Trematode Communities of the Appalachian Stream Snail, *Elimia proxima*: the Importance of Scale in Parasite Ecology Research

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ACADEMIC ABSTRACT

Understanding the ecological processes that impact parasite abundance and distribution is critically important for epidemiology and predicting how infectious disease dynamics may respond to future disturbance. Digenean trematodes (Platyhelminthes: Trematoda) are parasitic flatworms with complex, multi-host life cycles that include snail first-intermediate hosts and vertebrate definitive hosts. Trematodes cause numerous diseases of humans (e.g. schistosomiasis) and livestock (e.g. fascioliasis), and impact the ecology of wildlife systems. Identifying the ecological mechanisms that regulate these complex, multi-host interactions will advance both our understanding of parasitism and the dynamics of infectious disease. By examining patterns of infection in *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* snails, my dissertation research investigated the environmental factors and ecological processes that structure trematode communities in streams. First, I examined temporal variation in trematode infection of snails in five headwater streams. Over a three year period, I found no consistent seasonal patterns of trematode infection. There was consistency across sites in trematode prevalence, as sites with high prevalence at the beginning of the study tended to remain sites of high infection, relative to lower prevalence sites. Second, I examined landscape level variation in trematode infection by characterizing the regional distribution, abundance and diversity of *E. proxima* infections in 20 headwater streams. I found a broad scale spatial pattern in trematode communities due to regional turnover in dominant species. This pattern was correlated with elevation, but there were no significant relationships with other environmental variables. Additionally, molecular characterization of trematodes indicated the presence of cryptic (morphologically indistinguishable) species complexes within this system, and variation in genetic diversity among trematode types may reflect differences in host dispersal abilities. Third, I examined trematode infection within a single stream network across multiple headwaters and the mainstem. I found a decreasing downstream gradient of trematode prevalence related to several environmental variables including elevation, snail density, conductivity, and stream depth. Additionally, headwater communities were nested subsets of the communities found in the mainstem. By combining approaches at different temporal and spatial scales, my dissertation research increases our understanding of the processes that impact the abundance and distribution of parasites.

Trematode Communities of the Appalachian Stream Snail, *Elimia proxima*: the Importance of Scale in Parasite Ecology Research

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GENERAL AUDIENCE ABSTRACT

Understanding the ecology of wildlife parasite infection is critical both for public health and the conservation of global biodiversity. Digenean trematodes (Phylum: Platyhelminthes, Class: Trematoda) are parasitic flatworms that cause numerous diseases of humans (e.g. schistosomiasis) and livestock (e.g. fascioliasis), and can impact wildlife ecology. Trematodes have complex life cycles that involve multiple hosts. A typical trematode life cycle includes a series of three hosts: (1) a snail first-intermediate host; (2) an aquatic invertebrate or vertebrate second-intermediate host; and (3) a vertebrate final host. By identifying the ecological processes that are important in these complex, multi-host interactions, we can advance our understanding of parasites and infectious disease. Freshwater ecosystems serve as the transmission channels for many types of parasites, including trematodes, but we know relatively little about the ecology of parasites in streams. My dissertation research investigated the ecology of parasites in streams by examining patterns of trematode infection in stream snails, *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima*. First, I examined seasonal changes in trematode infection of snails in five headwater streams. Over a three year period, I found no consistent seasonal patterns of trematode infection. There was consistency across sites in the level of trematode infection (i.e. sites with high levels of infection at the beginning of the study tended to remain sites of high infection, relative to sites with lower levels of infection). Second, I examined variation in trematode infection across a regional spatial scale by examining the number and types of trematodes infecting snails in 20 headwater streams in southwestern Virginia and northwestern North Carolina. Across the region, I found a broad spatial pattern in the geographic distribution of trematodes due to changes in the dominant type of trematode infection. This pattern was related to elevation, but not to any other environmental variables we measured. Additionally, I obtained genetic sequences from these trematode samples, and this revealed the existence of additional trematode species that we could not distinguish based on visual examination of morphological features. Furthermore, differences in the genetic diversity of trematode species may be related to differences in the mobility of trematode host species. Third, I examined trematode infection at eight locations within a single stream. I found that trematode infection decreased from upstream to downstream, and that this pattern was related to several environmental variables including elevation, snail density, conductivity, and stream depth. By combining examinations of infection patterns over time and at different spatial scales, my dissertation research increases our understanding of the processes that impact parasite transmission in freshwater ecosystems.

Attributions

Chapter 2: Seasonal and annual variation in trematode infection of stream snails, *Elimia proxima*, in the southern Appalachian Mountains of Virginia

This chapter has been accepted with revisions by the Journal of Parasitology.

This article included six coauthors:

Jennie Wyderko, Virginia Tech, Blacksburg, VA: contributed to field and lab work

Jeronimo G. Da Silva Neto, Virginia Tech, Blacksburg, VA: contributed to field and lab work

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E. F. Benfield, Virginia Tech, Blacksburg, VA: contributed to the study design

Lisa K. Belden, Virginia Tech, Blacksburg, VA: contributed to the study design, lab work, analysis, and manuscript development

Chapter 3: Spatial turnover of dominant species drives regional variation in stream parasites

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Chapter 4: Upstream gradient of trematode infection in snails: environmental heterogeneity within a stream network counters the effects of downstream drift

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Table of Contents

Chapter 1: General Introduction

Digenetic trematodes (Phylum: Platyhelminthes, Class: Trematoda, Subclass: Digenea) are parasitic flatworms with complex multi-host life cycles. They are a medically, economically, and ecologically important class of parasites. Identifying the ecological mechanisms that regulate these complex, multi-host interactions will advance both our understanding of parasitism and the dynamics of infectious disease. *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* (Gastropoda: Pleuroceridae) is a stream snail that serves as first-intermediate hosts to a community of trematode species. The primary goal of this research was to identify the environmental factors and ecological processes that structure stream trematode communities by examining patterns of infection in *E. proxima*.

The importance of disease ecology

Traditional approaches to studying infectious disease have often neglected to explore the role of ecological processes in disease dynamics. Likewise, traditional ecology has often failed to include the impacts of parasites on ecosystems (Anderson and May 1982). Increasingly, disease agents are being recognized as important drivers of ecosystem function. In addition to regulating host population dynamics, parasites can alter interspecific competition, energy flow and community composition. For example, Wood et al. (2007) demonstrated that high levels of trematode infection in *Littorina littorea* snails can reduce snail grazing pressure in North Atlantic intertidal zones, significantly increasing the food resources available to other invertebrate fauna. Mouritsen and Poulin (2005) found similar parasite-mediated effects on energy flow and community composition in a New Zealand intertidal community. Efforts to incorporate parasites into food webs reveal that parasites play a major role in trophic dynamics. Lafferty et al. (2006) found that parasites were involved in 78% of all food web links in the Carpinteria Salt Marsh ecosystem; there were even more parasite-host links than predator-prey links. In a five year study that quantified parasite biomass in three Pacific coast estuaries, Kuris et al. (2008) found that parasite biomass was greater than that of top predators and similar to that of several groups of free-living organisms in these systems. Infectious-biomass is embedded in ecological processes; therefore, it is not surprising that parasites can have major impacts on ecosystem function.

Emerging infectious diseases (EIDs) are defined as diseases that are either new in a population or species, or have been previously identified, but are increasing in prevalence or geographic range (Morse 1995). EIDs increasingly threaten human health and economies, as well as global biodiversity (Daszak 2000; Jones et al. 2008). Recent examples of EIDs include HIV/AIDS and Lyme disease in humans, avian influenza in poultry, Rift Valley Fever in livestock, and chytridiomycosis in amphibians. Most human EIDs are zoonotic (transmitted from animals to people), and the majority of these zoonoses originate in wildlife (Jones et al. 2008). Diseases emerge when changes in wildlife systems affect the dynamics of host-pathogen relationships (Daszak 2000); therefore, identifying the ecological mechanisms that regulate host-parasite interactions in wildlife systems is critical for understanding the dynamics of EIDs.

Introduction to Trematodes

Trematodes (Phylum: Platyhelminthes, Class: Trematoda), also known as flukes or parasitic flatworms, are obligate endoparasites of mollusks and all classes of vertebrates (Bush et al. 2001). Digenetic trematodes (Subclass: Digenea) have remarkably diverse and complex life-cycles, usually involving a series of three hosts (Bush et al. 2001). Digeneans use intermediate hosts for several phases of asexual reproduction prior to sexual reproduction in definitive hosts (Whitfield 1993). The first-intermediate hosts of trematodes are always mollusks, usually snails (Haseeb and Fried 1997). Most trematodes exhibit a high degree of specificity for first-intermediate hosts, but use a wider range of species, vertebrate or invertebrate, for second-intermediate hosts (Whitfield 1993). Definitive hosts are almost always vertebrates (Haseeb and Fried 1997).

Trematodes cause numerous human diseases, the most important being schistosomiasis, a chronic disease that affects over 200 million people worldwide (Steinmann et al. 2006). Trematodes also have significant negative impacts on livestock (e.g. fasciolosis) and wildlife (Bush et al. 2001). There are over 24,000 species of trematodes, but only a small fraction of these parasites have been well-studied (Poulin and Morand 2004).

Elimia (= *Oxytrema* = *Goniobasis*) *proxima* (Gastropoda: Pleuroceridae) is a common, native inhabitant of Appalachian headwater streams that serves as the first-intermediate host to multiple species of trematodes, including parasites of fish, bats, birds and raccoons. *Metagonimoides oregonensis* is the most studied trematode of the *E. proxima* component community, and the life cycle has been described in detail. Adult *M. oregonensis* trematodes sexually reproduce in the small intestines of raccoon definitive hosts, and eggs are shed in feces (Burns and Pratt 1953; Ingles 1935). Eggs are ingested by stream snails (*Elimia spp.*). Within snails, trematodes reproduce asexually to create thousands of free-swimming cercariae that leave snails and infect amphibians [stream salamanders, e.g. *Desmognathus* spp. in the Southeast (Goater et al. 1987; Lang and Gleason 1967)]. The larval trematodes encyst in the muscle tissue of salamanders, and complete their life cycle when infected salamanders are consumed by raccoons (see Fig. 1). Slightly different life cycles have been described for *M. oregonensis* in different geographic regions where the trematode uses different intermediate hosts (Lang et al. 1968), suggesting there may be multiple species within the genus *Metagonimoides* across its range.

Metacommunity theory and the importance of scale

A primary goal of community ecology is to understand the processes that influence species interactions, abundance and distribution. Traditionally, ecological communities have been conceptualized and investigated as finite assemblages of species, structured primarily by species interactions and local environmental factors (Whittaker 1972, Tilman 1982). Within the past decade, this approach has been challenged by a renewed interest in the role of landscape features and dispersal in structuring communities. The development of metacommunity concepts has advanced our knowledge of the importance of both local and larger-scale processes in structuring communities (Leibold et al. 2004, Holyoak et al. 2005). Metacommunity paradigms incorporate environmental and spatial processes, but vary in the relative importance of species traits, environmental heterogeneity (i.e. local scale) and dispersal (i.e. larger scale) in structuring communities (Logue et al. 2011). In a synthesis of the primary factors considered in metacommunity ecology, Vellend (2010) related these community level processes as analogous to those in population genetics. He considered the four community level processes to be selection ("fitness" differences among species based on local conditions), dispersal, speciation (the appearance of new species), and drift (the stochastic element of population dynamics). This framework suggests that considering the relative importance of these factors in different systems can advance our understanding of ecological communities. The relative importance of processes that structure communities is likely to vary across habitat and organism type, as patterns of dispersal are determined in part by physical factors, such as landscape features and habitat connectivity, as well as the particular dispersal abilities of individual species (Cottenie 2005).

Overview of dissertation research

To advance our knowledge of what factors influence trematode community structure in stream networks, I completed a series of three studies. First, as reported in **Chapter 2**, I completed a three year seasonal survey of trematode infection in *E. proxima* in five streams in the southern Appalachian Mountains. I found no evidence of consistent seasonal peaks of trematode infection in *E. proxima*. There was consistency across sites in the prevalence of infection, as high prevalence sites tended remain sites of high infection, relative to lower prevalence sites. In **Chapter 3**, I present the results of a study examining landscape level variation in trematode infection at 20 headwater streams within the Upper New River Basin. I found a broad scale spatial pattern in *E. proxima* trematode communities due to regional turnover in dominant trematode species. While this pattern was correlated with elevation, there were no significant relationships with other environmental variables. Additionally, molecular characterization of larval trematodes indicated the existence of many cryptic (morphologically indistinguishable) species complexes, and variation in genetic diversity among trematode types may reflect differences in host dispersal abilities. Finally, in **Chapter 4**, I conducted a study describing infection within a single stream network in the Upper New River watershed. I found an increasing upstream gradient of trematode infection prevalence that may be explained by relationships to several environmental variables including elevation, snail density, conductivity, and stream depth. I also found that headwater communities were nested subsets of the communities found in the mainstem. In **Chapter 5**, I provide a synthesis of my research in this study system, including a brief discussion of research not included in data chapters, as well as recommendations for future research.

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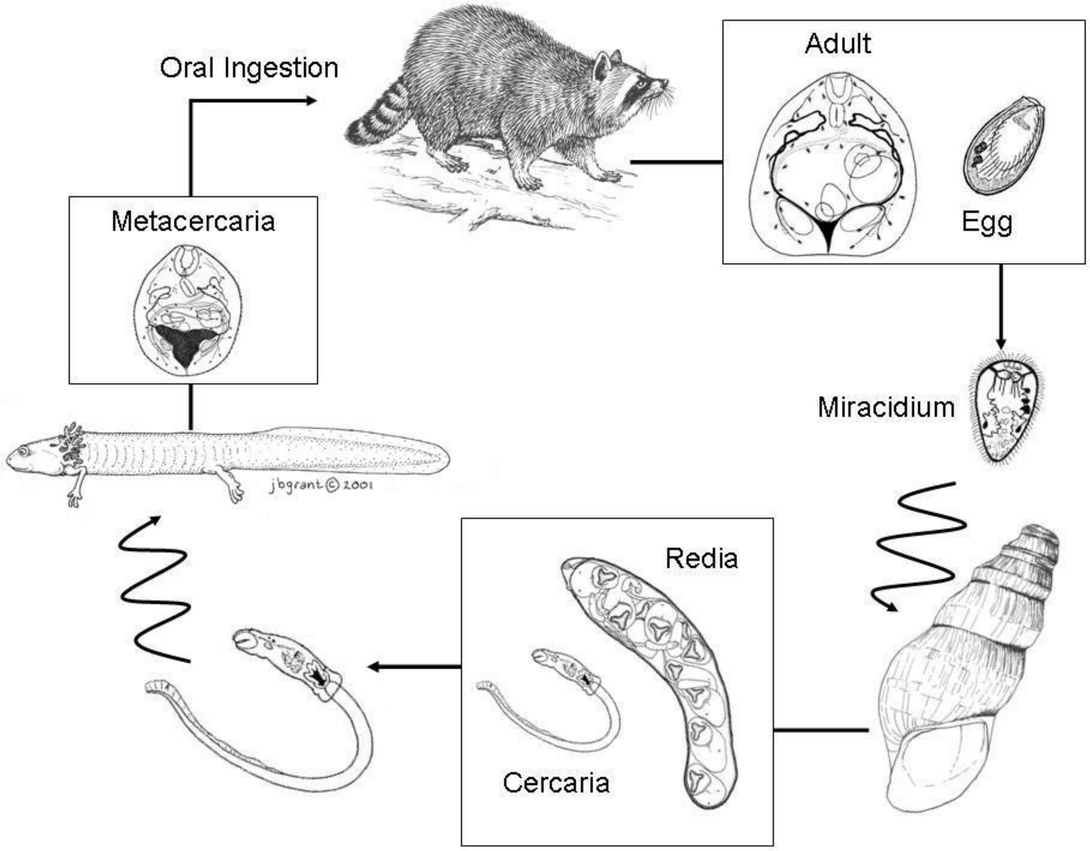

Figure 1. The life-cycle of *Metagonimoides oregonensis* in the Southeast. Adult *M. oregonensis* develop in raccoons, where they sexually reproduce. Trematode eggs are shed in raccoon feces and infect stream snails (*Elimia proxima*). Within snails, trematodes reproduce asexually to create thousands of free-swimming cercariae, which leave snails and penetrate stream salamanders (*Desmognathus* spp.). Within salamanders, trematodes develop into metacercariae, encysting in muscle tissue. The life-cycle is completed when infected salamanders are eaten by raccoons. Parasite drawings from Burns and Pratt (1953); snail drawing by Jeremy Wojdak.

Chapter 2: Seasonal and annual variation in trematode infection of stream snails, *Elimia proxima*, in the southern Appalachian Mountains of Virginia

Abstract

Understanding temporal variation of host-pathogen dynamics can be important for predicting disease risks and anticipating how disease systems may change in response to natural or human disturbances. Seasonal changes in weather, especially those associated with changes in temperature or precipitation, are often a key component of temporal changes in infection risk and can have important impacts on disease systems. However, these patterns can be difficult to track due to interannual variation and the need for longer-term, multi-year surveillance efforts. We assessed seasonal and annual changes in the trematode component community of first-intermediate host stream snails, *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima*, across five streams in the southern Appalachian Mountains. Over three years, we found no evidence of consistent seasonal peaks of trematode infection in *E. proxima*. There was some across site consistency in infection prevalence over four years, as high prevalence sites tended to maintain higher prevalence from year to year, relative to lower prevalence sites. Additionally, we examined the relationship between prevalence of first-intermediate host infection, weather variables and site-level factors, including snail density and water quality metrics. Trematode prevalence was negatively related to total precipitation, which may have been due to the movement of infectious parasite stages and infected hosts downstream during high flows. We found no strong relationship between trematode prevalence and snail density or any of the water quality metrics examined in this study, indicating that snail infection may be driven primarily by definitive host activity.

Introduction

Temporal variation in environmental conditions can have large impacts on host-pathogen interactions and affect infection rates in human, livestock, and wildlife disease systems (reviewed in Altizer et al., 2006). Seasonal changes are one key element of temporal variation that can drive disease dynamics. For example, peaks in human malaria are driven by increases in mosquito abundance and parasite development during warm, rainy seasons (Hoshen and Morse, 2004). Identifying temporal patterns in infection dynamics can inform treatments for controlling parasite infections, and is critical for advancing our understanding of how host-parasite systems might change in response to natural or human disturbance.

Digenetic trematodes (Phylum: Platyhelminthes, Subclass: Digenea) are parasitic flatworms with complex, multi-host life cycles. The basic digenean life cycle involves a series of three hosts, including a molluscan first-intermediate host and a vertebrate definitive host, while second-intermediate hosts may be either invertebrates or vertebrates, depending on the trematode species (Bush et al., 2001). Because of the range of hosts incorporated in their complex life cycles, trematodes play important roles in community and ecosystem level processes. Trematode infection can impact interspecific competition and community composition (Mouritsen and Poulin, 2005; Wood et al., 2007), as well as trophic dynamics and energy flow (Lafferty et al., 2006; Kuris et al., 2008).

