PATTERNS OF MULTIPARASITISM AND CONSEQUENCES OF CO-INFECTION IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

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DEDICATION

To my grandfather, Norwood Edgar “Sonny” Hirtle (1936 – 1999)

ABSTRACT

Individual hosts are often infected with multiple parasite species or strains simultaneously. Co-occurring parasites can profoundly impact each other and their hosts via interspecific interactions. To further our understanding of co-infection in wildlife, I censused the parasite communities of fathead minnows (*Pimephales promelas*) from southern Alberta over three years. Nearly all minnows were co-infected, and the larval trematodes *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. co-occurred more frequently than expected by chance. I exposed minnows to cercariae of these species to evaluate the effects of intensity dependence and co-infection on parasite development. While negative intensity-dependent growth occurred only for *O. ptychocheilus*, both species were significantly smaller post-encystment in co-infections than in mono-infections. Thus, *Ornithodiplostomum* spp. development is influenced by conspecifics and heterospecifics. Taken together, my results suggest that naturally co-occurring parasites in spatially segregated infection sites can influence one another's growth within their shared intermediate host, with possible ecological and evolutionary consequences.

PREFACE

"As we proceeded westward over the plateau, it became more elevated and other species began to take prominence, notably *Lupinus argentea* and *Potentilla fruticosa* covered miles of country to the exclusion of other species, and as both grew about eighteen inches in height, and had a bushy habit, the whole country, for a day's travel, was either blue or yellow or both, as either species prevailed or were intermixed. In all my wanderings I never saw any spot equal in beauty to the central plateau of the Cypress Hills."

—John Macoun, *Manitoba and the Great North-West*

"The parasitologists involved in this study brought human traits to the task: patience, care, breadth of knowledge, and willingness to get into the field and probably get quite dirty. The results are a clear statement of the way things are."

—John Janovy Jr., 2014, *Journal of Parasitology*, 100: 700-707

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CHAPTER 1: Introduction

1.1 Background

Parasites are ubiquitous, encompassing an estimated 40% of known species (Dobson et al., 2008) and individual hosts are frequently infected by multiple parasite species or strains simultaneously. This phenomenon of parasite co-infection masquerades under a variety of synonyms in the scientific literature, including double, concomitant, concurrent, or simultaneous infection, polyparasitism, and multiparasitism (Griffiths et al., 2011). Globally, multi-parasite infections are the rule, rather than the exception; in some regions, and in some species of host, co-infections are more common than single-species infections or no infection (Petney and Andrews, 1998). Multi-species infections in humans, for example, have been recorded in the tropical medicine literature for decades, especially following the return of American military personnel stationed in Asia during the Second World War (Stoll, 1947; Buck et al., 1978). Co-infections in human hosts are particularly pervasive in tropical and subtropical regions of the world (Tchuem Tchuenté et al., 2003; Fleming et al., 2006; Sayasone et al., 2011; Singh et al., 2017) where parasites co-occur spatiotemporally and are often co-endemic (Brooker and Clements, 2009).

However, parasite co-infection is not a uniquely human phenomenon. Multi-species infections are also common in wildlife hosts. Parasite co-infections have been recorded from all seven classes of vertebrates (Amphibia: Hopkins et al., 2016; Reptilia: Knapp et al., 2019; Chondrichthyes: Friggens and Brown, 2005; Agnatha: Katahira et al., 2014), although traditionally greater emphasis has been placed on the parasite communities of mammals (Lello et al., 2004), birds (Stock and Holmes, 1987) and bony

fishes (Holzer et al., 2005). Increasingly, scientists are revealing that parasite co-infections are common and affect diverse host taxa.

Co-occurring parasites can profoundly impact each other and their hosts. For example, HIV+ patients co-infected with malaria have higher viral burdens than those without malaria (Hoffman et al., 1999), and HIV+ malaria patients more commonly present with symptomatic disease than HIV- malaria patients (Whitworth et al., 2000). The underpinnings of this complex interaction are anchored in the cellular immune activation caused by HIV and malaria (Froebel et al., 2004). During co-infection, species may interact along the three axes that define the ecological niche of parasites: metabolic resources, infection sites, and immune responses elicited in the host (Pedersen and Fenton, 2007). These axes can be considered analogous to the bottom-up (resources and sites) and top-down (immune responses) controls that can often drive interaction between free-living species (Pedersen and Fenton, 2007). Further, top-down interactions can be synergistic (facilitative) or antagonistic (competitive; Behnke, 2008) depending on the nature of the host immune response. Parasites can encounter a permissive environment if a co-infecting species has polarized the host's immune response to one of the two cross-regulated T-helper cell phenotypes (T_H1 or T_H2), while parasites with immunodulatory capabilities can suppress a host's immune system to promote successful establishment by a second species. On the other hand, parasites can be mutually inhibited by cross-reactive immunity, a form of apparent competition. Studies in mice suggest that disease progression (Lamb et al., 2005), likelihood of successive establishment (Rodriguez et al., 1999), and parasite persistence (Budischak et al., 2015) depend on immune-mediated interspecific interactions.

These complex outcomes highlight the importance of studying multiparasitism in wildlife, despite its challenges. Ecologists are primarily concerned with parasite co-infections in wildlife for two reasons: understanding the effects of parasite co-infection on host condition and host population dynamics, and understanding how species' interactions structure parasite communities (Telfer et al., 2008). Untangling patterns and processes of infection in natural systems is difficult because genetic, environmental, and behavioural heterogeneity in hosts can influence parasite exposure and susceptibility to infection (Viney and Graham, 2013). However, seminal work has demonstrated that parasite co-infections influence disease risk (Telfer et al., 2010) and host mortality (Thumbi et al., 2014) in wildlife. Researchers have also suggested that parasite traits such as transmission and virulence (Clay and Rudolf, 2019) are altered during co-infection and thus co-infection may have ecological and evolutionary consequences for wildlife. Expanding our knowledge of the nature of the complex interactions between coinfecting parasites within wild hosts is critical for a paradigm shift towards multiparasitism in conservation biology, disease ecology, and veterinary medicine, especially during the current era of emerging infectious diseases (Daszak et al., 2000; Jones et al., 2008).

Host-parasite interactions have traditionally been investigated within a single host, single parasite framework. These studies have provided important insights into parasite establishment, reproduction, and survival. They have also reinforced that density-dependent affect these aspects of parasite population biology, as originally proposed by Anderson and May (1978). The number of conspecific parasites infecting an individual host can have direct effects on parasite fecundity and/or indirect effects on parasite growth and development (Keymer, 1982; Shostak and Scott, 1993). For example, the

relationship between intraspecific intensity dependence and growth is strongly supported for helminths (Sandland and Goater, 2000; Brown et al., 2003; Fredensborg and Poulin, 2005; Benesh and Valtonen, 2007; Saldanha et al., 2009; Cornet, 2011). There is also a long legacy examining functional responses of helminth parasites in the presence of heterospecifics, *e.g.*, niche partitioning (Holmes, 1962), establishment success (Dash, 1981), and fecundity (Holland, 1984; Wang et al., 2002). Conspecific and heterospecific parasites may alter the relationship between intensity, development, and fecundity in different directions (Lagrue and Poulin, 2008; Cattadori et al., 2014).

Still, a knowledge gap persists surrounding the influence of heterospecific parasites in co-infected wild hosts. Three main factors contribute to its persistence. Firstly, many studies of co-infection analyze naturally infected hosts rather than experimentally infected hosts, and thus do not control the initial infection intensities or the timing of exposure (*e.g.*, synchronous or sequential). Secondly, experimental studies often examine the effects of co-infection on parasite species with direct life cycles (*e.g.*, some nematodes), or species co-infecting their definitive host. Parasites with complex life cycles reproduce sexually within the definitive host but typically grow and develop within the intermediate host(s). Co-infection studies in the definitive host may attribute effects on fecundity directly to intensity dependence rather than to reduced growth or delayed development in the intermediate host (Fredensborg and Poulin, 2005). Lastly, studies investigating parasite growth in co-infected intermediate hosts are frequently conducted using relatively small hosts (Benesh, 2011). This may prematurely impose a resource limitation (*i.e.*, physical space) on the parasites which could obscure the effects of co-infection.

Trematode parasites infecting fishes may be ideal candidates for studies of multiparasitism in wild hosts. Trematodes infect marine, freshwater, and anadromous wild fishes as well as maricultured species (Bouillon and Dempson, 1989; Liu et al., 2010; Locke et al., 2010). Fish serve as second intermediate hosts (Faltýnková et al., 2016) or definitive hosts (Bartoli and Gibson, 2007) in the complex life cycles of many trematode species, although this thesis will concentrate on the former. The intermediate host represents a critical period in the transmission of trematodes; the low probability that parasites will contact and establish in motile hosts means that successful use of intermediate hosts is very important from an evolutionary perspective (Barger and Nickol, 1999). Trematodes often experience a several-fold increase in size during infection of the second intermediate host (Poulin and Latham, 2003). However, a second knowledge gap is the incidence of co-infection in fishes. Information on co-infection is scarce despite the abundance and diversity of parasites they encounter in spatially and hydrologically complex environments (Kotob et al., 2016; Chapman et al., 2021).

A host-parasite system that is amenable to both natural study and more controlled laboratory experimentation could address these knowledge gaps. Combining field and lab studies provides an integrative perspective to understanding co-infection (Ezenwa, 2016). For example, combined field-lab studies have revealed important findings about the impacts of co-infection on parasite transmission and disease risk in an amphibian host (Johnson and Buller, 2011; Johnson and Hoverman, 2012; Johnson et al., 2013). Studies of parasite co-occurrence in wild hosts have been largely descriptive and/or correlative (Bordes and Morand, 2011; Herczeg et al., 2021), and co-occurrence patterns from the field are insufficient evidence for interspecific interaction on their own (Blanchet et al.,

2020). Associations between co-occurring parasites can stem from factors other than parasite interactions, including host behaviour (Bush and Holmes, 1986), the host environment (Wu et al., 2019), and covariance in parasite transmission rates (Lotz et al., 1995). However, if parasite species co-occur more or less frequently than expected by chance alone in wild populations, lab studies can suggest mechanisms of interaction for these suspected associations.

In particular, the fathead minnow *Pimephales promelas* (Pisces: Cyprinidae) and its naturally occurring parasites are a tractable model system for examining patterns of multiparasitism in the field and consequences of co-infection in the laboratory. Abundant and widely distributed, these small-bodied omnivores often dominate the fish community in the shallow, eutrophic wetlands of the Prairie Pothole Region of North America (Held and Peterka, 1974; Laurich et al., 2003). In lentic waterbodies within the South Saskatchewan River watershed, fathead minnows typically co-occur with other small-bodied forage species like brook stickleback, Iowa darter, Northern redbelly dace, spottail shiner and larger piscivores like Northern pike, walleye, and rainbow trout (Nelson and Paetz, 1992). Minnow populations in Alberta are routinely co-infected with the metacercariae (larvae) of the sympatric trematodes *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp., as well as additional parasites (Sandland, 1999). Previous studies have shown that this trematode/minnow model system is highly amenable to experimental manipulation at field, lab, and mesocosm scales (Matisz and Goater, 2010; Stumbo et al., 2012). Furthermore, the singular effects of these species and the ecological conditions under which they occur have been extensively documented (Sandland et al., 2001; Shirakashi and Goater, 2001; Schleppe and Goater, 2004; Stumbo et al., 2012).

Ornithodiplostomum spp. (Digenea: Strigeoida) exemplify the striking complexity of the typical trematode life cycle. Fathead minnows are infected by motile, free-living cercariae that emerge into the water column following successive rounds of asexual reproduction within the first intermediate snail host, *Physa* spp. Within minnows, the larval parasites develop but do not reproduce; parasites in this stage are referred to as metacercariae. When minnows are predated by piscivorous ducks or wading birds like mergansers or herons (Hoffman, 1960) the adult trematodes reproduce sexually within the digestive tract of their final host. Their eggs are shed into the water column in the bird's feces, and free-living miracidia hatch from eggs to infect aquatic snails. *Ornithodiplostomum* sp. has not yet been described at the species level, but it is morphologically and ecologically similar to *O. ptychocheilus* (C. Goater, unpublished data). It is presumed to be a congeneric species because of these similarities but is distinguishable from *O. ptychocheilus* by its markedly different migration behaviour and infection site within minnows (Matisz and Goater, 2010; Matisz et al., 2010). *Ornithodiplostomum ptychocheilus* infects and encysts in the brain, while *Ornithodiplostomum* sp. infects the liver and encysts in the body cavity of minnows. This is consistent with previous reports regarding the site specificity of *O. ptychocheilus* from experimental infections (Hendrickson, 1978; Hendrickson, 1979). Our working hypothesis that *Ornithodiplostomum* sp. is a distinct, co-occurring species also reconciles earlier descriptions of *O. ptychocheilus* from both the brain and the body cavity within fathead minnows (Hoffman, 1954; Hoffman, 1958; Molnar et al., 1974).

This pair of co-occurring parasite species is ideally suited to studies of parasite co-infection. Metacercariae of both species infect upwards of 90% of minnows in southern

Alberta populations. They are also abundant, occurring at intensities ranging from 10 to more than 600 metacercariae per minnow (S. Hirtle, unpublished data). Minnows are easily caught with standard gear, facilitating the collection of large samples where each individual fish hosts its own parasite infracommunity. Thus, both host and parasites are readily sourced for controlled investigation into hypotheses derived from field observations (Kennedy, 2009).

1.2 Thesis objectives

In chapter 2, I conduct field surveys in southern Alberta to document the extent of multiparasitism in fathead minnows. I re-visit the same field sites in early summer for three years and capture sexually mature, male fish to explore spatiotemporal variation in parasite populations. I fully census the parasite infracommunity of 755 individual hosts, allowing me to ask important questions about how many parasite species co-occur in fathead minnows at different sites and whether these species co-occur regularly over time. For the first time, I complete a full species co-occurrence analysis to examine the strength and direction of associations between parasites of the fathead minnow. Crucially, I identify a pair of species that are consistently associated over time and space.

In Chapter 3, I experimentally infect naïve fathead minnows with the two trematode species that co-occur most frequently in natural infections. Individual minnows are exposed to known numbers of trematode cercariae that reflect intensities observed in the field. By controlling for factors such as parasite exposure dose, host exposure history, host size, and environmental conditions I can, for the first time, compare patterns of metacercariae growth and development in mono-infected versus co-infected fathead

minnows. More specifically, I explore the effects of intensity dependence (the presence of conspecifics) and co-infection (the presence of heterospecifics) on parasite growth and development in these experimental infections. This model host-parasite system allows me to ask whether regularly co-occurring species interact within individual hosts.

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CHAPTER 2: Patterns of multiparasitism in fathead minnows (*Pimephales promelas*) from southern Alberta, Canada reveal non-random species associations

2.1 Abstract

Parasites are ubiquitous in nature and individual hosts are infected with multiple parasite species or strains simultaneously. Multi-species infections have garnered increased attention within the last two decades as researchers discover that co-occurring parasites can profoundly impact each other and their hosts via synergistic or antagonistic interactions. Overall, there is limited knowledge on the incidence and effects of co-infection in aquatic animals, especially wild fishes. To further our understanding of co-infection, I fully censused the parasite infracommunities of sexually mature, male fathead minnows (*Pimephales promelas*; $n = 755$) from small to medium-sized lakes/ponds in southern Alberta, Canada between 2018–2020. The fifteen species assemblage of endoparasites identified here is the richest reported for this small-bodied forage fish. Prevalence and intensity were highly variable between years and sites for all species. Nearly all minnows (740/755; 98%) were co-infected with between two and nine parasite species. Non-random pairwise associations were detected within the overall parasite community on a landscape scale. In particular, there was a strong, positive association between intensities of the congeneric larval trematodes *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. Due to the persistence and consistency of this association in time and space, I suggest that it merits further experimental investigation to better understand its significance. My results highlight the highly context-dependent nature of host-parasite interactions.

2.2 Introduction

In nature, individuals are infected with multiple parasite species or strains simultaneously. These multi-species infections are considered the rule, rather than the exception, globally for humans and animals alike (Petney and Andrews, 1998; Bordes and Morand, 2011). Despite the phenomenon's multi-decadal recognition in tropical medicine (Buck et al., 1978), infectious disease researchers have historically investigated host-parasite interactions within a single-host, single-parasite framework (Hellard et al., 2015) that underestimates the impact of co-infection (Serrano and Millán, 2014). However, researchers have placed greater emphasis on co-infection and its consequences within the last twenty years (Ezenwa, 2016), revealing that co-occurring parasites profoundly impact each other and their hosts via interspecific interactions. These interactions alter host susceptibility to, and the duration of infection (Telfer et al., 2010; Clerc et al., 2019), disease progression and severity (Graham et al., 2005; Lamb et al., 2005), and host mortality (Thumbi et al., 2014). Interspecific interactions can also influence the success of parasite control strategies (Lello et al., 2004). Expanding our knowledge of the nature of interactions between co-infecting parasites in wild hosts is therefore critical for a paradigm shift towards multiparasitism in disease ecology, epidemiology, and veterinary medicine.

Despite the increased, recent focus on patterns and processes of parasite co-infection, some fundamental questions remain largely unanswered. For example, data on the frequency of co-infection is scarce (Griffiths et al., 2011). Even for vertebrate taxa with comparatively well- studied parasite communities like fishes, there is a paucity of data on the incidence of co-infection (Chapman et al., 2021). A second fundamental

question surrounds the strength, direction, and repeatability of parasite species' co-occurrences. Pairwise associations between species are typically inconsistent across space and time (Dezfuli et al., 2001; Poulin and Valtonen, 2002; Vidal-Martínez and Poulin, 2003) and consequently, intra- and interspecific interactions are not considered a major structuring influence for parasite communities in fish (Kennedy, 2009). It is important to note that we cannot infer interactions from associations. However, the characterization of pairwise associations provide researchers with important hypotheses that can then be tested experimentally (Stutz et al., 2018).

Here, I focus on the parasite community infecting fathead minnows (*Pimephales promelas*) in southern Alberta, Canada. The fathead minnow is one of the foremost small-fish models in aquatic toxicology in addition to being a major bait and forage species. These small-bodied cyprinids are abundant and widely distributed across North America (Scott and Crossman, 1973), often dominating the fish community in the shallow, productive lakes and wetlands that characterize the Prairie Pothole Region (Laurich et al., 2003). Minnow populations in Alberta are routinely co-infected with parasites (Sandland, 1999). In this study, I document the prevalence of multiparasitism in fathead minnows. I first characterize the overall parasite assemblage. Next, I assess the extent to which parasite prevalence and intensity vary within six populations of fathead minnows in southern Alberta over three consecutive years. Finally, I evaluate patterns of species co-occurrence within the overall sample of minnows by assessing the strength and direction of pairwise associations between co-occurring species.

2.3 Materials and Methods

2.3.1 Minnow sampling

I sampled fathead minnows (*Pimephales promelas*) from small to medium-sized lakes/ponds in southern Alberta, Canada (Fig. 2.1; Table 2.1). The sampling sites in this study are part of an ongoing, long-term survey of fish and their parasites in the region (Ahn, 2019). The sites can be considered broadly representative of lentic waterbodies in the region, where fathead minnows often dominate the fish community, and the adjacent landscape is characterized by anthropogenic alteration. Alterations include residential and recreational development (single-family detached homes, campgrounds, golf courses) and extensive conversion of the native dry mixedgrass prairie for cropland production. All sampling sites are artificial waterbodies maintained by stormwater runoff, damming, or irrigation water diversion. The four largest (McQuillan Reservoir, Gold Spring Park Pond, Reesor Lake, and Spruce Coulee Reservoir) are stocked annually with rainbow trout to support recreational angling.

I followed a sampling scheme designed to minimize the influences of seasonality and host age and sex on infection patterns. Sampling took place in late May and early-to-mid June between 2018–2020. Sexually mature, male minnows were collected with un-baited Gee traps set in approximately 50 cm of water at a distance of one to two m from the shoreline for 2–24 h (Sandland et al., 2001; Ahn, 2019). At the time of collection, mature, age-2 males were identified by the presence of breeding tubercles, fleshy protuberances on the snout that are a recognized secondary sexual character. Minnows were transported to the University of Lethbridge in aerated coolers, where sex and maturity were confirmed at necropsy by the presence of fully developed testes.

2.3.2 Censusing the parasite community

In the lab, minnows were euthanized and their parasite infracommunities were fully censused using standard necropsy techniques (*e.g.*, Desdevises et al., 1998). All fish were examined fresh and measured for fork and standard lengths (mm) using digital calipers, then weighed to the nearest 0.001g. Fish were first scanned for melanized, larval trematodes encysted in the epidermis and associated musculature. Next, the olfactory chambers were evaluated for the monogenean *Dactylogyrus olfactorius* (Lari et al., 2016). The brain and viscera were then removed and examined for larval and adult parasites under a stereomicroscope. The brain case and the body cavity were rinsed following the removal of their contents and these washes were checked for parasites that had separated from the meninges and/or the peritoenum during dissection. The lenses of both eyes were separated from the vitreous humour and crushed for the assessment of larval strigeoid trematodes. Finally, the spleen was removed and subjected to gentle pressure between a microscope slide and coverslip then viewed under transmitted light under a compound microscope. Parasites were identified based on morphology, infection site, and previous records from *P. promelas* in Alberta and Canada (*e.g.*, McDonald and Margolis, 1995; Sandland, 1999). I followed Marcogliese, Compagna, Bergeron, and McLaughlin's (2001) treatment of eyefluke taxonomy and recorded all unencysted trematode metacercariae recovered from the eye lenses as *Diplostomum* spp.

2.3.3 Statistical analyses

2.3.3.1 General patterns of infection

All statistical analyses were conducted in RStudio (version 1.4.1106) running R 4.0.4 (R Core Team, 2020). Quantitative descriptors of parasite populations follow Bush et al. (1997). For a given taxon, prevalence describes the proportion of infected fish, intensity describes the number of parasites per infected fish, and abundance describes the number of parasites per fish, both infected and uninfected. I report prevalence alone for the eimerian *Goussia degiustii* as I did not determine its intensity. I define a host population to consist of minnows sampled at a given site during a given year (Bolnick et al., 2020). Altogether, I sampled 755 minnows over the course of the study. I limited some analyses to the fish (n = 675) from sites sampled in all three years (excluding those collected at Stirling Fish Pond only in 2019 and 2020). Mean minnow standard lengths (Fig. 2.2) differed among years (Kruskal-Wallis $H = 7.76$, df = 2, $p < 0.05$) and sites ($H = 394.96$, df = 5, $p < 0.001$) and because of this, I included standard length as an explanatory variable in the following statistical analyses.

