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Histopathological and ultrastructural observations of metacercarial infections of *Diplostomum phoxini* (Digenea) in the brain of minnows *Phoxinus phoxinus*

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ABSTRACT: The spatial distribution and histopathological changes induced by metacercariae of the digenean trematode *Diplostomum phoxini* (Faust, 1918) in the brains of European minnows *Phoxinus phoxinus* (L.) from the River Endrick, Scotland, were studied by light and electron microscopy. Post-mortem examination of a sample of 34 minnows revealed that 50% (n = 17) of the population was infected with 13.7 ± 2.6 (mean ± SE; range 1 to 38) metacercariae per infected host. Serial histological sections of the infected minnow brains revealed that the metacercariae were unevenly distributed throughout the brain, with aggregations occurring in the cerebellum, the medulla oblongata and the optic lobes. In fish with highest intensities of infection, over 40% of the cerebellar area and about 30% of the medulla oblongata area were occupied by larvae. Metacercariae disrupt the integrity of brain tissue, with individuals being found in small pockets surrounded by cellular debris. Metacercariae were rarely encountered on the surface of the brain. Electron microscopic examination of infection sites revealed that the granular layer surrounding metacercariae was necrotic, exhibited nuclear degradation and was marked by vacuolation of the cytoplasm. Rodlet cells, the only inflammatory cell types recorded in this study, were found only in parasitized brains and in close proximity to the teguments of metacercariae. It is hypothesised that secretions released from the teguments of metacercariae are a counter response to protect the metacercariae from the fish brain's cellular defence mechanisms.

KEY WORDS: Brain infection · Digenean trematode · *Phoxinus phoxinus* · Rodlet cells · Histopathology · Ultrastructure

INTRODUCTION

Members of the strigeid digenean genus *Diplostomum* Nordmann, 1832 and the closely related genus *Tylodelphys* Diesing, 1850 are important parasitic pathogens of farmed and wild stocks of fish, and infections are known to induce blindness, emaciation, cranial deformation, poor growth and, in some species, host mortality (Shariff et al. 1980, Chappell 1995, Pylkkö et al. 2006).

A parasite's affinity or specificity for particular tissue/organs sites may result from a number of factors that may be driven by the specific nutritional and/or environmental requirements of the parasite (Crompton 1976), the consequences of inter-specific competition for resources and subsequent niche separation between species (Holmes & Price 1985), or by attempts to evade the host's chemical and cellular immune defences (Price 1987, Sukhdeho & Mettrick 1987, Sukhdeho & Sukhdeho 1994).

The current study on a population of *Diplostomum phoxini* (Faust, 1989)-infected *Phoxinus phoxinus* (L.) in the River Endrick, Scotland, found metacercariae principally within the cerebellum, the medulla oblon-

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gata and the optic lobes, centres of the brain that are responsible for host motor activity, sensory functions and vision (Barber et al. 2000, Shirakashi & Goater 2005). Until recently, metacercariae were generally regarded as immature, non-feeding and metabolically inactive stages waiting for transmission to the next/definitive host (Bush et al. 2001, Poulin & Latham 2003). However, a recent study by Goater et al. (2005) demonstrated via experimental infection that the metacercariae of *Ornithodiplostomum ptychocheilus* Faust, 1917 in *Pimephales promelas* Rafinesque, 1820 undergo a series of complex developmental changes associated with their feeding on host brain tissue.

A histological examination of *Phoxinus phoxinus* heads in the current study revealed the presence of rodlet cells only in infected brains, notably in the epithelium lining the ventricles of the optic lobes. It is believed that rodlet cells play a role within the piscine inflammatory response (Dezfuli et al. 2000, Manera & Dezfuli 2004, Reite 2005, Reite & Evensen 2006). We found rodlet cells only within the infected brain of a fish, and our findings are briefly compared with the information available on their presence in other fish-parasite systems (Dezfuli et al. 1998, 2000, 2002, 2003, Reite 1998, Bosi et al. 2005). While there have been numerous ultrastructural studies on metacercariae (Stein & Lumsden 1971, Strong & Cable 1972, So & Wittrock 1982, Faliex & Biagianti 1987, Wittrock et al. 1991, Goater et al. 2005), most focused on the development of the larval epithelial layer within the intermediate host, and only a few of these studies focused on the interactions between host tissues and the parasite (Baghaei 1981, So & Wittrock 1982, Faliex & Biagianti 1987, Wittrock et al. 1991). Fewer again used transmission electron microscopy (TEM) to examine the cysts of strigeoid digeneans (Bibby & Rees 1971, Mitchell 1974, Baghaei 1981, Goater et al. 2005).

