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Original investigations

Ultrastructural observations and radiometric assay on cercarial penetration and migration of the digenean *Diplostomum spathaceum* in the rainbow trout *Oncorhynchus mykiss*

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Abstract. The entrance into host tissues by the cercaria of the digenean *Diplostomum spathaceum* and the parasite's migration to the eyes of the fish host (post-penetration larvae) were studied by scanning and transmission electron microscopy at the ultrastructural level and by a radiometric assay in experimentally infected rainbow trout (*Oncorhynchus mykiss*). It was demonstrated that cercariae penetrated the host surface at several sites, although the major site involved the gill region. This observation was verified by the measurement of radioactivity in different regions of fish at intervals following their exposure to cercariae that had been radiolabeled with selenium in vivo. The migratory routes of the post-penetration-labeled cercariae (diplostomulum) were followed along with the distribution of radioactivity, which was altered with increasing time after exposure in the different regions of the host. A marked increase in radioactivity was noted, particularly in the eyes of the host, whereas the radiolabel decreased elsewhere. The results support the view that the cercariae of *D. spathaceum* contact the host by chance. The finding that the parasite mainly penetrates the host through the gills may be connected with fish ventilation hydrodynamics. The total amount of radioactivity retained in the fish gradually decreased with time, which may suggest that only a portion of the primarily atttached cercariae actually pentrated the fish. The majority of the penetrating parasites reached the eyes of the host, where they became established as metacercariae within 24 h, thus indicating migration directed to the eye region. This is facilitated by the functional morphology of the migrating stage of *D. spathaceum*.

Knowledge about the sites of entry and the subsequent routes of migration of parasites inside their hosts is fundamental to the understanding of the transmission dynamics of any endoparasite. This paper deals with these aspects of the digenean *Diplostomum spathaceum* in the rainbow trout *Oncorhynchus mykiss*. *D. spathaceum* is distributed worldwide, and it has been found in >125 species of freshwater fish. Rainbow trout is one highly susceptible host (Betterton 1974), and high infection densites of *D. spathaceum* metacercariae cause the parasitic disease diplostomosis, characterized in the chronic stage by eye cataracts that result in abnormal feeding and retarded growth (Palmieri et al. 1976).

One path of infection involves feeding on snails containing precocious metacercariae (Becker and Brunson 1966), but fish are believed to be more frequently infected via direct penetration of their integument by free-living cercariae. The chemical stimuli for invasion and the mechanism by which *D. spathaceum* cercariae attack and penetrate the integument of a fish host have been studied by Haas (1974a, b; 1975). The localization of and the subsequent migratory routes taken by the larvae inside various fish hosts have been studied histologically using light microscopy (LM, Ferguson 1943; Erasmus 1959; Betterton 1974; Ratanarat-Brockelman 1974). However, controversy remains over the invasion pattern of invading cercariae in rainbow trout, particularly the proportion that enter the fish directly through the cornea. Radiometry has been applied to overcome the problems encountered using traditional histological techniques to locate and follow the movements of *Schistosoma* and *Fasciola* species (Christensen et al. 1979; Christensen and Nansen 1981; Chandiwana 1988). Furthermore, ultrastructural information about the invasive process and the migratory routes of *Diplostomum* spp. remains incomplete. The purpose of the present study was to fill this lack of information on the penetration and subsequent migration routes taken by the cercariae of *D. spathaceum* in rainbow trout. Along with electron microscopy at the ultrastructural level, radioactivity counts in different regions of fish were made at regular intervals following exposure to cercariae that had been radiolabeled with selenium.

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Materials and methods

Parasite and snail material

Eggs of *Diplostomum spathaceum* were obtained from laboratory-infected herring gulls (*Larus argentatus*) maintained at the Department of Zoology, University of Aberdeen (kindly provided by Dr. S.K. Whyte and Mrs. L. Riley). In our laboratory, *Lymnea palustris* were infected with miracidiae as previously described by Whyte et al. (1988) and Höglund and Thuvander (1990). The cercariae were collected from shedding snails and were used for exposure of fish within 6 h. Pools from different snails were used, and the density of cercarial suspensions was determined from samples (6 × 100 µl) of the total volume stained with Lugol's iodine.

