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Interactions Between Digenean Trematodes and Human Land Use, Nutrient Cycling, and Climate in Colorado

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by

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Interactions Between Digenean Trematodes and Human Land Use, Nutrient Cycling, and Climate in Colorado

Thesis directed by Prof. Alan R. Townsend

Abstract: Human actions have changed the face of the planet and have modified how ecosystems develop and function. These changes affect multiple aspects of ecosystems which can in turn have deleterious effects on human well-being. Human-caused change has led to the collapse of fisheries, massive erosion of soils, loss of fertility, increased disease risk from complex life cycle parasites, and the endangerment of clean drinking water (among others). Specifically, anthropogenic activities have fundamentally changed how carbon (C), nitrogen (N), and phosphorus (P) cycle through and among ecosystems, both locally and globally. Altering the fluxes and pools of these elements, fundamental to all life on Earth, produces cascading effects that completely alter species interactions within ecosystems. In this thesis I used a series of vernal ponds on the eastern plains of Colorado to investigate interactions between nutrient cycling and parasites. First I demonstrated the likely N limitation of these ponds using both lab-based nutrient addition bioassays as well as field-based stoichiometric inference over the better part of a growing season. Both approaches yielded results consistent with widespread N limitation in these ponds. N limitation likely arises due to large inflows of anthropogenic P (manure, sewage, fertilizer) without correspondingly large N inflows, coupled with intrinsically low water column N fixation rates. Now, with the establishment of N limitation in these ponds, I determined the manner and degree of interaction between parasitic infection and nutrient cycling. The digenean trematode parasite *Cotylurus flabelliformis* commonly infects aquatic snails in these ponds and can reach high prevalences and abundances. Lab-based excretion trials using field-caught snails indicated that infection led to increased N fluxes from snail hosts via excretion. This N came both from the snails themselves (lower snail N content due to infection) as well as the periphyton (lower periphyton N content in higher infection

ponds). This infection-induced N excretion increased the flux of N from organic-bound reservoirs to the water column (where it is available to the rest of the ecosystem). Using a basic empirical modeling approach, I determined that this infection-induced N flux was on par with water column N fixation in these systems, with the potential to be much larger with depending on infection prevalences/abundances and snail densities. The N limited conditions present in these ponds due to anthropogenic P loading allow for parasite-mediated N to be significant at the ecosystem level. Anthropogenic change may also affect the prevalence and abundance of trematode parasites directly. Changes in molluscan-trematode communities were evaluated in freshwater habitats close to Crested Butte, CO over the last 50+ years. The first survey of snail infection was conducted over the summers of 1957, 1958, and 1959. I revisited two of these sites during the summers of 2011 and 2012 to evaluate the effects of environmental change on molluscan-trematode communities at two sites: Lake Grant (high disturbance) and the Kettles (low disturbance). Human disturbance led to changes in snail communities in Lake Grant, which decreased the resilience of parasite communities to year-to-year fluctuations in mean weather conditions. Conversely, an increase in snail biodiversity at the Kettles, possibly facilitated by cattle-derived nutrients or warmer springs, led to enhanced stability in trematode communities. Additionally, exceptionally high schistosome diversity was uncovered at this site, a first for high elevation ponds. Therefore, interaction between anthropogenic activities and infection can alter the role of parasites in ecosystems through both direct changes in prevalence and abundance as well as cascading effects involving nutrient cycling and species interactions.

Dedication

I dedicate this thesis to my wife Ruth. She has taught me what it is to love and to be loved. Her unyielding support and willingness to work alongside me through these years of graduate school has brought me through many difficult times. She spurs me on to seek true balance and joy in life. This dissertation signifies many late nights in the lab, long days in the field, and significant hours in front of a computer. I would just like to acknowledge the sacrifices and and hard work that my wife has lent to this effort. I truly could not do what I do without her.

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Chapter 1

INTRODUCTION TO THE THESIS

1.1 Anthropogenic Change

Anthropogenic activities over the last few centuries have drastically altered ecosystem structure and function on both local [255] and global [332] scales. Humans have fundamentally changed the cycling of carbon (C), nitrogen (N), and phosphorus (P) through the Earth system [333]. These changes are perpetuated throughout ecosystems through direct effects as well as species interactions [327]. Changes in land use (e.g. urban encroachment [101], deforestation [197], conversion to agriculture [8], etc.) has large effects on nutrient cycling, biodiversity, species distributions, and ecosystem structure and function [317].

1.2 Anthropogenic Effects on Nutrients

Changes to C, N, and P cycles have drastic cascading effects on a range of ecosystem parameters (biodiversity [317], phenology [145], species distributions [242], disease risk [155], primary producer quantity/quality [342], etc.) that are basic to ecosystem structure and function. Currently, anthropogenically-produced nutrients (N and P) are accumulating in the environment at an accelerating rate [132, 94, 134, 130]. Changes in land use (e.g. urban encroachment and agricultural development) are drastically changing biodiversity and changing nutrient cycling in surrounding

ecosystems [205]. In addition, anthropogenically-transported invasive species have completely restructured nutrient cycling of native ecosystems [104, 53, 174], as do natives [13, 114].

The ecological consequences of nutrient enrichment are not only determined by the total flux of N and P to a system, but also by the relative proportion of N to P contained within this flux (i.e. nutrient stoichiometry) [56]. Organisms assimilate N and P in specific ratios for optimal metabolic functioning [4]. For example, attached freshwater algal/bacterial/fungal communities (periphyton) require N and P at the ratio of 17:1 [122]. If nutrients are supplied to the periphyton at a lower N:P ratio (i.e. 17:4), the periphyton will assimilate N and P (at the ratio of 17:1) until bio-available N is exhausted, thus depleting N concentrations before all available P can be assimilated. Further periphyton production cannot take place until additional N is supplied to satisfy intrinsic stoichiometric constraints (N limitation).

1.3 Nutrient Enrichment and Pathogens

Mounting evidence indicates that anthropogenic alteration of N and P cycling, while beneficial for food production, has the potential to lead to significant changes in disease abundance and transmission [321, 211, 155]. N and/or P loading can lead to losses of biodiversity [333, 311] as well as eutrophication, which then leads to a reduction of habitat quality [131, 278, 299]. Ecological changes due to nutrient enrichment enhance pathogenicity of bacteria (bottulism [228]), plant-derived chemicals (harmful algal blooms [7]), viruses (West Nile virus and mosquito vector [263]), and parasites (trematode parasite *Ribeiroia ondatrae* and snail vector [151])). Complex life cycle parasites which require multiple hosts across multiple trophic levels are particularly susceptible to the effects of environmental change due to their increased exposure to change at multiple levels.

Digenean Trematode parasites require (i) a first intermediate host (usually an aquatic snail), (ii) optional subsequent intermediate hosts (invertebrate or vertebrate), and (iii) a vertebrate definitive host. Nutrients can alter patterns in disease transmission directly through changes in snail intermediate host food and habitat availability which can reduce infected snail host mortality and raise parasite output per snail host [151]. Nutrients may also reduce competition between suscepti-

ble and non-susceptible snail hosts, and in some cases, favor survival of the susceptible species over the non-susceptible species [96, 232]. The presence of non-susceptible snails decreases the infection rates of susceptible snails by drawing parasites away from susceptible snails to snails which cannot be infected [152, 12]. These effects may be compounded by climate change [315].

Additionally, trematode parasites themselves can affect nutrient cycling both directly and indirectly through their first intermediate snail hosts. Trematodes cause significant changes in snail host nutrient excretion fluxes, rates, and stoichiometry [15, 22] which directly influence ecosystem-level nutrient cycling [104]. Parasite-induced changes in nutrient cycling at the snail host level may be important at the ecosystem scale because: (1) aquatic snails dominate as benthic primary consumers, regulating nutrient flows through grazing effects and excretion/egestion [206, 104, 83] and (2) trematodes make up a large proportion of biomass within aquatic ecosystems [173, 116, 257]. Trematodes also affect snail host foraging efficiency and predation risk [23], snail host behavior [59], and snail host reproduction [304], which all can affect nutrient cycling.

It is imperative to fully understand mechanisms through which environmental change may enhance disease risk (especially diseases involving complex life cycle organisms). Not only do complex life cycle parasites cause disease, but they also are responsible for important ecosystem-level feedbacks. This thesis evaluates: the effects of anthropogenic change on nutrient limitation within aquatic ecosystems (**Chapter 2**), how this anthropogenically-imposed nutrient limitation regime affects the role of trematode parasites in ecosystem-level nutrient cycling (**Chapter 3**), and how a snail-trematode community responds to environmental change over a 50+ year time period (**Chapter 4**).

1.4 Nitrogen Limitation in Freshwater Ponds Induced by High Phosphorus Loads

The degree to which trematode parasites impact nutrient cycling depends which nutrient is limiting as well as the size of other nutrient pools and fluxes within the ecosystem. In **Chapter 2** I evaluate the nutrient limitation status of the small freshwater ponds which are the focus of the

first two chapters of this thesis. These ponds are set within an agriculturally-impacted landscape and receive large nutrient inputs from surrounding areas. The predominant theory put forth by Shindler et al. [277] maintains that P is almost always limiting in freshwater systems due to the ability of in-situ N fixation to ameliorate any N deficits (compared to P) imposed by nutrient supply stoichiometry. However, the study ponds (like many water bodies in the region [27]) receive large and consistent P loads from both domestic and agricultural sources [164, 253, 60, 272, 54]. P is received in such stoichiometric excess compared to N that it overcomes the ponds' intrinsic ability for N fixation, resulting in the widespread manifestation of N limitation across ponds.

1.5 Parasite-Induced Nitrogen Cycling is Relevant at the Ecosystem Scale

These ponds are are extremely sensitive to additional N sources due to this anthropogenically-induced state of N limitation described in **Chapter2**. In **Chapter3** I explore the degree to which trematodes are altering N cycling at the ecosystem level in these N limited ponds. Snails in these ponds are commonly infected with the second larval stage of the trematode *Cotylurus flabelliformis* [49]. Like other trematode species [15, 22], *Cotylurus flabelliformis* infection leads to higher N excretion rates from the snail host. Infected snails not only contain less N themselves, but also likely increase the rate of transfer of N from the periphyton (organic) to the water column (inorganic), which increases N availability to the entire pond ecosystem. In addition, when taking into account field-derived values for snail density, infection prevalence, and parasite abundance, the additional daily flux of N delivered to the ecosystem from infection-induced snails excretions is on the same scale as N fixation in these ponds (with the potential to be much greater as seen in other years at these sites with higher parasite abundances). Water column measurements and periphyton measurements are consistent with this hypothesis, with ponds with higher infection levels containing less N in the periphyton and more N in the water column.

1.6 Environmental Change and a 50+ Year Record of Snail Infection

In **Chapter 2** and **Chapter 3** I evaluated the effects of trematodes on ecosystem structure and function within an anthropogenically-altered landscape. In **Chapter 4** I evaluate the long-term effects of environmental change (local disturbance and changes in mean annual weather conditions) on high elevation snail-trematode communities around Crested Butte, CO. During the summers of 1957, 1958, and 1959 Hunter [140] surveyed high elevation ponds (>2700 m) around Crested Butte, CO to evaluate trematode prevalences and morphotype diversities within aquatic snails. This survey was undertaken in response to a 1956 outbreak of cercarial dermatitis ("swimmers itch") [138] caused by larval trematode parasites (schistosomes) released from their snail hosts. One of these sites (Lake Grant) has undergone a large amount of local disturbance with the installation of a dam, a golf course, and a large residential development since the work of Hunter [140]. In this current study, I re-surveyed both Lake Grant (high disturbance) as well as a set of water bodies that experiences much lower disturbance (the Kettles). Local disturbance at Lake Grant decreased snail biodiversity, leading to the destabilization of year to year parasite prevalences. Conversely, different environmental changes likely facilitated the expansion of one snail's range at the Kettles, therefore increasing snail biodiversity and year to year stability in infection prevalence. Thus the effects of environmental change differ depending on local disturbance patters. In general at these sites, longer growing seasons will result in higher parasite prevalences (in the absence of additional disturbance). In addition, an unprecedented amount of schistosome biodiversity for such high elevation ponds has been uncovered at these sites.

1.7 Conclusion

The work contained within this thesis evaluates how anthropogenic change and trematode parasite populations interact. These studies particularly focus on human changes to ecosystem-level nutrient cycling as well as parasite-mediated nutrient cycling. These studies will help to place parasites within an ecosystem context as well as evaluate how human change affects parasite

populations.

Chapter 2

NITROGEN LIMITATION OF POND ECOSYSTEMS ON THE PLAINS OF EASTERN COLORADO

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2.1 Abstract

Primary production in freshwater ecosystems is often limited by the availability of phosphorus (P), nitrogen (N), or a combination of both (NP co-limitation). While N fixation via heterocystous cyanobacteria can supply additional N, no comparable mechanism for P exists; hence P is commonly considered to be the predominant and ultimate limiting nutrient in freshwater ecosystems. However, N limitation can be maintained if P is supplied in stoichiometric excess of N (including N fixation). The main objective of this study was to examine patterns in nutrient limitation across a series of 21 vernal ponds in Eastern Colorado where high P fluxes are common. Across all ponds, water column dissolved inorganic N steadily decreased throughout the growth season due to biological demand while total dissolved P remained stable. The water column dissolved inorganic N to total dissolved P ratios suggested a transition from NP co-limitation to N limitation across the growth season. Periphyton and phytoplankton %C was strongly correlated with %N while %P was assimilated in excess of %N and %C in many ponds. Similarly, in nutrient addition bottle assays algae responded more strongly to N additions (11 out of 18 water bodies) than P additions (2 out of 18 water bodies)

and responded most strongly when N and P were added in concert (12 out of 18 water bodies). Of the ponds that responded to nutrient addition, 92% exhibited some sort of N limitation while less than 8% were limited by P alone. Despite multiple lines of evidence for N limitation or NP co-limitation, N fixation rates were uniformly low across most ponds, most likely due to inhibition by water column nitrate. Within this set of 18 water bodies, N limitation or NP co-limitation is widespread due to the combination high anthropogenic P inputs and constrained N fixation rates.

2.2 Introduction

Large-scale human alteration of nitrogen (N) [333, 93] and phosphorus (P) [17, 85, 194] cycles has substantially changed the absolute and relative supply rates of limiting nutrients in a broad variety of Earth's ecosystems. Eutrophication of freshwater aquatic ecosystems [334, 117, 133, 298, 299, 300] is one widespread consequence of such nutrient enrichment. Eutrophication can cause harmful algal blooms and bottom water hypoxia (dead zones) which in turn pose risks to fisheries resources, ecosystem services, and human health and recreation [7, 63]. Recent estimates for the United States suggest that cultural eutrophication has an annual cost of more than $2 billion per year [65]. As such, understanding the drivers and mechanisms of eutrophication has enormous societal and economic relevance [190, 279].

Phosphorus has long been identified as the ultimate limiting nutrient (and thus the causative agent of eutrophication when received in excess) within freshwater ecosystems [143, 72, 334], mainly because of strong observed relationships between total P and chlorophyll (an index of algal abundance) [267, 64]. Schindler [277] provided a mechanistic understanding of this relationship in a set of landmark multi-year whole lake nutrient enrichment experiments in which N derived from water column N fixation [133, 297, 181] and subsequent N regeneration and recycling from accumulated N in the sediment [239, 282] fully offset the N deficit induced by imbalanced experimental inputs of N and P over multiple growth seasons [283, 225]. This assertion that P is the eventual limiting nutrient in most freshwater ecosystems (the "P paradigm") has focused efforts to avoid or reverse eutrophication solely on P control and removal (see [279] for review).

However there is accumulating evidence of N limitation [79, 147, 32] or NP co-limitation [221, 66] of primary production within a wide variety of freshwater ecosystems, prompting an active debate about the use of P-only control to avoid/reverse eutrophication across diverse aquatic ecosystems [190, 282, 309, 47, 236, 292, 279]. N limitation or NP co-limitation can occur when N supply to the system is outpaced by P, (i.e. when rates of N fixation plus hydrologic supply are lower than the Redfield ratio of 16:1 - the optimal stoichiometry for phytoplankton growth [261]). This dynamic can occur in aquatic systems that receive low N:P ratio hydrologic inputs from sewage, concentrated animal feeding operations (CAFOs) and/or fertilizer sources coupled with an inability of N fixation to ameliorate these N deficiencies on relevant timescales [190, 292].

The South Platte River Basin - which spans Colorado, Wyoming, and Nebraska - is one such region. The Basin contains a number of off-stream reservoirs, many with accompanying vernal ponds and wetlands. These water bodies serve as agricultural water storage, recreational areas, wildlife habitat, and aquifer recharge sites. Nutrient concentrations in the South Platte River are among the highest measured by the NAWQA Program [60] due to the high incidence of municipal wastewater treatment plants, CAFOs, and irrigated/fertilized cropland across eastern Colorado in conjunction with a low base flow [253, 164, 272, 54]. These P-rich anthropogenic nutrient sources, along with high denitrification rates [208], explain the low DIN:TDP molar ratios (between 0.5 and 5.0) in the Basin's reservoirs and wetlands [305, 27]. In this area, and others like it, the sustained supply of high concentration, low N:P nutrient inputs could be fundamentally shifting pond nutrient limitation from a pre-anthropogenic P limited regime to an N limited regime [67]. Even if anthropogenic sources of P were reduced, the P accumulated in the sediments could maintain N limitation for years or decades to come as sediments return large quantities of P back to the water column [43, 313, 274, 179, 52]. Imbalanced P-rich N:P supply rates have induced stable N limitation in some cases (e.g., [147, 32, 187, 346, 229]) and weakened the correlation between indices of primary productivity (such as chlorophyll a (chl a)) and P [156, 256, 48] thereby emphasizing the capacity of high P fluxes to force systems into N limitation, leaving N fluxes as the key determinate of eutrophication.

The goal of this study was to examine patterns in nutrient limitation across a series of vernal ponds filled with South Platte River water. Information on nutrient limitation in aquatic ecosystems can aid managers in maintaining ecosystem health and avoiding the harmful effects of cultural eutrophication. We combined field-based observational measurements of water chemistry and other relevant environmental data with a series of laboratory experiments to assess nutrient limitation of primary production. Given the long and well-documented history of P-rich inputs [305], we predicted that the pond ecosystems would be N limited, and thus, that at least for the near-term, any attempts at avoiding eutrophication would need to focus on decreasing N availability.

2.3 Materials and Methods

2.3.1 Site Description

All data were collected within two complexes of shallow (<3m) vernal ponds located on the plains of Eastern Colorado. The Andrick Ponds State Wildlife Area and The Teal Hunting Lodge (40° 22'16.77" N, 104° 06'24.89" W; 13 ponds) comprise the western set of ponds while the Brush Prairie Ponds State Wildlife Area (40° 12'46.68" N, 103° 38'37.53" W; 8 ponds) is 40 km to the E-SE. All ponds are located on public land administered by the Colorado Division of Wildlife and all necessary permits were obtained for this study, which complied with all relevant regulations. Ponds at these sites form within shallow depressions in the uniformly sandy soil. They are replenished via irrigation ditches with water sourced from agricultural runoff and the South Platte River when water is available. All ponds at these sites often are filled to capacity in the late spring when irrigation water is abundant, but a lack of surplus water later in the season leads to gradual evaporation throughout the summer. Though some ponds may completely dry up at the end of the summer, others retain water (though at much lower levels). Because of the constant throughput of water in the spring, water residence times during the peak of the growth season may be short.

2.3.2 Field Data Collection and Analytical Analyses

Field campaigns were conducted during the growth seasons (end of May to mid August) of 2011 and 2012 to investigate pond nutrient limitation. In 2011, the water column and periphyton were sampled across 21 ponds to assess nutrient limitation from a stoichiometric perspective. We followed up on observed field patterns by collecting water samples from all previously surveyed ponds which held water in July 2012 (17 of 21 ponds) and conducted nutrient addition bottle assays and nitrogen fixation experiments to empirically determine the potential for phytoplankton to respond to N, P, and N+P additions as well as their potential to fix atmospheric nitrogen. We also measured seston C, N, and P in these samples. Sampling was limited by the ephemeral nature of the ponds as well as logistical considerations of pond management by the Colorado Division of Wildlife for waterfowl hunting.

During the 2011 growth season surface water samples were collected in triplicate weekly from each of the 21 ponds. Water was filtered with a GF/F (Whatman glass fiber filter, nominal pore size) into acid-washed polypropylene containers and frozen for later analyses. All surface water samples were analyzed for total dissolved organic carbon (TDOC), total dissolved nitrogen (TDN), soluble reactive phosphorus (SRP), NH_4^+, and NO_3^-+NO_2^-. TDOC and TDN were determined in all samples using a high temperature combustion TDOC/TDN analyzer (Shimadzu TOCvcpn, Kyoto, Japan). NO_3^- and SRP were analyzed colorimetrically on an Alpkem autoanalyzer (OI Analytical, College Station, TX, USA) using the cadmium reduction [118] and the ammonium molybdate ascorbic acid methods [172] respectively. NH_4^+ was analyzed colorimetrically on a BioTek Synergy 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Total dissolved phosphorus (TDP) in water samples was determined by a potassium persulfate/sodium hydroxide digestion to convert organic phosphorus to SRP and measured as above. Conductivity, pH, temperature, and Secchi depth were measured in each pond (Table A.1). In addition, a 6 liter composite water sample was collected from each pond on July 16, 2012 and prefiltered through a 153 μm Nitex screen. Four liters were used for the nutrient addition bottle assay experiment, 0.5 liters for the

nitrogen fixation syringe experiment, and 1.5 liters for a composite seston sample.

Periphyton samples were also collected from all 21 ponds surveyed in 2011. Periphyton was collected by selecting movable substrate (wood, dead reeds, aquatic macrophytes) every ∼30m around the entire circumference of the pond and placing these substrates with included periphyton into plastic bags and transporting them on ice to the lab. For ponds with circumferences larger than 300m, 10 equally spaced samples were taken around the circumference. Once in the lab substrate samples were mixed with DI water and rigorously shaken for 30 seconds to force as much periphyton as possible into suspension. This periphyton slurry was then filtered with a vacuum pump onto a Whatman GF/F filter. Seston samples were obtained from prefiltered (153 μm Nitex screen) water samples by filtering with a vacuum pump onto a Whatman GF/F filter. The seston and periphyton covered filters were dried at 60°C for 48 hours and placed in a freezer until analyzed for element composition.

Four equally-sized punches from the dried seston or periphyton covered filters were massed and packed in tins for C and N analyses. The average mass of 4 punches of a clean GF/F filter was subtracted from the dried seston or periphyton covered filter punches to determine the actual mass of seston or periphyton alone. Filter blanks were also prepared in the same was as the samples. Percentage of carbon (%C) and nitrogen (%N) was determined using a Carlo Erba EA 1110 elemental analyzer (CE Elantech, Lakewood, New Jersey, USA). Phosphorus (P) extraction was performed after [226, 301]. Four seston or periphyton punches were placed in 30 ml glass vials and ashed at 500°C. P was extracted using 5 ml of 1 N HCl heated to 80°C for 30 minutes and then diluted with 5 ml of DI water. P was determined in the diluted leachate using the colorimetric analysis above. All GF/F filters were heated to 500°C for 4 hours and rinsed with DI water to eliminate contamination and ensure nutrient-free conditions before use. Ten GF/F filter blanks were randomly selected for nutrient analyses. Nutrient levels were measured as described above, with all samples yielding nutrient levels below detection limits for %C, %N, and %P.