Previous research on larval trematode component communities of first-intermediate host snails revealed temporal variation in both prevalence and composition (Esch and Fernandez, 1994; Esch et al., 2001). Seasonal peaks of trematode infection have been described in various temperate freshwater snails (Fernandez and Esch 1991a, 1991b; Snyder and Esch 1993; Loy and Haas, 2001; Klockars et al., 2007; Faltýnková et al., 2008), one tropical freshwater snail (Namsanor et al., 2015), and several marine snails (Yoshino, 1975; Mouritsen et al., 1997; Kube et al., 2002). For host-trematode systems with short-lived snail hosts, seasonal infection patterns have often been explained as a function of the host snail phenology (recruitment, mortality, population turnover) and demography (size and age structure of snail population) (Fernandez and Esch, 1991a; Kube et al., 2002; Faltýnková et al., 2008). Additionally, both seasonal and longer-term variation in snail-trematode component communities has been related to fluctuations in egg input due to temporal heterogeneity in definitive host presence (Fernandez and Esch, 1991a, 1991b; Snyder and Esch, 1993; Mouritsen et al., 1997; Granovitch et al., 2000) and changes in rainfall patterns (Namsanor et al., 2015).

Most of the previous research on temporal variation in freshwater snail trematode infection has examined short-lived snail hosts (life spans of 3 to 18 months) in lentic (pond and lake) systems (Fernandez and Esch, 1991a, 1991b; Snyder and Esch, 1993; Klockars et al., 2007; Faltýnková et al., 2008). In this study, we investigated trematode community structure in a longer-lived lotic (stream) species, *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* (Pleuroceridae), a common native of Appalachian headwaters with a life span of 3 – 5 years (Dillon, 2000). A previous study on *E. proxima* conducted over 3 years in a single stream in North Carolina reported a winter peak of trematode infection (Lang, 1968). Here, we examined seasonal variation over 3 years and annual variation over 4 years in the trematode component community of *E. proxima* in five headwater streams in southwestern Virginia. Specifically, we addressed the following questions: (1) Are there seasonal patterns of trematode infection and if so, are these consistent across years?; (2) How does seasonal variation compare to annual variation in trematode infection?; (3) Is trematode infection in stream snails related to local (i.e. site-level) factors, such as water quality and snail density, and/or regional factors such as weather patterns?

Materials and Methods

We examined first-intermediate host trematode infection of *Elimia proxima* in five different headwaters (first and second order streams) in Floyd and Carroll Counties in the Appalachian Mountains in southwestern Virginia (Table 1). The distance between sites ranged from 4.5 km to 25 km. All streams were in the Upper New River Basin and ranged in mean width from two to six meters. Canopy cover varied across sites from completely closed to mostly open; substrate at all sites was predominately cobble. Sites were chosen to comprise a range of low to high *E. proxima* density and trematode infection prevalence (based on Dillon, 1982 and preliminary samples collected in 2009).

From February 2011 to August 2013, we sampled snails at each site 2 to 4 times per year to examine seasonal variation in prevalence of trematode infection (a total of 40 collections for all 5 sites over 3 years; Table 1). We visited each site a final time in summer, 2014, to examine annual variation in prevalence over a four-year period (a total of 20 collections for all 5 sites; Table 1). At each site, we established a 50 m reach, used throughout the entire study. On each visit, we quantified *E. proxima* density with a 1/3 m^2 quadrat sampler, placed at a minimum of 15 randomly selected points throughout the study reach. We also haphazardly collected a sample of ~100 *E. proxima* (all sizes) for trematode screening. In the laboratory, we measured the wet mass of each snail (high levels of variation in wear patterns of *E. proxima* shells prohibit the use of shell length as a suitable metric for snail size); before weighing, we dried snails with paper towels and removed any sediment attached to the shell.

To assess trematode infection, we dissected each snail under a dissecting microscope to examine the gonadal tissue and entire digestive tract for larval trematodes (sporocysts, rediae and cercariae). Using a compound microscope, trematode infections were categorized as one of seven types based on the morphology of cercariae described in Schell (1985), and primary sources when available: (1) *Metagonimoides oregonensis* (Cable, 1938; Lang and Gleason, 1967; Belden et al., 2012); (2) *Sanguinicola* sp. (Meade and Pratt 1965; Hoffman et al., 1985); (3) virgulate and ubiquita xiphidiocercariae (Hall, 1959, 1960; Lang, 1968); (4) cotylomicrocercous (Cable, 1938, 1939; Barger and Esch, 2000); (5) echinostome (Cable 1938); (6) monostome (Horsfall, 1930); and (7) vivax types (Cable, 1938) (Table 2). Molecular sequencing of trematode samples collected from *E. proxima* has revealed that virgulate and ubiquita type cercariae are not consistently and reliably distinguished morphologically (Zemmer unpublished); therefore, these types were grouped together as xiphidiocercariae for analyses.

We used a handheld meter (YSI Model 63, YSI, Inc., Yellow Springs, Ohio) to measure pH, water temperature, and specific conductance at each site on each visit. To examine potential relationships between regional weather patterns and trematode prevalence, we retrieved monthly mean air temperatures and monthly total precipitation for October 2010 to August 2014 from the National Climatic Data Center (NOAA; Station: Galax Water Plant, VA US COOP: 443272). Distance from the weather station to the study sites ranged from 28 to 53 km, making this the nearest station with data available for the full period of the study, and the elevation of this station was 719 m, similar to the mean elevation of the study sites (765 m).

Total trematode prevalence and *M. oregonensis* prevalence were analyzed as separate response variables. The number of snails infected with the other six types of trematodes was generally too low for accurate prevalence estimates given the sample size, so only *M. oregonensis* was analyzed individually. To test for consistent seasonal patterns of trematode infection, we used autocorrelation and partial autocorrelation functions (ACF and PACF) and lag plots to examine within site temporal autocorrelation in trematode prevalence, snail density and snail mass.

To compare seasonal variation in trematode infection to annual variation, we qualitatively compared within site ranges in prevalence for the respective study periods, as well as the maximum change in prevalence between collections. To address how trematode infection in stream snails is related to local and regional factors, we used generalized linear mixed models (GLMMs) with binomial error structures and logit-link functions, fit by maximum likelihood (following the approach described in Zuur et al., 2009). As sites were selected purposively for their initial differences in snail density and trematode prevalence, site was included as a random intercept in all models. Fixed effects in local factor GLMMs included mean snail density, temperature-compensated conductivity and water temperature. Snail mass in field surveys could represent both an explanatory and a response variable. Because snails may exhibit trematode-induced gigantism as a result of castration due to heavy infections in the gonadal tissue (Sousa, 1983; Mouritsen and Jensen, 1994; Sorensen and Minchella, 2001; Chapuis, 2009), snail mass was not included in models. Fixed effects in regional factor GLMMs were monthly mean air temperature and monthly total precipitation, including lagged values of one to three months of each measure. For GLMM selection, we chose best subsets of full models based on AICc (Burnham and Anderson, 2002). All GLMMs were constructed using the *lme4* package in R; all analyses were conducted in R version 2.15.1.

Results

Trematode prevalence

Seasonal and annual variation: We screened a total of 5,145 *E. proxima* and found 950 infected individuals. We found no evidence of temporal autocorrelation to indicate any pattern of seasonality in trematode prevalence (Figs. 1a and b). The total prevalence of infection (proportion of snails infected with trematodes of all types) at the highest prevalence site (Furnace Creek) ranged from 21.1 - 39.1%, with a mean prevalence of 29.8% (Figs. 1a and 2a, Table 1). At the lowest prevalence site (Cherry Creek), total prevalence of infection ranged from 2.6 - 16.1%, with a mean prevalence of 8.3% (Figs. 1a and 2a, Table 1). In general, variation in trematode prevalence across sites was consistent throughout the study period: sites with initially higher relative prevalence remained so, as did sites with initially lower relative prevalence (Figs. 1a and 2a).

Over the entire study period, *M. oregonensis* was the most common trematode infecting *E. proxima* (present in 567 out of 950 infected snails) and was found at every sampling time point at each site. It was also the most prevalent, and with three exceptions, composed the greatest number of infections in every collection, ranging in mean prevalence from 5.4% (Burkes Creek) to 16.4% (Adams Branch; Figs. 1b and 2b, Table 1). At all sites, the mean prevalence of *M. oregonensis* was higher than other trematode types (Table 1). *Sanguinicola* sp. had the second highest mean prevalence (7.2% at Furnace Creek), but was found at only two sites (Table 1). Virgulate and ubiquita xiphidiocercariae type infections were frequently encountered at each of the five sites (present in 39 out of 45 collections), with a mean prevalence ranging from < 1% (Cherry Creek) to 5.1% (Furnace Creek; Table 1). Cotylomicrocercous type infections were encountered at least once at each study site, but were less common (present in 28 out of 45 collections) and less prevalent, with a mean prevalence ranging from < 1% to 3.5% (Table 1). Additionally, three types of larval trematodes (echinostome, monostome, and vivax types) were encountered infrequently (Table 2).

Within site variation in trematode prevalence was greatest at the site with the second-highest mean total prevalence (Adams Branch): total trematode prevalence ranged from 12.4 to 37.4%, (SD = 7.3), *M. oregonensis* ranged from 8.2 to 21.6% (SD = 4.6) and xiphidiocercariae ranged from 1 to 11% (SD = 3.1) (Figs. 1a and b). Total trematode prevalence varied least at the lowest prevalence site (Cherry Creek, range of 2.6 to 16.1%, SD = 4.8), while *M. oregonensis* varied least at the highest prevalence site (Furnace Creek, range of 8.8 to 18.7%, SD = 2.9) (Figs. 1a and b). The greatest change in total trematode prevalence between consecutive collections was a 20% increase between summer and fall, 2011 (Adams Branch; Fig. 1a). The greatest change in total trematode prevalence between annual summer collections was a 13.1% decrease between 2011 and 2012 (Burkes Creek; Fig. 2a). There was no consistent pattern across sites in how the range of seasonal total infection compared to annual total infection. For *M. oregonensis*, the annual range was greater than the seasonal range at 4 of the 5 sites.

Snail density and water quality

Seasonal variation: We found no evidence of temporal autocorrelation to indicate a seasonal pattern of variation in mean snail density within sites (Fig. 1c). GLMM results indicated no significant relationship between snail density and total trematode infection; however, there was an overall signficant, but small, negative effect of snail density on the probability of *M. oregonensis* infection (a 1.8% decrease in odds of infection per 1 snail/m^2 increase in density, β = -0.0178, SE = 0.007; Table 3).

With the exception of one site (Furnace Creek), there was little within site variation in pH and temperature-compensated conductivity (Table 1). There was some across site variation in these variables and an overall small but significant, negative effect of conductivity on probability of total infection (a 2.5% decrease in the odds of infection per 1μS/cm increase in conductivity, β =-0.025, SE=0.008; Table 3) and *M. oregonensis* infection (a 2% decrease in odds of infection per 1μS/cm increase in conductivity, β = -0.021, SE = 0.011; Table 3). Additionally, there was a small overall negative effect of water temperature on total prevalence of infection (a 2.8% decrease in the odds of infection per 1degree C increase in water temperature, β = -0.028, SE= 0.009; Table 3), but not on *M. oregonensis* (Table 3).

Annual variation: There was little change in mean snail density between annual summer surveys, with one exception (Adams Branch, decrease of 25.9 snails/m^2 between summers 2011 and 2012; Fig. 2c). Mean snail density varied in a consistent pattern across 4 of the 5 sites (Furnace Creek, Chisholm Creek, Adams Branch and Cherry Creek), while one site (Burkes Creek) exhibited no change between years (Fig. 2c). Overall, there was no significant effect of snail density on either total or *M. oregonensis* infection (Table 3). There was an overall small but significant negative effect of conductivity on the probability of total infection (a 3.2% decrease in odds of infection per 1μS/cm increase in conductivity, β = -0.033, SE= 0.006; Table 3) and *M. oregonensis* infection (a 2.5% decrease in the odds of infection 1μS/cm increase in conductivity, β = -0.025, SE= 0.009; Table 3).

Snail mass

Seasonal and annual variation: We found no evidence of temporal autocorrelation to indicate a seasonal pattern of change in mean snail mass within sites (Fig. 1d). The greatest variation in mean snail mass between consecutive sampling points was a decrease of 0.072 g between spring and summer, 2012 (Burkes Creek; Fig. 1d and 2d).

Air temperature and precipitation

Seasonal variation: Air temperature was not significantly related to either total prevalence or *M. oregonensis* infection. Precipitation was negatively related to both total prevalence of infection and *M. oregonensis*. The optimal model for total prevalence of infection included both monthly total precipitation (a 1.9% decrease in the odds of infection per 1 inch increase in precipitation, β = -0.019, SE = 0.009; Table 3) and a three-month lag in precipitation (a 7.3% decrease in the odds of infection per 1 inch increase in precipitation, β= -0.073, SE = 0.222; Table 3). The optimal model for *M. oregonensis* included only a three-month lag in precipitation (a 6.1% decrease in the odds of infection per 1 inch increase in precipitation, β= -0.061, SE = 0.029; Table 3).

Annual variation: Total prevalence of infection was postively related to air temperature and negatively related to precipitation; the optimal model included a one-month lag in monthly mean air temperature (a 7.3% increase in the odds of infection per 1 degree F increase in temperature, β=0.073, SE = 0.204; Table 3) and monthly total precipitation (a 2.8% decrease in the odds of infection per 1 inch increase in precipitation, β=-0.028, SE = 0.011; Table 3). *M. oregonensis* infection was postively related to monthly mean air temperature (a 9.2% increase in the odds of infection per 1degree F increase in temperature, β= 0.092, SE = 0.032; Table 3), but was not significantly related to precipitation.

Discussion

The community structure of the larval trematodes infecting *E. proxima* at these study sites was similar to that observed by Belden et al., (2012) at sites within the Little Tennessee River Basin in North Carolina: *M. oregonensis* was ubiquitous and the most prevalent, while virgulate and cotylomicrocercous types were less common and less prevalent. To our knowledge, this study is the first to report several types of larval trematodes in *E. proxima* including *Sanguinicola* sp., echinostome, monostome, and vivax cercariae, although all of these types have been previously described in congeners (Horsfall, 1930; Cable, 1938; Meade and Pratt, 1965; Hunter and Wigington, 1972).

Previous research on temporal variation in freshwater snail trematode infection has largely focused on snails with short life spans (several months to 1.5 years) in lentic systems (Fernandez and Esch, 1991a, 1991b; Snyder and Esch, 1993; Klockars et al., 2007; Faltýnková et al., 2008). The seasonal peaks of trematode infection observed in a number of these short-lived species have been attributed largely to the phenology of the host snails (i.e. recruitment, mortality, population turnover) that makes infections more likely to occur during certain seasons (Fernandez and Esch, 1991a; Kube et al., 2002; Faltýnková et al., 2008). In contrast, the host snail examined in this study, *E. proxima*, has a life span of 3 to 5 years (Dillon, 2000), creating a much longer period during which snails may become infected. With annual recruitment, *E. proxima* populations consist of multiple cohorts, and if infections persist for several years, trematode prevalence in these snails is a product of infections accumulated over the past 3 to 5 years. While we do not have specific information regarding how long trematode infections persist in *E. proxima*, there is evidence from mark-recapture studies of several species of longer-lived snails that some larval trematode infections can persist for over 4 years (Sousa, 1993; Curtis and Tanner, 1999).

A previous study on *E. proxima* from a single North Carolina stream reported a peak of trematode infection in the winter (Lang, 1968). We found no significant pattern of seasonal variation in prevalence of infection; however, total infection was consistently higher in either winter or fall than in summer at 3 of the 5 sites (Furnace Creek, Adams Branch, and Chisholm Creek). Conversely, total infection was higher in summer than in fall at the other 2 sites (Burkes Creek and Cherry Creek; winter data for these 2 sites was not available; Fig. 1). Sites also differed in the degree and direction of annual fluctuations in trematode infection and how these compared to the range of infection among seasons, particularly for total trematode prevalence (Fig. 3). This site-level variation in temporal patterns suggests that local factors, such as the abundance and distribution of additional hosts, as well as local abiotic factors, may mediate the impacts of regional patterns in temperature and precipitation on first-intermediate host infection.

We found no strong relationships between trematode prevalence and the site-level variables measured in this study; however, sites likely differed in a number of unmeasured factors that may impact trematode prevalence (e.g. stream discharge and flashiness, sinuousity and microhabitat composition, surrounding land-use). Related to this, sites likely differed in degree of occupancy by definitive hosts, and numerous studies have underscored the importance of definitive hosts in structuring larval trematode communities in snails (Fernandez and Esch, 1991a, 1991b; Snyder and Esch, 1993; Mouritsen et al., 1997; Granovitch et al., 2000; Skirnisson et al., 2004; Hechinger and Lafferty, 2005; Byers et al., 2008). To identify the major drivers of parasite prevalence in this system, it may be necessary to quantify definitive host occupancy and explore the effects of additional abiotic variables.

This study differed from previous studies of temporal variation in larval trematode communities in several key ways: First, the host snail in this system, *E. proxima*, is longer-lived than most snail species examined in previous temporal variation studies. This may partially account for why we did not find a strong seasonal pattern of infection in this system. Although our study comprised observations over multiple years, this period may have been insufficient to identify patterns of temporal variation in the infection dynamics of a host with a life span of 3 to 5 years; a longer term study may be necessary. Second, we examined seasonal variation over a period of multiple years, while many of the previous studies reporting seasonal peaks of infection were conducted for a single year and do not provide information about the consistency of seasonal patterns over time. Research of larval trematode communities in lotic mollusks is sparse in comparison to research of lentic or marine intermediate host species, and infection dynamics in these systems may exhibit different patterns, even for the same class of parasites.

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Table 1. Physicochemical characteristics of each of the 5 study sites, mean snail density, and mean prevalence (± SE) of the 4 most common trematode types from all sampling points (February 2011 – August 2014). *Metagonimoides oregonensis* (META), virgulate and ubiquita type xiphidiocercariae (XIPH), cotylomicrocercous type (COTYL), *Sanguinicola* sp. (SANG).