Preparation for scanning electron microscopy

Fish exposed for about 15 min to approximately 500 cercariae each were prepared for scanning electron microscopy (SEM) by fixation for at least 2–4 h in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The specimens were then rinsed in cold 0.1 M phosphate buffer (pH 7.4) solution, post-fixed for 1 h in cold (5° C) 1% OsO_4 in the phosphate buffer, rinsed again in the buffer, and ultimately dehydrated through a graded series of ethanol. The ethanol was stepwise replaced by filtered freon TF, and the specimens were critical-point dried using CO_2 as the transitional fluid. Samples were mounted on stubs with double-stick tape or graphite paste (Leit-C), coated with gold-palladium using a Jeol JCF-1000 ion sputter and examined at 15 kV in a JSM-35 scanning electron microscope.

Preparation for transmission electron microscopy

For transmission electron microscopy (TEM), the fish were prepared as described above, However, some were kept for 1 h in a parasite-free storage tank prior to fixation. They were cut into small pieces and fixed as described above. The tissues were prestained with uranyl acetate in 70% ethanol for 1 h at 4° C, dehydrated in a graded series of ethanol, passed through propylene oxide and embedded in Agar 100. Ultra-thin sections were cut and collected on Formvar coated grids. Sections were stained with uranyl acetate and lead citrate before examination using a Philips transmission electron microscope (CM 10).

Preparation for light microscopy

Material for light microscopy (LM) was fixed and embedded as described for TEM. Serial sections of gills (3–5 µm) obtained from rainbow trouts following exposure to cercariae were removed with an LKB ultramicrotome using glass knives. The sections were stained with methylene blue for 2–3 min. The relative proportions of parasites occurring in different parts of the gills, i.e. in the gill arch and filaments, as well as in different compartments of the gills such as the gill epithelium, dermis, central venous sinus (CVS) and arteries were determined from the serial sections.

Radioisotope labeling of cercariae

A modification of the in vivo method for radiolabeling of *D. spathaceum* previously described by Christensen (1978) was used. Briefly, snails harbouring a patent infection were incubated for 4 days in water containing radioactive selenium ([^{75}Se]-methionine in an aqueous solution; code SC12, Amersham, 0.6–4 Ci/mmol at a concentration of 3 mCi per snail per ml). The snails were marked with nail polish and kept in the storage tank for an additional 11 days prior to harvesting of cercariae.

Fish exposure and determination of radioactivity

A total of 18 rainbow trouts (yearlings, 58–90 mm) from the Färnäs hatchery (Dalarna, Sweden) were exposed for 1 h to 17.5 ml cercarial suspension containing about 5,400 radiolabeled cercariae at a temperature of 15° C in a 10-l tank filled with continously aerated water. The fish were then transferred to a parasite-free tank from which six animals were sampled after 1, 12 and 24 h. To eliminate cercariae from the water, it was passed through a sieve (mesh size, 45 µm) and left for 48 h. Thereafter, three groups of two fish were sham-exposed as above so as to correct for background counts and for the uptake of radioactivity leaking from the snail into the water ("non-parasite radioactivity"). At the time of sampling, the eyes of each fish were removed and the rest of the body was dissected and separated into gills, forebody (head to pelvic fins), hindbody and internal organs. The different parts were placed in plastic capsules and analysed for radioactivity in a Searle gamma counter (Model 1185, Nuclear Chicago) that had been adjusted for measurement of ^{75}Se. All samples were counted for a period of 10 min and the results were corrected for "non-parasite radioactivity", background counts and radioactive decay. In addition, the eyes were dissected and the number of metacercariae were determined using a stereo-microscope.

Statistical analysis and definitions

Numerical data were statistically analyzed using analysis of variance (ANOVA) or the Kruskal-Wallis non-parametric test at the significance level of 0.05. Definitions were used according to Margolis et al. (1982).

