Ecosystem Impacts of Consumer Evolution:

Intraspecific Variation in the Elemental Phenotype of Aquatic Consumers

by

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ABSTRACT

Primary production in aquatic ecosystems is often limited by the availability of nitrogen (N) and/or phosphorus (P). Animals can substantially alter the relative availability of these nutrients by storing and recycling them in differential ratios. Variation in these stoichiometric traits, i.e., the elemental phenotype, within a species can link organismal evolution to ecosystem function. I examined the drivers of intraspecific variation in the elemental phenotype of aquatic consumers to test for the generality of these effects. Over a thermal gradient in Panamá, I found that average specific growth grate and body P content of the mayfly *Thraulodes* increased with environmental temperature, but that these patterns were due to site-specific differences rather than the direct effects of warmer temperature. In a meta-analysis of published studies, I found that in fishes intraspecific variation in dietary N:P ratio had a significant effect on excretion N:P ratio, but only when accounting for consumption. I tested for the effects of variation in consumption on excretion N:P ratio among populations of the fish *Gambusia marshi* in the Cuatro Ciénegas basin in Coahuila, Mexico. *G. marshi* inhabits warm groundwater-fed springs where it often co-occurs with predatory fishes and cool runoff-dominated wetlands which lack predators. Using stoichiometric models, I generated predictions for how variation in environmental temperature and predation pressure would affect the N:P ratio recycled by fishes. Adult female *G. marshi* excretion N:P ratio was higher in runoff-dominated sites, which was consistent with predators driving increased consumption rates by *G. marshi*. This result was supported by a diet ration manipulation experiment in which *G. marshi* raised on an *ad libitum* diet excreted N:P at a lower ratio than fish raised on a restricted diet ration. To further support the impacts of predation on phenotypic

diversification in *G. marshi*, I examined how body morphology varied among habitats and among closely related species. Both among and within species, predation had stronger effects on morphology than the physical environment. Overall, these results suggest that predation, not temperature, has strong effects on these phenotypic traits of aquatic consumers which can alter their role in ecosystem nutrient cycling through variation in consumption rates.

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CHAPTER 1

INTRODUCTION

Ecosystem ecology has frequently been argued to be the ecological discipline least informed by evolution (Holt 1995; Elser 2006; Matthews et al. 2011; Jeyasingh et al. 2014). Although heritable traits of single species can have strong effects at the ecosystem scale (Bailey et al. 2009; Post & Palkovacs 2009; Bassar et al. 2010), a widely-accepted predictive framework linking organic evolution to ecosystem function largely remains untested. Biological stoichiometry, the study of the balance of multiple chemical elements and energy in living systems, offers a promising theoretical framework for such studies because it is makes predictions using a common currency between individual organisms and ecosystem processes (Elser 2006). Jeyasingh et al. (2014) advanced this idea by defining the elemental phenotype of an organism as the content, acquisition, assimilation, allocation, excretion, and egestion of the chemical elements used in biological processes. In this sense, the elemental phenotype operates identically to the morphological phenotype and is thus subject to natural selection and drift. In order to use this framework, however, we first must get a better sense of the drivers of intraspecific variation in the elemental phenotype of organisms and the degree to which this variation is heritable. Considerable work on this subject has been conducted with primary producers (Sterner et al. 1992; Ågren & Weih 2012; Padfield et al. 2015), but less is known about drivers of intraspecific variation in the elemental phenotype of consumers. To advance our understanding of how ecosystem function is altered by the evolution of consumers, we must first examine these mechanisms.

Animal consumers are generally considered to be stoichiometrically homeostatic, i.e., their body elemental contents do not change with their diet (Sterner & Elser 2002). However, variation among individuals and especially among populations has been documented in many species of animals (summarized in Jeyasingh et al. 2014). Of these studies, nearly all found that phosphorus (P) content is substantially more variable among individuals than are the contents of other elements (Jeyasingh et al. 2014). In invertebrates, much of this variation has been successfully explained by the growth rate hypothesis, which states that organismal P content varies with maximum growth rate due to the elevated demand for ribosomal RNA (rRNA) production to meet rapid growth (Elser et al. 2003). Thus, selection for more rapid growth in turn leads to higher organismal P content and, potentially, reduced P recycling and greater potential sensitivity to stoichiometric food quality constraints (Sterner 1990; Sterner et al. 1992; Sterner & Hessen 1994; Elser et al. 2000). In vertebrates, however, the growth rate hypothesis is unlikely to explain variation in body P content because bone is a much larger pool of P than rRNA (Sterner & Elser 2002; Hendrixson et al. 2007). Empirical studies of vertebrate elemental contents have supported this hypothesized lack of relationship, with some evidence that body nitrogen: phosphorus ratio (N:P) actually increases with growth rate among species (Tanner et al. 2000; Liess et al. 2013; Benstead et al. 2014). We require a better understanding of what drives variation in growth rate and bone content in vertebrate animals to predict how their body elemental contents will respond to selection, but we also require a better understanding of how nutrient recycling of these key elements responds to these drivers in order to link this variation to ecosystem function.

The relative rates of nitrogen (N) and P recycled by animal consumers can drive variation in nutrient limitation and rates of primary production, particularly in aquatic ecosystems (Kitchell et al. 1979; Elser et al. 1988; Vanni 2002; Atkinson et al. 2013). The ratio of N:P resupplied by consumers varies with body elemental composition, diet elemental composition, and the maximum accumulation efficiency of the limiting nutrient (Sterner 1990). In Sterner's approach, this ratio encompasses both particulate egestion and dissolved excretion, but it is the latter that is most readily available to primary producers and microbes. In cases where consumers feed on similar diets and/or have large differences in body elemental demands, variation in body elemental composition can drive variation in excretion N:P (e.g., Sterner et al. 1992; Vanni et al. 2002). However, variation in body elemental contents has proven to be a poor predictor of variation in N:P excreted in intraspecific studies of fishes (El-Sabaawi et al. 2015; 2016; Tuckett et al. 2016), and field empirical studies have also found little support for dietary variation driving variation in N:P excreted by fishes (Verant et al. 2007; McManamay et al. 2011; Taylor et al. 2012). As a result, variation in N:P excreted by fishes seems to lie in variation in the N:P ratio of the diet as well as in the maximum accumulation efficiency of the limiting nutrient. However, little attention has been given to this latter parameter. In this dissertation, I explored drivers of intraspecific variation in the elemental phenotype of aquatic consumers to laid the groundwork for linking the evolution of these organisms to ecosystem function.

Outline of the Dissertation

In Chapter 2, I began by testing whether temperature-driven growth rate variation in Neotropical benthic consumers drives variation in body P content. Although

ectotherms generally grow more rapidly at warmer temperatures, P content tends to decrease with increasing acclimation temperature (Woods et al. 2003). These results run counter to the growth rate hypothesis, at least superficially (Cross et al. 2015). However, the growth rate hypothesis was formulated for growth at a constant temperature because increased growth at warmer temperatures is often driven by higher rates of protein synthesis by individual ribosomes rather than increased allocation to ribosomal RNA (Farewell & Neidhardt 1998; Elser et al. 2000). On the other hand, other sources of growth rate variation may covary with temperature. I directly addressed this question by measuring growth rates and body P content of a Neotropical grazer, the leptophlebiid mayfly *Thraulodes*, over an elevational and thermal gradient in Panamá as well as by conducting temperature manipulation experiments with *Thraulodes* and tadpoles of the toad *Rhinella* from the highest and lowest elevations of these study sites.

In Chapter 3, I turned to investigating variation in the stoichiometry of consumer-driven nutrient recycling by fishes. Although broad patterns in consumer excretion rates of N and P individually can be explained by body size, excretion N:P does not show strong allometric scaling (Allgeier et al. 2015; Vanni & McIntyre 2016), as would be expected if N and P excretion rates show similar allometric scaling themselves. Instead, intraspecific variation in excretion N:P may be due to variation in body N:P, diet N:P, and/or the maximum accumulation efficiency of the limiting nutrient (Sterner 1990). In this chapter, I specifically tested whether variation in dietary N:P and consumption rates drive variation in excretion N:P. Although difficult to measure in the field, a number of controlled aquaculture studies have directly manipulated dietary N:P and measured

consumption and excretion rates of N and P. I took advantage of the data generated by these studies to conduct a meta-analysis of these effects on excretion rates and N:P ratio.

In Chapter 4, I applied the questions examined in Chapter 3 to a natural ecosystem by investigating intraspecific variation in the fish *Gambusia marshi* in the Cuatro Ciénegas basin of Coahuila, Mexico. This basin consists of a large number of thermal springs, locally known as pozas, as well as a variety of runoff-fed wetlands and evaporative lagoons. Growth of irrigated agriculture in the region has led to declining groundwater flows into some springs, leading to transitions to runoff-dominated states. Runoff-dominated systems are more thermally variable and on average cooler, and these changes often lead to the extirpation of several fish species including the top predators, largemouth bass (*Micropterus salmoides*) and headwater catfish (*Ictalurus lupus*). Of the fish species in the region, *G. marshi* persists in both spring-fed and runoff-dominated systems, providing an opportunity to examine the effects of altered thermal regime and predation pressure on elemental phenotypic responses. I first modeled nutrient recycling N:P under different scenarios using the model of Sterner (1990) to establish predictions for how altered temperature and consumption would affect fish responses. I measured body C, N, and P contents and excretion rates of female *G. marshi* from nine sites in the Cuatro Ciénegas basin spanning this gradient in temperature and predation. I then conducted a common garden temperature manipulation experiment in the laboratory with populations from warm and cool low-predation habitats. Finally, I conducted a diet ration manipulation using fish from one source population.

In Chapter 5, I investigated the morphological phenotype of *Gambusia* to provide further evidence for how predation and the physical environment influence evolution of

these fishes. Due to morphological constraints on the caudal peduncle, fish face a trade-off between optimizing steady swim performance for endurance swimming and unsteady swim performance for burst swimming (Langerhans 2009). In lotic environments, fish should optimize steady performance to maintain position in the current and actively search for food, while in high predation environments fish should optimize unsteady performance to facilitate predator escape (Langerhans & Reznick 2010). In lotic environments with high predation, fish cannot optimize both types of swim performance; thus, the factor that most strongly affects fitness should be reflected in the phenotype. I addressed this question by examining specimens of *Gambusia marshi* across these gradients. In addition, I examined *G. alvarezi* and *G. hurtadoi* which inhabit predator-free springs as well as *G. panuco* which inhabits high predation lotic environments. I employed a geometric morphometric analysis to test the morphological predictions of Langerhans & Reznick (2010) and also specifically measured relative caudal peduncle area.

In the final chapter, I summarized the main conclusions of the research and synthesize the results. I discussed how these studies advance our understanding of stoichiometric theory and how it can be applied to understand ecosystem function in a changing world.

CHAPTER 2

DOES THE GROWTH RATE HYPOTHESIS APPLY ACROSS TEMPERATURES? VARIATION IN THE GROWTH RATE AND BODY PHOSPHORUS CONTENT OF NEOTROPICAL BENTHIC GRAZERS

Abstract

The growth rate hypothesis predicts that organisms with higher maximum growth rates will also have higher body phosphorus (P) content due to the increased demand for ribosomal RNA production needed to sustain rapid growth. However, this hypothesis was formulated for invertebrates growing at the same temperature. Within a biologically relevant temperature range, increased temperatures can lead to more rapid growth of ectotherms, suggesting that organisms in warmer environments might also contain higher P content per gram of dry mass. However, since higher growth rates at higher temperature may be supported by more rapid protein synthesis per ribosome rather than increased ribosome investment, increasing temperature might not lead to a positive relationship between growth and P content. I tested the growth rate hypothesis by examining two genera of Neotropical stream grazers, the leptophlebiid mayfly *Thraulodes* and the bufonid toad tadpole *Rhinella*. I measured the P content of field-collected *Thraulodes* as well as the stoichiometry of periphyton resources in six Panamanian streams over an elevational gradient spanning approximately 1100 m and 7 °C in mean temperature. I also measured *Thraulodes* growth rates using *in situ* growth chambers in two of these streams. Finally, I conducted temperature manipulation experiments with both *Thraulodes* and *Rhinella* at the highest and lowest elevation sites and measured differences in P content and growth rates. *Thraulodes* body %P increased

with temperature across the six streams, and average specific growth rate was higher in the warmer lowland stream. In the temperature manipulation experiments, both taxa exhibited higher growth rate and body %P in the lowland experiments regardless of experimental temperature, but growth rate and body %P of individuals were not correlated. Although I found that *Thraulodes* from warmer streams grew more rapidly and had higher body %P, my experimental results do not clearly support the application of the growth rate hypothesis across temperatures, instead supporting the hypothesis that organisms at warmer temperature achieve more rapid growth through more rapid protein synthesis rates per ribosome. Among the studied streams, other factors may play a larger role in driving variation in organismal P content.

Introduction

Somatic growth rate is a fundamental trait that integrates the effects of numerous environmental pressures (Arendt 1997). Somatic growth is a key component of organismal fitness and, as a result, is often strongly selected upon (Violle et al. 2007; Dmietriew 2011). Growth rate varies with a number of environmental factors including predation, disturbance, food quality, and temperature (Reznick & Endler 1982; Atkinson 1994; Dmietriew 2011). To achieve variation in growth rate over gradients in these variables, organisms must also vary in the biochemical processes that underlie growth. In most organisms except for retroviruses, new tissue is formed by the transcription of DNA into mRNA, which is then sent to ribosomes for protein synthesis which requires rRNA. Nucleic acids are relatively phosphorus-rich molecules (8.7%; Sterner & Elser 2002), and thus variation in their abundance in organismal cells could strongly affect phosphorus (P) demands. Although DNA content does not vary appreciably among individuals, RNA can vary considerably and thus represents a source of variation in whole organismal P content (Sterner & Elser 2002). Stemming from this logic, the growth rate hypothesis (GRH; Elser et al. 1996) was formulated to explain variation in organismal P content with growth rate.

The GRH states that differences in growth rate drive variation in organismal phosphorus (P) content (i.e., % dry mass) due to greater allocation to P-rich ribosomal RNA needed to support rapid growth (Elser et al. 1996; Elser et al. 2003; Hessen et al. 2013). The GRH has been supported by empirical tests in a wide variety of organisms, particularly microbes and small invertebrates growing under the same environmental conditions (Elser et al. 2003; Gillooly et al. 2005; Elser et al. 2006; see review in Hessen

et al. 2013). However, the GRH was formulated specifically for differences in invertebrate consumer growth under P limitation and at constant temperature (Elser et al. 2000a; Lukas et al. 2011; Hessen et al. 2013) and has not been well-tested beyond these conditions. Ectotherms generally grow more rapidly at warmer temperatures (Atkinson 1994) and their somatic RNA and P content might be expected to increase as well following the logic of the growth rate hypothesis (Cross et al. 2015). However, rapid growth at warmer temperatures is generally accomplished by more rapid protein synthesis per ribosome rather than increased ribosomal investment (Farewell & Neidhardt 1998), leading to the hypothesis that P content should not increase with growth rate over a gradient in temperature (Woods et al. 2003). Supporting this hypothesis, RNA content of many organisms is actually lower when acclimated to warmer temperatures (Woods et al. 2003). As a result, temperature-induced growth rate variation may not be useful in predicting consumer P demands under the growth rate hypothesis.

Changing thermal regimes could alter organismal P content through mechanisms other than the linkage between RNA and growth rate. Differences in temperature could alter P investment into other types of tissues, which may decouple growth rate and P content but could still lead to differences in somatic P storage (Sardans et al. 2012; Cross et al. 2015). This is particularly true in vertebrates, whose skeletal development involves a high P demand (Hendrixson et al. 2007). For this reason, the GRH was not expected to apply to vertebrates, a hypothesis supported by empirical tests with developing frogs (Sterner & Elser 2002; Liess et al. 2013). However, tadpoles reared at warmer temperatures still had higher P content at metamorphosis, suggesting that developmental temperature could affect skeletal ossification (Liess et al. 2013). On the other hand, more

rapidly growing fish exhibit reduced skeletal ossification (Arendt & Wilson 2000), suggesting that warmer temperatures could reduce somatic P content in vertebrates. Elucidating these effects is important as alterations to the thermal regimes of aquatic ecosystems could lead to shifts in P cycling through the consumer-mediated storage and transport of this often limiting nutrient.

Animal consumers can play an important role in ecosystem P dynamics through the storage, transport, and recycling of P and other biologically important nutrients (Vanni et al. 2013). Much empirical work on this topic has been conducted in tropical streams, where grazers can substantially affect aquatic ecosystems through their feeding, recycling, and storage of nutrients (e.g., Taylor et al. 2006; McIntyre et al. 2008; Capps & Flecker 2013). However, these consumer-mediated impacts are sensitive to changes in the biological community (Capps et al. 2015). Throughout the neotropics, for example, the species richness and biomass of stream-dwelling amphibians have been drastically reduced by disease-driven amphibian declines, particularly in cooler highland streams (Lips 1999). As a result, the dominant grazers in these affected streams are now aquatic insects, such as the immature stages of mayflies (Colón-Gaud et al. 2010; Rantala et al. 2015). This community shift has altered ecosystem function in affected streams (Connelly et al. 2008; Rugenski et al. 2012; Whiles et al. 2013), and can also affect riparian ecosystems through the movement of adults of both taxa to the terrestrial environment (Vanni et al. 2013). As a result, differential growth and P sequestration between aquatic insects and tadpoles in response to thermal variation could further alter the trajectory of stream and riparian ecosystems in the tropics affected by amphibian decline.

Here I investigate the relationships between consumer somatic P content and growth rate in two consumer taxa (a tadpole and a mayfly) across a thermal gradient in Panamá to test whether the growth rate hypothesis applies to temperature-induced growth rate differences. I conducted a survey of periphyton and consumer stoichiometry across a natural thermal gradient in combination with controlled temperature manipulation experiments. I predicted that organismal P content would not increase with growth rate over a thermal gradient based on the biochemical mechanisms by which more rapid growth is achieved at warmer temperatures. I especially did not expect this relationship in tadpoles, whose developing bones represent a substantial P reservoir. This work expands upon our understanding of the applicability of the growth rate hypothesis and sheds light on the ways in which temperature-induced growth rate differences may violate its assumptions.

Methods

Study Sites

I conducted this study in six streams in Panamá ranging in elevation from 52 m to 1156 m and in mean temperature from approximately 18 °C to 25 °C (Figure 2.1, Table 2.1). The three lowland streams were located in Parque Nacional Soberanía near Gamboa (Mendoza, Macho, and Frijolito), while the three higher elevation streams were located in Parque Nacional Omar Torrijos (Guabal), Bosque Protector Palo Seco (Castillo), and Reserva Forestal Fortuna (Chorro). I measured temperature and light incidence hourly during the dry season of 2014 (February-May) at all sites using HOBO Pendant loggers (Onset Computer Corporation; Bourne, MA). Organisms from two streams, Río Frijolito

and Quebrada Chorro, were studied more extensively through *in situ g*rowth measurements and temperature manipulation experiments.

Natural Thermal Gradient

At least six (range: 6-14) *Thraulodes* mayflies were collected from each stream by a combination of kick sampling with a D-frame net and hand searching on large rocks in riffles. The organisms were transported on ice and then frozen prior to determination of body P content. To examine whether variation in consumer P content could be driven by variation in food quality, I also measured carbon (C), nitrogen (N), and P contents (percentage of dry mass) of periphyton growing on rocks from all streams. Periphyton was collected from five replicate Loeb samples in both riffles and pools of each stream, and values were then averaged using methods described in Connelly et al. (2008) and Whiles et al. (2013).

I then measured *in situ* growth rates of *Thraulodes* using clear acrylic cylindrical chambers with fine mesh (500 μm) covering each end (described in Rugenski et al. 2012) in the two streams that provided the source populations for my experiments described below, Río Frijolito in Parque Nacional Soberanía and Quebrada Chorro in Reserva Forestal Fortuna. Mayflies were collected by hand by turning over rocks from stream riffles and then were placed into chambers containing four or five similar-sized individuals each with periphyton-covered rocks from the stream. I deployed five chambers per stream. Chambers were left in the stream for four days, after which mayflies were collected and re-measured. The length of mayflies was measured to the nearest 0.5 mm from photographs taken immediately before and after deploying and retrieving the chambers using imageJ, and converted to dry mass using a length-mass

regression for Leptophlebiidae (Benke et al. 1999). From these values I calculated specific growth rates (SGR) per day using the formula:

$$SGR = (\ln(Mass_{final}) - \ln(Mass_{initial})) / Time$$

Final sample size varied between sites based on number of mayflies recovered. I recovered approximately 60% of the mayflies initially placed into chambers at each site, but I excluded two chambers from the Soberanía site from further analysis because their corresponding labels were lost upon recovery.

Temperature Manipulation

For my temperature manipulation experiments, I collected grazers from the lowlands in Parque Nacional Soberanía and from Reserva Forestal Fortuna in the western highlands. I collected organisms from the stream with hand nets and transported them to coolers filled with 45 L of aged tap water (Soberanía) or aged stream water (Fortuna). Coolers were housed in a climate-controlled room in Soberanía (ambient air temperatures were too high to maintain cooler temperatures in pilot trials) with a 12:12 light:dark cycle for lowland experiments and on a covered outdoor patio at the Jorge L. Araúz research station in Fortuna for highland experiments where ambient temperatures were only slightly above stream temperatures (Figure 2.2). Light recordings inside coolers were also somewhat higher than those recorded in-stream, where zero light was recorded for 13-14 hours per day depending on weather. I kept coolers either at ambient temperature or heated them using 100 watt aquarium heaters (Eheim; Deizisau, Germany) to increase mean temperature by approximately 5 °C for three days. I employed recirculating aquarium pumps (Technological Aquatic Associated Manufacturing; Taiwan) to homogenously heat the water and simulate stream flow in both ambient and heated

coolers. Heaters were unplugged several hours after dark to mimic the slow overnight cooling of water experienced by the ambient treatments and the natural streams and then restarted in the morning after sunrise (Figure 2.2). The magnitude of the temperature manipulation was chosen based on the average sensitivity of stream temperature to changes in air temperature of 0.51°C/°C (Luce et al. 2014) and the range of predicted mean temperatures for Panamá under the A2 climate change scenarios (28-31°C, ensemble low and high, bottom and top 10% of all model predictions respectively, 2080-2099, World Bank Climate Change Knowledge Portal). Therefore, the experiment represents a short-term exposure to thermal regimes that these organisms may face for longer durations in the next century. I monitored water temperatures in the coolers using HOBO pendant loggers as above.

I placed five organisms in each cooler, conducting all treatments for a given species and site simultaneously. Organisms were fed periphyton *ad libitum* using either pre-colonized unglazed ceramic tiles from the stream of collection (Soberanía) or periphyton-covered rocks from the stream of collection (Fortuna). I replaced these with fresh tiles or rocks before starting with a new species and if periphyton appeared to have been substantially consumed during an experiment so that organisms could feed *ad libitum* and so the elemental composition of periphyton would not differ substantially from that in-stream. After three days, I measured change in body length as above, and for tadpoles, developmental stage (Gosner 1960). I held all organisms without food for approximately one hour to allow guts to clear so that body P content was not influenced by food in the gut. I then euthanized organisms and preserved them for determination of P content as described above. Half of the organisms were preserved for P content

analysis, while half were preserved for transcriptomic analysis (not included in this manuscript). Recovery rates were somewhat lower than field growth chambers. I recovered approximately 35% of mayflies and 60% of tadpoles at the Soberanía site and 50% of mayflies and 90% of tadpoles at the Fortuna site. My observations indicated that both mortality and emergence of adults contributed to my failure to recover all organisms. Most observed mortalities occurred within 12 h of transport to coolers, thus I believe these were likely due to handling stress rather than unfavorable conditions within coolers.

Laboratory Analyses

To measure body %P, tissue was dried to a constant weight at 50 °C. For mayflies and periphyton collected from streams, I digested homogenized tissues in 1N hydrochloric acid and then used inductively coupled plasma mass spectrometry to measure P content (Thermo iCAP 6300; ThermoFisher Scientific, Waltham, MA). Carbon and N contents of periphyton were measured using a CHN analyzer (Thermo Flash 2000 CHNS/O Elemental Analyzer; CE Elantech, New Brunswick, NJ). Phosphorus content of experimental organisms was measured using a similar protocol, but I instead used the colorimetric acid molybdate method following acid digestion (APHA 2005).

Statistical Analysis

I used ANCOVA with body length as a covariate to test how mayfly body %P varied with stream temperature. As specific growth rate is age- and size-dependent, I also used ANCOVA to test whether it varied in mayflies grown in chambers between the high-elevation cool stream and the low-elevation warm stream. For the temperature

manipulation experiment, I tested whether consumer P content varied among taxa, temperatures, sites, and the interaction of the latter two using ANOVA. I used separate ANOVAs for each species to test whether growth or development rates varied with temperature, site, and/or their interaction as the growth metrics were not directly comparable between the two genera. Although the metric for tadpoles is a measure more of development rate than growth rate, I could not reliably measure specific growth rate in developing tadpoles as they lost size and mass at later developmental stages. As faster rates of development also necessitate faster rates of protein synthesis, I believe this metric should produce results consistent with growth rate for the responses of interest. I also tested for a correlation between final Gosner stage and tadpole P content to test whether ontogenetic variation could explain variation in P contents. Finally, I tested for significance of correlations between consumer P content and specific growth rate (in mayflies) or development rate (in tadpoles) by testing whether Pearson's correlation coefficients differed from zero using t-tests. For all analyses, I visually assessed normality and heterogeneity of variance of model residuals using normal quantile and residuals vs. fitted value plots, respectively, and log-transformed data to meet assumptions as needed. All statistical analyses were performed using R version 3.2.2 (R Core Team, 2015).

Results

Natural Thermal Gradient

Mean water temperature during the dry season generally increased with decreasing elevation among the study sites, although the lowest site (Río Frijolito) was slightly cooler than the other two lowland sites (Table 2.1). The standard deviation of

water temperature varied somewhat among streams but not systematically with elevation or the mean temperature (Table 2.1). Mean incident light also varied among streams but again there was no evident pattern related to temperature or elevation (Table 2.1).

Among the six study streams, *Thraulodes* body P content did not vary with body size ($F_{2,42}$=0.98, p=0.382) but increased with mean water temperature ($F_{1,42}$=6.81, p=0.013) (Figure 2.3). In the *in situ* growth chambers, mean specific growth rate of mayflies decreased with initial body size (Size, $F_{1,11}$=17.08, p=0.002), but for a given size did not differ among sites (Site, $F_{1,11}$=4.09, p=0.068) (Figure 2.4). However, since mayflies were smaller on average in Río Frijolito, their average specific growth rate (0.059 mg*d^{-1}) was higher than those in Quebrada Chorro (0.035 mg*d^{-1}). I found no interactive effect of body size and stream site on specific growth rate ($F_{1,11}$=0.03, p=0.872).

