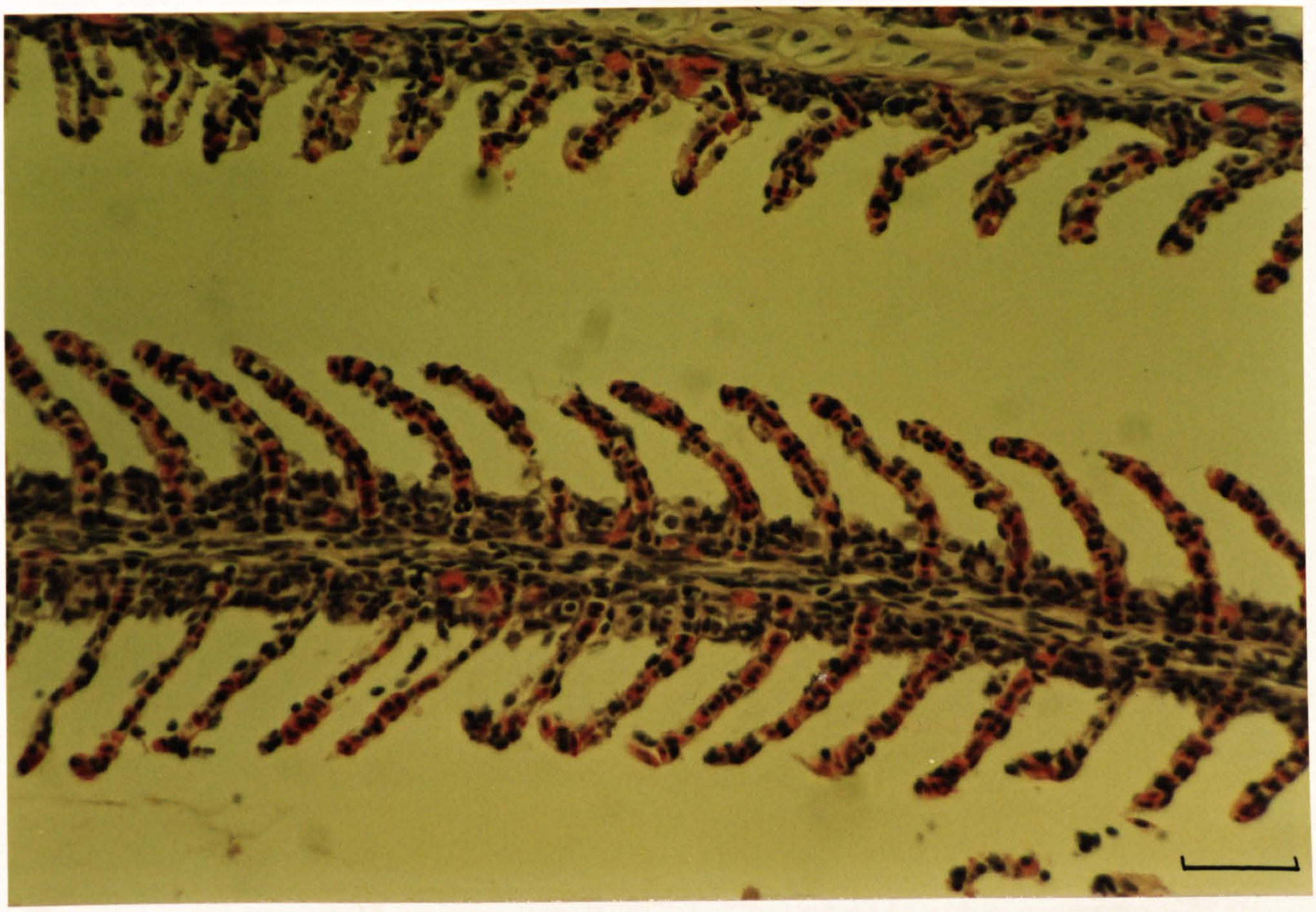


Plate 6.12 :- A light micrograph of a gill section from the control group, six weeks after the start of the experiment.
Scale bar = 0.1 mm

6. 4. Discussion

The immediate reaction of fish to the attachment of *D. vastator* is slight (Paperna, 1964). Paperna (1963b) reported that there was an increased mitosis not only close to the point of attachment but also in other areas and this ultimately results in the formation of hyperplastic tissue. Paperna did not report any other cellular reaction at the site of attachment.

It was observed in this study that the immature stages of *D. vastator* were mobile over the gills whereas the adults were permanently attached to a particular place. Molnàr (1980) found that during the first three days of infestation the mobile immature *D. vastator* localized on the surface of the gill epithelium. The immature *D. vastator* loosely attached by sinking their hamuli into the epithelial cells of secondary lamellae. The most conspicuous and clear response occurred at the apical margin of the edge of the primary lamellae of infected fish. The subsequent pathological changes in the gill extended along the primary gill filament towards the base and as a result the delicate respiratory surfaces were damaged. In badly affected fish the gill filaments merged into a large rounded mass of cuboidal epithelium which Paperna (1964) reported may occasionally elongate and protrude from the margins of the gill filament. In the present study a general tissue reaction developed in infected carp in addition to local lesions at the attachment sites. Cellular proliferation began in the inter-

lamellar gill epithelium, normally consisting of 2 - 3 cell layers. The epithelial cells are normally characterized by an abundance of nuclear chromatin, the amorphous shape of the nucleus and paucity of the cytoplasm, but in *D. vastator* infections these cells showed swollen, round pale-staining nuclei, and a turbid cytoplasm with signs of vacuolar degeneration. Similar changes were found in *D. lamellatus* infections (Molnàr, 1980). Hyperplasia of the epithelial cells is accompanied by massive proliferation of goblet cells which are arranged at the borders of the epithelial masses. Under pressure of the encroaching epithelial cells, the delicate vascular network losses its symmetry and is displaced proximally (Paperna, 1964). Subsequently the greater part of the vascular network was destroyed and the remaining traces formed symmetrical lacunae in the centres of the epithelial masses. In this study in some instances the cartilage was affected and the tips of the primary lamellae were damaged. A significant number of cells became necrotic on the surface of the primary lamellae among the normal cells separating adjacent gill filaments. Part of the filaments were fused by adhesion, but still showed a well defined structure. The hamuli of adult worms often penetrated as far as the cartilaginous supporting structure of the gill filament.

In carp fingerlings 20 - 25 mm long, infestation with 70 - 100 parasites may be fatal even before typical foci of hyperplasia appear (Paperna, 1964). In these cases, where the parasite sinks its hamuli into the respiratory epithelium,

a small area of hyperplasia forms around the parasite, which disrupts the normal functioning of the branchial respiratory epithelium and usually results in the death of the fingerlings. In lower level infections however, the fingerlings do not succumb to the infestation, gill tissue becomes more hyperplastic and is a less suitable location for the parasite which is forced off the gills (Paperna, 1964c). Similar responses in branchial epithelium has been described in infestations with other gill parasites, eg. *Dactylogyrus macracanthus* on *Tinca tinca* and *Nitschia sturionis* on the gills of *Acipenser nudiventris* (Paperna 1964). In this study of *D. vastator* infestations the gross tissue response was first observed at the tip of the gill filament and later spread to the basal region. However, no such tissue response was reported in carp infected with *D. extensus* or *D. anchoratus* (Paperna, 1964).

In infections with *A.vistulensis* on *Silurus glanis* from the fourth day of infection predominant regressive and progressive lesions were reported by Molnàr (1980). After three weeks of infection progressive lesions resulted chiefly in epithelial proliferation which destroyed the normal lamellar appearance of the gill filaments and in some cases led to the adhesion of gill filaments. These changes were very similar to those found in this study.

The histological changes in the gills of carp caused by the presence of *D. vastator* apparently makes the gills an unsuitable habitat for the parasites and

ultimately leads to their loss from the affected gills (Paperna, 1963b). Thus the formation of hyperplastic tissue on the gills of the host may be considered as a defence mechanism whereby the host can act against heavy infections. This defence mechanism may have a regulatory action on *D. vastator* populations.

Round cell infiltration was observed by Prost (1963) in carp infected with *D. extensus* but was not reported by Molnàr (1980) in sheat fish infected with *A. vistulensis*. In this study an increase in eosinophilic leucocytes, which is generally characteristic of parasitic infections, was not observed. Paperna (1964) and Kollmann (1972) reported that goblet cell proliferation is a characteristic of *D. vastator* infection. Molnàr (1972) suggested the mucus coating of infected gills originated from the disintegration of vacuolized, necrotic epithelial cells. In this study goblet cell proliferation was not apparent, but there was increased mucus production.

In these experiments, at the end of the 6 week experimental period infected carp showed abnormal behaviour. The fish were sluggish and were found at the water surface taking in air. This behaviour presumably directly results from the loss of functional gill tissue because of *D. vastator* infection and the inability of the fish to take up sufficient oxygen. If such fish do not die directly as a result of *D. vastator* infection, they would be very vulnerable to predators.

Chapter 7

Host response to *Dactylogyrus vastator* infections on carp

7.1. Introduction

Exposure of fish to parasites and pathogens often results in survivors becoming resistant to subsequent disease caused by the same pathogen or parasitic organism. In the case of parasites resistance is especially well developed against endoparasites rather than ectoparasites (Ellis, 1988). The piscine immune system is well developed and is normally quite efficient. Immunity represents a specific capability against a specific antigen (pathogen, parasite). Immunological reactions, in general, are characterized by a high degree of specificity. A particular antigen elicits the production of a specific antibody that will react only with that antigen or closely related antigens. Such similar antigens usually occur in closely related organisms (Schad, 1966). Foreign proteins of high molecular weight, notably polysaccharides or lipids, act primarily as antigens whose presence in the serum and other body fluids of fish stimulates the formation of specific antibodies, predominantly immunoglobulins (Schäperclaus, 1991b). Under the influence of an antigenic stimulation, the organism acquires an immunity which always signifies an individually acquired state of resistive capacity against certain parasites, their metabolic products or against other substances (Schäperclaus, 1991b). Humoral immunity is caused by the formation of circulating antibodies, the immunoglobulins. Cellular (cell mediated) immunity is based on the sensitization of lymphocytes and also

influenced in fish by non-specific and specific humoral factors such as enzymes and antibodies. Adaptive immunity is specific to the challenging pathogen or parasite and may persist for a long period of time (immune memory). This may relate to the changes in host lymphocyte populations reflected in the protein composition of blood sera.

Fish cultured at high densities are stressed by adverse environmental factors and their resistance to parasites and diseases may be severely compromised (Woo, 1992). Although many metazoan parasites of fish have been studied in relation to their life-cycle and pathogenicity or pathology, investigations on fish resistance mechanisms against parasite infections are relatively few (Williams & Hoole, 1992).

However, fish have been found to respond immunologically to a number of parasitic infections such as *Cryptocaryon irritans*, *Myxosoma cerebralis*, *Gyrodactylus bullatarudis*, *D. vastator*, *Neobenedenia melleni*, *Telogaster opisthorchis*, *Ligula intestinalis*, *Diphyllobothrium* spp and *Lernaea cyprinacea* (Woo, 1992).

There is evidence that antibodies may be present in skin and gill mucus, which might be important in immune responses to ectoparasites. This has been shown by Nigrelli & Breader (1934 cited in Shaharom Harrison, 1984) who

found that *Epibdella melleni* could survive longer in mucus from susceptible fish than in mucus from "immunised" fish. Immunoglobulins have been found in the serum and mucus of *Pleuronectes platessa* (Fletcher & Grant, 1969). Fletcher & White (1973) also demonstrated lysozyme activity and antibody production in the skin of *P. platessa*. Hines & Spira (1974) also demonstrated an epidermal immune response in fish.

The acquired resistance of fish to monogenean infection has been demonstrated in a number of cases, such as *Gyrodactylus alexanderi* on stickleback, *Gasterosteus aculeatus*, by Lester & Adams (1974), *Gyrodactylus bullatarudis* on *Poecilia reticulata* by Scott (1985) and with *Dactylogyrus vastator* on *Cyprinus carpio* by Paperna (1964) and Vladimirov (1971).