Results

Ultrastructural observations

The penetration of rainbow trout by *Diplostomum spathaceum* cercariae is demonstrated in Fig. 1. In all cases examined, the cercariae became attached with the ventral surface pointed towards the penetration site of the host surface. Furthermore, the spiny anterior end was always found to be more or less contracted, burrowing into the host tissue. The characteristic forked tail of the cercariae was always absent after attachment. The disruption of host tissues at the penetration site was minimal and the cercariae appeared to fold up the host epithelium without causing any damage (Fig. 1). Penetration was observed at several sites of the body surface, including the gill arch and filament, the skin and the fins.

TEM sections showed cercariae penetrating the basal lamina of the epithelium in the head region of the fish (Fig. 2). In Fig. 2A, the anterior end of a cercaria is burrowed into the elevated epithelium of the fish. The apical tuft of spines surrounding the mouth region obviously participated in the invasive process. It appeared that the spines were firmly attached to the basal lamina of a parasite that projected through the cytoplasmic layer of a more or less folded syncytial tegument con-

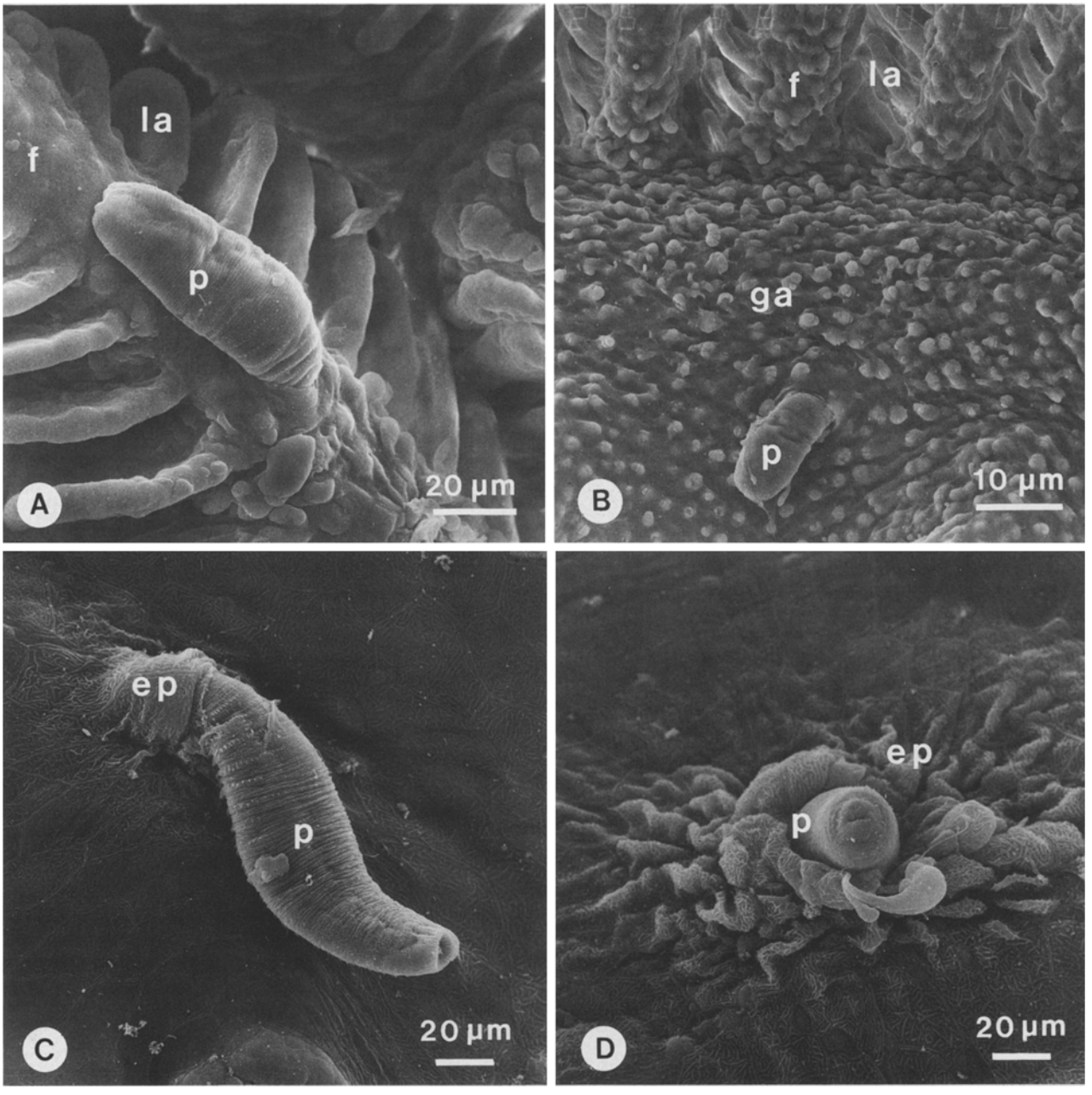