On July 16, 2012 surface water samples were collected from 17 ponds surveyed in 2011 (4 were completely dry) for chemical analyses listed above as well as for laboratory experimental analyses.

An additional 6 L composite water sample was collected from each pond and prefiltered through a 153μm Nitex screen. Four liters were used for the nutrient addition bottle assay experiment and 0.5 liters for the nitrogen fixation syringe experiment, which are both described below.

2.3.3 N and P Enrichment Experiment

A nutrient-enrichment bioassay experiment was performed in July 2012 using water from 17 of the 21 ponds surveyed in 2011 as well as an irrigation ditch supplying water to some of the ponds. Six liters of water was composited into a single sample from each pond on July 16, 2012 and prefiltered through a 153 μm Nitex mesh, with aliquots partitioned for measurement of water column N fixation. Our bioassays were modeled after the US Environmental Protection Agency protocol for algal assay bottle tests using natural assemblages of phytoplankton [230, 79, 9].

Treatments consisted of 4 repetitions each of (i) a control (no nutrient added), (ii) +N, (iii) +P, and (iv) +N+P. Nutrient treatments were 320 μmol/L N (i.e., 4480 μmol/L of NH_4NO_3) and 20 μmol/L P (i.e., 620 μmol/L of KH_2PO_4) in the single nutrient addition treatments and a combination of both in the +N+P treatment. The nutrient ammendments were adjusted to overcome the high P levels in the pond water and to achieve a Redfield N:P ratio of 16:1. All bottles (240 mL) were incubated in the lab at a constant temperature (25 to 26°C) for 5.5 days under a 16 h light:8 h dark cycle using natural spectrum grow lights (20-27μmol/m^2/s, Instant Sun natural spectrum fluorescent tube light, 2100 photopic lumens, 6280°K color temperature, 94.5 color rendering index). The bottles were shaken twice daily and randomized once daily. At the end of the experiment all water within each bottle (200 ml) was filtered onto GF/F filters and each filter was folded, placed in foil, and frozen for chlorophyll analysis (corrected for pheopigments via hydrochloric acid additions) [110, 349]. Chl a was determined using a FluoroMax-2 200-900nm spectrofluorometer (HORIBA Scientific Edison, NJ).

2.3.4 Water Column N Fixation

Nitrogen fixation rates were estimated by the acetylene reduction method [88] in water collected in 2012 from each of the 17 ponds and the irrigation ditch. Sixty ml polypropylene syringes were used as assay vessels after blank tests showed no leakage or in-situ ethylene production over our assay time period (3 to 4 hours) [160]. Four syringes were used for each pond (3 replicates, 1 control) after the methods of [27, 289]. An integrated water sample from each pond served as the sample water from which subsamples were drawn into each syringe. Each syringe was rinsed with sample water and 40 ml of sample water was drawn into the syringe, air was purged, and water volume in each syringe was adjusted to ∼30 ml. Five ml of acetylene (generated via hydrolysis of calcium carbide in DI water and stored in a bladder) was added to each replicate syringe, which was sealed using a valve and moderately agitated for 10 seconds. Five ml of air were added to each control syringe which were sealed and agitated in the same manner as those that received acetylene. Blanks were prepared using sterile DI water instead of sample water to account for background ethylene in the acetylene source.

The syringes were incubated for 3 to 4 hours at a constant temperature (25 to 26°C) under natural spectrum fluorescent grow lights (30-40μmol/m^2/s). These temperature and light conditions are common in the epilimnia (upper part of the water column) of many lakes [250, 163, 338]. At the end of the incubation, 20 ml of air was drawn into each syringe and each syringe was shaken vigorously for 30 seconds to equilibrate the liquid and vapor phases. Aqueous and vapor volumes were recorded following equilibration to account for partitioning of ethylene between aqueous and vapor phases [88, 69]. The incubation was halted by removing a sample of the headspace and placing it in a 5 ml vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) that had been previously manually evacuated to ensure that no other substances were present within the vacutainer. Ethylene was measured using a Shimadzu 14-A Gas Chromatograph equipped with a flame ionization detector (330°C) and a Poropak N column (110°C; Supelco, Bellefonte, Pennsylvania, USA) at an oven temperature of 80°C. For each sample a 3 ml aliquot of gas was

removed from the 5 ml vacutainer using an airtight glass syringe fitted with a valve that was closed prior to and following vacutainer sampling. This subsample was injected into the instrument by opening the valve and forcing all gas out of the syringe. Ethylene concentration was determined by comparing to a standard curve containing known ethylene concentrations. After accounting for variables affecting ethylene recovery (temperature and relative volume of headspace; [40]), ethylene production was converted to nitrogen fixation with a 4:1 ethylene/dinitrogen conversion ratio [160].

2.3.5 Statistical Analyses

Water column nutrient data were compared between the ditch and an aggregation of all pond data using t-tests. Correlations were examined using the Pearson product-moment correlation coefficient (r). N fixation rates were compared to DIN:TDP ratios using the MannWhitney U test. Where appropriate, values are displayed as means with ±1SE. Nutrient limitation was assessed using the ratio of DIN:TDP as measured in the water column during the 2011 growth season using the thresholds of Morris et al. [221]. According to [221] a water body is likely to be P limited if its DIN:TDP molar ratio is above ∼18 and N limited below ∼2.2. Co-limitation is thought to predominate in ponds with DIN:TDP molar ratios between ∼2.2 and ∼18. These thresholds were chosen specifically because of their widely accepted use and this study's explicit testing of the efficacy of different nutrient ratios in predicting nutrient limitation (though other thresholds exist - [20, 158]). The DIN:TDP ratio is a measure of nutrient supply to phytoplankton and has been demonstrated to be accurate 80% to 90% of the time in predicting limiting nutrients compared with results from bioassay experiments [221]. For N, the largest bioavailable pool tends to be DIN while P includes both SRP and dissolved organic phosphorus (DOP). DOP is bioavailable to phytoplankton because of the excretion of alkaline and acid phosphatases that enzymatically cleave phosphate groups off organic molecules [235]. The ambient DIN threshold above which water column N fixation becomes unfavorable (20 μg/L) was taken from work in adjacent pond systems by [27].

Periphyton and seston C:N, N:P, and C:P molar ratios were compared to modified Redfield

ratios from [122]. Because Redfield ratios are empirically developed stoichiometric ratios from deep ocean phytoplankton, slightly different ratios are expected for freshwater periphyton because of differences in physiology and life strategies. Hillebrand et al. [122] developed empirical stoichiometric ratios for optimal freshwater periphyton growth under balanced N:P supply rates (C:N:P = 119:17:1) which are stable against changes in abiotic conditions. Because optimal growth takes place at these modified Redfield ratios and periphyton's capacity to store P is enhanced when excess P is present in the environment (luxury P uptake - [207]) P accumulated in excess of C or N is indicative of excess P supply. The goodness of fit of periphyton/seston with the modified Redfield ratios of [122] were evaluated using coefficients of determination (R^2).

Results for the nutrient addition (+N, +P, +NP) bottle assays were compared qualitatively across treatments to determine broad-scale patterns in nutrient addition. A statistical approach, using a two-way ANOVA and post hoc pairwise comparisons following [74] was used to distinguish between various types of nutrient limitation. Single nutrient limitation was indicated by a significant chl a response to only one nutrient (N or P alone) addition in the 2-way ANOVA with no significant N-P interaction while an additive dual nutrient limitation was indicated by a significant chl a response to both N and P addition alone in the 2-way ANOVA with no significant N-P interaction. Sequential N co-limitation was indicated by a significant interaction in the chl a response to interaction in the 2-way ANOVA and post hoc pairwise comparisons where ChlNP > ChlN > ChlC = ChlP while sequential P co-limitation was indicated by a significant interaction in the chl a response to interaction in the 2-way ANOVA and post hoc pairwise comparisons where ChlNP > ChlP > ChlC = ChlN. Strict co-limitation with N and P was indicated by a significant interaction in the in 2-way ANOVA and a post hoc pairwise comparison indicating ChlNP as the only significant difference from the control. No significant terms in the 2-way ANOVA indicated a lack of response to nutrient enrichment. N fixation rates were computed as μmol per L per day assuming 10 hours of active fixation per day and uniform fixation with depth; rates were extrapolated to an annual flux assuming a 65 day growth season [133].

2.4 Results

2.4.1 Biogeochemical Patterns in N and P

Across the growth season the ditch had higher concentrations of all N and P species compared to the ponds except for DON (Figure 2.1A-D, TDP = 0.40±0.02, 0.20±0.01, p = 1.7e-11; DIP = 0.23±0.02, 0.15±0.007, p = 0.003; DIN = 1.41±0.15, 0.15±0.008, p = 4.4e-09; DON = 0.31±0.04, 0.76±0.02, p = 1.0e-10).

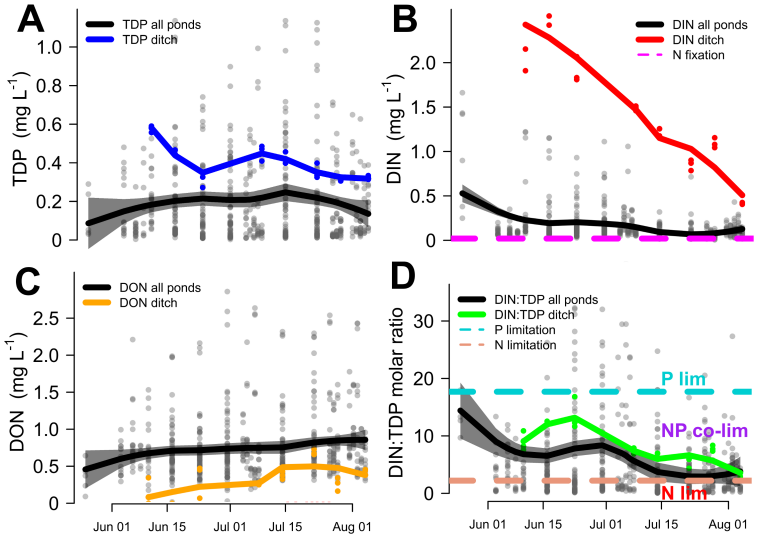

Figure 2.1: **Weekly water column biogeochemical measurements from 21 ponds indicate nitrogen limitation.** Water samples taken weekly throughout the 2011 growth season (late May to early August) from 21 ponds and an irrigation ditch providing water to the ponds. (A) TDP concentrations of the pond water (black) and the supply ditch (blue). (B) DIN concentrations (NO_3^- + NO_2^- + NH_4^+) of the pond water (black) and the supply ditch (red) compared to the threshold (magenta) from Bradburn et al. [27] above which DIN concentrations inhibit water column N fixation. (C) DON concentrations of the pond water (black) and the supply ditch (orange). (D) DIN:TDP molar ratios of the pond water (black) and the supply ditch (green) compared to the thresholds of Morris et al. [221] for P-limitation (above cyan line), NP co-limitation (below cyan line and above red line), and N-only limitation (below red line). Gray confidence intervals around the pond data are ±2 SE.

The mean DIN in the ditch was almost 10 times higher than the mean DIN in the ponds (Figure 2.1B) while the mean TDP was two times as high (Figure 2.1A). Consequently, the mean DIN:TDP ratio of the ponds was significantly (p=0.003) lower than that of the ditch (Figure 2.1D).

TDP concentrations in most ponds showed little variation over time (0.20±0.01), which was half the mean ditch concentration (0.40±0.02) (Figure 2.1A). In contrast, DIN concentrations in both the ditch and the ponds steadily declined throughout the growth season, reaching a low at the end of the summer (Figure 2.1B). DON concentrations were much higher than DIN, varied little (0.76±0.02), and were twice as high as the ditch mean (0.31±0.04, Figure 2.1C).

2.4.2 Indicators of Nutrient Limitation

2.4.2.1 Water Column Stoichiometry

DIN:TDP ratios of ditch water were within the thresholds of N and P co-limitation throughout the growth season (Figure 2.1D, [221]). In most ponds the DIN:TDP ratios strongly decreased throughout the season, driven by a decline in DIN concentrations. From a stoichiometric perspective, the decline in DIN:TDP values indicated a shift from N and P co-limitation to N limitation by the end of the growth season (Figure 2.1D). Water column TDOC was closely correlated with water column TDN ($r = 0.92$, $p < 2.2e\text{-}16$; Figure 2.2), while water column TDP exhibited a much weaker relationship with TDOC ($r = 0.27$, $p = 8.0e\text{-}11$; Figure 2.2).TDOC did not exhibit a strong trend across the season (Figure A.2).

2.4.2.2 Periphyton and Seston Stoichiometry

Periphyton stoichiometry and seston stoichiometry were measured during July 2011 and July 2012 respectively (Figure 2.3A-C). Organic C and N concentrations were tightly linked for both periphyton and seston ($r = 0.89$, $p < 2.2e\text{-}16$; Figure 2.3A). Periphyton/seston P had a much weaker relationship with N ($r = 0.18$, $p = 0.008$) and no significant relationship with organic C ($r = 0.12$, $p = 0.09$). The modified Redfield C:N ratio [122] was a good fit for field-derived periphyton/seston C:N ratios ($R^2 = 0.68$; Figure 2.3A), whereas field-derived N:P and C:P ratios were better fitted by their own means rather than the modified Redfield N:P and C:P ratios, emphasizing the modified Redfield ratios' poor fit with P-related field-derived ratios at this site ($R^2 = -0.13, -0.10$; Figures 3B, 3C). Seston and periphyton both commonly exhibited excess accumulation of P versus N (Figure

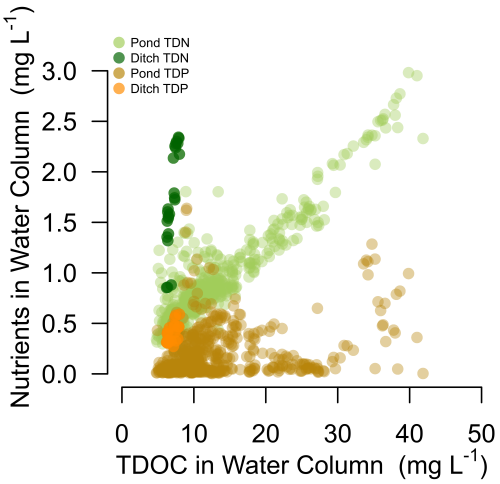

Figure 2.2: **Weekly water column measurements of TDOC vs. TDN and TDP from 21 ponds indicate N limitation.** Water samples taken weekly throughout the 2011 growth season (late May to early August) from 21 ponds (lighter points) and an irrigation ditch providing water to the ponds (darker points). TDN and TDP concentrations are higher in the ditch water than the pond water when compared to TDOC. There is a strong positive correlation between TDN and TDOC ($r = 0.92$, $p < 2.2e\text{-}16$) and a much weaker relationship between TDP and TDOC ($r = 0.27$, $p = 8.0e\text{-}11$) indicating N as limiting C fixation in the system. Alternatively the lack of a stong correlation between TDP and TDOC could arise from luxury P uptake decoupled from C fixation.

2.3B) and P versus C (Figure 2.3C) compared to the modified Redfield Ratio [261, 122] while rarely exhibiting excess N (Figure 2.3B) and excess C (Figure 2.3C) accumulation versus P.

2.4.2.3 Nutrient Addition Bioassay

N-only nutrient additions produced positive chl a responses (50% above control values) in 11 water bodies (Figure 2.4A) while P-only additions produced chl a responses in only 2 ponds (Figure 2.4B). When N and P were added in concert 13 ponds showed a positive response to nutrient enrichment (Figure 2.4C). Of the 18 water bodies tested in the 2012 bioassay (Figure 2.5), 7 ponds showed single nutrient limitation by N while only one showed single nutrient limitation by P. One pond showed additive dual nutrient limitation to N and P indicating that production would increase by adding either N or P alone or in concert. Two ponds showed sequential N co-limitation indicating that chl a showed a response to N addition and an even greater response to NP addition

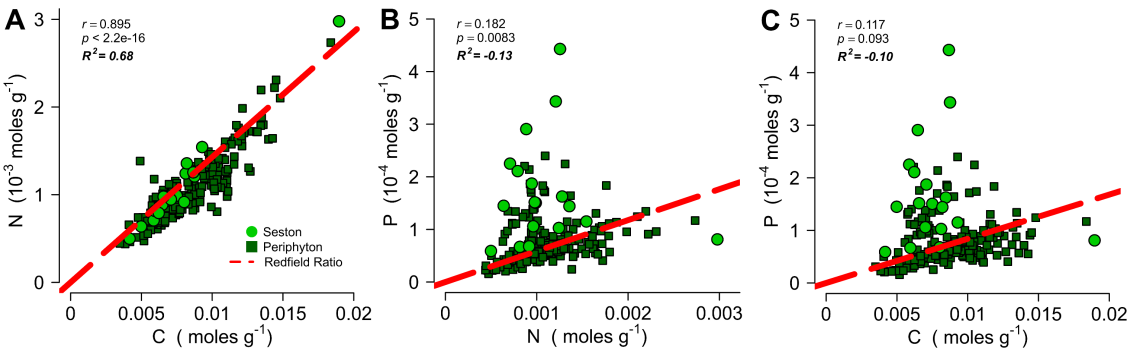

Figure 2.3: **Periphyton and seston stoichiometry within ponds.** Periphyton (dark green squares sampled July 2011) and seston (light green circles sampled July 2012) molar nutrient contents compared to the modified Redfield ratio (C:N:P = 119:17:1) for periphyton [122]. (A) C content is tightly coupled to N content (r = 0.89, p < 2.2e-16) and follows the modified Redfield ratio (R^2 = 0.68) indicating a strong dependence of C accumulation on N content. (B) P content is in excess of N content for many samples producing a much weaker correlation (r = 0.18, p = 0.008) and a poor fit to the modified Redfield ratio (R^2 = -0.13) and (C) P content is in excess of C content for many samples with no significant correlation and a poor fit to the modified Redfield ratio (R^2 = -0.10); both indicating luxury P uptake and a lack of dependence of C accumulation on P content.

but did not respond to P addition alone. In sequential N co-limitation P is only effective if N is added in concert but N is effective alone or in concert with P. One pond showed strict co-limitation with N and P. Of the bottles that responded to nutrient addition, all N only, N sequential, and NP co-limitations had DIN:TDP molar ratios less than 4.5, whereas P only and NP dual limitation occurred at DIN:TDP molar ratios greater than 4.5, indicating a higher threshold for shifting from N limitation to NP co-limitation than seen in [221]. N only, N sequential, and NP co-limitation was associated with higher TDP values (greater than 0.025 mg/L) whereas P only and NP dual limitation occurred at TDP < 0.025 mg/L. P only limitation occurred in only one pond (A1), which had a mean DIN:TDP molar ratio of 14.6.

2.4.3 N fixation

In general, N fixation rates were consistently low across all ponds (Figure 2.6) except for pond B5 which had an anomously high (8.9±0.005 μmol-N/L/day) N fixation rate compared to the other samples. N fixation rates in all other ponds were lower than many other lab-derived values under

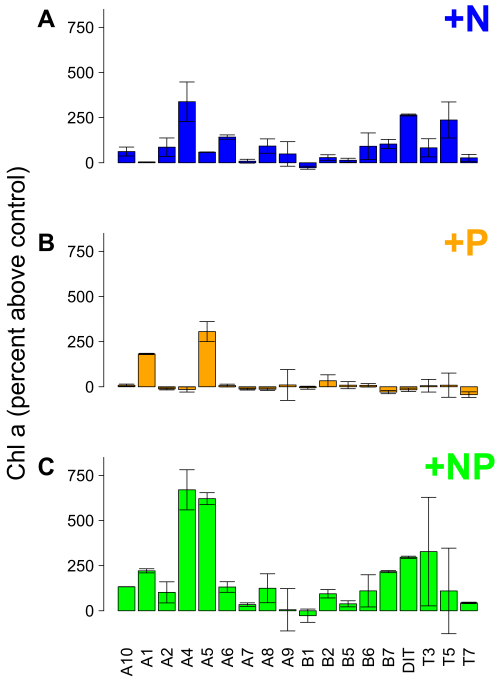

Figure 2.4: **Results from bottle nutrient addition experiments.** Percent change in chlorophyll a (chl a) after addition of (A) +N alone, (B) +P alone, and (C) +NP together, compared to controls (no nutrients added) from water taken from 17 ponds and the supply ditch in 2012. Chl a increased more than 50% above controls in: (A) 11 water bodies when N alone is added, (B) 2 water bodies when P alone is added, and (C) 13 water bodies when N and P are added together. While adding N alone induced chl a responses in most ponds, adding N and P in concert and the most consistent and largest magnitude effect.

similar light and stoichiometry levels [251], but are in line with estimates of N fixation from adjacent water bodies [27]. Similarly, [133] found a mean N fixation rate of ~0.52 μmol/L/day (range = 0.007 to 2.86 μmol/L/day) when looking across 20 freshwater field studies. If the observed N fixation rate of 0.065 μmol/L/day is extrapolated to a 65 day growth season N fixation contributes 0.06 mg of N per L per growth season, which is 12% of the mean standing stock of TDN in the 18 water bodies in this study (0.77 mg/L). There was no correlation between water column DIN:TDP and N fixation rates ($p = 0.7$), although N fixation rates were higher on average (0.14±0.03, 0.06±0.01; $p<0.05$) in ponds that had less than 4 μg/L NO_3^- (Figure 2.6). Water nutrient concentrations, N fixation rates, and nutrient limitation statuses are listed in Table A.2.

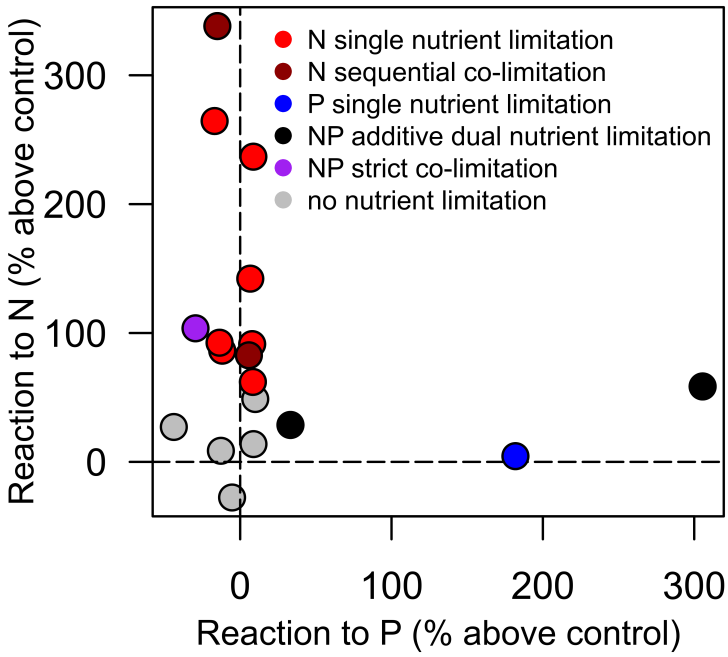

Figure 2.5: **Nature of nutrient limitation observed in the nutrient addition bottle experiments.** Percent change in chl a after addition of +N alone (y axis) and +P alone (x axis) compared to controls (no nutrients added) from water taken from 17 ponds and the supply ditch in 2012. Clustering along the y axis is indicative of N limitation while clustering on the x axis indicates P limitation. A two-way ANOVA was used to determine the type of nutrient limitation (colors) using the +N, +P, and +NP data after Elser et al. [74]. 12 of the 18 water bodies were limited by N alone or in combination with P, while only one pond exhibited P-only limitation. 5 ponds did not show a significant response to nutrient addition at the $p < 0.05$ level.

