LIFE IN THE EXTREME:

THE EVOLUTIONARY STOICHIOMETRY OF

POECILIA MEXICANA

By

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Abstract:

Since The Origin of Species, evolutionary biologists have studied the evolutionary response of organisms to ecological conditions to understand the mechanisms leading biological diversity. The integration of the fields of ecology and evolution is relatively new. The field of ecological stoichiometry is rooted in ecosystem ecology, but the abstraction of evolutionary change as a stoichiometric phenotype enables it as a powerful tool in their integration.

The two studies in this thesis are among the first to analyze the ecological consequences of evolutionary change within the stoichiometric framework. I combined the power of the ecological stoichiometry paradigm with a unique system of diverging populations of extremophile fish to test how evolutionary response to ecological conditions has altered the ecological function of these consumers. In my first chapter, I investigated the variation and convergences of the stoichiometric phenotype of *Poecilia mexicana*. This vigorous assessment included multiple years of field data, across spatially replicated populations, and a common-garden comparison. I found evidence for stoichiometric convergence in derived extremophile populations and variation in their ecological function.

In my second chapter, I tested for possible drivers of variation in the stoichiometric phenotype in two populations of *P. mexicana*. I used amino acids to create diets of excessive sulfur content, mimicking the ecological conditions in which divergent populations have evolved. With these diets, I tested whether derived populations of *P. mexicana* diverge from ancestral populations in their stoichiometric phenotype. While I found no patterns in nutrient excretion rates, there was evidence for differences between populations in plasticity of elemental demand regardless of dietary conditions.

My results clearly show that the stoichiometric phenotypic can be used as a measure of phenotypic change between populations. Furthermore, these results generated several predictions about the ecological consequences of such evolutionary change in the stoichiometric phenotype. Testing such predictions should refine stoichiometric theory in light of evolutionary theory, and thereby further our understanding of the intricate links between ecology and evolution.

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CHAPTER ONE

USING REPLICATED EVOLUTION IN EXTREMOPHILE FISH TO UNDERSTAND DIVERSIFICATION IN ELEMENTAL COMPOSITION AND NUTRIENT EXCRETION

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ABSTRACT

1. Ecological sources of selection are key drivers of evolutionary change in populations. Whether such evolutionary shifts alter ecological functions of organisms, such as nutrient recycling, remains poorly explored.
2. We used the framework of ecological stoichiometry to investigate whether divergent ecotypes of a livebearing fish, *Poecilia mexicana*, have also diverged in elemental composition. Further, we tested the potential ecological relevance (i.e., effects on nutrient release rates) of convergent evolution, by comparing replicated extremophile (derived) and non-extremophile (ancestral) populations.
3. First, we tested whether phenotypic convergence is manifested as changes in somatic stoichiometry by measuring carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) content of wild-caught individuals inhabiting sulfidic (extremophile) and nonsulfidic (non-extremophile) habitats. Secondly, we tested whether shifts in somatic stoichiometry impinge on the rates at which key nutrients (N and P) are excreted. Additionally, to determine the relative role of temporal variation and plasticity, we compared the C, N, P, and S stoichiometries of wild-caught individuals of one drainage over a three-year period as well as individuals of the same drainage, raised in a common garden, laboratory setting.
4. We found significant differentiation in somatic stoichiometry between ecotypes. Sulfidic ecotypes consistently exhibited higher S content. The significant interaction between ecotype and drainage in the content of C, N and P indicates that, despite the strong evidence for convergence, there are drainage specific (i.e., non-convergent) evolutionary mechanisms at play. Similarly, we found variation between ecotypes of

P. mexicana in the rates of NH_3 and soluble reactive phosphorus excretion. Nevertheless, we also found significant temporal variation and plasticity in somatic stoichiometry, indicating strong environmental influences.

5. Studying taxa that have undergone replicated evolution illuminate some of the evolutionary mechanisms that drive variation in somatic elemental composition. Applying stoichiometric principles to such variation, as we have done here, is a useful approach to understand the ecological relevance of evolutionary change.

INTRODUCTION

Biologists have long studied how populations adapt to local environmental conditions (Endler, 1986; Schluter, 2000). In contrast, the effects of evolutionary change on the ecological functions of populations have received increasing attention only recently, particularly because evolution was previously assumed to be too slow to have immediate ecological consequences (Pelletier, Garant & Hendry, 2009; Palkovacs, Wasserman & Kinnison, 2011; Post & Palkovacs, 2009; Fussmann, Loreau & Abrams, 2007; Yoshida *et al.*, 2003; Hairston *et al.*, 2005; Miner *et al.*, 2012). Nevertheless, contemporary evolution can be rapid (Carroll *et al.*, 2007; Hendry & Kinnison, 1999), and divergence in morphology, physiology, or behavior may lead to shifts in the ecological roles of populations in their respective habitats as a byproduct of adaptive evolution (Bassar *et al.*, 2012; Wallenstein & Hall, 2012). A major question in understanding the dynamic interactions between ecology and evolution is whether replicated populations exposed and adapting to similar environmental conditions experience similar changes in their ecological functions. There are numerous examples of convergent evolution in phenotypic traits when evolutionarily independent lineages are exposed to similar sources of selection (Rosenblum *et al.*, 2010; Ruber & Adams, 2001; Ojeda *et al.*, 1999; Endler, 1986; Schluter, 2000; Norton *et al.*, 2007; Losos *et al.*, 1998), but whether predictable phenotypic changes can lead to predictable modifications of ecological roles populations play in ecosystems remains largely unclear.

Ecological stoichiometry is a powerful framework that applies principles of atomic mass balance to understand ecological interactions (Sterner & Elser, 2002). Mass balance can be used to predict the stoichiometry of consumer-driven nutrient recycling by combining information on organismal stoichiometry of a species and the stoichiometry of environmental supply (Elser & Urabe, 1999). Elemental resources available to consumers are often imbalanced relative to consumer elemental requirements (Elser *et al.*, 2000). Thus, consumers must regulate internal elemental content via selective sequestration or release of elements in high demand or excessive supply (Sterner & Elser, 2002). In this manner, consumers act as elemental capacitors in their local habitat and influence elemental availability to other trophic levels, affecting important ecological processes such as primary production and community structure (Sterner, 1986; Sterner & Hessen, 1994; Schnidler & Eby, 1997; Urabe *et al.*, 2002; Vanni, 2002; Allen & Gillooly, 2009; Elser & Urabe, 1999).

Most studies consider consumer organismal stoichiometry as a fixed, species level trait (Karimi & Folt, 2006; Sterner & George, 2000; Hendrixson, Sterner & Kay, 2007; Vanni *et al.*, 2002; Torres & Vanni, 2007). Hence, individuals of the same species are expected to perform the same ecological function, and studies have primarily focused on the ecological effects of phenotypic differences among species. This has hindered an evolutionary perspective on ecological stoichiometry (Jeyasingh, Cothran & Tobler, 2014). Indeed, there is substantial evidence for intraspecific variation in stoichiometric composition and elemental processing for multiple taxa (Evans-White, Stelzer & Lamberti, 2005; Jeyasingh & Weider, 2007; El-Sabaawi *et al.*, 2012; Vrede *et al.*, 2011), which provides the basis for evolutionary processes to shape elemental traits of organisms. Selection on the elemental composition of organisms may be direct, if habitats differ in the environmental availability of elemental resources (Jeyasingh & Weider, 2005; Jeyasingh, Weider & Sterner, 2009) or indirectly in response to selection on traits that differ in elemental demands (Kay *et al.,* 2005; Bertram, Schade & Elser, 2006; Morehouse *et al.*, 2010; Goos *et al.*, 2014). In addition, intraspecific variation in organismal stoichiometry may be the result of phenotypic plasticity (Liess & Hillebrand, 2005; Jeyasingh, Cothran & Tobler, 2014). Analysis of intraspecific variation in consumer stoichiometry, its drivers, and the effects of selection on the elemental content of organisms will allow us to apply stoichiometric theory to predict evolutionary shifts in ecological functions of populations (Jeyasingh, Cothran & Tobler, 2014).

We quantified elemental composition and excretion in genetically isolated, locally adapted fish populations to test for intraspecific variation in elemental composition and concomitant variation in ecological functions. Specifically, we leveraged replicated populations of extremophile fish to address the following questions: (1) Do drastic differences in environmental conditions between extreme and adjacent benign habitats affect the elemental composition of consumers, and are there convergent differences in organismal stoichiometry across replicated population pairs? We expected extremophile populations to exhibit different somatic stoichiometries as compared to populations from non-extremophile populations. In addition, we predicted that the elemental composition of extremophile populations should be convergent, tracking convergence in traditional phenotypic traits. (2) How do genetic and plastic differences among populations interact to shape variation in organismal stoichiometry? If elemental content has a strong heritable basis, natural populations are expected to maintain stoichiometric differences temporally, even if there is variation in environmental elemental availability. Similarly, population differences in stoichiometry should be maintained in individuals raised under standardized environmental conditions. Alternatively, loss of population differences in common garden raised individuals would indicate that somatic stoichiometry is plastic and dependent on environmental conditions. (3) Are nutrient recycling rates affected by variation in consumer stoichiometry? Mass balance predicts that consumers differing in somatic stoichiometry should have distinct ecological functions (Sterner & Elser, 2002). Thus, we expected that population differences in somatic stoichiometry should be reflected in different elemental excretion rates between extremophile and non-extremophile populations.

MATERIALS AND METHODS

Study system

Poecilia mexicana – a species of livebearing fish of the family Poeciliidae – is common in a variety of freshwater environments of Mexico and Central America (Miller, Minckley & Norris, 2005). In southern Mexico, the species also occurs in toxic, hydrogen sulfide (H_2S) rich springs (Tobler *et al.*, 2011). Geographic and molecular genetic evidence indicates at least three evolutionarily independent colonizations of sulfide springs, giving rise to evolutionarily replicated pairs of sulfidic and non-sulfidic ecotype pairs in different river drainages (Tobler *et al.*, 2011; Palacios *et al.*, 2013). All sulfide spring populations are reproductively isolated and genetically distinct from ancestral populations in nonsulfidic habitats, despite small geographic distances and a lack of physical barriers preventing fish movement (Plath *et al.*, 2013; Tobler *et al.*, 2009a). However, only the sulfide spring ecotype in one of the river drainages (Pichucalco) has been described as a distinct species, *P. sulphuraria* (Alvarez, 1947), even though it phylogenetically nests within *P. mexicana*.

Sulfide populations of the fish are characterized by local adaptation and convergent phenotypic trait evolution (Palacios *et al.*, 2013; Tobler *et al.*, 2011; Riesch *et al.*, 2014; Pfenninger *et al.*, 2014). Replicated populations of the sulfidic ecotype have larger offspring, smaller brood sizes (Riesch *et al.*, 2014; Riesch *et al.*, 2010), larger heads and gills (Tobler *et al.*, 2011), and truncated abdomens and caudal peduncles (Tobler & Hastings, 2011). They demonstrate behavioral adaptations, spending less time foraging to preform aquatic surface respiration —i.e., skimming the water-air interface to maximize access to oxygenated water (Tobler *et al.*, 2009b). Reproductive isolation between sulfidic and nonsulfidic populations is possibly the result of the cost of physiological machinery necessary for high rates of sulfide detoxification in extreme sulfidic conditions (Tobler *et al.*, 2011).

A priori considerations suggest that both direct and indirect selection affect the elemental composition of *P. mexicana* ecotypes, facilitating testing for evolutionary differentiation in organismal stoichiometry and associated changes in ecological function. Sulfide springs discharge disproportional amounts of sulfur (S) into spring habitats, profoundly altering the stoichiometry of environmental supply. H_2S toxicity also affects resource availability for fish by reducing autochthonous photosynthetic production (Bagarinao, 1992) and supporting chemosynthetic primary production by sulfide-oxidizing bacteria (Schmidt *et al.*, 1987; Roach, Tobler & Winemiller, 2011). Accordingly, populations in sulfide springs differ in their diet from ancestral populations in nonsulfidic habitats (Tobler *et al.*, 2015) and should therefore be exposed to contrasting environmental availabilities of elemental resources. At the same time, adaptive trait divergence in sulfide spring fishes may ultimately have altered their elemental demand.