Fig. 1A–D. SEM micrographs of *Diplostomum spathaceum* cercariae penetrating rainbow trout. The anterior end is more or less contracted and has disappeared beneath the epithelial cells. Note that the characteristic forked tail of the cercaria is lost before penetration is begun; also observe the minimal damage inflicted on the host by the penetrating larva. **A** Penetration of the filament; **B** Penetration of the gill arch. **C, D** Penetration of the skin. *ep*, Epithelium; *f*, filament; *ga*, gill arch; *la*, lamella; *p*, parasite

taining electron-dense bodies (Fig. 2B–C). Below the basal lamina of the parasite, sets of longitudinal pro- and retracting muscle bundles were observed. Between the muscle bundles, which exhibited numerous mitochondria, a glandular region was seen. Electron-dense granules could be observed in the vicinity of the penetrating parasite, indicating the secretion of lytic products to facilitate entry into the host.

Inside the fish host, penetrating larvae were found both extra- and intravascularly (Fig. 3). Sagittal sections revealed migration through the dermis of the host and the parasite had evidently burrowed a tunnel through the host tissues (Fig. 3C). Backwardly directed spines of the parasite were often seen in close contact with the host cells (Fig. 3D); they probably aid migration achieved by alternate elongations and contractions of the body of the migrating larvae. The outermost cover of the parasite was a membranocalyx (Fig. 3D, inset).

Radiometric observations

In the eyes of the fish there was a significant gradual increase in radioactivity with time after exposure to