2.5 Discussion

Observational and experimental results indicate that N limitation is common in the 18 water bodies in this study, with P being supplied in stoichiometric excess of N compared to biological demand [261, 122]. While P limitation is widespread across many freshwater bodies [279], our results confirm the importance of strict or partial N limitation in water bodies in the South Platte River basin as suggested by others [187, 27, 305]. We can infer the existence of persistent strict or partial N limitation throughout the growth season at the study sites based on water nutrient stoichiometry (Figure 2.1A-D, Figure 2.2). This inference is confirmed by measurements of seston and periphyton stoichiometry (Figure 2.3A-C), estimates of water column N fixation rates, and the

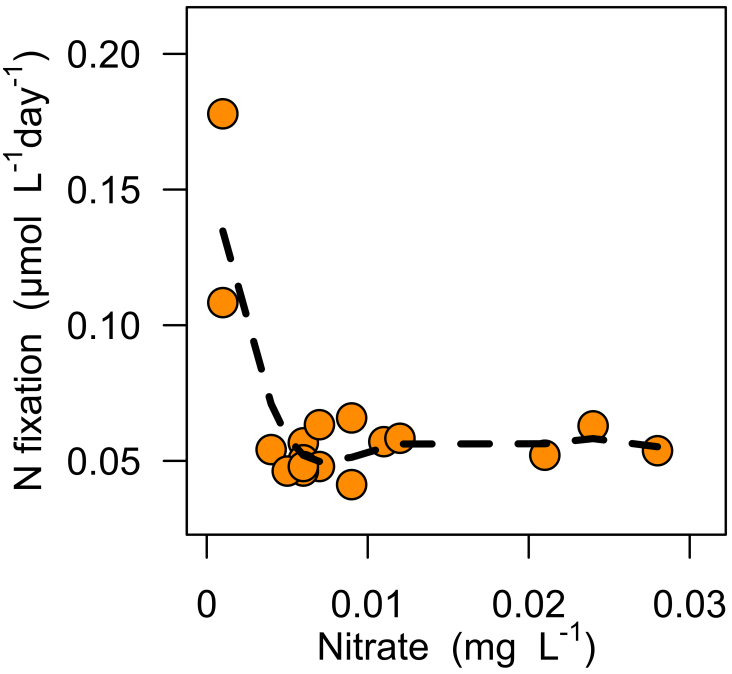

Figure 2.6: **Nitrate concentration threshold for loss of competitive advantage of water column nitrogen fixation.** Water Column N fixation rates were measured for water samples collected in July 2012. N fixation was not competitively advantageous when nitrate concentrations were higher than 4 μg/L. This result is broadly consistent with the results of Bradburn et al. [27] from Jackson Reservoir (an adjacent water body). The N fixation data from B5 were not included in this plot due to it being anomalously high compared to all other ponds.

results of nutrient addition bottle assays (Figures 4 and 5). On the plains of Eastern Colorado, P inputs appear to exceed both anthropogenic and *in situ* mechanisms for maintaining stoichiometric equilibrium, in ways that may have shifted many systems into N limitation.

2.5.1 Evidence for N limitation

2.5.1.1 Field Observations

Patterns in water column N and P chemistry suggest that soluble P is consistently in excess of autotrophic demand, suggesting a predominance of N limitation from a stoichiometric perspective (Figure 2.1D). The high P loads originate from the irrigation water, which is the predominant source of water and nutrients to the ponds in this study (Figure 2.1A-D). Groundwater is unlikely

to play a role in nutrient supply as the area of study does not contain any N or P-rich lithologies[3] and groundwater plays a minor role within this network of irrigation ponds. The ditches deliver large amounts of DIN and TDP during the beginning of the growth season (Figures 1A-B), but large TDP fluxes occur throughout the growth season (mean = 0.40±0.02 mg/L, Figure 2.1A) without much variation whereas DIN supply steadily decreases from 2.32 mg/L at the beginning of June to 0.42 mg/L in August (Figure 2.1B). As a result, the ditch water DIN:TDP decreases from 12±0.9 to 3±0.1 moving from NP co-limitation towards strict N limitation [221] (Figure 2.1D).

On average, ponds contain lower concentrations of DIN and TDP than the ditch indicating that they are nutrient sinks. However, while pond DIN never exceeds ditch DIN (Figure 2.1B), many ponds contain TDP concentrations higher than ditch TDP concentration for at least part of the season (Figure 2.1A). As with other indicators, these data suggest that while available N is quickly used in these systems and converted to organic N (Figure 2.1C), available P remains in the water column because it is supplied in excess of biological demand. Almost 85% of the TDN in these study ponds is DON, compared to only about 25% of TDP being DOP, and the tight positive correlation between water column TDOC and TDN (Figure 2.2) highlights the reliance of C fixation on N supply. The relationship between TDP and TDOC is much weaker, although we note that the tendency for luxury uptake is much higher for P than N [215].

Similarly, patterns in seston and periphyton stoichiometry also suggest widespread N limitation in these 18 water bodies (Figure 2.3). Periphyton and seston accumulate P in excess of the modified N:P and C:P Redfield ratios (Figure 2.3C, D). While this finding indicates excess available P in the water column and is suggestive of N limitation, it is not direct evidence of the latter due to the inherent flexibility of P assimilation in autotrophs [207]. However, the tight relationship between periphyton/seston C and N and the close agreement with the modified C:N Redfield ratio indicates that N availability controls C fixation specifically and hence primary production within the benthos in general (Figure 2.3A). Periphyton in the study ponds tends to be more efficient than phytoplankton at ameliorating N deficiency to achieve stoichiometries closer to the modified Redfield (Figure 2.3), possibly because of periphyton's faster and/or less constrained N fixation

rates [288, 18, 184, 199] or its ability to outcompete phytoplankton for available N in small ponds [11].

2.5.1.2 Experimental Evidence

The results of the nutrient enrichment bioassay also support N limitation or NP co-limitation in many of the ponds in this study (Figure 2.5). The threshold ratios of Morris and Lewis [221] indicate P-only limitation at DIN:TDP molar ratios above 18, N-only limitation at molar ratios below 2, and NP co-limitation between 18 and 2 (Figure 2.1D). Our results were broadly consistent with these thresholds showing P-only limitation at DIN:TDP ratios above 15, N-only limitation at ratios below 4, and NP co-limitation between 15 and 4. Of the 13 ponds that exhibited nutrient limitation, 12 involved some form of N limitation (Figure 2.5). We conducted nutrient addition bottle bioassays to confirm nutrient limitation suggested by water column stoichiometry measured throughout the season. The utility of bottle-style assay results alone can be limited due to the omission of crucial ecosystem processes (large free-ranging organisms, periphyton nutrient cycling, sediment/water interactions, etc.) [276]. The results of our nutrient addition bottle assays were broadly consistent with our measures of water column, periphyton, and seston stoichiometry and reflect likely N limitation or NP co-limitation in the water columns of these study ponds. However, whole-pond nutrient addition experiments to capture the full ecosystem complexity would be advisable to inform large-scale management actions in these ponds.

2.5.2 Why is N Limitation Prevalent?

The water column data (Figure 2.1), along with periphyton and seston stoichiometry (Figure 2.3) and nutrient addition bioassays (Figures 4 and 5) all suggest that N limitation both by itself and concurrently or reciprocally with P is widespread in this system of ponds. Such N limitation is most likely maintained by consistently imbalanced inputs of P versus N nutrient sources (Figure 2.1), as illustrated by the excess available P in the water column in conjunction with low DIN. Temporal differences in N and P supply (Figure 2.1) probably occur because (1) the main source

of DIN (fertilizer runoff) is maximized at the beginning of the growth season while more of the P-rich nutrient sources (sewage, manure) occur steadily throughout the year and (2) the reservoirs that supply the ditches concentrate P and sequester/volatilize N [305]. Under such P-rich, N-poor conditions, N fixation by cyanobacteria has been reported to make up N deficits [133, 297, 181], especially as N fixing cyanobacteria are expected to dominate when water column N:P is low [277, 225, 282] and TDP is abundant. However recent work suggests this may not be the case [237, 292, 135].

N fixation often cannot keep pace with regular and consistent nutrient inputs containing large amounts of P and proportionally small amounts of N [277, 346, 147, 229, 32, 187]. Also, in our ponds N fixation appeared to be inhibited even at low NO_3^- levels (4μg/L or higher, Figure 2.6). In nearby bodies Bradburn et al. [27] found that water column N fixation was inhibited at DIN concentrations above 20μg/L and Holl et al. [126] found similar inhibitory thresholds with $NO_3{}^-$ in marine cyanobacteria. Other factors such as turbulence, stratification, light availability, and minor elements (Fe and other trace metals) may also be important to N fixation rates in these systems. While DIN concentrations were uniformly low in the ponds in this study, all ponds maintained DIN concentrations above 20μg/L DIN (Figure 2.1B and Figure A.1). In combination with low N fixation rates, low N:P ratios could be exacerbated by high levels of denitrification of NO_3^- to N_2 gas due to warm water temperatures (Table A.1) [67, 271, 186] and high TDOC (Figure A.2), which would lower bioavailable N. Mean annual rates of denitrification in the South Platte river below Denver, Colorado, are extremely high (0.51.62 g N m^-2 d^-1 [258, 295]), five or more times higher than denitrification rates documented for many other US rivers [91, 159, 247]. If N losses due to denitrification outpace N inputs to aquatic systems, N limitation can be perpetuated if P remains available to support regenerated and new production [348, 192, 238].

There is no analogous removal pathway for P. Instead, excess P can accumulate in both organic (i.e. autotrophic luxury uptake, Figures 3B and 3C) and inorganic reservoirs (i.e. sorption to sediments), and regenerate as an internal source of P. TDP periodically exceeded TDP supplied from the ditch (Figure 2.1A), suggesting concentration of P within the ponds, perhaps

via evaporation [314] or sediment sources[6]. Additionally, periphyton and seston accumulated P in excess of N (Figure 2.3B), thus preventing P burial during the growth season and reinforcing high rates of internal P regeneration. Altogether, our results suggest that these pond systems will maintain relative N deficiency because of constrained N fixation rates (Figure 2.6) combined with high potential for denitrification, high hydrologic P relative to N loads, and a longer-term capacity for sediment P regeneration.

Broadly, our study suggests that widespread increases in anthropogenic P loading can cause freshwater systems to receive nutrient inputs that are stoichiometrically enriched in P relative to N (such as the ponds in this study), and thus push aquatic ecosystems into at least proximate N limitation. In theory, this N limitation is reversible, but regeneration of P stored in sediments can prevent N and P balance over multi-year timescales [43, 313, 274, 179, 52], even after P loads loads have been decreased.

2.6 Conclusion

The bulk of policy instruments and management strategies aimed at decreasing freshwater eutrophication in the U.S. and elsewhere focus on P management [293]. However, a number of recent studies [190, 309, 47, 236, 292] have highlighted the potential need for N control. In our study system, data suggest that remediation of N inputs would decrease eutrophication in the near-term. Substantial, long-term P control might eventually switch these systems into a P-limited state, but the legacy of past P inputs is likely to last for decades [274, 313, 43], meaning that any meaningful decrease of cultural eutrophication is almost certain to require a dual-nutrient strategy. Finally, we note that even where P-only control does successfully decrease eutrophication by inducing P limitation in previously N limited systems [98, 266], it can lead to higher water-column concentrations of reactive N species with consequent downstream impacts [87].

Chapter 3

TREMATODE PARASITE INFECTION OF AQUATIC SNAILS ALTERS NITROGEN CYCLING AT THE ECOSYSTEM SCALE

Submitted: Mischler J. A., P. T. J. Johnson, V. J. McKenzie, and A. R. Townsend (2014) Trematode parasite infection of aquatic snails alters nitrogen cycling at the ecosystem scale. Ecology Letters

Abstract: While parasites frequently influence host populations and communities, their effects on ecosystem-level processes remain relatively unexplored. Here, we combine field-derived snail infection patterns and nutrient cycling with laboratory-derived measurements of snail excretion within 18 nitrogen-limited ponds. Trematode metacercariae (*Cotylurus flabelliformis*) changed host snails' storage and excretion rates of nitrogen, carbon, and phosphorus, ultimately altering pond-scale nutrient cycling. Laboratory measurements of parasite abundance and snail nitrogen excretion revealed that infection enhanced fluxes of dissolved inorganic nitrogen (DIN), which often surpassed other sources of bioavailable N to the system and contributed substantially to the location of N "hotspots". In high infection ponds, snail-mediated fluxes of nitrogen from periphyton to the water column increased, leading to higher DIN water column concentrations and greater accumulation of total dissolved nitrogen over the growing season. Our data emphasize parasites' roles as ecosystem engineers: by altering the availability of limiting nutrients, parasites could play a widespread but largely unrecognized role in ecosystem nutrient cycling.

3.1 Introduction

Single species control over ecosystem level carbon (C), nitrogen (N), and phosphorus (P) cycling has been observed in field and mesocosm studies that include both native [13, 114] and invasive [104, 53, 174] species. Individual species can substatially alter nutrient cycling by achieving high abundances [104, 114] or by regulating crucial pathways of nutrient delivery [53, 174]. However, despite their potentially high biomass, relatively little is known about how parasites influence fundamental ecosystem processes. Parasites may function as 'hidden' ecosystem engineers [108], indirectly affecting nutrient cycling through drastic alteration of host abundance (whirling disease, Dutch elm disease, etc.), modulation of host behavior [224], and changes in species compositions [13].

While noteworthy examples of parasite-altered ecosystem properties exist for invasive diseases, relatively little is known about the cryptic roles of endemic parasites despite strong effects on host nutrient regeneration [15, 22]. If such effects exist, they may be important and widespread considering the ubiquity and high biomass of endemic parasites within ecosystems [173, 257]. The life history traits of parasitic helminths (specifically digenean trematodes) suggest they may have the capacity to modulate the availability of limiting nutrients at the ecosystem scale. Digenean trematodes are extremely common in terrestrial-aquatic ecotones with a biomass density that can be as much as five times larger than other parasite groups in the same ecosystem, and comparable to or greater than that of the insect orders [173, 257]. Because they have complex life cycles exploiting various members of an ecosystem including molluscs (often aquatic snails) as the first intermediate host, trematode parasites have the potential to alter ecosystem nutrient cycling via alterations in host metabolism and excretion.

Aquatic snails are commonly the dominant consumers in benthic ecosystems, regulating nutrient flows through grazing effects and excretion/egestion [206, 104, 83]. Consumer excretion is an important component of community and ecosystem-level biogeochemical cycling and can provide a large proportion of the nutrients required by algae and heterotrophic microbial communities

(periphyton) [330]. Within ecosystems, patchy organism distributions can create locally important "hotspots" of increased rates of consumer nutrient regeneration [210]. The often high biomass of both trematode parasites and their aquatic snail intermediate hosts, coupled with parasite-induced alterations to host metabolism and excretion (Appendix B), creates a high potential for trematode parasites to regulate nutrient fluxes within aquatic ecosystems. Nonetheless, few studies have sought to directly link infection-induced organism-level changes in host nutrient regeneration with ecosystem-level nutrient cycling.

Here, we combined field, experimental, and modeling approaches to investigate the direct links between endemic parasite infection and ecosystem-level nutrient cycling in a set of ponds in eastern Colorado. We focused on trematodes in general because of their demonstrated ability to alter intermediate host nutrient regeneration (Appendix B, [15, 22]). Because our study ponds are generally N limited, we used an empirical model to link measurements of organism-level N excretion rates from field-caught snails with infection abundance data from 18 shallow ponds across a natural infection gradient. Model outputs were used to assess how important parasite-mediated N fluxes (via altered snail excretion) may be to ecosystem-scale N cycling. Additionally we examined pond-level field correlations between infection and nutrients (both within the water column and the periphyton) to evaluate the capacity of parasites to alter ecosystem-level nutrient cycling.

3.2 Materials and Methods

3.2.1 Study site

Data were collected within two adjacent groups of shallow ponds located on the plains of Eastern Colorado - the Andrick Ponds State Wildlife Area and The Teal Hunting Lodge (40° 22'16.77" N, 104° 06'24.89" W) and the Brush Prairie Ponds State Wildlife Area (40° 12'46.68" N, 103° 38'37.53" W). These ponds form within shallow depressions in the uniformly sandy soil and are replenished via irrigation ditches with agricultural runoff and South Platte River water. Aside from parasite abundance, the ponds are otherwise similar with no other variables co-varying with

infection (snail density, snail biomass, water temperature, etc.). The ponds are filled to capacity in the late spring when water is abundant and gradually evaporate throughout the summer with occasional water inputs from the ditch. Because of the constant throughput of water in the spring, water residence times are often relatively short. Field measurements were constrained to dates between the end of May and the end of August due both to the ephemeral nature of the ponds and Colorado Division of Wildlife regulations regarding management for waterfowl hunting.

3.2.2 Parasite life cycle and Infection data

Our study concentrates on the metacercarial stage of the digenean trematode *Cotylurus flabelliformis* because they are native, ubiquitous, abundant, and parasitize dominant benthic consumers (aquatic snails). Metacercariae are a larval stage of *Cotylurus flabelliformis* and part of its complex life cycle. The life cycle begins with underwater incubation of *Cotylurus flabelliformis* eggs which then hatch into miracidia that seek out aquatic lymnaeid snails as the first intermediate host [49]. Following snail infection, the parasite develops into a sporocyst which reproduce asexually and generate free-swimming cercariae. These short-lived cercariae penetrate the second intermediate host (also a lymnaeid snail [49]) and undergo a period of rapid and drastic development into metacercariae during a temperature-dependent period of 12 to 42+ days [37]. These metacercariae remain encysted in the second intermediate snail host, absorbing and sequestering host-derived nutrients until ingested by the definitive host (a range of ducks including mallards and teal [38]) wherein they develop into mature worms within 2 to 6 days, reproduce sexually, and lay eggs that are passed via feces into water bodies, completing the life cycle.

During August 2010 and 2011, we systematically collected lymnaeid snails around the perimeter of each of the 18 ponds in our study area. Snails were collected using 1m dip net sweeps every ∼20m around the entire circumference of each pond to determine snail densities. Fifty snails were randomly selected per pond (from all snails collected in each pond) for evaluation of *Cotylurus flabelliformis* metacercarial abundance. In 2011, if fewer than 50 snails were collected during the perimeter dip net sweeps, we randomly collected additional snails by hand directly from the pond

so that at least 50 snails were collected per pond. Snails were placed in buckets (one for each pond) with unfiltered pond water, transported to the laboratory, and dissected within 36 hours of collection. We measured snail shell length using calipers, crushed snail shells using pliers, and removed snail foot tissue (which is not utilized by the parasite). The remaining snail tissue was teased apart using forceps and metacercariae per snail were counted using a dissecting microscope. We also noted other larval trematode infections when present.

We collected *Stagnicola elodes* snails (115) in August 2012 from a single pond at Andrick to determine how variations in *Cotylurus flabelliformis* metacercariae abundances within snails affect snail nutrient storage and excretion. Snails were hand-selected within the same size class by height (mean = 22.22±2.33 mm) and immediately individually placed within acid-washed and DI-rinsed 60 mL plastic centrifuge tubes with 40 mL of GF/F (Whatman glass fiber filter, nominal pore size) filtered pond water. We maintained water-filled tubes containing snails and snail-free controls (water only) at epilimnetic temperatures (21-25°C) in the dark for 3 hours and then removed all snails and all water from each tube. Water containing snail excretions was filtered (GF/F) and analyzed for dissolved nutrients. Snails were dissected to determine infection intensity as above and we retained the snail foot tissue and metacercariae for nutrient analyses. We collected all feces egested during the 3 hour excretion experiment for nutrient analyses. Possible variables that could have led to an underestimation of snail nutrient excretion are discussed in Appendix C.

3.2.3 Nutrient analyses

We filtered (GF/F) weekly surface water samples from pond benthic areas into acid-washed polypropylene containers to determine the evolution of nutrients in individual ponds over the 2011 growing season. Total dissolved organic carbon (TDOC) and total dissolved nitrogen (TDN) were measured using a high temperature combustion TDOC/TDN analyzer (Shimadzu TOCvcpn, Kyoto, Japan) and Nitrate+Nitrite (NO_3^-+NO_2^-) and soluble reactive phosphorus (SRP) were analyzed colorimetrically on an Alpkem autoanalyzer (OI Analytical, College Station, TX, USA) using the cadmium reduction and the ascorbic acid method respectively. We analyzed Ammonium (NH_4^+)

colorimetrically on a Synergy 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Total dissolved phosphorus (TDP) was determined using a potassium persulfate/soduim hydroxide digestion to convert organic phosphorus to SRP and measured as above. We measured conductivity, pH, temperature, and secchi depth in situ at each pond.

Periphyton samples were collected for nutrient analyses every 30m around pond perimeters by placing movable substrates (wood, macrophytes, dead reeds) into plastic bags, adding DI water, and rigorously shaking for 30 seconds to force periphyton into suspension. For ponds with circumferences larger than 300m, we collected 10 equally spaced samples around the perimeter. The suspended periphyton was filtered onto a GF/F, dried, and evaluated for %C and %N using a Carlo Erba EA 1110 elemental analyzer (CE Elantech, Lakewood, New Jersey, USA). Samples were analyzed for %P using the extraction methods of Mulholland et al. [226] and Solorzano et al. [301] and the SRP methods described above. We filtered seston samples onto GF/Fs from 1.5 liter prefiltered (153 μm Nitex mesh) composite surface water samples, dried them, and analyzed for %C, %N, and %P as described above. Snail foot tissues were ground to a powder and analyzed for %C, %N, and %P as described for periphyton. We collected metacercariae from snails, washed them in DI water, and divided them into three subsamples for %C, %N, and %P analyses. Snail feces were ground and analyzed them for %C and %N. Water containing snail excretions was analyzed for TDOC, TDN, SRP, NH_4^+, and NO_3^-+NO_2^- as described above.

