How the Evolution of Bony Traits Influences Resource Interactions in Threespine Stickleback

by

Daniel Durston
B.Sc, Lakehead University, 2014
BES, University of Waterloo, 2008

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of the Requirements for the Degree of

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Abstract

Evolution shapes ecosystems but the processes by which this occurs are not well understood. Adaptive change in resource expensive traits may underlie one such process, as evolution altering a species' resource needs may effect how that species interacts with ecosystem resources. For this, Ecological Stoichiometry (ES) may be a tractable framework, as it simplifies organisms into elemental ratios and then applies mass-balance to predict changes in diet and waste interactions. ES detects variation in resource expensive traits as variation in elemental ratios, and predicts compensation via parallel changes in diet (e.g. high phosphorous individuals consume high phosphorus diets) and/or offsetting changes in waste (e.g. high phosphorous individuals release low phosphorus waste). To test the utility of this framework and improve our understanding of eco-evolutionary dynamics, I studied variation in phenotypic traits, genetics, elemental content and resource interactions within and across natural populations of highly regarded eco-evolutionary model species threespine stickleback (*Gasterosteus aculeatus*). First, I related heritable variation in phosphorus rich bony traits and genetics commonly under natural selection with variation in elemental content (N:P) to determine the magnitude and basis of intraspecific variation in N:P. Second, I investigated the ecosystem consequences of variation in elemental content by determining whether stickleback compensate through changes in diet choice and excretion rates. I found stickleback vary widely in elemental composition (3.0 – 9.4:1 N:P) which models explained well with four bone related traits: bone mineralization, body size, lateral plating and pelvis size ($R^2 > 0.52$). Additional genetic models linked variation in *Eda* alleles (which underlie lateral plating) with a 12% shift in stickleback N:P. Stickleback compensated for this variation in N:P demand by altering diet choice rather than excretion rates, and by maximizing dietary inputs through changes in gut morphology. Within and across populations, high phosphorus stickleback consumed a larger proportion of high phosphorus prey and contained longer gastrointestinal tracts that more efficiency process diet resources. These results demonstrate that heritable variation in elemental composition is ecologically relevant with individual traits and genetics having large effects. As individuals compensated by altering resource acquisition rather than release, the direct ecological consequences of evolutionary change in these resource expensive traits is likely larger for food web structure and abundance than nutrient dynamics.

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Introduction

For much of the past century evolutionary biologists have focused their study on the ecological drivers of evolutionary change, rather than on how evolution affects ecology (Schoener 2011). This focus arose from the view that evolution is generally slow and thus any effects on ecology (evo-eco interactions) are also likely slow and of small effect (Schoener 2011). However numerous examples of meaningful evolutionary change over short periods have challenged this assumption (Reznick and Ghalambor 2001). The growing awareness of the potential for rapid evolution has led to the realization of a need for additional study into evo-eco interactions and raised the possibility of eco-evolutionary feedbacks, whereby evo-eco interactions shape subsequent evolution (Schoener 2011).

Recent work has provided numerous examples of evo-eco interactions, but our understanding of how these effects occur is still limited (Schoener 2011, Matthews et al. 2011, Jeyasingh et al. 2014). Many studies have linked genetic variation and evolved phenotypic differences with important changes in ecological properties, such as ecosystem structure, function and productivity (Palkovacs and Post 2009, Harmon et al. 2009, Bassar et al. 2010, Des Roches et al. 2013, Roy Chowdhury et al. 2014). This work has begun to demonstrate the importance and ubiquity of evo-eco interactions, but commonly lacks insight into the processes by which these interactions occur and thus we lack the ability to predict ecological change (Matthews et al. 2011). As such, the goal of this thesis is to investigate potential mechanisms underlying evo-eco interactions in the hopes of gaining a predictive understanding.

A potentially important form of evo-eco interactions may result from evolutionary change in resource expensive traits (Matthews et al. 2011, Leal et al. 2017). Here, evolution altering a species resource needs may consequently alter how that species interacts with ecosystem resource pools, such as diet sources and waste release (Matthews et al. 2011). Through change in these interactions, evolution may generate important ecological effects, such as changes in food web structure, prey abundance and nutrient dynamics (Matthews et al. 2011).

Ecological stoichiometry (ES) theory makes predictions for how this type of evolutionary change should alter ecological interactions and thus has been suggested as a potential bridge between ecology and evolution (Elser 2006, Jeyasingh et al. 2014). ES abstracts organisms into stoichiometry ratios (e.g. C:N, N:P) to provide a multi-dimensional picture of that organism's elemental requirements (Sterner and Elser 2002). Evolutionary change in trait investment can be detected here as change in organismal stoichiometry (OS), since variation in resource expensive traits (e.g. increased muscle) can disproportionately alter the abundance of some elements (e.g. % nitrogen) and thereby change stoichiometric ratios (e.g. lower C:N) (Jeyasingh et al. 2014). ES then applies the principle of mass balance to predict that changes in OS are balanced through changes in the stoichiometry of resource inflows (diet) or outflows (waste). Evolutionary change in OS is expected to be balanced by positively correlated changes in diet stoichiometry, such that required and acquired elements change in parallel, or by negatively correlated changes in waste stoichiometry, such that increases in elemental requirements are offset by decreases in elemental waste (Fig. 1) (Jeyasingh et al. 2014). My research tests the utility of this reductionist approach for insight into evo-eco interactions by asking whether heritable intraspecific variation in organismal stoichiometry is a useful predictor of intraspecific changes in diet and waste stoichiometry.

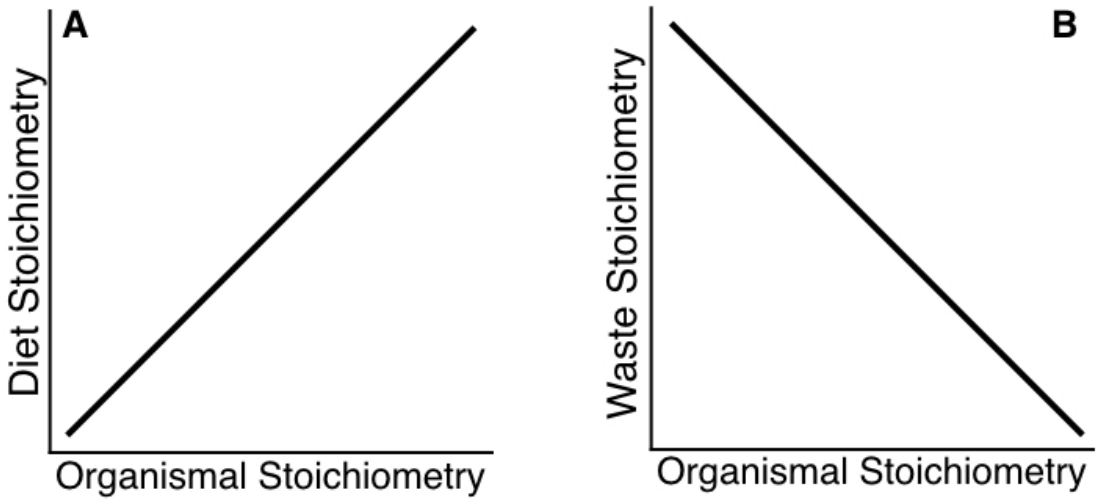

Fig. 1. Ecological stoichiometry predicts changes in organismal stoichiometry will be balanced by positively correlated changes in diet stoichiometry (A) and/or negatively correlated changes in waste stoichiometry (B).

Threespine stickleback (*Gasterosteus aculeatus*) are well suited to this question as they are a heavily studied model species within ecology and evolution, and contain conspicuous variation in resource expensive traits commonly under natural selection (Hendry et al. 2013). As a result of post-glacial colonization, threespine stickleback occupy many coastal lakes and rivers where they provide a replicated example of recent adaptive radiation (Withler and McPhail 1985, Lavin and McPhail 1986). Through natural selection, freshwater populations vary widely in phosphorus rich bony armour structures, including lateral armour plating, pelvic plating, pelvis spines and dorsal spines (Lavin and McPhail 1985). The ecological drivers of these evolutionary changes have been intensively studied and recent work has demonstrated ecological consequences from this evolution, but little is known about how these evo-eco interactions occur (Schluter 1993, Harmon et al. 2009, Reimchen et al. 2013). Here it is possible that evolutionary change in these expensive traits alters stickleback stoichiometry and this change in demand drives compensating changes in diet and waste interactions.

In chapter one of this thesis, I investigate whether heritable variation in stickleback bony traits generates meaningful variation in stickleback organismal stoichiometry. I investigate the extent to which stickleback elemental content (%P) and stoichiometry (N:P) vary, and use phenotypic traits and their underlying genetics to explain this variation. In my second chapter, I investigate the ecological consequences of evolutionary changes in bony traits by asking whether variation in these traits individually - and collectively as stickleback N:P - are good predictors of differences in diet choice and excretion rates. In doing so, I attempt to link heritable variation in traits commonly under strong natural selection with differences in resource interactions capable of driving ecological change.

Statement of Data Use

A subset of the phenotypic and composition data presented in chapter 1 is also included in chapter 2. This subset consists of all 10 freshwater locations out of the 14 total locations (Table 1) included in chapter 1.

Statement of Authorship

Dr. Rana El-Sabaawi (RE) and Daniel Durston (DD) designed this study. DD collected, modelled and analyzed the data, and wrote the first draft. RE and DD contributed substantially to revisions. Chapter 1 has been submitted to Functional Ecology, where it is in review with DD and RE as authors. Chapter 2 is planned for submission to Ecology Letters with the same authorship.

Chapter 1

Heritable bony traits and genetics drive intraspecific variation in vertebrate elemental content

Abstract

Differences between species in their elemental requirements are a major driver of differences in ecosystem interactions, but little is known about variation in elemental requirements within species. If intraspecific variation here is both substantial and heritable, it may underlie an important mechanism of evolutionary interplay with ecology. To investigate the magnitude and sources of intraspecific elemental variation in vertebrates, we sampled evolutionary model species *Gasterosteus aculeatus* (threespine stickleback) from 14 lakes in British Columbia, Canada. Fish were phenotyped, genotyped for *Eda* alleles underlying lateral plate variation and assayed for elemental content (C, N, P). We found stickleback vary widely in elemental composition (2.2 – 6.5 %P; 3.0 – 9.4:1 N:P), which phenotypic models explained well with bone related traits (bone mineralization, body size, lateral plating and pelvis size). Further genetic models linked variation in *Eda* alleles - which commonly undergo natural selection in wild populations – with a 12% shift in whole organism N:P. As many of these traits important to elemental composition are strongly heritable and relevant across vertebrates, we conclude that intraspecific variation in vertebrate elemental composition is likely to be widespread with large evolutionary potential.

Introduction

A central tenet of ecosystem ecology is that different species have unique and predictable effects on their abiotic environments (Tilman 1982, Sterner and Elser 2002). These interspecific differences have been well studied, but we know little about intraspecific diversity in ecosystem effects (Matthews et al. 2011, Jeyasingh et al. 2014). Recent work has found ecosystem effects do vary substantially within species, but the causes, magnitude and mechanisms by which this occurs are largely unknown (Harmon et al. 2009, Bassar et al. 2010, Rudman et al. 2015, El-Sabaawi et al. 2015). Differences in ecosystem effects arising from heritable variation may be particularly important, as natural selection here could generate meaningful evo-eco interactions and feedbacks (Matthews et al. 2011, Jeyasingh et al. 2014). Thus, a mechanistic understanding of intraspecific variation in ecosystem effects is needed to achieve a higher resolution understanding of ecosystem function and to determine the ecosystem consequences of evolutionary change.

A common mechanism by which species alter ecosystem function arises from differences in the elemental resources required to build their bodies, leading to differential resource uptake or release (Sterner and Elser 2002, Vanni 2002). Interspecific differences in elemental investment result in variation in the elemental composition and thus demand of an organism, with commensurate effects on the standing stocks and cycling rates of important nutrients (Vanni et al. 1997, Vanni 2002). If elemental composition also varies substantially within species, it too may underlie a widespread mechanism whereby phenotypic variation interacts with ecosystem processes (Matthews et al. 2011). Thus, understanding the magnitude of intraspecific variation in elemental composition and linking this with its causes and consequences are important steps towards understanding variation and evolution of ecosystem effects (Jeyasingh et al. 2014).

Elemental composition has been widely shown to vary substantially within species as a result of environmental and ontogenetic factors (Vrede et al. 2011, González et al. 2011, Cross et al. 2015, Boros et al. 2015). Conversely, heritable variation is less studied but could arise from genetic variation or gene by environment (G x E) interactions altering resource intensive traits (Jeyasingh et al. 2014). For invertebrates, RNA related traits (e.g. growth rate) and exoskeletal traits have large influences on phosphorus and nitrogen content respectively, while for vertebrates the skeletal system is the largest pool of phosphorous and thus bony traits are hypothesized to be the largest cause of variation in phosphorus composition (Sterner and Elser 2002, Hendrixson et al. 2007, Boros et al. 2015).

Previous work studying heritable differences in elemental composition has been limited to rearing multiple lineages within one or a limited set of environments to detect persistent differences (Liess et al. 2013, Roy Chowdhury et al. 2014, Tobler et al. 2016).While this work has confirmed heritable variation can be substantial, it hasn't provided links between compositional variation and specific traits or genes. Further, the relative importance of heritable vs. plastic variation is unknown as these studies have restricted plastic influences (Liess et al. 2013, Tobler et al. 2016). Important questions remain, including: (1) which traits drive variation in elemental composition, (2) how substantially do these traits alter elemental composition, (3) how important are heritable vs. plastic influences on these traits, and (4) can small genetic differences have large effects on elemental composition?

To investigate these questions, we studied the elemental composition of *Gasterosteus aculeatus* (threespine stickleback) - an important model species that has provided numerous widely

applicable insights into evolution and ecology (McKinnon and Rundle 2002, Hendry et al. 2013) This small fish varies phenotypically in numerous physical traits including phosphorus rich bony traits (Hagen and Gilbertson 1972, Bell 1987). As phosphorus is often a limiting element in freshwater ecosystems, bony trait variation may carry with it meaningful consequences for the availability and recycling rates of this element (Elser et al. 2007). Previous work has found the elemental composition of stickleback is highly variable, but the major traits and underlying causes of this variation are unknown (El-Sabaawi et al. 2016). Candidate bony traits include lateral armour plating and pelvic girdle size (Fig. 2). Both of these are strongly heritable and are frequently reduced in freshwater habitats through natural selection (Bell 1987, Colosimo et al. 2005, Chan et al. 2010). In particular, variation in lateral plating is reduced by selection on alleles at the Ectodysplasin locus (*Eda*), where low armour alleles commonly undergo positive selection in freshwater environments (Colosimo et al. 2005, Barrett et al. 2008). It is likely that evolutionary reductions in these bony traits decrease the phosphorus content of stickleback, altering its demand for important nutrients.

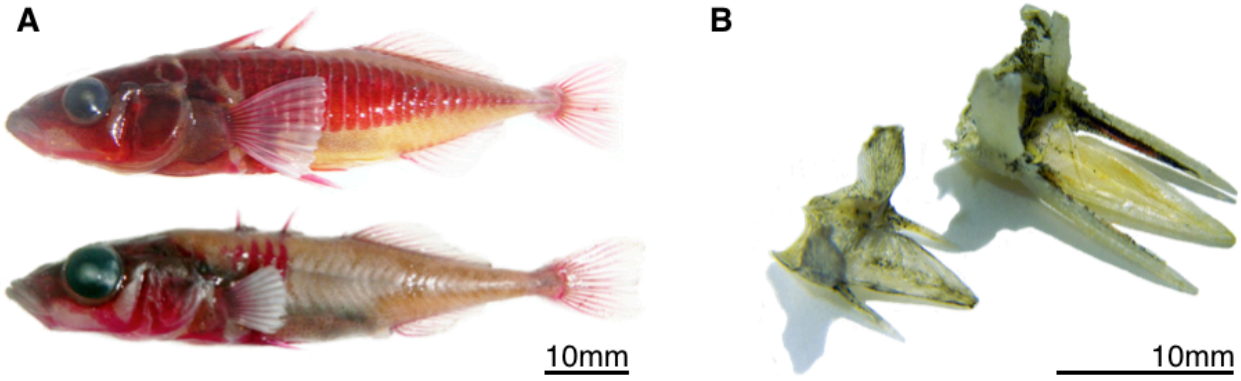

Fig. 2. Phenotypic variation in lateral plates (panel A) and pelvic girdle (panel B). Panel A fish are from Oyster Lagoon (top) and Trout Lake (bottom). Fish are stained with alizarin red which binds to bone calcium. In panel B, pelvic girdles were removed from similar sized fish (60-62mm standard length) to show differences in relative pelvis length, thickness and spine size. These girdles comprised a small (0.14) or large (0.26) proportion of standard length.