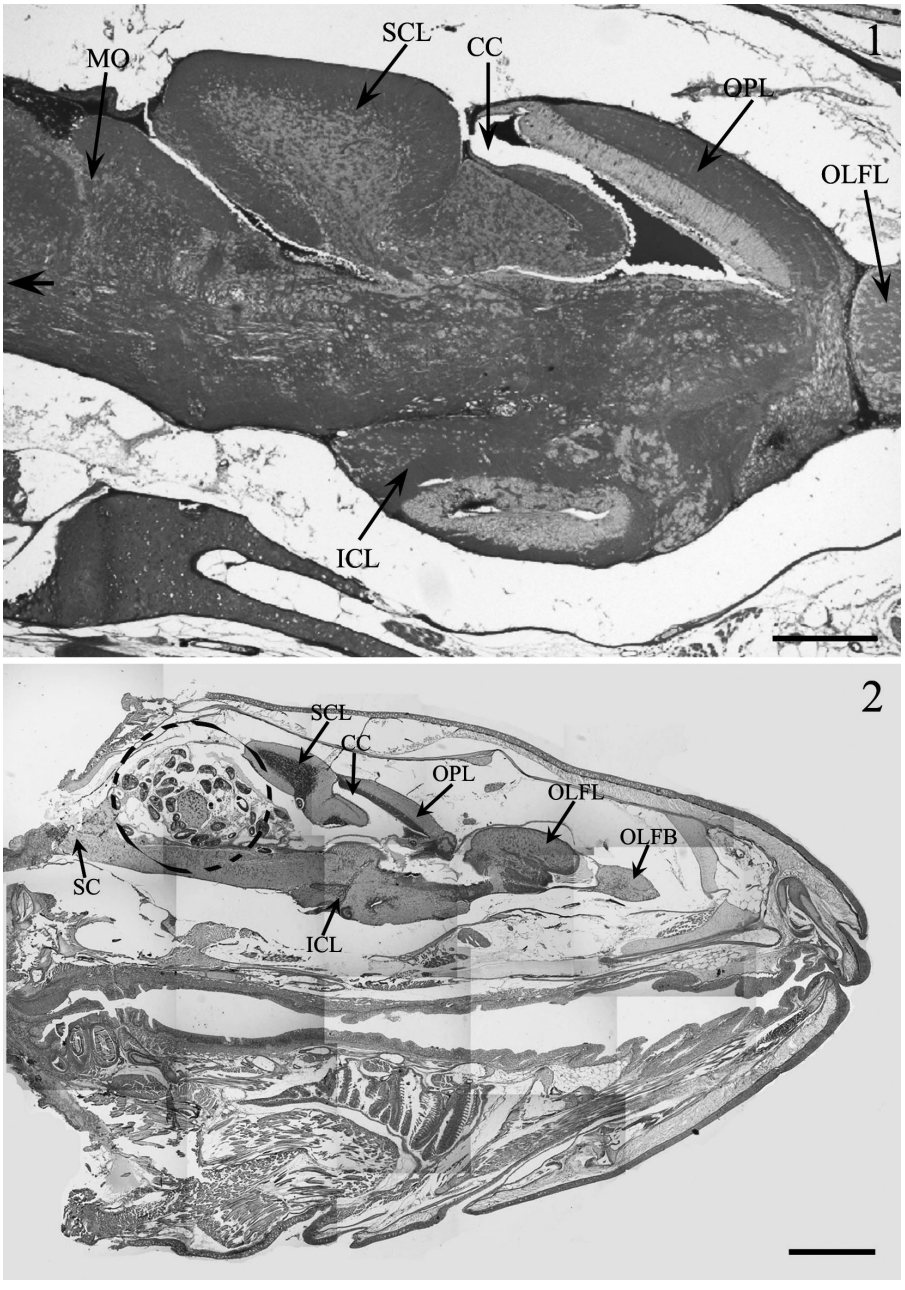
The present study provides information on (1) the occurrence of *Diplostomum phoxini* metacercariae in the brains of minnows *Phoxinus phoxinus*, with particular comments on the interface region between the host and the parasite; (2) light and electron microscopic observations on histopathological changes in brain tissue; (3) the location of metacercariae, the damage they cause in key regions of the brain and their effect on host vision and motor activity; and (4) the relationship between brain cells, inflammatory cells and *D. phoxini* metacercariae.

