Migration and site selection of *Ornithodiplostomum ptychocheilus* (Trematoda: Digenea) metacercariae in the brain of fathead minnows (*Pimephales promelas*)

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SUMMARY

The migration of subadult parasites to preferred sites within final hosts is well characterized. In contrast, the migration of larval stages of trematodes to specific sites within their second intermediate hosts is poorly understood. We used a serial necropsy approach to characterize the migration of *Ornithodiplostomum ptychocheilus* diplostomules from the point of cercarial penetration, to encystment within the outermost tissues of the brain of fathead minnows. Diplostomules utilized peripheral nerves to access the central nerve cord, or they used specific cranial nerves to directly access the brain. Within 3 h of exposure to cercariae, 46% of all diplostomules were observed within the medulla of the brain. Diplostomules subsequently utilized specific neural tracts to reach lateral regions of the outermost tissue layer of the optic lobes, the stratum marginale. Diplostomules remained in this layer during their 4-week growth phase, then shifted site to the adjacent meninges for encystment. Characterization of a habitat shift for developing versus encysted metacercariae helps explain the results of previous ecological studies that document transient changes in the effects of metacercariae on the surivival, behaviour, and anti-parasite defences of infected fish.

Key words: Strigeoid trematode, parasite navigation, parasite migration, meninges, optic lobes, cerebellum, habitat shift.

INTRODUCTION

Site selection is a central tenet of parasitology. Standard texts often provide striking examples of tissue, organ, and intracellular specificity for a wide variety of protozoan and metazoan parasites (Bush *et al*. 2001; Roberts *et al*. 2004). The historical attention devoted to patterns and explanations for parasite site selection likely stem from the implications that site specificity has on fundamental aspects of host-parasite biology. Thus, the specific sites occupied by parasites within their hosts can explain variation in features such as parasite-induced host pathology, parasite-induced alterations in host phenotypes (especially host behaviour) and expression of host immunity and other forms of host defence (reviews by Sukhdeo and Sukhdeo, 1994, 2004). Detailed knowledge of the sites parasites occupy has also been used to clarify aspects of parasite taxonomy and phylogeny (Williams, 1966; Sukhdeo and Sukhdeo, 1994).

Metacercariae of strigeoid trematodes tend to occupy specific sites within the tissues of their fish intermediate hosts (review by Erasmus, 1972). In the context of parasite migration and site selection, species that occupy the eyes, brain, heart, liver, and muscles have received considerable attention (Hoffman and Hoyme, 1958; Johnson, 1971; Hendrickson, 1979; Hoglund, 1991; Haas *et al*. 2007). However, the manner in which penetrating cercariae and their subsequent migrating stages (known as diplostomules) navigate through the complex tissues and organ systems of their hosts to reach these various sites remains poorly understood (reviews by Sukhdeo and Sukhdeo, 2004; Haas *et al*. 2007). Further, the temporal pattern of site selection is unknown for strigeoids and non-strigeoids alike. Thus, the metacercariae of strigeoids and many other trematodes require an obligate period of development within the second intermediate host prior to reaching the infective stage (Erasmus, 1972; Sandland and Goater, 2000; Conn *et al*. 2008). Whether or not the site(s) occupied by metacercariae change throughout this period of development has not been evaluated.

Metacercariae of the strigeoid *Ornithodiplostomum ptychocheilus* encyst within the optic lobes and cerebellum of the brain of fathead minnows, *Pimephales promelas* (Hendrickson, 1979; Sandland and Goater, 2000). Initial experimental studies established that cercariae penetrated the host epidermis anywhere along the host body, and then migrate to the brain via spinal and cranial nerves within 24 h (Hendrickson, 1979). Subsequent experimental work has shown that the migration of diplostomules is associated with the development of a protrusible proboscis that disappears following site selection within the outermost

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layer of the optic lobes (Conn *et al*. 2008). Developing metacercariae then undergo discrete phases of growth, encystment, and consolidation before reaching infectivity approximately 10 weeks after exposure to cercariae (Sandland and Goater, 2000; Shirakashi and Goater, 2005). However, we do not understand the manner in which individual *O. ptychocheilus* diplostomules navigate through the complex network of peripheral nerves to their ultimate site within the brain. We also do not know whether developing vs. encysted *O. ptychocheilus* metacercariae are associated with contrasting sites within the brain. The objective of the present study is to use serial necropsy (Wilson, 1994) to evaluate the routes and timing of migration of *O. ptychocheilus* diplostomules and metacercariae within the brain of fathead minnows.

MATERIALS AND METHODS

Infection procedure

The methods used to expose fathead minnows to *O. ptychocheilus* cercariae have been described by Sandland and Goater (2000). Trematode eggs were collected from the faeces of 1-day-old chickens that had been force-fed the brains of naturally infected minnows in June, 2007. The F1 offspring of field-collected pond snails, *Physa integra*, were exposed to miricidia that hatched from worm eggs collected through a series of filters and washes and incubated in aerated water. Cercariae were obtained approximately 4 weeks later by pooling together exposed snails in glass vials of dechlorinated water under artificial light for 2 h. Water containing cercariae was pooled into a graduated cylinder for immediate use. Triplicate cercarial counts in 1 ml aliquots were averaged to estimate the volume of water needed to produce required cercarial doses (Sandland and Goater, 2000).

Migration of O. ptychocheilus *diplostomules to the brain*

A combination of light microscopy and histology was used to evaluate the route of migration of *O. ptychocheilus* diplostomules from the epidermis to the brain. Juvenile fathead minnows (30 days old, between 1·5 and 2·0 cm standard length), obtained from a supply company, were maintained in aquaria and fed Tetramin fish flakes twice daily for 1 month prior to experimentation. On 5 August 2007, 33 fish were randomly selected from a stock tank. Individual fish were exposed to 200 cercariae in individual Petri-dishes filled with approximately 40 ml of dechlorinated water. Following a 15-min exposure period, fish were placed in a $30 \times 30 \times 60$ cm (H × W × L) aquaria and maintained on Tetramin fish flakes until euthanized at 15 min, 30 min, 1, 2, 3, 4, 8, 12, 16, 24, and 48 h post-infection (p.i.). Three fish were assigned at random to each interval. Minnow heads were removed, fixed in 10% neutral buffered formalin for 7 days, and decalcified in a 0·1 M EDTA titrant for a minimum of 14 days. Only 2 minnows at 16 h p.i. were successfully prepared for histology.

After dehydration in a graded ethanol series, samples were embedded in paraffin. Diplostomules of *O. ptychocheilus* are known to migrate along the longitudinal axis of the host (Hendrickson, 1979). Our main focus was on obtaining samples of brain tissue (together with the anterior-most part of the central nerve cord and the main cranial nerves) that would allow us to detect directed and anteriad migration of diplostomules towards the optic lobes. Preliminary studies indicated that sagittal sections of host brain tissue provided the best images of migrating diplostomules. Thus, most sections were obtained along a sagittal axis, from the operculum to the centre of the head. To provide additional information, 1 sample at each interval between 12 and 48 h p.i. was sectioned coronally (thickness = 10 μm) through the entire minnow head. Sections were stained with Mayer's haematoxylin and eosin Y, cover-slipped with permount, and examined using a light microscope.

