

**COST AND BEHAVIOURAL AVOIDANCE OF TREMATODE CERCARIAE
IN
FATHEAD MINNOWS**

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This thesis is dedicated to my father, Daniel Stumbo, who has been a significant keystone in my life. It is also dedicated to my grandparents, Orpha and Lynn Stumbo.

Abstract

Natural selection should favour host defenses that reduce a host's exposure to parasites or reduce their negative effects. One strategy that resolves the substantial costs of host immunity and/or tolerance is to avoid infective stages altogether. For fish, behavioural avoidance is well-known for defense against aquatic predators, but it is poorly known for defense against parasites. I used a model system that is amenable to experimental manipulation to test the behavioural avoidance hypothesis for fathead minnows exposed to the larvae of two of their common flatworm parasites. First, I showed that minnows exposed to a liver encysting trematode, *Ornithodiplostomum* sp. showed an increase in lipid peroxidation, an indicator of oxidative stress, persisting through worm development. Three lines of evidence provided support for the behavioural avoidance hypothesis. First, shoal area decreased in groups of minnows exposed to *O. ptychocheilus* cercariae compared to those exposed to cues from other aquatic threats. Second, average worm numbers were 50 % lower in fish confined to artificial shoals compared to non-shoaling minnows, indicating that shoaling reduces risk of exposure. The third experiment showed that minnows within the centre of shoal reduced their risk of infection by 67%. Taken together, these results demonstrate a cost of trematode infection on minnows, that minnows can detect infective larvae within the water column, and that social living reduces a hosts' risk of exposure.

“There is no better high than discovery.”

-E. O. Wilson

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Chapter 1: General Introduction

Parasites can impose direct and/or indirect costs on their hosts. Evidence for a range of costs comes from studies on countless host/parasite interactions. The methodology involved is as variable as the interactions themselves, ranging from computer models of host/parasite interactions (e.g. Anderson and May, 1978; May & Anderson, 1978), to empirical studies involving manipulated exposures (James *et al.*, 2008; Rohr *et al.*, 2009), to field-based studies (Heins *et al.*, 2000). Taken together, the results from these studies indicate that the magnitude of these costs on host fitness are highly variable, ranging from near absent to a level comparable to predation (Rohr *et al.*, 2009; Schmid-Hempel, 2009; Kortet *et al.*, 2010). Further, while some parasites have direct effects on host fitness through consumption or pathology of critical tissue (e.g. trematode larvae in snails, cestode larvae in fish; Bush *et al.*, 2001), others have more indirect effects on host features such as behaviour (Lefèvre *et al.*, 2009), cognition (Moore, 2002), personality (Kortet *et al.*, 2010), and appearance (Bakker *et al.* 1997). Regardless of the nature of the effects, so long as there is a reduction in host fitness, natural selection should favour the evolution of adaptive responses to reduce these effects on their host.

Given the diversity and magnitude of parasite-induced costs on host fitness, it follows that parasites should act as strong mediators of natural selection

for host defences. Studies completed over the past two decades have verified parasite-mediated selection on host personality traits (Kortet *et al.*, 2010), behaviours (Moore, 2002), life history traits (Sorensen & Minchella, 2001), immunity-based traits (Schmid-Hempel, 2003), genetic diversity (Blanchet *et al.*, 2009) and even host speciation (Maan *et al.*, 2008). Taken together, results from these studies indicate that when costs of infection are sufficiently high on hosts, parasite-mediated natural selection can be strong on a range of traits, and can act quickly.

Considering the cost of parasitic infection and the extent to which parasites can mediate natural selection, it is not surprising that hosts have developed various strategies to avoid or limit infection. Field studies from Wegner *et al.* (2003a) and experimental evidence from Wegner *et al.* (2003b) provide strong evidence that the presence of multiple parasites can select for an intermediate level of major histocompatibility complex (MHC) diversity best suited for combating infection. Further, a wild population of *Daphnia magna* infected with a pathogenic bacteria altered its clonal composition to favour resistant types (Duncan & Little, 2007) and striped bass introduced to the Pacific Ocean a century ago exhibit a higher tolerance to the Pacific shark-cestode *Lacistorhynchus dollfusi* than the original Atlantic population (Sakanari & Moser, 1990). In such cases, natural selection mediated by exposure to parasites favoured resistant or tolerant host phenotypes.

The complex immune system, especially of vertebrates, is an obvious target for parasite mediated natural selection. Many studies have documented the role of immunity in reducing the numbers of parasites in a host, or reducing their negative effects (review by Wakelin *et al.*, 2002). As the immune system is highly complex, immune responses to parasites are highly variable. This disparity is amplified by the variation between hosts, and the considerably large range of parasites possessing differing life histories. These immune responses can be classified as innate or acquired, although these are not necessarily independent (Hoffmann *et al.*, 1999). Responses involving inflammation, leukocytes (e.g. phagocytes), lymphokines, proteolytic cascades, and the MHC are a small fraction of immune components utilized in combating parasitic infection (Schmid-Hempel, 2009).

While less understood than immunity, recent studies have also documented the important role of host tolerance mechanisms in combating parasites. The ability to tolerate infection is a relatively passive strategy to combat parasite virulence (Miller *et al.*, 2006). Whereas immunity acts as a resistance mechanism, actively combating and limiting infection, tolerance mechanisms act to limit the impact of infection on host fitness (Schneider & Ayres, 2008). Although tolerance mechanisms are not well understood outside of plant research and are difficult to isolate from resistance, the genetic basis of these

mechanisms are becoming more apparent (Read *et al.*, 2008). Possible mechanisms of tolerance include investment in parasite burdened tissues, such as producing a thicker gut lining, an increased ability to replace red blood cells in organisms susceptible to malaria, or limiting immunopathology (Read *et al.*, 2008).

Immunology and tolerance are beneficial for combating infection, however utilizing these mechanisms may be costly. Results from studies completed over the past decade have confirmed that the maintenance and expression of immunity is costly to individual hosts. Such costs include, but are not limited to, immunopathology associated with fever, reproductive trade-offs, and metabolic expense (for review see Sheldon & Verhulst, 1996). Thus, mice resistant to rodent malaria had lower rates of reproduction compared to non-resistant mice (Råberg *et al.*, 2007). The same study showed that mice that were tolerant to infection had lower ability to mount effective resistance. Thus, both mechanisms of resistance are costly (Råberg *et al.*, 2007).

One way to resolve the costs of both immunity and tolerance is to avoid parasites altogether. Moore (2002) describes behavioural avoidance as the first line of defence to infection, although she also indicates that this defense mechanism is the least studied. Considering the costs associated with tolerance and immunity, a behavioural strategy of avoiding or limiting

exposure should be favoured by natural selection, provided that the cost of avoidance behaviour is not greater than the cost of increased infection (Hart, 1990; Moore, 2002; Wisenden *et al.*, 2009).

A number of studies have provided evidence for diverse behavioural avoidance strategies. These range from self and conspecific grooming and selective foraging, to altered mate selection, exothermic regulation (e.g. behavioural fever), and vector avoidance (Hart, 1992; Moore, 2002). Grooming behaviour has been strongly favored in mammalian and avian systems for the removal of ectoparasites and has been documented in many species including impala antelope (Mooring *et al.*, 1996), field mice (Stanko *et al.*, 2002), and mourning doves (Losito *et al.*, 1990). Although this behaviour is often costly (e.g. weight loss, reduced vigilance, and altered rest; review by Wilson, 2005), its common occurrence across a range of host-parasite interactions suggests that grooming behaviour is strongly favoured by natural selection.

While there is strong evidence for parasite avoidance, it is the least studied of the three response strategies. Evidence for behavioural avoidance of infection in aquatic systems is especially limited, primarily focusing on insect and amphibian hosts (Wisenden *et al.*, 2009). Female gray tree frogs adjust oviposition behaviour, discriminating against ponds with the snail

Pseudosuccinea columella, a trematode vector, with the highest discrimination against ponds with infected snails (Kiesecker & Skelly, 2000). *Rana* tadpoles reduced their activity in the presence of cercariae or predators, and reduced their activity most when presented with both threats (Thiemann & Wassersug, 2000). The range of transmission strategies within aquatic host/parasite interactions is notoriously diverse, ranging from ingestion of infected intermediate hosts, exposure to eggs or free-swimming larvae, and exposure via vectors.

Behavioural response of fish to parasites

Three forms of parasite avoidance behaviour in fish that have predominantly been studied are outlined in Hart (1990): avoidance of infective stages, repelling or removal of parasites, and preference for mates with low levels of parasites (for review see Barber *et al.*, 2000). Wisenden *et al.* (2009) provides a conceptual framework for these behaviours in the context of fish/parasite interactions. If potential parasitic threats are detectable before or during attack, fish may respond through behavioural avoidance of infective stages such as free-swimming infective larvae, contagious infective stages from infected conspecifics, or infected prey. If parasitic threats are not detectable prior to attack, potential hosts may develop behavioural management strategies such as chafing, cleaning and localized defecation.

Many parasites possess contagious infective stages, with transmission occurring via direct host/host contact (e.g. monogenean trematodes, parasitic arthropods). In these instances, avoidance of infected conspecifics should be favoured. For instance, female sticklebacks that distinguish infected males from non-infected males via dulled nuptial coloration avoid these potential mates (Milinski & Bakker, 1990). Kennedy *et al.* (1987) showed that female guppies prefer the company of non-parasitized males. Wedekind (1992) showed that the secondary sexual characteristics of male roach are altered by different species of parasites due to their contrasting effects on the host immune system. Wedekind (1992) also suggests that female roach may be able to ascertain infection status via these characteristics when choosing a mate. It has been hypothesised that parasites act as a strong selection pressure on sexual selection. Additional studies, however, have shown avoidance behaviour beyond mate choice. Dugatkin *et al.* (2004) showed that juvenile three-spined sticklebacks avoid conspecifics with the copepod ectoparasite *Argulus canadensis*. Ward *et al.* (2005) showed that three-spined sticklebacks avoid groups of conspecifics infected with *Glugea anomala*, a microsporidian that causes external growths on hosts. Taken together, these studies show that fish can determine the infection status of conspecifics and alter their behaviour, reducing contact with potentially infected hosts.

The avoidance of infected prey may be presented in terms of cost/benefits. Although the consumption of infected prey may present a parasite-induced metabolic cost, the nutrition obtained through consumption of infected prey may provide sufficient nutrition to offset this loss. Lafferty (1992) weighed the metabolic cost of infection against the metabolic gain of consuming infected fish, finding little evidence for selective pressure towards avoidance of infected prey. This is especially true in systems in which parasites alter the behaviour of hosts, facilitating consumption (Lafferty, 1992; Barber *et al.*, 2002; Wisenden *et al.*, 2009). Additionally, several studies have shown predator preference for infected versus uninfected prey. Bakker *et al.* (1997) showed that coloration observed in the crustacean *Gammarus pulex* when infected with an acanthocephalan parasite facilitated predation by three-spined sticklebacks. Seppälä *et al.* (2004) showed impaired predator avoidance behaviour in rainbow trout infected with a cataract inducing trematode, increasing predation susceptibility when compared to non-infected trout. Lafferty & Morris (1996) showed increased ‘conspicuous’ behaviour (i.e. ‘surfacing, flashing, contorting, shimmying, and jerking’) in trematode infected killifish, increasing predation by the definitive host. While the consumption of these prey will result in infection, the presence of the parasite reduces the effort required for predation. This reduced cost in energy acquisition may be indicative of commensalism between host and parasite.

Wisenden *et al.* (2009) stressed the importance of learned avoidance in regards to predator/host systems involving fish. Studies that have evaluated predation on infected prey focus on initial predation events. Although infected prey may be predominantly selected by naïve hosts, it is not clear if these hosts could learn to associate potential prey with infection. The authors additionally suggest that learned avoidance may occur when infection is observable in potential prey and pathology is experienced soon after ingestion.