Carp which have been infected with *D. vastator* eventually become highly refractive to subsequent challenges with the parasite (Paperna, 1964). When carp are infected with *D. vastator* they develop a relative super-invasion immunity, when not all the parasites from the primary infection have died out. The duration of this phase is 20 - 30 days at 18 - 20 °C and about 45 days at 14 - 16°C (Vladimirov, 1971). Subsequently, after the death of all the parasites from the first infection, the super-invasion becomes a post-invasion form of immunity. In general this immunity can be maintained for more than two months under optimum conditions (Vladimirov, 1971). The immunity depends

upon the intensity of infection and temperature. The increase in resistance of the infected fish is a result of activation of their protective mechanisms such as complement, properdine, phagocytic reaction and specific antibody formation (Vladimirov, 1971). The latter was found to be particularly important in the rejection of *D. vastator* where higher levels of antibody corresponded with lower numbers of parasites.

Paperna (1964) stated that cichlid fish have an acquired resistance to *Cichlidogyrus* infections, and he found that in the wild the cichlid fish often lost their infections. Lester & Adams (1974) demonstrated that stickleback were partially refractory to challenge infections with *G. alexanderi* one week after exposure of fish to a formalin bath which removed their primary infection. Eels, especially pigmented eels, show acquired resistance to the gill parasitic monogeneans *Pseudodactylogyrus anguillae* and *P. bini* (Slotved & Buchmann, 1993).

Nybelin (1925), Nordquist (1925) and Wunder (1929) observed that infestation with *D. vastator* was limited to carp up to 50 - 60 mm in length. As the fish approached a weight of 5 grams (length 60 - 70 mm) there was a sharp drop in infestation with *D. vastator* and eventually a total disappearance. Bauer (1959) concluded that the disappearance of the parasites from the larger carp was due to acquired immunity. Age of the host has long been known as an

important critical factor in determining susceptibility to infections by parasites (Sandground, 1929).

7.2. Materials and Methods

7. 2. 1. Experimental method

The 250 carp used in this study were received from the Munton and Fison PLC hatchery and had no known previous history of *D. vastator* infections. They were five weeks of age with a mean standard length of 3.47 cm and total weight of 2.014 grams. They were maintained in flow-through tanks at 17°C, this constant temperature being maintained by an immersed water heater. Fish were left in the tanks for two weeks to acclimatize before starting experiments. The fish were separated into two groups (experimental fish and control fish) for this experiment. One group of 150 fish was infected (challenged fish) by mixing them in a tank with 20 fish with *D. vastator* (average 14.5 worms/fish) infections. Infected and uninfected fish were left to cohabit for 10 weeks and 10 fish were checked twice a week to determine the number of *D. vastator* present. After 10 weeks the experimental fish had an average of 5.33 worms/fish. Control fish (100) and experimentally infected fish (100) were subjected to a formalin bath at 180 ppm for an hour, with adequate

aeration, to remove any infections. After one hour the fish were split and transferred to two experimental tanks of 68 cm x 57 cm x 34 cm at 17°C and left without any disturbance for a further two weeks before challenge infections. Fish were then subjected to infection as described above. Control fish were also divided between 2 tanks of the same size. After two weeks the original infected fish were removed. Samples of 20 fish from each tank were taken at 7, 21, 28, 35 and 42 days post-infection and the number of *D. vastator* counted. This experiment was repeated.

7. 2. 2. Analysis of the data

The experimental data were checked for normality, and if they showed a non-normal distribution they were subjected to non parametric STP, Dunns and Kruskal-Wallis tests. Normally distributed data was subjected to one-way anova.

7. 2.3. Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) was performed using a Mini-Protean II dual slab cell system (Biorad). Electrophoresis was carried out in the discontinuous buffer system of Laemmli (1970) with a 4% stacking gel and 12% separating gel.

7. 2.3.1. Stock solutions

The following stock solutions were prepared.

Acrylamide / bis solution

A solution of acrylamide (29.2g/ 100ml) (Sigma) and N'N'-bis-methylene-acrylamide (0.8g / 100 ml) (Biorad) was prepared and filtered using Whatman number 3 qualitative filter paper and left at 4°C. The solution was stored in the dark at 4°C and used within 30 days.

Sodium dodecyl sulphate (SDS)

A 10% w/v solution (Sigma) was prepared and stored at room temperature.

7.2.3.2. Preparation of separating gel

Acrylamide/bis stock solution and SDS stock solution were added to a 1.5M tris HCl buffer at pH 8.8, to yield a final concentration of 12%, 0.1% and 0.375M respectively. The mixture was deaerated to improve the polymerization reaction by using a vacuum pump for 15 minutes.

Freshly prepared ammonium persulphate solution(10% w/v) (Biorad) and N N' N'- tetramethylenediamine(TEMED) (Sigma) were then added to yield a final concentration of 0.05% (w/v) and 0.05% (v/v) respectively.

7.2.3.3. Preparation of stacking gel

Acrylamide/bis stock solution and SDS stock solutions were mixed with a 5% M Tris-HCl buffer at pH 6.8 to yield a final concentration of 4%, 0.1% and 0.125M respectively. The mixed volume was deaerated with the help of a vacuum pump for 15 minutes. Freshly prepared ammonium persulphate(10% w/v) and TEMED were then added to give a final concentration of 0.05% (w/v) and 0.1% (v/v) respectively.

7.2.3.4. Gel casting

After pouring the resolving monomer solution into the sandwich it was immediately overlaid with 500 µl of a 0.01 (w/v) SDS solution. The separating gel was allowed to polymerise for an hour. The stacking gel was cast after pouring off the overlaying SDS solution by adding more distilled water by a pasteur pipette. Well combs were inserted before pouring the stacking gels

where the samples were loaded for the electrophoresis.

7.2.3.5. Running conditions

Running time was approximately 45 minutes at a constant voltage setting of 200 V.

7.2.3.6. Samples used for electrophoresis

Blood from infected fish of the challenged and control groups was taken 35 days post-infection. The blood from naive fish also was taken. The fish from the control group had 23, 15, and 15 *D. vastator* respectively and the fish from the challenged group 6 and 9 *D. vastator* respectively. The tail of the fish was severed and blood collected in a small centrifuge tube. The collected blood was left in a fridge overnight at 4°C. On the following day the cells were separated off from the sera by centrifugation (Scotlab Micro Centaur, MSE, England) at 13,000g for 2 minutes; the supernatant was then transferred into another tube and served as a serum sample for electrophoresis. Each sample was mixed with an equal amount of electrophoretic sample buffer and mixed together in a centrifuge. Samples were boiled with sample buffer in a boiling water bath to denature the protein mixture into its polypeptide subunits. SDS

is an ionic detergent that is used as a dissociating agent and

B mercaptoethanol is a thiol reagent that cleaves disulphide bonds of the protein.

7.2.3.7. Molecular weight standards

In all gels, 5 µl of molecular weight standards (MWS) were loaded along with the samples. Low range SDS-PAGE MWS were used (Bio-Rad). MWS were diluted in sample buffer 1 : 20 for Coomassie blue staining.

Proteins included in the MWS were the following:

Rabbit muscle phosphorylase b - 97,400 Dalton

Bovine serum albumin - 66,200 Dalton

Hen egg white ovalbumin - 45,000 Dalton

Bovine carbonic anhydrase - 31,000 Dalton

Soybean trypsin inhibitor - 21,500 Dalton

Hen egg white lysozyme - 14,400 Dalton

7.2.3.8. Coomassie blue R-250 staining

This stain is used to visualize the protein bands in the gel clearly. 1% (w/v) of Coomassie Brilliant Blue (Bio-Rad) was diluted in fixative solution

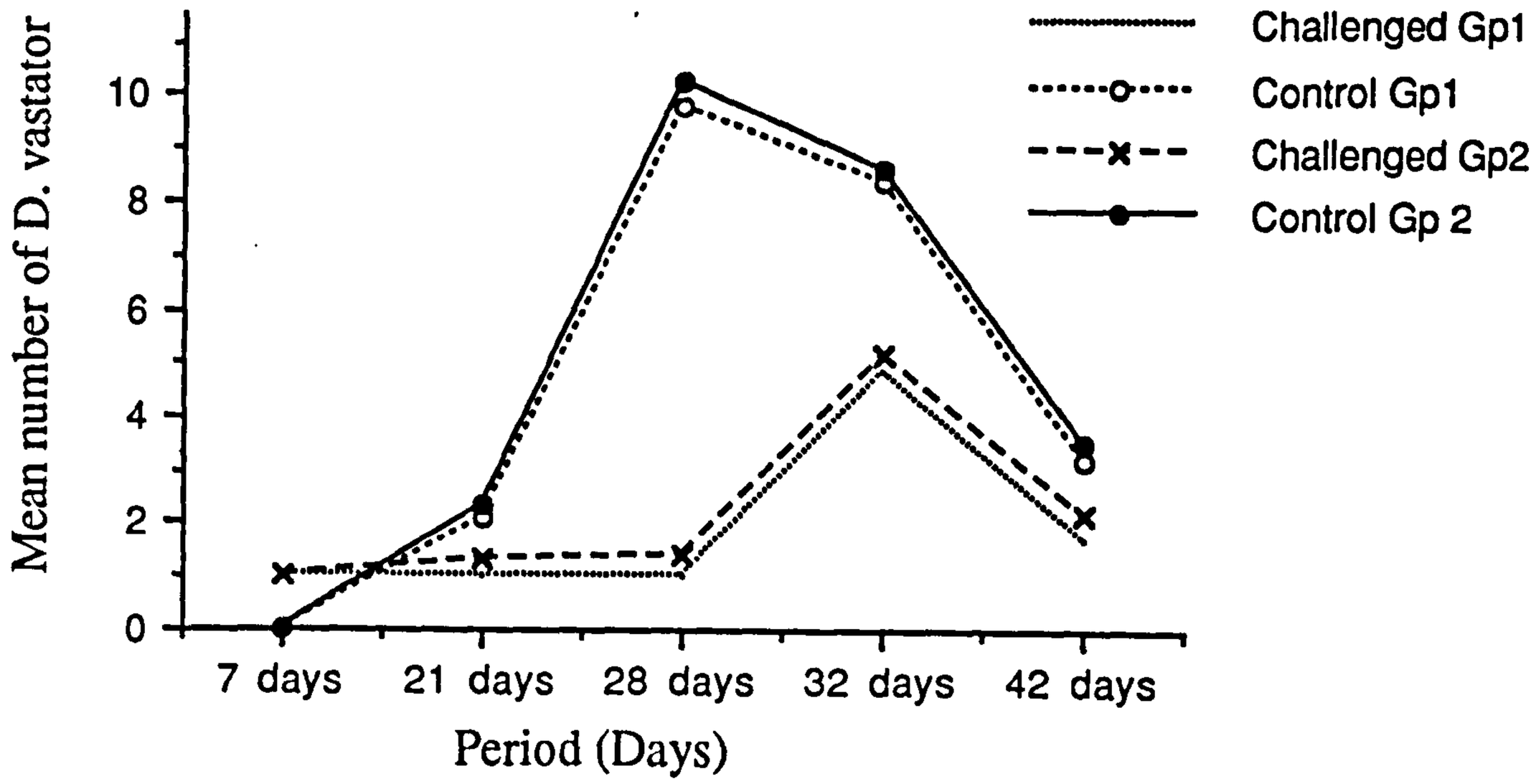
(40 % v/v methanol and 10 % v/v acetic acid). It was filtered through a number 3 Whatman filter paper to remove any insoluble material. Gels were stained overnight with 250 ml of the staining solution. The gels were destained with a 40% v/v methanol and 10% v/v acetic acid solution for 3 hours to remove the background colouration. Gels were then photographed by a Polaroid camera and stored in a polythene bag with distilled water.

7.3. Results

7.3.1. Challenge experiments

Most experimental carp became infected during the ten week infection period of the challenge. The changes in the *D. vastator* population over time in challenged fish differed abruptly from that in control fish. In control fish there was a continuous increase in mean intensity of *D. vastator* up to 28 days post-infection and a decline subsequently. The previously infected challenged fish did not show any real change in burdens with *D. vastator* up to 28 days post- infection. There was then a rise to a maximum mean intensity of infection at 32 days followed by a decline (Fig 7.1).

Fig 7.1 Variation in *D. vastator* number with time during challenge infection experiment.



The numbers of *D. vastator* from the two sets of challenged fish were analyzed statistically to determine any significant differences between them.