MATERIALS AND METHODS

Thirty-four minnows *Phonixus phoxinus*, were hand-netted from the River Endrick, Stirlingshire, Scotland (56° 03′ N, 4° 27′ W) and transferred to the laboratory in river water where they were maintained in aerated aquaria until being processed. Fish were subsequently sacrificed by exposure to a lethal dose of the anaesthetic MS-222 (Sandoz), and were then weighed (mean weight ± SE: 0.21 ± 0.01 g), measured (mean total length ± SE: 30.57 ± 0.55 mm) and processed for histology. After severing the spinal cord, 28 heads were removed and fixed in chilled (4°C) Bouin's fluid for 7 h before being transferred into chilled 70% alcohol for storage until being processed for paraffin embedding by routine methods using an automatic tissue processor (Shandon Citadel 2000 Tissue Processor, Shandon Southern Products). Histological sections (5 µm thick) were cut and stained with a haematoxylin and eosin (H&E), Azan-Mallory, periodic acid Schiff (PAS) or an alcian blue/PAS stain. Light photomicrographs were taken using a Nikon microscope Eclipse 80i. The dimensions of *Diplostomum phoxini* key features in the acquired images were subsequently determined using computerised image analysis software (Lucia G 4.8, Laboratory Imaging). For the TEM study, 6 *P. phoxinus* heads were fixed for 2 h in a chilled (4°C) 2% glutaraldehyde solution buffered at pH 7.2 with 0.1 M sodium cacodylate, and were then rinsed in 0.1 M sodium cacodylate buffer containing 6% sucrose for approximately 12 h. The minnow heads were then post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated through a graded ethanol series, transferred to propylene oxide and then embedded in an Epoxy-Araldite mixture. Semi-thin sections (1.5 µm) were cut on a Reichert Om U2 ultramicrotome using glass knives and stained with toluidine blue. Ultra-thin sections (90 nm) were contrasted in a 50% alcohol-uranyl acetate solution and lead citrate, and examined using a Hitachi H-800 electron microscope operated at 80 kV.