Social learning, as well as enhanced vigilance through social cues, are fundamental elements for the success of shoaling fish. This allows individuals to observe conspecifics for threat and foraging behaviour, taking immediate action as well as acquiring appropriate learned responses (Roberts, 1996; Brown & Laland, 2003). Fish often respond to threats with generalized responses that effectively work for predation and parasitic avoidance, and the same aspects of social living, such as shoaling, that provide anti-predator benefits may also provide anti-parasite benefits (Thiemann & Wassersug, 2000; Wisenden *et al.*, 2009). The bulk of studies relating shoaling and parasitism, nevertheless, focus on the effect of infection on shoaling behaviour (e.g. Barber *et al.*, 1995; Barber *et al.*, 1998; Krause & Godin, 1994; Krause & Godin, 1996; Seppälä *et al.*, 2008; Tobler & Schlupp, 2007). However, a meta-analysis performed by Cote and Poulin (1995) demonstrated

a negative correlation of mobile, non-contagious parasite encounters and host group size, and a positive correlation of infectious parasites and host group size. Poulin and FitzGerald (1989) also showed a decrease in attacks on individuals from the ectoparasites *Argulus canadensis* with increased stickleback group size, and that individuals possessed an increased affinity for shoaling in the presence of the parasite.

Thus, while there is evidence to support the idea that fish can alter behaviours associated with mate and prey selection, and can avoid contagious parasites, there is still much that is not known about fish avoidance behaviours. Most significantly, almost nothing is known regarding a fish's ability to detect and then directly or indirectly avoid free-swimming parasite larvae. This lack of knowledge is especially true for the infective stages of non-conspicuous parasites that are common in most water bodies, such as those involved in the life-cycles of myxozoans, microsporidians, and trematode cercariae. Some empirical evidence for avoidance of these stages does exist. Rainbow trout actively avoided refugia where trematode cercariae were present (Karvonen *et al.*, 2004). Evidence can also be drawn from tadpole systems. Green frog and wood frog tadpoles reduced their activity in the presence of cercariae (Thiemann & Wassersug, 2000).

Host-parasite system(s)

Studies involving naïve fish exposed to known numbers of free-living parasite stages are needed to fully understand avoidance behaviours in fish. One appropriate system involves fathead minnows (*Pimephales promelas*, Rafinesque) exposed to their suite of common strigeid trematodes. Field surveys have shown that these fish are common intermediate hosts for various strigeid trematode species (Sandland *et al.*, 2001), many of which are pathogenic. The diplostomid trematodes *Ornithodiplostomum ptychocheilus* (Faust), *Posthodiplostomum minimum* (MacCallum) and *Ornithodiplostomum* sp. (unpublished) are the most common of these trematodes in fathead minnows across their range, typically present in all fish in numbers ranging from approximately 10-1000 cysts/fish (Sandland *et al.*, 2001; Goater, unpublished observations). A wealth of background information exists for these three species on basic life-cycle maintenance in the laboratory (e.g. Sandland & Goater, 2000) and parasite development in fish (Matisz & Goater, 2010; Matisz *et al.*, 2010), making them ideal candidates for experimental manipulation. Additionally, infection is known to have negative effects on individual minnows (Sandland & Goater, 2001; Shirakashi & Goater, 2005; James *et al.*, 2008).

Ornithodiplostomum ptychocheilus, *P. minimum* and *Ornithodiplostomum* sp. have similar life cycles, using *Physa* spp. snails as the first intermediate host, fathead minnows as the second intermediate host, and piscivorous birds

as the definitive hosts (Miller, 1954; Hendrickson, 1979; Matisz and Goater, 2010). Following the methods outlined in Sandland & Goater (2000), this life cycle can be maintained in the laboratory. In short, one-day old chickens are fed fathead minnow brains containing *O. ptychocheilus* metacercariae or viscera containing *P. minimum* or *Ornithodiplostomum* sp. metacercariae. Eggs are then collected from the chicken's feces and incubated at room temperature. Laboratory-reared F₁ generation *Physa* sp. snails are exposed to the resulting miracidia. Rediae are then allowed to develop within the snail hosts for approximately 4 weeks. Once cercarial shedding starts to occur, infected snails are placed into glass vials for 2-3 hours, after which cercarial densities can be assessed for controlled exposures to individual fish.

Due to their robustness to a range of laboratory and field conditions and their ease of maintenance, fathead minnows are excellent model organisms in ecological and especially toxicological research (Denny, 1987; for review see Ankley & Villeneuve, 2006). In addition to the ease of cultivation of the parasite system outlined above, and the ability to perform controlled cercarial manipulations that are not usually available for most research of this type, fathead minnows provide an ideal model for experimental tests of various components of the behavioural avoidance hypothesis.

Thesis objectives

The first experimental study, presented in Chapter 2, evaluates the cost of cercarial infection in fathead minnows by assessing parasite-induced oxidative stress. The purpose of this experiment was to more carefully assess the physiological cost of trematode infection on minnows, and to extend the preliminary results of James *et al.* (2008) that described a crude effect of *Ornithodiplostomum* sp. on minnow growth. Minnows were exposed individually to dH₂O (control), *O. ptychocheilus*, or low (20) or high (100) doses of *Ornithodiplostomum* sp. cercariae. Livers were removed at 5, 10 or 28 days post-infection, and an assay was performed to determine the level of lipid peroxides, a commonly used indicator of oxidative stress. Results indicate cost of infection in relation to metacercariae development and parasite location within the minnow host.

In Chapter 3, three experiments were performed to test the role of shoaling as parasite avoidance strategy for fathead minnows exposed to trematode cercariae. This is the first experimental test of the shoaling hypothesis for protection against exposure. The first experiment assessed whether exposure to *O. ptychocheilus* cercariae altered the configuration of minnow shoals compared to exposure to other threats such as non-minnow infecting cercariae (*Plagiorchis elegans* (Rudolphi), a trematode that utilizes a range of insect larva as intermediate hosts; Genov & Samnaliev, 1984), and threats normally associated with predation. The second experiment determined

whether fish confined to artificial shoals reduced their risk of exposure to cercariae with free-running and more natural mesocosm environments. The last experiment focused on comparing the risk of cercarial exposure for minnows located within the centre of a shoal compared to those on the periphery.

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Chapter 2: Parasite-induced oxidative stress in liver tissue of fathead minnows exposed to trematode cercariae.

ABSTRACT

Evidence from field surveys support the idea that parasites can cause oxidative stress in their fish hosts, but there are few experimental tests of the oxidative stress hypothesis. I evaluated oxidative stress as an indicator of the physiological effects of larval trematodes on fish. Fathead minnows were exposed to the larvae (cercariae) of a trematode, *Ornithodiplostomum* sp., that develops in the liver and another, *O. ptychocheilus*, that develops in the brain. Following exposure to either species, there was a significant increase in lipid peroxidase (LPO) concentration, an indicator of oxidative stress, in liver tissue compared to controls. For *Ornithodiplostomum* sp., LPO concentration in liver tissue increased at 5 days post-infection and remained higher than controls up to 28 days. For *O. ptychocheilus*, LPO concentration in liver tissue was higher than controls at 5 days, but not thereafter. The sustained elevation in LPO concentration for the liver trematode can be explained by direct tissue damage caused by developing larvae in the liver or by immunopathology. The temporary increase in LPO concentration in liver tissue of *O. ptychocheilus* - infected minnows indicates a general physiological or immuno-pathological cost of infection.

Introduction

Aquatic organisms are exposed to a wide range of natural and anthropogenic stressors, each of which has the potential to disrupt their physiological homeostasis. One method that both field-based and laboratory-based researchers have increasingly used to evaluate the physiological costs of stress is to quantify by-products that are indicative of physiological stress. Among these tools is the assessment of chemically-reactive molecules that are associated with oxidative stress. Internal or external stressors such as UV radiation, heat exposure, contamination, tissue damage, and pathogens have been shown to cause elevated levels of reactive oxygen species (ROS), resulting in oxidative stress (Kelly *et al.*, 1998; Toyokuni, 2002). Oxidative stress damages cell lipids, DNA, and proteins, and has been shown to have direct and/or indirect links to liver lesions, inflammation, cancer, and reduced glutathione (Kelly *et al.*, 1998; Spector, 2000; Velkova-Jordanoska & Kostoski, 2005). By measuring biomarkers such as anti-oxidants (molecules that combat ROS) or lipid peroxidation (LPO, a consequence of ROS induced cell damage), the mechanisms mediating the damaging effects of stressors can be determined (Kelly *et al.*, 1998).

Results from studies involving fish confirm that oxidative stress is a frequent consequence of exposure to various stressors (Kelly *et al.*, 1998). This is especially the case for anthropogenic toxicants, which cause a significant increase in ROS in fish (reviews in Di Giulio *et al.*, 1989; Oost *et al.*, 2003).

Miller *et al.* (2007), for example, reported increased oxidative stress via reduced glutathione levels in juvenile rainbow trout subjected to acute selenium exposure. ROS induced damage resulting from selenium exposure has been suggested as a contributing cause of selenium toxicity, resulting in pathologies such as larval deformities (Spallholz *et al.*, 2004; Muscatello *et al.*, 2006). Similarly, results from a number of studies have shown that parasites can also cause oxidative stress in the tissues of infected fish. For example, Kurtz *et al.* (2006) experimentally exposed sticklebacks to the nematodes *Camallanus lacustris* and *Anguillicola crassus* and the trematode *Diplostomum pseudospathaceum*, resulting in increased oxidative stress in liver tissue. However, most of the evidence for parasite-induced oxidative stress is indirect, coming from field studies involving fish collected from sites that differ in exposure to a range of parasites. Farmed carp infected with a cestode parasite had elevated antioxidant levels compared with uninfected farmed carp (Dautremepuits *et al.*, 2003) and catfish, *Rhamdia quelen*, infected with a trematode had increased lipid peroxidation in muscle tissues (Belló *et al.*, 2000). Furthermore, exposure to mercury contamination and infection with the nematode *Raphidascaris acus* caused a synergistic effect on markers of oxidative stress in yellow perch (Marcogliese *et al.*, 2005). These results suggest that a variety of parasites can cause oxidative stress in a range of hosts.

Ornithodiplostomum sp. is an undescribed species of trematode that encysts within the body cavity of fathead minnows, *Pimephales promelas* (Matisz & Goater, 2010). Its congener *O. ptychocheilus* (Faust) encysts within the optic lobes of fathead minnows (Matisz *et al.*, 2010). In lakes and ponds in Alberta, these two species typically co-occur in the same individual fish (Sandland *et al.*, 2001; Goater unpublished observations). Both species utilize pond snails (*Physa* spp.) as first intermediate hosts, fathead minnows as second intermediate hosts, and piscivorous birds as definitive hosts. The migration and development of these worms in fathead minnows has been described by Matisz & Goater (2010) and Matisz *et al.* (2010). Between approximately 2-14 days post-infection, metacercariae develop rapidly within host tissues (optic lobe and liver parenchyma for *O. ptychocheilus* and *Ornithodiplostomum* sp., respectively), where they increase in body volume by at least 5X. Following this period of rapid development, metacercariae exit the tissues and encyst for the remainder of the fish's life.

Fathead minnows infected with *Ornithodiplostomum* spp. metacercariae exhibit a range of detrimental effects associated with the metacercariae growth-phase. James *et al.* (2008) demonstrated a metabolic cost, via reduced body condition, of *Ornithodiplostomum* sp. corresponding to the development phase within the liver. Sandland *et al.* (2001) showed cranial distortion and increased mortality due to developing *O. ptychocheilus* metacercariae.

Shirakashi & Goater (2001, 2002) showed compromised optomotor responses in *O. ptychocheilus*-infected minnows. Trematode-induced oxidative stress and associated pathology may provide a unifying mechanism to explain these diverse negative effects. The purpose of this experiment is to test for trematode-induced oxidative stress in liver tissue of fathead minnows exposed to either *Ornithodiplostomum* sp. or *O. ptychocheilus* cercariae. First, we evaluate whether temporary development of *Ornithodiplostomum* sp. within liver tissue causes long-term oxidative stress, and if these effects are dose-dependent. To assess if parasite-induced oxidative stress requires direct tissue contact, or is part of a generalized response to infection, we also evaluated oxidative stress in the liver tissue of minnows exposed to *O. ptychocheilus*.

Materials and Methods

Experimental Infections

Naïve minnows were exposed to known numbers of *Ornithodiplostomum* spp. cercariae following methods described in Sandland & Goater (2000). To initiate experimental infections, we collected young of the year (uninfected) minnows from Goldspring Pond, Alberta (49.09482, -111.991282), a population known to be infected with metacercariae of *O. ptychocheilus* and *Ornithodiplostomum* sp. In July 2009, 8 one-day old chickens were force-fed minnow brain or viscera containing large numbers of metacercariae. Three days later, eggs were present in the feces of the chickens. Eggs were collected

and processed following Sandland and Goater (2000). The F₁ generation of laboratory reared *Physa* sp. snails were exposed to the resulting miracidia, and reared under standard conditions. For exposures to cercariae, the dilution methods described in Sandland and Goater (2000) were used to estimate the volume of water containing known numbers of 2-hour old cercariae. Exposures of individual minnows occurred within 60 mm Petri dishes for a 2-hour period. Previous results in our laboratory indicate that approximately 80% of cercariae used in exposures are recovered subsequently as metacercariae.