We evaluated pond nutrient limitation using a nutrient-enrichment bioassay experiment [74] in which we apportioned 200 mL aliquots of prefiltered (153 μm Nitex mesh) pond water into 250 mL glass bottles and assigned treatments (4 repetitions each): (i) a control (no nutrient added), (ii) +N (320 μmol/L N (i.e., 4480 μmol/L) as ammonium nitrate (NH_4NO_3), (iii) +P (20 μmol/L P (i.e., 620 μmol/L) as potassium phosphate (KH^2PO_4)), and (iv) +N+P. Bottles were placed under natural spectrum grow lights (20-27μmol/m2/s) and incubated in the lab at a constant temperature (25 to 26°C) for 5.5 days under a 16 h light:8 h dark cycle. All water from these bottles was filtered (GF/F) to collect phytoplankton and chl a was measured using a FluoroMax-2 200-900nm spectrofluorometer (HORIBA Scientific, Edison, NJ, USA). We determined water

column N fixation rates via the acetylene reduction method [88] by incubating pond water (30-40μmol/m^2/s at 25 to 26°C) within polypropylene syringes (3 replicates, 1 control) for 3 to 4 hours [289, 27] and measuring ethylene concentrations using a 14-A Gas Chromatograph equipped with a flame ionization detector (330°C; Shimadzu, Kyoto, Japan) and a Poropak N column (110°C; Supelco, Bellefonte, Pennsylvania, USA). Ethylene production was converted to N fixation with a 4:1 ethylenedinitrogen conversion ratio.

3.2.4 Analyses

Our analyses were designed to: (i) determine the limiting nutrient in these ponds, (ii) resolve the effects of infection on host nutrient storage and excretion and, (iii) compare observed fluxes of the limiting nutrient from infection-altered host excretion to other pools and fluxes in our pond systems to determine the impact of *Cotylurus flabelliformis* on ecosystem-level nutrient cycling. We compared organism-level nutrient excretions to ecosystem-level nutrient pools and fluxes on a per volume basis (mg/L). All statistical analyses were conducted in R 3.0.1 (R Development Team 2013). We applied appropriate transformations to the data as needed to satisfy assumptions of equal variance and normality. Uncertainties are ±1SE about the mean or median.

3.2.4.1 Assessment of nutrient limitation

We determined which nutrients were likely limiting in these pond systems by comparing field-derived values to nutrient thresholds that are commonly identified in the literature (e.g. water column DIN:TDP - Morris et al. [221]; periphyton and phytoplankton N:P - [122]), as well as by conducting experimental nutrient addition bioassays. Bioassay results were analyzed using a two-way ANOVA and subsequent post hoc pairwise comparisons to determine significant responses to N and/or P nutrient addition as well as interactions (alpha=0.05) following Elser et al. [74].

3.2.4.2 Results of laboratory excretion trials

General linear models with snail shell length as an additional independent variable were used to test the effect of metacercariae abundance on the nutrient contents of snail host foot tissues, excretions, and feces. Additionally we used a linear regression to determine the effects of parasite abundance on DIN excretion. We applied a square root transformation to TDOC excretion data to satisfy normality and a gamma distribution was used to fit the SRP excretion data. Sources of variation among these snail-based data include: (a) snail forage periphyton stoichiometry, (b) metacercarial developmental stage and rate, and (c) timing of snail foraging relative to collection. Differences between median snail nutrient content and metacercaria nutrient content as well as between median fecal nutrient content and median periphyton nutrient content were determined using the Mann-Whitney U test.

3.2.4.3 Scaling up organism-level nutrient cycling to the whole pond

We constructed an empirical model comparing parasite-induced changes in DIN excretion rates to field-derived mean time-averaged DIN pools, irrigation ditch DIN fluxes, and water column N fixation rates on a per volume (mg/L) basis. Changes in N excretion rates per parasite were estimated using the linear model developed in the excretion trials (9.6e-05 mg of DIN per metacercaria per day). We used our field-derived and pond-averaged parasite abundances (0 to 16 parasites per snail per pond in 2011 and up to 165 parasites per snail per pond in 2012) and pond-averaged snail densities (0.3 to 1.2 snails per L) as well as local "hotspot" snail densities (up to 6 snails per L in single dip net sweeps) to scale up these organism-level effects to the ecosystem-level. Snails per unit area data were converted to snails per unit volume by assuming a uniform water depth of 0.1m in the benthic area where snails were collected, consistent with measurements and observations made during field collection.

Standing stock DIN was parameterized as the mean of DIN across ponds for the entire season (0.1$\pm$0.008 mg/L DIN) and daily fluxes of N (mg/L) from N fixation (0.0009 mg N) and the

irrigation ditch (early season = 0.014 mg DIN, late season = 0.0022 mg DIN) were also included. We estimated ditch DIN fluxes per volume by assuming that 80% of the total monthly evaporation (∼22% of total pond volume) is replaced by ditch water with known DIN concentrations (derived from field measurements). This is a conservative estimate based off the gradual decline of pond volumes throughout the season and the evaporation rate derived from the May-Sept average of pan measurements from a nearby site. As these ponds occupy very shallow depressions, we assumed a 1m uniform depth for all ponds, again this value is consistent with field observations. The daily N flux (per volume) from N fixation was estimated by taking lab-derived N fixation rates and assuming a daily 10 hour window of daylight. Additionally, we looked for infection effects on nutrient cycling in the field by examining relationships between mean per pond infection abundance and water column nutrients and periphyton nutrients using linear regression; we transformed water chemistry and periphyton data to satisfy normality. A linear mixed-effects model was used to combine infection abundances and DIN water column concentrations (inverse transformation) from 2010 and 2011, with year and mean snail biomass per area used as additional independent variables and site as the random effect.

3.3 Results

3.3.1 Nutrient Cycling

A comprehensive presentation and discussion of nutrient cycling in these ponds is described in Mischler et al. [214]; here we briefly summarize the nutrient cycling data as context for their links to parasite dynamics. Nutrient conditions were broadly consistent in indicating some degree of N limitation across ponds, especially during late summer (Figure 3.1A-B). DIN:TDP ratios of ditch supply water were within N and P co-limitation thresholds throughout the growing season (Figure 3.1A, [221]) and in most ponds the DIN:TDP ratios strongly decreased throughout the season (Figure 3.1A), driven by a decline in DIN concentrations (Figure 3.1B). Phytoplankton and periphyton both commonly exhibited luxury uptake of excess P [122] (Figure 3.1C). In experimental

bottle assays, 12 of the 13 ponds that responded to nutrient addition exhibited some degree of N limitation. N fixation rates averaged 0.065±0.004 μmol/L/day across ponds, consistent with other measures of low N fixation from adjacent water bodies [27].

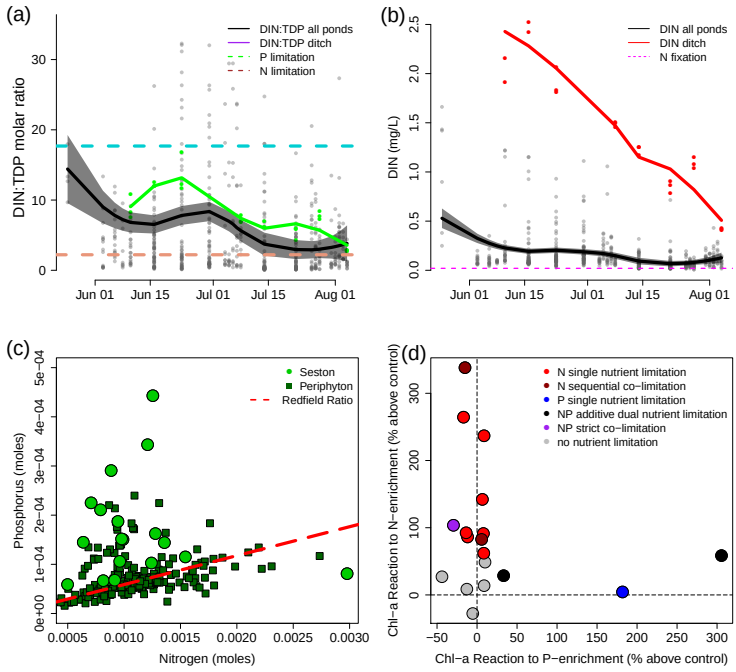

Figure 3.1: Evidence indicating widespread nitrogen limitation across ponds from water column DIN:TDP stoichiometry (a), water column DIN concentration (b), periphyton and phytoplankton N:P stoichiometry (c), and the results from nutrient addition bottle assays (d). Water column DIN:TDP stoichiometries (molar ratio) are compared to thresholds from Morris et al. [221] indicating NP co-limitation (between 18 and 2.2) and N only limitation (less than 1). Water column DIN concentrations are compared to the nitrogen fixation threshold determined by [27] in adjacent systems; nitrogen fixation is suppressed by DIN concentrations above 0.02 mg/L. Periphyton and phytoplankton N:P stoichiometries are compared to the modified Redfield Ratio of Hillebrand et al. [122] to determine which of the two elements are in relative excess. Bottle assay limitation statuses are determined according to Elser et al. [74].

3.3.2 Field Infection data

Lymnaeid snail host densities were highly variable at the local scale within and across ponds, ranging from 0 to 453 snails per m^2 (mean = 23±2.7). Mean snail densities per pond ranged from 0 to 123 snails per m^2. Across all ponds the average number of metacercariae per snail by pond

ranged from 0 to 16 parasites per snail in 2011, while a single pond surveyed in 2012 averaged 165 metacercariae per snail. Metacercariae (C:N:P = 101:13:1) were stoichiometrically distinct from their snail hosts (80:16:1) with metacercariae tending to be C-rich and P-rich/N-poor compared to snail hosts (Figure 3.2). %N (Figure 3.2B) and N:P (Figure 3.2E) were significantly higher (37%, 19%) in snails (9.6±0.2, 7.0±0.2) versus metacercariae (7.0±0.7, 5.9±0.6; p = 0.01, 0.03) while C:P (Figure 3.2D) and C:N (Figure 3.2F) were significantly lower (19%, 32%) in snail hosts (31.5±0.3, 4.5±0.06) versus metacercariae (38.7±3.0, 6.6±0.2; p = 0.01, 0.003).

Because parasites and snail hosts have different nutrient stoichiometries, parasites will preferentially take up host nutrients in accordance with their own nutrient demands (Appendix B), which in turn affects host nutrient stoichiometry. In the laboratory excretion trials, increased *Cotylurus flabelliformis* abundance correlated with changes in nutrients within host excretions (Figure 3.3A-C) and host foot tissues (Figure 3.3D-F). Host shell height was not correlated with host tissue or host excretion nutrients in any of the GLM models. Host TDOC and SRP excretion rates decreased with increasing parasite abundances (R^2=0.09, p=0.005; R^2=0.06, p=0.02) while host DIN excretion rates (Figure 3.3B) increased with parasite abundance (R^2=0.09, p=0.005). Increased N excretion led to a decrease in host foot tissue %N (Figure 3.3B) with parasite abundance (R^2=0.09; p=0.007). There were no significant effects of infection on fecal %C (Figure 3.3G) and %N (Figure 3.3H) content or fecal C:N stoichiometry (Figure 3.3I). Fecal C:N was 11% higher than periphyton C:N.

3.3.3 Parasitism and Ecosystem-Wide Nutrient Cycling

Because of widespread evidence for N constraints across these ponds, standing stocks and fluxes of bioavailable N (DIN) were compared to parasite-induced release of DIN from host snails to determine the relative importance of *Cotylurus flabelliformis* to ecosystem nutrient cycling. For the highest 2011 pond-wide mean infection abundances (16 metacercariae per snail), additional daily DIN released via infection ranges from 0.5% to 1.8% of standing N stocks on a per volume basis, depending on snail densities (0.3 to 1.2 snails per L; Figure 3.4). When we consider "hotspot"

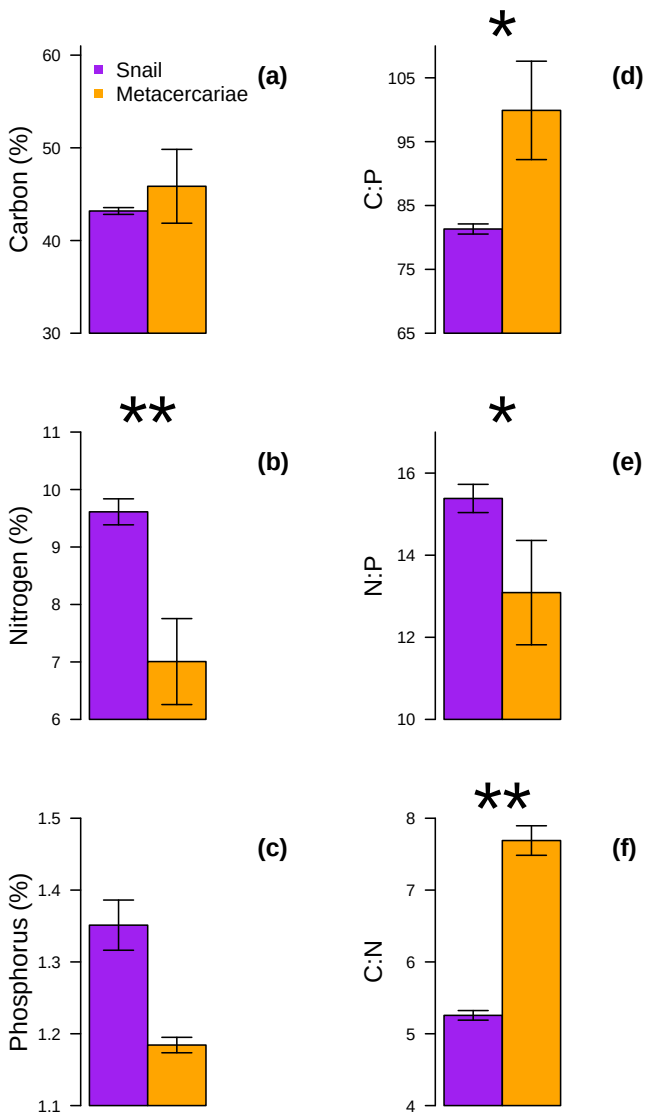

Figure 3.2: Field-collected per snail metacercariae abundance data (second stage larval trematode parasites) from field sites in Colorado. Median (1 SE) %C (a), %N (b), %P (c), C:P (d), N:P (e), and C:N (f) for metacercariae-free snail foot tissue (purple) and metacercariae alone (orange). Asterisks indicate a significant difference ($p < 0.05$ (*) and $p < 0.01$ (**)) between snail foot tissue nutrients and metacercariae tissue nutrients as determined by the Mann-Whitney U test. All percentages are based on dry mass and all ratios have been converted to molar ratios.

snail density values, additional parasite-induced N releases can exceed 9% of standing stocks. For higher metacercarial abundances (165 metacercariae per snail per pond was observed in 2012, and

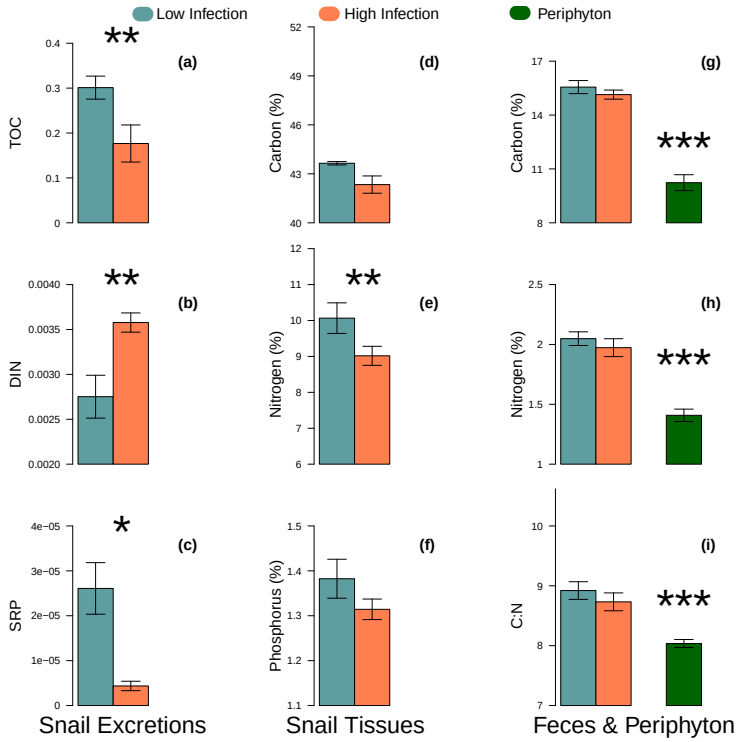

Figure 3.3: Median (± 1 SE) mg of TOC-C (a), DIN-N (b), and SRP-P (c) excreted per snail per day by snails in both the the high infection (red) and low infection (blue) groups during the excretion trial as well as median (± 1 SE) %C (d), %N (e), and %P (f) within snail foot tissues. Asterisks above the high and low infection group bars indicate a significant difference ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)) between snail excretion and tissue nutrients of the high and low infection groups as determined by the Mann-Whitney U test. The right-most column contains medians (± 1 SE) for %C (a), %N (b), and C:N (c) of snail feces egested during the 3 hour excretion trial by snails in both the the high infection (red) and low infection (blue) groups as well as the median (± 1 SE) periphyton (green) among all ponds. Asterisks above the periphyton bars indicate a significant difference between snail egesta nutrients in both the high and low infection groups combined and periphyton nutrients as determined by the Mann-Whitney U test. There were no significant differences in egesta nutrients between the low and high infection groups. All percentages are based on dry mass.

far higher values are reported from comparable systems (e.g. [38])), infection-induced changes in DIN excretion is 4.8% to 19% of standing stock DIN for pond-averaged snail densities and up to 95% of standing stock for 'hotspots". For comparison's sake, daily N supplied by water column N fixation is about 0.9% of standing stock while ditch-supplied DIN ranges from 14% to 2.2% of standing stock in the early season and the late season respectively.

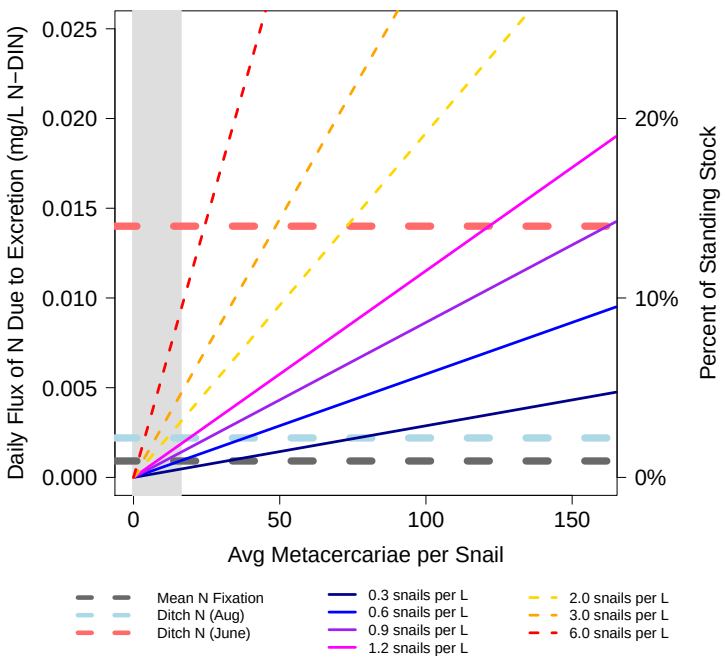

Figure 3.4: Model results quantifying the additional DIN-N excreted by snails due to the effects of Cotylurus flabelliformis metacercariae infection. The model was ran for a range of average infection intensities (x axis) and snail densities (colored lines) from within the range of field-derived values. Pond-averaged snails densities are denoted by solid lines and "hotspot" snail densities by dashed lines. Results were compared to average nitrogen fixation rates (dashed gray line), ditch nitrogen supply at the beginning of the season (dashed red line), ditch nitrogen supply at the end of the season (dashed blue line), and average standing stock of DIN in the water column across ponds. The shaded region denotes most pond-averaged infection abundances from 2011 whereas the maximum value on the x-axis denotes the lone pond-averaged abundance value from 2012

Field relationships between *Cotylurus flabelliformis* abundance and water column and periphyton N were also examined. In August 2010 and 2011 pond-wide mean metacercariae abundance positively correlated with water column DIN as measured in shallow benthic areas of the ponds ($p=0.003$, $R^2=0.39$; Figure 3.5A). DIN concentrations did not depend on snail biomass or year ($p>0.05$). Ponds with higher infection also tended to accumulate significantly more water column N (TDN) during the growing season ($p=0.045$, $R^2=0.18$; Figure 3.5B). The additional water column N in high-infection systems is most likely N previously immobilized in periphyton that was subsequently mineralized by snail grazing as indicated by reduced periphyton %N in high infection

ponds (p=0.018, R^2=0.275; Figure 3.5C). This is corroborated by the water column DIN data which show a negative relationship between water column DIN and periphyton %N (p=0.036, R^2=0.21; Figure 3.5D).

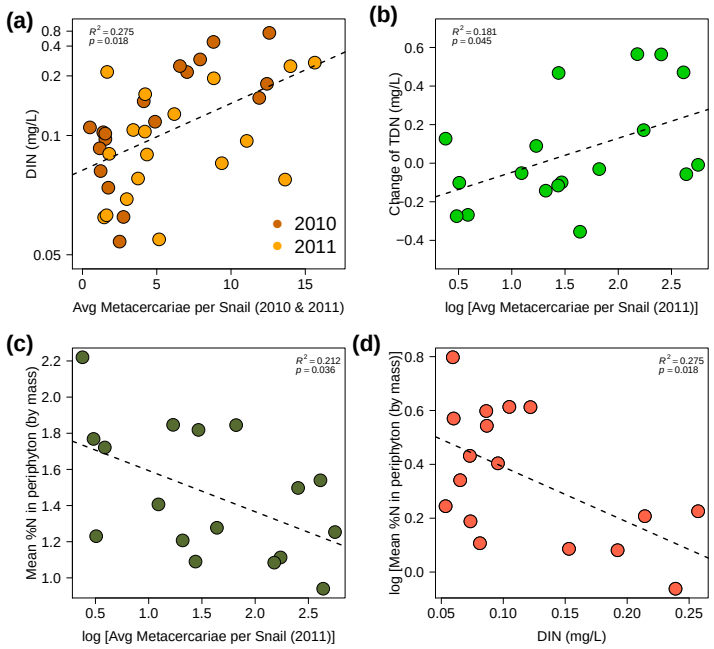

Figure 3.5: Relationships between pond-averaged snail infection intensity with Cotylurus flabelliformis metacercariae and water column DIN (a), change in water column TDN from the first two weeks to the last two weeks of the measuring interval (b), and periphyton %N (by mass) per pond as well as the relationship between water column DIN and periphyton %N (d). Data were transformed to satisfy normality and relationships were evaluated using linear regression. For the relationship between DIN and average snail infection intensity (both in 2010 and 2011 for the same sites) a linear mixed effects model was used with pond as the random variable. R^2 and p values are displayed in their respective plots.

3.4 Discussion

Benthic grazers in general [193] and aquatic snails in particular [206, 83] can control (and even dominate - [104]) nutrient cycling through grazing and excretion effects, which become intensified in biogeochemical "hotspots" [330]. However, the potential for parasites to affect ecosystem-level nutrient cycling through changes in the metabolism and excretion (Appendix B) of their aquatic

snail hosts is largely unexplored. In the context of invasive species, Vitousek et al. [331] outlined the conditions under which a single species would be most likely to affect ecosystem level processes. First, that species must have physiological or life history traits that modulate an element limiting to plant or microbial activity, and second, the species must have a sufficiently large biomass and/or distribution to have such modulation alter ecosystem-level budgets of the limiting element. In this study *Cotylurus flabelliformis* metacercariae accelerate host N excretion rates, causing enhanced fluxes of N from the periphyton to the water column - where N is in high biological demand (Figure 3.1; [214]). Sufficiently high host densities and parasite abundances enable these organism-level N fluxes to influence pond-level nutrient cycling under N-limited conditions.