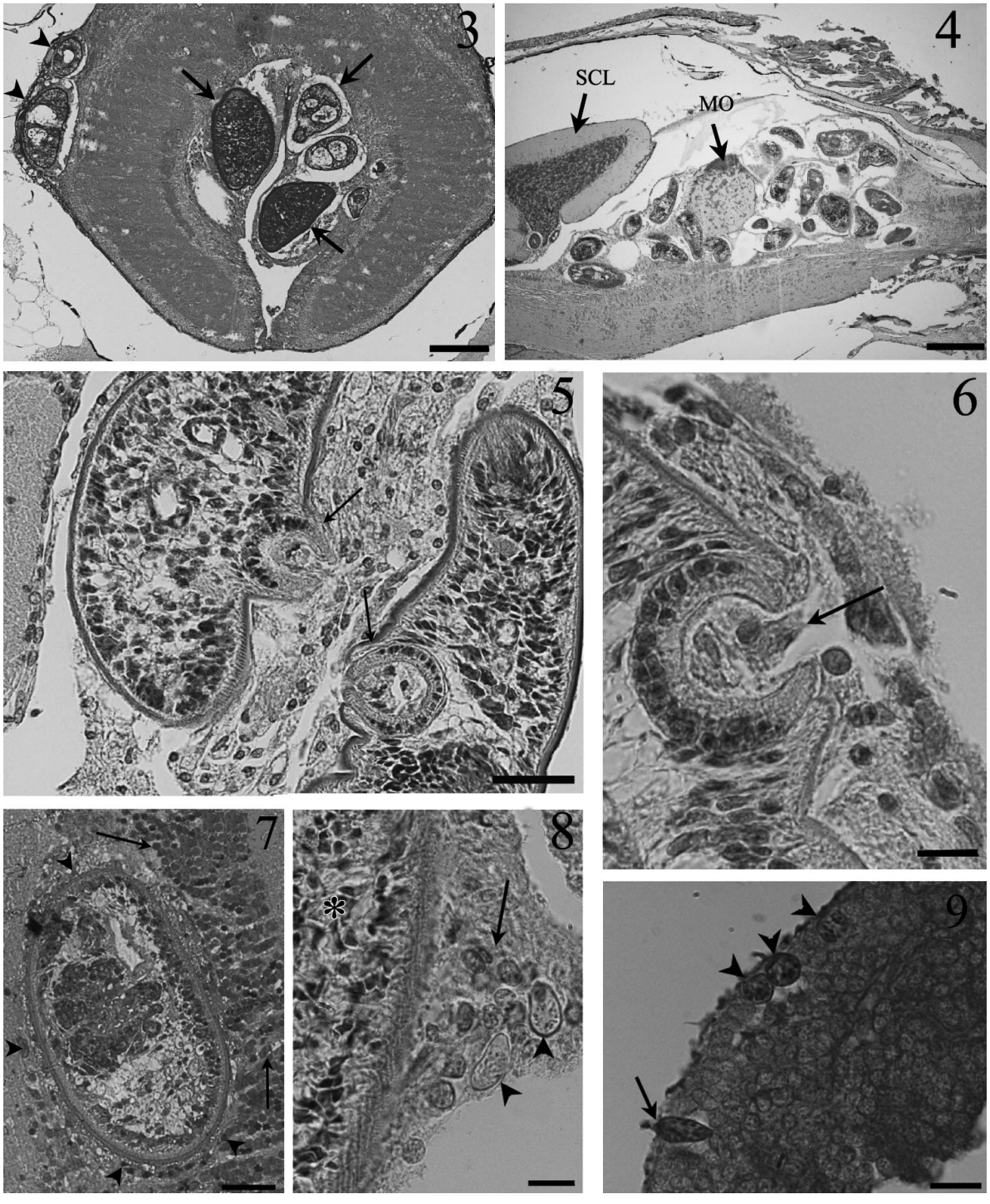
RESULTS

Post-mortem histological examination of 34 minnow heads revealed that 50% (n = 17) were infected with 13.7 ± 2.6 (mean ± SE; range 1 to 38) *Diplostomum phoxini* metacercariae per infected fish. Measurement (mean ± SE) of the metacercariae (239.75 ± 11.51 µm long by 103.05 ± 5.88 µm wide) indicated that the infections were well established (as opposed to being characterised by diplostomules that had recently entered the brain). Fig. 1 & 2 shows uninfected and *D. phoxini*-infected brains. The most frequently occupied regions of the brain included the medulla oblongata (cortex and centre, Fig. 3), the optic lobes, the cerebellum and its superior lobe, and the cavity between the superior lobe and the anterior part of the medulla oblongata. In addition to these areas, 3 fish were also



Figs. 1 & 2. Fig. 1. *Phoxinus phoxinus*. Sagittal section through an uninfected brain of *P. phoxinus*. Note the integrity of each region of the brain. OLFL: olfactory lobes; OPL: optic lobe; SCL: superior cerebellar lobe; CC: cerebellar cavity; ICL: inferior cerebellar lobe; MO: medulla oblongata. Short arrow shows the direction of the spinal cord. Scale bar = 250 µm. Fig. 2. Sagittal section through the entire head of a *P. phoxinus* with a heavy infection of *Diplostomum phoxini* metacercariae. A high proportion of the medulla oblongata and regions of the cerebellum have disappeared and are occupied by numerous metacercariae (dashed circle). OLFB: olfactory bulbs; SC: spinal cord. Scale bar = 500 µm

found to have metacercariae within the superior lobe of the cerebellum. However, the inferior lobe of the cerebellum, the pituitary, the olfactory lobes and the olfactory bulbs were largely free of metacercariae. In brains with more than 15 larvae, over 40% of the cerebellar area and about 30% of the medulla oblongata area were occupied by metacercariae; consequentially, a low proportion of the nervous tissue was found to remain within these regions (Figs. 2 & 4). Metacercariae within the medulla oblongata and the cerebellum were found within large cleared areas surrounded by brain cells and cellular debris.