The experiment was set up as an 'infection' x 'time period' factorial involving 108 naïve minnows separated at random into 9 groups. Treatments consisted of 36 fish exposed to dechlorinated water (control), 20 *Ornithodiplostomum* sp. cercariae (low), or 100 *Ornithodiplostomum* sp. cercariae (high) on 12 September 2009. Each group of 36 fish was separated at random into 3 groups of 12, corresponding to dates of dissection at 5, 10, or 28 days post infection (p.i.). At each of these intervals, two minnows were prepared for histological analysis to assess metacercariae development and intensity. Livers from the remaining fish were removed under a dissecting microscope and placed on ice in homogenization microcentrifuge tubes. Samples were stored at -80° C until analysis.

We incorporated an additional group of 36 minnows exposed to 100 *O. ptychocheilus* cercariae into the experimental design. A limited supply of fish and cercariae restricted our ability to complete a full 'time x dose x species' design. The 36 fish were necropsied in the same manner as described above at corresponding post-infection time intervals (i.e. 5, 10 and 28 days).

Lipid Peroxidation Assay

Lipid peroxidation in liver tissue was evaluated with the BIOXYTECH® LPO-586 Assay (OXIS International, Inc., Portland, USA; catalogue no. 21012) following the methods described in Miller *et al.* (2007).

Malondialdehyde (MDA) is the end product of the LPO process, and is used as an indicator for the concentration of LPO within tissue (Esterbauer *et al.*, 1991). LPO concentrations were quantified by the reaction of MDA with *n*-methyl-2-phenylindole at 45 °C and 586 nm, and LPO is expressed as μmol MDA/mg of protein.

Histopathology

Standard histological sectioning of *Ornithodiplostomum* sp. infected minnows was used to provide a qualitative assessment of ontogenic changes in metacercariae growth and site selection, and to verify infection levels at 5, 10 and 28 days p.i. Histological sections of two randomly-selected minnows were prepared from each of the six treatments. 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 (Matisz & Goater, 2010). Bodies were fixed in 10% neutral buffered formalin for 7 days, and decalcified in 0.1 M EDTA titrant for 14 days. These samples were dehydrated in ethanol prior to paraffin embedding. Each fish was serially sectioned along its sagittal plane (10 μ m thickness). Sections were deparaffinized and stained with Mayer's hematoxylin and eosin Y. All sections were examined by light microscopy using a Zeiss axiocam digital camera mounted onto a Zeiss axioskop 40 microscope.

Statistical Analysis

The data were log transformed prior to analyses to meet the assumptions of normality. A two-way ANCOVA was performed using Predictive Analytics SoftWare v.18 with 'time period' of liver dissections (5 days, 10 days, or 28 days p.i.) and treatment (control, *Ornithodiplostomum* sp. - low , or *Ornithodiplostomum* sp. - high) evaluated as fixed effects, and LPO (μ mol MDA/mg of protein) as the dependent variable. Minnow weight at necropsy was evaluated as a covariate. Least significant difference (LSD) pairwise comparisons were used to test differences between pairs of means within significant fixed effects. A second two-way ANCOVA was used to evaluate differences in LPO concentration between *O. ptychocheilus*-exposed and uninfected fish.

Results

Results from the first two-way ANCOVA showed that the concentration of LPO in liver tissue was not significantly affected by the interaction between time and infection ($F_{4, 69} = 1.37, p = 0.255$). LPO concentrations were also not affected by time period ($F_{2, 69} = 1.35, p = 0.246$). The covariate was not significant ($F_{1, 69} = 0.097, p = 0.757$). However, LPO concentration in liver tissue was significantly affected by infection ($F_{2, 69} = 5.66, p = 0.005$; Fig. 2.1). The LSD pairwise comparisons showed that the 39% difference in LPO concentration between controls and lightly infected hosts was significant ($p = 0.006$), as was the 35% difference between controls and heavily infected hosts ($p = 0.013$). The difference in LPO concentration between lightly- and heavily-infected hosts was not significant ($p = 0.779$). This suggests oxidative stress caused by *Ornithodiplostomum* sp. infection is independent of dose and time (Fig. 2.1).

The two-way ANCOVA containing the *O. ptychocheilus* treatment showed that the concentration of LPO in liver tissue was not significant relative to time period ($F_{2, 44} = 1.109, p = 0.339$), treatment ($F_{1, 44} = 0.253, p = 0.617$), or covariate ($F_{1, 44} = 0.121, p = 0.730$). The interaction between time period and treatment was significant ($F_{2, 44} = 3.900, p = 0.028$, Fig. 2.1) due to the peak in LPO concentration at 5 days, followed by a decline to levels similar to controls at later time periods.

Metacercariae counts (mean \pm s.e.) in low and high dose treatments were 15 ± 1.6 and 68.6 ± 6.4 , respectively. Large numbers of small, unencysted metacercariae were observed within the parenchyma of the liver at 5 days p.i. (Fig. 2.2a). At this time, a distinctive gap was evident between the metacercariae body and adjacent liver tissue. By 10 days p.i., unencysted metacercariae were still present within liver tissue. These metacercariae were larger than at 5 days p.i. and the gap between the parasite and the adjacent liver tissue had expanded (Fig. 2.2b). By 28 days pi, the metacercariae had grown substantially (Fig. 2.2c). Metacercariae were now absent from the parenchyma of the liver (although some were observed along its outer edge), located instead throughout the body cavity. All 28-day old metacercariae were enveloped by a distinctive cyst wall.

Discussion

These results provide the first direct evidence for metacercariae-induced alteration in lipid peroxidation in fish. Since LPO is a well-characterized indicator of oxidative stress, the results indicate that *Ornithodiplostomum* spp. infection causes elevated levels of reactive oxygen species in fathead minnow liver tissue. These results support findings from several indirect tests involving parasite induced oxidative stress in aquatic systems (e.g. Belló *et al.* 2000; Dautremepuits *et al.*, 2003). The results also support the use of the LPO assay for investigating interactions between parasites and environmental stressors under natural conditions.

This is the first study to evaluate biomarkers of oxidative stress over time relative to parasite development. The 35% and 39% increase in LPO concentration (low and high doses respectively) occurred 5 days after exposure to *Ornithodiplostomum* sp. cercariae, and remained elevated until at least 28 days post infection. At this time, all metacercariae were observed developing within the parenchyma of the liver, consistent with observations by Matisz & Goater (2010). Thus, our observations of maximum differences in LPO concentrations between infected and uninfected minnows coincided with the rate of maximum metacercariae development in the liver (Matisz & Goater, 2010). We do not know a mechanism by which encysted stages that are enveloped by a double-cyst wall could influence LPO concentrations within liver tissue, other than indirectly via host immunity. A likely explanation for the elevation in LPO up to 28 days post-infection is persistent cellular responses caused by developing larvae within tissues.

One explanation for the elevation in LPO involves direct damage to liver tissue caused by *Ornithodiplostomum* sp. Matisz & Goater (2010) and Matisz *et al.* (2010) showed extensive tissue damage to the liver during the developmental phase of *Ornithodiplostomum* spp., followed by rapid tissue repair. Although there is no direct evidence that metacercariae feed on tissue, the tegument of developing metacercariae is known to absorb nutrients for

development (Higgans *et al.*, 1979). The gap between tissue and developing *Ornithodiplostomum* sp. metacercariae expands with the apparent volumetric increase of the worm (Matisz & Goater, 2010). This suggests that tissue adjacent to developing worms is damaged, although perhaps only temporarily. When cellular damage occurs, platelets involved in tissue repair release ROS to recruit additional platelets, a process known as redox signaling (Palmer *et al.*, 1997). Thus, the damage that accrues during metacercariae development and migration could result in the oxidative stress observed in liver tissue. Additionally, these ROS may cause a chain reaction in which ROS produced during tissue repair would result in further cellular damage, causing additional ROS production by the host.

An alternative, although not necessarily independent, explanation for *Ornithodiplostomum* sp. induced oxidative stress is a host immune response. Stables & Chappell (1986) showed acquired immune response in rainbow trout exposed to *Diplostomum spathaceum* cercariae. Whyte *et al.* (1990) showed an effective trout immune response, via macrophage and antibody activation, to cercarial infection. Scharsack *et al.* (2007) showed increased immune activation via respiratory burst in sticklebacks exposed to parasites. Respiratory bursts occur when leukocytes come in contact with foreign organisms such as bacteria, fungi and parasites, resulting in the release of ROS (Muñoz *et al.*, 1998; Wang *et al.*, 2010) reducing the viability, and

affecting the development, of certain parasites (Allen & Fetterer 2002, Wilson *et al.* 1994). Kurtz *et al.* (2006) showed a positive correlation between the levels of immune activation and oxidative stress when sticklebacks were exposed to various endoparasites. These studies link immunopathology in response to parasitic infection, resulting in direct or indirect production of ROS.

The lack of a significant dose-dependent response in our study was a surprising result. However, using a similar parasite/host model, Shirakashi & Goater (2002) showed that the magnitude of behavioural changes induced by *O. ptychocheilus* metacercariae was also not dose-dependent. These results indicate a complex relation between worm intensity and host response to infection. One explanation for our results is a dose-independent immune response acting as a nonspecific defense against less virulent parasites. Additionally, we would expect increased tissue damage from the higher level of infection to result in additional LPO. This is not the case for this system, and a possible explanation may involve a plateau response.

Although *O. ptychocheilus* encysts within the optic lobe of fathead minnows, there was a significant infection by time interaction in the LPO concentration in liver tissue of *O. ptychocheilus*-infected fish. This indicates that induced oxidative stress can occur in non-target host tissue. Marcogliese *et al.* (2005)

demonstrated that natural infection of yellow perch with the muscle and skin encysting trematode *Apophallus brevis* correlates with increased oxidative stress in liver tissue. These results suggest endoparasites affect host physiology beyond the area in which they inhabit and may be linked to pathologies throughout the host. Marcogliese *et al.* (2005) suggests inflammation as a possible contributing factor for this non-targeted effect. Early development of *O. ptychocheilus* has been shown to result in inflammation of the meninges, indicating possible ROS production that may enter the vascular system, and hence the liver.

James *et al.* (2008) showed a significant reduction in the body mass of fathead minnows exposed to *Ornithodiplostomum* sp. cercariae at 17-days post exposure. Similarly, Sandland & Goater (2001) demonstrated 'parasite-induced phenotype' variation (i.e. cranial distortion) in juvenile minnows exposed to *O. ptychocheilus* and Shirakashi & Goater (2001, 2002) showed compromised optomotor response in *O. ptychocheilus* infected minnows. Taken together, these results indicate that developing metacercariae cause initial pathology that is manifested in features such as reduced growth, altered development, and altered behavior. The results of the present study indicate that one underlying mechanism is likely associated with lipid peroxidation, either due to direct damage to host tissue, or immunopathology.

In addition to providing support for the LPO assay as an indicator of stress due to parasitism, our experimental results also support field-based approaches that seek to uncover the importance of cumulative or combined stressors on natural fish populations. Marcogliese *et al.* (2005) showed that a combination of parasites and pollutants such as mercury lead to higher levels of ROS than either stressor alone. Jacobson *et al.* (2003) showed synergistic effects leading to lowered immune function in chinook salmon when exposed to polychlorinated biphenyls (PCBs) and the trematode *Nanophyetus salmincola*. Stress due to parasites is often neglected in studies of cumulative effects, but as our results indicate, specialist parasites that are a regular feature of most freshwater fish populations can reduce physiological performance (for review see Marcogliese & Pietrock, 2010), even at low rates of exposure. Oxidative stress, a consequence of exposure to multiple environmental stressors, including parasites and pollutants, may be an underlying mechanism of decreased fitness/performance.