The data were tested for normality of the homogeneity of variances for the two challenge groups at 7, 21, 28, 35 and 42 days post-infection. In all cases the F_{max} is less than the F_s test value, thus the variances are homogenous and data normally distributed. A one way anova was carried out to determine the significant differences between the data sets. The F_s test values at 7, 21, 28, 35 and 42 days are 0, 0.7080, 0.3962, 0.05217, 0.3323 respectively. Thus there is no significant difference between the two challenged groups.

Similarly with the control groups the data were found to be normally distributed and were therefore compared using a one way anova. The F_s test values at 7, 21, 28, 35 and 42 days post-infection are 0, 0.1432, 0.0296, 0.004095, 0.1146 respectively. Thus there is no significant difference between the two control groups.

The two challenged and two control groups were then combined for further analysis.

Table 7.1 :- The F values for homogeneity of variances and the Kruskal-Wallis test for the challenged and control fish groups.

Period	F_{test}	F_{max}	H value for Kruskal-Wallis test
21 days	2.49	2.46	5.408 *
28 days	24.07	2.86	26.141 ****
35 days	9.1099	3.28	0.61 NS
42 days	1.04	2.86	1.784 NS

NS Not significantly different

* Significantly different at probability level 0.05

**** Significantly different at probability level 0.05, 0.01, 0.005 and 0.001

Differences between challenged and control groups at the same sampling times were tested using the Kruskal-Wallis test, since the data was not normally distributed.

At 7 days post-infection control fish were apparently uninfected whereas challenged fish had a low mean intensity of infection. By 21 and 28 days post-

infection the control fish had a significantly greater number of *D. vastator* than the challenged fish ($H = 5.408$ $P < 0.05$, $H = 26.41$ $P < 0.001$). However, at 35 and 42 days post-infection the numbers of *D. vastator* were not significantly different between challenge and control fish ($H= 0.061$, $P > 0.05$, $H = 1.784$, $P > 0.05$) although control fish were more heavily infected (Table 7.1).

7.3.2. Gel electrophoresis

Gel electrophoresis of serum from challenged and naive fish showed a number of additional bands in the former. Seven additional bands were visualised in all challenged fish. By comparing their position on the gels in relation to the distance moved by molecular weight standards the bands from challenged fish were found to correspond to molecular weights of 29.09, 30.91, 33.64, 35.45, 36.36, 37.27 and 39.09 KD (Plate 7.1 & Fig 7.2).

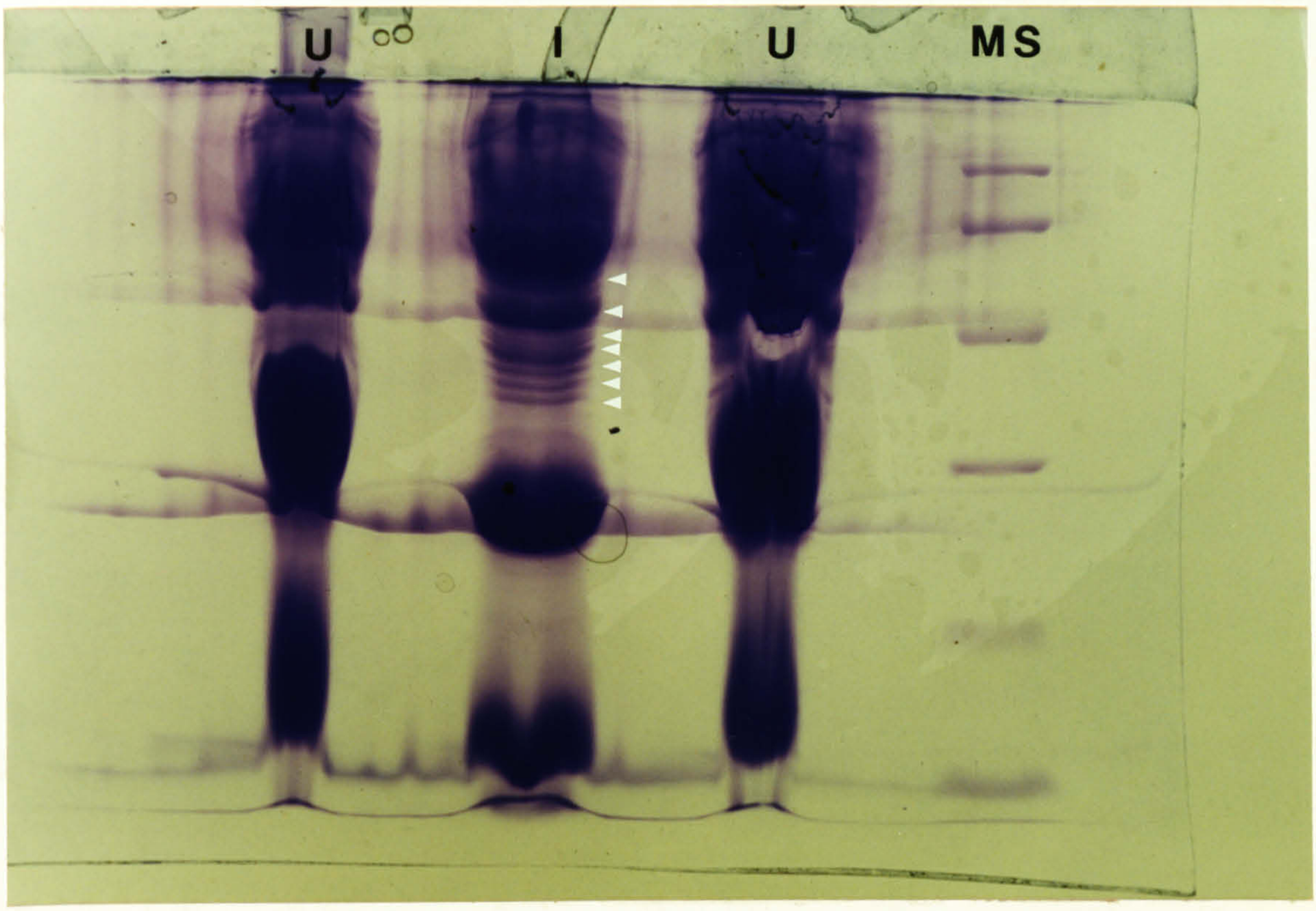
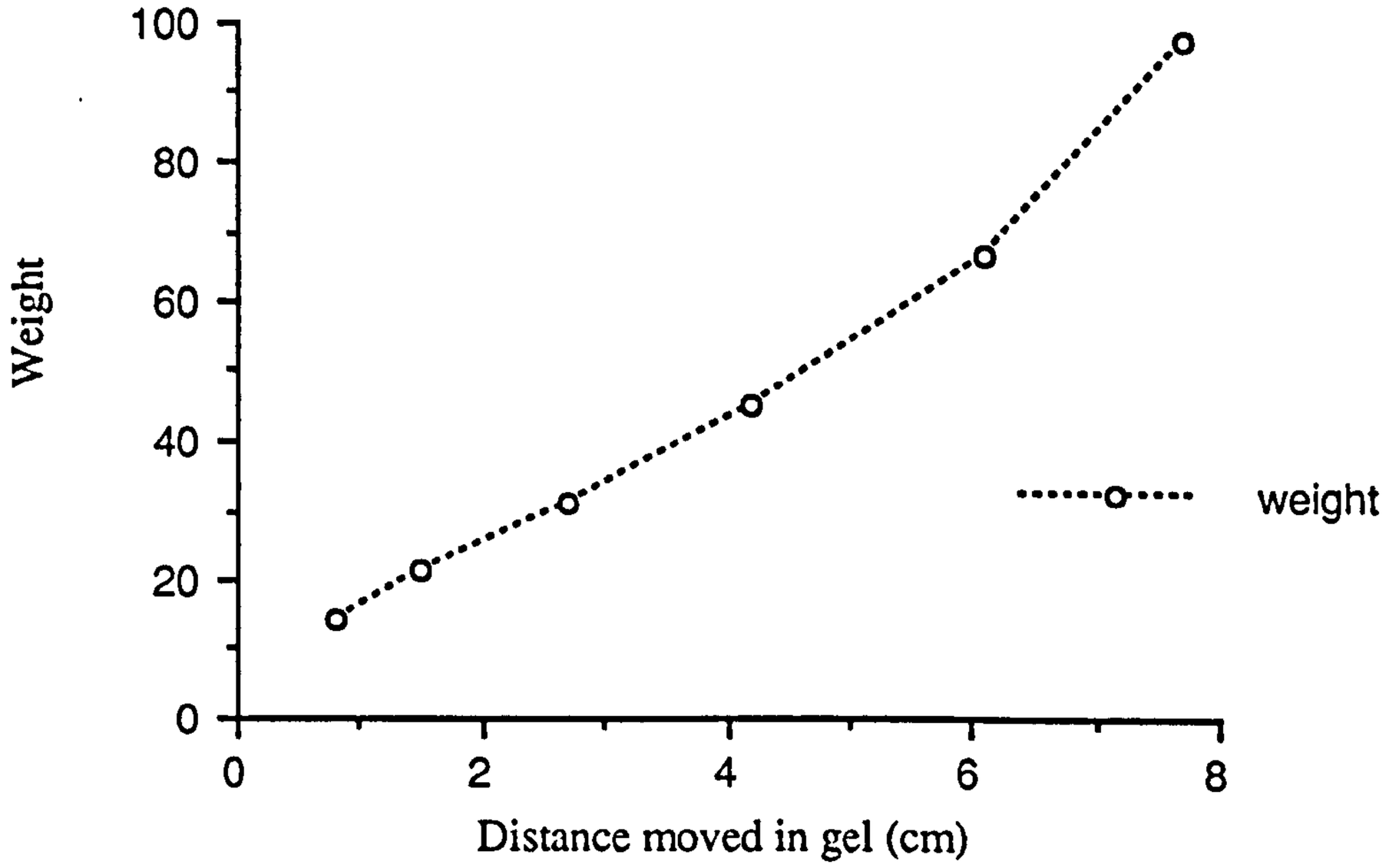


Plate 7.1 :- SDS - PAGE gel of blood sera from carp infected (I) infected with *Dactylogyrus vastator* and uninfected (U) carp stained with Coomassie blue. The arrows show the position of several additional bands.

Fig 7.2 :- The relationship between the molecular weight and distance moved in a gel electrophoresis



7.4. Discussion

7.4.1. Challenge experiments

From this experiment it is clearly evident that the carp acquired resistance against *D. vastator*. In general the pattern of population growth in *D. vastator* on *C. carpio* shows a sharp initial increase, reaching a maximum and followed by a decrease. This pattern was shown by the naive fish in these experiments reaching a maximum by day 28. In challenged fish however parasite numbers increased only slowly so that from days 7 - 28 there were significantly more *D. vastator* in the control fish. By day 35 parasite numbers had increased in the challenged fish so that a significant difference no longer existed, although control fish were more heavily infected. There is thus evidence from these experiments that the carp were able to mount an immune response against *D. vastator* which apparently lasted for 28 days.

Immunological reactions involve not only the tissue affected by the pathogen but the entire organism, mobilizing in the process both specific factors of immunity (Woo,1992). Vladimirov (1969) demonstrated the possible existence of an immune system in carp to *Dactylogyrus*. In a subsequent paper Vladimirov (1971) investigated the nature of the immune response. In infected fish an immune response apparently persists for not less than 2 months, rather

longer than in the present study. The extent of the immunity depends upon the degree of infection of the host, temperature conditions and the time after infection. Serum antibody formation and activation of the complement system seemed to be the main factors involved in resistance to the parasite. Two-year old carp infected with 400 - 450 *D. vastator* per fish showed antibody titres raised to 1 : 1536. In the serum of such fish the period of viability of larvae and adults of *D. vastator* was reduced by 15 - 25% and 10 - 20% respectively, compared to the serum of non-infested fish. Valdimirov also showed that these antibodies were highly specific for *D. vastator* compared with infections of *D. extensus* and the ciliate *I. multifiliis*. Non-specific defensive mechanisms including lysozyme activity, properdine and complement activity were also involved but seemed to be less important than serum antibodies. Valdimirov also found that it was possible to at least partially immunise carp against *D. vastator* by injection of fish with parasite derived antigens. However, grass carp (*Ctenopharyngodon idella*) infected with *Dactylogyrus lamellatus* did not show any response against challenge infections (Molnàr, 1971c). Acquired immunity of fish to further infection with monogenetic worms was described by Nigrelli (1937) in marine fish which acquired temporary immunity to *Epibdella melleni*. Attempts by Nigrelli & Breder (1934, cited by Paperna 1964) to evoke immunity by injecting antigenic material prepared either from the monogeneans or from immunised fish failed, but mucus of immunised fish killed *Epibdella melleni in-vitro* whereas the mucus from non-immunised fish did not kill the

parasite. Temporary 'self cure' of fish from their parasites was observed in *Tilapia nilotica* infected with *Cichlidogyrus tilapiae* (Paperna, 1963a). Scott & Robinson (1984) have reported that guppies are refractory to challenge infections immediately after recovery from initial infection with *Gyrodactylus bullatarudis*, indicating some sort of acquired host resistance. Lester & Adams (1974) reported a similar phenomenon. Scott (1985) elucidated the responses of guppies (*Poecilia reticulata*) against *Gyrodactylus bullatarudis* and found that previously infected guppies have a reduced number of parasites in later infections than non-primed guppies. Host response was detected in guppies against *G. bullatarudis* even 6 weeks after the initial infection (Scott, 1985). The variability of the response between individual fish was noted by Scott (1985). If parasite population changes in the host are mediated immunologically, then temperature may have important consequences for host-parasite relationships, since the development of an immune response in fish is very dependent on temperature (Woo, 1992). The current experiments were carried out at a constant temperature of 17°C to remove any effects of change of temperature on the host response to the parasitic infection.