Microsite selection of O. ptychocheilus *diplostomules within the brain*

Site-selection of developing metacercariae within the optic lobes was evaluated using histological techniques and light and transmission electron microscopy. Juvenile fathead minnows were exposed to 100 *O. ptychocheilus* cercariae for 3–4 h, on 20 August 2007 using the methods described above. At selected intervals post-exposure (4 days, 1, 2, 3, 4 and 6 weeks), 3 minnows from each time period were randomly selected for euthanization. These intervals were chosen to encompass the stages of *O. ptychocheilus* development from cercarial penetration to metacercarial encystment (Goater *et al*. 2005). Samples destined for light microscopy were fixed, decalcified, and embedded in paraffin as previously described. Serial 10 μm coronal sections of whole heads were prepared on gelatin-coated glass slides, and stained with Mayer's haematoxylin and eosin. The locations of individual metacercariae in selected regions of the brain were recorded at each interval. Particular attention was paid to locations of individual metacercariae within particular strata of the optic lobes as identified in Fig. 1.

For semi-thin light microscopy and transmission electron microscopy analyses, samples were prepared as follows. Brains were excised, trimmed, and optic lobes were fixed in fresh Karnovsky's solution for at least 24 h. Samples were rinsed overnight in a 0·1 M sodium cacodylate buffer (pH 7·2) and post-fixed for 1 h in 1% osmium tetroxide in the same buffer. After

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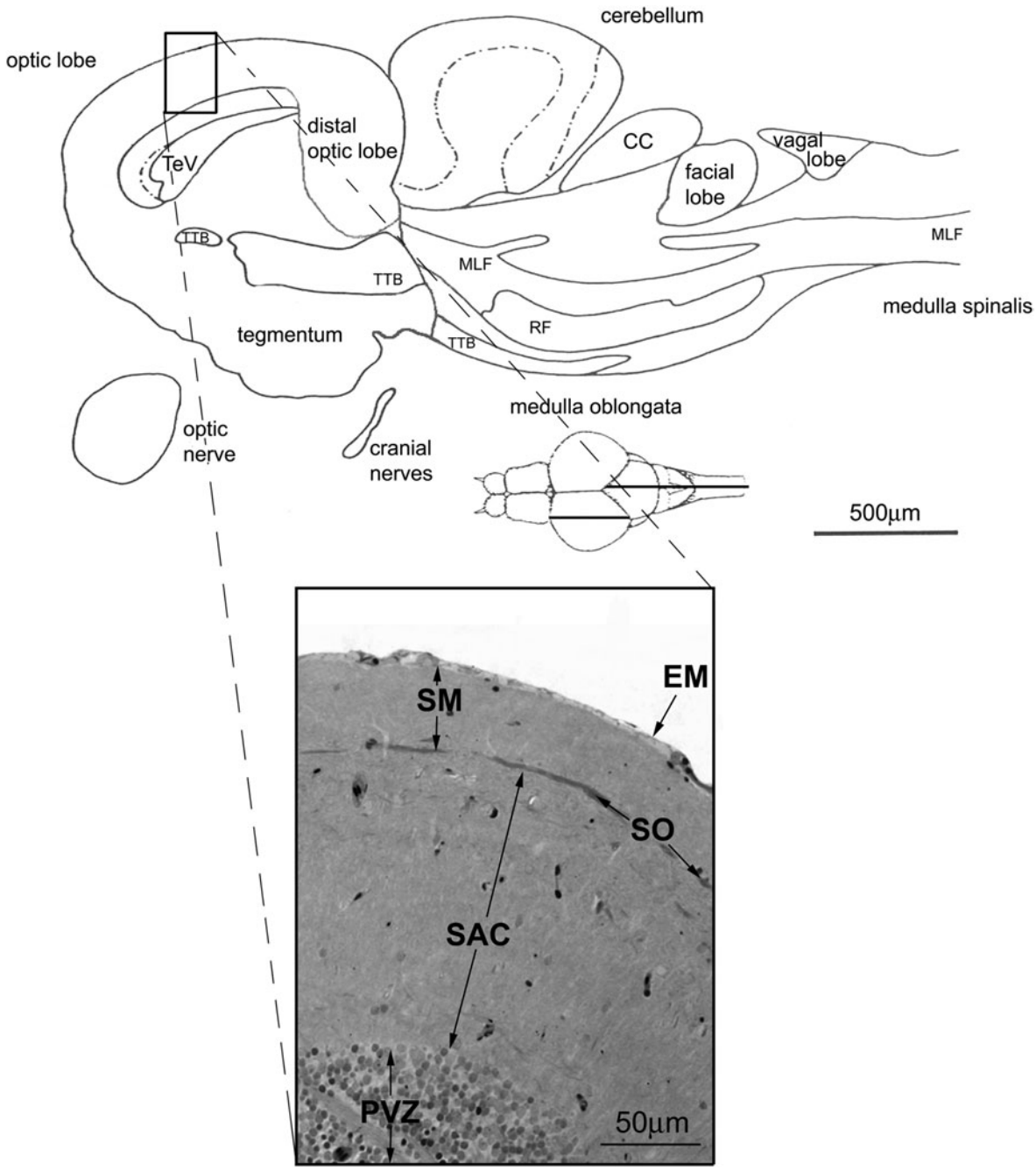

Fig. 1. Schematic sagittal diagram of selected brain tissues of *Pimephales promelas*: terminology follows Wullimann *et al*. (1996). The medulla spinalis is considered to be those tissues at the base of the brain, rostral to the spinal cord and caudal to the vagal lobe. The medulla oblongata is a collective term which includes the facial and vagal lobes, as well as the most dominant neural tracts of the teleost brain, the medial longitudinal fascicle (MLF), and the reticular formation (RF). The crista cerebelli (CC) is part of the cerebellum. The tegmentum consists of those tissues below the optic ventricle (TeV). The tectobulbar tract (TTB) extends from the medulla oblongata to the tegmentum, and into the optic lobes. The optic lobe is further characterized into 4 distinct layers, illustrated by the light micrograph in the boxed insert. This micrograph depicts the lamina of unexposed optic lobes of *P. promelas*. The periventricular zone (PVZ) is a dense granular region composed of cell bodies; the stratum opticum (SO) is characterized by thick, myelinated axons that originate in the retina. The outermost layer, the stratum marginale (SM), is characterized by thin unmyelinated fibres that run parallel to the optic tectum. Between these layers lies the stratum album centrale (SAC). The endomneninx (EM) is a secretory organ that covers the central nervous system.

dehydration through a graded ethanol series, tissue samples were embedded in Spurr's resin before being polymerized in an oven at 60 °C for 24 h. Semi-thin 1 μm coronal sections were cut on a Reichert OM-U2 ultramicrotome, mounted on glass slides coated with gelatin, flattened in an atmosphere of the clearing agent Hemo-De, and stained with 1% toluidine blue. Images were acquired using a Zeiss