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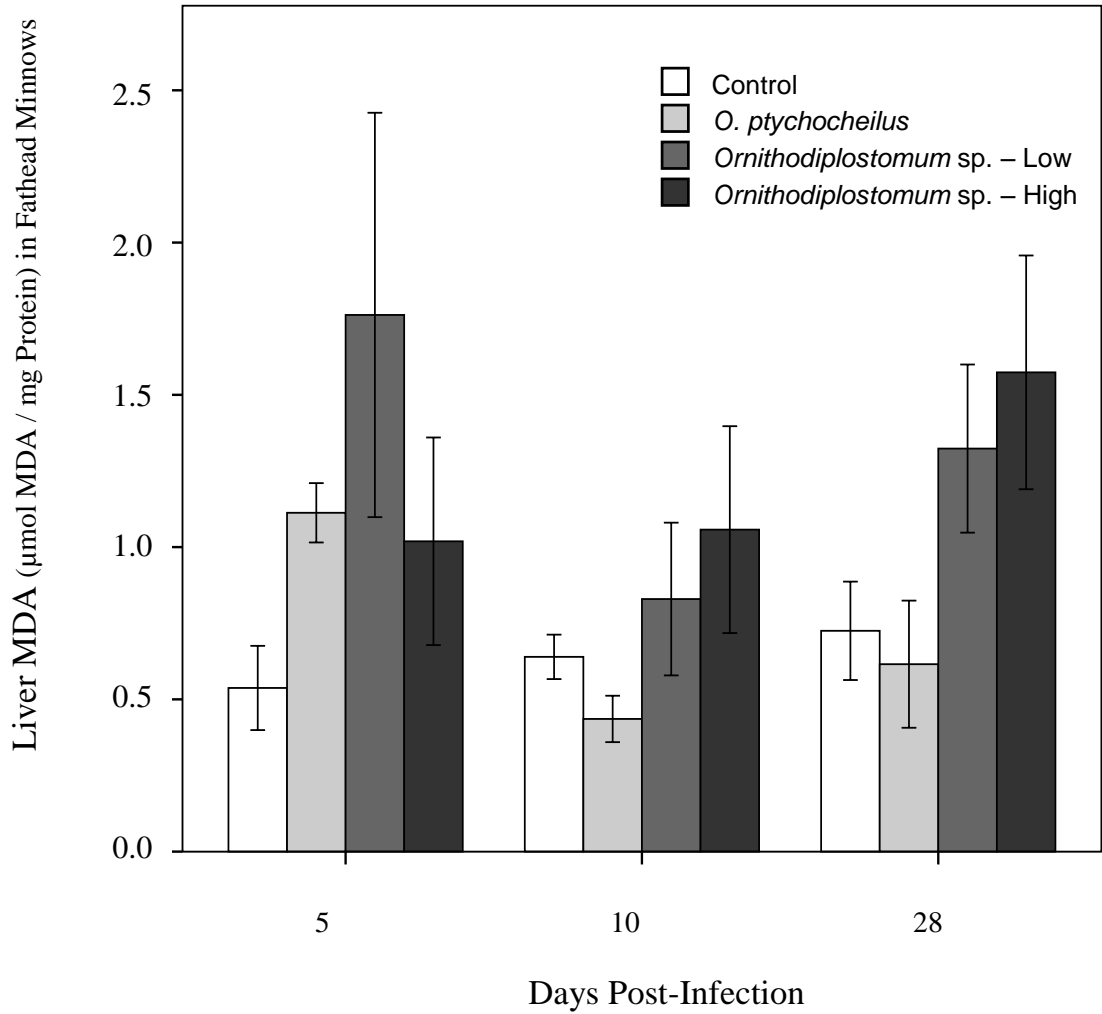


Figure 2.1. Mean (\pm se) concentration of LPO ($\mu\text{mol MDA} / \text{mg protein}$) at 5, 10 or 28 days post-infection in liver tissue of fathead minnows exposed to cercariae free water (Control), *O. ptychocheilus* cercariae, 20 *Ornithodiplostomum* sp. cercariae (Low), or 100 *Ornithodiplostomum* sp. cercariae (High).

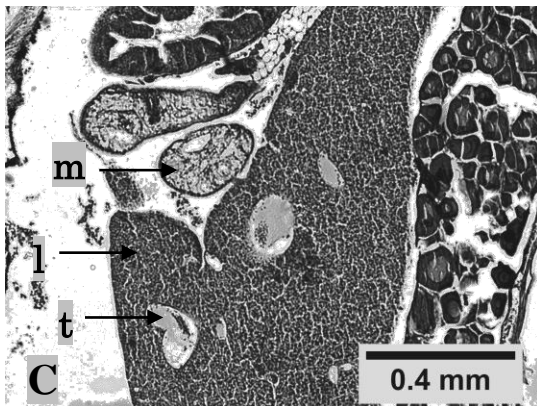
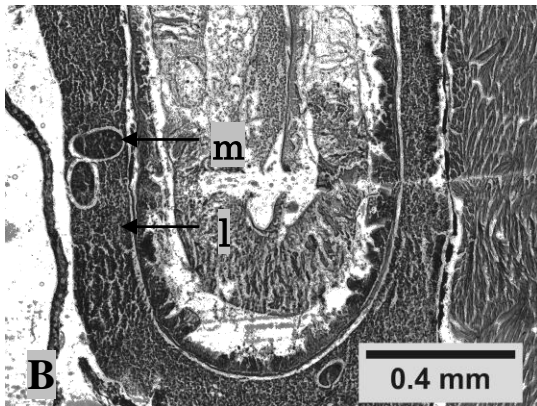
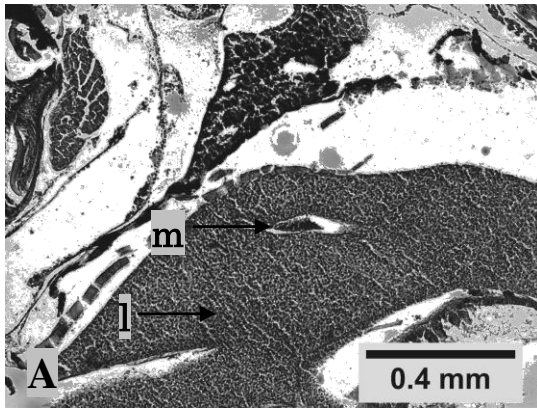


Figure 2.2. Histological cross-sections of fathead minnows infected with *Ornithodiplostomum* sp. at 5 days (A), 10 days (B), and 28 days (C) post-infection. Liver tissue (l), *Ornithodiplostomum* sp. metacercariae (m), and metacercariae migration tracks (t) are indicated by arrows.

Chapter 3: Safety in numbers: Shoaling as an anti-parasite defence in fathead minnows exposed to trematode cercariae

ABSTRACT

Individuals that live in groups can benefit from increased foraging success and decreased predation. There is evidence from ungulates exposed to biting flies that group living protects individuals from parasites, however, it is not known if analogous benefits accrue to aquatic animals exposed to infective stages of parasites. I tested for anti-parasite benefits of shoaling to fathead minnows exposed to infective stages (cercariae) of two common trematode species, *Ornithodiplostomum ptychocheilus* and *Posthodiplostomum minimum*. First, I evaluated shoal dimensions in groups of five minnows exposed to *O. ptychocheilus* cercariae in aquaria. Results showed that shoal area decreased 15-fold for groups exposed to cercariae compared to water controls. Second, I compared worm intensity between minnows experimentally confined to shoals versus experimentally isolated minnows within semi-natural outdoor mesocosms. Worm numbers were 3-fold higher for solitary versus shoaling minnows. Finally, results from a further infection trial showed that minnows located within the center of an artificial shoal had one third as many larval worms than non-shoaling minnows. Taken together, these results demonstrate that minnows can detect cercariae within the water column, form tight shoals in their presence, and occupy a central position within a shoal to reduce their risk of parasitism.

Introduction

Shoaling among prey fishes provides individual and group benefits associated with predator avoidance and foraging success. Such benefits tend to accrue in proportion to social cohesion among individuals within a shoal (Pitcher & Parrish, 1993). Risk dilution, predator confusion, and the ‘many eyes’ effect are three mechanisms that reduce individual risk during predation attempts (Pitcher & Parrish, 1993). In part, these foraging and antipredator benefits require attention to the social cues of shoalmates and consequently to acquisition of appropriate learned responses (Roberts, 1996; Brown & Lalands, 2003). For example, naïve individuals within a shoal can learn the location of successful foraging sites from experienced shoalmates and can associate olfactory and visual cues with predation risk. (Helfman & Schultz, 1984; Mathis *et al.*, 1996; Reeb, 2000).

For fish, the components of social living that make shoaling an effective anti-predator mechanism should hypothetically work to reduce the risk of parasite exposure, particularly for non-contagious parasites (Côté & Poulin, 1995; Wisenden *et al.*, 2009). Initial evidence for the anti-parasite benefits of group living comes from studies involving ungulate herds that are attacked by biting flies, such that individuals in closer proximity to conspecifics and those closer to the center of the herd are exposed to fewer flies (Helle & Aspi, 1983). A meta-analysis by Côté and Poulin (1995) demonstrated a negative correlation between encounters with mobile, non-contagious parasites and

host group size, and a positive correlation between contagious parasites and host group size. Poulin and FitzGerald (1989) showed a decrease in attacks on individuals from the ectoparasite *Argulus canadensis* with increased stickleback group size, and that individuals tended to shoal in the presence of the parasite. The degree to which shoaling reduces the risk of infection by other parasites, especially larval trematodes, has not been evaluated experimentally.

In this paper, we report the results from a series of experiments designed to test the hypothesis that shoaling reduces a fish's risk of exposure to trematode cercariae. Fathead minnows (*Pimephales promelas*, Rafinesque) are a group-living cyprinid fish abundant throughout most of North America. They are also a common intermediate host of various species of strigeid trematodes that utilize fish-eating birds as definitive host (Chubb, 1979). Infection occurs via penetration of cercariae through the host epidermis, typically en route to a specific site such as the eyes, brain, or muscle tissue, depending on the parasite species. Although the pathology caused by these trematodes to their hosts is context- and species-specific, several studies have documented a range of physiological and ecological effects on individuals (Sandland *et al.*, 2001; Shirakashi & Goater, 2005; James *et al.*, 2008; Chapter 2). Thus, in these and other host-parasite interactions, natural selection should favour evasive mechanisms to reduce infection risk, so long

as the parasites are detectable, they reduce host fitness, and the avoidance behaviour reduces exposure (Wisenden *et al.*, 2009). Results from several recent studies support this idea, indicating that fish reduce their overall activity and seek refuge in the presence of cercariae (Poulin *et al.*, 1991; Karyonen *et al.*, 2004). Cercariae penetration has also been shown to elicit an alarm response from shoal-mates via minnow chemical alarm cues released from ruptured epidermal cells, and individuals may obtain learned avoidance behaviour through chemo-association (Poulin *et al.*, 1999; Wisenden, 2000).

Ornithodiplostomum ptychocheilus (Faust) and *Posthodiplostomum minimum* (MacCallum) are diplostomid trematodes of fathead minnows that encyst within the brain or the body cavity, respectively (Schleppe, 2002). Both species use *Physa* snails as first intermediate host, fathead minnows as second intermediate host, and piscivorous birds as definitive hosts (Miller, 1954; Hendrickson, 1979). In Alberta lakes, the metacercariae of both species are found only in fathead minnows, typically infecting all minnows within a lake with 20-500 larvae/host (Sandland *et al.*, 2001; Schleppe, 2002). Both species are amenable to experimental manipulation under laboratory conditions. Previous experimental studies have shown that early developmental stages of *O. ptychocheilus* metacercariae reduce minnow growth rates (James *et al.*, 2008), alter visually-mediated behaviours (Shirakashi & Goater, 2005), and reduce survival (Sandland & Goater, 2001).

We performed three experiments to evaluate the anti-cercariae benefits of shoaling in fathead minnows. First, we compared the dimensions of minnow shoals in aquaria before and after they were exposed to cercariae. We evaluated whether minnows could distinguish cercariae from various stimuli within the water column, and if so, if they could respond behaviourally by decreasing overall shoal dimension. In a second experiment, we used outdoor mesocosms containing infected snails to evaluate the risk of exposure in shoaling versus solitary minnows. For this experiment, we used mesh cages that confined the minnows into an artificial shoal, but allowed free-swimming cercariae to pass through. Lastly, we used similar cages to confine minnows with or without peripheral conspecifics to determine if the central position in a shoal reduces an individual's risk of exposure to trematode larvae.