I assessed annual and between-pond variation in parasite prevalence (all minnows) and intensity (infected minnows only) with generalized linear models (GLM) for species with a prevalence ≥ 10%. These models predicted prevalence and intensity as a function of sampling site, year, and the interaction between sampling site and year with minnow standard length as a covariate (prevalence/intensity ~ site + year + site × year + minnow standard length). Prevalence was modelled with a binomial distribution and logit link function, while intensity was modelled with a negative binomial distribution and log link function. A Poisson distribution was deemed unsuitable for modelling helminth

intensities because parasites were typically highly overdispersed within samples of hosts. Following Young and Maccoll (2017), I pruned full models using a deletion approach to remove non-significant factors, and tested predictors in the minimum adequate models for significance with likelihood ratio tests. Negative binomial GLMs were run using the glm.nb function in the MASS package (Venables and Ripley, 2002) in R. I calculated the amount of deviance explained by the GLMs using an adjusted D^2 value that accounts for the number of observations and parameters in each model, rendering the values comparable across models. D^2 values were calculated using the Dsquared function in the modEvA package (Barbosa et al., 2013) in R.

Philometra sp. intensities were under-dispersed, so I used a zero-inflated negative binomial model to evaluate *Philometra* sp. abundance, rather than intensity. Zero-inflated models predict an outcome according to two sets of regressors, those that specify the outcome's occurrence (the zero-inflation component) and those that specify the frequency of its occurrence (the count component) (Beaujean and Grant, 2016). I specified the count component using identical predictors as in the GLMs while the zero-inflation component contained only the intercept (*Philometra* sp. abundance ~ site + year + site × year + minnow standard length | 1). I reasoned that the continuous covariate (minnow length) would not predict the occurrence of *Philometra* sp. since all minnows belonged to a single year-class and thus had equal probabilities of infection by a parasite with an annual life cycle. I ran the zero-inflated negative binomial model using the zeroinfl function in the pscl package (Jackman, 2020) and tested the significance of predictors with type II ANOVA in the car package (Fox and Weisberg, 2019) in R.

2.3.3.2 Parasite infracommunity structure

I used non-metric multidemnsional scaling (NMDS) to visualize parasite infracommunity structure. I calculated a Bray-Curtis dissimilarity matrix (based on abundance data) and a Sørensen dissimilarity matrix (based on presence-absence data) for parasite species that had ≥ 10% prevalence at one or more sites. Accordingly, six species were common to both matrices (*Ornithdiplostomum ptychocheilus*, *Ornithodiplostomum* sp., *Posthodiplostomum minimum*, *Diplostomum* spp., *Crassiphiala bulboglossa*, and *Philometra* sp.) while the Sørensen dissimilarity matrix additionally contained the eimerian *G. degiustii*. I excluded uninfected fish from NMDS analysis, and following Alfieri and Anderson (2019) I ln(x+1)-transformed zero-rich abundance data prior to analysis. Community-by-species distance matrices were calculated using the dissimilarity measures described above, and the number of dimensions (k) for each ordination were chosen through an iterative process until a solution was found that minimized stress. With respect to ordination techniques, stress refers to the degree to which the distance between samples in dimensional space corresponds to the multivariate distance between samples. Ordinations with a stress value < 0.2 are typically deemed acceptable, although ordinations with values < 0.1 are considered good, and ordinations with values < 0.05 are considered excellent (Clarke, 1993).

To complement the NMDS ordinations, I used multivariate statstics to compare community composition between sites and years. Specifically, I tested for compositional dissimilarity among sites and years using analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA). These tests are not performed on the ordination itself but rather on the ecological distance between samples

computed in the dissimilarity matrices. ANOSIM is a rank-based, non-parametric test that asks whether the distribution of samples is greater within a group than between groups. PERMANOVA tests a more specific hypothesis, that there are no differences in the position of the group centroids (Anderson and Walsh, 2013). PERMANOVA's test statistic compares the between-group sum of squares (distances from individual group centroids to the overall centroid) to the within-group sum of squares (distances from individual replicates to their group centroid). I ran 9999 permutations for ANOSIM and 999 permutations for PERMANOVA. These mutlivariate techniques were carried out using the metaMDS, anosim, and adonis functions in the vegan package (v.2.5-7) (Oksanen et al., 2020) in R.

2.3.3.3 Parasite species co-occurrence

I evaluated pairwise species co-occurrences using the probabilistic model developed by Veech (2013) and implemented in the R package cooccur (Griffith et al., 2016). Veech (2013) noted a positive relationship between the number of sampling sites and the percentage of non-random species pairs in their probabilistic method, suggesting that as a statistical test, the model has diminished power with reduced numbers of sites. Here, sites correspond to individual minnows. Minnow sample sizes at the population level are adequate for this analysis ($n > 10$) but still modest, and accordingly, I chose to assess species co-occurrence at the landscape scale by including minnows from sites sampled in all three years ($n = 675$). I constructed a presence-absence matrix in which parasite species were rows and individual minnows were columns. All routinely-censused parasite species present in at least one minnow (*i.e.*, prevalence > 0%) were included in

this matrix. I then summarized all pairwise species associations as positive, negative, or random. Positive associations were assigned to species pairs with a co-occurrence probability greater than the observed frequency of co-occurrence (P_{gt}), while negative associations were assigned to species pairs with a co-occurrence probability less than the observed frequency of co-occurrence (P_{lt}; Veech, 2013). Random associations were assigned to species pairs for which the predicted co-occurrence probabilities deviated from the expected co-occurrence by less than 10% of minnows.

I explored associations between the congeners *O. ptychocheilus* and *Ornithodiplostomum* sp. further because they frequently co-occurred. The measures of association are based on work by Johnson and Buller (2011). Using linear regression, I related the average abundance of both species at the population level. To examine whether covariation in abundance was confounded by host length, I regressed the species' abundances against minnow standard length, extracted the residuals, and correlated the species' residuals. I compared the frequency of their co-occurrence using chi-square analysis also at the level of individual hosts. At the within-host level, I evaluated whether minnows infected with one species (sp.1) were more or less likely to be infected with the second species (sp.2) using generalized linear mixed models (GLMM). Parasite prevalence for individual minnows was recorded as zero (uninfected) or one (infected). The GLMMs included minnow standard length as a covariate, while sampling site and year were included as random effects ($\text{prevalence}_{sp.1} \sim \text{prevalence}_{sp.2} +$ standard length + (1 | year) + (1 | site)). I ran GLMMs fit by maximum likelihood using the Laplace approximation with the glmer function in the lme4 package (Bates et al., 2015) in R.

2.4 Results

2.4.1 General patterns of infection

I recovered ten parasite species from fathead minnows, and observed all helminths as larvae. I also encountered five additional myxozoans for which minnows were not routinely censused: *Myxobolus hendricksoni*, *M. hyborhynchi*, *M. rasmusseni*, *Myxobolus* sp., and *Unicauda magna* (M. Tilley, unpublished data). The prevalence and mean intensity of each of the ten species, pooled across the three sampling years are summarized in Table 2.2. The complete species by site by year data matrix is provided in the appendix. Ninety-nine percent of minnows (754/755) were infected with at least one parasite species, and ninety-eight percent (740/755) were infected with two or more species. On average, minnows were co-infected with 4.4 ± 1.4 species (Fig. 2.3). Two larval trematodes (*O. ptychocheilus* and *Ornithdodiplostomum* sp.) dominated the overall parasite fauna, together accounting for 101,295 of 104,945 parasites recovered (96.5%; Fig. 2.4). The maximum intensity was 665 for *O. ptychocheilus* and 347 for *Ornithdodiplostomum* sp. The other helminth species exhibited strongly aggregated distributions with low mean intensities.

Species' prevalence and intensity varied by site, year, and the interaction between site and year (Table 2.3). Patterns in the prevalence and intensity of *Diplostomum* spp. provide a striking example. The prevalence of this species varied between zero to 100% across all seven sites. Even at sites where it was present, it was not present each year. For example, *Diplostomum* spp. was absent from the 2018 sample at Gold Spring Park Pond, present in 15% of fish in 2019, and then absent from the 2020 sample. Furthermore, the mean intensity ranged from 0.4 to 8.5 individual parasites per fish, although fish were

infected by as many as 33 individuals. In the GLMs, site was present in all thirteen minimum adequate models and was significant in all thirteen cases, while year was present in twelve of thirteen models and was significant in all twelve cases. The interaction term was absent from only three minimum adequate models *(O. ptychocheilus* prevalence*, G. degiustii* prevalence, and *P. minimum* intensity), but variation was attributed to site and year independently in these models. Minnow standard length positively predicted significant variation in prevalence and intensity. Overall, predictors in the minimum adequate models explained 19–50% and 37–89% of the variation in prevalence and intensity, respectively (Table 2.3).

2.4.2 Parasite infracommunity structure

The Bray-Curtis dissimilarity matrix was built using 666 infracommunities (six species) and the Sørensen dissimilarity matrix incorporated 673 infracommunities (seven species). I refer to these species sets as 'partial' and 'core' assemblages, respectively. The partial ordination had three dimensions and a stress value of 0.101 (Fig. 2.5). Infracommunity composition differed among sites (PERMANOVA $F_{5,660} = 141.31$, $p < 0.001$) and among years (PERMANOVA: $F_{2,663} = 15.28$, $p < 0.001$). Infracommunities were also more similar within sites/years than among sites/years (site: $R = 0.502$, $p = 0.0001$; year: $R = 0.060$, $p = 0.0001$) as indicated by ANOSIM. The core ordination had three dimensions and a stress value of 0.098 (Fig. 2.6). Infracommunity composition differed among sites (PERMANOVA $F_{5,667} = 56.14$, $p < 0.001$) and among years (PERMANOVA: $F_{2,670} = 52.10$, $p < 0.001$). Infracommunities were also more similar

within sites/years than among sites/years (site: R = 0.266, $p = 0.0001$; year: R = 0.122, $p = 0.0001$) as indicated by ANOSIM.

2.4.3 Parasite species co-occurrence

The presence-absence matrix from 675 fish contained ten parasite species, yielding 45 species pairs. Overall, the majority of pairs (32/45; 71.1%) were classified as randomly associated. All thirteen significant, non-random associations were positive (Fig. 2.7). Thus, these species co-occurred more often than expected by chance (Table 2.4). Positive associations occurred only between species with ≥ 10% prevalence (the core assemblage). The taxa with the highest and lowest number of positive associations were *C. bulboglossa* and *Diplostomum* spp. (five) and *Ornithodiplostomum* sp. (two). Of all possible pairs, *Ornithodiplostomum* sp. and *O. ptychocheilus* co-occurred in the greatest number of minnows (586/675; 86.8%) in the probabilistic analysis.

Overall, *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. co-occurred in 666/755 minnows (88.2%). At the population level, their mean abundances correlated positively (Fig. 2.8; $r = 0.87$, $p < 0.001$). In these 20 populations, *O. ptychocheilus* was consistently more abundant than *Ornithodiplostomum* sp. (paired *t*-test, $t = 3.60$, $p < 0.01$), with an overall mean (± SD) of 101.9 ± 120.1 individuals for *O. ptychocheilus* and 32.2 ± 56.2 for *Ornithodiplostomum* sp. When covariation in abundance was examined at the level of individual hosts, without the potential confound of host length, this relationship persisted (Fig. 2.9; $r = 0.73$, $p < 0.001$). Additionally, *O. ptychocheilus* and *Ornithodiplostomum* sp. were significantly more likely to co-occur in minnows than expected by chance (Fig. 2.10; Pearson's $\chi^2 = 1616.3$, $p < 0.001$). The

presence of one parasite positively predicted the presence of the other within minnows (*O. ptychocheilus* ~ *Ornithodiplostomum* sp.: $z = 2.41$, $p < 0.05$; *Ornithodiplostomum* sp. ~ *O. ptychocheilus*: $z = 3.34$, $p < 0.001$).

2.5 Discussion

Adult fathead minnows collected from water bodies in southern Alberta were infected by a rich parasite assemblage in which species' prevalence and intensity were highly variable between individual fish and between samples of fish. High spatial and annual variation in the parasite assemblage is consistent with the results of other studies of freshwater fish (reviewed by Kennedy, 2009). Nearly all minnows sampled in this study were co-infected with between two and nine parasite species. This finding is an important contribution because the incidence of co-infection in wild fishes is not widely known. This is particularly true for freshwater fishes, whose parasites have been neglected in favour of those infecting hosts such as elasmobranchs, coastal (inshore) species, and commercially valuable species targeted by fisheries (Scholz and Choudhury, 2014). My work is the first to leverage a full community-level analysis against parasite presence/absence data collected from fathead minnows. I found that parasite infracommunities were compositionally dissimilar among sites and years, and that species within the infracommunity tended to co-occur more frequently than expected by chance.

In particular, I described the strong, positive, and consistent association between *O. ptychocheilus* and *Ornithodiplostomum* sp. across seven sites and three years. Overall, these two larval trematodes co-occurred more frequently than expected, and the presence of one species positively predicted the presence of the other after controlling for sampling

site, year, and minnow size. The strength and direction of their association likely stem from their shared use of physid snails (*Physa* spp.) as first intermediate hosts (Schleppe and Goater, 2004; James et al., 2008). Positive associations are expected from species that share the same prior host (Johnson and Buller, 2011; Lagrue and Poulin, 2015; Stutz et al., 2018). There is mixed evidence that *O. ptychocheilus* can use *P. integra* as first intermediate host (Radabaugh, 1980; Hendrickson, 1986) in addition to natural infections in *P. gyrina*. Notwithstanding its degree of host specificity, *P. gyrina* and *P. integra* are sympatric and syntopic in many water bodies in south-central Canada (Pip and Franck, 2008). Thus, ecological conditions that promote the transmission of the cercariae of one trematode species should also promote the transmission of the other at the same site.

The parasite assemblage infecting fathead minnows in this study was relatively species rich compared to most other surveys for the species. Previous studies from central and midwestern North America report a maximum of eight species infecting fathead minnows (Bangham and Venard, 1946; Meyer, 1958; Hugghins, 1959; Dechtiar, 1972; Dechtiar and Christie, 1988; Dechtiar et al., 1988; Dechtiar et al., 1989; McDowell et al., 1992). The lone exception to these species-poor assemblages is the study by Sandland (1999), who recorded thirteen species infecting minnows in northern Alberta lakes. Like Sandland (1999), the parasite communities in this study were dominated by larval strigeid trematodes. Larval trematodes have been shown to dominate the parasite communities of other fishes in lentic habitats (Pracheil and Muzzall, 2009; Paterson et al., 2019). The dominance of trematodes in the minnow parasite assemblage counters the finding of Frandsen et al. (1989) that planktivorous Arctic charr had a cestode-dominated parasite fauna. While fathead minnows are known to feed on both zooplankton and zoobenthos

(Price et al., 1991), the species composition or density of zooplankton may be unsuitable or insuffient at our sites for widepsread transmssion of larval helminths which use copepods and amphipods as intermediate hosts.

The strigeid trematodes identified in this study all use piscivorous birds as definitive hosts. *Ornithodiplostomum ptychocheilus*, *Ornithodiplostomum* sp., and *P. minimum* are parasites of herons (Hoffman, 1960), *Diplostomum* spp. are parasites of gulls (Karvonen et al., 2006), and *C. bulboglossa* infect belted kingfishers (Berra and Au, 1978). Since these trematode species cycle bewteen freshwater and terrestrial hosts, their life cyles are termed allogenic. According to Esch et al. (1988) allogenic species explain most of the similarity in helminth communities within and between localities. Indeed, my ordination of Bray-Curtis dissimilarity showed that infracommunity composition differed among sites when the infracommunity was restricted to the five trematode species plus *Philometra* sp. The dominance of larval, allogenic species at my sites also aligns with Esch's (1971) finding that adult parasites are typically excluded from fish hosts in eutrophic lakes where birds are tertiary predators. While I did not empirically determine rates of bird visitaiton or minnow predation, I anecdotally observed piscivorous birds while sampling. These included American white pelicans, belted kingfishers, common mergansers, double-crested cormorants, great blue herons, grebes (*Podiceps* sp.), and gulls (*Larus* sp.). Visitation by a single pair of great crested grebes was sufficient for larvae of the trematode *Tylodelphys clavata* to establish in perch at Slapton Ley, a 70 hectare lake in southwest England (Kennedy and Burrough, 1977). Thus, the availability of avian definitive hosts can influence population dynamics of larval parasites in fish.

I observed considerable annual and between-pond variation in the prevalence and/or intensity of each species. The prevalences of *O. ptychocheilus* and *Ornithodiplostomum* sp. were consistenly high but their intensities fluctuated by as much as two orders of magnitude across all fish. Intensities of the other species were generally lower, more stable between years, and more consistent across sites. However, I saw annual changes in the prevalence of these other species. For example, *G. degiustii* prevalence increased between 2018 and 2019 at all sites. I also collected minnows infected with *M. rasmusseni* over the course of this study; although I did not routinely census minnows for *M. rasmusseni*, it was absent from annual surveys conducted prior to 2017 and may represent an emerging disease. Conspicuous ocular and buccal lesions in *M. rasmusseni*-infected minnows suggest that this mxyozoan may cause host pathology and subsequent host mortality (M. Tilley, unpublished data). Since its life history characteristics differ from other parasites in this study (minnows are the definitive host in an indirect, two-host life cycle), long-term monitoring is recommended to track the population dynamics of *M. rasmusseni* and any consequences of infection (*e.g.*, population-level declines in minnow abundance).

My GLM analysis supported site, year, and their interaction as significant predictors of prevalence and intensity. The interaction between site and year was significant in all ten minimum adequate models in which it appeared, highlighting that between-pond variation was dependent on annual variation. The largest reduction in deviance was attributed to the site term in nine of thirteen models. This suggests that local characteristics of each site are a major driver of parasite population dynamics, as has been concluded by others (Sandland, 1999; Byers et al., 2008; McDevitt-Galles et al., 2018).

Site-level characteristics that define the host's environment (extrinisic factors) are often determinants of rates of host contact with free-living parasite life stages or the survival of these life stages (Pietrock and Marcogliese, 2003). Examples of extrinsic factors known to influence parasite burdens in fish include lake surface area (Rossiter and Davidson, 2018), lake depth (Marcogliese and Cone, 1991), lake bottom type (Ondračková et al., 2004), and water temperature (Karvonen et al., 2013). I sought to remove major extrinsic and intrinsic factors from my analyses by sampling sexually mature, male minnows on an annual basis, yet I still observed unexplained variation, especially in prevalence. I suspect that additional factors not measured in this study are likely responsible for the residual variation in my models. Future studies should investigate interactions between fathead minnows and their environment, including the extrinsic factors listed above, to further explain variation in prevalence and intensity of the parasites recorded here.

I showed both positive and random pairwise associations among the parasite species that co-infect the fathead minnow. These patterns of co-occurrence illustrate that infections are often not independent of one another, while the over-dispersion I observed further indicates that parasites are distributed non-randomly among hosts. I found a positive association between two very prevalent species, similar to other authors (Faltýnková et al., 2011; Johnson and Buller, 2011) in addition to positive associations between less prevalent species pairs (*e.g.*, *Philometra* sp. and *C. bulboglossa*). Positive associations can arise if certain hosts represent higher-quality patches of habitat because of higher mobility or lower immunocompetence (Krasnov et al., 2011), if one species facilitates infection for a second species, or if species share common intermediate hosts or modes of transmission (Lotz and Font, 1991). Positive associations between larval

trematodes very likely result from their similar transmission strategies. Although the trematode species do not all share a common first intermediate host, lymnaeid, physid, and planorbid snails co-occur within the nutrient-rich littoral zones of southern Alberta lakes and ponds. Notably, I did not detect negative associations within the infracommunity. Negative associations arise primarily from competition, for example between species that infect the same host tissue (Dallas et al., 2019), although negative associations are typically outnumbered by positive associations in many host-parasite systems (Lotz and Font, 1991; Krasnov et al., 2011; Dallas et al., 2019). The absence of negative pairwise associations here suggests that competition within the multi-species infracommunity is either non-existent or undetectable using our chosen approach.

I searched for general patterns of association in the overall sample of fish, notwithstanding the spatial scale-dependent patterns of species co-occurrence illustrated for fish parasites by Bolnick et al. (2020). Despite the variation in parasite prevalence and intensity among years and sites reported here, I nevertheless felt justified in analyzing co-occurrence at the landscape scale. My chosen method of analysis classifies associations as random based on deviation between the number of observed and expected co-ocurrences. The threshold for classifying associations as random can be specified by the user, but ultimately is directly proportional to the total number of sites (hosts). My sample sizes were not equivalent between years or among sites, with the exception of 2020 when I sampled 40 minnows from all sites. Consequently, I felt it was inappropriate to compare the strength and direction of pairwise associations between populations.

I emphasize the utility of the probabilistic method developed by Veech (2013) for studying parasite associations in a pairwise manner. To my knowledge, this is the first

study to use this method on parasite communities. It has been previously applied to communities of free-living species including plants (Brazeau and Schamp, 2019) and birds (Scholer et al., 2018). The more widely employed C-score metric developed by Stone and Roberts (1990) is typically used as an aggregated index at the community level and may consequently obscure the fact that positive and negative associations can co-exist within a community (Cazelles et al., 2016). Pairwise tests of species association may be more valuable towards linking observed patterns with underlying mechanisms (Lavender et al., 2019). For example, researchers can predict expected co-occurrences *a priori* for specific species pairs and formally test these hypotheses (Veech, 2014). Nevertheless, the probabilistic method has its own advantages and disadvantages. Probabilistic analysis does not require data randomization to generate a null distribution as is necessary in matrix-level approaches, which eliminates a potential source of type I and II errors (Veech, 2013). Furthermore, the results of pairwise analysis are also potentially more intuitive to interpret since the probability of co-occurrence for a species pair is measured with *p* values (Scholer et al., 2018). However, Lavender et al. (2019) suggest that the probabilistic method should only be used to analyze co-ocurrence between intermediately incidient species (not the very rare or the very common). Ultimately, a comparative analysis should be conducted to compare species associations at the landscape (regional) scale versus at the level of individual populations. In the mean time, the species associations I describe at the landscape scale can be tested experimentally.

