

***ORNITHODIPLOSTOMUM* spp. METACERCARIAE IN FATHEAD MINNOWS:
MIGRATION, SITE SELECTION, AND HOST RESPONSE**

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for my grandmother, Elizabeth Dale Blair

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ABSTRACT

Site selection, and the means to access specific sites, is a keystone of parasitology. I evaluated migration and site selection behaviours of metacercariae of two congeneric species of strigeoid trematode throughout growth and encystment phases in the fathead minnow. Results showed that pre-encystment stages of *Ornithodiplostomum ptychocheilus* migrate along specific neural tracts to access sites in the optic lobes of the brain. Conversely, pre-encystment stages of *Ornithodiplostomum* sp. migrate via direct penetration, or via the vascular system to access visceral organs, especially the liver. Remarkably, both species have a bi-phasic pattern of development, with growth and encystment occurring in unique sites. Finally, I examined patterns of rodlet cell proliferation and maturation in response to growth and encystment phases of *O. ptychocheilus*. Cell densities were low ($<11/\text{mm}^2$) in brain tissue adjacent to 1 and 2 week old metacercaria, but peaked to approximately $210/\text{mm}^2$ at 6 weeks. These results shed new light on the potential function of these enigmatic cells.

“.....wouldn't that knowledge, suggesting as it does the possibility of unimaginable alternatives, wouldn't that knowledge be a bubble bath for your heart?”

Tom Robbins, Even Cowgirls Get the Blues

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Chapter 1: General Introduction

Parasitologists have had a long-standing interest in understanding and characterizing the specific sites that parasites occupy within individual hosts (review by Sukhdeo & Sukhdeo, 2002; 2004). This interest in parasite site selection stems from the pivotal role it plays in many aspects of host-parasite interactions, including parasite transmission dynamics, parasite-induced host pathology, and parasite taxonomy and phylogeny (Williams 1966; Sukhdeo & Sukhdeo 1994). Early studies on parasite site selection tended to focus on tissue-dwelling pathogenic parasites of humans such as *Ascaris* (migrating L4 larvae), *Trichinella* (migrating L1 larvae) and *Schistosoma* spp., (migrating schistomules) and similar parasites of domesticated animals such as *Fasciola hepatica*. Given the taxonomic breadth of these parasites, and their hosts, a general pattern of distinctive and highly specific site selection within individual hosts was rapid to emerge (Noble & Noble, 1976).

Following the early emphasis on characterizing specific sites of infection, subsequent studies tended to emphasize the identification of the causative factors, and consequences, of such strict site specificity. Several hypotheses to explain the phenomena were developed, including interspecific competition (Holmes, 1973), immune evasion (Crompton, 1973), enhancement of mating opportunities (Rhode, 1979), and enhancing specific nutritional requirements (Barber & Crompton, 1997). Additionally, research programs were designed to identify the precise routes of navigation from the initial point of entry, to the ultimate site of infection. For example, larvae of *A. lumbricoides* were

shown to use the circulatory system to access tissues of the lungs, heart, and liver (Cheng, 1964). Similarly, migrating larvae of *T. spiralis* accessed striated muscle tissues through the peripheral circulatory system (Cheng, 1964). Likewise, pre-adult stages of *F. hepatica* were shown to migrate along the abdominal wall of their final hosts to access the liver, before migrating to the gallbladder and bile duct (Sukhdeo & Sukhdeo, 2004). Thus, a substantial amount of research has been dedicated to elucidating the proximate and ultimate causes of extreme site specificity, particularly for those parasites that contribute to human and livestock disease.

These earlier studies on parasite site selection and migration tended to focus on pre-adult or adult stages of pathogenic and anthropogenic parasites. Comparatively little attention was paid to the resting stages of those parasites that possess complex and indirect life cycles. This bias was probably due to the early perception that the encysted larval stages of helminths such as acanthocephalans, cestodes, and trematodes were little more than a resting stage, with very limited metabolic interactions with their hosts (Lackie, 1975). More recent studies have shown this standard textbook view of ‚resting‘ stages is incorrect. The larval stages of several species of cestode have an extended pre-encysting stage (Shostak *et al.*, 2006) that is capable of extensive nutrient uptake from their second intermediate hosts (e.g. Hurd & Arme, 1984), often involving interaction with the host endocrine system (Kearns *et al.*, 1994). Similarly, some larval acanthocephalans have an obligate pre-encystment stage that involves extensive metabolic interactions with their crustacean intermediate hosts (Duclos *et al.*, 2006).

Trematode metacercariae in particular demonstrate a wide variety of encystment strategies in their intermediate hosts. At one extreme, species in the families Fasciolidae and Notocotylidae are known to encyst within a few hours on virtually any substrate, including vegetation. Experimental studies on the digenetic trematode *Zygomystyle lunata* have shown that cercariae begin encystment within 5 minutes of being placed on plastic, glass, and a variety of macrophytes (Peoples & Fried, 2008). At the other extreme, Strigeoid metacercariae are well known to undergo complex periods of growth and development within specific tissues of their second intermediate hosts before becoming infective (Erasmus, 1972). Numerous studies provide evidence that both glucose (Bibby & Rees, 1971; Higgins, 1979; Uglem & Larson, 1987; Larson *et al.*, 1988) and amino acids (Halton & Johnston, 1982; Higgins, 1979) are readily absorbed across the tegument of strigeoid metacercariae. Ultrastructural studies of the surface of the strigeoid *Ornithodiplostomum ptychocheilus* metacercariae indicate an extensive tegumental complex of microvilli and microlamellae during the pre-encystment stage (Goater *et al.*, 2005). These structures are most elaborate during highest metacercarial growth rates, and are absent upon encystment (Goater *et al.*, 2005). The transient nature of this morphological elaboration, together with evidence for delayed encystment (of up to 10 weeks, Shirakashi & Goater, 2005), provides strong evidence for an obligate, pre-encystment phase for these kinds of metacercariae (Sandland & Goater, 2000; Goater *et al.*, 2005; Conn *et al.*, 2008). Taken together, these lines of evidence suggest that understanding site selection within the context of metacercarial development is important, as trematode larvae, specifically strigeoids, are highly interactive with host tissues.

These recent studies show that many trematode metacercariae are remarkably site specific. These sites range from the lens of the eye (*D. flexicaudum*, Ferguson, 1943), the vitreous of the eye (*D. spathaceum*, Hoglund, 1991), to the choroid plexus of the optic lobes (*D. baeri eucaliae*, Hoffman & Hoyme, 1958) and the pericardium of the heart (*Cotylurus erraticus*, Johnson, 1971). However, we are only starting to understand the extent of this specificity. Moreover, our understanding of how parasites navigate to, and within, specific regions of the host is also poorly known, and is further augmented by the complexity of trematode life cycles. Both in vitro experimental (Grabe & Haas, 2004; Haas *et al.*, 2007), and theoretical approaches (Sukhdeo & Sukhdeo, 2004) have begun to address these gaps in knowledge. Thus far, experimental studies involve trematodes in which relatively little is known about their morphological and developmental traits, which are potential determinants of site specificity. Exploring the migratory and site selection behaviours of well-characterized and experimentally amenable trematodes in the context of their developmental features should provide some insight into specificity.

Ornithodiplostomum ptychocheilus (Digenea: Strigeida) is a model system for such studies, as this trematode is amenable to experimental manipulation. Furthermore, metacercariae of this species are widespread, with prevalence typically reaching 100%, and intensities ranging from 20 to 400 metacercariae per individual fish intermediate host (Sandland *et al.*, 2001; Schleppe 2002). As a result, numerous studies have described the migration and site selection processes, in addition to morphological structures, metacercarial development, and metacercarial-induced effects on behaviour. The life cycle of *O. ptychocheilus* represents a typical trematode life cycle; adults mate in the intestinal tract of piscivorous birds, such as mergansers and blue herons, and lay eggs

which are shed via the host faeces (Hoffman, 1960). Free-swimming miracidia infect the primary intermediate host, *Physa gyrina* (Hoffman, 1960). Subsequent cycles of asexual reproduction in the snail host produce cercariae, which are released into the water column and actively penetrate the epidermis of fathead minnows (*Pimephales promelas*).

Within hours after penetrating the host epidermis, *O. ptychocheilus* diplostomules migrate to the brain of fathead minnows via cranial and spinal nerves (Hendrickson, 1979). The migration process is believed to be aided by a transient, protrusible, spined structure at the anterior end called the prosoma (Conn *et al.*, 2008). Larvae localize primarily in the optic lobes, and secondarily in the cerebellum (Hendrickson, 1979; Radabaugh, 1980), where they undergo discrete growth, encystment, and consolidation phases before reaching infectivity (Sandland & Goater, 2000). The growth phase of *O. ptychocheilus* is characterized by an elaborate tegumental complex of microlamellae and microvilli that extends into adjacent host tissue (Goater *et al.*, 2005). Upon deposition of the cyst wall, this tegumental complex is reorganized into the familiar structure that characterizes the adult metacercarial stage (Goater *et al.*, 2005).

Ornithodiplostomum sp. is an unidentified species of digenetic trematode (Strigeidae), which is also amenable to experimental manipulation. Preliminary studies have determined that this species is morphologically, developmentally, and ecologically similar to *O. ptychocheilus* (Goater, unpublished observations). Originally, *Ornithodiplostomum ptychocheilus* was described as occurring in both the brain and viscera of fathead minnows (Hoffman & Hoyme, 1958; Sogandares-Bernal *et al.*, 1979; Radabaugh, 1980). However, results from experimental infections provide evidence that

they are distinct species (Hendrickson, 1979). *Ornithodiplostomum* sp. shows a unique preference for the primary intermediate host, *Physa integra* and shares the same second intermediate and definitive host as *O. ptychocheilus* (Goater, unpublished observations).

Despite the numerous studies on the migration, site selection, development, and morphology of *O. ptychocheilus* metacercariae, little is known about how diplostomules navigate within the brain to the outer edges of the optic lobes and cerebellum.

Additionally, while larvae are known to develop within the brain (Hendrickson, 1979; Radabaugh, 1980), multiple reports exist of these metacercariae on the surface of the brain, or in the cranial cavity (So & Wittrock, 1982; Shirakashi & Goater, 2001). This distinction is crucial, as damage caused to the neuronal networks of the brain should impact host physiology and biology much differently than damage to meninges, or parasitism of the cranial cavity. In contrast to *O. ptychocheilus*, little is known about the larvae of *Ornithodiplostomum* sp., beyond preliminary study that indicates a preference for metacercariae to encyst within the body cavity of fathead minnows (Goater, unpublished observations). The ability to experimentally manipulate *Ornithodiplostomum* sp. larvae offers a means to examine the specific migration route and habitat selection over the course of larval development. Thus, these trematodes provide an excellent opportunity to examine two congeneric species that have evolved radically different migration and site selection strategies.

One reason that parasitologists have focused so heavily on elucidating migration routes and the extent of site selection is because understanding these processes can lead to a better understanding of the potential costs that parasites incur on their hosts. Tissue-

dwelling parasites, particularly those in resting stages, often select sites that are critical to the growth, reproduction, and survival of their hosts. The infection of brain, liver, and lung tissue by *echinococcus* larvae, for example, have obvious implications for pathology in these tissues (Bush *et al.*, 2001), as do larvae in striated muscle tissues (Wu *et al.*, 2001). Tissue-dwelling trematode metacercariae are known to cause a wide variety of negative effects on their second intermediate hosts (e.g. Lemly & Esch, 1984; Ballabeni, 1994; Johnson *et al.*, 1999). Fathead minnows infected with metacercariae of *O. ptychocheilus* display an enlarged cranium that causes high host mortality (Sandland & Goater, 2001), and reduced visual performance (Shirakashi & Goater, 2005).

Given the extent and variety of costs imposed by parasites, natural selection should favour host responses that function to either limit exposure to parasites, or reduce the costs of infection. Somewhat surprisingly, the evidence that fish can detect and then avoid trematode cercariae is poor (review by Wisenden *et al.*, 2009). There is also little evidence for protective immunity against *O. ptychocheilus* cercariae due to prior infections (Sandland & Goater, 2000; Goater, unpublished observations). Finally, components of the epidermis of fathead minnows contain elements (likely components of mucus glands) that cause marked reduction in cercariae infectivity (James *et al.*, submitted manuscript), but this response is likely invoked by any general injury to the epidermis. Thus, the possibility exists that minnows have no means of contending with the presence of migrating, developing or encysted metacercariae, or other response mechanisms to attenuate parasite-induced damage must exist.

Preliminary studies on both *Ornithodiplostomum ptychocheilus* in the brain and *Ornithodiplostomum* sp. in the liver have found very distinctive cells adjacent to both developing and encysted metacercariae. These cells, known as rodlet cells, are found exclusively in fish, and are characterized by a thick fibrous capsule containing multiple elongated rods (rodlets). Since their discovery by Thelohan in 1892, who described them as a protozoan parasite (Laguesse, 1895) and Plehn (1906), whom ascribed to them a glandular function, a longstanding controversy as to their origin and function has persisted. The exogenous hypothesis considers them parasites, (Mayberry *et al.*, 1986) as ultrastructural studies have found the rodlets to be similar in appearance to infective stages of apicomplexan protozoans. Conversely, the endogenous hypothesis considers them to be normal fish cells, as they are present in lab-reared fry (Mazon *et al.*, 2007), and are not associated with tissue damage characteristic of the apicomplexans (Manera & Dezfuli, 2004).

The endogenous hypothesis has come into favour over the last 20 years, in part due to an abundance of studies that report elevated expressions of rodlet cells in response to stressors such as heavy metals, carcinogens, dermal wounding, and confinement (Iger & Abraham, 1997; Pawert *et al.*, 1998; Balabanova, 2000; Giari *et al.*, 2008). The most intense proliferation of rodlet cells is reported in tissues infected by protozoan and metazoan parasites (Leino, 1996; Sulmanovic, 1996; Mazon *et al.*, 2007; Koponen & Myers, 2000; Dezfuli *et al.*, 2007). Additionally, several recent studies have described an intimate association between rodlet cells and a variety of parasites (Dezfuli *et al.*, 2000; Reite *et al.*, 2005; Palenzuela *et al.*, 1999). Cumulatively, these studies suggest that rodlet cells play a role in the non-specific immune system of fishes. This is further supported by

numerous ultrastructural studies that document the secretory nature of these cells (Leino, 1974; Kramer & Potter, 2002).

Few studies have been designed to evaluate the specific cues leading to rodlet cell proliferation and recruitment. Those that do, tend to involve field-collected hosts (Leino, 1996; Dezfuli, *et al.*, 2000; 2007). Fewer still have attempted to quantify rodlet cell densities within parasitized and unparasitized tissues. In an experimental study involving a myxozoan infection in the kidney tubules of sunfish, Leino (1996) presents compelling evidence that tissue injury induced by myxozoans elicit a rodlet cell response in hosts. This conclusion was supported by Reite (2005). Additionally, because rodlet cells are well documented in a variety of tissues, (reviewed by Manera *et al.*, 2004) including the brain (Mitchell *et al.*, 1985; Dezfuli, 2007), these cells represent an ideal means to examine the role they play in parasite infections of an “immune-privileged region” such as the central nervous system (Cox, 1994; Sitja-Bombadilla, 2008).

Ornithodiplostomum ptychocheilus represents an ideal system to evaluate the expression of rodlet cells in the brain tissues of *P. promelas*. The brain of teleosts is well described in anatomical detail, and is suitable for histological examination. Secondly because this host-parasite interaction is amenable to experimental manipulation, and the developmental phases of *O. ptychocheilus* have been extensively studied, I can test two predictions regarding the linkage between rodlet cells and helminth infection. First, if rodlet cells are produced in response to metacercariae, hosts exposed to cercariae should contain significantly more rodlet cells in infected host tissue compared to unexposed controls. Secondly, if rodlet cells provide protection against infection, particularly during

the critical pre-encystment phase, then their density and maturation should parallel the developmental sequence of *O. ptychocheilus*. Thus, employing this system in rodlet cell research can provide quantitative data on rodlet cell expression in response to helminths, and insight into potential cues that lead to rodlet cell proliferation.

Thesis Objectives

The first experimental study was designed to evaluate the migration route and microsite selection of *O. ptychocheilus* in the brain of fathead minnows. While previous studies have examined the migration route of *O. ptychocheilus* diplostomules to the brain of these fish, nothing is known about how diplostomules navigate within the brain to specific sites within the optic lobes and cerebellum. In this study, I used the method of serial autopsy, followed by standard histological analyses, to evaluate changes in microsite selection from the point of cercarial penetration (host epidermis, 0-1 hour post infection (p.i.)), through the migration (1 hour p.i.-48 hours p.i.) and development phases (4 days p.i.-4 weeks p.i.) to the encystment phase (4+ weeks p.i.).

In the third chapter, I provide the first examination of the migration and site selection of *Ornithodiplostomum* sp. Here, I followed the same procedures as in Chapter 2 to evaluate microsite selection throughout migration, development, and encystment phases. In addition to characterizing migration and microsite selection, I also used these data to compare the navigation and site-selection of these two closely related, sympatric and syntopic species of trematode.

My final chapter provides the first quantitative assessment of rodlet cell densities in fish experimentally exposed to trematode cercariae. Additionally, this is the first study to examine temporal changes in rodlet cell density over the course of parasite development. This study tested several important predictions regarding the putative function of rodlet cells. First, I tested the simple hypothesis that the density of rodlet cells in parasitized tissues would be greater than the density of rodlet cells in unparasitized tissues. Second, I examined the correspondence between metacercariae-induced tissue damage, parasite development and the proliferation and maturation of rodlet cells. If rodlet cells are a host response to tissue damage, then I would expect greatest densities during maximum parasite-induced tissue damage. Thus, I predicted greatest densities of rodlet cells during the pre-encystment phase, between 1-4 weeks post-infection (p.i.). I tested this idea by evaluating changes in rodlet cell density in unexposed minnows, and minnows exposed to cercariae at 1, 2, 4, 6, and 9 weeks p.i. Finally, I assessed the stage of maturity of rodlet cells in hosts bearing a 4 day, 3 week and 6 week old parasite infections.

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Chapter 2: Migration and site selection of *Ornithodiplostomum ptychocheilus* (Trematoda: Digenea) in the brain of fathead minnows (*Pimephales promelas*)

INTRODUCTION

Site selection is an essential tenet of parasitology. Striking examples of tissue, organ, and intracellular specificity are well-characterized among protozoan and metazoan parasites (Roberts *et al.*, 2008). The impacts of parasite site selection are recognized in parasite-induced effects on host tissues, host phenotypes, parasite ecology, and parasite taxonomy and phylogeny (Sukhdeo & Sukhdeo, 1994). Thus, defining the migration route of parasites, from the initial point of entry to the final site of development, has been a central focus of parasitological research. These routes are well documented for those parasites pathogenic to humans and their domestic stock, such as *Ascaris lumbricoides* (Bethony *et al.*, 2006), *Fasciola hepatica* (Sukhdeo & Sukhdeo, 2004), and *Schistosoma mansoni* (Wheater & Wilson, 1979). Despite ample attention on parasite site selection, the manner in which parasites successfully navigate and locate specific habitats within the host is poorly understood (Sukhdeo & Sukhdeo, 2004; Haas *et al.*, 2007). Additionally, the conclusions drawn from studies in the past warrant a closer examination of parasite migration in a more integrated and experimental framework (Wilson, 1994).

Specific tissue-dwelling parasites should generally exhibit greater active migratory behaviours than those parasites which reside in the lumen of the gut or visceral cavity. Given the extreme site specificity of tissue-dwelling strigeoid trematodes, it is not surprising that these parasites have received considerable attention in migration studies. For example, the migration routes of *D. flexicaudum* (lens of the eye in rainbow trout and

fathead minnows, Ferguson, 1943), *D. spathaceum* (vitreous of the eye of rainbow trout, Hoglund, 1991), *D. baeri eucaliae* (choroid plexus of the optic lobes in stickleback, Hoffman & Hoyme, 1958), *C. erraticus* (pericardium of rainbow trout, Johnson, 1971) and *O. ptychocheilus* (optic lobes of fathead minnows, Hendrickson, 1979) in their intermediate fish host have been examined in considerable detail. However, very few studies have been designed to specifically evaluate the navigation of migrating stages of strigeoids through complex tissues.

Metacercariae of the strigeid trematode *Ornithodiplostomum ptychocheilus* encyst within the optic lobes of the brain of fathead minnows (*Pimephales promelas*) (Sandland & Goater, 2000). Initial experimental studies established that cercariae penetrate the host epidermis, then migrate to the brain of fathead minnows via spinal and cranial nerves within 24 hours after initial penetration (Hendrickson, 1979). The migratory process is believed to be aided by a transient, spined, protrusible structure (Conn *et al.*, 2008). Site selection occurs primarily within the optic lobes, where developing metacercariae undergo discrete phases of growth, encystment, and consolidation before reaching infectivity (Sandland & Goater, 2000). The specific location of metacercarial cysts have been described both in and on neural tissues (Radabaugh, 1980; So & Wittrock, 1982; Sandland & Goater, 2000). However, the manner in which individual diplostomules navigate and migrate through, and from, the complex network of peripheral nerves to their ultimate site within the optic lobes is unknown.

It seems unlikely that migrating diplostomules access site-specific regions within the brain, such as the optic lobes, via random movement through the extraordinarily

complex neural tissues of their fish hosts. Rather, it would stand to reason that over successive generations, diplostomules have evolved a highly efficient migratory path. Thus, the objective of the present study was to elucidate the details on the migration pathways employed by *O. ptychocheilus* within the brain. The neuroanatomy of cyprinids has been mapped in extraordinary detail (Wulimann *et al.*, 1996), providing a solid background of the particulars on the environment in which *O. ptychocheilus* diplostomules must migrate. In addition, this study will provide specific characterization of the final resting sites of both developing and encysted metacercariae.

MATERIALS AND METHODS

General infection procedure

The methods used to experimentally infect fathead minnows with *O. ptychocheilus* cercariae have been previously described by Sandland and Goater (2000). Briefly, day old chicks were force-fed the brains of naturally infected minnows on June, 2007. Trematode eggs were collected from chick faeces through a series of filters and washes and incubated in aerated water at 20° C. Miracidia that hatched from eggs were exposed to juvenile snails from the F1 generation of field-collected *Physa gyrina*. Snails were maintained in the laboratory in de-chlorinated water and fed boiled lettuce *ad libitum*. Cercariae were obtained approximately 4 weeks later by pooling together exposed snails in glass vials of dechlorinated water under artificial light for 2 hours. Water containing cercariae was pooled into a graduated cylinder for immediate use.