I also measured patterns in periphyton stoichiometry among the six study streams to determine if resource quality variation could explain my results. Periphyton stoichiometry did not follow any clear elevational or thermal trends. The periphyton in the mid-elevation Quebrada Castillo was relatively nutrient-rich, with considerably higher P content than in the other streams, while the low-elevation Río Macho periphyton was relatively nutrient-poor, having the lowest C, N, and P contents of all six streams (Table 2.2). Periphyton C:P and N:P ratios were higher in Río Macho and the highland Quebrada Chorro than in the other four streams (Table 2.2), indicating greater potential for P-limitation of consumer growth in these two streams.

Temperature Manipulation

In mayflies, specific growth rate was higher at the warmer lowland site Soberanía (Site, $F_{1,20}$=8.93, p=0.007) and in the warmer temperature treatment (Treatment, $F_{1,20}$=8.56, p=0.008). Tadpoles used in the experiments were initially at later Gosner developmental stages at the lowland Soberanía site, and thus ultimately reached later Gosner stages by the end of the experiment (Table 2.3). In tadpoles, development rate was also higher at the lowland site (Site, $F_{1,35}$=63.21, p<0.001) as well as in the warmed treatments (Treatment, $F_{1,35}$=35.84, p<0.001), and there was also a significant interaction effect of site and temperature (Interaction, $F_{1,35}$=39.73, p<0.001) such that tadpoles developed more rapidly in warmer water at Soberanía, but developmental rate did not vary with temperature at Fortuna.

Body %P was not significantly correlated with final Gosner stage in tadpoles among sites and treatments (r=0.40, t_{13}=1.59, p=0.137). Similar to specific growth rate, body %P was higher at the lowland site in both taxa (Site, $F_{1,25}$=20.83, p<0.001). In contrast, body %P did not vary with temperature manipulation (Treatment, $F_{1,25}$=2.72, p=0.112) nor did it vary among species (Species, $F_{1,25}$=0.01, p=0.904) (Figure 2.5). Body P content was not significantly correlated with ln-transformed development rate in tadpoles (r=0.32, t_{13}=1.40, p=0.238) or ln-transformed specific growth rate in mayflies (r=0.42, t_{10}=1.47, p=0.173) (Figure 2.6).

Discussion

Variation in the somatic P content of invertebrates is consistently explained by variation in rRNA production needed for protein synthesis to achieve somatic growth under the growth rate hypothesis (GRH), but the limits to its applicability, particularly in regards to developing vertebrates, have not been fully tested. In this study I sought to test

the GRH in vertebrate and invertebrate benthic grazers over a thermal gradient in Panamá. Although my results meet predictions of the growth rate hypothesis, they do not support its application in explaining somatic P content variation with temperature. Across the six streams, average P content of *Thraulodes* increased with average water temperature. Since average specific growth rates of *Thraulodes* were also higher in the warmer lowland stream than the cooler highland stream, these data conform to the predicted relationship between growth rate and somatic P content under the GRH. However, neither body P content nor growth rate increased in either taxon with increased temperature in the manipulative experiment. Instead, both body %P and growth rates were higher in the lowland site experiments regardless of temperature treatment (Figure 2.25). My results suggest that, at least in *Thraulodes*, body P content increases with growth rate among sites, but that growth rate variation is not driven by temperature variation. This result aligns with the assumption of the GRH that it does not apply to growth rate variation driven by varying temperatures. Instead, I suggest that other factors drive variation in growth rate and body P content among these sites.

The GRH was formulated with an assumption that organisms were growing at the same temperature (Elser et al. 1996; Elser et al. 2000). We know from previously published work examining temperature-induced growth variation that it might not lead to variation in somatic P content if individual ribosomes synthesize proteins more rapidly, requiring a lower amount of ribosomal RNA to support rapid protein synthesis at higher temperatures (Farewell & Neidhardt 1998; Gillooly et al. 2005). In fact, eukaryotic RNA content tends to decrease in warm-acclimated organisms (Woods et al. 2003). Therefore, in taxa for which RNA is the primary pool of somatic P, including the mayflies and early-

stage tadpoles I studied, it is unlikely that increasing growth rates with warming temperatures will lead to higher somatic P content. If grazer P requirements do not change with warming temperatures, this will buffer them from potential P-limitation due to increased algal C:P with warming (Yvon-Durocher et al. 2010). Further, the accumulation of P in consumer biomass can represent a substantial P sink in aquatic ecosystems, particularly when those consumers have biphasic life cycles and migrate to terrestrial ecosystems as adults (Vanni et al. 2013; Tiegs et al. 2016); thus, the fact that grazer P content is invariant with temperature has implications for ecosystem-scale fluxes of P in these streams. However, growth rate and P content of these grazers did vary among streams both in field collections and in my experiment; thus, it is important to discuss what may have driven this variation.

Though it may be clear that temperature-driven effects did not affect the somatic P content of the grazers I studied, it is less clear why body P content and growth rate of both taxa varied among sites. As site-specific differences in P content were consistent in the temperature manipulation experiment in both *Thraulodes* and *Rhinella*, they may reflect some other important difference between lowland (<200 m) and highland (>500 m) streams (Figure 2.3). For example, the amphibian pathogen *Batrachochytridium dendrobatidis* is present across this entire elevational gradient, but has only led to catastrophic amphibian declines in highland streams (La Marca et al. 2005; Kilburn et al. 2010). If insect grazer densities increase in highland streams following amphibian declines, more intense intraspecific competition could lead to reduced individual growth rates. However, after reductions in tadpole densities following declines in one of my study streams, Río Guabal, densities of *Thraulodes* and other grazing invertebrate taxa

did not significantly change (Colón-Gaud et al. 2010). Since *Thraulodes* should have less competition for algal resources in post-decline streams, I believe this would lead to increased growth rates if it affected them at all. Differences in the severity of amphibian decline do not effectively explain *Thraulodes* growth differences among sites, but other differences, such as disturbance frequency, resource quality, and predation pressure among streams may provide more insights.

The presence or absence of fishes was a defining characteristic between the upland and lowland streams in this study. The lowland streams in my study host diverse insectivorous fish communities including the aquatic insect-feeding genera *Astyanax*, *Brycon*, *Andinoacara*, *Geophagus*, *Pimelodella*, *Trichomycterus*, and *Gobiomorus* (Kramer & Bryant 1995), while the upland streams either lack fish (Chorro and Castillo) or host only the primarily terrestrial insectivore *Brachyrhaphis* (Guabal) (Colón-Gaud et al. 2010). While all streams host predatory invertebrates such as pseudothelphusid crabs, dragonflies, and dobsonflies (Colón-Gaud et al. 2010; Múrria et al. 2015), fish and invertebrate predators can often invoke contrasting responses in prey due to differences in feeding strategies (Peckarsky & McIntosh 1998; Touchon & Warkentin 2008). Many fishes are visual predators and feed more actively during daylight hours while many of the invertebrate predators such as crabs and odonates are nocturnally active and rely more heavily on olfactory and tactile cues (Flecker 1992; Peckarsky & McIntosh 1998; Maitland 2003). Unfortunately, which predators have greater effects on prey fitness is highly prey species-specific (e.g., Touchon & Vonesh 2016), and I have insufficient data to know whether fish predators have stronger fitness costs than invertebrate predators in *Thraulodes* and *Rhinella*.

Elevated predation pressure selects for more rapid growth and development to maturity in many organisms including mayflies and amphibians (Reznick & Endler 1982; Peckarsky et al. 2001; Vonesh & Warkentin 2006), so variation in predation pressure could explain higher growth rate and P content of lowland taxa if fish predators induce stronger selection for rapid growth. Selection for rapid growth and development to maturity in Arctic *Daphnia* populations developing under short growing seasons explained higher P content in those populations relative to temperate populations when reared at the same temperature (Elser et al. 2000), and a similar scenario induced by predation risk may have occurred in my study. Although predation pressure is not a strong predictor of variation in body stoichiometry in Trinidadian guppies (El-Sabaawi et al. 2012), its effects on somatic P content via growth rate may be stronger in invertebrates and tadpoles. The latter develops P-rich bones only at later Gosner stages. As the streams I studied host genetically isolated populations of *Thraulodes* (Múrria et al. 2015), these effects could represent a potential evolutionary tradeoff in response to exposure to fish predators and merit further study.

Another possibility for the elevated P content of *Rhinella* at the lowland site is that it is an artifact of my experimental design. Tadpoles at the lowland site developed more rapidly and reached later stages by the end of my study (Table 2.3; Figure 2.5); thus, ontogenetic variation could explain variation in P content (Main et al. 1997; Pilati & Vanni 2007). This is particularly true of developing Anurans, which exhibit extreme ontogenetic changes in P content as bone ossification begins at later Gosner stages (Tiegs et al. 2016). In the related European toad (*Bufo bufo*), tadpole bones begin ossification at Gosner stage 38 (Dunlap & Sanchiz 1996). Some tadpoles at the lowland site reached

Gosner stage 38 by the end of the experiment, but I only included P content values for tadpoles at stages less than 38 in my analysis. The stage at which *Rhinella alata* or *R. marina* begin ossification is not known, but the fact that P content was not significantly correlated with final Gosner stage indicates that minimal ossification occurs before stage 38 in these species. Even if ontogenetic variation did not strongly influence my results, however, these changes are likely to affect the P content of developing tadpoles in natural ecosystems.

While my results provide insight into stoichiometric theory, they also have implications for Neotropical stream ecosystems facing disease-driven amphibian declines and future climatic change. The effects of the loss of amphibian communities on aquatic ecosystem functions have been well-studied (Connelly et al. 2008; Rugenski et al. 2012; Whiles et al. 2013), but both amphibians and many aquatic insects have terrestrial adult stages. The movement of organisms from aquatic ecosystems to the terrestrial environment can represent a significant nutrient sink to aquatic ecosystems and an important subsidy to their surrounding terrestrial counterparts (Sabo & Power 2002; Hoekman et al. 2011; Vanni et al. 2013; Capps et al. 2015). Variation in P content among populations of *Rhinella* and *Thraulodes* can lead to variation in P export to the surrounding terrestrial ecosystem. However, the biphasic life cycles of these organisms could lead to dramatic changes in the P content of adults (Tiegs et al. 2016), which I did not consider in this study. *Rhinella* metamorphs in particular may exhibit strong differences in P content from pre-ossification tadpoles if bone density is unrelated to tadpole growth rates. Further, since the total consumer biomass remains significantly

lower in post-decline streams (Whiles et al. 2013), this variation is unlikely to offset the P fluxes lost to amphibian extirpations.

One final caveat is that dietary stoichiometry of the study organisms likely varied among sites following variation in periphyton elemental contents (Table 2.2). The GRH is only successful at predicting P content variation under P-limited consumer growth, because consumers can store P in tissues aside from ribosomal RNA when excess is available (Hessen et al. 2013). This is particularly true of vertebrates, in which bone tissue may serve as a flexible pool of body P (Benstead et al. 2014). As a result, the particularly high C:P ratio of periphyton in Fortuna experiments (Chorro) relative to that in Soberanía (Frijolito) could explain why somatic P content of both taxa was not significantly correlated with growth rate (Figure 2.6) if consumer growth were limited by P in Fortuna experiments and C in Soberanía experiments. A more detailed investigation of how threshold elemental ratios (TERs; Sterner & Hessen 1994) vary with temperature is required to examine this mechanism. Variation in ontogeny and resource stoichiometry contributed to variation in consumer P content among sites in this study; thus, a carefully controlled study examining the effects of reduced competition for resources and predation pressure on the P content of these grazers is needed to understand the mechanisms behind the patterns I observed.

Although global climate change is expected to have a number of effects on stream ecosystems (Cross et al. 2015), my results do not provide any evidence that warming temperatures themselves will alter nutrient storage and export by individual consumers. Instead, a mechanistic understanding of the drivers of variation in these stoichiometric traits is needed to predict how they will respond over environmental

gradients. I suggest that more work investigating the drivers of growth rate variation and the relationship between juvenile growth and adult elemental composition in these taxa is needed to elucidate how consumers with biphasic life cycles such as these will alter nutrient dynamics in changing ecosystems.

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Table 2.1. Temperature, light incidence, and elevation of the six study streams in Panamá.

Site	Mean Temp (°C)	SD Temp	Mean Light (lx)	Elevation (m)
1. Quebrada Chorro	18.3	0.83	43.4	1156
2. Río Guabal	21.2	0.54	242.6	679
3. Quebrada Castillo	21.3	0.87	156.0	533
4. Río Frijolito	24.7	0.93	156.5	52
5. Río Macho	25.1	0.60	373.8	134
6. Río Mendoza	25.3	0.86	370.0	80

Table 2.2. Elemental contents and ratios of periphyton from the six sampled streams. Stoichiometric ratios are presented in molar form.

Site	%C	%N	%P	C:N	C:P	N:P
1. Qbda Chorro	18.83	2.30	0.08	9.54	606.96	63.60
2. Río Guabal	9.04	0.90	0.11	11.71	211.92	18.10
3. Qbda Castillo	14.45	1.69	0.21	9.97	177.44	17.80
4. Río Frijolito	16.07	1.86	0.12	10.07	345.33	34.29
5. Río Macho	8.33	0.79	0.03	12.29	716.01	58.25
6. Río Mendoza	10.65	1.03	0.12	12.05	228.86	18.99

Table 2.3. Gosner developmental stages of *Rhinella* tadpoles used in the temperature manipulation experiments.

Site	Temperature	Initial Avg.	Initial Range	Final Avg.	Final Range
Fortuna	Ambient	24.75	24-25	26.00	25-27
Fortuna	Warm	25.00	25-25	25.67	25-27
Soberanía	Ambient	29.60	29-31	31.20	30-34
Soberanía	Warm	30.75	29-32	33.67	31-36

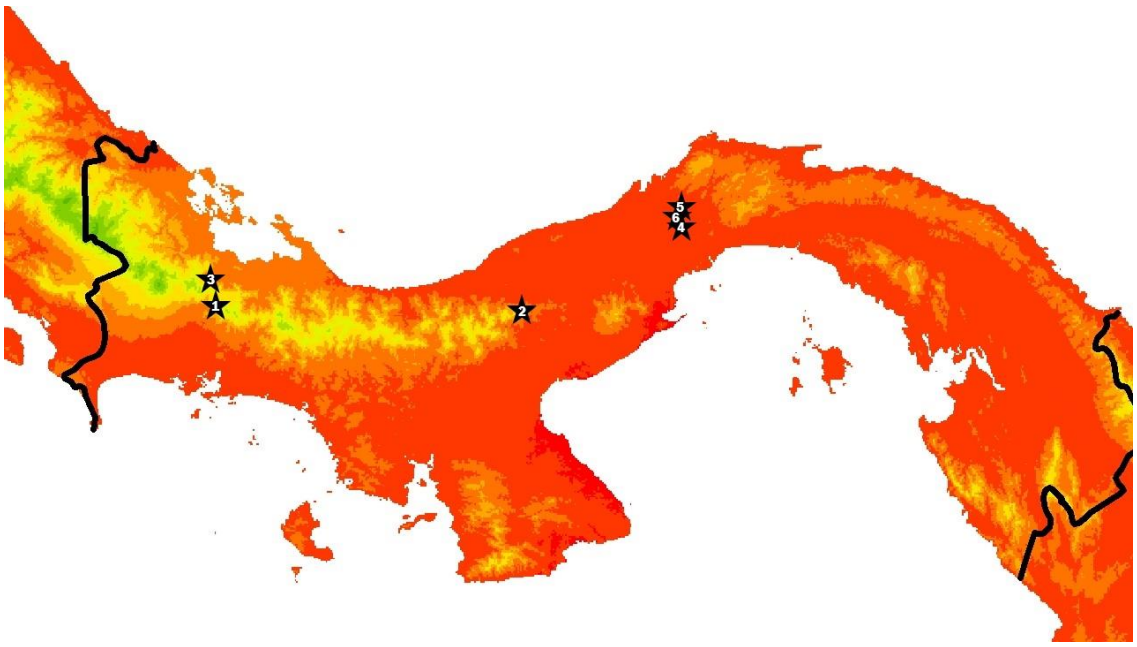

Figure 2.1. Map of Panamá with stars indicating the locations of the six study streams. The color gradient shows mean annual air temperature from WORLDCLIM at 30 arcsecond resolution, with green to red indicating colder to warmer. Sites are ranked from coldest to warmest and correspond to site numbers in Tables 1 and 2. Site 1 corresponds to where organisms were collected for Fortuna and Site 4 corresponds to where organisms were collected for Soberanía temperature manipulation experiments.

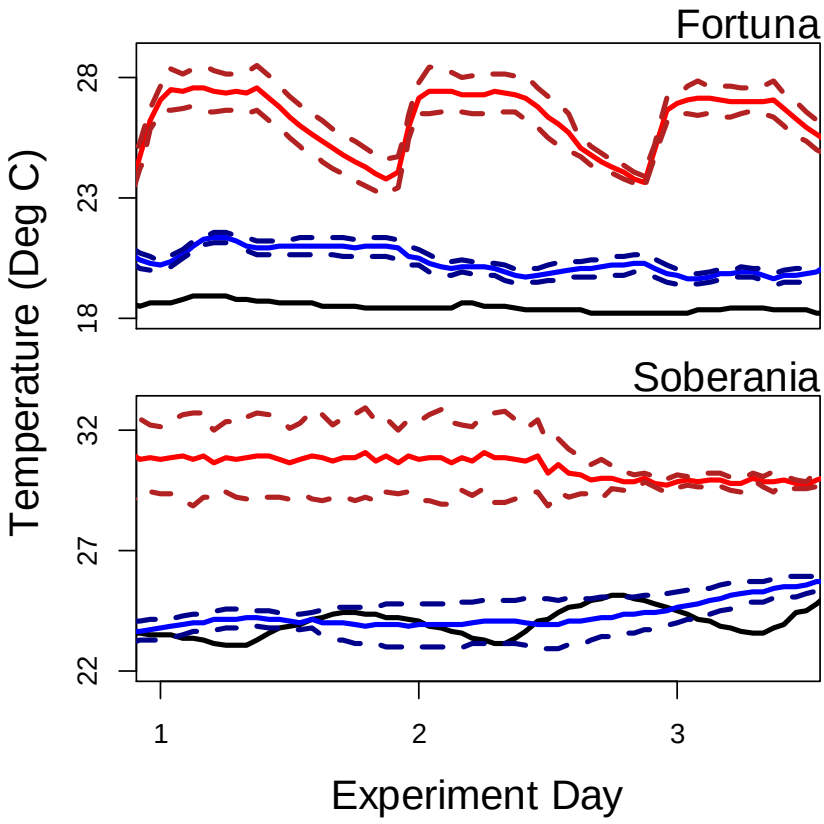

Figure 2.2. Temperature regimes in source streams (black lines), ambient treatments (blue lines), and heated treatments (red lines) during the course of temperature manipulation experiments. For experimental treatments, solid lines represent average temperatures and dashed lines represent ± one standard deviation.

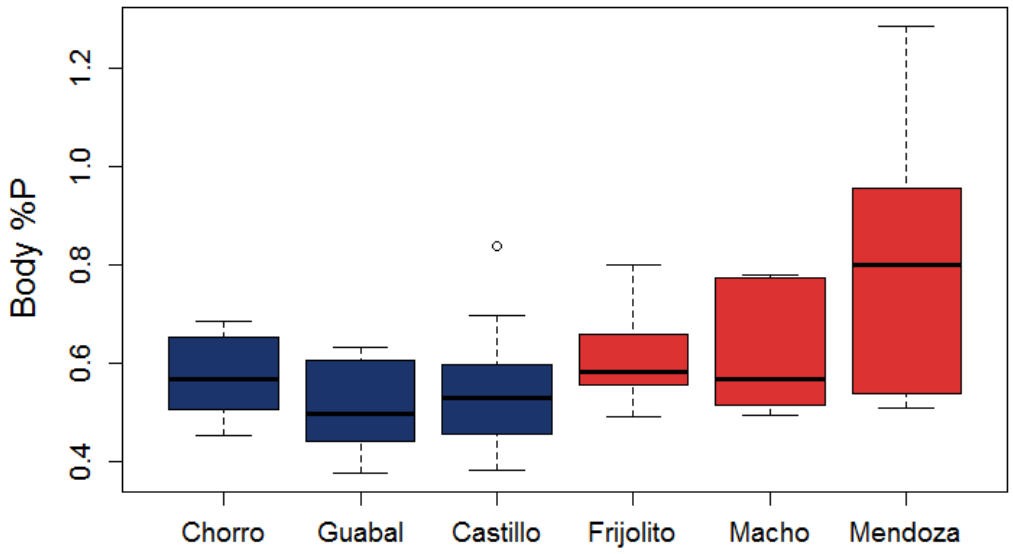

Figure 2.3. Body %P by dry mass of six populations of *Thraulodes* mayflies in Panamá. Streams are ordered from coolest to warmest. Ln-transformed body %P did not vary with mayfly size ($F_{2,42}$=0.98, p=0.382) but did increase with increasing average water temperature ($F_{1,42}$=6.81, p=0.013). This plot shows untransformed data for easier biological interpretation. In the boxplot, the thick line represents the median, the edges of the boxes represent the upper and lower quartiles, and the whiskers represent 1.5 times the interquartile range above and below the upper and lower quartiles.

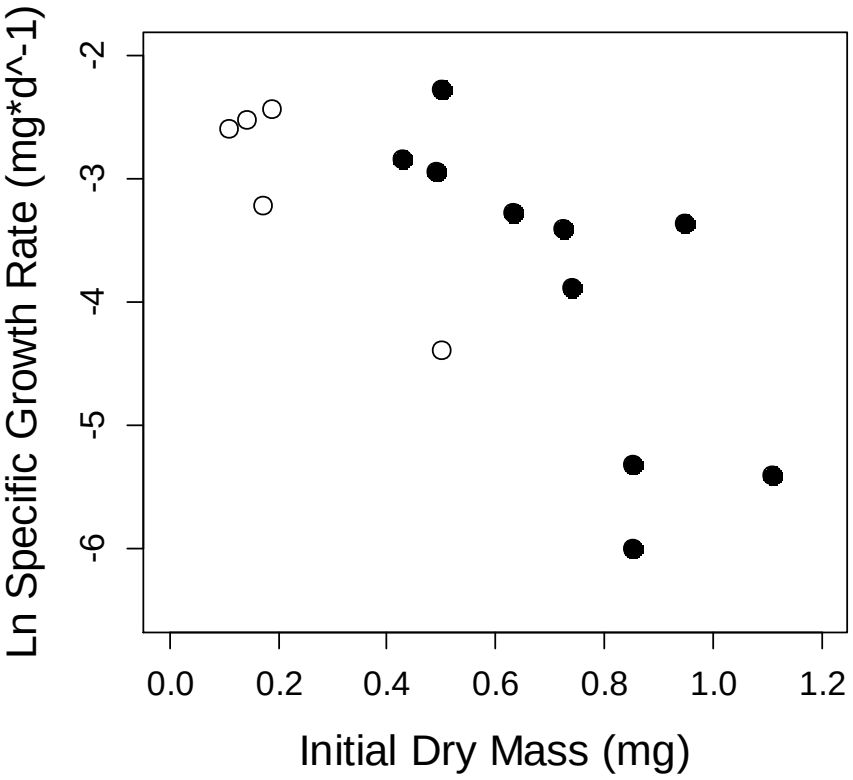

Figure 2.4. Specific growth rate of *Thraulodes* measured from *in situ* chambers at two sites. Points in white are the lowland stream, Frijolito, and points in black are the highland stream, Chorro. Each point is an individual mayfly. There was a significant effect of the covariate initial dry mass ($F_{1,11}$=17.08, p=0.002), but no site effect on specific growth rate after controlling for body size ($F_{1,11}$=4.09, p=0.068).

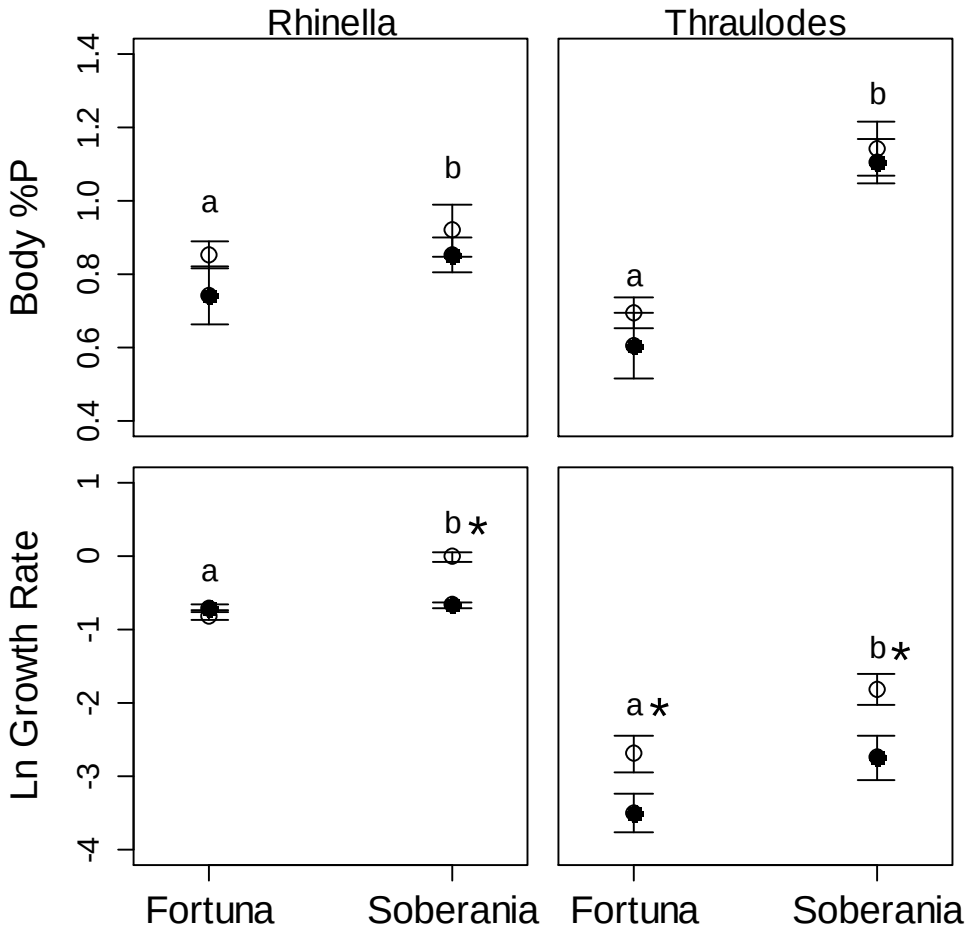

Figure 2.5. Average body %P by dry mass and growth rate of *Rhinella* (left panels) and *Thraulodes* (right panels). Growth of *Rhinella* is represented as Δ Gosner stage * d^{-1}, and for *Thraulodes* as specific growth rate based on body length (mg * d^{-1}). White points are heated temperature treatments and black points are ambient temperature treatments. Error bars are ± one standard error. Letters above points represent significant differences in the response variable among sites and asterisks next to letters represent significant effects of temperature treatment at that site.