For infection to alter N cycling, it must first change the ways in which N is stored and excreted from the host organisms. Such changes in host nutrient excretion and storage can be caused by physiological alterations associated with infection including: (i) differential nutrient demand between parasite and host, (ii) increased stress/metabolic demand, and (iii) utilization of alternative metabolic pathways to sustain parasite and host (Appendix B). Differences in nutrient stoichiometry between parasites and hosts govern nutrient flows both between hosts and parasites and ultimately between host/parasite systems and the larger ecosystem (Appendix B). Larval trematode parasites typically exhibit a larger proportion of C-rich carbohydrates (specifically glycogen) in relation to their hosts [36, 33], C-reserves which they draw directly from their hosts [106].

Cotylurus flabelliformis metacercariae (C:N:P = 100:13:1, Figure 3.2) appear to have an enhanced demand for C and a reduced demand for N compared to the snail host (C:N:P = 82:16:1, Figure 3.2) based on stoichiometric differences (Appendix B). In addition, the density of metabolic units (MUs) per unit mass within the snail-metacercariae phenotype increases with metacercariae abundance (Appendix B), thereby increasing C demand for glycogenolysis to maintain a higher metabolic rate and P demand for construction of P-rich MUs (Appendix B). As a result, infected snails excrete comparatively less C and P to the environment (Figures 3A and 3C). The physiological stress from infection likely further accelerates metabolic rate (Appendix B). The end result of these

effects is an increasing metabolic rate and an increasing demand for metabolically-available C (and possibly P) with increasing parasite abundances.

By contrast, infected snails excrete significantly more bioavailable N to the surrounding environment (Figure 3.3). Within this C-starved snail-metacercariae phenotype, the increase in N excretion (Figure 3.3B) and the reduction in snail tissue %N (Figure 3.3E) associated with higher infection is consistent with increased protein catabolism for use in gluconeogenesis in response to elevated metabolic demand (Appendix B). As host proteins are catabolized to maintain the elevated metabolic state of infection, most of the nitrogen-rich fraction of these proteins is not required by the parasite for growth and maintenance, and thus is excreted into the environment. Indeed, snail tissue %N decreases faster than snail tissue %C with increasing infection (figure E) indicating a highly mobile and metabolically-available N pool within the snail tissue. Fecal C:N ratios were significantly higher than periphyton C:N ratios indicating higher assimilation rates of N versus C from the periphyton due to non-metabolically available C fractions (lignin, etc.) in the periphyton [35].

Taken as a whole, our excretion trial data, combined with the infection field survey, suggests that parasite-induced changes in host nutrient cycling and storage may alter ecosystem-level nutrient cycling. Measurements of nutrient pools *in situ* (Figure 3.1A-C) along with nutrient addition bioassays (Figure 3.1D) indicate high biological N demand across our ponds, while our excretion trials highlight increased host N (as DIN) excretion rates as a result of increasing parasite abundances (Figure 3.3B). However, the overall influence of parasites on ecosystem-level N cycling will depend on the density of infected hosts, parasite abundances within hosts, and the relative magnitude of the infection-induced N flux compared to other pools and fluxes of N in the system. Water column DIN pools for these ponds are low, with most ponds staying below 0.2 mg/L DIN for the entire growing season (Figure 3.1B). Major fluxes of DIN into these systems are constrained to the ditch water inputs (Figure 3.1B) and nitrogen fixation (Figure 3.1C). Internally the rate of conversion of organically stored periphyton N to bioavailable DIN in the water column depends on dominant primary consumer (aquatic snails) grazing and excretion rates.

In order to explore the potential effects of parasite infection on ecosystem-level N cycling, we used a simple model that combines the empirically-derived relationship between snail infection, metacercarial abundance and N excretion (Figure 3.4) with data on snail density. For 2011, pond-averaged metacercarial abundances and pond-averaged snail densities result in parasite-mediated N flux rates to the water column that are as much as two times greater (depending on snail density) than N contributed via water column N fixation and 23% to 82% of N contributed by the ditch at the end of the growing season (Figure 3.4). Moreover, "hotspot" snail density values can contribute parasite-mediated N at rates up to 10 times greater than N fixation and over 4 times greater than late season ditch N (Figure 3.4).

Moreover, metacercarial abundance in 2011 was on the low end of ranges reported in the literature; reports exist of infection rates up to 1,000 metacercariae per snail [38]. Indeed, in 2012 metacercarial abundances were much higher (165 metacercariae per snail per pond), and when coupled with pond-averaged snail densities generated parasite-mediated N supply rates 20 times greater than water column N fixation, over 8 times that of late season ditch N, and 35% more than early season ditch N. Under these infection rates, maximum "hotspot" estimates were over 100 times that of N fixation, 43 times late season ditch N, and 6.8 times early season ditch N (Figure 3.4). Thus, while the overall effect will undoubtedly vary with snail density, infection rate and fluctuations in metabolic activity, our calculations strongly suggest that metacercarial infection of aquatic snails can substantially alter the N cycle. We note that such alteration does not introduce new N into the ecosystem, but it does greatly increase the rate at which internal sources of N become available in the water column, in effect creating an acceleration that results in a richer N cycle.

If parasite-mediated N release is a major source of bioavailable N to these ponds, this should be apparent in pond-level measurements. At the pond scale, water column DIN increased with infection intensity in 2010 and 2011 (Figure 3.5A) resulting in increased TDN accumulation in the water column across the season (Figure 3.5B), consistent with a parasite-mediated N source. In addition, periphyton %N decreased with increasing infection intensity (Figure 3.5C) and is inversely

related to water column DIN (Figure 3.5D). Snails with higher metacercarial abundances free up periphyton-stored N at a faster rate (Figure 3.3B). If infection-induced compensatory feeding rates (Appendix B) outpace the periphyton's ability to assimilate N (through N fixation or uptake from sediments), then N will shift from the periphyton pool (decrease in %N - Figure 3.5C) to the water column (increase in DIN - Figure 3.5A), transforming a pond's N budget from negative (due to N drawdown across the season - Figure 3.1B) to positive (Figure 3.5D) over the growing season. Thus, *Cotylurus flabelliformis* metacercariae appear to be ecosystem engineers in these ponds [108], acting as "nutrient pumps" [83] that increase the N flux from the periphyton pool to the water column pool. In turn, water column N enrichment could enhance the ability of these parasites to infect, reproduce, and spread through direct (snail forage quality/quantity, pond attractiveness to waterfowl) and indirect (changes in biodiversity, species shifts, interspecific and intraspecific competition, etc.) means [211].

3.5 Conclusion

Here we have presented a system where parasite-induced changes to host metabolism modulate the flux of a limiting nutrient (N). These parasite-induced changes can produce nutrient cycling "hotspots" which in turn exert a significant influence at the community and even ecosystem level (Figure 3.4). We note that the effects we report here are from a common larval form (metacercaria) of a native parasite species (*Cotylurus flabelliformis*) found throughout North America. Because of the pervasiveness of parasites in a variety of ecosystems [173], many such systems may contain 'hidden' effects of parasites on nutrient cycling. While multiple factors must converge to translate organism-level effects of infection to the ecosystem level, such situations are not expected to be rare. Because parasites cause many human and livestock diseases worldwide (malaria, schistosomiasis, fascioliasis, etc.) they are often targeted for eradication programs, but this work suggests that eradication of parasites from ecosystems may have unintended effects on ecosystem structure and function.

Chapter 4

EFFECTS OF ENVIRONMENTAL CHANGE ON A MOLLUSCAN-TREMATODE COMMUNITY OVER A LONG-TERM PERIOD IN HIGH ELEVATION AQUATIC ECOSYSTEMS (CRESTED BUTTE, CO)

Abstract: Currently, various environmental changes are occurring throughout diverse ecosystems and at multiple spatial and temporal scales. However, few long-term studies have been conducted to evaluate the effects of environmental change on parasites, even though the prevalence and abundance of parasites in ecosystems can have large effects on disease risk as well as broad-scale ecosystem structure and function. Complex life cycle parasites (such as digenean trematodes) are particularly susceptible to the effects of environmental change due to their reliance on multiple hosts across different trophic levels to complete their life cycles. A long-term study of snail-trematode communities was conducted at two high elevation sites (Lake Grant and the Kettles) spanning the last 50+ years. During this time interval, Lake Grant has undergone significant anthropogenic disturbance (damming, urban encroachment, golf course installation) while the Kettles have been used for cattle grazing throughout. At the same time, climate trends indicate a broad-scale spring warming and advancement of the first date of bare ground in the spring at these sites. The aim of this study was to compare the prevalence of trematode parasites in aquatic snails (the first intermediate host) across sites and across eras (past vs. present) to evaluate the effects of environmental change on snail-trematode communities. Snails were sampled in 1957, 1958, 1959, 2011, and 2012 and examined for trematode parasites. Snail populations shifted over time, with Physids becoming much

more common in the present at both sites, possibly because of their high tolerance of disturbance and general environmental preferences. Snail biodiversity increased at the Kettles and decreased at Lake Grant over time. Trematode prevalences responded to year to year changes in mean weather conditions, with higher prevalences in years with warmer springs and earlier snow-free dates as well as increased trematode community stability in higher diversity snail host communities. Also, unprecedented avian schistosome diversity has been observed at this high elevation site, with diversity changing with mean annual weather conditions. These results indicate that snail-trematode host communities are susceptible to environmental change and disturbance. Anthropogenic activities may have substantial and long-lasting effects on disease risk and ecosystem structure and function through modulation of host-trematode communities.

4.1 Introduction

Parasites have long been known to be pathogenic organisms capable of causing disease in their hosts [90, 153, 103]. However, there is a growing body of evidence indicating that parasites also fulfill important ecological roles [318, 46, 136]. Parasites often influence ecosystems through alterations in their hosts (e.g. host behavior [176, 319, 21, 182], host metabolism and nutrient cycling [15, 183, 22], host reproduction [304], etc.). The roles of parasites in food webs [175] are also receiving increased attention due to their large collective biomass, especially in aquatic systems [173, 257].

While trematodes can exert strong controls on host behavior and physiology (and hence ecosystems), the abundance and distribution of potential host organisms can in turn determine parasite abundance and biodiversity [115]. Therefore, environmental variables (e.g. weather, pollution, disturbance, predation, etc.) that affect host phenology, biodiversity, distribution, and abundance, or parasite transmission efficiencies [316], will ultimately affect parasite prevalence [34, 296]. Parasite responses to environmental changes have the potential to enhance the effects of environmental change on ecosystem structure and function as well as alter disease risk [241], and thus have implications for ecosystem management.

Shifts in environmental factors disproportionately affect complex life cycle parasites due to their reliance on multiple hosts spanning across various trophic levels [107]. The life cycles of digenean trematode parasites include a first intermediate host (usually an aquatic snail) and a vertebrate definitive host (within which the adult parasite matures and reproduces). In many trematode species additional invertebrate or vertebrate intermediate hosts are used. Parasites are transmitted from the first intermediate host snail to the next host via a free-swimming larval parasite stage (the cercaria). Ultimately, trematode eggs are released from the definitive host to the ecosystem (via feces, etc.) where they hatch and infect an aquatic snail to complete their life cycle. The occurrence of one trematode species indicates the presence of all other organisms in its life cycle as well as the conditions required for transmission [201]. Accordingly, aquatic snail communities with high trematode prevalences and biodiversity indicate high diversity and abundance of free-living organisms in the same ecosystem [142, 141]. Any environmental changes that affect host biodiversity, abundance, and distribution, or trematode transmission, will affect trematode prevalence and diversity in snail hosts and possibly inhibit the long-term stability of snail-trematode communities (which likely play a role in overall ecosystem health [50]).

For example, in the Carpinteria Salt Marsh (California USA) wading bird diversity is correlated with trematode diversity in aquatic snails [115]. Habitat restoration in an area of this salt marsh in 1997 (improved tidal flow, channel construction, and replanting native estuarine vegetation) resulted in the quadrupling of trematode prevalence in snails over 6 years [142]. Similarly, the environmental effects of Hurricane Isidore decimated trematode communities in a coastal lagoon (Yucatan Peninsula, Mexico), with fish-snail-trematode communities taking several years to fully recover from the disturbance [1]. Morely et al. [220] observed a large decrease in trematode prevalences in snails in response to the dredging of a UK canal, with prevalences exhibiting only a modest recovery over the next 12 years. The long-term (1936 to 1994) study of Keas et al. [157] in a northern Michigan lake detailed decreases in trematode diversity and prevalence in aquatic snails due to changes in vertebrate host diversity and abundance caused by the effects of gradual urban encroachment.

Because host-trematode community recovery times can be long, and environmental changes can be either abrupt or gradual, long-term studies examining changes in trematode communities in response to environmental change are are needed. Here we investigate changes in trematode prevalences and trematode morphotype diversities between the summers of 1957, 1958, and 1959 and 2011 and 2012 at 2 high elevation (2752m and 2877m) sites located around the town of Crested Butte, CO. One site (Lake Grant) has experienced a large amount of residential construction as well as the building of a golf course and the installment of a dam, while another site (Kettles), is located on protected land and has seen very little direct anthropogenic disturbance over the last 50+ years.

These ponds were first surveyed for digenean trematodes in aquatic snails as part of a study by [140] in the summers of 1957, 1958, and 1959 in response to an isolated and rare (for that time period) outbreak of cercarial dermatitis in 1956 in local townspeople at Lake Grant [138]. These cases of cercarial dermatitis developed after larval trematode parasites (avian schistosome cercariae, family *Schistosomatidae*), mistakingly penetrated human skin instead of bird skin [167]. Each penetration site develops into an irritated red swelling due to an allergic response. Since the work of [138], reports of schistosomes in the Southwest U.S. have been sporadic [139, 243]. Recently, [29] conducted a survey of water bodies in New Mexico and Colorado and found a wide variety of avian schistosomes present throughout that state. Additionally, two heavily used Colorado swimming areas (Prospect Lake and Cherry Creek Reservoir) have recently experienced outbreaks of cercarial dermatitis. In addition to measuring schistosome prevalence, we also have generated the first known record of high elevation schistosome species using molecular techniques to identify schistosome species at these sites.

Materials and Methods

During the summers of 2011 and 2012 snails were collected within the vicinity of the Rocky Mountain Biological Laboratory (RMBL) at Gothic and the town of Crested Butte, both in Gunnison County, Colorado. This study is a continuation of an earlier study by Hunter [140] which

was conducted during the summers of 1957, 1958, and 1959. Hunter [140] collected 3,491 snails of three different species from Lake Grant and the Kettles and examined them for larval digenean trematode parasites across each of the three years' growing seasons (June to August). Lake Grant and the Kettles were chosen as sites for revisit in this study because they could be unambiguously located using information from Hunter [140]. In our current study 3,886 snails of three different species were collected 50+ years later across the summers of 2011 and 2012 (June to August) from these same 2 sites and evaluated for larval digenean trematode parasites. Additionally, we extracted DNA from all schistosomes collected from our study sites and sequenced them to determine schistosome species identities. All snail collection and examination methods are modeled after Hunter [140] to ensure comparability between the two studies. Also, we collected water samples from all water bodies surveyed, and evaluated them for total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP). Temperature, conductivity, and pH were measured in-situ. Climate data were gathered from RMBL and the National Oceanic and Atmospheric Administration (NOAA), bird arrival data were obtained from RMBL, and bird diversity and abundance data were obtained from the Breeding Bird Survey (BBS) and the Christmas Bird Count (CBC). All statistical analyses were completed in R (version 2.15.1) and data were transformed when needed to satisfy the conditions of selected statistical tests.

Site Description

Collections were made from two sites: (1) Lake Grant (38.868268°W, −106.947508°N, 2752 m) and (2) a set of four adjacent kettle ponds at RMBL (together called "Kettles"; pond 1 = 38.943205°W, −106.976719°N; pond 2 = 38.944766°W, −106.976054°N; pond 3 = 38.944866°W, −106.977427°N; pond 4 = 38.946109°W, −106.975614°N; 2877 m). Lake Grant has undergone considerable anthropogenic alterations in the last 50+ years. During the study of Hunter [140] Lake Grant was a shallow 1.2 ha spring-fed lake surrounded primarily by grazing land. Currently Lake Grant has been expanded to nearly 8.5 ha via the construction of a dam and is surrounded by a housing development and golf course. In contrast, the Kettles are located on protected

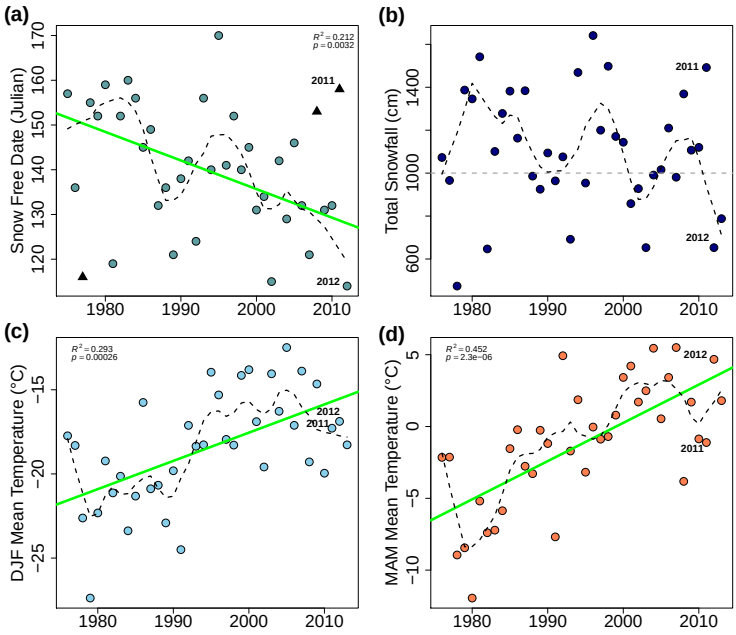

Figure 4.1: **Climate data from Rocky Mountain Biological Lab (1975 to 2013).** Advance of the first date of bare ground during the spring thaw (snow-free date, p=0.0032, R^2=0.212). Triangles mark points that were excluded from the linear regression analysis because they were outside Cook's Distance, if points are included relationship is no longer significant. (a), Total annual snowfall (Sept to June) (b), Increase in the mean December, January, and February temperature (p=0.00026, R^2=0.293) (c), Increase in the mean March, April, and May temperature (p=2.3e-06, R^2=0.452) (d). All date were collected by the RMBL station manager.

RMBL land and thus have remained largely unchanged since the late 1950s. The Kettles are a series of four small vernal pools ranging from 0.06 ha to 2.37 ha and up to 0.3 meters in depth. These ponds are fed by snow-melt and runoff from the surrounding slopes and are heavily utilized as water sources for grazing cattle. All water bodies provide feeding and nesting habitats for a variety of birds, with Mallards (*Anas platyrhynchos L.*), Spotted Sandpipers (*Actitis macularia L.*), Redwing Blackbirds (*Agelaius phoeniceus*), and Brewer's Blackbirds (*Euphagus cyanocephalus Wagler.*) commonly appearing in the 1950s and Mallards and Great Blue Herons (*Ardea herodias*) being commonly seen currently.

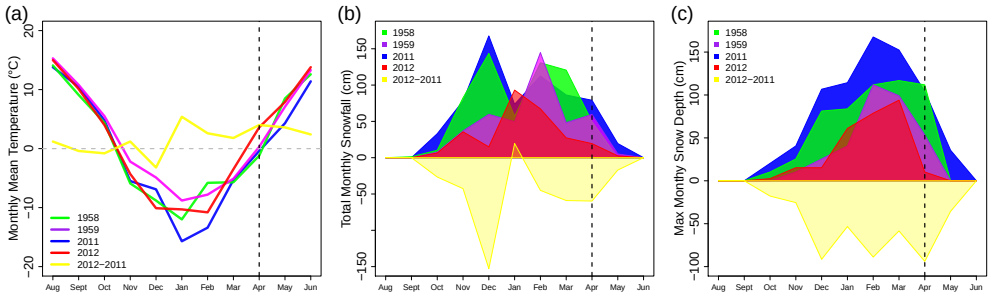

Figure 4.2: **Weather data (NOAA) for Crested Butte, CO from 1957/58 (green), 1958/59 (purple), 2010/11 (blue), and 2011/12 (red) (Aug to June).** Mean monthly temperature values. Jan to June of 2012 was warmer than Jan to June of 2011 (yellow line) (a), Mean monthly snowfall. 2011/12 snowfall was lower than 2010/11 snowfall (except for Dec, yellow polygon) (b), Maximum monthly snow dept. 2011/12 snow depth was lower than 2010/11 snow depth for every month (yellow polygon). April snow depth in 2011 was much deeper than in 2012 (black dashed vertical line) (c)

Climate Data

Climate data (mean monthly snowfall, max monthly snow depth, and mean monthly temperature) were downloaded from the NOAA National Climate Data Center (NCDC) for Crested Butte, CO for the time interval of 1933 to 2013. Also climate data from from RMBL (snow free date, mean monthly temperature, mean monthly snowfall) were obtained for the time interval from 1975 to 2013. The NCDC Crested Butte data were used to compare year-to-year weather conditions during the collections of [140] (1957/1958 and 1958/1959) and this study (2010/11 and 2011/12). We also evaluated the RMBL climate data for long-term trends via linear regression. Any data outside Cook's distance were omitted from the analyses to exclude extreme values that exerted disproportional leverage on the regression analysis. Where data are removed from the analyses, significance is discussed both with and without these data.

Water Nutrients

During each of the four collection time periods (late June, early July, late July, and early August), surface water samples were collected in triplicate from Lake Grant and the Kettles in both 2011 and 2012. Water was filtered with a GF/F (Whatman glass fiber filter, nominal pore size)

into acid-washed polypropylene containers and frozen for later analyses. All surface water samples were analyzed for total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP). TDN was determined in all samples using a high temperature combustion TDOC/TDN analyzer (Shimadzu TOCvcpn, Kyoto, Japan). Total dissolved phosphorus (TDP) in water samples was determined by a potassium persulfate/sodium hydroxide digestion to convert organic phosphorus to soluble reactive phosphorus (SRP) and SRP was measured colorimetrically on an Alpkem autoanalyzer (OI Analytical, College Station, TX, USA) using the ammonium molybdate ascorbic acid methods [172]. TDN and TDP concentrations were combined across 2011 and 2012 for each site and compared between sites using a Student's t test.

Snail Collection, Dissection, and Infection

Snails were collected in accordance with the methods of Hunter [140] to ensure comparability between studies. Collections were made during each of four time periods during the summers of 2011 and 2012: late June, early July, late July, and early August. Snails were collected by walking around the circumference of the ponds or wading and picking up snails from the pond bottom, vegetation, or rocks. Snails were placed in buckets and returned to the lab where their shells were crushed with pliers within 36 hours of collection and their tissues dissected under a dissecting microscope using forceps in search of trematode cercariae. Trematode cercariae were collected and identified to morphotype using a compound microscope according to [275]. Whenever schistosome cercariae were encountered, trematode tissue specimens were collected using a new low density polyethylene disposable pipette and placed within vials and preserved using 90% Ethanol for later genetic identification.