TriPLICATE cercarial counts in 1mL aliquots were averaged to estimate the volume of water required to produce required cercarial doses, following dilution techniques described in Sandland and Goater (2000).

Migration of O. ptychocheilus to the brain

A combination of light-microscopy and histology was used to assess the route of migration of *O. ptychocheilus* metacercariae (diplostomules) from the epidermis to the brain. Juvenile fathead minnows (30 days old, between 1.5-2.0 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 haphazardly selected from the stock tank. Individual fish were exposed to 200 cercariae in individual petri-dishes filled with de-chlorinated water. Following a 15 min exposure period, fish were placed in a 30 x 30 x 60cm (H x W x L) aquaria and maintained on tetramin fish flakes until sacrifice at 15min, 30min, 1, 2, 3, 4, 8, 12, 16, 24, and 48 hours post infection (p.i.). The relatively high dose of cercariae ensured a high recovery of migrating diplostomules, despite the brief exposure time. 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 0.1M EDTA titrant for a minimum of 14 days. Only 2 minnows at 16 hours p.i. were successfully prepared for histology.

After dehydration in a graded ethanol series, samples were embedded in paraffin. *O. ptychocheilus* diplostomules are known to migrate along the longitudinal axis of the

host (Hendrickson, 1979). Therefore most sections were cut along a serial sagittal axis, from the operculum to the center of the minnow head. One sample at each interval between 12-48 hours p.i. was serially sectioned coronally (thickness=10 μ m) through the entire minnow head. Preliminary evidence suggested that most diplostomules would be migrating through brain tissue during this interval. Thus, coronal sections were cut to provide an alternate visual perspective of migrating diplostomules in neural tissue. Sections were stained with Mayer's hematoxylin and eosin Y, coverslipped with permount, and examined using a light microscope. To quantitatively evaluate diplostomule migration through host neural tissues, the brain was divided into selected and identifiable lobes, nerves, and the most dominant neural tracts (Fig. 2.1).

The approximate locations of individual metacercariae were recorded in each section of each sample, following the semi-quantitative method in Barber and Crompton (1997). Several lateral and medial sagittal sketches of the cyprinid brain from Wulliman (*et al.*, 1996) neuroanatomical atlas were modified to form a single image. Individual positions of diplostomules between 3-24 hours p.i. were coalesced onto this image. The resulting figures provided a schematic indication of the directed movement of diplostomules through neural tissues over time.

Site Selection of O. ptychocheilus within the brain

Site-selection of developing metacercariae within the optic lobes was evaluated using histological techniques in addition to light and transmission electron microscopy.

Juvenile fathead minnows were exposed to 100 *O. ptychocheilus* cercariae for 3-4 hours, on 20 August, 2007 using the methods described above. Fish were then returned to aquaria and sustained on tetramin fish flakes. At various intervals post exposure (4 days, 1, 2, 3, 4 and 6 weeks), 3 minnows were randomly selected for sacrifice. These intervals were selected to encompass the stages of metacercarial development described in Goater *et al.* (2005), from penetration to encystment. Samples destined for light microscopy were fixed, decalcified, and embedded in paraffin as previously described. Serial coronal sections (thickness=10 μ m) of whole heads were prepared on gelatin coated glass slides, and stained with Mayer's hematoxylin 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 (Fig. 2.1) (Laufer & Vanegas, 1974).

For ultrathin 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 hours. Samples were rinsed overnight in a 0.1M sodium cacodylate buffer (pH 7.2) and post-fixed for 1 hour in 1% osmium tetroxide in the same buffer. After dehydration through a graded ethanol series, tissue samples were embedded in Spurr's resin. Samples were polymerized in an oven at 60 degrees centigrade for 24 hours. Semi-thin coronal sections (thickness=1 μ m) were cut on an OM-U2 ultramicrotome, mounted on glass slides coated with gelatin, dehydrated in an atmosphere of Hemo-De, and stained with 1% toluidine blue. Images were acquired using a digital camera. Ultrathin sections (thickness=0.1 μ m) were cut along the coronal plane on an OM-U2 ultramicrotome and stained with 4% uranyl acetate for 20 minutes, and

Reynolds lead citrate for 5 minutes (Reynolds, 1963). Sections were photographed using at an accelerating voltage of 75 kV.

Analyses

Evaluation of changes in mean proportions of diplostomules in the optic lobes, cerebellum, and endomeninx between 4-42 days p.i. were analyzed using the Kruskal-Wallis non-parametric test.

RESULTS

15 minutes to 3 hours post infection

Diplostomules were observed in subdermal connective, adipose, and hypodermal tissues as early as 15 min p.i. (Fig. 2.2a). At this time, diplostomules were also observed within the muscle layers associated with the head region (Fig. 2.2b). At each interval between 15 min and 4 hours p.i., diplostomules were observed within several cranial nerves, including the octaval nerve (Fig. 2.2c, d), the optic nerve (Fig. 2.2 e, f), an unidentified nerve that runs adjacent to a pseudobranch associated with the gills (Fig. 2g), and the anterior lateral line nerve (Fig. 2.2h). Multiple diplostomules were located within the optic tract (Fig. 2.2i). No diplostomules were found in the eyes, although a small number occupied tissues within the orbital cavity.

3-48 hours post infection

Mapping the locations of individual diplostomules within specific regions of the brain indicated the dynamic and non-random nature of migration (Fig. 2.3). Between 3-8 hours p.i., 36% of the total number of diplostomules observed was found in the optic lobes and cerebellum (Fig. 2.3). At this time, 40% appeared to be scattered haphazardly throughout the medulla oblongata of the brain, and 10% in the tegmentum. The remaining proportion was recorded in cranial nerves, the hindbrain, and ventricles. By 12-16 hours p.i., 88% of all diplostomules had localized in the optic lobes and cerebellum, with 6% in the tegmentum and the remaining 4% in the medulla oblongata (Fig. 2.3). At 48 hours p.i. 98% of all diplostomules were observed in the optic lobes and cerebellum.

The specific locations of the relatively small numbers of diplostomules found in the medulla and the tegmentum between 3-8 hours p.i. illustrate that larvae tended to migrate along specific neural tracts (Fig. 2.4a-d). Most notably, 30% of diplostomules in the medulla oblongata occupied the medial longitudinal fascicle (MLF) (Fig. 2.3; Fig. 2.4b), 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. 2.3). Of those diplostomules in the tegmentum between 3-8 hours p.i., 36% occupied the tecto-bulbar tract (TTB). Proportions in the TTB between 12-16 hours p.i. were comparable at 30%. (Fig. 2.3; Fig. 2.4c, d).

Diplostomules tended to be found in the lateral-most regions of the optic lobes (Figs. 2.3; 2.4e). Of the 30% of diplostomules occupying the optic lobes between 3-8

hours p.i., about half were found in the extreme lateral portions of the tectum (Fig. 2.3, Fig. 2.4e). Between 12-16 hours p.i. and 24-48 hours p.i., relatively equal proportions of diplostomules were located in the lateral-most regions of the optic lobes (64% and 62% respectively) (Fig. 2.3; Fig. 2.4e).

Diplostomules between 12 and 16 hours p.i were localized primarily within the optic lobes, and secondarily in the cerebellum (Fig. 2.3). This trend continued for diplostomules between 24-48 hours p.i. (Fig. 2.3). Consistently, across 3-8 hours p.i, 12-16 hours p.i, and 24-48 hours 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 hours p.i. (89%, 80%, & 85% respectively) (Fig. 2.3.).

4 to 42 days post-infection

Differences in the proportion of metacercariae located in specific brain tissues over time were highly significant (Fig 2.5). Overall, there was a steady decline in the proportion of metacercariae found in the optic lobes from 4 days p.i. to 28 days p.i (chi-square 10.13; df=4, p=0.038). The decline in the proportion of metacercariae in the cerebellum was similar over the same period (chi-square 13.23, df=4, p=0.010). Both of these declines corresponded to significant increases in the proportion of metacercariae located within adjacent meninges between 14-42 days p.i. (chi-square 9.56, df=3,

$p=0.023$). By 42 days p.i., virtually all metacercariae were found in the endomeninx layers surrounding the optic lobes and cerebellum (Fig. 2.5).

Micosite selection within the optic lobes was evident between 4-14 days, with a marked preference for the stratum marginale (Fig. 2.6). At 4 days p.i., 74% of diplostomules in the optic lobes were in this outermost layer, with 21% in the stratum album central, and the remaining 5% in the periventricular zone. Similar proportions of diplostomules were found at 7 days p.i. in the stratum marginale (76%) and stratum album centrale (23.6%). Once the microhabitat shift began at 2 weeks p.i., 90% of diplostomules were located in the stratum marginale, 5.6% in the stratum album central, and 4.5% in the periventricular zone. By 3 and 4 weeks p.i., those few diplostomules remaining in the optic lobes were observed in the stratum marginale (Fig. 2.6).

The shift in microhabitat from the tissues of the optic lobes to the adjacent meninges was characterized by obvious morphological changes that occurred in the meninges (Fig. 2.6), and at the host-parasite interface (Fig. 2.7). While light micrographs at 4 days p.i depict the tegument of diplostomule as smooth (Fig. 2.7a), abundant microlamellae and microvilli are visible in transmission electron micrographs (Fig. 2.7b). The fuzzy appearance of diplostomules in light micrographs at 2 weeks p.i. (Fig. 2.7c) is a result of the proliferation and elongation of the microvilli, which extend perpendicularly from the parasite surface into the adjacent gap (Fig. 2.7d). Concurrent with the increasing elaboration of the tegumental surface is a widening of the gap between host and metacercarial tissues. Host tissues at the leading edge of the gap are visibly less cohesive than more distant tissues unaccompanied by infiltration of

microlamellae (Fig. 2.7d). At 2 weeks p.i, disrupted host tissues contain an abundance of host macrophages (Fig. 2.7c, d). At this time, the boundary between host meninges and brain tissues was unclear, although some diplostomules still occupied brain tissues (Fig. 2.7c, Fig 2.6c). At 4 weeks p.i the metacercariae are clearly embedded in the meninges of the host, which appeared greatly inflamed (Fig. 2.7e). Meningeal inflammation, which was first visible at 2 weeks p.i., was most evident between 4-6 weeks p.i (Fig. 2.6e, f). Adjacent host tissues were flush with the cyst wall, and appeared intact and consolidated (Fig. 2.7f). Both microvilli and the gap between host and parasite tissues are absent (Fig. 2.7f).

DISCUSSION

The results of the present study add to the mounting evidence that some, perhaps all, strigeoid metacercariae have elaborate, complex interactions with host tissues. Previous results have determined that *O. ptychocheilus* metacercariae undergo a well-defined and extensive pattern of growth and development before becoming infective (Sandland & Goater, 2000). More recent, studies have described the presence of transient migratory (Conn *et al.*, 2008) and tegumental structures associated with migration and development (Goater *et al.*, 2005). My results indicate that *O. ptychocheilus* diplostomules migrate along specific neural tracts in the brain to access the outer edges of the optic lobes and cerebellum. Upon encystment, metacercariae shift from the tissues of the brain, to the adjacent meninges. Thus, from diplostomule migration to metacercarial

encystment, *O. ptychocheilus* larvae display a wide range of complex and interactive behaviours with their host.

Histological evidence indicates that those cercariae that penetrate regions of the minnow body caudal to the head access the brain via the spinal cord. Thus, between 3-8 hours p.i., high proportions of diplostomules were observed in caudal regions of the medulla oblongata. While my results cannot assess how migrating diplostomules accessed the central nervous system throughout the main body of fathead minnows, histological observation of the head region of fathead minnows support Hendrickson's (1979) conclusion that access to the central nervous system is accomplished through peripheral nerves. Results based on counts of migrating diplostomules also support Hendrickson's claims that access to the brain also occurs via cranial nerves.

The vertebrate spinal cord consists of an outer layer of longitudinally coursing axons, and an inner layer of their associated motor neurons, all surrounding the central canal (Speare & Frasca, 2006). Large bundles of axons coursing in the same direction (fascicles) could represent the basis for a navigational cue from any point in the spinal cord to the brain. These fascicles provide a clear directional path toward the brain. They also contain few cell bodies, presumably making travel easier. Those diplostomules that access the brain from cranial nerves would similarly travel along fascicles. Once larvae reach the brain, however, neural anatomy becomes more complex (Wullmann *et al.*, 1996). The brain contains many sub-regions with cell bodies, dense cellular granular layers, and ventricles that would act to complicate potential navigation routes from the brain stem to the outer edges of the optic lobes and cerebellum. Navigation along fibrous

tracts within the brain would be a means to avoid such brain tissues and ventricles and, in some cases, would provide direct access to preferred sites. Thus the mechanism to migrate to the brain could be co-opted for navigation within the brain.

Two fibrous tracts in particular are hypothesized to play a role in providing cues to migrating diplostomules, based on their occupation by numerous diplostomules, and their anterior-posterior orientation. The medial longitudinal fascicle (MLF) is the most dominant fiber system in the brain of fish (Wullimann *et al.*, 1996). Axons 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, where it would likely be encountered by the majority of migrating diplostomules. At 4 hours p.i., over 40% of all diplostomules in the medulla oblongata and hindbrain were observed migrating along the MLF. Flanking the MLF from the caudal medulla oblongata all the way to the tegmentum is another substantial series of fibers, collectively called the reticular formation. Thus, a significant portion of medulla oblongata tissues consist of large neural pathways coursing in a longitudinal direction, which have the potential to aid migrating diplostomules in a rostro-caudal direction towards preferred habitats within the brain.

The tectobulbar tract (TTB) is another fiber system that descends from the optic lobes (Wullimann *et al.*, 1996). Several portions of this tract detach, and course in both medial and lateral anterior-posterior directions. Between 4-16 hours p.i., 25-36% of all diplostomules in the tegmentum are observed migrating along the TTB. The alternate route to the optic lobes is a longer, anterior-central migration directly through the

tegmentum. This would result in the optic lobes being accessed via the most anterior point, which was rarely observed. The use of the TTB to access the optic lobes could explain the relatively low numbers of diplostomules in the tegmentum. Those diplostomules that migrate along the TTB to the optic lobes would spend very little time in the tegmentum, as only several hundred micrometers separate the anterior portion of medulla oblongata from the TTB (Wulimann, *et al.*, 1996). This could be traversed from 3-18 minutes, based on calculations of rates of migration of *O. ptychocheilus* larvae by Hendrickson (1979), a narrow window that is unlikely to be captured by this study's post-infection intervals. Additionally, the potential use of the TTB as a migration route could explain the consistently high proportions of diplostomules occupying the most distal regions of the lateral lobes, as this tract provides direct access to these regions (Fig. 2.8).

Diplostomules that access the brain via cranial nerves would begin their navigation within the host brain at a different position than those that access the brain via the spinal cord. Thus, these diplostomules would find themselves occupying the lobes in which the cranial nerves they migrate along terminate, before moving onto the cerebellum and optic lobes. Therefore, the presence of diplostomules within these lobes of the brain can provide insight regarding potential migration routes. The vagus nerve, which has been previously documented as a route for migrating *O. ptychocheilus* diplostomules (Hendrickson, 1979), terminates in the vagal lobe. As the vagal lobe was on average occupied by 10% of those diplostomules within the medulla oblongata between 3-8 hours p.i., it follows that some of these diplostomules may have previously been migrating along the vagus nerve. Similarly, this study, and several others (Hendrickson, 1979; Radabaugh, 1980), report diplostomules in the hypothalamus and inferior lobes of

the tegmentum. Some proportion of these diplostomules likely accessed this region by the optic nerve and tract. This idea is supported by the presence of many diplostomules that were observed in both the optic nerve and optic tract. While the high numbers of diplostomules that were observed in the crista cerebella cannot be explained by migration along cranial nerves (this region is not fed by cranial nerves), these proportions could represent those migrating diplostomules which are *en route* to the cerebellum, rather than the optic lobes.

Micro-site segregation of *O. ptychocheilus* diplostomules in the optic lobes and cerebellum is a consistent finding. In two other experimental studies the partitioning of diplostomules occurs primarily in the optic lobes (54% and 56 %), and secondarily the cerebellum (21 % and 24%) (Hendrickson, 1979; Radabaugh, 1980, respectively). It is unknown why some diplostomules migrate to cerebellum, while others to the optic lobes. Density-dependant site segregation is one possibility, with the cerebellum acting as a spill-over site in high-intensity infections. This is unlikely, however, as the partitioning of diplostomules between the optic lobes and cerebellum was first observed as early as 3-8 hours p.i.. Just as diplostomules use the TTB to access the optic lobes, another neural tract could serve to provide access to the cerebellum. Thus, the segregation of diplostomules in the optic lobes and cerebellum may result from differential migration along particular fibrous tracts-one leading to the optic lobes, and another leading to the cerebellum. The unifying feature of the two primary locations of *O. ptychocheilus* larvae is their tendency to migrate to the outermost portions of these regions. It may be adaptive for the parasite to select a location within the host brain that will accommodate

substantial larval growth, thus reducing host mortality. Neural tissue adjacent to the meninges could fulfill this requirement, as larvae have a region to “spill” into.

The optic lobes of teleosts are composed of numerous functionally and morphologically distinct strata (Vanegas, 1983). The majority of *O. ptychocheilus* diplostomules migrated to the outermost stratum marginale of the optic lobes. This layer is composed of fine, unmyelinated fibers which run parallel to the tectal surface. Diplostomule distribution across the stratum marginale was not uniform, as expected. Rather, there was a marked preference for the lateral regions of the optic lobes. As suggested previously, the consistently high proportions of diplostomules found in these lateral regions is hypothesized to be a result of diplostomule migration along the TTB, which provides direct access to the lateral-most optic lobes.

My results are the first to describe the role of the fish endomeninx during the development of metacercariae. This structure, which is considered analogous to the pia-arachnoid layer found in mammals (Momose *et al.*, 1988), surrounds the central nervous system in three distinct layers. The innermost layer, which functions to secrete cerebrospinal fluid, contains large extracellular spaces, fibroblasts, collagen fibrils, macrophages, blood vessels, red blood cells, and leucocytes (Momose *et al.*, 1988; Schwartz *et al.*, 1993; Hoffman & Schwartz, 1996). Evidence based upon TEM observations indicate that this layer undergoes marked inflammation in association with the development of metacercariae. Above the innermost layer lies the intermediate layer, whose tightly packed cells provide protection from infection, through maintenance of the

blood-cerebrospinal fluid barrier (Momose *et al.*, 1988; Hoffman & Schwartz 1996; Speare & Fraca, 2006).

Massive meningeal inflammation (endomeningitis; Speare & Frasca, 2006) coincides with metacercarial encystment, and infiltration of the meninges. Slight swelling of the meninges was first visible at 2 weeks p.i., with inflammation apparently peaking at 4 weeks p.i. Fibroblasts, proliferated from the expanding meninges (So & Wittrock, 1982) were observed to encapsulate pre-encysted metacercarie, forming part of the outer cyst wall (So & Wittrock, 1982). While several studies have observed developing trematode metacercariae to lie under the meninges or dura mater of other trematode-infected fish (Rees, 1955; 1957; Hoffman & Hoyme, 1958; Muzzal & Kilroy, 2007), very few studies describe a direct association of metacercariae with the meninges (Sweeting, 1976; Karlsbakk, 1995). Reports of endomeningitis due to trematode infection are scanty. Infection by *D. mordax* metacercariae provoked a “moderate inflammatory reaction” of the meninges in silversides (Siegmund *et al.*, 1997). By 9 weeks p.i. meningeal inflammation has largely subsided, though the meninges remain slightly swollen.

Although the mechanism behind the observed micro-habitat shift of *O. ptychocheilus* metacercariae between the stratum marginale of the optic lobes and the inner layer of the endomeninx is unknown, it seems unlikely to be due to active migration. An ultrastructural study by Conn *et al.* (2008) describes a transient proboscis-like structure (prosoma) borne by *O. ptychocheilus* diplostomules 4-14 days post infection that is believed to play a role in their penetration and migration through brain tissue. However, once the cyst wall appears, this structure disappears. Further, the

transformation of the tegument from an elaborate complex of invasive structures to the smoothed cyst wall, concomitant with the re-scaffolding of damaged axons, could cause the expulsion of metacercariae from brain tissue; much like a splinter is expelled from skin. Thus, the observed habitat shift could be a by-product of host tissue recovery processes.

Regardless of the precise mechanism leading to the shift in micro-habitat, site selection in the endomeninx offers several clear advantages for metacercarial development. The inner layer of the endomeninx is broad, and contains large extracellular spaces. Thus, metacercarial growth is not constrained by space. This region is additionally very rich in glucose, and therefore would presumably satisfy nutrition requirements (Hoffman & Schwartz, 1996). Finally, the inner endomeningeal layer is capable of a fibroblastic response (Hoffman & Schwartz, 1996), enabling the development of the fibrocyte-derived cyst wall characteristic of so many metacercariae. (Mitchell, 1974; So & Wittrock, 1982; Larson *et al.*, 1988; Wittrock *et al.*, 1991).

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Figure 2.1: Schematic sagittal diagram of selected tissues in the brain of *Pimephales promelas*. Terminology follows Wullmann *et al.* (1996). The medulla spinalis is considered as 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, and includes 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 a light micrograph in the boxed insert. The light micrograph depicts the lamina of unexposed optic lobes of *Pimephales promelas*. The periventricular zone (PVZ) is a dense granular region composed of cell bodies; the stratum opticum (SO) is characterized by thick, myelinated axons of retinal origin. The outermost layer, the stratum marginale (SM), is characterized by thin unmyelinated fibers that run parallel to the optic tectum. Between these layers lies the stratum album centrale (SAC). The endomeninx (EM) is a secretory organ that covers the central nervous system.