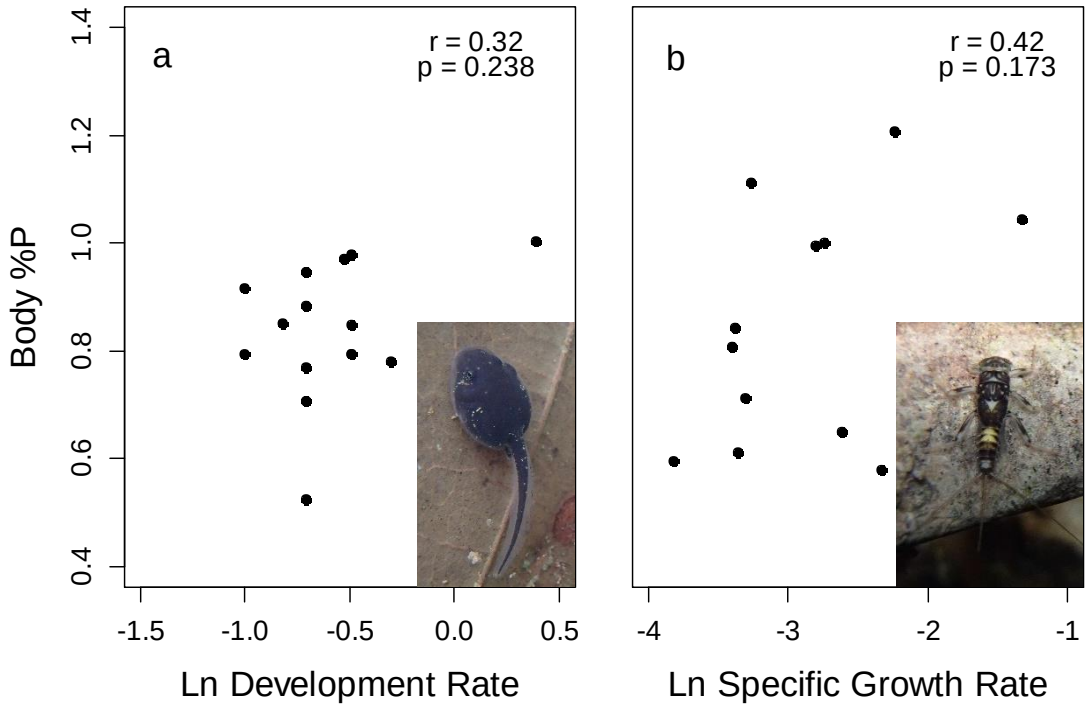

Figure 2.6. Correlations of body P content with growth rate in *Rhinella* (a) and *Thraulodes* (b) from temperature manipulation experiments. For *Rhinella*, growth is represented as Δ Gosner stage * day^{-1}, while in *Thraulodes* it is represented as specific growth rate based on body mass. Neither correlation coefficients were significantly different from zero ($p>0.05$).

CHAPTER 3

DIET COMPOSITION AFFECTS THE RATE AND N: P RATIO OF FISH EXCRETION

Abstract

Nutrient recycling by fish can be an important part of nutrient cycles in both freshwater and marine ecosystems. As a result, understanding the mechanisms that influence excretion elemental ratios of fish is of great importance to a complete understanding of aquatic nutrient cycles. As fish consume a wide range of diets that differ in elemental composition, stoichiometric theory can inform predictions about dietary effects on excretion ratios. I conducted a meta-analysis to test the effects of diet elemental composition on consumption and nutrient excretion by fish. I examined the relationship between consumption rate and diet N:P across all laboratory studies and calculated effect sizes for each excretion metric to test for significant effects. Consumption rate of N, but not P, was significantly negatively affected by diet N:P. Effect sizes of diet elemental composition on consumption-specific excretion N, P and N:P in laboratory studies were all significantly different from 0, but effect size for raw excretion N:P was not significantly different from zero in laboratory or field surveys. My results highlight the importance of having a mechanistic understanding of the drivers of consumer excretion rates and ratios. I suggest that more research is needed on how consumption and assimilation efficiency vary with N:P and in natural ecosystems in order to further understand mechanistic processes in consumer-driven nutrient recycling.

Introduction

Consumers can play an essential role in nutrient cycles in marine and freshwater ecosystems by controlling the storage and fluxes of key nutrients such as nitrogen (N) and phosphorus (P) (Kitchell et al. 1979; Elser et al. 1988; Vanni 2002; McIntyre et al. 2007; Allgeier et al. 2013). Through the excretion of dissolved inorganic nutrients, consumers can supply significant amounts of limiting nutrients to primary producers and decomposers (McIntyre et al. 2008; Small et al. 2011). While a considerable body of literature has developed around investigations of the importance of consumers to nutrient cycles in aquatic ecosystems, a mechanistic understanding of what influences rates and elemental ratios of nutrients excreted by consumers has lagged behind. Consumers can create biogeochemical hotspots simply by achieving locally high biomass (McIntyre et al. 2008; Atkinson et al. 2013; Capps & Flecker 2013), but the digestion, metabolism, storage and retention of consumed nutrients in consumer bodies, in combination with overall biomass, control the role individual species play in altering ecosystem function (Vanni et al. 2002; Small et al. 2011; Capps & Flecker 2013; Vanni et al. 2013). As a result, both the elemental composition of an organism and its diet should impact the rates and ratio at which it excretes nutrients (Sterner 1990; Elser & Urabe 1999; Sterner & Elser 2002). While the effect of organismal elemental composition on nutrient recycling by aquatic vertebrates has been investigated (e.g., Vanni et al. 2002; Hood et al. 2005), empirical studies of the impacts of diet elemental composition on excretion ratios have provided mixed results. The positive relationship between diet N:P and excreted N:P predicted by Sterner (1990) has been found in *Daphnia*, crayfish and mottled sculpin (*Cottus bairdi*) (He & Wang 2008; McManamay et al. 2011), but no significant

relationship has been found for a number of other species of fish and invertebrates (Schindler & Eby 1997; Verant et al. 2007; McManamay et al. 2011; Taylor et al. 2012). I investigate the impacts of diet on consumer excretion ratios in fish, a group of consumers that is both abundant in aquatic ecosystems and exhibits a great diversity of dietary strategies over which to examine excretion responses.

Fish are both abundant and diverse in many aquatic ecosystems, and as a result they have been frequently identified as the most important nutrient recyclers or retainers in a diverse range of aquatic systems (e.g., McIntyre et al. 2007; Small et al. 2011; Allgeier et al. 2013; Capps & Flecker 2013). Fish are diverse taxonomically as well as functionally, with known diets ranging widely in elemental composition from plant and algal detritus to invertebrates and other vertebrates (González-Bergonzoni et al. 2012). While some fish species are highly specialized to feed on specific foods, many fish are omnivorous to some degree and thus may consume diets that vary widely in quality over time or ontogeny (e.g., Grimm 1988; Pilati & Vanni 2007; González-Bergonzoni et al. 2012). Diets that are animal-based are generally relatively higher in P content than plant- or algae-based diets (e.g., Green et al. 2002), thus the impacts of animal- vs. plant- or algae-based diets on organismal physiology are informed by the mass balance of multiple chemical elements and energy in ecological systems employed by ecological stoichiometry (Sterner & Elser 2002). Following a mass balance model of fish growth assuming no difference in growth rate between diets, the difference between the amount of a given nutrient in the diet and that used by the consumer will equal the total released, which includes both nutrients excreted as dissolved inorganic and organic molecules and those egested as particulate waste (Kitchell et al. 1974; Sterner 1990; Schindler & Eby

1997; Figure 3.1). Therefore, fish excretion ratios should be proportional to diet elemental composition across a gradient of food elemental ratios unless fish differentially assimilate N and P (Sterner 1990; Schindler & Eby 1997; Sterner & Elser 2002). However, if fish differentially excrete and egest waste products, these ratios may not be directly proportional. Such a scenario arises when assimilation efficiency changes with diets of varying composition.

To assess how diet composition affects fish excretion ratios, direct manipulations of organismal diets in a controlled setting are required. Here I review the literature for studies in which multiple diets were fed to fish in a controlled setting and consumption rates and excretion rates and/or ratios were measured. Specifically, I draw on the field of experimental aquaculture research which represents a rich source of data on physiological responses of consumers to differing diets, the value of which is only beginning to be recognized by ecologists (Boersma & Elser 2006; Benstead et al. 2015). I employ a meta-analysis using standardized effect sizes to quantify how both consumption and composition of diet may affect excretion ratio in fish. Finally, I discuss the implications of the results from controlled settings to nutrient recycling in natural ecosystems.

Methods

I used a meta-analytic approach to determine if fish consumption rates and nutrient excretion ratios are influenced by the N and P composition of their diet. I used the ISI Web of Science database to search the peer-reviewed literature for studies of fish where diet was directly manipulated and a dissolved excretion response was measured. While faecal egestion is undoubtedly important in the N and P budgets of organisms (Figure 3.31; Halvorson et al. 2015), I focus on dissolved excretion because it is in this

form that excreted nutrients can have significant immediate impacts at the ecosystem scale (e.g., Kitchell et al. 1979; McIntyre et al. 2008; Small et al. 2011). I included studies that measured mass-specific excretion as a rate and those that reported it only as a loading per unit of fish biomass. I performed this search using the terms *fish*, *diet* and *excretion*. My search included articles published between 1970 and 2013. This search initially returned >600 articles, which were cursorily examined by title to determine whether they were likely relevant to the meta-analysis; for example, articles discussing only modeled excretion and growth or the use of fishmeal as a feed for other animals were disregarded. I identified 74 articles that appeared to be relevant by suggesting some type of study of fish N and P excretion among different diets which were then searched in greater detail to determine whether they met my criteria of inclusion. Studies included in the meta-analysis were those that were conducted on fish from a single population, included multiple diets that were directly manipulated or measured over natural gradients, measured N and P composition and fish consumption rates of those diets and measured N and/or P excretion in some form. In the few instances where my search returned multiple studies of a single species by the same research group, I selected only one of them with a random number generator to avoid violating test assumptions of independence. I categorized studies as those with direct diet manipulations in laboratory settings and those that measured natural variation diets in field settings and also noted whether dietary P was manipulated by varying the level of organic or inorganic P. I found no studies that conducted diet manipulation experiments in a natural setting.

As raw excretion rates may be influenced by differences in diet elemental composition and changes in consumption rate caused by diet differences, I used linear

models of mass-specific consumption rate (g * g fish^{-1} * day^{-1}) of N, P and total food consumption predicted by diet N:P to calculate and test for significance of effect sizes. From these models, I calculated effect size as the Pearson correlation coefficient *r*, which I transformed to *Z*-scores using the Fisher transformation (Rosenthal & DiMatteo 2001). Then, I tested whether mean effect sizes differed from 0 using *t*-tests with Bonferroni corrections to adjust α when performing multiple comparisons with the same dependent variable (Rice 1989; Rosenthal & DiMatteo 2001). I then calculated consumption-specific excretion measurements for each study by dividing N, P and N:P excreted by the mass-specific consumption rate when feeding on a given diet and used the above methods to calculate effect sizes for both consumption-specific and raw N, P and N:P excreted as a response to diet N:P in diet manipulation studies. Field surveys did not measure consumption rates and some did not report N and P excretion data individually, thus I could not calculate consumption-specific and single nutrient excretion effect sizes for those studies.

To assess whether effect sizes may have been influenced by other factors aside from diet composition, I tested study heterogeneity in the effect size measures. First, I used Cochran's *Q* to test for significance of study heterogeneity for each effect size measure. Cochran's *Q* follows a χ^2 distribution and is a widely used and relatively conservative test of study heterogeneity in meta-analyses (Takkouche et al. 1999). For those effect sizes with significant heterogeneity, I fit linear regression models for each effect size measurement as a response to difference in N:P between the diet end-members, average water temperature during the experimental period, initial fish mass and experimental duration (Rosenthal & DiMatteo 2001). My sample size was not sufficient

to estimate the interaction terms between all of these variables thus I examined only main effects. I assessed homoscedasticity and normality of residuals visually for each model with a plot of model residuals vs. fitted values and a normal probability plot, respectively. I could not construct linear regression models for field studies due to a lack of data presented in those manuscripts and small sample size. All analyses were conducted in the software R v2.15 (R Core Team 2013).

Results

Of the 74 candidate papers identified as possibly relevant, I found 19 independent studies that met my criteria for inclusion in the meta-analysis (Table 3.1). Of these, two studies featured only two experimental diets; these studies were excluded from the meta-analysis because effect sizes could not be calculated from two data points. Of the remaining 17 studies, 15 were diet manipulation experiments conducted in controlled laboratory facilities and two were field surveys conducted over natural gradients of diet elemental composition. Of the diet manipulations, 12 studies manipulated the levels of animal vs. plant-based protein while three studies directly manipulated dietary P content by adding phosphate salts to the same base diet; however these three studies did not measure N excretion. The majority of laboratory studies fed fish to apparent satiation, although several fed fish at specific levels based on fish body mass (Ballestrazzi et al. 1994; Green et al. 2002; Sumagaysay-Chavoso 2003; Yang et al. 2011). The laboratory studies included involved 10 fish species in seven families while the field studies included involved seven fish species in seven families (Table 3.1). Resource N:P ratios (by mass) ranged from 2.5 to 56 in laboratory studies (mean=8.2, SD=8.3) and from 2.4 to 174 in field studies (mean=44.7, SD=42.4). All field studies measured excretion N:P,

but only 12 of 15 laboratory studies presented N excretion data that allowed me to calculate N:P ratios of excretion. Additionally, all laboratory studies measured average initial fish mass, the average water temperature and the length of the experimental period between when the diet switch began and when excretion was measured.

I first examined whether consumption rates differed with diet composition. Total mass-specific consumption was not significantly affected by diet N:P (two-tailed *t*-test, t=-1.796, ν=11, P=0.10). Mass-specific consumption rate of N was also unaffected by diet N:P (two-tailed *t*-test, t=-0.270, ν=11, P=0.480) across studies but mass-specific P consumption rate significantly decreased with increasing diet N:P (two-tailed *t*-test, t=-3.650, ν=11, P=0.004) (Figure 3.2).

Diet effects on excretion ratios were similar for laboratory and field studies; however, I had fewer results for field studies due to the lack of consumption and separated N and P excretion data. For diet manipulation studies, effect size of diet N:P was significantly below 0 for P excretion (two-tailed *t*-test, t=-2.606, ν=14, P=0.021), and positive, but not significantly different from 0 for N excretion (two-tailed *t*-test, t=1.381, ν=11, P=0.195) (Figure 3.3). However, effect sizes for consumption-specific excretion of both P (two-tailed *t*-test, t=-2.244, ν=14, P=0.042) and N (two-tailed *t*-test, t=2.915, ν=11, P=0.014) were significantly different from 0 (Figure 3.3). Mean effect size of diet elemental composition on excretion N:P was not significantly different from 0 in diet manipulation studies (two-tailed *t*-test, t=2.00, ν=11, P=0.071) nor field surveys (two-tailed *t*-test, t=-0.002, ν=6, P=0.999), but was significantly different from 0 when corrected for consumption in diet manipulations (two-tailed *t*-test, t=2.42, ν=11, P=0.034) (Figure 3.4). Of all excretion response effect sizes in diet manipulation studies, only raw

P excretion exhibited significant heterogeneity (Q=23.82, ν=11, P=0.014). However, this heterogeneity was not significantly related to temperature, body mass, experimental duration or the difference in diet elemental composition (P>0.35 for all slopes). Additionally, there was significant heterogeneity in the response of N:P excretion in field studies (Q=12.83, ν=6, P=0.046), but I could not further explore any potential sources of this heterogeneity with the data available.

Discussion

In this study I synthesize a variety of empirical studies to show that diet can influence the ratio of dissolved nutrients excreted by aquatic consumers and suggest mechanisms by which it may do so. I found that dietary composition can have significant impacts on fish excretion ratios in controlled aquaculture settings. In particular, fish feeding on low N:P diets with higher amounts of animal protein excreted at a lower N:P ratio when accounting for the amount consumed (Figure 3.4). While these effects were strong in laboratory studies, other sources of variation must be examined to improve our mechanistic understanding of consumer-driven nutrient recycling in the field.

The mass-balance used in ecological stoichiometry (Sterner & Elser 2002) provides a simple framework for making predictions about organismal growth and nutrient recycling (Elser et al. 1988; Sterner 1990; Elser & Urabe 1999; Elser et al. 2001). In a mass-balance model of organismal growth, an animal should excrete and/or egest the excess nutrients consumed beyond what is needed for somatic growth and reproduction (Kitchell et al. 1974; Sterner & Elser 2002; Figure 3.1). As animals often exhibit strong stoichiometric homeostasis, their body elemental composition should not change substantially with diet; therefore excess consumed nutrients should be excreted or egested

(Sterner & Elser 2002). Some recent studies have suggested fish can be stoichiometrically flexible in some cases (McManamay et al. 2011; El-Sabaawi et al. 2012; Benstead et al. 2015), thus offering a potential explanation for the lack of strong correspondence of diet to excretion ratios in prior field studies (Schindler & Eby 1997; McManamay et al. 2011). However, in finding that consumption-specific excretion of N and N:P increases and P decreases with increasing diet N:P, my results support the predictions of stoichiometric theory. By accounting for consumption rates, we have gained new insights into how diet affects excretion ratios, insights that we could not from field studies for which consumption is extremely challenging to measure.

My results highlight the importance of consumption to excretion ratios. Most importantly, I found that while fish excretion rate of N did not significantly differ with diet composition, the excretion rate of N per gram of food consumed did (Figure 3). In contrast, excretion of P significantly decreased with increasing dietary N:P both independent of consumption and per gram consumed (Figure 3.3). This result could stem from fish eating less total food when feeding on high N:P diets and/or the fact that those diets had less P. The fact that mass-specific P consumption declined with increasing diet N:P is likely a consequence of most studies manipulating diet N:P primarily by manipulating P rather than N contents. As dietary P contents of fish can vary substantially through space and time (e.g., Mehner et al. 1998; Zandonà et al. 2011), this mechanism certainly impacts fish excretion ratios in natural settings. Further, mass-specific consumption rates tended to decline with increasing dietary N:P (P=0.10), thus this mechanism may be important in some, but not all situations. If fish consume less material when feeding on high N:P foods, and they also excrete more N and less P per

gram of diet consumed, then the ratio of N:P excreted will be altered through both direct and consumptive effects of diet stoichiometry. However, the underlying fact that both N and P excretion per gram consumed differed with diet N:P ratio is itself an interesting result that merits further examination.

In many of these studies, and often in natural systems, shifts in diet elemental composition co-occur with differences in the abundance of animal, plants or algae in the diet. In systems where consumers are largely consuming entirely one group of diet items, such as zooplankton feeding on phytoplankton, dietary N:P alone should largely determine how diet impacts excretion ratios (e.g., Sterner 1990). However, when animals consume diets with co-varying elemental composition and protein sources, these confounding sources of variation can produce differing effects on excretion ratios. Differences in the biochemical form of nutrients present could alter assimilation efficiency, which could in turn lead to differential egestion and excretion of individual nutrients. Although previous researchers have assumed constant assimilation efficiencies across diets in fish, this assumption is unrealistic for fish that consume diets consisting of multiple food types (Lall 1991). Since excess undigested nutrients should be egested as particulate waste products (Wotton & Malmqvist 2001; Halvorson et al. 2015), concurrent changes in digestibility with diet N:P could confound effects of diet on dissolved excretion rates. For example, variation in protein digestibility among plant- or algae-based and animal-based diet items could lead to differences in the amount of N egested as opposed to excreted without substantially affecting the amount of P egested or excreted (Robbins et al. 2005). However, P digestibility often differs between plants, algae and animals because plants often contain large amounts of P in phytate or phytic

acid, which is difficult for many fish to digest (Lall 1991). In my study a large number of plant-based diets were treated with phytase to increase P digestibility, thus I expected effects of P digestibility to be lower in magnitude than those of N digestibility. However, this digestibility difference is likely important to consumers in natural settings where fish cannot easily digest phytic acid. My results support this prediction, as consumption-specific excretion rates of both N and P differed with diet N:P (Figure 3.3), suggesting that N and P assimilation efficiency differed when feeding on high N:P plant-based diets vs. low N:P fishmeal-based diets. If the proportion and elemental ratios of material egested and excreted differ as a function of diet elemental composition and/or protein source, no strong relationship between diet elemental composition and excretion ratios may be observed (McManamay et al. 2011). As a result, my results support the idea that factors other than diet N:P such as protein digestibility, phytate contents and consumption rates must be taken into account when assessing the impacts of diet on consumer excretion ratios.

In spite of the considerable interest in excretion ratios such as N:P due to the importance of stoichiometric ratios of nutrients supplied to primary producers (e.g., Elser et al. 1988; Sterner et al. 1992), studies of excretion ratios are complicated by the fact that physiological regulation of N and P is largely controlled separately in fish. The majority of P consumed by fish and other vertebrates is used for bone mineralization (Lall 1991; Hendrixson et al. 2007; Huitema et al. 2012), yet a large amount of N consumed is used for the synthesis of protein (Sterner & Elser 2002). However, stoichiometric theory offers a link between these disparate physiological pathways. Since fish are generally stoichiometrically homeostatic over an individual life stage (Sterner &

Elser 2002), those excess nutrients not assimilated must be excreted and/or egested. Therefore, the ratio of what is consumed to what is needed by a fish can still be used to predict excretion ratios even if the individual pathways of those elements within the organism are not tightly connected. Another potential factor that may confound dietary effects on excretion is that excretion rates of N and P scale differently with body mass (Torres & Vanni 2007). If consumers grow at different rates when feeding on diets of differing elemental composition, differences in body mass alone could account for differences in excretion ratios (Villéger et al. 2012). I was unable to correct for the different allometries of N and P excretion because the units in which excretion was reported varied between studies, but all studies reported excretion as some function of fish mass. I believe that my conclusions are robust to the lack of an allometric correction in my analyses since specific growth rate was not significantly affected by diet N:P in the studies analyzed. However, P-limitation of growth in fish is possible at ecologically relevant dietary P levels (Hood et al. 2005; Benstead et al. 2015), thus I do believe that organismal growth and size differences caused by feeding on different diets could lead to differences in excretion ratios in natural settings.

Physiological responses to differing diets that are not accounted for in field studies of diet effects on excretion ratios may explain the difficulty of translating laboratory results into field settings. While heterogeneity in the only effect size measured in field studies, excretion N:P, was significantly greater than 0, only one of the six effect size measurements, raw P excretion, exhibited significant heterogeneity in laboratory studies. One source of this discrepancy may be the lack of correspondence between measured resources and actual fish diets. There are considerable difficulties associated

with measuring the true elemental composition of the diet consumed and assimilated in the field. If the resources sampled by the researchers do not specifically match what the fish are consuming and assimilating, conclusions about the effect of diet on excretion ratios may be invalid (Hood et al. 2005). This may be particularly true of omnivorous fish, which may consume different proportions of animals, plants and algae at different sites or times of the year (e.g., Grimm 1988). Further, local selection pressures such as the degree of predation can lead to differences in fish dietary habits and life history traits between sites (Zandonà et al. 2011; El-Sabaawi et al. 2012). While differences between fish in each treatment were controlled for in aquaculture studies by selecting all fish from one population, such as a single hatchery source and keeping all fish under the same conditions aside from the diet they were fed, field studies often compare individuals from separate populations.

Evolutionary differences between populations in the field studies may also represent a covariate that cannot be separated from diet differences, thus complicating interpretation. That is, comparisons of diet differences of a given species between sites, e.g., different streams or lakes, represent populations of that species that likely experience at least some degree of genetic separation. Therefore, differences in genotypes between populations cannot be ruled out as a confounding variable in these studies. While stoichiometric theory predicts that individuals of a given animal species and life history stage should have a given C:N:P stoichiometric composition (Sterner & Elser 2002), this does not apply across organisms with differing genotypes. Indeed, P homeostasis is known to be genetically controlled in developing fish (Huitema et al. 2012). Therefore, differential selection pressures between populations may affect a fish's response to diet

quality. Differences in selection pressures such as temperature, salinity, resource quality and predation pressure also drive evolution of organismal traits and life histories that can affect body elemental composition (e.g., Zandonà et al. 2011; El-Sabaawi et al. 2012; Liess et al. 2013). Since interpopulation differences may be a source of unmeasured variance in studies across natural gradients, linking evolutionary divergence to consumer-driven nutrient recycling represents a promising area of future research.

Since Vanni (2002) reviewed the importance of nutrient recycling by consumers in freshwater ecosystems, we have gained a greater appreciation for the role animals play in the way nutrients cycle through ecosystems. Indeed, many studies have investigated how important the transportation and transformation of nutrients by consumers can be to ecosystem function (McIntyre et al. 2007; Layman et al. 2011; Small et al. 2011; Atkinson et al. 2013). However, more work is needed to improve our understanding of the mechanisms that influence consumer excretion rates and ratios. My results suggest that diet is one of these mechanisms, but relatively few studies have examined the effects of diet composition on consumer-driven nutrient recycling in the field (McManamay et al. 2011). I show that dietary N:P can affect excretion ratios across several fish species when correcting for consumption (Figure 3.4). As raw N excretion was not significantly affected by dietary N:P (Figure 3.3), I hypothesize that differences in protein digestibility can weaken the relationship between dietary N:P and excreted N:P for consumers that feed on both animal and plant or algal material. While the application of stoichiometric theory provides a promising framework through which to investigate consumer impacts on ecosystem function, effective testing of stoichiometric theory may require that future work examining dietary effects on excretion rates and ratios should consider not only

dietary N:P but specifically the forms in which these nutrients are present in the diet, how much is consumed and how efficiently consumers assimilate dietary elements. Additionally, it is worth investigating whether evolutionary differences between populations impact intraspecific consumer nutrient recycling rates. While my study suggests that dietary composition can play a significant role in altering excretion rates and ratios, more careful tests of this effect in the field across a range of diets are needed before the impact of resource quality changes on consumer-driven nutrient recycling and its importance to ecosystem function can be fully understood and integrated into conceptual and theoretical frameworks.