Studies on the infection of fish with glochidia, *Ichthyophthirius* and *Benedenia* show increasing mucus production related to the antiparasitic activity of the host (Paperna, 1964, Vladimirov, 1969). The glochidia or larvae of freshwater mussels live parasitically within a cyst formed by the epidermis of

the gills or external parts of the parasitised fish. Reuling (1919 cited in Sandground, 1929) counducted experiments on the parasitisation of the large blackmouth bass (*Micropterus salmoides*) by glochidia of *Lampsilis luteola* and showed that, after two or three heavy infections, the glochidia were sloughed off shortly after encysting. The acquired immunity was found to apply not only to the glochidia of *L. luteola* but also to other species of *Lampsilis*. He added that *in-vitro* tests showed that the serum of immune fish contained lysin which destroyed the glochidia and that the immunity was not concerned with purely mechanical factors.

7.4.2. Gel electrophoresis

The blood serum contains a variety of proteins including storage and transport proteins, enzymes, hormones and proteins associated with host cellular defence reactions (Hurd & Arme, 1984). Wang & Patton (1968) used a discontinuous electrophoresis to detect separate bands. The appearance of specific antibodies in fish invaded by *D. vastator* has been reported before by earlier studies (Paperna, 1964; Vladimirov, 1971) but not visualised. Challenged fish had additional bands as visualised in Plate 7.1 compared to uninfected fish. Pathophysiological changes in fish are often detected after the time when parasite numbers are presumably greatest, and thus the elevation of serum protein concentration may not be proportional to the number of parasites

present in the host. Additional protein bands found in parasitised fish are of host origin, and not derived from the parasite (Hurd & Arme, 1984). It has been suggested for other parasite systems that the host response to invading organisms, involving blood cells or humoral responses, may be responsible for changes in blood serum proteins (Hurd & Arme, 1984).

Summary and Conclusions

Temperature plays a major role in the population biology of helminth parasites of fish including *D. vastator* populations. Paperna (1964) reported the optimum temperature for *D. vastator* to lie between 24 - 28°C whilst Bauer *et al.* (1973) reported the range to be 22 - 24°C. However the present study demonstrated experimentally that at different temperatures the pattern of population change over time is similar but that maximum numbers occur at different times at different temperatures. Parasite abundance was highest at 19°C and lowest at 12°C. At 22°C parasite abundance was intermediate between the other temperatures. A water temperature of 19°C therefore appears to be closest to the optimum for the *D. vastator* used in this study, although this is a little lower than the optimum temperature reported by other authors.

Monogenean taxonomy is mostly dependent on the morphology and measurement of the scelerotised hard parts of the worm, especially the hamuli. In this study it was found that measurements of the sclerites of *D. vastator* reared at different temperatures varied significantly. If measurements are crucial in determining the taxonomic status of a specimen then species identity may be confused if the material in question is from a population at a temperature different from that of those used for original descriptions. It is thus essential to consider the role of temperature on morphological characters before making

a conclusion as to a parasite's identification. The right and left hamuli of *D. vastator* are not of the same size. The hamuli measurements of parasites reared at 12°C are larger than those reared at 19°C. The length of the hamuli in *D. vastator* is inversely related to water temperature. Thus, in any identification procedures it is necessary to consider the length of the hamuli together with the temperature of the environment to avoid possible errors.

The ultrastructure of *D. vastator* is similar to that of other closely related gill monogeneans in some of its characters such as tegument, cephalic lobes, hamuli, reproductive organs and accessory glands. An ultrasonication technique was used to produce debris-free hamuli which were then viewed using the scanning electron microscope. By virtue of this novel technique it is possible to study the functional morphology of the hamuli and to discriminate the hamuli of immature worms from those of adult worms. The hamuli have external and internal root processes which join to form a shaft. The hamuli have longitudinal striations which may possibly aid in a firm attachment by the worm to the host. Marginal hooks have a blade and spike. These sclerites help in firmly anchoring the opisthaptor.

Many monogeneans and crustaceans are known to be restricted to specific parts of the gill apparatus of their hosts. Some authors have suggested that the water current flowing through the gills may have an influence on the parasite

spatial distribution (Paling, 1968; Wootten, 1974; Hanek & Fernando, 1978a,b; Buchmann, 1988a,b, 1989). In this study, there were no significant differences in the distribution of *D. vastator* over the different gill arches, left & right side and internal and external gill filaments. There was a significant difference between the ventral proximal sector and the dorsal distal sector. This may reflect differential water flow over the gills. The fact that *D. vastator* showed relatively little spatial separation in this study may reflect the small size of the carp used, which perhaps have a more uniform flow of water over the parts of the gill apparatus. Where *D. vastator* did occur parasites were often clustered together and this may reflect the need to successfully mate.

Length frequency distributions of parasites are useful in distinguishing parasites which have recently invaded the host. An experiment was carried out using carp fry to determine how the length-frequency distribution of a *D. vastator* population changed over time. The length frequencies of *D. vastator* generally showed a bell shaped distribution. Initially the fish had a larger number of smaller sized worms. After 4 weeks there were fewer worms but these were larger. The smallest worms found were probably newly invaded. The lower numbers of larger worms found may reflect an age related mortality that serves to keep parasite numbers at a low level.

Dactylogyrus vastator was found to oviposit readily under *in-vitro* conditions but the rate observed was higher than in reported *in-vivo* observations. The oviposition rates were determined at three different temperatures (11°C, 13°C, 19°C) and were found to be inversely related to temperature. For *D. vastator* the rate of egg laying was 2.027 eggs hour^{-1} worm^{-1} at 11°C, 1.97 eggs hour^{-1} worm^{-1} at 13°C and 1.45 eggs hour^{-1} worm^{-1} at 19°C. Egg formation within the parasite was described and was generally similar to that reported for other monogeneans.

Dactylogyrus vastator is known to cause serious pathology to carp fry and fingerlings. Experimental infections showed that although immature worms were mobile over the gills adults were sedentary. The presence of the parasites causes a proliferation of gill tissue with fusion of gill lamellae. The hyperplastic response is caused initially by the attachment of the parasite's opisthaptor. The heavily infected fish showed abnormal behaviour, they were sluggish and were found at the water surface gulping air. This is probably a result of gill damage and an inability to take up enough oxygen through the usual respiratory mechanism and will eventually lead to death of fish.

Challenge infections showed that carp developed an acquired immunity to *D. vastator*. Providing exposed carp had significantly lower numbers of *D. vastator* than naive controls when challenged with the parasite and the rate

of increase in parasite numbers was much slower. Gel electrophoresis showed that immune fish had several extra bands of serum proteins which may represent antibodies against the parasite.

References

Adams, A. M. (1986). The parasite community on the gills of *Fundulus kansae* (Garman) from the South Platte River, Nebraska (U.S.A). *Acta Parasitologica Polonica*. **31**: 47 - 54.

Agapova, A. I. (1948). K voprosu izucheniya parazitarnykh zabolevanii molodiryb Alma-Atinskogo karpovogo pitomnika (Investigations on parasitic diseases of fry in the Alma-Ata carp nursery). *Izvestiya AN Kazakshoi SSR, Seriya Parazitologicheskaya.* **6**: 140 - 145.

Amlacher, E. (1970). *Taschenbuch der Fischkrankheiten (Pocket Book of Fish Diseases).* VEB Gustav. Fischer Verlag, Jena.

Anderson, M. (1981). The change with host age of the composition of the Ancyrocephaline (Monogenea) populations of parasites on thick - lipped grey mullets at Plymouth. *Journal of the Marine Biological Association of the United Kingdom.* **61** : 833 - 842.

Andrewartha, H. G. (1970). *Introduction to the study of animal populations.* 2nd ed., Chapman & Hall, London.

Andrewartha, H. G. and Brich, L. C. (1954). *The distribution and abundance of animals.* University of Chicago.

Arme, C. and Halton, D. W. (1972). Observations on the occurrence of *Diclidophora merlangi* (Trematoda : Monogenea) on the gills of whiting, *Gadus merlangus. Journal of Fish Biology.* **4** :27 - 32.

Baer, J. G and Euzet, L. (1961). Traité de Zoologie : Class des Monogénes In: Grassé, P. P. (Ed.). *Traité de Zoologie.* **4**: 244 - 325.

Bates, R. M. and Kennedy, C. R. (1990). Interactions between the acanthocephalans *Pomphorhynchus laevis* and *Acanthocephalus anguillae* in rainbow trout: testing an exclusion hypothesis. *Parasitology.* **100**: 435 - 444.

Bauer, O. N. (1954). Biology of the monogenetic trematode *Dactylogyrus vastator* Nybelin. *Trudy Leningradskogo Obshchestva Estestvoispytatelei.* **72**(4): 9 - 15.

Bauer, O. N. (1959). Ecology of the parasites of freshwater fish. Inter-relationships between the parasite and its habitat. *Izvestiya Gosudarstvennogo Instituta Ozernogo i Rechnogo Rybnogo Khozyaistva.* **49**: 1 - 206. (In Russian : Translation Israel Program for Scientific Translations).

Bauer, O. N. (1988). Epizootiological significance of Monogeneans. In: *Investigation of Monogeneans in the USSR*; Skarlato, O. A. (ed) Russian translation series 62 Publ. 1988. A. A. Baltema Vse soyuznoa simpozium po monogeneyama (1976; Leningrad) pp 137 - 142.

Bauer, O. N., Musselius, V. A; Sterlkov and Yu, A. S. (1973). *Diseases of pond fish.* Moscow: Izdatel 'sbvo "kolos" (In Russian: translation Israel Program for Scientific Translations).

Bauer, O. N. and Nikol'skaya (1954). Biology, development and piscicultural Importance of *Dactylogyrus solidus* Achmerow. *Trudy Problemnykh i Tematiheskikh Soveshchanii ZIN AN SSSR.* **4**: 99- 109.

Beveridge, I. and Smith, K .(1985). An ultra-structure study of the cirrus and vagina of *Phyllobothrium vagans* (Cestoda: Tetraphyllidea). *Zeitschrift fur Parasitenkunde.* **71**: 609 - 616.

Björkman, N. and Thorsell, W. (1964). On the ultra-structure of the ovary of the liver fluke (*Fasciola hepatica*). *Zeitschrift fur Zelforschung.* **63**: 538 - 549.

Bråten, T. (1968). An electron microscope study of the tegument and associated structure of the procercoid of *Diphyllobothrium latum* (L). *Zeitschrift für Parasitenkunde.* **30**: 95 - 103.

Bray, R. A. and des Clers, S. A. (1992). Multivariate analyses of metrical features in the *Lepidapedon elongatum* (Lebour, 1908) species - complex (Digenea, Lepocreadiidae) in deep and shallow water gadiform fishes of the NE Atlantic. *Systematic Parasitology.* **21**: 223 - 232.

Brüggemann, J. (1986). Feinstruktur der Protonephridien von *Paromalostomum proceracauda* (Plathelminthes, Macrostomida). *Zoomorphology.* **106**: 147 - 154.