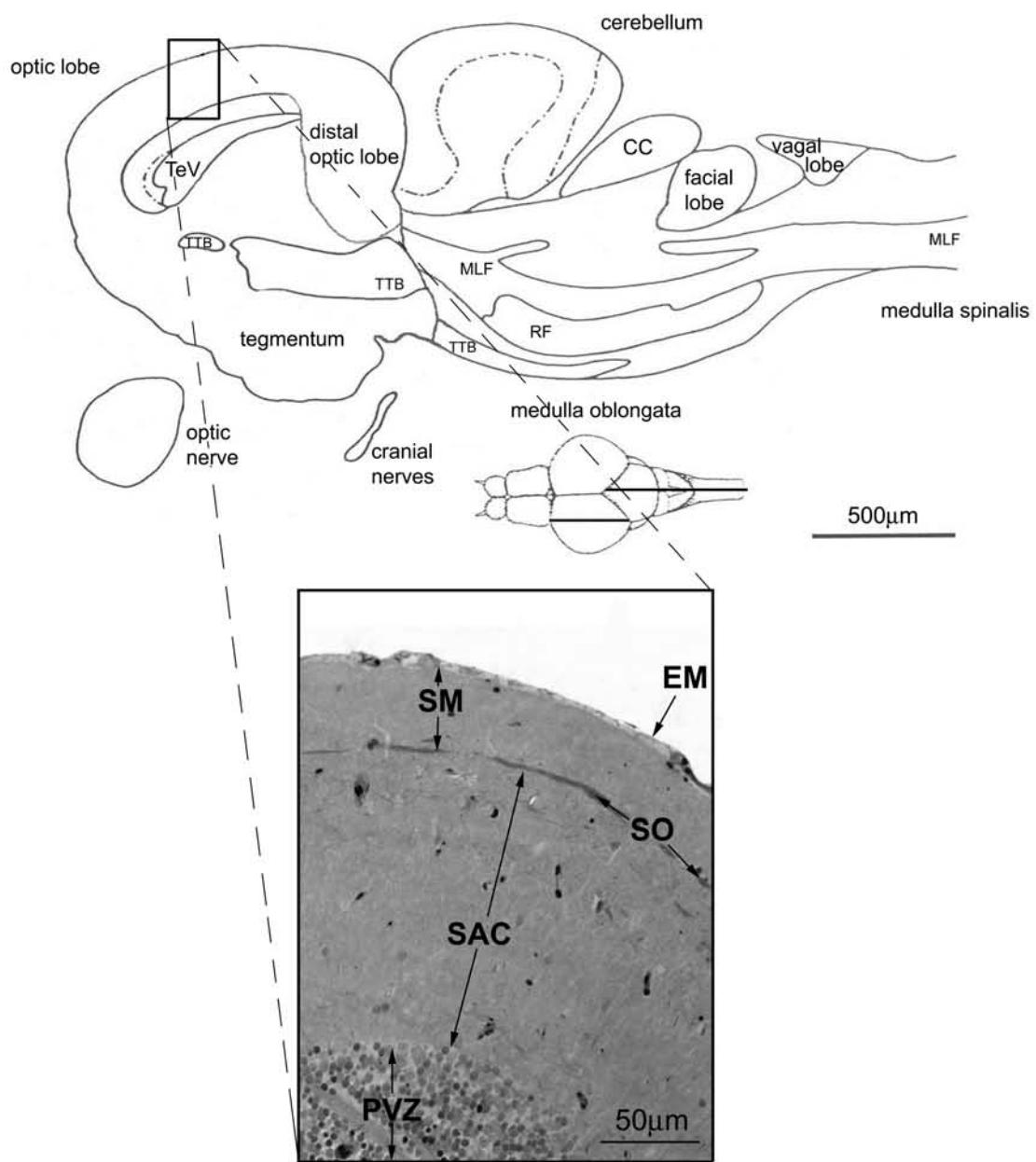


Figure 2.1

Figure 2.2: Light micrographs of migrating diplostomules of *Ornithodiplostomum ptychocheilus* in brain tissues of experimentally infected *Pimephales promelas* at 15 min to 4 hours post-infection. At 15 min p.i. diplostomules (arrows) were observed in host adipose (a) and muscle (b) tissues. The boxed regions in figures c and e represent regions magnified in figures d and f. As early as 15 minutes 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 at 4 hours p.i. deeper in the optic tract within the brain itself (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).

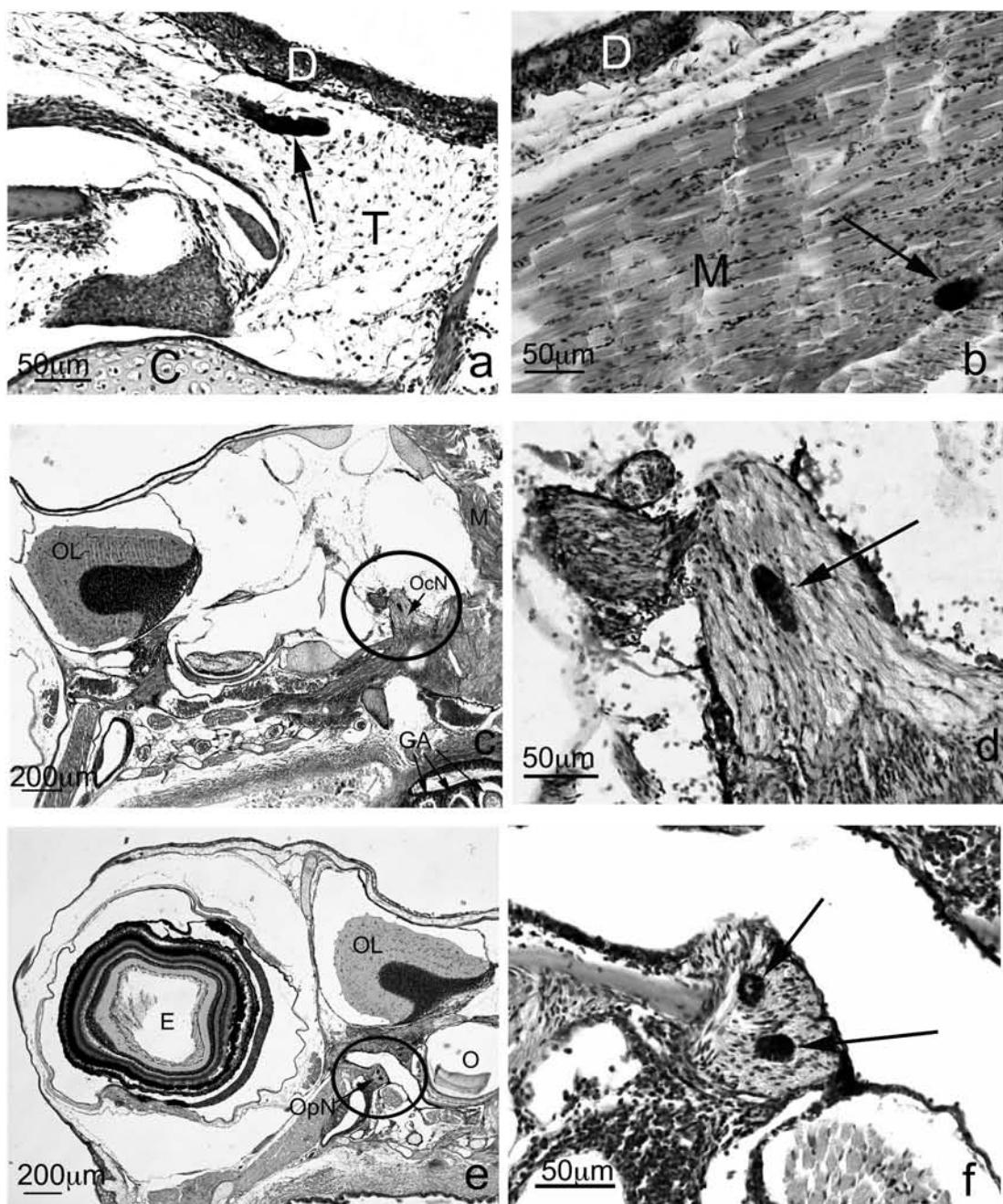


Figure 2.2

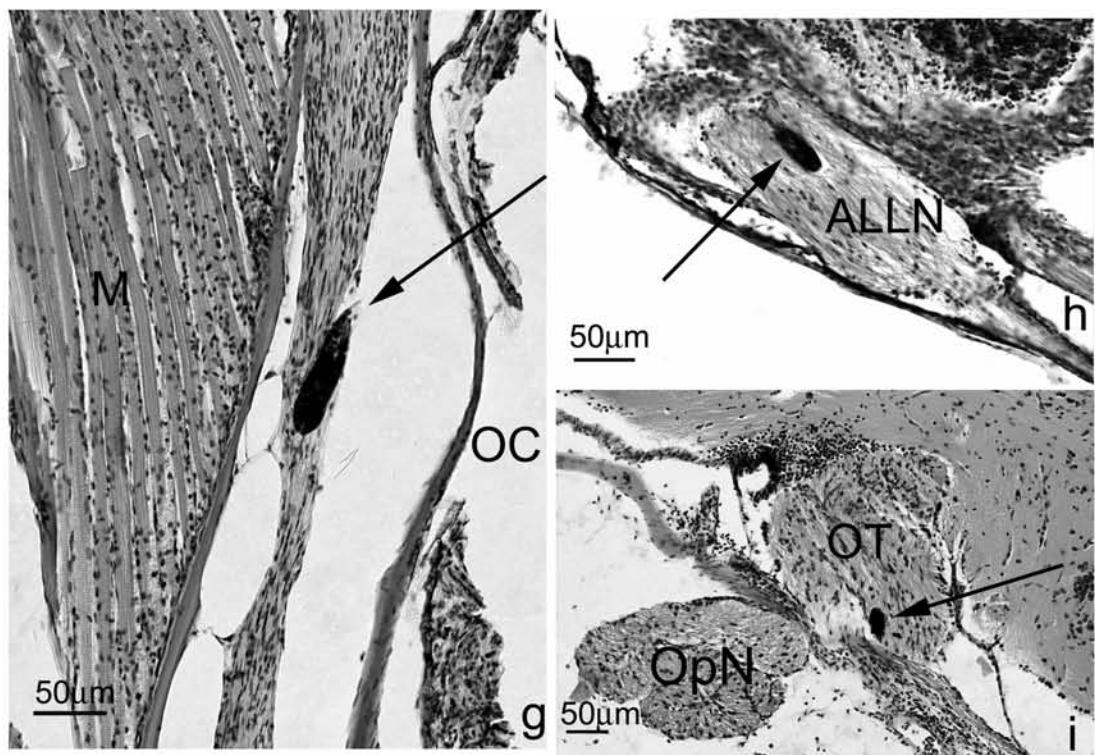


Figure 2.2 continued

Figure 2.3: Schematic sagittal diagram of the brain of *Pimephales promelas* experimentally infected with cercariae of *O. ptychocheilus*. Demarcations of selected sub-regions of the brain are described in Figure 2.1. The approximate locations of all observed metacercariae are depicted by unique symbols according to the length of time parasite migration has been underway. The diagram summarizes metacercariae site location pooled among 3 fish processed at each time interval. The individual diplostomule locations across all fish per interval that were migrating for 3-8 hours post-infection (p.i.) were depicted on a single diagram (a); similarly diplostomules migrating from 12-16 (b) and 24-48 (c) hours p.i. were grouped together.

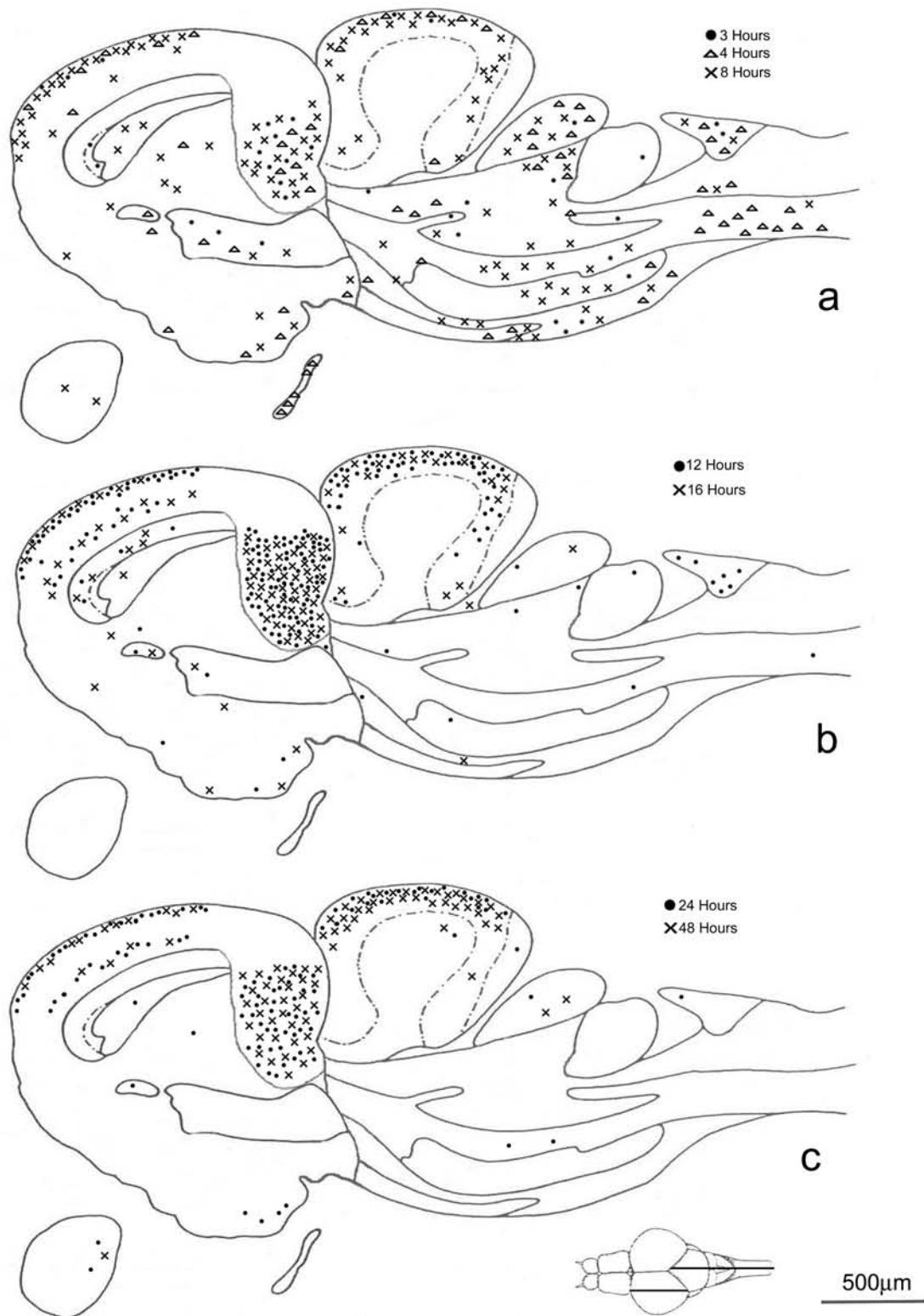


Figure 2.3

Figure 2.4: Light micrographs of *O. ptychocheilus* diplostomules in brain tissue of *Pimephales promelas* between 4-24 hours post-infection. Diplostomules (arrows) were observed migrating along major neural tracts within the brain (a; tractus preTECToMAMaILLaRIS) 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 preTECToMAMaILLaRIS (TPM); medial longitudinal fascicle (MLF); stratum marginale (SM); Stratum album central (SAC); periventricular zone (PVZ); tectobulbar tract (TTB).

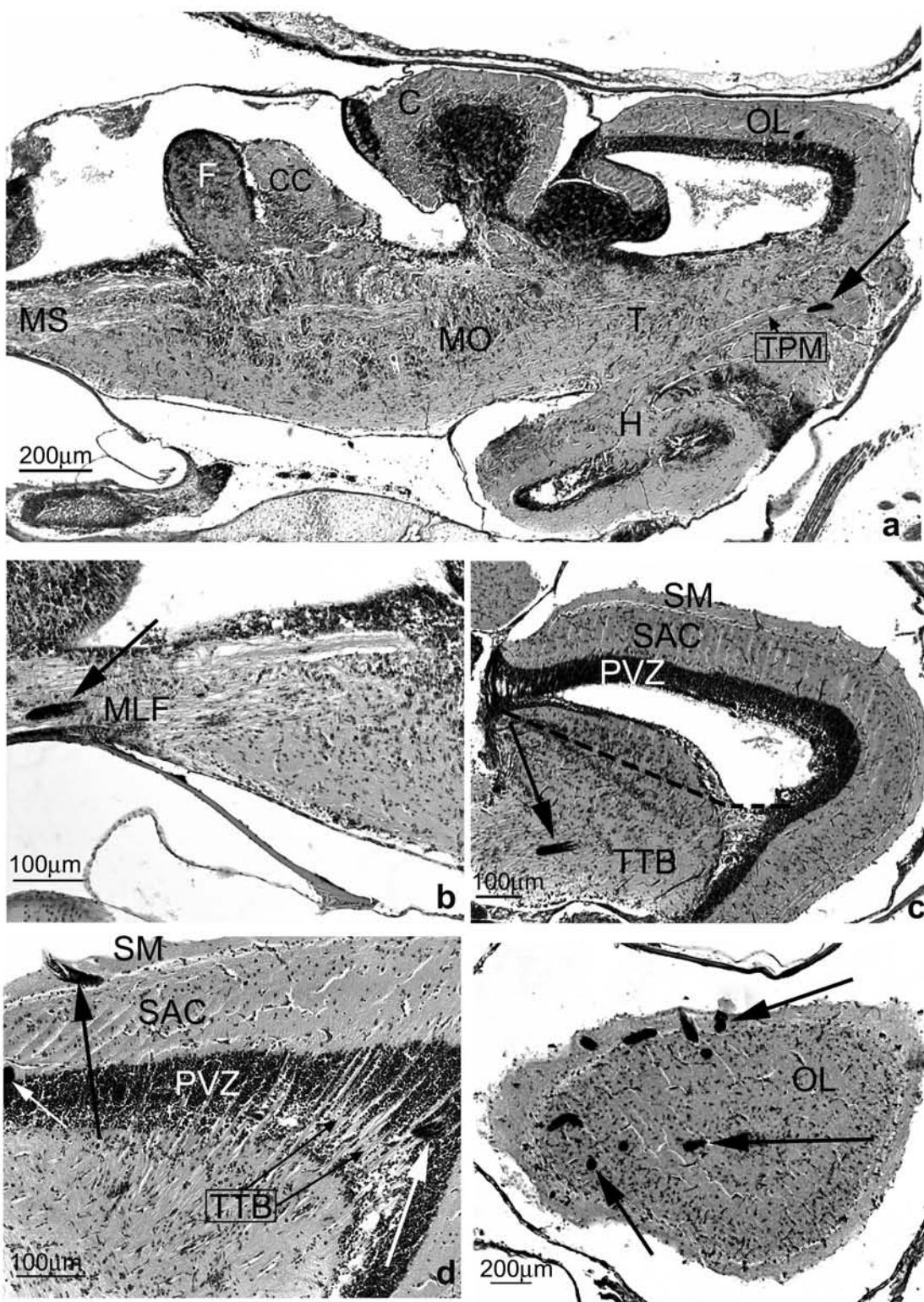


Figure 2.4

Figure 2.5: Mean temporal changes in the proportion of metacercariae in the brain and endomeninx of minnows experimentally infected with *O. ptychocheilus* cercariae.

Metacercaria from 3 fish were examined at each interval. Bars represent standard error.

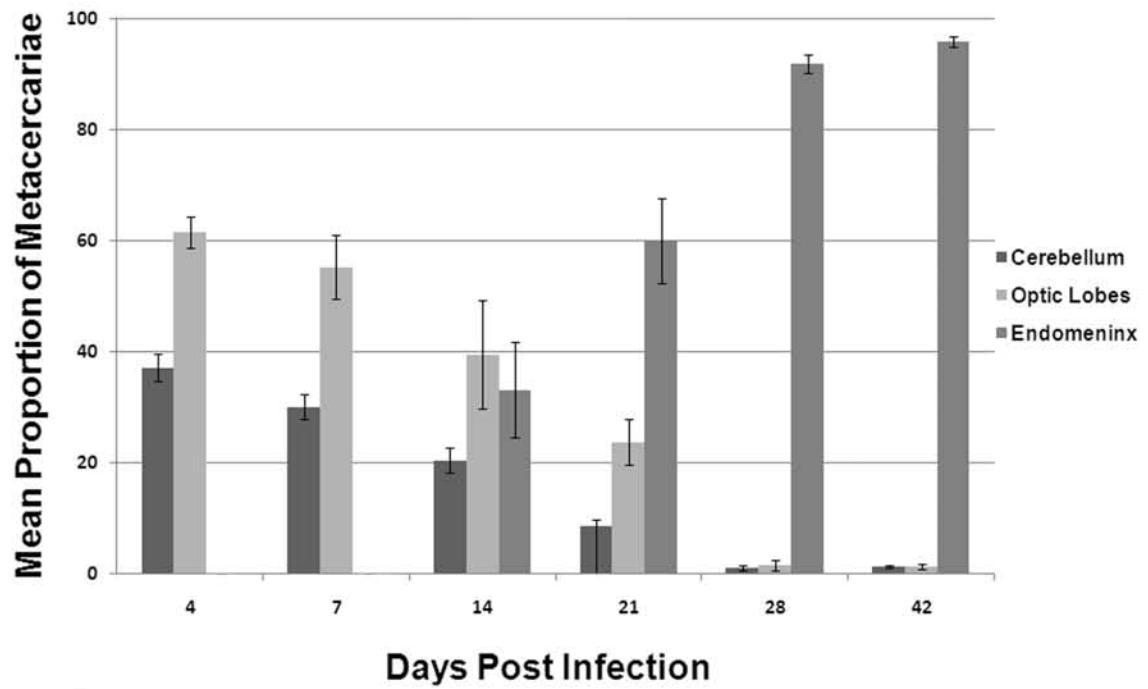


Figure 2. 5

Figure 2.6: Coronal sections of uninfected (a) and experimentally infected (b-f) minnows exposed to cercariae of *O. ptychocheilus*. Diplostomules range in age from 1 week (b), 2 weeks (c), 4 weeks (d), 6 weeks (e), to 9 weeks old (f). Diplostomules between 1-2 weeks are identified with arrows; meninges in unexposed minnows and between 1-2 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 μ m.