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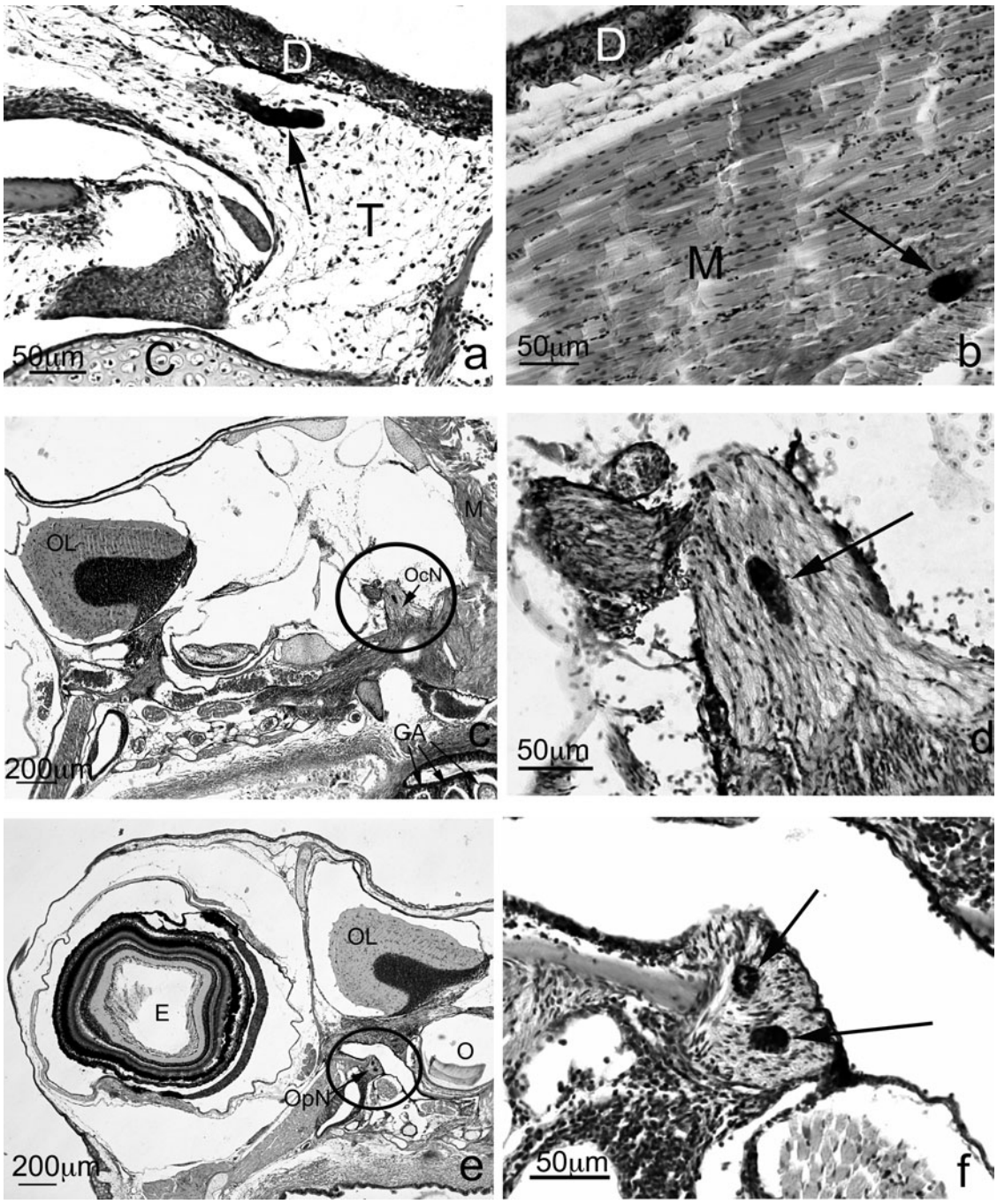

Fig. 2. (Cont.)

axiocam digital camera mounted onto a Zeiss axioskop 40 microscope. Semi-thin 0·1 μm sections were cut along the coronal plane and stained with 4% uranyl acetate for 20 min, and Reynolds lead citrate for 5 min. Sections were photographed using an Hitachi H-7500 TEM at an accelerating voltage of 100 keV.

Analyses

Modification of the techniques used to illustrate the location of brain flukes developed by Barber and Crompton (1997) were used to characterize temporal changes in diplostomule distribution within the brain during the migration phase. Thus, the approximate location of individual diplostomules between 3 and 24 h p.i. was mapped onto schematic images of the cyprinid brain, modified from Wullimann *et al.* (1996), to provide a semi-quantitative assessment of putative migration (Fig. 1). For quantitative analyses, we evaluated changes in the mean proportion of metacercariae located in specific tissues between selected time intervals with Kruskal-Wallis non-parametric tests.

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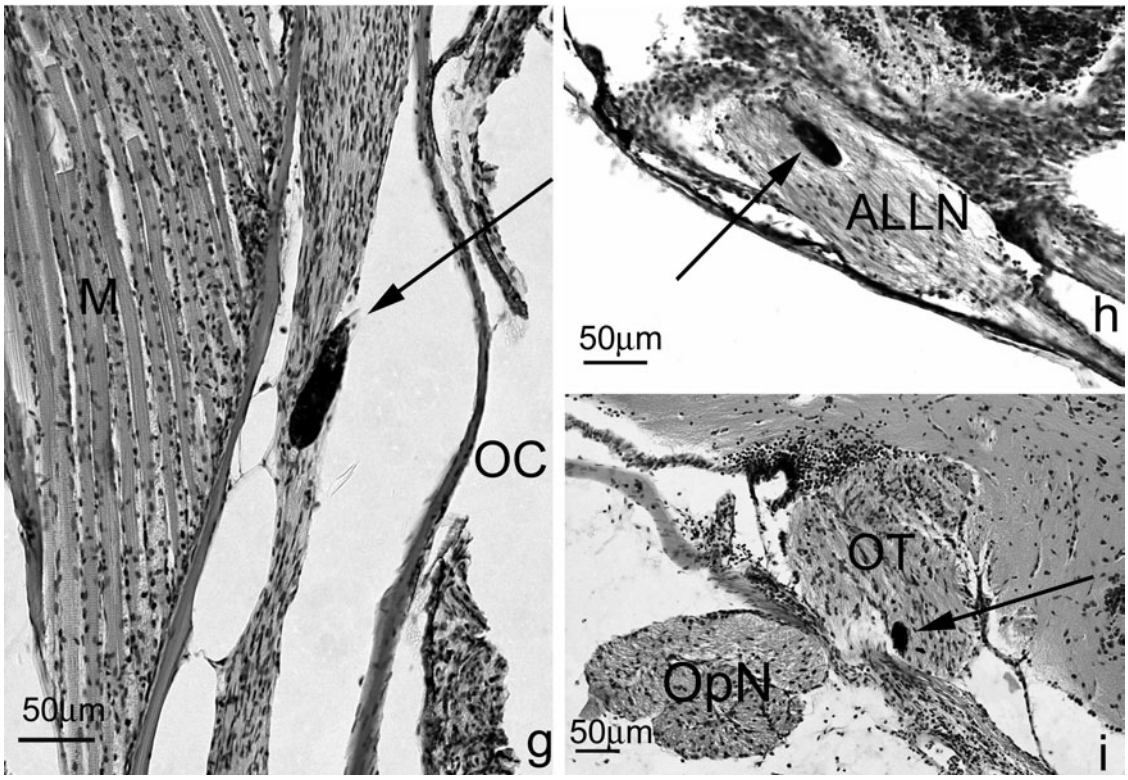