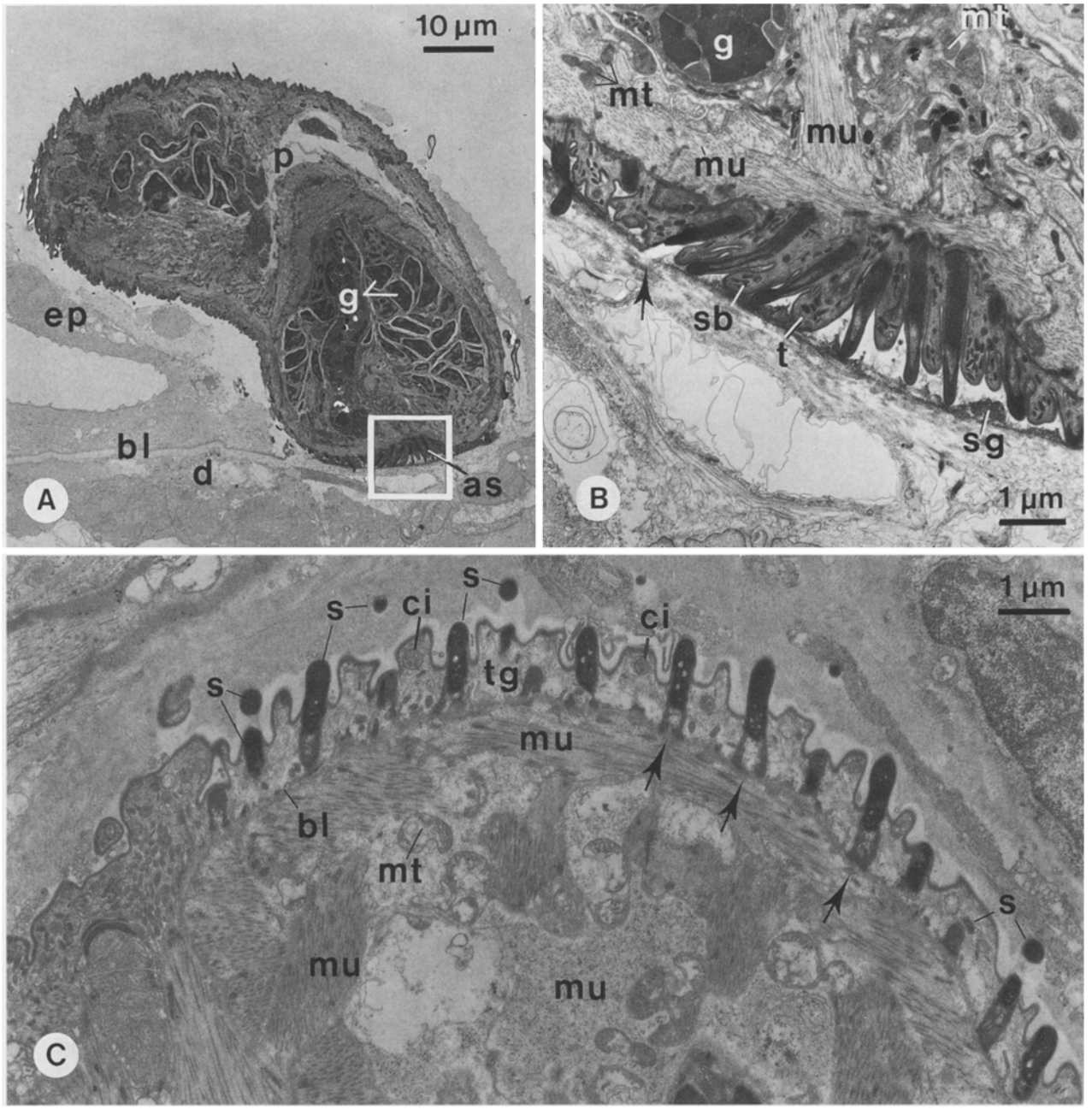

Fig. 2 A–C. TEM micrographs of *Diplostomum spathaceum* in rainbow trout. **A** Sagital section showing a cercariae penetrating the skin. The host epithelium is elevated and surrounded the parasite. **B** Magnification of the site of the basal lamina in a position corresponding to that indicated in **A**. The basal lamina is ruptur (*arrow*). **C** Transverse section of the anterior end of a migrating larvae in the dermis of the gill arch. Note the action and anchorage of the spines (*arrows*) and the muscular layers of the parasite. *as*, Apical tuft of spines; *ci*, cilia; *ep* epithelium; *d*, dermis; *g*, glandular region; *mt*, mitochondria; *mu*, musculature; *p*, parasite; *s*, spine; *sb*, secretory body; *sg*, secretory granule; *t*, tegument

water containing radiolabeled cercariae (ANOVA, $P<0.001$) (Fig. 4A). This increase in radioactivity correlated with an increase in the number of established metacercariae in the lenses of the same fish (Kruskal-Wallis test, $P<0.001$; Fig. 4B). At the same time, a corresponding decrease in radioactivity was noticed in the gill region (ANOVA, $P<0.001$), the forebody (ANOVA: $F=16.1$, $df=2$, $P<0.001$) and the hindbody (ANOVA, $P<0.001$), whereas the level of radioactivity remained constant throughout the experimental period in the internal organs (ANOVA, not significant). At 24 h post-exposure, the majority of the penetrating larvae were recovered as established metacercariae in the eyes of the hosts. Notably, the sum of the radioactivity measured in all parts of the fish decreased continuously during the study (ANOVA, $P<0.001$); at 1 h post-exposure, a total of