While armour traits may be major determinants of stickleback elemental composition, they may also trade off against other phosphorus intensive traits, such as bone mineralization, to mitigate changes in composition. As well other traits including lipid stores, muscle stores and body size may also influence composition. Carbon rich lipids and nitrogen rich muscle contain little phosphorus and could dilute whole body phosphorus proportions, while body size could alter composition though skeletal allometry where bone as a proportion of body mass increases with body size (Casadevall et al. 1990, Sterner and Elser 2002).

To investigate links between elemental composition, phenotypic traits and causative drivers, we first compared fish within two phenotypically and genetically diverse wild populations (Kennedy, Miami). By comparing fish within locations, we were able to minimize the confounding environmental and genetic differences that may exist in a comparison between locations. Fish from both locations were collected, characterized phenotypically, genotyped for *Eda* alleles underlying lateral plate variation and assayed for elemental composition (C, N & P). For each location, elemental variation was modelled against phenotypic traits and genetics to determine composition-phenotype and composition-genotype relationships.

Additionally, we sampled 12 more stickleback populations from a diverse range of environments to determine whether these phenotypic and genetic relationships with elemental composition apply broadly across environments, and to gain insight into the extent of compositional variation present from a broader range of genetics and environmental influences. These environments included eutrophic sloughs, large oligotrophic lakes, brackish river mouths and marine inlets. By establishing a minimum estimate of the magnitude of intraspecific variation in composition, we were able to determine the importance of some traits to the total variation. Our hypotheses were

that bony traits and their underlying genotypes would be substantial predictors of fish elemental composition, with less bony fish exhibiting lower phosphorus content (lower %P, higher N:P). We also expected several other patterns: C-rich lipid stores would have a major dilutive effect on %P but not N:P, body size would be positively related to phosphorus content, and bone mineralization would be negatively correlated with other bony traits and positively correlated with phosphorus content. Overall, we expected traits known to have high heritability would explain a large portion of the total intraspecific variation in composition such that natural selection can meaningfully alter stickleback composition.

Methods

During May - July 2015 we collected a total of 432 threespine stickleback (*Gasterosteus aculeatus*) from 14 locations in southwestern British Columbia, Canada (Table 1). 22-25 fish were sampled from each location except for Garden Bay Lake (13), Cranby Lake (16), North Lake (18), Trout Lake (36), Oyster Lagoon (47), Miami River (61) and Kennedy Lake (71). Fish were collected with cheddar cheese baited minnow traps deployed for three hours and sacrificed in accordance with our animal use protocol (University of Victoria AUP 2015-006) and collection permits (BC MFLNRO NASU15-164904, DFO XR-30-2015).

Phenotypes for each fish were quantified including standard length, head length, body depth, pelvis length (combined anterior and posterior process) and lateral plate count (average of both sides) (Table 2). Head length, body depth and pelvis length were converted to proportions of standard length to reduce covariance with body size. Fish were sexed internally and genetically via IDH genotyping (Peichel et al. 2004). Additionally, the 7th lateral armour plate was removed from the left side of each fish to measure bone mineralization (defined as bone %P) as this plate

is highly conserved across populations. Pure mineral bone (Hydroxyapatite or $Ca_{10}(PO_4)_6(OH)_2$) is 18.5% P by weight. Digestive and reproductive tissues were discarded prior to elemental analysis as standard procedure (El-Sabaawi et al. 2012).

Table 1. Environmental characteristics. Marine and brackish habitats contain predominantly marine fish. SRP is soluble reactive phosphorus. Spc. Cond. is specific conductivity (uS/cm).

Location	Location (Lat/Long)	Water Type	Area (ha)	pH	SRP (ug/L)	Spc. Cond. (uS/cm)
Cowichan Lake	48°50'4.70"N, 124° 7'7.69"W	Fresh	6214	7.7	0.0	48
Cranby Lake	49°41'30.05"N, 124°30'24.08"W	Fresh	45	7.6	0.0	104
Dougan Lake	48°42'50.33"N, 123°36'38.17"W	Fresh	10	7.6	2.6	185
Englishman Lagoon	49°19'32.09"N, 124°17'19.48"W	Marine	-	8.1	1.9	20200
Garden Bay Lake	49°38'49.14"N, 124° 1'18.00"W	Fresh	59	7.9	1.7	56
Harrison Lake	49°23'8.57"N, 121°49'7.73"W	Fresh	22192	7.5	1.2	45
Kennedy Lake	49° 7'35.27"N, 125°25'38.24"W	Fresh	6542	7.4	1.1	44
Koksilah River	48°45'33.29"N, 123°39'7.92"W	Brackish	-	7.0	31.9	1706
Miami River	49°18'5.67"N, 121°46'47.48"W	Fresh	6.8	6.8	5.5	156
North Lake	49°44'51.85"N, 123°58'11.97"W	Fresh	36	7.0	4.5	37
Oyster Lagoon	49°36'49.44"N, 124° 1'49.69"W	Marine	-	8.0	21.3	35514
Sooke River	48°23'34.31"N, 123°42'38.81"W	Brackish	-	7.3	1.8	1393
Sproat Lake	49°17'4.69"N, 124°58'32.46"W	Fresh	4233	7.3	1.2	52
Trout Lake	49°30'26.11"N, 123°52'34.58"W	Fresh	6.5	7.2	2.0	54

Table 2. Mean and range of phenotypic variation at study locations. Lateral plate count is the average of both sides of the fish. Head length and pelvis length are proportions of standard length (SL). Pelvis length is the combined ventral length of the anterior and posterior process. Bone mineralization is %P of the 7th lateral plate.

Location	***n***	**Standard Length (mm)**		**Lateral Plate Count**		**Head Length**		**Pelvis Length**		**Bone Min. (%P)**	
		μ	Range	μ	Range	μ	Range	μ	Range	μ	Range
Cowichan	25	52.2	43-60	7.2	6-9	.29	.27-.32	.23	.20-.25	11.3	10.8-11.7
Cranby	16	47.7	29-72	5.2	4-7	.32	.28-.34	.16	.14-.18	10.8	9.3-11.7
Dougan	21	50.0	40-63	4.6	3-6	.30	.28-.34	.17	.15-.20	10.3	9.3-11.4
Englishman	25	59.1	44-66	33.2	5-36	.28	.26-.32	.23	.17-.26	11.3	9.7-11.5
Garden Bay	13	48.1	44-52	7.2	6-9	.29	.26-.30	.20	.18-.22	11.0	9.8-12.1
Harrison	24	50.0	45-59	33.8	31-36	.29	.26-.32	.24	.22-.27	10.7	9.7-11.3
Kennedy	71	50.4	36-62	21.5	5-36	.30	.28-.33	.24	.21-.27	11.1	9.8-11.9
Koksilah	25	52.6	39-59	33.9	32-35	.29	.26-.32	.24	.23-.27	11.1	9.8-11.8
Miami	61	49.0	38-56	24.6	6-35	.29	.26-.33	.24	.18-.26	11.2	10.1-11.9
North	18	43.8	39-51	33.4	32-34	.30	.28-.32	.21	.19-.22	11.8	11.5-12.1
Oyster	47	55.1	38-61	33.1	27-35	.29	.24-.32	.23	.21-.25	11.3	10.4-12.4
Sooke	25	53.6	33-62	33.9	32-36	.29	.26-.33	.23	.21-.25	11.0	10.0-11.8
Sproat	25	55.1	46-65	8.0	6-13	.29	.27-.32	.23	.21-.26	11.2	10.0-12.0
Trout	36	42.9	25-59	4.2	2-6	.30	.28-.33	.16	.13-.19	10.5	9.2-11.8
All Locations	432	50.7	25-72	21.3	2-36	.29	.24-34	.22	.13-.27	11.1	9.2-12.4

Fish were freeze dried for 72 hours using a LABCONCO 77545-00-J and ground with a Retsch MM400 mixer mill after recording dry mass. Phosphorus content (%P) was determined as the mean of two 9-11 mg subsamples of the whole body ground tissue. These samples were ashed at 550°C for 8 hrs and digested with 1N HCl at 105°C for 2 hrs before assay with a Mandel UVmini-1240 spectrophotometer using an acid molybdate method (Murphy and Riley 1962, Boros and Mozsár 2015). The mean coefficient of variance was <1% between fish replicates and extraction efficiency was >99% for bonemeal (NIST 1486) and spinach (NIST 1570a) standards.

Fish were further analyzed for %C and %N with a 1 mg subsample of whole body ground tissue. Samples were run on a Finnigan Delta Plus Advantage mass spectrometer at the University of Victoria with a dogfish muscle standard (NRC Canada DORM-4). All elemental ratios were determined as molar ratios. C:N was used as a measure of condition rather than length-mass residuals as variation in bony traits also influences mass (Wilder et al. 2016).

DNA was extracted using Promega Wizard SV96 kits. Stn382 and IDH primers were used to target *Eda* and sex for PCR. Amplified DNA was run via electrophoresis with ethidium bromide on 2% agarose gel. *Eda* genotype was recorded for 381 fish, omitting Garden Bay Lake, Sproat Lake and half of the Oyster Lagoon fish because our DNA extraction kit was limited to 384 samples. *Eda* alleles are classified as C (complete armour) or L (low armour), giving genotypes of LL, LC and CC.

Data analysis was done in R (R Core Team 2016). Two types of normally distributed models were constructed: location-specific GLMs to test patterns within Kennedy Lake or Miami River,

and full dataset GLMMs to investigate compositional variation across all locations. In full dataset models location was always included as a random effect. For both model types, we investigated %P and log transformed N:P as response variables. After checking global model terms for collinearity via VIF scores, we performed model searches with the MuMIn package based on AICc scores (Grueber et al. 2011, Bartoń 2016, Fox et al. 2016). Global models for each search contained 8 candidate main effects: standard length, condition (C:N), head length, body depth, pelvis length, sex, bone mineralization and left plate count (replaced by *Eda* genotype in genetic + phenotype models, see Fig. S1 for phenotype-genotype relationship). A correlation matrix for these main effects is provided in Fig. S2. All main effects were standardized to a mean of 0 and a standard deviation of 0.5 to allow comparison of coefficients as a measure of effect size in GLMMs (Gelman 2008). For location-specific models, the best model was selected based on AICc and partial η^2 effect sizes were determined with the lsr package (Navarro 2015) with thresholds of >.01 (small effect), >0.06 (medium effect) and >.14 (large effect) (Richardson 2011). For full dataset models, MuMIn was used to average the top ranking models (ΔAICc < 5) and effect sizes were based on coefficients (Bartoń 2016). Figures were developed using the visreg package (Breheny and Burchett 2016).

Results

At both Kennedy Lake and Miami River we observed substantial phenotypic variation in lateral plating, with counts ranging from less than 7 to over 34 plates (Table 2). These populations also varied in standard length (<40mm to >55mm), pelvis length (<0.21 to >0.26 of standard length) and bone mineralization (<10.1 % to 11.9 %P; Table 2). Additional populations included in the full dataset contained wider variation in standard length (25 to 72mm) and bone mineralization

(9.2 to 12.4 %P), as well as further reductions in lateral plating (2 to 36 plates) and pelvis length (0.13 to 0.27 of standard length).

Composition of *G. aculeatus* was highly variable within the Miami and Kennedy populations with phosphorus varying from 3.1 – 6.2% and 3.2 – 6.1% respectively, while N:P ranged from 3.3 – 6.3:1 (Miami) and 3.4 – 6.4:1 (Kennedy) (Table 3). Across all 14 locations, composition varied even more widely as phosphorus, nitrogen and carbon spanned ranges of 2.2 - 6.5%, 7.0 – 12.2% and 30.8 – 49.6% respectively between individuals and 3.3 – 5.0%, 8.1 – 11.4% and 35.2 - 42.6% between population means (Table 3). For the full dataset, N:P ranged threefold amongst all individuals (3.0 – 9.4:1) and twofold among population means (3.9 – 7.7:1; Table 3). Phosphorus was consistently the most variable element with a mean population coefficient of variation of 14.0% compared to 6.2% for nitrogen and 7.5% for carbon (Table 3).

Table 3. Elemental composition by location for phosphorus, nitrogen and carbon. For each element we have shown % (mean of site individuals), standard deviation (SD) and coefficient of variation (CV). Elemental ratios are molar ratios of population means for individual elements.

Location	Phosphorus			Nitrogen			Carbon			N:P	C:N	C:P
	%	SD	CV	%	SD	CV	%	SD	CV			
Cowichan	5.0	0.5	9.7	10.2	0.3	3.4	35.2	1.8	5.0	4.5	4.0	18.0
Cranby	3.6	0.4	11.6	10.8	0.3	2.8	39.0	1.5	3.9	6.6	4.2	28.1
Dougan	3.3	0.4	13.1	11.3	0.8	6.9	40.9	2.5	6.1	7.7	4.2	32.3
Englishman	4.9	0.9	17.7	8.7	0.6	7.0	37.0	4.0	10.7	3.9	5.0	19.6
Garden Bay	3.3	0.5	14.2	9.6	0.5	4.7	42.6	2.2	5.1	6.4	5.2	33.3
Harrison	4.4	0.8	18.3	9.4	0.7	7.9	39.0	3.7	9.6	4.8	4.8	23.1
Kennedy	4.7	0.7	15.4	9.6	0.7	7.2	37.7	3.3	8.8	4.5	4.6	20.5
Koksilah	4.7	0.6	13.1	9.3	0.8	8.7	37.5	2.6	7.0	4.4	4.7	20.8
Miami	4.8	0.8	15.7	9.7	0.6	6.6	36.4	3.6	10.0	4.4	4.4	19.4
North	4.5	0.7	14.6	9.4	0.4	4.6	38.1	3.2	8.4	4.6	4.7	21.6
Oyster	3.8	0.7	19.3	8.1	0.6	7.6	42.5	4.3	10.0	4.7	6.1	28.5
Sooke	4.5	0.6	13.6	9.6	0.8	8.6	40.2	3.4	8.5	4.7	4.9	22.9
Sproat	4.4	0.5	11.2	9.9	0.8	7.7	37.6	2.7	7.3	5.0	4.4	21.9
Trout	3.5	0.3	9.0	11.4	0.4	3.3	41.7	1.7	4.1	7.3	4.3	30.9
All Locations	4.2	0.6	14.0	9.8	0.6	6.2	40.0	3.0	7.5	5.0	4.6	23.1

Location Specific Phenotypic – Composition Models

The best models for %P at both Kennedy and Miami explained most of the variation with 5 - 7 phenotypic traits ($R^2_{Adj} > 0.75$; Table 4). The best %P models at both locations had condition as the largest effect (Partial $\eta^2 = 0.45 - 0.51$) with lateral plating, pelvis length, bone mineralization, sex and standard length as medium to large effects (Partial $\eta^2 = 0.07 - 0.30$; Table 4). Percent phosphorus was reduced in high condition fish and increased with standard length, pelvis length, lateral plate count and bone mineralization (Fig. 3). Sex had differing effects at each site, with males higher in %P at Miami and lower at Kennedy.

Table 4. Best models based on AICc for %P and N:P at Kennedy and Miami. N:P was log transformed prior to modelling.

	Kennedy Lake			**Miami River**		
Term	Est.	*P*-value	Par. η^2	Est.	*P*-value	Par. η^2
% P Model		$R^2_{Adj} = .75$			$R^2_{Adj} = .81$	
Standard Length	.54	<0.001	.27	.56	.006	.13
Condition (C:N)	-1.06	<0.001	.51	-1.22	<0.001	.45
Sex (Male)	-.26	.012	.10	.34	.016	.10
Body Depth	-.36	.015	.09			
Pelvis Length	.86	.002	.14	.54	.015	.10
Lateral Plate Count	.26	.014	.09	.26	.050	.07
Bone Mineralization	.65	<0.001	.30	.44	.008	.12
N:P Model		$R^2_{Adj} = .67$			$R^2_{Adj} = .66$	
Standard Length	-.064	<0.001	.33	-.090	<0.001	.23
Condition (C:N)	.029	.036	.07	.058	.009	.12
Sex (Male)	.027	.010	.10			
Body Depth	.029	.049	.06			
Head Length				-.044	.002	.16
Pelvis Length	-.085	.003	.13			
Lateral Plate Count	-.029	.007	.11	-.053	<0.001	.22
Bone Mineralization	-.083	<0.001	.40	-.054	.005	.14

Best models for N:P at Kennedy and Miami also explained most of the variation ($R^2_{Adj} > 0.66$) but condition had a much reduced effect (Partial $\eta^2 = 0.07 - 0.12$; Table 4). Instead standard length, lateral plate count and bone mineralization were larger effects at both sites (Partial $\eta 2 = 0.11 - 0.40$). Additionally, pelvis length had a medium effect at Kennedy (Partial $\eta^2 = 0.13$) while

head length had a large effect at Miami (Partial $\eta^2 = 0.16$). At Kennedy only, males were higher in N:P than females. In all cases, N:P declined with increases in standard length and bony traits (Fig. S3).