Buchmann, K. (1988a). Epidemiology of pseudodactylogyrosis in an intensive eel-culture system. *Diseases of Aquatic Organisms.* **5** : 81 - 85.

Buchmann, K. (1988b). Interaction between the gill parasitic monogeneans *Pseudodactylogyrus anguillae* and *P. bini* and the host *Anguilla anguilla. Bulletin of the European Association of Fish Pathologists.* **8** : 98 - 99 .

Buchmann, K. (1988c). Spatial distribution of *Pseudodactylogyrus anguillae* and *P. bini* (Monogenea) on the gills of European eels *Anguilla anguilla. Journal of Fish Biology.* **32** : 801 - 802.

Buchmann, K. (1989). Microhabitats of monogenean gill parasites on European eel (*Anguilla anguilla*). *Folia Parasitologica.* **36** : 321 - 329.

Bullock, T. H. and Horridge, G. A. (1965). *Structure and function in the nervous system of invertebrates.* Vol 1. W.H Freeman & Company, San Francisco and London.

Burton, P. R. (1966a). A comparative electron microscopic study of cytoplasmic microtubules and axial unit tubules in a spermatozoan and a protozoan. *Journal of Morphology.* **120** : 397 - 424.

Burton, P. R. (1966b). Substructure of certain cytoplasmic microtubules : an electron microscopic study. *Science.* **154** : 903 - 905.

Burton, P. R. (1966c). The ultra structure of the integument of the frog bladder - fluke *Gorgodernia* sp. *Journal of Parasitology.* **52** : 926 - 934.

Burton, P. R. (1972). Fine structure of the reproductive system of a frog lung fluke. III. The spermatozoon and its differentiation. *Journal of Parasitology.* **58** : 68 - 83.

Bychowsky, B. E. (1933). Die Bedutung der monogenetischen Trematoden fűr die Erforschung der systematischen Beziehungen der Karpfenfische. *Zoologischer Anzeiger.* **102** : 243 - 251.

Bychowsky, B. E. (1957). *Monogenetic trematodes, their classification and phylogeny.* 509 pp. Moscow, Leningrad. (English ed. 1961. Edited by Hargis, W. J. Translation by AIBS. 481 pp).

Bychowsky, B. E. and Nagibina, L. F. (1967). On intermediate hosts in monogeneans (Monogenoidea). *Parasitologiya* **I(2)** : 117 - 123.

Cerfontaine, P. (1896). Contribution a l'étude des Octocotylides. *Archives de Biologie, Paris.* **6** : 497 - 560.

Cerfontaine, P. (1898). Contribution a Í'étude des Octocotylides. IV. Nouvelles observations sur le genere *Dactylocotyle* et description du *Dactylocotyle luscae. Archives de Biologie, Paris.* **15** : 301 - 328.

Charles, G. H. and Orr, T. S. C. (1968). Comparative fine structure of outer tegument of *Ligula intestinalis* and *Schistocephalus solidus. Experimental Parasitology.* **22** : 137 - 149.

Cheshina, A. S. (1954). Zabolevaniya ryb v poslevoennye gody v BSSR (Diseases of fishes in post war years in BSSR). *Trudy Problemnykh i Tematicheskikh Soveshchanii ZIN.* **4** : 39 - 42.

Chubb, J. C. (1977). Seasonal occurrence of helminths in freshwater fishes. Part 1. Monogenea. *Advances in Parasitology.* **15** : 133 - 199.

Chubb, J. C. (1979). Seasonal occurrence of helminths in freshwater fishes. *Advances in Parasitology.* **17** : 141 - 313.

Cone, D. K. and Burt, M. D. B. (1985). Population biology of *Urocleidus adspectus* Muller, 1936 (Monogenea) on *Perca flavescens* in New Brunswick. *Canadian Journal of Zoology.* **63** : 272 - 277.

Cone, D. K. and Burton, M. B. (1981). The surface topography of *Benedenia* sp. (Monogenea : Capsalidae). *Canadian Journal of Zoology.* **59** : 1941 - 1946.

Cone, D. K. and Cusack, R. (1989). Infrapopulation dispersal of *Gyrodactylus colemanensis* (Monogenea) on fry of *Salmo gairdneri. Journal of Parasitology.* **75** : 702 - 706.

Davis, R. E. and Roberts, L. S. (1983). Platyhelminths - Eucestoda. In : Adiyodi , K. G. and Adiyodi, R. G. (eds) *Reproductive Biology of Invertebrates.* Vol I : Oogenesis, oviposition and oosorption, Chap 4. John Wiley & Sons Ltd, Chichester, New York & Toronto. pp 109 - 133.

Dogiel, V., Petruschewsky, G. and Poljansky, G. (1958). *Die Gudlagen der Parasitologie der Fische (Fundamentals of Fish Parasitology)* Verlag der Leningrader Staatlichen Universität.

Drury, R. A. B and Wallington, E. A. (Eds) (1980). *Carleton's Histological Technique.* Fifth edition, Oxford press, pp 511.

Dupont, F. and Crivelli, A. J. (1988). Do parasites confer a disadvantage to hybrids? A case study of *Alburnus alburnus* x *Rutilus rutilis* a natural hybrid of lake Mikri Prespa, Northern Greece. *Oecologia.* **75** : 587 - 592.

Dyk, V. (1944). More attention to the parasites of the carp fry. *Ceskomoravskỳ rybàř.* **23** : 29 - 33.

Dzika, E. (1987). Annual occurrence dynamics of common Monogenean species on the gills of bream from the lake Goslawskie (Poland). *Acta Parasitologica Polonica.* **32** : 121 - 137.

Dzika, E. and Szymanski, S. (1989). Co-occurrence and distribution of Monogenea of the genus *Dactylogyrus* on gills of the bream *Abramis brama* L. *Acta Parasitologica Polonica.* **34** : 1 - 14.

Ebrahimzadesh, A. (1966). Histologische untersuchungen beriiden feinbau des oogenotop bei digegen Trematoden. *Zeitschrift fur Parasitenkunde.* **27** : 127 - 168.

Ehlers, U. (1986). Comments on a phylogenetic system of the Platyhelminthes. *Hydrobiologica.* **132** : 1 - 12.

Ellis, A. U. (1986). General principles of fish vaccination. In: *Fish vaccination.* Academic Press, London.

El-Naggar, M. M. (1992). Ultrastructure observations on the marginal hooklets of the monogenean gill parasite *Cichlidogyrus halli typicus. International Journal for Parasitology.* **22** : 613 - 619.

El-Naggar, M. M. and Kearn, G. C. (1983a). The tegument of the monogenean gill parasites *Dactylogyrus amphibothrium* and *D. hemiamphibothrium. International Journal of Parasitology.* **13** : 579 - 592.

El-Naggar, M. M. and Kearn, G. C. (1983b). Glands associated with the anterior adhesive areas and body margins in the skin parasitic monogenean *Entobdella soleae. International Journal for Parasitology.* **13 :** 67 - 81.

El-Naggar, M. M. and Kearn, G. C. (1989). Haptor glands in the gill parasitic, ancyrocephaline monogenean *Cichilidogyrus halli typicus* and the report of a possible prokaryotic symbiont. *International Journal for Parasitology.* **19** : 401 - 408.

El-Naggar, M. M., Khidr, A. A. and Kearn, C. G. (1990). Ultrastructural observations on the oviduct, Mehlis glands and ootype of the monogenean *Cichlidogyrus halli typicus* (Price & Kirk, 1967) Paperna, 1979. *International Journal for Parasitology.* **20** : 203 - 209.

Erasmus, D. A. (1973). A comparative study of the reproductive system of mature, immature and 'unisexual' female *Schistosoma mansoni. Parasitology.* **67** : 165 - 183.

Ergens, R. and Lom, J. (1970). *Puvodci Parasitàrnich Nemoci Ryb.* Akademia 383pp.

Erwin, B. E. (1984). *Studies on the reproductive system of a trematode parasite Bucephaloides gracilescens.* Ph.D Thesis, Queen's University, Belfast.

Erwin, B. E. and Halton, D. W. (1983). Fine structural observations on spermatogenesis in a progenetic trematode, *Bucephaloides gracilescens. International Journal for Parasitology.* **13** : 413 - 426.

Euzet, L. Swiderski, Z. and Mokhtar-Maamouri, F. (1981). Ultra structure comparee du spermatozoide des Cestodes. Relations avec la phylogenese. *Annales de Parasitologie* **56** : 247 - 259.

F.A.O (1990). *Aquaculture production (1985 - 1988).* F.A.O Fisheries Circular No. 815. Revision 2 pp 136.

Ferguson, H. W. (1989). *Systemic pathology of fish, A text and atlas of comparative tissue responses in diseases of teleosts.* Iowa State University Press / Ames.

Fischthal, J. H. and Allison, L .N. (1940). *Acolpenteron uretocetes* n.g, n.sp, a monogenetic trematode from the ureters of black basses. *Journal of Parasitology.* **26** : 34 - 35.

Fletcher, T. C and Grant,, P. T. (1969). Immunoglobulins in the serum and mucus of the plaice (*Pleuronectes platessa*). *Journal of Biochemistry.* **115** : 65 - 66.

Fletcher, T.C. and White, A. (1973). Lysozyme activity in the plaice (Pleuronectes platesssa). Experimentia. **29** : 1283 - 1285.

Frankland, H. M. T. (1955). The life history and bionomics of *Diclidophora denticulata* (Trematoda: Monogenea). *Parasitology.* **45** : 313 - 351.

Gibson, D., Taskinen, J. and Valtonen, T. (1992). Studies on bucephalid digeneans parasitising molluscs and fishes in Finland. II. The description of *Rhipidocotyle fennica* n. sp. and its discrimination by principal components analysis. *Systematic Parasitology.* **23** : 67 - 79.

Golovin, P. P. (1987). Young carp susceptibility to *Dactylogyrus vastator* infection. *Actual problems in fish parasitology: 2nd International symposium of Ichthyoparasitology, 27 Sept - 3 Oct 1987. Tihany, Hungary 1987* p. 21.

Golovina, N. A. and Golovin, P. P. (1988). Pathogenicity of *Dactylogyrus vastator* for young carp and methods of its evaluation. *Parasites of fresh water fishes of North - West Europe : International symposium within the program of the Soviet-Finnish cooperation 10 - 14 January 1988.* 47 - 54.

Grabda-Kazubska, B. ; Baturo-Warszawnska, B. and Poimańska, T. (1966). Dynamics of parasite infestation of fish in lakes Dgal Wielki and Warniak in connection with introduction of phytophagous species. *Acta Parasitologica Polonica.* **32** : 1 - 28.

Grabda, J. and Grabda, E. (1966). Neguvon w walce Z daktylogyroza karpi. *Gospod rybna.* 6.

Grant, W. C., Harkema, R. and Muse, K. E. (1976). Ultrastructure of *Phryngostomoides procyonis* Harkema 1942 (Diplostomatidae) I. Observations on the male reproductive system. *Journal of Parasitology.* **62** : 39 - 49.

Grant, W. C., Harkema, R. and Muse, K. E. (1977). Ultrastructure of *Pharyngostomoides procyonis* Harkema 1942 (Diplostomatidae) II. The female reproductive system. *Journal of Parasitology.* **63** : 1019 -1030.

Gresson, R. A. R. (1964). Oogenesis in the hermaphroditic Digenea (Trematoda). *Parasitology.* **54** : 409 - 421.

Gresson, R. A. R. (1965). Spermatogenesis in the hermaphroditic Digenea(Trematoda). *Parasitology.* **55** : 117 - 125.

Gupta, B. C., Parshad, V. R. and Guraya, S. S. (1987a). Histochemical studies on egg shell formation in *Paramphistomum cervi* (Digenea : Paramphistomatidae). *Journal of Helminthology*. **61** : 59 - 64.

Gupta, B. C., Parshad, V. R. and Guraya, S. S. (1987b). Morphological and histochemical observations on the vitelline cells of developing and adult *Paramphistomum cervi* (Trematoda : Digenea). *Journal of Helminthology*. **61** : 297 - 305.

Gussev, A. V. (1973). Freshwater Indian monogenoidea. Principles of systematics, analysis of the world faunas and their evolution. *Indian Journal of Helminthology*. **25-26** : 1 - 241

Gussev, A. V. (1988). Methodology in Monogenean studies. Methods and terminology in Monogenean studies. *Papers presented at The All Union Symposium on Monogeneans*. 169 - 178.