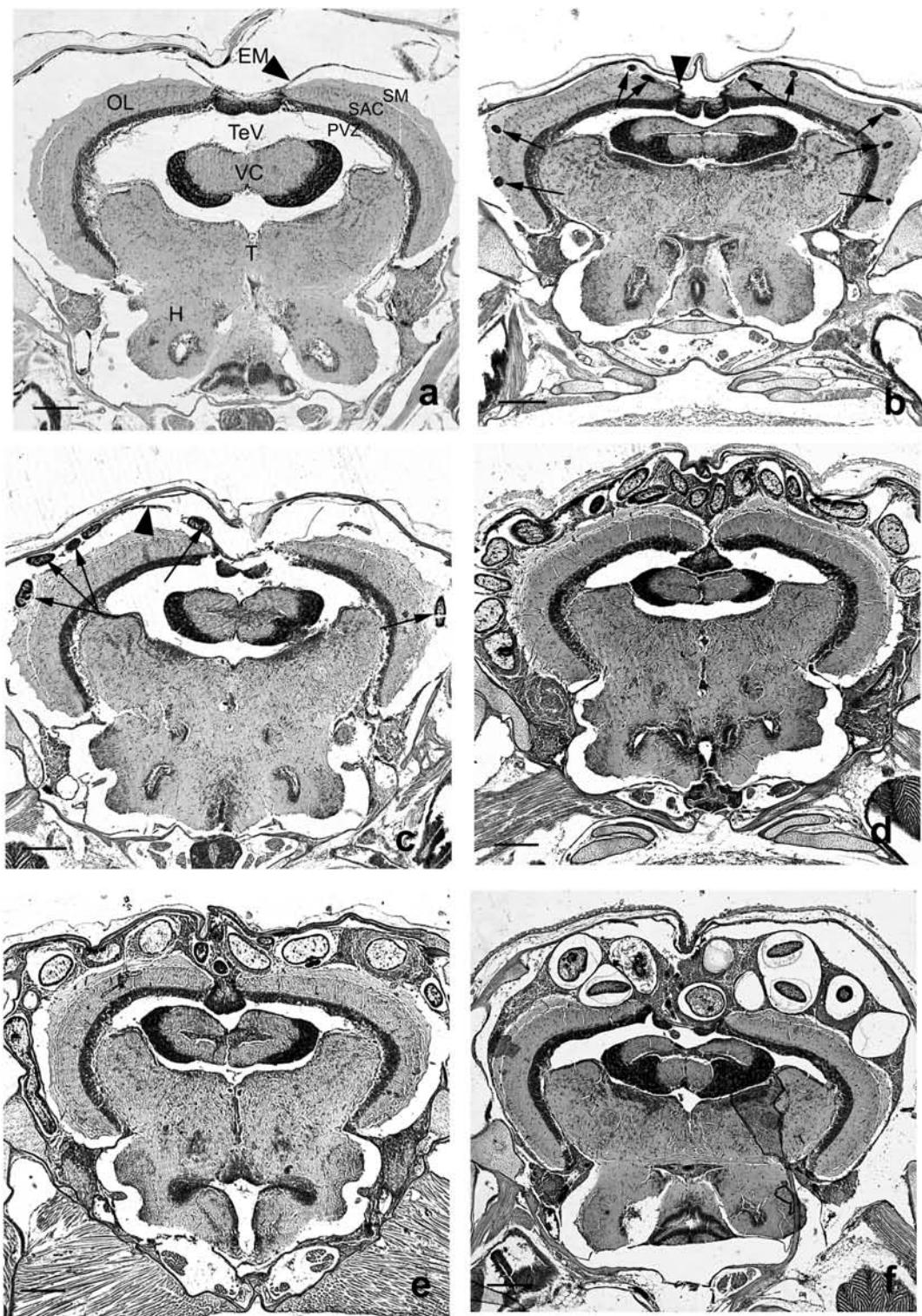


Figure 2.6

Figure 2.7: Light and transmission electron micrographs of brain tissues of minnows experimentally infected with *O. 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).

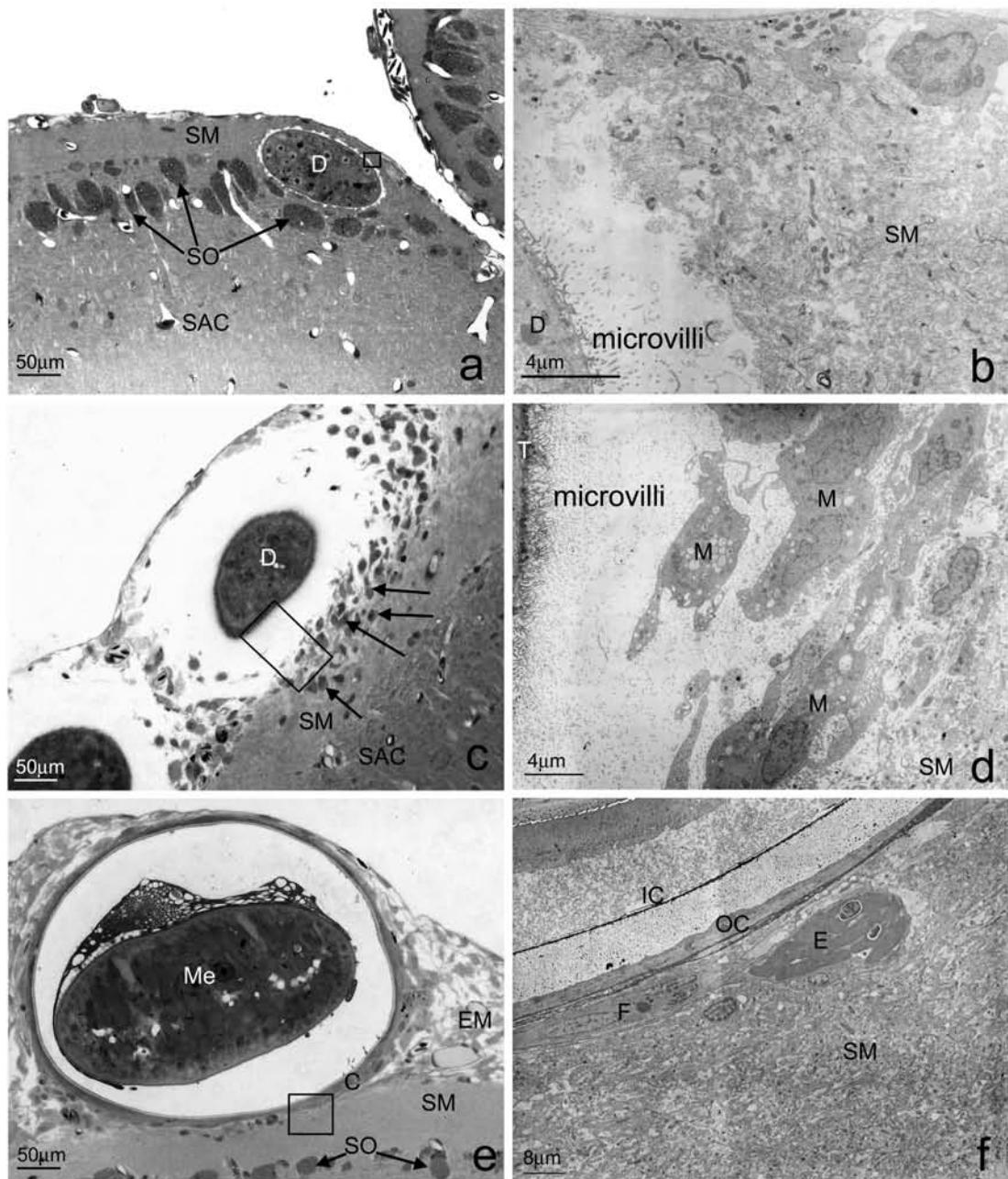


Figure 2.7

Figure 2.8: Schematic diagram illustrating a hypothetical migration route of *O. ptychocheilus* cercariae to the optic lobes in the brain of minnows. Medial longitudinal fascicle (MLF); reticular fascicle (RF); tectobulbar tract (TTB); optic lobes (OL); stratum marginale (SM); stratum opticum (SO); stratum album central (SAC); periventricular zone (PVZ).

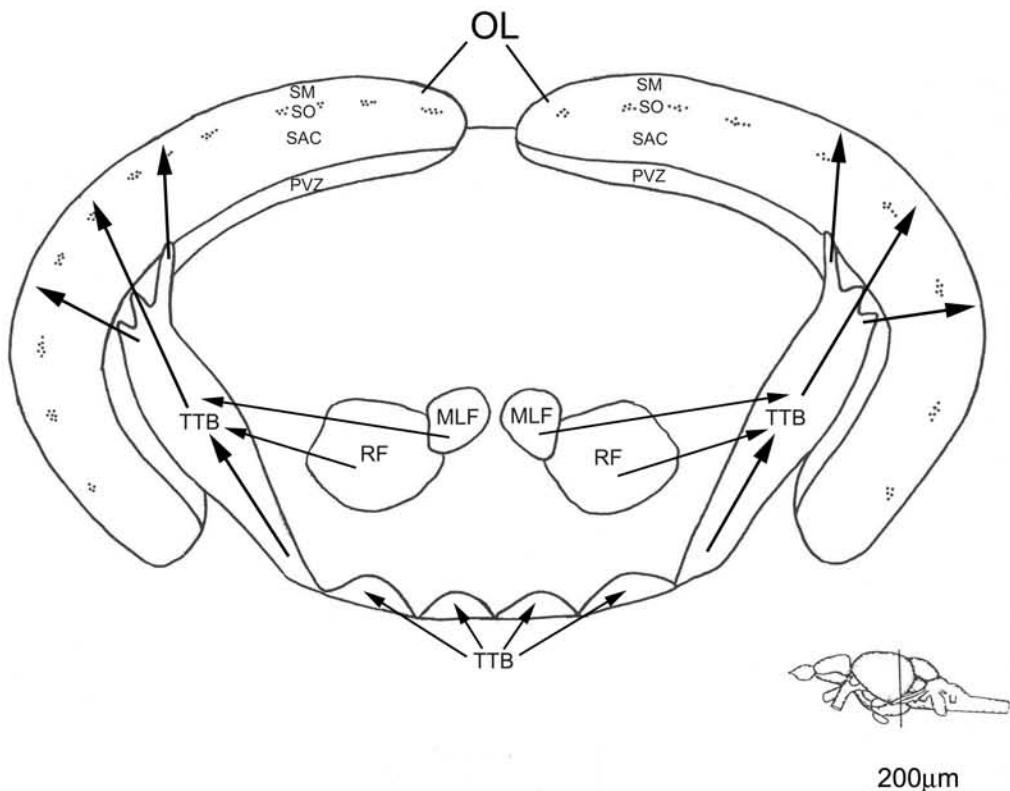


Figure 2.8

**Chapter 3: Migration, site selection, and habitat shift of *Ornithodiplostomum* sp.
metacercariae (Trematoda: Digenea) in fathead minnows (*Pimephales promelas*)**

INTRODUCTION

The resting stages of digenetic trematodes often encyst within a wide variety of tissues within their second intermediate hosts (reviews in Chapters 1 and 2). The manner in which they access these sites and migrate to them is a defining feature of individual trematode species (Chapter 1; Sukhdeo & Sukhdeo, 2004; Haas, 2007). For example, metacercariae of the strigeoid trematode *Ornithodiplostomum ptychocheilus* undergo a well-characterized pattern of growth, development, and encystment in the brain of fathead minnows (Sandland & Goater, 2000). Diplostomules migrate to the brain via spinal and cranial nerves within 24 hours of epidermal penetration (Hendrickson, 1979, Chapter 2). Further, specific neural tracts within the brain are utilized by diplostomules to localize along the perimeter of the optic lobes and cerebellum (Chapter 2). The growth phase is characterized by an elaborate tegumental complex of microlamellae and microvilli that extend into adjacent host tissues (Goater *et al.*, 2005). Upon deposition of the cyst wall, this tegumental complex is absent, and host tissues are flush against the outer cyst (Goater *et al.*, 2005). Recent studies involving *O. ptychocheilus* metacercariae reveal a microhabitat shift in developing diplostomules from the neural tissues to the adjacent host meninges (Chapter 2). This is an important result because it provides the first indication that the obligate growth and encystment phases of some metacercariae are associated with different microhabitats in the host. It is unknown if this feature is unique to *O. ptychocheilus*, or is shared by related species.

Metacercariae of *Ornithodiplostomum* sp. typically encyst in the body cavity of fathead minnows (Goater, unpublished observations). It follows that the pattern of migration and site selection should differ from *O. ptychocheilus*, which utilizes the nervous system. Preliminary studies have determined that *Ornithodiplostomum* sp. is morphologically, developmentally, and ecologically similar to its congener, *O. ptychocheilus* (James *et al.*, 2008; Goater, unpublished observations). Like *O. ptychocheilus*, it is amenable to experimental manipulation. The objectives of this study are to 1) characterize migration and site selection of minnows experimentally infected with *Ornithodiplostomum* sp., and 2) to compare the migration route and site selection preferences of *Ornithodiplostomum* sp. with *O. ptychocheilus*.

MATERIALS AND METHODS

General infection procedure

Methods used to experimentally infect fathead minnows with *Ornithodiplostomum* sp. are similar to those outlined in Sandland and Goater (2000) and described for *O. ptychocheilus* in Chapter 2. One-day old chickens were force-fed the viscera of fathead minnows naturally infected with *Ornithodiplostomum* sp. metacercariae. Trematode eggs were collected and isolated from chick faeces using a series of filters and washes, as described in Chapter 2. Eggs were incubated in dechlorinated water at 20° C. The F1 generation of field collected physid snails (*P. gyrina*) were exposed to hatched miracidia. Cercariae were collected approximately 4 weeks later using the dilution techniques that are described in Chapter 2 and following Sandland & Goater (2000).

Migration of Ornithodiplostomum spp. to the visceral cavity

This component mirrors methods used to assess the migration route of *O. ptychocheilus*, as outlined in Chapter 2. The source of minnows used in the infections was the identical to those used in Chapter 2. One month prior to infection, juvenile minnows (30 days old, between 1.5-2.0 cm standard length) were maintained in aquaria and fed tetramin fish flakes twice daily. On 12 August 2007, 30 fish were randomly selected from a stock tank. Individual minnows were isolated in petri dishes containing de-chlorinated water and exposed to 200 cercariae. After 15 minutes, fish were transferred to a 30 x 30 x 60cm (H x W x L) aquarium containing aerated water. The relatively high dose of cercariae ensured a high recovery of migrating diplostomules, despite the brief exposure time. Fish were fed tetramin flakes twice daily until sacrifice. Three fish were randomly assigned to each interval post exposure (30 min, 1, 2, 3, 4, 8, 12, 16, 24, and 48 hours). To allow for complete fixative penetration, minnow heads were removed immediately rostral to the operculum, and tails were removed just caudal to the visceral cavity. Bodies were fixed in 10% neutral buffered formalin for a minimum of 7 days, and decalcified in 0.1M EDTA titrant for a minimum of 14 days. Only two of three minnows survived to 48 hours post exposure.

Samples were dehydrated in ethanol prior to paraffin embedding. Each fish was serially sectioned along its sagittal axis (thickness=10 μ m). Sections were deparaffinized and stained with Mayer's hematoxylin and eosin Y. All sections were examined using light microscopy; selected sections were photographed using a digital camera.

The central aim of the study was to evaluate the navigation of *Ornithodiplostomum* sp. diplostomules through various tissues and organs over time. For visualization of diplostomule migration, selected sections throughout individual fish were sketched using a camera Lucida. Cumulatively, these sketches were used to create a single diagram that depicted a multidimensional view of the interior and exterior tissues and organs of fathead minnows (Fig. 3.1). The locations of individual diplostomules were plotted onto this image, provided a schematic perspective of diplostomule migration at various lateral positions within the host.

Site selection of Ornithodiplostomum sp. in the body cavity

Site selection of *Ornithodiplostomum* sp. over time was assessed using histology and light microscopy. On 13 August 2007, 18 juvenile fathead minnows were exposed to 100 *Ornithodiplostomum* sp. cercariae for 3 hours, as previously described. The cercarial dose reflects similar intensities of *Ornithodiplostomum* sp. recorded in field collected minnows. Fish were returned to aerated aquaria and fed tetramin flakes twice daily. At various intervals post exposure (4, 7, 12, 14, 21, 28 days), 3 fish were randomly selected for sacrifice in a lethal dose of clove oil. Preliminary studies have shown that the developmental process for *Ornithodiplostomum* sp. is similar to *O. ptychocheilus* (unpublished observations). Therefore, the intervals were selected to encompass the growth, development, and encystment stages of metacercariae. Fish heads and tails were removed, while ensuring the visceral cavity was not disturbed. Samples were fixed in 10% neutral buffered formalin, decalcified in 0.1M EDTA titrant, embedded in paraffin,

and sectioned (thickness=10 μ m) as described above. Sections were stained with Mayer's hematoxylin and Eosin Y. Locations of metacercariae were recorded over time.

Analyses

The association between the proportion of diplostomules occupying the muscular tissues and time post-infection was analyzed using Spearman's Rho. Evaluation of changes in mean proportions of diplostomules in the liver, pancreas, on the surface of the swim bladder, and in the body cavity between 4-28 days p.i. were analyzed using the Kruskal-Wallis non-parametric test.

RESULTS

0.5-48 hours post-infection

The characteristic shape and size of recently penetrated diplostomules made them easily discernable within variety of host tissues (Fig. 3.2). At 30 minutes p.i., large numbers of diplostomules were observed within sub-dermal muscles (Fig. 3.2a). Their orientation parallel to individual muscle fibers (Figs. 3.2a, b) indicated that diplostomules tended to navigate along muscle fibers, rather than perpendicular to them. Many diplostomules were observed migrating along myotemes (Fig. 3.2a) and between muscle fibers (Fig. 3.2b). As early as 2 hours p.i., young diplostomules were observed exiting muscle layers and directly penetrating the peritoneum (Fig. 3.2c). Diplostomules at this time were also observed migrating along, or inside the peritoneal lining within the visceral cavity (Fig. 3.2d).

Young, migrating diplostomules were also recorded within the cardiovascular system as early as 1 hour p.i. The most commonly invaded blood vessels were the dorsal aorta (Fig. 3.2e), and those blood vessels associated with the surface of the swim bladder (Fig. 3.2f). In addition, diplostomules were observed closely associated with cardiac tissues (Fig. 3.2g) and occupying large blood-filled atria of the heart (Fig. 3.2h).

Once inside the visceral cavity, diplostomules were observed migrating along the interfaces between organ surfaces (Fig. 2i). Most notably, many diplostomules were detected directly penetrating (Fig. 2j) and migrating within hepatic tissues of the liver (Fig. 2k). Direct penetration of pancreatic tissues (Fig. 2l), and those tissues associated with the swim bladder was observed less frequently.

Body maps that indicated the location of diplostomules in host tissues and organs between 0.5-48 hours p.i. provided a kinetic context for the individual observations made with light microscopy (Fig. 3). The resulting evidence suggests a highly directed and efficient migratory process for *Ornithodiplostomum* sp. Several diplostomules were observed in the epidermis of the host at 30 minutes p.i., although at this time most occupied deeper muscular layers. There was a significant correlation between time post-infection, and the proportion of diplostomules in the muscular tissues (Spearman's rho=0.97; p<0.01; df=29). Peak proportions of diplostomules occupied the muscles at 0.5-2 hours p.i. (84%), followed by a gradual decrease to 65% between 3-4 hours p.i., 24% between 8-16 hours p.i., and less than 1% between 24-48 hours p.i. (Fig. 3.3a-d). Overall, high proportions of diplostomules in the muscles were recorded in the dorsal regions of muscle tissues. Approximately 33% of diplostomules in the muscles between

0.5-2 hours p.i. were observed here, with proportions increasing to about 50% between 3-4 hours p.i., and 8-16 hours p.i. (Fig. 3.3a-d).

Clear site selection within the visceral cavity was first visible between 3-4 hours p.i (Fig. 3.3). While the majority of diplostomules were still in the muscular tissues at this time, there was a noticeable proportion (15%) of diplostomules observed within the liver (Fig. 3.3b). By 8-16 hours p.i., the proportion of diplostomules in the liver increased to 42%, while the pancreas and the surface of the swim bladder each contained approximately 10% of the overall diplostomulum population. This preference, primarily for the liver, and secondarily for pancreatic tissues and the surface of the air bladder, continued until 88% of all diplostomules occupied liver tissues, with equal proportions (approximately 6%) remaining in the pancreas and on the swim bladder at 48 hours p.i. (Fig. 3.3d).

The proportion of diplostomules in the blood vessels and cardiac tissues remained relatively low between 0.5-2 hours p.i. (3%). This increased to 6% of diplostomules at 3-4 hours p.i., and 9% between 8-16 hours p.i. Proportions peaked at 12 hours p.i., when 17% of diplostomules were observed within the cardiovascular system. The majority of diplostomules recorded in the blood system between 8-16 hours p.i. were associated with the blood vessels of the swim bladder (Fig. 3.3c, d). By 48 hours p.i., diplostomules were absent from the cardiovascular system, although those diplostomules on the surface of the swim bladder remained closely associated with blood vessels (Fig. 3.3d).

4-28 days post-infection:

Temporal differences in the proportions of metacercariae observed in the liver and body cavity between 4-28 days p.i. were highly significant (Fig. 3.4). A sharp decline in the proportion of metacercariae in the liver tissues (chi-square, 15.61; df=5; p=0.008) was concomitant with a significant increase in the proportion of metacercariae occupying the body cavity (chi-square, 16.35; df=5; p=0.006). Decreases in the proportion of metacercariae in pancreatic tissues was also significant (chi-square 11.36; df=5; p=0.045), while proportions on the surface of the swim bladder remained approximately static (chi-square=5.96, df=5; p=0.31). By 4 weeks p.i., virtually all metacercariae occupied the body cavity (Fig. 3.4).

A small gap between the diplostomule body and adjacent liver tissues was visible at 4 days p.i. (Fig. 3.5a). By 1 week p.i., both the diplostomules and the surrounding gap increased in size, with diplostomules remaining embedded in liver tissues (Fig. 3.5b). At 12 days p.i., the association between diplostomules and liver tissues was less distinct. While multiple diplostomules were embedded within host liver tissues, others were observed on the periphery of the liver lobes. Frequently, most individual diplostomules remained in direct contact within liver tissues, while others were observed within the visceral cavity (Fig. 3.5c). Those diplostomules which were no longer in tissues often appeared on the surface of organs in the visceral cavity (Fig. 3.5c), particularly on the liver and pancreas. By 4 weeks p.i., approximately 90% of metacercariae were found on the surfaces of visceral organs including the gut, liver, pancreas, swim bladder, and abdominal lining (Fig. 3.5d). At this time, about 66% of are encysted. The surface of

organs bearing *Ornithodiplostomum* sp. often appeared confluent with metacercarial cysts.