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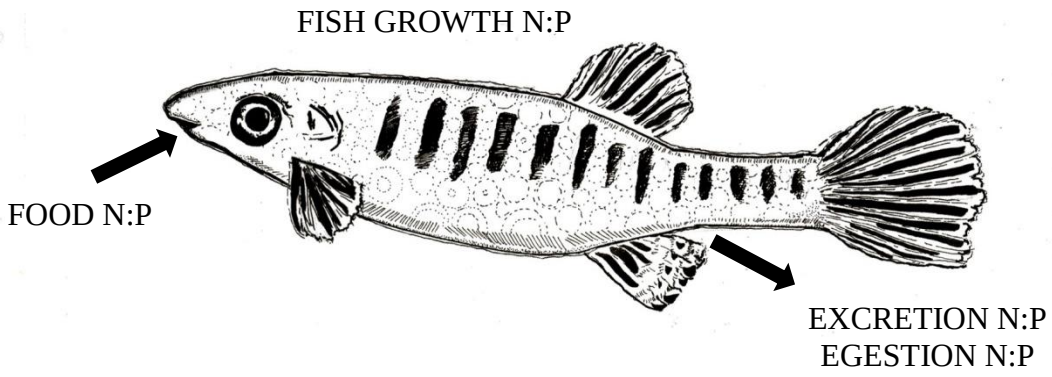
Table 3.1. Species and family identities of fish in studies included in the meta-analysis. Reference numbers are as follows: (1) Sukumaran et al. 2009; (2) Sumagaysay-Chavoso 2003; (3) Jahan et al. 2002; (4) Kaushik et al. 2004; (5) Ballestrazzi et al. 1994; (6) Tantikitti et al. 2005; (7) Yang et al. 2009; (8) Green et al. 2002; (9) Bureau & Cho 1999; (10) Rodehutscord et al. 2000; (11) Hossain et al. 2007; (12) Sarker et al. 2007; (13) Storebakken et al. 1998; (14) Sarker et al. 2011; (15) Dias et al. 2009; (16) Small et al. 2011; (17) McManamay et al. 2011.

Species	**Family**	**Reference(s)**
Diet Manipulations		
Catla catla	Cyprinidae	1
Chanos chanos	Chanidae	2
Cyprinus carpio	Cyprinidae	3
Dicentrarchus labrax	Moronidae	4,5
Lates calcarifer	Latidae	6
Oncorhynchus mykiss	Salmonidae	7,8,9,10
Pagrus major	Sparidae	11,12
Salmo salar	Salmonidae	13
Seriola quinqueradiata	Carangidae	14
Sparus aurata	Sparidae	15
Field Studies		
Alfaro cultratus	Poeciliidae	16
Astatheros alfari	Cichlidae	16
Astyanax aeneus	Characidae	16
Atherinella hubbsi	Atherinopsidae	16
Chrosomus erythrogaster	Cyprinidae	17
Cottus bairdi	Cottidae	17
Oncorhynchus mykiss	Salmonidae	17



EXCRETION N = FOOD N – (FISH GROWTH N + EGESTION N)

EXCRETION P = FOOD P – (FISH GROWTH P + EGESTION P)

Figure 3.1. Mass balance model of N and P budgets for a fish. This model represents a conceptual simplification of the major nutrient fluxes in consumers (Kitchell et al. 1974; Sterner 1990).

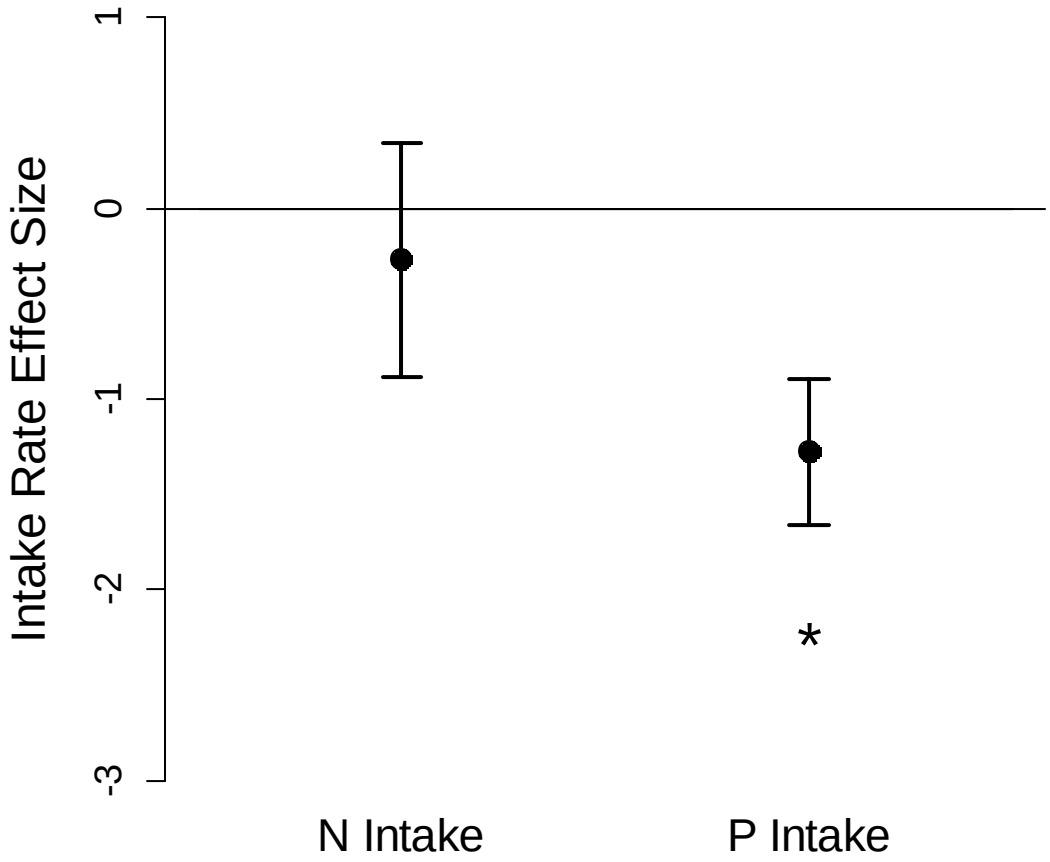


Figure 3.2. Effect size of diet N:P on intake (g * g fish^{-1} * day^{-1}) of N and P in diet manipulation studies. Effect size, η^2, was measured as the treatment sum-of-squares divided by total sum-of-squares from a linear model then transformed into a *Z* score for ease of analysis. Bars with * indicates effect size significantly different from zero based on a two-tailed *t*-test. Column lengths indicate mean effect sizes and error bars represent 95% confidence intervals.

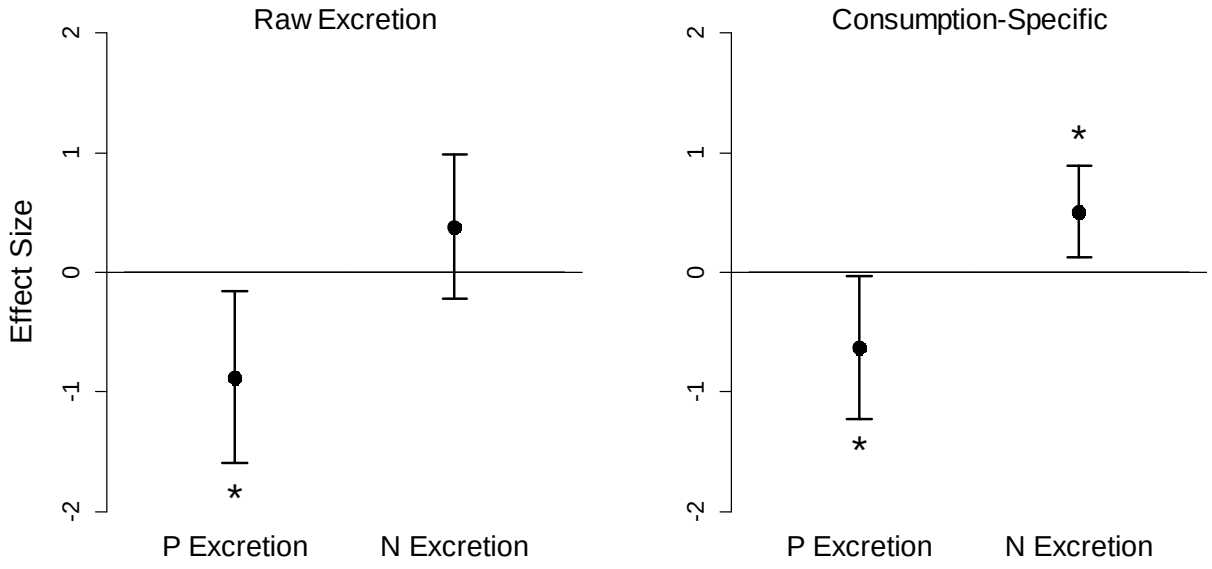


Figure 3.3. Effect size of diet N:P on raw and consumption-specific N and P excretion in diet manipulation studies. Effect size, η^2, was measured as the treatment sum-of-squares divided by total sum-of-squares from a linear model then transformed into a *Z* score for ease of analysis. Consumption-specific excretion was calculated as the excretion measure presented in the study divided by mass-specific consumption rate. Points with * indicates effect size significantly different from zero based on a two-tailed *t*-test. Points indicate mean effect sizes and error bars represent 95% confidence intervals.

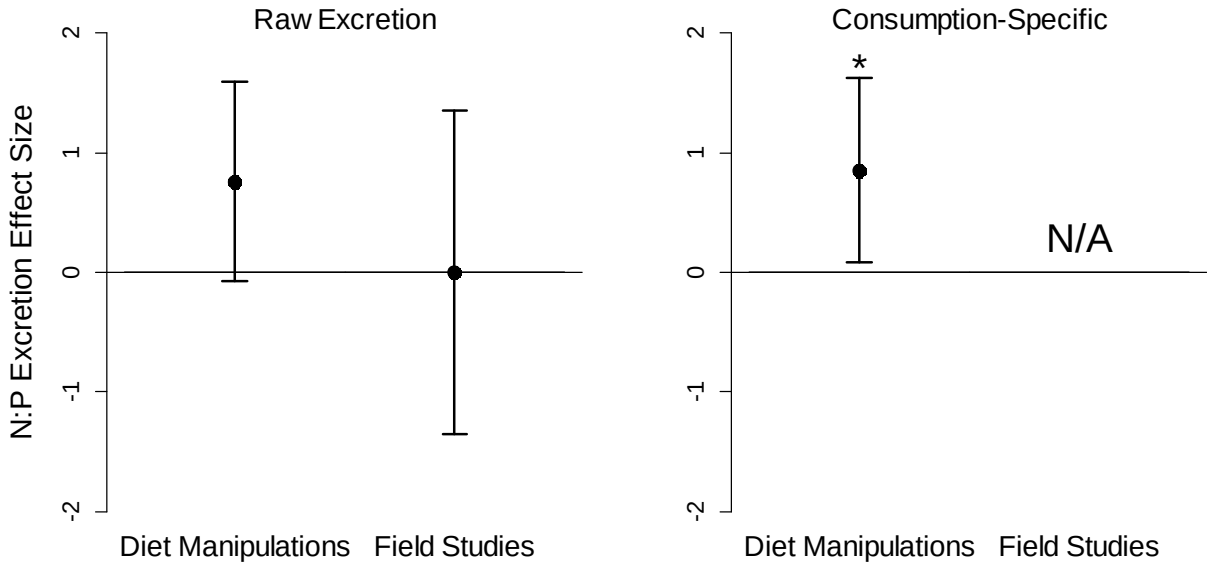


Figure 3.4. Mean ± 95% confidence interval of effect size of diet N:P on excretion N:P. Effect size, η^2, was measured as the treatment sum-of-squares divided by total sum-of-squares from a linear model then transformed into a *Z* score for ease of analysis. Consumption-specific effect sizes are missing in field studies because those studies did not measure consumption rate. Points with * indicates effect size significantly different from zero based on a two-tailed *t*-test. Points indicate mean effect sizes and error bars represent 95% confidence intervals.

CHAPTER 4

CONSUMPTION UNDERLIES THE EFFECTS OF PREDATION ON NUTRIENT RECYCLING BY A DESERT FISH

Abstract

Consumer-driven nutrient recycling can have substantial effects on primary production and patterns of nutrient limitation in aquatic ecosystems by altering the relative supplies of the key nutrients nitrogen (N) and phosphorus (P). Thus, study of intraspecific variation in nutrient excretion rates and N:P ratio can link organismal evolution to ecosystem function if that variation is driven by heritable, evolving traits. However, the mechanisms that explain variation in recycling N:P are not well-understood. I examined the relative importance of potential drivers of intraspecific variation in nutrient recycling by the fish *Gambusia marshi* among aquatic habitats in the Cuatro Ciénegas basin of Coahuila, Mexico where *G. marshi* inhabits warm thermal springs with high predation pressure and cooler, surface runoff-fed systems with low predation pressure. I hypothesized that variation in consumption rates among these habitats would drive intraspecific differences in excretion rates and N:P ratio. I found that female fish in groundwater-fed sites were smaller, had lower body %C, and higher body %P. Further, these fish excreted N at a lower rate and P at a higher rate, leading to a lower excretion N:P ratio. Laboratory experiments revealed that developmental temperature alone did not explain variation in excretion N:P when fish were fed *ad libitum*. However, experimentally restricting diet ration in the lab led to reduced rates of P excretion and thus a higher excretion N:P ratio. Reduced consumption under reduced predation pressure had stronger consequences for P retention and excretion among

populations than did variation in body stoichiometry. These results highlight the utility of stoichiometric models for predicting variation in consumer-driven nutrient recycling within a phenotypically variable species.

Introduction

Primary production in aquatic ecosystems is often limited by the supply of nitrogen (N) and/or phosphorus (P) (Elser et al. 2007). These nutrients originate largely from the surrounding watershed for uptake and growth by primary producers but are also recycled to primary producers in dissolved form via excretion by aquatic consumers (Kitchell et al. 1979; Vanni 2002). This consumer-driven nutrient recycling can create biogeochemical hotspots in aquatic ecosystems and supply a substantial fraction of the nutrient demand by primary producers and decomposers over a growing season (McIntyre et al. 2008; Small et al. 2011). Furthermore, consumers can recycle N and P differently due to differential retention of N and P into body tissues (Elser & Urabe 1999) and thus regulate the identity of the nutrient (N or P) limiting to primary producers (Elser et al. 1988). These stoichiometric traits have been defined as the elemental phenotype of an organism (Jeyasingh et al. 2014). Intraspecific variation in the elemental phenotype can serve as a key link in understanding how organismal evolution can affect ecosystem function (Elser 2006; Jeyasingh et al. 2014; Leal et al. 2017). For this to be true, intraspecific variation in the elemental phenotype must be driven by heritable traits subject to natural selection and/or genetic drift (Jeyasingh et al. 2014). Evidence that nutrient storage and excretion are heritable traits affected by natural selection and drift does exist (Elser et al. 2000; El-Sabaawi et al. 2015; 2016), but the mechanisms linking various selective pressures to variation in nutrient recycling remain relatively unstudied.

Changes in the consumer community can have dramatic effects on the fluxes of N and P in aquatic ecosystems (Elser et al. 1988; Capps & Flecker 2013; Capps et al. 2015); thus, much of our knowledge on drivers of variation in excretion rates and ratios comes

from interspecific comparisons (but see DeMott et al. 1998; Jeyasingh et al. 2015; El-Sabaawi et al. 2015; 2016). Among species, excretion rates of N and P individually scale with body mass due to their dependence on metabolic rate but body mass is a poor predictor of the N:P ratio excreted by consumers (Allgeier et al. 2015; Vanni & McIntyre 2016). Stoichiometric theory predicts that the N:P recycled by consumers in general depends on consumer body N:P, diet N:P, and *L*, the maximum accumulation efficiency of the limiting nutrient (Sterner 1990). Empirical studies have demonstrated that variation in consumer nutrient demands can explain variation in excretion N:P among species (Elser et al. 1988; Vanni et al. 2002), but it remains unclear whether these same predictors can be used to explain *intra*specific variation in excretion rates and N:P ratio.

Unlike in interspecific studies, intraspecific variation in body N:P is, at best, a weak predictor of excretion N:P of aquatic consumers (El-Sabaawi et al. 2015; 2016; Tobler et al. 2016; Tuckett et al. 2016). Instead, variation in diet and consumption rate appear to be more consistent drivers of intraspecific variation in excretion N:P (Moody et al. 2015; El-Sabaawi et al. 2016). High consumption rates are needed to meet elevated energetic demands and, in growing consumers, this can result in more rapid somatic growth. As fish bone acts as a flexible P reservoir (Lall & Lewis-McCrea 2007; Benstead et al. 2014); growing fish face a tradeoff between allocating P to bone for structural support vs. ribosomal RNA (rRNA) needed for growth (Sterner & Elser 2002). Supporting this notion, some fishes exhibit reduced ossification of bones and scales under rapid growth (Arendt et al. 2001). However, more rapidly growing fish also exhibit elevated C:P and N:P ratios of mass gain, indicating that rapidly growing fishes are less efficient at retaining dietary P (Azevedo et al. 1998; Downs et al. 2016). If P assimilation

efficiency is reduced at higher consumption and growth rates, then this effect could drive variation in nutrient recycling N:P ratio by fishes.

Individual energetic demands and consumption rates vary with many factors, but one potential driver of variation in consumption is predation pressure. Predators may increase consumption rates and alter nutrient recycling by prey due to the energetically demanding stress responses through what has been called the "general stress paradigm" (Hawlena & Schmidt 2010) or reduce prey consumption and excretion rates by limiting foraging activity (Dalton & Flecker 2014). Empirical studies of aquatic organisms (e.g., Dalton & Flecker 2014) suggest the latter, but this result has not been well-tested at the scale of natural populations of aquatic consumers. Further, this body of work has focused on C and N dynamics; P should be integrated into this framework given its broader relevance in aquatic ecosystems.

I tested how variation in predation pressure and temperature affect intraspecific variation in nutrient recycling rates and ratios in a desert fish species, *Gambusia marshi*. *G. marshi* is widespread in the Cuatro Ciénegas basin in Coahuila, Mexico, where it inhabits thermal groundwater-fed springs with predators in addition to cooler, surface runoff-dominated wetlands and lagoons that tend to lack predators (Minckley 1969). Growth of irrigated agriculture in the region has led to declines in the groundwater table and spring discharge, which has in turn reduced temperatures and piscivore populations in runoff-dominated habitats (Contreras-Balderas 1984; Minckley 1992; Souza et al. 2006). I hypothesized that variation in predation pressure and temperature would drive patterns in fish excretion N:P through changes in P retention and assimilation efficiency (*L* in Sterner 1990) because variation in this parameter can have large effects on recycled

N:P. In this study, I explore the mechanisms by which shifts from groundwater- to runoff-dominated systems alter *G. marshi* consumption and nutrient recycling.

Methods

Study System

The Cuatro Ciénegas basin (Figure 4.1) hosts hundreds of spring pools as well as numerous evaporative marshes and lagoons (Minckley 1969). These ecosystems together support high proportions of endemic fishes, aquatic invertebrates, and microbiota (Minckley 1969; Souza et al. 2006). Springs are geothermally heated and range in temperature from 29-34 °C, while evaporative systems are on average cooler and much more thermally variable (Corman & Ramos unpublished data; Table 4.1). As groundwater discharge into some springs has declined following intensification of irrigated agriculture in the region, springs are increasingly fed by surface runoff and have become physically and biologically more similar to evaporative environments. Finally, the surface waters of Cuatro Ciénegas are deficient in P and primary production is strongly P-limited (Minckley & Cole 1968; Elser et al. 2005; Corman et al. 2016).

Although not endemic to the basin, *Gambusia marshi* is restricted to Cuatro Ciénegas and its outflow, the Río Salado de Nadadores (Minckley 1962). Within its range, it is found in nearly all aquatic habitats in Cuatro Ciénegas that can support fish (Minckley 1969). In some of these locations it co-occurs with the predatory largemouth bass (*Micropterus salmoides*), headwater catfish (*Ictalurus lupus*), and the piscivorous morph of the Cuatro Ciénegas cichlid (*Herichthys minckleyi*), while in others it occurs with no other fish or with only small fish incapable of preying upon adults (such as the bolsón pupfish (*Cyprinodon atrorus*) and the Cuatro Ciénegas killifish (*Lucania*

interioris) (Minckley 1969)). Like other members of the genus *Gambusia*, *G. marshi* is viviparous and sexually dimorphic. *G. marshi* generally consume terrestrial arthropods and zooplankton, but also consume some amounts of detritus and plant matter (Meffe 1985; Hernández et al. 2016). The species is highly morphologically variable, with predation driving variation in body shape among populations (Minckley 1962; Chapter 5).

Nutrient Recycling Model

I first generated quantitative predictions for how *Gambusia* nutrient recycling N:P ratio would vary between cool, low-predation sites and warm, high-predation sites using the model of Sterner (1990). In this model, which assumes strict consumer stoichiometric homeostasis, recycled N:P (*s*) varies with consumer body N:P (*b*), diet N:P (*f*), and the maximum accumulation efficiency of the limiting nutrient (*L*). As *s* also encapsulates egestion, which is generally lower in N:P than excretion (Médale et al. 1998; Rodehutscord et al. 2000), *s* should be an underestimate of excretion N:P into dissolved pools. When *f* is less than *b*, growth is N-limited and this relationship is characterized by equation 1:

1) $s = f * (1 - L) / (1 - L * f / b)$

When *f* is greater than *b*, growth is P-limited and this relationship is instead characterized by equation 2:

2) $s = f / (1 - L) - b * L / (1 - L)$

To model recycled N:P by *Gambusia marshi*, I assumed *b* did not change between environments and that instead variation was due to variation in *L* and/or *f* (Table 4.2). In

the closely related guppy (*Poecilia reticulata*), body N:P varies among populations but not systematically with predation pressure; as I had no prior data on *Gambusia* body stoichiometry, I substituted 6 for *b* as it fell near the median of the distribution of body N:P among guppy populations (El-Sabaawi et al. 2012). This value was slightly lower than values I measured for *Gambusia marshi* in the field (Table 4.3), but this relatively small difference did not affect my conclusions. To calculate *L*, I used data from juvenile *Gambusia affinis* grown at 25 and 30 °C under different diet rations (Wurtsbaugh & Cech 1983). Fish in these experiments were fed oligochaete worms (*Tubifex sp.*); thus, to calculate the %P of food consumed I used the only value of oligochaete worm %P I found in the peer-reviewed literature: the average of oligochaetes from Lake Constance in Central Europe (Fink et al. 2006). As dietary N:P was higher than body N:P, I calculated *L* for P accumulation under each temperature-ration combination using growth rates from Wurtsbaugh & Cech (1983). To calculate P accumulation, I incorporated the flexibility of body P content in fishes by calculating the slope of the relationship between the C:P of mass gain and fish growth rate described in bluegill sunfish (*Lepomis macrochirus*) by Downs et al. (2016). Although Sterner's (1990) model assumes strict stoichiometric homeostasis, my incorporation of the flexible P content of fishes does not violate this assumption because I did not allow *b* to vary within a particular fish, but rather accounted for differences in P assimilation efficiency by varying *L* among fishes.

I modeled N:P recycled under three hypotheses: A) if cool- and warm-water populations both consume moderate rations, B) if cool-water fish consume a restricted ration and warm-water fish consume moderate ration, and C) if cool-water fish consume moderate ration and warm-water fish consume a restricted ration. The first hypothesis

establishes whether temperature by itself could explain differences in nutrient recycling N:P (Figure 4.2A), while the second and third hypotheses aim to distinguish between the predictions of Hawlena & Schmidt (2010) and Dalton & Flecker (2014). In the second hypothesis, predators increase consumption rates of *Gambusia* relative to predator-free sites (Figure 4.2B), while in the third hypothesis predators reduce consumption rates relative to predator-free sites (Figure 4.2C). Although these models make several assumptions that are not entirely realistic, they provide a baseline prediction for the direction in which temperature and predation should affect the stoichiometry of consumer-driven nutrient recycling by *G. marshi*.

Field Survey

I compiled data on the physical and biological variation among nine sites in Cuatro Ciénegas from previous work (Corman & Ramos unpublished data; Figure 4.1; Table 4.1) and from museum collections. I classified sites as groundwater-fed or runoff-dominated based on thermal regimes. Specifically, groundwater-fed sites had average temperatures above 29 °C and runoff-dominated sites had average temperatures below 27 °C. I also surveyed sites through a combination of snorkeling and seining to confirm whether predators persisted in the study period of 2013-2016 and then categorized them as high predation (having populations of *M. salmoides*, *I. lupus*, and/or the piscivorous morph of *H. minckleyi*) or low predation (lacking these three species/forms). One site, Churince, was classified as low predation even though it historically supported these three species because only a few individuals of *M. salmoides* and non-piscivorous *H. minckleyi* were observed during the study period.

To examine elemental phenotypic variation among sites, I collected fifteen adult female *G. marshi* from each of the nine sites using seine and/or hand dip nets in May 2013. I specifically focused on females because, unlike males, they exhibit indeterminate growth and also invest more heavily in reproduction. Thus, I expected that I would be more likely to detect effects of environmental variation in females than in males. All fish were collected and measured between 10 A.M. and 4 P.M. when they were actively feeding. Fish were held for less than five minutes before being placed into bags filled with 250 mL of filtered water (Whatman 0.45 μm glass fiber filter; GE Healthcare, Little Chalfont, England) from the collection site to measure excretion rates of ammonium (NH_4^+) and soluble reactive phosphorus (SRP). Excretion rates were measured according to the methods of Small et al. (2011) and compared against control bags that underwent the same procedure with no fish. In earlier pilot sampling I established additional controls into which I briefly dipped fish into the water and then removed them to test for the effects on nutrient concentration of simply adding a fish. I did not continue this practice as I found no significant difference from fishless controls. Excretion water samples were kept on ice in the field and frozen upon return to the laboratory for later analysis. I then euthanized fish with an overdose of MS-222, dissected guts and reproductive tissues, froze the fish carcasses in the laboratory, and later dried them at 60 °C for subsequent body stoichiometry analysis.