Sample collection

Specimens were collected by seine (1.8m x 4.6m, 3.2 mm^2 mesh) from one sulfidic and one nonsulfidic site from each of the Pichucalco, Puyacatengo, and Tacotalpa drainages in June 2012, representing samples from evolutionarily independent ecotype pairs (see Fig. 1 and Table S1 for details on localities). The fish were transported to a nearby field station in insulated coolers and maintained in aerated 40L tanks filled with water from the original collection site, and fed commercial fish flake. Additional specimens were collected from one ecotype pair (Tacotalpa drainage) in 2011 and 2013 to analyze temporal variation in somatic stoichiometry.

Descendants of *P. mexicana* of the two Tacotalpa ecotypes (approximately seven generations removed from wild captured adults) were also collected from a greenhouse at the University of Oklahoma. Comparisons between common garden raised and wild caught individuals allowed for testing whether the somatic stoichiometry of these populations are at all plastic. Six males and six females were isolated from each population, totaling 24 common garden raised individuals. These populations were raised in green house stock tanks located at the University of Oklahoma, and fed on growing algal stocks and supplementary fed commercial fish flake food (I. Schlupp, pers. comm.).

Quantifying somatic tissue stoichiometry

To quantify intraspecific variation in elemental composition between the two ecotypes of *P. mexicana*, we measured C, N, P, and S content of somatic tissues in three replicate drainages (see table S1 for sample sizes from each locality). The fish were euthanized via cervical dislocation (Nickum *et al.*, 2004), sexed, blotted dry, and measured for standard length (mm). The intestinal tract was removed to prevent ingested, but unassimilated, materials from skewing the estimation of somatic stoichiometry. Because allocation of nutrients to reproductive tissue in livebearing fishes can vary greatly depending on the reproductive state, we also removed the gonads. The fish were then dried in a food dehydrator (Nesco, Two Rivers, WI) at ~77ºC for a minimum of 48 hours. Dry mass of each fish (without intestine or gonad) was then measured (0.01 g accuracy), and each fish was homogenized with a mortar and pestle. The homogenized samples were placed in a drying oven (Thermo Scientific, Langenselbold, Germany) at 60ºC for a minimum of 48 hours to remove excess moisture. Subsamples between 2.0 and 2.6 mg were measured with 0.0001 mg accuracy using microbalance (XP2U, Mettler Toledo, USA) and analyzed for % C, N, and S with a varioMICRO (Elementar Americas, Inc., Mt. Laurel, New Jersey). P content (%) was determined using a modified sulfuric acid digestion method (APHA, 1992).

Testing for convergence across geographically isolated populations

We tested for potential convergence in somatic stoichiometry in the replicated populations collected in 2012. Due to variation among elemental means and standard errors, we *z*-transformed measurements of each element, then subjected the data to a principal component analysis (PCA) based on a covariance matrix. The PCA retained two components with eigenvalues >1 explaining 68.19% of variation (Table S2). PC scores were then used as dependent variables in a multivariate analysis of covariance (MANCOVA) with $\log_{10}$-transformed dry mass as a covariate. Drainage and ecotype were used as fixed factors. Although not the primary focus of the study, we also included sex in all models to account for variation in sex ratios in our samples from different populations. *F*-values were approximated with Wilks' lambda, and effect sizes were estimated using partial eta squared (η_p^2). Overall, this analytical approach allows for quantifying the relative contribution of convergent and non-convergent patterns of stoichiometric differentiations; the ecotype term reflects shared (i.e., convergent) aspects of elemental composition, while the ecotype by drainage interaction represents unique (i.e., non-convergent) patterns of stoichiometric differentiation in each drainage (Langerhans & DeWitt, 2004). We also examined patterns of variation for each element using analysis of covariance (ANCOVA) models with factors as described above. Percentages of C, N, P, and S were logit-transformed prior to the ANCOVA analyses. We expect convergent trends in somatic stoichiometry — C, N, P and S content — in populations from similar habitats (i.e. replicated ecotypes) across the three tested drainages.

Testing for temporal variation in somatic stoichiometry

To contrast spatial and temporal variation in wild populations of *P. mexicana* across three collection years, we analyzed the logit transformed values of C, N, P and S content of the two ecotypes of the Tacotalpa drainage using a multivariate generalized linear mixed model with ecotype and sex of the individual as fixed factors. Logit transformation was necessary because elemental content can only range from 0 to 100%, violating statistical assumptions. Year of capture was included in the analysis as a random factor. The wet mass ($\log_{10}$-transformed) of the individuals was used as a covariate in the model. We reduced model complexity by eliminating non-significant ($P \geq 0.05$) interaction terms. If intraspecific variation in somatic stoichiometry was at all genetically determined across the two ecotypes, we expected for the population of origin ecotype to remain a significant determinant of somatic stoichiometry and capture year to be non-significant.

Testing for variation in somatic stoichiometry between wild caught and common garden raised fish

We tested for potential plasticity in somatic stoichiometry (C, N, P and S content) by comparing wild-caught *P. mexicana* of the Tacotalpa drainage with common garden laboratory raised descendants of the same populations using a MANCOVA with logit transformed elemental contents of C, N, P and S as response variables. Fixed factors included sex, whether the individual was laboratory raised or wild-caught and ecotype. Wet mass ($\log_{10}$-transformed) was included as a covariate. If plasticity is a contributing factor to the determination of somatic stoichiometry, we expected the laboratory/wild-caught term to be significant in the model. In both analyses, if population differences in somatic stoichiometry are maintained, (i.e., some genetic determination and divergence in somatic stoichiometry between ecotypes), we expected ecotype to be significant.

We also used discriminant function analysis (DFA) to test whether common garden individuals could be assigned to the correct population of origin based on elemental composition. We used the residuals of a preparatory MANCOVA removing the effects of sex and mass as dependent variables in the DFA. Ecotype was used as a grouping variable. Discriminant functions were built based on wild-caught individuals (training data). We then analyzed the classification success of common garden raised individuals (testing data). The purpose of the DFA was to obtain a quantitative measure of the ability of elemental signatures in assigning laboratory raised individuals to the correct population of origin based on natural variation in elemental content. If the classification success was 100%, variation in C, N, P, and S content has a strong genetic basis. If C, N, P, and S content were completely plastic, we would expect classification to be random based on the number of groups in the analysis (classification success ≤50%). With we conducted all statistical analyses using IBM SPSS Statistics 20, with the exception of temporal plasticity (SAS 9.2).

Excretion trials

We measured NH_3 and soluble reactive phosphorus (SRP) recycling rates in sulfidic and nonsulfidic ecotypes to determine the potential ecological consequences of local adaptation in the *P. mexicana* complex of the Grijalva system. To test for variation in elemental excretion and whether this variation is predictable across multiple pairs of sulfidic and nonsulfidic habitats in different drainages, a subset of specimens collected for the elemental analyses (specimens from 2012 collecting season, all three drainages, N_{total}= 95), and their excretion of SRP and NH_3 was measured prior to euthanasia and drying for estimation of somatic stoichiometry. The laboratory equipment available did not allow excretion measurements in sulfidic water, preventing the more ideal measurement of excretion rates in the field (Whiles *et al.*, 2009). Thus, nutrient excretion measurements for all fish were collected in similar conditions that are admittedly different from natural conditions, particularly in comparison to sulfidic streams. We chose NH_3 and SRP because primary producers depend on them as nutritional resources; thus, they have important ecological significance (Schaus *et al.*, 1997; Mehner *et al.*, 2005; Tátrai & Istvánovics, 1986). They were starved overnight (12-14 hours) to allow for evacuation of guts, minimizing overestimation of nutrient release by defecation, coprophagy, and stress due to the acclimated transition between sulfidic and nonsulfidic water. After the group fasting period, fish were placed individually into a plastic container with 150 ml water from the acclimation tub for a period between 30-46 minutes. Variation in incubation time was due to smaller individuals needing to be incubated for longer periods than larger individuals to ensure detectability of excreted nutrients (McIntyre *et al.*, 2008). We attest that variation in incubation time was not ideal, as we had to assume that excretions rates did not vary significantly between 30 and 46 minutes, but the alternative of decreasing water volume may risk stress on the fish, subsequently affecting excretion. We used a Portable Spectrophotometer (Hach DR 2800, Loveland, CO, USA) to measure SRP and NH_3 in the water samples after the incubation period following manufacturer's instructions. Soluble reactive phosphorus and NH_3 values from the excretion trials were converted to mg SRP or NH_3 excreted hr^{-1}, correcting for length of trial and volume of water. To test for differences in excretion between sexes and ecotypes, data were analyzed with ANCOVA models as detailed above for elemental composition. We reduced model complexity by eliminating non-significant ($P \geq 0.05$) interaction terms.

RESULTS

Convergence between replicated sulfidic and nonsulfidic ecotypes

Results from PCA indicated substantial intraspecific variation in somatic stoichiometry of *P. mexicana* inhabiting sulfidic and nonsulfidic habitats (Fig. 2). Ecotypes segregated along PC axis 1, which was negatively correlated with C content and positively with N, P, and S content (Table S2), with nonsulfidic ecotypes generally having higher C content than sulfidic populations. Axis 2, — which was positively correlated with C and N content and negatively correlated with P content (Table S2) — primarily uncovered variation among nonsulfidic populations. The segregation of ecotypes along the first PC axis was also reflected in the results of the MANCOVA; ecotype explained the bulk of variation in elemental composition indicating a strong pattern of convergence across replicated pairs of sulfidic and nonsulfidic populations (Table 1A). Mass, sex, drainage and most interaction terms explained relatively small amounts of variation. Nonetheless, they were significant predictors of elemental composition, highlighting the multitude of factors influencing intraspecific variation. Notably, the significant interaction between ecotype and drainage demonstrates that – despite the strong evidence for convergence – there also are drainage specific (i.e., non-convergent) aspects of elemental differentiation. Examination of the PC plot (Fig. 2) suggests that these non-convergent changes pertain to both the magnitude and direction of stoichiometric differentiation between ecotypes of each drainage.

Examination of individual elements reveals additional insights into patterns of convergent and non-convergent changes in somatic stoichiometry. Somatic C content was consistently lower in sulfidic ecotypes than nonsulfidic ecotypes, although the magnitude of the difference varied between drainages (Fig. 3A; Tables S3 and S4). Sulfidic females of all three rivers contained less C than nonsulfidic females (Table S3). This same trend was found in the males of the Tacotalpa and Puyacatengo drainages — but not the Pichucalco — which had relatively more C in sulfidic males than nonsulfidic males. Furthermore, larger individuals tended to have greater C content.

The effect of ecotype on somatic N content was inconsistent between drainages (Fig. 3B and Table S3). Sulfidic ecotypes contain less N in the Pichucalco, more in the Puyacatengo and had no difference in the Tacotalpa. Males contained more somatic N in sulfidic environments and females containing less. N content had a significant inverse relationship with mass.

Similar to C, ecotype significantly affected somatic P content in *P. mexicana*, both on its own and in combination with geographic location (i.e., drainage of origin), demonstrating consistent albeit weak signs of stoichiometric convergence (Table S3). Specifically, sulfidic populations of the Tacotalpa and Puyacatengo rivers had greater P content than nonsulfidic populations (Fig. 3C). Also similar to C content, there was no difference in P content of the Pichucalco population pair, which is explanatory of this drainage signaling the least divergence in the principal component analysis. Furthermore, the most dramatic difference was in the Tacotalpa population pair (Fig. 3C and Table S3).

Of the four elements measured, S content showed the clearest patterns of stoichiometric convergence between ecotypes. Sulfidic ecotypes from all three drainages showed significantly more S in the somatic tissue relative to nonsulfidic ecotypes. Nevertheless, the magnitude of the effect varied between drainages (Fig. 3D), similar to results on C, N, and P. The Tacotalpa drainage had the most drastic difference in sulfur content between sulfidic and nonsulfidic ecotypes while the Pichucalco had the least.