						No. of snail collections					% Infected snails				
Stream name	**Width (m)**	**Elev (m)**	**pH**	**Conductivity (µS/cm)**	**Snails m^{-2} ± SE**	**Win**	**Spr**	**Sum**	**Fall**	**Total**	**META**	**XIPH**	**COTYL**	**SANG**	**TOTAL**
Furnace Crk.	6.10	778	6.77 - 7.30	31.7 - 39.8	8.5 ± 1.3	3	1	4	2	10	14.5 ± 0.9	5.1 ± 0.8	3.5 ± 1.0	7.2 ± 1.5	29.8 ± 1.8
Adams Br.	2.24	728	7.30 - 7.57	51.9 - 58.3	17.8 ± 3.4	1	2	4	2	9	16.4 ± 1.5	4.2 ± 1.0	1.9 ± 0.5	-	23.0 ± 2.4
Chisholm Crk.	5.37	812	7.09 - 7.44	44.8 - 50.8	16.3 ± 2.4	3	1	4	2	10	11.1 ± 1.3	3.3 ± 0.9	1.5 ± 0.4	2.1 ± 0.6	18.2 ± 1.8
Burkes Crk.	2.73	821	7.30 - 7.60	60.4 - 73.4	2.8 ± 0.5	0	2	4	2	8	5.4 ± 1.2	2.0 ± 0.5	1.1 ± 0.5	-	9.8 ± 1.6
Cherry Crk.	4.40	685	7.23 - 7.57	63.2 - 70.6	10.6 ± 1.6	0	2	4	2	8	6.5 ± 1.5	0.6 ± 0.4	0.1 ± 0.1	-	8.3 ± 1.7

Table 2. Types of trematode cercariae from first-intermediate host infections of *Elimia proxima* (for all collections), including likely second-intermediate and definitive hosts.

Type	**Family(s)**	**Second intermediate hosts***	**Definitive hosts***	**No. of sites**	**Range in prevalence (%)**
Metagonimoides oregonensis	Heterophyidae	Amphibians	Raccoons, mink	5	1.4 - 21.6
Sanguinicola sp.	Sanguinicolidae	None	Fish	2	0 - 18
Virgulate and ubiquita xiphidiocercariae	Lecithodendriidae, Collyriclidae	Aquatic insects	Bats, birds	5	0 - 11
Cotylomicrocercous	Opecoelidae	Aquatic insects	Fish	5	0 - 10.4
Echinostome	Echinostomatidae	Snails, amphibians	Mammals, birds	3	0 - 2.3
Monostome	Notocotylidae	None; encysts on vegetation	Birds (anseriforms)	1	0 - 1.4
Vivax	Cyathocotylidae	Fish	Piscivorous birds	1	0 - 0.5

* As described in: Ingles (1935), Burns and Pratt (1953) for *M. oregonensis*; Meade and Pratt (1965), Hoffman et al. (1985) for *Sanguinicola* sp.; Seitner (1945), Hall (1959) for virgulate type; and Schell (1985) for cotylomicrocercous, echinostome, monostome and vivax types

Table 3. AICc scores for null (intercept only) and optimal GLMMs for seasonal variation study (February 2011 – August 2013) and optimal GLMMs for annual variation study (summer surveys, 2011 - 2014).

Models	**AICc**	**ΔAICc**
Seasonal total prevalence of infection: site level variables		
Site	238.7	
Conductivity + Water temperature + Site	225.0	13.8
Seasonal total prevalence of infection: regional weather variables		
Site	295.2	
Precipitation + Precipitation three-month time lag + Site	282.4	12.8
Seasonal M. oregonensis prevalence: site level variables		
Site	201.5	
Snail Density + Conductivity + Site	197.5	4.0
Seasonal M. oregonensis prevalence: regional weather variables		
Site	201.5	
Precipitation three-month lag + Site	199.4	2.0
Annual total prevalence of infection: site level variables		
Site	170.51	
Conductivity + Site	164.64	5.87
Annual total prevalence of infection: regional weather variables		
Site	170.51	
Temperature one-month time lag + Precipitation + Site	158.91	11.60
Annual M. oregonensis prevalence: site level variables		
Site	142.25	
Conductivity + Site	139.37	2.88
Annual M. oregonensis prevalence: regional weather variables		
Site	142.25	
Temperature + Site	136.58	5.67

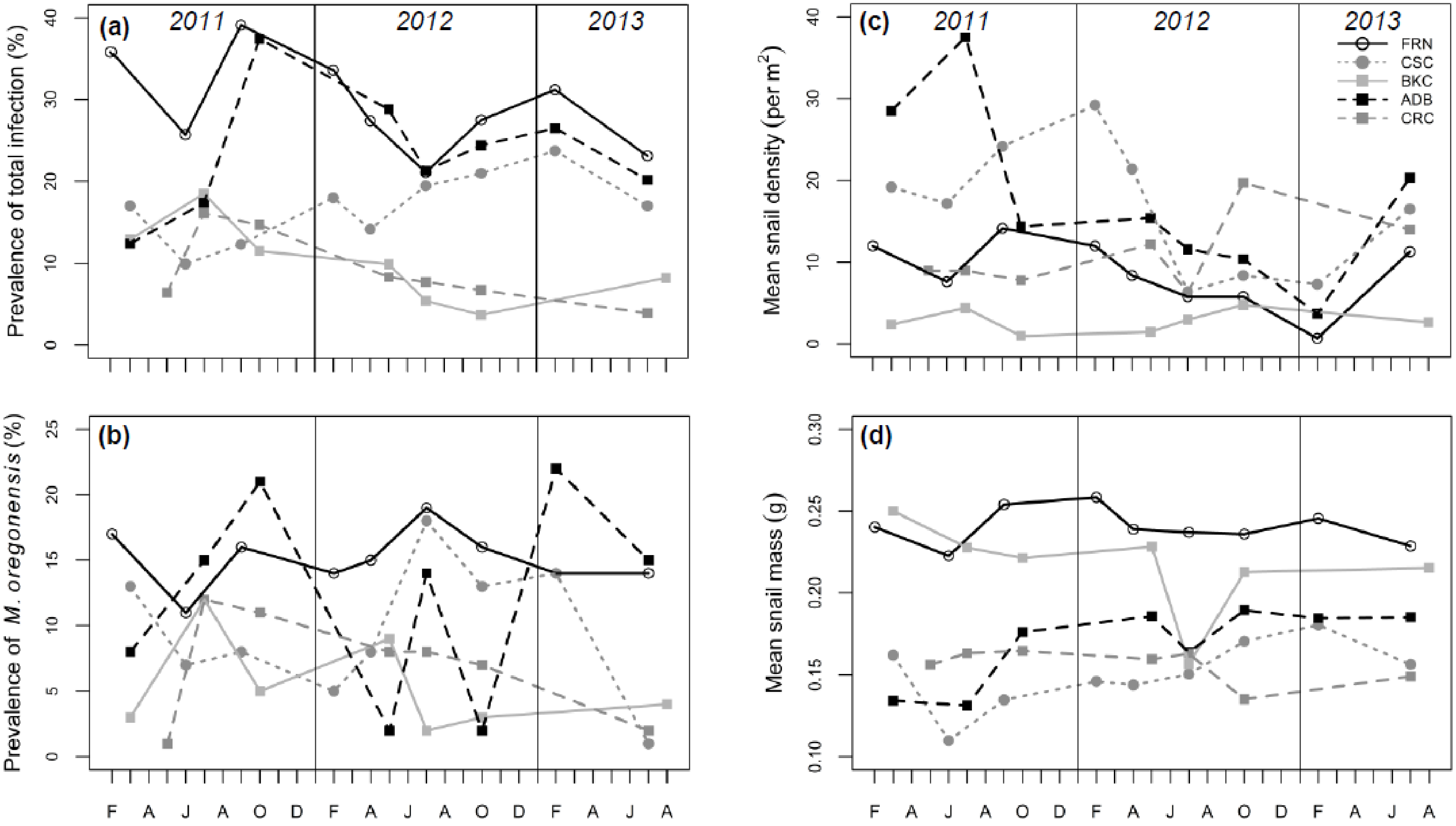

Figure 1. Seasonal variation (February 2011 to August 2013) in *Elimia proxima*: (a) overall trematode infection, (b) *Metagonimoides oregonensis* infection, (c) mean snail density and (d) mean snail mass. Furnace Creek (FRN), Chisholm Creek (CSC), Burkes Creek (BKC), Adams Branch (ADB), Cherry Creek (CRC).

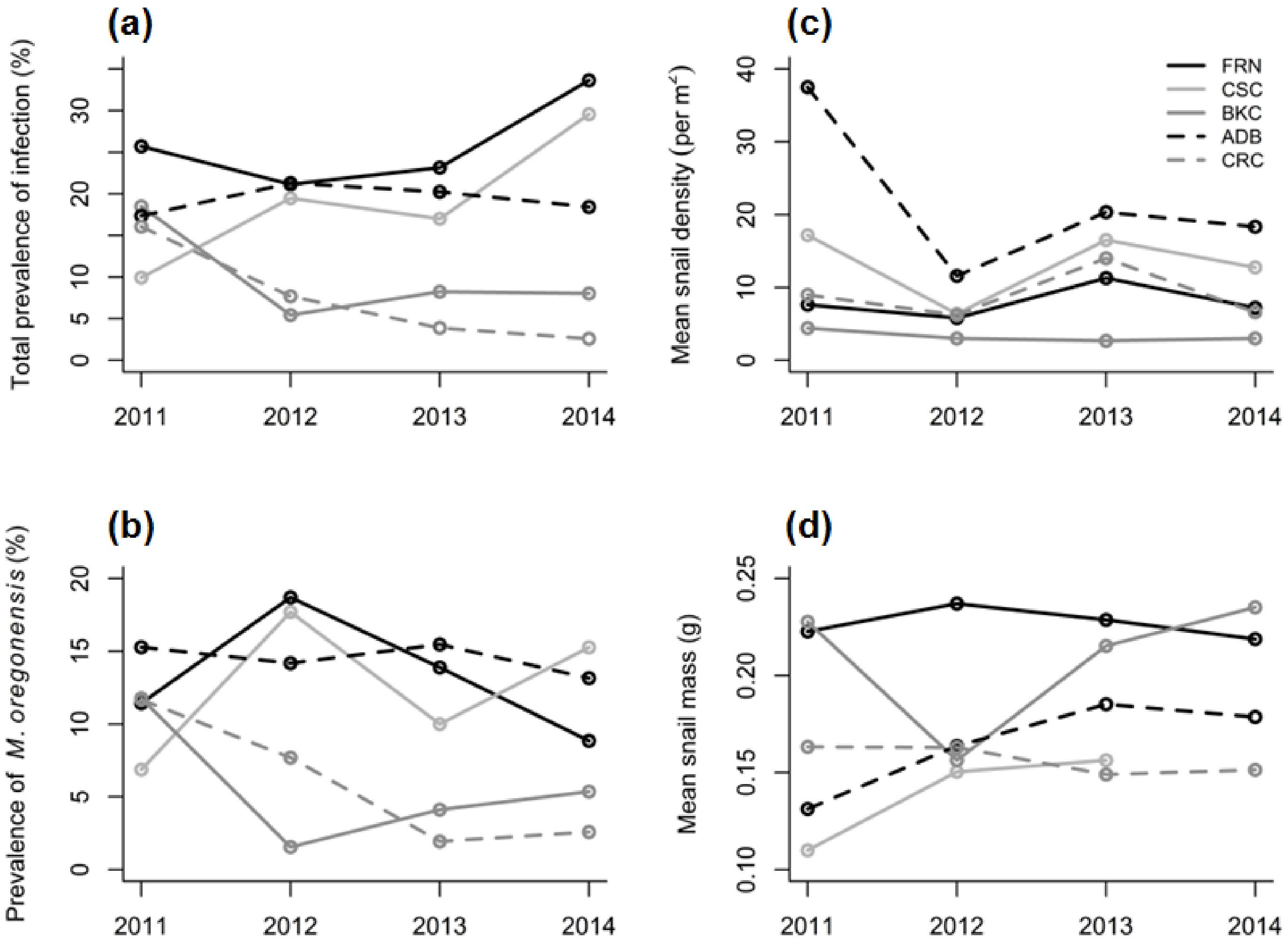

Figure 2. Inter-annual variation (2011 to 2014) in summer samples of *Elimia proxima*: (a) overall trematode infection, (b) *Metagonimoides oregonensis* infection, (c) mean snail density and (d) mean snail mass. Furnace Creek (FRN), Chisholm Creek (CSC), Burkes Creek (BKC), Adams Branch (ADB), Cherry Creek (CRC).

Chapter 3: Spatial turnover of dominant species drives regional variation in stream parasites

Abstract

By considering the roles of both local environmental factors and dispersal across regional spatial scales, the metacommunity concept has enhanced our knowledge of the processes that structure ecological communities. Digenean trematodes are parasitic flatworms with complex life cycles requiring multiple hosts. For most parasites, including trematodes, the dispersal of hosts is typically greater than that of any free-living parasite stages, and differences in host dispersal abilities can drive variation in patterns of parasite abundance and diversity at local and regional scales. Here, we investigated the role of local factors in shaping spatial patterns of larval trematode communities across a regional scale. We examined first-intermediate host trematode infection of *Elimia proxima* snails in 20 Appalachian headwater streams distributed within the Upper New River Basin in North Carolina and Virginia. We conducted all analyses at two levels of biological characterization: morphotype, based on visual identification of larval trematodes, and haplotype, based on molecular identification. We identified five trematode morphotypes, and within those we identified 30 unique haplotypes belonging to nine families. We found a broad scale spatial pattern in *E. proxima* trematode communities due to regional turnover in the dominant trematode species, and this pattern was significant for both morphotype and haplotype defined communities. While this pattern correlated with elevation, we did not find any significant relationships with other environmental factors, indicating that dispersal may be an important driver of community structure in this system. Additionally, we found evidence of multiple species within 3 of the 5 morphotypes. This variation in the levels of genetic diversity within morphotypes may reflect differences in host dispersal abilities. Incorporating this information in future research could elucidate relationships that may be masked by the grouping together of morphologically similar species.

Introduction

A primary goal of community ecology is to understand the processes that influence species interactions, abundance and distribution. Traditionally, ecological communities have been conceptualized and investigated as finite assemblages of species, structured primarily by species interactions and local environmental factors. Within the past decade, this approach has been challenged by a renewed interest in the role of landscape features and dispersal in structuring communities. The development of metacommunity paradigms has extended the study of ecological communities to include processes occurring at both local and regional scales (Leibold et al. 2004, Holyoak et al. 2005). The four main metacommunity paradigms (species sorting, mass effects, patch dynamics and the neutral model) all incorporate environmental and spatial processes, but vary in the relative importance of species traits, environmental heterogeneity (i.e. local scale) and dispersal (i.e. regional scale) in structuring communities (Logue et al. 2011).

By some estimates, there are many more parasitic than free-living species (see Bush et al. 2001), but parasites are largely underrepresented in ecological research compared to their free-living hosts. Understanding the ecological processes that impact parasite abundance and distribution is critically important for epidemiology and predicting how infectious disease dynamics may respond to future disturbance. One group of parasites that has become a common focus of ecological studies is digenean trematodes (Phylum: Platyhelminthes), which are parasitic flatworms, also known as flukes. Trematodes have complex life cycles that typically involve a series of three hosts, although there is substantial diversity in life cycle patterns. Adult trematodes sexually reproduce in vertebrate definitive hosts and release eggs in the host feces. Eggs hatch into larvae that infect mollusks as first-intermediate hosts and reproduce asexually to generate free-living cercariae. Cercariae leave the mollusk, penetrate and encyst inside organs of an invertebrate or vertebrate second-intermediate host. The life cycle is completed when an infected second-intermediate host is consumed by the definitive host (Bush et al. 2001).

Ecological studies of trematodes often focus on larval forms. But larval forms can lack distinguishing morphological features, making visual identification especially challenging, which can lead to poor taxonomic resolution (Poulin and Leung 2010). When species are grouped together based on morphological similarity, patterns of host specificity and spatial distribution can be obscured (Leung et al. 2009, Locke et al. 2010). To adequately capture parasite diversity, especially in preliminary research of novel study systems, molecular identification is often necessary.

For most parasites, including trematodes, the dispersal of hosts is typically greater than that of any free-living parasite stages; consequently, differences in the dispersal abilities of host species can drive variation in patterns of parasite abundance and diversity at local and regional scales (Criscione and Blouin 2004, Prugnolle et al. 2005, Louhi et al. 2010, Blasco-Costa and Poulin 2013). For parasites with complex life cycles, such as trematodes, distribution is dependent on the co-occurrence of all hosts, as well as environmental conditions suitable for hosts and free-living larval stages. Indeed, previous research on larval trematode communities has established roles for both local and regional processes in determining community structure. Several studies have found that effects of local factors, such as surrounding land use and water quality, influence larval trematode diversity and prevalence (Johnson et al. 2007, Soldánová et al. 2010, Ciparis et al. 2013, Richgels et al. 2013, Hofmann et al. 2016). Additionally, intermediate hosts are often less mobile than definitive hosts. If the definitive host is a fish, a trematode life cycle may be entirely aquatic and limited to within site dispersal (autogenic life cycle; Esch et al. 1988); however, if the definitive host is terrestrial (e.g. a mammal or bird) trematodes may disperse with their hosts across the landscape (allogenic life cycle). The structure of larval trematode communities at local sites is often highly linked to the regional distribution and abundance of definitive hosts (Fernandez and Esch 1991, Snyder and Esch 1993, Mouritsen et al. 1997, Granovitch et al. 2000, Smith 2001, Skirnisson et al. 2004, Hechinger and Lafferty 2005, Byers et al. 2008, Thieltges et al. 2009, 2013, Blasco-Costa et al. 2013, Levakin et al. 2013).

Due to the dendritic structure of stream networks and the effects of continuous, downstream current, the infection dynamics of lotic parasites may differ from those of other aquatic systems. Most of the previous research on the processes that drive variation in larval trematode communities has been conducted in either lentic (Fernandez and Esch 1991, Snyder and Esch 1993, Klockars et al. 2007, Faltýnková et al. 2008, Soldánová et al. 2010, 2011, 2012, Richgels et al. 2013) or marine systems (Byers et al. 2008, Levakin et al. 2013). In this study, we characterized the regional diversity and abundance of trematodes infecting stream snails *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* (Gastropoda: Pleuroceridae). *Elimia proxima* is a common, native inhabitant of Appalachian headwater streams (Dillon 2000) that serves as first-intermediate host to a number of trematode species, some with autogenic life cycles and some with allogenic life cycles. Here, our main objective was to examine the roles of local factors and dispersal in shaping spatial patterns of lotic parasite distribution across a regional scale.