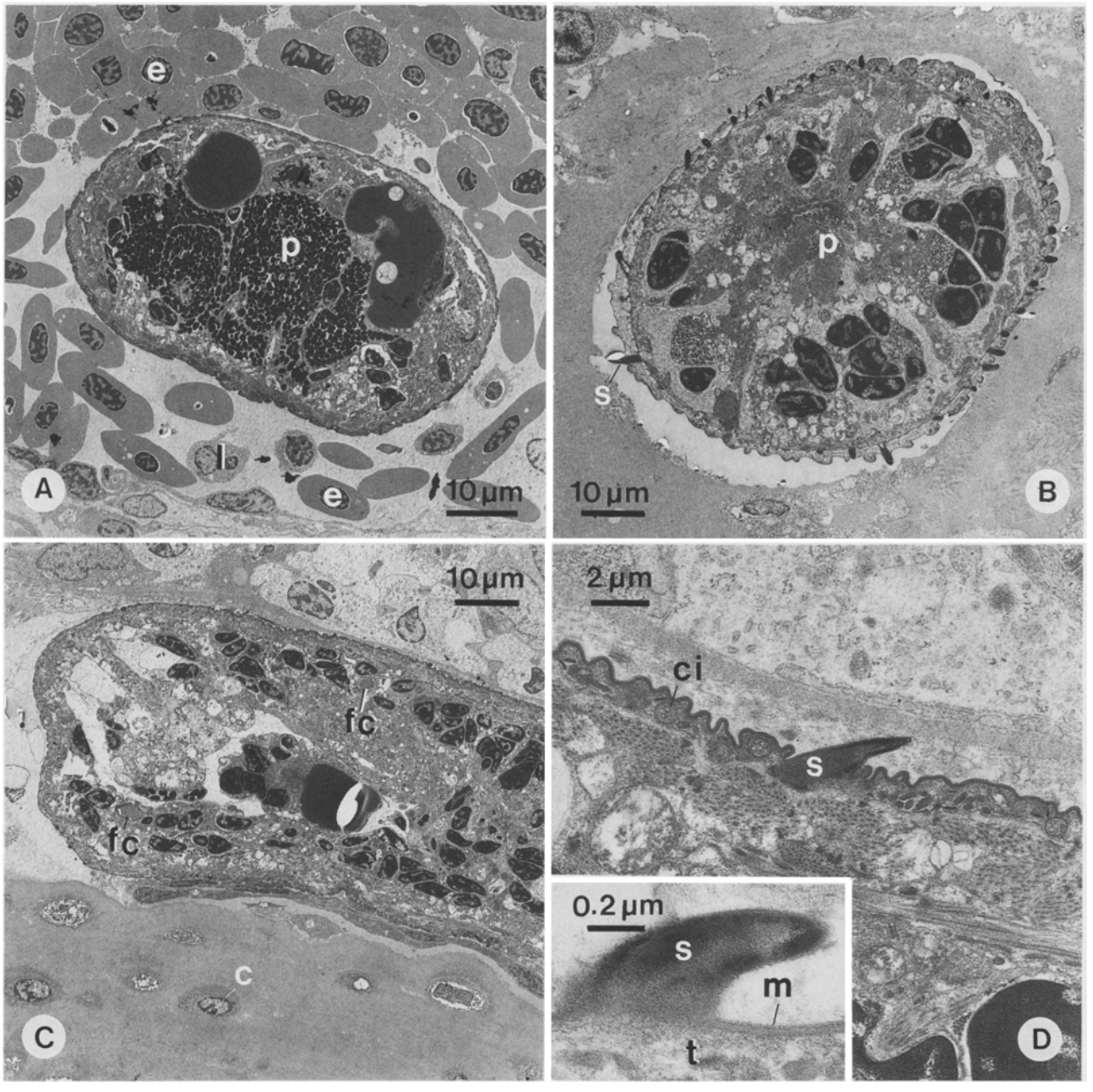

Fig. 3A–D. TEM micrographs of *Diplostomum spathaceum* in rainbow trout. **A** Transverse section of a parasite surrounded by blood cells within a gill blood vessel. **B** Transverse section of a larva migrating along a gill ray. **C** Sagittal section through a larva migrating in the dermis of the gill arch. **D** Detail of migrating larva. *Inset*: a backward-directed spine and the membanocalyx of the parasite tegument. *c*, Chondrocyte; *ci*, cilia; *e*, erythrocyte; *fc*, flame cell; *l*, leukocyte; *m*, membranocalyx; *p*, parasite; *s*, spine

107 ± 12 cpm was retained in the whole fish, which decreased to 75 ± 9 and to 41 ± 9 cpm after 12 and 24 h, respectively.