Testing the role of plasticity in somatic stoichiometry

We examined the role of plasticity in the determination of somatic stoichiometry in *P. mexicana* using individuals of the Tacotalpa drainage in two ways: (1) by comparing the somatic stoichiometry of wild-caught individuals across three collecting seasons (2011-2013) and (2) by comparing the somatic stoichiometry of wild-caught and common garden raised individuals. Year of capture had a highly significant effect on elemental content, suggesting a high degree of temporal plasticity in somatic stoichiometry (Tables 1B). While the ecotype main effect was non-significant (P = 0.0915), it suggested a trend towards differentiation in somatic stoichiometry between sulfidic and nonsulfidic ecotypes of *P. mexicana*. No interaction terms were significant. Sex and mass did not affect elemental composition of these populations.

MANCOVA and DFA of common garden, laboratory raised *P. mexicana* individuals of the Tacotalpa populations suggest that plasticity plays some role in the somatic phenotype of these livebearer populations; however, differences in somatic stoichiometry between populations were still present (Tables 1C, S5 and Fig. S1). DFA tests the ability to correctly assign individuals to ecotype based on their elemental makeup. If common garden raised individuals are correctly assigned to ancestral ecotype, DFA supports fixed differences in somatic stoichiometry between ecotypes. Overall, laboratory raised individuals were correctly classified into ancestral population 84.6% of the time, greater than that of random assignment (50%), indicating a large plastic component, but also genetic contributions to somatic stoichiometry in these fish (Table S6). MANCOVA results also support both genetic and plastic components in the determination of somatic stoichiometry in *P. mexicana* of the Tacotalpa (Table 1C), but between subjects effects show that the role of plasticity in the determination of somatic stoichiometry varies by element (Table S5B).

Elemental Excretion Rates

Congruent with somatic tissue stoichiometry, elemental excretion of N and P varied among populations; however, ecotype explained comparatively less variation for both N and P excretion (Table 2). Drainage and its interaction with ecotype explained the greatest amount of variation in elemental excretion rates for N and P excretion, respectively (Table 2). Nevertheless, ecotype also explained significant amounts of variation in both N and P signaling possible convergent effects of ecotype on elemental excretion. While the Puyacatengo and Pichucalco nonsulfidic populations excreted greater amounts of N than their sulfidic population pairs, the Tacotalpa population showed no difference in N excretion rate, driving a significant interaction between ecotype and drainage (Fig. 4A and Tables 2 and S4). Only the Pichucalco drainage showed stark differences in the rate of P excretion between ecotypes (Fig. 4B). Specifically, the sulfidic population of this drainage excreted P at a greater rate than that of the nonsulfidic population, but this trend was not observed in either the Puyacatengo or Tacotalpa ecotype comparisons (Tables 2 and S4). This resulted in ecotype explaining less of the variation in P excretion rates than was found in N excretion.

DISCUSSION

Our analyses of somatic stoichiometry in sulfidic and nonsulfidic populations of the fish, *P. mexicana,* uncovered substantial intraspecific variation in elemental content and excretion as well as convergent patterns of elemental differentiation between replicated population pairs. While comparison of somatic stoichiometries across collection years and a common garden experiment provided evidence for variation in somatic stoichiometry due to plasticity, discriminant function analysis showed ecotype differences were partially maintained in the laboratory. This suggests both genetic and environmental drivers of elemental content. Convergence within ecotypes was not as evident in elemental excretion.

Implications of intraspecific variation in consumer stoichiometry

Food sources in sulfidic and nonsulfidic habitats vary considerably in elemental composition (Fig. S2); hence, differences in elemental composition between ecotypes may be driven by the stoichiometry of supply. Nevertheless, this does not necessarily exclude the role of population difference in elemental demand. We found high plasticity in somatic stoichiometry across collection years. Flexibility in elemental composition may relieve some of the cost of maintaining stoichiometric homeostasis in novel or dynamic environments (Hood & Sterner, 2010), resulting in a possible fitness benefit (Gonzalez *et al.*, 2010). However, we must consider that the individuals tested for the laboratory common-garden were a mix of fish from different families of the same population kept in breeding stock tanks over several generations. Thus, familial lineage was not incorporated into our analysis. Such an undertaking would be ideal to further test plasticity in somatic stoichiometry of *P. mexicana*, but was beyond the scope of this study. Our temporal data only tested the Tacotalpa drainage, which was more recently colonized by *P. mexicana* than other drainages of the Grijalva (Palacios *et al.*, 2013). Furthermore, it is possible that our sample sizes did not capture the full spectrum of genetic variation each collection year, which may have lead to increased variation in means of elemental content among years. Future comparison of similar data on the Pichucalco and Ixtapangajoya, which were colonized previous to the Tacotalpa (Tobler *et al.*, 2011), should provide important insights into the evolution of *P. mexicana* stoichiometry.

Convergence in somatic stoichiometry

The sulfidic populations of *P. mexicana* sampled in this study convergently evolved modifications in morphological and physiological traits (Tobler *et al.*, 2011). Such evolutionary differentiation from ancestral nonsulfidic populations is expected to result in changes in the elemental signature of sulfidic ecotypes due to differences in elemental demand, as different biomolecules and structural components comprising living organisms vary in elemental composition (Sterner & Elser, 2002). Overall, our data from replicated sulfidic and nonsulfidic populations suggest that stoichiometric composition of *P. mexicana* converges depending on the habitat. Nevertheless, all elements sampled in this study revealed the magnitude of stoichiometric differences to be somewhat distinct between drainages, suggesting considerable non-convergent mechanisms impacting the evolution of consumer stoichiometry. This is not only reflected in differences in the magnitude of differentiation, but also highly idiosyncratic patterns in somatic N content. This finding is not completely unexpected, given that each sulfidic population was established independently, and are reproductively isolated from each other (Tobler *et al.*, 2011). Different genetic backgrounds of invading *P. mexicana*, stochastic processes such as genetic drift, and variation in environmental parameters not measured (Kaeuffer *et al.*, 2011) may have led to phenotypic divergence and associated shifts in elemental composition. Additionally, substantial differences in elemental availability between contrasting environments may act as a direct source of selection on a population's elemental demands.

Evolutionary shifts in nutrient excretion

Although rates of nutrient excretion did not exhibit strong convergence compared to somatic stoichiometry, there were significant differences in both N and P excretion among populations and drainages (Fig. 4). Stoichiometric theory predicts elements in high demand within the organism to have reduced excretion rates in comparison to those in lower demand (Sterner & Elser, 2002). In the case of N excretion, we note that the ecotypes of the Tacotalpa drainage had similar N contents as well as N excretion rates, implying that N content and recycling has less variation than the other two drainages

(Figs 3 and 4). Whether or not this is due to the more recent colonization of Tacotalpa drainage by *P. mexicana* than the other two drainages remains to be investigated. In respect to the N excretion trends of the Pichucalco, we suspect inundation of nitrogenous wastes by surrounding agricultural use. If true, it is logical that N content of the sulfidic population of this drainage would be greater than the nonsulfidic population, especially given that N content of the Tacotalpa populations have a high degree of plasticity (Table S5). However, a relatively low N excretion rate by the sulfidic population indicates an uncoupling between N content and N excretion (Figs. 3 and 4). Further investigation is needed to uncover what may be driving such contrasting patterns in N excretion between these populations of *P. mexicana*; however, these data clearly demonstrate intraspecific variation in N content and recycling rates.

P content and release also demonstrated unexpected results. The populations of the Pichucalco, which demonstrated the least difference in P content between ecotypes, was the only population pair which showed drastic differences in P excretion (Figs 3 and 4). Analysis directly comparing body stoichiometry to P excretion rates yielded non-significant results, indicating an uncoupling of somatic stoichiometry and recycling stoichiometry. Our analysis did not explicitly examine differences in food resource stoichiometry. It is possible that similarities in excretion rates between ecotypes, even with differences in tissue stoichiometries, are a result of large differences in elemental intake (see Fig. S2). Comparing excretion rates between ecotypes over a range of food stoichiometries would further elucidate the relationship between shifts in somatic stoichiometry and ecological function in this complex, which is under current study. Although such detail is not a prerequisite for stoichiometric models predicting nutrient excretion of species, attention to the mechanisms that underlie such mismatches between somatic and recycling stoichiometry among populations may be of evolutionary significance. Furthermore, due to the effects of hydrogen sulfide on the accuracy of our readings, we were unable to measure nutrient excretion on individuals in native stream water immediately after capture. The extreme conditions on the sulfidic ecotype caused concern of physiological effects of placing these individuals directly into nonsulfidic water. Thus, our excretion data was collected after a period of acclimation while sulfide levels gradually reduced in holding container. We acknowledge the possible impeding ramifications of this caveat on interpreting ecological consequences of intraspecific variation in nutrient excretion rates in this species. Nevertheless, intraspecific variation and evolutionary shifts in the stoichiometry of *P. mexicana* suggest that such shifts can be ecologically relevant. Fish are known to be important moderators in the movement of nutrients through and among aquatic ecosystems (Vanni, 2002) and may even create points of high nutrient concentrations in densely populated areas (McIntyre *et al.*, 2008). Whether intraspecific variation and evolutionary shifts in somatic and recycling stoichiometry become ecologically relevant remains a plausible hypothesis that can be rigorously tested using the *P. mexicana* system.

Conclusions

Overall, we found evidence for convergence in elemental composition, indicating that phenotypic change in a population can manifest as shifts at the elemental level of organization. Our data also shows the importance of considering plasticity in elemental content within the framework of ecological stoichiometry. Additionally, we found that intraspecific variation in somatic stoichiometry did not necessarily correspond to variation in nutrient excretion, indicating divergent physiological strategies of elemental use among the study populations. Together, these results demonstrate the power of studying taxa that have undergone replicated evolution for a evolutionary understanding of variation at the most fundamental level of biological organization. Such information will be pivotal in integrating existing stoichiometric models predicting ecological functions of species with evolutionary genetic models of speciation to better understand the dynamic interplay between ecology and evolution.

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TABLES

Table 1. Results of multivariate analyses of C, N, P and S content of the somatic tissue of *Poecilia mexicana.* A. MANCOVA testing for convergence in somatic stoichiometry across ecotypes from individuals collected in 2012 — based on principal component scores. B. Multivariate GLMM on logit transformed carbon, nitrogen, phosphorus and sulfur content testing for temporal variation in somatic stoichiometry *P. mexicana* of the Tacotalpa drainage collected over three field seasons (2011-2013). C. MANCOVA on logit transformed carbon, nitrogen, phosphorus and sulfur contents testing for plasticity in somatic stoichiometry by comparing wild-caught and laboratory raised *P. mexicana* of the Tacotalpa drainage. Statistically significant effects are in bold.

A. Variation in somatic stoichiometry based on principal component scores of C, N, P and S content.					
Effect	***F***	**Hyp. *df***	**Error *df***	***P***	η_p^2
Intercept	2.700	2	172	0.070	0.030
Dry mass	**3.643**	**2**	**172**	**0.028**	**0.041**
Drainage	**3.981**	**4**	**344**	**0.004**	**0.044**
Ecotype	**31.083**	**2**	**172**	**< 0.001**	**0.265**
Sex	0.412	2	172	0.663	0.005
Drainage × Ecotype	**7.021**	**4**	**344**	**< 0.001**	**0.075**
Drainage × Sex	0.873	4	344	0.480	0.010
Ecotype × Sex	**3.820**	**2**	**172**	**0.024**	**0.043**
Drainage × Ecotype × Sex	**2.499**	**4**	**344**	**0.042**	**0.028**
B. Temporal variation in somatic stoichiometry of *P. mexicana* of the Tacotalpa					
Year	**0.36**	**2**	**0**	**<0.0001**	
Ecotype	**2.86**	**1**	**486**	**0.0915**	
Sex	0.00	1	486	0.9589	
Log Wet Mass	0.02	1	468	0.8819	
C. Plasticity in somatic stoichiometry in common garden raised *P. mexicana* of the Tacotalpa					
Intercept	**4177.067**	**4**	**136**	**<0.001**	**0.992**
Sex	0.931	4	136	0.448	0.027
Wild/Lab	**22.655**	**4**	**136**	**<0.001**	**0.400**
Sulfidic/Nonsulfidic (NS)	**24.186**	**4**	**136**	**<0.001**	**0.416**
Log Wet Mass	**1.201**	**4**	**136**	**0.313**	**0.034**
Sex x Sulfidic/NS	1.443	4	136	0.223	0.041
Sex x Wild/Lab	1.008	4	136	0.406	0.029
Wild/Lab x Sulfidic/NS	**3.576**	**4**	**136**	**0.008**	**0.095**

Table 2. Results of the analyses of covariance (ANCOVA) on elemental excretion of A. NH_3 and B. soluble reactive phosphorus extraction in *P. mexicana* populations. Significant effects are in bold.