Halton, D. W. (1979). The surface topography of a monogenean, *Diclidophora merlangi* revealed by scanning electron microscopy. *Zeitschrift fur Parasitenkunde*. **61** : 1 - 12.

Halton, D. W. and Hardcastle, A. (1976). Spermatogenesis in the monogenean *Diclidophora merlangi*. *International Journal for Parasitology*. **6** : 43 - 53.

Halton, D. W. and Hardcastle, A. (1977). Ultrastructure of the male accessory ducts and prostate gland of *Diclidophora merlangi* (Monogenoidea). *International Journal for Parasitology*. **7** : 393 - 401.

Halton, D. W., Stranock, S. D. and Hardcastle, A. (1974). Vitelline cell development in monogenean parasites. *Zeitschrift fur Parasitenkunde*. **45** : 45 - 61.

Halton, D. W., Stranock, S. D. and Hardcastle, A. (1976). Fine structural observation on oocyte development in monogeneans. *Parasitology*. **73** : 13 - 23.

Hanek, G. and Fernando, C. H. (1978a). Seasonal dynamics and spatial distribution of *Cleidiodiscus stentor* Muller 1937 and *Ergasilus centrarchidarum* Wright 1882, gill parasites of *Ambloplites rupestris* (Raf). *Canadian Journal of Zoology*. **56** : 1244 - 1246.

Hanek, G. and Fernando, C. H. (1978b). Seasonal dynamics and spatial distribution of *Urocleidus ferox* Muller 1934, a gill parasite of *Lepomis gibbosus* (L). *Canadian Journal of Zoology*. **56** : 1241 - 1243.

Hanek, G. and Fernando, C. H. (1978c). Spatial distribution of gill parasites of *Lepomis gibbosus* (L) and *Ambloplites rupestris* (Raf). *Canadian Journal of Zoology*. **56** : 1235 - 1240.

Hanzelova, V. and Žitňan, R. (1983). The seasonal dynamics of the invasion cycle of *Dactylogyrus vastator* Nybelin 1924 (Monogenea) in the carp fry. *Helminthologica.* **20** : 137 - 150.

Hargis, W. J. (1953). Monogenetic trematodes of Westhampton Lake fishes. III. Part 1. Comparative morphology of the species encountered. *Journal of Parasitology.* **39** : 88 - 105.

Hendelberg, J. (1983). Platyhelminthes - Turbellaria. In Adiyodi , K. G. and Adiyodi, R. G. (eds) *Reproductive Biology of Invertebrates.* II. Spermatogenesis and sperm function. John Wiley & Sons Ltd. Chichester, New York & Toronto. pp 75 - 104.

Hines, R. S. and Spira, D. T. (1974). Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.) V. Acquired immunity. *Journal of Fish Biology.* **6** : 373 - 378.

Holmes, J. C. (1986). The structure of helminth communities. In: *Parasitology - Quo Vadit ? Proceedings of the Sixth International Congress of Parasitology.* (ed. Howell, M. J.), pp 203 - 208. Camberra : Australian Academy of Science.

Howells, R .E. (1969). Observations on the nephridial system of the Cestode *Moniezia expansa* (Rud 1805). *Parasitology.* **59** : 449 - 459.

Hughes, G. M. (1961). Gill ventilation in fishes. *Report of the Challenger Society.* **3** : 13.

Hughes, G. M. and Shelton, G. (1958). The mechanism of gill ventilation in three freshwater teleosts. *Journal of Experimental Biology.* **35** : 807 - 823.

Hurd, H. and Arme, C. (1984). Pathophysiology of *Hymenolepis dimnuta* infections in *Tenebrio molitor* : effect of parasitism n haemolymph proteins. *Parasitology.* **89** : 253 - 262.

Imada, R. and Muroga, K. (1978). *Pseudodactylogyrus microrchis* (Monogenea) on the gills of cultured eels. II Oviposition, hatching and development on the host. *Bulletin of the Japanese Society of Scientific Fisheries.* **44** : 571 - 576.

Irwin, S. W. B. and Maguire, J. G. (1979). Ultrastructure of the vitelline follicles of *Gorgoderina vitelloba* (Trematoda : Gorgoderidae). *International Journal for Parasitology.* **9** : 47 - 53.

Irwin, S. W. B. and Threadgold, L. T. (1970). Electron-microscope studies on *Fasciola hepatica* VIII. The development of the vitelline cells. *Experimental Parasitology.* **28** : 399 - 411.

Ivasik, V. M. (1953).Carp parasites and the diseases they cause in fish farms of the Zapadnaya region of the Ukrainian S.S.R. *Trudy Nauchno-Issledovatel'skogo*

Instituta Prudovogo i Ozerno-Rechnogo Rybnogo Khozyaistva. USSR. **9**: 85 - 112

Izyumova, N. A. (1953). The biology of *Dactylogyrus vastator* Nybelin and *D. solidus* Akhmerov in carp farms. *Avtoreferat Dissertatsii Zoologi Institut Akaddemiya. Nauk SSSR.* 1 - 7.

Izyumova, N. A. (1956). Materials on the biology of *Dactylogyrus vastator* Nybelin. *Parazitologiya.* **16**: 229 - 243.

Izyumova, N. A. and Zelencov, N. I. (1969). Nabljunija nad razvitiem *Dactylogyrus extensus* Müller et v Cleave, 1932. *Parazitologija. Leningrad,* **3(6)** : 528 - 531.

Izyumova, N. A., Zharikova, T. I., Mashtakov, A. V. and Stepanova, M. A. (1982). Nekotorye faktory opredeljajushhie chislen nost i struktury populjacii daktilo girid karpovykh ryb. In : *Gelminty v presnevodnykh biocenozakh*, K. M. Ryzhikov (ed), Izd. " Nauka ", Moskva. 17 - 31.

Jahn, T. L. and Kuhn, L. R. (1932). The life history of *Epibdella melleni* MacCallum, 1927 a monogenetic trematode parasitic on marine fishes. *Biological Bulletin.* **62** : 89 - 111.

Kanaev, A. I. and Lyaiman, E. M. (1959). Epizootiologicheskoe sostoyanie stada ryb v prudovykh khozyaistvakh RSFR (Epizootiological state of fish in fish farms of the RSFSR). *Trudy Soveshchanii Ikhtiologicheskaya Komissiya, Akademiya, NakusSSR.* **9** : 28 - 33.

Kane, M. B. (1966). Parasites of Irish fishes. *Scientific Proceedings of the Royal Dublin Society. B.* **1** : 205 - 220.

Katheriner, L. (1894). Die Gattning *Gyrodactylus* v. Nordmann. *Arbeiten aus dem Zoologisch - Zootomischen Institut in Wurzbürg.* **10** : 125 - 166.

Kearn, G. C. (1963). The egg, oncomiracidium and larval development of the *Entobdella soleae*, a monogenean skin parasite of the common sole. *Parasitology.* **53** : 435 - 447.

Kearn, G. C. (1967). Experiments on host - finding and host - specificity in the skin monogenean skin parasite *Entobdella soleae. Parasitology.* **57** : 585 - 605.

Kearn, G. C. (1968). The development of the adhesive organs of some diplectanid tetraonchid and dactylogyrid gill parasites (Monogenea). *Parasitology.* **58** : 149 - 163.

Kearn, G. C. (1976). Body surface of fishes. In; Kennedy, C. R. (ed) *Ecological Aspects of Parasitology*. The North Holland Publ. Co., Amsterdam, The Netherlands. 185 - 208.

Kearn, G. C. (1985). Observations on egg production in the monogenean *Entobdella soleae*. *International Journal for Parasitology*. **15** : 187 - 194.

Kearn, G. C. (1986). The eggs of Monogeneans. *Advances in Parasitology*., **25** : 175 - 273.

Kennedy, C. R. (1985). Site segregation by species of Acanthocephala in fish, with special reference to eels, *Anguilla anguilla*. *Parasitology*. **90** : 375 - 390.

Kino, H. (1984). Parasite density and the fecundity of *Angiostrongylus cantonensis* in rats. *Parasitology*. **89** : 275 - 285.

Kollmann, A. (1970). *Dactylogyrus vastator*, Nybelin, 1924 (Trematoda : Monogenoidea) als Krankheitserreger auf den Kiemen des Karpfens (*Cyprinus carpio* L.) (*Dactylogyrus vastator* Nybelin, 1924 Trematoda : Monogenoidea) as a pathogen on the gills of common carp (*Cyprinus carpio*) Part 1. *Zeitschrift fur fisherei und deren Hilfswissenschaften. Berlin.* **18** : 129 - 150.

Kollmann, A. (1972). *Dactylogyrus vastator* Nybelin 1924 (Trematoda, Monogenoidea) as a pathogen on the gills of carp (*Cyprinus carpio*. L.).(*Dactylogyrus vastator* Nybelin, 1924 (Trematoda, Monogenoidea) als Krankeitserreger auf den kiemen des karpfens (*Cyprinus carpio*, L.) Part 3. Teil. *Zeitchrift fur Wissenschafliche Zoologie, Leipzig*. **185** : 1 - 54.

Koskivaara, M., Valtonen, E. T. and Vuori, K. M. (1992). Microhabitat distribution and coexistence of *Dactylogyrus* species (Monogenea) on the gills of roach. *Parasitology*. **104** : 273 - 281.

Kritsky, D. C. (1978). The cephalic glands and associated structures on *Gyrodactylus eucaliae* Ikezaki and Hoffman,1957 (Monogenea : Gyrodactylidae). *Proceedings of the Helminthological Society of Washington*. **45** : 37 - 49.

Kritsky, D. C. and Kruidenier, F. J. (1976). Fine structure and the development of the body wall in the monogenean *Gyrodactylus eucaliae* Ikezaki and Hoffman, 1957. *Proceedings of the Helminthological Society of Washington*. **43** : 47 - 58.

Ktari, M. H. (1971). *Recherches sur la reproduction et le developpement de quelques monogenes (Polyopisthocotylea) parasites de poissons marins*. These. Academie de Montpellier, Universite de Sciences et Techniques du Languedoc.

Kulemina, I. V. (1987). Size variability of the adhesive elements in some species of *Gyrodactylus*. In: *Investigations of Monogeneans of the USSR*. Skarlato, O.A. (ed) Russian Translation Series 62. publ 1988 Balkema, A.A. (Vseseyuznoe Simpozium po Monogeneyam, 1976: Leningrad), 34-37.

Kümmel, C. (1958). Das Terminalorgan der Protonephridien, Feinstruktur und Detung der Funktion. *Zeitschrift fur Naturforschelle*. **13 b** : 677 - 679.

Kümmel, C. (1959). Feinstruktur der Wimperflamme in den protonephridien. *Protoplasma*. **51** : 371 - 376.

Lee, D. L (1966). The structure and composition of the helminth cuticle. *Advances in Parasitology*. **4** : 187 - 254.

Lester, R. J. G. and Adams, J. R. (1974). *Gyrodactylus alexanderi* : reproduction, mortality and effects on its host *Gasterosteus aculeatus*. *Canadian Journal of Zoology*. **52** : 827 - 833.

Llewellyn, J. (1956). The host - specificity, micro - ecology, adhesive attitudes and comparative morphology of some trematode gill parasites. *Journal of the Marine Biological Association of the United Kingdom*. **35** : 113 - 127.

Llewellyn, J. (1957). The larvae of some monogenetic trematode parasites of Plymouth fishes. *Journal of the Marine Biological Association of the United Kingdom*. **36** : 243 - 259.

Llewellyn ,J and Owen, I. L. (1960). The attachment of the monogenean gill parasite *Discocotyle sagittata* Leuckart to the gills of *Salmo trutta* L. *Parasitology*. **50** : 19 - 30.

Löser, E. V. (1965a). Der feinbau des oogenotop bei cestoden. *Zeitschrift fur Parasitenkunde*. **25** : 413 - 458.

Löser, E. V. (1965b). Die eibildung bei cestoden. *Zeitschrift fur Parasitenkunde*. **25** : 556 - 580.

Lucký, Z. (1964a). Investigations into the parasites of the carp. 1. Remarks concerning the seasonal dynamics and the pathogeny of the monogenetic trematodes (Monogenoidea) of the carps in some fisheries in Moravia. *Sborn Vysoké Školy Zenmědělske Brne, Bruno*. **12** : 239 - 269.