Materials and Methods

Study sites

We examined first-intermediate host trematode infection of *Elimia proxima* snails in 20 Appalachian headwater streams distributed across 4 counties in southwestern Virginia and 2 counties in northwestern North Carolina in summer, 2011 (Table 1; Fig. 1). Distance between sites ranged from < 1 km to 132 km. All sites were first, second or third order streams in the Upper New River watershed and ranged in mean width from 1.5 to 9.85 m. Surrounding land use, canopy cover and substrate composition varied across sites from completely closed reaches in forests with predominately cobble substrate, to mostly open reaches in agricultural grazing land with predominately silt substrate. Elevation of sites ranged from 673 to 952 m. Sites were chosen to comprise a range of low to high *E. proxima* density (based on Dillon 1982 and preliminary samples collected in 2009).

Snail density and environmental measurements

At each site, we established a 50 m reach and quantified *E. proxima* density with a 1/3 m^2 quadrat sampler placed at a minimum of 15 randomly selected points throughout the study reach. We used a handheld meter (YSI Model 63, YSI, Inc., Yellow Springs, OH) to measure pH, water temperature and specific conductance, and collected water samples to measure phosphorous and nitrogen concentrations; eutrophication could contribute to higher snail densities and increased trematode infection (Johnson et al. 2007). To quantify nutrient levels, we measured total phosphorous and total nitrogen with standard colorimetric assays (APHA 2005) using a Lachat flow-injection autoanalyzer (Hach Company, Loveland, CO) at the Virginia Tech Ecosystem Research Group Analytical Lab.

Trematode collection

At each of the 20 sites, we haphazardly collected a sample of ~120 *E. proxima* (all sizes) for trematode screening (total N=2,515 snails). In the laboratory, we measured the wet mass of each snail after removing any sediment attached to the shell. We used a dissecting microscope to examine the gonadal tissue and digestive tract of each snail for larval trematodes (sporocysts, rediae and cercariae). Using a compound microscope, we identified trematode infections as one of five morphotypes based on the morphology of cercariae described in Schell (1985) and primary sources. Morphotypes included: (1) *Metagonimoides oregonensis* (Cable 1938, Lang and Gleason 1967, Belden et al. 2012); (2) *Sanguinicola* sp. (Meade and Pratt 1965, Hoffman et al. 1985); (3) virgulate and ubiquita xiphidiocercariae (Hall 1959, 1960, Lang 1968); (4) cotylomicrocercous (Cable 1938, 1939, Barger and Esch 2000); and (5) monostome (Horsfall 1930) (Table 2). From every infected snail, we also collected larval trematode samples in 95% ethanol (stored at -20^{o}C) for molecular identification.

Molecular identification

To confirm visual identification and examine genetic variation within morphotypes, we sequenced an ~1,400 base pair region of the 28S large subunit rRNA gene following the methods described in Olson et al. (2003) with modifications. For each infected snail (N=548, excluding

13 snails with dual infections = 535), DNA was extracted from a single sporocyst or redia, or when no other parasite tissue was available, we pooled ~ 5 cercariae from the snail. Prior to DNA extraction, we rinsed tissue twice in Milli-Q water to remove ethanol and transferred samples in 2 µl of Milli-Q water into 0.2 ml tubes. For DNA extraction, we added 98 µl of 6.7% Chelex and 1% Proteinase K solution and incubated tubes at 56°C for 2 hours, followed by an 8 minute boil at 100°C. We amplified DNA with PCR using forward primer LSU-5 and reverse primer 1500R (Olson et al. 2003). Each 25 µl reaction comprised 3 µl of DNA sample, 0.4 µM of each primer and 1x GoTaq® G2 Hot Start Colorless Master Mix (Promega Corp., Madison, WI) containing GoTaq® Hot Start DNA polymerase, reaction buffer at pH 8.5, 400 µM of each dNTP, and 4mM $MgCl_2$. Thermal cycling parameters for PCR were: an initial denaturation (3 minutes at 95°C); 40 cycles of amplification (45 seconds at 94°C, 30 seconds at 54°C and 2 minutes at 72°C); and a final extension period (7 minutes at 74°C). Amplicons were visualized using gel electrophoresis. PCR products were cleaned with a QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions and eluted with Milli-Q water to a final volume of 30 µl. Purified samples were sequenced with PCR primers. We aligned sequences in Geneious v. 8.0.4 (Biomatters Ltd., Auckland, New Zealand) and assigned samples to haplotype (i.e. sequence). Haplotypes were distinguished by the presence of any single-nucleotide polymorphism and identified via BLAST search in GenBank. Sequences of each haplotype were deposited in NCBI Genbank.

Statistical analysis

To visualize relationships between sites based on trematode community composition we conducted principal coordinates analysis (PCoA) with both Bray-Curtis (prevalence of trematode types) and Jaccard (presence/absence of trematode types) dissimilarity matrices. We conducted distance-based redundancy analysis (db-RDA) to test for both species-environment relationships and spatial variation in trematode community composition. The environmental variables examined included *E. proxima* density, stream width, pH, specific conductance, total nitrogen, total phosphorous, and elevation. Variables comprising a gradient of broad to fine scale spatial structure were extracted from geographic coordinates using principle coordinates of neighbor matrices (PCNM) (Borcard and Legendre 2002, Dray et al. 2006). For both sets of potential explanatory variables, we used forward stepwise model selection based on adjusted R^2 values. We then used variation partitioning to determine the proportions of across site variation in trematode communities that could be explained explicitly by environmental variables, spatial variables, or spatially structured environment variables. To determine if community patterns remained the same when examined at different levels of taxonomic resolution, all analyses were conducted for both morphotype (based on visual identification) and haplotype (based on molecular identification) defined communities. Cryptic (morphologically indistinguishable) species diversity within morphotypes could potentially obscure spatial patterns of distribution and host specificity (Donald et al. 2004, Miura et al. 2005, Leung et al. 2009, Detwiler et al. 2010, Locke et al. 2010). All analyses were conducted using the *labdsv* and *vegan* packages in R v. 3.2.4.

Results

Trematode prevalence

Out of 2,515 *E. proxima* screened, 548 snails were infected with trematodes. Trematodes were present at all sites, and total prevalence of infection ranged from 10% (Chisholm Creek) to 49% of snails infected (Little Wilson) (Table 1; Fig. 2). Both *M. oregonensis* and xiphidiocercariae infections were encountered at all 20 sites. *M. oregonensis* ranged in prevalence from 2% (Long Branch) to 20% (Francis Mill), while xiphidiocercariae infections were less prevalent and ranged from < 1% (Chisholm and West Fork Dodd Creeks) to 10% of snails infected (East Fork Crooked Creek). Cotylomicrocercous type infections were also common (encountered at 17 out of 20 sites) and comprised the highest prevalence of any single type of infection with 38% of snails at one site (Little Wilson) infected. Infections with other trematode types were rare; *Sanguinicola* sp. was present at three sites with a maximum prevalence of 6% (Furnace Creek), and monostome type cercariae were present at one site with a prevalence of 4% (Cherry Creek).

Molecular characterization and haplotype diversity

We obtained partial 28S rRNA gene sequences for 491 larval trematodes, from which we identified 30 unique haplotypes belonging to nine families (Table 2; Fig. 3). For most morphotypes, 1 to 2 haplotypes comprised >80% of all infections. For cotylomicrocercous type infections, we identified nine unique haplotypes, all most closely matched (max identity 97 – 99%) to *Plagiocirrus loboides* (Opecoelidae). Haplotype divergence within a 1,265 bp fragment ranged from 1 to 51 base pairs (0.08 – 4.0%). For infections visually identified as *M. oregonensis*, we identified seven haplotypes. Six of these haplotypes most closely matched (max identity 98 – 99%) *Metagonimoides oregonensis* (Heterophyidae) and varied at 1 to 17 sites within a 1,293 bp fragment (0.08 – 1.28% divergence). The seventh haplotype most closely matched (max identity 97%) *Clonorchis sinensis* (Opisthorchiidae) and differed from *M. oregonensis* haplotypes by 83 to 88 base pairs (6.4 – 6.75% divergence).

From virgulate and ubiquita type xiphidiocercariae infections, we identified eight haplotypes most closely matched to four species from three different families. Four of these haplotypes matched species within the family Lecithodendriidae: three haplotypes most closely matched (max identity 97%) *Paralecithodendrium parvouterus* and one most closely matched (max identity 97%) *Lecithodendrium linstowi*. Divergence among these haplotypes within a 1,242 bp fragment ranged from 6 to 65 base pairs (0.48 – 5.23%). Two other xiphidiocercariae haplotypes most closely matched (max identity 96%) *Collyriclum faba* (Collyriclidae), variable at 1 site within a 1,287 bp fragment (0.08% divergence). The remaining two xiphidiocercariae haplotypes most closely matched (max identity 92 – 93%) *Allassogonoporus amphoraeformis* (Pleurogenidae), variable at 4 sites within a 1,282 bp fragment (0.31% divergence).

We identified a single haplotype each for *Sanguinicola* sp. and monostome type infections: respectively, these matched (max identity 85%) *Sanguinicola cf. inermis* (Aporocotylidae) and (max identity 98%) *Notocotylus* sp. (Notocotylidae). Additionally, we identified four unique haplotypes from immature or unknown types of infections, which included: one additional haplotype most closely matched (max identity 97%) to *Plagiocirrus loboides* (Opecoelidae); one haplotype that matched (max identity 99%) multiple sequences within Echinostomadiae; and two haplotypes that each matched (max identity 94%) additional species within Pleurogenidae, *Collyricloides massanae* and *Parabascus duboisi*.

Snail density, environmental and spatial variables

Snail density and water quality varied across sites (Table 1). Stream pH ranged from 6.32 (Piney Fork) to 7.97 (Francis Mill Creek), with a mean of 7.22 ± 0.1, and conductivity ranged from 18 µS/cm (Howell Creek tributary) to 152.3 µS/cm (Francis Mill Creek), with a mean of 64.87 ± 7.4 µS/cm. Snail density was highly skewed (skewness = 1.57), ranged from 0.2 snails/m^2 (Big Wilson Creek) to 37.5 snails/m^2 (Adams Branch), and averaged 10.23 ± 2.36 snails/m^2. While TP ranged from 7 ppb (Big Wilson Creek) to 75 ppb (Long Branch), 75% of sites fell within 25 to 30 ppb. Similarly, TN ranged from 207 ppb (Adams Branch) to 3,342 ppb (Long Branch), but 50% of sites fell within 500 to 850 ppb.

From PCNM analysis, we obtained 12 positive eigenvectors used as spatial variables in db-RDA to test for spatial structuring of trematode communities. Several environmental variables were correlated with the spatial variable, PCNM1, indicating broad scale spatial patterns in these variables: elevation (r = 0.5, p = 0.026); TN (r = 0.49, p = 0.029); and snail density (r = -0.55, p = 0.012).

Variation in community structure

Metagonimoides oregonensis was the most prevalent infection at 11 of the 20 sites, while cotylomicrocercous type was most prevalent at 9 sites (Table 1). The prevalence of these two types was highly negatively correlated (r = -0.897, p < 0.001), and PCoA ordinations of morphotype and haplotype prevalence showed that variation in trematode communities was driven primarily by dominant species (Figs. 4 and 5). For PCoA based on Bray-Curtis distance of morphotype defined communities, the first two principal coordinates accounted for 92% of the variance in trematode communities (Fig. 4). The first principal coordinate accounted for 77% of the total variance, and was most correlated with cotylomicrocercous type species (r = 0.97) and *M. oregonensis* (r = -0.95). The second principal coordinate accounted for an additional 15% of the total variance, and was most correlated with virgulate and ubiquita type species (r = -0.86). Similarly, in the PCoA of morphotype prevalence based on presence-absence (Jaccard), the first principal coordinate accounted for 64% of the variance, and was most correlated with cotylomicrocercous type species (r = 0.97) and *M. oregonensis* (r = -0.95), while the second principal coordinate accounted for an additional 14% of the variance, and was most correlated with virgulate and ubiquita type species (r = -0.88). In PCoA of haplotype defined communities, a greater number of principal coordinates was needed to capture the variance in composition. For Bray-Curtis distance based PCoA of haplotype communities, the first two principal coordinates accounted for 47% of the variance in trematode communities. The first principal coordinate accounted for 31% of the total variance, and was most correlated with a cotylomicrocercous haplotype (r = 0.75) and a *M. oregonensis* haplotype (r = -0.74). The second principal coordinate accounted for an additional 16% of the total variance, and was most correlated with a virgulate haplotype (r = 0.71). For Jaccard based PCoA of haplotype defined communities, the first principal coordinate accounted for 23% of the variance, and was most correlated with a *M. oregonensis* haplotype (r = -0.78) and a cotylomicrocercous haplotype (r = 0.77), while the second principal coordinate accounted for an additional 12% of the variance, and was most correlated with virgulate and ubiquita type species (r = -0.82).

Trematode communities defined by morphotypes were spatially structured at the broadest scale (PCNM1) based both on prevalence (Bray-Curtis: adj. R^2= 0.215, p=0.003) and presence-absence of morphotypes (Jaccard: adj. R^2= 0.243, p=0.002) (Fig. 6; Table 3). PCNM1 was positively correlated with cotylomicrocercous morphotype prevalence (r = 0.673, p = 0.001) and negatively correlated with *M. oregonensis* morphotype prevalence (r = -0.627, p = 0.003) (Fig. 6). There were no significant relationships between any of the measured environmental variables and variation in morphotype communities. While elevation was positively correlated with cotylomicrocercous type prevalence (r = 0.473, p = 0.035), it was not significant in db-RDA of morphotype communities.

Trematode communities defined by haplotypes were also structured at the broadest spatial scale (PCNM1) and related to additional broad scale spatial variables (PCNM2 and PCNM3) based on haplotype prevalence (Bray-Curtis: adj. $R^2 = 0.199$, p = 0.001) and presence-absence of haplotypes (Jaccard: adj. $R^2 = 0.206$, p=0.001) (Fig. 6; Table 3). For both dissimilarity metrics, variation in haplotype communities was significantly related to altitude. Elevation explained only a small proportion of the total variation (adj. R^2= 0.05; Table 3) and was not independent of spatial variation, as elevation was positively correlated with PCNM1 (r = 0.50, p = 0.026). No other environmental variables were significantly related to haplotype community variation.

Discussion

Spatial structure of trematode communities

We found that trematode component communities of *E. proxima* exhibited a broad scale spatial pattern due to regional turnover in the dominant trematode species for both morphotype and haplotype defined community structure. Sites in the southwestern part of the study area (Ashe and Alleghany Cos., NC, and Grayson and Carroll Cos., VA) were characterized by high prevalence of cotylomicrocercous type infections. Conversely, cotylomicrocercous type infections were either absent or had relatively low prevalence at sites located at higher latitudes, primarily in the northeastern part of the study region, where the prevalence of *M. oregonensis* was high (Floyd, Carroll, and Wythe Cos., VA). This spatial pattern was correlated with elevation, but elevation was a significant predictor variable in constrained ordination only of haplotype defined communities. This suggests that the significance of elevation in explaining community variation emerged as a result of capturing additional heterogeneity within communities by using haplotype level resolution. Indeed, three rare haplotypes were encountered only at the highest elevation sites, and an additional three rare haplotypes occurred only at the lowest elevation sites. It is not clear whether this relationship with elevation is related to dispersal or due to relationships with environmental factors that are aggregated by elevation (e.g., canopy cover, flow regime, land use).

Effects of environmental factors on community structure

Other than the effect of spatially structured environment (elevation), we did not find evidence that variation in community structure was driven by any of the local environmental factors we measured. This contrasts with the results of previous studies in lentic systems, where cultural eutrophication has driven increases in first-intermediate host biomass and subsequent trematode infection (Johnson et al. 2007, Richgels et al. 2013). One simple explanation for this contrast is differences in the habitat requirements of the first-intermediate hosts in lentic systems, pulmonate (lung-breathing) snails that thrive in environments with high levels of autochthonous matter resulting from high nutrient inputs, despite subsequent oxygen depletion. *Elimia proxima* is a prosobranch (gill-breathing) species adapted to the high levels of dissolved oxygen found in headwater streams, so we would not expect to find persistent populations at sites that are highly impacted by eutrophication.

Trematode diversity

One motivation for molecular identification of trematodes was to confirm visual identification. This is especially important for studies of larval trematodes, as identifying morphological features of larval stages is often difficult. Traditionally, trematodes have been identified on the basis of adult morphology and life cycle information, which requires collection of individuals from definitive hosts. Here, molecular identification revealed that 2 of the 3 morphotypes (*M. oregonensis* and virgulate type) comprised trematodes from multiple families. A second motivation for genetic analysis was that previous studies of larval trematodes have revealed that cryptic (morphologically indistinguishable) species diversity can obscure patterns of spatial distribution and host specificity (Donald et al. 2004, Miura et al. 2005, Leung et al. 2009, Detwiler et al. 2010, Locke et al. 2010). These studies demonstrate the importance of using molecular analysis to identify the presence of cryptic species. The D1- D3 region of the large subunit rRNA (28S) gene is often used as a marker to resolve phylogenies and identify cryptic species within Digenea (Barker et al. 1993, Littlewood and Johnston 1995, Razo-Mendivil et al. 2006, Miller and Cribb 2007, Blasco-Costa et al. 2010a, Shylla et al. 2013, Herrmann et al. 2014); sequence divergence at the 28S gene as low as 0.4% (Miller and Cribb 2007, Herrmann et al. 2014) or 0.8% (Blasco-Costa et al. 2010b) is evidence of cryptic speciation. Based on the divergence of haplotypes identified in this study, we conclude that there is support for the existence of cryptic species within each of the three morphotypes (cotylomicrocercous type, *M. oregonensis* and virgulate type) containing multiple haplotypes. Pairwise sequence divergence of 0.7% - 4.0% between eight of nine cotylomicrocercous haplotypes suggests this morphotype may comprise up to eight species. For the seven haplotypes identified from *M. oregonensis* infections, sequence divergence of 1.0 – 1.3% suggests the presence of at least two unique species within Heterophyidae, plus a third species from Opisthorchiidae (6.4 – 6.7% divergence from heterophyid haplotypes). Finally, sequence divergence between virgulate haplotypes, suggests this morphotype may comprise up to five species: one species most closely related to Collyriclidae; one species most closely related to Pleurogenidae; and an additional three species within Lecithodendriidae (sequence divergence of 0.5 – 5.2%). Additional support for species delimitation could be provided by molecular analysis at a second locus.