Effect	***F***	***df***	***P***	η_p^2
A. NH_3 excretion				
Intercept	**123.413**	**1**	**< 0.001**	**0.601**
Dry mass	**21.459**	**1**	**< 0.001**	**0.207**
Drainage	**9.780**	**2**	**< 0.001**	**0.193**
Ecotype	**15.147**	**1**	**< 0.001**	**0.156**
Sex	**1.359**	**1**	**0.247**	**0.016**
Drainage × Ecotype	**6.745**	**2**	**0.031**	**0.141**
Drainage × Sex	0.117	2	0.890	0.003
Ecotype × Sex	0.804	1	0.372	0.010
B. Soluble reactive phosphorus excretion				
Intercept	**15.327**	**1**	**< 0.001**	**0.162**
Dry mass	2.143	1	0.147	0.026
Drainage	**13.757**	**2**	**< 0.001**	**0.258**
Ecotype	**5.578**	**1**	**0.021**	**0.066**
Sex	0.130	1	0.720	0.002
Drainage × Ecotype	**16.031**	**2**	**<0.001**	**0.289**
Drainage × Sex	0.170	2	0.844	0.004
Ecotype × Sex	0.167	1	0.684	0.002

FIGURES

Figure 1. Map of sampling sites of the Grijalva River system of Tabasco, Mexico. Filled symbols represent sulfidic environments and hollow symbols, nonsulfidic. The Ixtapangajoya River, local roads (grey lines) and towns of the areas (grey shapes) are included for reference. Figure adapted from Palacios *et al.* (2013).

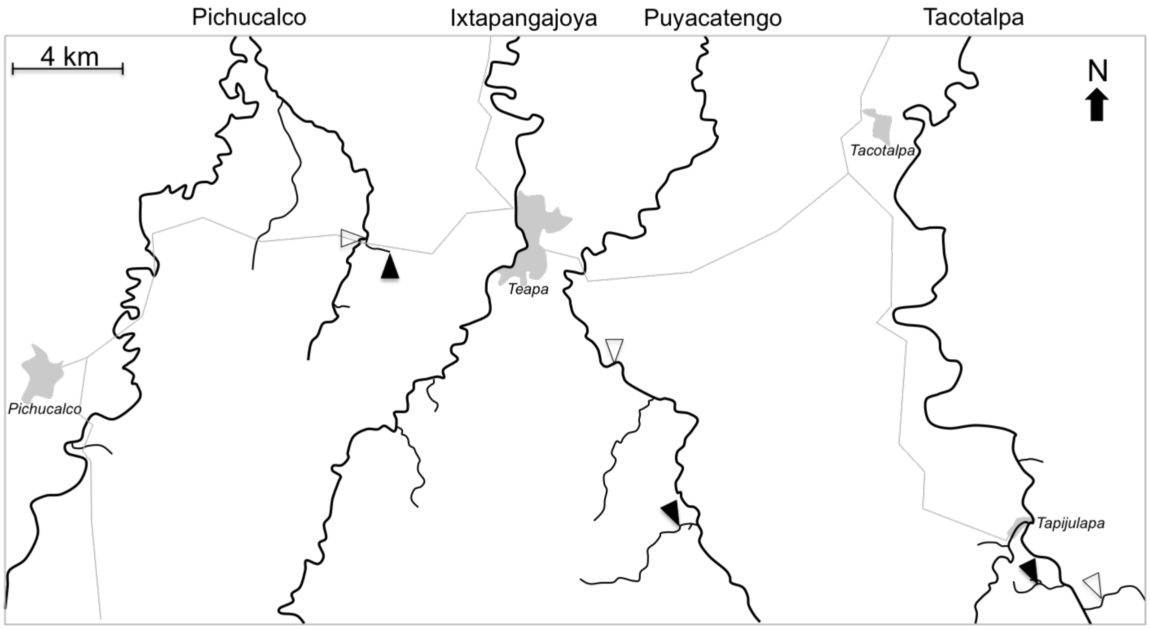


Figure 2. Principal component analysis of *z*-transformed percent content of carbon, nitrogen, phosphorus and sulfur of the somatic tissue of sulfidic (black points) and nonsulfidic (white points) ecotypes of *Poecilia mexicana*. Data collected from 2012 field season *P. mexicana* samples.

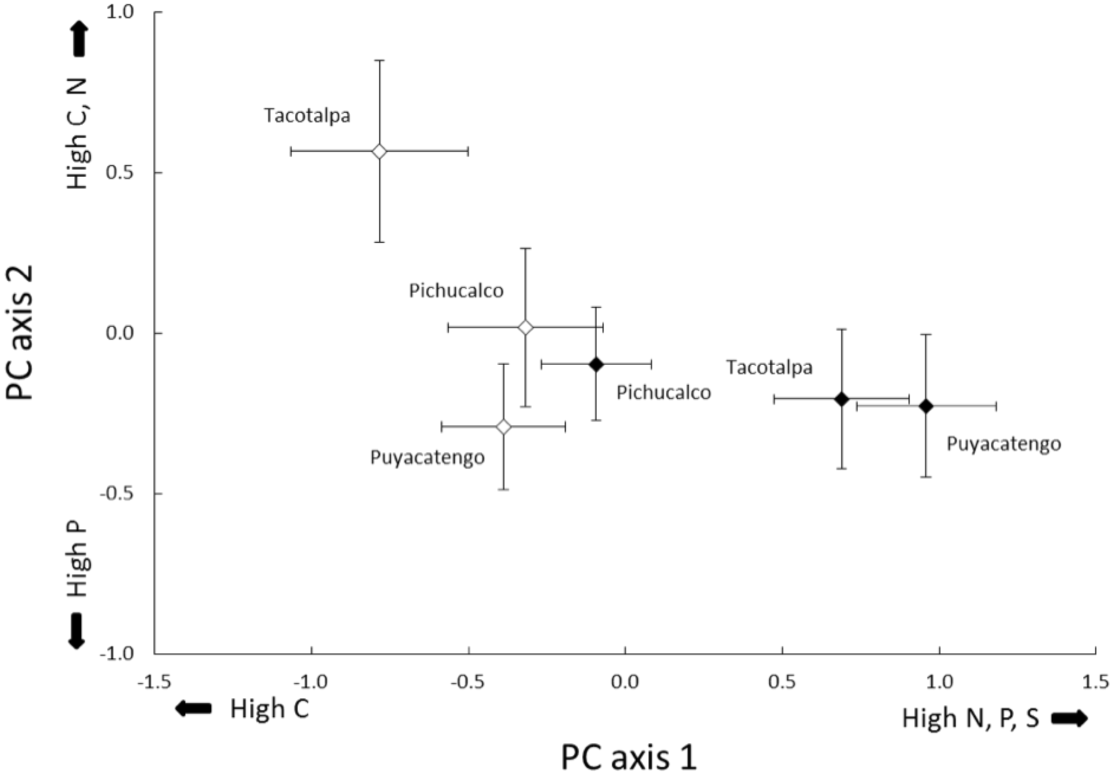


Figure 3. Estimated marginal means ± SE of A. carbon, B. nitrogen, C. phosphorus, and D. sulfur content of somatic tissue of *P. mexicana* collected during the 2012 field season from sulfidic and nonsulfidic population pairs from the Tacotalpa (circles), Puyacatengo (squares), and Pichucalco (triangles) river drainages.

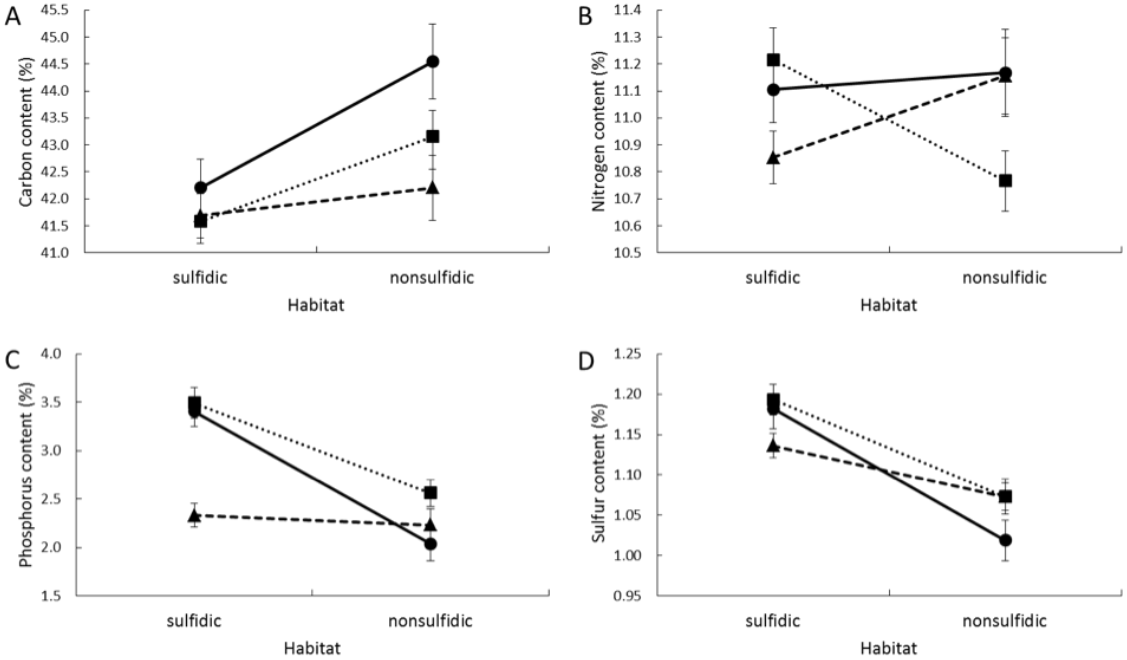
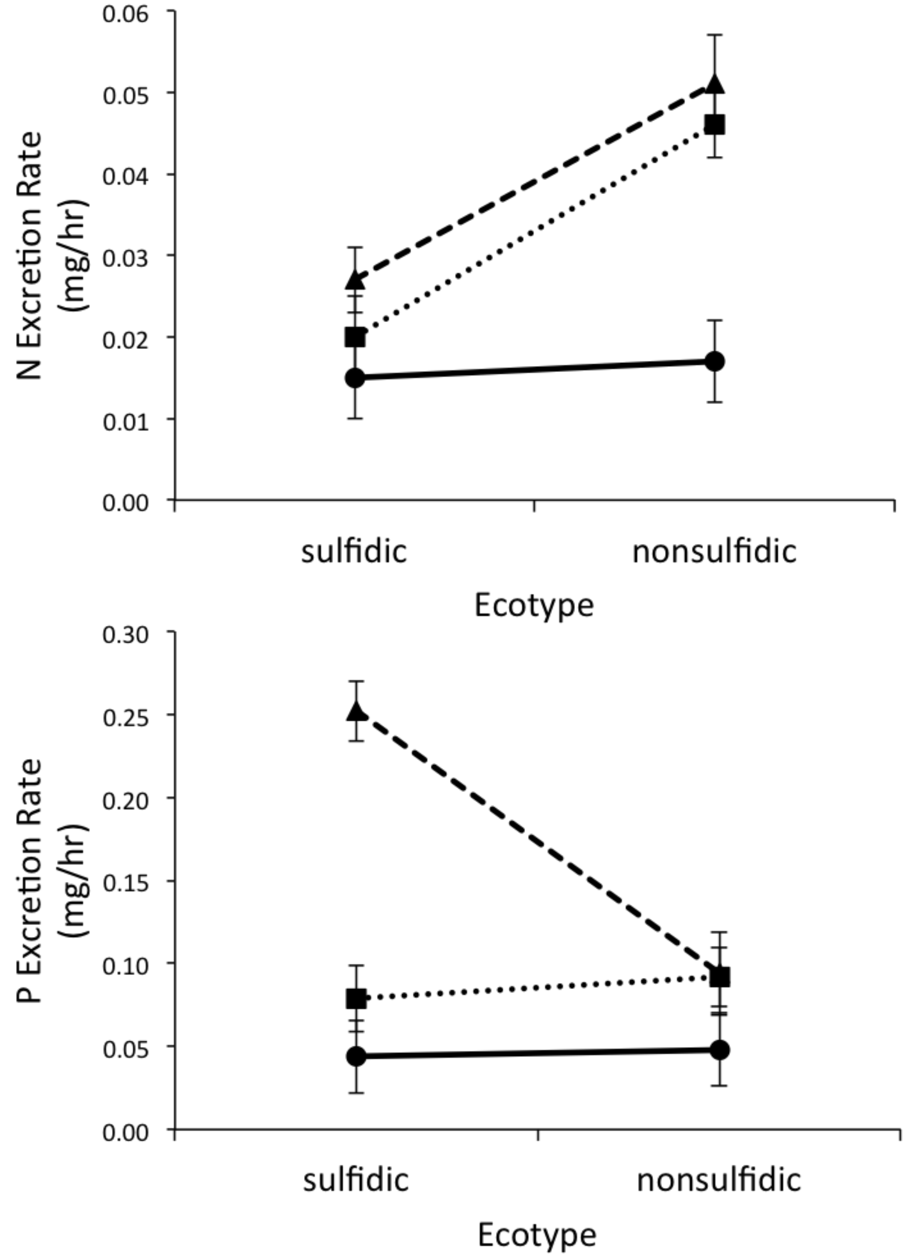