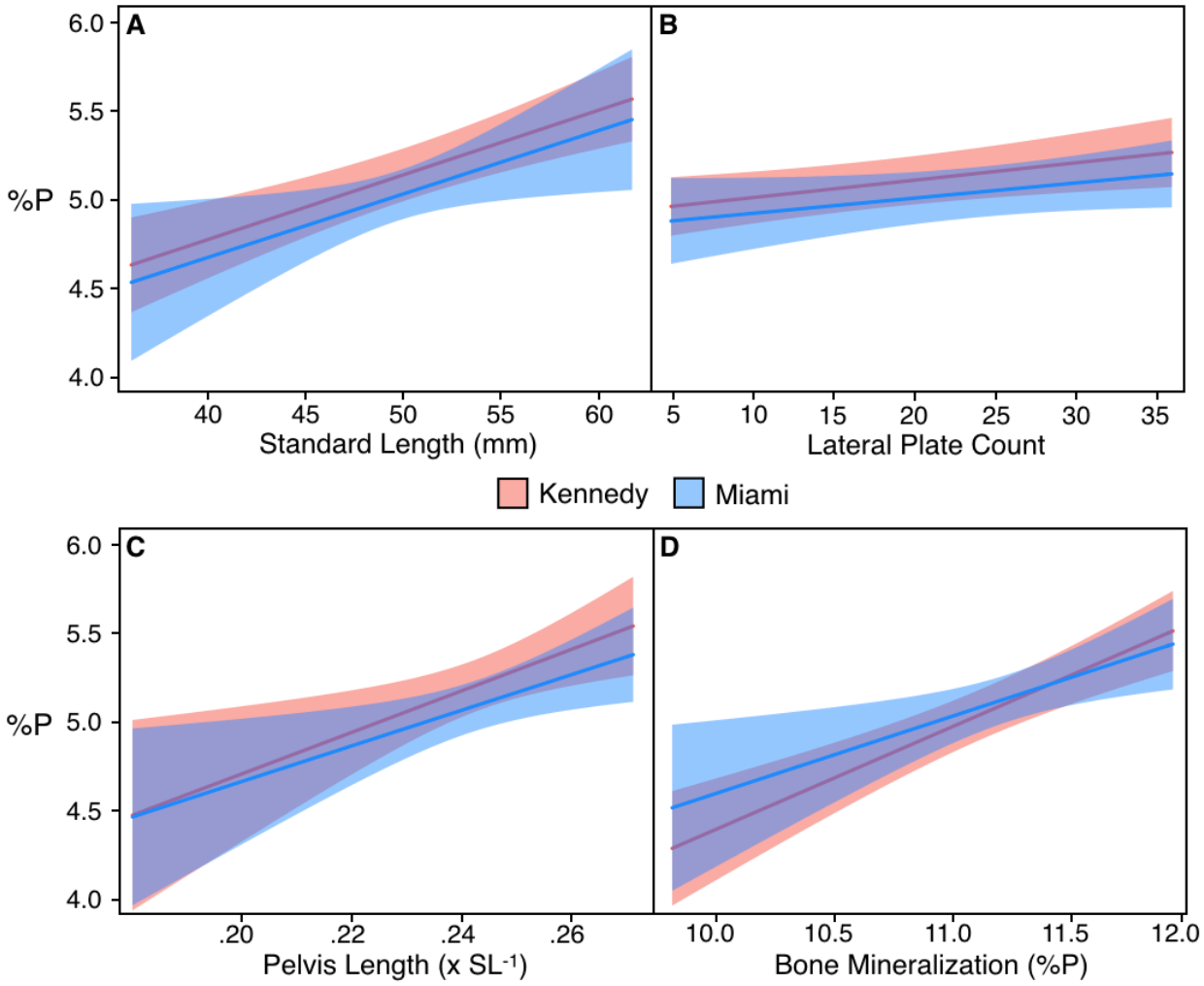

Fig. 3. Relationships between phenotypic traits and %P at Kennedy and Miami. Plots are outputs from the location specific GLMs (Table 4). %P rises significantly with standard length (Panel A; SL), lateral plate count (B), pelvis length (C) and bone mineralization (D). Shaded regions depict 95% confidence ranges.

Full Dataset Phenotype - Composition Models

The GLMMs for all 14 locations in the full dataset investigated a wider range of variation in composition (%P, N:P) and phenotypic traits than location-specific models, with several populations containing large reductions in mean pelvis size and/or bone mineralization (e.g. Dougan and Trout Lakes; Table 5).

For %P, top GLMMs explained most of the variation (R^2_{Marg} of 0.73 - 0.75) with 6-8 traits (Table 5). In the averaged model, the most important traits were condition, standard length and three bony traits (lateral plates, pelvis size and bone mineralization), where %P declined with condition and increased with the other bony traits (Fig. S4). Of these, condition had the largest effect with an averaged standardized coefficient of -1.20 compared to averaged coefficients of 0.27 – 0.50 for the others (Table 5).

Top GLMMs for N:P also explained most of the variation with the same traits as %P models (R^2_{Marg} of 0.52 - 0.54; Table 5). We found N:P decreased with standard length, pelvis length, left plate count and bone mineralization, while increasing with condition and body depth (all $p < 0.001$; Fig. 4). Effect sizes were more similar than %P models, with pelvis length (-0.058) and condition (0.058) as the most important effects based on averaged standardized coefficients. Standard length, lateral plating and bone mineralization had nearly as large coefficients (-0.035 to -0.052; Table 5) while body depth, sex and head length had smaller effects (-0.020 to 0.004).

Table 5. Top GLMMs for %P and log transformed N:P with location as a random effect. Top models were selected based on ΔAIC < 5 from best model (lowest AIC). Averaged coefficients are full model averages. Model terms are standard length (SL), condition (Cond.), body depth (BD), head length (HL), sex, pelvis length (PL), lateral plate count (LPC) and bone mineralization (BM).

%P Top Models				**Coefficients**							
Rank	ΔAIC	R^2_{Marg}	Weight	SL	Cond.	BD	HL	Sex (M)	PL	LPC	BM
1	0	.74	.494	.377	-1.201	-.086	.127		.517	.266	.330
2	1.5	.73	.239	.366	-1.192		.112		.463	.275	.348
3	3.5	.73	.085	.389	-1.208	-.079	.125	+	.513	.264	.327
4	3.8	.75	.073	.386	-1.208			+	.507	.264	.342
5	4.0	.74	.068	.385	-1.207		.088	+	.482	.268	.341
6	5.0	.75	.041	.389	-1.209	-.044		+	.531	.261	.335
%P Averaged Model				SL	Cond.	BD	HL	Sex (M)	PL	LPC	BM
Importance				1.00	1.00	.60	.92	.24	1.00	1.00	1.00
Coefficient				.377	-1.200	-.051	.111	.012	.500	.268	.336
Adj. Std. Error				.041	.046	.055	.053	.042	.082	.063	.042
Significance				<.001	<.001	.35	.038	.77	<.001	<.001	<.001
N:P Top Models				**Coefficients**							
Rank	ΔAIC	R^2_{Marg}	Weight	SL	Cond.	BD	HL	Sex (M)	PL	LPC	BM
1	0	.53	.368	-.052	.058	.009	-.022		-.060	-.035	-.046
2	0.2	.52	.330	-.052	.057		-.020		-.055	-.036	-.047
3	2.0	.54	.138	-.052	.058		-.017	+	-.057	-.035	-.047
4	2.1	.53	.129	-.052	.058	.009	-.021	+	-.061	-.035	-.046
5	4.7	.53	.035	-.052	.058			+	-.063	-.035	-.047
N:P Averaged Model				SL	Cond.	BD	HL	Sex (M)	PL	LPC	BM
Importance				1.00	1.00	.50	.97	.30	1.00	1.00	1.00
Coefficient				-.052	.058	.004	-.020	-.001	-.058	-.035	-.046
Adj. Std. Error				.005	.006	.006	.007	.006	.011	.008	.005
Significance				<.001	<.001	.47	.005	.82	<.001	<.001	<.001

Genetic – Composition Models

Significant relationships between *Eda* genotype and fish N:P were found when *Eda* genotype was used in place of lateral plate phenotype in location specific and whole dataset models (Table 6). *Eda* had a large effect on N:P at Miami ($p < 0.001$, Partial $\eta^2 = 0.23$) and a medium effect at Kennedy ($p = 0.04$, Partial $\eta^2 = 0.07$) and across the full dataset ($p < 0.001$, Partial $\eta^2 = 0.12$). All models found low armour genotypes (LL) are higher in N:P than full armour (CC) genotypes (Fig. 5). Effect sizes and significance for the other model terms were similar to phenotype only models (Table 6).

Table 6. Top GLMs for log transformed N:P with *Eda* genotype in place of lateral plate count. Model terms are standard length (SL), C:N (CN), body depth (BD), head length (HL), pelvis length (PL) and bone mineralization (BM).

	Kennedy Lake (R^2_{Adj} = .66)			**Miami River** (R^2_{Adj} = .66)			**All Locations** (R^2_{Adj} = .81)		
Term	Est.	*P*-value	Par. η^2	Est.	*P*-value	Par. η^2	Est.	*P*-value	Par. η^2
SL	-.066	<0.001	.32	-.084	<0.001	.20	-.053	<0.001	.23
CN	.027	.052	.06	.056	.012	.11	.046	<0.001	.14
Sex (Male)	.026	.015	.09						
BD	.029	.057	.06				.021	<0.001	.04
HL				-.043	.003	.16	-.019	<0.001	.04
PL	-.078	.008	.11				-.111	<0.001	.43
Eda (LC)	.007	.550	.07	.023	.046	.23	.021	.004	.12
(LL)	.026	.040		.059	<0.001		.048	<0.001	
BM	-.082	<0.001	.38	-.053	.007	.13	-.060	<0.001	.25

Discussion

We found the elemental composition of stickleback is highly variable, with elemental ranges within this species similar to those reported for diverse sets of fish taxa (Vanni et al. 2002, Hendrixson et al. 2007, Benstead et al. 2014). As expected, most of this variation was related to investment in phosphorus rich bone. Lateral plating, pelvis length and bone mineralization are directly related to bone investment, while the relationship between composition and standard length is consistent with skeletal allometry, where bone comprises a larger portion of vertebrate mass as body size increases (Casadevall et al. 1990). Similar body size-composition relationships have been found in several other teleost species (Davis and Boyd 1978, Hendrixson et al. 2007, Pilati and Vanni 2007, Boros et al. 2015).

In %P models, condition (C:N) had by far the largest effect (Tables 4 and 5). These observed declines in %P with increased condition are likely the product of a dilutive mechanism where gains in carbon rich lipids increase body mass and consequently reduce phosphorus as a percentage, despite leaving the mass of phosphorus unchanged. Since this condition effect does

not alter phosphorus mass (which shapes demand for phosphorus), percentage analyses such as this give distorted insight into the variability and trait basis of an organism's elemental requirements. Clearer insight can be had by evaluating elements in ratios with other important or invariable elements, as we have done with N:P models (Sterner and Elser 2002). Gains in carbon rich lipids dilute phosphorus and nitrogen equally, such that N:P is not affected by this dilutive mechanism. Further, because nitrogen is fairly stable (Table 3), N:P models predominantly give insight into variation in phosphorus mass.

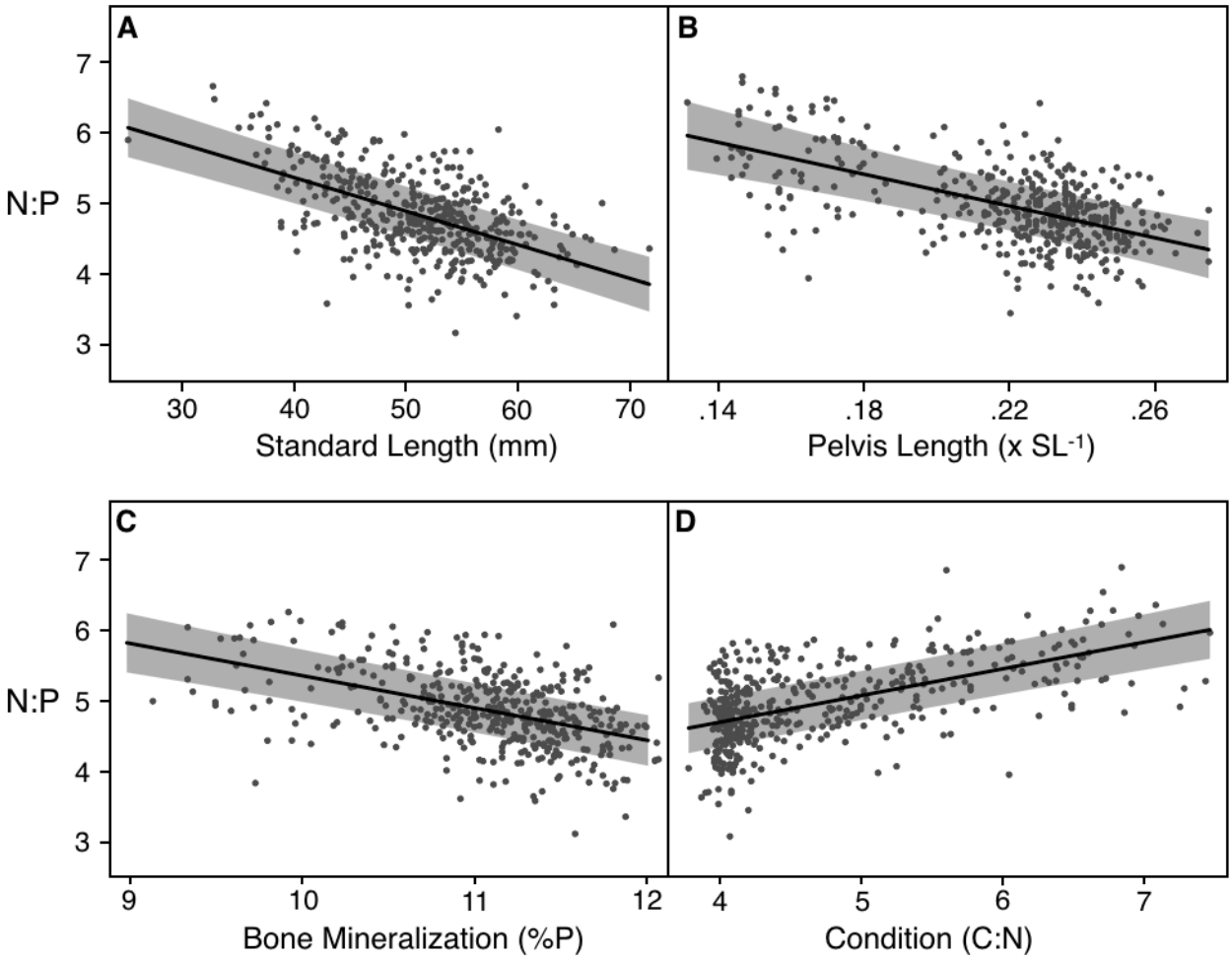

Fig. 4. Relationships between phenotypic traits and N:P for the best full dataset GLMM (Table 5). N:P is untransformed for visualization. N:P declined significantly ($p < 0.001$) with standard

length (Panel A; SL), pelvis length (B) and bone mineralization (C), while rising with condition (D). Shaded regions depict 95% confidence ranges.

As expected, the effect of condition (C:N) in N:P models was much reduced such that bony traits (standard length, bone mineralization, lateral plating, pelvis size) were the major drivers of intraspecific variation in N:P (Table 5). Condition was still an important predictor, but such a pattern is likely the result of the high energetic expense of bone investment where fish investing less in phosphorus rich bone are higher in condition (Giles 1983, Marchinko and Schluter 2007, Barrett 2010). Thus, condition is positively correlated with stickleback N:P but this is a confounded byproduct of the relationship between bony traits and N:P, such that the importance of these bony traits is likely underestimated even here. Even still, it is clear that variation in bony traits is the major cause of variation in the N:P content.