Morphotypes differed in both the level of sequence divergence among respective haplotypes, as well as the distribution of individual haplotypes across the study region. This may reflect differences in the dispersal abilities of definitive host species. There was a relatively high level of genetic diversity within the cotylomicrocercous morphotype, potentially representing up to 8 species, and 4 of these were present at only a single site. This may be a result of the autogenic life cycle of these parasites: fish definitive hosts have different constraints on dispersal than terrestrial hosts, which can result in greater geographic isolation and higher genetic diversification (Criscione and Blouin 2004, Prugnolle et al. 2005, Louhi et al. 2010, Blasco-Costa and Poulin 2013). In contrast, within the *M. oregonensis* morphotype, there was less sequence divergence among the 6 heterophyid haplotypes, which potentially represent 2 species. These trematodes are allogenic parasites and may disperse across the landscape with their terrestrial hosts, raccoons, which could also explain the broader distribution of these haplotypes across the study region. Note, two of these 6 haplotypes were present at only a single site, but their low divergence from other haplotypes does not suggest speciation. Finally, 3 of the 4 lecithodendriid haplotypes within the virgulate morphotype potentially represent separate species, and one of these haplotypes was encountered at a single site, while the others were more broadly distributed. These are also allogenic parasites, most likely with bats as definitive hosts, so we would expect these trematodes to be broadly distributed, but not necessarily to show a high degree of speciation. It is possible, that the difference in genetic diversity we see between the 2 allogenic morphotypes is due to a greater diversity in definitive host species (i.e. multiple species of bats) among the lecithodendriid trematodes, versus a single definitive host species, raccoons, in the heterophyids.

In conclusion, we found a broad scale spatial pattern in *E. proxima* trematode communities due to regional turnover in the dominant trematode species, and this pattern was significant for both morphotype and haplotype defined communities. While this pattern was correlated with elevation, we did not find any significant relationships with other local factors, indicating that dispersal may be an important driver of metacommunity structure in this system. Additionally, we found that each morphotype potentially comprises multiple species, and that genetic diversity within morphotypes may reflect dispersal abilities related to allogenic versus autogenic life cycles. If each morphotype comprises multiple species, incorporating this information in future research could elucidate relationships that may be masked by the grouping together of morphologically similar species.

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Table 1. Physicochemical, *E. proxima* density and trematode infection data for the 20 streams within the Upper New River Basin. Stream width (W), specific conductance (TCC), total phosphorus (TP), total nitrogen (TN) and elevation (Elev). Trematode morphotypes: cotylomicrocercous type (COTYL); *Metagonimoides oregonensis* (META); virgulate and ubiquita xiphidiocercariae (XIPH); *Sanguinicola* sp. (SANG); and monostome type (MONO).

				TCC		TP	TN		Snails m^{-2}	% Infected snails					
Site ID	**Name**	**County**	**W (m)**	**(µS/cm)**	**pH**	**(ppb)**	**(ppb)**	**Elev (m)**	**± SE**	**META**	**XIPH**	**COTYL**	**SANG**	**MONO**	**TOTAL**
ADB	Adams Br.	Carroll, VA	2.24	56.33	7.57	25.13	207	735	37.5 ± 8.6	15.3	2.1	0.0	-	-	17.4
BKC	Burkes Crk.	Floyd, VA	2.73	72.20	7.50	35.63	837	823.7	4.4 ± 1.4	11.8	1.7	2.5	-	-	18.5
BVR	Beaver Crk.	Ashe, NC	4.35	99.17	7.76	25.73	957	853.3	2.3 ± 1.3	2.4	2.4	9.6	-	-	14.4
BW	Big Wilson Crk.	Grayson, VA	8.80	77.90	7.64	6.76	702	806.5	0.2 ± 0.2	5.7	5.7	18.9	-	-	35.8
CK	Crooked Crk. Trib	Carroll, VA	5.22	31.70	6.83	9.89	507	786.3	29.4 ± 6.4	2.4	4.9	13.0	-	-	22.0
CRC	Cherry Crk.	Carroll, VA	4.40	67.27	7.46	28.28	522	682.1	9.0 ± 2.1	10.2	1.5	0.7	-	3.6	16.1
CSC	Chisholm Crk.	Floyd, VA	5.37	48.70	7.30	26.03	567	811.1	17.2 ± 3.6	6.9	0.8	0.8	1.5	-	9.9
ECK	East Fork Crooked Crk.	Carroll, VA	5.68	27.23	6.64	26.03	627	840	15.0 ± 3.5	4.6	10.1	13.8	-	-	29.4
FNM	Francis Mill Crk.	Wythe, VA	4.80	152.30	7.97	26.93	477	673.3	1.1 ± 0.6	20.4	9.5	0.0	-	-	29.2
HEL	Little Helton Crk.	Ashe, NC	1.50	87.23	7.03	28.58	642	836.9	3.7 ± 1.0	3.6	4.3	7.1	-	-	15.7
HWL	Howell Crk. Trib	Floyd, VA	3.99	18.00	6.62	28.43	732	882.6	5.0 ± 1.4	18.0	1.8	0.0	1.8	-	21.6
LBR	Long Br.	Ashe, NC	1.50	99.90	6.84	74.63	3342	832.5	2.2 ± 0.9	2.4	3.2	1.6	-	-	10.3
LW	Little Wilson Crk.	Grayson, VA	3.15	112.40	7.86	29.03	1647	803.6	4.0 ± 1.1	7.0	4.9	38.0	-	-	49.3
PIN	Piney Fork	Alleghany, NC	3.49	49.77	6.32	27.98	1272	866.2	11.0 ± 4.2	4.7	2.3	2.3	-	-	14.1
FRN	Furnace Crk.	Floyd, VA	6.10	31.73	7.06	26.78	402	779.8	7.6 ± 2.3	11.4	8.6	1.0	5.7	-	25.7
ROA	Roan Crk.	Ashe, NC	6.25	36.97	6.68	25.43	432	824.2	6.9 ± 1.5	4.7	2.4	7.1	-	-	15.0
STH	Sutherland Crk.	Ashe, NC	7.00	51.13	7.46	26.33	582	952.4	4.8 ± 1.1	7.4	6.6	5.8	-	-	23.1
TOD	Todd Park Crk.	Ashe, NC	8.35	63.73	7.52	27.08	732	897.8	8.5 ± 2.1	4.6	6.1	22.9	-	-	33.6
TRP	Three Top Crk.	Ashe, NC	9.85	44.30	7.34	53.18	822	877.5	4.6 ± 1.6	7.1	4.0	9.5	-	-	22.2
WFD	West Fork Dodd Crk.	Floyd, VA	2.90	69.53	6.92	28.28	837	744.3	30.2 ± 8.0	7.9	0.8	1.6	-	-	14.2

Table 2. Larval trematodes infecting *E. proxima* as a first-intermediate host across 20 sites, including the results of BLAST searches (conducted May 5, 2016) of each haplotype identified via molecular analysis.

	Potential hosts*						**Matches to GenBank sequences**		
Morphotype of cercariae	**Second intermediate**	**Definitive**	**No. of sites**	**Prev (%)**	**No. of haplotypes**	**No. of samples**	**Species (% Identity)**	**Accension No.**	**Family(s)**
Cotylomicrocercous	Aquatic insects	Fish	17	0 - 38	1	119	*Plagiocirrus loboides* (99%)	EF523477.1	Opecoelidae
					6	45	*Plagiocirrus loboides* (98%)	EF523477.1	Opecoelidae
					2	5	*Plagiocirrus loboides* (97%)	EF523477.1	Opecoelidae
Metagonimoides oregonensis	Amphibians	Raccoons, mink	20	2 - 20	1	70	*Metagonimoides oregonensis* (99%)	JQ995473.1	Heterophyidae
					5	117	*Metagonimoides oregonensis* (98%)	JQ995473.1	Heterophyidae
	Fish	Piscivorous mammals			1	5	*Clonorchis sinensis* (97%)	JF823989.1	Opisthorchiidae
Virgulate and ubiquita xiphidiocercariae	Aquatic insects	Bats, birds	20	< 1 - 10	3	28	*Paralecithodendrium parvouterus* (97%)	AY220617.1	Lecithodendriidae
					1	7	*Lecithodendrium linstowi* (97%)	AF151919.1	Lecithodendriidae
					2	29	*Collyriclum faba* (96%)	JQ231122.1	Collyriclidae
					1	6	*Allassogonoporus amphoraeformis* (93%	AF151924.1	Pleurogenidae
					1	38	*Allassogonoporus amphoraeformis* (92%	AF151924.1	Pleurogenidae
Sanguinicola sp.	None	Fish	3	0 - 6	1	8	*Sanguinicola cf. inermis* (85%)	AY222180.1	Aporocotylidae
Monostome	None; encysts on vegetation	Birds (anseriforms)	1	0 - 4	1	5	*Notocotylus* sp. BH-2008 (98%)	EU712725.1	Notocotylidae
Immature or unknown type	variable	variable			1	1	*Echinostomatidae* sp. 1 (99%)	GU270100.1	Echinostomatidae
					1	2	*Plagiocirrus loboides* (97%)	EF523477.1	Opecoelidae
					1	5	*Collyricloides massanae* (94%)	KP682451.1	Pleurogenidae
					1	1	*Parabascus duboisi* (94%)	AY220618.1	Pleurogenidae

*Life cycles described in: Ingles 1935, Burns and Pratt 1953 (*M. oregonensis*); Hoffman et al. 1985 (*Sanguinicola* sp.); Seitner 1945, Hall 1959 (virgulate type); Schell 1985 (cotylomicrocercous and monostome types).

Table 3. Variation partitioning of community composition for morphotype and haplotype defined communities. Adjusted R^2 is the fraction of variation in community composition explained by environmental (E) or spatial (S) variables. Pure E is the variation explained by environmental variables alone, S str. E is the variation explained by spatially structured environmental variables, Pure S is the variation explained by spatial variables alone, and E + S is the total variation explained by environmental and spatial variables combined.

Community type	Dissimilarity distance	Environmental variables	Spatial variables (PCNM eigenvectors)	Fraction of variation explained (adj. R^2)						
				Total E	Total S	Pure E	S str. E	Pure S	Total E + S	Unexplained
Morphotype	Jaccard	Elevation	PCNM 1	0.085	0.243*	0	0.085	0.158*	0.243*	0.764
	Bray-Curtis	Elevation	PCNM 1	0.074	0.215*	0	0.074	0.141*	0.215*	0.796
Haplotype	Jaccard	Elevation	PCNM 1, 2, 3	0.045*	0.206*	0.009	0.036	0.17*	0.215*	0.785
	Bray-Curtis	Elevation	PCNM 1, 2, 3	0.047*	0.199*	0.007	0.04	0.159*	0.206*	0.794

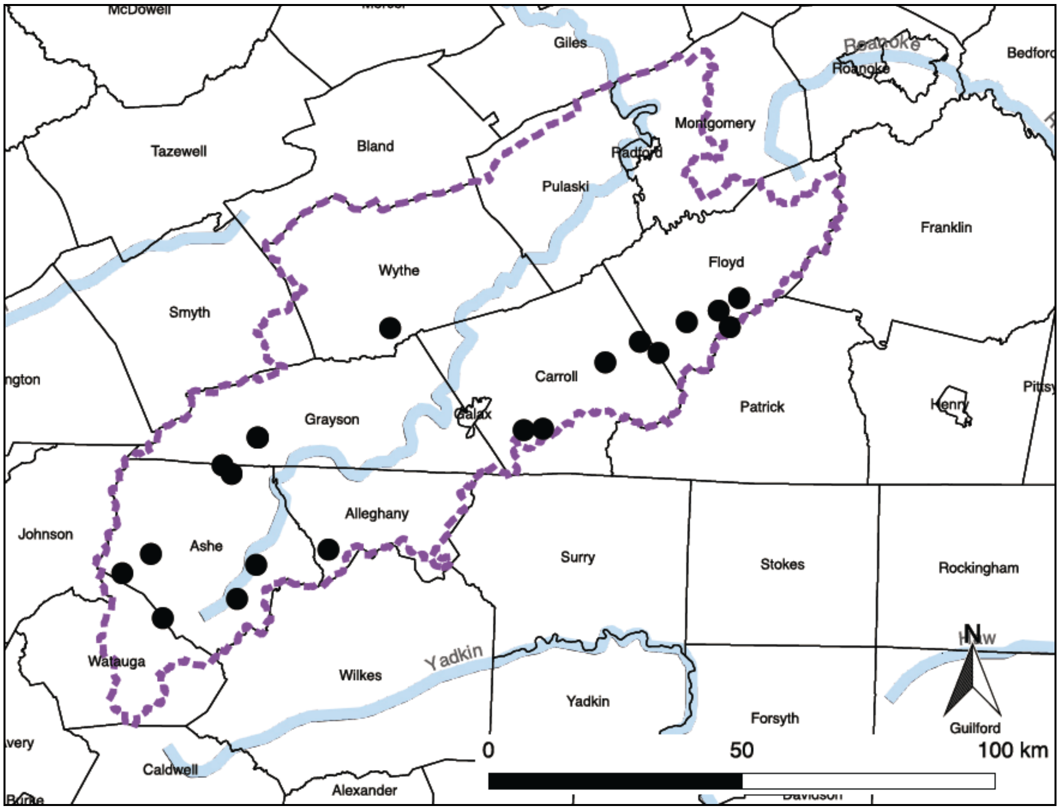

Figure 1. Twenty study sites in southwestern Virginia and northwestern North Carolina in the Upper New River Basin (demarcated by dashed purple line).

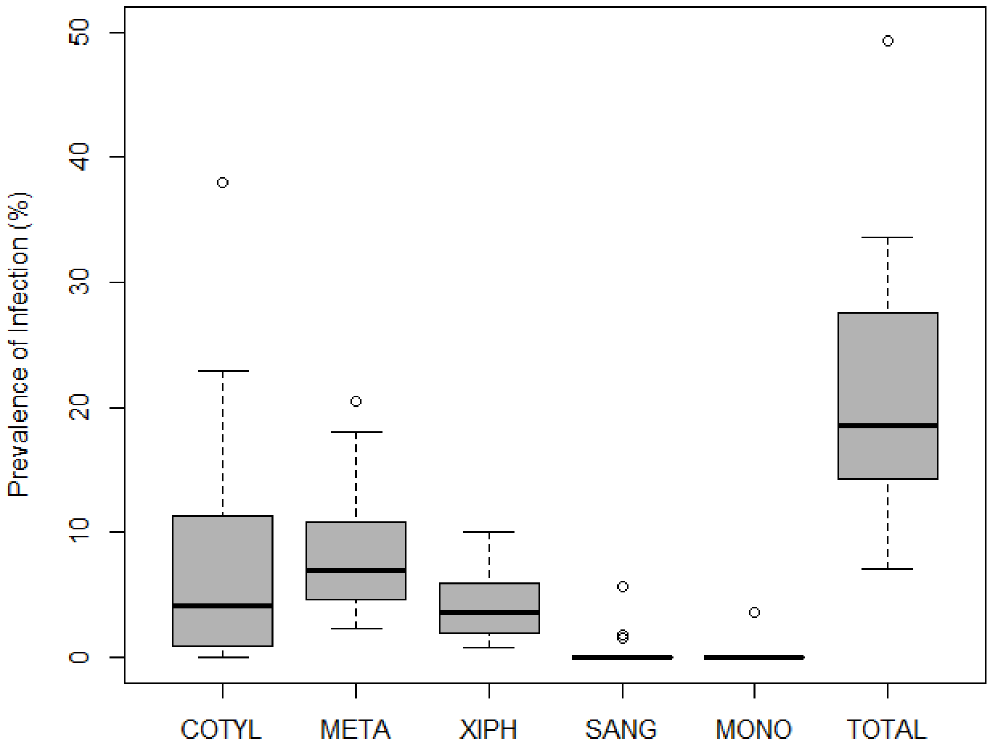

Figure 2. Median and range of prevalence of each trematode morphotype across all 20 sites**.** Whiskers represent 1.5 interquartile range. Trematode morphotypes: cotylomicrocercous type (COTYL); *Metagonimoides oregonensis* (META); virgulate and ubiquita xiphidiocercariae (XIPH); *Sanguinicola* sp. (SANG); and monostome type (MONO).

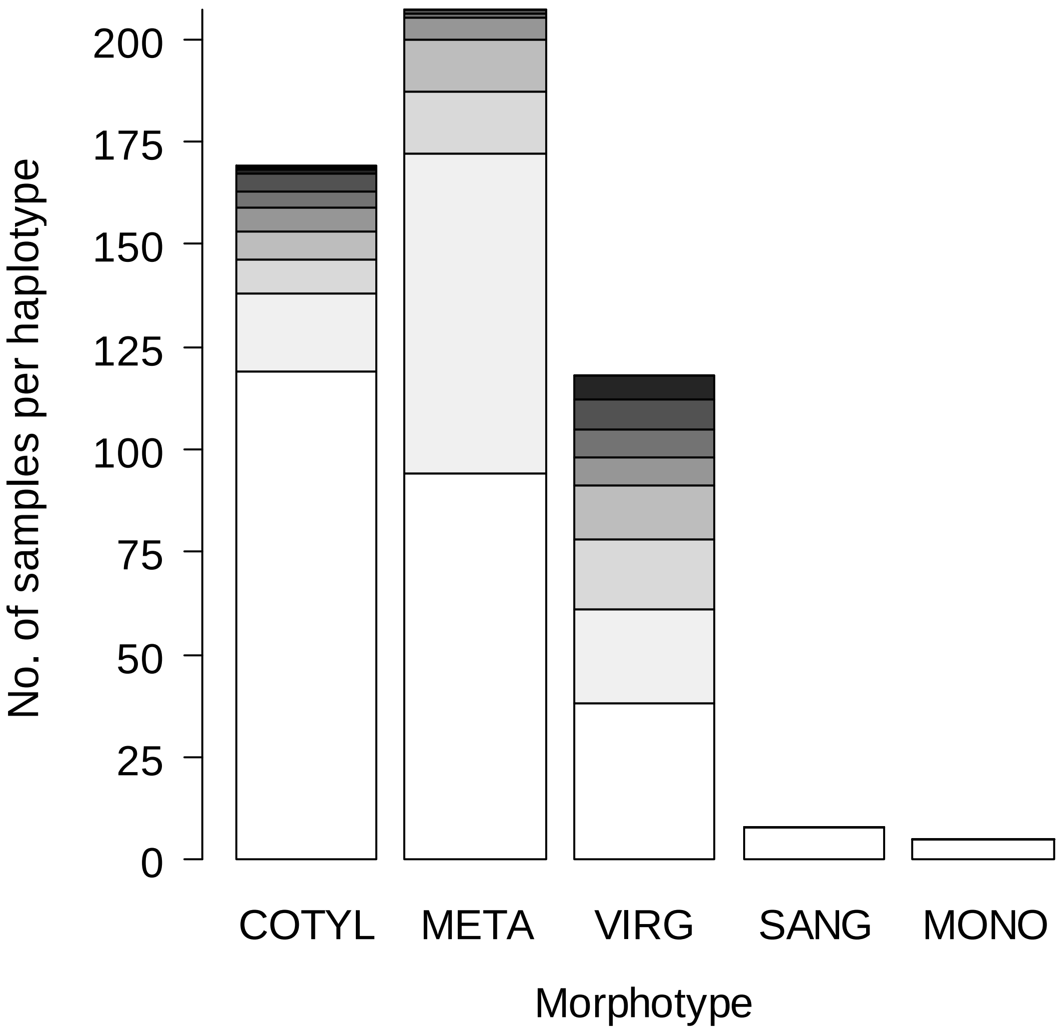

Figure 3. Number of trematode samples (total N = 491 samples) per unique haplotype within each of the five morphotypes. Abbreviations and total number of unique haplotypes identified for each morphotype are as follows: cotylomicrocercous type (COTYL; n = 9 haplotypes); *Metagonimoides oregonensis* (META; n = 7 haplotypes); virgulate and ubiquita xiphidiocercariae (XIPH; n = 8 haplotypes); *Sanguinicola* sp. (SANG; n = 1 haplotype); and monostome type (MONO; n = 1 haplotype). Different shades within bars show the proportion of samples per unique haplotype.