Fig. 2. Light micrographs of migrating *Ornithodiplostomom ptychocheilus* diplostomules in brain tissues of *Pimephales promelas* 15 min to 4 h post-infection (p.i.). At 15 min p.i., diplostomules (arrows) were present in host adipose (a) and muscle (b) tissues. The encircled regions in (c) and (e) represent regions magnified in (d) and (f). As early as 15 min p.i., diplostomules were present in various cranial nerves including the oculomotor nerve (c and d), the optic nerve (e and f), the anterior lateral line nerve (h), and other unidentified nerves (g). Diplostomules were also observed deeper in the optic tract at 4 h p.i. (i). Adipose tissues (T); cranium (C); epidermis (D); muscular tissues (M); otolith (O); gill arches (GA); optic lobes (OL); optic nerve (OpN); oculomotor nerve (OcN); anterior lateral line nerve (ALLN); optic tract (OT).

RESULTS

First period post-infection (*15 min–3 h*)

Diplostomules were observed in connective, adipose, and hypodermal tissues as early as 15 min p.i. (Fig. 2a). At this time, diplostomules were also observed within the muscle layers associated with the head region (Fig. 2c). Between 15 min and 4 h p.i., diplostomules were observed within several cranial nerves, including the octaval nerve (Fig. 2c, d), the optic nerve (Fig. 2e, f), an unidentified nerve that runs adjacent to a pseudobranch associated with the gills (Fig. 2g), and the anterior lateral line nerve (Fig. 2h). Several diplostomules were located within the optic tract (Fig. 2i).

Second period post-infection (*3–48 h*)

Between 3 and 8 h p.i., 36%, 40% and 10% of the total number of diplostomules were observed within the optic lobes and cerebellum, medulla oblongata, and tegmentum, respectively (Fig. 3a). The remaining diplostomules were located within cranial nerves, the hindbrain, and ventricles. By 12–16 h p.i., 88% of all diplostomules were located in the tissues of the optic lobes and cerebellum, with 6% in the tegmentum and the remaining 4% in the medulla oblongata (Fig. 3b). At 24–48 h p.i., 92% of all diplostomules were observed in the optic lobes and cerebellum (Fig. 3c).

The specific locations of the relatively small number of diplostomules found in the medulla and the tegmentum between 3 and 8 h p.i. illustrate that larvae tended to migrate along specific neural tracts (Fig. 4a–d). Most notably, 30% of diplostomules in the medulla oblongata occupied the medial longitudinal fascicle (MLF) (Figs 3a and 4b), while approximately 10% were in the reticular formation (RF). Other noteworthy locations of diplostomules in the medulla oblongata were in the vagal lobe (10%), and the crista cerebella (12%) (Fig. 3). Of those diplostomules in the tegmentum between 3 and 8 h p.i., 36% occupied the tecto-bulbar tract (TTB). Proportions in the TTB between 12 and 16 h p.i. were comparable at 30% (Figs 3a and 4c, d).

Diplostomules tended to be found in the lateralmost regions of the optic lobes (Figs 3 and 4e). Of the 30% of diplostomules occupying the optic lobes

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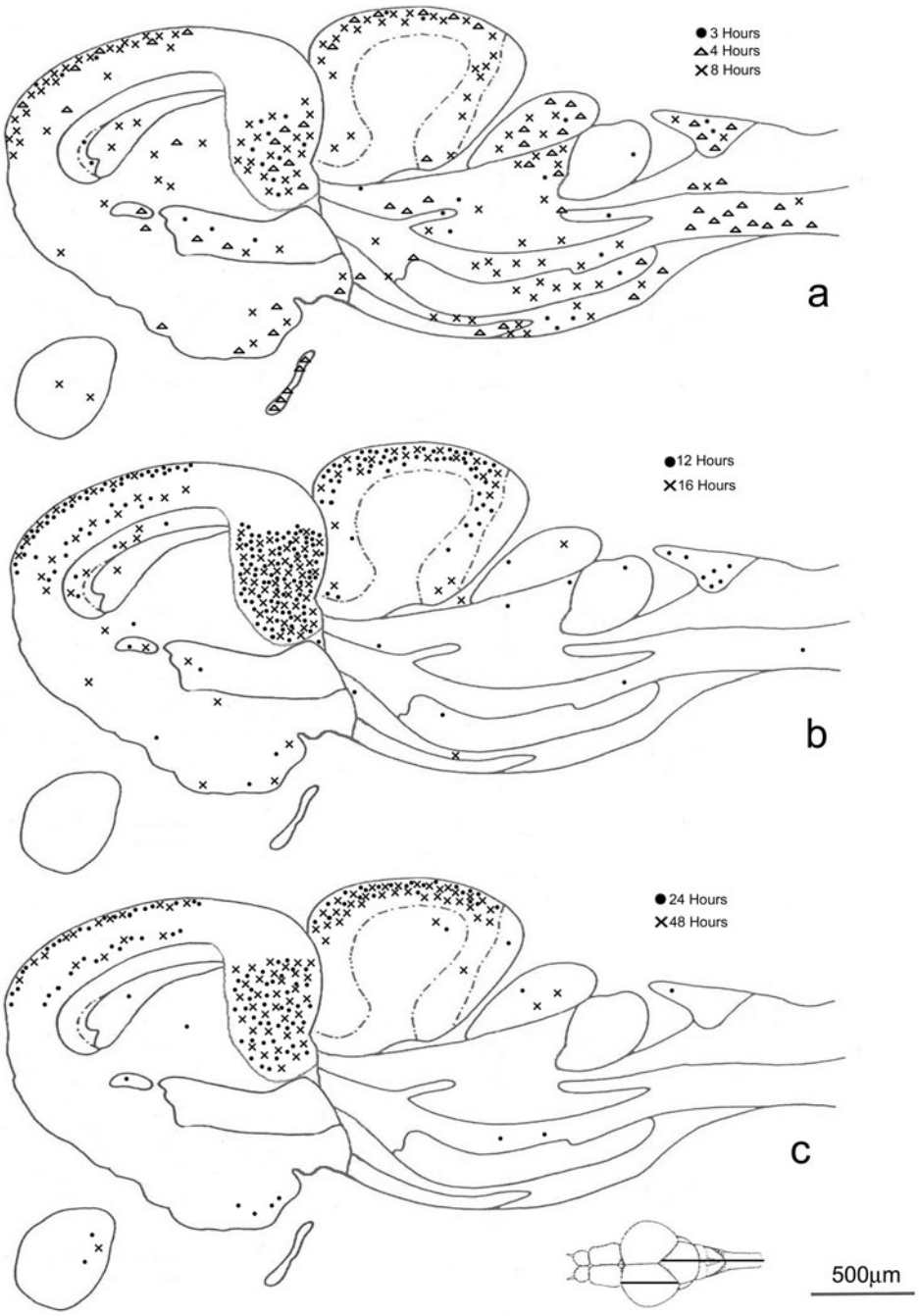