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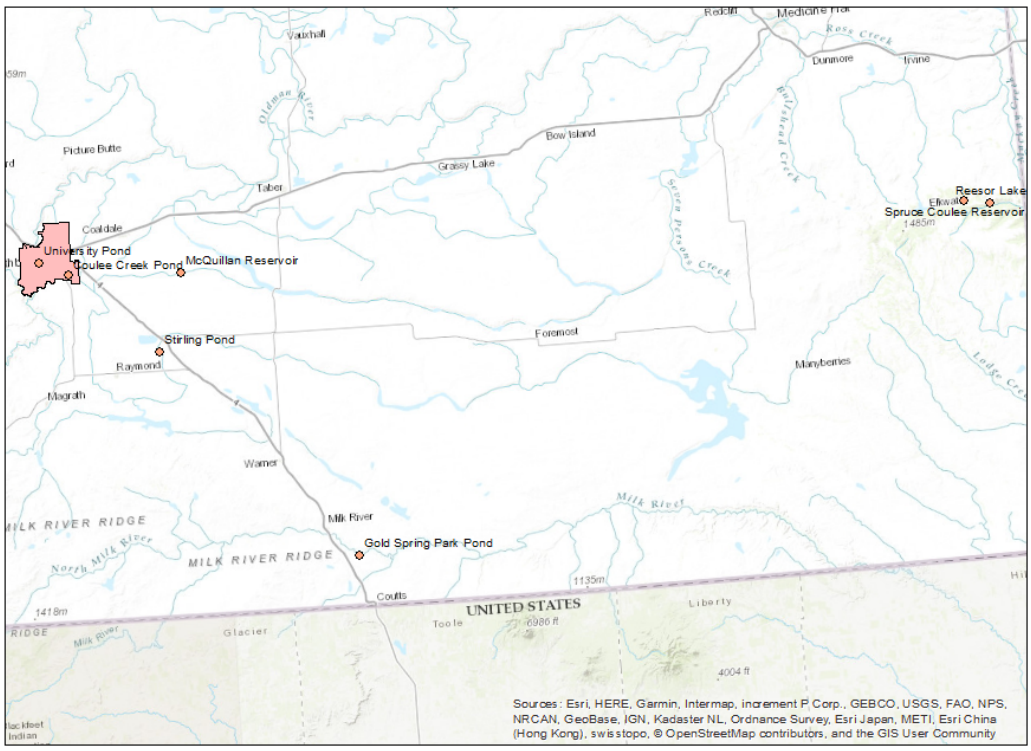

Figure 2.1. Sample locations for fathead minnows (*Pimephales promelas*) in southern Alberta collected for the evaluation of parasite assemblages. Sampling sites are indicated by the orange dots and the city of Lethbridge is represented by the red polygon.

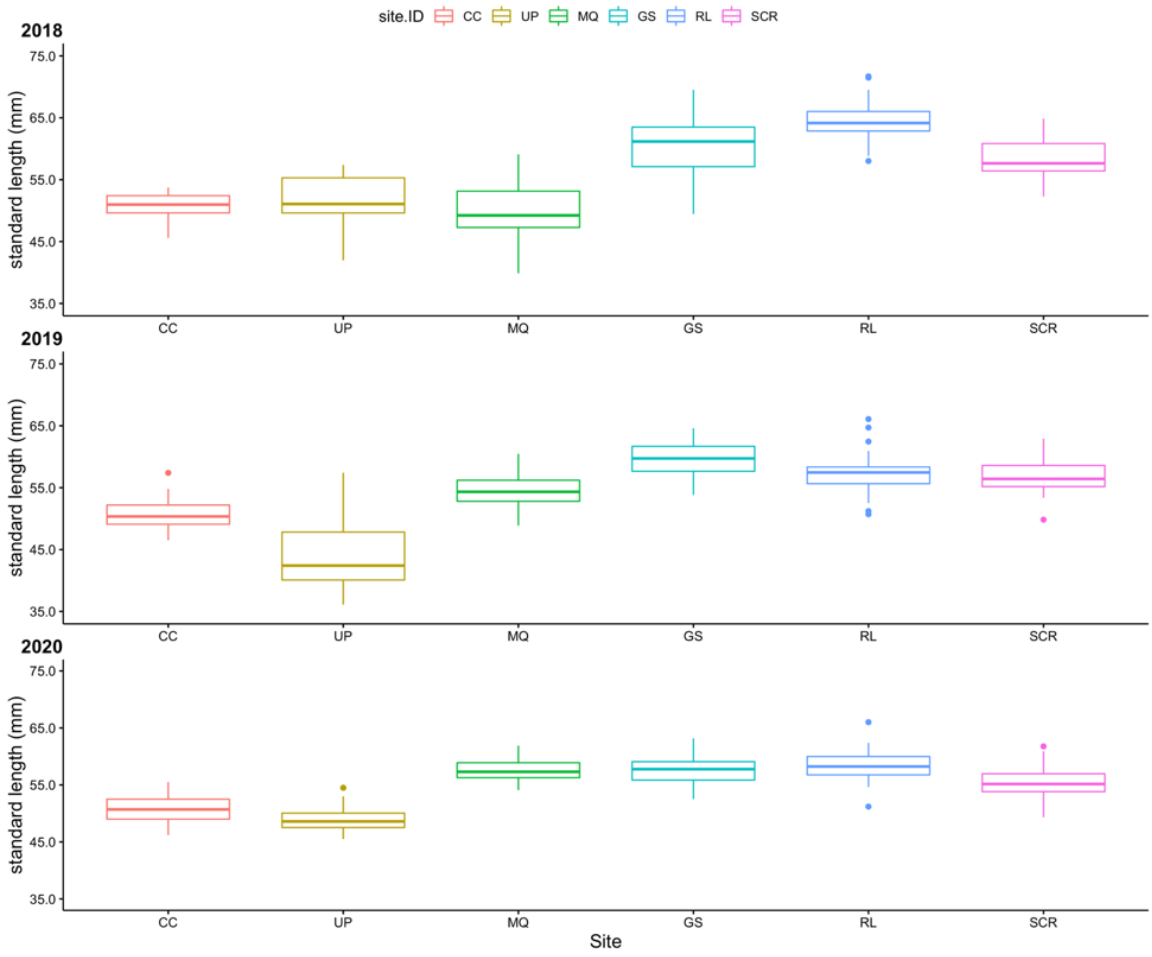

Figure 2.2. Mean standard lengths (mm) of fathead minnows (*Pimephales promelas*) collected in southern Alberta, Canada between 2018–2020. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; UP: University Pond.

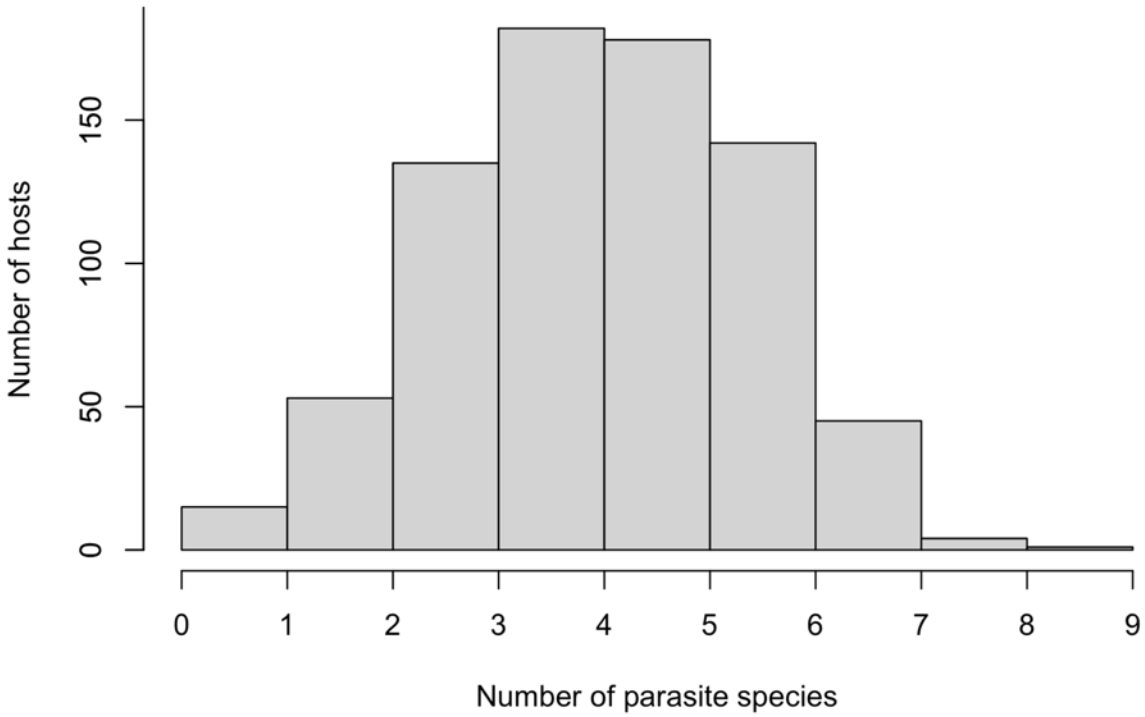

Figure 2.3. The number of parasite species concurrently infecting sexually mature, male fathead minnows (*Pimephales promelas*) sampled from seven sites in southern Alberta, Canada between 2018–2020.

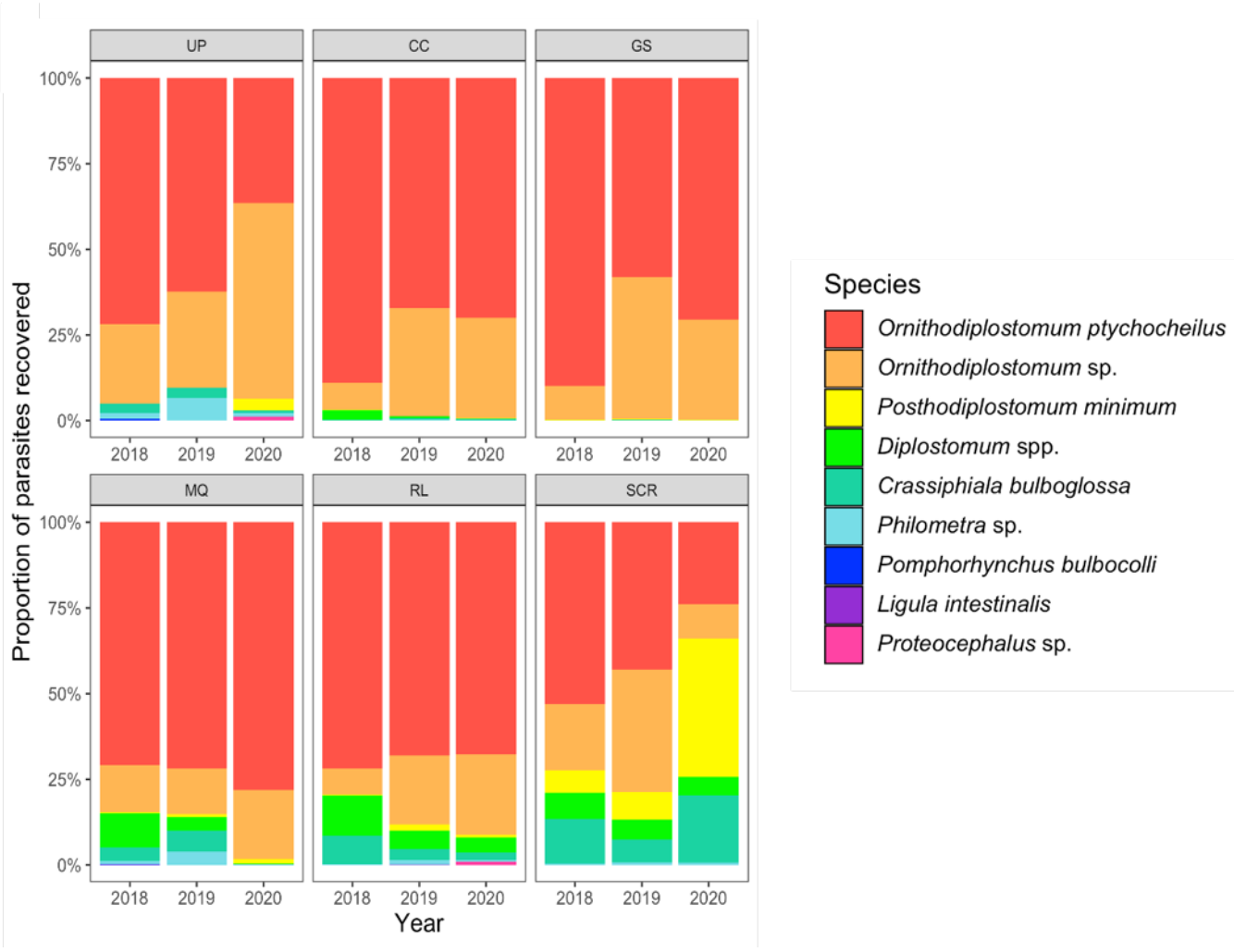

Figure 2.4. Relative abundances of parasite species recovered in fathead minnows (*Pimephales promelas*) from southern Alberta, Canada between 2018–2020.

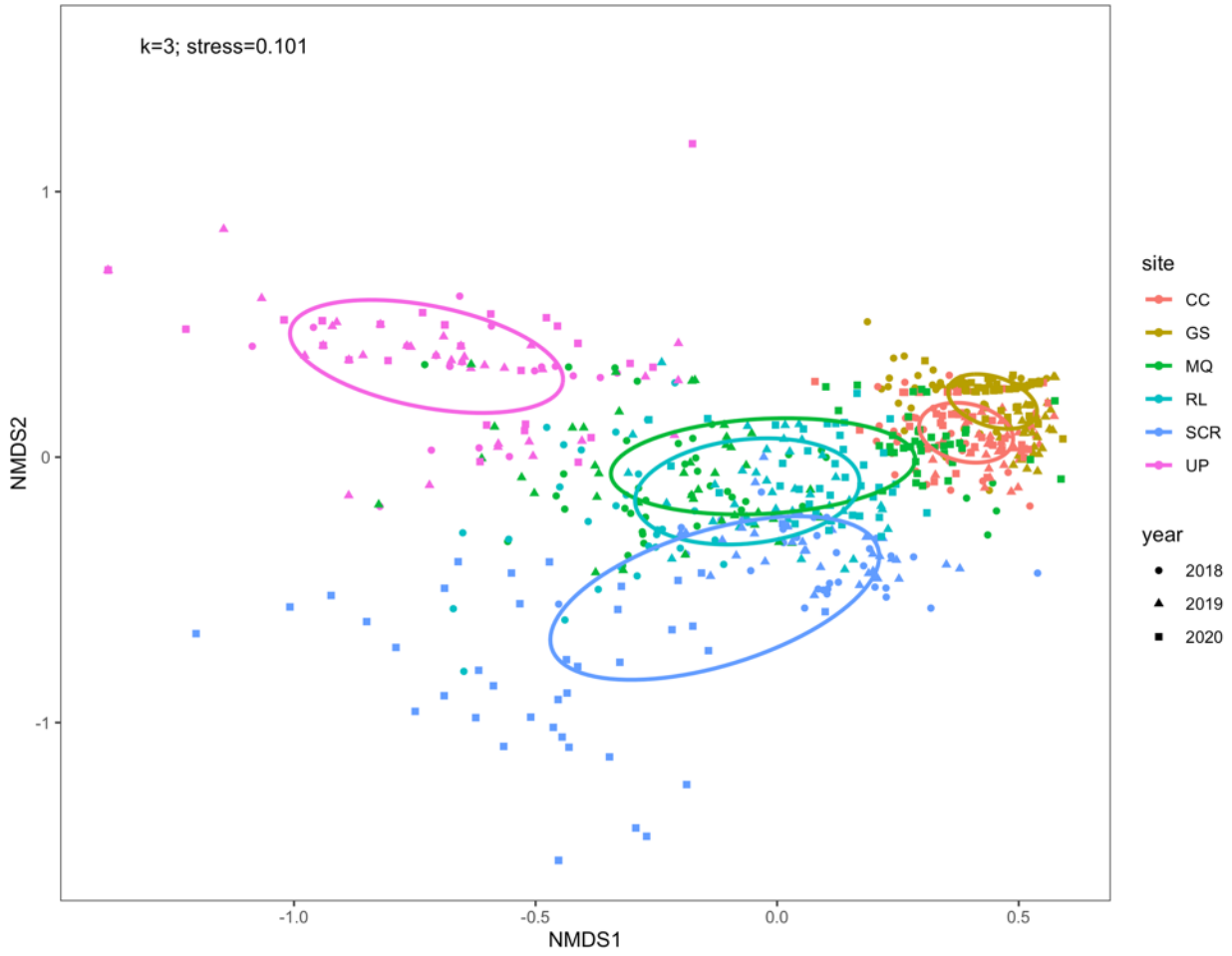

Figure 2.5. Nonmetric multidimensional scaling (NMDS) ordinations of parasite infracommunities of fathead minnows (*Pimephales promelas*) from southern Alberta, Canada between 2018–2020. Infracommunity distances are based on Bray-Curtis dissimilarities for six species. Infracommunities are limited to taxa with ≥ 10% prevalence at one or more sites. Ellipses represent 95% confidence intervals enclosing all points in each site. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; UP: University Pond.

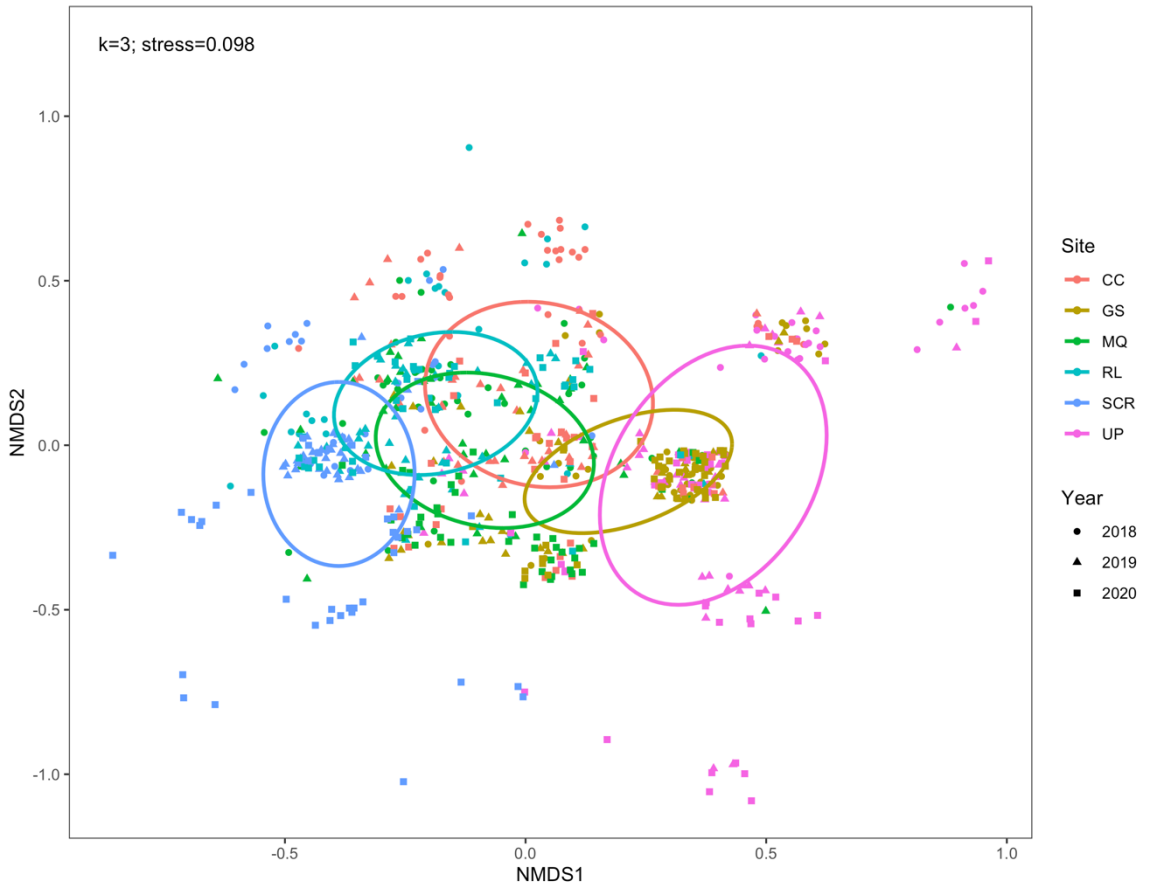

Figure 2.6. Nonmetric multidimensional scaling (NMDS) ordinations of parasite infracommunities of fathead minnows (*Pimephales promelas*) from southern Alberta, Canada between 2018–2020. Infracommunity distances are based on Sørensen dissimilarities for seven species. Infracommunities are limited to taxa with ≥ 10% prevalence at one or more sites. Ellipses represent 95% confidence intervals enclosing all points in each site. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; UP: University Pond.

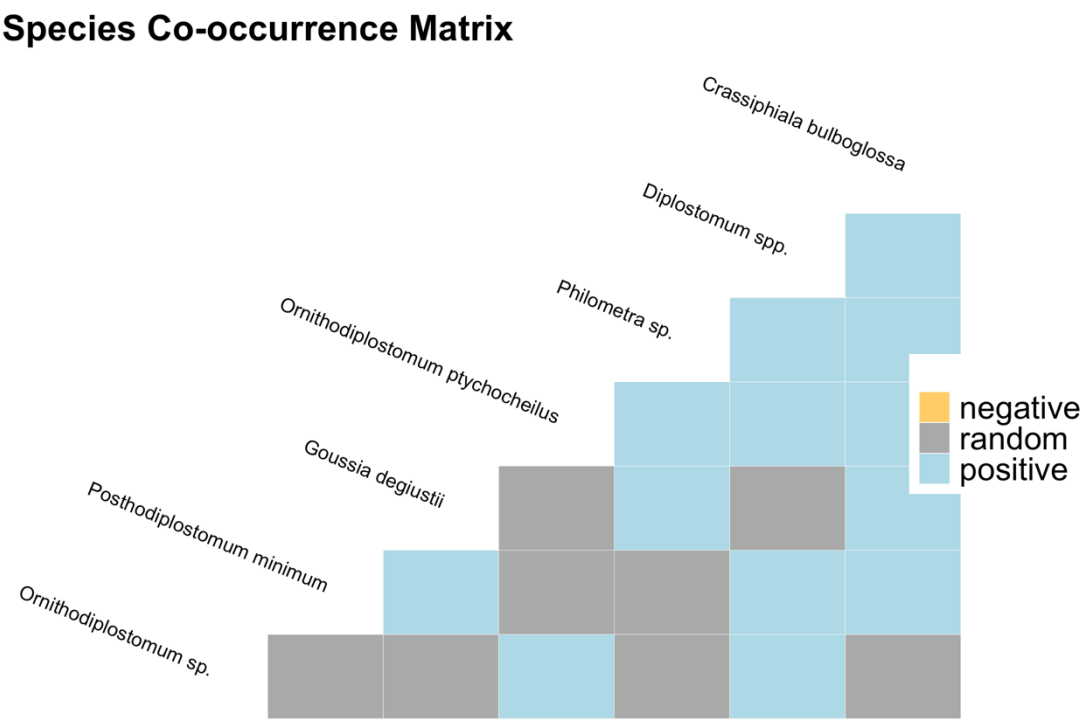

Figure 2.7. Species co-occurrence matrix for all possible pairwise comparisons between parasites infecting fathead minnows (*Pimephales promelas*) from six sites in southern Alberta between 2018–2020. Data from all sites and years were pooled.