Year by year differences in snail infection risk were analyzed for Lake Grant and the Kettles separately using logistic regressions and subsequent pairwise comparisons (Tukey's HSD). The exact identities of individual kettle ponds were not clear from the information contained in Hunter [140], thus all 4 kettle ponds were combined into the single "Kettles" site. We also compared the odds of snail infection across sites (Kettles vs. Lake Grant) via a logistic regression (interaction between

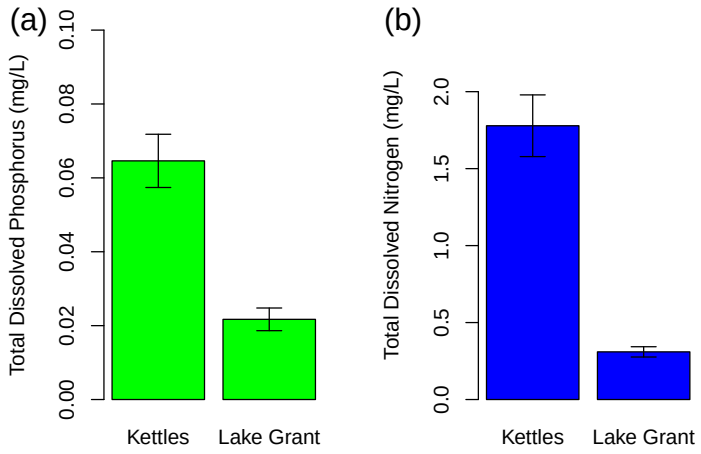

Figure 4.3: **Water column dissolved nutrient data from 2011 and 2012 water samples from Lake Grant and the Kettles.** Total dissolved phosphorus water column concentrations (a), Total dissolved nitrogen water column concentrations (b), Both differences are significant ($t(9)=3.0365$, $p < 0.05$; $t(7)=4.0073$, $p < 0.05$) and error bars signify $\pm$1SE.

era ([140] vs. this study) and water body (Kettles vs. Lake Grant)) and subsequent pairwise comparisons (Tukey's HSD). Time series' of snail infection prevalences were constructed for total trematode infection as well as schistosome-only infection in 2011 and 2012 to match the 1959 time series of Hunter [140].

We also evaluated changes in parasite morphotype and snail biodiversity. Hunter [140] recorded three snail species across all ponds - *Physa propinqua* (now *P. gyrina* or *P. acuta*), *Helisoma trivolvis* (now *Planorbella trivolvis*), and *Lymnaea palustris* (now *Lymnaea (Stagnicola) elodes*) - belonging to three different families (*Physidae*, *Planorbidae*, *Lymnaeidae*). In the present era, four distinct snail species were found - *P. gyrina*, *P. acuta*, *Gyraulus parvus*, and *L. elodes* - belonging to three different families (*Physidae*, *Planorbidae*, *Lymnaeidae*). However, *Gyraulus parvus* was omitted from analyses as they are small (2 or 3 mm diameter) and their distribution sporadic in space and time, thus it is unclear if the methods of Hunter [140] would have been sufficient to

locate these snails. In the current era, the only water bodies that contained more than one species of snail were the Kettles (*P. acuta* and *L. elodes*), thus snail and infection data from the Kettles were used to determine changes in snail diversity between 2011 and 2012 (years with very different environmental conditions) as well as the effects of snail identity on infection success. We used a proportion test to determine if relative abundances of *P. acuta* and *L. elodes* varied between 2011 (high snowfall, late snow-free date) and 2012 (low snowfall, early snow-free date). Additionally, because snail family has the potential to affect infection rates, a logistic regression with pairwise comparisons (Tukey's HSD) was used to determine if the odds of a snail becoming infected in the Kettles is dependent on family (*Physa* vs. *Lymnaea*), and if this dependency varies by weather conditions (2011 - high snowfall, late snow-free date; 2012 - low snowfall, early snow-free date). A logistic regression with pairwise comparisons (Tukey's HSD) was also used to determine if snail family affects infection success among trematode parasite morphotypes within the Kettles. We also looked separately at the odds of schistosome infection in all snails in all ponds by year using a logistic regression with pairwise comparisons (Tukey's HSD). We used the ShannonWiener index to evaluate changes in parasite morphotype diversity across years for both Lake Grant and the Kettles.

Bird Data

Bird data were collected from RMBL, the BBS (number of birds seen or heard along a 24.5 mile route), and the CBC (number of birds seen in a 24 km diameter count circle per party hours). Each spring (beginning in 1975) the arrival dates of 16 bird species have been recorded at RMBL. Bird arrival data were compared to first snow free date via linear regression to determine if bird arrival dates were dependent on the emergence of bare ground in the spring. Additionally, the trend in Robin arrival dates by year was evaluated via linear regression. Data from thirty-six bird species from the BBS which commonly use riparian or aquatic habitats were analyzed for longterm trends (1968 to 2012) using a standard hierarchical modeling approach [270]. BBS data were from the Southern Rockies/Colorado Plateau region. BBS credibility measures (red, yellow, blue) were

Table 4.1: Linear regression results for relationships between bird spring arrival date at Rocky Mountain Biological Lab and first snow-free date (first open ground date in spring) from 1975 to 2012.

Bird Species	**p value**	**R^2**	**Mean Arrival**	**Arrival Variance**	**Wintering Area**
Ruby-crowned Kinglet	0.00003	0.37	03-May	6.9	S. USA
Fox Sparrow	0.00003	0.44	23-Apr	11.8	S. USA
Yellow Warbler	0.0002	0.37	23-May	6.1	S. Am., C. Am.
Dark-eyed Junco	0.0004	0.28	08-Apr	12.6	Resident
Brown-headed Cowbird	0.0005	0.42	22-May	12.0	S. USA, Mex.
White-crowned Sparrow	0.002	0.22	12-May	4.8	Resident
Red-naped Sapsucker	0.002	0.24	25-Apr	12.7	SW. USA, Mex.
Broad-tailed Hummingbird	0.005	0.19	15-May	5.8	Mex.
Northern Flicker	0.04	0.09	10-Apr	7.2	Resident
Mountain Bluebird	0.04	0.11	06-May	20.2	Resident
American Robin	0.2	0.01	26-Mar	8.1	Resident
Red-winged Blackbird	0.3	0.003	20-Apr	16.2	Resident
Yellow-rumped Warbler	0.7	<0.001	16-May	8.5	S. USA, Mex., C. Am.
Tree Swallow	0.7	<0.001	27-Apr	10.1	C. Am.
Cliff Swallow	0.8	<0.001	13-May	6.9	S. Am.
Stellar's Jay	0.9	<0.001	13-Apr	13.6	Resident

assigned to each bird species trend as a metric of reliability. The red category indicates that (1) The regional abundance is less than 0.1 birds/route (very low abundance), (2) The sample is based on less than 5 routes for the long term, or is based on less than 3 routes for either subinterval (very small samples), or (3) The results are so imprecise that a 5%/year change would not be detected over the long-term (very imprecise). The yellow category indicates that (1) The regional abundance is less than 1.0 birds/route (low abundance), (2) The sample is based on less than 14 routes for the long term (small sample size), (3) The results are so imprecise that a 3%/year change would not be detected over the long-term (quite imprecise), or (4) The sub-interval trends are significantly different from each other (P less than 0.05, based on a z-test). This suggests inconsistency in trend over time). The blue category indicates that the data contain at least 14 samples in the long term, are of moderate precision, and are of moderate abundance on routes.

Additionally, we qualitatively compared changes in Mallard and Canada Goose (*Branta canadensis*) abundances due to the vastly different weather conditions in 2010/11 vs. 2011/12

in the vicinity of Crested Butte at both low elevation sites and high elevation sites during the time of our snail collections in this study (2010/11 - high snowfall, late snow-free date; 2011/12 - low snowfall, early snow-free date) from both the BBS (low elevation = McPhee Reservoir, 2114 m; high elevation = Tincup, 3096 m; late Dec to early Jan) and the CBC (low elevation = Cortez, 1971 m; high elevation = Aspen, 2532 m; June). These sites were close to Crested Butte and contained sufficient numbers of Canada Geese and Mallards that were consistently present to allow qualitative comparison. Canada Geese and Mallards were chosen because (1) they are confirmed hosts of avian schistosomes and other trematodes, (2) they are migrant waterfowl species observed at our field sites during collection, and (3) they are common bird types that are not likely to be confused or missed by volunteers with both the BBS and the CBC.

Schistosome Genetic Analyses

DNA was extracted from 10 ethanol-preserved cercariae from each sample using HotShot Lysis [322]. DNA was amplified using the polymerase chain reaction (PCR) (Takara Ex Taq kit, Takara Biomedicals, Otsu, Japan) for mitochondrial gene cox1 (732 bp) using previously published primers [29, 28]. Sequencing reactions were performed using the Applied Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City, California, USA). Sequences obtained were compared to available sequences in GenBank (Appendix 1). The cox1 gene fragments were used in phylogenetic analyses using Bayesian inferences (BI) with MrBayes [137, 265] and Maximum Likelihood (ML) using the software PAUP* 4.0b10. For the BI analysis of the cox1 dataset, the parameters were unlinked; data was partitioned by gene, for codons one and two Nst=2 and for codon three Nst=6 rates=gamma, ngammacat=4. Four chains were run simultaneously for 5,000,000 generations, trees were sampled every 100 cycles, the first 5,000 trees with preasymptotic likelihood scores were discarded as burnin, and the retained trees were used to generate a 50% majority-rule consensus tress and posterior probabilities. Nodal support for ML was estimated using the bootstrap method with 100 replicates. The sequences obtained were deposited in GenBank.

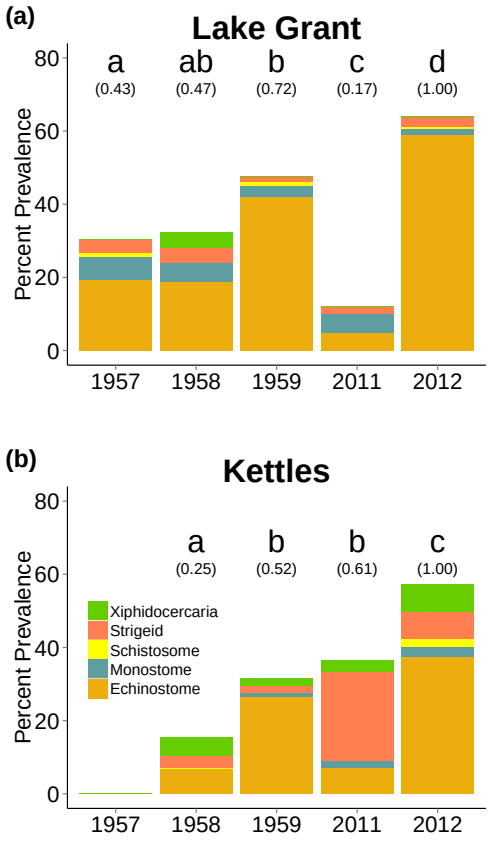

Figure 4.4: **Snail infection prevalence and morphotype biodiversity.** Snail infection prevalence and parasite morphotype diversity for Lake Grant (a) and the Kettles (b). Letters above bars indicate statistically significant differences in snail infection risk (logistic regression and Tukey's HSD ($p<0.05$). Numbers in parentheses above bars are the antilogged regression coefficients and indicate the odds of a snail being infected in each year compared to the odds of snail infection in 2012. Different colors signify different trematode morphotypes.

4.2 Results

4.2.1 Climate and Nutrients

Climate data from RMBL indicate that, over the last 38 years, the snow-free date has advanced on average 24 days ($p=0.0032$, $R^2=0.212$; Figure 4.1a) if extreme years are omitted, otherwise there is no significant relationship. Monthly snowfall exhibits a downward trend over this same period (Figure 4.1b, $p > 0.05$), though the trend is not significant and exhibits high year

to year variability (Figure 4.1b).The snow-free date for any given year depends on that season's total snowfall (p=6e-13, R^2=0.38). The snow-free date in 2011 is much later than most other years over the last decade (Figure 4.1a), whereas the 2012 snow free date is much more representative of other contemporary years (Figure 4.1a). DJF (Figure 4.1c) and MAM (Figure 4.1d) temperatures at RMBL are both warming significantly over this time period (p=2.3e-6, R^2=0.452, 7°C, Figure 4.1b; p=0.00026, R^2=0.293, 11.47°C; Figure 4.1d) with 2012 MAM temperatures being considerably warmer than in 2011. Temperatures are independent of snowfall ($p > 0.05$).

Overall in Crested Butte, August to June temperatures were qualitatively similar across all four time periods (1957/58, 1958/59, 2010/11, 2011/12), with 2012 spring temperatures warming slightly faster (Figure 4.2a). In 2012, January to June temperatures were slightly warmer than in 2011 (Figure 4.2a - yellow line). The 2010/11 winter was exceptionally snowy compared to 2011/12 (one of the least snowy years on record (Figure 4.1b)) with snowfalls in 1957/58 and 1957/59 being between these two extremes (Figure 4.2b). The total snowfall in 2010/11 was 649.7 cm, which is almost 2.5 times greater than the snowfall in 2011/12 (266.5 cm) (Figure 4.2b - yellow shading). Both the 1957/58 and 1958/59 snowfall totals (595.7 cm and 411.2 cm) were below those of the 2010/11 season and were 2.2 and 1.5 times larger than the low snowfall season of 2011/12 (Figure 4.2b). These differences in snowfall translated to changes in snow depth and spring melting rate (hence April snow depth - Figure 4.2c). In 2011 there was 104.1 cm of snow on the ground in Crested Butte in April whereas there was only 10.2 cm (over 10 times less) in April 2012 (Figure 4.2c - yellow shading). In April 1958 and 1959 snow depths were 111.8 cm and 53.3 cm (11 times and 5.2 times more than in 2012) respectively. Water column nutrients in the Kettles were significantly higher than in Lake Grant (Figure 4.3). TDP (Figure 4.3a) water column concentrations in Lake Grant (0.022±0.002 mg/L) were significantly lower ($t(9)=3.0365$, $p < 0.05$) than those in the Kettles (0.065±0.005 mg/L). Similarly, TDN (Figure 4.3b) water column concentrations in Lake Grant (0.31±0.02 mg/L) were significantly lower ($t(7)=4.0073$, $p < 0.05$) than those in the Kettles (1.78±0.13).

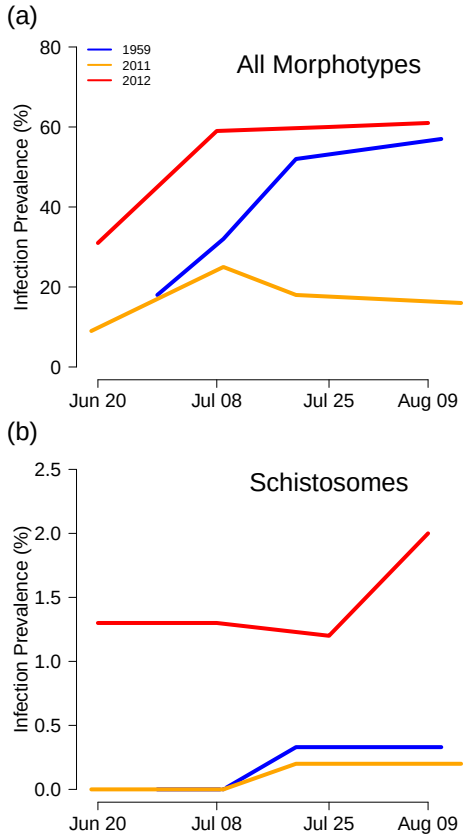

Figure 4.5: **Times series' of infection prevalence in 1959, 2011, and 2012.** The time series' of infection prevalences in snails for all morphotypes combined (a) and schistosomes only (b) for 1959 (blue line), 2011 (orange line) and 2012 (red line).

4.2.2 Snail Infection

Snail infection risk varied from year to year and between ponds (Figure 4.4). Year to year variations in infection were present in both Lake Grant (Figure 4.4a) and the Kettles (Figure 4.4b). At Lake Grant, infection risk in 2012 (64% prevalence; low snowfall, early snow-free date) was significantly greater ($p < 0.05$) than any other year surveyed (Figure 4.4a) while the 2011 infection risk (12% prevalence; high snowfall, late snow-free) was 0.17 times that of 2012. Prevalences in 1957 (30% prevalence), 1958 (32% prevalence), and 1959 (48% prevalence) were similar to each other, with 1959 having a significantly higher ($p < 0.05$) infection risk than 1957. At the Kettles (Figure 4.4b), again the infection risk in 2012 (57% prevalence) was significantly higher ($p < 0.05$)

than 2011 (36% prevalence, 0.61 times the 2012 risk), as well as 1959 (31% prevalence, 0.52 times the 2012 risk) and 1958 (15% prevalence, 0.25 times the 2012 infection risk). The infection risk in 1958 was the lowest recorded for the Kettles, while the infection risk during the year of highest prevalence in the Kettles recorded by Hunter [140] (31% prevalence, 1959) was indistinguishable from the infection risk during 2011 (Figure 4.4b, $p > 0.05$). Across sites, the chances of a snail becoming infected in Lake Grant vs. the Kettles depended on era ($p < 0.05$). In the past, snail infection risk was higher in Lake Grant than in the Kettles, but in the present infection risk is higher in the Kettles ($p < 0.05$).

Additionally, infection time trends behaved qualitatively different depending on the year (Figure 4.5). In 1959 total infection prevalence began at 18% (all morphotypes) during the middle of June and rose steadily from the end of June until the middle of July, after which it leveled off at about 57% (Figure 4.5a - blue line). In 2011 (high snowfall, late snow-free date) infection prevalence started out much lower than in 1959 (9%) and increased steadily to a maximum (25%) at the beginning of July (mainly due to increased strigeid infections) after which it decreased to 16% by the beginning of August (Figure 4.5a - orange line). In 2012 the prevalence time trend was similar to 1959, although accelerated, with infection prevalence starting at 31% and increasing steadily until the beginning of July and leveling off to a maximum value of 61% at the beginning of August (Figure 4.5a - red line). The strigeids that were common during 2011 in the *Physids* in the Kettles are 96% similar to *Ichthycotolyrus sp.* (28S, GenBank). While this is not close enough to identify these strigeids as an *Ichthycotolyrus sp.*, they are certainly closely related.

4.2.3 Bird Populations

Of the 16 bird species monitored at RMBL, the annual arrival times of 10 of these birds significantly covary with snow-free date ($p < 0.05$, $R^2 = 0.37$ to 0.11) (Table 4.1). Of the six bird species whose annual arrivals did not depend on snow-free date, one (American Robin - *Turdus migratorius*) had arrival dates that significantly advanced with year ($p < 0.05$, R^2=0.4066) (Figure 4.6). Bird arrival dates did not depend on mean arrival date, arrival date variance, or over-

wintering geographic area for any species (Table 4.1). CBC and BBS Mallard and Canada Goose data (late Dec/early Jan, June) from low elevation sites and CBC and BBS Mallard and Canada Goose data from high elevation sites were collected from both the 2010/11 (high snowfall, late snow-free date) and 2011/12 (low snowfall, early snow-free date) seasons to qualitatively compare the effects of drastically different weather conditions on bird abundances and visitation for both the summer (BBS) and the winter (CBC) (Table D.1). At the low elevation sites there was not much difference between 2010/11 bird numbers and 2011/12 bird numbers, indicating that the large weather difference experienced across these two years did not appreciably affect bird numbers at low elevations. At higher elevations bird numbers were 6 to 9 times greater during the winter of 2011 (low snowfall) and 3 to 7 times greater during the summer of 2012 (early snow-free date) compared to the 2010/11 season (high snowfall, late snow-free date) (Table D.1).

BBS data were also used to determine if the overall trends of 36 selected birds, (36 waterfowl, wading birds, etc.) that commonly use riparian and aquatic habitats, vary significant over the time period 1968 to 2012 (Table D.2). These bird data were collected in June from multiple sites in the Colorado Plateau/Southern Rockies region (including RMBL). Of these 36 birds, 21 show some sort of positive trend. Of those that show a positive trend, 8 have a CI which does not include zero, and only 2 have a significant positive trend with credibility measures are taken into account (Table D.2). Fifteen bird species show a negative trend. Of those that show a negative trend, 5 have a CI's which do not include zero, and these same 5 have a significant negative trend with credibility measures are taken into account (Table D.2). Specifically, the Canada Goose and the Common Merganser have shown 6.36% and 3.15% increases respectively over the region and the Killdeer, Spotted Sandpiper, Wilson's Snipe, Red-Winged Blackbird, and Brewer's Blackbird have shown 5.47%, 1.42%, 1.75%, 2.44%, and 2.84% decreases respectively. All other bird species surveyed show no significant change in abundance over time.

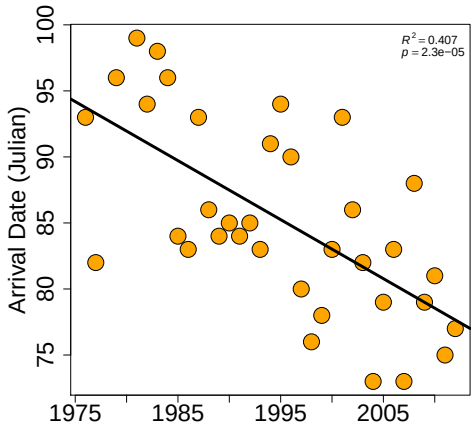

Figure 4.6: **Robin arrival dates (1975 to 2013).** Trend in the advancing arrival date of the American Robin (*Turdus migratorius*) at Rocky Mountain Biological Lab using linear regression (p=2.3e-05, R^2=0.407).

4.2.4 Snail Populations

Qualitative changes in snail biodiversity are evident in these ponds between the 1950s and the present (Figure 4.7). Hunter [140] recorded that Lake Grant contained two families of snails (*Planorbidae* and *Physidae*). Currently, Lake Grant only contains snails in the family *Physidae* (Physids) (Figure 4.7). In contrast, Hunter [140] recorded only snails of the family *Lymnaeidae* (Lymnaeids) in the Kettles, whereas now they contain both Lymnaeids and Physids (Figure 4.7). In addition, snails of the family *Planorbidae* (*Gyraulus parvus*) were found sporadically in the Kettles in 2011 (not 2012), but it is unclear if the methods of Hunter [140] would have been sufficient to detect them. Physids now make up a larger proportion of snail biomass within these ponds than in the 1950s, so any difference in infection susceptibility in Physids versus Lymnaeids could translate into a change in pond-wide infection. The proportion of snails in the Kettle ponds that are Physids depends on the year ($p < 0.05$). The proportion of Physids to total snails was 2.94 times higher in 2011 (high snowfall, late snow-free date) compared to 2012 (low snowfall, early snow-free date). Within the Kettles, there is no significant difference in the overall susceptibility of Physids or Lymnaeids ($p > 0.05$), though there is a difference in the odds of infection between Physids and Lymnaeids depending on the year ($p < 0.05$). In 2011 (high snowfall, late snow-free

date, a higher proportion of Physids) Physids were more likely to be infected, whereas in 2012 (low snowfall, early snow-free date, lower proportion of Physids) Lymnaeids were more likely to be infected ($p < 0.05$). Snail family also had a significant effect on trematode morphotype. Strigeids were more likely to infect Physids whereas Echinostomes were more likely to infect Lymnaeids ($p < 0.05$). All other trematode morphotypes showed no preference for snail family.