Laboratory Experiments

Of the two primary differences that I observed between spring-fed and runoff-fed sites, I decided to experimentally test whether temperature differences rather than differences in predation pressure were responsible for observed differences in the

elemental phenotype as it was feasible to manipulate temperature in the laboratory. To accomplish this, I conducted a controlled laboratory common garden experiment with two populations. I controlled for potential evolutionary effects of piscivore exposure by choosing populations from two sites lacking piscivores: one cool site, Los Hundidos, and one warm site, La Becerra. Wild fish were collected in February 2015 with seine and hand dip nets and allowed to acclimate to laboratory conditions in the ASU animal care facility for two months. Fish were then randomly paired within the same population to produce F1 offspring for the full-factorial common garden experiment. *Gambusia* can store sperm for periods greater than two months (Robbins et al. 1987), and thus females used in this experiment may have carried sperm from other males. However, recent matings contribute disproportionately to the parentage of offspring (Robbins et al. 1987). Further, it is extremely unlikely that previous mates came from populations that were genetically distinct from those used in experimental pairings. As a result, I do not expect that this would have any confounding effects on the results.

Individual broods of F1 offspring from both populations were randomly assigned to one temperature treatment, cool (~25 °C) or warm (~32 °C), and held at that temperature over the course of development. Temperatures were maintained with 100-watt aquarium heaters (Eheim; Deizisau, Germany) and recorded and checked regularly with HOBO pendant loggers (Onset Corporation; Bourne, MA). In this experiment, fish were fed *ad libitum* with TetraMin tropical flakes (Spectrum Brands; Blacksburg, VA). C, N, and P contents of the flake diet were analyzed as described for fish below. Average molar C:N:P stoichiometry of the diet was 192:23:1.

When fish reached maturity, I measured excretion rates of F1 fish using a modified version of the field methods described above. Due to the smaller size of newly matured lab-raised fish relative to wild adult fish, excretion trials were conducted with only 45 mL of water but all other procedures were the same. Based on pilot experiments, I determined that fish did not excrete measurable amounts of P until 4 h post-feeding while NH_4^+ excretion rate measurements measured after 4 h did not differ from measurements 1 h post-feeding; thus, I measured fed excretion rates of all fish 4 h after feeding. Following excretion rate measurements, F1 fish were euthanized for analysis of body stoichiometry as described above except for a small subset of fish, which were paired with fish from another brood from the same population and temperature treatment to produce F2 offspring. To eliminate inbreeding effects, the fish paired to produce F2 offspring shared neither parent. I measured excretion rates and body stoichiometry of F2 fish following the same methods above. Due to logistical issues, I did not raise any F2 fish from the warm La Becerra population at 32 °C.

I also conducted a separate experiment in which I manipulated diet ration to test directly if consumption plays a role in controlling fish excretion rates and stoichiometric ratios. In this experiment, I used only F1 fish from the La Becerra population held at 25 °C. Four broods of fish were fed the same TetraMin flake diet as in the common garden experiment, two which were fed *ad libitum* every day and two of which were only fed every other day during the course of development. When fish reached maturity, I measured fish excretion rates and body stoichiometry using the same methods described above.

Analytical Methods

I quantified concentrations of soluble reactive phosphorus (SRP) in excretion samples using the ammonium molybdate method (APHA 2005). Samples collected in the field were measured within 3 d at the field laboratory, while samples collected in the laboratory experiment were measured within 4 h of collection. Methods for quantifying NH_4^+ concentrations in excretion samples differed between field and laboratory samples. For field-collected samples, I brought frozen water samples to the Goldwater Environmental Laboratory at Arizona State University where they were analyzed on a flow injection analyzer (Lachat QuikChem 8000; Lachat Instruments, Loveland, CO) for both NH_4^+ and NO_3^- concentrations. As I rarely found measurable NO_3^- concentrations in excretion samples, subsequent laboratory samples were measured only for NH_4^+ using the fluorometric method (described by Taylor et al. 2007) and these samples were fixed with reagent within thirty minutes of collection. To measure body stoichiometry of dried fish, I first homogenized them using either a Wiley mill (field samples) or a ball mill (laboratory samples). I then analyzed C and N contents on a CHN analyzer and P contents on a spectrophotometer using the ammonium molybdate method after hydrochloric acid digestion (field samples) or on an inductively-coupled plasma mass spectrometer (laboratory samples) following digestion in HCl.

Statistical Analysis

I first tested whether *Gambusia* body mass differed among populations by testing for a significant correlation between average fish dry body mass and average water temperature among the nine sites. I also estimated the slope of this relationship to quantify the metabolic scaling of body size with water temperature. Next, I calculated length-mass regressions of the form $M=a*L^b$ from the collected specimens as no such

regression has previously been published for *G. marshi*. Due to collinearity (as determined by variance inflation factors greater than ten) between water temperature and predation pressure, I characterized these differences by analyzing stoichiometric responses in the field between groundwater vs. runoff-dominated systems. Specifically, I tested whether somatic elemental composition, nutrient excretion rates, and excretion N:P ratio varied with water source as fixed categorical effects using ANCOVA, with body mass as a covariate and site as a random effect. Excretion rates (measured as μg per hour per individual) and molar ratios were ln-transformed to meet the assumption of normally-distributed residuals. For all of these analyses I also ln-transformed body mass.

Although both males and females were produced and raised in laboratory experiments, I present only the results for mature female fish for consistency in comparing with field results. For fish from laboratory experiments, I analyzed differences in body elemental composition and excretion rates among populations and treatments as fixed effects with body mass as a covariate using ANCOVA as for field-collected fish. For all analyses, the assumptions of normality and heterogeneity of variance of model residuals were inspected visually using Q-Q plots and residuals vs. fitted value plots, respectively. All statistical analyses were performed in the R computing environment v. 3.2.2 (R Core Team 2015).

Results

Nutrient Recycling Model

Models indicated that temperature alone did not have strong effects on predicted recycling N:P of *Gambusia* on a given diet (Figure 4.2A). Instead, variation in consumption rate had much stronger effects. Fish that consumed more food had a

substantially lower values of *L* (Table 4.2) and therefore were predicted to recycle nutrients at a lower N:P ratio when feeding on a given diet (Figure 4.2). This result held regardless of the temperature at which consumption was higher (Figure 4.2).

Field Survey

Average *Gambusia* mass decreased with average temperature among the nine sites (Pearson's correlation test: $r=-0.855$, $t_7=-4.36$, $p=0.003$; Figure 4.3). Length-weight relationships also varied among populations, as the 95% confidence intervals of both the slope and intercept differed between cool and warm sites (Table S4.1). Specifically, fish from cooler, runoff-fed sites sites were heavier at a given length than fish from warm, groundwater-fed sites. Somatic contents of C, N, and P all varied with water source and exhibited site-specific effects but none varied with body mass nor exhibited a mass-temperature interaction (Table 4.3; Table S4.2). At groundwater-fed sites, fish had lower body %C and %N but higher body %P (Figure 4.4). Following these results, body C:P and N:P ratios were significantly higher in fish from runoff-dominated sites (Table 4.3; Figure S4.1).

NH_4^+ and SRP excretion rates significantly increased with body mass but excretion N:P did not (Table S4.3). N excretion rates scaled approximately isometrically with mass (allometric coefficient=0.96) while P excretion rates were closer to the predicted ¾ scaling power of metabolic theory (allometric coefficient=0.79). Consistent with a statistically insignificant effect of body mass, excretion N:P exhibited a very small mass scaling exponent (allometric coefficient=0.17). Controlling for body mass, fish from warmer, groundwater-fed sites excreted NH_4^+ at lower rates and SRP at higher rates

(Figure 4.4). As a result, fish at groundwater-fed sites excreted nutrients at a lower N:P ratio (Figure 4.4).

Laboratory Experiments

In the *ad libitum* temperature manipulation experiment, female size at maturity was determined by interacting effects of source population and treatment temperature (Table S4.4). Fish from the cool (25 °C) Los Hundidos population exhibited greater plasticity in their growth response to developmental temperature than fish from the warmer (32 °C) La Becerra population. However, fish from La Becerra matured at smaller sizes than the Los Hundidos fish regardless of temperature (Figure 4.5). F1 fish also matured at smaller sizes and showed a stronger developmental temperature response than F2 fish. In contrast to field results, fish raised at 32 °C had higher body %C than fish raised at 25 °C (Table S4.5). However, fish from the cool source population had higher body %C than fish from the warm source population across both temperatures (Table S4.5). Fish raised at 32 °C also had lower %N than fish raised at 25 °C but %P did not vary with any of the independent variables (Table S4.5). NH_4^+ excretion rate varied with fish size, source population, and generation, but not temperature (Figure 4.6; Table S4.6). P excretion rate also varied with source population, generation, and their interacting effect but not with size or temperature (Figure 4.6; Table S4.6). Fish from the warmer site, La Becerra, excreted higher rates of both N and P regardless of temperature. F2 fish also excreted NH_4^+ at higher rates and P at lower rates than F1 fish (Table S4.6). Although the excretion N:P ratio varied with fish size in this experiment, it did not vary with generation, developmental temperature, or source population (Figure 4.6; Table S4.6).

In the diet restriction experiment, mature female body %C increased with fish size ($F_{1,12}$=19.82, p=0.001), while both body %N ($F_{1,12}$=11.03, p=0.006) and %P ($F_{1,12}$=7.01, p=0.021) decreased with size. However, the size of fish analyzed for somatic elemental contents did not differ among dietary treatments (Mann-Whitney test, U=26.5, p=0.955) and there were no effects of diet treatment on somatic contents of any elements after correcting for size (p>0.05). Fish NH_4^+ excretion rate did not vary with fish size ($F_{1,14}$=3.52 p=0.082) or diet treatment ($F_{1,14}$=0.004, p=0.949), but fish fed a restricted diet excreted P at a lower rate after correcting for size differences ($F_{1,14}$=13.29, p=0.003). As a result, fish fed the restricted diet also excreted nutrients at a higher N:P ratio than fish fed *ad libitum* ($F_{1,14}$=13.31, p=0.003; Figure 4.7).

Discussion

Variation in the elemental phenotype within a species can have strong effects on the availability of key nutrients to microbes and primary producers. Here, I tested the hypothesis that variation in consumption rates due to temperature and predation pressure could in turn drive variation in the N:P ratio of fish excretion. I found that female *Gambusia marshi* excreted N at lower rates and P at higher rates in warmer groundwater-fed environments with high predation pressure in spite of the fact that these fish also had higher somatic P content. I demonstrated experimentally that this variation was not driven by temperature but rather by variation in consumption. Supporting my second alternative hypothesis, my results suggest that prey fish consume food at higher rates at high predation sites than at low predation sites, thereby driving variation in excretion stoichiometry among sites.

I found significant differences in fish body elemental composition among sites, but these were not consistent in the laboratory and did not explain variation in excretion N:P. Intraspecific variation in body N:P is a poor predictor of excretion stoichiometry in fishes (El-Sabaawi et al. 2015, 2016; Tuckett et al. 2016), indicating that variation in diet and/or the maximum accumulation efficiency for the limiting nutrient (*L*) have stronger effects among natural populations (Moody et al. 2015). These results indicate that variation in consumption rate manifested in *L* drove patterns in excretion N:P among populations. Although *L* is not frequently considered in studies of variation in the N:P ratio excreted within (e.g., El-Sabaawi et al. 2015; 2016; Tobler et al. 2016; Tuckett et al. 2016) or among species (e.g., Vanni et al. 2002), its variation can have strong effects on the ratio of these nutrients recycled by consumers. Considering the importance of Sterner's (1990) model in making my predictions, it is worth considering how my application of the model may have influenced my conclusions.

Initially, the *L* parameter represents the maximum accumulation efficiency for the nutrient limiting consumer growth, and was used by Sterner (1990) to model nutrient recycling by strictly homeostatic zooplankton feeding on phytoplankton. In my application of the model, I incorporated variation in the efficiency of converting dietary P to body P with growth rate (Azevedo et al. 1998; Downs et al. 2016). If I had assumed a consistent P content of new somatic growth, Sterner's (1990) model predicts that *L* would actually increase slightly with consumption rate and, as a result, recycled N:P would also increase for a given diet. However, the predicted differences in recycling N:P under this assumption are minimal relative to what I found. For example, if P content of new somatic growth did not vary with growth rate, fish feeding on the experimental diet

(N:P=23) at 25 °C should have recycled N:P at ratios of 42.2 and 40.4 when fed *ad libitum* and a restricted ration, respectively. Instead, I found that average excretion N:P for fish fed *ad libitum* was 18.8 and for fish fed a restricted diet, 72.5. Although I found clear differences in excreted N:P even when fish fed on the same diet and varied little in elemental composition, this could result from the difference between the excretion N:P I measured and recycled N:P as predicted by the model.

An important caveat in the application of Sterner's (1990) model is that it estimates recycled N:P, which encompasses the nutrients egested in addition to those excreted. Fish feces are relatively low in N:P compared to dissolved waste products (Médale et al. 1998; Rodehutscord et al. 2000); thus, if the proportion of nutrients egested decreases with diet intake then the total recycled N:P may not vary much or may increase with consumption. Although I did not measure egestion in this study, this trend is supported by the literature (e.g., Médale et al. 1998; Bureau & Cho 1999; Rodehutscord et al. 2000), suggesting that variable consumer P content with growth rate may not be needed to predict my results. However, I included the flexibility of fish P content in my calculation of *L* because it would be unrealistic to assume that P content of new tissue does not change with growth rate (Lall & Lewis-McCrea 2007; Benstead et al. 2014; Downs et al. 2016). It is clear that more work is needed to understand the relative contributions of variable P assimilation efficiency and the proportion of nutrients egested to variation in N:P excreted by fishes. As a result, we must either rethink our interpretation of the parameters in Sterner's (1990) model to apply it to dissolved excretion of fishes or devise a modification that includes terms explicitly describing these phenomena.

My results align with predictions of the general stress paradigm (Hawlena & Schmitz 2010; Schmitz et al. 2010; Leroux et al. 2012) in that fish from populations with high predation pressure exhibited higher consumption and nutrient excretion rates. However, my results are in contrast to studies of other aquatic consumers that did not find support for the predictions of the general stress paradigm (Costello & Michel 2013; Dalton & Flecker 2014). These studies manipulated predation pressure in controlled laboratory settings alone. In natural populations, predation may have effects at larger scales that are missed in these types of experiments. For example, predators tend to reduce prey densities, thereby alleviating intraspecific competition among prey individuals (Svanbäck & Bolnick 2007; Araújo et al. 2014). In Bahamian *Gambusia*, populations from fragmented tidal creeks lacking piscivorous fish exhibited higher population densities, reduced individual growth rates, and a broader dietary breadth than conspecifics coexisting with piscivores (Araújo et al. 2014). In addition to broadening dietary breadth, individuals from high-density populations may also exhibit reduced per capita consumption rates (Svanbäck & Bolnick 2007). Supporting this proposed mechanism, higher proportions of *Gambusia marshi* have empty stomachs in low predation sites compared to high predation sites (Meffe 1985; Hernández et al. 2016). However, increased diet breadth could also partially explain my results if fish in low predation environments were consuming more low quality, high C:P food items such as detritus. Available diet data for *G. marshi* do not clearly indicate variation in diet composition with predation pressure (Meffe 1985; Hernández et al. 2016); thus, the evidence suggests that differences in overall consumption were the primary source of variation in excretion stoichiometry among sites.

Recent work has documented strong allometric effects on individual excretion rates of N and P, but not N:P ratio (Allgeier et al. 2015; Vanni & McIntyre 2016). My work supports these effects and indicates that stoichiometric theory, not metabolic theory, explains variation in the N:P ratio excreted by consumers. Excretion of individual nutrients varies with metabolic rate and, therefore, the allometric coefficients for N and P excretion rates are relatively similar. If excretion rates of both nutrients scale similarly with body mass, there is no reason to expect that the N:P ratio should as well. As variation in resource supply stoichiometry leads to shifts between N- and P-limited primary production, it is this ratio that is most relevant for examining the ecosystem effects of consumer-driven nutrient recycling (Elser et al. 1988; 1999). I argue that stoichiometric models are needed to predict how intraspecific variation in the elemental phenotype is relevant at the ecosystem scale. The positive effect of increased consumption by individual fish on P excretion rate was strong in these fish inhabiting a strongly P-limited ecosystem; thus, this effect provides a potential mechanism for scaling up from individuals to ecosystems.

My results have implications for understanding some of the ecosystem consequences of ongoing food web perturbations, such as “trophic downgrading”, the widespread reduction in the number of trophic levels present in a community (Estes et al. 2011). Many studies examining these effects focus on changes in the community composition following trophic downgrading; however, reduced predation pressure also has well-known effects on intraspecific phenotypic variation (e.g., Reznick & Endler 1982; Langerhans et al. 2004; Langerhans 2009). We are only beginning to understand the ways in which these intraspecific shifts can affect nutrient fluxes and ecosystem

function (Bassar et al. 2010; Schmitz et al. 2010; El-Sabaawi et al. 2015). I found strong effects of the presence of top predators on nutrient fluxes from individual prey fishes but scaling these impacts up to the whole ecosystem requires metrics such as volumetic excretion rates by the entire fish community (McIntyre et al. 2008; Capps et al. 2015). Variation in population density as well as sex ratios can have strong ecosystem effects in sexually dimorphic consumers such as *Gambusia* (Fryxell et al. 2015). More detailed work on the ecosystem-scale implications of these changes is needed to understand how reduced predation pressure alters ecosystem processes. This is particularly true in arid-land aquatic ecosystems, where food chain length predictably varies with hydrologic variability (Sabo et al. 2010; Bogan & Lytle 2011). Despite its protected status, springs in Cuatro Ciénegas are now facing the threat of increased hydrologic variation as irrigated agricultural growth lowers the water table in aquifers that feed them (Contreras-Balderas 1984; Minckley 1992; Souza et al. 2006). In these dynamic and endangered ecosystems, ecosystem function could be dramatically altered by subsequent changes in community structure and consumer physiology as various fish taxa are lost.

This work sheds light on the mechanisms through which variation within a species can lead to altered nutrient fluxes in aquatic ecosystems. Furthermore, the common garden experiment demonstrates that some aspects of the elemental phenotype, particularly individual nutrient excretion rates and ratios, have genetic components that may be driven by differences in adaption to local thermal regimes. As thermal regimes are altered by global climate change, the degree of organismal thermal adaptation will modulate how organisms mediate nutrient fluxes in the ecosystems they inhabit. Understanding these mechanisms will improve our ability to link the evolution of

organisms to ecosystem function (Elser 2006; Jeyasingh et al. 2014; Leal et al. 2017). Continued work on this subject will vastly improve our ability to predict how ecosystem function varies over gradients in biotic and abiotic conditions across the landscape.

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Table 4.1. Description of the nine study sites sampled in the field survey. Temperatures were recorded along water edges where *Gambusia* are most abundant on at least 3 and as many as 66 dates during the months of February-October and the years 2011-2016. Water column TDN:TDP is presented as a molar ratio and was measured at least 3 and as many as 24 times during the same period. The dotted line indicates the separation between groundwater-fed and runoff-dominated sites.

Site	Water Source	Avg. Temperature (°C)	Predation	TDN:TDP
Escobedo	Groundwater	33.35	High	199
Mojarral	Groundwater	32.53	High	149
La Becerra	Groundwater	31.80	Low	501
Santa Tecla	Groundwater	30.23	High	372
Anteojo	Groundwater	29.97	High	26
Las Teclitas	Groundwater	29.76	High	287
Los Gatos	Runoff	26.23	Low	34
Los Hundidos	Runoff	25.65	Low	25
Churince	Runoff	25.19	Low	113

Table 4.2. Data used to calculate parameters for the model of nutrient recycling N:P. *L* = maximum accumulation efficiency for phosphorus, *b* = fish body N:P. Data for fish growth are from juvenile *Gambusia affinis* (Wurtsbaugh & Cech 1983), and the value of *b* is the median value of *Poecilia reticulata* from Trinidad (El-Sabaawi et al. 2012).

Temperature	Ration	P consumed (mg)	P gain (mg)	*L*	*b*
25 °C	Restricted	0.027	0.009	0.34	6
25 °C	Moderate	0.088	0.012	0.14	6
30 °C	Restricted	0.026	0.008	0.33	6
30 °C	Moderate	0.099	0.012	0.12	6

Table 4.3. Average body elemental composition of adult female *Gambusia marshi* from nine sites in the Cuatro Ciénegas basin. All percentages are by dry mass and all ratios are molar.

Site	Water Source	%C	%N	%P	C:N	C:P	N:P
Escobedo	Groundwater	38.23	10.67	3.47	4.18	28.86	6.91
Mojarral	Groundwater	39.80	9.97	3.48	4.66	29.98	6.41
La Becerra	Groundwater	45.41	10.78	2.21	4.92	53.37	10.84
Santa Tecla	Groundwater	39.49	10.73	3.26	4.30	32.41	7.51
Anteojo	Groundwater	38.67	10.34	3.54	4.37	28.84	6.61
Las Teclitas	Groundwater	40.33	10.14	3.51	4.65	29.99	6.45
Los Gatos	Runoff	41.13	11.47	3.05	4.19	35.28	8.44
Los Hundidos	Runoff	44.95	10.87	2.59	4.83	45.73	9.46
Churince	Runoff	43.61	10.19	2.65	5.00	42.77	8.56

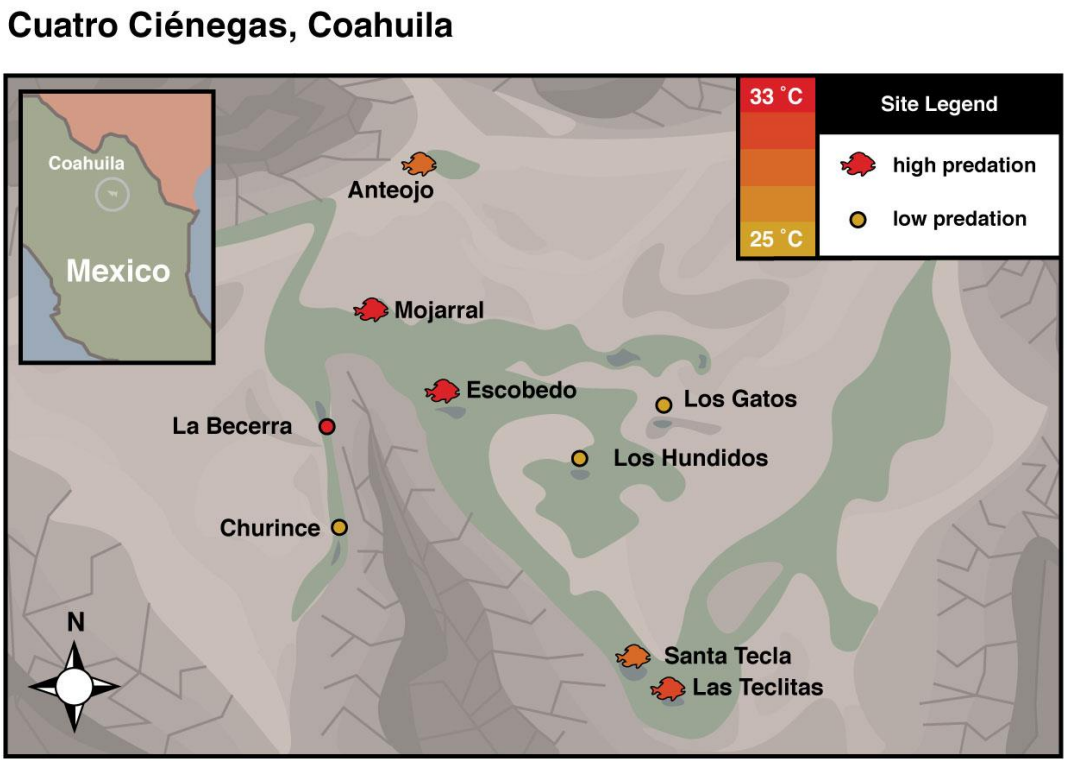

Figure 4.1. Map of the Cuatro Ciénegas basin in Coahuila, Mexico. The nine study sites included in the field survey are shown according to average water temperature and predation pressure, with groundwater-fed sites being those above 29 °C and runoff-dominated sites being those below 27 °C on average.

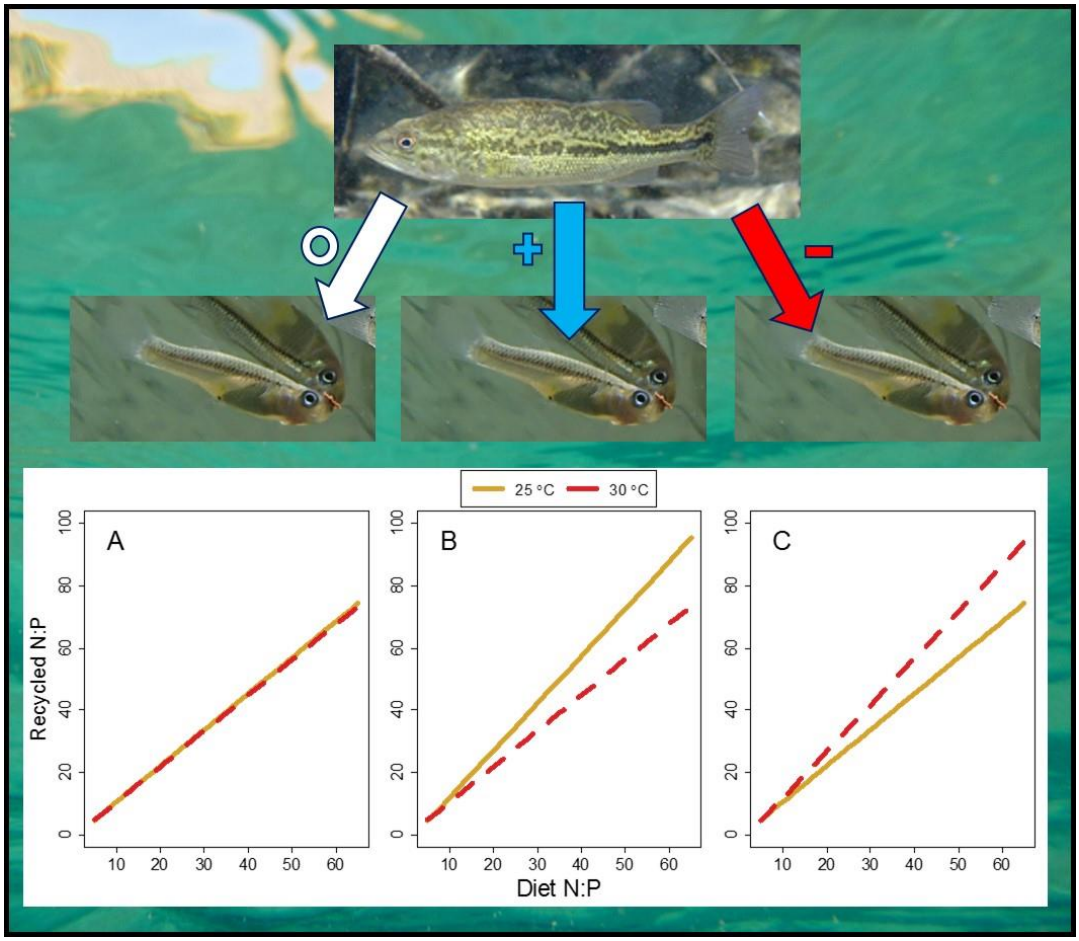

Figure 4.2. Conceptual diagram and modeled recycled N:P for three scenarios under which predation pressure is reduced with water temperature: A) predators have no effect on *Gambusia* consumption rate, so fish grown at 25 °C and 30 °C both consu me approximately half *ad libitum* ration, B) predators increase *Gambusia* consumption rates, so fish grown at 25 °C consume a restricted ration, and C) predators reduce *Gambusia* consumption rates, so fish grown at 30 °C consume a restricted ration. Recycled N:P was modeled according to Sterner (1990). Model results are based on *Gambusia affinis* growth and consumption data from Wurtsbaugh & Cech (1983). See methods for a more detailed description of the model. Largemouth bass image © Michael Tobler, *Gambusia marshi* image © Eric Moody.