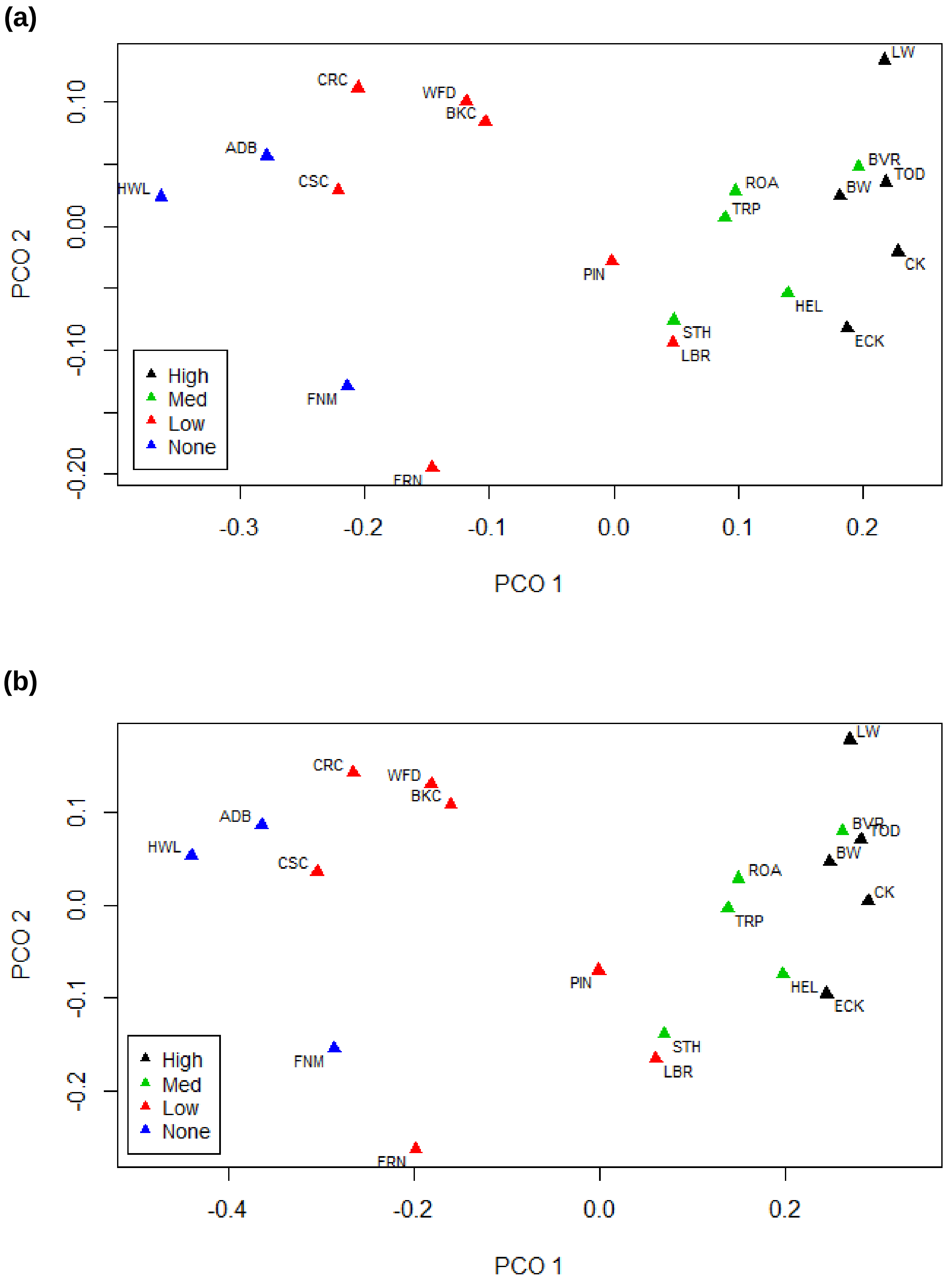

Figure 4. PCoA of morphotype defined communities based on (a) Bray-Curtis and (b) Jaccard dissimilarity matrices. Sites are labeled with abbreviations (see Table 1) and categorized by prevalence of cotylomicrocercous type infections defined as: High > 10%; Medium = 5 – 10%; and Low < 5% of snails infected. "None" indicates absence of cotylomicrocercous type.

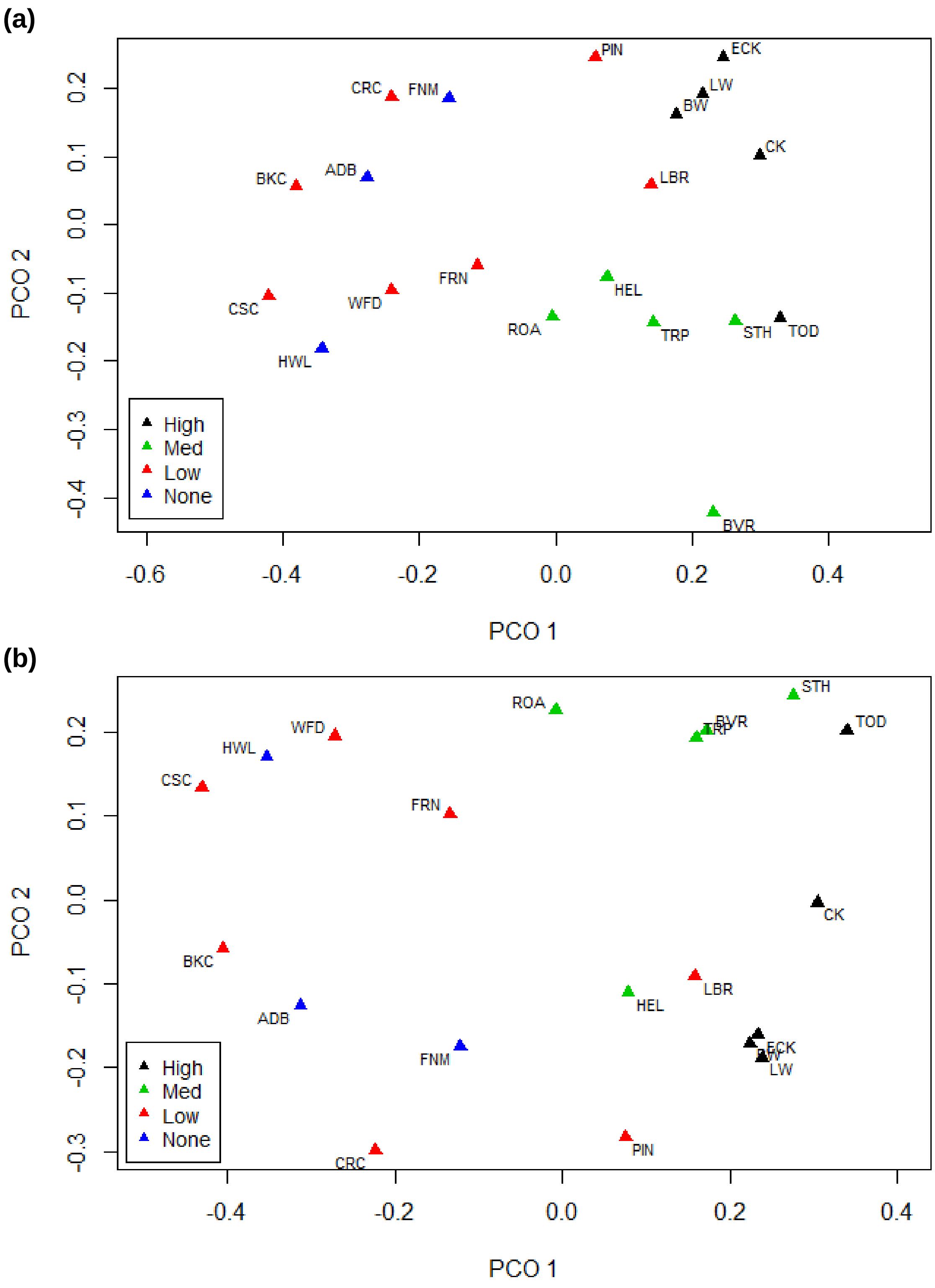

Figure 5. PCoA of haplotype defined communities based on (a) Bray-Curtis and (b) Jaccard dissimilarity matrices. Sites are labeled with abbreviations (see Table 1) and categorized by prevalence of cotylomicrocercous type infections defined as: High > 10%; Medium = 5 – 10%; and Low < 5% of snails infected. “None” indicates absence of cotylomicrocercous type.

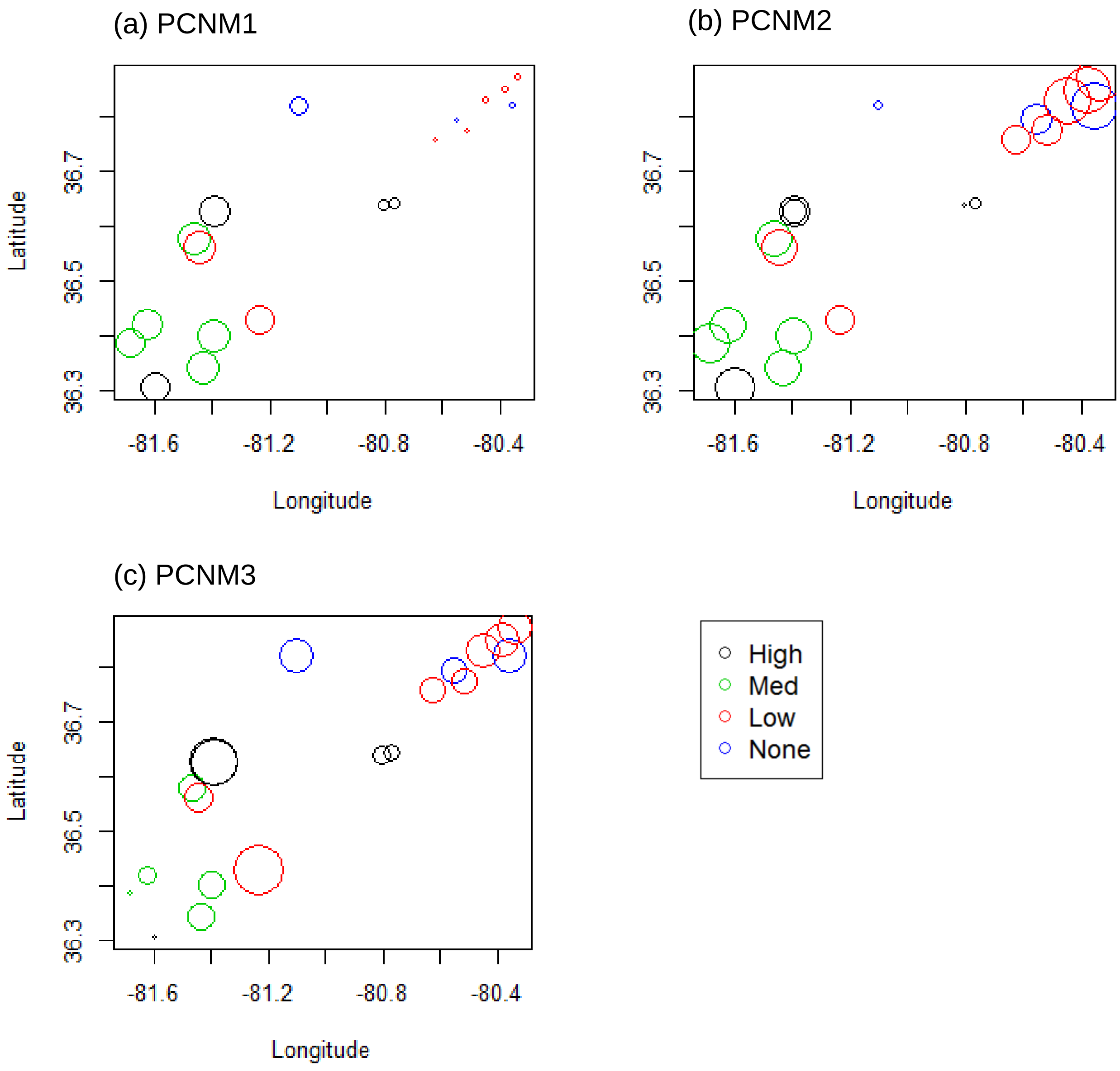

Figure 6. Maps representing significant spatial variables (eigenvectors calculated using principal coordinate analysis of neighbor matrices, PCNM) used in variation partitioning. Point size is proportional to the absolute value of the spatial filter at the site. Color corresponds to prevalence of cotylomicrocercous type infections at that site, categorized as: High > 10%; Medium = 5 – 10%; and Low < 5% of snails infected. Note that the scale of the spatial gradient described by a PCNM filter decreases with increasing PCNM number.

Chapter 4: Upstream gradient of trematode infection in snails: environmental heterogeneity within a stream network counters the effects of downstream drift

Abstract

Examining the role of dispersal in community structure has become a focal point in community ecology as metacommunity concepts have advanced our understanding of the importance of both local and larger scale processes. Lotic systems are central to the dispersal of many pathogens, including many species of freshwater trematodes. Digenean trematodes are parasitic flatworms with multi-host, complex life cycles. Patterns in the distribution and diversity of trematodes are often driven by the dispersal abilities of hosts. Here, we examined the influence of site-level factors and dispersal on shaping spatial patterns of larval trematode communities within a dendritic stream network. We characterized trematode component communities of the first-intermediate host *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* (Gastropoda: Pleuroceridae) at eight different locations, including headwater and mainstem sites, within a single stream network in the Upper New River watershed. We identified four types of trematodes based on morphological examination, which subsequent molecular identification indicated may actually be 7 unique species. Due to downstream drift of free-living parasite stages and infected hosts, we expected to find an increasing downstream gradient of trematode prevalence. Instead, we found the opposite pattern: overall trematode prevalence increased from mainstem to headwater sites revealing an increasing upstream gradient of infection. Trematode prevalence was positively related to snail density and elevation, and negatively related to stream depth. The observed upstream gradient of infection may be due to relationships with site-level factors. Additionally, we found diversity in headwater streams to be nested subsets of mainstem communities, which could be due to downstream drift within the dendritic network structure. Our results suggest that species-environment relationships may determine metacommunity structure of parasites within stream networks.

Introduction

Dispersal has emerged as a key topic in community ecology in recent decades as metacommunity concepts have advanced our knowledge of the importance of both local and regional processes in structuring communities (Leibold et al. 2004; Holyoak et al. 2005). The four main metacommunity paradigms (species sorting, mass effects, patch dynamics and the neutral model) vary in the relative importance ascribed to species traits, environmental heterogeneity (i.e. local scale) and dispersal (i.e. regional scale) in structuring communities (Logue et al. 2011). The relative importance of processes that structure communities is likely to vary across habitat and organism type, as patterns of dispersal are determined in part by physical factors, such as landscape features and habitat connectivity, as well as the particular dispersal abilities of individual species (Cottenie 2005).

The majority of the research on metacommunity dynamics, and the role of dispersal in shaping community structure, has focused on systems with discrete habitat patches, such as ponds or forest fragments (see review by Logue et al. 2011). In contrast, stream and river networks represent a very different spatial structure than distinct patches due to dendritic connectivity and single direction flow. Indeed, stream ecologists have long considered the importance of flow and its impact on dispersal in terms of the drift paradox, the concept that populations of invertebrates can be maintained at upstream sites despite the continual downstream movement of individuals (Muller 1982). However, it is only recently that the broader metacommunity framework has started to be applied in lotic systems (Heino 2005, Muneepeerakul et al. 2008, Heino and Mykrä 2008, Brown and Swan 2010, Patrick and Swan 2011, Heino et al. 2012, Grönroos et al. 2013, Widder et al. 2014).

Lotic systems are central to the transmission of many pathogens, including many species of freshwater trematodes. Trematodes (Phylum: Platyhelminthes, Subclass: Digenea), also known as flukes or parasitic flatworms, are obligate endoparasites of mollusks and all classes of vertebrates (Bush et al. 2001). They have remarkably diverse, complex life cycles, usually involving a series of three hosts, although there is substantial variation in life histories. Adult trematodes sexually reproduce in vertebrate definitive hosts and release eggs in the host feces. Eggs hatch into larvae that infect mollusks as first-intermediate hosts and reproduce asexually to generate free-swimming cercariae. Cercariae leave the mollusk, penetrate and encyst inside an aquatic invertebrate or vertebrate second-intermediate host. The life cycle is completed when an infected second-intermediate host is consumed by the definitive host (Bush et al. 2001). If all hosts are aquatic organisms (e.g. snails, aquatic invertebrates and definitive host fish), a trematode life cycle may be entirely aquatic (autogenic life cycle; Esch et al. 1988); however, if the definitive host is a mammal or bird, trematodes may disperse across the landscape with their hosts (allogenic life cycle).

For most parasitic organisms, including trematodes, the movement of hosts is greater than that of any free-living parasite stages, and thus hosts likely contribute to most longer-distance dispersal (Bush et al. 2001; Prugnolle et al. 2005; Louhi et al. 2010). Because intermediate hosts are often less mobile than definitive hosts, the structure of larval trematode communities at local sites is often correlated with the distribution and abundance of definitive hosts (Fernandez and Esch 1991, Snyder and Esch 1993, Mouritsen et al. 1997, Granovitch et al. 2000, Smith 2001, Skirnisson et al. 2004, Hechinger and Lafferty 2005, Byers et al. 2008, Thieltges et al. 2009, 2013, Blasco-Costa et al. 2013, Levakin et al. 2013). Therefore, there may be differences in the dispersal patterns of trematode species, depending on whether they have autogenic or allogenic life cycles (Blasco-Costa et al. 2012; Grönroos et al. 2013).