The surprising variability in bone mineralization and its large effect in composition models suggests that factors determining mineralization have a major influence on vertebrate composition. Variation in mineralization arising from genetic differences would substantially alter resource demand, while plastic variation is potentially an important buffer between nutrient intake, demand and release. Studies with other fish species have observed plastic reductions in bone mineral as a result of severely calcium and phosphorus deficient diets (Ye et al. 2006, Nwanna and Schwarz 2007). Similar limitation could exist in resource constrained stickleback populations and result in trade-offs between mineralization and other bony traits if investment elsewhere is achieved by withdrawing or withholding from mineralization. However, we find no correlations between mineralization and other bony traits within the Kennedy and Miami populations ($R^2 < 0.03$), providing no support for this trade-off hypothesis.

We do, however, find positive correlations among population means for bone mineralization and lateral plate count ($R^2 = 0.26$), as well as pelvis length ($R^2 = 0.30$). The result is a pattern where populations with the strongest evolutionary reductions in other bony traits also have the lowest bone mineralization (Table 2). This is consistent with a hypothesis of calcium or phosphorus limitation at low armour sites, but resource limitation does not appear likely as these fish have evolutionarily reduced demand and yet occupy habitats where stickleback typically consume a relative phosphorus rich littoral diet (Schluter and McPhail 1992, Schluter 1993). It is more likely that the observed population level reductions in bone mineralization are the result of the same selection pressure that reduces other bony traits in these small, low visibility habitats: more successful flight from predators enabled by reduced mass (Reimchen et al. 2013). Such adaptation would require heritable variation in bone mineralization which is uninvestigated in fish but other vertebrate studies have found high levels of heritability (Prentice 2001, Tse et al. 2009). Further study into the causes of variation in bone mineralization may yield important insights into vertebrate composition, how this interacts with nutrient dynamics, and the causes and mechanisms of evolutionary loss in bone mineralization seen in many vertebrate families (Currey 2008, Cohen et al. 2012).

A remaining question is how important are heritable, environmental and plastic influences on stickleback composition. Here we find clear links between *Eda* genotype and composition. In all models, *Eda* had a medium to large effect, with low armoured genotypes higher in N:P (Table 6). Across all locations, LL genotypes were a mean of 0.57 (12%) higher in N:P - a shift equal to 9% of the total observed range of individual N:P variation (3.0 – 9.4:1) (Fig. 5). Thus a single genetic difference contributes a substantial portion of the total variation in composition.

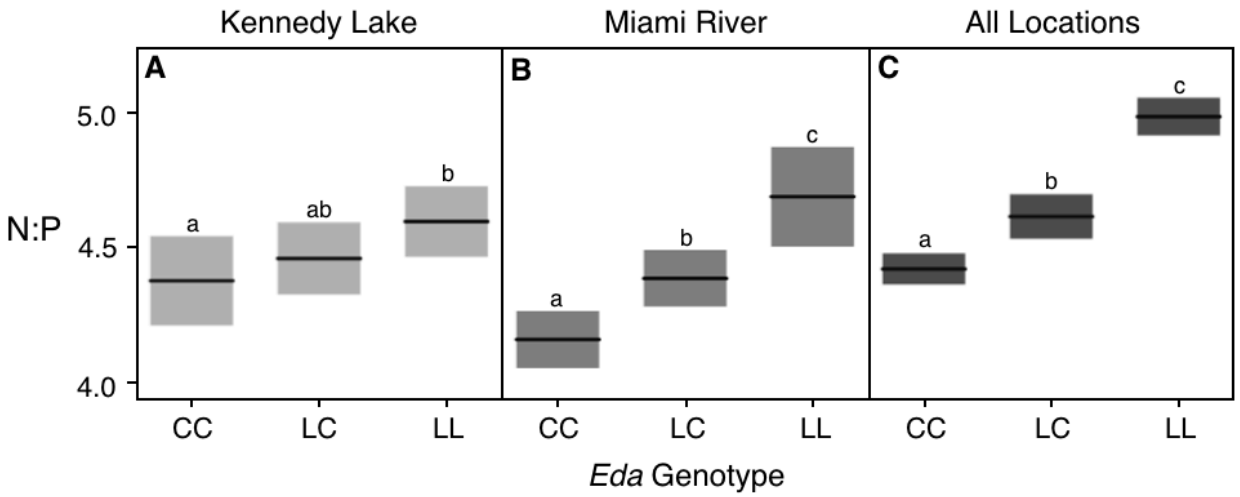

Fig. 5. Mean N:P by *Eda* genotype from GLM models at Kennedy Lake (A), Miami River (B) and the full dataset (C). Genotypes not sharing a letter are significantly different (see Table 6). Full armour genotypes (CC) had significantly lower N:P ratios than low armoured genotypes (LL) at all locations and were significantly different from heterozygotes (LC) Miami and all locations. Shaded regions depict +/- 1 SE.

Pelvis size and standard length – two other important predictors of N:P – are also known to have large heritable components (Shapiro et al. 2004, Leinonen et al. 2011, Reimchen et al. 2013). Within marine populations, variation in pelvis length has been shown to be largely heritable , while deletions in the regulatory regions for the *Pitx1* gene are known to be responsible for the major pelvic reductions seen in some freshwater populations (Shapiro et al. 2004, Chan et al. 2010, Leinonen et al. 2011). Variation in body size (standard length) has also been shown to be heritable and natural selection on this variation has resulted in more than two fold differences in mean adult standard length between populations (Reimchen 1991, Leinonen et al. 2011, Reimchen et al. 2013). Thus, despite the uncertain nature of variation in bone mineralization, it is clear that most of the traits with large effects on elemental composition have a substantial heritable basis such that that natural selection here can and has meaningfully altered stickleback composition (Barrett et al. 2008).

Environmental and ontogenetic factors can also influence composition, most readily through effects on body size and condition. Ontogenetic differences in body size clearly alter fish N:P, as will phenological patterns in the type of tissue investment (e.g. lipid stores vs. growth) (Chellappa et al. 1989). Although we have not captured the full range of body size here, the composition-body size relationship that does exist suggests that juvenile resource demands may be quite different than adults. As stickleback are a short lived species with a defined breeding season, these ontogenetic and phenological patterns are likely to generate cyclical patterns in elemental composition and demand (Ostlund-Nilsson et al. 2006).

Environmental differences between locations may also alter composition through plastic influences on the major traits important to elemental composition. Our results find that phenotype-composition relationships are consistent across diverse environments (Fig. 3 and 4). Environmental differences could induce plastic shifts along these relationships, but much of this variation is heritable or related to ontogeny, such that location induced plasticity is not likely to have a major influence on these compositionally important traits. Other differences between environments (captured by location as a random effect) explained only a modest portion of the total variation, despite potentially including both plasticity and local adaptation. Accordingly, we find between-location environment induced plasticity is unlikely to be a major determinant of composition. Rather, it is likely that the indirect effects of location on composition through natural selection (such as on lateral plating) exceeds that from plasticity, and that within-location influences such as ontogeny and phenology are more important than between-location environmental differences.

As we find heritable variation in elemental composition is substantial, it potentially forms an integral part of how ecology and evolution interact. Through heritable variation in composition,

ecology can influence evolution if individuals with unsuitably high resource demands are selected against in resource limited environments (Kay et al. 2005). Conversely, evolution of composition for any reason will affect ecology as individuals with differing requirements must consume or release nutrients differently to meet their unique demand (Matthews et al. 2011). Accordingly, we find that bony traits and their underlying genetics are likely to be important drivers of intraspecific variation in vertebrate interactions with nutrient dynamics.

Conclusions

Our work demonstrates that elemental composition is highly variable and can be explained with phenotypic traits and genetics. We find compositional variation in a vertebrate species is well explained by a small set of bone related traits (lateral plating, pelvis length, bone mineralization and body size) as well as condition. As most of these bony traits are strongly heritable and known to be under natural selection, we conclude that the elemental composition of this species can and has evolved substantially. Whether this evolution of elemental composition has predictable impacts on ecosystem nutrient availability and dynamics remains an important area of future study.

Chapter 2

Stickleback compensate for heritable variation in organismal stoichiometry with diet shifts rather than altered excretion.

Abstract

Research into eco-evolutionary dynamics has shown evolutionary change can be rapid with meaningful influence on ecology, but the mechanics of how evolution drives changes in ecology are largely unknown. For this, Ecological Stoichiometry (ES) may provide insight by simplifying organisms into elemental ratios and applying mass-balance to make predictions for how change in an organism's elemental content should influence resource interactions. ES predicts evolutionary change in elemental content will be compensated by parallel changes in diet (e.g. higher phosphorous individuals will consume higher phosphorus diets) and/or opposing changes in waste (e.g. higher phosphorous individuals will release lower phosphorus waste). We tested these hypotheses by comparing heritable bony trait driven variation in the phosphorus content of threespine stickleback (*Gasterosteus aculeatus*) with differences in dietary phosphorus and excretion rates using 10 natural freshwater populations. We found stickleback compensate for heritable variation in elemental content by altering diet choice and further maximizing dietary resources through changes in gut morphology. Within and across these environments, high phosphorus stickleback consumed a larger proportion of high phosphorus littoral prey and contained longer gastrointestinal tracts to more efficiency process dietary resources. Conversely, phosphorus excretion was unaffected by stickleback phosphorus content and only varied in relation to sex based differences in reproductive investment. These results demonstrate that evolutionary change in elemental content can be balanced through changes in resource

acquisition rather than release, such that evolution may have larger influences on food web structure and abundance than for nutrient dynamics.

Introduction

The ecological drivers of evolutionary change (eco-evo interactions) have long been an important area of study, but evolution also has large effects on ecology (evo-eco interactions) (Schoener 2011). In combination, these interactions potentially generate on-going and complex eco-evolutionary dynamics (Schoener 2011, Reznick 2013). With increasing evidence that natural selection can be meaningful over short periods, a robust understanding of eco-evolutionary dynamics is needed for insight into future ecological change (Schoener 2011). Recent studies have begun this work by demonstrating a diversity of ecological symptoms that can arise from evolutionary change, such as changes in ecosystem structure and function (Harmon et al. 2009, Bassar et al. 2010, Rudman et al. 2015, El-Sabaawi et al. 2015). However robust insight also requires an understanding of the mechanisms by which this occurs, and such knowledge is presently lacking (Schoener 2011, Jeyasingh et al. 2014).

Evolutionary change in a species' resource needs potentially underlies a widespread form of evo-eco interactions (Elser 2006, Matthews et al. 2011). Here, evolution of resource intensive traits such as growth rate, energy stores or costly structures (e.g. antlers, exoskeletons) is hypothesized to alter a species resource needs and consequently, how that species interacts with ecosystem resources (Jeyasingh et al. 2014). Species experiencing evolutionary changes in demand are expected to compensate by altering resource acquisition and release interactions, with potentially large ecological consequences (Jeyasingh et al. 2014). Evolutionary change leading to modified resource acquisition interactions may drive important changes in food web structure and

abundance, while changes in resource release interactions could alter nutrient translocation and cycling rates (Elser 2006, Matthews et al. 2011, Jeyasingh et al. 2014).

Ecological Stoichiometry (ES) is a framework capable of mechanistically integrating evolutionary change in a species' resource needs with ecology (Jeyasingh et al. 2014, Leal et al. 2017). ES uses elements to reduce organisms and resource pools (diet, waste) into stoichiometric ratios (e.g. N:P) and applies mass balance accounting to predict how change in one compartment is reciprocated by the others (Sterner and Elser 2002). This reductionist approach has provided numerous insights into interspecific ecological interactions, but its value for investigating eco-evolutionary dynamics remains uncertain (Vanni et al. 2002, Cross et al. 2005, Stephens et al. 2015, Tobler et al. 2016, Tuckett et al. 2016). Under an ES framework, evolutionary change in resource expensive traits can be detected as change in that species' organismal stoichiometry (hereafter OS, such as organism N:P). Per mass balance, evolutionary change in OS should be balanced by parallel changes in resource acquisition (e.g. high N:P individuals consume high N:P diets) and/or opposing changes in resource release (e.g. high N:P individuals excrete low N:P waste) (Sterner and Elser 2002, Jeyasingh et al. 2014).

Previous studies investigating evolutionary change in OS have commonly found heritable differences between phenotypes and genotypes (Liess et al. 2013, Roy Chowdhury et al. 2014, Tobler et al. 2016). However, attempts to use intraspecific variation in OS to predict differences in ecosystem effects via excretion have consistently found no relation (Tobler et al. 2016, El-Sabaawi et al. 2016, Tuckett et al. 2016). We think this is because organisms may also compensate for changes in demand by altering diet and physiology, which are rarely addressed in these studies (Moody et al. 2015, Vanni and McIntyre 2016). No previous evolution specific

studies on OS and excretion have additionally considered differences in diet stoichiometry, consumption rate and assimilation efficiency - any of which can decouple OS from excretion (Moody et al. 2015, Tobler et al. 2016, El-Sabaawi et al. 2016, Tuckett et al. 2016). Increases in consumption rate should cause waste stoichiometry to more closely resemble diet stoichiometry as the influence of OS wanes, while increases in assimilation efficiency could balance demand by retaining otherwise egested rather than excreted resources (Moody et al. 2015, Vanni and McIntyre 2016). Thus, to gain clearer insight into how species compensate for evolutionary change in OS, we conducted a test with threespine stickleback (*Gasterosteus aculeatus*) that allows and considers variation in diet stoichiometry, consumption rate, assimilation efficiency and excretion stoichiometry in relation to heritable differences in OS.

Threespine stickleback are a widely used model species across ecology and evolutionary biology (Hendry et al. 2013). This small teleost contains striking variation in several defensive armour structures which are often reduced through natural selection in freshwater populations (Hagen and Gilbertson 1972, McKinnon and Rundle 2002, Barrett et al. 2008). As these armour structures are constructed of phosphorus rich bone, phenotypic differences in lateral plating and pelvis size - as well as genetic differences underlying lateral plate variation – result in large differences in OS (Durston and El-Sabaawi In Review). Two other bone related traits (bone mineralization, body size) also explain variation in OS, such that collectively these four traits explain most of the wide variation in stickleback OS (3.0 – 9.4:1 N:P) (Durston and El-Sabaawi In Review).

Stickleback diet also varies widely within and among freshwater populations to include a diverse range of littoral and pelagic invertebrate prey (Lavin and McPhail 1986, Schluter and McPhail

1992). Littoral diets contain mostly chironomids, ostracods and amphipods, while pelagic diets are dominated by calanoid copepods (Lavin and McPhail 1986, Schluter and McPhail 1992, Schluter 1993, 1995, Day and McPhail 1996). These diet types differ widely in stoichiometry, as pelagic calanoid copepods are very low in phosphorus content (0.65 %P; 27 - 38:1 N:P) compared with all littoral prey (1.0 – 1.1 %P; 16 - 21:1 N:P) (Andersen and Hessen 1991, Sterner and Elser 2002, Cross et al. 2003, Frost et al. 2006). Thus, stickleback in general are N:P imbalanced with their diet but especially so for low N:P stickleback on high N:P pelagic diets. These imbalances create costs for the individual, such as increased consumption requirements to avoid deficits of relatively sparse nutrients and physiological costs from regulating excesses of other nutrients (Simpson et al. 2004, Boersma and Elser 2006). As such, compensatory responses in diet choice to minimize imbalances are expected under ES and foraging theory, and have been observed in many species (Buck et al. 2003, Simpson et al. 2004, Berner et al. 2005, Raubenheimer and Jones 2006, Cease et al. 2016, Vanni and McIntyre 2016). At present, this plasticity in diet choice has only been observed as compensation for changes in diet quality, but might also be employed to compensate for evolutionary change in demand (Simpson et al. 2004). If true, low N:P stickleback should consume a larger proportion of littoral prey to reduce dietary imbalances.