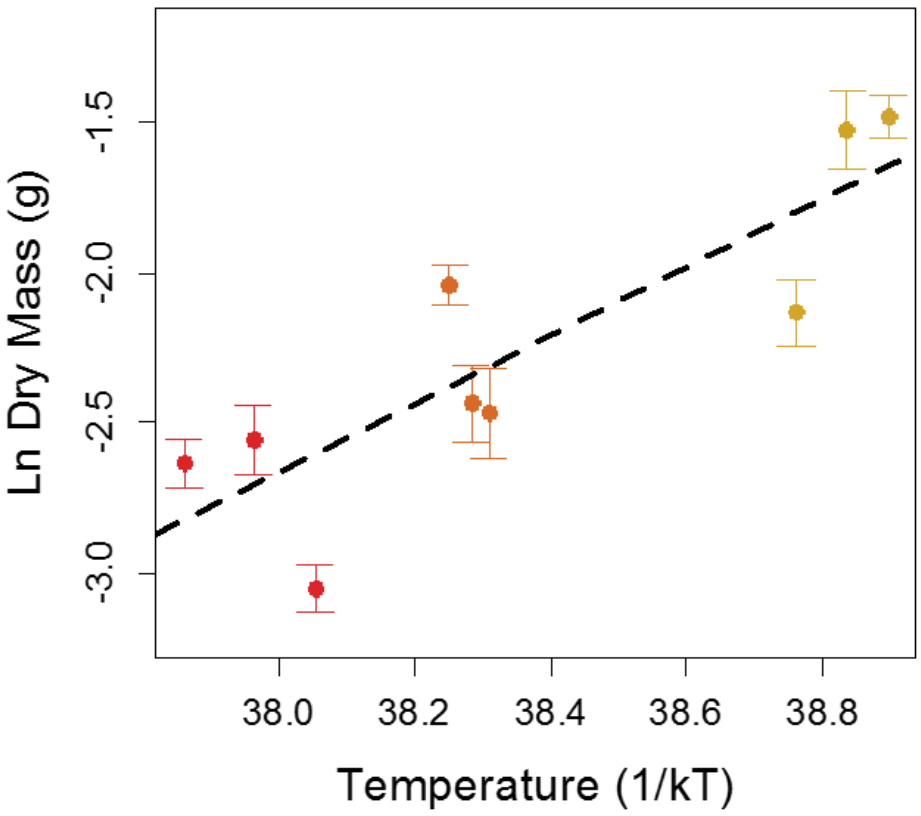

Figure 4.3. Scaling of average adult female body mass with average temperature among nine sites. Temperature is measured in K. The parameter *k* represent Boltzmann's constant. Average body mass was negatively correlated with average temperature among sites (Pearson's correlation test: $r=-0.855$, $t_7=-4.36$, $p=0.003$). Error bars represent one standard error.

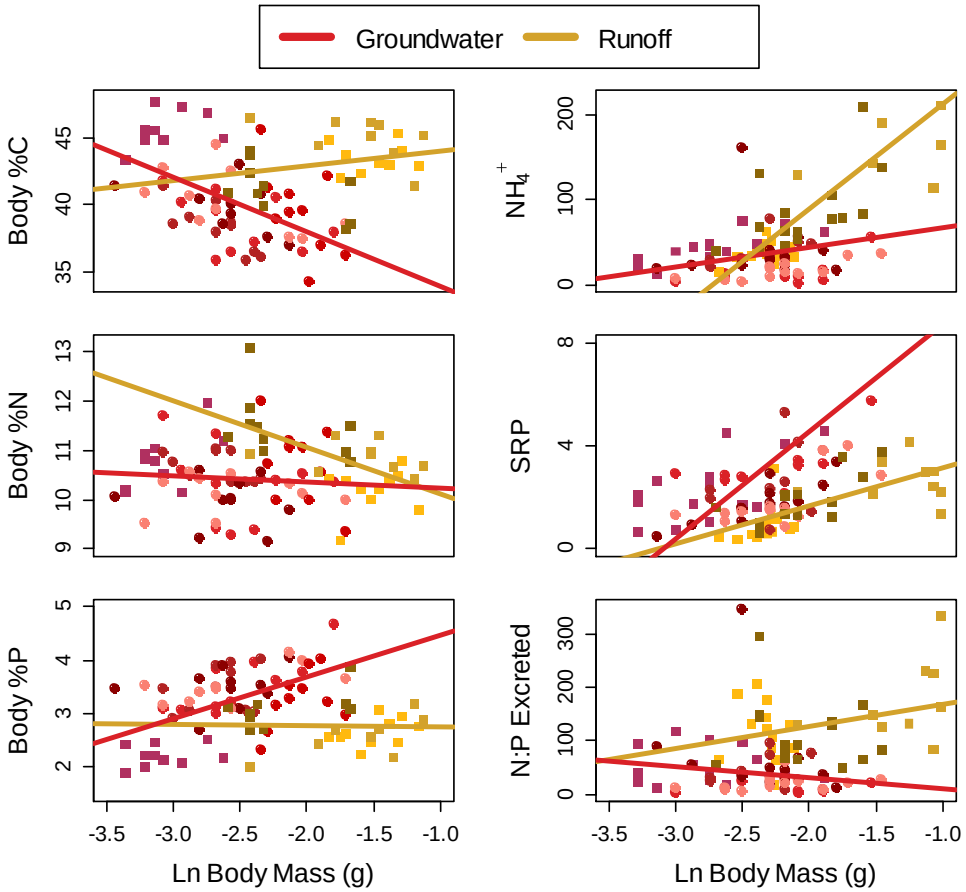

Figure 4.4. Body stoichiometry and excretion rates of female *Gambusia marshi* collected from nine sites in the Cuatro Ciénegas basin of Coahuila, Mexico. Body elemental contents are plotted as percent dry mass. Individual nutrient excretion ratess are plotted as µg*individual^-1*hour^-1, N:P is plotted as the molar ratio, and all values are plotted in their original scale for ease of interpretation. Each color represents a different site, with red colors being warm site and yellow colors being cool sites. Squares indicate sites without predators and circles indicate sites with predators. Trendlines indicate differences between groundwater-fed sites (red) and runoff-dominated sites (yellow).

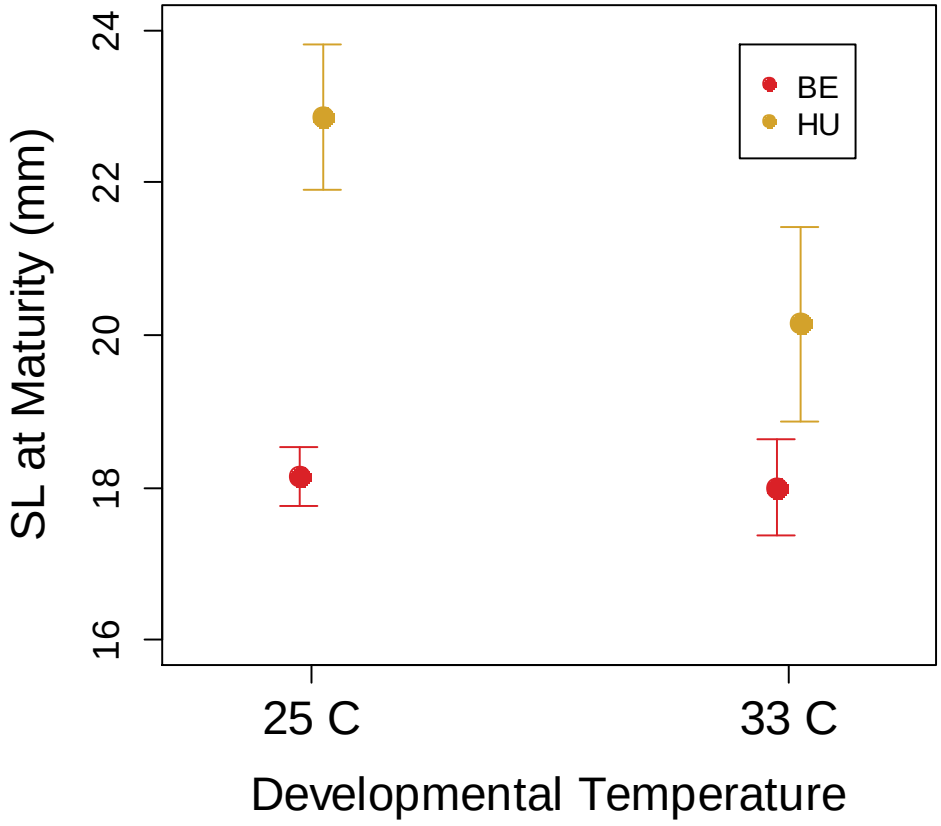

Figure 4.5. Standard length at maturity of F1 female *Gambusia marshi* from two populations raised at different temperatures and fed *ad libitum* in the laboratory. Yellow points represent the cool source population, Los Hundidos, and red points represent the warm source population, La Becerra. Error bars represent one standard error.

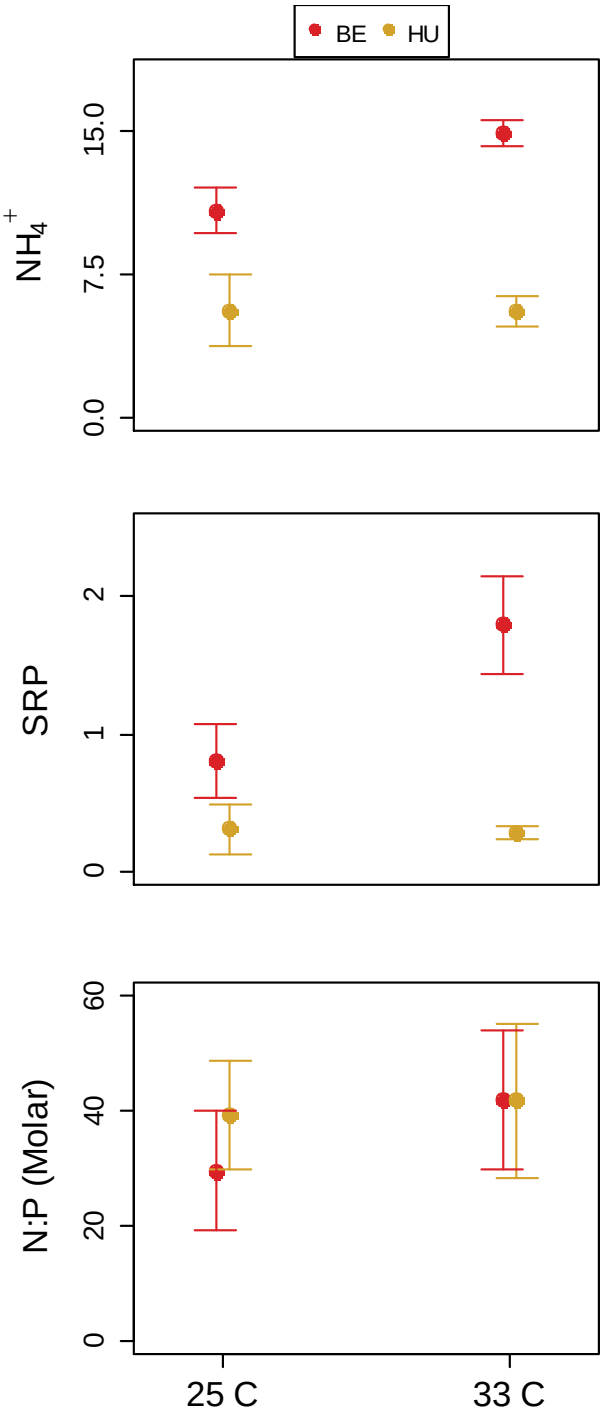

Figure 4.6. Least squares means of NH_4^+ and SRP excretion rates (µg*hr^-1) and N:P ratio (molar) by mature female *Gambusia marshi* from two populations raised at 25 and 32 °C. Both F1 and F2 data are combined in this figure. HU=Los Hundidos (cool source population) and BE=La Becerra (warm source population). N and P excretion rates both varied with source population and generation, but no responses varied with developmental temperature. Error bars represent one standard error.

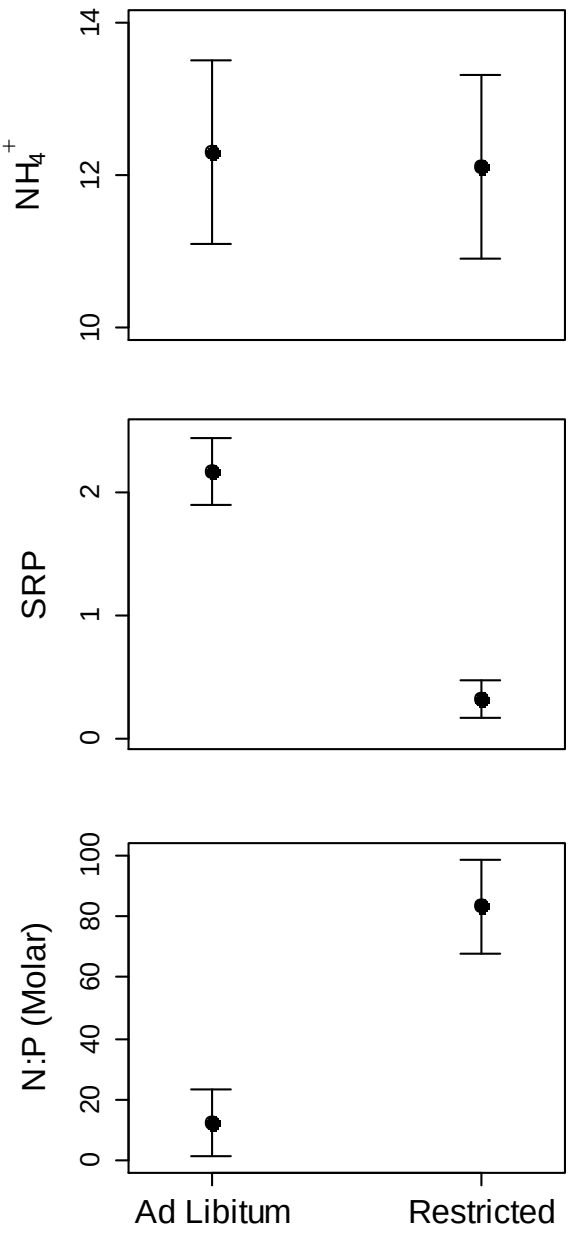

Figure 4.7. Least square means of NH_4^+ and SRP excretion rates (µg*hr^-1) and N:P ratio (molar) by mature F1 female fish from the La Becerra population raised at 25 °C fed *ad libitum* vs. a restricted diet. Accounting for differences in size at maturity between treatments, fish fed a restricted diet excreted P at a lower rate and N:P at a higher ratio. Error bars represent one standard error.

SUPPLEMENTARY MATERIALS

Table S4.1. Length-mass regressions of the form $M=a*L^b$ for cool and warm population female fish. The units of the regression parameters are mm and g, and the regressions are based on wet standard length and dry mass. The 95% confidence intervals reported are for the regression between ln-transformed mass and untransformed standard length to linearize the relationship and allow for ease of interpretation.

Temperature	a	b	95 % CI Intercept	95% CI Slope
Cool	4*10^-5	2.4394	(-4.66, -3.60)	(0.06, 0.09)
Warm	5*10^-6	2.9772	(-5.96, -5.26)	(0.10, 0.13)

Table S4.2. ANCOVA table of body stoichiometry of female *Gambusia marshi* collected from nine sites in the Cuatro Ciénegas basin in Coahuila, Mexico.

%C					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.69	0.69	0.20	0.654
Water Source	1	253.84	253.84	73.98	**<0.001**
Site	7	380.04	54.29	15.82	**<0.001**
Mass*Water Source	1	0.90	0.90	0.26	0.609
Residual	78	267.65	3.43		
%N					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.01	0.01	0.02	0.884
Water Source	1	5.88	5.88	18.76	**<0.001**
Site	7	11.73	1.68	5.35	**<0.001**
Mass*Water Source	1	0.05	0.05	0.14	0.706
Residual	78	24.43	0.31		
%P					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.06	0.06	0.50	0.484
Water Source	1	8.90	8.90	78.98	**<0.001**
Site	7	13.22	1.89	16.75	**<0.001**
Mass*Water Source	1	0.01	0.01	0.02	0.882
Residual	78	8.79	0.11		

Table S43. ANCOVA table of excretion rates and stoichiometry of female *Gambusia marshi* collected from nine sites in the Cuatro Ciénegas basin in Coahuila, Mexico.

NH_4^+					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	27.10	27.10	87.85	**<0.001**
Water Source	1	14.63	14.63	47.43	**<0.001**
Site	7	33.84	4.83	15.67	**<0.001**
Mass*Water Source	1	0.01	0.01	0.04	0.850
Residual	98	30.23	0.31		
SRP					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	18.43	18.43	111.48	**<0.001**
Water Source	1	18.57	18.57	112.30	**<0.001**
Site	7	12.40	1.77	10.72	**<0.001**
Mass*Water Source	1	0.01	0.01	0.01	0.930
Residual	98	16.20	0.17		
N:P (Molar)					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.83	0.83	1.56	0.214
Water Source	1	66.18	66.18	124.92	**<0.001**
Site	7	33.76	4.82	9.11	**<0.001**
Mass*Water Source	1	0.01	0.01	0.01	0.924
Residual	98	51.92	0.53		

Table S4.4. ANOVA table of size at maturity of adult female *Gambusia marshi* raised in the *ad libitum* laboratory temperature manipulation experiment.

Factor	**df**	**SS**	**MS**	**F**	**p**
Temperature	1	20.27	20.27	5.83	**0.023**
Population	1	95.74	95.74	27.55	**<0.001**
Generation	1	123.80	123.80	35.63	**<0.001**
Temperature*Population	1	36.65	36.65	10.55	**0.003**
Residual	28	97.30	3.48		

Table S4.5. ANCOVA table of body stoichiometry of adult female *Gambusia marshi* raised in the *ad libitum* laboratory temperature manipulation experiment. All interaction terms were included in the model, but only the significant effects are presented in the table.

%C

Factor	df	SS	MS	F	p
Mass	1	7.10	7.10	4.07	0.063
Temperature	1	8.12	8.12	4.66	**0.049**
Source Population	1	28.92	28.92	16.58	**0.001**
Generation	1	2.59	2.59	1.48	0.243
Residual	14	24.41	1.74		

%N

Factor	df	SS	MS	F	p
Mass	1	0.29	0.29	0.79	0.388
Temperature	1	2.59	2.59	7.15	**0.018**
Source Population	1	0.74	0.74	2.03	0.176
Generation	1	1.16	1.16	3.20	**0.095**
Mass*Temperature	1	3.20	3.20	8.81	**0.011**
Residual	14	5.08	0.36		

%P

Factor	df	SS	MS	F	p
Mass	1	0.13	0.13	1.03	0.330
Temperature	1	0.04	0.04	0.34	0.572
Source Population	1	0.01	0.01	0.09	0.767
Generation	1	0.23	0.23	1.84	0.200
Residual	12	1.48	0.12		

Table S4.6. ANCOVA table of excretion rates and stoichiometry of adult female *Gambusia marshi* raised in the *ad libitum* laboratory temperature manipulation experiment. All interaction terms were included in the model, but only the significant effects are presented in the table.

NH_4^+					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.62	0.62	5.80	**0.028**
Temperature	1	0.01	0.01	0.08	0.785
Source Population	1	1.48	1.48	13.87	**0.002**
Generation	1	2.23	2.23	20.88	**<0.001**
Residual	16	1.71	0.11		
SRP					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	0.28	0.28	0.81	0.381
Temperature	1	0.21	0.21	0.61	0.446
Source Population	1	4.30	4.30	12.66	**0.003**
Generation	1	6.28	6.28	18.50	**0.001**
Population*Generation	1	3.44	3.44	10.14	**0.006**
Residual	16	5.43	0.34		
N:P					
Factor	**df**	**SS**	**MS**	**F**	**p**
Mass	1	1.67	1.67	5.68	**0.030**
Temperature	1	0.14	0.14	0.48	0.496
Source Population	1	0.71	0.71	2.42	0.139
Generation	1	1.01	1.01	3.45	0.082
Population*Generation	1	2.19	2.19	7.45	**0.015**
Residual	16	4.70	0.29		

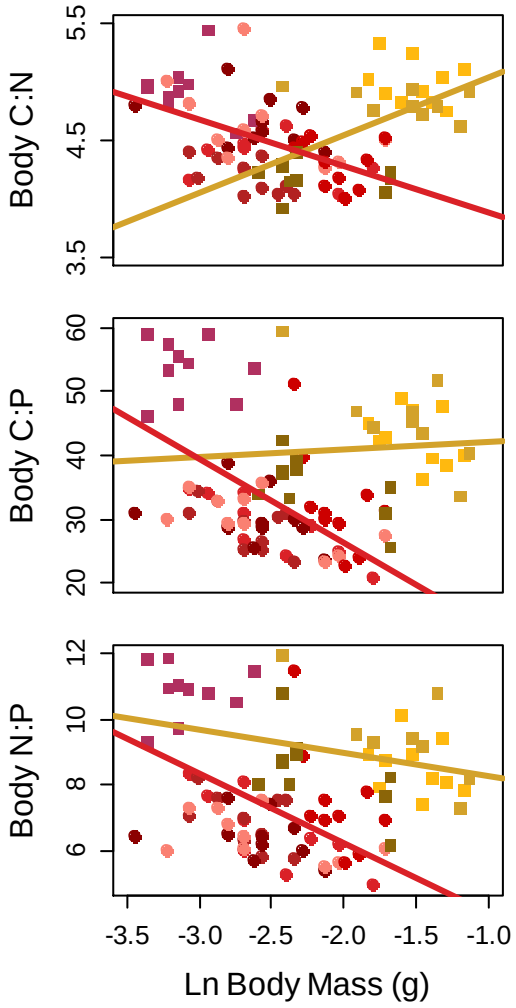

Figure S4.1. Body elemental ratios of female *Gambusia marshi* collected from nine sites in the Cuatro Ciénegas basin. Each color represents a different site, with red colors being warm site and yellow colors being cool sites. Squares indicate sites without predators and circles indicate sites with predators. Trendlines indicate differences between groundwater-fed sites (red) and runoff-dominated sites (yellow).

CHAPTER 5

PREDATION, NOT WATER FLOW VELOCITY, CAUSES MOST VARIATION IN BODY MORPHOLOGY AMONG AND WITHIN GAMBUSIA SPECIES IN NORTHEASTERN MEXICO

Abstract

Fish morphology is often thought to emerge from a trade-off between optimizing sustained or burst swimming performance. Phenotypically, this tradeoff is principally manifested through variation in the relative size of the caudal peduncle. High-flow environments tend to select for relatively small caudal peduncle area to maximize sustained swimming performance while predators tend to select for relatively large caudal peduncle area to maximize burst swimming performance. When prey species encounter high predation pressure in high-flow environments, however, it is unclear which aspect of performance will be optimized. I investigated this question using four *Gambusia* species in northeastern Mexico: the riverine *G. panuco*, the spring endemics *G. alvarezi* and *G. hurtadoi*, and *G. marshi*, found in a variety of habitats with and without predators. I employed a geometric morphometric analysis to test how body shapes of both male and female fish differ among species and habitats and with predators. I found that riverine and spring species diverged morphologically, with the habitat generalist *G. marshi* exhibiting a variable, intermediate body shape. Within *G. marshi*, morphological variation was explained by piscivore presence in both sexes, suggesting that predators exert a stronger selective force on *Gambusia* morphology than does flow velocity. Supporting this hypothesis, fish from sites with piscivores present, whether high- or low-flow, had larger relative caudal peduncle areas. I also found that sexual

dimorphism in size among species was higher in spring environments regardless of predator presence, highlighting sex-specific effects of some environmental factors. These results are of particular importance because many desert spring fishes are threatened by declining groundwater levels and introduced species, both of which could alter their morphology if they persist under altered environmental conditions.

Introduction

Trade-offs in prey life history and performance in the presence or absence of predation pressure can lead to phenotypic evolution via gene frequency changes (Stearns 1989; Ghalambor et al. 2004; Langerhans 2009). On the other hand, prey species can also cope with predation risk through phenotypic plasticity with little to no change in gene frequencies between generations (Brönmark & Miner 1992; Boersma et al. 1998; Touchon & Warkentin 2008). For species that span selection gradients, such as variation in the degree of predation pressure, high levels of gene flow across the gradient can inhibit adaptive divergence (Garant et al. 2007). However, the fact that both phenotypic plasticity and adaptive genetic divergence can produce similar phenotypic responses allows for the maintenance of phenotypic traits that maximize fitness among contrasting environments even in the face of gene flow among them (Fitzpatrick et al. 2015; Mäkinen et al. 2016). Further, plasticity itself can have a genetic basis, potentially further obscuring the effects of changing allele frequencies and phenotypic plasticity on the phenotype within genetically mixed populations (West-Eberhard 2003). Together, phenotypic plasticity and adaptive genetic divergence shape an organism's phenotypic response to selection pressures such as predation.