Figure 4. Estimated marginal means ± SE of A. NH_3 and B. soluble reactive phosphorus excretion of sulfidic and nonsulfidic population pairs from the Tacotalpa (circles), Puyacatengo (squares), and Pichucalco (triangles) river drainages. Measurements were taken during 2012 field season.



SUPPORTING INFORMATION

USING REPLICATED EVOLUTION IN EXTREMOPHILE FISH TO UNDERSTAND DIVERSIFICATION IN ELEMENTAL COMPOSITION AND NUTRIENT EXCRETION

Danielle M. Alba, Lenin Arias-Rodríguez, Punidan D. Jeyasingh, and Michael Tobler

Table S1. Male and female sample sizes collected from sulfidic and nonsulfidic populations of the Grijalva system with site locations. N in parenthesis represents sample size of the subset used for both somatic content and excretion trials.

	Sulfide	**GPS Coordinates**	**N_{male}**	**N_{female}**
Pichucalco				
Baños del Azufre	+	17.552, -92.999	11(4)	25(12)
Vet Station	-	17.557, 93.008	5(2)	27(14)
Puyacatengo				
La Lluvia	+	17.464, -92.895	9(4)	26(12)
Vincente Guerrero	-	17.509, 92.915	11(4)	21(12)
Tacotalpa				
El Azufre I	+	17.442, 92.751	6(2)	26(14)
Arroyo Bonita	-	17.427, -92.775	3(2)	29(14)

Table S2. Results of the principal component analysis on somatic elemental composition (carbon, nitrogen, phosphorus and sulfur) of sulfidic and nonsulfidic *P. mexicana* collected in three replicated drainages during 2012. Provided are component loadings, eigenvalues, and percent variance explained for both axes.

	PC axis 1	**PC axis 2**
C	-0.444	0.688
N	0.503	0.728
P	0.675	-0.318
S	0.829	0.195
Eigenvalues	1.751	1.188
% variance explained	40.620	27.568

Table S3. Results of the analyses of covariance (ANCOVA) on A. carbon, B. nitrogen, C. phosphorus, and D. sulfur content of somatic tissue of wild caught sulfidic and nonsulfidic *P. mexicana* caught from three replicated drainages during 2012. Elemental contents were logit transformed prior to analysis. Significant effects are in bold.

Effect	*F*	*df*	*P*	η_p^2
A. Carbon				
Intercept	**6.170**	**1**	**< 0.001**	**0.973**
Dry mass	0.568	1	0.452	0.003
Drainage	**3.429**	**2**	**0.035**	**0.037**
Ecotype	**11.529**	**1**	**0.001**	**0.060**
Sex	0.071	1	0.790	< 0.001
Drainage × Ecotype	1.027	2	0.360	0.011
Drainage × Sex	0.610	2	0.545	0.007
Ecotype × Sex	**4.453**	**1**	**0.036**	**0.024**
Drainage × Ecotype × Sex	**3.132**	**2**	**0.046**	**0.033**
B. Nitrogen				
Intercept	**9918.321**	**1**	**< 0.001**	**0.982**
Dry mass	**10.472**	**1**	**0.001**	**0.054**
Drainage	0.647	2	0.525	0.007
Ecotype	0.056	1	0.814	< 0.001
Sex	3.173	1	0.077	0.017
Drainage × Ecotype	**10.881**	**2**	**< 0.001**	**0.106**
Drainage × Sex	1.417	2	0.245	0.015
Ecotype × Sex	**4.846**	**1**	**0.029**	**0.026**
C. Phosphorus				
Intercept	**163.447**	**1**	**< 0.001**	**0.502**
Dry mass	0.588	1	0.444	0.004
Drainage	**8.672**	**2**	**< 0.001**	**0.097**
Ecotype	**4.246**	**1**	**0.041**	**0.026**
Sex	0.585	1	0.446	0.004
Drainage × Ecotype	**7.847**	**2**	**0.001**	**0.088**
Drainage × Sex	0.593	2	0.554	0.007
Ecotype × Sex	0.036	1	0.850	< 0.001
D. Sulfur				
Intercept	**6174.920**	**1**	**< 0.001**	**0.971**
Dry mass	**6.925**	**1**	**0.009**	**0.036**
Drainage	2.521	2	0.083	0.027
Ecotype	**45.553**	**1**	**< 0.001**	**0.199**
Sex	2.591	1	0.109	0.014
Drainage × Ecotype	**9.237**	**2**	**< 0.001**	**0.092**
Drainage × Sex	0.565	2	0.569	0.006
Ecotype × Sex	0.027	1	0.869	< 0.001

Table S4. Estimated marginal means of carbon, nitrogen, phosphorus and sulfur content and NH_3 and soluble reactive phosphorus excretion rates of sulfidic and nonsulfidic *P. mexicana* across drainages of the Grijalva river system. Data collected from 2012 samples.

Element	**Sulfide**	**Drainage**	**Content (%)**	**$SE_{content}$**	**Excretion (mg/hr)**	**$SE_{excretion}$**
C	-	Tacotalpa	44.542	0.691		
		Puyacatengo	43.154	0.481		
		Pichucalco	42.208	0.602		
	+	Tacotalpa	42.203	0.527		
		Puyacatengo	41.584	0.513		
		Pichucalco	41.686	0.415		
N	-	Tacotalpa	11.167	0.161	0.017	0.005
		Puyacatengo	10.766	0.112	0.046	0.004
		Pichucalco	11.156	0.141	0.051	0.006
	+	Tacotalpa	11.105	0.123	0.015	0.005
		Puyacatengo	11.215	0.120	0.020	0.005
		Pichucalco	10.853	0.097	0.027	0.004
P	-	Tacotalpa	2.04	0.173	0.048	0.022
		Puyacatengo	2.563	0.137	0.092	0.018
		Pichucalco	2.231	0.168	0.094	0.025
	+	Tacotalpa	3.403	0.152	0.044	0.022
		Puyacatengo	3.493	0.152	0.079	0.020
		Pichucalco	2.331	0.124	0.252	0.018
S	-	Tacotalpa	1.019	0.025		
		Puyacatengo	1.073	0.017		
		Pichucalco	1.073	0.022		
	+	Tacotalpa	1.182	0.019		
		Puyacatengo	1.193	0.019		
		Pichucalco	1.136	0.015		

Table S5. Between-subjects effects table of MANCOVA analysis on plasticity in somatic stoichiometry (C, N, P and S content) of *P. mexicana* collected from ecotypes of the Tacotalpa drainage. Data were logit transformed prior to analysis. Mass is the log of the wet mass of the individual. Significant effects are in bold.

Source	**Dep. Variable**	***df***	***F***	***P***	η_p^2
Corrected Model	**C**	**15**	**15.552**	**<0.001**	**0.439**
	N	**15**	**5.837**	**<0.001**	**0.227**
	P	**15**	**3.513**	**0.002**	**0.150**
	S	**15**	**11.047**	**<0.001**	**0.357**
Intercept	**C**	**1**	**10725.353**	**<0.001**	**0.987**
	N	**1**	**13909.864**	**<0.001**	**0.990**
	P	**1**	**299.967**	**<0.001**	**0.683**
	S	**1**	**7626.508**	**<0.001**	**0.982**
Sex	C	1	<0.001	1.000	<0.001
	N	1	1.860	0.175	0.013
	P	1	0.258	0.612	0.002
	S	1	0.864	0.354	0.006
Wild/Lab	**C**	**1**	**87.991**	**<0.001**	**0.388**
	N	**1**	**26.964**	**<0.001**	**0.162**
	P	1	2.501	0.116	0.018
	S	**1**	**5.021**	**0.027**	**0.035**
Sulfidic/NS	**C**	**1**	**6.134**	**0.014**	**0.042**
	N	**1**	**6.074**	**0.015**	**0.042**
	P	**1**	**4.850**	**0.029**	**0.034**
	S	**1**	**45.143**	**<0.001**	**0.245**
Wet Mass	C	1	0.323	0.571	0.002
	N	1	0.923	0.338	0.007
	P	1	0.793	0.375	0.006
	S	1	0.106	0.745	0.001
Sex x Sulfidic/NS	C	1	0.014	0.907	0.000
	N	1	1.711	0.193	0.012
	P	1	1.868	0.174	0.013
	S	1	2.730	0.101	0.019
Sex x Wild/Lab	C	1	0.082	0.776	0.001
	N	1	1.486	0.225	0.011
	P	1	0.164	0.686	0.001
	S	1	3.607	0.060	0.025
Wild/Lab x Sulfidic/NS	C	1	0.014	0.905	0.000
	N	1	0.126	0.723	0.001
	P	1	2.016	0.158	0.014
	S	**1**	**9.380**	**0.003**	**0.063**

Table S6. Results of discriminant function analysis (DFA). Cross validation (i.e., assignment of common garden raised individuals to ancestral population based on carbon, nitrogen, phosphorus and sulfur contents of somatic tissues) correctly assigned 84.6% of laboratory reared individuals.

	Function 1
Residual C	-0.131
Residual N	-0.512
Residual P	0.516
Residual S	0.878
Eigenvalue	0.490
% of variance	100.0
Cumulative %	100.0
Canonical Correlation	0.573
Wilks' lambda	0.671
X^2	47.452
Df	4
P	< 0.001

Figure S1. Estimated marginal means ± SE generated from a MANCOVA of somatic stoichiometry (C, N, P, and S content) of laboratory raised common garden *P. mexicana* and wild caught individuals of the populations of the Tacotalpa. Wild caught *P. mexicana* include individuals caught over three years (2011-2013). Gray bars represent wild-caught *P. mexicana* and white, common garden reared.