Fig. 3. Schematic sagittal diagram of the brain of *Pimephales promelas* experimentally infected with *Ornithodiplostomum ptychocheilus* cercariae. Demarcations of selected subregions of the brain are described in Fig. 1. The approximate locations of all observed metacercariae are depicted by unique symbols according to time since exposure to cercariae. The diagram summarizes metacercariae site locations pooled among 3 fish processed at (a) 3–8 h post-infection (p.i.), (b) 12–16 h p.i. and (c) 24–48 h p.i.

between 3 and 8 h p.i., about half were found in the extreme lateral portions of the tectum (Figs 3a and 4e). Between 12–16 h p.i. and 24–48 h p.i., relatively equal proportions of diplostomules were located in the lateral-most regions of the optic lobes (64% and 62% respectively) (Figs 3b, c and 4e).

At 12–16 h post-infection, diplostomules were localized primarily within the optic lobes, and secondarily in the cerebellum (Fig. 3b). This trend continued for diplostomules between 24 and 48 h p.i. (Fig. 3). Consistently, across 3–8 h p.i., 12–16 h p.i., and 24–48 h p.i., about 95% of metacercariae in the cerebellum occupied the outermost layer. Diplostomules in the optic lobes also showed a preference for the outermost layer, the stratum marginale, between 3–8, 12–16, and 24–48 h p.i. (89%, 80% and 85% respectively) (Fig. 3).

Third period post-infection (4–42 days)

The observed preference of diplostomules for the outermost layers of the optic lobes and cerebellum

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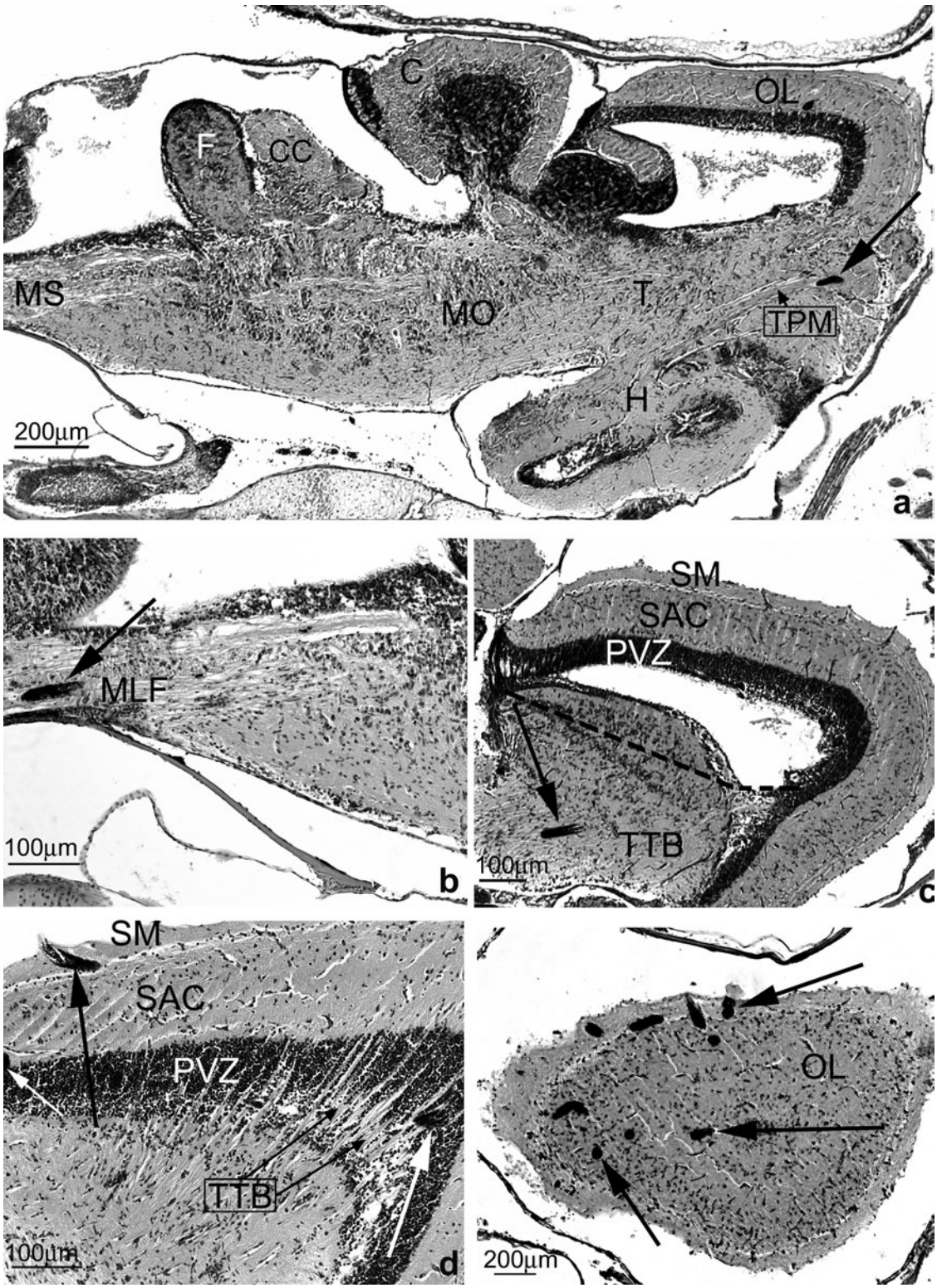

Fig. 4. Light micrographs of *Ornithodiplostomum ptychocheilus* diplostomules in brain tissue of *Pimephales promelas* between 4 and 24 h post-infection. Diplostomules (arrows) were observed migrating along major neural tracts within the brain (a; tractus pretectomamillaris) including the MLF (b), and the tecto-bulbar tract (d, e). Diplostomules were frequently observed in the extreme distal regions of the optic lobes (e). Medulla spinalis (MS); cerebellum (C); crista cerebellis (CC); facial lobe (F); medulla oblongata (MO); hypothalamus (H); tegmentum (T); optic lobe (OL); tractus pretectomamillaris (TPM); medial longitudinal fascicle (MLF); stratum marginale (SM); Stratum album central (SAC); periventricular zone (PVZ); tectobulbar tract (TTB).

remained until approximately 14 days p.i. (Fig. 5). Specifically, there was a steady decline from approximately 60% and 40% in the optic lobes and cerebellum, respectively, to less than 5% by 28 days p.i. The precipitous drop in the overall proportion of diplostomules located in these tissues was