Figs. 3 to 9. *Phoxinus phoxinus*. Different regions of *Diplostomum phoxinus*-infected brains. Fig. 3. *D. phoxini* metacercariae can be seen in the centre (arrows) and on the periphery (arrowheads) of the medulla oblongata, and are sometimes surrounded by a clear area. Scale bar = 250 µm. Fig. 4. In a heavily *D. phoxini*-infected brain, only a small proportion of the cerebellum (SCL) and medulla oblongata (MO) regions were found intact. Scale bar = 250 µm. Fig. 5. Attachment of 2 metacercariae to host brain tissue, with detached pieces of host nervous tissue within their ventral/oral suckers (arrows). Scale bar = 30 µm. Fig. 6. A high magnification of the ventral sucker on a metacercaria. Note the presence of nervous elements within the sucker (arrow). Scale bar = 10 µm. Fig. 7. Metacercaria encystment within the optic lobe. Host cells (arrowheads) are attached to the larval tegument; arrows show granule cells of optic lobe. Scale bar = 30 µm. Fig. 8. Two rodlet cells (arrowheads) within the granule cells (arrow) surrounding a metacercaria (*). Scale bar = 10 µm. Fig. 9. Rodlet cells (arrowheads) in the surface of the ventricle of the optic lobe. One rodlet cell (arrow) is in the process of discharging its contents. Scale bar = 10 µm

Parasites were commonly observed attached to the host tissue by their ventral and/or oral suckers, or were observed with detached pieces of brain tissue within them (Figs. 5 & 6). Single metacercaria, in 2 separate fish, were also noted embedded within the tissues of an olfactory bulb (Fig. 7). Although infections in the optic lobes were observed to be lower than those seen in the cerebellum or the medulla oblongata, metacercariae found here and within the medulla oblongata (Fig. 7) were observed to be in intimate contact with granule cells of the brain. Granule cells, which are typically dark in appearance and possess circular-shaped nuclei and a diffuse cytoplasm, form the granular layer of the optic lobe ventricles (Fig. 7).

The presence of rodlet cells scattered among the granule cells of the optic lobes of infected fish only (Figs. 8 & 12) is of particular interest. In each case, the rodlet cells were observed on the surface of the ventricles of the optic lobes and in close proximity to a metacercaria, where some rodlet cells were seen to be discharging their contents (Fig. 9). Rodlet cells were also commonly encountered within the periphery of the cerebellum in all *Diplostomum phoxini*-infected brains. The rodlet cells, which appeared to be large in dimension and measured 12.12 ± 0.19 µm (major axis) by 8.59 ± 0.16 µm (minor axis), typically contained a high number of club-shaped structures (rodlets) that possess a crystalline core and a vesicular, foam-like cytoplasm that did not contain any other evident organelles (Fig. 12).

Thirteen of the 28 minnows heads prepared for wax embedding and 4 of the 6 heads prepared for the TEM study were infected with metacercariae: 1 to 38 parasites per infected head were observed in the former study, and 1 to 3 in the latter. From these samples, only metacercariae within the optic and olfactory lobes were observed to make intimate contact with host tissue. A TEM study of these regions suggested that the region where the parasite interfaces with the host can be broken down into 2 zones. The inner zone, which is adjacent to the metacercaria, possesses a large number of white vacuoles (Fig. 10). In histological sections prepared for light microscopy, this zone appears as large white spaces surrounding metacercariae (Figs. 3 & 4). Within the optic lobe, the inner zone of granule cells, which are in close proximity to a metacercaria, are notably flattened and in a degenerative state typified by severe vacuolation and organelle regression (Figs. 10 & 11). The nuclei of the cells in this region also appear necrotic, show karyolysis, chromatin margination and condensation (Fig. 11), and in some cases nuclear swelling (Fig. 11).