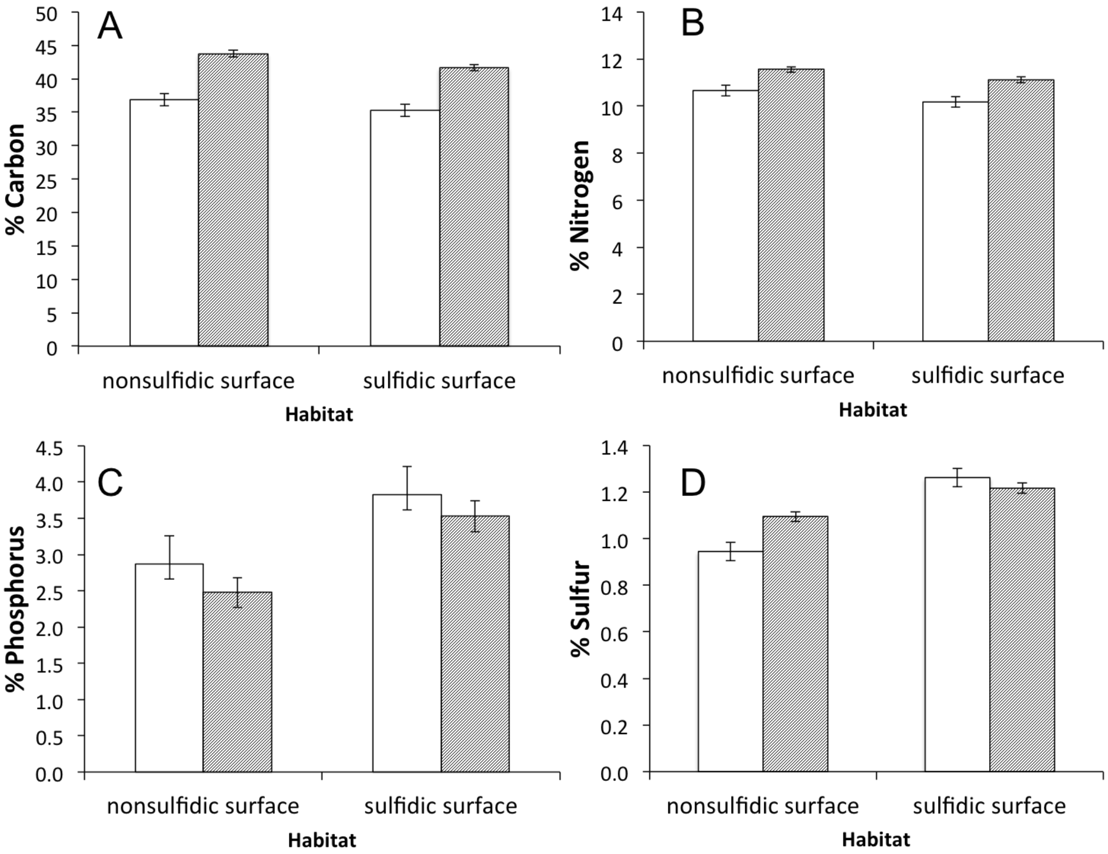
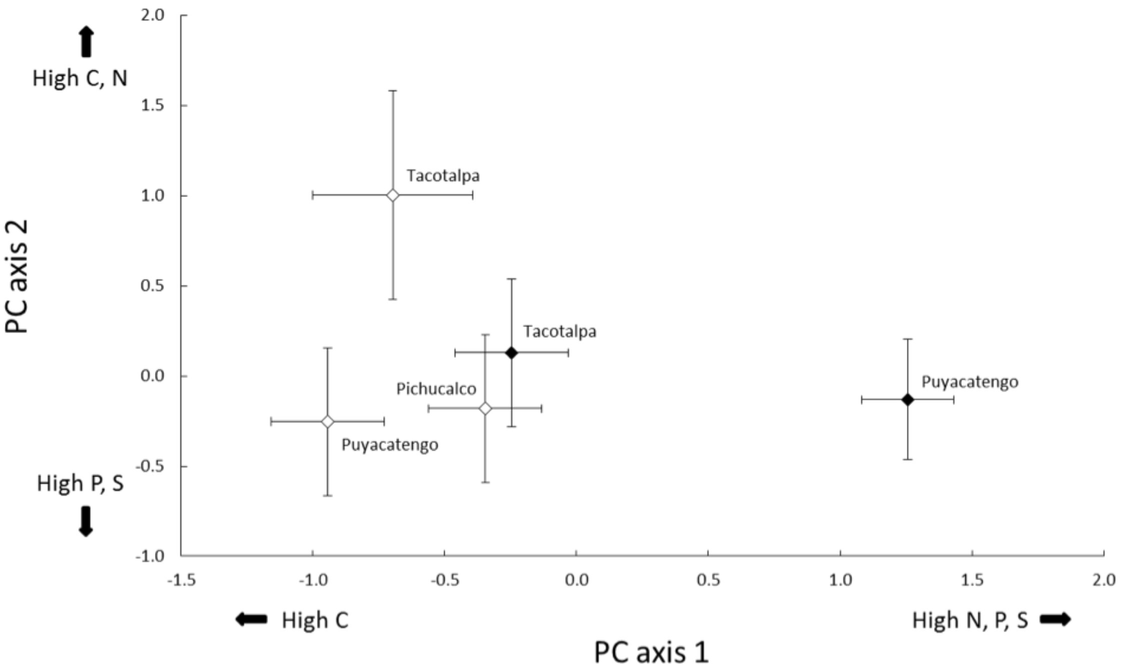


Figure S2. Principal component analysis of the percent elemental content of C, N, P and S of primary producer materials composing the diet of sulfidic (black points) and nonsulfidic (white points) *P. mexicana* ecotypes in their relative habitats. Samples of periphyton and detritus were collected from streams and analyzed for C, N, P and S content with an Elementar microvario cube and modified sulfuric acid digestion. Values were *z*-transformed prior to principal component analysis. Samples of the sulfidic Pichucalco drainage were not available for analysis.



CHAPTER TWO

EFFECTS OF HIGH DIETARY SULFUR CONTENT ON THE SOMATIC STOICHIOMETRY, LIFE HISTORY, AND ECOLOGICAL FUNCTION OF AN EXTREMOPHILE FISH

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ABSTRACT

The field of Ecological Stoichiometry offers integration of ecological and evolutionary processes through the quantification of elemental phenotypes and elemental interactions between organisms. Recently, research has documented variation in elemental phenotype within species, but the drivers of this variation are yet unknown. In this study, we used divergent populations of *Poecilia mexicana* locally adapted to distinct conditions of sulfur (S) availability to test whether dietary S availability or stoichiometric homeostasis accounts for variation in S somatic stoichiometry. We used the amino acids leucine and methionine to manipulate the C:S of two high S diets fed to two ecotypes of *P. mexicana* in the laboratory. We also tested whether dietary treatment and/or evolutionary divergence have altered sulfate recycling rates in these populations. We found strong differences in ecotypes in their somatic S and C composition; however, trends in S content contradict previous findings in wild-caught individuals. Interestingly, there was no effect of dietary S content on somatic stoichiometry or sulfate excretion rates. We think testing both S limitation and S excess may lead to better explanation of the variation in elemental phenotype and nutrient recycling of these diverging populations.

INTRODUCTION

Our understanding of the ecological consequences of evolutionary change has been greatly improved through the study of ecological stoichiometry. Ecological stoichiometry uses the law of the conservation of mass to quantify and predict the nutritional interactions between organisms (Sterner & Elser, 2002). The field abstracts these organisms by their composite elemental ratios and uses their rates of acquisition, assimilation and release of elemental resources to model the movement of mass through ecosystems. This has allowed for a predictive perspective on ecology. Recent work has also highlighted the potential of ecological stoichiometry in understanding evolutionary processes (Elser, 2006; Jeyasingh, Cothran & Tobler, 2014). Stoichiometric parameters (e.g., organismal elemental composition) can quantify summed phenotypic change. For example, directional selection favoring exaggerated traits should be reflected in organismal stoichiometry, as the expression of any trait requires atomic resources (Sterner & Elser, 2002). In combination, the use of ecological stoichiometry in quantifying nutritional interactions and phenotypic variation creates a powerful tool to understanding eco-evolutionary dynamics (Palkovacs, Wasserman & Kinnison, 2011; Bassar *et al.*, 2010; Harmon *et al.*, 2009).

Recent research has found substantial variation in stoichiometric traits. Stoichiometric phenotype varies across trophic levels, species, populations and even sexes (Goos *et al.*, 2014; Karimi & Folt, 2006; Liess *et al.*, 2013). Interspecific variation in organismal stoichiometry has long been assumed as part of the paradigm of the field, but evidence for intraspecific variation has furthered our understanding of ecological processes. What is less clear is how this intraspecific variation arises. Consumers in particular are assumed to have homeostatic elemental compositions, but there is substantial evidence that organismal stoichiometry may be more plastic than previously thought. Stoichiometric plasticity may be advantageous in situations of instability if consumers are flexible in elemental consumption (Sistla & Schimel, 2012). We expect that consumer stoichiometry may respond to conditions of nutritional availability and that plasticity in the organismal stoichiometry of a consumer affects its elemental interactions with other species.

Previous work has demonstrated intraspecific variation in somatic stoichiometry and elemental excretion of populations of the consumer, *Poecilia mexicana* that inhabit the tributaries of the Grijalva River in Tabasco, Mexico (Alba *et al.* in review). These tributaries are characterized by springs that inundate sections of the tributaries with hydrogen sulfide, leading to great spatial variation in the sulfur (S) content of dietary resources. Both the *P. mexicana* and the dietary resources of sulfidic environments have increased S content relative to nonsulfidic environments, but whether increased S of the sulfidic *P. mexicana* is homeostatically maintained (e.g., by assimilation or excretion) as a result of adaptation, or as a plastic response to differences in elemental availability remains unanswered.

In this study, we subjected sulfidic and nonsulfidic ecotypes of *P. mexicana* to variation in dietary S and addressed the following questions: (1) Do the sulfidic and nonsulfidic ecotypes maintain their differences in somatic S content observed in wild populations when S is excessive in the diet? Because the sulfidic ecotype has evolved in conditions of excessive S availability, we expect them to show greater homeostasis in S content compared to the nonsulfidic ecotype. (2) Does the excessive intake of dietary S lead to increased rates of S excretion by either ecotype? We expect that if sulfidic *P. mexicana* are adapted to high dietary S, they will regulate organismal S content through excretion of sulfate. Thus, we expect sulfidic *P. mexicana* to show marked increase in sulfate excretion between the two dietary treatments. We expect nonsulfidic *P. mexicana* will be unable to regulate S content through sulfate excretion, and sulfate excretion to not differ between the dietary treatments. (3) Does the sulfidic ecotype outperform the nonsulfidic ecotype in terms of growth under conditions of high S intake? Over consumption of S may decrease fitness in populations not adapted to such conditions. We expect the sulfidic ecotype to have higher growth rates than the nonsulfidic ecotype with high S diets.

METHODS

Sulfide spring fish as a study system

Hydrogen sulfide acts as a mitochondrial inhibitor and also creates an environment of hypoxia in water (Bagarinao, 1992). Adaptation to the toxic conditions of sulfidic habitats has led to replicated evolution of an extremophile ecotype of *P. mexicana* (Tobler *et al.*, 2011; Palacios *et al.*, 2013). Extremophile populations are reproductively isolated from their nonsulfidic conspecifics (Tobler *et al.*, 2009a; Plath *et al.*, 2010). The sulfidic ecotype has also repeatedly evolved several adaptations to extreme conditions, including larger head and gill size (Tobler *et al.*, 2011), increased aquatic surface respiration performance to increase oxygen uptake (Tobler *et al.*, 2009b), reduced fecundity and increased individual neonate mass (Riesch, Plath & Schlupp, 2010). The extreme environmental conditions in sulfide springs have been documented to reduce photosynthetic primary production (Bagarinao, 1992) and alter community composition in comparison to nonsulfidic springs inhabited by the nonsulfidic ecotype; while both ecotypes feed on available periphyton and detritus (Tobler, 2008), the periphyton of the sulfidic habitat is rich in sulfide-reducing bacterial mats which are an important dietary resource to the sulfidic ecotype (Roach, Tobler & Winemiller, 2011).

The specimens used in this study were from laboratory stock tanks of *P. mexicana* of one sulfidic and one nonsulfidic population of the Tacotalpa tributary of the Grijalva system, which have been bred in captivity for approximately 2 - 4 generations. Specifically, the ancestors of the experimental fish were collected in the Arroyo Bonita (nonsulfidic site) and Arroyo El Azufre (sulfidic site) near or within the Villa Luz Eco-Park (Tapijulapa, Tabasco). Both of these streams directly flow into the Tacotalpa River.

The stock tanks consisted of one 40-gallon tank of *P. mexicana* of the Bonita population and two 40-gallon tanks of *P. mexicana* of the El Azufre population, with males and females housed together. Laboratory stock tanks were maintained in nonsulfidic water. Stock tank fish are kept on a diet of commercial fish flakes fed *ad libitum* twice daily and received weekly 20-gallon water changes. The daily light cycle was set to 12 hours light, 12 hours dark and the room was kept at ~27° C..