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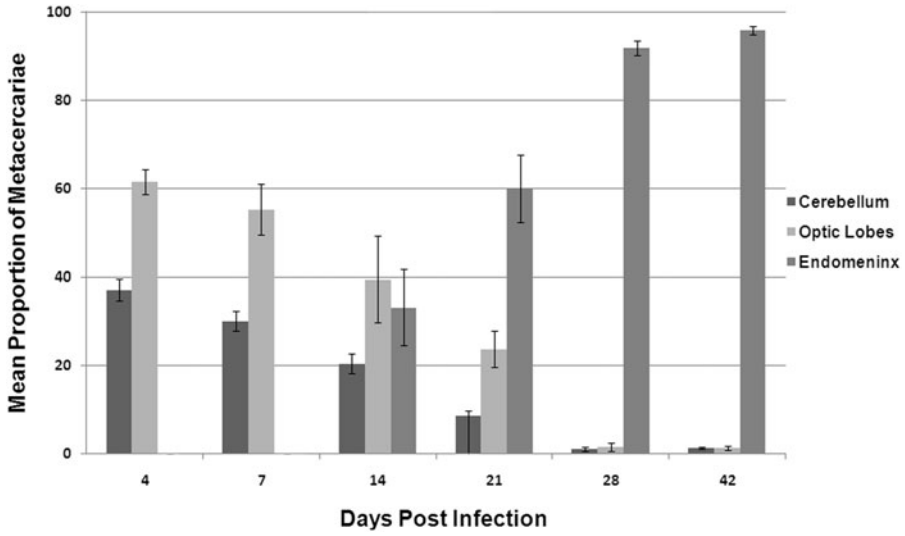

Fig. 5. Temporal changes in the mean proportion of metacercariae in the optic lobes, cerebellum, and endomeninx of minnows experimentally infected with *Ornithodiplostomum ptychocheilus* cercariae. Metacercaria from 3 fish were examined at each interval. Bars represent standard error.

paralleled by a significant increase in the proportion of diplostomules in the adjacent endomeninx between 14 and 42 days p.i. (Chi-square 9·56, D.F. = 3, $P = 0{\cdot}023$). By 42 days p.i., almost all metacercariae were found in the endomeninx layers surrounding the optic lobes and cerebellum (Fig. 5).

Microsite selection within the optic lobes was evident between 4 and 14 days, with a marked preference for the stratum marginale (Fig. 6). At 4 and 7 days p.i., 74% of diplostomules in the optic lobes were in this outermost layer; the remainder were in the layer immediately below the marginale (stratum album central) and in the periventricular zone. By 2 weeks p.i., 90% of diplostomules were located in the stratum marginale of the optic lobes, 6% in the stratum album central, and 4% in the periventricular zone. By 4 weeks p.i., virtually all diplostomules were absent from the optic lobes and had shifted location to the adjacent meninges (Fig. 6d). The meningeal layer between 2 and 9 weeks p.i. appeared massively inflamed. The habitat shift from the tissues to the meningeal layer coincided with the development of the outer cyst wall surrounding individual metacercariae (Fig. 6d).

The microhabitat shift that began at 4 weeks p.i. was characterized by morphological changes that occurred at the host-parasite interface (Fig. 7). At 4 days p.i., the tegument of the diplostomule is composed of a complex network of microlamellae and microvilli that appears to produce a noticeable space at the host-parasite interface (Fig. 7b). These microvillar elaborations to the tegument elongate at 2 weeks p.i., extending perpendicularly from the parasite surface into the adjacent gap between parasite body and host tissue (Fig. 7c). At this time, infiltration of microvilli into adjacent tissue in the stratum marginale appears disassociated and discontinuous (Fig. 7d). Macrophages are numerous within adjacent host tissue during this pre-encystment period (Fig. 7c, d). Metacercariae are embedded in the inflamed meninges at 4 weeks p.i. (Fig. 7e). Meningeal inflammation, which was first visible at 2 weeks p.i., was most evident between 4 and 6 weeks p.i (Fig. 6e, f). At this time, the stratum marginale was flush with the cyst wall, and cells appeared normal (Fig. 7f). Tegumental microvilli were absent and the space between host and metacercariae tissues had disappeared (Fig. 7f).

DISCUSSION

The biphasic nature of *O. ptychochelus* development within the optic lobes of fathead minnows has previously been described (Sandland and Goater, 2000; Goater *et al.* 2005; Conn *et al.* 2008). Thus, diplostomules enter an obligate period of rapid growth within brain tissues, afterwhich they enter their characteristic resting phase. The present study extends upon these earlier results in 2 important ways. First, the 2 phases of development are associated with contrasting habitats, the former within host brain tissue, the latter within adjacent meninges. We are not aware of other studies demonstrating a habitat shift for trematode metacercariae associated with their development. Second, our results indicate that *O. ptychocheilus* diplostomules navigate within specific neural tracts within the medulla of the brain *en route* to specific microsites within the optic lobes or cerebellum. Taken together, these results confirm the nature of site specificity of strigeoid trematodes (Erasmus, 1972), while additionally demonstrating that metacercariae of some species of trematode develop rapidly within such sites, and that they shift sites in parallel with their stage of development.

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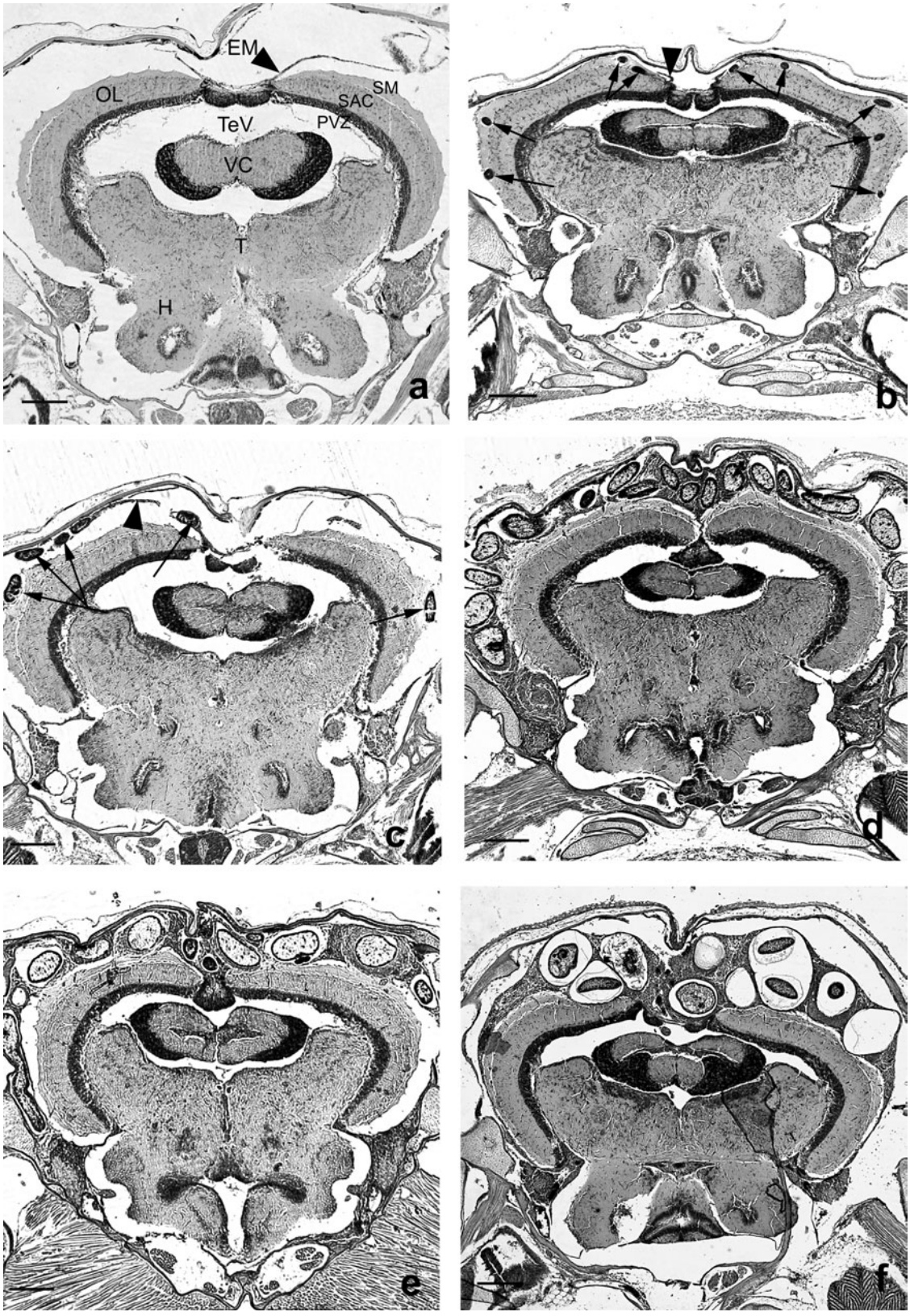