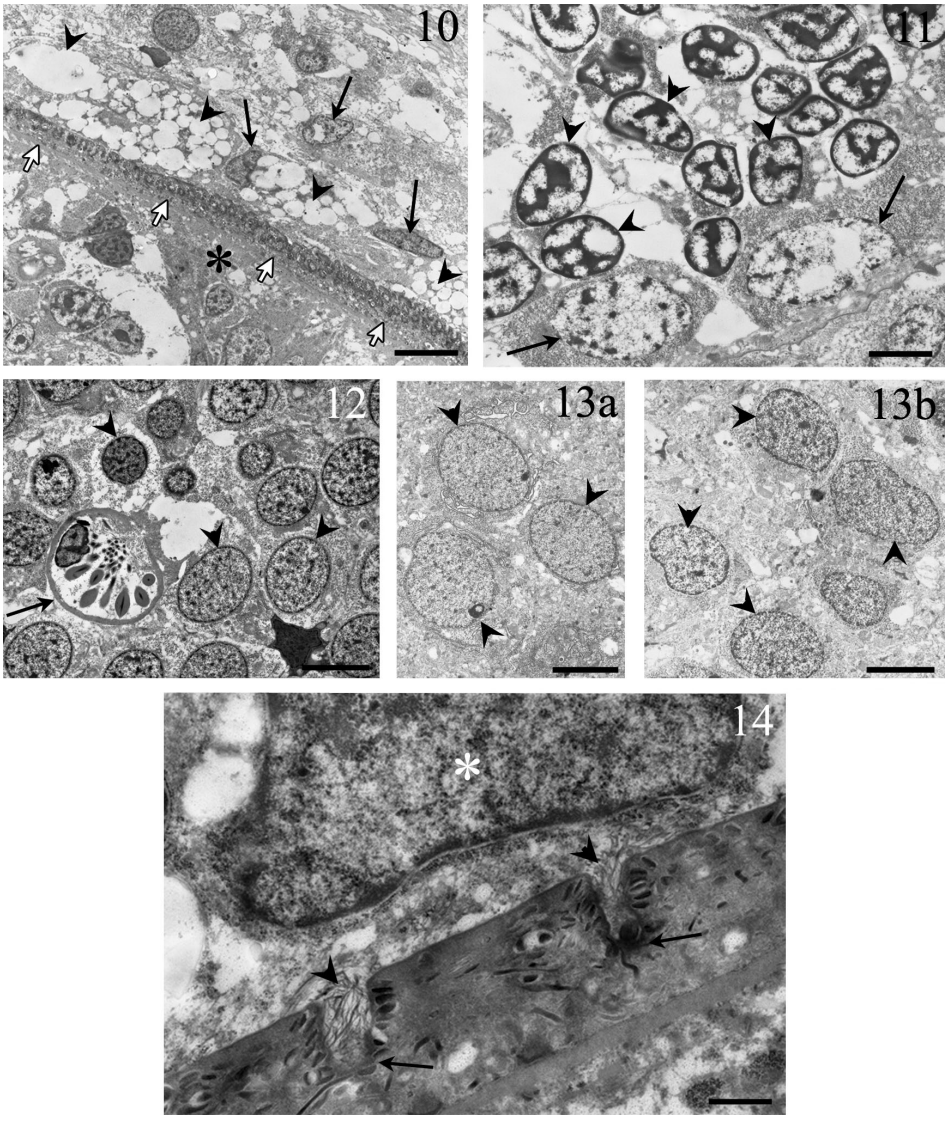
By contrast, the outer zone of the host-parasite interface appears more compact in density and contains several granule cells in a state of degeneration. The ultrastructural changes observed in the olfactory lobe were similar to those observed for the optic lobe, with cells in a degenerative state that decreased in severity with distance from the metacercariae (Fig. 13a). The characteristics of the granule cells that are located at a distance from metacercariae and show few histopathological changes (Fig. 13a) are similar to the granule cells observed in the brains of uninfected fish (Fig. 13b). The only observed difference between these 2 groups of cells was that the granule cells in the uninfected fish brains contained more glycogen.