Several general features of the morphology of *Ornithodiplostomum* sp. in the minnow host were revealed with LM analyses. Diplostomules had two distinguishable body parts; the main larger cylindrical body (opithosoma) and a smaller organ protruding from the anterior end (prosoma) (Fig. 3.6). Numerous thin extensions from the tegument gave diplostomules a fuzzy appearance (Fig. 3.6). A large gap between the diplostomule body and liver tissues contained cellular debris, including solitary hepatocytes, red blood cells, and a filamentous matrix (Fig. 3.6).

DISCUSSION

Several lines of evidence indicate that the development of *Ornithodiplostomum* sp. metacercariae within their second intermediate host is a complex, multi-stage process, similar in many aspects to the development of metacercariae of *O. ptychocheilus* (Sandland & Goater, 2000; Goater *et al.*, 2005; Conn *et al.*, 2008). The migration phase for *Ornithodiplostomum* sp. appears to correspond to the presence of a spined, protrusible proboscis that disappears by approximately 2 weeks p.i. This penetrative organ is similar in appearance to the prosoma described by Conn *et al.* (2008) for *O. ptychocheilus*. Additionally for *O. ptychocheilus*, the subsequent growth phase that occurs between 1-4 weeks p.i. corresponds to an elaborate network of microvilli on the surface of the metacercarial tegument (Goater *et al.*, 2005). Upon encystment at 4 weeks p.i., this

elaboration of the tegument is absent, and the tegumental surface appears smooth (Goater *et al.*, 2005). Taken together, these results indicate that both species represent one extreme in the variation of metacercarial development strategies within the intermediate host.

Despite the broad similarities in the development strategies between *O. ptychocheilus* and *Ornithodiplostomum* sp., there exist striking differences in their migratory and site selection behaviours. Unlike *O. ptychocheilus*, which travel exclusively via the nervous system to site-specific regions (Hendrickson, 1979; Chapter 2), *Ornithodiplostomum* sp. diplostomules were frequently observed migrating within blood vessels. Thus, diplostomules of *Ornithodiplostomum* sp. possess similar migration strategies as a wide variety of strigeoids that use the circulatory system as a migration route (*D. flexicaudum*, Ferguson, 1943; *D. spathaceum*, Hoglund, 1991; *C. erraticus*, Johnson, 1991). Such interspecific variation in migration strategies within related taxa is probably quite rare among trematodes. However, an example is found in the family Schistosomatidae, where the circulatory system is the dominant pathway employed by migrating schistosomules, although migrating cercariae of *Trichobilharzia regenti* use the peripheral nerves to access the spinal cord (Blažová & Horák, 2005).

My results indicate that *Ornithodiplostomum* sp. diplostomules use two quite distinct migration paths to access sites of development in the visceral tissues. The most commonly employed pathway is a direct penetration through sub-dermal connective and muscular tissues to the visceral cavity. This is evidenced by the high number of diplostomules observed migrating through muscle tissues and penetrating the peritoneal

lining. Given the thinness of the peritoneum, and the expedient migration of *Ornithodiplostomum* sp. diplostomules, the frequency at which this event was observed suggests this to be the central means to access the visceral cavity. Studies on the migration of the sub-adults of the trematode *Fasciola hepatica* suggest these worms locate the liver by migrating along the abdominal wall (Sukhdeo & Sukhdeo, 1994). High numbers of diplostomules observed migrating along or associated with the peritoneum suggest that *Ornithodiplostomum* sp. may similarly use this lining to navigate within the host's visceral cavity. Additionally, diplostomules were frequently observed penetrating the surface of hepatic and pancreatic tissues to access these organs. Thus, the study provides evidence that some proportion of *Ornithodiplostomum* sp. diplostomules exclusively employ a tissue-penetrating strategy.

The second migratory route employed by *Ornithodiplostomum* sp. diplostomules is a combination of tissue penetration, and migration within the circulatory system. Evidence suggests that migration through muscular tissues is still accomplished by tissue penetration, as very few diplostomules were observed in blood vessels within these tissues. As early as 1 hour p.i. diplostomules were observed within specific blood vessels. The most prominently occupied blood vessels were those surrounding the swim bladder, and secondarily the dorsal aorta. Diplostomules were also observed penetrating the cardiac tissues, and inside the atria of the heart. Blood from the heart is pumped first to the gills, before bifurcating anteriorly to the carotid arteries of the head region, and posteriorly to the dorsal aorta (Farrell, 1993). The dorsal aorta, via the coeliaco-mesenteric artery, feeds vessels associated with the swim bladder, intestines, and stomach (Farrell, 1993). The hepatic portal system, which is surrounded in part by the pancreas,

delivers nutrient rich blood from the stomach and intestines directly to the liver (Farrell, 1993). Thus, those diplostomules associated with the heart are in a position to follow the flow of blood to the dorsal aorta. Diplostomules observed in the dorsal aorta could either continue on to the vessels on the surface of the swim bladder, or circuitously access the tissues of the pancreas and liver via the hepatic portal system. High proportions of diplostomules associated with the blood vessels around the swim bladder suggest the former scenario occurs more frequently than the latter. While relatively few diplostomules were observed in blood vessels within the liver and pancreas, high proportions of diplostomules in these tissues suggest that some diplostomules might access these tissues using the circuitous hepatic-portal route.

These two alternative migration strategies may explain the broad variation in development sites within the viscera. Thus, the final site of diplostomule localization could be dependent upon whether the blood system is employed as part of the migration route. Those diplostomules that use the blood stream may localize on the swim bladder, while those that opt for direct tissue penetration might localize in the lobes of the liver, and secondarily the pancreas. This could account for the particularly close association with the blood vessels exhibited by those diplostomules on the surface of the swim bladder. A similar explanation for the micro-site segregation of *O. ptychocheilus* diplostomules was suggested in Chapter 2. Consistently, *O. ptychocheilus* diplostomules migrate primarily to the stratum marginale of the optic lobes, and secondarily to the outer layers of the cerebellum (Chapter 2). It was hypothesized that this partitioning could be the result of differential migration routes along particular neural tracts.

My interpretation of two alternative migration routes requires two important caveats. First, due to the size constraints of these small hosts, they were not perfused with fixative. Consequently, blood vessels were not ideally preserved. Additionally, the removal of the host head and tail largely drained the cardiovascular system. These factors made assessment of the presence of diplostomules in blood vessels more challenging, as empty blood vessels were challenging to identify. However, the presence of numerous diplostomules within blood vessels conclusively demonstrates the potential use of the circulatory system for navigation. A second caveat is represented by the selected intervals post-infection, which may have missed crucial periods of diplostomule migration in the blood. Specifically, those periods directly preceding diplostomule penetration of the peritoneal lining may have indicated the presence of diplostomules in blood vessels, which would not support tissue penetration as the primary migration route. However, due to size constraints, it seems unlikely that migrating diplostomules would use smaller blood vessels, such as those segmental arteries in the muscle tissues. Rather, diplostomules were more frequently observed migrating between muscle fibers, and along myotemes. More generally, a well recognized limitation of the histological sampling-after-autopsy approach used in this study is the assumption that the frequency of diplostomule observation in specific tissues parallels the overall importance of that tissue in the migratory process. As Wilson (1994) points out, this assumption overlooks the potential of highly efficient, and thus transient, routes employed by migrating parasites.

My results are the first to show a clear shift in microhabitat for a trematode metacercariae. Thus, there was a period of growth and development associated with sites

such as the liver and pancreas, followed by an encystment phase in the visceral cavity. The timing of this shift was remarkably similar to its congener, *O. ptychocheilus* (Chapter 2). In that species, diplostomules develop in the outer edges of the tissues in the optic lobes and cerebellum, and shift to the adjacent meninges upon encystment (Chapter 2). Taken together, my results confirm that the development process, from site selection to infectivity, is even more complex for some species of strigeoids than previously believed.

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Figure 3.1: Schematic drawing of selected visceral organs and tissues of the fathead minnow. The lateral muscles are sketched below the main body to expose organs within the visceral cavity. Relevant lateral and medial organs have been sketched simultaneously to depict a multidimensional view of the organs and tissues. Visceral cavity organs, particularly the liver and pancreas, are exaggerated in size.

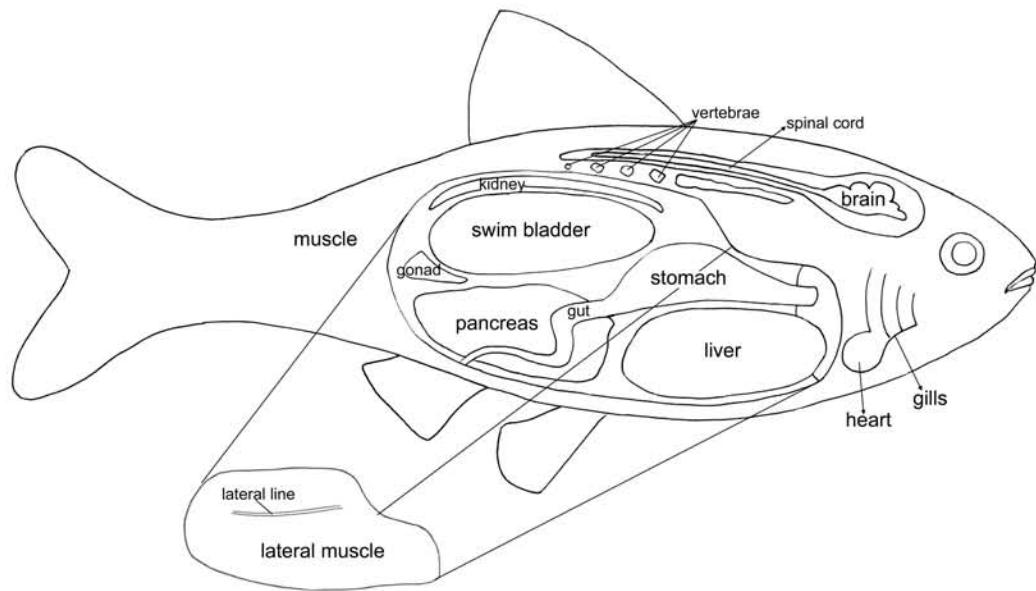


Figure 3.1

Figure 3.2: Light micrographs of migrating diplostomules of *Ornithodiplostomum* sp. in various tissues and organs of experimentally infected *Pimephales promelas* between 2 to 16 hours p.i.. Between 2-3 hours p.i. diplostomules (arrows) were observed migrating along myotemes (a) and between muscle fibers (b). Diplostomules were observed penetrating (c) and migrating inside, or along the peritoneal lining of the abdominal cavity (d). At 3 hours p.i., diplostomules were observed in various locations within the cardiovascular system including the dorsal aorta (e), blood vessels associated with the swim bladder (f), cardiac tissues (g), and blood-filled atria of the heart (h). Frequently, diplostomules were observed migrating along tissue interfaces (i), and penetrating and migrating within liver (j and k) and pancreatic tissues (l). Muscle (M); peritoneum (Pe); visceral cavity (VC); epidermis (D); kidney (K); dorsal aorta (DA); blood vessels (BV); red blood cells (RBCs); liver (L); pancreas (P); gut (G); cardiac tissues (C); swim bladder (SB). Scale bar=100 μ m.

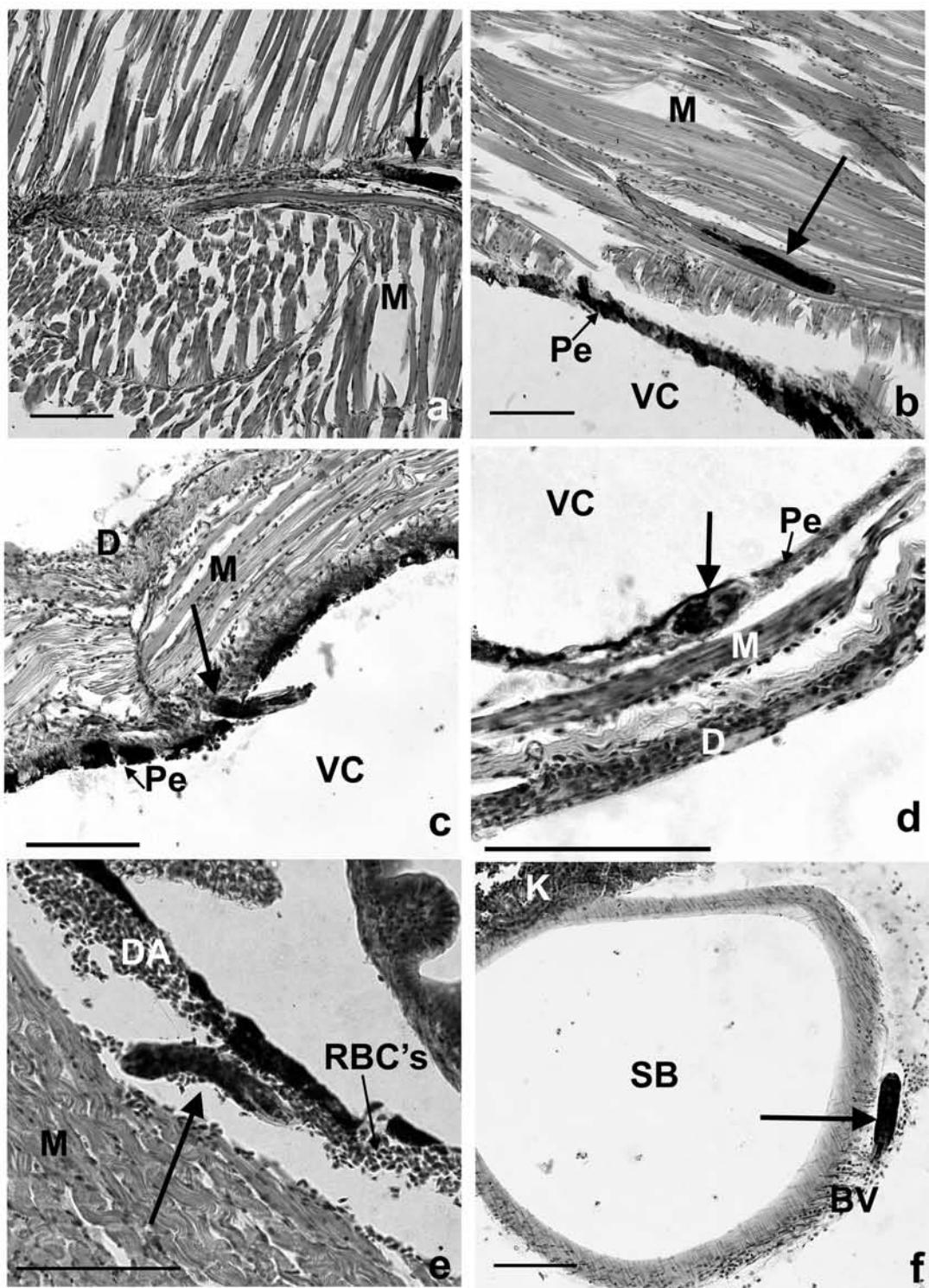


Figure 3.2

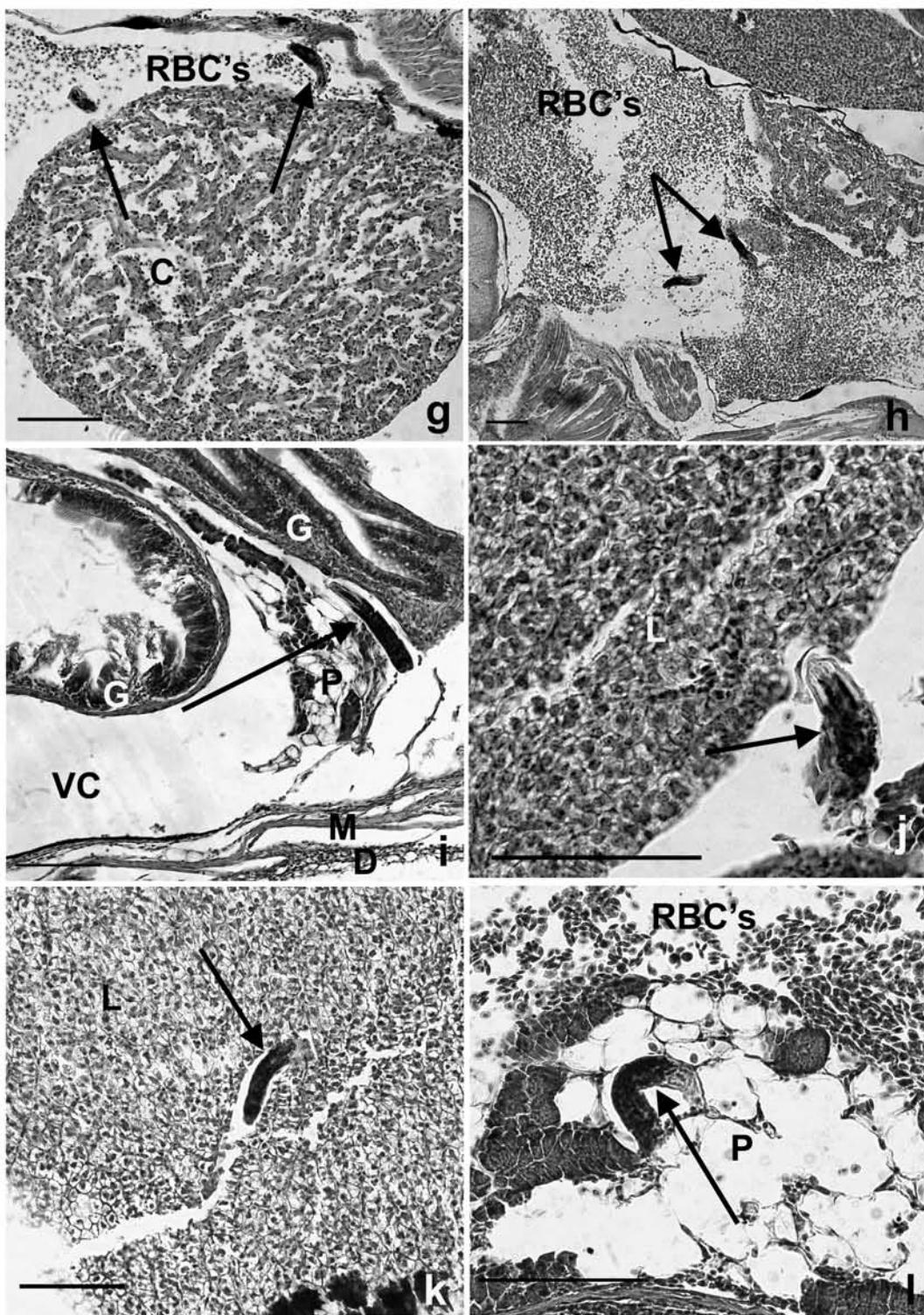


Figure 3.2 continued

Figure 3.3: Schematic sagittal diagram of the body of fathead minnows (*Pimephales promelas*) experimentally infected with *Ornithodiplostomum* sp. between 0.5-48 hours post-infection. Labels of specific tissues and organs are outlined in Figure 3.1. The approximate locations of all observed metacercariae are depicted by unique symbols according to the time elapsed since exposure. Arrows denote those diplostomules observed within blood vessels. The diagrams summarize the location of metacercariae pooled across three fish per interval, at specific times post exposure; 0.5-2 hours post-infection (p.i.) (a), 3-8 hours p.i. (b), 12-16 hours p.i., and 24-48 hours p.i.