4.2.5 Parasite Diversity

Changes in parasite morphotype diversity were tested for on a year-by-year basis using the Shannon Wiener diversity index (H). For years during which data were available from both water bodies (1958, 1959, 2011, 2012) there was no difference in morphotype diversity either by year, by pond, or by the interaction of the two ($p > 0.05$, two-way ANOVA), thus suggesting that the diversity of trematode morphotypes was relatively stable across the last 50+ years. However, schistosome prevalence in 2012 was significantly higher ($p < 0.05$) than in 2011, 1959, or 1958 (Figure 4.5b). In August, schistosome prevalence peaked at 2% during 2012 but only reached 0.2%, 0.33%, and 0.1% in 2011, 1959, and 1958 respectively. Schistosome biodiveristy was quite high at this site in 2012 (Figure 4.8). In 1959 the only avian schistosome reported from the Crested Butte area (Lake Grant) was *Trichobilharzia physellae*. Two schistosome species were found in 2011: *Trichobilharzia physellae* (found in *Physa gyrina*, known to use birds from the genera *Aythya*, *Bucephala*, and *Mergus*, and is found throughout North America) and an unnamed avian schistosome (found in *Gyraulus sp.*, known to infect *Aix sponsa*, *Bucephala albeola*, and *Aythya marila* in NM, PA, and Manitoba). In 2012, at least six distinct schistosome species (all from the genus *Trichobilharzia*) were recorded (Figure 4.8): *Trichobilharzia D* (*Stagnicola sp.*, *Anas sp.*, so far only other confirmed location is Manitoba), *Trichobilharzia E* (*Stagnicola sp.*, unknown definitive host), *Trichobilharzia querquedulae* (*Physa acuta*, *Anas discors*/*Anas clypeata*/*Anas cyanoptera*), *Trichobilharzia physellae* (see above), *Trichobilharzia sp1* (unknown lineage), and *Trichobilharzia sp2* (unknown lineage). All spacings between species were significant with > 95% bootstrap support using minimum evolution algorithms (Figure 4.8).

4.3 Discussion

Environmental change alters trematode parasite prevalences over multi-year time scales [203, 157, 220, 1]. Changes in host-trematode communities have implications for ecosystem structure and function as well as disease risk. This study evaluates the possible effects of environmental variations (human development, mean annual weather variations (Figure 4.1 and Figure 4.2), and nutrients from cattle grazing (Figure 4.3)) on snail-trematode communities within the context of broadly changing climate conditions (Figure 4.1). Lake Grant has experienced a large amount of anthropogenic disturbance (dam building, residential encroachment, golf course development) between the study of Hunter [140] and the current study, while the area containing the Kettles has remained grazing land. Year to year mean weather conditions have fluctuated widely at both Lake Grant and the Kettles (Figure 4.1 and Figure 4.2), with the 2010/11 winter snowfall being higher than all other years in this study and the 2011/12 winter snowfall being the lowest (Figure 4.2b). Snow-free date has been advancing since 1975 and is the dominant control on the growing season start date at this site [145]. In fact, the phenology of a number of organisms around RMBL are shifting in response to changing climate conditions [145, 144, 42].

4.3.1 Environmental effects on Snail Infection

At both Lake Grant and the Kettles infection prevalences varied within ponds by year and by era (past = 1958 and 1959, present = 2011 and 2012). The winter of 2010/11 was accompanied by higher snowfalls (Figure 4.2b) and greater April snow depths (Figure 4.2c) compared to the winter of 2011/12. The higher snowfall (and later snow-free date) of 2010/11 was associated with significantly lower snail infection prevalences in the 2011 growing season in both the Kettles and Lake Grant compared to the 2012 growing season (Figure 4.4). A weaker but similar pattern was observed in the data of Hunter [140], with the high snow year (1957/58) being associated with lower prevalences than the low snow year (though the difference at Lake Grant is not significant). Therefore, years with early snow-free dates (longer growing seasons driven by lower snowfall) are

associated with higher trematode prevalences in snails, both in the past and to a larger extent in the present.

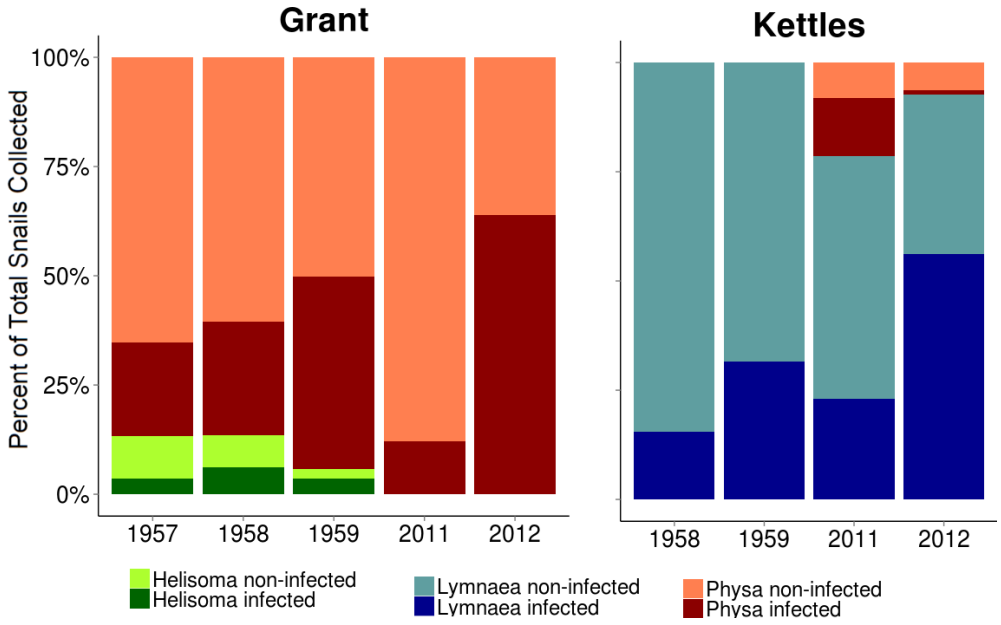

Figure 4.7: **Snail Biodiversity and Infection.** Snail biodiversity and infection in both Lake Grant and the Kettles. The number of snails per family are expressed as a proportion of the total snails collected per site. Each color represents a snail family, with the darker shade representing infected snails and the lighter shade representing uninfected snails.

Years with earlier snow-free dates also tend to have earlier bird arrival dates (Table 4.1) and probably earlier snail emergence dates. Therefore, in such years (i.e 2011/12) parasite egg deposition and snail infection will also be expected to occur earlier. The initial late June values for snail infection prevalence in 2012 (Figure 4.5a) were higher than those in higher snowfall years (1959 and 2011). Additionally, maximum snail infection prevalences in 2012 were slightly higher than in 1958 (moderate snowfall) and substantially higher than in 2011 (high snowfall) underscoring the importance of year to year changes in weather conditions to parasite prevalence. Longer parasite transmission seasons result in an increase in per snail parasite production per season which has implications for parasite-mediated ecosystem effects, food webs, and disease risk.

However, some bird species (e.g. American Robins, Figure 4.6) do not appear to respond to local snow-free date, and instead may arrive at RMBL based on weather conditions or food

availability at lower elevations. During high snow years (late snow-free date years), migrating birds may bypass the high elevation water bodies around Crested Butte and RMBL altogether if they are still frozen, and forage at lower elevation sites on their way north [145]. For example, less Mallards and Canada Geese were observed at high elevation sites around Crested Butte during 2011 vs. 2012, whereas low elevation site populations were largely unchanged (Table D.1). This "bypass effect" may also be partially responsible for the low snail infection prevalences (Figure 4.2) in 2011 (high snowfall, late snow-free date). Additionally, the time trend of infection in 2011 never remotely reached the equilibrium values of 1959 and 2012 (Figure 4.5a), suggesting that the delivery of trematode eggs in 2011 was reduced.

4.3.2 Trematode Hosts and Environmental Change

The presence of multiple functionally-similar species with different environmental tolerances enhances ecological resilience to environmental change [245, 195]. In the case of snail-trematode communities, the presence of multiple snail species with varying environmental tolerances but similar susceptibilities ensures that overall snail host populations (and hence trematode populations) will be stable over a range of environmental conditions. Since 1958 and 1959 snail communities have shifted in both Lake Grant and the Kettles, with Physids becoming a larger proportion of total snails at both sites (Figure 4.7). Physids are generalists that are able to survive in a wide range of habitats in the absence of more competitive species, possessing exceptional habitat breadth compared to distributional studies of other freshwater taxa [326].

Snail biodiversity has decreased in Lake Grant, which contained both Planorbids and Physids in 1957, 1958, and 1959, but now only contains Physids (Figure 4.7). Increased human disturbance (residential encroachment and dam emplacement) may have favored the expansion of the disturbance tolerant Physid species *Physa acuta* at the expense of the Planorbid species *Planorbella trivolvis* [326]. The deepening of Lake Grant via a dam (thus making the water colder) could have also provided a competitive advantage to the Physids as Planorbids prefer warmer waters [329]. This decrease in snail diversity is associated with a decrease in year-to-year infection stability. In

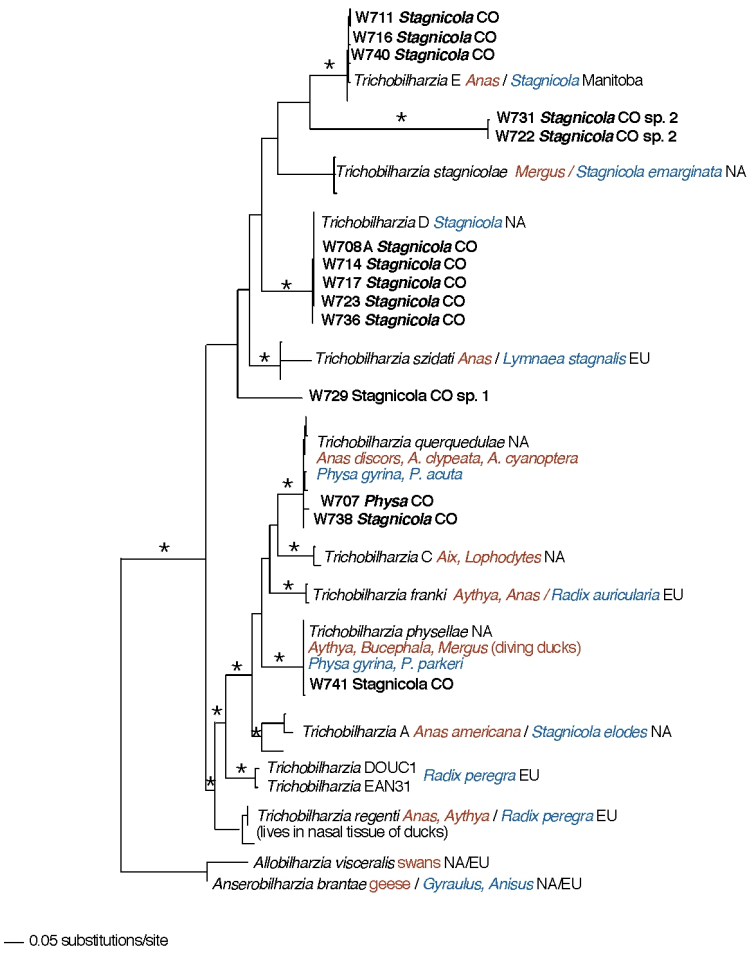

Figure 4.8: **Schistosome diversity.** Cox1 tree of schistosomes identified from the Kettles and Lake Grant. Maximum liklihood tree with asterisks indicating >95% bootstrap support using minimum evolution algorithms. Samples collected in this study are in bold, known bird hosts are in orange. If only genus is listed this indicated either (1) the designated schistosome utilizes all species within that genus or (2) the designated schistosome has tentatively been found in a few members of that genus. Snail hosts are listed in blue. The overall geographic distribution is listed after snail host (NA=North America, EU=Europe).

the present era, Lake Grant exhibited large annual fluctuations in prevalences (greater than 80%) induced by changes in mean annual weather conditions, while year to year prevalence values in the past were much more stable (Figure 4.4a).

Conversely, the Kettles have shifted from Lymnaeid-only communities to containing both Lymnaeids and Physids (and occasionally Planorbids) (Figure 4.7). Warming trends (Figure 4.1), variable year to year weather conditions (Figure 4.2), and/or disturbance and enhanced nutrient

delivery via cattle (Figure 4.3) could have facilitated Physid expansion into the Kettles. The presence of both Lymnaeids and Physids has induced increased infection stability when faced with large swings in environmental conditions (mean annual weather in 2010/11 and 2011/12) because both Lymnaeids and Physids are equally likely to be infected by trematodes in the Kettles. Environmental conditions in 2011 (high snowfall, late snow-free date) led to increases in the proportion of snails that were Physids, thus trematodes were more successful at infecting Physids. In 2012 (low snowfall, early snow-free date) a larger proportion of the snails were Lymnaeids and trematodes were more successful at infecting Lymnaeids. Accordingly, because strigeids are more likely to infect Physids, and echinostomes are more likely to infect Lymnaeids, strigeid prevalence was increased in the Kettles in 2011 relative to 2012 (Figure 4.4). These changes in infection stability in response to environmental change led to the Kettles having a higher infection risk in the present than Lake Grant, a change from the past era when Lake Grant had higher infection risk. Morphotype diversity was not significantly different in the 1950s than in the current era, although this is not directly indicative of definitive host diversity as morphotypes are much broader than species designations.

4.3.2.1 Schistosomes

The first recorded outbreak of cercarial dermatitis at this site occurred in 1956 [140]. Researchers at RMBL have noticed a marked issue with cercarial dermatitis in the kettle ponds for at least the last 20 years, though incidence of the condition is highly variable from year to year (Wissinger pers. comm.). The prevalence of schistosomes in snail hosts in 1959 and 2011 were statistically identical whereas the 2012 prevalence was significantly higher than either (Figure 4.4, Figure 4.5b). The remarkable diversity of schistosomes at this high elevation site is unprecedented (Figure 4.8) and is much more indicative of a high latitude community ([30], Brant unpublished data). The higher diversity of schistosomes present in 2012 vs. 2011 may be due to the proposed "bypass effect" present in 2011 caused by the late snow melt. While this high diversity and high prevalence of schistosomes suggests a high diversity of definitive hosts, BBS data indicate a largely unchanged regional bird diversity (Table D.2) over the last 45 years (though there are significant

increases in Canada Geese and Common Mergansers). However, the BBS data are too coarse to capture local changes in bird populations with elevation, which could be underlying these patterns. More research is needed to determine the effects of local and global change on schistosome communities at high elevations.

4.4 Conclusion

Environmental change has the potential to alter snail-trematode communities and thus disease risk and ecosystem structure and function. Overall, the generalist snail family *Physa* has become more common at these sites over the last 50+ years. There are indications that human disturbance at Lake Grant may have shifted snail (and possibly bird) host communities such that trematode community resilience to environmental change (annual mean weather conditions) has been impaired. The Kettles meanwhile seem to have acquired increased trematode community stability via an increase in snail biodiversity due to the colonization of Physids. In general, fluctuations in mean annual weather conditions seem to have caused changes to trematode prevalences both in the past and the present, either through longer parasite transmission seasons or by the passing-by of migratory birds during years when snow cover decreased the availability of forage ("bypass effect"). Also, these sites contain extremely high schistosome diversity for the region and the risk of contracting cercarial dermatitis from these ponds can be high, depending on the year. While these data are suggestive of widespread change in snail-trematode communities at these sites, more in-depth mechanistic research is needed to follow-up on these initial patters.

Chapter 5

CONCLUSION

In this thesis I have demonstrated that parasites can change how nutrients cycle through ecosystems, thus altering their environment [108]. I have also demonstrated that environmental change has the potential to alter parasite prevalence, with potential feedbacks into ecosystem structure and function [141]. These interactions operate within the context of widespread and often dramatic anthropogenic alterations of ecosystems. It is crucial to understand the mechanisms underlying relationships between parasites and environmental change in order to manage ecosystems in a way that minimizes disease risk to humans while maintaining important links between parasites and ecosystem structure and function. Feedback mechanisms are particularly important as they can often be difficult to foresee and may apply significant leverage to ecosystems, thus producing non-linear responses to environmental change.

Parasites' effects on ecosystems are expected to vary both in character and magnitude depending on: the dominant type of host-parasite interaction (e.g. changes in behavior, reproduction, survival, excretion), the host's functional role (e.g. dominant organism, keystone species, ecosystem engineers, trophic level), and ecosystem characteristics (e.g. limiting nutrients, species assemblages/interactions, existing energy flows). For example, parasitic nematomorphs cause infected crickets to seek out water. These parasite-modulated terrestrially-derived nutrient subsidies are a preferred food for an endangered trout [269]. Because trout preferred crickets in this system, parasites induced cascading effects both for trout as well as benthic invertebrates, etc. Similarly,

isopods infected with an acanthocephalan parasite exhibited reduced litter consumption [119]. Parasites had the greatest effect on isopod-processed detritus when prevalence was high, and had a diminished effect when prevalence was lower and fish predation of isopods was high, with significant ecosystem-level effects expected in systems where isopods are dominant detritivores. Ecosystem-level effects will almost always occur in systems where parasites drastically affect species interactions and assemblages (e.g. avian malaria in Hawaii reduces/eliminates bird populations - [**?**]) or exert strong controls on primary production (exclusion of fungal parasitic pathogens increases primary production in a grassland ecosystem - [**?**]).

Cotylurus flabelliformis metacercariae have the potential to act as ecosystem engineers in these ponds [108], modulating the rate at which snails "pump" nutrients from the periphyton to the water column [83]. Most notably, parasites enhance the bioavailabilty of N in the water column (originally derived from periphyton N fixation and direct uptake from sediments) as well as decrease detrital N fluxes (through reduced snail body %N). Parasite-mediated N contributes significantly to total bioavailable water column N in these ponds when compared to other sources (the ditch, water column N fixation) and existing standing stocks. At the height of the growing season, increased biological N demand and decreased ditch N loading in these ponds leads to decreased water column N. At the same time, parasite-mediated N is expected to increase due to accumulated infections and temperature-driven increases in metabolic rate, thus becoming a progressively larger factor in pond N budgets. This N stimulates planktonic production which in turn has the potential to increase waterfowl visitation, thus providing a positive feedback in which increased parasite populations facilitate recruitment of definitive host organisms, which further increase parasite populations. Conversly, parasite-induced decreases in snail body %N decreases detrital %N (especially in systems with high snail biomass) thus reducing protein delivery to microbial decomposers, which could reduce detritus decomposition rates [114]. Additionally, altered snail excretion and body nutrient content could have a number of other cascading effects (e.g. periphyton quantity and stoichiometry, changes in species assemblages and populations, interspecific and intraspecific competition, etc.) relevant to ecosystem structure and function [211]; depending on the ecosystem context.

Therefore, parasites must be viewed within their ecological context to truly understand interactions between anthropogenic land use and parasites. In this case, human-caused N limitation provided a context within which parasite-mediated N had significant ecosystem-level ramifications. These types of interactions have broader implications, such as the changing snails and parasite fauna around Crested Butte, CO. A working knowledge of mechanistic links involving parasites, humans, and the environment is crucial evaluating how human-caused change will alter ecosystem structure and function.

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Appendix A

Additional Figures for Chapter 1

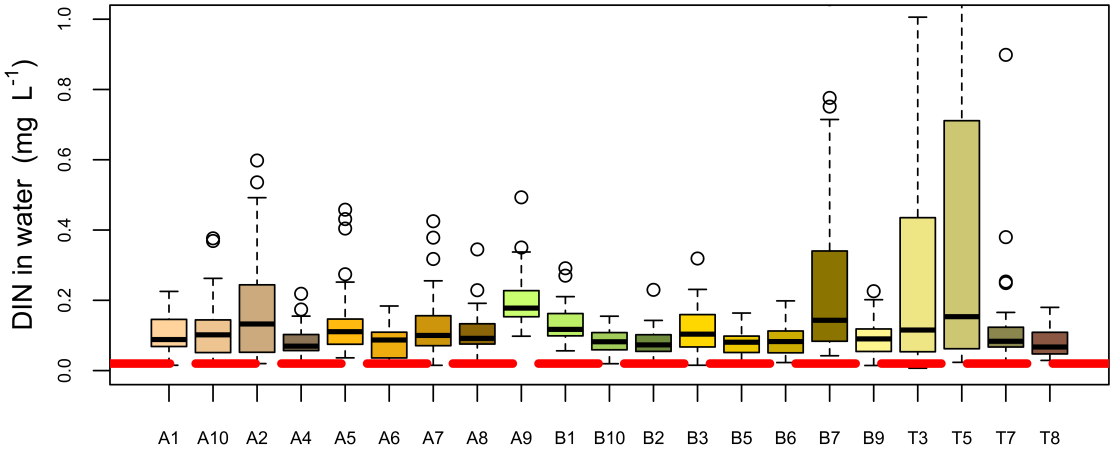

Figure A.1: **Water column dissolved inorganic nitrogen (DIN) values per pond compared to the experimental N fixation threshold determined by Bradburn et al. [27].** Each box contains per pond DIN values across the growth season. The red dashed line is the DIN threshold (20 μg/L) above which water column N fixation rates decrease substantially. This threshold was determined by field experiments by Bradburn et al. [27] using adjacent water bodies. While DIN concentrations were low in many of the study ponds, DIN levels were consistently maintained above this 20 μg/L threshold suggesting a water column N control on N fixation within the study ponds.