Lucký, Z. (1964b). Study on parasites of carp fry. *Sobrn Vysoke Skoly Zenmedelske Brně, Series B*, **12** : 139 - 167.

Lucký, Z. (1984). Investigation on invasive diseases of the herbivorous fish fry and their treatment. *Symposia Biologica Hungaria*. **23** : 173 - 180.

Lumsden, R. D. (1966). Cytological studies on the adsorptive surfaces of cestodes. I. The fine structure of the strobilar integument. *Zeitschrift fur Parasitenkunde*. **27** : 355 - 382.

Lumsden, R. D. and Byram, J. (1967). The ultra structure of cestode muscle. *Journal of Parasitology*. **53** : 326 - 342.

Lumsden, R. D., Gonzales, G., Mills, R. R. and Viles, J. M. (1968). Cytological studies on the absorptive surfaces of cestodes. III. Hydrolysis of phosphate esters. *Journal of Parasitology*. **54** : 524 - 535.

Lyaiman, E. M. (1948). New data on epizootology of dactylogyrosis. *Rybnoe Khozyaistva* **12** : 32 - 36.

Lyaiman, E. M. (1951a). Influence of water temperature on reproduction of the monogenetic trematode *Dactylogyrus vastator. Trudy Mosrybvtuza* **4** : 190 - 196.

Lyaiman, E. M. (1951b). The seasonal incidence of invasion of carp with parasites. *Trudy Moskovskii tekhn institut rybn prom i khoz im Mikoyana*. **4** : 197 - 204.

Lyons, K. M. (1968). A comparison of the adult epidermis of some monogeneans : The development of the outer layer in *Entobdella soleae*. *Parasitology*. **58** : 14.

Lyons, K. M. (1969a). Compound sensilla in monogenean skin parasites. *Parasitology*. **59** : 625 - 636.

Lyons, K. M. (1969b). Sense organs of monogenean skin parasites ending in a typical cilium. *Parasitology*. **59** : 431 - 443.

Lyons, K. M. (1970). The fine structure and function of the adult epidermis of two skin parasitic monogeneans, *Entobdella soleae* and *Acanthocotyle elegans*. *Parasitology*. **60** : 39 - 52.

Lyons, K. M. (1971). Comparative electron microscope studies on the epidermis of the blood living juvenile and gill living adult stages of *Amphibdella flavolineata* (Monogenea) from the electric ray *Torpedo nobiliana*. *Parasitology*. **63** : 181 - 190.

Lyons, K. M. (1973a). Epidermal fine structure and development in the oncomiracidium larva of *Entobdella soleae* (Monogenea). *Parasitology*. **66** : 321 - 333.

Lyons, K. M. (1973b). Scanning and transmission electron microscope studies on the sensory sucker papillae of the fish parasite *Entobdella soleae* (Monogenea). *Z. Zellforsch*. **137** : 471 - 480.

Maamouri, F. (1979). Étude en Microscopié electronique de la spermiogénése et du spermatozoide de *Phyllobothrium gracile* Weld, 1855 (Cestoda, Tetraphyllidea, Phyllobothriidae). *Zeitschrift fur Parasitenkunde*. **59** : 245 - 258.

MacArthur, R. H. (1972). *Geographical ecology. Patterns in the distribution of species*. Harper & Row, New York.

Macdonald, S. and Jones, A. (1978). Egg-laying and hatching rhythms in the monogenean *Diplozoon homoion gracile* from the Southern barbel (*Barbus meridionalis*). *Journal of Helminthology*. **52** : 23 - 28.

Mahendrasingam, S. (1989). *Studies on the developmental biology of Trilocularia acanthiaevulgaris (Cestoda, Tetraphyllidea).* Ph.D Thesis. The Queen's university of Belfast, Northern Ireland.

Malmberg, G. (1970). The excretory systems and the marginal hooks as a basis for the systematics of *Gyrodactylus* (Trematoda, Monogenea). *Arkiv for Zoologi.*, **223** : 1 - 235.

Mizelle, J. D. (1974). Studies on monogenetic trematodes. XXXIV. A population study of the gill parasites of *Lepomis cyanellus* Rafinesque. *The Wasmann Journal of Biology*. **32** : 207 - 214.

Mo, T. A. (1991a). Seasonal variations of opisthaptoral hard parts of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea : Gyrodactylidae) on parr of Atlantic salmon *Salmo salar* L. in the river Batinfjordselva, Norway. *Systematic Parasitology*. **19** : 231 - 240.

Mo, T. A. (1991b). Variations of opisthaptoral hard parts of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea : Gyrodactylidae) on rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) in a fish farm, with comments on the spreading of the parasite on south eastern Norway. *Systematic Parasitology*. **20** : 1 - 9.

Mo, T. A. (1991c). Variation of opisthaptoral hard parts of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea:Gyrodactylidae) on parr of Atlantic salmon *Salmo salar* L. in laboratory experiments. *Systematic Parasitology*. **20** : 11 - 19.

Mo. T. A. and Appleby, C. (1990). A special technique for studying haptoral sclerites of monogeneans. *Systematic Parasitology*. **17** : 103 - 108.

Molnár, K. (1971a). Studies on gill parasitosis of the grass carp *Ctenopharyngodon idella* caused by *Dactylogyrus lamellatus* Achmerow 1952. I. Morphology and biology of *Dactylogyrus lamellatus. Acta Veterinaria Academie Scientica Hungaricae*. **21** : 267 - 289.

Molnár, K. (1971b). Studies on gill parasitosis of the grass carp *Ctenopharyngodon idella* caused by *Dactylogyrus lamellatus* Achmerow 1952. II. Epizootiology. *Acta Veterinaria Academie Scientica Hungaricae*. **21** : 361 - 375.

Molnár, K. (1971c). Studies on gill parasitosis of the grass carp *Ctenopharyngodon idella* caused by *Dactylogyrus lamellatus* Achmerow 1952. III. Therapy and control. *Acta Veterinaria Academie Scientica Hungaricae*. **21** : 377 - 382.

Molnár, K. (1972). Studies on gill parasitosis of the grass carp *Ctenopharyngodon idella* caused by *Dactylogyrus lamellatus* Achmerow 1952. IV. Histopathological changes. *Acta Veterinaria Academie Scientica Hungaricae*. **22** : 9 - 24.

Molnár, K. (1980). A histological study on ancylodiscoidosis in the sheatfish (*Silurus glanis*). *Helminthologica*. **17** : 117 - 126.

Morris, G. P. and Halton, D. W. (1971). Electron microscope studies of *Diclidophora merlangi* (Monogenea : Polyopisthocotylea). II. Ultrastructure of the tegument. *Journal of Parasitology*. **57** : 49 - 61.

Morris, G. P. and Threadgold, L. T. (1967). A presumed sensory structure associated with the tegument of *Schistoma mansoni*. *Journal of Parasitology*. **53** : 537 - 539.

Newton, W. D. (1974). The accessory cell and the yolk halo of the oocyte of the fresh water turbellarian *Hydrolimax grisea* (Platyhelminthes, Plagiostomidae). *Biological Bulletin*. **147** : 618 - 629.

Nguenga, D. (1988). A note on infestation of *Oreochromis niloticus* with *Trichodina* sp. and *Dactylogyrus* sp. *The second international symposium on tilapia in aquaculture, ICLARM Conference Proceedings*. **15** : 623.

Nieland, M. L. and Weinbach, E. C. (1968). The bladder of *Cysticercus fasciolaris* : electron microscopy and carbohydrate content. *Parasitology*. **58** : 489 - 496.

Nilakarawasam, N. (1993). *Ecological studies on the parasites of* Etroplus suratensis *(Bloch) (Pisces:Cichilidae) with special reference to* Enterogyrus spp *(Monogenea:Ancyrocephlinae)*. Ph.D Thesis, University of Stirling, Scotland, United Kingdom.

Nordquist, H. (1925). Untersokningar över *Dactylogyrus* sjuk domen hos karpynglet och betingelserna för en säkrare sattkarpproduktion. *Skrifter utgivna av Södra Sveriges Fiskeriförening Lund*. **(1 - 2)** : 4 - 41.

Nybelin, O. (1924). *Dactylogyrus vastator* n. sp. *Arkiv för zoologi*. **16**, 1 - 2.

Nybelin, O . (1925). *Dactylogyrus* - Studies vid Aneboda Fiskeriförsökstation. *Skrifter utgivna av Södra Sveriges Fiskeriförening Lund* . **(1 - 2)** : 42 - 72.

Odum, E. P. (1963). *Ecology*. New York; Holt, Rinehart & Wilson, Modern Biology series.

Owen, I. L. (1963). The attachment of the monogenean *Diplozoon paradoxum* to the gills of *Rutilus rutilus* L. I. Micro-habitat and adhesive attitude. *Parasitology*. **53** : 455 - 461.

Pan, Sc - T. (1980). The fine structure of the miracidium : *Schistosoma mansoni*. *Journal of Invertebrate Pathology*. **36** : 307 - 372.

Paling, J. E. (1968). A method estimating the relative volumes of water flowing over the different gills of a freshwater fish. *Journal of Experimental Biology*. **48** : 533 - 544.

Paling, J. E. (1969). The manner of infection of trout gills by the monogenean parasite *Discocotyle sagitta*. *Journal of Zoology. London*. **159** : 293 - 309.

Paperna, I. (1963a). Dynamics of *D. vastator* Nybelin (Monogenea) population on the gills of carp fry in fish ponds. *Bamidgeh*. **15** : 31 - 50.

Paperna, I. (1963b). Some observations on the biology and ecology of *Dactylogyrus vastator* in Israel. *Bamidgeh*. **15** : 8 - 28.

Paperna, I. (1964). Host relation to infestation of carp with *Dactylogyrus vastator* Nybelin 1924 (Monogenea). *Bamidgeh*. **16** : 129 - 141.

Pasztor, V. M. and Kleerekoper, H. (1962). The role of the gill filament musculature in teleosts. *Canadian Journal of Zoology*. **40** : 785 - 802.

Pianka, E. R. (1974). *Evolutionary ecology*. Harper & Row, New York.

Pojmańska, T. and Dzika, E. (1987). Parasites of bream *Abramis brama* L. from the lake Goslawskie (Poland) affected by long-term thermal pollution. *Acta Parasitologica Polonica*. **32** : 139 - 161.

Pool, D. W. and Chubb, J. C. (1987). *Dactylogyrus extensus* Muller and Cleave 1932 and *Dactylogyrus vastator* Nybelin 1924 on gills of common carp *Cyprinus carpio* L. *Bulletin of the European Association of Fish Pathologists*. **7** :15 - 17.

Prost, M. (1963). Investigations on the development and pathogenicity of *Dactylogyrus anchoratus* (Duj 1845) and *D. extensus* Muller et V. Cleave 1932 for breeding carps. *Acta Parasitologica Polonica*. **11** : 11 - 47.

Reda, E. S. A. (1988). An analysis of parasite fauna of bream, *Abramis brama* L. in Vistula near Warszawa in relation to the character of fish habit. II. Seasonal dynamics of infestation. *Acta Parasitologica Polonica*. **33** : 35 - 58.

Remley, L. W. (1942). Morphology and life history studies of *Microcotyle spinicirrus* McCallum 1918, a monogenetic trematode parasitic on the gills of *Aplodinotus grunniens*. *Transactions of the American Microscopical Society*. **61** : 141 - 155.

Ricklefs, R. E. (1980). *Ecology*. 2nd ed. Sunbury-on-Thames; Nelson.

Roberts, R. J. (1978). *Fish Pathology*. MacMillan Publishing Co. Inc. New York.

Robinson, R. D. and Halton, D. W. (1982). Fine structural observations on spermatogenesis in *Corrigia vitta* (Trematoda : Dicrocoeliidae). *Zeitschrift fur Parasitenkunde*. **68** : 53 - 72.

Rohde, K. (1965). Studies on the genus *Polystomoides* Ward 1917 (Monogenea). I. Description of 4 Malaya species, a key to the known species and a comparison of the subcuticular layers in *Polystomoides* and some digenetic trematodes. *Zoologishes Jahrbuch Systematesche*. **92** : 345 - 368.