In this study, we examined the effects of dendritic network structure and downstream drift on the metacommunity dynamics of larval trematodes within a stream network. The snail, *Elimia* (= *Oxytrema* = *Goniobasis*) *proxima* (Gastropoda: Pleuroceridae), a common, native inhabitant of Appalachian headwater streams, is the first-intermediate host of multiple species of trematodes including some with autogenic life cycles and some with allogenic life cycles. Here, we characterized *E. proxima* trematode communities at eight different locations, including headwater and mainstem sites, within a single stream network. We hypothesized that there would be an increasing downstream gradient of trematode prevalence and diversity due to the continuous movement of free-living parasite stages and infected hosts downstream. Furthermore, we expected allogenic trematodes to have a less pronounced downstream gradient of prevalence and diversity than autogenic trematodes due to greater definitive host mobility of mammals or birds across the landscape. Additionally, we predicted that diversity in headwater streams would be nested subsets of mainstem communities due to the effects of downstream drift within a dendritic network structure.

Materials and Methods

Study sites

During summer, 2014, we examined first-intermediate host trematode infection of *Elimia proxima* snails at 8 sites within the Burks Fork branch of the Big Reed Island Creek drainage in Carroll County, VA (located in the Upper New River Basin). We sampled 2 sites in the Burks Fork mainstem and 6 headwater sites in 3 different headwaters (Table 1, Fig. 1). Mainstem (third order) sites were open canopy with predominately cobble substrate and ranged in mean width from 9.8 to 12.4 m. Mainstem sites ranged in elevation from 684 to 705 m and were separated by 5.5 km in-stream distance (3.4 km Euclidean distance). Headwater sites (first or second order streams) were closed canopy with predominately cobble substrate and ranged in mean width from 1.3 to 3.2 m. Headwater sites ranged in elevation from 726 to 876 m, and were separated from the first mainstem site (farthest downstream) by a minimum of 2.6 km in-stream distance (2.1 km Euclidean distance) and a maximum of 13 km in-stream distance (6.1 km Euclidean distance).

Snail sampling and environmental measurements

At each site, we established a 50 m study reach, quantified snail (*E. proxima*) density with quadrat sampling at 30 points, and collected a random sample of ~115 snails for laboratory dissection (total N = 946 snails). We used handheld meters to measure pH, specific conductance, water temperature and dissolved oxygen (YSI Models 63 and 550A, YSI, Inc., Yellow Springs, OH), and estimated stream discharge using a flow meter (Flo-mate 2000, Marsh-McBirney, Inc., Frederick, MD) and cross-sectional measurements.

Trematode collection

In the laboratory, we measured the wet mass of each snail after removing any sediment attached to the shell. We dissected each snail and used a stereo microscope to screen gonadal tissue and the digestive tract for larval trematodes (sporocysts, rediae and cercariae). Trematodes were then examined with a compound microscope and identified as one of four morphotypes based on cercarial morphology. Morphotypes included: (1) *Metagonimoides oregonensis* (Cable 1938; Lang and Gleason 1967; Belden et al. 2012); (2) small and (3) large virgulate xiphidiocercariae (Hall 1959, 1960; Lang 1968); and (4) cotylomicrocercous (Cable 1938, 1939; Barger and Esch 2000) (Table 2). To confirm visual identification, we collected larval trematode samples in 95% ethanol (stored at -20^{o}C) from each infected snail for molecular identification.

Molecular identification

For a subset of samples from each site, we sequenced an ~1,400 base pair region (D1 - D3) of the 28S large subunit rRNA gene, following modifications of methods described in Olson et al. (2003). For DNA extraction, we selected a single sporocyst or redia from each infection. Prior to extraction, trematode tissue was double rinsed in Milli-Q water to remove ethanol and transferred in 2 µl of Milli-Q water into 0.2 ml tubes with 98 µl of 6.7% Chelex and 1% Proteinase K solution. Tubes were then incubated at 56°C for 2 hours, followed by an 8 minute boil at 100°C. We amplified DNA via PCR with forward primer LSU-5 and reverse primer 1500R (Olson et al. 2003). Each 25 µl reaction contained: 3 µl of DNA; 0.4 µM of each primer; and 1X GoTaq G2 Hot Start Colorless Master Mix (Promega Corp., Madison, WI) containing GoTaq Hot Start DNA polymerase, reaction buffer at pH 8.5, 400 µM of each dNTP, and 4mM $MgCl_2$. PCR parameters included: initial denaturation (3 minutes at 95°C); 40 amplification cycles (45 seconds at 94°C, 30 seconds at 54°C and 2 minutes at 72°C); and final extension (7 minutes at 74°C). Amplicons were visualized using gel electrophoresis and cleaned with QIAquick PCR Purification kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. Purified samples were sequenced with the same primers used for PCR (LSU-5 and 1500R) at the Biocomplexity Institute of Virginia Tech (Blacksburg, VA). Sequences were aligned in Geneious v. 8.0.4 (Biomatters Ltd., Auckland, New Zealand) and matched to haplotypes established from a previous survey of regional haplotype diversity (Zemmer unpublished doctoral dissertation, Chapter 3). Haplotypes were distinguished by the presence of any single-nucleotide polymorphism and were taxonomically identified via BLAST search in GenBank.

Statistical analysis

To examine species-environment relationships and to test for an increasing downstream gradient of trematode infection, we used generalized linear models (GLMs) with binomial error structures and logit-link functions to model morphotype prevalence. Total trematode prevalence, as well as the prevalence of each morphotype, was modelled separately to determine if infection patterns differed for allogenic trematodes (*M. oregonensis*, small and large virgulate types) versus autogenic trematodes (cotylomicrocercous type). Predictor variables for species-environment relationship models included *E. proxima* density, pH, specific conductance, dissolved oxygen, stream depth and site elevation. For GLM selection, we chose best subsets of full models based on AICc or QAICc. We used the in-stream distance from the mainstem site located farthest downstream (MN1) as the predictor variable in models testing for a downstream gradient of infection.

To examine relationships between site-level factors and variation in trematode community composition, we conducted redundancy analysis (RDA) on Hellinger transformed morphotype prevalence. Site-level factors included *E. proxima* density and all abiotic variables measured. We used forward stepwise model selection based on adjusted R^2 values. To assess the relationship between spatial distribution and variation in trematode community composition, we conducted an additional RDA with the predictor variables Euclidean and in-stream distance from the mainstem site located farthest downstream (MN1). All analyses were conducted in R v. 3.2.4 using the *AICcmodavg* and *vegan* packages.

Results

Trematode prevalence

Out of 946 *E. proxima* examined, 284 snails were infected. Total prevalence of infection ranged from 8.9% (MN1) to 55.6 % of snails infected (ADB3; Table 1, Fig. 2). *M. oregonensis* was present at all 8 sites and ranged in prevalence from 2.4% (MN1) to 33.9% (BB2). Small virgulate infections were present at 6 of 8 sites and comprised the highest prevalence of infection at a single site at 42.1% (AB3). Large virgulate infections were encountered at 6 of 8 sites with a maximum prevalence of 5.8% (BB2). Cotylomicrocercous type infections were present at 5 of 8 sites with a maximum prevalence of 15.3% (BB1).

Molecular identification

We obtained partial 28S rRNA gene sequences for 227 larval trematodes (out of 284 total infections), from which we identified 7 unique haplotypes belonging to 4 families (Table 2). For *M. oregonensis* infections, we identified 2 haplotypes, both of which most closely matched (max identity 98 – 99%) *Metagonimoides oregonensis* (Heterophyidae). The two *M. oregonensis* haplotypes varied at 14 sites across a 1,293 bp fragment (1.08% divergence). From small virgulate infections, we identified two haplotypes, both of which most closely matched (max identity 96 – 97%) *Paralecithodendrium parvouterus* (Lecithodendriidae). The two small virgulate haplotypes varied at 19 sites across a 1,142 bp fragment (1.66% divergence). We identified two additional haplotypes from large virgulate infections that most closely matched two different species, (max identity 94%) *Collyricloides massanae* and (max identity 92%) *Allassogonoporus amphoraeformis,* both within the family Pleurogenidae. For cotylomicrocercous type infections, we identified a single haplotype matched (max identity 99%) to *Plagiocirrus loboides* (Opecoelidae).

Snail density and trematode-environment relationships

As expected, elevation decreased from headwater to mainstem sites, while stream width, depth and discharge all increased (Table 1). Dissolved oxygen at all sites was at or near saturation and did not differ appreciably across sites. Mean snail density at sites ranged from 0.8 (± 0.4) snails/m^2 at mainstem site MN1 up to 46.1 (± 8.0) snails/m^2 at headwater site BB2 (Table 1). Snail density covaried positively with elevation and negatively with stream width, depth, and discharge. Snail density was most strongly correlated with stream width (adj. $R^2 = 0.92$, p= 0.0001, Fig. 3).

Total prevalence of infection was not significantly related to snail density. The optimal GLM of total prevalence of infection included only elevation as an explanatory variable (β=0.012, SE=0.002, $p < 0.001$; Fig. 4). However, total prevalence of infection was also negatively related to pH (β=-1.264, SE=0.459, p=0.04), conductivity (β=-0.027, SE=0.004, p=0.002) and mean maximum depth (β=-0.093, SE=0.013, $p < 0.001$), but these relationships did not remain significant when included in a GLM with elevation as an independent variable.

The prevalence of *M. oregonensis* was positively related to snail density (β=0.034, SE=0.009, p=0.01; Fig. 4) and negatively related to mean maximum depth (β= -0.09, SE=0.033, p=0.036; Fig. 4); however, neither of these relationships remained significant when modeled with the other explanatory variable. Small virgulate prevalence was positively related to elevation (β= 0.015, SE=0.006, p=0.009) and negatively related to conductivity (β= -0.05, SE=0.026, p=0.035). For both the large virgulate and cotylomicrocercous types, there were no significant relationships with snail density or any of the environmental factors measured.

In-stream infection gradient and variation in community composition

Total prevalence of infection decreased from headwater to mainstem sites (Table 1), and there was a significant positive relationship between total prevalence of infection and in-stream distance to mainstem (β= 0.185, SE=0.033, p=0.001). The same pattern was observed qualitatively for both *M. oregonensis* and small virgulate infections, but there were no significant relationships with in-stream distance for any of the individual morphotypes.

Trematode community composition also varied across sites. The trematode communities of the headwater sites located farthest upstream (AB3 and BB2), as well as another headwater site (HD1), were dominated by either *M. oregonensis* or small virgulate infections, whereas cotylomicrocercous infections were completely absent. Cotylomicrocercous infections were most prevalent at sites with intermediate depth, 6.4 to 7.7 cm (BB1 and AB2), and comprised the majority of infections at the mainstem site located farthest downstream (MN1). In RDA, elevation was the only environmental variable that significantly explained variation in trematode communities (adj. $R^2 = 0.359$, $p = 0.022$), and neither spatial variable (Euclidean or in-stream distance) was significant.

Discussion

Trematode diversity

Because larval stages often lack distinguishing morphological features, visual identification of larval trematodes can be difficult. Previous studies of larval trematodes have revealed that cryptic (morphologically indistinguishable) species diversity can obscure host specificity and life cycles, as well as patterns of spatial distribution (Donald et al. 2004; Miura et al. 2005; Leung et al. 2009; Detwiler et al. 2010; Locke et al. 2010). These studies highlight the utility of molecular identification in studies of larval trematodes. For the large subunit rRNA (28S) gene, sequence divergence as low as 0.4 to 0.8 % (Miller and Cribb 2007, Blasco-Costa et al. 2010, Herrmann et al. 2014) is evidence of cryptic speciation. In addition to two unique species matches within the large virgulate haplotype, sequence divergence between the two *M. oregonensis* haplotypes (1.08%), as well as the two small virgulate haplotypes (1.66%), suggests cryptic speciation in these groups. If these are unique species, as our results suggest, the *E. proxima* component community in this stream network contains at least 7 unique species of trematodes. In a previous regional survey of *E. proxima* that included a site within this stream network, we identified 19 potentially unique species of trematodes (Zemmer, unpublished doctoral dissertation, Ch. 3). Six of the seven haplotypes identified in this stream network were previously identified in the regional survey, and we discovered one new small virgulate haplotype in this study.

Trematode relationships with local factors

We found relationships between trematode infection and site-level factors for two of the four trematode types. *Metagonimoides oregonensis* was positively related to snail density and negatively related to stream depth. While a positive relationship between host density and directly transmitted parasites is often expected (Arneberg et al. 1998), for complex life cycle parasites, such as trematodes, this relationship is often less clear. Previous research in freshwater systems has found positive (Voutilainen et al. 2009), negative (Puurtinen et al. 2004), or no association (Lagrue and Poulin 2008; Ciparis et al. 2013) between trematode infection in snails and snail density. For *M. oregonensis,* a positive relationship with snail density suggests that other factors affecting prevalence are not limiting snail infection. Namely, that egg input from raccoon definitive hosts is high, amphibian second-intermediate hosts are abundant, and that stream flow is conducive to transmission at both the miracidial and cercarial stages. All of these factors are also likely to decrease with increasing stream depth. For the small virgulate morphotype, prevalence was negatively related to conductivity, which may be due to a reduction in aquatic insect second-intermediate hosts as stream order and conductivity increase. Small virgulate prevalence was also positively related to elevation, but this relationship is more difficult to interpret, because many environmental factors (e.g. land use, canopy cover, water temperature, stream flow patterns) covary with elevation.

These results differ from the results of our regional survey (Zemmer, unpublished doctoral dissertation, Ch. 3), in which we did not find any relationships between environmental variables and trematode prevalence. However, for both studies, elevation was the only factor to explain variation in trematode communities. It remains unclear whether the relationship with elevation is the result of aggregated environmental factors that we cannot isolate and/or that were not measured.

Decreasing downstream gradient of infection and shifts in community composition

We did not find an increasing downstream gradient of infection prevalence, as hypothesized. Instead we observed the opposite pattern, with trematode prevalence at its highest in the headwater sites farthest upstream (AB3 and BB2) and decreasing with in-stream distance to mainstem site 1 (MN1). This pattern was significant only for total prevalence of infection, but we observed this same pattern for *M. oregonensis* and small virgulate infections. Cotylomicrocercous infections exhibited a less linear pattern of distribution. Cotylomicrocercous trematodes were not present at the headwater sites farthest upstream (AB3 and BB2), presumably because the stream depth was too low for fish definitive hosts. These parasites reached highest prevalence at intermediate headwater sights (AB2 and BB1) and remained present in mainstem sites. This suggests that sites with intermediate stream depth are the sites of greatest overlap between the aquatic insect second-intermediate hosts and fish definitive hosts for this parasite. The prevalence of large virgulate infections was generally low, and we did not observe a clear spatial pattern in the prevalence of this type within the network. While we did not observe an increasing downstream gradient of overall infection as predicted, we did find that diversity in headwater streams consisted of nested subsets of mainstem communities. This could be due to the effects of downstream drift if *E. proxima* found inhabiting mainstem sites are from source populations upstream.

In conclusion, we found a decreasing downstream gradient of infection from headwaters to mainstem. In addition, we found several significant trematode-environment relationships that indicate heterogeneity in site-level factors is driving the metacommunity structure within the stream network. Our observations appear to most closely fit the species sorting paradigm, but mass effects may also be important, particularly in the structure of trematode communities in mainstem sites.

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Table 1. Physicochemical and trematode infection data for 8 study sites in Big Reed Island Creek drainage in Carroll Co., VA, sampled during summer, 2014. Mean stream width (W), mean maximum depth (D), discharge (Q), specific conductance (TCC), dissolved oxygen (DO). *Metagonimoides oregonensis* (META), small virgulate morphotype (SVIRG), large virgulate morphotype (LVIRG), and cotylomicrocercous morphotype (COTYL).

										% Infected snails				
Site ID	**Site type**	**W (m)**	**D (cm)**	**Q (m^3/s)**	**TCC (µS/cm)**	**pH**	**DO (mg/L)**	**Elev (m)**	**Snails m^{-2} ± SE**	**META**	**SVIRG**	**LVIRG**	**COTYL**	**TOTAL**
MN1	Mainstem	9.8	22.0	1.23	56.3	7.17	9.2	684	0.8 ± 0.4	2.4	0.0	0.8	4.9	8.9
MN2	Mainstem	12.4	17.7	1.03	55.2	7.50	8.4	705	1.1 ± 0.6	12.4	1.8	0.0	2.7	17.7
HD1	Headwater 1	2.1	4.7	0.00	92.2	7.66	8.7	752	8.5 ± 1.9	15.8	0.0	0.8	0.0	16.7
AB1	Headwater 2	2.2	11.5	0.06	57.4	7.30	9.2	726	18.3 ± 2.9	13.2	1.8	0.9	3.5	18.4
AB2	Headwater 2	2.2	6.4	0.01	47.7	6.99	8.9	799	24.7 ± 2.6	15.3	9.9	1.8	13.5	40.5
AB3	Headwater 2	2.4	2.4	0.02	29.2	6.62	9.3	876	11.4 ± 1.6	19.0	42.1	0.0	0.0	55.6
BB1	Headwater 3	3.2	7.7	0.04	57.8	7.33	8.6	751	7.7 ± 1.3	11.9	2.5	2.5	15.3	31.4
BB2	Headwater 3	1.3	3.5	0.01	45.6	7.16	8.9	829	46.1 ± 8	33.9	13.2	5.8	0.0	49.6

Table 2. Larval trematodes infecting *E. proxima* as a first-intermediate host, including the results of BLAST searches (conducted May 5, 2016) of each haplotype identified via molecular analysis.

	Potential hosts*					Matches to GenBank sequences		
Morphotype of cercariae	**Second intermediate**	**Definitive**	**No. of sites**	**Prev (%)**	**No. of haplotypes**	**Species (Max Identity %)**	**Accension No.**	**Family(s)**
Metagonimoides oregonensis	Amphibians	Raccoons, mink	8	2.4 - 33.9	2	*Metagonimoides oregonensis* (98 - 99)	JQ995473.1	Heterophyidae
Small virgulate	Aquatic insects	Bats	7	0 - 42.1	2	*Paralecithodendrium parvouterus* (96 - 97)	AY220617.1	Lecithodendriidae
Large virgulate	Aquatic insects	Birds, rodents	6	0 - 5.8	1	*Collyricloides massanae* (94)	KP682451.1	Pleurogenidae
	Aquatic insects	Bats			1	*Allassogonoporus amphoraeformis* (92)	AF151924.1	Pleurogenidae
Cotylomicrocercous	Aquatic insects	Fish	5	0 - 15.3	1	*Plagiocirrus loboides* (99)	EF523477.1	Opecoelidae

*Life cycles described in: Ingles 1935, Burns and Pratt 1953 (*M. oregonensis*); Hoffman et al. 1985 (*Sanguinicola* sp.); Seitner 1945, Hall 1959 (virgulate type); Schell 1985 (cotylomicrocercous and monostome types).