In fishes, one common way predation affects the prey phenotype is through a morphological trade-off between optimizing steady vs. unsteady swimming performance (Hendry et al. 2006; Langerhans 2009; Langerhans & Reznick 2010). Fish exposed to predators are thought to optimize their ability to rapidly escape predators by maximizing unsteady swim performance, i.e., locomotion involving changes in velocity and/or direction such as fast starts and rapid turns (Hendry et al. 2002; Langerhans 2009).

Morphological traits that enhance unsteady swimming performance include a flexible body with a small head, a large caudal peduncle, a high proportion of white muscle, and a large, low-aspect ratio caudal fin (summarized in Langerhans 2009). As most fish use these same structures in both steady and unsteady swimming maneuvers, they therefore face a trade-off in optimizing one type of swimming performance at the expense of the other (Langerhans 2009; Langerhans & Reznick 2010). The effects of this tradeoff on prey phenotype can be quite strong, but it is still unclear how the phenotype responds to multiple opposing pressures (Langerhans & Reznick 2010; Landy & Travis 2015). In some cases, fishes respond to variation in predation pressure through variation in life history traits instead of or in addition to optimizing unsteady swimming performance (Reznick & Endler 1982; Fu et al. 2015; Sharpe et al. 2015). This may especially be the case for fishes that also face strong selection pressure to optimize steady swimming performance. For fishes living in lotic environments with rapid flow, increased steady swimming performance can improve a fish's ability to forage actively and maintain position in the current (Langerhans 2008; Langerhans & Reznick 2010; Foster et al. 2015; Senay et al. 2015). Fish exposed to predators in high-flow environments face a trade-off in which the factor with a greater effect on fitness should ultimately be represented in the fish's phenotype.

Such a scenario arises in desert springs and rivers, where spring-fed pools are often too small and/or isolated to support or allow the colonization of larger-bodied predators (e.g., Hubbs 1995; Hubbs 2003). As a result, prey species living among these habitats can face high-flow conditions with predators, low-flow conditions without predators, and occasionally, low-flow conditions with predators. Among fishes that have

colonized spring systems in the western United States and Mexico, members of the families Poeciliidae are among the commonest and most diverse (Hubbs 1995). In particular, members of the genus *Gambusia* are well-represented in spring systems in the Chihuahuan desert of the southwestern United States and northeastern Mexico (Minckley 1962; Hubbs 2003). *Gambusia* are an ideal group in which to study the evolution of fishes in spring environments because multiple species have colonized spring environments while closely related congeners remain in stream environments where they coexist with predators (Rauchenberger 1989; Hubbs 2001). Here, I investigated whether the colonization of springs in the Chihuahuan desert by *Gambusia* has led to a convergence in morphology among species and explore the mechanisms by which it may have happened.

I hypothesized that variation in the morphological phenotype of *Gambusia* both among and within species is driven by variation in predation pressure rather than variation in flow velocity. As the relative importance of these two drivers of phenotypic variation has not been previously assessed, my hypothesis is based on several lines of evidence from prior work on the effects of predation on fishes. Although many heterotrophs including most fishes must actively forage for food, many fishes exhibit foraging behavior that reduces encounters with predators (e.g., Fraser & Gilliam 1987; Werner & Hall 1988), thus trading off the ability to acquire more food in favor of minimizing predation risk. These results suggest that predation has stronger fitness costs than does acquiring fewer resources. Supporting this hypothesis, several species of *Gambusia* from various environments exhibit phenotypic convergence consistent with optimized unsteady swimming performance in high predation environments (Langerhans

et al. 2004; Langerhans et al. 2007). Finally, the phenotype within a species can respond to reduced predation pressure over a relatively small number of generations (Heinen-Kay et al. 2014). As a result, I predicted that regardless of flow environment, species exposed to fish predators would exhibit more streamlined body shape with a relatively large caudal peduncle area to maximize burst swimming performance. I also predicted that morphological variation would follow a similar pattern within a species that inhabits both high- and low-predation sites.

To test this hypothesis, I studied the morphology of four species of *Gambusia* from northeastern Mexico. Of these, *G. alvarezi* and *G. hurtadoi* are spring obligate sister species, each living only in one isolated spring and its outflow (Hubbs & Springer 1957; Hubbs 2003). *G. panuco* are widespread in streams in the Río Pánuco basin of northeastern Mexico (Rauchenberger 1989). *G. marshi*, the sister species of *G. panuco*, is found in a variety of springs, wetlands, and spring-fed rivers within the Cuatro Ciénegas basin and its outflow the Río Salado de Nadadores (Minckley 1962; Lydeard et al. 1995). These species also differ in exposure to fish predators, allowing us to potentially separate the effects of predation and other factors associated with spring environments. The two spring obligate species live entirely without piscivorous fish (Hubbs & Springer 1957), *G. panuco* primarily coexist with piscivores (Obregón-Barboza et al. 1994), and *G. marshi* coexist with predators in streams and some springs but not wetlands (Minckley 1969). To understand how these factors may shape the evolution of fish morphology in desert springs, I investigated whether fish living in similar environments have converged on a similar phenotype. Specifically, I employed a geometric morphometric analysis to examine patterns in morphological convergence among species living in springs and

under similar predation pressures, and tested whether these patterns differ among sexes. My results shed light on how desert spring environments shape the evolution of fish populations and I discuss how changing environmental conditions may affect these threatened species.

Methods

I examined 347 specimens of *Gambusia alvarezi*, *G. hurtadoi*, *G. marshi*, and *G. panuco* collected between 1982 and 2013 and catalogued in the fish collection at La Universidad Autónoma de Nuevo León (UANL) (Table S5.1). All study species live in inland waters within Caribbean drainages of Northern Mexico (Figure 5.1). Although several populations I included were catalogued as *G. regani*, they were identified as such based on *G. panuco* being a junior synonym of *G. regani* (Obregón-Barboza et al. 1994). These individuals were collected in Veracruz, while *G. regani sensu stricto* is found only in southern Tamaulipas (Rauchenberger 1989). I follow Rauchenberger (1989) and Lydeard et al. (1995) in considering *G. panuco* to be a valid species, thus I refer to the individuals identified as *G. regani* by Obregón-Barboza as *G. panuco*. All specimens collected before 2004 were fixed in formalin and preserved in 50% isopropyl alcohol, while specimens collected in 2004 or later were fixed and preserved in 70% alcohol. I tested for effects of preservation method on morphology using ANOVA with a random effect of locality using specimens collected at two localities both before 2004 and after 2004: *G. hurtadoi* from Ojo de Hacienda Dolores and *G. marshi* from the Río Salado de Nadadores. I limited my analysis to specimens collected more recently to minimize any potential effects of recent evolution in these populations that may be ongoing and only used specimens collected before 2000 when no more recent specimens were available for

a particular population. In all cases I used the most recently collected samples from a given locality.

For *G. marshi*, I classified each locality as either a spring, river, or wetland based on my familiarity with the sites and key words in the locality description (i.e., *manantial* and *poza* for spring, *río* for river, and *humedal* for wetland). For each locality, I also examined fish collection records at UANL and Arizona State University for specimens of the three common piscivorous fish species that overlap in range with *G. marshi*: *Herichthys minckleyi*, *Ictalurus lupus*, and *Micropterus salmoides*. Although the piscivorous form of the trophically polymorphic *H. minckleyi* is less common than the other forms within populations (Cohen et al. 2005), their distribution among sites is not well-studied so I included all sites for which *H. minckleyi* was recorded as having piscivores. Many of these sites also support *I. lupus* and/or *M. salmoides*, so I do not expect that their inclusion as piscivores substantially biased my results.

For each lot, I randomly selected up to fifteen adults of each sex to be measured and photographed. Most lots had fewer than fifteen adult males and females, so all individuals that could clearly be identified as adults were photographed (Table S5.1). Standard length of all fish was measured to the nearest millimeter using calipers, and I then photographed fish against a white graph paper background using a Canon Powershot SX530 digital camera. Eleven morphological landmarks were selected based on those used in a prior study of *Gambusia affinis* (Langerhans 2009; Figure 5.2) and were digitized using the software tpsDig (Rohlf 2015a). The landmarks selected were (1) tip of upper jaw, (2) posterodorsal tip of skull, (3) anterior insertion point of dorsal fin, (4) posterior insertion point of dorsal fin, (5) dorsal insertion point of caudal fin, (6) midpoint

of caudal fin, (7) ventral insertion point of caudal fin, (8) posterior insertion point of anal fin, (9) anterior insertion point of anal fin, (10) junction of operculum with ventral midline, and (11) center of orbital. I then applied geometric morphometric methods to analyze variation in body shape (Rohlf & Marcus 1993). As poeciliids are sexually dimorphic, with males having a characteristically anterior positioning of the modified anal fin (Rosen & Gordon 1953), I analyzed sexes separately to include individual aspects of morphological variation within each sex. In each analysis, landmarks were aligned to a constant axis to correct for differences in rotation and scale in photographs using least-squares superimposition, and the weight matrix of these landmarks was then analyzed with a principal components analysis using the tpsRelw software to produce relative warps (Rohlf 2015b). I calculated relative caudal peduncle area as the area of the polygon bounded by landmarks 4-8 and dividing by the total body area measured from all lateral landmarks following Langerhans et al. (2004). Sexual size dimorphism between males and females was calculated for each lot both as the ratio of average female: male standard length and the ratio of average female: male centroid size from the geometric morphometric analysis.

I first analyzed whether morphology differs among species separately in each sex using MANCOVA on the relative warps with species as a fixed effect and centroid area as a covariate. Significant differences between species were assessed using the Pillai-Bartlett approximation of F. To more closely examine how morphology differs among sites and habitats, I performed a principal components analysis on the sums of squares and cross products matrix for the species term in the MANCOVA and from it I calculated scores on the divergence eigenvector (*d*) following Langerhans (2009). Briefly, *d*

summarizes the maximum multivariate variation in body shape that could be attributed to species-specific differences while controlling for other model terms such as body size. The use of *d* allows for a more intuitive single variable that can be used to test for divergence among species and habitat types rather than testing for variation in all relative warps. I tested whether *d* differs among species using a two-way ANOVA with a random factor for clade (i.e., the *panuco-marshi* group or the *alvarezi-hurtadoi* group) to correct for species relatedness. I then conducted Tukey's post-hoc tests if ANOVA results were significant. I similarly tested whether relative caudal peduncle area differs among species, habitat types, and piscivore presence as well as between sexes using a two-way ANOVA. I then used the same analytical methods to test whether morphology differed with habitat type and predation pressure within *G. marshi*, except I did not include any attempt at phylogenetic correction because genetic relationships among localities are unknown. For this analysis, I examined specimens from 23 sites (Table 5.5S1).

Further, to determine whether fish could reliably be classified as being from springs or rivers based on *d*, I performed a linear discriminant analysis on fish from these two habitat types among all four species. Assumptions of normality and heterogeneity of variance of residuals were tested using normal quantile plots and residuals vs. fitted values plots, respectively. All statistical analyses were performed in R v. 3.2.2 (R Core Team 2015).

Results

I first tested whether fish morphology differed with preservation method using two localities for which I had specimens fixed in both formalin and alcohol. I found no effect of preservation method on morphology of male (ANOVA, $F_{1,16}$=0.512, p=0.485) or

female (ANOVA, $F_{1,35}$=0.034, p=0.856) specimens. On the other hand, both male (MANCOVA, Pillai-Bartlett $F_{18,116}$=1.84, p=0.028) and female (MANCOVA, Pillai-Bartlett $F_{18,257}$=5.89, p<0.001) fish differed in multivariate shape with centroid size, indicating significant allometric effects on fish morphology. Further, the landmark positions of males (MANCOVA, Pillai-Bartlett $F_{54,354}$=5.423, p<0.001) and females (MANCOVA, Pillai-Bartlett $F_{54,777}$=17.671, p<0.001) differed among species. The divergence eigenvector, *d*, explained 79% of variation in body shape of males and 96% of variation in body shape of females. Examining *d* (Figure 5.3) revealed interspecific differences in morphology of males (ANOVA, $F_{2,134}$=20.82, p<0.001) and females (ANOVA, $F_{2,275}$=84.59, p<0.001) among species, with significant effects of clade in males (ANOVA, $F_{1,134}$=19.60, p<0.001) and females (ANOVA, $F_{1,275}$=442.59, p<0.001). Males of each species differed from each other in morphology except for *G. hurtadoi*, which was not significantly different from its sister species *G. alvarezi* (Tukey's post-hoc test, p=0.321) or from *G. marshi* (Tukey's post-hoc test, p=0.508). For males, low *d* scores were associated primarily with a deeper anterior body, more posterior position of the anal fin (gonopodium), and shorter caudal peduncle compared to fish with high *d* scores (Figure 5.4). Similarly, in females all species differed from each other except for the spring-dwelling species pair of *G. hurtadoi* and *G. alvarezi* (Tukey's post-hoc test, p=0.954, Figure 5.3). Female *d* scores appeared to follow the opposite pattern of that in males (i.e., females with low *d* scores were from predator-free environments), so I analyzed and present here the inverse of these values for ease of comparison with results for males. For females, low *d* scores were associated with less streamlined bodies with

shorter caudal peduncles compared to the deeper bodies of fish with high *d* scores (Figure 5.4).

I then explicitly tested whether relative caudal peduncle area differed among sexes and species. Males had significantly larger relative caudal peduncle area than females (ANOVA, $F_{1,412}$=1192.40, p<0.001), with a significant difference among clades (ANOVA, $F_{1,412}$=29.12, p<0.001) and species as well (ANOVA, $F_{1,412}$=76.36, p<0.001). There was no significant interaction effect of species and sex (ANOVA, $F_{1,412}$=3.12, p=0.078), as *G. panuco* drove this pattern by having a larger relative caudal peduncle area in both sexes (Figure 5.5).

In both sexes, *G. marshi* had intermediate scores on the divergence vector between the spring species and the riverine *G. panuco*. Within *G. marshi*, however, results differed between sexes. In males, fish morphology differed among habitat types (ANOVA, $F_{2,88}$=7.88, p=0.001). Males from rivers had higher *d* scores than those in wetlands (Tukey's post-hoc test, p=0.001) and springs (Tukey's post-hoc test, p=0.019), similar to patterns seen among species between river and spring habitats (Figure 5.6). Male morphology also differed with piscivore presence in the same direction as it differed among species inhabiting different predation environments (ANOVA, $F_{1,88}$=15.28, p<0.001, Figure 5.6). In contrast, female *G. marshi* did not differ in morphology among habitat types (ANOVA, $F_{2,152}$=1.48, p=0.230), but did differ with piscivore presence (ANOVA, $F_{1,152}$=83.10, p<0.001). The interaction term was not significant in either sex (p>0.05). As with interspecific patterns, female fish from sites with piscivores had higher *d* scores than fish from sites without piscivores (Figure 5.6).

Similarly, *G. marshi* males (t_{91}=-4.59, p<0.001) and females (t_{155}=-4.79, p<0.001) had larger relative caudal peduncle areas at sites with piscivores present (Figure 5.7).

Among species, the linear discriminant analysis correctly classified male fish from springs 81% of the time and from rivers 67% of the time. In spring fish, 83% of misclassifications were *G. marshi* and 17% were *G. hurtadoi*, while in rivers 59% were *G. marshi* and 41% were *G. panuco*. For females, the percentages of correct classification were 80% for spring fish and 78% for river fish. In springs, all misclassifications were *G. marshi,* while in rivers 57% were *G. marshi* and 43% were *G. panuco*. Sexual size dimorphism among species, as measured both by standard length and by centroid area, was greatest in *G. hurtadoi,* followed by *G. alvarezi*, *G. marshi*, and *G. panuco* (Table 5.1). Within *G. marshi,* results differed slightly depending on whether standard length or centroid size was used. Using standard length, females were disproportionately larger than males in springs compared to wetlands or rivers. Using centroid area, sexual size dimorphism was similar in springs and rivers, with wetlands still showing lower sexual size dimorphism. Using centroid size, sexual size dimorphism in *G. marshi* was also much more similar to that in *G. panuco* than when using standard length, indicating that although female *G. marshi* are longer than males, males may have a more robust body shape than those of *G. panuco*. In contrast to body morphology, sexual size dimorphism in *G. marshi* did not differ markedly with piscivore presence whether it was measured by standard length or by centroid size.

Discussion

Variation in the phenotype among genetically distinct species occurring in environments differing in predation pressure and water flow velocity can provide insight

into the drivers of intraspecific variation. I tested the hypothesis that variation in the morphological phenotype is driven by variation in predation pressure by examining phenotypic variation within *Gambusia marshi* and among several of its congeners that inhabit similar environments. Body morphology of both sexes varied between spring- and river-dwelling species, and these patterns suggest that predation has a stronger influence on fish phenotype than does the physical environment in this set of species. Riverine populations of *G. marshi* and *G. panuco* exhibited phenotypes consistent with greater unsteady swimming performance, a trait that would be favored under increased predation pressure but not under higher flow velocity (Langerhans & Reznick 2010). Further supporting this hypothesis, the morphology of *G. marshi* diverged with predation pressure to a greater extent than with flow velocity or other physical characteristics.

Variation among species was consistent with my predictions, but the phenotype of these species may be constrained by the phylogenetic relationships among them. *G. alvarezi* and *G. hurtadoi*, the two species found only in predator-free springs, are also sister species and may be most similar in morphology for this reason alone (Rauchenberger 1989). Indeed, there were clade-specific differences in both *d* scores and in relative caudal peduncle area, suggesting that a shared common ancestry does constrain morphological variation among these species. However, *G. marshi* and *G. panuco* are also sister species which differed significantly in body morphology (Rauchenberger 1989; Lydeard et al. 1995) (Figure 5.3). Further, I found that populations of *G. marshi* living with piscivores showed similar trends to those among *Gambusia* species suggesting that these morphological differences are at least in part caused by predation effects (Langerhans et al. 2004; Langerhans 2009). An examination of

morphological variation within *Gambusia marshi* is informative in understanding how fish from a single lineage can diversify phenotypically after colonizing a diversity of habitats.

In addition to effects on body shape, I also found differences in sexual size dimorphism among species and habitats (Table 5.1). In particular, fish living in springs had greater dimorphism in size measured by standard length regardless of the presence of predators. However, this effect did not hold when using centroid size, in which case only the two spring endemic species exhibited a larger size dimorphism than *G. panuco* and *G. marshi* in any habitats. This may be explained by the longer, continuous growing period in thermal springs allowing females with indeterminate growth to outgrow males to a greater degree than in more variable rivers and wetlands (Stearns 1983). Larger body size in spring-dwelling females could also be explained by higher quality resources in springs than in wetlands and rivers. These fish consume primarily terrestrial invertebrates (Meffe 1985), so variation in resource quality may depend more upon water permanence and riparian vegetation than characteristics of the aquatic environment. Riparian ground-dwelling arthropod communities along arid watercourses do vary with surface water permanence (Moody & Sabo In Press), and biomass of some species can be reduced with reduced surface water as well (McCluney & Sabo 2014). The perennial nature of springs relative to wetlands and even rivers in the study region could lead to more stable and higher quality resource inputs to fish living in those environments, further allowing females to outgrow males to a greater extent.

In male *G. marshi*, habitat still had a significant effect on body morphology in addition to that of predation (Figure 5.6). One potential explanation for the observed

sexual difference in *G. marshi* is that pregnancy can place a constraint on body morphology such that female fish from populations experiencing differences in predation pressure appear more similar to each other than do males (Wesner et al. 2011; Ingley et al. 2014). Ingley et al. (2014) found that immature *Brachyrhaphis* females actually diverged more with predator presence than reproductive adults and suggested that morphological constraints due to carrying live young could be the cause. Other environmental factors may also have greater selective effects on males than females (Hendry et al. 2006). For example, temperature variables had stronger effects on divergence in body morphology of males between *G. sexradiata* and *G. yucatana* (Jourdan et al. 2016). If thermal effects on male morphology are strong in *G. marshi*, this could explain differences in their morphology among habitats that vary in temperature. Unsteady swim performance in males is also used in forced copulations, which is a more common strategy in high predation environments in other species of poeciliids (Godin 1995; Heinen et al. 2013). As a result, variation in courtship behavior among habitats could also explain sexual differences. Examining sex-specific differences in the strength of selection on body morphology from various environmental pressures will improve our understanding of sexual dimorphism in fishes such as these.

Understanding the combination of factors that together shape the morphology of desert *Gambusia* is becoming increasingly relevant as the desert spring habitats where they live are among the most threatened in the world (Pister 1974; Souza et al. 2006; Contreras-Balderas et al. 2008). Indeed, two species of *Gambusia* that formerly lived in springs in Texas have already been declared extinct within the last century (Peden 1973; Miller et al. 1989) and numerous other spring-dwelling *Gambusia* are listed as

vulnerable, threatened, or endangered on the IUCN Red List including *G. alvarezi* and *G. hurtadoi* (both vulnerable). Although the status of *G. marshi* has not been assessed, it too is predicted to be threatened (Kopf et al. In Press). These systems are variably threatened by the introduction of novel predators and the extinction of native ones (Contreras-Balderas 1984; Sabo et al. 2010), and small fishes such as *Gambusia* may undergo morphological changes if they persist under novel conditions. While increased environmental variability due to spring failure would likely extirpate other spring fishes in Cuatro Ciénegas (e.g., Carson et al. 2008), *G. marshi* persists in a wide variety of habitats (Minckley 1962). However, my results suggest that the loss of predators would alter the phenotype of the remaining fish, likely reduce the standing genetic variation related to morphology, and therefore potentially alter their ability to respond morphologically to future changes.

As individual populations of these fishes become increasingly threatened, it is increasingly important to understand what factors drive phenotypic divergence among populations and species. The fact that I found a morphological contrast among species in high predation environments and species in low predation environments that is consistent with other *Gambusia* species (Langerhans et al. 2004; Langerhans et al. 2007) supports the hypothesis that predation is a strong and consistent force driving selection on the phenotype of these fishes. As some of these traits under selection are incorporated into female mate preference, morphological and behavioral changes in the mating system of Bahamian *Gambusia* in environments of varying predation pressure can lead to ecological speciation in these fishes (Langerhans et al. 2007; Heinen et al. 2013; Heinen-Kay & Langerhans 2013). In the Cuatro Ciénegas basin, *G. marshi* are frequently able to

move among high-predation and low-predation environments, but future work is needed to address whether phenotypic divergence among these population is reinforced by assortative mating.

My results support the hypothesis that predation pressure can exert strong effects on prey species without extirpating them (Reznick & Endler 1982; Boersma et al. 1998; Langerhans et al. 2004; Ingley et al. 2014). Across dramatic gradients of flow, temperature, salinity, and diel fluctuation in those variables, I found that predation still exerted at least as strong of an effect on fish morphology. As these patterns emerged both within and among species, I believe predation is a major selective force acting upon the evolution of *Gambusia* as has been previously suggested (Langerhans et al. 2004; 2007; Heinen-Kay & Langerhans 2013; Heinen-Kay et al. 2014). Changes in predation pressure could dramatically alter the phenotype of these species and their resultant role in the food webs of the springs they inhabit. While I have shown clear morphological differences among populations, uncertainties remain regarding how these morphological differences affect the way these fish interact with their environment. My work on morphological variation lays the groundwork for future studies to examine the evolutionary-ecological dynamics of predator-prey interactions in these fragile yet fascinating spring ecosystems.

Acknowledgements

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marking landmarks on images. The manuscript was greatly improved by comments on earlier drafts from James Collins, Krista Capps, James Elser, Justa Heinen-Kay, and the Sabo lab. I thank the ASU School of Life Sciences Research and Training Initiatives fund for providing funding for EKM to travel to Monterrey to complete this work.

Table 5.1. Sample size and sexual size dimorphism of *Gambusia* species living in springs, rivers, and wetlands in Northeastern Mexico. SL = standard length.

Species	Habitat	Sample Size	Avg Female SL (mm)	Avg Male SL (mm)	Female: Male SL Ratio	Female: Male Centroid Ratio
G. alvarezi	spring	22	29.20	21.86	1.34	1.29
G. hurtadoi	spring	46	29.18	20.58	1.42	1.36
G. marshi	spring	143	33.22	24.16	1.38	1.18
G. marshi	wetland	62	30.85	24.10	1.28	1.12
G. marshi	river	45	30.52	23.41	1.28	1.18
G. panuco	river	110	28.68	23.66	1.22	1.15

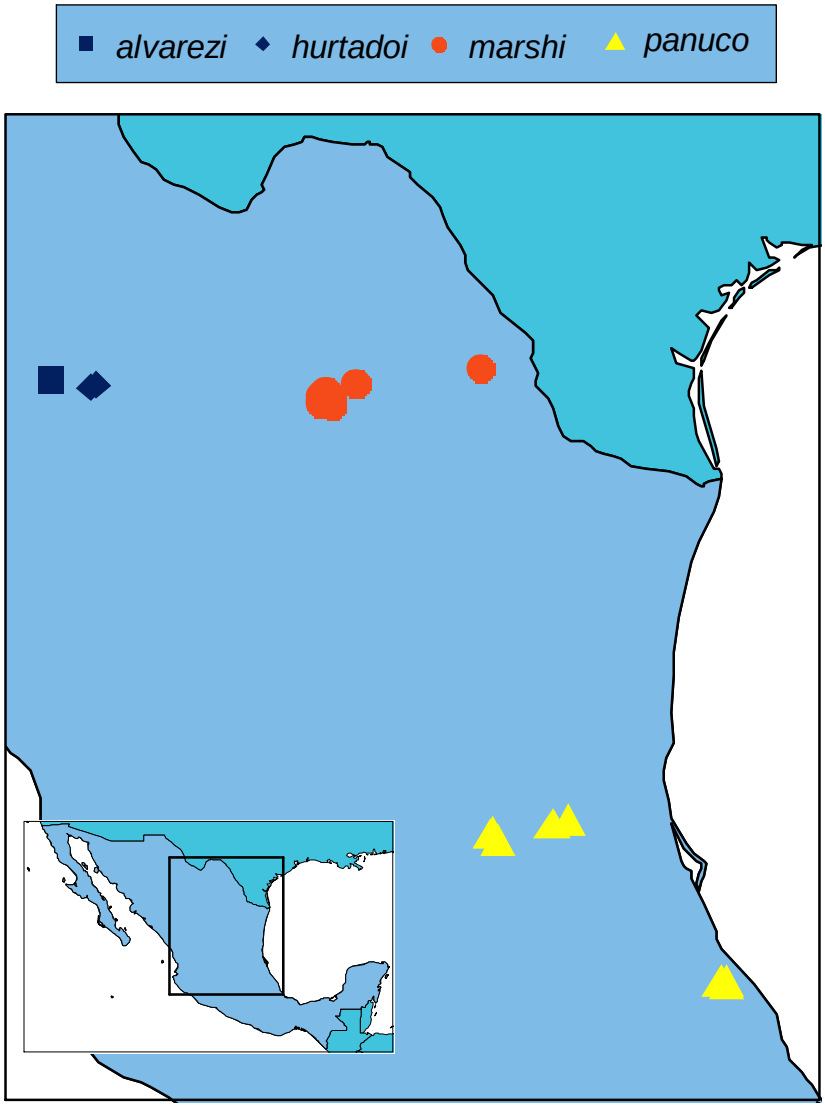

Figure 5.1. Collection localities in Northeastern Mexico from which I analyzed specimens. See Table S5.1 for more information on specific lot numbers, sample sizes, and collection localities.