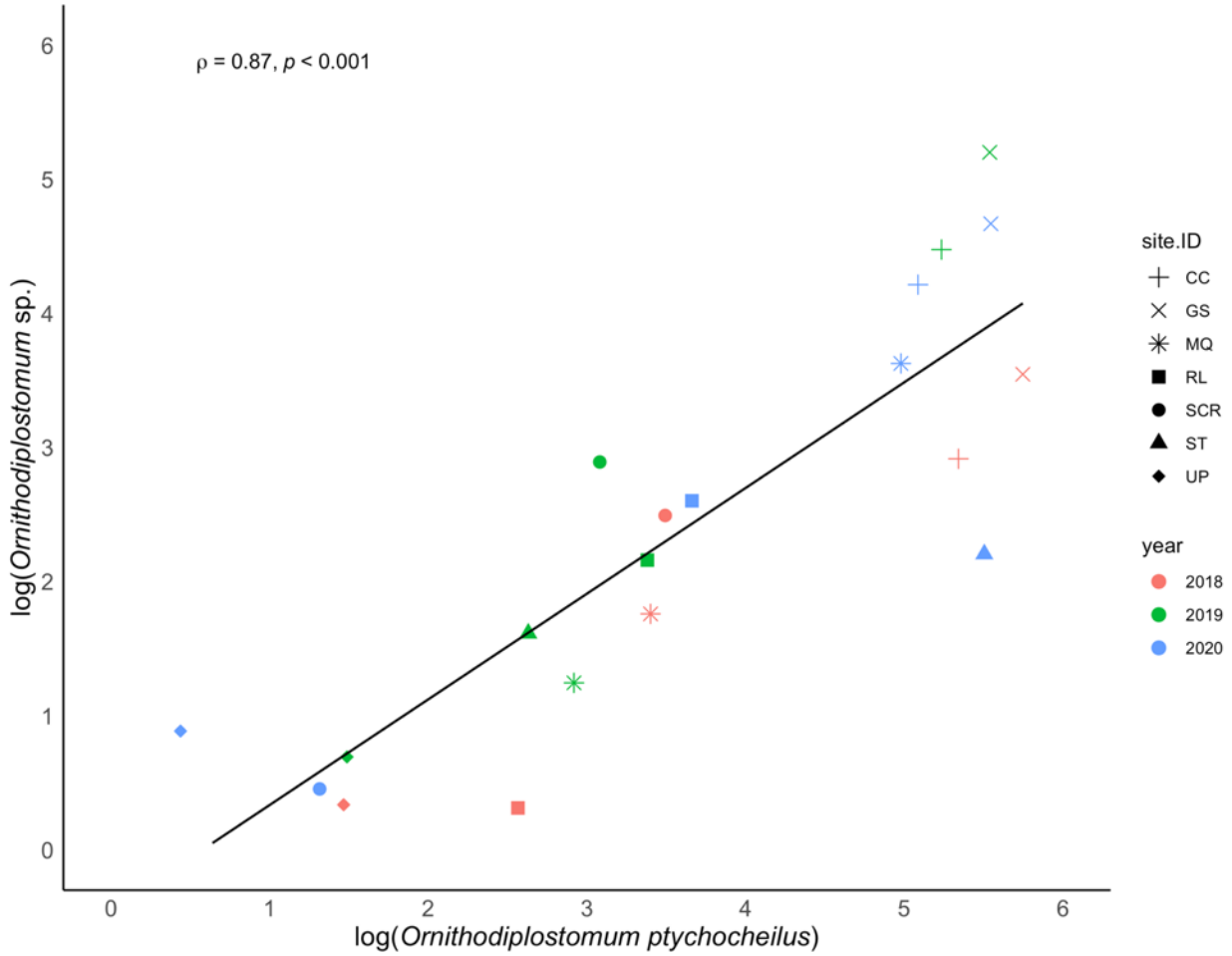

Figure 2.8. Spearman correlation between mean abundances of the two trematodes *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. in populations of fathead minnows (*Pimephales promelas*) collected in southern Alberta between 2018–2020. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; ST: Stirling Lions' Fish Pond; UP: University Pond.

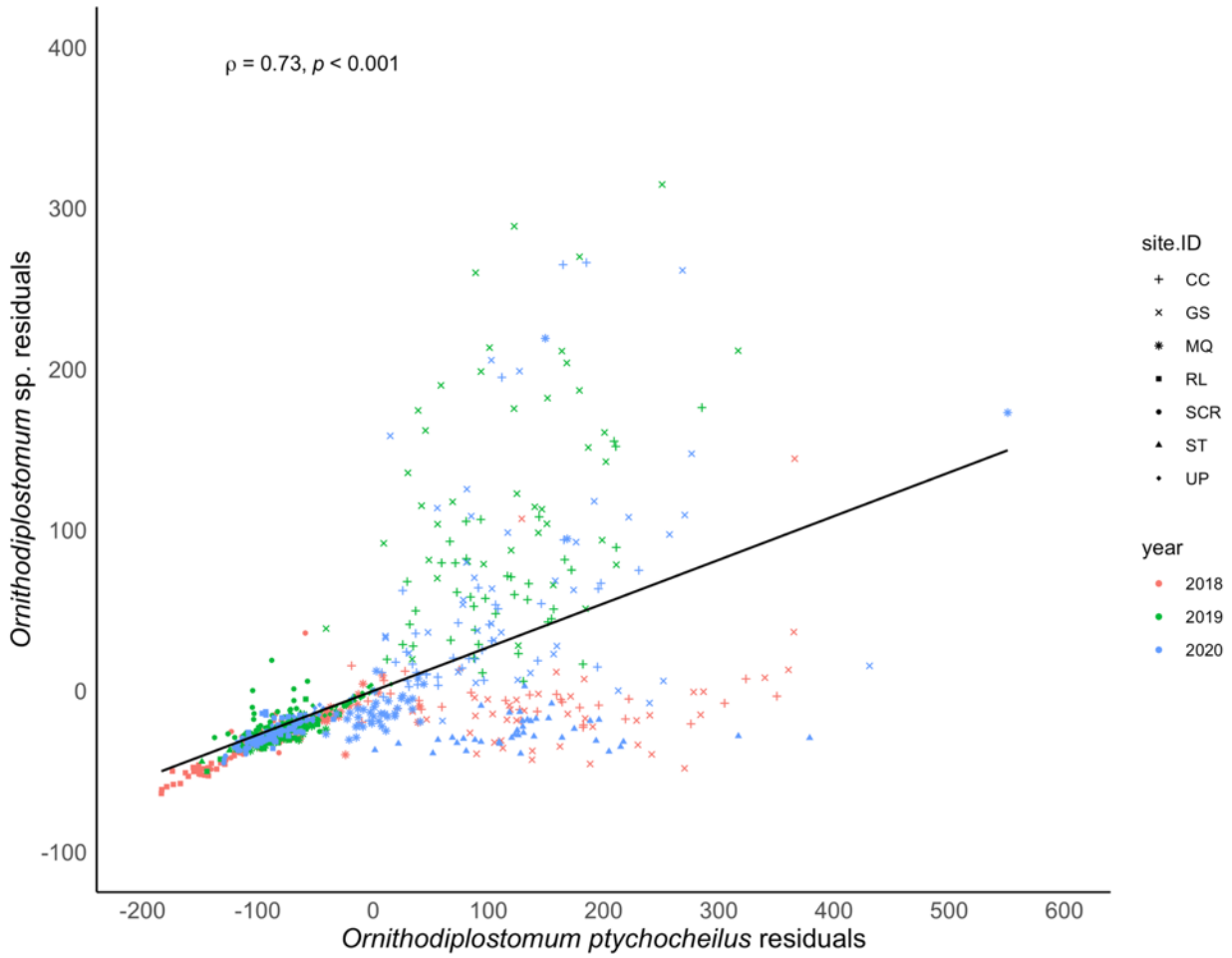

Figure 2.9. Spearman correlation between residuals from the abundance-host standard length relationships for *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. in fathead minnows (*Pimephales promelas*) from southern Alberta between 2018–2020. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; ST: Stirling Lions' Fish Pond; UP: University Pond.

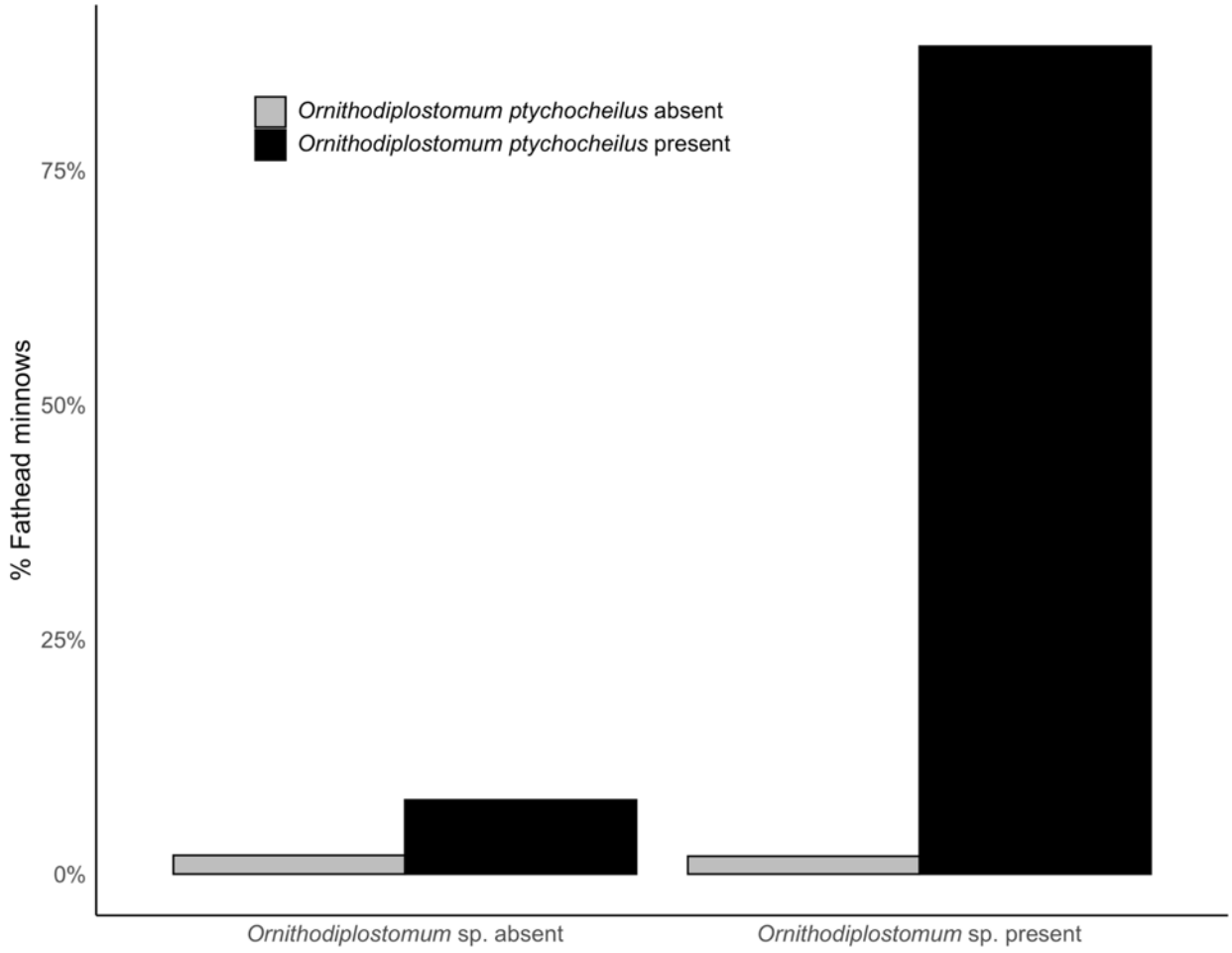

Figure 2.10. Percentage of fathead minnows (*Pimephales promelas*) infected by *Ornithodiplostomum ptychocheilus*, *Ornithodiplostomum* sp., or both species.

Table 2.1. Geographic coordinates of sampling sites expressed in decimal degrees.

Site	Latitude	Longitude
University Pond	49.680528	-112.870672
Coulee Creek Stormwater Pond	49.656625	-112.784628
McQuillan Reservoir	49.647114	-112.459358
Gold Spring Park Pond	49.096131	-111.995817
Reesor Lake	49.664333	-110.105672
Spruce Coulee Reservoir	49.672761	-110.180889
Stirling Lions' Fish Pond	49.500775	-112.536664

Table 2.2. Intensity (mean ± SD) and prevalence (%) of parasites infecting fathead minnows (*Pimephales promelas*) collected in southern Alberta, Canada between 2018–2020. Intensity is not provided for eimerian *Goussia degiustii* because it was not enumerated at necropsy. Host sample size (*n*) is provided next to the site abbreviations. CC: Coulee Creek Stormwater Pond; GS: Gold Spring Park Pond; MQ: McQuillan Reservoir; RL: Reesor Lake; SCR: Spruce Coulee Reservoir; ST: Stirling Lions' Fish Pond; UP: University Pond.

Species	Site						
	CC (110)	GS (121)	MQ (122)	RL (107)	SCR (105)	ST (80)	UP (110)
Ornithodiplostomum ptychocheilus	183.9 ± 78.9 (100%)	274.7 ± 94.9 (100%)	64.0 ± 81.0 (100%)	28.3 ± 15.4 (100%)	20.0 ± 16.8 (90%)	129.7 ± 128.2 (100%)	4.1 ± 3.3 (83%)
Ornithodiplostomum sp.	61.6 ± 53.0 (100%)	107.0 ± 84.4 (100%)	16.8 ± 34.1 (92%)	9.5 ± 6.9 (89%)	12.4 ± 12.7 (81%)	7.1 ± 4.2 (100%)	2.8 ± 2.1 (70%)
Posthodiplostomum minimum	1.2 ± 0.4 (15%)	2.1 ± 1.5 (26%)	2.4 ± 2.0 (39%)	1.5 ± 0.8 (32%)	5.8 ± 4.7 (85%)	3.9 ± 7.3 (60%)	1.2 ± 0.4 (5%)
Diplostomum spp.	5.9 ± 7.1 (42%)	1.3 ± 0.5 (5%)	3.5 ± 6.4 (52%)	2.7 ± 1.6 (86%)	3.6 ± 3.3 (74%)	4.9 ± 2.8 (99%)	– (0%)
Crassiphiala bulboglossa	2.1 ± 1.2 (55%)	2.0 ± 1.4 (18%)	2.4 ± 1.7 (50%)	2.3 ± 1.5 (62%)	4.8 ± 4.7 (96%)	1.4 ± 0.5 (6%)	1.1 ± 0.3 (12%)
Philometra sp.	1.4 ± 0.9 (15%)	1.5 ± 1.0 (14%)	1.6 ± 0.8 (30%)	1.1 ± 0.4 (27%)	1.2 ± 0.4 (19%)	2.4 ± 3.1 (36%)	1.2 ± 0.5 (18%)
Goussia degiustii	– (62%)	– (87%)	– (95%)	– (89%)	– (90%)	– (98%)	– (67%)

Species	Site						
	CC (110)	GS (121)	MQ (122)	RL (107)	SCR (105)	ST (80)	UP (110)
Pomphorhynchus bulbocolli	– (0%)	1.0 ± NA (1%)	1.0 ± 0.0 (3%)	– (0%)	– (0%)	1.0 ± NA (2%)	1.0 ± NA (1%)
Ligula intestinalis	– (0%)	1.0 ± NA (1%)	1.0 ± 0.0 (2%)	1.0 ± NA (1%)	– (0%)	– (0%)	– (0%)
Proteocephalus sp.	– (0%)	– (0%)	– (0%)	2.6 ± 2.4 (8%)	– (0%)	– (0%)	1.0 ± 0.0 (2%)

Table 2.3. Summary of associations between the prevalence and intensity of parasite species by site, year, their interaction, and fathead minnow standard length using GLM analysis. Factors absent from minimum adequate models are denoted by a dash. *Philometra* sp. intensities were under-dispersed, so a zero-inflated negative binomial model was used to evaluate *Philometra* sp. abundance. *P* values are as follows: NS > 0.05; * < 0.05; ** < 0.01; *** < 0.001.

	Site		Year		Site × Year		Standard length		% Deviance explained
	χ^2	*P*	χ^2	*P*	χ^2	*P*	Estimate ± SE	*P*	
O. ptychocheilus prevalence	72.00	***	27.09	***	–	–	0.1859 ± 0.0808	*	35.3
O. ptychocheilus intensity	4858.70	***	52.00	***	738.50	***	0.0612 ± 0.0058	***	80.4
Ornithodiplostomum sp. prevalence	89.99	***	2.05	NS	81.95	***	–	–	43.2
Ornithodiplostomum sp. intensity	1868.95	***	285.61	***	310.18	***	0.0570 ± 0.0089	***	89.3
P. minimum prevalence	197.14	***	50.02	***	77.73	***	–	–	36.2
P. minimum intensity	151.59	***	12.35	**	–	–	0.0674 ± 0.0167	***	48.2
Diplostomum spp. prevalence	347.41	***	80.47	***	40.13	***	0.1197 ± 0.0423	*	50.0
Diplostomum spp. intensity	41.44	***	50.59	***	62.96	***	0.0862 ± 0.0154	**	37.2
C. bulboglossa prevalence	243.27	***	22.87	***	29.03	**	–	–	29.8
C. bulboglossa intensity	118.59	***	26.36	***	41.07	***	0.0293 ± 0.0127	*	39.4
Philometra sp. prevalence	13.94	*	105.36	***	26.50	**	0.0783 ± 0.0344	NS	19.3
Philometra sp. abundance	17.63	**	55.50	***	20.47	**	0.0659 ± 0.0245	*	NA
G. degiustii prevalence	68.99	***	208.53	***	–	–	–	–	42.9

Table 2.4. Number of observed and expected co-occurrences for significant, non-random species associations between parasites infecting fathead minnows (*Pimephales promelas*) from southern Alberta, Canada. Minnows from sites sampled in all three years (n = 675) were included in the analysis. P_{lt} and P_{gt} represent the probability that each species pair co-occurs less than or greater than expected by chance, respectively. Cb: *Crassiphiala bulboglossa;* Ds: *Diplostomum* spp.; Gd: *Goussia degiustii;* Op: *Ornithodiplostomum ptychocheilus*; Osp: *Ornithodiplostomum* sp.; Ph: *Philometra* sp.; Pm: *Posthodiplostomum minimum*.

Species 1	Species 2	Sites with species 1	Sites with species 2	Observed # of sites with both species	Probability both species occur at a site	Expected # of sites with both species	P_{lt}	P_{gt}
Op	Osp	646	600	586	0.851	574.2	1.00000	0.00000
Op	Ds	646	286	284	0.406	273.7	1.00000	0.00002
Op	Cb	646	324	316	0.459	310.1	0.99355	0.01850
Op	Ph	646	139	138	0.197	133.0	0.99894	0.00949
Osp	Ds	600	286	263	0.377	254.2	0.99012	0.01903
Pm	Ds	225	286	117	0.141	95.3	0.99987	0.00024
Pm	Cb	225	324	134	0.160	108.0	0.99999	0.00001
Pm	Gd	225	553	208	0.273	184.3	1.00000	0.00000

Species 1	Species 2	Sites with species 1	Sites with species 2	Observed # of sites with both species	Probability both species occur at a site	Expected # of sites with both species	P_{lt}	P_{gt}
Ds	Cb	286	324	197	0.203	137.3	1.00000	0.00000
Ds	Ph	286	139	78	0.087	58.9	0.99991	0.00018
Cb	Ph	324	139	79	0.099	66.7	0.99257	0.01239
Cb	Gd	324	553	278	0.393	265.4	0.99569	0.00767
Ph	Gd	139	553	128	0.169	113.9	0.99995	0.00016

CHAPTER 3: Experimental co-infections reveal intra- and interspecific effects on the growth of *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. (Trematoda: Digenea) metacercariae in fathead minnows (*Pimephales promelas*)

3.1 Abstract

Parasites are ubiquitous in nature and individual hosts are regularly infected with multiple parasite species or strains simultaneously. Co-occurring parasites can profoundly impact each other and their hosts via synergistic or antagonistic interspecific interactions within hosts. Co-infection studies in mice indicate that these interactions can mediate aspects of infection and disease. These complex outcomes together with the experimental intractability of many host-parasite systems have led to contention regarding the significance of multiparasitism in wild hosts. Here I show that the development of the larvae of two species of digenean trematode (*Ornithodiplostomum* spp.) is influenced by both conspecific and heterospecific intensities in their fathead minnow intermediate host. Following exposure to infective cercariae, both species showed consistent and complex patterns of development that involved a two to four week period of rapid growth, followed by consolidation and encystment phases between four and ten weeks. Negative intensity-dependent growth occurred for *O. ptychocheilus* metacercariae in the brain of minnows, but not for *Ornithodiplostomum* sp. metacercariae in the liver. Furthermore, both species' body sizes were larger post-encystment in mono-infections than in co-infections. My results demonstrate that naturally co-occurring parasites in spatially segregated infection sites can influence each another's growth, although the mechanism for this subtle yet symmetric interaction remains unclear.

3.2 Introduction

Digeneans (Trematoda: Digenea) are obligate parasitic flatworms, numbering upwards of some 25,000 species (Esch et al., 2002) that include taxa relevant to both clinical and veterinary medicine. Digenean trematodes are renowned for their complex life cycles, which typically involve three hosts. When digeneans infect fish second intermediate hosts, cercariae may penetrate at specific sites, like the primary gill lamellae (Paller and Uga, 2008) or more generally at sites characterized by irregularities in the host body surface, like the base of the fins and the opercula and between scales (Hendrickson, 1979). At this stage in development, the larval trematodes are known as metacercariae. Metacercariae were previously thought to be a quiescent, resting stage due to the restricted nutrient absorption and ingestion imposed by a complex cyst wall present in fully developed metacercariae (Poulin and Latham, 2003). This view has been largely overturned in recognition that metacercariae of some diplostomid species possess a dynamic tegumental ultrastructure thought to be involved in feeding that 1) supports increases in somatic growth and 2) disappears at encystment (Podvyaznaya, 1999; Goater et al., 2005). Metacercariae represent an important stage in the digenean life cycle, and their patterns of growth and development can inform us about the overall host-parasite interaction.

Metacercariae of strigeid trematodes undertake extensive within-host migrations to the site of encystment (Conn et al., 2008). Furthermore, some strigeids undergo subtle microhabitat shifts from sites where they grow to nearby, secondary sites associated with encystment. For example, *Ornithodiplostomum ptychocheilus* transitions from within the optic lobes or cerebellum of the brain to the adjacent meninges (Matisz et al., 2010).