Rohde, K. (1971a). Phylogenetic origin of trematodes. *Parasitologische Schriftenreihe. Jena*. **21** : 17 - 27.

Rohde, K. (1971b). Untersuchungen an *Multicotyle purpisi* Dawes 1941 (Trematoda : Aspidogastrea) VIII. Elektronen mikroskopischer Bau des Exkretionssystems. *International Journal for Parasitology*. **1** : 275 - 286.

Rohde, K. (1972). The Aspidogastrea, especially *Multicotyle purpisi* Dawes 1941. *Advances in Parasitology*. **10** : 77 - 151.

Rohde, K. (1973). Ultrastructure of the protonephridial system of *Polystomoides malayi* Rohde and *P. renschi* Rohde (Monogenea : Polystomatidae). *International Journal for Parasitology*. **3** : 329 - 333.

Rohde, K. (1976). Monogenean gill parasites of fish of *Scomberomorus commersoni* Lacepede and other mackerel on the Australian east coast. *Zeitschrift fur Parasitenkunde*. **51** : 171 - 182.

Rohde, K. (1977). A non- competitive mechanism responsible for restricting niches. *Zoologischer Anzeiger*. **199** : 164 - 172.

Rohde, K. (1979). A critical evaluation of intrinsic and extrinsic factors responsible for niche restriction in parasites. *American Naturalist*. **114** : 648 - 671.

Rohde, K. (1980). Some aspects of the ultrastructure of *Gotocotyle secunda* Tripathi 1954 (Monogenea : Gotocotylidae) and *Hexostoma euthynni* Meserve 1938 (Monogenea : Hexostomatidae). *Angewarette Parasitologie*. **21** : 32 - 48.

Rohde, K. (1986). Ultrastructure of the flame cells and protonephridial capillaries of the Temnocephala; Implications for phylogeny of the parasitic platyhelminthes. *Zoologischer Anzeiger*. **216** : 39 - 47.

Rohde, K. and Georgi, M. (1983). Structure and development of *Austramphilina elongata* Johnston 1931 (Cestodaria : Amphilinidea). *International Journal for Parasitology*. **13** : 273 - 287.

Rohde, K. and Watson, N. (1986a). Ultrastructure of spermiogenesis and sperm of *Austramphilina elongata* (Platyhelminthes, Amphilinidea). *Journal of Submicroscopical Cytology*. **18** : 361 - 374.

Rohde, K. and Watson, N. (1986b). Ultrastructure of the sperm ducts of *Austramphilina elongata* (Platyhelminthes, Amphilinidae). *Zoologischer Anzeiger*. **217 1 / 2, S.** : 23 - 30.

Rohde, K., Watson,N. and Roubal, F. (1989). Ultrastructure of the protonephridial system of *Dactylogyrus* sp. and an unidentified Ancyrocephaline (Monogenea : Dactylogyridae). *International Journal for Parasitology*. **19** : 859 - 864.

Sandground, . (1929). A consideration of the relation of host specificity of helminths and other metazoan parasites to the phenomena of age resistance and acquired immunity. *Parasitology*. **21** : 227 - 255.

Sarig, S. (1968). A review of diseases and parasites of fishes in warm water ponds in the near East and Africa. *F. A. O Fish Culture Bulletin*. **44** : 278 - 289.

Sarig, S. (1971). Diseases of fishes : the prevention and treatment of diseases of warm water fishes under sub-tropical conditions, with special emphasis on the intensive fish farming. In: *Diseases of Fishes* (Eds, Snieszke, S. F. and Axelrod, H. R.) pp. 1 - 127. Neptune, New Jersey : T. F. H. Publications Inc.

Sarig, S., Lahav, M and Shilo, M. (1965). Control of *Dactylogyrus vastator* on carp fingerlings with Dipterex. *Bamidgeh*. **17** : 47 - 52.

Satchell, G. H. (1984). Respiratory toxicology of fishes. In: *Aquatic Toxicology*, Vol.2, ed. L.J. Weber, pp 1 -50. New York : Raven Press.

Saunders, R. L. (1961). The irrigation of the gills in fishes. I. Studies of the mechanism of branchial irrigation. *Canadian Journal of Zoology*. **39** : 637 - 653.

Schad, G. A. (1966). Immunity, competition, and natural regulation of helminth populations. *The American Naturalist*. **100** : 359 - 564.

Schäperclaus, W. (1991a). *Fish diseases*. Volume 1. Oxonian Press Pvt Ltd, New Delhi.

Schäperclaus, W. (1991b) *Fish diseases*. Volume 2. Oxonian Press Pvt Ltd, New Delhi.

Scott, M. E. (1985). Experimental epidemiology of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*) : short and long term studies. In: Rollinson, D & Anderson, R. M. (Eds.). *Ecology and Genetics of Host-Parasite Interactions*, New York : Academic Press.

Scott, M. E. (1987). Temporal changes in aggregation : a laboratory study. *Parasitology*. **94** : 583 - 595.

Scott, M. E. and Robinson, M. A. (1984). Challenge infections of *Gyrodactylus bullatarudis* (Monogenea) on guppies, *Poecilia reticulata* (Peters), following treatment. *Journal of Fish Biology*. **24** : 581 - 586.

Shaharom-Harrison, F. (1984). *Monogenetic trematodes (Dactylogyridae : Ancyrocephalinae) on the gills of* Tilapia *(a warm water cultured fish) with special reference to* Cichlidogyrus sclerosus *Paperna and Thurston 1969*. Ph.D Thesis, University of Stirling, Scotland, United Kingdom.

Shaharom-Harrison, F. (1986). The reproductive biology of *Dactylogyrus nobilis* (Monogenea : Dactylogyridae) from the gills of big head carp (*Aristichthys nobilis*). *Proceedings of the First Asian Fisheries Forum*, Manila, Philippines. **26 - 31 May** : 265 - 268.

Shaw, M. K. (1987). *Schistosoma mansoni* : Vitelline gland development in females from single sex infections. *Journal of Helminthology*. **61** : 153 - 259.

Shelton, G. (1970). The regulation of breathing. In: *Fish Physiology*. Edited Hoar. W. S. and Randall. D. J. Vol. IV. pp 293 - 359 .

Shinn, A. P. (1994). *The application of new biosystematic techniques in the discrimination of the genus* Gyrodactylus *(Monogenea) on salmonid fish*. PhD Thesis. University of Stirling, 340pp.

Shinn, A. P. ; Gibson, D. I. and Sommerville, C. (1993). An SEM study of the haptoral sclerites of the genus *Gyrodactylus* Nordmann 1832 (Monogenea) following extraction by digestion and sonication techniques. *Systematic Parasitology*. **25** : 135 - 144.

Slotved, H. C. and Buchmann, K. (1993) Acquired resistance of the eel, *Anguilla anguilla* L., to challenge infections with gill monogeneans. *Journal of Fish Diseases*. **16** : 585 - 591.

Smirnova, K. V. (1947). K parazitologii ryb Alma - Atinskogo karpovogo rybopitomnika (Parasitology of fishes of Alma - Ata carp nursery). *Uchenye Zapiski Ural'skogo Gosudarstvennogo Pedgogicheskogo Institutata Imeni Pushkina*. **1** : 1 - 3.

Smyth, J. D. (1954). A technique for the histochemical demonstration of polyphenoloxidase and its application to egg - shell formation in helminths and byssus formation in *Mytilus*. *Quarterly Journal of the Microscopical Society*. **95** : 139 - 152.

Symth, J. D. and Clegg, J. A. (1959). Parasitological Review Section - Egg - shell formation in trematodes and cestodes. *Experimental Parasitology*. **8** : 286 - 323.

Suydam, E. l. (1971). The micro - ecology of three species of monogenetic trematodes of fishes from the Beaufort - Cape Haltteras area. *Proceedings of the Helminthological Society of Washington.* **38** : 240 - 246.

Thoney, D. A and Hargis, W. J. (1991). Monogenea (Platyheminthes) as hazards for fish in confinement. *Annual Review of Fish Diseases.* **1** : 134 - 153.

Thurston, J. P. (1968). The larva of *Oculotrema hippopotami* (Monogenea : Polystomatidae). *Journal of Zoology, London.* **154** : 475 - 480.

Tinsley, R. C. (1983). Ovoviviparity in platyhelminth life-cycles. *Parasitology.* **86** : 161 - 196.

Ubelaker, J. E. (1983). The morphology, development and evolution of tapeworm larvae. In : Arme, C. and Pappas, P. W. (eds) *Biology of the Eucestoda.* Academic Press, London. **1** : 235 - 296.

Uspenskaya, A. V. (1962). Vliyanie *Dactylogyrus vastator* Nybelin, 1924 na organizm karpa (Effect of *Dactylogyrus vastator* Nybelin, 1924 on the organism of carp). *Zoologicheskii Zhurnal.* **40(1)** : 10 - 12.

Vladimirov, V. L. (1969). Concerning some immunity factors in fish. *Izvestiya Gosniorkh,* **69** : 173 - 181.

Vladimirov, V. L. (1971). Immunity of fishes during dactylogyrosis. *Parasitologiya.* **5** : 51 - 58.

Wagener, G. R. (1860). Ueber *Gyrodactylus elegans* V.Nordm. *Archiv fur Anatomie, Physiologie und Wissenschaftliche, Medicin. Berlin.* : 768 - 797.

Wang, C. M. and Patton, R. L. (1968). The separation and characterization of the haemolymph proteins of several insects. *Journal of Insect Physiology.* **14** : 1069 - 1075.

Wilcoxon, F. and Wilcox, R. A. (1964). *Some rapid approximate statistical procedures.* Lederle Laboratories, Peral River, NY. 59pp.

Wiles, M. (1968). The occurrence of *Diplozoon paradoxum* Nordmann, 1832 (Trematoda : Monogenea) in certain waters of Northern England and its distribution on the gills of certain Cyprinidae. *Parasitology.* **58** : 61 - 70.

Williams, M. A. and Hoole, D. (1992). Antibody response of the fish *Rutilis rutilis* to the metacestode of *Lingula intestinalis. Diseases of Aquatic Organisms.* **12** : 83 - 89.

Wilson, R. A. (1969). The fine structure of the protonephridial system in the miracidium of *Fasciola hepatica. Parasitology.* **59** : 461 - 467.

Wilson, R. A. and Webster, L. A. (1974). Protonephridia. *Biological Review*. **49** : 127 - 160.

Woo, P. T. K. (1992). Immunological responses of fish to parasitic organisms. *Annual Review of Fish Diseases*. **2** : 339 - 366.

Wootten, R. (1974). The spatial distribution of *Dactylogyrus amphibothrium* on the gills of ruffe *Gymnocephalus cernua* and its relation to the relative amounts of water passing over the parts of the gills. *Journal of Helminthology*. **48** : 167 - 174.

Woskoboinikoff, M. M. and Balabai, P. P. (1936). Vergleichenede experimentelle Untersuchung des Atmungsapparates bei Knochenfischen. Vorläufige Mitteilung ũber die Ergebnisse der Beobachtugen in den Sommern 1933 und 1934. *Travaux du Musee Zoologie de la Academie Scientifique Ukraine*. **10** : 145 - 155.

Wright, R. R. and MacCallum, A. B. (1887). *Sphyranura osleris*, a contribution to American helminthology. *Journal of Morphology*. **1** : 1 - 48.

Wunder, W. (1926). Die *Dactylogyrus* Krankheit der Karfenbrutnd iher Ursache und iher Bekämpfung. *Bilogisches Zentralblatt Erlagen and leipzig*. **46(16)** : 748 - 755.

Wunder, W. (1929). Die *Dactylogyrus* Krankheit der Karpfenbrwrt, ihre Ursache.U.ihre Bekämpfung. *Zeitschrift fur Fischerei.* **27(4)** : 511 - 545.

Wunder, W. (1949). Wirbelsäulenverkür zung als rassebildends Merkmal biem Aischgründer Karpfen (Shortening of the vertebral column as a character of the Asian carp race). *Verhandlungen der Deutschen Zoologischen Kiel*. 294 - 297.

Yamaguti, S. (1940). Studies on the helminth fauna of Japan. Part 31. Trematodes of fishes VII. *Systema Helminthum*. Interscience publication.

Zitnan, R. (1974). Acclimatization of fish in the carpathian region of Czechoslovakia and the role of helminths in this process. *Ichthyologica*. **6** : 143 - 155.