Materials and Methods

Experiments were performed at the University of Lethbridge, AB, Canada during summer 2010. All minnows used in these experiments originated from a small pond located in southern Alberta (Stirling Lions Club Fish Pond, AB; 49.501, -112.537). I collected large numbers of young-of-the-year (YOY) fish in June, 2010. A sub-sample of 20 YOY indicated a background infection with *O. ptychocheilus* metacercariae (mean = 6.9 ± 1.8). Minnows were maintained within two separate 1200-L outdoor mesocosms (108-cm diameter, 120-cm height; Pearson & Goater 2008) prior to experiments.

I used the methods described in Sandland and Goater (Sandland & Goater, 2000) for experimental infections. One-day-old chickens were fed minnow brains containing *O. ptychocheilus* metacercariae or minnow viscera containing *P. minimum* metacercariae from fish collected from a local lake. Trematode eggs were collected from the chicken feces 3-5 days after exposure to metacercariae. Eggs were incubated following Sandland and Goater (Sandland & Goater, 2000) and the resulting miracidia were exposed to the F1 generation of laboratory-reared *Physa* snails. Exposed snails were housed in 2-L plastic containers at 20° C, with a 16:8 L:D photoperiod and fed boiled romaine lettuce daily. For experiments 1 and 3, which required exposing minnows to known doses of cercariae, infected snails were placed into glass vials of dechlorinated water under direct light to promote cercarial release. The cercariae from all infected snails were pooled into a 100-mL flask and diluted to 100-mL with dH₂O. Estimates of the numbers of cercariae/mL were calculated from 3, 1.0-mL aliquot samples. The volume containing the required number of cercariae for each dose was then estimated.

Shoal dimensions following exposure to cercariae

The aim of this experiment was to determine if the presence of cercariae in the water column caused minnows to increase shoal cohesion. I also evaluated whether minnows can distinguish infective cercariae from other potential novel stimuli in the water column, including other (non-infective)

species of cercariae. I exposed groups of five minnows to one of the following 6 stimuli: 60-mL dechlorinated water (control), 500 2-3 hour old *O. ptychocheilus* cercariae in 60-mL water, 500 2-3 hour old *Plagiorchis elegans* (Rudolphi) cercariae in 60-ml water (this trematode uses a range of insect larvae as intermediate hosts; Genov & Samnaliev, 1984; naturally infected snails were obtained from Park Lake, Alberta, 49.807388,-112.927995), minnow chemical alarm cues (using minnow skin fillets; Wisenden, 2000), 0.25-g of cleaned and sieved standard playground sand (125-400 μm), 3 drops vanilla extract. The last two stimuli were used to control for the potential effects of presenting a novel physical and chemical cue, respectively. Eight trials were performed daily over a period of 6 d within four 37-L tanks divided in half with black Plexiglas. Order effects were controlled by rotating stimuli among the 8 replicate tanks.

Five minnows were selected at half-hazardly for each trial and placed into each half tank 20 h prior to the trials to allow for acclimation to the tank apparatus. A stimulus injection hose was wedged into a sponge filter producing a stream of air bubbles to mask pressure change during stimulus injection and to allow for rapid dispersion of each stimulus. Just prior to each trial, 30-mL of water was withdrawn through the stimulus hose and discarded to remove any stagnant water within the hose. Another 30-mL was acquired and retained to completely flush test stimuli from the injection hose

during stimulus injection. Each trial was video recorded from behind a black cloth blind for 11 min, comprising a 5-min pre-stimulus period, a 1-min stimulus injection period, and a 5-min post-stimulus period. The dimensions of each 5-minnow shoal were calculated using ImageJ software every 15 s during the pre- and post-stimulus periods. The 2-dimensional area (x and y coordinates) was determined by calculating the perimeter of the polygon made from lines that joined the heads of each fish. A paired samples t-test was used to compare means of pre- and post-stimulus polygon area to determine if a change in shoaling cohesion had occurred. The average total area of the pre-stimulus polygon was subtracted from the average post-stimulus polygon to assess change in shoal dimensions following stimulus injection. A one-way ANOVA was used to compare treatment means and Tukey post-hoc comparisons were used to test for differences ($P < 0.05$) between selected pairs of stimuli.

Risk of parasitism within artificial shoals

The aim of this experiment was to determine if, under semi-natural conditions, minnows in shoals reduce their risk of infection relative to solitary minnows. I confined minnows to mesh cages that were placed into four outdoor mesocosms (1200-L; 108-cm diameter x 120-cm height) where they fed on abundant phytoplankton and zooplankton communities (Pearson & Goater, 2008). Three days prior to the addition of the caged minnows, two

Physa gyrina snails that I had confirmed were infected with *P. minimum* were added to each mesocosm.

Minnows were assigned at random to one of three cage types: 5 minnows in a 1000-cm³ cage, representing the shoaling treatment (L-5; shoaling), a single minnow in a 1000-cm³ cage (L-1; non-shoaling), and single minnow in a 200-cm³ cage (S-1; non-shoaling). The purpose of the using the small and large cages for the single fish was to control for proportional volume or overall available volume.

Cages were constructed with Trical plastic netting (3-mm mesh) and fastened together with monofilament fishing line. Each cage was naturally buoyant and was suspended just below the water surface, weighted down with a metal weight tied to the bottom. Three L-5 and four each of the S-1 and L-1 cages were placed into half-hazard positions within each mesocosm (Fig 3.1). The period of potential infection lasted 3-d. Thereafter, surviving minnows were housed in 2-L plastic containers labelled according to their cage and mesocosm of origin. Fish had a 16:8 L:D photoperiod and were fed commercial flake food *ad libitum* for 16 d, after which fish were necropsied and metacercarial intensity was assessed. Data from individual minnows in the L-5 treatment were not independent therefore mean metacercarial intensity was determined for each shoaling cage. A 1-way ANCOVA was used to

compare means between non-shoaling treatments (S-1, L-1), with the 4 mesocosms treated as blocks and minnow length at time of necropsy treated as a covariate. A 1-way ANCOVA was used to compare means between shoaling and non-shoaling treatments, with the 4 mesocosms treated as blocks and minnow length at time of necropsy treated as a covariate.

Risk of cercariae exposure relative to position within a shoal

The aim of this experiment was to determine if minnows confined to a central position within a shoal obtained fewer *P. minimum* metacercariae than solitary minnows. For this experiment, I constructed artificial shoals from the same materials as described above, configured to have a single central minnow and 5 peripheral ones (Fig. 3.2). The shoaling configuration consisted of a focal minnow in the central cage, with an additional minnow in each peripheral cage. The solitary configuration consisted of a focal minnow in the central cage and none in the peripheral cages. The purpose of the empty peripheral cages around the central minnow was to control for cage effects so that individual cercariae had to pass through the same number of cage walls to reach the focal minnow in the center cage.

Both shoaling units were placed on one side of a 31.5-L plastic container and 75 2-h old cercariae were added to the opposite side of the container 4-h later. Trials were run for 4 h, after which minnows were housed in 2-L plastic containers with a 16:8 L:D photoperiod and fed commercial flake food *ad*

libitum for 16-d, and then assessed for metacercariae intensity. Trials were run over 3-d for a total of 7 replicates. A paired samples t-test was used to assess mean difference between minnows with versus without peripheral conspecifics. Individual plastic containers were treated as replicates.

Results

Shoal dimensions following exposure to cercariae

There was no significant change in polygon area between pre- and post-stimulus for control ($t_7 = 0.50$, $p = 0.634$) or vanilla extract ($t_7 = 1.15$, $p = 0.288$) treatments. However, there was a significant change in pre- and post-stimulus area for *O. ptychocheilus* ($t_7 = 4.63$, $p = 0.002$), *P. elegans* ($t_7 = 3.21$, $p = 0.015$), alarm substance ($t_7 = 5.12$, $p = 0.001$), and sand ($t_7 = 2.53$, $p = 0.039$) following stimulus injection.

The average change in shoal polygon area was strongly affected by treatment (one-way ANOVA, $F_{5, 42} = 15.735$, $p < 0.001$, Fig. 3.3). A Tukey HSD post-hoc showed a significantly greater decrease in shoal area of fish exposed to *O. ptychocheilus* cercariae than for fish in the water controls ($p = 0.018$), and the vanilla extract and sand treatments ($p < 0.001$). However, the change in size of *O. ptychocheilus*-exposed shoals was not significantly different from shoals exposed to minnow alarm substance ($p > 0.999$). Results of post-hoc comparisons showed no significant differences in mean change in shoal size between the control, vanilla extract, and sand stimuli ($p > 0.50$). There was a

significant increase in shoal area for shoals exposed to *P. elegans* cercariae compared to other stimuli ($p < 0.05$) except for sand ($p = 0.224$).

Risk of parasitism within artificial shoals

Of the 100 minnows used in this experiment, 86 survived the exposure and the metacercariae development periods, with 5% escaping confinement prior to collection and 9% mortality after collection. These minnows were not included in the analyses. There was extensive variation in *P. minimum* intensity between and within mesocosms, with minnows harbouring between 8 and 311 metacercariae (Table 3.1). Metacercarial intensity ranged from 37 to 311 for solitary minnows (large and small cages) and from 8 to 178 for minnows in the artificial shoals. A frequency distribution of worm numbers/host emphasizes this variation (Fig. 3.4).

When mesocosms were treated as blocks and minnow body length as a covariate, metacercariae intensity was not significantly different between the two non-shoal treatments, i.e. the small versus large containers that contained a single minnow (two-way ANCOVA, $F_{1, 21} = 0.04$, $p = 0.948$). There was also no significant effect of the covariate ($F_{1, 21} = 0.07$, $p = 0.792$), blocks ($F_{3, 21} = 0.04$, $p = 0.173$), or the interaction between treatment and blocks ($F_{3, 21} = 0.47$, $p = 0.706$). Because mean metacercariae intensity was the same between the different sized containers that contained a single fish, I pooled these data to focus our analysis on shoaling versus non-shoaling fish. With

the mesocosms treated as blocks and minnow body length as a covariate, metacercariae intensity was significantly different between shoaling and non-shoaling treatments (two-way ANCOVA, $F_{1, 33} = 7.23$, $p = 0.011$, Fig. 3.5), with fish harbouring 70.0 ± 6.9 metacercariae in shoaling configurations and 132.4 ± 13.8 in non-shoaling configurations. Body length was not a significant predictor of metacercarial intensity ($F_{1, 33} = 0.01$, $p = 0.911$). Metacercariae intensities were highly variable among the 4 mesocosms, but differences between the mesocosms were not significant ($F_{3, 33} = 0.72$, $p = 0.548$) and there was no interaction between treatments and blocks ($F_{3, 33} = 1.90$, $p = 0.148$).

Risk of cercariae exposure relative to position within a shoal

Metacercarial intensity was significantly higher in minnows with no peripheral conspecifics compared to minnows with conspecifics in peripheral locations (paired sample t-test, $t_6 = 3.89$, $p = 0.008$, Fig. 3.6). Minnows without peripheral conspecifics harboured 8.9 ± 1.5 metacercariae, whereas minnows with peripheral conspecifics harboured 2.9 ± 0.3 metacercariae. Though not included in statistical analysis due to possible cage effects, mean metacercarial intensity for peripheral fish was 11.5 ± 1.8 .

Discussion

Results from our first experiment indicated that fathead minnows distinguish infective cercariae from other stimuli and exhibit a behavioural

response that is comparable in magnitude to an alarm response stimulated by cues indicative of predation. The ability to discern threatening stimuli from non-threatening stimuli is critical to determining behavioural avoidance strategies to a wide range of infective stages (Wisenden *et al.*, 2009). In general, when the numbers of fish in an artificial shoal were kept constant, the dimensions of the shoal decreased significantly during exposure to *Ornithodiplostomum* sp. cercariae. This decrease was greater than it was for novel chemical or physical stimuli, indicating a specific behavioural response independent of a sudden introduction of a visual or olfactory stimulus. The opposite behavioural response was observed when minnows were exposed to non-minnow cercariae, further indicating that the adjustment in shoal cohesion in response to *Ornithodiplostomum* sp. is not a general response to 'any' cercariae within the water column.