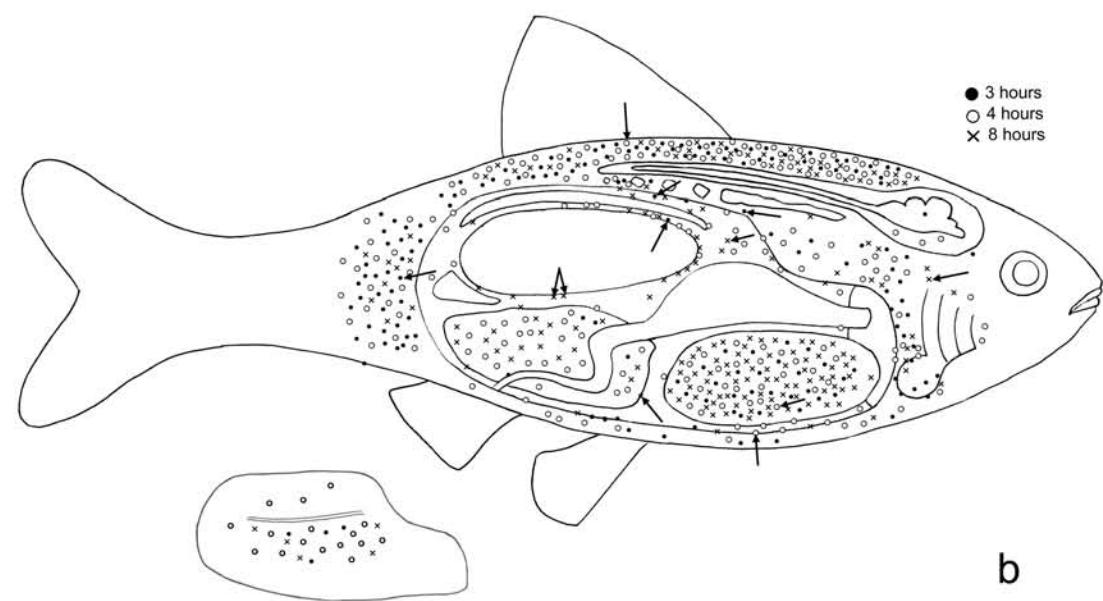
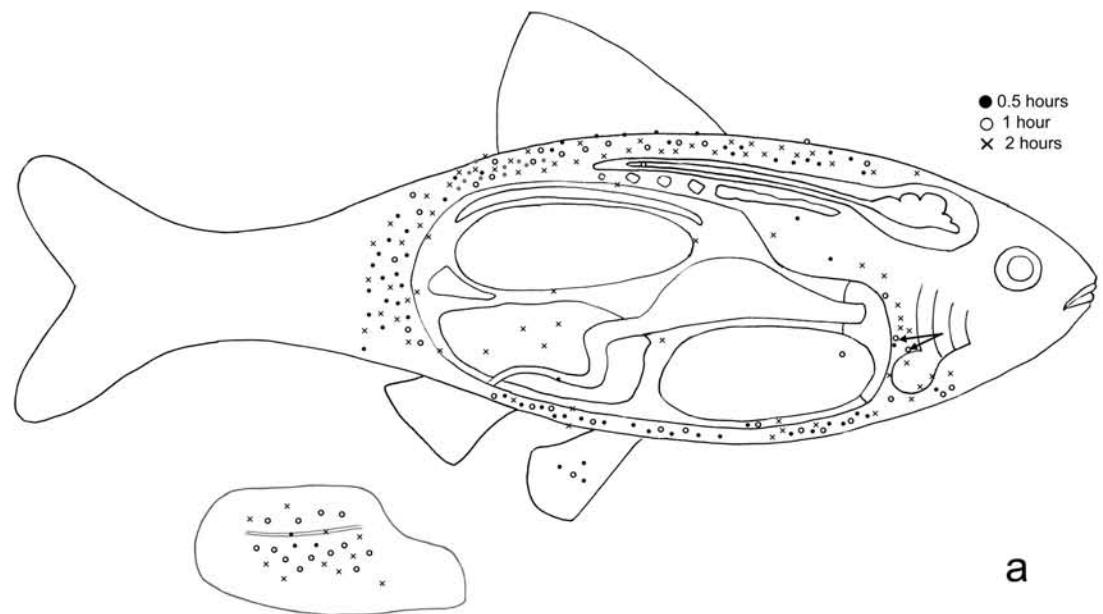
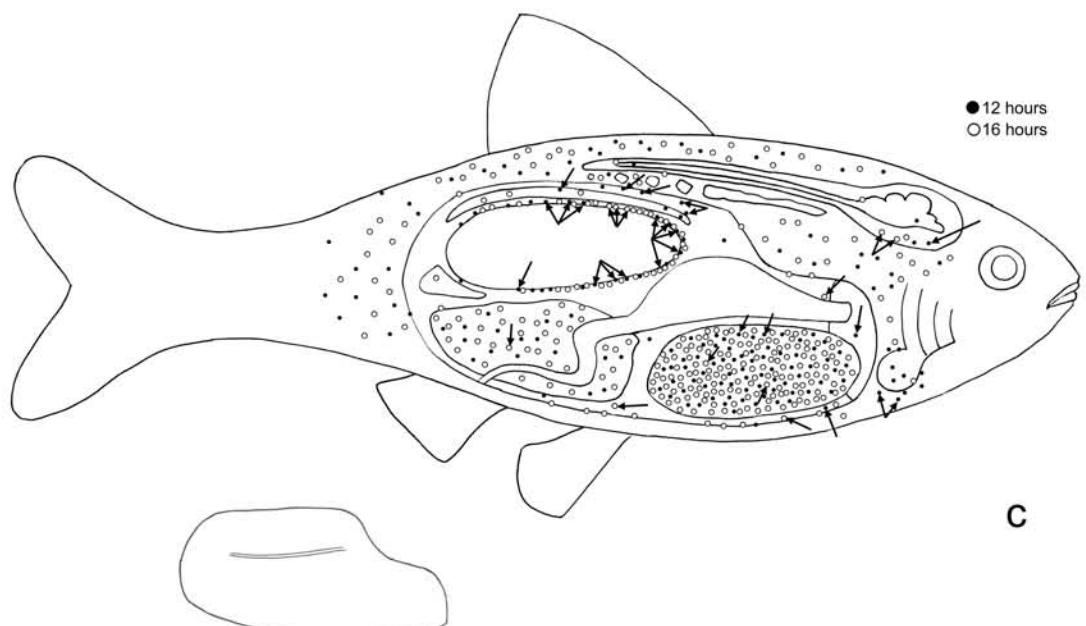
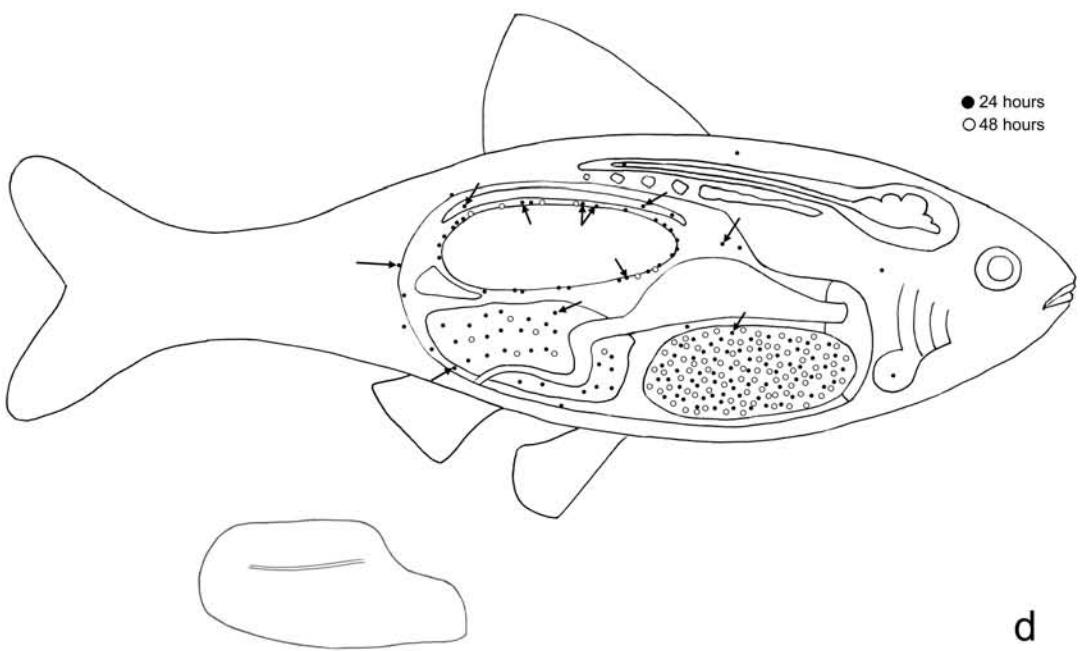


Figure 3.3



C



d

Figure 3.3 continued

Figure 3.4: Mean temporal changes in the proportion of metacercariae in the liver, pancreas, on the swim bladder, and body cavity of minnows experimentally infected with *Ornithodiplosotmum* sp. cercariae. Metacercariae from 3 fish were examined at each interval. Bars represent standard error.

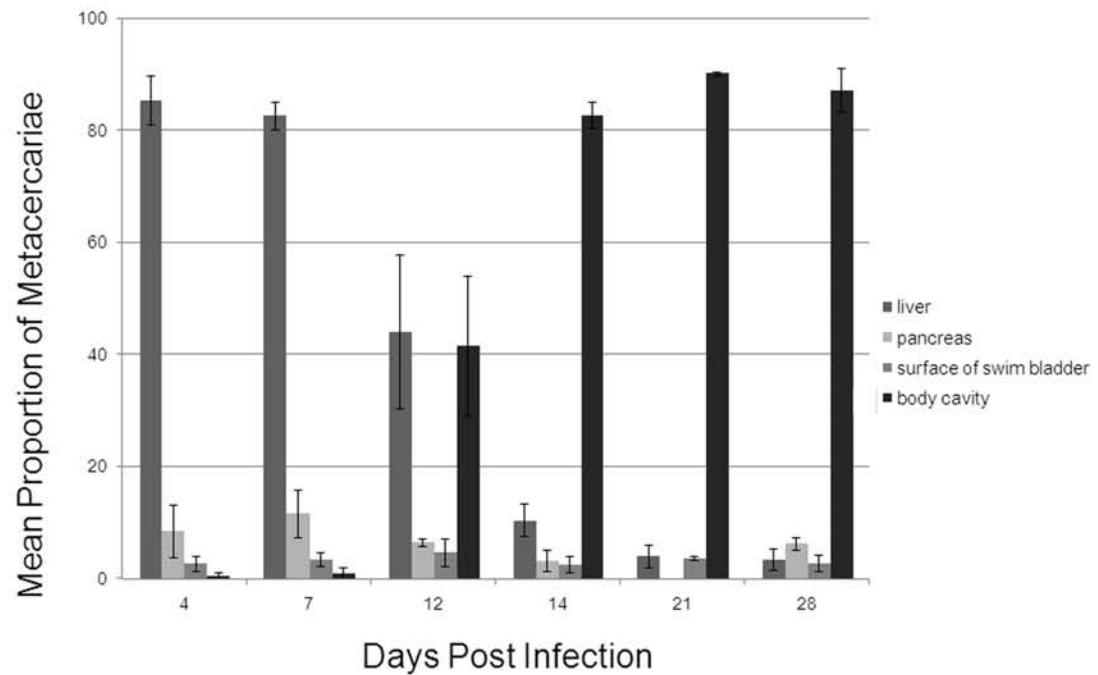


Figure 3.4

Figure 3.5: Light micrographs of *Ornithodiplostomum* sp. diplostomules in the body of *Pimephales promelas* experimentally exposed to cercariae. At 4 days post-infection (p.i.) migration tracks (arrowhead) caused by diplostomules (arrows) are still visible (a). Diplostomules at 1 week p.i. remained embedded within liver tissues (b). By 12 days p.i., several diplostomules were observed both within, and on the periphery of the liver (c). At 4 weeks p.i., metacercariae were most frequently observed on the surface, and between visceral organs in the body cavity (d). Liver (L), muscle (M), epidermis (D), kidney (K), ovaries (O), loops of the gut(G), pancreas (P), spleen (S), swim bladder (SB). Scale bar=200 μ m.

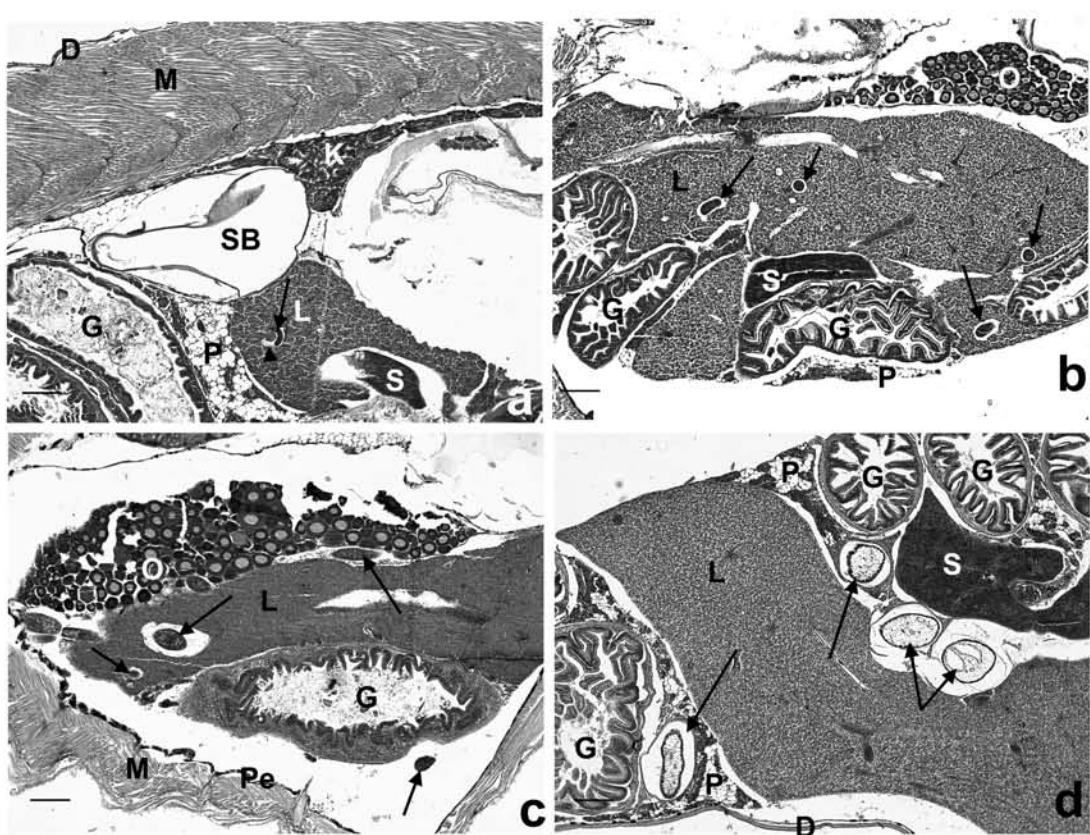


Figure 3.5

Figure 3.6: Light micrograph of a diplostomule at 12 days post-infection in the liver tissues of a fathead minnow (*Pimephales promelas*) experimentally exposed to *Ornithodiplostomum* sp. cercariae. Liver (L), red blood cells (RBCs), prosoma (P), opithosoma (O).

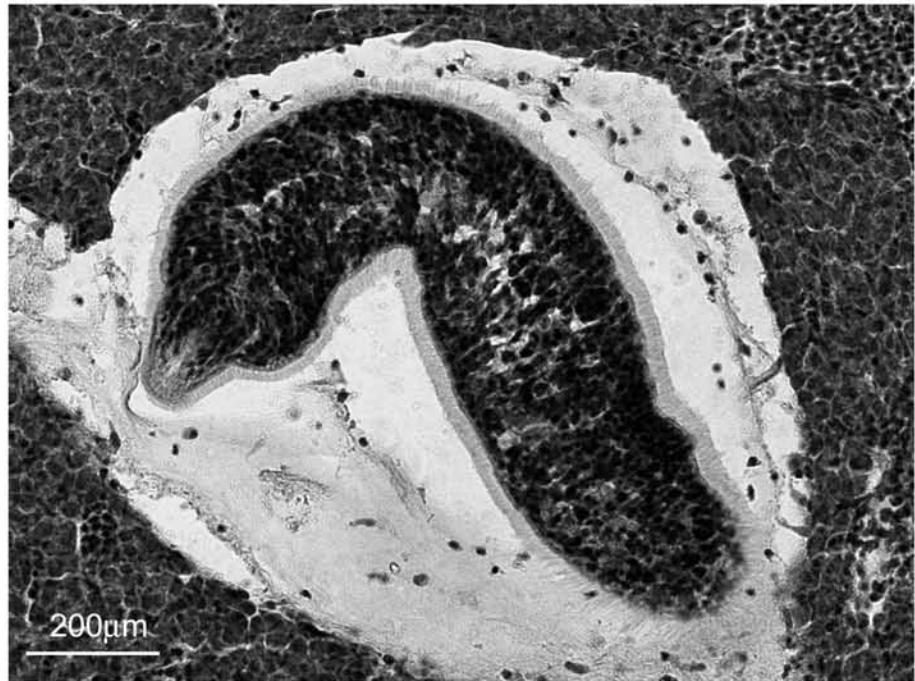


Figure 3.6

Chapter 4: Temporal changes in density and maturation of rodlet cells in brain tissue of fathead minnows (*Pimephales promelas*) exposed to trematode cercariae

INTRODUCTION

Rodlet cells are large, distinctive cells found exclusively in fish (see reviews by Manera & Dezfuli, 2004; Reite & Evensen, 2006). They are named after the elongated rods located within the cytoplasm of the cell. These cells are most often associated with epithelial surfaces of virtually all fish tissues (review by Morrison & Odense, 1978). Rodlet cells undergo a well-characterized developmental process whereby the rodlets are synthesized, while rodlet cells migrate to epithelial surfaces. Mature rodlets are then secreted at epithelial surfaces into the adjacent lumen and extracellular spaces (Leino, 1974; Desser & Lester, 1975; Kramer & Potter, 2002). Despite numerous studies focused on rodlet cell ultrastructure, their precise function is unknown. Most recently, rodlet cells have been hypothesized to be a component of the fish immune system (reviews by Manera & Dezfuli, 2004; Reite & Evenson, 2006). This contention is based on their proliferation in response to physiological stressors (Iger & Abraham, 1997; Giari, 2008), and on multiple observational studies that report a correlation between rodlet cell density and the presence of parasites (Leino, 1996; Dezfuli, 1998; 2000; 2007; 2008; Kopenen & Myers, 2000).

Although results of observational studies indicate a functional linkage between parasite infection and rodlet cell proliferation (Leino, 1996; Dezfuli 1998; 2007; Reite & Evenson, 2006), direct evidence from experimental studies is absent, especially involving metazoan helminth-fish interactions. Additionally, the consensus view that rodlet cells

are associated with host defense against parasites (Reite & Evenson, 2006; Mazon *et al.*, 2007), with parasite-induced tissue damage a likely cue for recruitment and proliferation (Leino, 1996; Reite, 2005), is based on observational studies. In part, these limitations are due to the lack of appropriate model systems that are amenable to experimental manipulation. One specific shortcoming is our poor understanding of the temporal pattern of rodlet cell proliferation following initial exposure to infective stages of parasites.

Experimental assessment of the time course of rodlet cell proliferation should shed light on the role rodlet cells play in host defense against parasites, and their response to parasite-induced tissue damage. Metacercariae of *Ornithodiplostomum ptychocheilus* encyst in the brain of fathead minnows, *Pimephales promelas*. Experimental studies have indicated a complex pattern of metacercarial development following exposure of fish to cercariae (Sandland & Goater, 2000; Goater *et al.*, 2005; Conn *et al.*, 2008). Migrating diplostomules localize in the optic lobes and cerebellum within 24 hours, followed by a 3-4 week period of rapid growth and development. At this stage, the entire larval tegument undergoes a transformation from a smooth surface to being enveloped in an elaborate complex of microvilli (Goater *et al.*, 2005). Damage to adjacent host tissues is greatest when tegumental elaborations are at a maximum (Goater *et al.*, 2005). At approximately 4 weeks, metacercariae enter an encystment phase in which the tegument is completely reorganized (Goater *et al.*, 2005) and the interior tissues consolidate into the familiar resting stage. Host tissues adjacent to metacercarial cysts appear to be reconstituted (Goater *et al.*, 2005). In the course of these studies, rodlet cells were frequently observed in close proximity to metacercariae at various stages of development (Goater *et al.*, 2005), similar to the descriptions of rodlet cells in the brains

of European minnows infected with metacercariae of *Diplostomum phoxini* (Dezfuli *et al.*, 2007).

The purpose of this study is to evaluate temporal changes in the proliferation and maturation of rodlet cells in the optic lobes of minnows infected with metacercariae of *O. ptychocheilus*. The ability to experimentally manipulate this host-parasite system (Goater *et al.*, 2005) provides an opportunity to directly test several predictions regarding rodlet cells and their linkage to helminth infective stages. Firstly, if rodlet cells are produced in response to helminth infection, hosts exposed to cercariae should contain significantly more rodlet cells in infected host tissue compared to unexposed controls. Secondly, if rodlet cells constitute a defensive cell that proliferates in response to parasite-induced tissue damage, density of rodlet cells should be high when destruction of host tissues is greatest.

MATERIALS AND METHODS

Infection procedure

The methods used to infect fathead minnows with *O. ptychocheilus* cercariae followed Sandland & Goater (2000) and as described in Chapter 2. Briefly, day old chickens were fed the brains of field-collected infected minnows on 5 June 2007. Eggs were collected from the feces of chicks through a series of washes and filters as described in Sandland & Goater (2000). The F1 generation of field collected snails, *Physa gyrina*, reared in the laboratory in dechlorinated water and fed boiled lettuce ad libitum, was exposed to the hatched miricidia. Cercariae were collected approximately 4 weeks later

by placing infected snails in glass vials filled with dechlorinated water under a light source.

Juvenile fathead minnows (30-days-old, approximately 1.5-2.0 cm standard length) obtained from a supply company were acclimatized in aerated water and fed tetramin fish flakes twice per day for approximately 7 days prior to experimental infections. Thirty fish were randomly selected from a stock tank and placed into individual petri dishes containing de-chlorinated water. Twenty seven fish were exposed on 15 August 2007, to 100 cercariae for 3-4 hours; the remaining 3 fish were sham-exposed in water. Cercariae originated from 8 experimentally-infected snails that had been individually isolated for a maximum of 3 hours. The cercariae from the 8 snails were pooled and the total number present in 100mL was estimated. The volume of water containing 100 cercariae was estimated via dilution (Sandland & Goater, 2000) and then added to individual petri dishes that contained fish. Fish were housed in separate 30 x 30 x 60cm (H x W x L) aquaria of aerated water and maintained on tetramin fish flakes until sacrifice.

Evaluation of rodlet cell density

The time course to evaluate temporal changes in rodlet cell density was selected to encompass the period between the initial developing diplostomule stages that occur within the brain tissue (diplostomulum) and the encysted metacercariae located within the adjacent meninges (Goater *et al.*, 2005; Chapter 2). Thus, minnows were selected for dissection at 1, 2, 4, 6, and 9 weeks post infection (p.i). Three minnows were assigned at random to each interval. There were also three sham-exposed controls. Minnows were

sacrificed by immersion in a lethal dose of clove oil. The brains were removed with fine-forceps using a dissecting microscope. To permit fixative to fully penetrate brain tissue, the olfactory bulbs and medulla oblongata were removed with a scalpel. Tissue samples were fixed in fresh Karnovsky's solution for a minimum of 24 hours and rinsed overnight in 0.1 M sodium cacodylate buffer (pH 7.3) followed by 1 hour post fixation in 1% osmium tetroxide in the same buffer (Goater et al., 2005). Samples were dehydrated in a graded ethanol series prior to embedding in a graded ethanol: Spurr's resin series. Samples were then polymerized in an oven at 60 degrees centigrade for 24 hours.

Semi-thin coronal sections (thickness=1 μ m) were cut approximately every 50 μ m on a Reichart OM-U2 ultramicrotome. The distance between sections was sufficient to ensure that individual cells were not sectioned twice. Sections were mounted on glass slides coated with gelatin, dehydrated on a slide warmer in an atmosphere of HemoDe, and stained with 1% toluidine blue. Rodlet cells on all sections between the start of the periventricular zone (PVZ) of the optic tectum and the cerebellum were counted. These two landmarks were selected to ensure that similar regions of the brain were consistently evaluated in each fish. Depending on the size of the optic lobes, the numbers of sections that were evaluated from an individual fish ranged from 6 to 15. Counts were made at 400x magnification following methods adapted from Dezfuli *et al.* (2008). Rodlet cells that occurred within brain tissues were distinguished from those in the meninges to provide an estimate the proportion of rodlet cells in the migration phase.

If rodlet cell density and maturation rate are associated with the presence of metacercariae, then the density of rodlet cells may be associated with the total number or

size of metacercariae in a host. To test this relationship, metacercarial counts (= parasite intensity, Bush *et al.*, 1997) were made concurrently with rodlet cell counts. The total area occupied by metacercariae in each fish was assessed by estimating the area occupied by metacercariae in each section. Individual images of all sections were acquired using a digital camera at 50x magnification. The area of brain tissue and area occupied by metacercariae (mm^2) was assessed relative to the summed total area of optic lobe and cerebellum for each section using Image J software.