Fig. 6. Coronal sections of uninfected (a) and experimentally infected (b–f) minnows exposed to *Ornithodiplostomum ptychocheilus* cercariae. Diplostomules range in age from 1 week (b), 2 weeks (c), 4 weeks (d), 6 weeks (e), to 9 weeks (e). Diplostomules between 1 and 2 weeks are identified with arrows; meninges in unexposed minnows and between 1 and 2 weeks are identified by arrowheads. Valvula cerebella (VC); optic ventricle (TeV); optic lobes (OL); stratum marginale (SM); stratum album centrale (SAC); periventricular zone (PVZ); endomeninx (EM). Scale bar = 200 μm.

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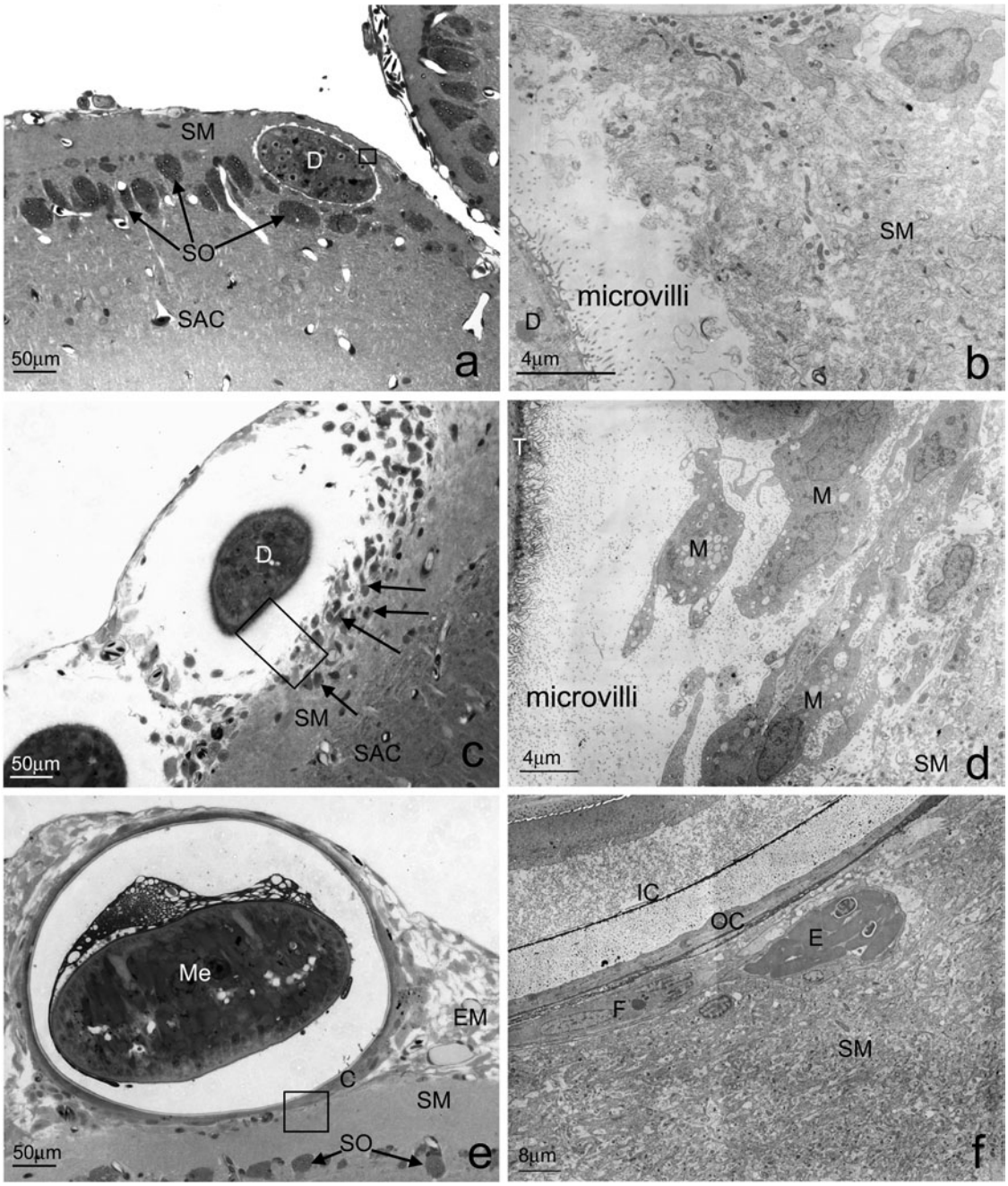

Fig. 7. Light and transmission electron micrographs of brain tissues of minnows experimentally infected with *Ornithodiplostomum ptychocheilus* cercariae. The boxed regions on the light micrographs represent the approximate location of the transmission electron micrographs on the right hand side. At 4 days p.i., diplostomules are located in the outermost stratum marginale of the optic lobes (a). A minute gap is visible between the diplostomule and adjacent host tissues of the stratum marginale (b). A cross-section of a diplostomule at 2 weeks p.i. illustrates an increase in the gap between diplostomule and host tissue (c, d). Numerous macrophages (arrows, 'M') were observed at the leading edge of host tissue at this time (c, d). At 4 weeks p.i. metacercariae were encysted in the host meninges (e); host tissues were flush against the outer cyst wall (f). Diplostomule (D); stratum marginale (SM); stratum opticum (SO); stratum album central (SAC); endomeninx (EM); macrophages (M); cyst (C); metacercariae (Me); inner cyst (IC); outer cyst (OC); fibroblast (F); erythrocytes (E).