Several mechanisms could lead to the detection of cercariae, such as direct olfactory detection or direct visual observation. James *et al.* (2008) determined that fathead minnows that had previous exposure to live (infective) *Ornithodiplostomum* sp. cercariae reduced their activity when re-exposed to frozen (non-infective) cercariae. The minnows used in our experiment, however, had no or at least limited previous exposure to *O. ptychocheilus* cercariae. As for many other species of trematode cercariae, penetration of *O. ptychocheilus* through the epidermis causes a characteristic

shaking and twitching by the host, probably due to general discomfort. This response may appear as a potential threat to conspecifics and cause the observed contraction of the shoal during exposure. Further, Poulin *et al.* (1999) presented evidence that cercarial penetration may elicit an alarm response in conspecifics by rupturing club cells in the fish epidermis and releasing chemical alarm cues (i.e. *Schreckstoff*). Our results are consistent with the observations of Poulin *et al.* (1999) because the intensity of alarm response in our experiment was similar for minnows exposed to *O. ptychocheilus* and minnows exposed to chemical alarm cues.

The contrasting shoaling response of minnows exposed to the two different species of cercariae was striking. In the case of *O. ptychocheilus* cercariae, minnows formed tighter shoals relative to water controls, whereas those exposed to *P. elegans* cercariae distributed themselves throughout the aquaria. One important implication of these results is that minnows can not only directly or indirectly detect cercariae, but can also distinguish between species and then alter their evasive behaviours accordingly. The cues that minnows use to distinguish among species of cercariae are unknown. One possibility is that the process of penetration of even small numbers of *O. ptychocheilus* may provide the only necessary cue to shoal, whereas in the absence of penetration, cercariae may be perceived as food. Size-selected

foraging on cercariae has recently been demonstrated in a number of fish/trematode interactions (e.g. Kaplan *et al.*, 2009).

Results from our third experiment support the hypothesis that fish occupying an interior position within a shoal reduce their exposure to trematode cercariae. This is the first experimental evidence to show that position within a shoal can reduce rates of cercariae exposure. Helle & Aspi (1983) showed that *Rangifer tarandus* (reindeer) closer to the interior of a herd and those closer to herd mates were attacked less by biting flies than those on the exterior of a herd. Similarly, Grosholz (1994) showed that *Transennella tantilla* (marine mussels) attached to peripheral edges of a cluster experienced higher rates of exposure to cercariae of *Parvatrema borealis* compared to those attached to central locations. In this case, hosts on the perimeter of host aggregations were considered “sinks,” acting to dilute the availability of cercariae to the interior hosts. In the case of minnow shoals, a similar mechanism may explain the significant reduction in exposure of central minnows to *O. ptychocheilus* cercariae.

Many factors contribute to the natural spatial positioning of shoaling fish. Most studies have focused on predation risk and energy requirements as key factors for determine positioning choice within a shoal. Peripheral and front positions provide greater foraging opportunities, and are often utilized by

individuals with greater energetic needs, such as larger or malnourished fish (Bumann & Krause, 1993; Krause, 1993). Predation risk, however, is greater for fish occupying these locations (Bumann *et al.*, 1997) and a trade-off arises to balance threat and energy needs (Krause, 1993). These results suggest that the spatial structure of a shoal is dynamic, although some studies have shown a “sustained position preference” over prolonged periods of time, with innate behavioural traits (e.g. shy versus bold fish) influencing spatial positioning, and a tendency for larger individuals to occupy positions at the front of a shoal (Bumann & Krause, 1993; Bumann *et al.*, 1997; Ward *et al.*, 2004; Leblond & Reeb, 2006). Although our results demonstrate a clear linkage between spatial position within a shoal and risk of exposure to cercariae, follow-up studies are needed to clarify the temporal component of shoal positioning under natural conditions relative to the temporal pattern of release of cercariae from snails.

The results from empirical studies and from field surveys have shown that some shoaling fish infected with metacercariae tend to occupy peripheral shoal positions (Krause & Godin, 1994; Barber & Huntingford, 1995). This pattern is typically attributed to adaptive alterations in host behaviour and to conspecific segregation that can facilitate transmission to definitive hosts. Further, Ward *et al.* (2002) and Barber & Huntingford (1995) suggest that metabolic costs associated with infection may also explain the tendency for

more heavily infected fish to occupy peripheral positions in a shoal. An alternative possibility suggested by our results is that fish in peripheral positions are more at risk of exposure to free-swimming cercariae. It is not possible to distinguish these alternatives until we know more about the stability of shoal positioning within natural ponds and lakes. Indeed, it is possible that all three mechanisms operate within natural shoals to determine the tendency for peripheral fish to have higher numbers of parasites. The action of multiple mechanisms would help explain the remarkably high variation in worm counts that we observed between individual minnows in the mesocosm experiment.

Results from the mesocosm experiment provide evidence that shoaling, beyond reducing infection risk for central fish, reduces the overall metacercariae intensity of a shoal as a whole. These results imply that all or most fish within a shoal benefit from shoal membership relative to solitary fish. These data support results from field collections performed by Ward *et al.* (2002) where randomly selected individuals collected from within *Fundulus diaphanus* (banded killifish) shoals had fewer cysts than non-shoaling conspecifics. One explanation for this pattern is the encounter-dilution effect, observed also in ungulate systems where the numbers of blood-sucking flies does not increase with herd size and the number of individuals infected by each parasite is limited (Mooring & Hart, 1992).

Shoaling may provide an anti-parasite benefit to individual fish in the same manner as anti-predator behaviour; however, it cannot be assumed the parasites themselves would experience a disadvantage to this behaviour as a predator would. I have shown that shoaling fish receive fewer metacercariae on average than non-shoaling minnows (Fig. 3.5). It can be speculated, however, that cercariae may benefit via a greater number of potential hosts. The number of successful infections may increase while simultaneously diluting the number of cercariae that will infect an individual fish. Though this is similar to Poulin & FitzGerald (1989), which showed that increased group size diluted the number of attacks of a crustacean ectoparasite per individual, but did not have a negative effect on parasite success, the mechanisms of trematode systems may differ in such a way to actually increase parasite fitness. This may be due to the overwhelming numbers of cercariae that a shoal may encounter, or the attack mechanisms of this larval trematode. Further experimental or modeling studies should be performed to address this question.

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Table 3.1. Summary infection characteristics for the effects of shoaling on mean *P. minimum* metacercariae intensities between mesocosms and between treatments. L-5 = minnows in artificial shoals; S-1 = solitary minnows in small cages; L-1 = solitary minnows in large cages.

Pond	Treatment	n	mean \pm se	range	median	CV (%)
1	L-5	3	71 \pm 4	63 - 76	70	9
	S-1	3	197 \pm 65	86 - 311	195	57
	L-1	4	172 \pm 41	72 - 272	173	48
Pond Summary		10	149 \pm 29	63 - 311	125	61
2	L-5	3	48 \pm 8	36 - 64	43	31
	S-1	5	112 \pm 24	66 - 192	90	49
	L-1	5	160 \pm 35	83 - 263	133	49
Pond Summary		13	105 \pm 21	36 - 263	83	71
3	L-5	3	75 \pm 12	60 - 99	67	29
	S-1	3	137 \pm 28	81 - 167	162	35
	L-5	4	110 \pm 54	37 - 267	68	99
Pond Summary		10	105 \pm 23	37-267	82	70
4	L-5	3	88 \pm 20	49 - 116	98	40
	S-1	4	79 \pm 17	52 - 130	68	44
	L-1	3	100 \pm 41	56 - 181	62	71
Pond Summary		10	86 \pm 25	47 - 181	63	51

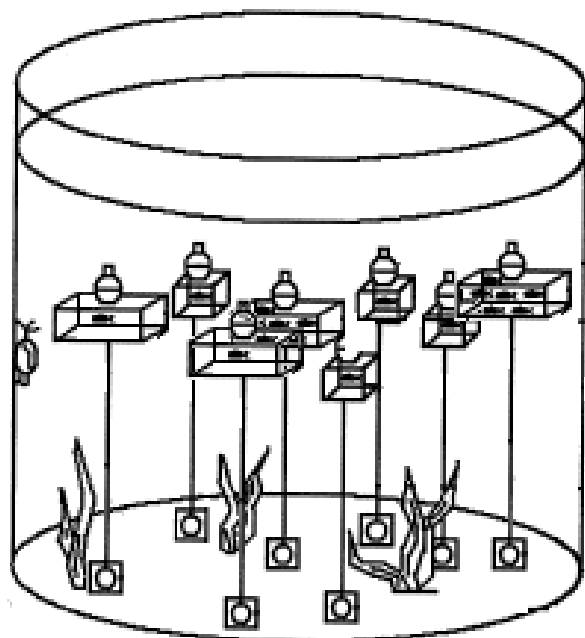


Figure 3.1. Schematic representation of the design of the mesocosms used to evaluate the role of shoaling in reducing a minnow's risk of cercariae exposure.

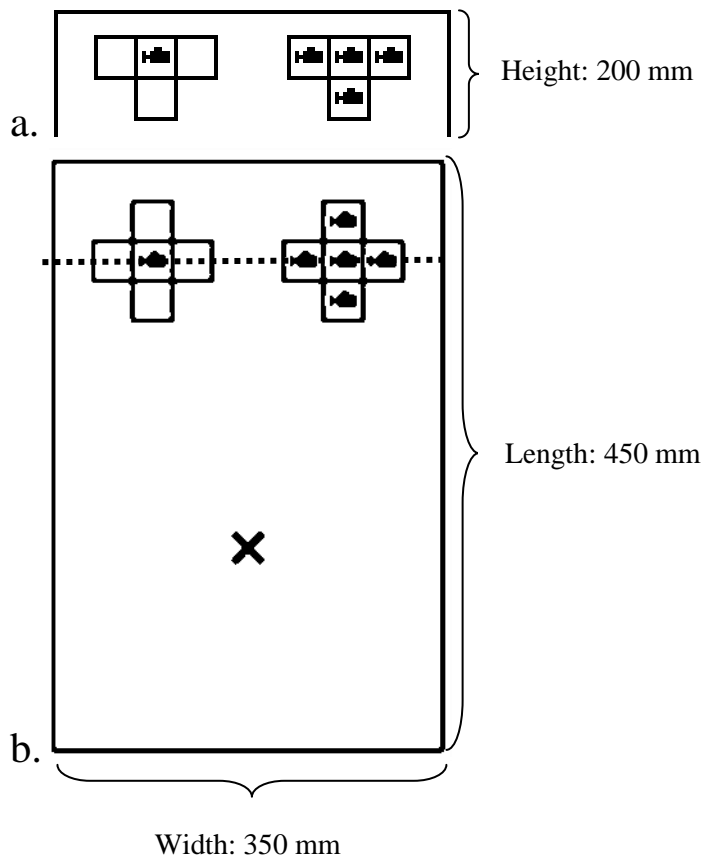


Figure 3.2. Schematic representation of experimental setup for the effect of position within an artificial shoal on the risk of a minnow's exposure to trematode cercariae. a) Side cross section view signified by dashed line in (b). b) Bird's eye view. "X" represents point where cercariae were introduced.

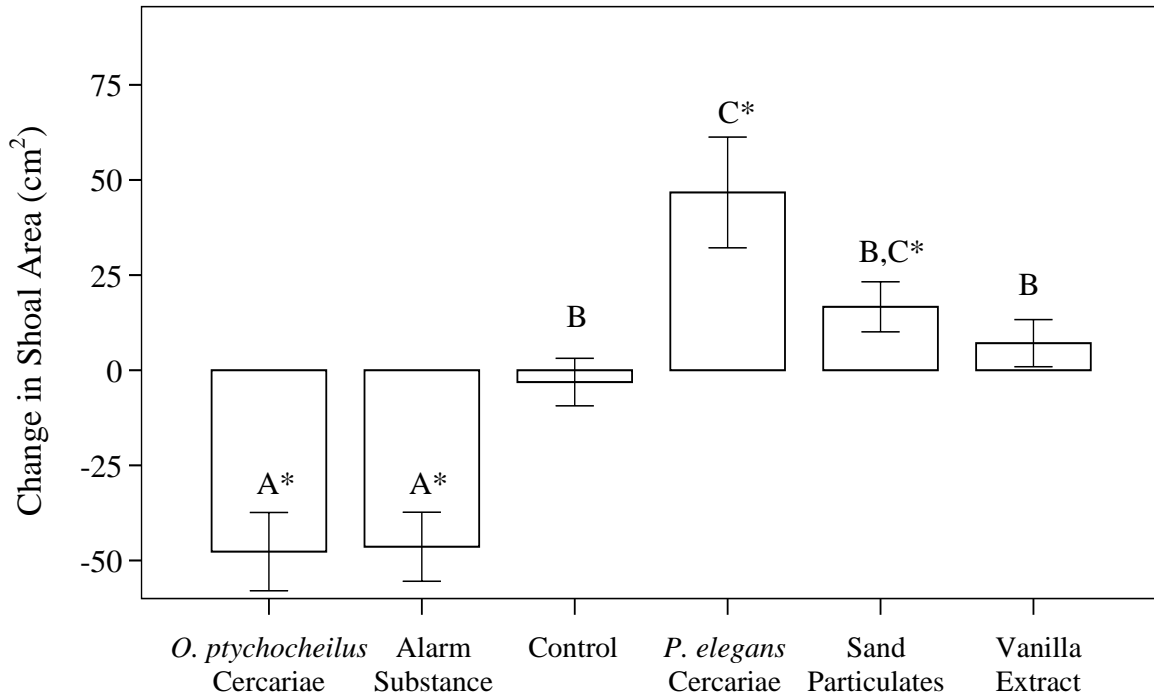


Figure 3.3. Mean (\pm se) change in shoal area for minnows exposed to various stimuli, including *O. ptychocheilus* cercariae. Significant change in shoal area after stimulus injection is indicated by *.