Matisz et al. (2010) proposed that this microhabitat shift is assumed to accommodate metacercarial encystment, perhaps by placing developing trematodes in proximity to cellular components like fibroblasts thought to be involved in the formation of the secondary, host-contributed cyst wall (So and Wittrock, 1982). This microhabitat shift is also demonstrated for a congeneric species, *Ornithodiplostomum* sp., which transitions from within tissues of the liver and pancreas to spaces in the body cavity along, or in loose association with, the peritoneum (Matisz and Goater, 2010).

Metacercarial growth within second intermediate hosts has implications for adult fitness. The size of adult worms is positively related to their size as metacercariae (Poulin and Latham, 2003). Larger metacercariae also produce more eggs than their smaller conspecifics (Fredensborg and Poulin, 2005). Metacercariae size is thus a direct determinant of parasite reproduction and survival. The results of studies completed on other larval helminths such as acanthocephalans suggests that size at transition (*i.e.*, from intermediate to definitive hosts) is positively related to infection success (Steinauer and Nickol, 2003). These results indicate that reduced larval size in second intermediate hosts might impact the success of adult trematodes in the definitive host.

Parasite size is also related to the number of conspecific and heterospecific individuals that co-occur within an individual host (Peoples and Poulin, 2011; Dianne et al., 2012). These intraspecific and interspecific relationships cannot be overlooked, as parasites co-occur with conspecifics, and fishes are routinely co-infected by multiple parasite species or strains simultaneously (Holzer et al., 2005; Pracheil and Muzzall, 2009). Intraspecific intensity dependence is strongly supported for helminths (Benesh and Valtonen, 2007; Cornet, 2011) including trematodes (Sandland and Goater, 2000; Brown

et al., 2003; Fredensborg and Poulin, 2005; Saldanha et al., 2009). Interspecific interactions may be asymmetric, where one species' growth is influenced but the other species' is not, or symmetric, where both species influence one another's growth. Helminth interspecific interactions are not widely studied outside of the host gastrointestinal tract (Poulin, 2001), and studies examining interactions between larval helminths are rare (Poulin et al., 2003) perhaps because of the long-standing paradigm (described above) that these stages are metabolically inactive. Relationships between larval helminth size and intensity within the second intermediate host often concentrate on relatively small hosts like amphipods, copepods, or isopods (Benesh, 2011).

The fathead minnow *Pimephales promelas* (Pisces: Cyprinidae) and its naturally occurring assemblage of trematode parasites are a tractable model system for examining the consequences of co-infection in individual hosts. In this study, I evaluate the effects of intensity dependence and co-infection on parasite development for two trematode species that co-occur in wild-caught fish, *Ornithodiplostomum ptychocheilus* and its congener, *Ornithodiplostomum* sp. (Chapter 2). I experimentally exposed naïve minnows to cercariae of a single species at one of two intensities (mono-infections) and of both species at the same intensity (co-infection). I assess the effects of the presence of conspecifics and heterospecifics, for both species, on metacercariae development during distinct growth, encystment, and consolidation phases.

3.3 Materials and Methods

3.3.1 Source and maintenance of naïve fish

In Chapter 2 I show that fathead minnow populations in southern Alberta are frequently co-infected with both species of *Ornithodiplostomum* at intensities often exceeding 100 parasites per fish. This precluded the use of field-collected minnows for experimental exposures. Thus, to source naïve hosts I collected fathead minnow eggs and reared juvenile minnows under cercariae-free conditions. Fathead minnow eggs were collected at McQuillan Reservoir (49.647114, -112.459358) on July 17, 2019. McQuillan Reservoir is a shallow, eutrophic irrigation reservoir managed as a rainbow trout fishery approximately 25km east of Lethbridge. Eggs were collected from clutches that were attached to substrate and were transported to the University of Lethbridge in aerated coolers. Eggs were then transferred into four plastic 250L containers housed within a gated, fenced area on campus. Following hatch, juvenile minnows were transferred to larger, 1200L tanks for rearing (Stumbo et al., 2012). Each of the larger containers contained 800 g of air-dried reeds (*Typha* L.) as a source of cover that was inoculated with locally-collected concentrated zooplankton and amphipods as an *ad libitum* food source for fish. Beginning in November 2019, minnows were overwintered indoors in flow-through, 9L tanks (water T: 22°C) and fed a maintenance diet of ground Tetramin fish flakes and freeze-dried bloodworms. Minnows were returned to the outdoor containers in May 2020, and the maintenance diet was continued until the experiment started. The capture of approximately size-matched minnows from the containers was via Gee traps and dip nets and took place two days in advance of experimental infections.

Minnows were acclimated in aquaria in between capture and infection on 14 August 2020.

3.3.2 Infection procedure

The experimental protocols herein were approved by the University of Lethbridge's Animal Welfare Committee (protocol #1806) in adherence with guidelines established by the Canadian Council on Animal Care. Methods used to infect minnows follow Sandland and Goater (2000). Cercariae were gathered from infected adult *Physa gyrina* collected from a local stormwater drainage pond that was known to contain minnows infected with both species of trematode (Chapter 2; Ahn, 2019). Individual snails were placed in glass vials containing a small volume of dechlorinated water and exposed to artificial light for approximately three hours to stimulate cercarial shedding. *Ornithodiplostomum ptychocheilus* (OP) cercariae were collected from two snails and *Ornithodiplostomum* sp. (BC) cercariae were collected from nine snails. Cercariae from individual snails were pooled according to species and placed in graduated cylinders with volume adjusted to 100mL (Sandland and Goater, 2000). Average cercarial concentrations in these suspensions were estimated by counting the numbers of cercariae present in three 1mL aliquot samples. The counting procedure required approximately one hour; thus, the cercariae used in experimental infections were a maximum of four hours old.

Minnows were individually exposed to either 50 or 100 cercariae of OP or BC (referred to as 'mono-infection'), or 50 cercariae of both species (50+50; referred to as 'co-infection'). These exposure intensities fall within the range of natural intensities

recorded from field-caught fish (Sandland et al., 2001) without being so high as to induce localized epidermal hemorrhaging. Twenty-five fish were assigned at random to each of the infection treatments. Fifteen fish were designated as controls and were given a sham exposure of 1mL of parasite-free water. Individual minnows were exposed to cercariae for two hours, during which they were housed in covered dishes containing 40mL of dechlorinated water in a darkened room.

Following exposure, minnows were transferred to cercariae-free water. Fish were randomly assigned to be necropsied at one of five experimental endpoints at two, four, six, eight, or ten weeks post-infection (wpi). These endpoints were selected to capture the phases of metacercarial development (growth, encystment, and consolidation) described in previous studies of *Ornithodiplostomum* spp. in their second intermediate host (Sandland and Goater, 2000; Matisz and Goater, 2010). Minnows were fed ground Tetramin flake food once per day and were maintained in aerated, flow through 9L tanks under a constant 16:8 light:dark photoperiod (water T: 22°C) in the Aquatic Research Facility within the Alberta Water and Environmental Science Building.

3.3.3 Necropsy

On the date of necropsy, each host's total and standard lengths (mm) and weight (g) were measured. Fish were euthanized via a stunning blow to the skull followed by cervical dislocation. Intact brains and viscera were removed from each fish and placed on an individual microscope slide. To achieve consistent fixation of living worms, approximately two mL of 70% ethanol was dripped onto the tissue (Sandland and Goater, 2000). The tissue containing metacercariae was then teased apart with dissecting needles.

A coverslip was carefully applied without manual pressure to this fixed tissue. Additionally, the brain case and the body cavity were rinsed following the removal of their contents to isolate metacercariae that had separated from the meninges and/or the peritoneum during dissection (Weinersmith et al., 2014); any such metacercariae were fixed in the same manner as described above.

Immediately following fixation, slides were examined for metacercariae under a compound microscope. A random subsample of unobstructed metacercariae (average: 15/sample; range: 6 – 43) was photographed with a digital camera. Maximum length and width was measured for each metacercariae from digital photographs using ImageJ V2.1.0 (Schindelin et al., 2012). Metacercarial volume was calculated according to the formula for an oblate spheroid, $V = \frac{4}{3}\pi(r_1^2)(r_2)$, where r_1 is half the maximum width and r_2 is half the maximum length (Fredensborg and Poulin, 2005). Metacercarial volume was selected as the most conservative growth metric because it accounted for the less-uniform shape of unencysted metacercariae along two axes (*i.e.*, length and width) rather than one (length). Metacercarial length and volume were positively correlated ($r[2,321] = 0.77$, $p < 0.05$).

3.3.4 Statistical analyses

All statistical analyses were conducted in RStudio (version 1.4.1106) with R 4.0.4 (R Core Team, 2020). Prior to analysis, data were assessed for normality and homogeneity of variance with Shapiro-Wilk's and Levene's tests, respectively. Parasite volume measurements were $\log_{10}$-transformed to meet the assumptions of normality.

Spearman correlations were used to test for relationships between metacercariae intensity and metacercariae volume in mono-infected fish exposed to 50 or 100 cercariae, and between metacercariae intensity and host length. I evaluated the effects of metacercariae intensity and infection frequency (mono-infection versus co-infection) on the mean proportion of metacercariae recovered following exposure to cercariae, the mean proportion of encysted metacercariae, and mean metacercariae volume. Effects of infection intensity on these responses were assessed by comparing parasites in fish exposed to 50 and 100 cercariae. The effects of co-infection were assessed by comparing metacercariae recovery, encystment, and volume in fish exposed to 50 and 50+50 cercariae.

Metacercariae recovery was the number of metacercariae in each fish divided by the cercariae exposure dose. In co-infected hosts, recovery was calculated separately for each species (*e.g.*, OP recovery was the proportion of OP cercariae exposure rather than the total (OP+BC) cercariae exposure). Differences in mean percent recovery were assessed with a pair of two-way ANOVAs. The first tested for the effects of species, infection intensity, and the interaction, and the second tested for the effects of species, infection frequency, and their interaction.

Metacercariae encystment was the number of encysted metacercariae in each fish divided by the total number counted at necropsy. Proportion encystment was assessed with beta regression (Ferrari and Cribari-Neto, 2004), since arcsine square-root and logit data transformations failed to resolve their non-normal distributions. Beta regression, most often used for rates, proportions, or inequality indices, models a continuous variable linked to a linear predictor like in generalized linear models (GLM), but the continuous

variable is modelled with a beta probability distribution (Douma and Weedon, 2019). I rescaled encystment values of zero and one according to Smithson and Verkuilen (2006) so they fit within the required open interval (0,1). Separate regressions related encystment to the fixed effects of 1) species, infection intensity, and their interaction and 2) species, co-infection, and their interaction. Regression modelling was carried out with the betareg package (Cribari-Neto and Zeileis, 2010) in R using a logit link and maximum likelihood estimation, and pairwise *post-hoc* contrasts between differences of least-square means were performed with the emmeans package (Lenth, 2021).

I used linear mixed modelling to evaluate the effects of infection intensity and infection frequency on $\log_{10}$-transformed metacercariae volume. This approach allowed us to consider the main, fixed effects of interest, in addition to random effects that would accommodate the non-independence generated by measuring multiple trematodes infecting the same host (Harrison et al., 2018). The effects of infection intensity and infection frequency were examined in separate sets of models. All models were fit with the lmer function within lme4 (Bates et al., 2015) using restricted maximum likelihood estimation to minimize type I error rates (Luke, 2017). Models included host total length as an additional fixed factor, while fish, and metacercariae nested within fish were included as random factors. Fixed factors were tested with *F*-tests (type III analysis of variance) and Satterthwaite's approximation using the anova function within lmerTest (Kuznetsova et al., 2017). A random intercept model was applied to the random factors, which were evaluated with likelihood ratio tests using the rand function within lmerTest. This random intercept model allowed model intercepts to vary among fish and

metacercariae within fish. Tukey's tests were used *post-hoc* to compare mean volumes between levels of the fixed factors.

3.4 Results

3.4.1 General patterns of infection in exposed fish

Minnow mortality was negligible during the ten week experiment; two experimentally infected fish died prior to sampling. In the remaining 123 exposed minnows, prevalence was 100% for both OP (n = 72) and BC (n = 75). Mean metacercariae intensities for OP and BC for each treatment are provided in Table 3.1. At exposure, average total length (TL) of minnows was 45.0 ± 6.9 mm (mean ± SD, range: 30.3 – 60.2 mm). The correlation between host length and metacercariae intensity was not significant for either species of parasite (OP: $r_s = 0.01$, $p = 0.781$; BC: $r_s = -0.03$, $p = 0.283$). The overall pattern of encystment was consistent for both species. All metacercariae were unencysted at two wpi whereas by six wpi, 99.2% of the 1,271 metacercariae recovered at necropsy were encysted. All metacercariae were encysted by eight wpi.

3.4.2 Effects of infection intensity

Mean metacercariae recovery was not affected by the interaction between species and intensity (F[1,94] = 1.14, $p = 0.288$). Mean recovery was significantly higher for BC than for OP (Fig. 3.1; F[1,94] = 19.4, $p < 0.001$). Overall, metacercariae recovery was 11% higher for BC (mean: 65.5 ± 13.0 %) compared to OP (mean: 54.4 ± 12.1%).

Metacercariae recovery was not significantly different in fish exposed to 50 or 100 cercariae (Fig. 3.1; $F[1,94] = 1.81$, $p = 0.182$).

Mean metacercariae encystment at four wpi (Fig. 3.2) was not affected by the interaction between species and intensity ($F[1,\infty] = 1.92$, $p = 0.166$). Rates of encystment were significantly different between BC and OP (Fig. 3.3, $F[1,\infty] = 34.41$, $p < 0.001$) but not between fish exposed to 50 or 100 cercariae ($F[1,\infty] = 1.23$, $p = 0.267$). Mean metacercariae encystment was 12.5% higher for BC (LS mean: 97.2 ± 0.8%) than for OP (LS mean: 84.7 ± 2.0%). Pairwise contrasts between least-square means confirmed significant differences in encystment between species in fish exposed to 50 cercariae ($p = 0.004$) and between species in fish exposed to 100 cercariae ($p < 0.001$). In fish exposed to 50 cercariae encystment was 9.5% higher for BC (LS mean: 96.9 ± 1.2%) than for OP (LS mean: 87.4 ± 2.6%), while in fish exposed to 100 cercariae encystment was 15.4% higher for BC (LS mean: 97.5 ± 1.0%) than for OP (LS mean: 82.1 ± 3.1%).

Peak metacercariae volume occurred at four wpi for both species (Figs. 3.4, 3.5). For OP, maximum volume at four wpi resulted from significant increases from two wpi (50: $p < 0.001$; 100: $p < 0.001$). Similarly, there was an increase in BC volume between two and four wpi in the 50 cercariae exposure while BC volume in the 100 cercariae exposure decreased between two and four wpi, although neither change in volume was significant. Between four and six wpi, both species experienced significant declines in volume (OP – 50: $p < 0.001$; 100: $p < 0.001$; BC – 50: $p < 0.001$; 100: $p < 0.001$). At ten wpi, OP metacercariae were 31-52% smaller and BC metacercariae were 34-46% smaller than at four wpi. Within the overall data set that included 1,607 metacercariae, metacercariae volumes varied significantly between fish exposed to the same cercariae

dose for both species, and variation in metacercariae volume within a given host was only significant for OP (Table 3.2). The effect of intensity on volume was significant at two, four, eight, and ten wpi for OP (Fig. 3.4) and at two, six, and eight wpi for BC (Fig. 3.5).

The relationship between metacercariae intensity and metacercariae volume was further assessed with Spearman correlations at ten wpi when the maximum length of time for metacercariae to potentially interact with each other and with the host had elapsed. At ten wpi I found patterns of negative intensity-dependent growth for OP ($r_s = -0.38$, $p < 0.001$) in experimental mono-infections ranging from 19-54 individuals, whereas individual BC volume was independent of intensity ($r_s = -0.07$, $p = 0.371$) in mono-infections ranging from 22-79 individuals (Fig. 3.6).

3.4.3 Effects of co-infection

Co-infection had complex effects on recovery, encystment, and growth for both species (Figs. 3.7-3.9). Overall, mean metacercariae recovery was not affected by the interaction between species and the presence of a congener ($F[1,94] = 0.64$, $p = 0.424$). Mean recovery was significantly higher for BC than for OP (Fig. 3.2, $F[1,94] = 16.90$, $p < 0.001$), similar to the effects of intensity. Overall, metacercariae recovery was 10% higher for BC (mean: 68.0 ± 13.7 %) compared to OP (mean: 58.0 ± 12.2%). Additionally, mean metacercariae recovery was significantly higher in co-infected fish than in mono-infected fish ($F[1,94] = 11.87$, $p < 0.001$). Recovery in co-infected fish was 9% higher (mean: 66.8 ± 13.0%) than in mono-infected fish (mean: 58.5 ± 13.7%).

The interaction between species and co-infection had a significant effect on mean metacercariae encystment at four wpi (Fig. 3.7; $F[1,\infty] = 5.56$, $p = 0.018$). Co-infection had contrasting effects on encystment for the two species. For OP, co-infection was associated with a 2.5% increase in encystment, while for BC co-infection was associated with a 11.0% decrease. Following pairwise contrasts between least-square means, there was a significant difference in encystment between species in mono-infection ($p = 0.037$) and between co-infection and mono-infection for BC ($p = 0.016$). Encystment in mono-infected fish was 9.5% higher for BC (LS mean: 96.1 ± 1.6%) than for OP (LS mean: 86.6 ± 3.3%). BC encystment in mono-infected fish was 11% higher (LS mean: 96.1 ± 1.6%) than in co-infected fish (LS mean: 85.1 ± 3.5%).

Within the overall data set that included 1,350 metacercariae, both species' volumes differed significantly between fish exposed to the same treatment; Table 3.3). Volumes also differed significantly ($p < 0.05$) for BC metacercariae within the same host, at ten wpi. The effect of co-infection on metacercariae volume was significant for both species. OP metacercariae were 26% larger in co-infections than in mono-infections at four wpi ($p < 0.001$), while OP metacercariae were 17% larger in mono-infections than in co-infections at ten wpi ($p = 0.02$; Fig. 8). BC metacercariae were 7% larger in mono-infections than in co-infections at eight wpi ($p < 0.001$; Fig. 3.9).

3.5 Discussion

My work is the first to examine intensity dependence of BC metacercariae in fathead minnows, and I reaffirm the negative intensity dependent growth of OP previously described by Sandland and Goater (2000). The developmental trajectory of BC

reflected that of OP, comprising distinct phases of growth, consolidation, and encystment. BC metacercariae were larger than OP during the growth phase, which was characterized by considerable variation in volume for both species. Encystment was largely synchronous within and between species and metacercariae volumes remained relatively stable between six and ten weeks post-infection following consolidation. Interspecific effects on metacercariae growth were observed post-encystment in a symmetric manner, where the presence of heterospecifics at a distant infection site resulted in smaller parasite volumes compared to conspecifics in mono-infected fish. Taken together, my results show that the development of *Ornithodiplostomum* spp. metacercariae in their second intermediate host is influenced by both conspecific and heterospecific intensities.

My results show that BC metacercariae follow a complex pattern of development. Metacercariae initially experienced an extended growth phase, then a rapid period of encystment, and finally a consolidation phase during which parasite volumes stabilized to approximately half their maximum. The sequence and the timing of these phases paralleled the growth and development of the congener, OP. My results support Matisz and Goater's (2010) finding that complex developmental trajectories are adopted by parasites infecting sites other than the central nervous system. I observed encysted metacercariae free within the visceral cavity, typically associated with the peritoneum near the intestine, liver, spleen, swim bladder, or kidney. This is consistent with Matisz and Goater's (2010) description of a microhabitat shift by developing metacercariae from sites within tissues to the visceral cavity, corresponding to encystment. I did not evaluate infectivity of BC metacercariae to a suitable avian definitive host, but OP infectivity in chickens increases from zero at four wpi to 36% at ten wpi (Shirakashi and Goater, 2005).

Based on their overall similarities in development, BC likely requires a similar window of obligate development prior to infectivity. While an eight to ten week requirement is longer than those compiled by Dönges (1969) for all other diplostomid metacercariae that encyst after a growth phase, he suggests the time frame may have an adaptive value since trematodes can use the metacercariae stage to bridge ecologically unfavourable periods (*e.g.*, the seasonal absence of the definitive host).

Patterns of intensity-dependent parasite growth are typically attributed to competition between conspecifics. Since early descriptions of the "crowding effect" (Read, 1951), the phenomenon has been demonstrated for various developmental stages of many helminth species (Fong et al., 2017). This intraspecific phenomenon is often presumed to be exploitative competition for the finite resources available to parasites within individual hosts. In particular, parasites are thought to compete for nutrients or space. Identifying the proximate cause of competition between OP conspecifics was not an objective of the present study, but my results on intensity-dependent growth are consistent with the results of other experimental (Shostak et al., 2008) and field studies (Brown et al., 2003) of parasites in their intermediate hosts. Perhaps most importantly, my results confirm those in Sandland and Goater (2000), despite the fact I infected minnows older and larger than those used in the earlier study, likely rendering space and nutrients less limiting.

I saw the first evidence of negative, intensity-dependent growth for OP at two wpi (Fig. 3.10), while metacercariae are within their peak growth phase, as well as at ten wpi. Contrastingly, I saw evidence of negative, intensity-dependent growth for BC at two wpi, but at ten wpi growth was independent of intensity. Intensity-dependent growth may

persist for OP because metacercariae are still competing for resources at ten wpi, or the pattern of growth may be the result of carry-over effects from earlier in development (pre-encystment). Carry-over effects manifest when an organism's performance or characteristics are influenced by conditions experienced during a prior stage in life history or development (O'Connor et al., 2014), and are well recognized for species with complex life cycles. Trematodes experience carry-over effects across life history stages (Fredensborg and Poulin, 2005), so perhaps they experience similar effects within life history stages. Ultimately, selection optimizes parasite growth and development in intermediate hosts against overall parasite survival and fecundity (Keymer, 1982; Michaud et al., 2006). Density-dependent growth regulation for OP may serve to avoid pathological disruption of tissues in the central nervous system, while density-independent growth for BC by ten wpi may indicate that the liver and/or body cavity is a more resource-rich environment than the central nervous system.

This study is one of few that examines interspecific effects on trematode growth through the lens of controlled, experimental infections. My results are among the first to demonstrate a significant and symmetrical effect of co-infection on larval parasite growth. Along with detectable effects of co-infection on metacercariae recovery, metacercariae volume of one species was affected by the presence of the other, and vice versa. One plausible explanation for these symmetric effects is cross-reactive immunity. OP and BC are closely related species and may share cross-reactive antigens. Rellstab et al. (2013) showed that infection with one genotype of the strigeid *Diplostomum spathaceum* was sufficient to induce cross-immunity against subsequent genotypes in rainbow trout. Relatively little is known about the teleost immune response to strigeid metacercariae, but

studies do support a functional response in fishes involving the innate arm of the immune system (Kalbe and Kurtz, 2006; Rauch et al., 2006). The targets of immune surveillance are thought to be penetrating cercariae, migrating diplostomules, and unencysted metacercariae (Stables and Chappell, 1986; Wood and Matthews, 1987). Thus, OP and BC would risk immune detection and attack from the time of infection until approximately four wpi. Cercariae experienced lower success penetrating the epidermis of Arctic charr compared to during their within-host migration (Voutilainen et al., 2010); a potent response localized at the epidermis could explain why I observed metacercariae recovery less than 100%. However, the host immune response to closely related species may be different from the response to different genotypes of the same species.