Conversely, if stickleback are incapable of compensatory responses in diet choice or if the costs of dietary imbalances are not meaningful relative to the other ecological factors which determine diet choice, then differences in OS should be manifested through changes in consumption rate, assimilation efficiency or excretion stoichiometry. If stickleback modify excretion rates, high N:P (low phosphorus) stickleback should exhibit low excretion N:P, as surplus phosphorus released. Consumption rate and assimilation efficiency are difficult to measure in natural

populations, but gut morphology can provide insight here (Karasov and Douglas 2013). Fish are known to lengthen their gastrointestinal tracts through plastic or evolutionary responses to low digestibility diets, increased consumption volumes or increased assimilation efficiency (Relyea and Auld 2004, Olsson et al. 2007, Wagner et al. 2009, Karasov and Douglas 2013, Sullam et al. 2014). Thus if high N:P stickleback compensate for decreased phosphorous demand via reduction in consumption rate or assimilation efficiency, we expect to see decreases in gut length that are unrelated to diet type (Karasov and Douglas 2013, Sullam et al. 2014)

To investigate these potential OS driven changes in resource interactions, we sampled two polymorphic stickleback populations (Kennedy Lake and Miami River) which are exceptionally diverse in OS while sharing a common environment, providing an excellent opportunity to study OS driven changes in resource interactions. We measured nitrogen and phosphorus excretion rates, gut morphology, OS (as N:P) and diet choice (using $\delta^{13}C$ stable isotopes as pelagic prey are more depleted in ^{13}C than littoral prey) (France 1995, Post 2002, Bolnick et al. 2008, Matthews et al. 2010). To assess whether differences found within these two phenotypically and genetically diverse populations apply broadly across stickleback populations, we sampled eight additional stickleback populations from a diverse range of environments (Table 1), which are more phenotypically similar within populations but collectively represent an even wider range of evolutionary divergence in OS relevant traits (Table 7).

Methods

We collected 312 threespine stickleback from 10 freshwater locations (Table 1) in British Columbia, Canada using the trapping methods described in chapter 1.

Excretion

Upon collection, fish were transferred to 550ml clear plastic containers for 120 minutes to record excretion rates. Each container contained 500mL of filtered (0.2 microns) source water and was temperature controlled +/- 1°C to the trapped habitat. Water samples were withdrawn via a syringe at 0, 40, 80 and 120 minutes after cycling the syringe several times to homogenize the water. Animals were then sacrificed in accordance with our animal use protocol (University of Victoria AUP 2015-006).

Excretion samples were assayed for phosphorus and nitrogen (as ammonium) content using spectrophotometry and fluorometry respectively (Murphy and Riley 1962, Holmes et al. 1999). Excretion rates were calculated for each of the three periods (0-40 min, 40–80 min, 80–120 min) to check for effects of stress and fasting (Whiles et al. 2009). Nitrogen excretion rates were stable over the three periods, while phosphorus showed steady but consistent decline over the three periods, so we averaged the rates for the three periods for each fish to determine excretion N:P. Data were discarded if the coefficient of variation across the three periods exceeded 35% (nitrogen) or 50% (phosphorus). All elemental ratios are molar ratios.

Phenotyping

Fish were phenotyped as described in chapter 1. Additionally, we noted the presence of any internal parasites (predominately *Schistocephalus solidu*s). Gut mass (wet) was converted to a proportion of fish dry mass also to account for covariance with body size. Gut fullness was calculated from gut length – gut mass residuals based on the gut length – gut mass relationship from the full dataset.

Table 7. Phenotypic variation across 10 freshwater study locations (a subset of the 14 locations presented in Table 2). Lateral plate count is the average of both sides of the fish. Head length and pelvis length are proportions of standard length (SL). Pelvis length is the combined ventral length of the anterior and posterior process. Bone mineralization is %P of the 7th lateral plate.

Location	Standard Length (mm)		Lateral Plate Count		Head Length		Pelvis Length		Bone Min. (%P)	
	μ	Range	μ	Range	μ	Range	μ	Range	μ	Range
Cowichan	52.2	43-60	7.2	6-9	.29	.27-.32	.23	.20-.25	11.3	10.8-11.7
Cranby	47.7	29-72	5.2	4-7	.32	.28-.34	.16	.14-.18	10.8	9.3-11.7
Dougan	50.0	40-63	4.6	3-6	.30	.28-.34	.17	.15-.20	10.3	9.3-11.4
Garden Bay	48.1	44-52	7.2	6-9	.29	.26-.30	.20	.18-.22	11.0	9.8-12.1
Harrison	50.0	45-59	33.8	31-36	.29	.26-.32	.24	.22-.27	10.7	9.7-11.3
Kennedy	50.4	36-62	21.5	5-36	.30	.28-.33	.24	.21-.27	11.1	9.8-11.9
Miami	49.0	38-56	24.6	6-35	.29	.26-.33	.24	.18-.26	11.2	10.1-11.9
North	43.8	39-51	33.4	32-34	.30	.28-.32	.21	.19-.22	11.8	11.5-12.1
Sproat	55.1	46-65	8.0	6-13	.29	.27-.32	.23	.21-.26	11.2	10.0-12.0
Trout	42.9	25-59	4.2	2-6	.30	.28-.33	.16	.13-.19	10.5	9.2-11.8
All Locations	49.4	25-72	16.8	2-36	.30	.24-34	.21	.13-.27	11.0	9.2-12.4

Elemental and Isotopic Analysis

Fish were assayed for carbon, nitrogen and phosphorus content as described in chapter 1. Additionally, fish tissues were analyzed for % carbon, $\delta^{13}C$ and % nitrogen using a 1 mg subsample of whole body ground tissue. These samples were run on a Finnigan Delta Plus Advantage mass spectrometer at the University of Victoria with a dogfish muscle standard (NRC Canada DORM-4).

Genotyping

DNA was extracted using Promega Wizard SV96 kits. Due to resource limitations we excluded fish from Garden Bay Lake and Sproat Lake. In total we obtained *Eda* genotypes for 273 of the 312 fish. Stn382 primers were used to target *Eda*, while IDH primers were use to target a master sex determining locus to genetically sex the fish (Peichel et al. 2004). Amplified DNA was run via electrophoresis with ethidium bromide on 2% agarose gel.

Data Analysis

All data analysis was done in R (R Development Core Team 2016) using normal distributions. To investigate whether high stickleback N:P consume a larger proportion of pelagic prey, we used univariate regression, GLMs and GLMMs to investigate whether differences between pelagic (light $\delta^{13}C$) and littoral (heavy $\delta^{13}C$) diets were explained by fish N:P and traits that influence N:P. First, we first compared stickleback N:P and $\delta^{13}C$ within locations using univariate regressions, and across the full dataset using a GLMM containing only fish N:P plus location as a random effect to account for isotopic baseline and other differences between locations. Next, we created global models with a large set of candidate main effects for $\delta^{13}C$ variation within the Kennedy Lake and Miami River populations ("location specific" GLMs) and across all locations ("full dataset" GLMMs). The "full dataset" GLMMs included location as a random effect. For each of these three datasets, we created two sets of candidate main effects (six global $\delta^{13}C$ models in total) which were sex, parasitized status (Y/N), eye diameter, body depth and outer jaw length along with either stickleback N:P or factors known to influence stickleback N:P (*Eda* genotype, standard length, pelvis length and bone mineralization) (Durston and El-Sabaawi In Review). All continuous main effects were standardized to a mean of 0 and a standard deviation of 0.5 to allow comparison of coefficients as an measure of effect size for GLMMs (Gelman

2008). Partial η^2 was calculated using the lsr package (Navarro 2015) as a measure of effect size for GLMs with thresholds of >0.01 (small effect), >0.06 (medium effect) and >0.14 (large effect) (Richardson 2011). Global models were checked for collinearity via VIF scores, with all scores less than 3 (Fox et al. 2016). All six global models were then exhaustively searched and a best model was selected for each based on AICc (Bartoń 2016).

To test whether high N:P stickleback have shorter, lighter or less full gastrointestinal tracts, we compared variation in three response variables (gut length, gut mass and gut fullness) with stickleback N:P for the three datasets (Kennedy, Miami, all locations). We used univariate regressions within Kennedy and Miami and a GLMM for the full dataset containing stickleback N:P plus location as a random effect.

To investigate whether excretion stoichiometry offsets changes in stickleback OS, we constructed location specific global GLMs for Kennedy and Miami, and full dataset global GLMMs for all 10 locations (again with location as a random effect). For these three datasets, we investigated three response variables: phosphorus excretion rate, nitrogen excretion rate and log-transformed excretion N:P (nine global excretion models in total). All nine excretion global models contained standard length, fish N:P, condition (C:N), sex, parasitized status (Y/N), gut fullness and outer jaw length as main effects. Additionally, the full dataset GLMMs added temperature as a main effect. Again, continuous main effects were standardized to a mean of 0 and a standard deviation of 0.5 to allow comparison of coefficients as an measure of effect size for GLMMs (Gelman 2008). Within GLMs we used Partial η^2 as a measure of effect size (Richardson 2011, Navarro 2015). We checked all models for collinearity via VIF scores, which again were always less than

3 (Gelman 2008, Fox et al. 2016). Global models were exhaustively searched and a best model selected based on AICc (Bartoń 2016). Figures were developed using the visreg and ggplot2 packages (Wickham et al. 2016, Breheny and Burchett 2016).

Results

Diet

Significant relationships between stickleback N:P and diet were found in simple regressions (Fig. 6) and best models for Kennedy Lake and Miami River (Table 8). Consistently, low phosphorus (high N:P) fish were more depleted in $\delta^{13}C$, representing a higher proportion of pelagic dietary carbon. In simple regressions, fish N:P explained a substantial amount of the within population $\delta^{13}C$ variation at Miami River ($R^2 = 0.45$; $p < 0.001$) and Kennedy Lake ($R^2 = 0.33$; $p < 0.001$). Elsewhere, we found significant and consistently negative relationships between fish N:P and $\delta^{13}C$ at four of the eight other sites ($R^2 = 0.26 – 0.48$; Fig. S5) despite reduced variation in fish N:P.

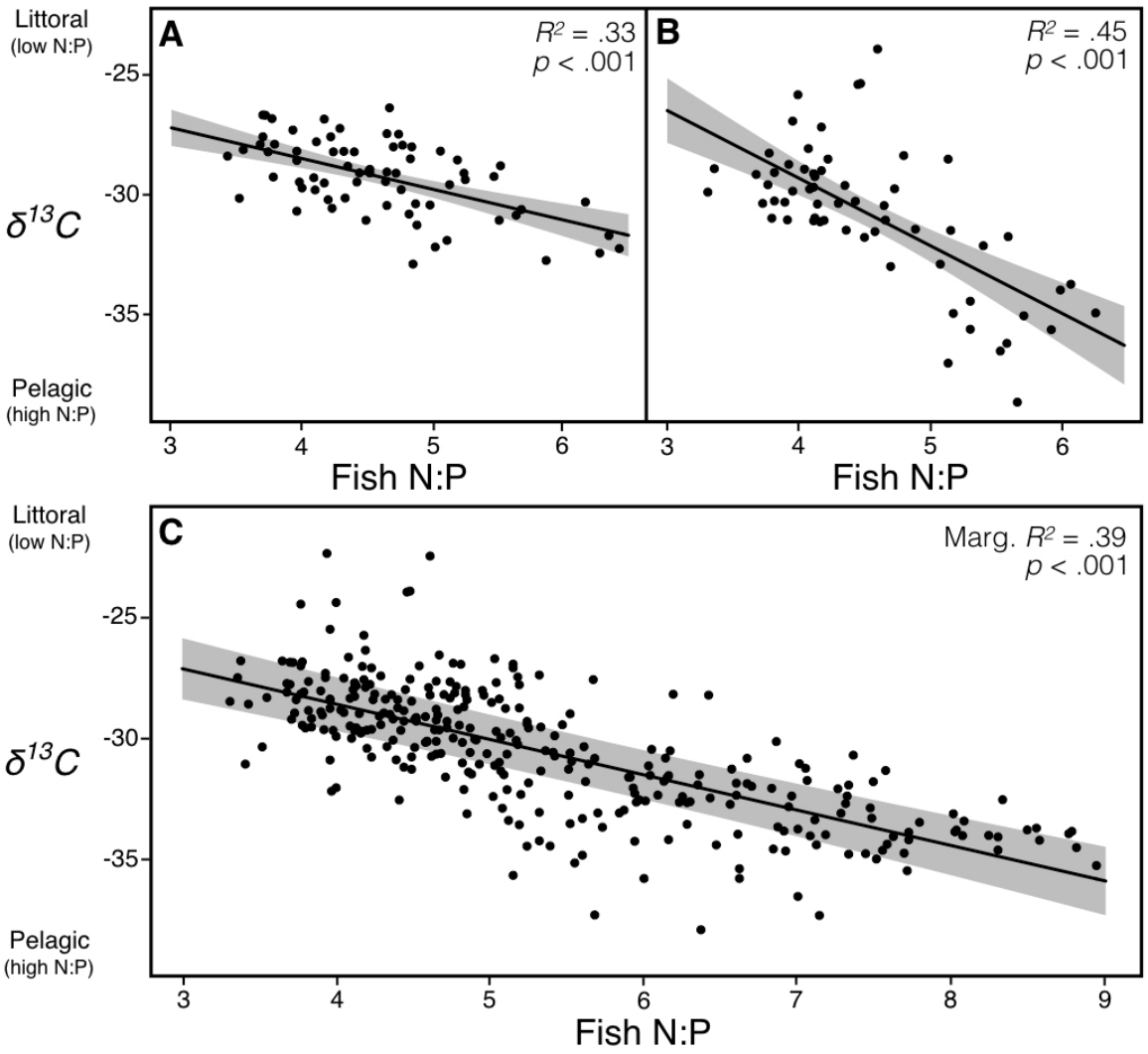

Fig. 6 – Relationships between $\delta^{13}C$ and organismal stoichiometry (N:P) at Kennedy Lake (A), Miami River (B) and across all 10 locations (C). Fish with high N:P ratios were more depleted in $\delta^{13}C$ in all cases. Kennedy and Miami plots are simple regressions, while the all locations plot is the output of a GLMM containing fish N:P plus location as a random effect to account for isotopic baseline differences. Shaded areas depict 95% confidence ranges.

In the best location-specific GLMs for $\delta^{13}C$, fish N:P was consistently the largest effect term with large partial η^2 effect sizes of 0.32 (Kennedy) and 0.46 (Miami) (Table 8). The best $\delta^{13}C$ model at Kennedy also included outer jaw length as a non-significant term, while the best Miami model retained only fish N:P (Table 8). The best full dataset GLMM found fish N:P was again the

largest main effect (-3.6 coefficient; $p < 0.001$) with this best model also containing sex (-0.8 coefficient; $p = 0.001$) and eye diameter (0.5 coefficient; $p = 0.092$) (Table 8).

Table 8. Best models for $\delta^{13}C$ using fish N:P at Kennedy Lake, Miami River and all 10 locations.

	Kennedy Lake (n = 71)			**Miami River** (n = 61)			**All Locations** (n = 312)	
Term	Est.	*p*-value	Par. η^2	Est.	*p*-value	Par. η^2	Est.	*p*-value
***δ*^{13}*C* Model**	$R^2_{Adj} = .34$			$R^2_{Adj} = .45$			$R^2_{Marg} = .37$	
Fish N:P	-3.17	<0.001	.32	-7.19	<0.001	.46	-3.55	<0.001
Outer Jaw Length	.52	.118	.04					
Eye Diameter							.48	.092
Sex (M)							-0.81	.001

When N:P influencing traits and genotypes were substituted for fish N:P in global models, best models found significant relationships between $\delta^{13}C$ and three of these factors: *Eda* genotype, bone mineralization and standard length (Table 9). Consistently, phenotypes and genotypes known to be lower in N:P were more depleted in $\delta^{13}C$ (Fig. 8). Low plated LL *Eda* genotypes were more depleted than fully plated CC *Eda* genotypes within Kennedy Lake ($p = 0.006$), Miami River ($p = 0.006$) and across all locations ($p < 0.001$), as were fish with reduced bone mineralization and smaller body size.

Table 9. Best models for $\delta^{13}C$ using N:P influencing traits in place of fish N:P at Kennedy Lake, Miami River and all 10 locations.