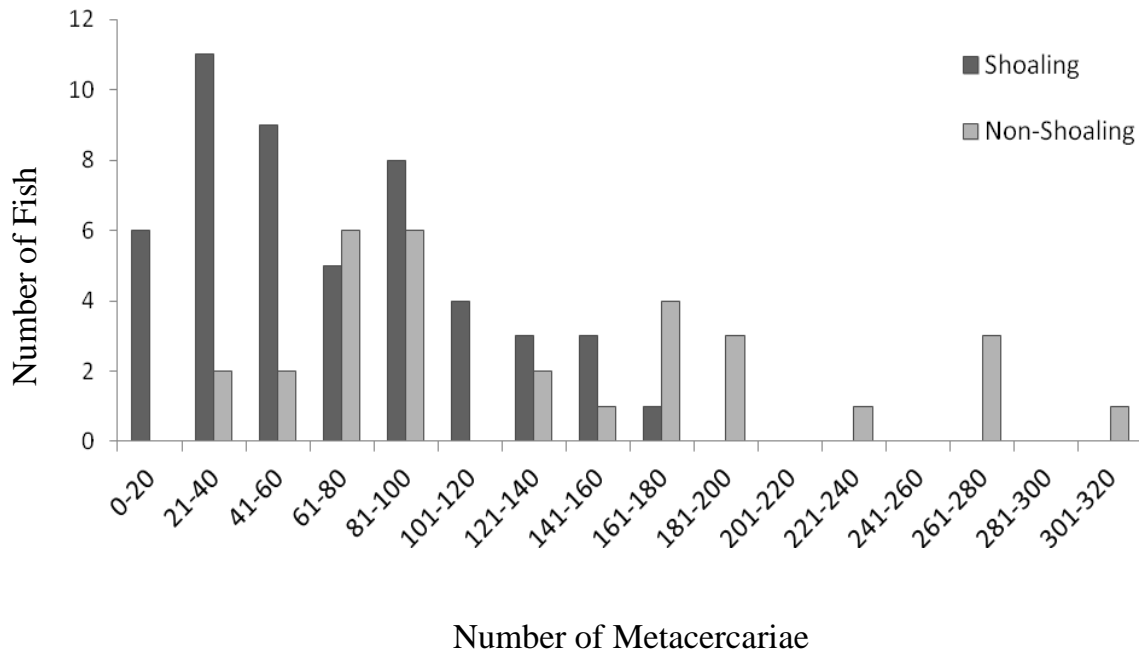


Figure 3.4. Frequency distribution of *Posthodiplostomum minimum* metacercariae in fathead minnows confined to non-shoaling or shoaling configurations.

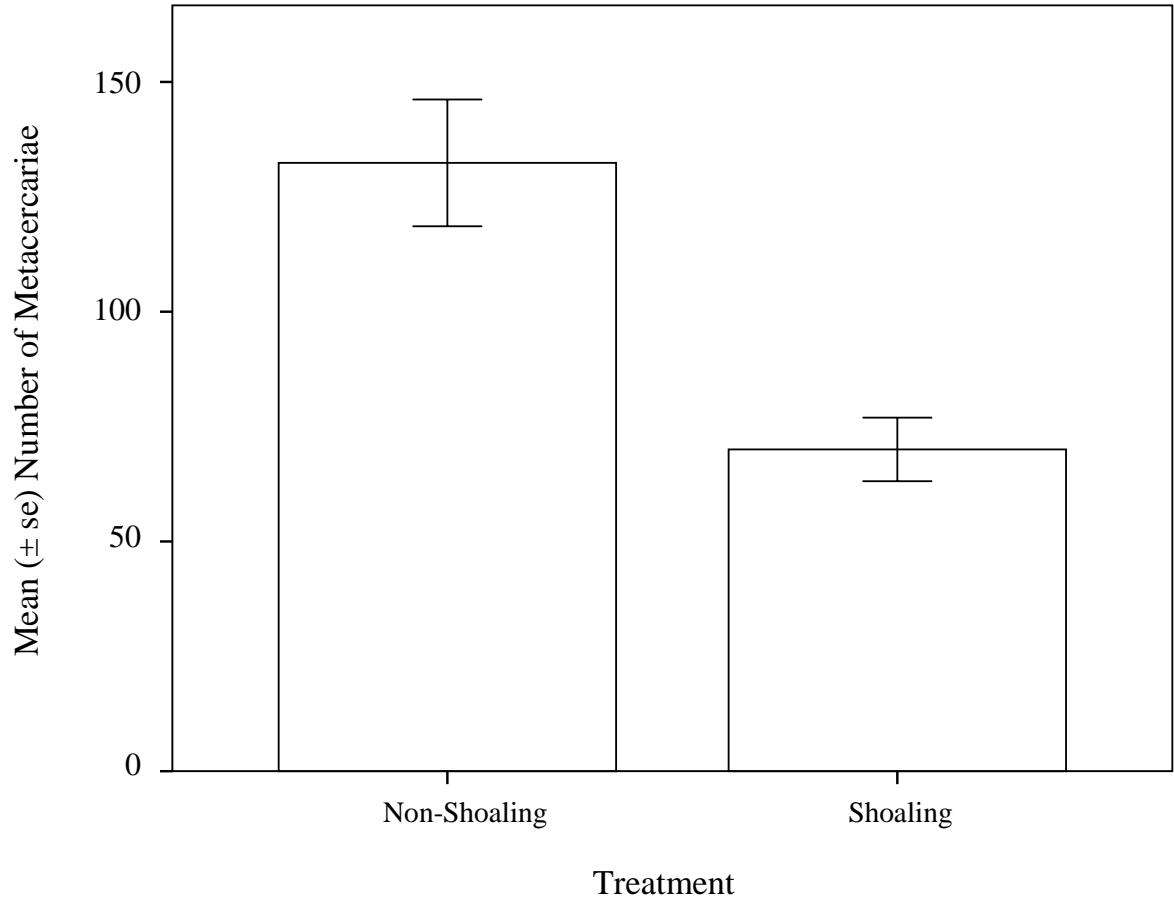


Figure 3.5. Mean (\pm se) *Posthodiplostomum minimum* metacercariae intensity in fathead minnows confined to non-shoaling or shoaling configurations.

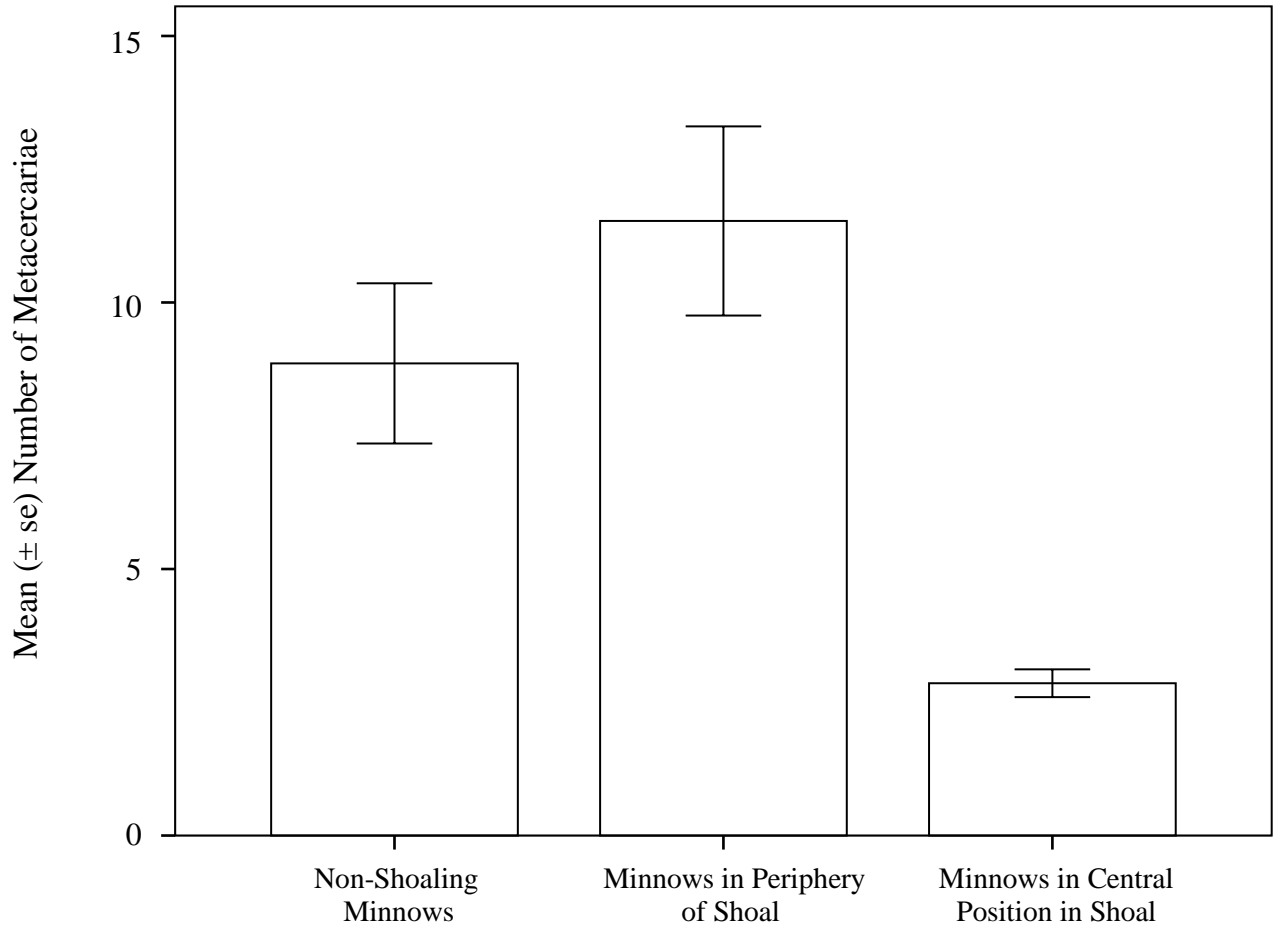


Figure 3.6. Mean (\pm se) *Posthodiplostomum minimum* metacercariae

intensity in fathead minnows confined in 6-chambered housing units.

Minnows were either in the central chamber with no peripheral fish (non-shoaling) or with peripheral fish (shoaling), or in the peripheral chambers (shoaling).

Chapter 4: General Conclusions

Wisenden *et al.* (2009), following earlier suggestions by Hart (1994), identified three requirements needed to classify a host behaviour as a parasite avoidance trait: the parasite must have a negative effect on host fitness, the potential host must be able to directly or indirectly detect the parasite, and the behaviour must mitigate infection through reduced exposure or limiting the cost to fitness. The experiments described in this thesis were designed, in part, to address these three requirements within a model trematode/fish interaction.