Table A.1: **Conductivity, pH, temperature, and dissolved oxygen measurements throughout the 2011 growing season.**

Site	Date	C1 (μS)	C2 (μS)	C3 (μS)	pH1	pH2	pH3	T1 (°C)	T2 (°C)	T3 (°C)	DO1 (ppm)	DO2 (ppm)	DO3 (ppm)
B9	06/08/01	1159	1194	1142	8.82	7.87	8.87	21.2	20.5	20.2	5.39	4.17	7.57
T5	05/25/11	984	1115	1119	9.01	8.56	8.33	14.3	12.4	12.1	15.26	15.73	13.28
T7	05/25/11	1307	1555	1400	7.2	8.05	8.08	19	24.6	17.4	2.1	6	9.08
B1	06/03/11	1101	1096	1081	9.04	8.87	8.65	19.3	18.8	17.7	13.27	14.72	8.45
B2	06/03/11	1310	1289	1281	8.14	8.22	8.22	20.5	19.2	19	7.1	7.28	7.27
B3	06/03/11	840	877	889	9.65	9.73	9.89	22.8	20.4	21.1	16.28	16.07	17.08
B6	06/03/11	1170	1161	1157	8.85	8.72	8.75	16.3	15.4	15.9	8.75	7.81	6.71
B7	06/03/11	999	985	988	10.11	9.88	10.36	23.5	24.1	25	18	16.33	18
T8	06/04/11	1366	1376	1368	8.71	8.42	8.32	19.3	19.2	20.6	2.84	2.41	5.93
A1	06/06/11	1816	1564	1529	7.6	8.68	8.88	19.3	20.6	21	5.14	7.6	8.68
A2	06/06/11	1208	1156	1182	7.73	8.04	7.7	22.8	21.3	20.2	5.94	8.08	4.57
A4	06/06/11	1776	1779	1849	7.74	8.08	8.21	27.7	26.7	26.3	12.1	7.4	12.31
A5	06/06/11	1638			7.67			28			4.05		
A9	06/06/11	1264	1368	1222	7.86	7.96	7.83	22.6	13.3	14.2	3.53	2.07	3.09
A10	06/06/11	3730	3770	3860	8.3	8.19	7.94	26.6	27.7	26	14.55	8.75	7.6
B5	06/08/11	1274	1283	1284	8.36	8.3	8.13	22.2	22.2	22	9.93	9.65	6.7
B10	06/08/11	1392	1387	1413	8.15	8.41	8.41	28.5	26.2	24.3	10.42	18	11.92
A6	06/10/11	2720	2800	2800	8.14	7.89	7.9	19.4	20.6	20.2	8.68	8.93	4.2
A7	06/10/11	2450	2500	2490	7.82	7.93	8.04	17.7	17.4	16.7	4.61	3.56	2.65
A8	06/10/11	2950	3040	2980	8.15	8.07	8.19	18.1	16.4	17.6	10.37	5.92	11.19
T3	06/10/11	1069	1128		9.15	9.14	9.15	20.2	19.8	20.8	14.51	13.32	14.4
T5	06/10/11	1212	1088	1106	8.71	9.02	9.97	27.5	24.3	22.7	9.49	9.38	18
T7	06/10/11	1249	1498	1374	8.19	7.48	8.12	22.5	24.6	24.1	1.91	4.19	6.93
T8	06/10/11	1321	1349	1322	8.61	8.67	8.47	22.6	23.5	22.1	9.95	10.32	10.67
B7	06/16/11	530	511	510	8.56	8.66	9.16	20.1	20.4	20.5	9.58	4.54	5.02
B3	06/16/11	555	556	554	9.22	9.35	9.37	22.1	22	22.3	9.81	10.54	10.35
B1	06/16/11	1031	1065	1050	9.07	9.04	9.03	21	20.8	20.6	8.66	6.76	6.99
B2	06/16/11	1328	1329	1331	8.19	7.98	8.05	20.3	20.9	20.5	3.28	2.73	3.6
B6	06/16/11	1095	1106	1119	9.02	9.03	9.06	22.5	22.3	27	9.86	9.54	8.57
B9	06/16/11	1179	1184	1182	8.88	8.68	8.61	21.6	21.8	21.5	4.69	5.34	5.92
B5	06/16/11	1277	1281	1270	8.6	8.61	8.58	22.1	22.5	22.8	10.43	10.41	11.02
B10	06/16/11	1388	1384	1383	8.33	8.64	8.66	23.1	23.4	22.8	13.68	15.22	14.45
A1	06/16/11	1532	1538	1528	8.52	8.82	8.45	22.3	22.5	23.1	3.71	7.05	6.98
A2	06/16/11	1234	1265	1256	7.55	7.53	7.57	22.9	22.3	22.7	1.84	4.79	2.19
A7	06/16/11	1438	1475	1460	7.92	8.1	8.02	21.5	21.9	21.6	4.88	6.11	5.3
A10	06/16/11	4370	4280	4290	8.34	8.36	8.5	24.8	23.2	23.1	7.38	7.33	7.5
A8	06/16/11	3070	3100	3210	8.18	8.36	8.34	22.3	23.6	21.6	9.05	12.63	10.11
A9	06/16/11	1332	1308	1343	7.75	7.56	7.63	18.8	19.2	18	3.84	4.54	5.29
A4	06/16/11	1784	1751	1795	8.53	8.29	8.23	23.8	25.1	24	11.97	11.79	13.95
A5	06/16/11	1639	1620	1583	7.71	7.83	7.82	23.9	24.6	23.2	9.98	15.18	4.06
A6	06/16/11	2700	2830	2720	8.05	7.95	7.91	22.9	25.2	25.2	10.05	9.58	9.72
T5	06/16/11	1087	1093	1136	9.14	9.27	8.74	25.7	25.6	24.1	6.78	9.03	7.64
T7	06/16/11	1336	1264	1319	7.71	8.48	8.46	28.7	23.5	23.7	15.37	11.17	8.23
T8	06/16/11	1264	1310	1282	9.19	9.2	9.41	24	22.9	23.1	13.02	12.92	12.94
T3	06/16/11	1089	1111	1094	9.47	9.45	9.44	23.1	22.7	22.6	14.76	15.27	15.42
B6	07/03/11	1205	1229	1241	8.88	8.3	8.83	25.1	24.4	24.8	3.15	1.63	3.93
B5	07/04/11	1322	1336	1346	9.15	9.29	9.32	30.5	30.2	29.8	13.17	18	18
B6	07/04/11	1144	1211	1220	9.44	9.54	9.59	26.9	25.8	25.5	14.14	12.45	11.4
B9	07/04/11	1372	1434	1380	8.82	8.84	9.66	33.3	31.7	31.6	18	18	18
B10	07/04/11	1511	1586	1571	10.31	10.44	10.31	28.9	28.6	28.2	18	18	18
A1	07/05/11	1728	1853	1845	7.79	7.83	7.94	33	27.1	26.5	9.08	4.53	1.93
A2	07/05/11	1291	1390	1375	8.13	8.15	8.03	25.7	25	24.9	2.19	2.67	2.33
A7	07/05/11	2220	2250	2300	8.33	8.35	8.3	24.8	24.7	24.7	7.53	7.13	7.12
A10	07/05/11	4070	4660	4720	8.72	8.16	7.86	28.4	27.5	27.7	1.94	4.05	1.04
B1	07/06/11	1560	1284	1279	8.04	8.83	9.15	28.6	26.1	26.1	3.25	3.62	9.47
B2	07/06/11	1328	1369	1335	9.15	9.17	9.36	30.4	29.5	29.2	18	18	18
B3	07/06/11	455	484	470	9.83	10.19	10.14	23.8	23	22.6	15.51	16.53	16.14
B7	07/06/11	496	492	495	8.46	8.64	8.51	22.9	23.7	21.7	7.33	8.37	5.27
A4	07/07/11	1943	1957	1961	7.82	7.85	7.82	28.7	28.5	28.3	2.72	3.11	2.55
A5	07/07/11	1850	2110	1879	8.85	7.87	8.14	30.1	29	28.9	1.15	4.27	2.17
A6	07/07/11	2720	2850	2750	8.45	8.36	8.55	29.4	29.5	29.2	10.04	9.21	6.3
A8	07/07/11	3130	3140	3260	8.69	8.85	8.65	23.3	23	22.9	1.29	1.74	0.58
A9	07/07/11	1418	1445	1443	8.62	8.05	7.98	12.2	15.5	11.4	2.13	2.35	1.61
T3	07/08/11	1049	1101	1136	9.88	10	9.86	28.5	28.9	28.1	13.7	18	12.59
T5	07/08/11	1059	1118	959	8.56	8.54	8.46	27.9	27.9	27.3	2.56	4.21	2.59
T7	07/08/11	1233	1239	1292	7.74	7.69	7.53	19.5	19.3	19.7	0.35	0.24	0.27
T8	07/08/11	1345	1384	1353	8.42	8.4	8.6	20	20.1	18.2	6.7	7.5	4.65
B5	07/31/11	1243	1293	1311	8.86	8.74	8.7	29.1	28.5	28.8	12.8	11.85	11.63
B9	07/31/11	1671	1658	1690	8.83	8.86	8.61	28.2	29.7	29.1	1.76	2.29	1.31
B10	07/31/11	1309	1334	1381	9.07	9.77	9.8	31.8	31.5	30.8	18	18	18
A1	08/01/11	1746	1752	1763	7.69	7.83	7.89	26.1	23.2	24.7	2.24	1.92	4.26
A2	08/01/11	1368	1395	1395	8.1	8.13	8.2	24.4	24.6	24.5	5.18	4.48	5.13
A7	08/01/11	1922	1930	1946	8.65	8.68	8.66	27.8	26.4	27.9	7.91	9.64	9.63
A10	08/01/11	3510	3600	3570	8.59	8.6	8.67	30.8	30.3	30.7	12.8	13.22	12.93
A4	08/02/11	1769	1948	1982	7.88	8.71	8.59	29.7	29.7	30	14.11	18	15.11
A5	08/02/11	2280	2190	2230	7.49	7.7	7.53	28.4	28.2	28.3	5.11	7.12	6.88
A6	08/02/11	2570	2630	2560	7.72	7.7	8.07	29	29.4	29.2	0.75	0.74	1.32
A8	08/02/11	3380	3380	3360	8.32	8.29	8.37	24.2	23.8	24	3.55	3.54	2.25
A9	08/02/11	1468	1481	1487	7.35	7.38	7.33	17.1	16.8	16.4	0.9	1.03	0.78
B1	08/03/11	1056	1107	1122	8.66	8.43	8.26	26.5	26.5	26.4	14.77	9.33	8.46
B2	08/03/11	1270	1270	1265	10.29	10	10.17	31.1	32.3	31.3	18	18	18
B3	08/03/11	725	532	614	8.71	8.76	9.02	23.8	23.8	23.8	5.74	11.78	9.53
B7	08/03/11	1133	1082	1093	7.81	7.87	7.85	23.2	23.3	23.3	4.51	8.55	4.56
T3	08/04/11	1271	1284	1306	9.35	9.58	9.55	26.1	27.2	26.6	12.21	18	18
T5	08/04/11	1143	1181	1151	7.79	7.58	7.68	24.3	24	25.9	0.61	0.38	1.28
T7	08/04/11	1235	1232	1235	7.3	7.35	7.36	19	19.1	19.3	0.26	0.5	0.4

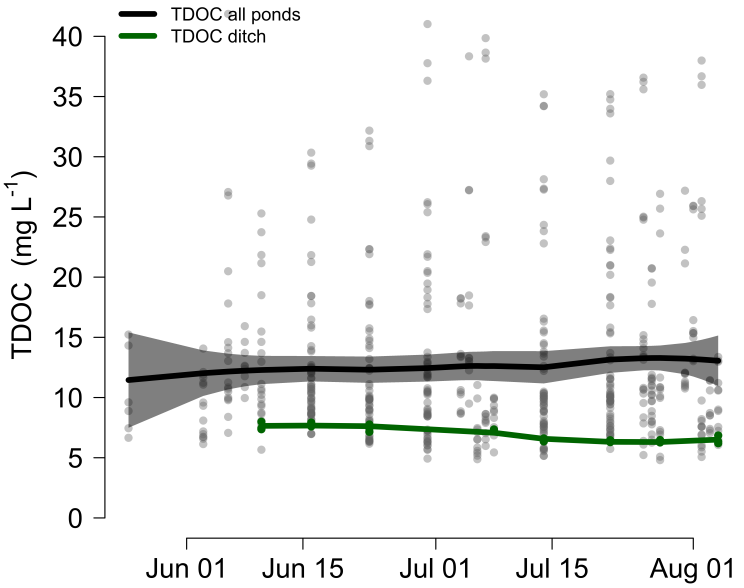

Figure A.2: **Total dissolved organic carbon (TDOC) concentrations for all ponds across the growth season.** TDOC measurements across the growth season remained steady (mean = 13±0.3 mg/L) with no systematic changes across the growth season. The ponds maintained TDOC concentrations above those of the ditch indicating the ditch is not a significant source of TDOC to the ponds.

Table A.2: **Water Chemistry and Seston Data for Waters Used in 2012 Experiments.**

pond name	Nitrate-N mg/L	Ammonium-N mg/L	DIN mg/L	TDP mg/L	DIN:TDP mass	Seston N:P molar	N fixation rate μ mol N/L/day	Nutrient Limitation
A1	0.028	0.085	0.113	0.0172	6.6	6.6	0.054	P
A2	0.004	0.046	0.050	0.0582	0.9	7.9	0.054	N
A4	0.006	0.011	0.017	0.0398	0.4	12.1	0.057	Nseq
A5	0.011	0.070	0.081	0.0124	6.5	3.0	0.057	NPdual
A6	0.006	0.027	0.034	0.2321	0.1	9.1	0.050	N
A7	0.021	1.460	1.481	0.0508	29.2	3.5	0.052	–
A8	0.009	0.045	0.053	0.7180	0.1	13.4	0.066	N
A9	0.009	0.058	0.067	0.0579	1.2	5.1	0.041	–
A10	0.007	0.004	0.011	0.0279	0.4	9.4	0.048	N
B1	0.012	1.848	1.860	0.1762	10.6	8.5	0.058	–
B2	0.006	0.055	0.061	0.0206	3.0	3.1	0.046	NPdual
B5	0.007	0.075	0.082	0.0702	1.2	36.7	8.896	–
B6	0.024	0.417	0.441	0.6165	0.7	4.4	0.063	N
B7	0.005	0.050	0.055	0.0299	1.8	6.4	0.046	NPco
DITCH	0.001	0.007	0.008	0.217336	0.0	12.2	0.108	N
T3	0.001	0.059	0.060	0.0340	1.8	2.8	0.178	Nseq
T5	0.006	0.057	0.064	0.1762	0.4	3.8	0.048	N
T7	0.007	0.061	0.067	0.0204	3.3	13.5	0.063	–

Appendix B

Metabolic Theory, Ecological Stoichiometry, and Host-Parasite Interactions

Nutrients (C,N,P) are used by organisms both as physical building blocks and as a means to produce energy [4]. Integrating the theory of ecological stoichiometry and the metabolic theory of ecology can provide a framework within which to evaluate the interactions between nutrient demand and metabolism that lead to changes in ecosystem-level nutrient cycling. For example, herbivores exhibit metabolic changes as a result of predation-induced stress (increased cardiovascular activity, increased blood pressure, increased respiration and ventilation, and the stimulation of protein catabolism and gluconeogenesis [113, 114]) which have consequences for ecosystem-level nutrient cycling (lower body C:N stoichiometry and elevated excretion of nitrogen-rich compounds [113]). Parasite-host and predator-prey interactions have often been considered analogous [260]; therefore ecological stoichiometry can also be useful in examining proposed nutrient-mediated interactions between parasites, hosts, and primary producers, while metabolic theory can be used to describe the implications of fundamentally different metabolic rates imposed by infection for nutrient consumption and energy flow [4].

The supply ratios of consumer-derived nutrients depends on consumer stoichiometry [78]. While primary producers are homeostatically flexible, consumers (snails, parasites, etc.) are more homeostatically rigid - seeking to achieve an optimum body stoichiometry for growth, maintenance, and reproduction (the threshold elemental ratio - TER [310, 105]). Snail body TERs determine the ratios of nutrients assimilated from periphyton versus excreted/egested back to the ecosystem [206, 83]. Similarly, parasite TERs determine which nutrients will be in demand from the host,

with implications for host body and host excretion stoichiometry.

While consumer TER determines *which* nutrients are recycled back to the ecosystem and in what proportions, metabolic rate determines the *rate* at which nutrients are recycled back to the ecosystem. Infection induces fundamental changes in host vital rates and behavior, shifting hosts into an alternate phenotype [99] that is often metabolically and stoichiometrically distinct from the original uninfected phenotype. The density of metabolic units in an organism (mitochondria, ribosomes, etc.) is inversely related to organism mass [4]. Metacercariae are expected to have a higher density of these metabolic units (MUs) than their hosts. Therefore, increased parasite abundances lead to higher MU densities in the snail-metacercariae phenotype, which in turn lead to higher metabolic rates. Increased MU densities lead to higher demand for P (P-rich MUs - Growth Rate Hypothesis [73]) and metabolically available C in the modified snail-metacercariae phenotype.

In the snail-metacercariae phenotype the infection-induced metabolic rate is not in equilibrium with the original snail stoichiometry and TER. Increasing metacercariae abundances amplify this mismatch, forcing the snail to maintain a metabolic rate which is sub-optimial for its intrinsic TER. In response to the increased metabolic demands, snail hosts resort to compensatory feeding [23]. Starved snails routinely increase both feeding rate and nutrient assimilation efficiency [35]. When compensatory feeding is not sufficient to keep hemolymph glucose levels from dropping below their homeostatic threshold [15, 248] snails must metabolize their own tissues either through the degradation of glycogen (glycogenolysis) or the catabolism of proteins to fuel gluconeogenesis. In digenean trematode infections the intermediate host commonly exhibits an increase in nitrogenous products of excretion in the hemolymph [15, 323] and an increase in nitrogen excretion [15, 22]. These metabolic changes are a result of the host's use of protein catabolism to offset the glucose starvation induced by infection [15, 248]. Infected snails also tend to have reduced carbohydrate [248] and protein [249] tissue contents as a result of parasite metabolic demands from larval trematodes in multiple life stages. Therefore, parasites alter host nutrient stoichiometry through preferential uptake of nutrients and alter host metabolic function through increased MU densities

in the host/parasite phenotype, while infection-induced host stress exacerbates these metabolic impacts. The integration of metabolic theory and ecological stoichiometry provides a framework to achieve a more complete understanding of parasite alterations to energy and nutrient flows within ecosystems.

Appendix C

Sources of Underestimation of the Excretion Effect of Infection

A number of factors could have led to an underestimate in our determination of the effects of infection intensity on snail DIN-N excretion. Nutrient excretion rates were determined using wild-caught snails from a single pond within a single size class grazing on natural periphyton communities prior to capture and held at a constant temperature throughout the 3 hour excretion period. Snail N excretion rates depend to a large extent on the stoichiometry of the periphyton they are grazing [22], temperature [2], and body mass [330]. Periphyton %N can be highly variable within a single pond, therefore wild-caught snails grazing on wild periphyton communities will be ingesting periphyton with variable N contents thus contributing to variable snail N excretion rates. The magnitude of the effect of infection on snail N excretion is therefore tied to periphyton N content. Both snail N excretion [2] and *Cotylurus flabelliformis* development rate within the snail second intermediate host increases with higher temperatures [37]. The rate of metacercariae development could impact the N excretion rate through acceleration or deceleration of snail metabolic rate and stress. Also, metacercarial developmental stage may influence N excretion. In this study most metacercariae observed were fully grown and either fully encysted or partially encysted. Earlier developmental stages during which rapid growth occurs may induce more extreme metabolic stresses on the snail host leading to increased N excretion. Additionally although a relatively short time window of 3 hours was used to quantify snail excretion rates to minimize the effects of starvation on excretion estimates, it is possible that N excretion rates were underestimated due to starvation [330]. Also, because snails were separated from their food source and thus not actively grazing

during excretion measurements, any additional N excretion derived from increased feeding rates (and not increased metabolism alone) would be underestimated. Parasite-induced N enrichment can also have secondary effects on nutrient storage and cycling through alteration of community composition and consumer productivity which can alter the pathways and magnitude of nutrient flows [57].

Appendix D

Bird Abundance and Trend Data

Table D.1: Abundances of Canada Geese (*Branta canadensis*) and Mallards (*Anas platyrhynchos*) at high and low elevation sites during years of vastly different mean weather conditions (2010/11 and 2011/12) as well as the change in abundance across years. Population abundances of Canada Geese and Mallards were very low at high elevations surrounding Crested Butte both during the winter of 2010/11 (high snowfall, late first snow-free date) and the following summer of 2011 compared to abundances in the winter of 2011/12 and the summer of 2012. Lower elevation populations remained constant across both years.

		Low Elevation		**High Elevation**	
Bird Species	**Time Period**	**Winter**	**Summer**	**Winter**	**Summer**
Canada Goose	2010-11	16.3967	4	1.7273	0
	2011-12	12.7273	4	10.5556	7
Change	**2010-11 / 2011-12**	**0.78**	**1**	**6.11**	**7+**
Mallard	2010-11	11.9669	12	1.5455	3
	2011-12	10.8858	13	14.1111	10
Change	**2010-11 / 2011-12**	**0.91**	**1.08**	**9.13**	**3.33**

Table D.2: Results from the Breeding Bird Survey (BBS) for the Southern Rockies/Coloroado Plateau Region for the time period from 1968 through 2012. Data are listed with number of routes (N), trend, 95% confidence interval, and a credibility measure (blue most credible, red least credible). Trends with a single asterisk denote significant trends with low credibility while a double asterisk denotes moderate or high credibility.

Bird Species	**N**	**Trend**	**(95% CI)**	**Credibility**
Canada Goose	85	6.36**	(1.79, 10.13)	yellow
Gadwall	58	2.54	(-0.31, 5.65)	yellow
American Wigeon	19	5.96*	(0.32, 12.12)	red
Mallard (all forms)	156	-0.4	(-1.75, 1.02)	blue
Blue-winged Teal	27	-3.35	(-8.23, 3.02)	red
Cinnamon Teal	52	0.01	(-3.28, 3.48)	yellow
Northern Shoveler	19	-0.13	(-5.08, 5.08)	red
Northern Pintail	26	-4.37	(-8.71, 0.37)	yellow
Green-winged Teal	73	-1.23	(-4.04, 1.60)	yellow
Redhead	22	7.71*	(2.27, 13.60)	red
Ring-necked Duck	29	4.8	(-0.77, 10.56)	red
Lesser Scaup	26	4.47	(-2.84, 11.95)	red
Common Merganser	44	3.15**	(0.03, 6.18)	yellow
Ruddy Duck	26	3.18	(-2.51, 8.68)	red
Pied-billed Grebe	30	4.3	(-1.74, 10.20)	red
Eared Grebe	22	6.17	(-2.21, 14.28)	red
Western and Clark's Grebe	27	7.92*	(0.94, 15.73)	red
Double-crested Cormorant	18	22.33*	(11.65, 38.34)	red
American White Pelican	16	16.45*	(5.65, 27.85)	red
Great Blue Heron	78	2.47	(-0.32, 4.61)	red
Snowy Egret	8	1.44	(-8.23, 12.81)	red
Black-crowned Night-Heron	22	-7.01	(-13.84, -0.93)	red
White-faced Ibis	18	11.86	(-1.01, 28.90)	red
American Coot	60	1.13	(-2.01, 4.16)	yellow
Sandhill Crane	26	8.01*	(2.60, 14.57)	red
Killdeer	168	-5.47**	(-6.25, -4.59)	blue
Mountain Plover	10	-4.77	(-14.24, 4.68)	red
American Avocet	16	-4.24	(-10.12, 2.07)	red
Spotted Sandpiper	117	-1.42**	(-2.63, -0.23)	yellow
Long-billed Curlew	10	0.04	(-4.92, 5.65)	red
Wilson's Snipe	114	-1.75**	(-2.81, -0.63)	blue
Wilson's Phalarope	26	-3.19	(-6.99, 0.66)	yellow
Ring-billed Gull	16	-4.74	(-15.28, 6.25)	red
California Gull	23	2.81	(-2.20, 11.28)	red
Red-winged Blackbird	161	-2.44**	(-3.01, -1.90)	blue
Brewer's Blackbird	177	-2.84**	(-3.61, -2.08)	blue