Evaluation of rodlet cell maturation

A further aim was to assess temporal changes in rodlet cell maturation. For this component, material was prepared for evaluation with a transmission electron microscope (TEM). Three fish dissected at 4 days, 3 and 6 weeks p.i., and were fixed and prepared as described above. For each individual sample, one ultrathin section ($90\mu\text{m}$) was cut along the coronal plane of the optic lobes on an OM-U2 ultramicrotome and stained with 4% uranyl acetate for 20 minutes, and Reynolds lead citrate for 5 minutes (Reynolds, 1963). Using an Hitachi TEM, whole sections were methodically scanned at a minimum of 5000x. The developmental status of each cell encountered in each section was designated as immature, mature, or discharging, according to detailed descriptions provided by Leino (1974), Bielek (2002), and Kramer & Potter (2002). Specifically, immature rodlet cells were those cells possessing a thin fibrous capsule and rodlets without dense cores, with the cytoplasm dominated by rough ER, Golgi complexes, and a relatively large electron-lucent nucleus (Fig. 4.1a). Mature cells were those with a well developed fibrous capsule, multiple rodlets possessing dense cores, and a small basal nucleus containing

electron dense deposits. The apex of the cell possessed numerous mitochondria, vacuoles, and ribosomes (Fig. 4.1b and c). Discharging rodlet cells possessed similar characteristics of mature rodlet cells, in addition to a distinct cytoplasmic bleb protruding from the cell apex, with cell contents visibly being expelled (Fig. 4.1d). Photographs were taken at an accelerating voltage of 100 kV.

Analyses

Rodlet cell counts were variable between individual hosts, and between sections within a host. For hypothesis testing involving density data, the Kruskal-Wallis non-parametric test was used for comparisons between multiple means, while the Mann-Whitney U Test was used for comparisons between selected pairs of means. Proportional data on rodlet cell location (tissue vs. meninges), were arcsine (square root) transformed to normality. Datasets of the proportion of rodlet cells in the tissues, and rodlet cell maturation were analyzed with a one-way ANOVA, with multiple comparisons made using Tukey-Kramer HSD test. Correlations between metacercarial intensity and both rodlet cell density and the proportion of rodlet cells in the tissues used Pearson's parametric tests; density data were log-transformed, and proportional data were arcsin (square root) transformed. Correlations between metacercarial size and rodlet cell density, metacercarial size and the proportions of rodlet cells in the tissues, and rodlet cell density and the proportion of rodlet cells in the tissues were made using Spearman's non-parametric tests.

RESULTS

At 1 week p.i, diplostomules occupied the outermost layer of the optic lobes (Fig. 4.2a). The space immediately adjacent to developing diplostomules suggested substantial disruption to host tissues. Several rodlet cells were visible in the adjacent meninges and in the brain tissues (Fig. 4.2a). At 6 weeks p.i (Fig. 4.2b), metacercariae were fully encysted, and occupied the visibly inflamed meninges. Host tissues were flush against the cyst wall, and appeared normal. Rodlet cell densities were visibly greater, with a substantial portion in the brain tissues. Most rodlet cells appeared to be mature (Fig. 4.2b).

Analysis of temporal changes in median rodlet cell density indicated there was a complex temporal pattern of proliferation over the 9 week period (Fig. 3; Chi Square = 14.89 df = 5; p = 0.011). Densities were low in hosts exposed 1 and 2 weeks p.i., and these densities were not significantly different from controls. There was a marked increase in rodlet cell densities at 4 weeks p.i, reaching maximum levels at 6 weeks p.i. The increases between 2 and 4 weeks p.i (Chi Square = 3.86; df = 1; p = 0.050) and between 4 and 6 weeks p.i were significant (Chi Square = 3.86; df = 1; p = 0.050). The decline in rodlet cell density between 6 and 9 weeks p.i. was also significant (Chi Square = 3.86; df = 1 p=0.050).

There was a significant effect of time on the total proportion of rodlet cells found in the tissues of the optic lobes ($F_{1,5} = 3.52$; df = 5; p = 0.034). Unexposed hosts and hosts at 1 week p.i had lowest proportions of rodlet cells in their tissues. At 2 weeks p.i., 14% of rodlet cells were in the tissues. At 4 weeks p.i, the proportion of rodlet cells in the

tissues doubled to 33%. The greatest populations of tissue-dwelling rodlet cells were recorded at 6 weeks p.i., although none of these increases were significant (Fig. 4.4).

The proportion of mature rodlet cells varied significantly as metacercaria developed ($F_{2,6} = 9.96$; $p= 0.012$), with a 45% increase in proportion of mature rodlet cells between 4 days and 6 weeks p.i. (Fig. 4.5). At 4 days and 3 weeks p.i, the proportion of mature rodlet cells ranged between 27-56%, which was not significant. Relatively equivalent proportions of rodlet cells were in the discharging phase at 4 day, 3 week, and 6 weeks p.i. ($F_{2,6}=2.37$; $p=0.17$).

There were significant positive correlations between the space occupied by metacercariae in the optic lobes and both rodlet cell density (Spearman's rho = 0.84; $p<0.0001$; $n=18$) and the proportion of rodlet cells in the tissues (Spearman's rho = 0.67; $p=0.002$; $n=18$). Metacercariae intensity was not correlated with either rodlet cell density ($F_{1,16}=0.5279$; $p=0.48$) or the proportion of rodlet cells in the tissues ($F_{1,16}=1.1892$; $p=0.29$). Rodlet cell density was positively correlated with the proportion of rodlet cells in the tissues (Spearman's rho=0.70; $p=0.001$; $n=18$).

DISCUSSION

The 30-fold increase in rodlet cell density between uninfected and infected minnows at 6 weeks p.i. provides direct evidence that rodlet cell proliferation results from exposure to cercariae. The positive correlation between the total area occupied by metacercariae in the brain and rodlet cell density provides an additional line of evidence

for an association between the presence of metacercariae and the presence of rodlet cells. Thus, a single exposure to trematode cercariae leads to a massive increase in rodlet cell numbers in tissue adjacent to metacercariae. Although these results confirm those from observational studies on helminth-fish interactions (Reite, 2005; Dezfuli *et al.*, 2007), and are in line with one experimental study involving a protozoan (Kinetoplastida: tryplanoplasma) in carp (Mazon *et al.*, 2007), they are the first to experimentally demonstrate a close linkage between the occurrence and density of rodlet cells with a helminth infection.

The results are also the first to characterize a distinct temporal pattern of rodlet cell proliferation following exposure to trematode cercariae. Factors such as host breeding condition (Jordanova *et al.*, 2007) and inherent seasonality (Kopenen & Myers, 2000; Leino, 1996) have been shown to contribute to temporal variation in rodlet cell counts. Additionally, observations in rodlet cell numbers in response to a variety of stressors (exposure to manure, heavy metals, acidified water, distilled water, polluted water and elevated temperatures) were found to increase as post-exposure time (up to 30 days) progressed (Iger & Abraham, 1997). However, the 30-fold difference between maximum and minimum rodlet cell densities in fathead minnows following exposure to cercariae is striking. The present study observed rodlet cell densities two orders of magnitude greater than the single previous experimental study involving rodlet cells and parasites (Mazon *et al.*, 2007). These results demonstrate the need for additional experimental studies with multiple observations over time, as rodlet cell expression varies temporally in response to both intrinsic and environmental factors.

A temporal pattern in the proportion of rodlet cells in the brain tissues of hosts infected with *O. ptychocheilus* metacercariae was also confirmed. The increased proportion of rodlet cells in the tissues between 4 and 9 weeks p.i. indicate rodlet cell migrations are underway at this time. Further, the positive correlation between rodlet cell density and the proportion of rodlet cells in the tissues suggests rodlet cells are being recruited from additional regions in the brain beyond those immediately infected. It is believed that rodlet cells are borne in the basal layers of epithelial tissues (Leino, 1974; Desser & Lester, 1975), whereupon they migrate to the lumen or epithelial surfaces before expelling their contents (Iger & Abraham, 1990). A probable source of the migrating cells observed in this study is the epithelia lining the optic ventricle.

The proportion of mature rodlet cells increased from approximately 20% at 4 days p.i., to 70% at 6 weeks p.i.. Relatively equal proportions of rodlet cells in the discharging phase between 4 days and 6 weeks is likely due to the expedient nature of the discharging process (Schmatenborg, 2007). Although this aspect of the study examined only a small portion of the infected brain of individual fish, and sample sizes were low, this data suggests that most rodlet cells in hosts bearing 4-9 week-old infections are mature. Previously, only one study has observed the developmental stage of rodlet cells in concert with parasite infection or physiological insult (Leino, 1996). Although no data exists on rates of rodlet cell development, or potential cues relating to development, Iger and Abraham (1997) demonstrate rodlet cell recruitment as early as 1 hour at the site of mechanically-induced dermal wounding. Descriptions and images of the cells in their study indicate they are mature. Therefore, it seems unlikely that immature rodlet cells in hosts bearing 4 day old parasites are constrained by developmental lag.

The temporal pattern of rodlet cell proliferation provides limited observational evidence regarding rodlet cell function. As outlined in the introduction, rodlet cells are believed to constitute a host defense against parasites, although my results do not support this notion. The encystment process involves the deposition of host fibroblasts, proliferated from the expanding meninges, around the parasite (So & Wittrock, 1982). If rodlet cell secretions are capable of damaging parasites or dampening parasitic infections, we would expect maximum densities of mature rodlet cells before parasites are protected in a double-layered cyst wall. The most striking evidence in opposition to the theory that rodlet cell functions in host defense against parasites is the absence of dead, or damaged *O. ptychocheilus* metacercariae. Based on these observations, the present study does not find evidence to support the hypothesis that rodlet cells play a defensive role against metacercariae.

Several authors have suggested that parasite-induced tissue damage is a likely cue for the proliferation of rodlet cells (Leino, 1996; Reite, 2005). Based on these suggestions, it could be postulated that maximum rodlet cell density in the optic lobes of minnows would parallel the known developmental sequence of *O. ptychocheilus* metacercariae (Goater *et al.*, 2005; Conn *et al.*, 2008). Thus, peak densities should occur approximately between 2-4 weeks p.i., when pre-encysting diplostomules are feeding and causing maximum tissue disruption. Contrary to this prediction, proliferation began at 4 weeks p.i., when metacercariae have begun to encyst, with densities peaking at 6 weeks p.i., well after the maximum period of damage to the brain.

One explanation for this result is that rodlet cell proliferation and maturation may not be stimulated by tissue damage, but rather the process of encystment. Beginning at 2 weeks p.i., diplostomules shift sites from brain tissues to the adjacent meninges (Chapter 2). At 4 weeks p.i., metacercariae are encysted in the meninges, with meningeal inflammation beginning at approximately the same time. One noticeable feature of the inflamed meninges is the abundance of fibroblasts, an observation consistent with other studies (Chang & Plumb, 1996). Fibroblasts are known to play a critical role in the formation of the outer cyst wall of *O. ptychocheilus* (So & Wittrock, 1982) and other metacercariae (Mitchell, 1974; Wittrock et al., 1991). Fibroblasts are considered the most active secretory cells of the teleost meninges, synthesizing various molecules and proteins of the extracellular matrix (Hoffman & Schwartz, 1996). Thus, it is possible that fibroblasts, or their secretions, are capable of stimulating the proliferation and recruitment of rodlet cells.

At 4 weeks p.i., when rodlet cell proliferation begins, several features of the host response suggest tissue repair processes are underway. Observed meningeal inflammation, increased vascularization (personal observation) and cellular proliferation are characteristic features of tissue healing in all vertebrates (Galliot *et al.*, 2008). At 4 weeks p.i., diplostomules are no longer an obstacle in the brain, as the shift of diplostomules from brain tissue to the adjacent meninges is complete (Chapter 2). The invasive tegumental structures of *O. ptychocheilus* metacercariae are absent, and host tissues adjacent to cysts appear identical to that of controls (Goater *et al.*, 2005). The transient nature of the damage to neural tissues is not surprising, given teleost neuro-regenerative capacities (Zupanc, 2001). As mentioned previously, fibroblast proliferation

is a hallmark host response. These cells secrete substances called ependymins which have been implicated in the neural repair process (Schmidt & Shashoua, 1988; Thormodsson *et al.*, 1992 Hoffmann & Schwarz, 1996; Shashoua *et al.*, 2001; Adams *et al.*, 2003).

More research, however, is required to assess the activity of these cells and their secretions in the context of *O. ptychocheilus* development.

Given that rodlet cell proliferation seems to coincide with host tissue repair, rodlet cell secretions may play some role in the healing process. This hypothesis is supported by a preliminary study that reported greater rodlet cell numbers accompanying reconstruction of liver tissues, rather than the pathology caused by exposure to a carcinogen (Courtney & Couch, 1988). The orchestrated cascade of biochemical and cellular events related to immunity and tissue repair responses overlap. Experimental studies that distinguish the role rodlet cells play in these two processes are necessary to evaluate rodlet cell function.

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Figure 4.1: Transmission electron micrographs of rodlet cells in various stages of development in the optic lobes of fathead minnows experimentally infected with cercariae of *Ornithodiplostomum ptychocheilus*. Immature rodlet cells (a) possess a thin fibrillar cell wall (F). Rough ER with dilated cisternae (ER), golgi complexes (G), and a large electron-luscent nucleus (N) dominate the cytoplasm. Several developing rodlets are visible (R). The cell wall of mature rodlet cells (b) is visibly thickened. Appreciable heterochromatin is deposited in the basal nucleus. Rodlets are more numerous, and tend to display a more electron-dense core. Mitochondria (M) and vesicles accumulate at the apex of the cell; vacuoles are found throughout the cytoplasm. Prior to discharge (c), the fibrous capsule thins at the cell apex, though a plasma membrane is still visible (arrowhead). Junctional complexes (arrows) are frequently observed at the apex. Contents of the discharging rodlet cells (d) are expelled at the cell apex into the lumen. Scale bar = 1.7 μ m.

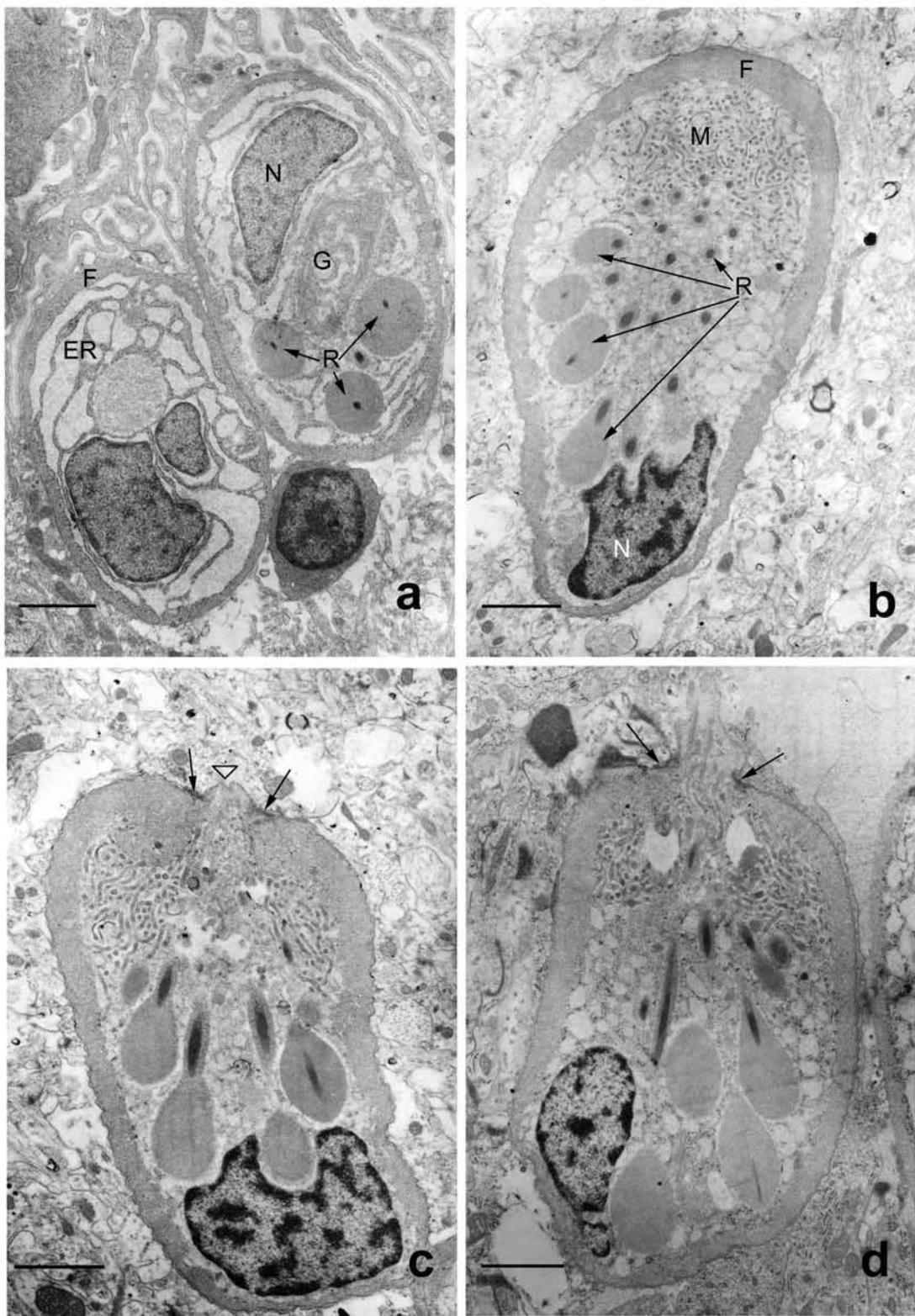


Figure 4.1

Figure 4.2: Light micrographs of the optic lobes of fathead minnows experimentally infected with cercariae of *Ornithodiplostomum ptychocheilus*. At 1-week post-infection (a), the diplostomulum (D) is found in the outer edges of the tissues of the optic lobes (T). A large gap between the host and parasite indicates substantial disruption of host tissues. Several rodlet cells (arrows) are found in the endomeninx (EM) and brain tissue. At 6-weeks p.i. (b) the metacercaria (M) is fully encysted (C) in the host meninges. At this time, rodlet cells are significantly greater in number, with a large proportion found in the brain tissues. Scale bar =200 μ m.

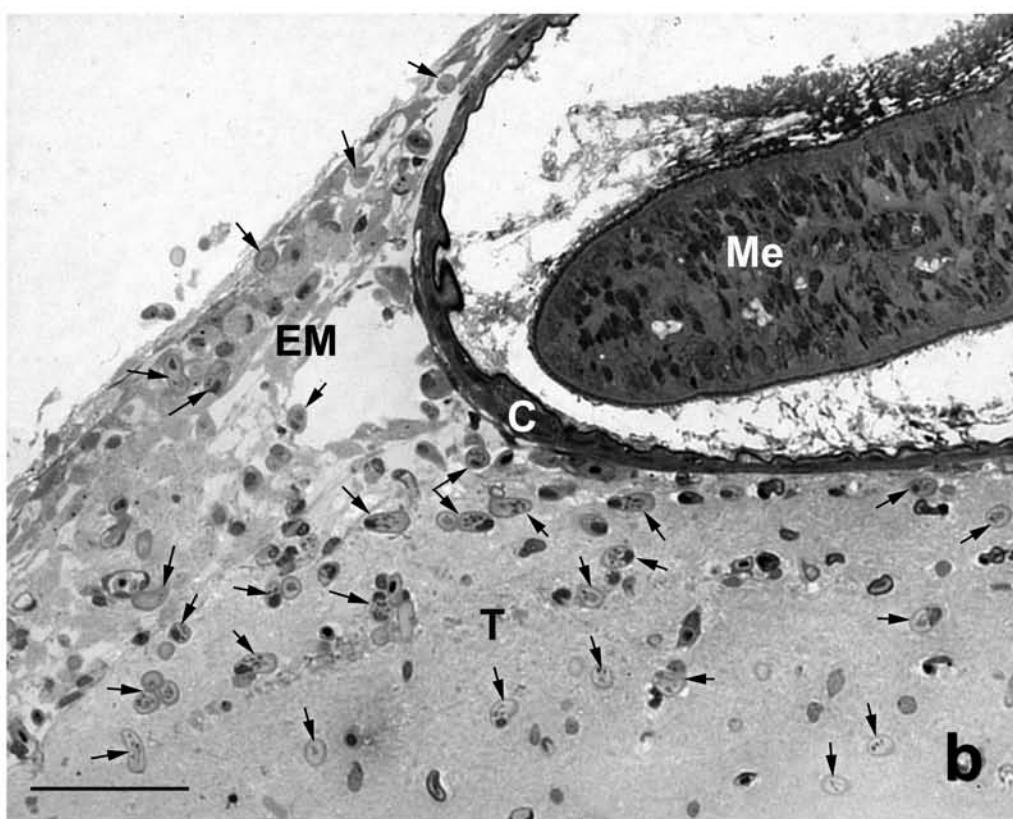
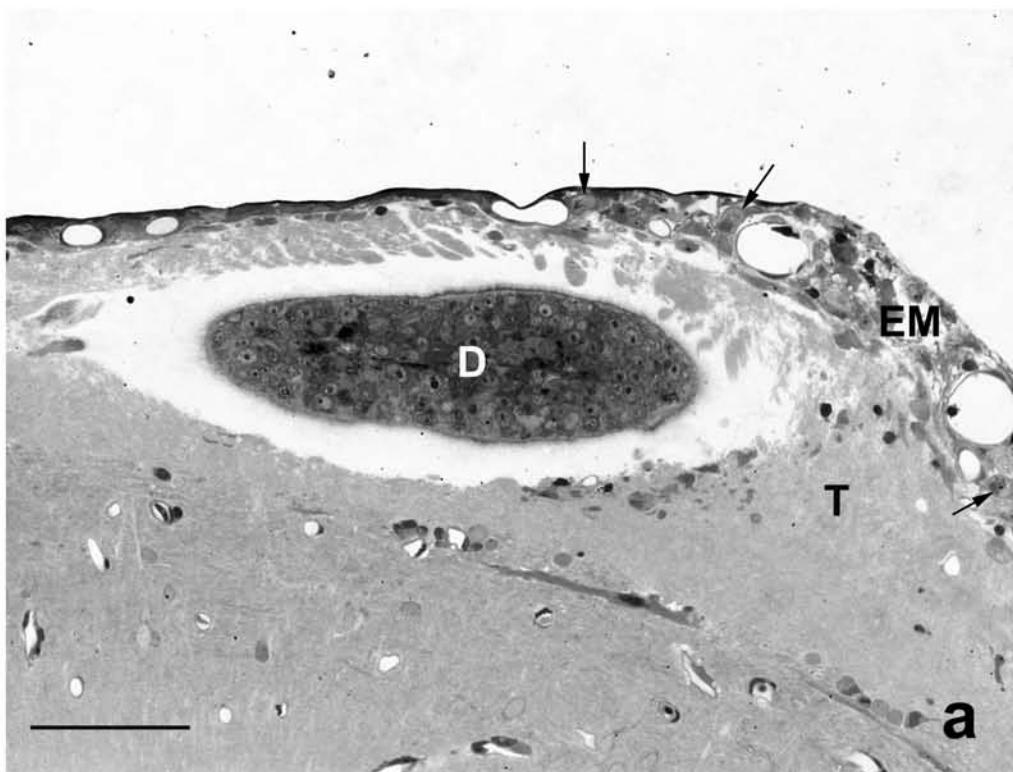


Figure 4.2

Figure 4.3: Box and whisker plots of temporal changes in median rodlet cell density in the optic lobes of minnows experimentally infected with cercariae of *Ornithodiplostomum ptychocheilus*. Unexposed controls are designated „c”. Boxes encompass the 25th to 75th interquartile ranges in rodlet cell density among 3 hosts dissected at each time interval. The extensions to the boxes represent overall range; line extensions represent the range of average densities.