	Kennedy Lake (n = 71)			**Miami River** (n = 61)			**All Locations** (n = 273)	
Term	Est.	*p*-value	Par. η^2	Est.	*p*-value	Par. η^2	Est.	*p*-value
***δ*^{13}*C* Model**	R^2_{Adj} = .31			R^2_{Adj} = .49			R^2_{Marg} = .13	
Standard Length	1.07	.010	.10	5.97	<0.001	.35	1.37	<0.001
Bone Mineralization	1.49	.002	.14	1.77	.045	.07	0.72	.009
Eda LC genotype	-0.18	.660	.13	-1.14	.063	.14	-.67	.084
LL genotype	-1.19	.006		-2.30	.006		-1.85	<0.001
Sex (M)	-0.92	.031	.07				-1.16	<0.001
Eye Diameter							1.28	<0.001
Outer Jaw Length	0.84	.081	.05					

Gut Length, Mass and Fullness

At Kennedy, Miami and across all locations, stickleback N:P was negatively related to gut length (Fig. 7 A-C). High N:P stickleback had significantly shorter gastrointestinal tracts within Miami River (R^2 = 0.24; p < 0.001), Kennedy Lake (R^2 = 0.12; p = 0.004), and across all locations (R^2_{Marg} = 0.15, p < 0.001) (Fig 7 A-C). Gut mass showed no relationship with stickleback N:P within the Kennedy and Miami populations but was lower for high N:P stickleback in the full dataset GLMM (Fig. 7 D-F). High N:P stickleback at Kennedy and Miami had significantly higher gut fullness as a result of reduced gut length but not mass, whereas gut fullness showed no relationship with stickleback N:P in the full dataset (Fig. 7 G-I).

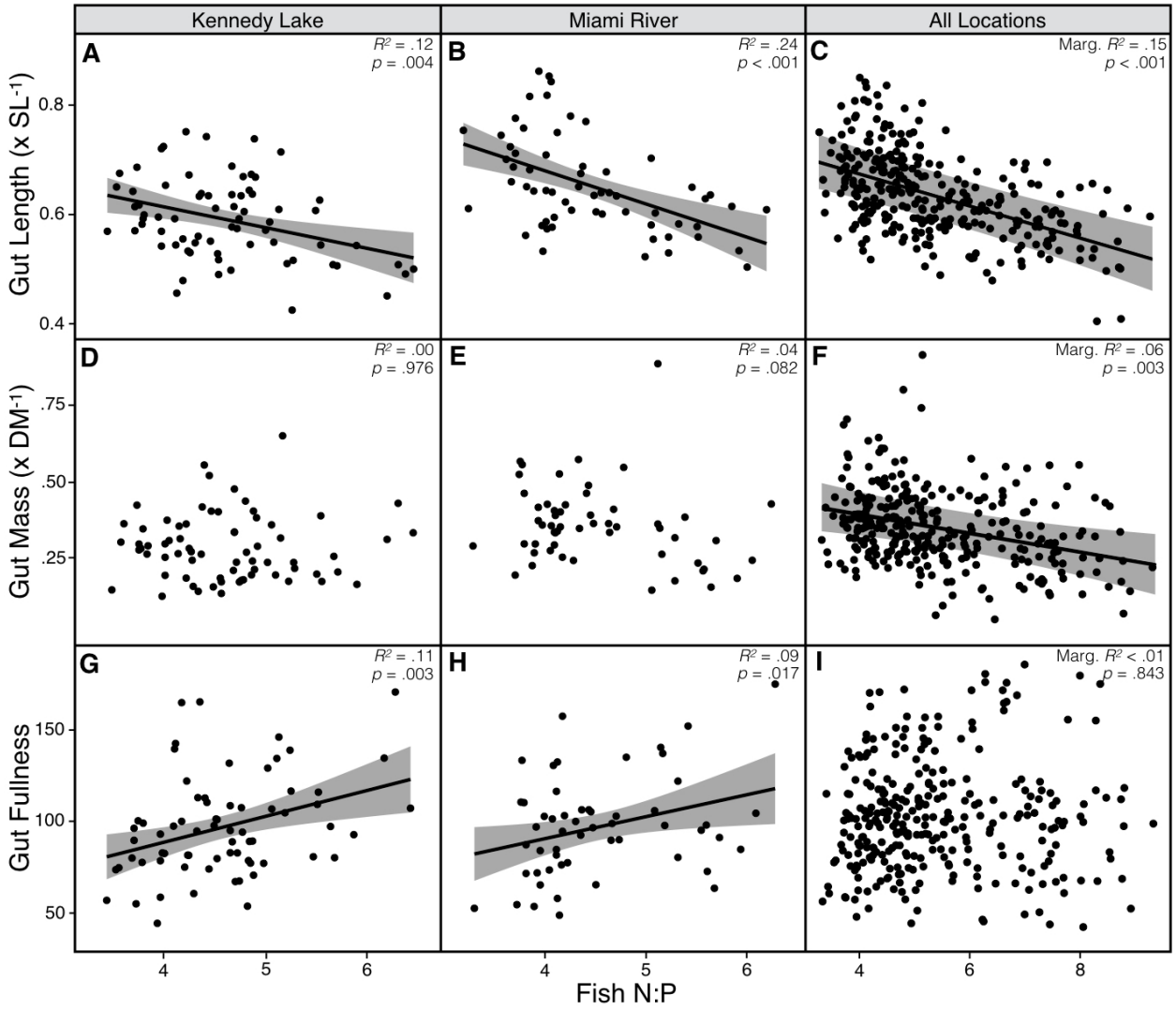

Fig. 7 – Relationships between stickleback OS (N:P) and gut traits. Gut length and mass are expressed as proportions of standard length (SL) and dry mass (DM) respectively. Kennedy and Miami plots are simple regressions, while all locations plots are the output of GLMMs comparing each gut trait to fish N:P plus location as a random effect. In all instances, gut length significantly declined with increased in stickleback N:P. Shaded areas depict 95% confidence ranges.

Excretion

Phosphorus and nitrogen excretion rates were highly variable within Kennedy Lake and Miami River, and across all locations (Table 10). Mean phosphorus excretion was 0.21 - 0.22 μg/min in all three cases, while mean nitrogen excretion ranged from 0.9 – 1.3 μg/min. Excretion N:P

varied widely from 3 – 45:1 across all locations, with median N:P ratios of 10:1 (Kennedy), 13:1 (Miami) and 10:1 (all locations) (Table 10).

Table 10. Rates (μg*minute^{-1} (min)) and stoichiometry of stickleback excretion. Elemental ratios are molar ratios.

	Phosphorus (μg/min)		**Nitrogen (μg/min)**		**Excretion N:P**	
	Range	Mean	Range	Mean	Range	Median
Kennedy Lake	0.05 – 0.51	0.21	0.4 – 2.3	0.9	4-26:1	10:1
Miami River	0.08 – 0.48	0.21	0.6 – 1.9	1.3	5-43:1	13:1
All Locations	0.02 – 0.66	0.22	0.2 – 2.4	1.0	3-45:1	10:1

The best excretion models at Miami and Kennedy explained a substantial portion of the variation in both phosphorus and nitrogen excretion (R^2_{Adj} = 0.47 - 0.56; Table 11), as did the best all locations GLMMs (R^2_{Marg} = 0.50 - 0.52; Table 11). These models found significant increases in nitrogen and phosphorus excretion with increased gut fullness (all models) and standard length (Kennedy and all locations models) (Table 11). Standard length was not retained in the best Miami models. All three phosphorus models found higher phosphorus excretion in females, but no differences between sexes in nitrogen excretion. The all locations GLMMs also found significant increases in nitrogen excretion and non-significant increases in phosphorus excretion with temperature (Table 11).

Models for excretion N:P explained only a modest portion of the variation ($R^2_{Adj/Marg}$ = 0.10 - 0.22, Table 11). In all cases, fish N:P was not retained in the best models for excretion N:P. The only consistent pattern across the best excretion N:P models was significantly lower excretion N:P in females (Fig. 9). We also found an effect of standard length only at Kennedy and an effect of temperature across all locations, with nitrogen excretion rising faster than phosphorous excretion such that N:P rises with temperature (Table 11).

Table 11. Best models based on AICc for excretion rates and ratios at Kennedy Lake, Miami River and for all locations combined. Only excretion N:P was log transformed.

	Kennedy Lake (n = 62)		**Miami River** (n = 43)		**All Locations** (n = 253)	
Term	Est.	*p*-value	Est.	*p*-value	Est.	*p*-value
Phosphorus Excretion	R^2_{Adj} = .56		R^2_{Adj} = .47		R^2_{Marg} = .50	
Standard Length	.159	<0.001			.142	<0.001
Sex (Male)	-.046	.008	-.069	.015	-.069	<0.001
Gut Fullness	.048	.003	.107	<0.001	.045	<0.001
Fish C:N	-.039	.111				
Fish N:P			-.144	.001		
Temperature					.028	.091
Nitrogen Excretion	R^2_{Adj} = .48		R^2_{Adj} = .50		R^2_{Marg} = .52	
Standard Length	.484	<0.001			.551	<0.001
Gut Fullness	.310	<0.001	.352	<0.001	.211	<0.001
Fish C:N	-.241	.022			-.195	<0.001
Fish N:P			-.928	<0.001		
Parasitized (Yes)	.230	.086				
Temperature					.190	.002
N:P Excretion	R^2_{Adj} = .22		R^2_{Adj} = .15		R^2_{Marg} = .10	
Standard Length	-.156	<0.001				
Sex (Male)	.075	.029	.173	.006	.135	<0.001
Temperature					.054	.115

Discussion

Understanding how organisms compensate for evolutionary changes in trait investment is an essential component of understanding eco-evolutionary dynamics (Leal et al. 2017). We find organismal trait investment is not compensated for by modifying excretion rates, as widely expected, but rather by targeting more balanced diets and modifying physiological traits to optimize these inputs (Tobler et al. 2016). We observed stickleback which invest more in phosphorus rich bony traits target higher phosphorus littoral diets and maximize these resources through longer gastrointestinal tracts which enable higher consumption rates or assimilation efficiency. Within both Kennedy Lake and Miami River populations, stickleback N:P explained intraspecific variation in diet choice and gut length but not excretion stoichiometry, indicating that changes in resource acquisition and processing rather than release, are the principal ways in which variation in stickleback OS affects ecosystems. Our full dataset with eight additional stickleback populations provided additional support for this finding, as stickleback OS continued

to explain inter and intra population differences in diet choice and gut morphology but not excretion across a diverse range of phenotypic and environmental variation.

When we replaced stickleback OS in diet models with phenotypic and genetic factors known to drive variation in OS, we consistently found less bony traits and genotypes associated with pelagic diets (Fig. 8) (Durston and El-Sabaawi In Review). At both Kennedy Lake and Miami River we found diet choice was predicted by *Eda* genotype, with low plated genotypes more depleted in $\delta^{13}C$ than fully plated genotypes. Similarly, fish with reduced bone mineralization or smaller body size consumed a higher proportion pelagic prey. The causes of variation in bone mineralization and body size among adult stickleback are not fully known, but body size has a large heritable component and differences in bone mineralization are thought to be more heritable than plastic (see Chapter 1). Pelvis size was the only N:P influential trait that was not a predictor of diet choice, but the Kennedy and Miami populations contained only a narrow portion of the phenotypic variation in this trait that is observed elsewhere (Table 7) (Lescak et al. 2013). Overall, the consistent direction of the *Eda*, body size and bone mineralization patterns, as well as the greater explanatory power of stickleback N:P than these individual factors combined (based on model fit, see Tables 8 and 9), suggests that variation in OS is the underlying cause of this intraspecific variation in diet choice.

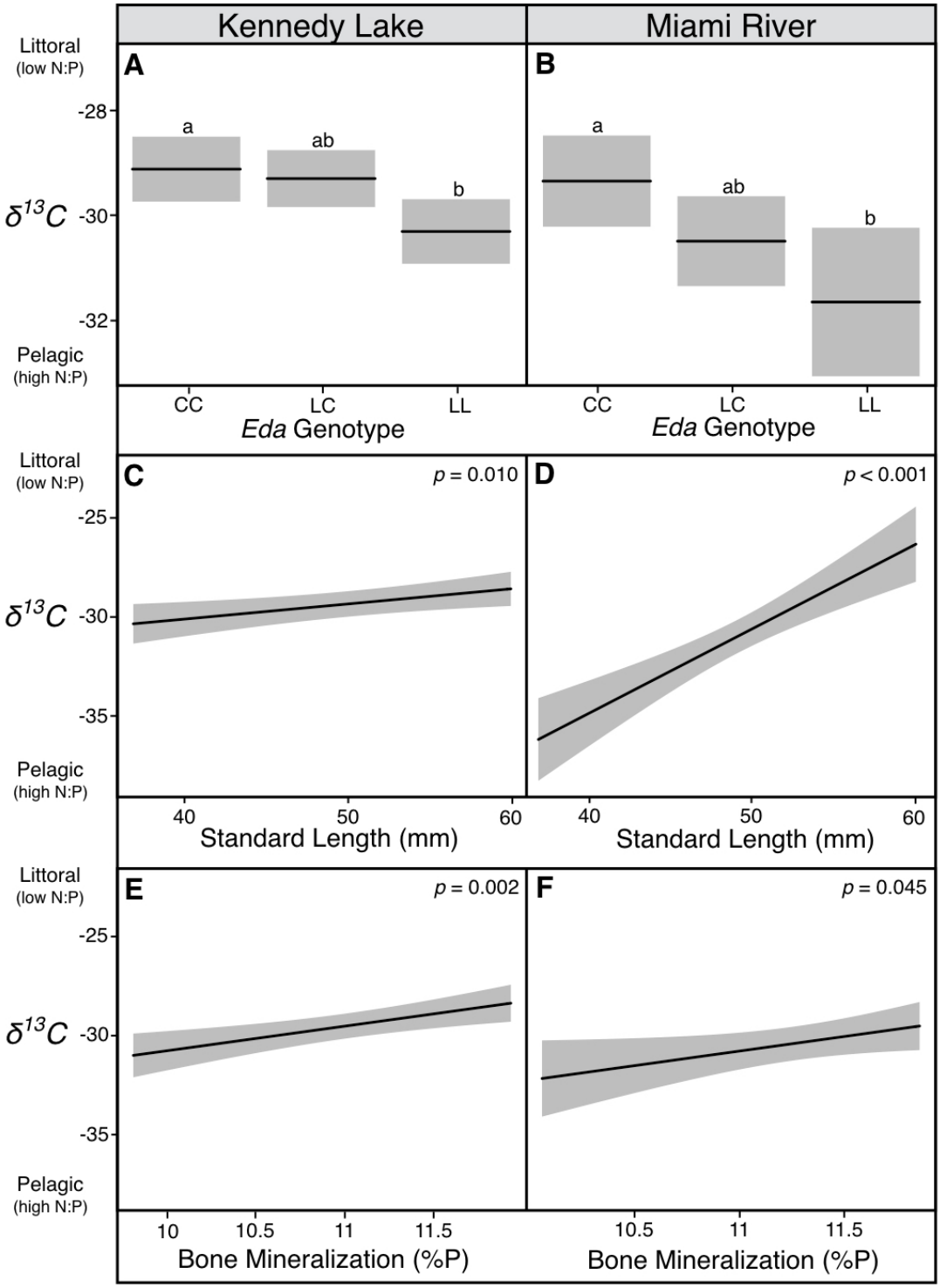

Fig. 8 – Modelled relationships between $\delta^{13}C$ and phenotypic traits or genotypes with high N:P influence. Plots are the outputs of the best GLM for $\delta^{13}C$ at each location (Table 9). Low armour genotypes (LL) were significantly more depleted in $\delta^{13}C$ than full armour genotypes (CC) at both Kennedy Lake ($p = 0.009$) and Miami River ($p = 0.006$). Similarly, significant depletions in $\delta^{13}C$ occurred with increases in phenotypic traits that elevate fish N:P (standard length, bone mineralization). Shaded areas depict 95% confidence ranges.

Alternative explanations for the relationships between stickleback OS and diet include tissue specific patterns of $\delta^{13}C$ enrichment correlated with N:P, or diet influenced plasticity in OS (Matthews and Mazumder 2004). The latter is unlikely, as we relate $\delta^{13}C$ in part to genetics and previous work has shown much of the variation in stickleback OS is heritable (Durston and El-Sabaawi In Review). It is possible that OS plasticity strengthens these patterns, as the causes of variation in bone mineralization are largely unknown, but there is insufficient room for plasticity to be the major driver (Durston and El-Sabaawi In Review). Tissue specific fractionation is also unlikely to be a large influence, as the bony traits which are the major determinants of stickleback N:P contain little carbon and stickleback N:P is not strongly correlated with traits that do (e.g. condition) (Durston and El-Sabaawi In Review). Further, previous work also at Kennedy Lake has found a similar relationship between *Eda* genotype and $\delta^{13}C$ using only muscle tissue rather than whole body samples, ruling out tissue specific fractionation at least in this case (Marchinko et al. 2014).