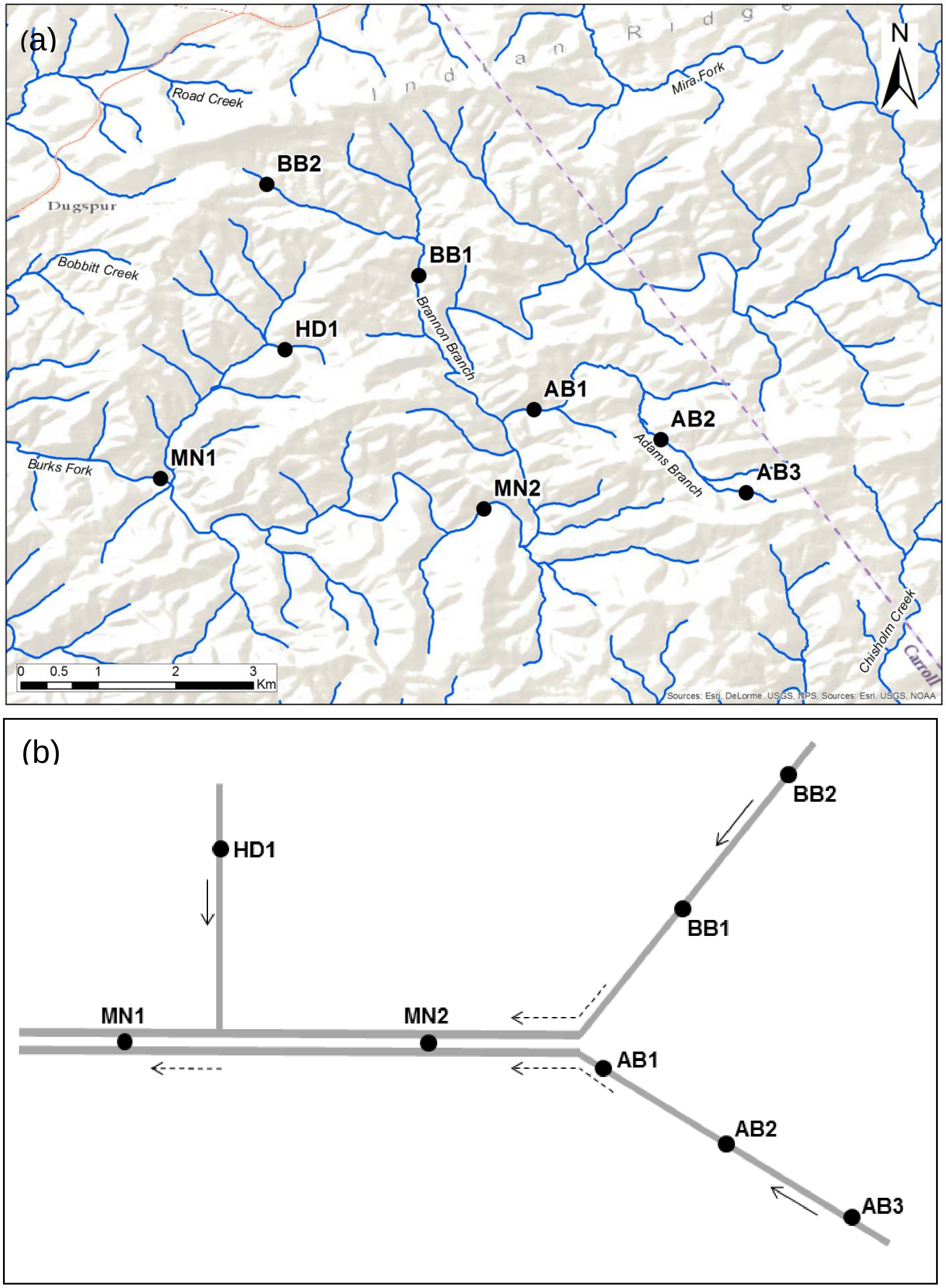


Figure 1. (a) Eight study sites in the Big Reed Island Creek drainage in Carroll Co., VA, and (b) simplified stream network diagram indicating relative position of streams within network configuration (arrows indicate direction of flow). Mainstem sites (MN) and headwater sites (HD, AB, BB).

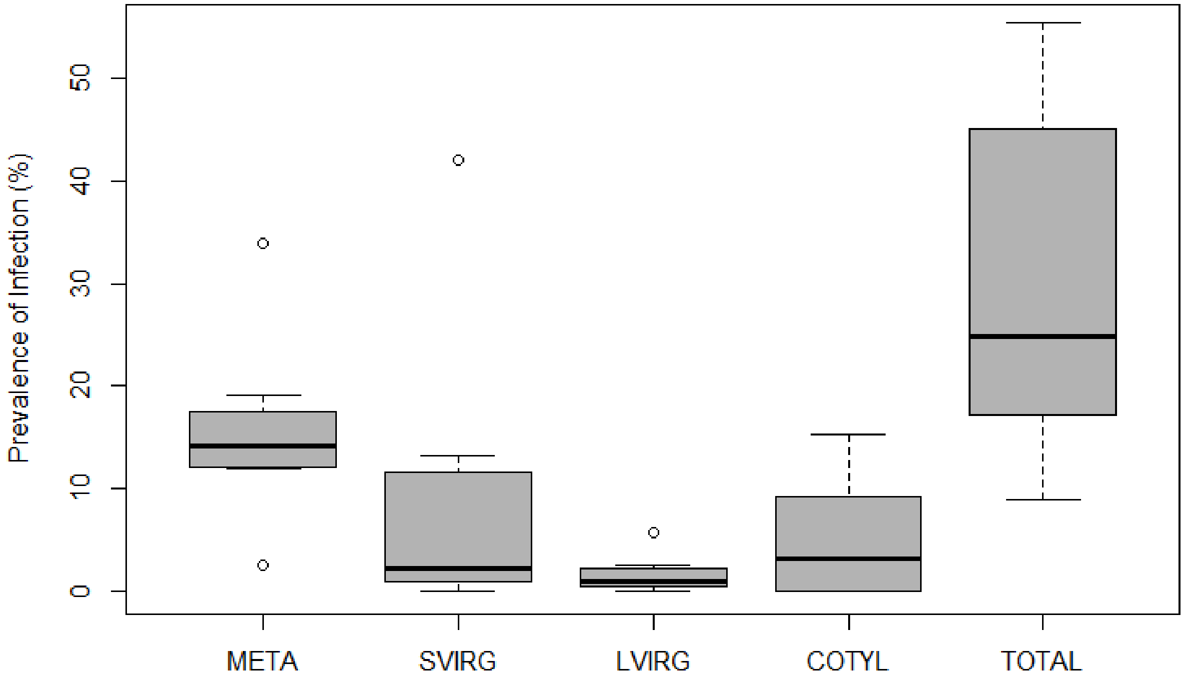


Figure 2. Median and range of prevalence for each trematode type across all sites**.** Whiskers represent 1.5 interquartile range. *Metagonimoides oregonensis* (META), small virgulate (SVIRG), large virgulate (LVIRG) and cotylomicrocercous type (COTYL).

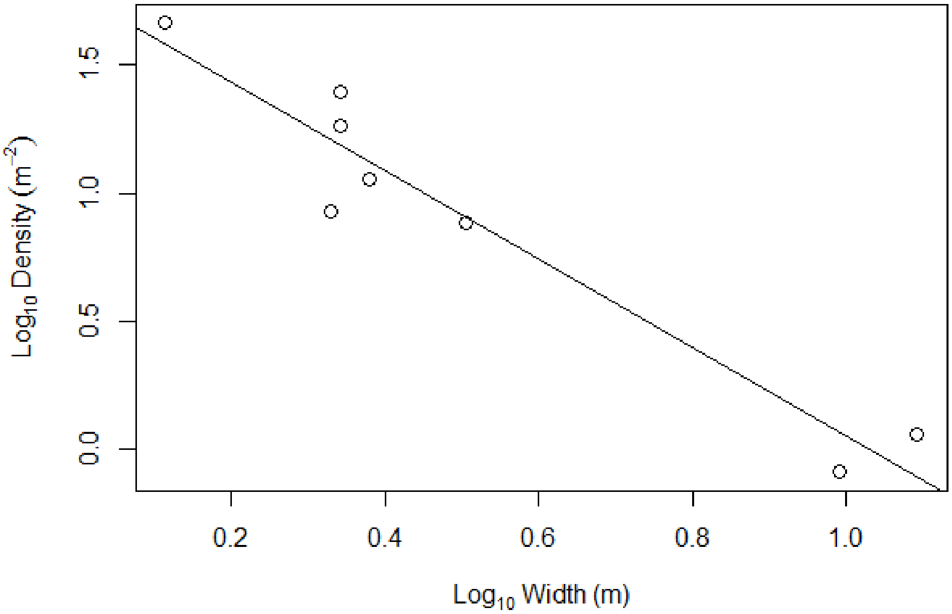
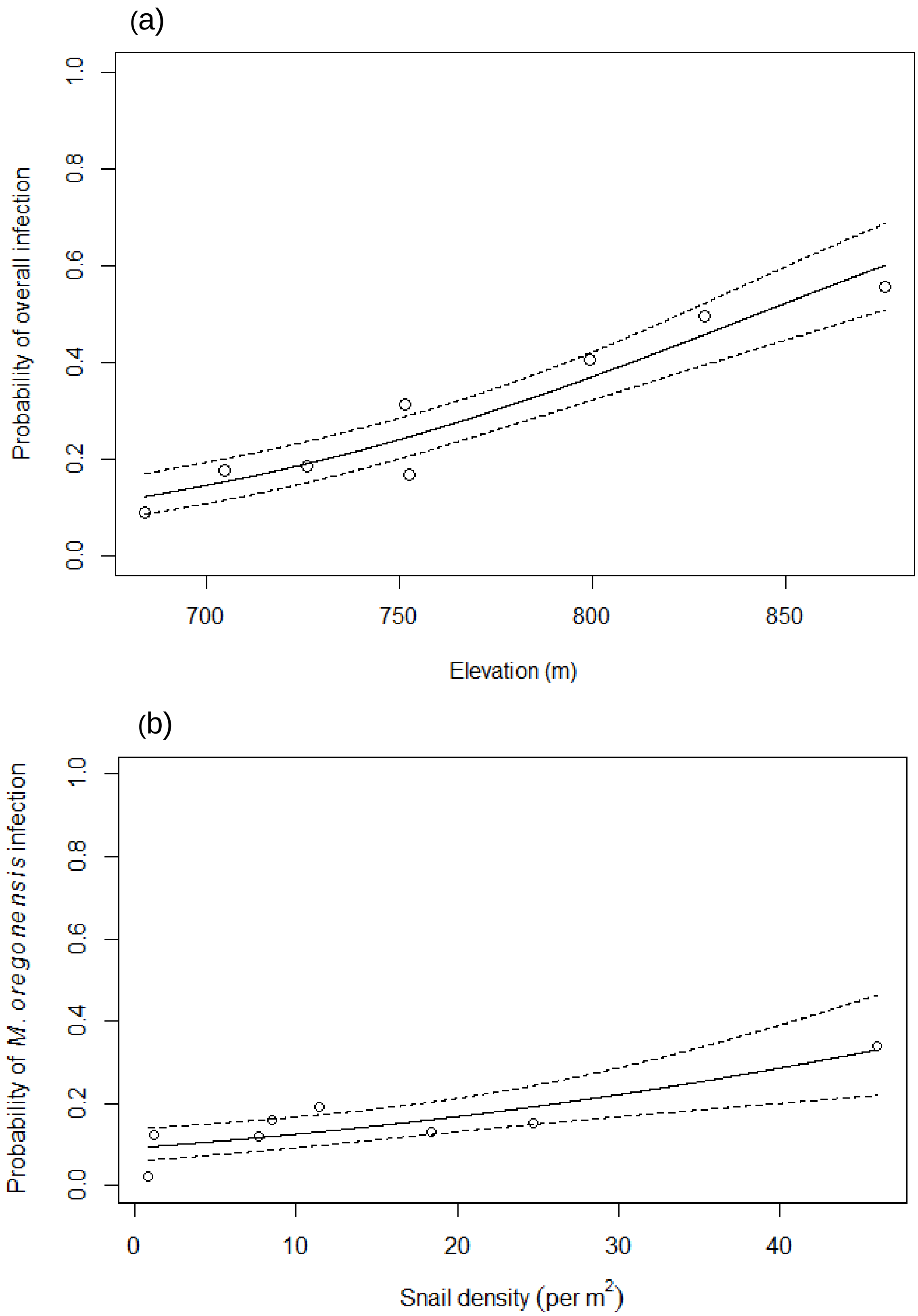


Figure 3. Relationship between the mean snail density and mean stream width at each site.


(a)
Probability of overall infection
0.0
0.2
0.4
0.6
0.8
1.0
700
750
800
850
Elevation (m)
(b)
Probability of *M. oregonensis* infection
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Snail density (per m^2)

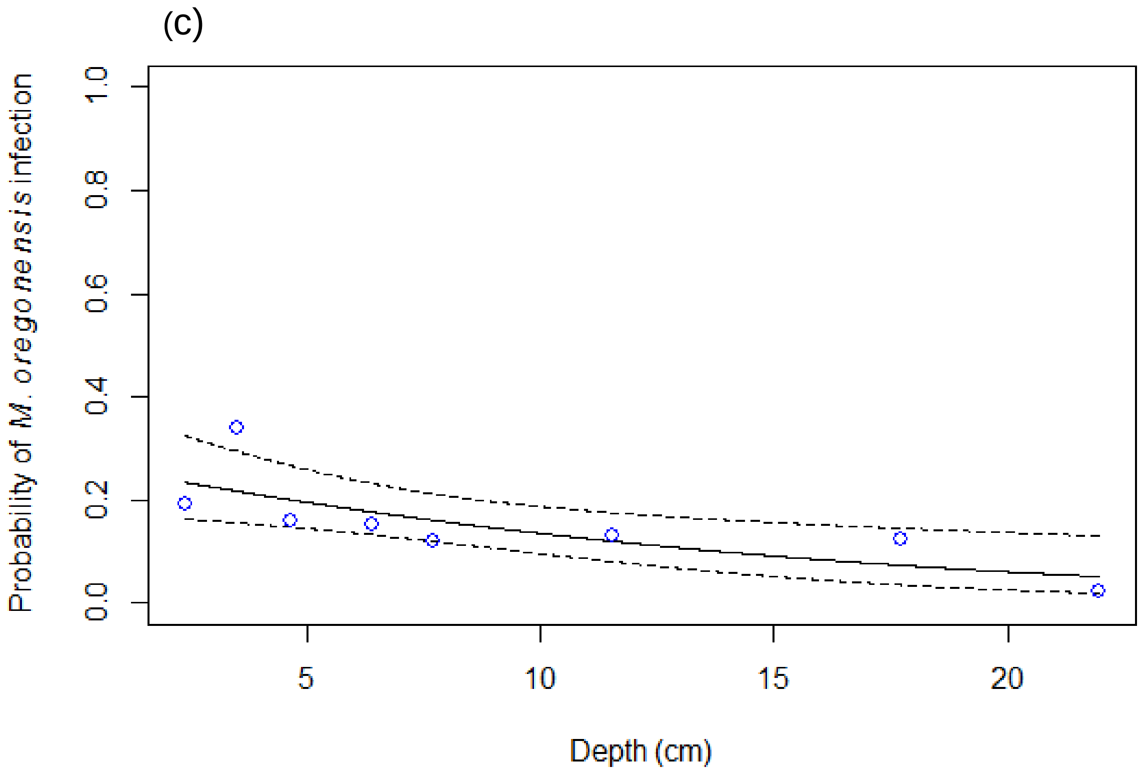


Figure 4. Fitted values (solid line) and 95% confidence bands for GLMs representing the relationships between (a) total trematode prevalence and elevation, (b) *M. oregonensis* prevalence and snail density, and (c) *M. oregonensis* prevalence and stream depth. Points represent observed values.

Chapter 5: Synthesis

Ecological processes can occur rapidly or over long periods, and patterns observed at small scales may differ at larger scales. To address questions in ecology, it is necessary to consider the issue of scale. In my investigation of the infection dynamics of trematode communities in streams, I incorporated approaches at multiple temporal and spatial scales. In Chapter 2, I present evidence from a 3-year study that indicates trematode infection in stream snail species may not be highly impacted by seasonal changes. This suggests that definitive hosts in this system are likely resident species rather than hosts with seasonal variation in abundance at sites. Certainly, this is not the case for all lotic systems and seasonal variation may drive patterns of infection at larger scales, especially in areas with migratory flyways. It is possible that a study longer than 3 years could reveal patterns of temporal variation in the infection dynamics of a host with a lifespan of 3 to 5 years, especially if infections are persistent.

In Chapter 3, I characterized the abundance and diversity of trematode infection across a regional scale. I did not find evidence of significant species-environment relationships. The results suggest that dispersal may be the dominant force in structuring trematode communities at this scale. However, at the scale of a single stream network (Chapter 4), species-environment relationships emerged significant and appeared to largely counter the force of downstream drift. Additional observations spanning multiple stream networks are needed to assess the generalizability of these patterns.

One general dynamic of first-intermediate host trematode communities is that they are highly linked to definitive host abundance and diversity (Fernandez and Esch 1991, Snyder and Esch 1993, Mouritsen et al. 1997, Granovitch et al. 2000, Smith 2001, Skirnisson et al. 2004, Hechinger and Lafferty 2005, Byers et al. 2008, Thieltges et al. 2009, 2013, Blasco-Costa et al. 2013, Levakin et al. 2013). To explore the definitive host component of this system, I tried several approaches that could be useful for future research. First, I experimentally infected rats with *M. oregonensis* metacercariae from dissected salamanders. While I had limited success, it is possible that increasing the dose of metacercariae and screening for eggs beginning several days after infection (Lang et al. 1974) could improve the chance of collecting enough eggs to conduct experimental research. Second, to assess definitive host occupancy at study sites, I conducted camera trapping and collected raccoon feces, which I subsequently screened for trematode eggs. With the development of methods to use these techniques quantitatively and ensure equal sampling effort across study sites, both of these approaches could be useful for future research focused on definitive hosts.

There are an estimated 24,000 species of trematodes (Poulin and Morand 2004). The species that have been studied the most are parasites of medical and economic importance (e.g. *Schistosoma* spp., *Fasciola* spp., *Clonorchis* spp.). Only a small fraction of the trematodes that infect wildlife have been studied. Because we know less about the life histories of most parasites than we do about their free-living hosts, exploring new study systems involving parasites can be especially challenging. Yet, parasites are an important component of the ecology in natural systems, with impacts at multiple levels of ecological organization (Mouritsen and Poulin 2005; Lafferty et al. 2006; Wood et al. 2007; Kuris et al. 2008; Sato et al. 2011), and it is critical that we continue to advance our knowledge of these taxa.

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