The nearly 10% higher recovery in co-infected fish relative to mono-infected fish is difficult to explain. This result was also symmetric, such that more metacercariae of both species were recovered in co-infected fish than in mono-infected fish. This could be suggestive of a facilitative, interspecific interaction, or merely heterogeneity in cercariae host search success. The mechanism by which cross-reactive immunity would influence metacercariae growth is unknown, but one hypothesis is that the effects are mediated through pathways related to oxidative stress. Stumbo et al. (2012) demonstrated that both OP and BC induce lipid peroxidation, an indicator of oxidative stress, in the liver of fathead minnows. Oxidative stress has been demonstrated to be a product of host immune activation in other host-parasite systems (Scharsack et al., 2007). The resultant environment may not provide optimal conditions for growth, and constraining growth could be a strategy for co-infected hosts to tolerate infections.

These results must be interpreted relative to natural patterns of parasite transmission. The key difference between experimental and natural infections is that minnows received a single, synchronous cercariae exposure in this study while metacercariae development occurs in the presence of already-established, encysted conspecifics in wild populations (Sandland et al. 2001). However, my experimental exposures would represent the experience of young-of-the-year minnows that have not yet accumulated metacercariae. A second limitation of my work is that I did not control for clonal diversity after pooling cercariae from multiple (two or more) snails to gather sufficient numbers of parasites for exposure. Clonal diversity could result in competition between parasites during infection. For example, *Diplostomum spathaceum* cercariae from mixed-genotype infections in snails had higher success infecting immunologically-naïve rainbow trout (*Oncorhynchus mykiss*) than cercariae from single-genotype infections (Karvonen et al., 2012). Klemme et al. (2016) also found that co-infecting genotypes were more successful than single genotypes at infecting previously-infected hosts, and emphasized the importance of infection history in experimental exposures since hosts are only immunologically-naïve for a short fraction of their lifespan in the wild.

My study design shows that I can draw robust conclusions about within-host parasite development from these results. The design addresses several concerns raised by Poulin (2010) in his meta-analysis of experimental cercarial infections. Chiefly, my exposures produce infection intensities within the range observed in naturally infected minnows; indeed, intensities established in this study are relatively low considering natural intensities can exceed 500 metacercariae per fish in some lakes (Chapter 2). I did

not observe negative dose-dependency in the proportions of cercariae recovered as metacercariae. The absence of dose-dependent recovery suggests that the surface area of an individual minnow is sufficiently large that cercariae do not interact with one another during host encounter (Poulin, 2010). The two-hour cercariae exposure was sufficient for successful infection of fathead minnows. Metacercariae were initially recovered in this study at two weeks post infection, but OP diplostomules can be found within connective, adipose, and hypodermal tissues as early as fifteen minutes post-infection (Matisz et al., 2010) and BC diplostomules can be detected within sub-dermal muscles as early as thirty minutes post-infection (Matisz and Goater, 2010). Altogether, I show that the fathead minnow and its parasites are a tractable model system for exploring the broader significance of parasite co-infections in other wild hosts, such as endangered and threatened fishes and those considered for the diversification of finfish aquaculture.

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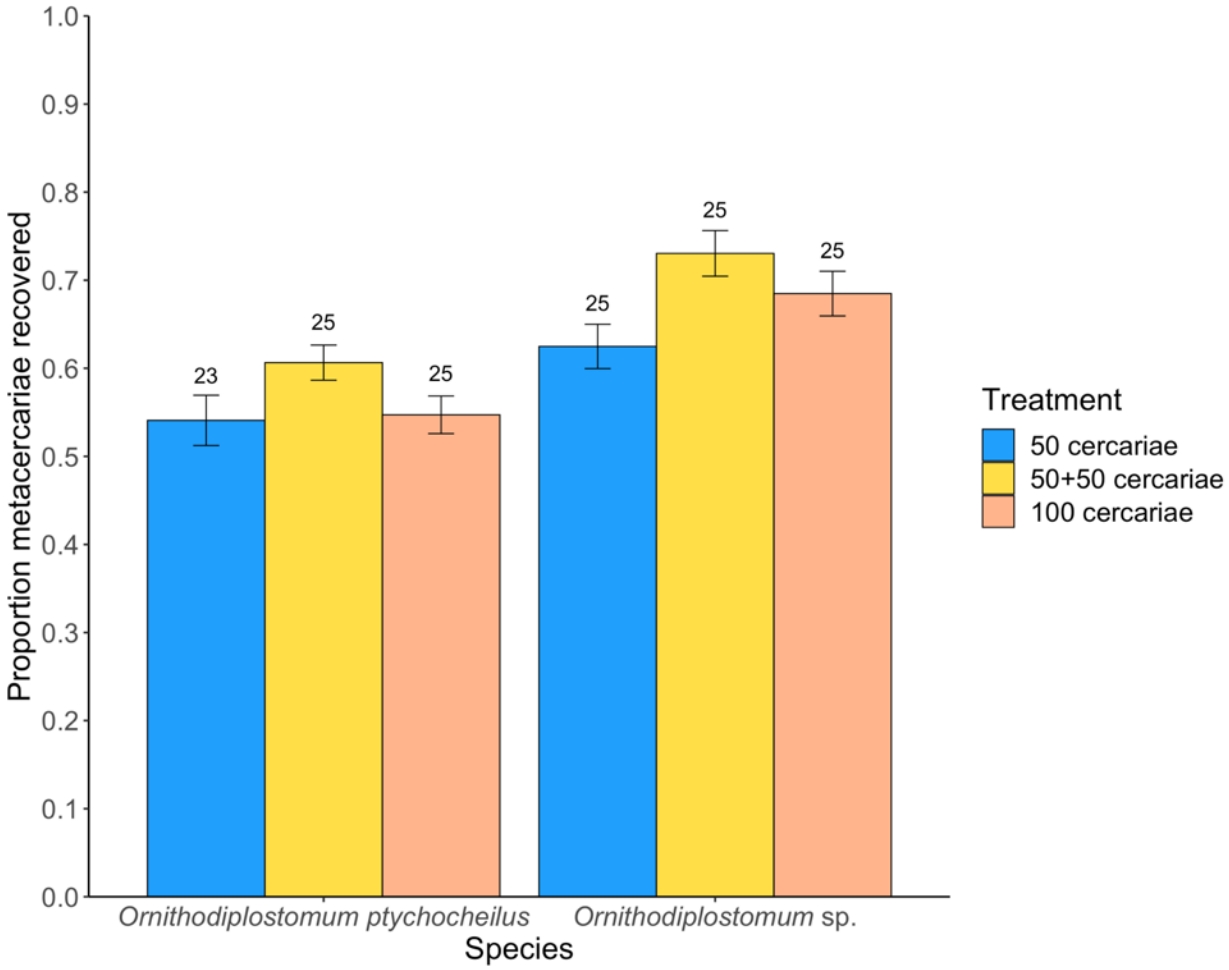

Figure 3.1. Proportion of trematode cercariae recovered as metacercariae in experimentally infected fathead minnows (*Pimephales promelas*). For co-infected hosts (50+50 cercariae) recovery was calculated in a species-specific manner, as a proportion of the 50 cercariae of that species rather than the total 100 cercariae. Recovery was pooled across experimental endpoints for minnows exposed to the same treatment. Host sample sizes are presented above the error bars.

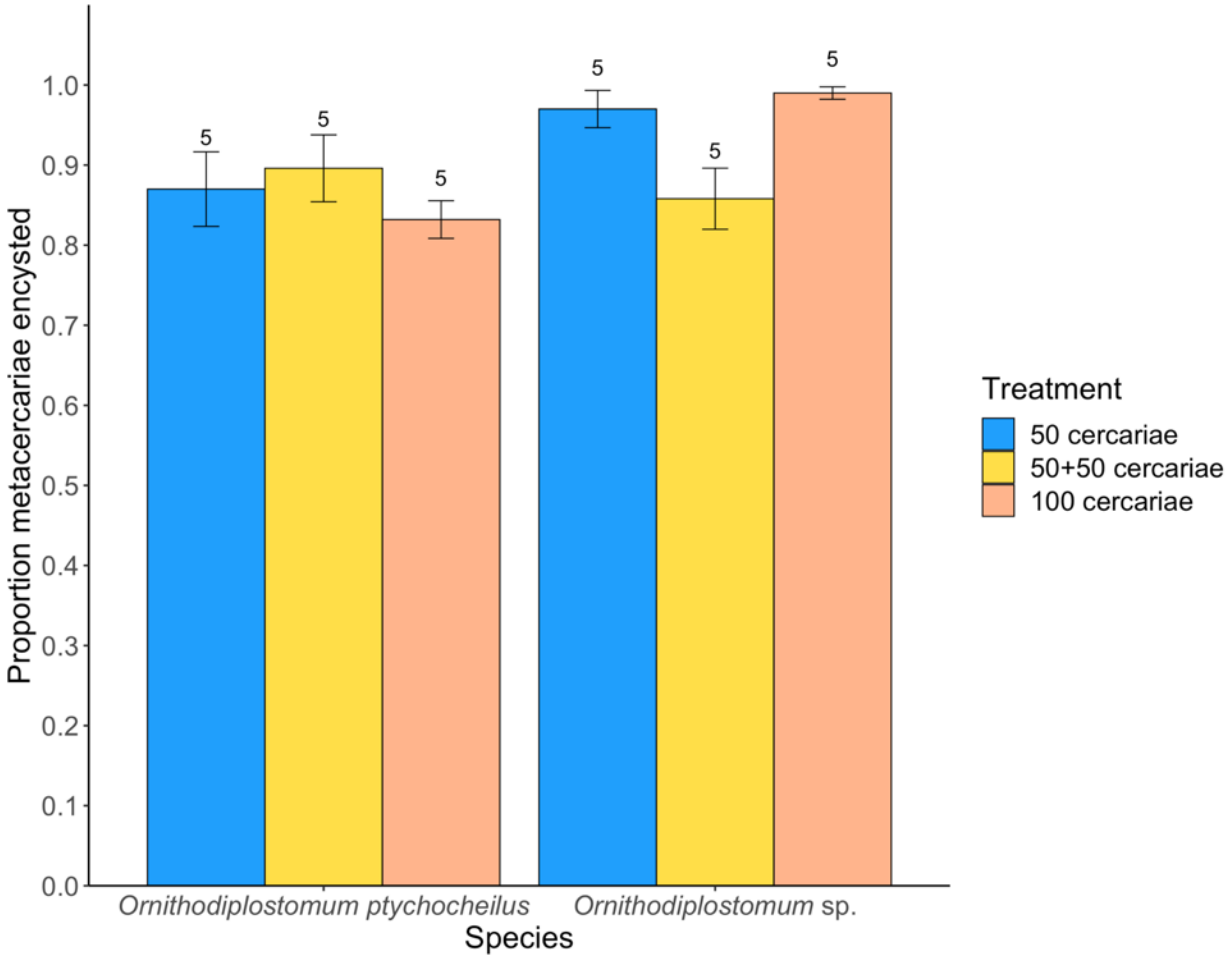

Figure 3.2. Proportion of encysted trematode metacercariae relative to the total number recovered at necropsy at four weeks post-infection in experimentally infected fathead minnows (*Pimephales promelas*). For co-infected hosts (50+50 cercariae) encystment was calculated in a species-specific manner, as a proportion of the 50 cercariae of that species rather than the total 100 cercariae. Host sample sizes are presented above the error bars.

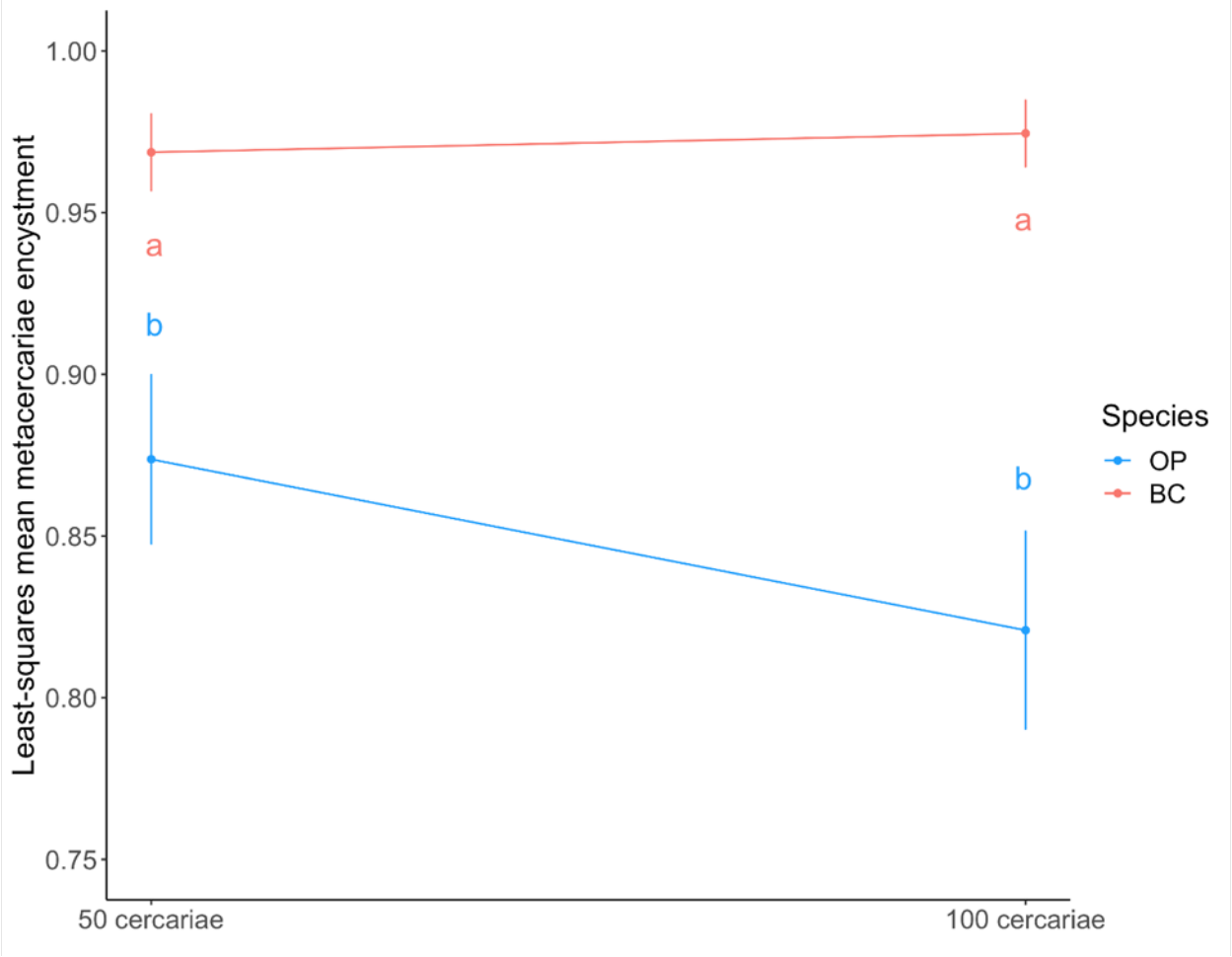

Figure 3.3. Interaction plot between trematode species and infection intensity for fathead minnows exposed to 50 or 100 cercariae. Different letters represent significant differences ($p < 0.05$) based on pairwise contrasts of least-square means using Tukey's adjustment for multiple comparisons. OP: *Ornithodiplostomum ptychocheilus*; BC: *Ornithodiplostomum* sp.

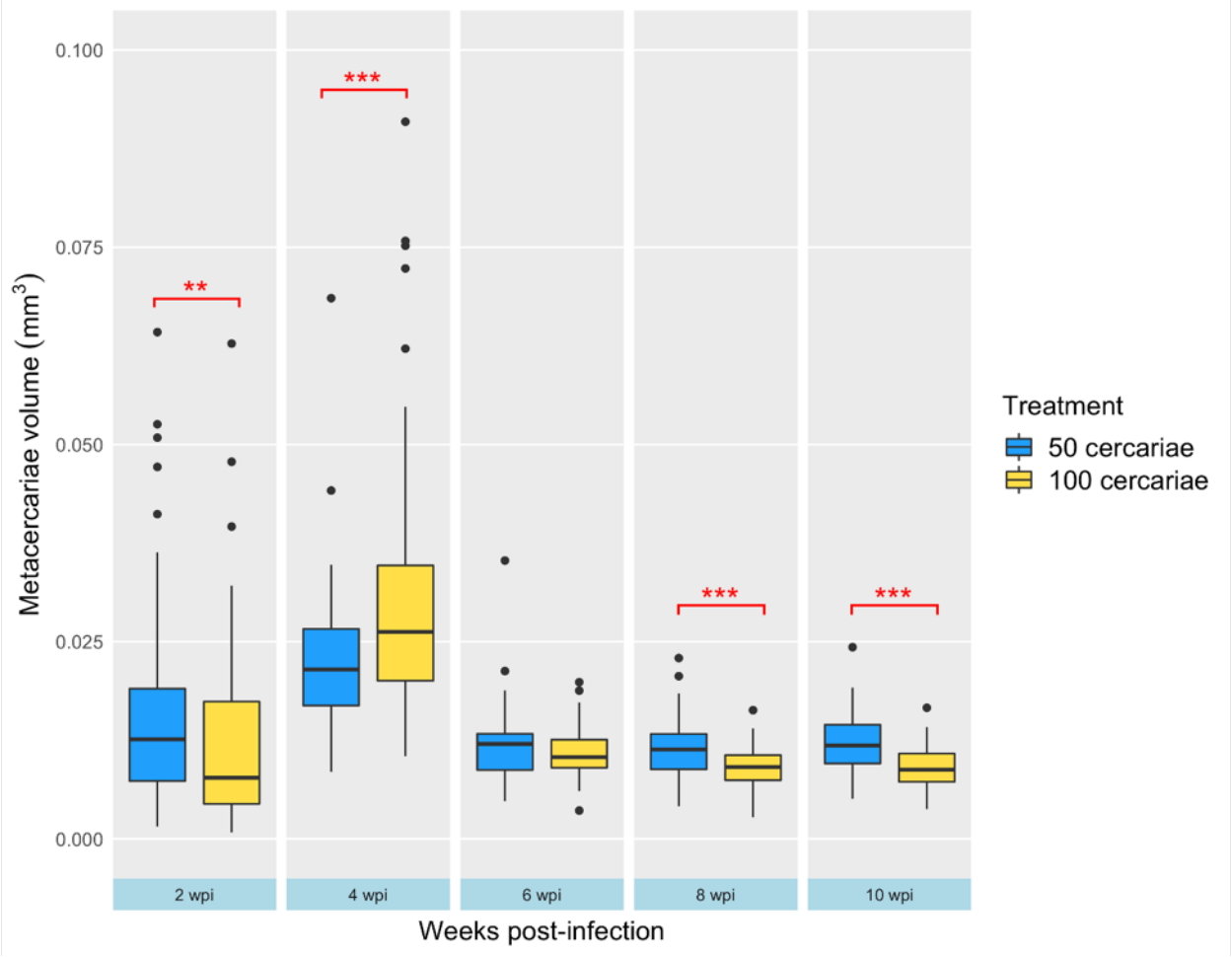

Figure 3.4. Growth of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows exposed to either 50 (blue) or 100 (yellow) cercariae. Parasite volume was evaluated at two, four, six, eight, and ten weeks post-infection (wpi). Arcs indicate a significant infection intensity effect while asterisks represent *p* values: * <0.05; ** <0.01; *** <0.001.

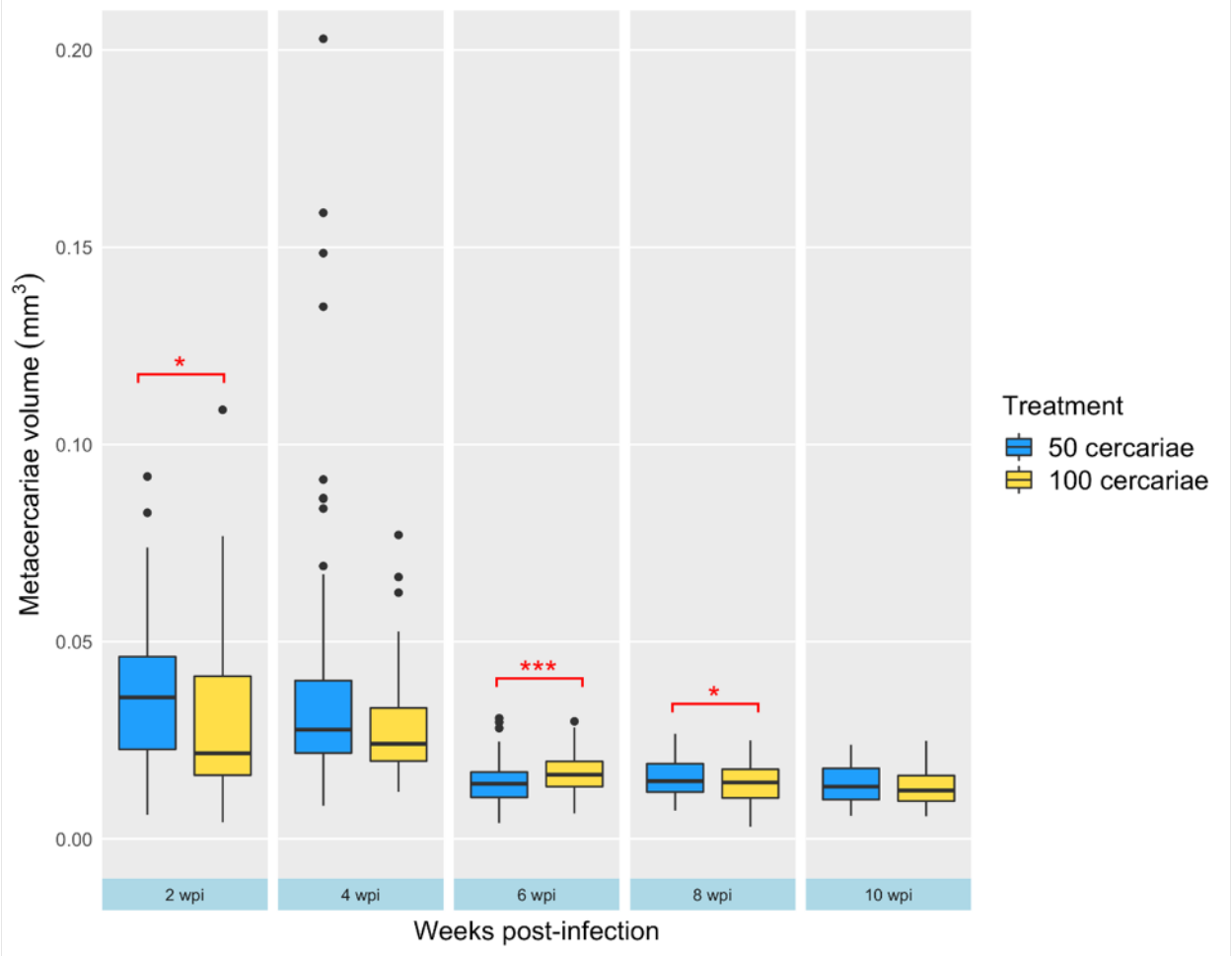

Figure 3.5. Growth of *Ornithodiplostomum* sp. metacercariae in fathead minnows exposed to either 50 (blue) or 100 (yellow) cercariae. Parasite volume was evaluated at two, four, six, eight, and ten weeks post-infection (wpi). Arcs indicate a significant infection intensity effect while asterisks represent *p* values: * <0.05; ** <0.01; *** <0.001.