Diet Manipulation

The two experimental C:S diets of this study were made of a mixture of amino acids and aquatic gel diet for omnivorous fish (Mazuri, Richmond, IN, USA). Metazoans cannot directly assimilate inorganic S and thus must acquire it through ingested amino acids

(Nimni, Han & Cordoba, 2007). The diets were made with a mixture of 20% percent amino acid and 80% percent gel diet by mass. The high S treatment contained only gel food and methionine – an S-containing amino acid – and exhibited a dietary C:S ratio of 20.50 ± 1.12. The lower S diet was a mixture of gel, methionine, and leucine. The latter is similar in structure to methionine, but lacks S, resulting in a dietary C:S ratio of 54.59 ± 2.58. In comparison, the natural dietary C:S of the sulfidic and nonsulfidic ecotypes are 85.60 ± 29.34 and 414.13 ± 43.43, respectively (Alba *et al.,* in review); thus, both diets are relatively high in S compared to wild diets. S content of both experimental diets were verified using an Elementar Microvario cube (Mount Laurel, NJ, USA). The diets were frozen during the duration of the study and thawed just before feeding. To standardize availability, the diets were pressed into 96 well plates to create gel pellets (average mass 67.8 ± 6.9 mg dry mass).

Experimental Set-up

Neonate fry were collected from laboratory stock tanks of *P. mexicana* and placed in groups of two to four fry per experimental tank (10 gallon). Restrictions on space and available tanks prevented individual housing of experimental fish, and *P. mexicana* tend to not thrive in solitary conditions (*M. Tobler, personal observation*). Placement of fry and assignment of treatment was randomized in experimental tanks, which were filled with nonsulfidic water. The fish were fed one food pellet per day or a period of six hours. Any remaining gel after the six-hour period was removed.

After forty days of feeding neonates on assigned dietary treatments, they were blotted dry with paper towel, and measured for mass (0.01 g accuracy) and standard length, and sexed. Initial measurements started on day forty because of the fragility of neonate fry to handling stress. After measurement (approximately 3 minutes), the fish were returned to their experimental tanks. Data on sex, standard length, and mass were taken again every ten days through day ninety of the experiment. In addition to these measurements of growth, sulfate excretion was measured on days sixty and ninety. Due to grouping of fish within experimental tanks, they were not individually identified. Any fish that died over the experimental period were removed from the tank.

Excretion Measurements

Excretion trials began between one and four hours after feeding. To measure sulfate excretion rates, an individual fish was placed in a glass jar (approximately 200 ml in size) with 50 ml of dechlorinated, non-sulfidic water for a period of thirty minutes (McIntyre *et al.*, 2008). After the thirty-minute period, the fish was removed from the water, and measured for mass and standard length, and sexed as described above before being placed back into its experimental tanks. The water was frozen until it could be thawed and filtered prior to water nutrient analysis (McIntyre *et al.*, 2008). The water was filtered in a two-step process: first, using a course mesh to remove large particles (e.g., feces), and second, with a fine filter to remove small particles and bacteria from the sample. Sulfate content was measured using a Hach DR2800 Spectrophotometer and sulfate TNT plus low range vials per manufacturer instructions (Loveland, CO, USA).

Organismal Stoichiometry

After day 90 of excretion trials and growth measurements, the fish were euthanized via cervical dislocation (Nickum *et al.*, 2004), dissected to remove the intestinal tract, gonads and liver (Alba *et al.,* in review), and prepared for elemental analysis of body stoichiometry. The intestinal tract was removed to prevent any unassimilated dietary materials from affecting body stoichiometry measurements. Intestinal length (mm) was measured prior to preparation for separate analysis. The gonads (particularly embryotic sacs) of *P. mexicana* vary greatly in size over the reproductive cycle, potentially causing great variation in somatic stoichiometry, as they are rich in carbon-based lipids. Thus, we removed them prior to elemental analysis and address only somatic stoichiometry in these fish (Alba *et al.,* in review).

The somatic tissues were desiccated in a driving oven (Thermo Scientific, Langenselbold, Germany) at 60° C for a minimum of 48 hours, measured for dry mass, and then pulverized with motar and pestle, and dried again for another 48 hours minimum. Subsamples of the homogenized powder were measured to 0.0001mg accuracy with a microbalance (XP2U, Mettler Toledo, USA). These subsamples were then analyzed for C and S content (%) with an Elementar Microvario cube per manufacturer's instructions (Elementar Americas, Inc., Mt. Laurel, New Jersey).

Statistical Analysis

The effects of ecotype (i.e., sulfidic or nonsulfidic population) and dietary C:S on the S and C contents of *P. mexicana* were analyzed using an ANCOVA. The percentages of S and C by mass were logit transformed prior to analysis to correct for elemental content percentages only spanning from 0 to 100, which violates statistical assumptions. Population and treatment were included as fixed factors, and the $\log_{10}$ of the dry mass of the fish was included as a covariate.

The effects of ecotype and dietary treatment on sulfate excretion on days 60 and 90 were analyzed using two ANCOVAS with dietary treatment and ecotype as fixed factors. The $\log_{10}$ of mass — dry mass for day 90 data, wet mass for day 60 data — was included as a covariate, as excretion rate is expected to change with animal size. We included somatic C:S as a covariate in the analysis of day 90 excretion to test for a relationship between body stoichiometry and sulfate excretion. Somatic C:S was not available for day 60, and so was not included as a covariate.

We then used two ANCOVAs to determine the relationship between ecotype and dietary S content on the response variables of dry mass corrected intestinal length (mm/g) and percent mass gained over the study period. Percent mass gained was calculated as follows,

$$\%\, mass\ gained = [(Wet\ mass_{day\ 90} - Wet\ mass_{day\ 40}) \div Wet\ mass_{day\ 40}] \times 100.$$

Mass gained was logit transformed prior to analysis. Ecotype and dietary treatment were included as fixed factors and the $\log_{10}$ of the dry mass of the fish as a covariate in both models.

All analyses were done in SPSS 20 (IBM), reducing model complexity by removing non-significant ($P \geq 0.05$) interaction terms.

RESULTS

We found that ecotype had a major effect on both somatic sulfur and carbon content (P = 0.001 and < 0.001, respectively), regardless of diet (Table 1). Contrary to previous work

(Alba *et al*., in review), the sulfidic ecotype contained less S than the nonsulfidic ecotype (Fig. 1). The mass of the fish also contributed to trends in somatic stoichiometry, with larger fish generally having less S by mass than smaller fish ($P < 0.001$).

We found no effect of dietary C:S or ecotype on either the day 90 or day 60 sulfate excretion rates (Table 2, Fig. 2). Similarly, neither somatic C:S nor mass of the fish had a relationship with sulfate excretion on either trial day.

ANCOVAs on the effects of dietary C:S and ecotype on intestinal length and growth (% mass gained from days 40 to 90 of the experiment) show no difference between dietary treatments on either trait (Table 3). There was no difference between ecotypes in their growth over the study period. Nevertheless, there was a marginally significant ($P = 0.052$) difference in intestinal length between the ecotypes, with the sulfidic ecotype having shorter intestines (Fig. 3).

DISCUSSION

Our results on somatic S content between the *P. mexicana* ecotypes demonstrate clear differences between these diverging lineages in the regulation of ingested S; however, a lack of pattern in sulfate excretion makes inferences into the mechanism of this regulation difficult. When considering consumers as important regulators elements in ecosystems, the evolutionary history of a population may be an important consideration in making predictions on their elemental phenotype (Jeyasingh, Cothran & Tobler, 2014; Elser, 2006). The high S content of the nonsulfidic ecotype on diets with excessive S suggests that they are unable to cope with high S diets in comparison to the sulfidic ecotype. It is also possible that they demonstrate adaptive plasticity in storing S, when it is available in excess. In the wild, the sulfidic and nonsulfidic *P. mexicana* of the Tacotalpa have somatic S contents near 1.182% and 1.019%, respectively (Alba *et al.,* in review). In comparison to the results of this study, the sulfidic ecotype maintained S contents more similar to their wild counter parts than the nonsulfidic ecotype. Whether this is because sulfidic ecotypes are generally more homeostatic than nonsulfidic ecotypes, or because the diets of the wild sulfidic ecotype are more S rich are both possible explanations from our previous understanding of plasticity in these populations (Alba *et al.,* in review). To our knowledge, this work is the only study of phenotypic differences in S stoichiometry in consumers exposed to excessive S resources. Thus, the plausibility of these hypotheses requires future testing.

Both the diets used in this study were designed to be in excess of S in comparison to the S demand of either ecotype. Our lack of significant differences between treatments could be due to dietary threshold where excessive S is no longer assimilated into the body (Fron *et al.*, 1990), and thus is released in fecal matter, which we did not test in this study. Our lack of differences in sulfate excretion rates would not support homeostasis being maintained solely by excretion alone, at least not for these dietary C:S treatments. The two treatments included in this work may have been too similar in C:S to tease apart any differences in sulfate excretion. We had originally included a third dietary treatment with less S than either treatment presented in this study, which was more similar to expected S demands by the fish (C:S 111). Unfortunately, unexpectedly low production of neonates during the study period severely limited our replication and we did not include these data in the analysis.

The majority of studies on the effects of S availability on organisms investigate effects of excess or limitation of S on crops and domesticated livestock; limited sulfur resources tends to reduce crop vigor and result in neurological and developmental defects in animals (Hawkesford, 2007). Future studies comparing similar responses of sulfidic and nonsulfidic *P. mexicana* to S limited diets may further explain how evolution to extreme environments has altered the nutritional requirements of the derived sulfidic ecotype (Soloway, 2006). In the process of adapting to their novel environment, sulfidic *P. mexicana* may have become dependent on high S levels in the diet. Limiting S availability to the two ecotypes may demonstrate fitness differences if nonsulfidic ecotypes are better adapted to lower S diets — differences our dietary treatments were unable to identify.

Nevertheless, our finding that nonsulfidic *P. mexicana* of the Tacotalpa increased in S content, surpassing the sulfidic ecotype which has higher S content both in the wild and in previous common garden studies, leads to questions on how evolutionary divergence may alter the elemental homeostasis of organisms, even within species. Elemental homeostasis is typically described at the level of an ecological functional group with higher trophic levels assumed to be more homeostatic (Sterner & Elser, 2002). These data in combination with previous data on stoichiometric regulation in *P. mexicana* revealed considerable variation in stoichiometric homeostasis within species. Comparing these findings with those from diets of broader differences in dietary stoichiometry may lead to further elucidation of how species adapt to novel conditions of dietary resource availability, an important consideration given widespread anthropogenic nutrient loading and global climate change.

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Table 1. Results of the ANCOVA on somatic S and C contents of sulfidic and nonsulfidic ecotypes of *P. mexicana* on dietary treatments of C:S 55 and C:S 20. Sulfur and carbon contents (% by mass) were logit transformed prior to analysis. The $\log_{10}$ of dry mass of the fish was included as a covariate. Significant terms are in bold. $n_{total} = 56$ (29 high S treatment, 27 low S treatment, 18 nonsulfidic, 38 sulfidic)

Source	***F***	***df***	***P***	η_p^2
A. Somatic S content				
Corrected Model	**7.184**	**3**	**0.001**	**0.368**
Intercept	**418.074**	**1**	**< 0.001**	**0.919**
Diet C:S	0.035	1	0.853	0.001
Ecotype	**12.037**	**1**	**0.001**	**0.245**
Mass	**17.623**	**1**	**< 0.001**	**0.323**
Error		37		
Total		41		
Corrected Total		40		
B. Somatic C content				
Corrected Model	**6.111**	**3**	**< 0.001**	**0.268**
Intercept	0.003	1	0.954	0.000
Diet C:S	1.700	1	0.198	0.033
Ecotype	**15.987**	**1**	**< 0.001**	**0.242**
Mass	0.035	1	0.853	0.001
Error		50		
Total	54			
Corrected Total	53			

Table 2. ANCOVA results on sulfate excretion rates of A. day 90 and B. day 60 excretion trials of sulfidic and nonsulfidic *P. mexicana* on diets of C:S 20 and C:S 55. Fish somatic C:S and $\log_{10}$ mass were included as covariates in the analysis. Significant effects are in bold. Reduction of sample size due to mis-reading by Hach.