Relative proportion of parasites in the gills

Results of the differential counts of parasites in the gills are shown in Fig. 5. A majority of the parasites found in the gills were localized in the gill arch. Most were distributed between the dermis of the arch and the filament, outside the circulatory system. However, they were frequently observed in the vicinity of the central venous space close to the gill rakers at the base of the primary filament.

Discussion

Electron microscopy and radiometry demonstrated that the cercariae of *Diplostomum spathaceum* attaches to the body surface of rainbow trout at several sites, with a

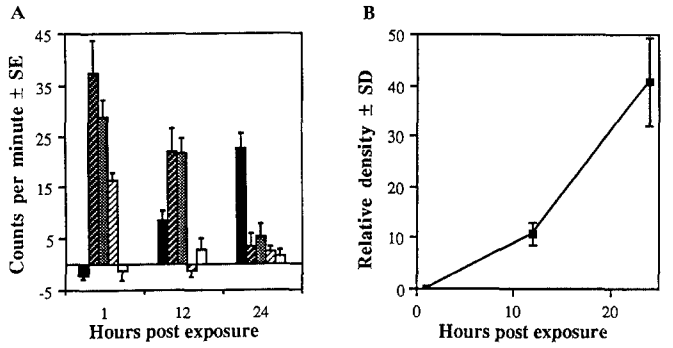

Fig. 4. **A** Radioactivity retained in different parts of the fish at intervals following exposure to radiolabeled cercariae as described in Materials and methods (means ± SE for 6 fish). **B** The relative density of metacercariae in the lenses of both eyes at intervals following exposure to cercariae (means ± SE for 6 fish). ■ Eyes; ▨ Gills; ▩ Forebody; ▨ Hindbody; □ Internal organs

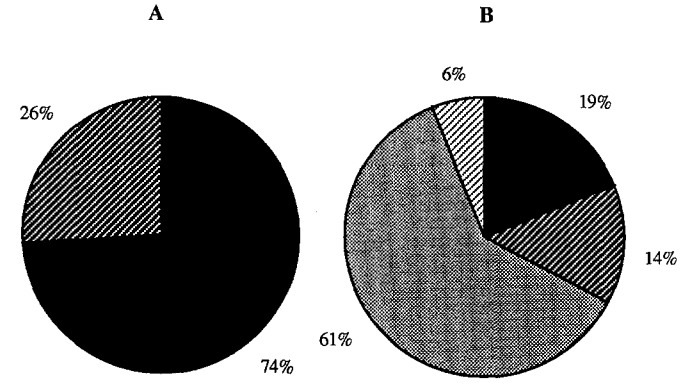

Fig. 5A, B. Relative proportion of *Diplostomum spathaceum* as determined by LM on serial sections of rainbow trout gills at up to 1 h following exposure to cercariae. **A** The distribution between arch and filament. **B** The distribution in compartments of the gills. Terminology is used according to Laurent (1983), $n = 72$. **A** ■ Arch; ▨ Filament; **B** ■ Artery; ▨ Central venous sinus; ▩ Dermis; ▨ Epidermis

marked preference for entry through the gills and the head region of the host. Although this basically agrees with results previously presented by Erasmus (1959) and Ratanarat-Brockelman (1974), a higher proportion of diplostomula were found within the gills. Also, the TEM and SEM micrographs show new details of the penetration and migration process of the diplostomula. The similarity between the pattern of entry of *D. spathaceum* cercariae into the fish host as determined by examination of histological sections and the radiometric result of the present pattern study emphasizes the usefulness of the radioactive tracer method in this parasite-host system.