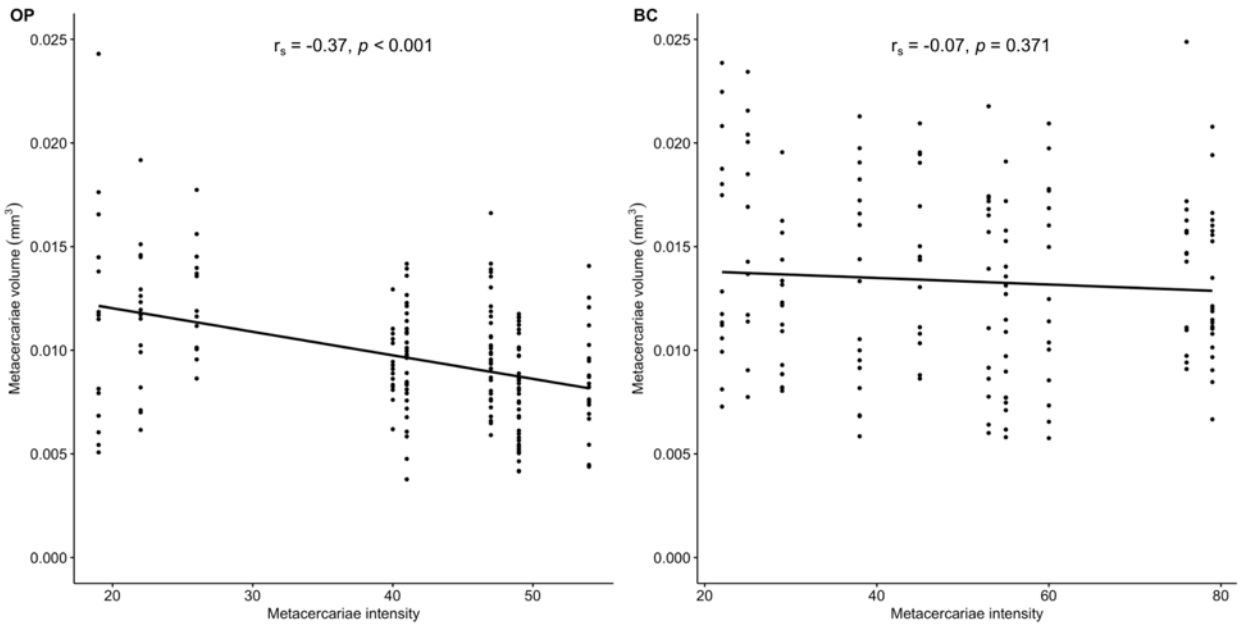

Figure 3.6. Patterns of intensity-dependent growth for *Ornithodiplostomum ptychocheilus* (OP) and *Ornithodiplostomum* sp. (BC) metacercariae recovered from experimentally infected fathead minnows (*Pimephales promelas*) at ten weeks post-infection. Intensity refers to the number of metacercariae recovered from minnows during necropsy.

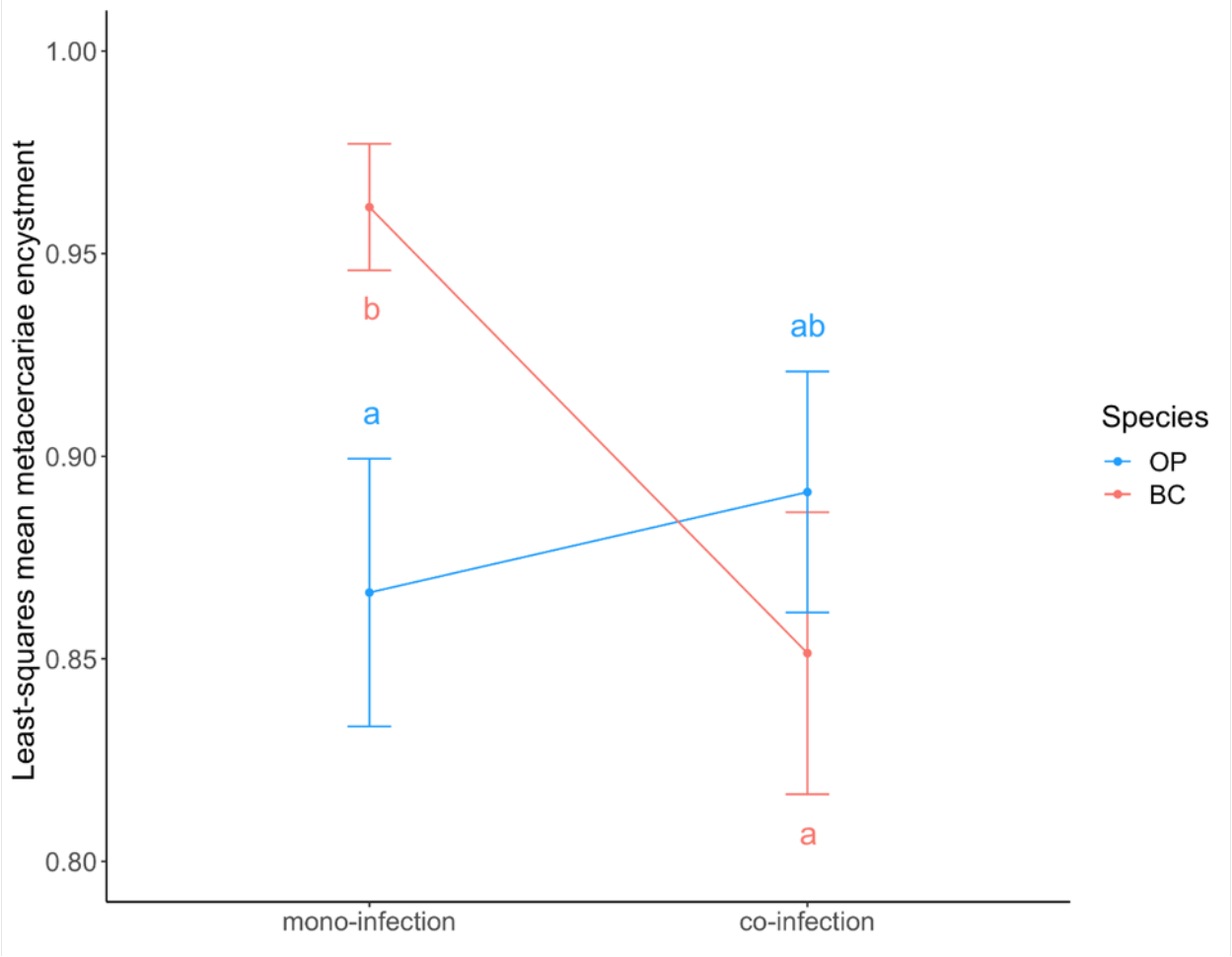

Figure 3.7. Interaction plot between trematode species and exposure conditions (mono- or co-infection) for fathead minnows exposed to 50 or 50+50 cercariae. Different letters represent significant differences ($p < 0.05$) based on pairwise contrasts of least-square means using Tukey's adjustment for multiple comparisons. OP: *Ornithodiplostomum ptychocheilus*; BC: *Ornithodiplostomum* sp.

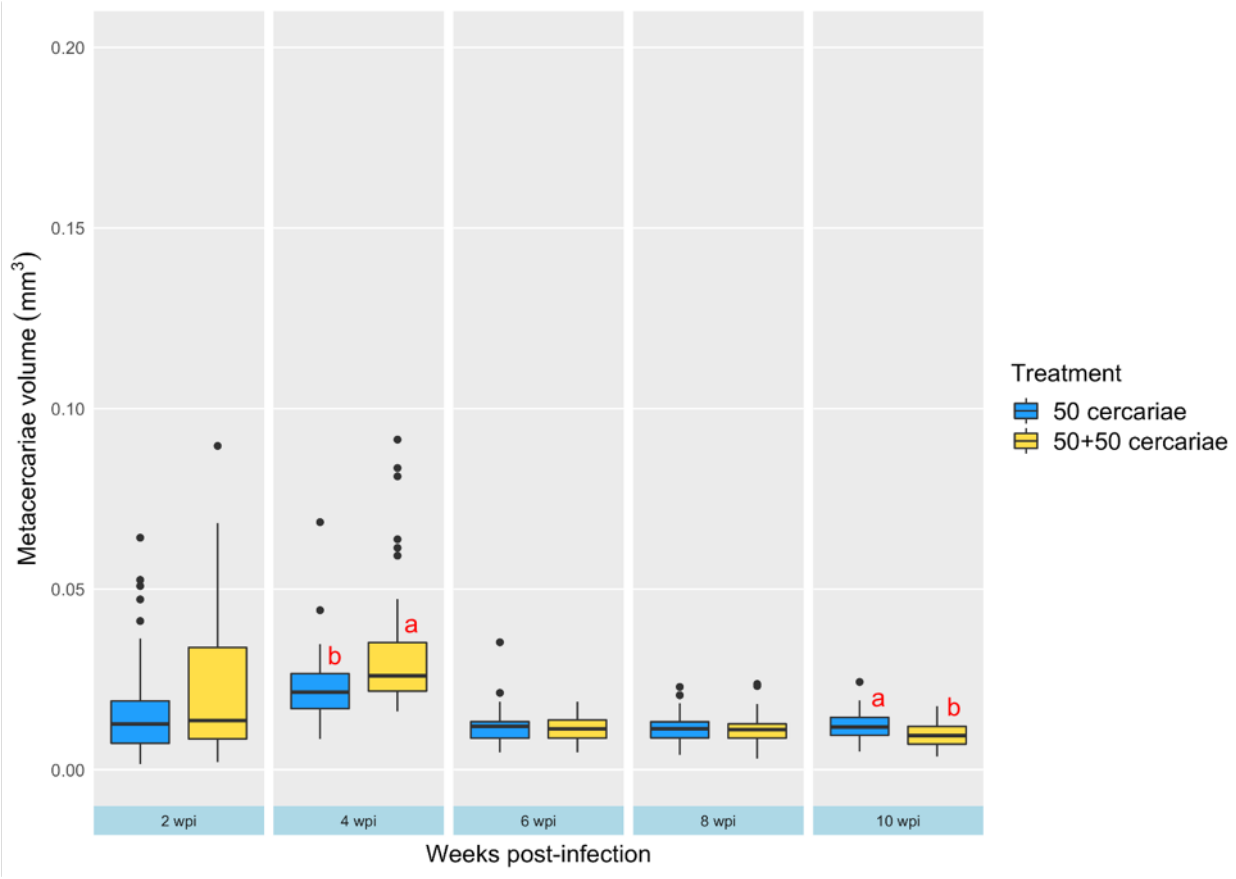

Figure 3.8. Growth of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows mono-infected with 50 cercariae or co-infected with 50 cercariae plus 50 cercariae of the congeneric trematode *Ornithodiplostomum* sp. Parasite volume was calculated for metacercariae recovered at two, four, six, eight, and ten weeks post-infection (wpi). Means with different letters represent a significant infection frequency effect ($p < 0.05$).

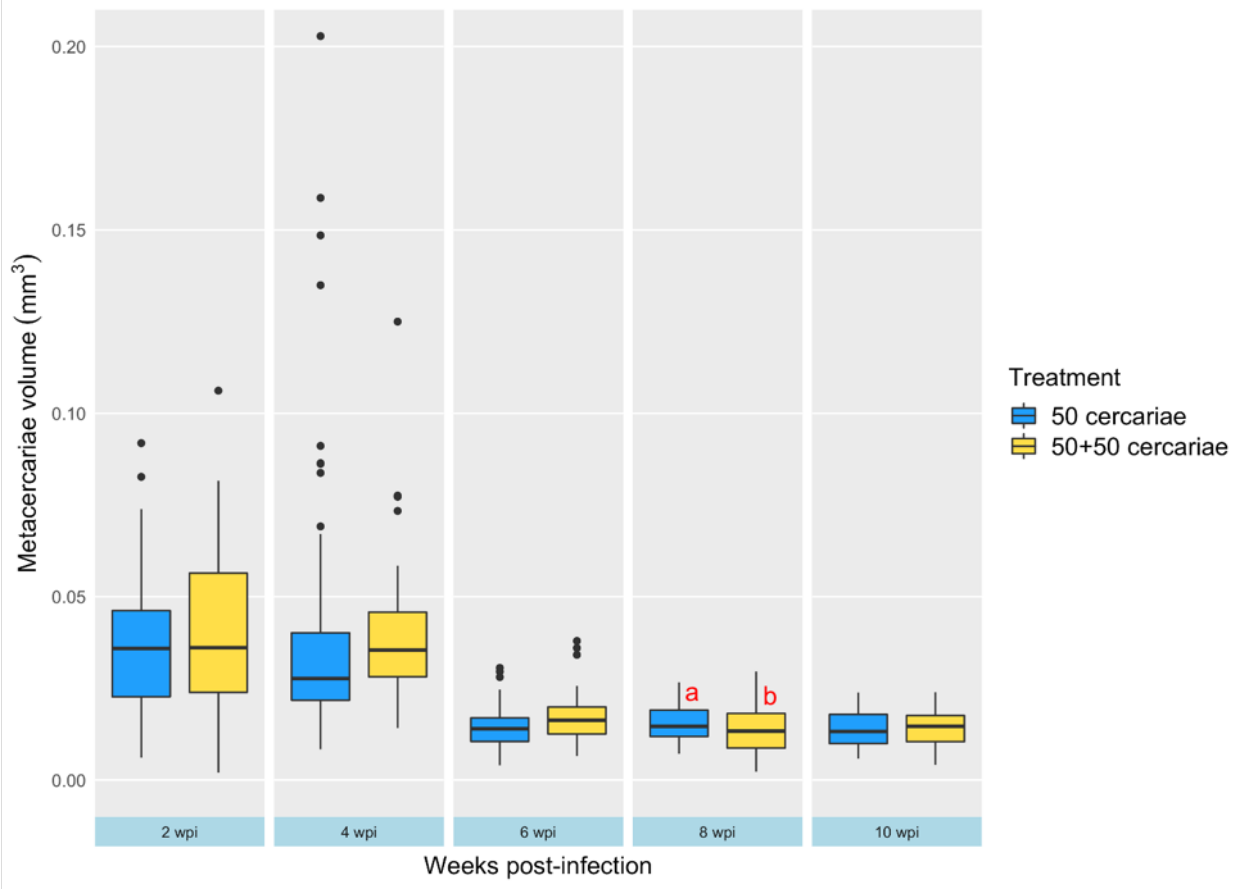

Figure 3.9. Growth of *Ornithodiplostomum* sp. metacercariae in fathead minnows mono-infected with 50 cercariae or co-infected with 50 cercariae plus 50 cercariae of the congeneric trematode *Ornithodiplostomum ptychocheilus*. Parasite volume were calculated for metacercariae recovered at two, four, six, eight, and ten weeks post-infection (wpi). Means with different letters represent a significant infection frequency effect ($p < 0.05$).

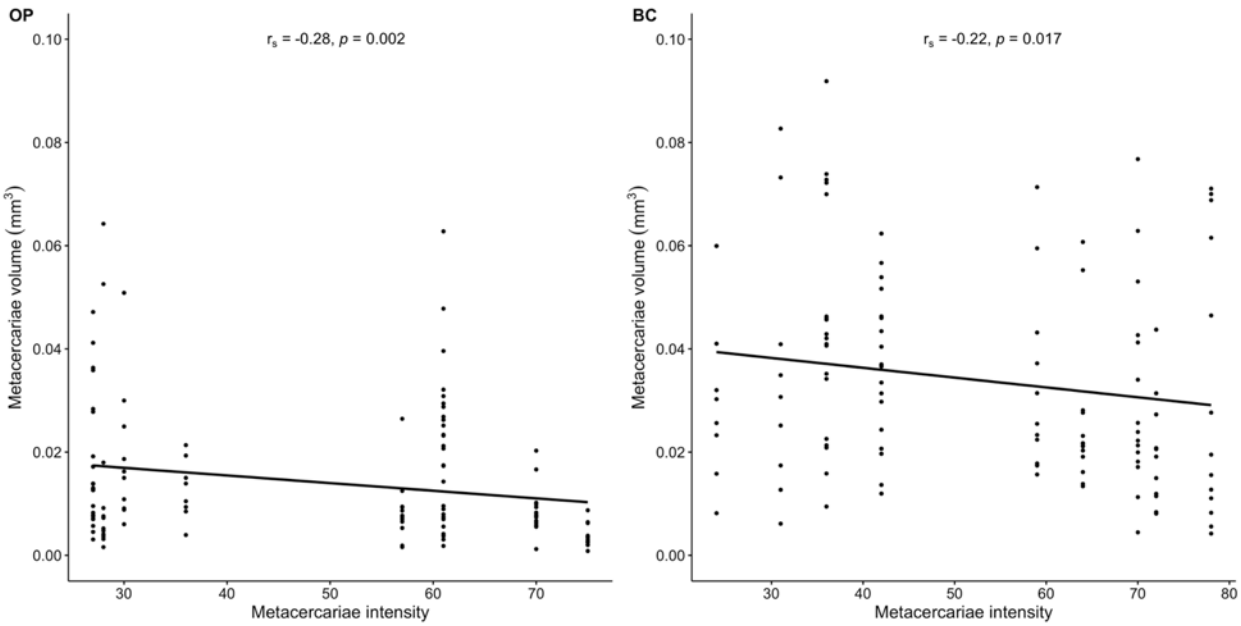

Figure 3.10. Patterns of intensity-dependent growth for *Ornithodiplostomum ptychocheilus* (OP) and *Ornithodiplostomum* sp. (BC) metacercariae recovered from experimentally infected fathead minnows (*Pimephales promelas*) at two weeks post-infection. Intensity refers to the number of metacercariae recovered from minnows during necropsy.

Table 3.1. Mean intensities of two trematodes in experimentally infected fathead minnows (*Pimephales promelas*). Mono-infected fish were exposed to either 50 or 100 cercariae of one species and co-infected fish were exposed to 50 cercariae of both species. OP: *Ornithodiplostomum ptychocheilus*; BC: *Ornithodiplostomum* sp.; SD: standard deviation.

Treatment	OP (SD; range)	BC (SD; range)
50 cercariae	27.0 (6.5; 13-42)	31.5 (6.4; 22-45)
50+50 cercariae	29.9 (4.6; 22-43)	38.4 (7.0; 24-52)
100 cercariae	54.0 (9.8; 32-75)	71.4 (12.9; 45-96)

Table 3.2. Summary ANOVA statistics for the effects of infection intensity (50 or 100 cercariae) on the volume of *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. metacercariae in fathead minnows. Effects are those among different infection intensities, and among different fish receiving each intensity. *P* values are as follows: * <0.05; ** <0.01; *** <0.001; *n* = number of metacercariae measured at each week post-infection (p.i.).

	ANOVA effects					
Weeks p.i.	*Ornithodiplostomum ptychocheilus*			*Ornithodiplostomum* sp.		
	n	Intensity	Fish(intensity)	*n*	Intensity	Fish(intensity)
2	120	$F(1,78.59) = 8.40^{**}$	**	119	$F(1,89.67) = 5.32^{*}$	
4	167	$F(1,87.95) = 14.26^{***}$		129	$F(1,50.69) = 2.96$	
6	158	$F(1,152.69) = 0.07$	***	214	$F(1,92.18) = 12.91^{***}$	
8	158	$F(1,151.79) = 24.36^{***}$	***	202	$F(1,199.00) = 4.91^{*}$	
10	182	$F(1,52.60) = 12.43^{***}$		158	$F(1,125.14) = 0.87$	

Table 3.3. Summary ANOVA statistics for the effects of co-infection (50 or 50+50 cercariae) on the volume of *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. metacercariae in fathead minnows. Effects are those among different treatments, and among different fish receiving each treatment. *P* values are as follows: * <0.05; ** <0.01; *** <0.001; *n* = number of metacercariae measured at each week post-infection (p.i.).

	ANOVA effects					
Weeks p.i.	*Ornithodiplostomum ptychocheilus*			*Ornithodiplostomum* sp.		
	n	Treatment	Fish(treatment)	*n*	Treatment	Fish(treatment)
2	93	$F(1,93) = 3.45$		113	$F(1,72.99) = 0.01$	
4	134	$F(1,133.97) = 26.69^{***}$		124	$F(1,86.67) = 1.12$	
6	147	$F(1,147) = 0.05$		137	$F(1,134.95) = 3.84$	***
8	142	$F(1,140.36) = 0.01$	**	181	$F(1,178.01) = 21.86^{***}$	***
10	127	$F(1,123.04) = 5.28^{*}$		152	$F(1,83.11) = 0.09$	

CHAPTER 4: Discussion

4.1 Synthesis

In this thesis, I explored various aspects of parasite co-infection in fathead minnows (*Pimephales promelas*). In the first data chapter I described patterns of multiparasitism in wild fish, while in the second data chapter I evaluated the consequences of experimental co-infection in wild-origin fish raised under controlled conditions. My field surveys indicated that sexually mature, male minnows are routinely co-infected with multiple parasite species. The two most prevalent and abundant of these species, a pair of larval trematodes, co-occurred more frequently than expected by chance. Despite their consistently strong, positive association in wild fish, my experimental results suggested a negative interspecific interaction between these two species in co-infected fish. This interaction manifested as a subtle, but symmetric, functional response whereby both species were significantly smaller post-encystment when heterospecifics were present (co-infection) than when they were absent (mono-infection).