Our results indicate that *O. ptychochelus* diplostomules navigate along specific fibrous tracts within the brain to access preferred sites. The medial longitudinal fascicle (MLF) is the most dominant fibre system in the brain of fish (Wullimann *et al.* 1996). Axons associated with this fibre system proceed posteriorly from the MLF nucleus, which is immediately ventral to the optic ventricle, towards the caudal end of the medulla oblongata. The tract then projects into the spinal cord. At 4 h p.i., over 40% of all diplostomules in the medulla oblongata and hindbrain were observed within the MLF, and many

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were orientated in an anterior-posterior direction. Flanking the MLF from the caudal portion of the medulla oblongata to the tegmentum is another substantial series of fibres, collectively called the reticular formation. Thus, neural pathways within the medulla oblongata that course in a longitudinal direction likely support the migration of diplostomules in a rostro-caudal direction towards preferred habitats within the brain.

There are no direct axonal connections between the MLF and the optic lobes. Thus, migrating diplostomules must utilize additional routes to complete migration from the brain stem to their preferred site within the outermost strata of the optic lobes, the stratum marginal. Access to the optic lobes from the central nerve cord and brain stem of cyprinids is possible through additional fibre systems. For instance, the tectobulbar tract (TTB) descends from the stratum album layer of the optic lobes via numerous projections that connect to dorsal and anterior regions of the tegmentum (Wullimann *et al*. 1996). Approximately 10–20 of these tracts are visible with light microscopy coursing ventro-dorsally beneath the outer-most layers of both lobes of the optic tectum. Many diplostomules were observed within this fibrous system between 4 and 16 h p.i. The use of the TTB to access the optic lobes would explain the relatively low numbers of diplostomules observed within the tegmentum itself. It would also explain why many diplostomules were observed within the SM and SAC layers of the optic lobes, but rarely within more ventral regions such as the PVZ or ventricles. Further, the use of the TTB would explain the preference of diplostomules for lateral regions of the stratum marginal in the optic lobes. These results indicate that migration of most diplostomules occurs along precise neural paths within the minnow brain, particularly those that link the ventral regions of the brain with lateral regions of the optic lobes.

Most migrating diplostomules accessed the brain via the central nerve cord and brain stem. However, some were observed within a variety of cranial nerves. Hendrickson (1979) made similar observations. It is likely that these diplostomules originated from cercariae that penetrated the head region of the host. We could not determine whether the ultimate site of those diplostomules that utilize specific cranial nerves differs from those that reach the tegmentum via the central nervous system. However, diplostomules that utilize cranial nerves are likely to encounter sites in which the nerves they migrate along terminate before advancing to the optic lobes or cerebellum. For example, approximately 10% of those diplostomules observed within the medulla oblongata between 3 and 8 h p.i. were observed within the vagal lobe. These diplostomules likely accessed this site via the vagus nerve. Similarly, those metacercariae observed within the hypothalamus and inferior lobes of the tegmentum likely migrated along the optic nerve and/or optic tract. These results raise the possibility that cercariae that penetrate the head region and have relatively straightforward access to specific cranial nerves develop and encyst in different sites than those that penetrate more posteriorly on the fish's body.

Although most diplostomules were observed within the stratum marginal of the optic lobes, 27% were located along the outer edges of the cerebellum. Similar segregation between the optic lobes and cerebellum has been observed in other experimental studies involving *O. ptychocheilus* (Hendrickson, 1979; Radabaugh, 1980). Density-dependent site segregation could explain these results, with the cerebellum acting as a spill-over site in high-intensity infections. However, we observed partitioning of diplostomules between the optic lobes and cerebellum as early as 3 h p.i., making this scenario unlikely. Further, we never observed diplostomules exiting the optic lobes and entering alternative sites, nor did we observe direct penetration of the cerebellum. A second possibility is that site segregation could result from migration along distinct fibrous tracts; those diplostomules that migrate along the TTB may ultimately reside in the optic lobes, while diplostomules that do not follow the TTB come to rest in the cerebellum.

The second main result of this study is the demonstration of a shift in microhabitat associated with the growth *versus* encysted phases of metacercarial development. Thus, all developing *O. ptychocheilus* diplostomules were found within specific layers of the optic lobes or cerebellum, whereas nearly all encysted forms were located within the adjacent non-tissue layer, the endomeninx. This structure is composed of 3 distinct layers that surround the entire central nervous system of fish (reviews by Momose *et al*. 1988; Schwartz *et al*. 1993). This complex structure is considered analogous to the pia-arachnoid layer of mammals, whose primary function is to secrete cerebrospinal fluid and maintain the blood-brain barrier (Schwartz *et al*. 1993).

Transmission electron microscopy indicated that metacercariae occurred within the innermost layer of the endomeninx. This layer is characterized by the presence of large extracellular spaces, together with fibroblasts, collagen fibrils, macrophages, leucocytes and blood vessels (Schwartz *et al*. 1993). Our qualitative observations indicated that the movement of pre-encysted metacercariae out of the stratum marginal began as early as 2 weeks, with the majority completing the shift by 4 weeks p.i. This habitat shift coincided with massive inflammation (endomeningitis: Speare and Frasca, 2006) of the inner layer of the endomeninx. By 9 weeks p.i. inflammation had largely subsided. Microsite selection in the endomeninx is likely associated with accessibility to host components involved in the encystment

process, such as fibroblasts and collagen (So and Wittrock, 1982) and/or to the large and expandable spaces available to accommodate cyst development.

Although the mechanism behind the observed microhabitat shift of *O. ptychocheilus* metacercariae between the stratum marginale of the optic lobes and the inner layer of the endomeninx is unknown, it is unlikely to be due to active migration. The disappearance of the penetrative proboscis at 2 weeks p.i. (Conn *et al*. 2008) makes it unlikely that encysted metacercariae are capable of additional movement. The transformation of the diplostomule tegument from an elaborate complex of microvilli to the smoothed cyst wall would facilitate the passive expulsion of metacercariae from tissues of the optic lobes and cerebellum.

Previous studies on this system have shown that the pre-encystment phase is associated with distortion of the entire cranium, leading to high host mortality relative to uninfected controls (Sandland and Goater, 2001). Similarly, the pre-encystment phase is associated with marked reduction in visual performance and overall host activity, while the encystment phase is not (Shirakashi and Goater, 2005). Negative effects on host performance due to developing *versus* encysted metacercariae have been demonstrated for other species of trematode (Ballabeni and Ward, 1993; James *et al*. 2008). We should also expect differences in host response to developing *versus* encysted metacercariae. Few studies have been designed to address differences in host immune responsiveness to metacercariae at different stages of development. However, Matisz *et al*. (2009) showed a 30-fold difference in the density of rodlet cells in brain tissue immediately adjacent to encysted *versus* developing *O. ptychocheilus* metacercariae. These cells are thought to play a role in regulating the host inflammatory response. Taken together, the combined results from several experimental studies indicate that the shift in microhabitat associated with the developing *versus* resting stages of metacercariae has important ecological implications for this and similar host-parasite interactions.

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