A. Day 90 sulfate excretion, $n_{total} = 38$

Source	***F***	***df***	***P***	**η_p^2**
Corrected Model	0.716	4	0.590	0.115
Intercept	3.647	1	0.069	0.142
Dietary C:S	0.539	1	0.471	0.024
Ecotype	0.083	1	0.776	0.004
Mass	1.024	1	0.323	0.044
Somatic C:S	0.324	1	0.575	0.015
Error		22		
Total		27		
Corrected Total		26		

B. Day 60 sulfate excretion

Source	*F*	*df*	*P*	**η_p^2**
Corrected Model	1.589	3	0.210	0.123
Intercept	**59.455**	**1**	**<0.001**	**0.636**
Dietary C:S	1.636	1	0.210	0.046
Ecotype	0.384	1	0.539	0.011
Mass	0.086	1	0.772	0.003
Error		34		
Total		38		
Corrected Total		37		

Table 3. ANCOVAs of life history traits of sulfidic and nonsulfidic ecotypes of *P. mexicana* from the Tacotalpa on two dietary C:S treatments. A. Dry mass corrected intestinal length. B. Logit transformed percent growth from days 40 to 90 of experiment. Growth was measured with fish blotted dry (i.e., wet weight). Significant terms in bold. $n_{total} = 56$

A. Intestinal Length

Source	***F***	***df***	***P***	η_p^2
Corrected Model	0.799	2	0.455	0.032
Intercept	**340.818**	**1**	**< 0.001**	**0.874**
Diet C:S	1.179	1	0.283	0.024
Ecotype	0.929	1	0.340	0.019
Error		49		
Total		52		
Corrected Total		51		
B. % Growth				
Corrected Model	1.246	3	0.438	0.087
Intercept	**0.537**	**1**	**< 0.001**	**0.014**
Diet C:S	0.236	1	0.971	0.006
Ecotype	0.571	1	0.231	0.014
Error		40		
Total		43		
Corrected Total		42		

Figure 1. Estimated marginal means ± SE of the somatic sulfur (above) and carbon content (below) of the sulfidic (shaded) and nonsulfidic (un-shaded) ecotypes of *P. mexicana* of the Tacotalpa raised in laboratory conditions on diets of C:S 20 and C:S 55. Log_{10} of dry fish mass was included as a covariate.

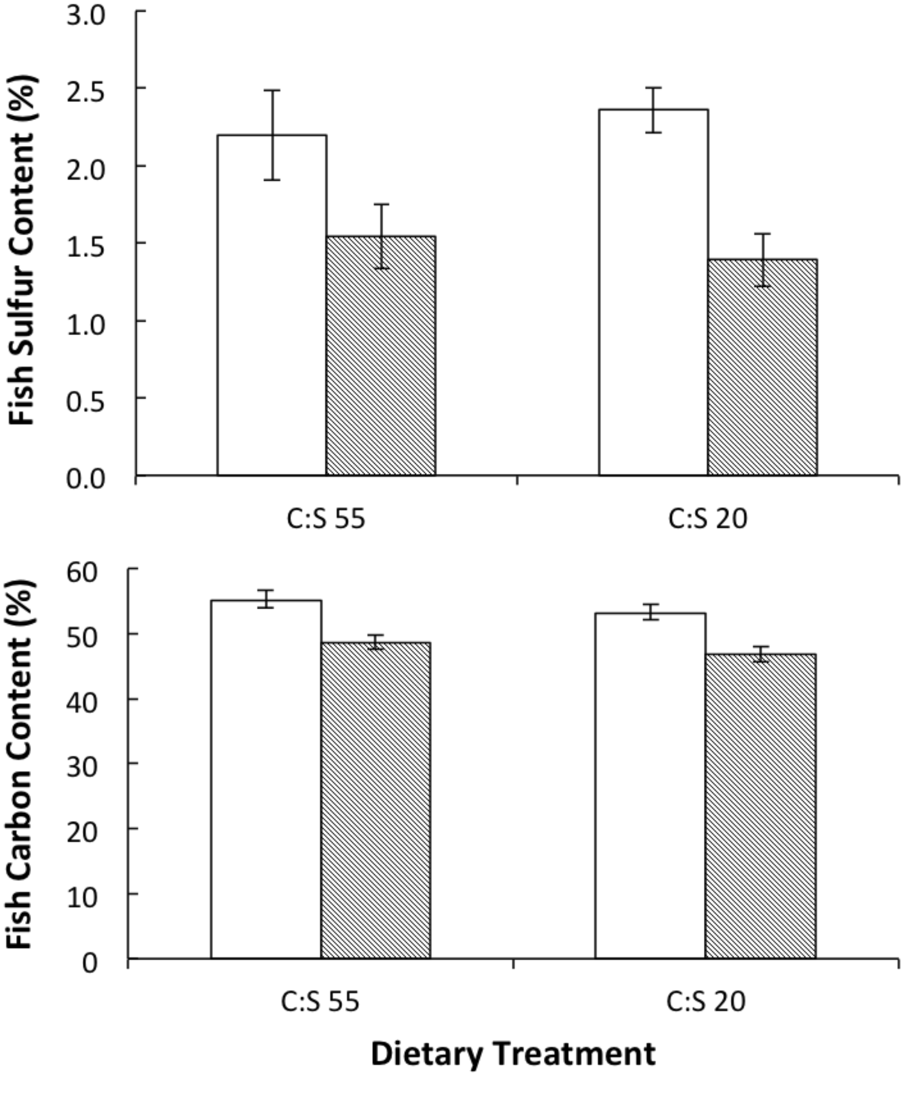


Figure 2. Estimated marginal means ± SE of mass corrected sulfate excretion by sulfidic (shaded) and nonsulfidic (un-shaded) ecotypes of *P. mexicana* on two treatments of dietary C:S. Top: excretion after 90 days on dietary treatments. Bottom: excretion after 60 days on dietary treatments. Difference in scale of excretion is due to wet mass being used for day 60 and dry mass for day 90.

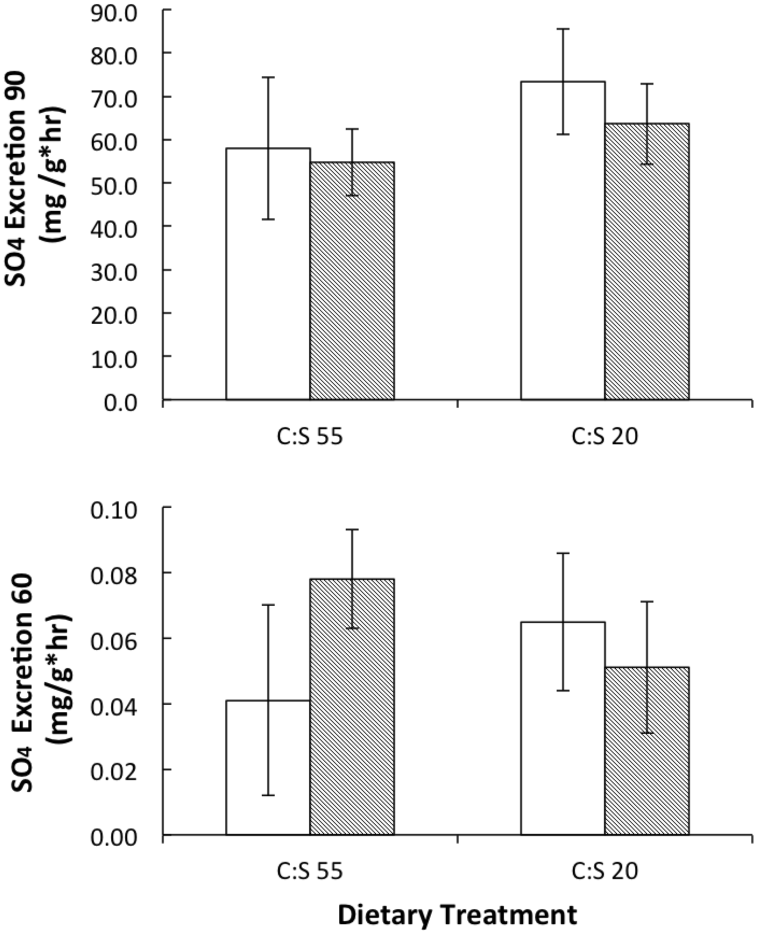
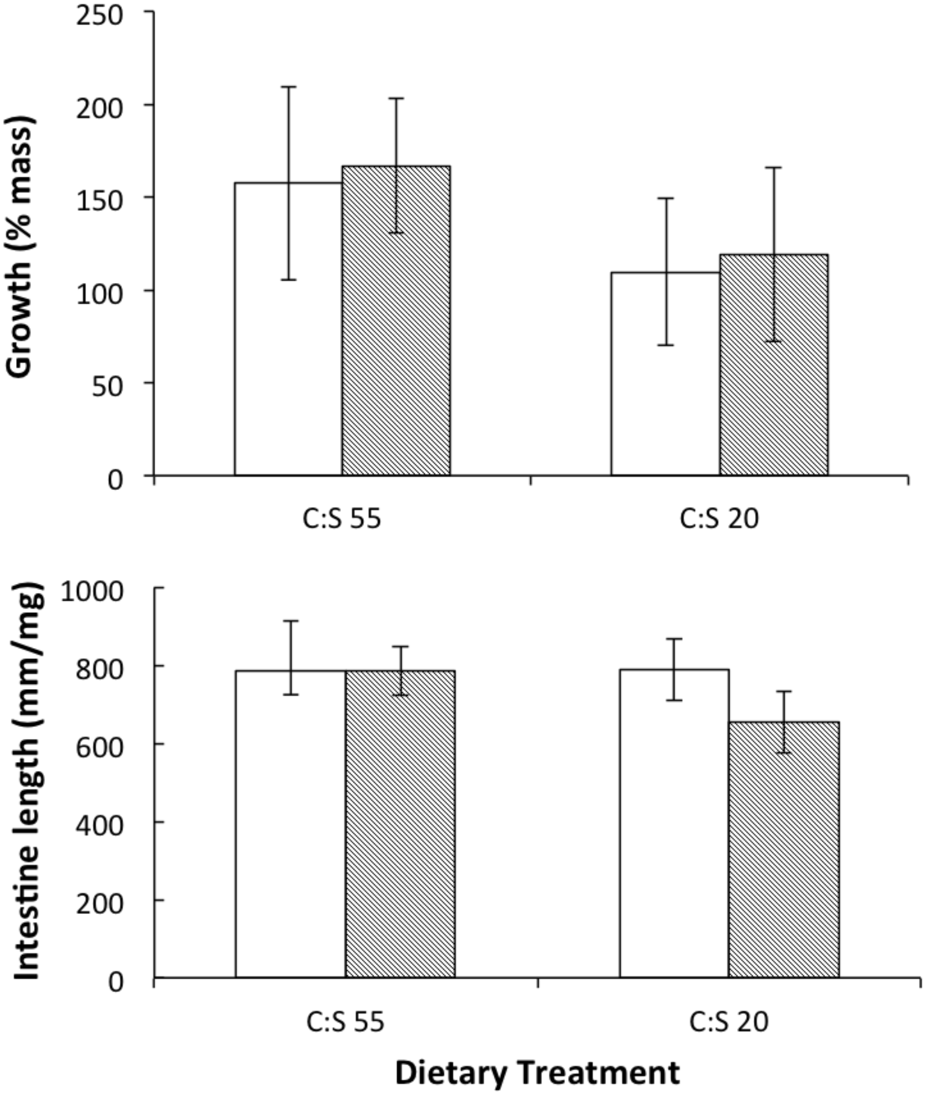


Figure 3. Estimated marginal means ± SE of life history traits of sulfidic (shaded) and nonsulfidic (un-shaded) ecotypes of *P. mexicana* from the Tacotalpa drainage on two C:S diets. Top, percent growth from days 40 to 90 of experiment. Bottom, dry mass corrected intestine length.



VITA

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