The combined results from experiments described in Chapters 2 and 3 show that the requirements for parasite avoidance are met in the fathead minnow/*Ornithodiplostomum* spp. interaction. Thus, exposure to cercariae of *Ornithodiplostomum* sp. was associated with immediate and prolonged oxidative stress in minnow liver tissue. This result confirms those from other experimental studies on infected minnows (Sandland & Goater, 2001; Shirakashi & Goater, 2005; James *et al.*, 2008) and indicates that cercariae penetration, migration, development, and/or encystment reduces the performance of individual fish, and may therefore reduce host fitness. It follows that natural selection should favour behavioural mechanisms to reduce a minnow's exposure to *Ornithodiplostomum* spp. cercariae. One of the key results from Chapter 3 is the combined evidence that minnows can

detect and distinguishing infective cercariae from various stimuli, including non-infective cercariae and that minnows shoaling behaviour reduces exposure.

The experiment on parasite-induced oxidative stress in Chapter 2 provided three key results. First, minnows exposed to *Ornithodiplostomum* sp. cercariae had elevated concentrations of reactive oxygen species in their liver tissue compared to unexposed controls. Since ROS is a well-established indicator of oxidative stress (Kelly *et al.*, 1998), these results provide the first experimental verification that exposure to even low doses of cercariae can lead to long-term physiological costs. The measurable effect in minnows in the low-dose treatment indicates oxidative stress as a method for evaluating the cost of low-intensity infections, and future studies may focus on the extent of this phenomenon (i.e. exposure to 1, 5, 10, 15 and/or 20 cercariae), and if similar results occur in minnows infected with varying intensities of *O. ptychocheilus*. Second, the increase in ROS was associated with the obligate developmental stage of *Ornithodiplostomum* sp. Thus, the elevated concentrations of ROS that persisted to 28 days post infection were most likely attributable to carry-over effects of earlier damage caused by developing worms. Lastly, off-target effects of *O. ptychocheilus* infection showed a significant interaction between time and treatment compared to controls. Marcogliese *et al.* (2005) alluded to a similar off-target effect,

suggesting inflammation as a possible mechanism. If this is the case for *O. ptychocheilus* infection, the parasite may be causing inflammation while imbedded within the minnow optic lobe, as well as causing pathological effects in non-targeted tissue. Analysis of oxidative stress in additional tissue such as brain, muscle, and pancreas would give valuable insight into *O. ptychocheilus* pathology.

Individual fathead minnows are infected with up to 12 different species of strigeid trematode (Sandland et al., 2001). In southern Alberta, individuals are almost always infected with both *Ornithodiplostomum* sp. and *O. ptychocheilus* (Goater, personal communication). Since both *Ornithodiplostomum* sp. and *O. ptychocheilus* cause a spike in oxidative stress, one key follow-up line of enquiry will be to assess the additive versus synergistic affect of multi-species infections. Hosts from various taxa infected with multiple parasite species show increased cost compared to individual infection (see Bordes *et al.*, 2011 for review). For example Davidar & Morton (2006) showed purple martins infected with either the haematozoan *Haemoproteus prognei* or an unidentified filarial nematode exhibited little or no cost when present alone in a host, but 90% mortality when infected with both. Romansic *et al.* (2011) showed higher mortality in the Pacific tree frog when infected with both the trematode *Ribeiroia* sp. and the fungus *Batrachochytrium dendrobatidis*, than individuals infected with one species.

Given that even a non-target species (*O. ptychocheilus*) increased the concentration of ROS in liver tissue, it suggests that the structure of the entire parasite community may be an important determinant of overall levels of oxidative stress. The fathead minnow/ *Ornithodiplostomum* spp. interaction is an ideal model to test this hypothesis.

The key direction for future studies addressing parasite induced oxidative stress is the determination of proximate mechanisms. Although I cannot definitively state the mechanism responsible for my observed results, the results from previous studies on oxidative stress in fish indicates tissue damage and immunopathology as the two most likely possibilities. The *Ornithodiplostomum* spp. systems provide an ideal opportunity for distinguishing pathology from immunopathology with careful experimentation. The determination of immuno-activation via respiratory burst assays (Muñoz *et al.*, 1998) in infected individuals would provide a foundation for further manipulation. Further, various means exist for testing minnows experiencing immuno-suppression. Male fathead minnows, for example, experience impaired immune function during the breeding season due to increased testosterone production (Smith, 1973; Halbgewachs *et al.*, 2009). Immune function can also be artificially suppressed via cortisol injection (Halbgewachs *et al.*, 2009) or controlled stress (Pickering & Pottinger, 1989). Immune function is likely to be limited in brain tissue

relative to liver tissue, due to the presence of the blood/brain barrier. These scenarios, in combination with the ease of manipulation of *Ornithodiplostomum* cercariae, provide interesting possibilities for experiments designed to distinguish the role of immunopathology in determining parasite-induced oxidative stress.

The observed increase in the level of ROS has important implications for the cost of trematode infection in fish. First, *Ornithodiplostomum* spp. has been shown to result in altered development, reduced growth, and altered behaviour in fathead minnows (Sandland & Goater, 2001; Shirakashi & Goater, 2001, 2002; James *et al.*, 2008). The mechanisms underlying these costs are ambiguous and increased oxidative stress may act as a unifying contributing or indicative factor. For example, Sandland & Goater (2001) demonstrated cranial distortion in *O. ptychocheilus*-infected minnows due to inflammation associated with metacercariae development. Presumably, such inflammation would likely lead to the production of ROS. Similarly, the mechanism behind the reduced growth of *Ornithodiplostomum sp.*-infected minnows demonstrated by James *et al.* (2008) may be associated with elevated ROS levels, or the damage and repair of liver tissue. A second implication is the presence of the ROS themselves. While previously shown costs of trematode infection may be symptomatic of elevated ROS, the presence of ROS will express additional costs to infected hosts such as

cellular damage (Spector, 2000). Additional studies need to focus on the extent to which ROS may alter a host's fitness via physiological damage or facilitated transmission to definitive hosts, possible links of ROS to other previously shown costs, and the metabolic cost of combating ROS through antioxidant production and damage repair.

The results in Chapter 3 are the first to show that minnows can recognise a cercarial threat, and respond in a manner comparable to a predation threat. The key unknown here lies in the mechanism of recognition, though a response to tactile discomfort due to cercariae penetration, or the release of alarm substance from cells ruptured during penetration (Poulin *et al.*, 1999) are likely explanations. In either case, the number of minnows that need to experience this cue to elicit a response by the group in shoalmates is not known. When common minnows (*Phoxinus phoxinus*) observe a predation event, a threat response is elicited in conspecifics that cannot directly observe the predator (Maguran & Higham, 1988). The transfer of observed threat is so strong in group-living fish that a response to a potential threat can be transferred between individuals of differing species (Krause, 1993). In addition to visual observation of neighbouring fish behaviour, individuals may be alerted to potential threats via chemical cues. These cues may be associated with the release of alarm substance (Wisenden, 2000) or an alarm pheromone (Jordão & Volpato, 2000) released from one or more fish that have

detected a potential threat. It may be the case that for free-swimming cercariae only a few, and perhaps only one, fish is required to perceive a threat or experience cercarial penetration to elicit an alarm response in the remaining shoalmates. As above, this hypothesis is testable with the minnow/*Ornithodiplostomum* spp. model system.

The change in shoal dimensions observed in minnows exposed to *P. elegans* cercariae was a surprising result. When these non-infective cercariae were introduced to the aquaria, the minnows distributed themselves throughout the water column. Cercariae have recently been suggested as a previously overlooked, yet essential part of community food webs, with a number of aquatic species reported to feed on this larval stage (Schotthoefer *et al.*, 2007; Hopper *et al.*, 2008; Kaplan *et al.*, 2009). Additionally, fish shoal cohesion is altered with regards to feeding area, with fish increasing or decreasing shoal cohesion to fully inhabit the available foraging area (Keenleyside, 1955; Morgan, 1988). Thus, minnows foraging on cercariae would be expected to disperse throughout the limited foraging area of an aquarium to maximize individual acquisition. If the minnows in my experiment were foraging on *P. elegans* cercariae, this is the first controlled experiment demonstrating cercariae feeding behaviour by fish. Future studies need to confirm that cercariae are actually being ingested, possibly via gut content analysis aided

by the use of vital fluorescent dyes (e.g. Keeney *et al.*, 2008 for trematode cercariae), or labelling with various radioisotopes (Reid *et al.*, 1977).

While the results from chapter 3 indicate that minnows can distinguish infective (*O. ptychocheilus*) from non-infective (*P. elegans*) cercariae, the mechanism for this differentiation are unknown. A possible explanation is innate recognition of infective cercariae. This is doubtful considering the numerous species of cercariae, infective and non-infective, that a minnow may encounter within the water column, and the different species compositions between water bodies. James *et al.* (2008) demonstrated that naïve minnows did not change activity or water column depth when exposed to a cercariae cue, presenting a visual and olfactory introduction without infectivity. Thus, innate recognition of cercariae is unlikely. A second possibility is that naïve/juvenile minnows may consider all mobile, detectable cercariae as a potential food source. While the minnows in my experiment appeared to immediately distinguish cercariae species, the aquaria presented limited space for cercariae dispersal. In a natural environment, minnows may observe a cercariae cloud and attempt to feed, at which point they may or may not detect the cercariae as a potential threat. If this is true, a potential trade-off may arise. Will minnows attempt to feed on infective cercariae if alternative energy sources are limited? If so, will the metabolic gain outweigh the cost of infection?

Results from the mesocosm experiment showed that average metacercariae intensity was significantly less in shoaling individuals than non-shoaling individuals. Few studies have experimentally demonstrated a reduction in parasitism as a result of grouping in fish (e.g. Poulin & FitzGerald, 1989), and it is the first such evaluation in a trematode system. Although the intent of the mesocosms was to test this hypothesis in a semi-natural environment, a potential drawback of this design was the restriction in movement due to cage design. Given the increased vigilance of shoaling fish, I speculate that reduction of metacercariae intensity would be more pronounced in a natural environment. This would be consistent with results from Ward *et al.* (2002), who showed that killifish sampled from shoals had fewer metacercariae than singletons. It cannot be determined however, if the higher burdens in non-shoaling killifish observed in Ward *et al.* (2009) were due to their solitary nature or if higher burdens caused isolation. Future studies may address this concern by infecting individuals within a shoal and characterizing shoalmate interactions. Furthermore, the vigilance of shoals of varying sized and solitary minnows in response to infected snails, and therefore cercarial “hot spots” may be observed in natural and artificial water bodies.

Although results from experiment 2 showed that shoaling minnows reduced their exposure, the results raise the question whether shoaling provides

greater benefit for certain individuals within a shoal. The third shoaling experiment determined that a central position within a shoal provided an increased benefit to solitary behaviour. Here, the peripheral fish likely acted as cercariae 'sinks,' leading to reduced exposure of the central fish to cercariae. As shoal size is highly variable in a natural population, future studies need to address the relation of peripheral fish to fish within the shoal. As a shoal size increases, the ratio of outer fish to inner fish should decrease, reducing the ratio of 'sink' fish to 'protected' fish. The cohesion of shoals may also be addressed in this manner. What proximity is required for peripheral fish to act as 'cercarial sinks,' benefiting shoalmates within the spatial configuration?

This is the first controlled experiment to demonstrate spatial benefits in a fish parasite system. A shortcoming of this experiment, however, is the lack of fluidity of the experimental shoal. Although in chapter 3, I make an argument for somewhat stagnant shoal positioning, natural shoals are unlikely to be as static as they are within the artificial cages. Tissue marking in fish has become more accessible, and provides one opportunity to address this shortcoming. Providing individuals within a shoal with unique marks, followed by video analysis will also allow for assessment of changes in positioning behaviour. Additionally, this method would allow the observer to correlate infection with individual activity level and change in activity.

Shoaling may provide an anti-parasite benefit to individual fish in the same manner as anti-predator behaviour. Yet it is equally plausible that the parasites themselves benefit from host shoaling behaviour. I have shown that shoaling minnows recruit fewer metacercariae, on average, than non-shoaling minnows. Yet the cercariae themselves benefit from shoaling since there are more hosts available to penetrate within a shoal. Thus, it is conceivable that parasites benefit by infecting fish in shoals because it maximizes the numbers of hosts available for infection while simultaneously diluting the number of cercariae that will infect an individual fish. Poulin & FitzGerald (1989) made a similar argument to explain that increased stickleback group size diluted the number of attacks of a crustacean ectoparasite per individual, but did not have a negative effect on parasite success. The extension of this idea for trematodes is that infecting shoaling fish may actually increase parasite fitness. This may be due to the overwhelming numbers of cercariae that a shoal may encounter, or the attack mechanisms of this larval trematode. Further experimental or modeling studies should be performed to address this question.

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