Close examination of the host-parasite interaction zone also revealed the occurrence of a high number of secretory vesicles (Fig. 14). These vesicles, which are arranged regularly within the tegument of the metacercariae, open into the interfacing zone, where they can be seen releasing their cellular contents, which consist of a loose microfilamentous material (Fig. 14). In many instances, the secreted material was observed in contact with the host brain cells, which appeared to be in a partial or total degenerative state (Fig. 14).

DISCUSSION

The current study found that the sub-sample of *Phoxinus phoxinus* collected from the River Endrick population were infected by *Diplostomum phoxini* that favor the cerebellum, the medulla oblongata and the optic lobe regions of the brain. Metacercariae colonizing these sites are suitably placed to alter the sensory and motor functions of the host. For example, the cerebellum of fish is believed to be linked to a variety of cognitive functions (Rodriguez et al. 2005), and its removal from a dogfish *Mustelus canis* Mitchill resulted in a marked reduction in motor and sensory performance (Karamyan 1956). The medulla oblongata is known to control a range of involuntary responses such as breathing, digestion and heart rate (Bernstein 1970), while the optic lobes are the primary vision-processing centre in teleost fishes (Guthrie 1986); herein, visual information regarding movement, color and shape are analyzed, and stimuli from the lateral line system are received (Springer et al. 1977, Kortrschal et al. 1991).

An investigation by Barber & Crompton (1997) that examined the distribution of *Diplostomum phoxini* in the brains of 2 populations of minnows found that the 2 populations favoured particular regions of the brain and almost totally neglected others. The finding from Barber & Crompton's (1997) study suggests that individual *D. phoxini* or populations of *D. phoxini* may favour certain sites over others, which—once damaged—facilitates their transmission to the final host by compromising the anti-predatory behaviour and performance of their fish host.



Figs 10 to 14. *Phoxinus phoxinus*. Electron micrographs of uninfected and *Diplostomum phoxini*-infected brains. Fig. 10. High magnification of the host-parasite interface zone. Numerous white vacuoles (arrowheads) close to the metacercaria tegument (*) are evident, as are a number of flattened, degenerating nuclei (arrows). A number of secreting bodies within the tegument of the metacercaria are also visible (white arrows). Scale bar = 5 µm. Fig. 11. High magnification of the region between *D. phoxini* and *P. phoxinus*. Micrograph shows a large number of necrotic nuclei (arrowheads) with karyolysis, chromatin margination and condensation. Two very swollen nuclei (arrows) in close proximity to the parasite are highlighted. Scale bar = 2.5 µm. Fig. 12. Peripheral zone of a *D. phoxini*-infected cerebellum in close proximity to a metacercaria. A rodlet cell (arrow) is evident among several brain cells with swollen nuclei (arrowheads) and rarefied cytoplasm. Scale bar = 4.3 µm. Fig. 13a. Brain cells (arrowheads) distant from a metacercaria. Cells appear less degenerated and have only slightly swollen nuclei. Scale bar = 4.8 µm. Fig. 13b. Granule cells (arrowheads) in an uninfected brain. Brain cells possess nuclei of normal shape and size with more cytoplasm. Scale bar = 3.7 µm. Fig. 14. High magnification of the tegument of *D. phoxini* and a brain cell. Two secreting bodies (arrows) of parasite tegument can be seen discharging microfilamentous material (arrowheads) into the zone between the metacercaria and brain cells of the host. A degenerated nucleus (*) is also highlighted. Scale bar = 0.8 µm