The results of Chapter 2 demonstrate the utility of detailed, systematic field surveys towards identifying potentially important species associations. Quite simply, we would not know which or how many parasite species co-infect minnows in southern Alberta without this detailed census of parasite infracommunities. Nevertheless, detecting co-infections is challenging (Clark et al., 2016) because parasites are often small and inconspicuous, and consequently overlooked in wildlife surveys (Jaenike and Perlman, 2002). We may fail to detect or underestimate co-infection in individual hosts when

sampling designs are limited to the use of minimally invasive methods. Reports of multiparasitism often describe intestinal multiparasitism, due to non-invasive collection of stool samples which permit researchers to examine the gastrointestinal parasite community (Steinmann et al., 2010). Only three of the ten parasite species I recorded presence/absence data for in Chapter 2 infect the gastrointestinal tract of fathead minnows. Where possible, analyzing multiple types of biological samples (*e.g.*, blood, urine, cerebrospinal fluid, tissues) and integrating microscopy and molecular diagnostic tools (Valkiūnas et al., 2006) will help precise the occurrence of mixed-species infections.

By determining the prevalence of infection and co-infection in natural populations, field surveys represent the first opportunity to identify associations of interest (Hellard et al., 2012) although they can only be used as a starting point to evaluate interspecific interactions in combination with manipulative field studies or experimental studies (Herczeg et al., 2021). Researchers are increasingly identifying interactions from associations found in field surveys. For example, Clerc et al. (2019) confirmed the negative interaction between parasites of wood mice first suggested by Knowles et al. (2013), and Lello et al. (2018) predicted the interaction of parasites in sheep according to the associations between taxonomically and functionally similar parasites in wild rabbits reported by Lello et al. (2004). Like Johnson and Buller (2011) I report a positive association between two very prevalent species, but I also report associations between species pairs that include a less prevalent species (*e.g.*, *Crassiphiala bulboglossa*). Without systematic, detailed surveys that document all parasites in all sites in fathead minnows, I would not have identified these associations.

The consistently high prevalence and especially the substantial variation in intensity observed for *Ornithodiplostomum ptychocheilus*, and to a lesser extent *Ornithodiplostomum* sp. in Chapter 2 are extremely intriguing. Indeed, parasitologists and ecologists alike have long been interested in understanding what drives variation in infection in natural populations (Rossiter and Davidson, 2018). Poulin (2006) indicates that parasites infecting fishes typically display wider inter-population variation in prevalence than intensity because extrinsic factors should have a larger influence on prevalence. This is contrary to my observations for *O. ptychocheilus*. I am not the first to find *O. ptychocheilus* infecting 100% of minnows; Sandland et al. (2001) showed that prevalence can reach 100% in young-of-the-year (YOY) minnows while Wisenden et al. (2012) also documented 100% prevalence in fish of unknown age. Authors have previously suggested that 100% prevalence coupled with intensities ranging into the hundreds is indicative that infection by *O. ptychocheilus* has minimal impact on minnows (Wisenden et al., 2012) or alternately, that minnows have ineffective defenses against infection (Matisz, 2009). Additionally, high prevalence and intensity may indicate extremely successful transmission of cercariae from snails to minnows.

Kennedy (1987, 1990) proposed that metacercariae abundance in fish is principally governed by transmission events, and that variation in abundance stems from variation in the duration of transmission events. At northern latitudes the length of the cercariae transmission window is limited (Faltýnková et al., 2009), but a prolonged, warm autumn should extend it (Schleppe, 2002). The annual cohort of juvenile *Physa gyrina* are infected early in life, susceptible at shell sizes of 3-5 mm (Schleppe and Goater, 2004), and cercariae emergence likely begins as early as July. Beyond the approximate start of

the transmission window, many knowledge gaps remain about how successful transmission of cercariae from snails to minnows is promoted in natural settings. Studies suggest that the abundance and density of snails, infection prevalence in snails, and diel patterns of cercariae emergence and how these correspond to diel patterns of fish activity all relate to metacercariae intensity in fish (Ondračková et al., 2004; Harrod and Griffiths, 2005; Karvonen et al., 2005; Faltýnková et al., 2009). *Physa gyrina* shed cercariae diurnally (Schleppe and Goater, 2004), possibly synchronous with minnows' diurnal patterns of activity in inshore habitats (Price et al., 1991). Another knowledge gap that should be given priority is whether *Physa* spp. have differential susceptibility to *O. ptychocheilus* and *Ornithodiplostomum* sp. For example, if one congener preferentially infected *P. gyrina* and the other preferentially infected *P. integra* (C. Goater, unpublished data), differential first intermediate host usage would uncouple co-transmission of cercariae to fish. Then, research could focus on the ecological and evolutionary consequences of co-infection in the remaining hosts in the complex life cycle. Studies in experimental ponds with young-of-the-year minnows and known densities of snails experimentally infected with miracidia could help resolve these questions.

The experimental co-infections in Chapter 3 were conducted with species that infect different sites within the host, but parasites can also infect the same site. For example, *Plasmodium* spp. co-occur within individual erythrocytes and ectoparasitic monogeneans co-occur on the gills of fish. Spatial (niche) segregation serves to minimize within-host competition and promote species co-existence (Lotz and Font, 1991; Krasnov et al., 2006; Rynkiewicz et al., 2015). For example, the nine *Dactylogyrus* species infecting the gills of roach were distributed in a segregated manner on different gill arches

or different segments (proximal, medial, distal) of a single arch (Šimková et al., 2001). Parasites infecting the same tissue should face more competition than parasites infecting different tissues, especially if they also exploit the same resources (Dallas et al., 2019). However, parasites infecting different sites within the host can exploit the same resource and thus experience competition as has been demonstrated for *Plasmodium chabaudi* and *Nippostrongylus brasiliensis*, which both deplete red blood cells (Griffiths et al., 2015). Meanwhile, parasites which infect different tissues and exploit different resources are thought to compete via immune-mediated mechanisms (Graham, 2008; Johnson and Buller, 2011). The interaction I detected between *O. ptychocheilus* in the brain and *Ornithodiplostomum* sp. in the body cavity further supports the idea that species in spatially segregated infection sites potentially influence one another. This idea provides future directions for additional experimental studies. For example, hypotheses of resource limitation during co-infection could be tested with *Ornithodiplostomum* sp. and *Posthodiplostomum minimum*, which co-occur within the liver of fathead minnows.

Cross-reactive immunity is a plausible mechanism that would explain the smaller size of encysted metacercariae in fish co-infected with *O. ptychocheilus* and *Ornithodiplostomum* sp. Cross-reactive immunity is considered to be a form of apparent competition, where the host immune system is the common enemy of multiple parasite species (Mideo, 2009). Cross-reactive immunity may be immunologically specific, where antigens shared by closely related species elicit an adaptive immune response, or non-specific, triggering a systemic innate immune response (Dineen et al., 1977). *Ornithodiplostomum ptychocheilus* and *Ornithodiplostomum* sp. are closely related species and may share cross-reactive antigens. Cross-reactive immunity has been

proposed to explain the decreases in infection success and parasite persistence in trematode co-infections of amphibians (Johnson and Buller, 2011; Johnson and Hoverman, 2012; Hoverman et al., 2013), but the hypothesis has never been formally tested in that system. Cross-reactive immunity has been shown to have costs but is also thought to confer benefits (Fairlie-Clarke et al., 2009), for example in multi-species malaria infections (Haghdoost and Alexander, 2007).

The selective pressures shaping that the host immune response to trematode cercariae may differ according to the prevalence of infection in snails, lake size, and host habitat, as suggested by Rellstab et al. (2013). These three factors, in part, determine the rate of host-parasite encounter and thus transmission. For example, if the prevalence of infection in snails is high, then fish could encounter many different parasite genotypes (one per infected snail). In response to this diversity, selection should favor the development of a non-specific immune response. However, fish could encounter the same parasite genotype repeatedly if infected snails shed cercariae for a prolonged period of time. If the duration of cercarial shedding were longer than the time fish require to mount a specific, acquired immune response, selection should favor the development of a specific immune response (Rellstab et al., 2013). Relatively little is known about the teleost immune response to strigeid metacercariae, but existing studies support an innate immune response (Kalbe and Kurtz, 2006; Rauch et al., 2006). Ultimately, studies designed to characterize the immune response are needed. Key immunological components must be identified before the hypothesis of cross-reactive immunity in co-infections can be tested.

The results of Chapter 3 might be relevant to the success of adult trematodes in the definitive host. Metacercariae were smaller post-encystment in co-infected hosts than in mono-infected hosts, and metacercariae size has been shown to be a determinant of parasite fitness since larger metacercariae produce more eggs than their smaller conspecifics (Fredensborg and Poulin, 2005). In other helminths, size at transition (*i.e.*, from intermediate to definitive hosts) is positively related to infection success (Steinauer and Nickol, 2003). Additional studies have shown that co-infection reduces transmission (Tang et al., 2020) and delays infectivity relative to mono-infection (Barger and Nickol, 1999). This work could be extended to ask whether there are downstream consequences of the effects shown in intermediate hosts for traits like infectivity and fecundity. In the case there were negative consequences, then natural selection should favour mechanisms to reduce negative effects within the larval stage.

During the experimental co-infections conducted in Chapter 3, I exposed minnows to cercariae of *O. ptychocheilus* and *Ornithodiplostomum* sp. simultaneously. In nature, hosts can be exposed to multiple parasite species simultaneously or sequentially and in the latter case, priority effects can impact the outcome of co-infection. Priority effects have been demonstrated in several experimental co-infection studies (de Roode et al., 2005; Hoverman et al., 2013; Natsopoulou et al., 2015). In these studies, the sequence in which the parasite species infect the host influenced parasite infection success, abundance and density, growth, and persistence. I did not test for priority effects in this system because they are likely not ecologically relevant. There is no evidence that suggests that fathead minnows are not exposed to cercariae of *O. ptychocheilus* and *Ornithodiplostomum* sp. simultaneously in nature. Even if these species infected different

physid hosts, sympatric and syntopic *Physa* spp. are likely subject to very similar environmental influences.

Ultimately, the fathead minnow and its naturally co-occurring parasites are an experimentally tractable model for understanding the consequences of co-infection in individual hosts. This demonstrated tractability means that it is possible to address follow-up questions regarding the phenomenon of multiparasitism. Follow-up questions could include the effects of simultaneous versus sequential parasite exposure, the effects of single versus repeated parasite exposure, the effects of host size and age, and the effects of co-infection on host condition and development. Trematodes in particular are well suited to experimental co-infection studies because they do not reproduce within their second intermediate hosts and consequently each individual parasite represents an independent, quantifiable infection event (Johnson and Buller, 2011). Additional larval trematodes identified in my field survey could be incorporated into future experimental studies to expand the study of within-host interactions beyond two species, which would increase the relevance of the results (Knowles et al., 2013).

In summary, individual fathead minnows in southern Alberta are frequently co-infected by multiple parasite species simultaneously. The consistent, positive association between *O. ptychocheilus* and *Ornithodiplostomum* sp. in natural infections likely results from similar transmission strategies. The subtle but symmetric negative interaction between these congeners in experimental co-infections contrasts with the results of the field surveys. This contrast has been observed previously by researchers studying trematode co-infection in amphibian hosts (Johnson and Buller, 2011), and potentially indicates that patterns of cercariae transmission are independent of within-host

interactions between metacercariae. The rich and diverse parasite community hosted by the fathead minnow may positively reflect the health of minnow populations in southern Alberta (Fenton, 2008). Continued monitoring of infection prevalence and co-infection prevalence will further inform us about the spatial and temporal consistency of species associations within the parasite infracommunity.

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APPENDIX

Supplementary Table 1. Prevalence (%) and mean abundance (SD; range) of parasites infecting fathead minnows (*Pimephales promelas*) in southern Alberta, Canada. NE: not enumerated; NS: not sampled.

Site	Parasite species	Prevalence (n)			Mean abundance (SD; range)		
		2018	2019	2020	2018	2019	2020
University Pond	*Ornithodiplostomum ptychocheilus*	86.7 (30)	95.0 (40)	67.5 (40)	4.33 (4.1; 0-14)	4.43 (3.0; 0-13)	1.55 (1.8; 0-7)
	Ornithodiplostomum sp.	70.0 (30)	67.5 (40)	72.5 (40)	1.4 (1.2; 0-4)	2 (2.3; 0-10)	2.43 (2.5; 0-9)
	Posthodiplostomum minimum	0.0 (30)	0.0 (40)	12.5 (40)	–	–	0.15 (0.4; 0-2)
	Diplostomum spp.	0.0 (30)	0.0 (40)	0.0 (40)	–	–	–
	Crassiphiala bulboglossa	13.3 (30)	20.0 (40)	2.5 (40)	0.17 (0.5; 0-2)	0.2 (0.4; 0-1)	0.03 (0.2; 0-1)
	Philometra sp.	10.0 (30)	37.5 (40)	5.0 (40)	0.1 (0.3; 0-1)	0.48 (0.7; 0-3)	0.05 (0.2; 0-1)
	Goussia degiustii	13.3 (30)	85.0 (40)	90.0 (40)	NE	NE	NE
	Pomphorhynchus bulbocolli	3.3 (30)	0.0 (40)	0.0 (40)	0.03 (0.2; 0-1)	–	–
	Ligula intestinalis	0.0 (30)	0.0 (40)	0.0 (40)	–	–	–
	Proteocephalus sp.	0.0 (30)	0.0 (40)	5.0 (40)	–	–	0.05 (0.2; 0-1)
Coulee Creek Stormwater Drainage Pond	*O. ptychocheilus*	100.0 (30)	100.0 (40)	100.0 (40)	208.63 (101.9; 70-433)	187.5 (61.5; 69-370)	161.75 (66.5; 59-319)
	Ornithodiplostomum sp.	100.0 (30)	100.0 (40)	100.0 (40)	18.47 (10.4; 2-46)	87.88 (39.2; 29-203)	67.65 (62.6; 6-295)
	P. minimum	13.3 (30)	0.0 (40)	32.5 (40)	0.17 (0.5; 0-2)	–	0.4 (0.6; 0-2)
	Diplostomum spp.	73.3 (30)	47.5 (40)	12.5 (40)	6.63 (8.5; 0-25)	1.5 (2.4; 0-10)	0.28 (0.9; 0-6)
	C. bulboglossa	43.3 (30)	72.5 (40)	47.5 (40)	0.67 (0.8; 0-2)	1.65 (1.4; 0-5)	1 (1.4; 0-5)
	Philometra sp.	0.0 (30)	40.0 (40)	2.5 (40)	–	0.55 (0.9; 0-4)	0.03 (0.2; 0-1)

Site	Parasite species	Prevalence (n)			Mean abundance (SD; range)		
		2018	2019	2020	2018	2019	2020
Coulee Creek Stormwater Drainage Pond	*G. degiustii*	3.3 (30)	82.5 (40)	85.0 (40)	NE	NE	NE
	P. bulbocolli	0.0 (30)	0.0 (40)	0.0 (40)	–	–	–
	L. intestinalis	0.0 (30)	0.0 (40)	0.0 (40)	–	–	–
	Proteocephalus sp.	0.0 (30)	0.0 (40)	0.0 (40)	–	–	–
McQuillan Reservoir	*O. ptychocheilus*	100.0 (42)	100.0 (40)	100.0 (40)	29.98 (35.0; 5-161)	18.5 (7.8; 5-39)	145.23 (92.8; 69-665)
	Ornithodiplostomum sp.	92.9 (42)	82.5 (40)	100.0 (40)	5.81 (8.4; 0-39)	3.48 (3.1; 0-11)	37.6 (49.8; 7-263)
	P. minimum	16.7 (42)	15.0 (40)	87.5 (40)	0.17 (0.4; 0-1)	0.18 (0.4; 0-2)	2.55 (2.1; 0-9)
	Diplostomum spp.	71.4 (42)	67.5 (40)	17.5 (40)	4.21 (7.7; 0-33)	1.03 (0.4; 0-4)	0.23 (0.5; 0-2)
	C. bulboglossa	69.0 (42)	55.0 (40)	25.0 (40)	1.69 (1.7; 0-6)	1.6 (2.1; 0-9)	0.33 (0.6; 0-3)
	Philometra sp.	19.0 (42)	60.0 (40)	10.0 (40)	0.33 (0.8; 0-3)	1 (1; 0-3)	0.1 (0.3; 0-1)
	G. degiustii	88.1 (42)	97.5 (40)	100.0 (40)	N NE	NE	NE
	P. bulbocolli	7.1 (42)	0.0 (40)	2.5 (40)	0.07 (0.3; 0-1)	–	0.03 (0.2; 0-1)
	L. intestinalis	4.8 (42)	0.0 (40)	0.0 (40)	0.05 (0.2; 0-1)	–	–
	Proteocephalus sp.	0.0 (42)	0.0 (40)	0.0 (40)	–	–	–
Gold Spring Park Pond	*O. ptychocheilus*	100.0 (41)	100.0 (40)	100.0 (40)	313.02 (101.7; 115-529)	254.13 (76.2; 74-465)	255.9 (91.1; 134-585)
	Ornithodiplostomum sp.	100.0 (41)	100.0 (40)	100.0 (40)	34.68 (34.9; 1-185)	181.43 (72.3; 61-347)	106.55 (63.0; 13-295)
	P. minimum	17.1 (41)	42.5 (40)	20.0 (40)	0.27 (0.7; 0-3)	0.95 (1.3; 0-5)	0.48 (1.4; 0-8)
	Diplostomum spp.	0.0 (41)	15.0 (40)	0.0 (40)	–	0.2 (0.5; 0-2)	–
	C. bulboglossa	22.0 (41)	27.5 (40)	5.0 (40)	0.54 (1.3; 0-7)	0.5 (0.9; 0-4)	0.05 (0.2; 0-1)
	Philometra sp.	0.0 (41)	32.5 (40)	10.0 (40)	–	0.43 (0.7; 0-2)	0.2 (0.8; 0-5)
	G. degiustii	65.9 (41)	95.0 (40)	100.0 (40)	NE	NE	NE
	P. bulbocolli	0.0 (41)	0.0 (40)	2.5 (40)	–	–	0.03 (0.2; 0-1)

Site	Parasite species	Prevalence (n)			Mean abundance (SD; range)		
		2018	2019	2020	2018	2019	2020
Gold Spring Park Pond	*L. intestinalis*	2.4 (41)	0.0 (40)	0.0 (40)	0.02 (0.2; 0-1)	–	–
	Proteocephalus sp.	0.0 (41)	0.0 (40)	0.0 (40)	–	–	–
Stirling Lions' Fish Pond	*O. ptychocheilus*	NS	100.0 (40)	100.0 (40)	NS	13.88 (7.1; 3-31)	245.6 (74.6; 134-516)
	Ornithodiplostomum sp.		100.0 (40)	100.0 (40)		5.03 (2.6; 1-12)	9.08 (4.4; 2-23)
	P. minimum		37.5 (40)	82.5 (40)		0.48 (0.7; 0-2)	4.23 (7.9; 0-49)
	Diplostomum spp.		100.0 (40)	97.5 (40)		5.13 (2.8; 1-13)	4.55 (2.9; 0-10)
	C. bulboglossa		5.0 (40)	7.5 (40)		0.05 (0.2; 0-1)	0.13 (0.5; 0-2)
	Philometra sp.		32.5 (40)	40.0 (40)		0.43 (0.7; 0-3)	1.33 (2.9; 0-15)
	G. degiustii		97.5 (40)	97.5 (40)		NE	NE
	P. bulbocolli		0.0 (40)	2.5 (40)		–	0.03 (0.2; 0-1)
	L. intestinalis		0.0 (40)	0.0 (40)		–	–
	Proteocephalus sp.		0.0 (40)	0.0 (40)		–	–
Reesor Lake	*O. ptychocheilus*	100.0 (30)	100.0 (37)	100.0 (40)	13 (6.6; 2-26)	29.38 (12.4; 11-60)	38.9 (12.7; 13-63)
	Ornithodiplostomum sp.	63.3 (30)	97.3 (37)	100.0 (40)	1.37 (1.6; 0-7)	8.68 (4.9; 0-23)	13.5 (6.9; 3-32)
	P. minimum	6.7 (30)	51.4 (37)	32.5 (40)	0.07 (0.2; 0-1)	0.78 (0.9; 0-4)	0.53 (0.9; 0-3)
	Diplostomum spp.	86.7 (30)	83.8 (37)	87.5 (40)	2.1 (1.5; 0-6)	2.29 (1.4; 0-6)	2.48 (2.1; 0-8)
	C. bulboglossa	73.3 (30)	51.4 (37)	62.5 (40)	1.53 (1.4; 0-5)	1.38 (1.9; 0-9)	1.3 (1.3; 0-4)
	Philometra sp.	3.3 (30)	51.4 (37)	22.5 (40)	0.03 (0.2; 0-1)	0.59 (0.6; 0-2)	0.25 (0.5; 0-2)
	G. degiustii	60.0 (30)	100.0 (37)	100.0 (40)	NE	NE	NE
	P. bulbocolli	0.0 (30)	0.0 (37)	0.0 (40)	–	–	–
	L. intestinalis	0.0 (30)	2.7 (37)	0.0 (40)	–	0.03 (1.2; 0-1)	–
	Proteocephalus sp.	0.0 (30)	0.0 (37)	22.5 (40)	–	–	0.58 (1.5; 0-8)

Site	Parasite species	Prevalence (n)			Mean abundance (SD; range)		
		2018	2019	2020	2018	2019	2020
Spruce Coulee Reservoir	*O. ptychocheilus*	100.0 (30)	100.0 (35)	75.0 (40)	32.87 (16.7; 5-94)	21.77 (12.1; 5-65)	3.73 (6.3; 0-30)
	Ornithodiplostomum sp.	100.0 (30)	100.0 (35)	50.0 (40)	12.1 (14.5; 1-84)	18.03 (11.1; 6-60)	1.58 (2.3; 0-7)
	P. minimum	66.7 (30)	91.4 (35)	92.5 (40)	3.97 (5.2; 0-23)	4.06 (3.3; 0-14)	6.35 (5.2; 0-25)
	Diplostomum spp.	96.7 (30)	94.3 (35)	40.0 (40)	4.8 (4.3; 0-21)	2.94 (2.4; 0-9)	0.83 (1.2; 0-5)
	C. bulboglossa	100.0 (30)	100.0 (35)	90.0 (40)	8.1 (5.9; 1-36)	3.37 (2.2; 1-9)	3.08 (3.7; 0-20)
	Philometra sp.	16.7 (30)	34.3 (35)	7.5 (40)	0.2 (0.5; 0-2)	0.4 (0.6; 0-2)	0.1 (0.4; 0-2)
	G. degiustii	66.7 (30)	100.0 (35)	100.0 (40)	NE	NE	NE
	P. bulbocolli	0.0 (30)	0.0 (35)	0.0 (40)	–	–	–
	L. intestinalis	0.0 (30)	0.0 (35)	0.0 (40)	–	–	–
	Proteocephalus sp.	0.0 (30)	0.0 (35)	0.0 (40)	–	–	–