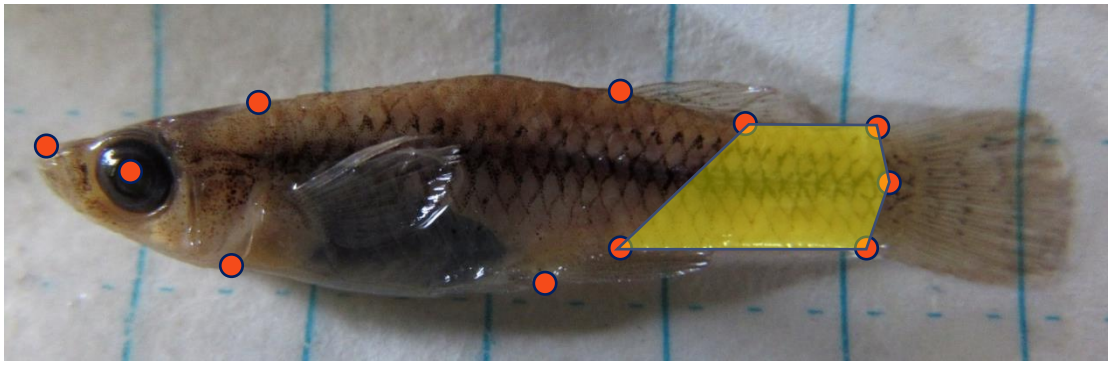

Figure 5.2. A female specimen of *Gambusia marshi* showing the positions of the eleven landmarks used in the geometric morphometric analysis. The same landmarks were used in males. The yellow shaded area represents the caudal peduncle area.

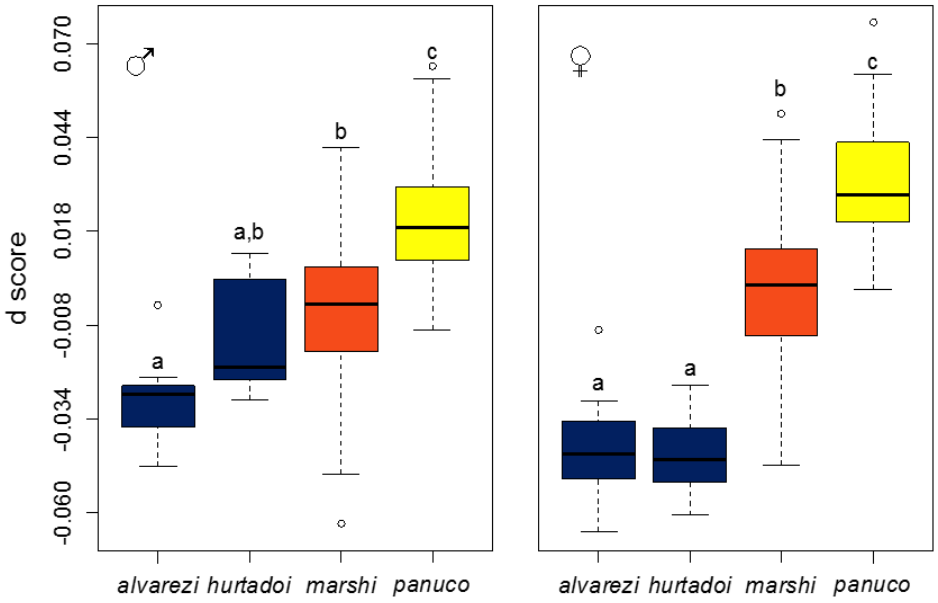

Figure 5.3. Scores on the *d* vector for four *Gambusia* species. Males (left panel) were analyzed separately from females (right panel), so *d* values for one sex do not reflect the same morphology as those for the other sex. Letters above boxes indicate statistically significant differences between species within a sex based on Tukey's post-hoc tests following significant differences among species determined by ANOVA.

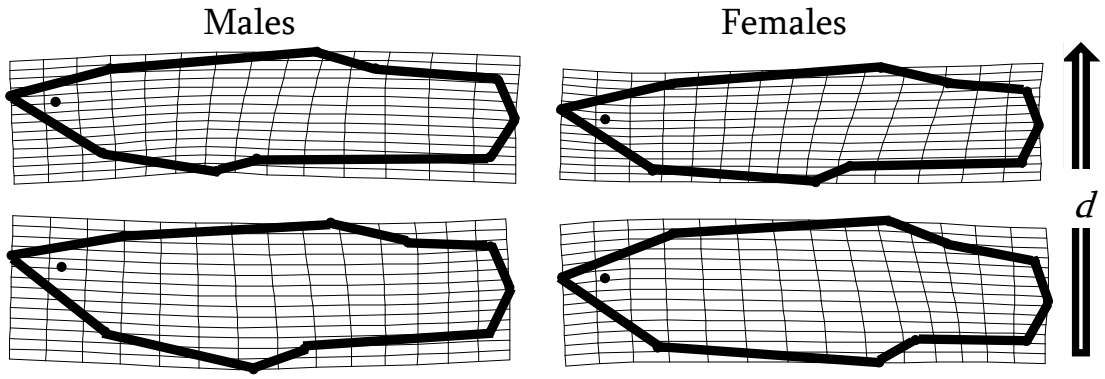

Figure 5.4. Thin plate splines showing morphological differences in males and females. For each sex the illustrations represent positions of landmarks for fish with the highest (top panel) and lowest (bottom panel) scores on the *d* vector among all species. Landmarks (except orbital) are connected with lines to illustrate how body shape varies with *d*.

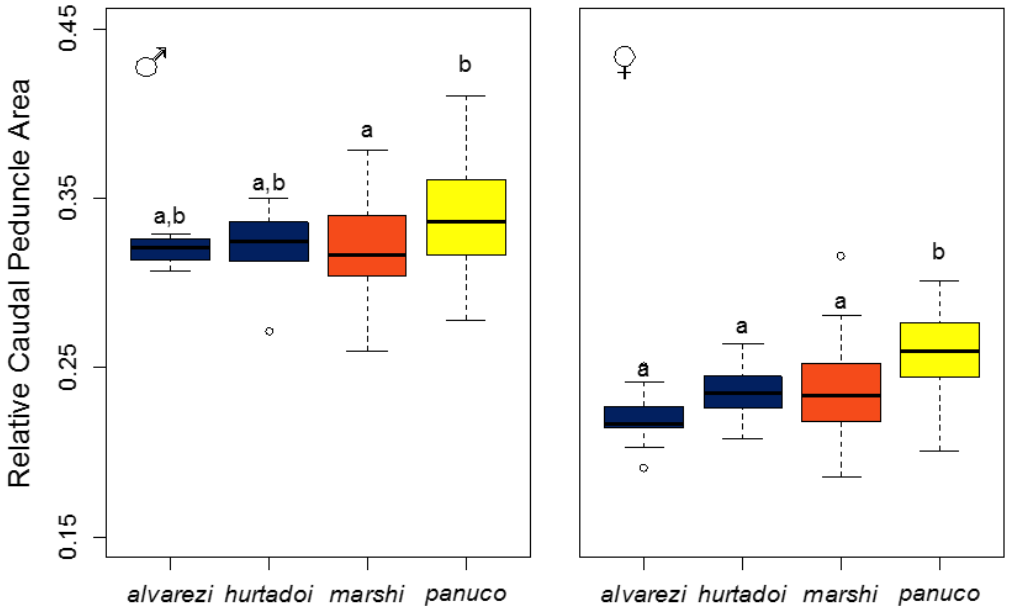

Figure 5.5. Relative caudal peduncle area for males (left panel) and females (right panel) of four *Gambusia* species. Values represent the lateral area of the caudal peduncle divided by lateral area of the entire fish body. Letters above boxes indicate statistically significant differences between species within a sex based on Tukey's post-hoc tests following significant differences among species determined by ANOVA.

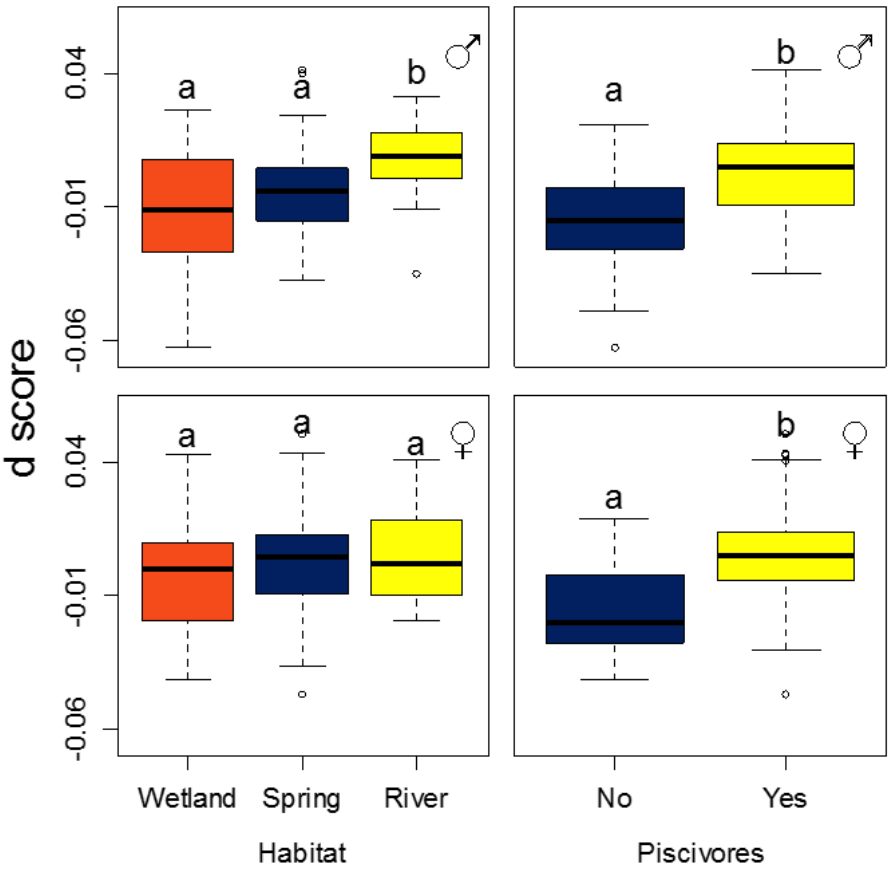

Figure 5.6. Scores on the *d* vector for *Gambusia marshi*. Males (top panels) were analyzed separately from females (lower panels), so *d* values for one sex do not reflect the same morphology as those for the other sex. Letters above boxes indicate statistically significant differences between habitats or predation within a sex based on Tukey's post-hoc tests following significant differences among species determined by ANOVA (for habitats) or based on t-tests (for piscivores).

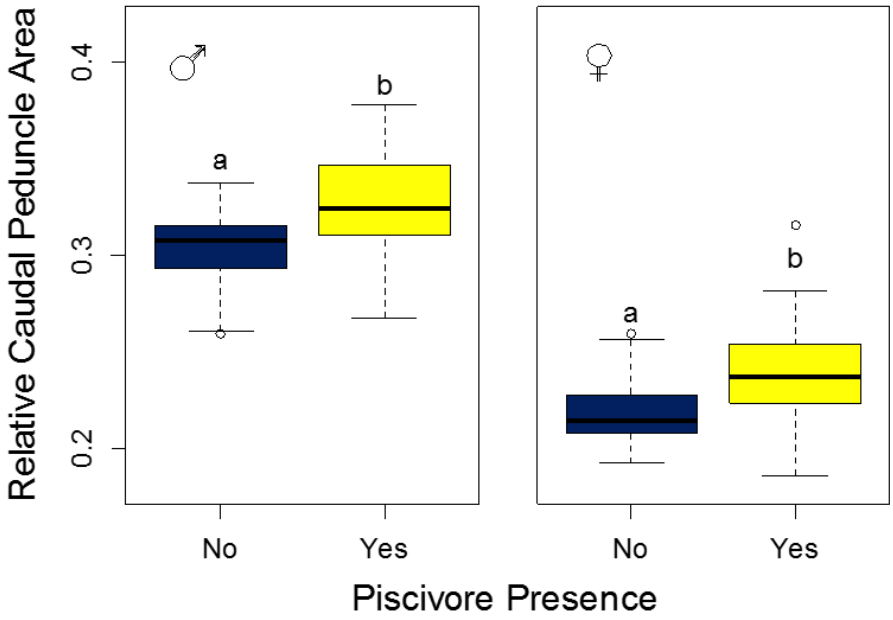

Figure 5.7. Relative caudal peduncle area for males (left panel) and females (right panel) of *Gambusia marshi*. Values represent the lateral area of the caudal peduncle divided by the lateral area of the entire fish body. Letters above boxes indicate statistically significant differences between species within a sex based on t-tests.

SUPPLEMENTARY MATERIALS

Table S5.1. Lots examined in the geometric morphometric analysis. All lots are catalogued in the UANL fish collection. Numbers represent the number of males and females examined, respectively.

Species	**Lot**	**Locality**	**State**	**No.**
G. alvarezi	19141	Ojo in San Gregorio	Chih.	7/15
G. hurtadoi	5544	Río Florido in Jiménez	Chih.	2/3
G. hurtadoi	18726	Ojo de Hacienda Dolores	Chih.	5/16
G. hurtadoi	21074	Ojo in Villa Lopez	Chih.	2/13
G. marshi	8216	Río Salado at Pte. Anáhuac	N.L.	7/10
G. marshi	11869	Laguna Grande	Coah.	3/3
G. marshi	15107	Poza Churince	Coah.	5/5
G. marshi	15311	Poza Juan Santos	Coah.	2/3
G. marshi	15352	Poza El Anteojo	Coah.	2/7
G. marshi	15532	Poza near Pozas Azules	Coah.	4/5
G. marshi	15539	Laguna Churince	Coah.	6/6
G. marshi	15420	Poza La Tecla	Coah.	0/4
G. marshi	15505	La Tecla	Coah.	0/4
G. marshi	15558	Poza Escobedo	Coah.	2/10
G. marshi	15560	Poza 600 m W. of Escobedo	Coah.	6/7
G. marshi	15562	Manantial in Ejido El Venado	Coah.	4/10
G. marshi	15575	Las Playitas	Coah.	2/5
G. marshi	15586	Laguna W. of Las Playitas	Coah.	5/9
G. marshi	15598	Ejido El Venado/San Pablo	Coah.	8/10
G. marshi	15632	Poza El Mojarral	Coah.	4/4
G. marshi	15647	Poza N. of Escobedo	Coah.	1/2
G. marshi	15654	Laguna Doble in Los Hundidos	Coah.	2/10
G. marshi	15659	Poza in Los Hundidos	Coah.	9/10
G. marshi	15682	Poza in San Marcos	Coah.	4/6
G. marshi	17664	Río Salado at Cariño de la Montaña	Coah.	3/0
G. marshi	19842	Río Salado at km 39 Pte. por MX 57	Coah.	5/9
G. marshi	21711	Humedales in La Angostura	Coah.	6/5
G. panuco	6649	Laguna La Media Luna SE Río Verde	S.L.P.	3/8
G. panuco	6761	Río Valles 1 km from Ciudad Valles	S.L.P.	5/8
G. panuco	6769	Río Gallinas 800 m E. of Rascón	S.L.P.	3/8
G. panuco	9516	Arroyo El Potrero	Ver.	6/12
G. panuco	9521	Arroyo Zanjas in Arena de la Palma	Ver.	2/9
G. panuco	9525	Río Solteros in Congregación Solteros	Ver.	2/10
G. panuco	15204	Río Calabaza at Pte. Plazuela	S.L.P.	3/7
G. panuco	15216	Balneario Anteojitos S. of Porfirio Díaz	S.L.P.	5/13

CHAPTER 6

CONCLUDING REMARKS

Summary of Major Findings

Chapter 2

In this work I aimed to test the growth rate hypothesis in Neotropical benthic grazers over a natural thermal gradient in Panamá. I found that growth rate and body P content both increased with temperature in the leptophlebiid mayfly *Thraulodes* across this gradient. However, controlled temperature manipulation experiments at one cool site and one warm site did not support the conclusion that temperature itself drove this relationship. In both *Thraulodes* and tadpoles of the toad *Rhinella,* growth rate and P content were not significantly correlated among sites and temperatures. Instead, differences among the two sites explained more variation in consumer growth rate and P content. While these results support the hypothesis that variation in P content is driven by variation in growth rate due to the elevated demand for ribosomal RNA production, they do not support the application of this conclusion to differences in growth rate due to variation in temperature.

Chapter 3

The ratio of nitrogen to phosphorus (N:P) excreted by a consumer is predicted to vary with dietary N:P, consumer N:P, and *L*, the maximum accumulation efficiency of the limiting nutrient (Sterner 1990). I examined the contribution of two of these metrics, dietary N:P and *L*, to variation in excretion N:P by conducting a meta-analysis of published dietary manipulation experiments with fishes. Fish excreted more N and less P when fed a diet higher in N:P ratio, but only when accounting for consumption. These

results suggest that the amount consumed can affect the ratio of N:P excreted through variation in maximum accumulation efficiency (*L* in Sterner 1990). In field studies, I found no significant effect of dietary N:P on excretion N:P. This may be due to the inability to measure consumption in the field as well as the difficulty of accurately describing diet N:P from gut contents and/or stable isotope studies of diet.

Chapter 4

After establishing the importance of consumption to driving variation in N:P excreted by fishes (Chapter 3; Moody et al. 2015), I sought to test this hypothesis in a field system. In *Gambusia marshi*, adult female fish had both higher body N:P and higher excretion N:P in cooler runoff-fed sites than in warmer groundwater-fed sites. Model results suggested that this could be explained by decreased individual consumption rates at these sites, but not due to temperature alone. In a temperature manipulation experiment, developmental temperature had no effect on excretion N:P of mature females from two populations. On the other hand, reduced diet ration led to increased excretion N:P, supporting model predictions. As runoff-fed sites also tended to have lower piscivore densities and higher *Gambusia* densities than groundwater-fed sites, I hypothesize that this variation is due to increased intraspecific competition and thus lower food consumption rates under the lack of predation pressure.

Chapter 5

Having found effects of predation on the elemental phenotype of *Gambusia marshi* (Chapter 4), I then tested whether predation pressure affects the morphological phenotype of these fish to support its potential importance in driving phenotypic evolution in this system. *Gambusia* face a morphological trade-off in optimizing burst

swim performance to escape predation or optimizing sustained swim performance to actively forage and maintain position in flowing water because both types of performance rely on the relative size of the caudal peduncle (Langerhans 2009). I conducted a geometric morphometric analysis using museum specimens of *G. marshi* from high- and low-predation sites as well as the low-predation *G. alvarezi* and *G. hurtadoi* and the high-predation *G. panuco*. Both within and among species, fish from high-predation sites had more streamlined bodies and larger relative caudal peduncle area as would be predicted if burst swim performance is optimized. This was true even of fish in riverine environments, where sustained swim performance is needed to forage in the current. These results suggest that in these species of *Gambusia*, predation has stronger effects on fitness than variation in the physical environment.

Overarching Themes

Intraspecific variation in the elemental phenotype can link organismal evolution to ecosystem function (Jeyasingh et al. 2014; Leal et al. 2017). In this dissertation I examined several aspects of intraspecific variation in the elemental phenotype of aquatic consumers. I studied several different systems to expand the generality of the hypotheses I have tested. I studied benthic grazers in Panamá, *Gambusia* in Northeastern Mexico, and various fishes from the published literature over gradients of biotic and abiotic conditions to test what drives variation in the morphological and elemental phenotype of these consumers. In spite of the differences among these systems, my results suggest similar processes underlie consumer phenotypic variation in diverse aquatic ecosystems.

Although water temperature is a variable of considerable interest due to widespread changes in climate over the past century, my results indicate it has little effect

on the elemental fluxes of aquatic ectothermic consumers. In fact, almost none of the aspects of variation in the elemental phenotype I tested could be attributed to variation in temperature. Temperature did not affect P content of mayflies and tadpoles in Neotropical streams (Chapter 1), the N:P ratio excreted by fishes in laboratory experiments (Chapter 2; Moody et al. 2015), or the N and P excretion rates and N:P ratio excreted by *Gambusia marshi* (Chapter 3). Further, variation in the morphology of *G. marshi* was also unrelated to variation in habitats that differ in temperature (Chapter 4). These results do not imply that changing thermal regimes will not affect ecosystem function; altered thermal regimes in fact have many effects on aquatic ecosystems (e.g., Yvon-Durocher et al. 2010; Jeppesen et al. 2014; Cross et al. 2015). In order to understand the potential impacts of global climate change, we must understand mechanistically how ecosystem function responds to these changes. My results indicate that thermal effects on nutrient fluxes into and out of ectothermic consumers are negligible in relation to other potential drivers of those processes.

Unlike variation in temperature, this work collectively suggests strong effects of biotic interactions on nutrient storage and recycling by consumers. In Neotropical grazers, site-specific differences between lowland and highland streams explained more variation in consumer body P content than did temperature (Chapter 1). Unlike highland streams, these lowland streams are inhabited by predatory fishes and were also less severely affected by amphibian declines that have reduced amphibian species richness and biomass, thus creating highly contrasting biotic environments for the grazers living among them (Kramer & Bryant 1995; Colón-Gaud et al. 2010; Kilburn et al. 2010). I found that variation in consumption rate can drive variation in excretion rates of N and P

as well as the N:P ratio excreted by fishes (Chapter 3, Chapter 4), which likely also reflects variation in the biotic environment. In particular, reduced consumption in *Gambusia marshi* at runoff-fed sites may be driven by increased intraspecific competition and reduced metabolic rates in these low predation environments. This is further supported by the strong effects of predation pressure on the morphology of these fish and their congeners (Chapter 5). Although previous studies have examined the effects of biotic interactions on the elemental phenotype of consumers (e.g., Schmitz et al. 2010; Dalton & Flecker 2014; El-Sabaawi et al. 2015), this work offers a general framework characterizing these effects in aquatic ecosystems.

Of these contributions, my dissertation particularly advances our ability to predict the elemental phenotype of consumers under altered environmental conditions using the theory of ecological stoichiometry (Sterner & Elser 2002). Recent work has questioned the applicability of this theory in making predictions about allometric scaling of consumer-driven nutrient recycling (Allgeier et al. 2015; Vanni & McIntyre 2016), but my work supports various predictions of ecological stoichiometry in explaining intraspecific variation in the N:P ratio of nutrients excreted by consumers (Sterner 1990). Unlike the metabolic theory of ecology, ecological stoichiometry theory can successfully predict variation in this response which can alter nutrient limitation of primary producers in aquatic ecosystems (Elser et al. 1988; Atkinson et al. 2013). My work has especially shown that a key to the ability of ecological stoichiometry to predict excreted N:P lies in L, the maximum accumulation efficiency of the limiting nutrient, which is rarely considered in studies of consumer-driven nutrient recycling. Stronger consideration of

this parameter will improve our understanding of how consumers affect ecosystem nutrient dynamics.

Implications and Future Directions

My aim with this research has been not only to advance our basic understanding of stoichiometric theory and consumer-driven nutrient recycling, but also to investigate the functioning of ecosystems that are particularly threatened by global change. Throughout the Neotropics, disease-driven amphibian declines have had drastic effects on the consumer communities of headwater streams, which in turn have led to dramatic changes in these ecosystems (Lips 1999; Whiles et al. 2013). In the Cuatro Ciénegas basin, as in many aridland springs, spring discharge is being reduced by groundwater pumping for irrigated agriculture which has locally extirpated a number of spring-adapted species (Minckley 1992; Souza et al. 2006; Carson et al. 2008). My results suggest that these environmental changes could alter the way these consumers operate in their environment.

Among Panamanian headwater streams, *Thraulodes* and *Rhinella* had higher body P content in lowland streams where predators are numerous and the chytrid fungus pathogen has had minimal effects on the amphibian communities relative to highland streams (Chapter 1). These results are consistent with the growth rate hypothesis, suggesting that increased predation pressure and more intense interspecific competition for algal resources drives higher growth rates in these lowland populations and/or species. Amphibian declines have apparently relaxed these selection pressures on highland taxa, and as a result they have lower body P content. As algal nutrient contents have changed little since amphibian declines, individual grazers may consume surplus P

and recycle P back into the environment at higher rates (Rugenski 2013; Connelly et al. 2014). Similarly, changes in the biotic community affect nutrient recycling by *Gambusia marshi* in Cuatro Ciénegas. Individual fish excrete more P and less N in groundwater-fed sites, where water TDN:TDP ratios also tend to be higher and P-limitation of algal growth potentially most intense (Chapter 4). While these effects on individual nutrient fluxes are strong, it is the volumetric excretion rates by the entire community that can drive variation in ecosystem function (Capps et al. 2015). If the total biomass of the community is reduced by amphibian declines or by spring failure, variation in the excretion rates of individuals may be irrelevant. In Panamanian streams, for example, amphibian declines reduced volumetric nutrient excretion rates of tadpoles and macroinvertebrates by 98% and 80%, respectively (Rugenski 2013). In the face of such dramatic changes in biomass, changes in nutrient excretion rates by individuals will not scale up to the ecosystem level. However, events such as disease-driven amphibian declines have become an example of this phenomenon because they are such an extreme case. In other systems where variation in biotic interactions are less extreme, variation in individual nutrient excretion rates could be more easily scalable to ecosystem level functions.

While this work does not establish how these changing environmental conditions alter ecosystem functions, it does provide mechanistic clarity into how environmental changes affect multiple phenotypic traits of consumers that can have implications for ecosystem functions. To better understand how environmental drivers affect these fluxes at the ecosystem scale, however, we must examine how traits such as consumer population density, size structure, and sex ratio are affected by environmental change. A

combined understanding of these effects at the individual scale, as I have investigated here, and at the population scale will be needed to make predictions about how environmental change will alter nutrient recycling by consumer populations.

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APPENDIX A

PERMISSION FOR INCLUSION OF PUBLISHED WORK

All co-authors have granted permission for published work to be included.

Chapter 3, the only previously published chapter, is attributed to the journal Freshwater Biology.