Almost all the *Diplostomum phoxini* metacercariae encountered in the current study were fully developed, i.e. were not migrating diplostomules that had just entered the brain. Bush et al. (2001) and Poulin & Latham (2003) assumed that, developmentally and ecologically, this part of the digenean life-cycle is a resting stage. However, this suggestion was most likely based on ultrastructural and cytochemical observations made on the cyst wall of fully developed metacercariae. Based on ultrastructural examinations of the metacercarial tegument, it was suggested that the opportunities for absorption across the tegument

and for ingestion via the mouth were likely to be low (Stein & Lumsden 1971, Erasmus 1972, Bock 1988, Wittrock et al. 1991). The recent research of Goater et al. (2005) provided morphological evidence to suggest that certain species of metacercariae undergo complex developmental changes associated with feeding in their intermediate host. The observation of detached brain tissue within the oral suckers of attached metacercariae in the present study would suggest that this species feeds on host tissues. The work of Bibby & Rees (1971) on extracorporeal digestion in *D. phoxini* metacercariae and the finding of acid phosphatase throughout the metacercaria would suggest that intracellular digestion is occurring and that the parasites are feeding. Based on the results of an experimentally infected sample of minnows, Goater et al. (2005) suggested that minnows are either able to tolerate a certain level of damage to their brains or are able to regenerate or repair brain tissue. Post-mortem examination of minnows in the current study revealed that the minnows had light infections (range 1 to 38) compared with those (1300 metacercariae) reported by Rees (1955). Therefore, the observed levels of infection and the results from the current study correspond to the findings of Goater et al. (2005).

Only a few records relating to the host's immune reaction in response to *Diplostomum* spp. infections exist (Bortz et al. 1984, Stables & Chappell 1986). Similarly, the host cellular reaction in *Diplostomum*-based histopathological studies has been largely overlooked (Rees 1955, Hoffman & Hoyme 1958, Shariff et al. 1980, Baghaei 1981). One of the main objectives of the current study was to look closely at the host–parasite interaction and to characterise the cells present within the interfacing zone. One interesting observation was the presence of rodlet cells within this zone, the only host inflammatory cells documented in this sub-sample of *D. phoxini*-infected *Phoxinus phoxinus* brains. Rodlet cells are, as far as we are aware, exclusive to fish and are associated with the epithelial tissues of virtually every fish species (Morrison & Odense 1978). Recent research by a number of workers lends support to the theory that rodlet cells have an endogenous fish origin (Manera & Dezfuli 2004, Giari et al. 2006, Reite & Evensen 2006, Dezfuli et al. 2007). Although their precise function is unknown, it is thought that rodlet cells are a type of inflammatory cell akin to other piscine inflammatory cells (e.g. mast cells/eosinophilic granule cells, epithelioid cells and mesothelial cells) (Dezfuli et al. 2000, Reite 2005, Reite & Evensen 2006). Most of the rodlet cells that were found in the current study were observed in the epithelial lining of the ventricles of the optic lobes and in sites close to the tegument of metacercariae. This observation fits well with Leino's (1996) suggestion that rodlet cells react to the presence of parasites on the epithelial surface, and that cellular secretions are possibly antibiotic in nature. This theory is also supported by the work of others that have investigated fish protozoan (Leino 1996, Dezfuli et al. 2004) and metazoan parasitic agents, all of which appear to induce the recruitment of rodlet cells to the site of infection and/or to adjacent tissues (Dezfuli et al. 1998, 2000, 2003, Palenzuela et al. 1999, Bosi et al. 2005, Reite 2005, Reite & Evensen 2006, the present study).

While there have been numerous studies on the ultrastructure of metacercariae (Stein & Lumsden 1971, Strong & Cable 1972, Higgins 1980, Bock 1988, Wittrock et al. 1991), only a few have focused on that of diplostomatids (Bibby & Rees 1971, Mitchell 1974, Goater et al. 2005). Of these, the detailed study by Bibby & Rees (1971) on the epidermis of *Diplostomum phoxini* metacercariae described 3 types of secretory bodies that were regularly arranged in the outer epidermal layer, but did not comment on the possible function of the secretions. The numerous secretory bodies found in the tegument of *D. phoxini* in the present study were observed to release a loose, microfilamentous material into the zone between the host and the parasite. In addition to these bodies, many bodies that had already emptied their contents were also evident, usually in that part of the parasite's tegument adjacent to a host cell (Fig. 3f). The brain cells in this region characteristically bore flattened nuclei and were in a partial or complete degenerative state. However, without knowing the precise chemical nature of the secreted product (which we are investigating within our laboratory at the time of writing), we can only hypothesise that the compound is secreted to protect the metacercaria from the host's cellular response.

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