Stickleback N:P was also a predictor of gut length - providing additional evidence that OS mediates stickleback interactions with ecosystem resources (Fig. 7). We consistently found high N:P stickleback had shorter gastrointestinal tracts than low N:P fish (Fig. 7). In other species, variation in gut length arises as a plastic or evolutionary response to changes in diet digestibility, consumption rate or assimilation efficiency (Olsson et al. 2007, Karasov and Douglas 2013, Sullam et al. 2014). Here we find gut length is much better predicted by OS (Fig. 7) than diet (Kennedy R^2 <0.01, Miami $R^2 = 0.11$, all locations $R^2_{Marg} = 0.08$), indicating that the observed differences in gut length represent changes in consumption rate or assimilation efficiency rather than diet type. Further supporting changes in gut length as an additional compensatory response,

we find the combination of diet ($\delta^{13}C$) and gut length predicts stickleback N:P substantially better (Miami $R^2_{Adj} = 0.53$; Kennedy $R^2_{Adj} = 0.39$) than either term alone (Table 8, Fig. 7 A-C).

Parsing out consumption rate and assimilation efficiency as causes of gut length variation is difficult using ephemeral metrics of gut mass and fullness. Changes in gut length from decreased assimilation efficiency should increase gut fullness as similar food volumes are processed in less space, while compensation in gut length for changes in consumption rate should leave gut fullness unaffected (Karasov and Douglas 2013). Here we find gut fullness is higher in high N:P fish at Kennedy and Miami - indicating low phosphorus fish have reduced assimilation efficiency - but this finding does not hold across the larger dataset where perhaps both causes are at play. Regardless of this uncertainty, changes for either reason reduce the acquisition of surplus resources as expected for high N:P fish and demonstrate that stickleback modify physiological or behavioural traits to optimize resource inputs.

Unlike diet and gut morphology, we found no evidence of a relationship between stickleback OS and excretion, indicating the aforementioned changes in diet type and gut morphology fully compensate for variation in OS. Such a finding is notable, as the vast majority of inter and intraspecific studies on ecosystem effects from OS variation have focused on excretion but usually with low explanatory power (Allgeier et al. 2015, Tobler et al. 2016, Tuckett et al. 2016, Vanni and McIntyre 2016). Our results provide evidence that changes in resource acquistion and processing, rather than release, are used to compensate for variation and evolution of OS and should be considered alongside variation in waste release dynamics.

While OS was unrelated to excretion, we did observe significantly higher phosphorus excretion (+25-40%) in females, yet with similar nitrogen excretion such that female excretion N:P was significantly lower (Fig. 9). These observations were made during the breeding season, where females invest heavily in low phosphorus reproductive tissues (1.1 %P, unpublished data) while males invest only in higher phosphorus somatic tissues (2.2 – 6.5 %P) (Chapter 1). We suspect this difference in investment underlies the observed differences in excretion, such that short term differences in reproductive investment are reflected in excretion rates even while long term differences in somatic investment are not.

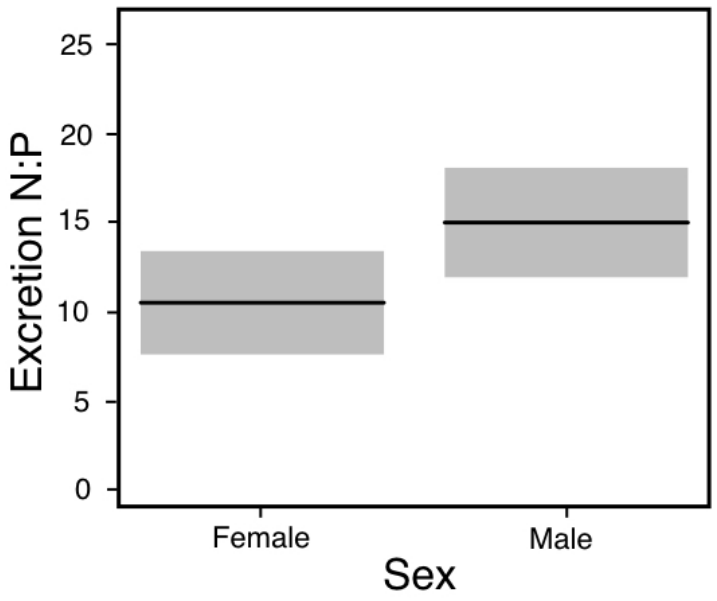

Fig. 9 – Modelled relationships for excretion N:P showed male stickleback excrete at significantly higher molar N:P ratios than females (14.7:1 vs 10.7:1; $p < 0.001$; Table 11). Shaded areas depict 95% confidence ranges.

Species can theoretically modulate four factors to balance resource accumulation with OS investment: diet type, consumption rate, assimilation efficiency and excretion (Sterner and Elser 2002, Moody et al. 2015, Vanni and McIntyre 2016). Of these, increasing excretion as compensation for reduced demand is the most costly solution as it involves competing for and

assimilating - but then releasing - surplus resources (Simpson et al. 2004). Less costly is reducing assimilation efficiency or consumption rate, but the optimal solution is modifying diet choice such that diet resources are balanced with OS (Simpson et al. 2004, Boersma and Elser 2006). Thus while previous studies have largely focused on excretion – perhaps on the assumption that species have limited ability to modify other resource interactions – there is good evidence that a wide range of taxa can modify diet choice and theory suggests this is the optimal response (Buck et al. 2003, Simpson et al. 2004, Berner et al. 2005, Raubenheimer and Jones 2006, Allgeier et al. 2015, Cease et al. 2016, Vanni and McIntyre 2016). In combination with our results, we think it is likely that the ecological effects of evolutionary change in OS are larger for resource acquisition interactions than excretion.

While excretion rates were unaffected by OS, we did find large differences in phosphorus excretion and excretion N:P in relation to reproductive investment (Table 11). We suspect this is because changes in diet choice and gut length are poorly suited responses to short term changes in demand, whereas excretion can be rapidly modified (Day and McPhail 1996, Olsson et al. 2007). A switch between littoral and pelagic prey reduces stickleback foraging efficiency for months until restored by morphological and behavioural plasticity (Day and McPhail 1996). Similarly, plastic changes in gut morphology occur slowly and have substantial energetic costs (Olsson et al. 2007). Thus, both of these compensatory responses are likely too slow and expensive to modify for a relatively short breeding season. Conversely, excretion can be modified rapidly with little in the way of apparent costs - as even here we see excretion varies in relation to ephemeral differences in gut fullness (Table 11). Thus, switching costs favour excretion compensation for short term changes in investment, while diet shifts and altered gut morphology are more efficient ways of compensating for long term differences in investment from OS. We

suspect this is a general principle and expect the direct consequences of OS evolution are larger for food web structure and prey abundance than for nutrient dynamics. We recommend future work consider all of these potential evo-eco interactions including their costs, plasticity and adaptive potential in response to varying magnitudes and durations of investment.

Conclusions

We find stickleback compensate for heritable variation in organismal stoichiometry with changes in diet choice and gut morphology, rather than modified excretion rates. Conversely, short term differences in reproductive investment are reflected in excretion stoichiometry, which is likely a less efficient but more easily modified means of balancing resource demands. By compensating for OS using resource acquisition mechanisms rather than excretion, we find OS evolution may generate previously underappreciated ecological consequences including effects on food web structure and abundance. The utility of organismal stoichiometry in predicting these diet shifts supports ecological stoichiometry as a useful framework for understanding eco-evolutionary dynamics when all facets of resource interactions are taken into account.

Conclusions

Ecological stoichiometry has been heavily studied for more than two decades but over this time intraspecific variation in organismal stoichiometry has largely been dismissed or attributed to environmental plasticity. Only recently have studies demonstrated heritable variation and evolved differences between lineages. My work expands upon this by mapping large differences in organismal stoichiometry with variation in individual phenotypic traits and alleles. Here I find four bony traits (lateral plating, pelvis size, bone mineralization and body size) that explain the majority of variation in stickleback N:P. Additionally, I show allelic variation for a single gene underlying lateral plating can meaningfully alter stickleback N:P. As natural selection on several of these traits and genetics is known to be rapid, these results show organismal stoichiometry can also evolve rapidly (Barrett et al. 2008).

Whether this variation and evolution of organismal stoichiometry has ecological consequences was the primary goal of this thesis. Here I found organismal stoichiometry does influence ecosystem interactions but not in the expected manner. Like several other recent studies, I expected evolutionary changes in organismal stoichiometry would be reflected in excretion stoichiometry (Tobler et al. 2016, Tuckett et al. 2016). However excretion was unrelated to organismal stoichiometry, and instead I found organisms compensate using previously undemonstrated mechanisms of altered diet choice and gut morphology. More specifically, stickleback with elevated phosphorus requirements were found to consume more phosphorus rich prey, and also had modified gut morphology to more efficiently process dietary resources.

Significance for Ecological Stoichiometry

These results have important implications for Ecological Stoichiometry (ES) as they demonstrate a need to look beyond excretion rates. Both inter and intraspecific studies have commonly focused on excretion without consideration of diet, but typically with low predictive power (Allgeier et al. 2015, Vanni and McIntyre 2016). These results provide an explanation for this by demonstrating animals also compensate for differences in organismal stoichiometry by modifying diet choice. It is likely that past studies have focused on excretion because it is widely recognized as something organisms can modulate easily and it is ecologically important, but these show excretion is not the only compensating response organisms use (Vanni and McIntyre 2016).

The observed differences in gut morphology suggest that organisms also modify resource processing traits - such as consumption rate - to balance changes in demand. This illustrates the need to not only consider the stoichiometry of biological compartments, but also the magnitude of flows between them. Even when diet and organism stoichiometry are invariable, differences in consumption rate will alter waste stoichiometry. Similarly, differences in growth rate should alter excretion stoichiometry even in the absence of changes in organismal stoichiometry. Accordingly, the central equation of ES (Equation 1) is unreliable if flow rates between compartment also change, which is common (Carrillo et al. 2001, González et al. 2014).

Equation 1: $Diet_{Stoich} = Organism_{Stoich} + Waste_{Stoich}$

In chemistry, stoichiometry equations consist not only of ratios but also contain information on the amounts of each component to achieve mass balance. In light of evidence here and elsewhere that growth and consumption rates vary meaningfully, the same should be done for ES to include

information on rates of change for each resource pool (Equation 2). This need to consider both ratios and amounts has been articulated previously but continues to be largely disregarded, seemingly because several of these components are difficult to measure and may require novel experimental approaches (Schindler and Eby 1997).

Equation 2: $Diet_{Stoich} \; x \; Consumption_{Rate} = Organism_{Stoich} \; x \; Growth_{Rate} + Waste_{Stoich} \; x \; Waste_{Rate}$

By considering variation in both the rates of change and stoichiometry of each compartment, this equation is mass balanced without implicit assumptions. Consideration of rates does make investigation more difficult, but it is likely necessary if ES is to achieve robust predictive ability (Schindler and Eby 1997, Moody et al. 2015). Also of interest, equation 2 demonstrates that changes in growth rate should influence waste stoichiometry even in the absence of changes in organismal stoichiometry. This potential direct effect of growth rate on waste stoichiometry has never been parsed from the widely credited indirect effect, where growth rate influences waste stoichiometry because phosphorus rich RNA required for growth affects organismal stoichiometry, and this affects excretion (Sterner and Elser 2002). As this example shows, a lack of consideration of rates creates the risk of attributing correct patterns to incorrect mechanisms.

Significance for Eco-Evo Dynamics

These results provide evidence that changes in nutrient dynamics may not be the primary way in which evolution of resource expensive traits influences ecology. Rather, if evolutionary change in these traits is balanced by modifying diet choice and consumption rates, then the effects on food web structure and abundance are likely much larger. While more work is needed on the

generality of this finding, this result is potentially of great importance to our understanding of eco-evolutionary dynamics.

As a corollary to this, understanding how trait evolution affects ecology may provide additional insight into subsequent natural selection (or eco-evolutionary feedbacks). If evolutionary change in expensive traits alters diet choice, this may generate further selection on diet related traits such as foraging behaviour and trophic morphology. Diet choice is a complex decision influenced by many factors including prey availability and competition, but if variation in resource demand also influences diet choice then further evolution should generate correlations between resource expensive traits and foraging traits. Correlations between diet choice and foraging traits have already been observed in many habitats, some of which may be the result of eco-evolutionary feedbacks as postulated here (Matthews et al. 2010)

Future Work

An important area of subsequent research is to link these observed changes in resource interactions (e.g. diet choice, consumption rate) with changes in ecosystems. A mesocosm study comparing fish with different resource demands (OS) could provide stronger evidence that variation in resource expensive traits influences ecology through differences in diet choice and consumption rates. In a study where high, medium and low N:P stickleback are introduced to equivalent environments, I expect mesocosms with low N:P fish will be less prey abundant and contain a disproportionate amount of pelagic prey.

An interesting follow up study would be to study natural selection in these potentially modified habitats to investigate eco-evolutionary feedbacks. Here I expect selection on trophic traits to

match changes in diet choice, but also selection favouring opposite organismal stoichiometry from the original population as these fish can best utilize resources previously under exploited by the original inhabitants. This could lead to stabilizing selection favouring intermediates in both organismal stoichiometry and trophic traits, or divergent selection favouring specialization in both trophic morphology and organismal stoichiometry.

Lastly, it would be valuable to conduct similar research across a diverse range of species to determine the generality of these findings. Determining whether similar mechanisms of compensation are used in other species would support these findings as a general pattern underlying eco-evolutionary dynamics.

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Appendix 1: Chapter 1 Supplementary Information

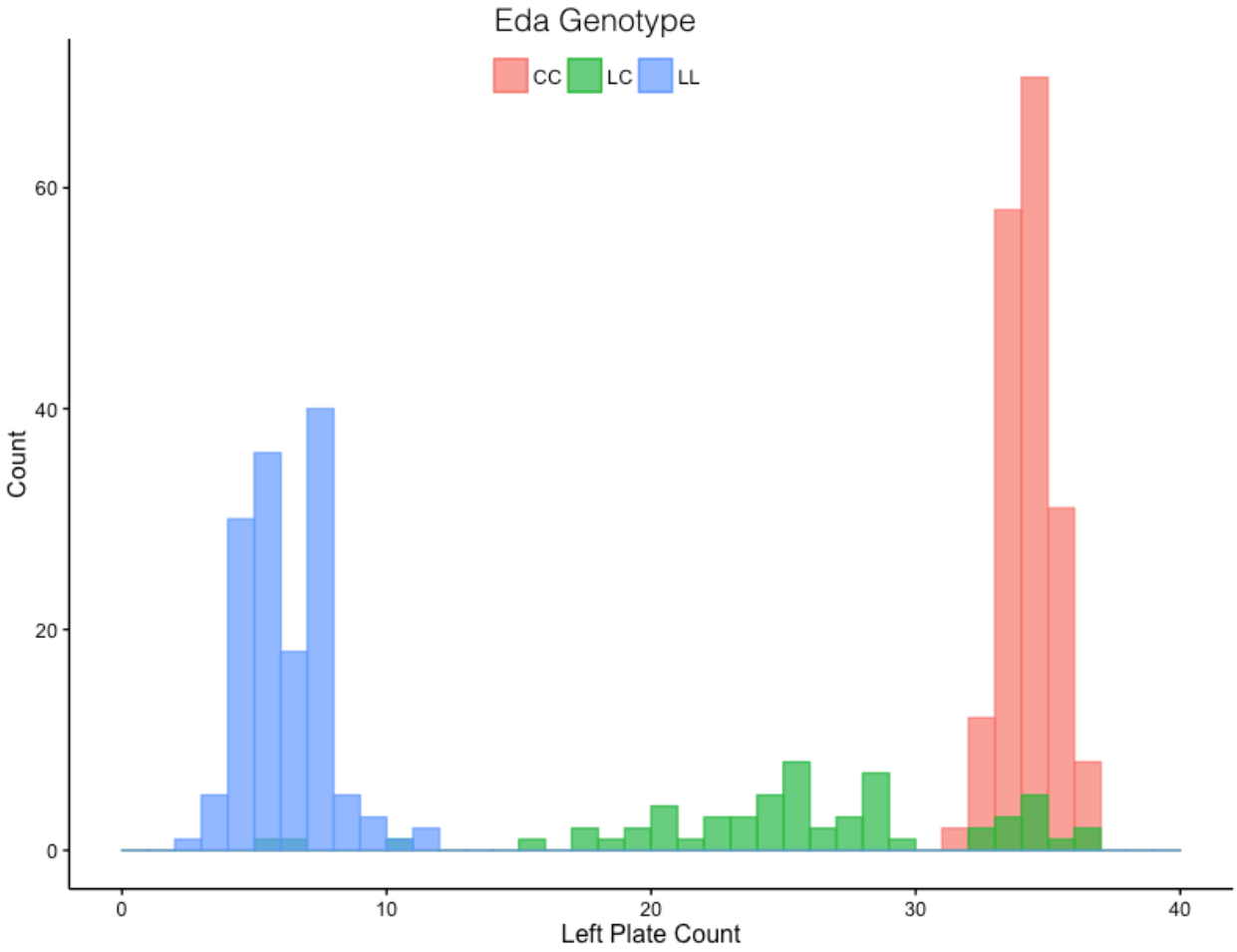

Fig. S1. Phenotype-genotype relationship for *Eda* genotype and lateral plate count (average of both sides).