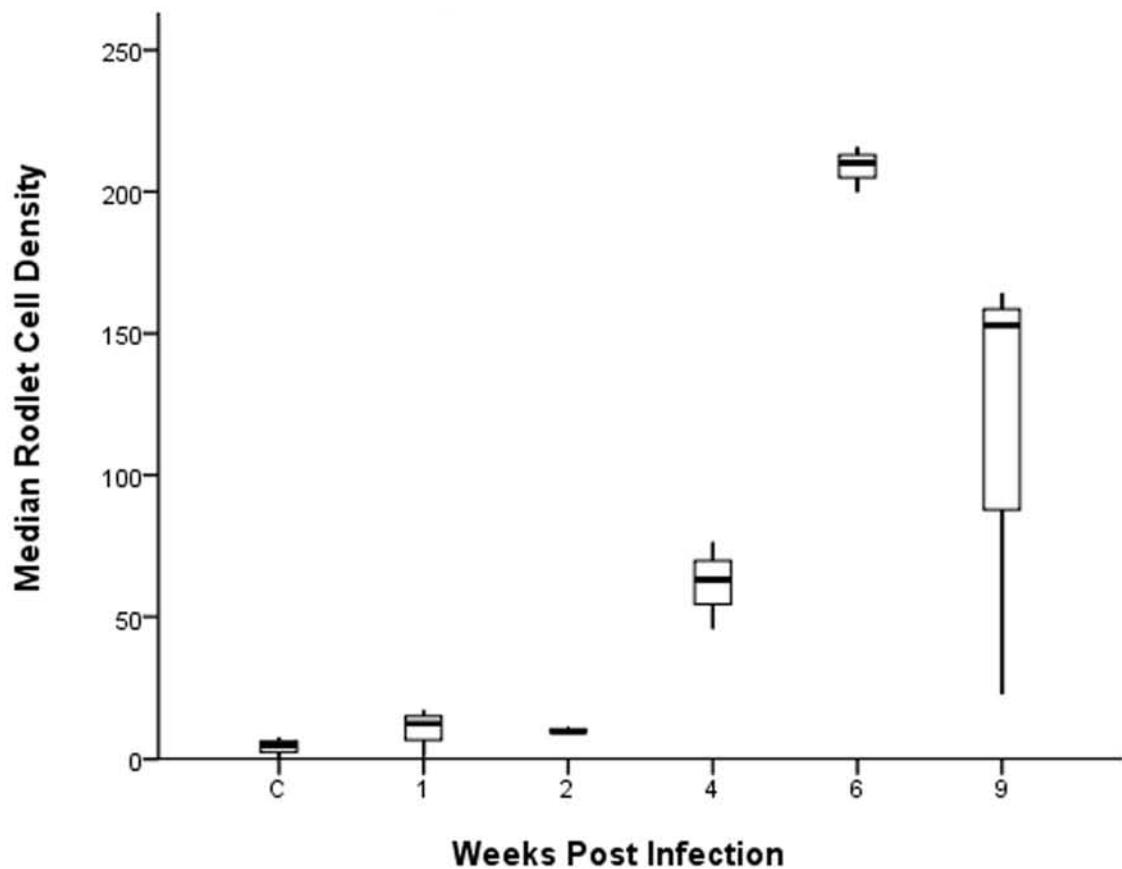


Figure 4.3

Figure 4.4: Box and whisker plots of temporal changes in the proportion of rodlet cells in the tissues of the optic lobes of minnows experimentally infected with cercariae of *Ornithodiplostomum ptychocheilus*. Unexposed controls are designated „c”. Boxes encompass the 25th to 75th interquartile ranges in rodlet cell density among 3 hosts at each time interval. The extensions to the boxes represent overall range; line extensions represent the range of average densities.

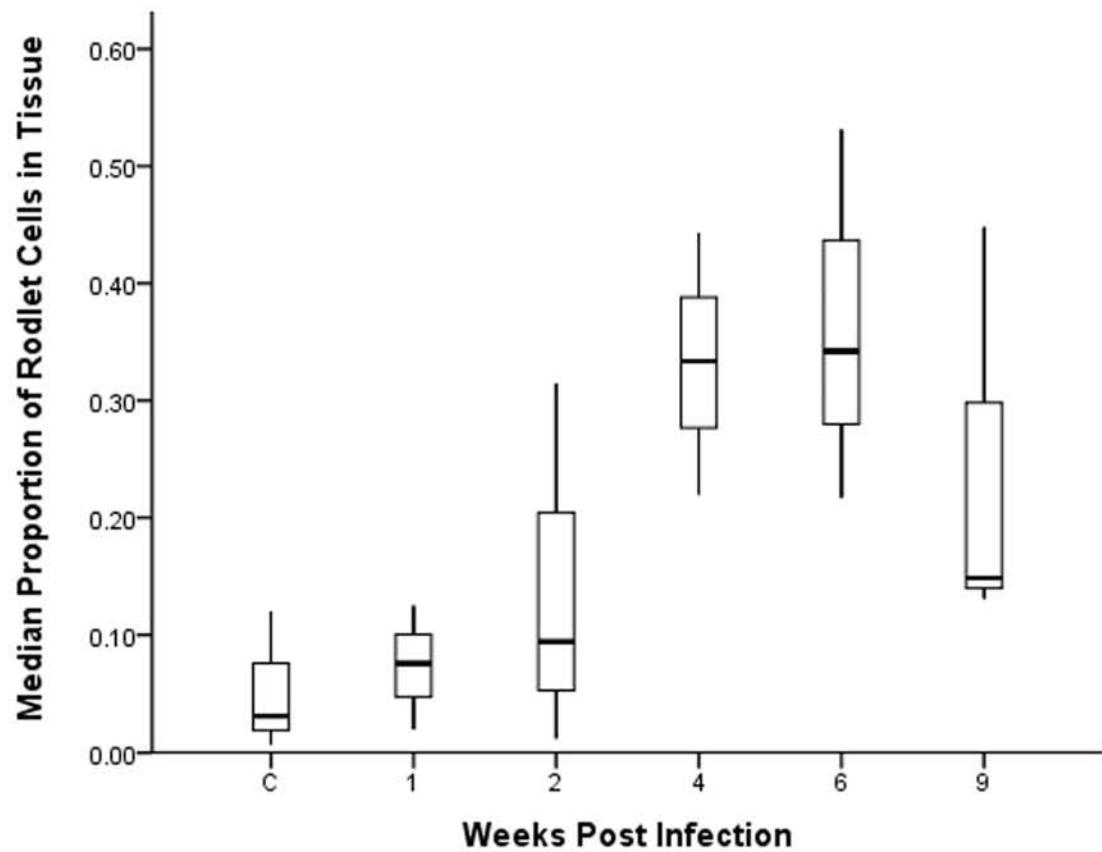


Figure 4.4

Figure 4.5: Mean (\pm SE) proportion of mature and discharging rodlet cells in the optic lobes of minnows experimentally infected with cercariae of *Ornithodiplostomum ptychocheilus*. One semi-thin section from three hosts bearing 4 day, 3 week, and 6 weeks p.i. were examined at each interval. Different letters above bars indicate statistical significance ($p < 0.05$) following one way ANOVA comparisons with Tukey-Kramer HSD.

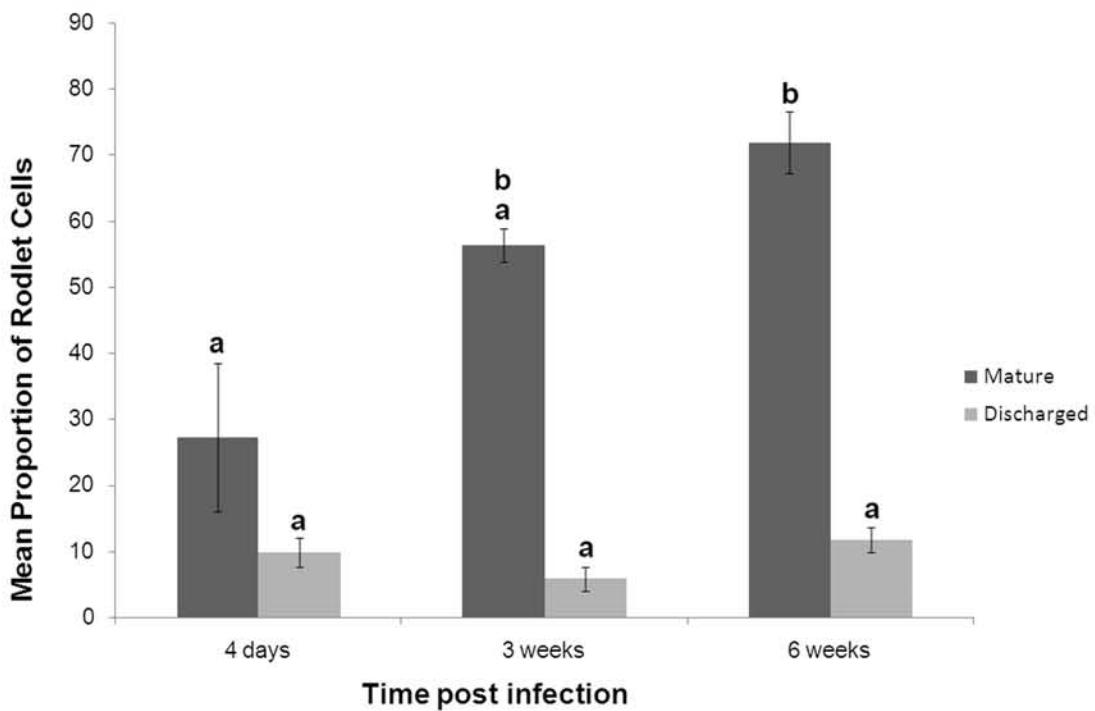


Figure 4.5

Chapter 5: General Conclusions

There is already a strong body of evidence which suggests the traditional view of metacercariae as an inert resting stage is incorrect (outlined in Chapter 1). Recent studies on *O. ptychocheilus* have indicated this stage represents a complex and interactive period of growth and development (Sandland & Goater, 2000; Goater *et al.*, 2005; Conn *et al.*, 2008). The results of my thesis further emphasize that the period of time from initial cercarial penetration, to the final stages of metacercarial development, involve highly complex interactions with host tissues, and elicit host cellular responses. My results are especially important because they indicate that these patterns of migration, growth, and development of metacercariae are no longer confined to a unique brain-encysting strigeoid model. Rather, elaborate migrations, and periods of growth and development are features shared by other strigeoids, and perhaps in varying degrees, to other trematodes.

Sandland & Goater (2000) describe a bi-phasic period of development involving pre-encysted and encysted forms of metacercariae. The results of my thesis both support this concept, and expand upon the idea that migration, growth, and development of strigeoid metacercarie is even more complicated than previously believed. First, results from Chapter 3 indicate that metacercariae of *Ornithodiplostomum* sp. possess similar patterns of growth and development as *O. ptychocheilus* metacercariae. Thus, there exists an obligate pre-encystment stage, characterized by a tegumental complex that invades adjacent host tissues. Upon encystment, the tegument is reorganized, and follows typical descriptions indicated by many studies on metacercariae (Goater *et al.*, 2005). Most importantly, my results are the first to show that this bi-phasic period of growth and

development occurs in distinct habitats within the host. Thus, results from Chapter 3 indicate that pre-encysting stages of *Ornithodiplostomum* sp. develop in the hepatic tissues, while encysted forms continue development within the visceral cavity. Similarly, Chapter 2 demonstrates that the initial *O. ptychocheilus* growth phase occurs in the outer layers of the optic lobes and cerebellum, while encysted metacercariae develop in the inner layers of the meninges. Perhaps the blood brain barrier, which lies immediately above the inner layer of the endomeninx, effectively constrains *O. ptychocheilus* metacercariae from a similarly dramatic shift.

The existence of a pre-encysted tissue-dwelling phase, followed by a shift out of the tissues during encystment, has significant implications. Previous studies have associated the pre-encystment phase with specific negative effects on individual hosts; developing *O. ptychocheilus* metacercariae cause substantial disruption and damage to adjacent brain tissues (Goater *et al.*, 2005). Similarly, visual acuity was significantly reduced in those fathead minnows bearing developing, pre-encysted *O. ptychocheilus* larvae (Shirakashi & Goater, 2005). Upon encystment, adjacent host neural tissue appears healthy and consolidated (Goater *et al.*, 2005), and visual acuity of hosts was identical to that of controls (Shirakashi & Goater, 2005). Studies also suggest that pre-encysted, growing stages of *D. phoxini* (Ballabeni, & Ward, 1993) and *Ornithodiplostomum* sp. (James *et al.*, 2008) reduce host growth. Thus, the bi-phasic habitat shift described in Chapters 2 and 3 provide a mechanism to explain these transient effects of tissue dwelling trematodes in their intermediate hosts.

In addition to the growth and development stages of *O. ptychocheilus* and *Ornithodiplostomum* sp., the diversity and complexity of migratory patterns is also greater than previously believed. While there are some similarities between the migration behaviours of *rnithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp., there exists strong inter-specific variation in the migration route employed. *O. ptychocheilus'* exclusive use of the nervous system to migrate to the brain was initially reported by Hendrickson (1979), and subsequently supported by results in Chapter 2. The use of specific neural tracts by *O. ptychocheilus* diplostomules to navigate within the brain lends weight to Sukhdeo & Sukhdeo's (2004) theory that the internal anatomy of the host is an important consideration when explaining parasite site-finding behaviour. Unlike its congener, *Ornithodiplostomum* sp. diplostomules are not restricted to one navigation route. Rather, evidence from Chapter 3 suggests that most migrating diplostomules access sites within the visceral cavity via tissue-penetration, while some diplostomules employ the circulatory system in their migration. Such divergent strategies between two closely related species of trematode have not been reported before. The use of phylogenetic analyses on *Ornithodiplostomum* spp., and related species such as *Diplostomum* spp. and *Posthodiplostomum* spp., would be useful in determining the origin of alternative migration strategies, and would also aid in the identification of intermediate stages of trematode migration.

In both systems, there exists some degree of intra-specific variation in diplostomule site selection within individual hosts. There are two possibilities that would explain how diplostomules arrive at different sites, whether in the optic lobes and cerebellum for *O. ptychocheilus*, or various visceral tissues for *Ornithodiplostomum* sp..

First, the specific sites selected by diplostomules could be a fixed behaviour (eg. Sukhdeo & Sukhdeo, 2004). Thus, the second generation of metacercariae which had developed in the liver, pancreas, or on the surface of the swim bladder, would in turn migrate to the respective organs their parents occupied. Similarly, metacercariae from the cerebellum would produce offspring that would migrate to the cerebellum, while the F1 generation of diplostomules with optic lobe-dwelling parents would select this region. This idea would be straightforward to test, and would provide valuable insight into the relative importance fixed behaviours play in parasite navigation. The role of fixed behaviours is a central tenet of the postulations by Sukhdeo & Sukhdeo (2004), which describes the “third environment” as a platform in which these fixed behaviours can flourish. Conversely, the partitioning of same-species diplostomules within a host may be based on the particular migration route employed (outlined in Chapters 2 and 3). While manipulating known migration tracts in the host would provide a means to test this alternative, this idea would be more difficult to test.

Studying the behavior of migrating and developing trematode larvae has obvious challenges and limitations. The nature of endoparasites makes it impossible to study the behaviours of parasites in their natural context. The most dominant method of study, sampling after autopsy, was used in Chapters 2 and 3. Wilson (1994) advises caution in interpreting data involving sampling after autopsy. Specifically, he notes that the absence of migrating cercariae in a particular tissue is not evidence that these tissues are not involved in the migration route. In fact, it may be that cercarial migration through the specific tissue is so efficient, that it is rarely observed at the various intervals post-infection. In contrast, a high proportion of migrating parasites in a particular tissue is

often (and logically) interpreted to be evidence of a migratory route. Thus, based on one set of results, it is possible that opposing conclusions can be reached.

The final component of my thesis described a quantitative host response to trematode infection, and provides several central advances to the study of rodlet cells in fish. Results from Chapter 4 provide the first experimental evidence for a functional linkage between rodlet cell proliferation and exposure to trematode cercariae. Furthermore, this is the first experimental study to report an increase in rodlet cells due to a helminth infection. The temporal pattern of rodlet cell proliferation over the course *O. ptychocheilus* development was highly significant, with a substantial expression of rodlet cells occurring after encystment (Chapter 4). Thus, in addition to the bi-phasic pattern of metacercarial growth and development, there exists a corresponding bi-phasic host-response involving rodlet cells.

Although the function of rodlet cells remains unresolved, interpretation of results from Chapter 4 offer a tantalizing alternative to traditional theories. Thus, it was suggested that because rodlet cell proliferation is concomitant with several hallmarks of tissue repair (reviewed in Chapter 4), these cells might similarly play a role in repair processes. While the precise composition of rodlet cell secretions is still unknown, the envelope surrounding the dense rodlet core contains alkaline phosphatase (Iger & Abraham, 1990). The biological significance of this enzyme is not fully understood, although it is known to play a significant role in cellular division and differentiation (Rai & Mittal, 1983), and bears potential as a marker for wound healing (Alpaslan *et al.*, 1997). Clearly, elucidating the composition of rodlet cell secretions would be a valuable

step in determining rodlet cell function. More generally, our understanding of rodlet cells would benefit from examining their responses in concert with other cells involved in host responses. More studies that link processes of encystment, tissue repair, fibroblasts, and rodlet cell responses are needed.

If rodlet cells do play a role in tissue repair, their expression could be particularly enhanced in highly regenerative tissues. Thus, this could account for the high expression of rodlet cells reported in the brain tissues, which are exceptionally regenerative in teleosts (Zupanc, 2001). Hepatic tissues are also regarded as highly regenerative, thus making future studies with rodlet cell proliferation over the course of *Ornithodiplostomum* sp. development an important next step.

Several lines of evidence suggest that mechanisms to resist *O. ptychocheilus* infection are ineffective in fathead minnows. This is supported by the high prevalence (approximately 100%) of *O. ptychocheilus* in fathead minnow populations (Sandland *et al.*, 2001). Indeed, a recent review by Wisendon *et al.* (2009) finds little evidence that fish are able to detect, and avoid trematode cercariae. Additionally, fathead minnows do not appear to possess mechanisms to combat infection. Evidence suggests that prior exposure to *O. ptychocheilus* does not confer protective immunity to fathead minnows (Sandland & Goater, 2000; Goater, unpublished observations). Furthermore, there exists no record of dead or damaged *O. ptychocheilus* metacercariae in the Goater lab (Goater, unpublished observations). Finally, results from Chapter 4 do not support the hypothesis that rodlet cells can damage or dampen *O. ptychocheilus* metacercariae. Without means to resist or combat infection, it follows that natural selection should favour those hosts

that are better suited to tolerate the presence of established parasites. Thus, additional mechanisms for tissue repair could compensate for the absence of infection-avoidance strategies, and the lack of acquired or innate immune mechanisms effective against trematodes. While indirect evidence to suggest that rodlet cells play a role in tissue repair is extremely limited, the potential for rodlet cells to enhance host tolerance for parasite infections is very enticing.

The multi-faceted approaches employed in parasitology have always been one of the field's greatest strengths. Future investigation involving *O. ptychocheilus* and *Ornithodiplostomum* sp. should follow these integrative and versatile approaches. Functional morphological studies offer valuable insight into parasite behaviours; indeed, scanning electron microscopy has altered our view of the metacercarial tegument. Ultrastructural studies of the metacercarial tegument prior to encystment indicate a highly dynamic, interactive, and invasive structure (Stein & Lumsden 1971; Strong & Cable, 1972; Koie, 1977; 1992). The presence of cercarial glands, eyespots, and various mucoid and lytic secretions speaks to the potential range of behaviours employed by trematode larvae (Bruce *et al.*, 1970). Additional methods to study endoparasitic behavior include agar-filled choice chambers (Grabe & Haas, 2004; Haas, *et al.*, 2007) and the radiolabelling of cercariae (Hoglund, 1991). Preliminary studies involving *O. ptychocheilus* and fathead minnows have shown that live fathead minnows are suitable subjects for nuclear MR imaging. In concert with radiolabelled cercariae, this technology would allow the first opportunity to study behavior of live parasites within a host. Finally, parasite-induced alteration of host behaviour is another potential avenue for research with this system. Results from Chapter 2 offers insight into those host behaviours that would

be ideal candidates for study. Thus, those sites selected by the majority of developing *O. ptychocheilus* metacercariae are downstream from a region in the brain that is primarily responsible for an orienting mechanism, the dorsal light reflex, in fish (Gibbs & Northmore, 1996). Thus, an abundance of developing diplostomules could severely compromise this behaviour. This research could further examine potential negative effects on the host caused by the pre-encystment tissue-dwelling phase.

In conclusion, the results of my thesis speak to the enormous complexity of migration, site selection, growth, and development of two species of strigeoid trematode. It appears that host responses to these features are similarly complex. While both *O. ptychocheilus* and *Ornithodiplostomum* sp. represent ideal models to continue examining host-parasite interactions, host responses, and parasite behaviours, future research will certainly show that such complexities are not confined to these systems.

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