The range of fish hosts for *D. spathaceum* is wide, and the cercariae locate, attach to and penetrate a variety of vertebrates (Davis 1936; Shigin 1986). This may be interpreted, as there is not true evidence for the existence of a specific response to one or the other chemical gradient and chemo-attraction does not seem to play a significant role in host location. However, once *D. spathaceum* cercariae come in contact with the host, they creep around and search the host's body surface for a suitable penetration site (Erasmus 1959). Chemical stimulation that triggers the cercariae to penetrate the fish has been suggested by Haas (1974a, b; 1975). However, if the cercariae could select the site for entry, penetration directly through the cornea of the eye would probably be favoured to enable the parasite to evade an immunological response against the larvae migrating post-penetration (Whyte et al. 1987, 1989; Bortz et al. 1988; Höglund and Thuvander 1990). Although direct penetration by the cercariae through the cornea of the host has been experimentally demonstrated (Ferguson 1943), it does not occur frequently. More probably, the cercariae reach the host by chance and the penetration sites are more or less accidental. The aggregation of penetrants in the gill region found in the present study could be explained by the passive sweeping of cercariae into the gill chamber of the host via the inhalant respiratory water current.

The anterior end of penetrating parasites were always seen to be more or less burrowed into the host tissue. The mechanism of attachment and penetration seems to differ from that of *Schistosoma*, whereby the cercariae first adhere to the host by means of a sticky mucus secreted from the post-acetabular glands (Bruce et al. 1970; Stirwalt and Dorsey 1974). However, like *Schistosoma*, the cercariae of *D. spathaceum* seem to penetrate the host skin via both chemical and mechanical aids. They have a membranocalyx as well, which probably protects the parasite against the host defense system. The total amount of radioactivity retained in the fish gradually decreased with time. It seemed that a proportion of the attached parasites failed to penetrate the fish; This finding may have been due to sampling artifacts. However, the probabilities of sampling the six most heavily infected fish in the first round and the following six individuals in the second round are almost zero ($P = 0.000054$ and 0.0011, respectively). Thus, the results more probably reflected a true situation in that the total number of parasites attached to and present in the fish actually decreased. A second possible explanation for the decreased radioactivity content is that the isotope was metabolized by and eliminated from the fish; however, the biological half-life of selenium methionine in fish has been demonstrated to be 27 days (Kleinow and Brooks 1986).

Previous investigators of diplostomatoid cercariae in various hosts have differed in their views on the migratory route taken by the larvae post-penetration. Ferguson (1943) and Betterton (1974) both concluded that migration to the eyes of the larvae of *D. flexicaudum* in rainbow trout and *D. spathaceum* in 3-month-old trout (*Salmo trutta*) principally followed the circulatory system. In contrast, Larson (1965) and Ratanarat-Brockelman (1974) excluded the circulatory system as a migration pathway for the larvae of *D. flexicaudum* in the black bullhead (*Ictalurus melas*) and for *D. spathaceum* in the minnow (*Phoxinus phoxinus*). Hoffman and Hoyme (1958) and Erasmus (1959) suggested that the cercariae of *D. baeri eucaliae* and "cercariae X" migrate through

muscles and connective tissues of brook trout (*Salvelinus fontinalis*) and sticklebacks (*Gasterosteus aculeatus*). In the present study, larvae were found both within and outside the circulatory system of the host. Although a majority of larvae were located in the dermis of the gills, they were often observed in the vicinity of the circulatory system. Irrespective of the migratory route taken, the majority of parasites became established as metacercariae within 24 h after the fish had been exposed to cercariae in ambient water. This indicates a comparatively rapid migration that seems to be directed to the eyes of the fish. The most probable explanation for this is that larvae reaching the eye region of the host are actively and/or passively transported with the blood in the circulatory system.

The location of host rainbow trout by the cercariae of *D. spathaceum* most probably occurs by chance. The main site of entry is the gill region, presumably due to ventilation hydrodynamics. Once inside the fish, the majority of cercariae appear in the eye region within 24 h. Migration in connective tissue and blood vessels is presumably non-random, mechanically directed forwards by muscle action and spines and by some kind of histolysis, and may be due to chemical cues.

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