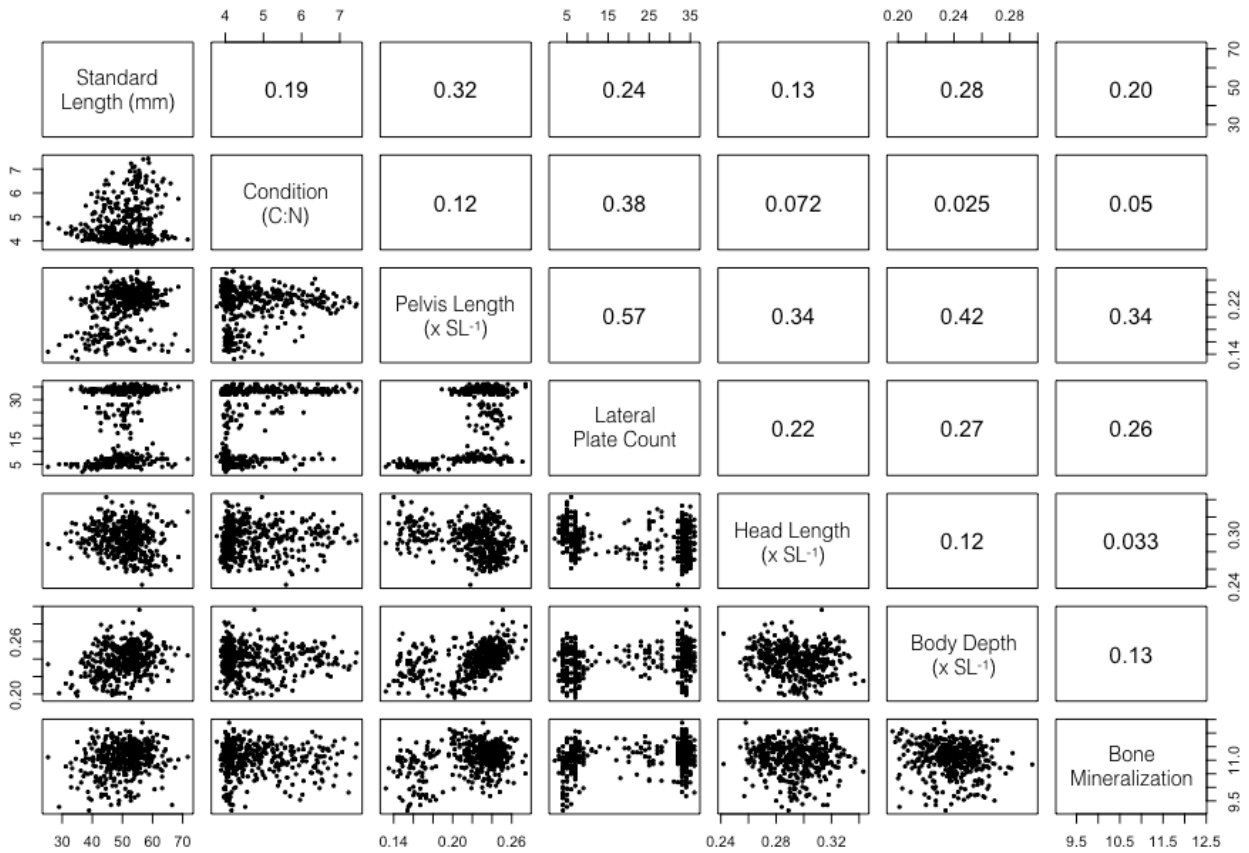

Fig. S2 Correlation matrix of phenotypic candidate model terms for the full dataset. In all models, variation inflation factor (VIF) scores were always less than 4.

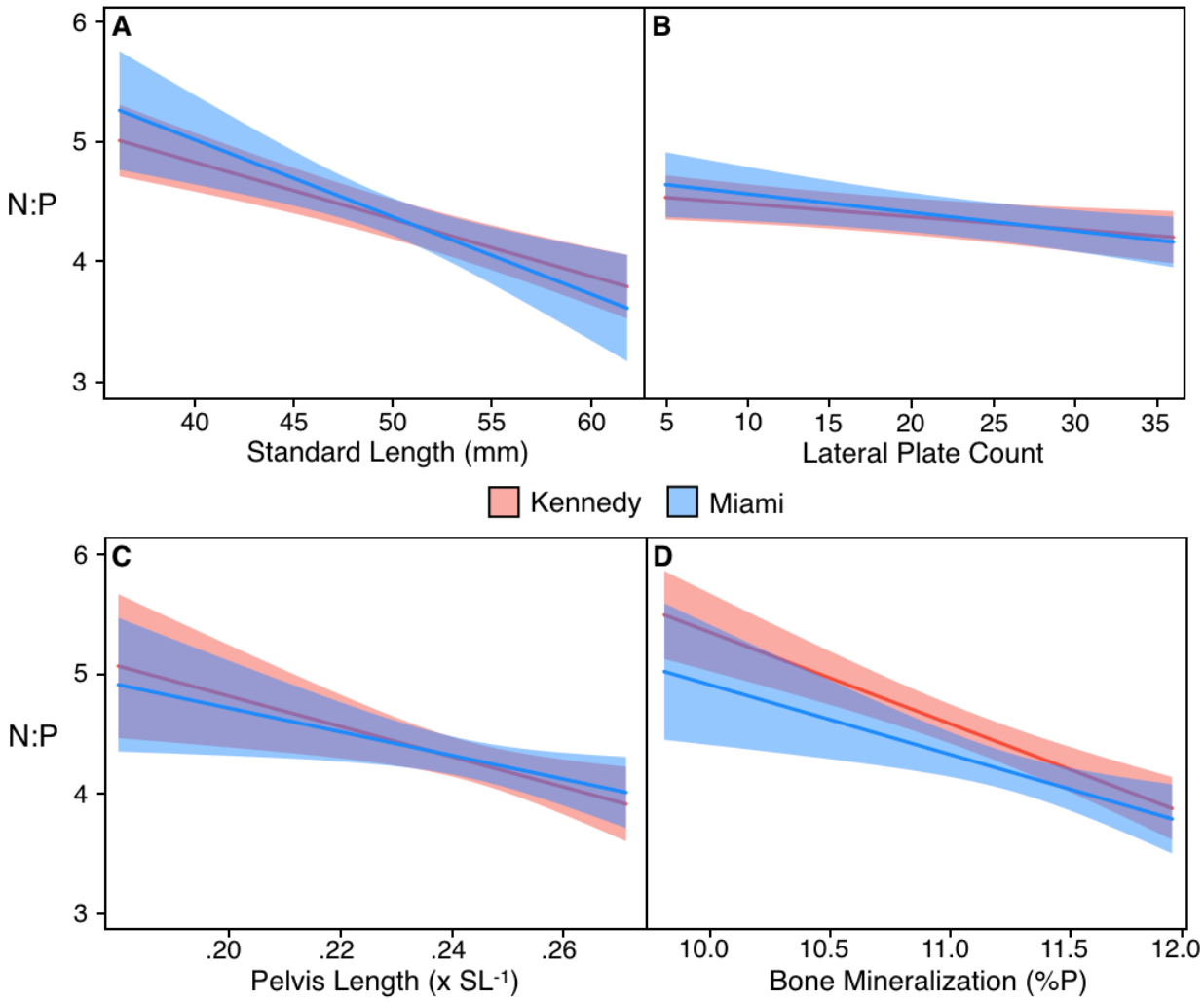

Fig. S3. Relationships between phenotypic traits and N:P at Kennedy and Miami. Plots are outputs from the location specific GLMs (Table 1). N:P declines significantly at both sites with standard length (Panel A; SL), lateral plate count (B) and bone mineralization (D). N:P declines significantly with pelvis length only at Kennedy. Shaded regions depict 95% confidence ranges.

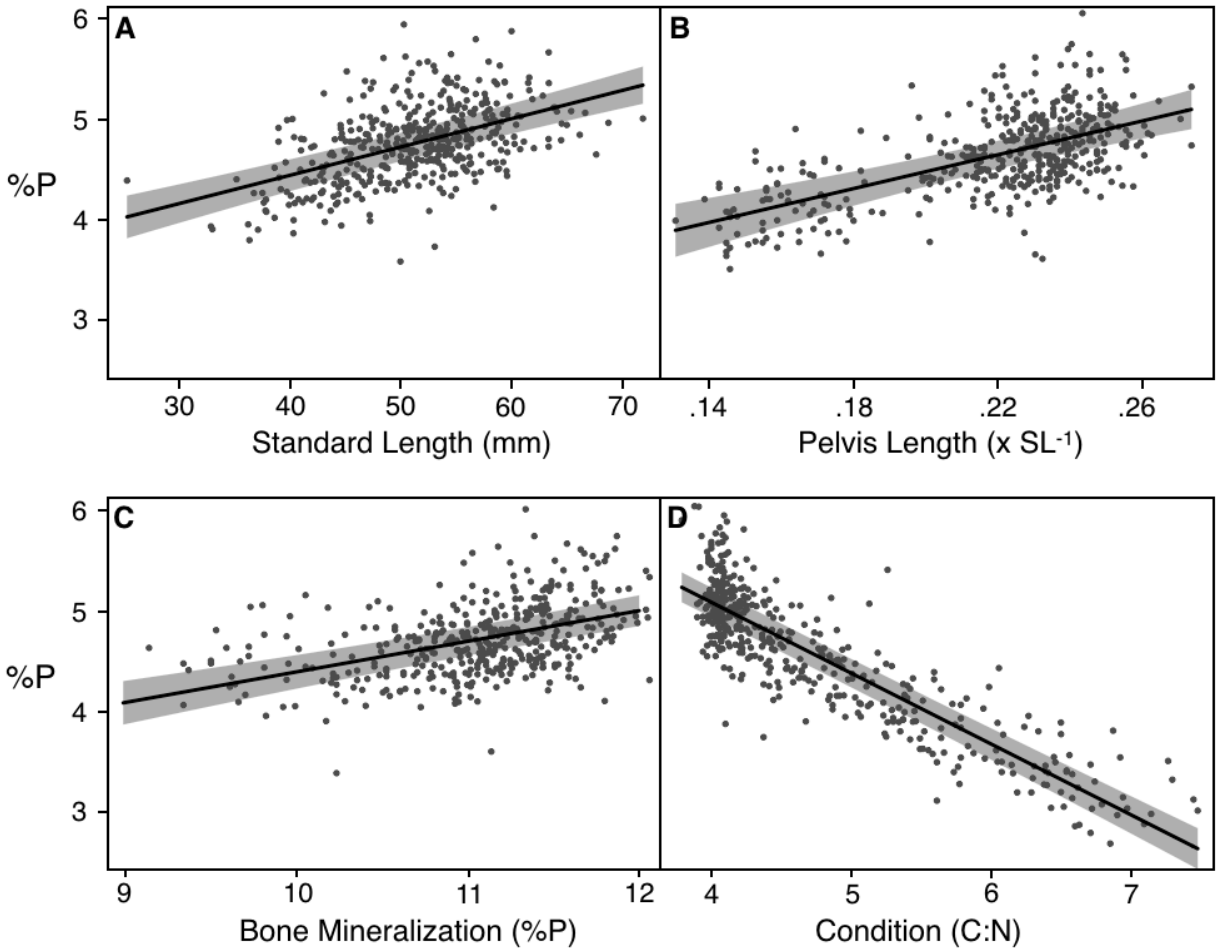

Fig. S4. Relationships between %P and phenotypic traits for the best full dataset GLMM (Table 2). %P increased significantly ($p < 0.001$) with standard length (SL), pelvis length and bone mineralization while decreasing with increases in condition (C:N). Shaded regions depict 95% confidence ranges.

Appendix 2: Chapter 2 Supplemental Information

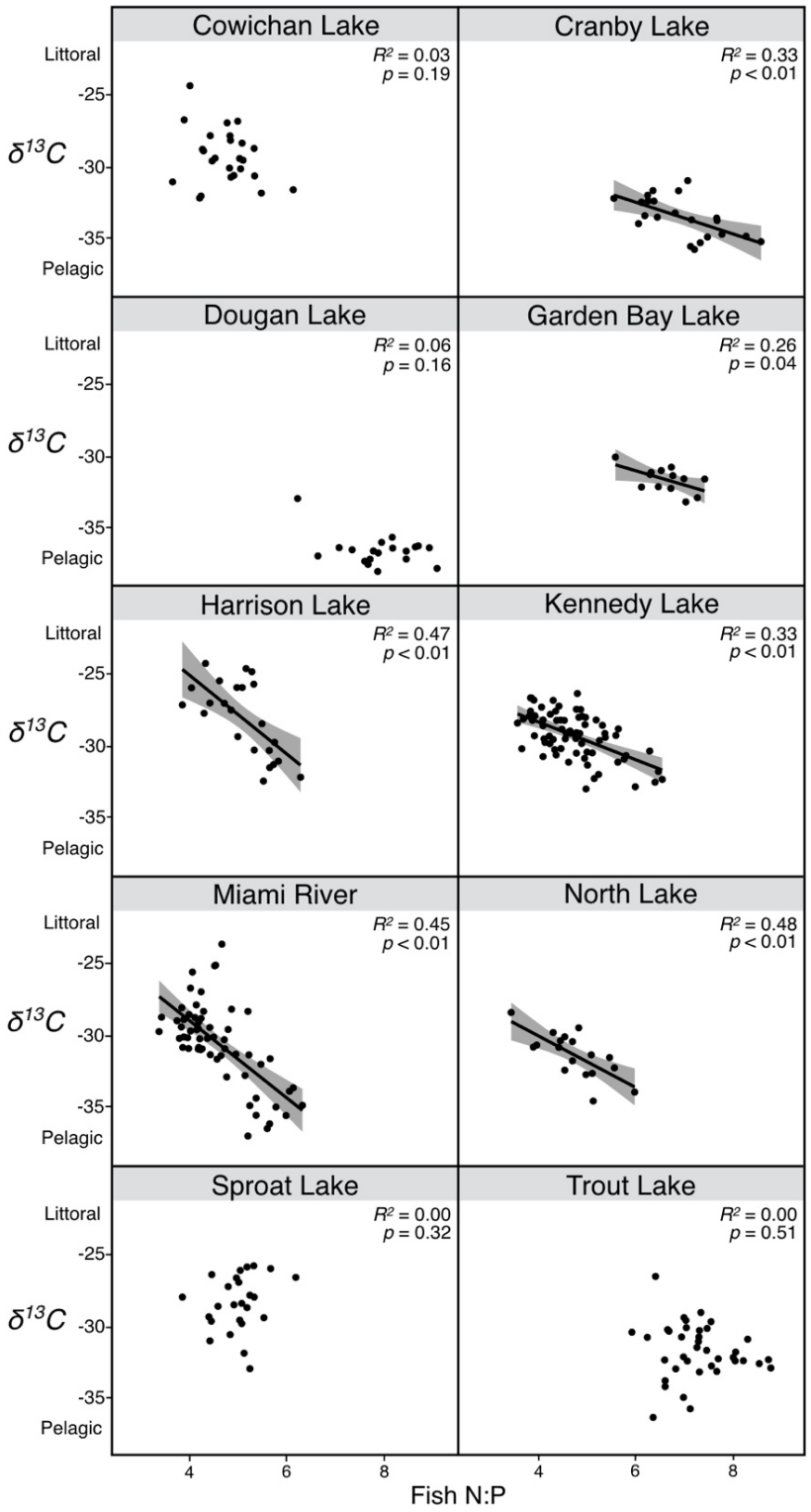

Fig. S5 – Relationships between diet type ($\delta^{13}C$) and fish organismal stoichiometry (N:P) at all 10 freshwater sampling locations. Shaded areas depict 95% confidence ranges.